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How to cite:

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Version: [\[not recorded\]](#)

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.1097/NEN.0b013e318197eca7>

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Expression of chemokines and their receptors by human brain endothelium:

Implications for multiple sclerosis

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Running title: Chemokines and receptors on human brain endothelium

Acknowledgements: We are grateful to Dr R. Selway and staff at King's College Hospital, London, the UK Multiple Sclerosis Tissue Bank, and the MRC London Brain Bank for Neurodegenerative Diseases (Dr. Safa Al-Sarraj, Clinical Director) for provision of human tissues for use in our investigations. This work was supported by The Multiple Sclerosis Society of Great Britain and Northern Ireland.

Role of Authors: EAS carried out most of the experiments for her PhD thesis under the direct supervision of IAR, JG and DKM; PR carried out the analysis of chemokine expression in post mortem tissue sections; HAD and FMC provided technical support for the immunogold electron microscopy studies. The manuscript was written by EAS, PR, DKM and IAR.

Abstract

Leukocyte migration into the CNS is mediated by chemokines, expressed on the surface of brain endothelium. This study investigated the production of chemokines and expression of chemokine receptors by human brain endothelial cells (HBEC), *in vitro* and *in situ* in multiple sclerosis tissue. Four chemokines (CCL2, CCL5, CXCL8 and CXCL10), were demonstrated in endothelial cells *in situ*, which was reflected in the chemokine production by primary HBEC and a brain endothelial cell line, hCEMC/D3. CXCL8 and CCL2 were constitutively released and increased in response to TNF α and/or IFN γ . CXCL10 and CCL5 were undetectable in resting cells but were secreted in response to these cytokines. TNF α strongly increased the production of CCL2, CCL5 and CXCL8, while IFN γ up-regulated CXCL10 exclusively. CCL3 was not secreted by HBECs and appeared to be confined to astrocytes *in situ*. The chemokine receptors CXCR1 and CXCR3 were expressed by HBEC both *in vitro* and *in situ*, and CXCR3 was up-regulated in response to cytokine stimulation *in vitro*. By contrast, CXCR3 expression was reduced in silent MS lesions. Brain endothelium expresses particularly high levels of CXCL10 and CXCL8, which may account for the predominant TH1-type inflammatory reaction seen in chronic conditions such as multiple sclerosis.

Keywords: Inflammation; blood-brain barrier; brain endothelium; multiple sclerosis; chemokines.

Introduction

Chemokines are low molecular weight chemotactic cytokines that regulate leukocyte trafficking into tissues and are thought to be key mediators of the inflammatory response. Over 40 different chemokines, binding to 18 different chemokine receptors, have been identified to date [1]. The position of the first and second conserved cysteine residues determines the basis for the classification of chemokines into four families (CC, CXC, CX₃C and XC or the α , β , δ and γ subfamilies in the old nomenclature, respectively). Many chemokines can bind to different receptors within the same sub-group and most receptors can bind to several different chemokines. This is thought to ensure the redundancy of the system [2]. Indeed, most of the knock-out mice for chemokines or chemokine receptors are viable with the exception of CXCL12 and its receptor CXCR4 [3-5].

Multiple Sclerosis (MS) is considered to be an autoimmune disorder of unknown aetiology, in which a strong TH1-type immune response develops in the CNS, associated with production of the inflammatory cytokines interferon- γ (IFN γ), tumour necrosis factor- α (TNF α), and transforming growth factor- β 2 (TGF β 2). Activated T-lymphocytes and macrophages cross the blood-brain barrier (BBB) and target myelin, creating demyelinated plaques which are associated with areas of chronic active inflammation [6]. A range of chemokines and their receptors are highly expressed on infiltrating leukocytes as well as on CNS resident cells of MS lesions. These include CCL2, CCL3, CCL5, CCL19, CCL21 and CXCL8 and 10 and their cognate receptors [7-11]. Amongst these, the increased expression of CXCL10 and its receptor, CXCR3, has been shown to be a prominent feature of reactive astrocytes [12] and infiltrating CD3⁺ T cells within lesions [13]. This observation is important for understanding disease pathology because CXCL10 is an IFN γ -inducible chemokine and its receptor CXCR3 is highly expressed on active IFN γ -producing TH1 cells. Consequently, once a TH1-type response has developed in the CNS, the induction of IFN γ -inducible chemokines will selectively attract further TH1 cells to the site of inflammation, thus reinforcing the chronic

inflammation. In addition, CXCL8 expression has been shown to be increased in MS, although its exact role in MS pathogenesis remains unknown [14]

Of the CC chemokines, CCL2, CCL3, CCL5 and their receptors, CCR1 CCR2 and CCR5 show increased expression in MS lesions [15] on microglia and astrocytes or on the infiltrating lymphocytes and macrophages. The increase of CCL2, CCL3 and CCL5 also occurs in experimental autoimmune encephalomyelitis (EAE), an animal model for MS [16], and it was thought that CCL2 signalling could be important in attracting macrophages to sites of neuroinflammation. This view is supported by the observation that CCL2 knock-out mice showed a reduced clinical EAE score [17] and knock out of its receptor CCR2 prevented mice from developing EAE in one study [18]. Other important CC chemokines in MS pathogenesis are CCL19 and CCL21 which have been shown to be involved in T lymphocyte migration into the CNS in EAE and MS [7].

Most studies of chemokine expression in MS or EAE have focussed on chemokine production by CNS parenchymal cells or infiltrated leukocytes, assuming that chemokines expressed in the parenchyma will be transported to the luminal surface of the endothelium. However, the potential for chemokines to move to the lumen is very limited, because brain endothelia normally have continuous tight junctions, preventing paracellular diffusion of proteins. Moreover, transcytosis is also limited in brain endothelium, as the cells have relatively few transport vesicles, by comparison with other endothelia. For these reasons, chemokine production by brain endothelial cells themselves may be particularly important in regulating leukocyte traffic into the CNS (i.e. they may represent the primary source of chemokines that interact directly with circulating leukocytes). As endothelia from different tissues secrete different subsets of chemokines, it potentially has a major role in determining the characteristics of inflammation in each tissue [19], and this is particularly true for brain endothelium.

The aim of this study was to identify the pattern of chemokine expression by human brain endothelium both in the resting state and in response to inflammatory cytokines. In the study, we used primary human brain endothelial cells (HBEC) and an immortalised human brain endothelial cell line hCMEC/D3 [20]. The chemokines investigated in this study included a subset of chemokines potentially involved in MS pathogenesis, namely CXCL8, CXCL10, CCL2, CCL3 and CCL5, as there is evidence that they may be produced by vascular endothelium [19]. The findings *in vitro* were related to observations of chemokine expression by the endothelial cells of cerebral capillaries *in situ* in normal CNS and in areas of neuroinflammation in MS.

In addition, the expression of chemokine receptors on brain endothelium *in vitro* and *in situ* was investigated. The functional significance of endothelial chemokine receptors is not fully understood [21]. They may be involved in clearance of chemokines from the cell surface, by endocytosis [22-24]. It has also been proposed that they are involved in transcytosis [25, 26]. Alternatively, some chemokines have angiogenic or angiostatic properties, so the presence of receptors could allow these cells to respond appropriately to signals for proliferation [27]. Berger and colleagues [28] have previously demonstrated the expression of CXCR1, CXCR3, CCR2 and CCR5 by cultured human brain endothelial cells but there is little comparative data on cells *in situ* [25]. We therefore examined MS tissue using immunohistochemistry and immunogold labelling combined with transmission electron microscopy to identify specific chemokine receptors (CXCR1 and CXCR3), in order to establish whether our findings *in vitro* reflect the expression of the receptors *in situ*. By identifying the sets of chemokines and their distribution, the work indicates which of the chemokines present in neuroinflammation are localised in endothelium, where they can control leukocyte transmigration.

Materials and methods

Human brain tissue and cell culture

Human brain tissue from the frontal or temporal cortex was obtained with ethical approval, either post-mortem from patients diagnosed clinically and neuropathologically with multiple sclerosis through the UK Multiple Sclerosis Tissue Bank (London), and the MRC London Brain Bank for Neurodegenerative Diseases (Institute of Psychiatry, London), as indicated in Table 1 (10 female, 3 male, mean age of 55.6 ± 4.37 years, range 34-92 years of age), or from three epilepsy patients undergoing temporal lobe resection (King's College Hospital, London), according to local ethical committee guidelines (Protocol 99-002). Tissues obtained from the frontal and temporal lobes of MS patients contained demyelinated plaques and/or non-affected (non-demyelinated) regions (Table 1). Brain tissues were used for immunohistochemical and electron microscopic analyses (froze, formalin-fixed and paraffin-embedded materials) as indicated in Table 1. Fresh brain tissue was available from five cases (Table 1, cases 4, 5, 6, 7 and 9) for setting up primary brain endothelial cultures (from the non-affected white matter in two cases, from a silent lesion in one case, and from the white matter of two other cases in which lesion activity had not been determined at post-mortem).

Primary human brain endothelial cells were isolated according to previously described techniques [29], with the modification that cells were cultured in medium containing 0.5 $\mu\text{g/ml}$ puromycin for 5 days to remove contaminating cells [30]. For isolating primary HBEC, 1 cm^3 tissue blocks were required in order to obtain sufficient capillaries to generate pure endothelial cultures. In MS brain tissue, the blocks were taken from areas that contained MS lesions (as determined by naked eye) but that also contained periplaque areas and surrounding NAWM. HBEC reached confluence 2-3 weeks post-isolation and were used between passages 2 and 4. Purity was confirmed by staining for Von Willebrand factor. HBEC and the hCMEC/D3 cell line [20], were cultured in EBM-2 (Cambrex, Wokingham, UK) supplemented with VEGF, IGF, bFGF, hydrocortisone, ascorbate, gentamycin and 2.5 % fetal bovine serum (FBS), but were rested in the same medium without growth factors for at least 48 hours before assay.

Typing of lesions in MS brain tissue samples

Lesions within the white matter in MS tissues were characterised according to (i) classical histological labelling with luxol fast blue, (ii) immunostaining with myelin-associated proteins (myelin basic protein and proteolipid protein) (neuropathological samples), and/or (iii) immunostaining patterns for CD68 and MHC class II antigens [31]. Active (demyelinating) lesions are hypercellular and can be characterised by infiltrating MHC-II positive macrophages throughout the lesions; chronic active lesions are demyelinated with hypocellular centers, but are hypercellular at the periphery, with activated macrophages located primarily at the edge of the lesion and chronic inactive or 'silent' lesions are hypocellular and lack MHC-II positive cells [31].

Sectioned materials were taken to methanol/hydrogen peroxide solution, permeabilised with 0.1% triton X100 in PBS for 1 h, and non-specific binding sites were blocked using 10% normal rabbit serum for 1 h. The primary antibodies (CD68, clone PGM1, 1/20 and MHC class II, clone CR3/43, from DakoCytomation, UK) were incubated on the slide overnight at RT, and processed according to established immunoperoxidase protocols (Dako 3-step ABC-HRP protocol, DakoCytomation, UK), using 1:100 biotinylated rabbit anti-mouse IgG as secondary antibody. In the sampled tissues, 'active' lesions were identified by the abundance of CD68 positive/MHC class II positive mononuclear phagocytes within a lesion core; 'chronic active' lesions as CD68 positive/MHC class II positive primarily at the edge of the lesion, and CD68 positive/MHC class II negative within the centre; 'silent' lesions were CD68 negative/MHC class II negative [31] (suppl. Fig. 1). A total of 13-16 active/chronic active lesions from five cases, and silent lesions from six cases (Table 1) were investigated for chemokine and chemokine receptor expression *in situ*.

Immunoperoxidase staining of chemokines *in situ*

The immunoperoxidase staining procedure for chemokines and chemokine receptors *in situ* (tissue samples) was performed as previously described [32] with minor modifications.

Formalin-fixed and fresh-frozen tissue blocks were sectioned serially at 10/20 μm thickness using a cryostat, and collected on superfrost plus slides. Frozen sections were air-dried for 90 minutes RT, immersed in methanol containing 2.5% of a 30% hydrogen peroxide solution, incubated in Hanks Balanced Saline Solution (HBSS) containing 1% bovine serum albumin, 1% of a [1M] stock of MgCl_2 and CaCl_2 , and 0.01% Tween 20. Sections were next incubated with 10% appropriate normal sera (rabbit, or swine) made up in phosphate buffered saline solution (PBS) for 90 minutes, prior to incubation with primary antibody solution for 24-36 hours at 4°C (1/500, 1/100 or 1/100 dilutions of mouse monoclonal IgG1 antibodies for CXCL8, CCL2 and CCL3, respectively; 1/1000 or 1/500 dilution of rabbit polyclonal IgG antibodies for CXCL10 and CCL5, respectively; PeproTech, London, UK). Negative controls were included, where the primary antibody step was omitted and sections incubated with normal sera alone. Following three 5-minute washes in PBS, sections were incubated in relevant biotinylated secondary antibodies (rabbit anti-mouse IgG, 1:100; swine anti-rabbit IgG, 1:100, DakoCytomation Ltd., UK) for 120 minutes, washed in three changes of PBS and incubated with Avidin-Biotin-Horseradish Peroxidase Complex (ABC-HRP Standard Kit, Vector Laboratories, UK) made up according to the manufacturer's instructions, for a further 90-120 minutes. After a final three washes in PBS, the sections were reacted with 0.5mg/ml 3,3'-Diaminobenzidine (DAB) made up in PBS to which 20 μl of hydrogen peroxide was added, and the reaction timed for optimal staining (3-5 minutes). Nuclei were weakly counterstained with Harris' Haematoxylin solution if required, and sections differentiated in 0.1% acid alcohol solution (10 sec) before being rinsed under running tap water. Sections were then dehydrated in a graded series of alcohol (50%, the 75%, 90%, 95%, and 100% ethanol), cleared in xylene, and coverslipped using DPX mountant (VWR Ltd., Dorset, UK). Slides were viewed using a brightfield microscope. Formalin-fixed, paraffin-embedded tissue blocks were also sectioned at 3 μm using a base-sledge microtome, dewaxed in xylene and transferred to 100% alcohol, before immersion in methanol/hydrogen peroxide solution and immunohistochemical staining as described above.

Immunofluorescence labelling of chemokine receptors *in situ* and in culture

For chemokine receptor staining, fresh tissues were also fixed using 2% para-formaldehyde (PAF) and 3.75% acrolein in 0.1M PB for 1 h and left overnight in 2% PAF only. The sections were cut at 50µm using a Vibratome (Leica Microsystems, Milton Keynes, UK). For immunolabelling to detect vWF/GFAP and CXCR1/CXCR3, an antigen retrieval step was included, by incubating for 20 min in 0.01M citrate buffer, pH 6, at 95°C. All sections were then permeabilized with 0.1% triton X100 in PBS for 1 h. Non-specific binding was blocked using 10% normal goat serum for 1 h. Sections were incubated with mouse anti-CXCR1/CXCR3 (10µg/ml, R&D, Oxon, UK) or their respective isotype-matched antibody controls for 1 h, and after 3 washes, followed by Cy3-conjugated goat anti-mouse IgG (1/100, Chemicon, Hampshire, UK). Sections were then incubated with a rabbit polyclonal anti-human vWF (1/800, Sigma, Dorset, UK) or anti-GFAP (1/100, Chemicon, Hampshire, UK) antibodies in PBS overnight at RT, and detected using goat anti-rabbit-Ig conjugated to FITC (1/200, Chemicon, Hampshire, UK), incubated for 1 h. Sections were mounted using Mowiol. Negative controls were included, where sections were incubated with normal sera in place of primary antibodies.

For characterisation of chemokine/ receptor expression *in vitro*, cultured endothelial cells were first grown to 50 % confluence on collagen-coated Labtek multiwell chambered slides (Nunc, Scientific Laboratory Supplies, Nottingham, UK), and fixed using 4% paraformaldehyde in PBS for 10 min. The cells were permeabilised using 0.1 % triton X-100 in PBS for 5 min. Non-specific binding sites were then blocked using 10% normal goat serum in PBS for 1 h, followed by 1 h incubation with primary antibodies at room temperature. Chemokine receptor expression was determined using a panel of phycoerythrin or fluorescein-conjugated mouse monoclonal antibodies specific for CCR1, CCR2, CCR5, CXCR1, CXCR2 and CXCR3 (R&D systems, Oxon, UK) or their respective isotype-matched antibody controls as previously described (15). These were applied for 1 h at RT at the

recommended dilutions. After 3 washes, the slides were mounted with a glass coverslip with Dako Fluorescent Mounting Medium (DakoCytomation Ltd, Cambridgeshire, UK) and viewed with a fluorescence microscope (Olympus BX61).

Immunogold labelling and electron microscopy

For chemokine receptor staining, fresh tissue was fixed using 2% p-formaldehyde and 3.75% acrolein in 0.1M phosphate buffer (PB) for 1 h and left overnight in 2% p-formaldehyde. The sections were cut at 50µm using a vibratome. The floating sections were treated with 1% sodium borohydride in PB for 30 min and then freeze-thawed to permeabilise the tissue. The sections were incubated in Cryoprotectant solution and then rapidly immersed in chloriduofluoromethane, followed by liquid nitrogen and then in three successive PB washes. Blocking solution was added for 30 min, and sections were incubated with primary mouse antibody (mouse IgG_{2A} anti-human CXCR1, 5µg/ml or mouse IgG₁ anti-human CXCR3, 1µg/ml, R&D, Oxon, UK) or their respective isotype-matched antibody controls for 24 h at RT and a further 24 h at 4°C in 0.1%BSA in TS. Non specific binding of the secondary antibody was blocked with the incubation/washing buffer for 10 min after a rinse with PBS. Sections were then incubated for 2 h with goat anti-mouse IgG secondary antibody (dilution 1/50) conjugated to colloidal gold (British Biocell International, Cardiff, UK) in the incubation/washing buffer. After a 5 min wash with the incubation/washing buffer and three rinses with PBS, bound (1 nm) gold particles were fixed using 2% glutaraldehyde in PBS for 10 min followed by a wash in a 0.2M citrate buffer solution. The sections were reacted with a silver enhancement solution (British Biocell International, Cardiff, UK). The silver enhancement reaction was stopped by two washes in the citrate buffer and three washes with PB. The immunogold stained tissue was post-fixed for 1 h in 2% osmium tetroxide in PB for electron microscopic viewing. They were then dehydrated in a series of ethanol solutions (concentration 50%, 70%, 80%, 95% for 5 min, and twice in 100% for 10 min) and twice in propylene oxide for 10 min before being embedded in Epon 812 between sheets of Aclar

plastic. Epon polymerisation was carried out by incubation at 60°C over 48 h, and a sample of the tissue was mounted on the tips of Epon Block. Ultra-thin sections of 70 nm were cut with a diamond knife (Diatone, TAAB, Gillingham, UK) and collected on copper mesh grids. The sections on grids were counterstained with uranyl acetate for 20 min and lead citrate for 6 min, and examined with a Joel JEM1010 electron microscope attached to a Gatan Bioscan digital camera (Joel, Welwyn Garden City, UK). Micrographs at X8000 magnifications were taken, scanned with Epson perfection 4870 photo, and visualised with Photoshop 5.5 programme. For quantification of the density area (number of gold particles/ μm^2), the negative film of the photograph was scanned and used to count the number of gold particles within endothelial cells. The surface of the endothelial cells was determined from the digital images, using NIH image programme on a Macintosh G4. The subcellular localisation of gold particles in the cells was recorded as cytoplasmic, luminal or abluminal.

RT-PCR

RNA was isolated from primary HBEC grown to confluence in a 25cm² flask coated with collagen. Total RNA was extracted using 1 ml of TRIzol LS (Invitrogen, Paisley, UK) according to the manufacturer's protocol and stored at -80 °C. For the reverse transcription (RT) procedure, the protocol given by the supplier, Promega (ImProm-IITM Reverse transcriptase) was followed. Briefly, 2 μg of RNA was mixed with 1 μg of random primers at 70°C for 5 min then annealed on ice. Reverse transcriptase was carried out by addition of 40 μl RT mix at 25°C for 5 min followed by 60 min at 40 °C. To terminate the reaction, the mix was incubated for a further 15 min at 70 °C. The negative control for each sample consisted of RNA samples annealed with random primers without the Reverse Transcriptase. The resulting cDNA was either stored at -20°C, or taken directly into the PCR amplification procedure.

The PCR amplification steps were performed by adding 4 μl of cDNA from the RT reaction to 20 μl of PCR mix (50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, and 0.1% Triton® X-

100), 1.5mM MgCl₂, 0.2mM of dNTPs, 1µM of each primer and 1U of Taq polymerase) and placed into an iCycler PCR machine (Bio-Rad Laboratories, Hertfordshire, UK). PCR conditions were 5 min at 94°C, followed by 40 cycles (94°C, 45 s; Ta indicated below, 45 s, 72°C, 60 s) finishing at 72°C for 7 min. Primers, Ta and size of PCR product were as follows: CCR1, F5'ACGAAAGCCTACGAGAGTG3', R5'GGTGAACAGGAAGTCTTGG3', Ta 50 °C, 240 bp; CCR2 F5'GATTACGGTGCTCCCTGTC3', R5'GCCACAGACATAAACAGAATC3', Ta 50, 496 bp; CCR5, F5'GCTGAGACATCCGTTCCCCTACA3', R5'GGTGACCGTCCTGGCTTTTA3', Ta 58 °C, 477 bp; CXCR1 F5'GTGATGCTGGTCATCTTATACAG3', R5'TTGTTTGGATGGTAAGCCTGG3', Ta 52 °C, 230 bp; CXCR2 F5'CGAAGGACCGTCTACTCATC3', R5'AGTGTGCCCTGAAGAAGAGC3', Ta 53 °C, 519 bp; CXCR3 F5'GGAGCTGCTCAGAGTAAATCAC3', R5'GCACGAGTCACTCTCGTTTTTC3', Ta 53 °C, 200 bp. Each sample was run in parallel with cyclophilin primers, acting as a positive control [33]. PCR products were visualized using agarose gel electrophoresis.

Capture ELISA

Primary HBEC (passage 2) were cultured on collagen-coated 24 well plates until confluent and treated for 48 h with cytokines at the following concentrations: TGF-β at 25ng/ml, TNFα at 25ng/ml and IFNγ at 100ng/ml (R&D Systems, Oxon, UK). Culture supernatants were then collected and frozen at -20°C until further analysis. Chemokines were measured by sandwich ELISA (R&D systems, Oxon, UK), according to the manufacturer's protocols. The standard range for all four chemokines tested was between 0.03 and 8 ng/ml. Only absorbance values within the linear part of the standard curve corresponding to supernatants and/or diluted supernatants were used for each experiment. For experiments on polarized secretion of chemokines, transwell polyester membrane inserts [Corning Costar, UK (0.4 µm pore, 12mm diameter)] were first coated with rat collagen [Sigma-Aldrich, UK, (0.005 % w/v, 1 h at room

temperature)]and then with human fibronectin [Sigma-Aldrich, UK (5 µg/ml, 1 h)]. Primary HBEC cells were grown to confluence ($\sim 1 \times 10^5$ cells/cm²) with a culture media change every 2-3 days and incubated for 2 days post confluence prior to treatment.

Flow Cytometry

Chemokine receptor expression on hCMEC/D3 cells in the absence or presence of cytokines (25ng/ml TNF α and 100ng/ml IFN γ for 24 h) was determined using a panel of fluorescently labelled antibodies (R&D Systems, Oxon, UK) as previously described [33]. Briefly, hCMEC/D3 cells were grown to confluence, washed and trypsinized using 0.25% trypsin/EDTA (Invitrogen, UK). Cells were fixed using 1ml 4% *p*-formaldehyde in PBS for 10 min at 4°C and then centrifuged at 300xg for 5 min. Cells were then permeabilised using 0.1% Triton-X-100 in PBS for 1 min at room temperature, centrifuged at 300xg for 5 min, resuspended in 1 ml of blocking solution (0.1 mg/ml human IgG /10% normal goat serum in PBS), and incubated for 30 min at 4°C. Cells were counted and resuspended at $8 \cdot 10^6$ cells/ml. For the assay, 25µl of the cell suspension ($2 \cdot 10^5$ cells) was added 10µl of appropriate antibodies at the manufacturer's recommended concentrations. Appropriate isotype-matched controls were used. Cells were incubated with antibodies for 1 h at 4°C, then washed once using PBS and resuspended in 0.4 ml PBS for analysis. Flow cytometry data was acquired and analysed using the FACScalibur flow cytometer and CellQuest™ software (Becton Dickinson, UK).

Statistical analysis

Significance was determined by a one way or two way ANOVA, followed by a post hoc Tukeys t-test. $P < 0.05$ was considered significant. For CXCL8 production by HBEC, the data were not normally distributed and a Kruskal-Wallis and Mann-Whitney test was used.

Results

Expression of chemokines by human cerebral endothelium in multiple sclerosis

Endothelial cells in non-demyelinated, normal-appearing white matter (NAWM) and cortical grey matter showed a vesicular staining for CCL2 (MCP-1: Fig. 1A) and CXCL8 (IL-8: Fig. 1H). Higher power magnification shown in Figs. 1A' and 1A'' illustrate this expression more clearly for CCL2. These chemokines as well as CCL3 (MIP-1 α), CCL5 (RANTES) and CXCL10 (IP-10) were detected on the surface of endothelial cells and/or on surrounding cells but there were differences in the pattern of expression between normal and plaque tissue. While CCL2 heavily stained endothelial cells in large vessels and surrounding cells within the demyelinated plaque (Fig. 1C), expression in adjacent non-affected cortical areas (white and grey matter) was more patchy on the vasculature, and was sometimes associated with perivascular cells (Fig. 1B, asterisk). The localisation of CXCL8 closely matched that described for CCL2 within the plaque area (Fig. 1I) and in adjacent non-affected regions (not shown). Similarly, CCL5 showed a finely-peppered, patchy distribution at the surface of endothelial cells (Fig. 1F). By contrast, CCL3 staining in the plaques was primarily detected on fine processes radiating perpendicularly away from the blood vessels (Fig. 1D). This pattern of immunoreactivity can be seen to a much lesser extent on endothelial cells of blood vessels in non-affected areas (Fig. 1E). The pattern of staining for CCL3 suggests that it is primarily associated with astrocyte processes and end-feet rather than the endothelium or other perivascular cells. CXCL10 is more diffusely expressed on vascular endothelium in the demyelinating plaque (Fig. 1G). These results implicate human brain endothelial cells as a source of chemokines *in vivo*, both in the resting state (CCL2, CXCL8) and in inflammation (CCL2, CCL5, CXCL8, CXCL10).

Chemokine secretion by primary human brain endothelial cells and hCMEC/D3 cells

In order to determine whether human brain endothelial cells can secrete chemokines, the chemokine levels constitutively released into the culture medium by cultured primary human brain endothelial cells (HBEC) isolated from 5 MS donors and 3 epileptic donors and by the

hCMEC/D3 cell line were determined by ELISA (Fig. 2). Out of the five chemokines tested only CCL2 and CXCL8 were constitutively produced by all cells tested. This finding corroborates the observation that only these two chemokines are present in the NAWM *in situ*. Basal CCL2 levels released by primary HBEC from MS brain tissue in the range 1.4 - 22.7 ng/ml (n=5), was similar to that produced by HBEC from temporal lobe resected tissue (4.4 - 14.3 ng/ml (n=3)). CXCL8 constitutive levels were more variable as primary HBEC from MS brain tissue ranged to from 3.2 - 206 ng/ml (n=5) and HBEC from epileptic temporal lobe tissue which ranged from 45 - 142 ng/ml (n=3). Endothelium from both sources may be considered 'resting', since they had been removed from potential micro-environmental proinflammatory stimuli for at least one week *in vitro* before assay.

Following cytokine stimulation, chemokine release into the culture medium increased to similar levels from primary HBEC of both MS brain tissue and temporal lobe resections (Fig. 2). However, specific cytokines or combinations of cytokines differentially affected chemokine secretion. The most potent activator of CXCL8 and CCL2 production by primary HBEC was TNF α alone, although the effect of TNF α on CXCL8 secretion was effectively blocked by co-incubation with either IFN γ or TGF β . Both CXCL10 and CCL5 were maximally induced by IFN γ in combination with TNF α although in the case of CXCL10, IFN γ alone was sufficient to induce a considerable increase in CXCL10 production. CCL3 was not released into the culture medium either basally or following any of the conditions tested (data not shown), correlating with the results *in situ*, where CCL3 appeared to be associated with astrocytes rather than endothelium.

The hCMEC/D3 cell line, largely exhibited the same pattern of chemokine secretion as primary HBEC with two exceptions: 1) the basal levels of CCL2 and CXCL8 secretion was lower than those observed for primary HBEC and 2) TNF α alone was sufficient to induce CXCL10 synthesis (Fig. 2).

Since chemokines are presented to circulating leukocytes, we investigated whether primary HBEC cells grown on filters were polarized in their secretion either constitutively or following stimulation with TNF α and IFN γ . Constitutive CCL2 and CXCL8 levels were similar in both the upper and lower chambers (Fig.3). By contrast, following stimulation with TNF α and IFN γ , the concentrations of all chemokines were higher in the upper than in the lower chamber although increased apical levels were statistically significant only for CCL2, CXCL8 and CXCL10 (Fig.3). These results indicate that cytokine-induced chemokine secretion was preferentially directed to the apical side of the endothelium.

Chemokine receptor expression by cultured human brain endothelial cells

It is possible that chemokines released into the circulation act on brain endothelial cells in an autocrine manner or that receptors are involved in clearance of free chemokines from plasma. We therefore investigated the expression by cultured brain endothelial cells of the chemokine receptors to which the chemokines investigated above bind, namely CXCR1-3 and CCR1, 2 and 5. Using semi-quantitative RT-PCR, primary HBEC expressed CCR1, CCR5 and CXCR1-3 mRNA but not CCR2 (Fig. 4a). At the protein level, CXCR1 and 3 were present at high levels, whereas levels of CXCR2 and CCR5 were lower and CCR1 and 2 were not detected by immunocytochemistry (Fig. 4b). CXCR1 and CXCR3 appeared to be localised mainly intracellularly, in particular around the nucleus. The pattern of chemokine receptor expression did not differ between cells originating from MS brains and those obtained from epileptic patients (data not shown).

The pattern of chemokine receptor expression of primary HBEC was similar to that observed in the hCMEC/D3 cell line, with CXCR1 and CXCR3 showing the highest levels of expression amongst the chemokine receptors tested (16). We therefore used this cell line to quantify changes in chemokine receptor expression induced by cytokines using FACScan

analysis. Following incubation of hCMEC/D3 with TNF α and IFN γ for 24 h, only CXCR3 expression was significantly increased ($p < 0.05$, $n = 3$) whereas CCR1 expression was induced ($p < 0.05$, $n = 3$). No changes in expression were observed for CXCR1, CXCR2, CCR2 or CCR5 (Fig. 5).

Chemokine receptor expression by human brain endothelium *in situ*

Since CXCR1 and CXCR3 appeared to be expressed at high levels by cultured HBEC and CXCR3 was upregulated by cytokine stimulation, a detailed analysis of the expression of these two chemokine receptors by HBEC was performed *in situ* in MS brain sections. Active (CD68+, MHC class II+) and silent (CD68+, MHC class II-) lesions were compared with normal appearing white matter (NAWM) (CD68-, MHC class II-) in terms of expression of CXCR1 and CXCR3 by immunohistochemistry. In 50 μ m sections, double labelling with von Willebrand factor, an endothelial marker, and either CXCR1 or CXCR3 (Fig. 6a) revealed chemokine receptor staining associated with blood vessels in NAWM. In addition, other cells within the brain parenchyma which were negative for von Willebrand factor were also positively labelled for CXCR1 and CXCR3. Since human astrocytes have been previously shown to express CXCR1 and CXCR3 (29), double labelling with either CXCR1 or CXCR3 and GFAP, a specific marker for astrocytes, was carried out to determine whether the positive chemokine receptor expression by blood vessels was due to associated astrocytic end-feet. A clear co-localisation of CXCR1 (Fig. 6b) and CXCR3 (not shown) with GFAP was observed in the astrocytic processes surrounding blood vessels. In addition, cells enclosed within the astrocytic end-feet were also positively labelled for CXCR1 (Fig. 6b) and CXCR3 (not shown), although whether these cells were endothelial cells could not be ascertained. In MS lesions, CXCR1 (not shown) and CXCR3 (Fig. 6c) positive staining was diffuse throughout the lesion, probably due to astrogliosis and/or leukocyte infiltration.

Subcellular localisation of CXCR1 and CXCR3 by immunogold labelling and electron microscopy

To determine whether endothelial cells expressed chemokine receptors *in situ*, the immunogold technique using monoclonal antibodies to CXCR1 and CXCR3 was carried out on sections of MS brain tissue. Immunogold labelling with the monoclonal CXCR1 and CXCR3 antibodies revealed gold particles along the plasma membrane and cytoplasm of the endothelial cells, pericytes and astrocytic end-feet (Fig. 7a,b,d). Leukocytes, whether infiltrated or interacting with the endothelial cells on the luminal side, were also labelled for CXCR1 (Fig. 7c) and, to a lesser extent, CXCR3 (not shown).

In the context of endothelial cells, labelling was easily identified at both the luminal and abluminal plasma membranes and within the cytoplasm of the capillary endothelial cells (Fig. 7d). The total number of gold particles on endothelial cells per surface area for CXCR1 and CXCR3 did not vary significantly between NAWM, active lesions and silent lesions, with the exception of CXCR3 in silent lesions which was significantly lower than in NAWM or active lesions (Table 2). In NAWM, CXCR1 and CXCR3 antigenic sites were mainly localized at the cytoplasm, in particular for CXCR1 with approximately 87% of gold particles located in the cytoplasm compared to 65% for CXCR3 (Table 2). The distribution of antigenic sites between the luminal and abluminal membranes was different for CXCR1 and CXCR3, with a higher percentage of gold particles on the luminal membrane compared to the abluminal membrane for CXCR1, whereas the opposite was observed for CXCR3 (Table 2). The endothelial subcellular distribution of CXCR1 in MHC class II⁺ (active) and MHC class II⁻ (silent) lesions did not differ significantly from that observed in NAWM. By contrast, a significant reduction in CXCR3-immunolabelling on the luminal membrane and a significant increase in CXCR3-immunolabelling within the cytoplasm was detected in MHC class II⁺ lesions (Table 2). Control experiments carried out to assess labelling specificity showed a negligible number of gold particles randomly distributed when the primary antibody was omitted.

Discussion

Many chemokines are strongly expressed in the CNS during multiple sclerosis [11]. However, it is thought that the set of chemokines which are expressed on the luminal surface of the endothelium controls the pattern of leukocyte migration into each tissue including the CNS. Chemokines may be synthesised by the endothelium [19] or produced within tissues and transported across the endothelium in transport vesicles including caveolae [22, 26]. Transcytosis is more likely to be important in tissues such as the lung, where the bulk transport systems are well developed [34]. Conversely, in the brain, where the endothelial barrier is strong and transcytosis limited, chemokine secretion by the endothelium itself is likely to be more important. Endothelia from different tissues vary in their chemokine secretion profiles and the rate of chemokine clearance from the cell surface [35]. Moreover, chemokine binding to the cell surface depends on the glycocalyx: brain microvascular endothelium has a particularly high negative charge, due to its sulphated glycosaminoglycans, which can interact with and retain positively-charged chemokines [36]. For these reasons, identifying chemokine production by brain endothelium is particularly important for understanding the distinctive patterns of leukocyte migration that occur in the CNS.

This study has shown that CCL2 and CXCL8 are produced and secreted by resting brain endothelium *in vitro* and by cerebral endothelium in normal-appearing brain tissue *in situ*. These chemokines, together with CXCL10 and CCL5 are induced following activation by inflammatory cytokines in endothelial cells *in vitro*, and in areas of inflammation and demyelination in MS tissue. Chemokine production by brain endothelium is indeed distinct from other endothelial subtypes, including primary microvascular endothelium from lung, dermis and liver and saphenous vein endothelium [19]. Our results are in agreement with previous reports demonstrating production of CCL2 and CXCL8 by HBEC isolated from temporal lobe of epileptic patients under resting conditions [37] and following stimulation with cytokines [38] or with supernatants derived from allogeneic or myelin basic protein reactive TH1 cells [39]. In this study, the chemokine profiles from primary brain endothelium

were similar, regardless of whether the cells came from MS patients or temporal lobe resection, and were broadly similar to the results with the cell line hCMEC/D3, suggesting that, in MS, HBEC do not show increased chemokine production *per se* but rather respond normally to the inflammatory environment they are exposed to by releasing chemokines. However, the level of production of CXCL8 and CXCL10 by brain endothelium is high by comparison with non-brain endothelia [19]. The finding with CXCL10 is notable, since this chemokine acts on CXCR3, which is strongly expressed on activated TH1 cells, precisely the population that is thought to drive the neuropathology of MS.

In contrast to other chemokines, CXCL8 secretion by HBEC was more variable either in resting or cytokine-stimulated cells. However, both EP and MS HBEC appeared to secrete variable levels of CXCL8 depending on the individual donor rather than on tissue type. This variability in CXCL8 expression has been observed by others in response to the HIV-1 protein tat [40] and in endothelial cells from non-CNS tissues in response to cytokines [19]. It is possible that the number of cell divisions is a critical factor regulating the storage of CXCL8 in non-brain endothelial cells, as previously demonstrated [41]. In primary cultures, the number of cell divisions at the time of the assay could have varied between donors as the yield of capillary fragments and hence the number of cell divisions to attain confluence is dependent on a number of factors (post-mortem time, quantity of tissue, cause of death, among others). Another possibility involves inter-individual variation amongst different donors. Indeed, CXCL8 plasma levels of healthy blood donors varied greatly in a recent study [42] and variability has also been demonstrated between different ethnic groups [43].

The downregulatory effect of IFN γ and TGF β on the TNF α -induced upregulation of CXCL8 secretion merits attention. Indeed, TGF β is considered to be an anti-inflammatory cytokine and its expression has been detected in active MS lesions [44]. However, CXCL8 inhibition by TGF β was only partial suggesting that it may not exert its anti-inflammatory effects in the

presence of high levels of pro-inflammatory cytokines. The inhibitory effect of IFN γ on TNF α -induced CXCL8 production has been reported in other cell types such as monocytes [45]. The activation of NF κ B and AP-1 by TNF α , required for CXCL8 transcription, is inhibited in the presence of IFN γ in endothelial cells [46]. The suppression of CXCL8 production by IFN γ may thus be considered a protective effect against CXCL-8 mediated neutrophil infiltration. Another important finding from the present study is that brain endothelium does not produce CCL3 *in vitro*. This is consistent with the observations *in situ*, which suggest that astrocytes, rather than endothelium are the main source of CCL3. These results stress the importance of correlating *in vitro* and *in situ* studies when investigating MS pathogenesis.

How does chemokine expression by HBEC relate to leukocyte infiltration? Apical release of chemokines by brain endothelium *in vitro* may rather reflect the rapid dilution of this chemokine by the blood stream *in vivo*. Indeed, chemokines released in the circulation would be rapidly degraded by proteases or their actions neutralized by decoy chemokine receptors such as DARC expressed by erythrocytes [47]. Alternatively, chemokines released by endothelial cells may bind to the endothelial glycocalyx either on the secretory cell itself or on other endothelial cells further along the capillary wall trapping immune cells within the inflamed area either at the lumen or within the perivascular space. This may apply to CXCL10, as high levels of its receptor, which might serve to bind this chemokine, are detected on the abluminal side of brain endothelial cells *in situ*. Indeed, previous studies stress the importance of chemokines in directing leukocyte trafficking into the CNS. In chronic relapsing EAE in mice, disease severity correlated with CCL3 production during the initial acute phase, but more closely with CCL2 levels during relapse [48]. These observations and the fact that CCL2-null mice do not develop EAE indicate a potential role for CCL2 in the development of neuroinflammation [18]. Whether CCL2 is essential in EAE has however been questioned by other studies which show that EAE can develop in a number of CCR2-

deficient mice [49]. In this model neuropathological examination showed a higher proportion of neutrophils and fewer macrophages than in normal animals, which suggests that macrophages can be partly replaced by neutrophils in producing the EAE pathology although it still implies that CCL2/CCR2 are important in monocyte migration into the CNS. In multiple sclerosis the levels of CCL3 in the CSF have a weak positive relationship to the level of cells present [50] but this may merely reflect the fact that inflammation will activate astrocytes to produce CCL3, and does not necessarily imply that CCL3 is required to drive leukocyte transmigration. Indeed other evidence suggests that CCL2 is also important in controlling monocyte migration in MS and that migrating cells lose their CCR2 receptor as they transmigrate [10].

There is also considerable evidence that at least some endothelial-derived chemokines regulate leukocyte trafficking into the CNS, specifically of TH1 cells. Supernatants from TH1, but not TH2, cells induce production of CXCL10, CXCL8 and CCL2 by HBEC [39]. Using an *in vitro* human BBB model, Prat et al. [37] demonstrated that antibody neutralization of CCL2 considerably reduced migration of T lymphocytes isolated from MS patients across HBEC. Indeed, CCL2 has been shown to be crucial for the TH1 immune response in EAE [17]. These studies were expanded to show, using intravital microscopy, that treatment with anti-CCL2 or anti-CCL5 antibodies prevent leukocyte adhesion, but not rolling, in EAE [51]. Our results have shown that brain endothelium itself is a major source of CCL2 as well as CXCL10, both of which have been implicated in the development of TH1-type inflammatory reactions in MS.

The expression of chemokine receptors on brain endothelium as determined by fluorescence microscopy and FACS analysis shows some similarity to other endothelia, with high expression of CXCR1 and CXCR3. Our findings are generally in agreement with Berger et al [28] who showed expression of CXCR1-3 by HBEC, although the expression of CXCR2 was low in the present study. In addition, we confirmed a low expression of CCR5 in agreement

with other *in vitro* [28, 52] and *in situ* [53] studies. By contrast, although CCR2 expression has previously been reported in HBEC [28], and more recently also in mouse BEC at the protein and mRNA level [54], we could not demonstrate CCR2 expression either at the protein or transcript level in our study. Species differences in CCR2 expression cannot be ruled out at present. In the study of Berger et al [28], HBEC were positive by immunofluorescence using goat polyclonal antibodies to CCR2A but not with those to CCR2B, whereas we used a more specific CCR2 monoclonal antibody. In addition, Andjelkovic et al [52] showed binding sites to CCL2 in isolated human brain capillaries, although this may either be due to other cell types expressing CCR2 such as perivascular cells/pericytes, or to other endothelial chemokine receptors (i.e. DARC).

The function of endothelial chemokine receptors has not been defined. Interestingly, CXCR1 and CXCR3 bind to CXCL8 and CXCL10 respectively, which would allow secreted chemokines to act in an autocrine fashion. Previous studies have suggested that signalling via CXCR1 is angiogenic, whereas signalling via CXCR3 is angiostatic [27]. Another potential function for endothelial chemokine receptors is to clear the plasma of free chemokines, so that leukocytes do not become activated unless they are triggered by chemokines held on the endothelial glycocalyx. Finally, it has been proposed that chemokine receptors could be involved in transport of chemokines across endothelium. Our observation that the subcellular localization of CXCR3 is altered in MS lesions compared to NAWM is suggestive of this hypothesis. Indeed, there appears to be an increase in the intracellular pool of CXCR3, and a decrease in the abluminal membrane in MS lesions. The fact that CXCL10 induces the internalisation of its receptor [55] might account for the observed increase in the intracellular pool of CXCR3 as CXCL10 is increased in MS lesions and could explain the decrease of the receptor on the abluminal side. CCR2 has been suggested to act as a means of transport for its chemokine ligand, CCL2 across the BBB [56]. It is possible that the same case applies for CXCR3 and its ligand, as they are greatly increased in the lesions, mainly in the active lesions. Indeed, we have shown that CXCR3 is upregulated by cytokine treatment in a human

brain endothelial cell line. However, our *in vitro* results should be approached with caution as hCMEC/D3 cells do not retain all characteristics of the BBB phenotype, such as high transendothelial resistance [20], and the pattern of chemokine expression was somewhat different between primary HBEC and the immortalised cell line. Whether CXCR3 transports CXCL10 across human brain endothelium remains to be determined.

This study has shown that human brain endothelium cultured *in vitro* expresses a similar pattern of chemokines and chemokine receptors to that seen *in situ*. It has shown that brain endothelium responds to cytokine stimulation by secreting chemokines, and does so with the same response pattern as other microvascular endothelium. However it also highlights the high secretion of CXCL8 and CXCL10, in comparison with other endothelia, which may explain the distinctive TH1-pattern of chronic inflammation in the brain, seen in diseases such as MS.

Acknowledgements

We are grateful to Dr R. Selway and staff at King's College Hospital, London, the UK Multiple Sclerosis Tissue Bank, and the MRC London Brain Bank for Neurodegenerative Diseases (Dr. Safa Al-Sarraj, Clinical Director) for provision of human tissues for use in our investigations. This work was supported by The Multiple Sclerosis Society of Great Britain and Northern Ireland.

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Figure legends

Fig. 1: Chemokine expression in multiple sclerosis brain tissue

Representative photographs taken from sections of the frontal lobe of cases with MS (Table 1) immunolabelled to detect CCL2, CCL5, CXCL8 or CXCL10. [A, A', A'', H]: paraffin-embedded sections at 3µm thickness. [B-G, I]: frozen sections cut at 20µm thickness [A, A', A'', B, H] are taken from non-demyelinated areas. [C-G, I] are taken from demyelinating plaque areas. [A'] and [A''] represent high power photographs of endothelial cells expressing CCL2 from the same case as shown in [A]. [A' and A''] show higher power photographs of endothelial cells expressing CCL2. Polarised expression is indicated by an arrowhead in [A']. The (unlabelled) nucleus of an endothelial cell is indicated by the adjacent (*e*) in [A'']. The asterisk in [B] denotes a perivascular cell expressing CCL2, associated with the external (parenchymal) blood vessel wall. The scale bar represents approx. 115µm in [A], 20µm in [A', A''], 195µm in [B], 410µm in [C], 150µm in [D], 95µm in [E,F], 85µm in [G], 100µm in [H] and 335µm in [I]. Nuclei have not been counterstained.

Fig. 2: Chemokine release into culture medium by primary HBEC and the hCMEC/D3 cell line under basal conditions and following stimulation by cytokines for 48 h.

Supernatants from confluent monolayers of HBEC or hCMEC/D3 cells were collected after 48 h stimulation with TNF α (50 ng/ml), IFN γ (100 ng/ml) and TGF β (25 ng/ml) or a combination of them and assayed by sandwich ELISA for (a) CXCL8 (b) CXCL10 (c) CCL2 and (d) CCL5. Results presented are means \pm S.E.M. of 3-5 different experiments for HBEC isolated from MS brain tissue (white), 3 for HBEC isolated from temporal lobe resections from epileptic patients (grey) and 3 for the hCMEC/D3 cell line (black) with duplicate wells. Significant differences were determined by ANOVA followed by a post hoc t-test for CCL2, CCL5 and CXCL10 and by a Kruskal-Wallis and Mann-Whitney test for CXCL8. * significant difference ($p < 0.05$) versus the control; Δ significant difference ($p < 0.05$) of sample

treated with a combination of cytokines versus TNF- α treated sample; • significant difference ($p < 0.05$) of sample treated with a combination of cytokines versus IFN γ treated sample.

Fig 3: Chemokine release into the apical and baso-lateral culture medium by primary HBEC grown on filters under basal conditions and following stimulation by cytokines.

Supernatants from confluent monolayers of human brain endothelial cells grown on filters were collected at 48 h from both the apical (white) and the basolateral sides (grey) and assayed by sandwich ELISA for CXCL8, CXCL10, CCL2, and CCL5. Results presented are means \pm S.D. from two different experiments with duplicate wells. Significant differences were determined by ANOVA followed by a post hoc t-test. * significant difference ($p < 0.05$) versus the control; • significant difference ($p < 0.05$) between concentration at the apical chamber and the basal chamber.

Fig. 4. Chemokine receptor expression by primary HBEC detected by (a) semi-quantitative RT-PCR and (b) immunocytochemistry. cDNA for CCR1, CCR5, CXCR1, CXCR2 and CXCR3 was detected in primary HBEC but not that of CCR2 (+). Cyclophilin was used as positive control and to ensure equal cDNA loading for each sample. Negative controls (-) of samples incubated in the absence of RT were included. Results are representative of 2 experiments. (b) Immunofluorescence staining of primary HBEC for CCR5, CXCR1, CXCR2 and CXCR3 (Texas red; bottom). CXCR1 and CXCR3 showed a predominant surface staining extending over the cell body. CXCR2 and CCR5 showed predominantly vesicle staining. Scalebar = 20 μ m.

Fig. 5. Chemokine receptor expression by hCMEC/D3 cells under basal conditions and following stimulation by cytokines for 24 h. (a) Representative FACScan histograms show the expression of unstimulated hCMEC/D3 cells (black line) and hCMEC/D3 cells stimulated with 500ng/ml TNF α and 10ng/ml IFN γ for 24 h (grey line). hCMEC/D3 cells were fixed

with 4% PAF, permeabilised with triton X-100, labelled with FITC- or PE- labelled antibodies specific for CXCR1, CXCR2, CXCR3, CCR1, CCR2 and CCR5, and analysed by FAScan. Cells labelled with FITC- or PE- labelled isotope-matched antibodies are represented as grey filled histograms. The histograms are of one experiment representative of three. **(b)** Results are expressed as the mean fluorescence for chemokine receptor expression by unstimulated hCMEC/D3 cells (white) and hCMEC/D3 cells stimulated with 500ng/ml TNF α and 10ng/ml IFN γ (grey) for 24 h. Control values have been subtracted. Significance was tested on normalised values by ANOVA with a post hoc t-test (* p<0.05). Results presented are the mean \pm S.E.M. of three different experiments.

Fig. 6. Immunofluorescent detection of CXCR1 and CXCR3 in human NAWM and MS lesions. Fixed 50 μ m thick sections cut with a vibratome were permeabilised with triton X-100 and then processed for detection of either CXCR1 (b) or CXCR3 (a, c) (in red) and either von Willebrand factor (a), an endothelial cell marker, or GFAP (b,c), an astrocyte marker, (in green) by immunohistochemistry in NAWM (a, b) and in a chronic active MS lesion (c). Merged images show the localisation of CXCR1 and CXCR3 within the neurovascular unit. Results are representative of experiments from six donors. Scale bar= 40 μ m

Fig. 7. Electron micrographs of CXCR1 and CXCR3-labelled MS brain tissue. Fixed 50 μ m thick sections were processed for detection of CXCR1 and CXCR3 using silver-enhanced gold particles and visualized by electron microscopy. (A) Brain capillary endothelial cells and the perivascular end-feet of astrocytes show CXCR1-antigenic sites positively labelled with gold particles in NAWM. (B) In a chronic active lesion (MHC class II +), both brain capillary endothelial cells and pericytes are labelled for CXCR1. Inset represents dashed area at higher magnification showing positive immunogold labelling for CXCR1 on the luminal surface of brain endothelial cells. (C) Leukocytes interacting with brain endothelial cells within the lumen (asterisk) are highly positive for CXCR1 in a chronic active lesion (MHC class II +).

(D) In a silent lesion, CXCR3 is distributed mainly within the cytoplasm of endothelial cells (some within endosomes) but also on the luminal (asterisk) and abluminal membranes.

Abbreviations: AE=astrocytic end-feet; EC= endothelial cell; E= erythrocyte; L=leukocyte;

P=pericyte. Scale bar= 1 μ m

Expression of chemokines and their receptors by human brain endothelium:

Implications for multiple sclerosis

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Running title: Chemokines and receptors on human brain endothelium

Acknowledgements: We are grateful to Dr R. Selway and staff at King's College Hospital, London, the UK Multiple Sclerosis Tissue Bank, and the MRC London Brain Bank for Neurodegenerative Diseases (Dr. Safa Al-Sarraj, Clinical Director) for provision of human tissues for use in our investigations. This work was supported by The Multiple Sclerosis Society of Great Britain and Northern Ireland.

Role of Authors: EAS carried out most of the experiments for her PhD thesis under the direct supervision of IAR, JG and DKM; PR carried out the analysis of chemokine expression in post mortem tissue sections; HAD and FMC provided technical support for the immunogold electron microscopy studies. The manuscript was written by EAS, PR, DKM and IAR.

Abstract

Leukocyte migration into the CNS is mediated by chemokines, expressed on the surface of brain endothelium. This study investigated the production of chemokines and expression of chemokine receptors by human brain endothelial cells (HBEC), *in vitro* and *in situ* in multiple sclerosis tissue. Four chemokines (CCL2, CCL5, CXCL8 and CXCL10), were demonstrated in endothelial cells *in situ*, which was reflected in the chemokine production by primary HBEC and a brain endothelial cell line, hCEMC/D3. CXCL8 and CCL2 were constitutively released and increased in response to TNF α and/or IFN γ . CXCL10 and CCL5 were undetectable in resting cells but were secreted in response to these cytokines. TNF α strongly increased the production of CCL2, CCL5 and CXCL8, while IFN γ up-regulated CXCL10 exclusively. CCL3 was not secreted by HBECs and appeared to be confined to astrocytes *in situ*. The chemokine receptors CXCR1 and CXCR3 were expressed by HBEC both *in vitro* and *in situ*, and CXCR3 was up-regulated in response to cytokine stimulation *in vitro*. By contrast, CXCR3 expression was reduced in silent MS lesions. Brain endothelium expresses particularly high levels of CXCL10 and CXCL8, which may account for the predominant TH1-type inflammatory reaction seen in chronic conditions such as multiple sclerosis.

Keywords: Inflammation; blood-brain barrier; brain endothelium; multiple sclerosis; chemokines.

Introduction

Chemokines are low molecular weight chemotactic cytokines that regulate leukocyte trafficking into tissues and are thought to be key mediators of the inflammatory response. Over 40 different chemokines, binding to 18 different chemokine receptors, have been identified to date [1]. The position of the first and second conserved cysteine residues determines the basis for the classification of chemokines into four families (CC, CXC, CX₃C and XC or the α , β , δ and γ subfamilies in the old nomenclature, respectively). Many chemokines can bind to different receptors within the same sub-group and most receptors can bind to several different chemokines. This is thought to ensure the redundancy of the system [2]. Indeed, most of the knock-out mice for chemokines or chemokine receptors are viable with the exception of CXCL12 and its receptor CXCR4 [3-5].

Multiple Sclerosis (MS) is considered to be an autoimmune disorder of unknown aetiology, in which a strong TH1-type immune response develops in the CNS, associated with production of the inflammatory cytokines interferon- γ (IFN γ), tumour necrosis factor- α (TNF α), and transforming growth factor- β 2 (TGF β 2). Activated T-lymphocytes and macrophages cross the blood-brain barrier (BBB) and target myelin, creating demyelinated plaques which are associated with areas of chronic active inflammation [6]. A range of chemokines and their receptors are highly expressed on infiltrating leukocytes as well as on CNS resident cells of MS lesions. These include CCL2, CCL3, CCL5, CCL19, CCL21 and CXCL8 and 10 and their cognate receptors [7-11]. Amongst these, the increased expression of CXCL10 and its receptor, CXCR3, has been shown to be a prominent feature of reactive astrocytes [12] and infiltrating CD3⁺ T cells within lesions [13]. This observation is important for understanding disease pathology because CXCL10 is an IFN γ -inducible chemokine and its receptor CXCR3 is highly expressed on active IFN γ -producing TH1 cells. Consequently, once a TH1-type response has developed in the CNS, the induction of IFN γ -inducible chemokines will selectively attract further TH1 cells to the site of inflammation, thus reinforcing the chronic

inflammation. In addition, CXCL8 expression has been shown to be increased in MS, although its exact role in MS pathogenesis remains unknown [14]

Of the CC chemokines, CCL2, CCL3, CCL5 and their receptors, CCR1 CCR2 and CCR5 show increased expression in MS lesions [15] on microglia and astrocytes or on the infiltrating lymphocytes and macrophages. The increase of CCL2, CCL3 and CCL5 also occurs in experimental autoimmune encephalomyelitis (EAE), an animal model for MS [16], and it was thought that CCL2 signalling could be important in attracting macrophages to sites of neuroinflammation. This view is supported by the observation that CCL2 knock-out mice showed a reduced clinical EAE score [17] and knock out of its receptor CCR2 prevented mice from developing EAE in one study [18]. Other important CC chemokines in MS pathogenesis are CCL19 and CCL21 which have been shown to be involved in T lymphocyte migration into the CNS in EAE and MS [7].

Most studies of chemokine expression in MS or EAE have focussed on chemokine production by CNS parenchymal cells or infiltrated leukocytes, assuming that chemokines expressed in the parenchyma will be transported to the luminal surface of the endothelium. However, the potential for chemokines to move to the lumen is very limited, because brain endothelia normally have continuous tight junctions, preventing paracellular diffusion of proteins. Moreover, transcytosis is also limited in brain endothelium, as the cells have relatively few transport vesicles, by comparison with other endothelia. For these reasons, chemokine production by brain endothelial cells themselves may be particularly important in regulating leukocyte traffic into the CNS (i.e. they may represent the primary source of chemokines that interact directly with circulating leukocytes). As endothelia from different tissues secrete different subsets of chemokines, it potentially has a major role in determining the characteristics of inflammation in each tissue [19], and this is particularly true for brain endothelium.

The aim of this study was to identify the pattern of chemokine expression by human brain endothelium both in the resting state and in response to inflammatory cytokines. In the study, we used primary human brain endothelial cells (HBEC) and an immortalised human brain endothelial cell line hCMEC/D3 [20]. The chemokines investigated in this study included a subset of chemokines potentially involved in MS pathogenesis, namely CXCL8, CXCL10, CCL2, CCL3 and CCL5, as there is evidence that they may be produced by vascular endothelium [19]. The findings *in vitro* were related to observations of chemokine expression by the endothelial cells of cerebral capillaries *in situ* in normal CNS and in areas of neuroinflammation in MS.

In addition, the expression of chemokine receptors on brain endothelium *in vitro* and *in situ* was investigated. The functional significance of endothelial chemokine receptors is not fully understood [21]. They may be involved in clearance of chemokines from the cell surface, by endocytosis [22-24]. It has also been proposed that they are involved in transcytosis [25, 26]. Alternatively, some chemokines have angiogenic or angiostatic properties, so the presence of receptors could allow these cells to respond appropriately to signals for proliferation [27]. Berger and colleagues [28] have previously demonstrated the expression of CXCR1, CXCR3, CCR2 and CCR5 by cultured human brain endothelial cells but there is little comparative data on cells *in situ* [25]. We therefore examined MS tissue using immunohistochemistry and immunogold labelling combined with transmission electron microscopy to identify specific chemokine receptors (CXCR1 and CXCR3), in order to establish whether our findings *in vitro* reflect the expression of the receptors *in situ*. By identifying the sets of chemokines and their distribution, the work indicates which of the chemokines present in neuroinflammation are localised in endothelium, where they can control leukocyte transmigration.

Materials and methods

Human brain tissue and cell culture

Human brain tissue from the frontal or temporal cortex was obtained with ethical approval, either post-mortem from patients diagnosed clinically and neuropathologically with multiple sclerosis through the UK Multiple Sclerosis Tissue Bank (London), and the MRC London Brain Bank for Neurodegenerative Diseases (Institute of Psychiatry, London), as indicated in Table 1 (10 female, 3 male, mean age of 55.6 ± 4.37 years, range 34-92 years of age), or from three epilepsy patients undergoing temporal lobe resection (King's College Hospital, London), according to local ethical committee guidelines (Protocol 99-002). Tissues obtained from the frontal and temporal lobes of MS patients contained ~~both~~ demyelinated plaques and/or non-affected (non-demyelinated) regions (Table 1). Brain tissues were used for immunohistochemical and electron microscopic analyses (froze, formalin-fixed and paraffin-embedded materials) as indicated in Table 1. Fresh brain tissue was available from five cases (Table 1, cases 4, 5, 6, 7 and 9) for setting up primary brain endothelial cultures (from the non-affected white matter in two cases, from a silent lesion in one case, and from the white matter of two other cases in which lesion activity had not been determined at post-mortem).isolating cerebral endothelial cells and astrocytes (fresh material) or for immunohistochemical and electron microscopic analyses (frozen, formalin fixed and paraffin-embedded materials) (see Table 1).

Primary human brain endothelial cells were isolated according to previously described techniques [29], with the modification that cells were cultured in medium containing 0.5 $\mu\text{g/ml}$ puromycin for 5 days to remove contaminating cells [30]. For isolating primary HBEC, 1 cm^3 tissue blocks were required in order to obtain sufficient capillaries to generate pure endothelial cultures. In MS brain tissue, the blocks were taken from areas that contained MS lesions (as determined by naked eye) but that also contained periplaque areas and surrounding NAWM. HBEC reached confluence 2-3 weeks post-isolation and were used between passages 2 and 4. Purity was confirmed by staining for Von Willebrand factor. HBEC and the hCMEC/D3 cell line [20], were cultured in EBM-2 (Cambrex, Wokingham, UK) supplemented with VEGF, IGF, bFGF, hydrocortisone, ascorbate, gentamycin and 2.5 % fetal

bovine serum (FBS), but were rested in the same medium without growth factors for at least 48 hours before assay.

Typing of lesions in MS brain tissue samples

Lesions within the white matter in MS tissues were characterised according to (i) classical histological labelling with luxol fast blue, (ii) ~~immunolabelling-immunostaining~~ with myelin-associated proteins (myelin basic protein and proteolipid protein) (neuropathological samples), and/or (iii) ~~immunolabelling-immunostaining~~ patterns for CD68 and MHC class II antigens [31]. Active (~~demyelinating~~) lesions are ~~hypercellular and can be~~ characterised by infiltrating MHC-II positive macrophages ~~throughout the lesions but no demyelination~~; chronic active lesions are demyelinated with ~~hypocellular centers, but are hypercellular at the periphery, with~~ activated macrophages located ~~primarily~~ at the edge of the lesion and ~~chronic inactive or~~ 'silent' lesions ~~are hypocellular and~~ lack MHC-II positive cells [31].

Sectioned materials were taken to methanol/hydrogen peroxide solution, permeabilised with 0.1% triton X100 in PBS for 1 h, and non-specific binding sites were blocked using 10% normal rabbit serum for 1 h. The primary antibodies (CD68, clone PGM1, 1/20 and MHC class II, clone CR3/43, from DakoCytomation, UK) were incubated on the slide overnight at RT, and processed according to established immunoperoxidase protocols (Dako 3-step ABC-HRP protocol, DakoCytomation, UK), using 1:100 biotinylated rabbit anti-mouse IgG as secondary antibody. In the sampled tissues, 'active' lesions were identified by the abundance of CD68 positive/MHC class II positive mononuclear phagocytes within a lesion core (~~in the absence of significant demyelination~~); 'chronic active' lesions as CD68 positive/MHC class II positive primarily at the edge of the lesion, and CD68 positive/MHC class II negative within the centre; 'silent' lesions were CD68 negative/MHC class II negative [31] (~~suppl. Fig. 1~~). A total of 13-16 active/chronic active lesions from five cases, and silent lesions from six cases (Table 1) were investigated for chemokine and chemokine receptor expression *in situ*.

Immunoperoxidase labelling-staining of chemokines *in situ*

The immunoperoxidase labelling-staining procedure for chemokines and chemokine receptors *in situ* (tissue samples) was performed as previously described [32] with minor modifications.

Formalin-fixed and fresh-frozen tissue blocks were sectioned serially at 10/20 µm thickness using a cryostat, and collected on superfrost plus slides. Frozen sections were air-dried for 90 minutes RT, immersed in methanol containing 2.5% of a 30% hydrogen peroxide solution, incubated in Hanks Balanced Saline Solution (HBSS) containing 1% bovine serum albumin, 1% of a [1M] stock of MgCl₂ and CaCl₂, and 0.01% Tween 20. Sections were next incubated with 10% appropriate normal sera (rabbit, or swine) made up in phosphate buffered saline solution (PBS) for 90 minutes, prior to incubation with primary antibody solution for 24-36

hours at 4°C (1/500, 1/100 or 1/100 dilutions of mouse monoclonal IgG1 antibodies for CXCL8, CCL2 and CCL3, respectively; 1/1000 or 1/500 dilution of rabbit polyclonal IgG antibodies for CXCL10 and CCL5, respectively; PeproTech, London, UK). Negative controls were included, where the primary antibody step was omitted and sections incubated with normal sera alone. Following three 5-minute washes in PBS, sections were incubated in

relevant biotinylated secondary antibodies (rabbit anti-mouse IgG, 1:100; swine anti-rabbit IgG, 1:100, DakoCytomation Ltd., UK) for 120 minutes, washed in three changes of PBS and incubated with Avidin-Biotin-Horseradish Peroxidase Complex (ABC-HRP Standard Kit, Vector Laboratories, UK) made up according to the manufacturer's instructions, for a further 90-120 minutes. After a final three washes in PBS, the sections were reacted with 0.5mg/ml 3,3'-Diaminobenzidine (DAB) made up in PBS to which 20 µl of hydrogen peroxide was added, and the reaction timed for optimal staining (3-5 minutes). Nuclei were weakly counterstained with Harris' Haematoxylin solution if required, and sections differentiated in 0.1% acid alcohol solution (10 sec) before being rinsed under running tap water. Sections were then dehydrated in a graded series of alcohol (50%, the 75%, 90%, 95%, and 100% ethanol), cleared in xylene, and coverslipped using DPX mountant (VWR Ltd., Dorset, UK). Slides were viewed using a brightfield microscope. Formalin-fixed, paraffin-embedded tissue

blocks were also sectioned at 3µm using a base-sledge microtome, dewaxed in xylene and transferred to 100% alcohol, before immersion in methanol/hydrogen peroxide solution and immunohistochemical [labelling-staining](#) as described above.

Immunofluorescence labelling of chemokine receptors *in situ* and in culture

For chemokine receptor staining, fresh tissues were also fixed using 2% para-formaldehyde (PAF) and 3.75% acrolein in 0.1M PB for 1 h and left overnight in 2% PAF only. The sections were cut at 50µm using a Vibratome (Leica Microsystems, Milton Keynes, UK). For immunolabelling to detect vWF/GFAP and CXCR1/CXCR3, an antigen retrieval step was included, by incubating for 20 min in 0.01M citrate buffer, pH 6, at 95°C. All sections were then permeabilized with 0.1% triton X100 in PBS for 1 h. Non-specific binding was blocked using 10% normal goat serum for 1 h. Sections were incubated with mouse anti-CXCR1/CXCR3 (10µg/ml, R&D, Oxon, UK) [or their respective isotype-matched antibody controls](#) for 1 h, and after 3 washes, followed by Cy3-conjugated goat anti-mouse IgG (1/100, Chemicon, Hampshire, UK). Sections were then incubated with a rabbit polyclonal anti-human vWF (1/800, Sigma, Dorset, UK) or anti-GFAP (1/100, Chemicon, Hampshire, UK) antibodies in PBS overnight at RT, and detected using goat anti-rabbit-Ig conjugated to FITC (1/200, Chemicon, Hampshire, UK), incubated for 1 h. Sections were mounted using Mowiol. Negative controls were included, where sections were incubated with normal sera in place of primary antibodies.

For characterisation of chemokine/ receptor expression *in vitro*, cultured endothelial cells were first grown to 50 % confluence on collagen-coated Labtek multiwell chambered slides (Nunc, Scientific Laboratory Supplies, Nottingham, UK), and fixed using 4% paraformaldehyde in PBS for 10 min. The cells were permeabilised using 0.1 % triton X-100 in PBS for 5 min. Non-specific binding sites were then blocked using 10% normal goat serum in PBS for 1 h, followed by 1 h incubation with primary antibodies at room temperature. Chemokine receptor expression was determined using a panel of phycoerythrin or

fluorescein-conjugated mouse monoclonal antibodies specific for CCR1, CCR2, CCR5, CXCR1, CXCR2 and CXCR3 (R&D systems, Oxon, UK) [or their respective isotype-matched antibody controls](#) as previously described (15). These were applied for 1 h at RT at the recommended dilutions. After 3 washes, the slides were mounted with a glass coverslip with Dako Fluorescent Mounting Medium (DakoCytomation Ltd, Cambridgeshire, UK) and viewed with a fluorescence microscope (Olympus BX61).

Immunogold labelling and electron microscopy

For chemokine receptor staining, fresh tissue was fixed using 2% p-formaldehyde and 3.75% acrolein in 0.1M phosphate buffer (PB) for 1 h and left overnight in 2% p-formaldehyde. The sections were cut at 50µm using a vibratome. The floating sections were treated with 1% sodium borohydride in PB for 30 min and then freeze-thawed to permeabilise the tissue. The sections were incubated in Cryoprotectant solution and then rapidly immersed in chloriduofluoromethane, followed by liquid nitrogen and then in three successive PB washes. Blocking solution was added for 30 min, and sections were incubated with primary mouse antibody (mouse [IgG_{2A}](#)-anti-human CXCR1, 5µg/ml or mouse [IgG₁](#)-anti-human CXCR3, 1µg/ml, R&D, Oxon, UK) [or their respective isotype-matched antibody controls](#) for 24 h at RT and a further 24 h at 4°C in 0.1%BSA in TS. Non specific binding of the secondary antibody was blocked with the incubation/washing buffer for 10 min after a rinse with PBS. Sections were then incubated for 2 h with goat anti-mouse [IgG](#) secondary antibody (dilution 1/50) conjugated to colloidal gold (British Biocell International, Cardiff, UK) in the incubation/washing buffer. After a 5 min wash with the incubation/washing buffer and three rinses with PBS, bound (1 nm) gold particles were fixed using 2% glutaraldehyde in PBS for 10 min followed by a wash in a 0.2M citrate buffer solution. The sections were reacted with a silver enhancement solution (British Biocell International, Cardiff, UK). The silver enhancement reaction was stopped by two washes in the citrate buffer and three washes with PB. The immunogold stained tissue was post-fixed for 1 h in 2% osmium tetroxide in PB for

electron microscopic viewing. They were then dehydrated in a series of ethanol solutions (concentration 50%, 70%, 80%, 95% for 5 min, and twice in 100% for 10 min) and twice in propylene oxide for 10 min before being embedded in Epon 812 between sheets of Aclar plastic. Epon polymerisation was carried out by incubation at 60°C over 48 h, and a sample of the tissue was mounted on the tips of Epon Block. Ultra-thin sections of 70 nm were cut with a diamond knife (Diatone, TAAB, Gillingham, UK) and collected on copper mesh grids. The sections on grids were counterstained with uranyl acetate for 20 min and lead citrate for 6 min, and examined with a Joel JEM1010 electron microscope attached to a Gatan Bioscan digital camera (Joel, Welwyn Garden City, UK). Micrographs at X8000 magnifications were taken, scanned with Epson perfection 4870 photo, and visualised with Photoshop 5.5 programme. For quantification of the density area (number of gold particles/ μm^2), the negative film of the photograph was scanned and used to count the number of gold particles within endothelial cells. The surface of the endothelial cells was determined from the digital images, using NIH image programme on a Macintosh G4. The subcellular localisation of gold particles in the cells was recorded as cytoplasmic, luminal or abluminal.

RT-PCR

RNA was isolated from primary HBEC grown to confluence in a 25cm² flask coated with collagen. Total RNA was extracted using 1 ml of TRIzol LS (Invitrogen, Paisley, UK) according to the manufacturer's protocol and stored at -80 °C. For the reverse transcription (RT) procedure, the protocol given by the supplier, Promega (ImProm-IITM Reverse transcriptase) was followed. Briefly, 2 μg of RNA was mixed with 1 μg of random primers at 70°C for 5 min then annealed on ice. Reverse transcriptase was carried out by addition of 40 μl RT mix at 25°C for 5 min followed by 60 min at 40 °C. To terminate the reaction, the mix was incubated for a further 15 min at 70 °C. The negative control for each sample consisted of RNA samples annealed with random primers without the Reverse Transcriptase. The resulting cDNA was either stored at -20°C, or taken directly into the PCR amplification procedure.

The PCR amplification steps were performed by adding 4 μ l of cDNA from the RT reaction to 20 μ l of PCR mix (50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, and 0.1% Triton® X-100), 1.5mM MgCl₂, 0.2mM of dNTPs, 1 μ M of each primer and 1U of Taq polymerase) and placed into an iCycler PCR machine (Bio-Rad Laboratories, Hertfordshire, UK). PCR conditions were 5 min at 94°C, followed by 40 cycles (94°C, 45 s; Ta indicated below, 45 s, 72°C, 60 s) finishing at 72°C for 7 min. Primers, Ta and size of PCR product were as follows: CCR1, F5'ACGAAAGCCTACGAGAGTG3', R5'GGTGAACAGGAAGTCTTGG3', Ta 50 °C, 240 bp; CCR2 F5'GATTACGGTGCTCCCTGTC3', R5'GCCACAGACATAAACAGAATC3', Ta 50, 496 bp; CCR5, F5'GCTGAGACATCCGTTCCCCTACA3', R5'GGTGACCGTCCTGGCTTTTA3', Ta 58 °C, 477 bp; CXCR1 F5'GTGATGCTGGTCATCTTATACAG3', R5'TTGTTTGGATGGTAAGCCTGG3', Ta 52 °C, 230 bp; CXCR2 F5'CGAAGGACCGTCTACTCATC3', R5'AGTGTGCCCTGAAGAAGAGC3', Ta 53 °C, 519 bp; CXCR3 F5'GGAGCTGCTCAGAGTAAATCAC3', R5'GCACGAGTCACTCTCGTTTTTC3', Ta 53 °C, 200 bp. Each sample was run in parallel with cyclophilin primers, acting as a positive control [33]. PCR products were visualized using agarose gel electrophoresis.

Capture ELISA

Primary HBEC (passage 2) were cultured on collagen-coated 24 well plates until confluent and treated for 48 h with cytokines at the following concentrations: TGF- β at 25ng/ml, TNF α at 25ng/ml and IFN γ at 100ng/ml (R&D Systems, Oxon, UK). Culture supernatants were then collected and frozen at -20°C until further analysis. Chemokines were measured by sandwich ELISA (R&D systems, Oxon, UK), according to the manufacturer's protocols. The standard range for all four chemokines tested was between 0.03 and 8 ng/ml. Only absorbance values within the linear part of the standard curve corresponding to supernatants and/or diluted

supernatants were used for each experiment. For experiments on polarized secretion of chemokines, transwell polyester membrane inserts [Corning Costar, UK (0.4 μm pore, 12mm diameter)] were first coated with rat collagen [Sigma-Aldrich, UK, (0.005 % w/v, 1 h at room temperature)] and then with human fibronectin [Sigma-Aldrich, UK (5 $\mu\text{g}/\text{ml}$, 1 h)]. Primary HBEC cells were grown to confluence ($\sim 1 \times 10^5$ cells/ cm^2) with a culture media change every 2-3 days and incubated for 2 days post confluence prior to treatment.

Flow Cytometry

Chemokine receptor expression on hCMEC/D3 cells in the absence or presence of cytokines (25ng/ml $\text{TNF}\alpha$ and 100ng/ml $\text{IFN}\gamma$ for 24 h) was determined using a panel of fluorescently labelled antibodies (R&D Systems, Oxon, UK) as previously described [33]. Briefly, hCMEC/D3 cells were grown to confluence, washed and trypsinized using 0.25% trypsin/EDTA (Invitrogen, UK). Cells were fixed using 1ml 4% *p*-formaldehyde in PBS for 10 min at 4°C and then centrifuged at 300xg for 5 min. Cells were then permeabilised using 0.1% Triton-X-100 in PBS for 1 min at room temperature, centrifuged at 300xg for 5 min, resuspended in 1 ml of blocking solution (0.1 mg/ml human IgG /10% normal goat serum in PBS), and incubated for 30 min at 4°C. Cells were counted and resuspended at $8 \cdot 10^6$ cells/ml. For the assay, 25 μl of the cell suspension ($2 \cdot 10^5$ cells) was added 10 μl of appropriate antibodies at the manufacturer's recommended concentrations. Appropriate isotype-matched controls were used. Cells were incubated with antibodies for 1 h at 4°C, then washed once using PBS and resuspended in 0.4 ml PBS for analysis. Flow cytometry data was acquired and analysed using the FACScalibur flow cytometer and CellQuest™ software (Becton Dickinson, UK).

Statistical analysis

Significance was determined by a one way or two way ANOVA, followed by a post hoc Tukeys t-test. $P < 0.05$ was considered significant. For CXCL8 production by HBEC, the data were not normally distributed and a Kruskal-Wallis and Mann-Whitney test was used.

Results

Expression of chemokines by human cerebral endothelium in multiple sclerosis

Endothelial cells in non-demyelinated, normal-appearing white matter (NAWM) and cortical grey matter showed a vesicular staining for CCL2 (MCP-1: Fig. 1A) and CXCL8 (IL-8: Fig. 1H). Higher power magnification shown in Figs. 1A' and 1A'' illustrate this expression more clearly for CCL2. These chemokines as well as CCL3 (MIP-1 α), CCL5 (RANTES) and CXCL10 (IP-10) were detected on the surface of endothelial cells and/or on surrounding cells but there were differences in the pattern of expression between normal and plaque tissue. While CCL2 heavily stained endothelial cells in large vessels and surrounding cells within the demyelinated plaque (Fig. 1C), expression in adjacent non-affected cortical areas (white and grey matter) was more patchy on the vasculature, and was sometimes associated with perivascular cells (Fig. 1B, asterisk). The localisation of CXCL8 closely matched that described for CCL2 within the plaque area (Fig. 1I) and in adjacent non-affected regions (not shown). Similarly, CCL5 showed a finely-peppered, patchy distribution at the surface of endothelial cells (Fig. 1F). By contrast, CCL3 staining in the plaques was primarily detected on fine processes radiating perpendicularly away from the blood vessels (Fig. 1D). This pattern of immunoreactivity can be seen to a much lesser extent on endothelial cells of blood vessels in non-affected areas (Fig. 1E). The pattern of staining for CCL3 suggests that it is primarily associated with astrocyte processes and end-feet rather than the endothelium or other perivascular cells. CXCL10 is more diffusely expressed on vascular endothelium in the demyelinating plaque (Fig. 1G). These results implicate human brain endothelial cells as a source of chemokines *in vivo*, both in the resting state (CCL2, CXCL8) and in inflammation (CCL2, CCL5, CXCL8, CXCL10).

Chemokine secretion by primary human brain endothelial cells and hCMEC/D3 cells

In order to determine whether human brain endothelial cells can secrete chemokines, the chemokine levels constitutively released into the culture medium by cultured primary human brain endothelial cells (HBEC) isolated from 5 MS donors and 3 epileptic donors and by the

hCMEC/D3 cell line were determined by ELISA (Fig. 2). Out of the five chemokines tested only CCL2 and CXCL8 were constitutively produced by all cells tested. This finding corroborates the observation that only these two chemokines are present in the NAWM *in situ*. Basal CCL2 levels released by primary HBEC from MS brain tissue in the range 1.4 - 22.7 ng/ml (n=5), was similar to that produced by HBEC from temporal lobe resected tissue (4.4 - 14.3 ng/ml (n=3)). CXCL8 constitutive levels were more variable as primary HBEC from MS brain tissue ranged to from 3.2 - 206 ng/ml (n=5) and HBEC from epileptic temporal lobe tissue which ranged from 45 - 142 ng/ml (n=3). Endothelium from both sources may be considered 'resting', since they had been removed from potential micro-environmental proinflammatory stimuli for at least one week *in vitro* before assay.

Following cytokine stimulation, chemokine release into the culture medium increased to similar levels from primary HBEC of both MS brain tissue and temporal lobe resections (Fig. 2). However, specific cytokines or combinations of cytokines differentially affected chemokine secretion. The most potent activator of CXCL8 and CCL2 production by primary HBEC was TNF α alone, although the effect of TNF α on CXCL8 secretion was effectively blocked by co-incubation with either IFN γ or TGF β . Both CXCL10 and CCL5 were maximally induced by IFN γ in combination with TNF α although in the case of CXCL10, IFN γ alone was sufficient to induce a considerable increase in CXCL10 production. CCL3 was not released into the culture medium either basally or following any of the conditions tested (data not shown), correlating with the results *in situ*, where CCL3 appeared to be associated with astrocytes rather than endothelium.

The hCMEC/D3 cell line, largely exhibited the same pattern of chemokine secretion as primary HBEC with two exceptions: 1) the basal levels of CCL2 and CXCL8 secretion was lower than those observed for primary HBEC and 2) TNF α alone was sufficient to induce CXCL10 synthesis (Fig. 2).

Since chemokines are presented to circulating leukocytes, we investigated whether primary HBEC cells grown on filters were polarized in their secretion either constitutively or following stimulation with TNF α and IFN γ . Constitutive CCL2 and CXCL8 levels were similar in both the upper and lower chambers (Fig.3). By contrast, following stimulation with TNF α and IFN γ , the concentrations of all chemokines were higher in the upper than in the lower chamber although increased apical levels were statistically significant only for CCL2, CXCL8 and CXCL10 (Fig.3). These results indicate that cytokine-induced chemokine secretion was preferentially directed to the apical side of the endothelium.

Chemokine receptor expression by cultured human brain endothelial cells

It is possible that chemokines released into the circulation act on brain endothelial cells in an autocrine manner or that receptors are involved in clearance of free chemokines from plasma. We therefore investigated the expression by cultured brain endothelial cells of the chemokine receptors to which the chemokines investigated above bind, namely CXCR1-3 and CCR1, 2 and 5. Using semi-quantitative RT-PCR, primary HBEC expressed CCR1, CCR5 and CXCR1-3 mRNA but not CCR2 (Fig. 4a). At the protein level, CXCR1 and 3 were present at high levels, whereas levels of CXCR2 and CCR5 were lower and CCR1 and 2 were not detected by immunocytochemistry (Fig. 4b). CXCR1 and CXCR3 appeared to be localised mainly intracellularly, in particular around the nucleus. The pattern of chemokine receptor expression did not differ between cells originating from MS brains and those obtained from epileptic patients (data not shown).

The pattern of chemokine receptor expression of primary HBEC was similar to that observed in the hCMEC/D3 cell line, with CXCR1 and CXCR3 showing the highest levels of expression amongst the chemokine receptors tested (16). We therefore used this cell line to quantify changes in chemokine receptor expression induced by cytokines using FACScan

analysis. Following incubation of hCMEC/D3 with TNF α and IFN γ for 24 h, only CXCR3 expression was significantly increased ($p < 0.05$, $n = 3$) whereas CCR1 expression was induced ($p < 0.05$, $n = 3$). No changes in expression were observed for CXCR1, CXCR2, CCR2 or CCR5 (Fig. 5).

Chemokine receptor expression by human brain endothelium *in situ*

Since CXCR1 and CXCR3 appeared to be expressed at high levels by cultured HBEC and CXCR3 was upregulated by cytokine stimulation, a detailed analysis of the expression of these two chemokine receptors by HBEC was performed *in situ* in MS brain sections. Active (CD68+, MHC class II+) and silent (CD68+, MHC class II-) lesions were compared with normal appearing white matter (NAWM) (CD68-, MHC class II-) in terms of expression of CXCR1 and CXCR3 by immunohistochemistry. In 50 μ m sections, double labelling with von Willebrand factor, an endothelial marker, and either CXCR1 or CXCR3 (Fig. 6a) revealed chemokine receptor staining associated with blood vessels in NAWM. In addition, other cells within the brain parenchyma which were negative for von Willebrand factor were also positively labelled for CXCR1 and CXCR3. Since human astrocytes have been previously shown to express CXCR1 and CXCR3 (29), double labelling with either CXCR1 or CXCR3 and GFAP, a specific marker for astrocytes, was carried out to determine whether the positive chemokine receptor expression by blood vessels was due to associated astrocytic end-feet. A clear co-localisation of CXCR1 (Fig. 6b) and CXCR3 (not shown) with GFAP was observed in the astrocytic processes surrounding blood vessels. In addition, cells enclosed within the astrocytic end-feet were also positively labelled for CXCR1 (Fig. 6b) and CXCR3 (not shown), although whether these cells were endothelial cells could not be ascertained. In MS lesions, CXCR1 (not shown) and CXCR3 (Fig. 6c) positive staining was diffuse throughout the lesion, probably due to astrogliosis and/or leukocyte infiltration.

Subcellular localisation of CXCR1 and CXCR3 by immunogold labelling and electron microscopy

To determine whether endothelial cells expressed chemokine receptors *in situ*, the immunogold technique using monoclonal antibodies to CXCR1 and CXCR3 was carried out on sections of MS brain tissue. Immunogold labelling with the monoclonal CXCR1 and CXCR3 antibodies revealed gold particles along the plasma membrane and cytoplasm of the endothelial cells, pericytes and astrocytic end-feet (Fig. 7a,b,d). Leukocytes, whether infiltrated or interacting with the endothelial cells on the luminal side, were also labelled for CXCR1 (Fig. 7c) and, to a lesser extent, CXCR3 (not shown).

In the context of endothelial cells, labelling was easily identified at both the luminal and abluminal plasma membranes and within the cytoplasm of the capillary endothelial cells (Fig. 7d). The total number of gold particles on endothelial cells per surface area for CXCR1 and CXCR3 did not vary significantly between NAWM, active lesions and silent lesions, with the exception of CXCR3 in silent lesions which was significantly lower than in NAWM or active lesions (Table 2). In NAWM, CXCR1 and CXCR3 antigenic sites were mainly localized at the cytoplasm, in particular for CXCR1 with approximately 87% of gold particles located in the cytoplasm compared to 65% for CXCR3 (Table 2). The distribution of antigenic sites between the luminal and abluminal membranes was different for CXCR1 and CXCR3, with a higher percentage of gold particles on the luminal membrane compared to the abluminal membrane for CXCR1, whereas the opposite was observed for CXCR3 (Table 2). The endothelial subcellular distribution of CXCR1 in MHC class II⁺ (active) and MHC class II⁻ (silent) lesions did not differ significantly from that observed in NAWM. By contrast, a significant reduction in CXCR3-immunolabelling on the luminal membrane and a significant increase in CXCR3-immunolabelling within the cytoplasm was detected in MHC class II⁺ lesions (Table 2). Control experiments carried out to assess labelling specificity showed a negligible number of gold particles randomly distributed when the primary antibody was omitted.

Discussion

~~Acute inflammation in the CNS is characterised by inflammatory cell infiltrates, mainly activated T helper cells and macrophages, but relatively few neutrophils. As chronic inflammation develops, activated T cells and macrophages are still prevalent, but resting CD4+ T cells and CD8+ T cells are present in increasing numbers.~~ Many chemokines are strongly expressed in the CNS during multiple sclerosis [11]. However, it is thought that the set of chemokines which are expressed on the luminal surface of the endothelium controls the pattern of leukocyte migration into each tissue including the CNS. Chemokines may be synthesised by the endothelium [19] or produced within tissues and transported across the endothelium in transport vesicles including caveolae [22, 26]. Transcytosis is more likely to be important in tissues such as the lung, where the bulk transport systems are well developed [34]. Conversely, in the brain, where the endothelial barrier is strong and transcytosis limited, chemokine secretion by the endothelium itself is likely to be more important. Endothelia from different tissues vary in their chemokine secretion profiles and the rate of chemokine clearance from the cell surface [35]. Moreover, chemokine binding to the cell surface depends on the glycocalyx: brain microvascular endothelium has a particularly high negative charge, due to its sulphated glycosaminoglycans, which can interact with and retain positively-charged chemokines [36]. For these reasons, identifying chemokine production by brain endothelium is particularly important for understanding the distinctive patterns of leukocyte migration that occur in the CNS.

This study has shown that CCL2 and CXCL8 are produced and secreted by resting brain endothelium *in vitro* and by cerebral endothelium in normal-appearing brain tissue *in situ*. These chemokines, together with CXCL10 and CCL5 are induced following activation by inflammatory cytokines in endothelial cells *in vitro*, and in areas of inflammation and demyelination in MS tissue. Chemokine production by brain endothelium is indeed distinct from other endothelial subtypes, including primary microvascular endothelium from lung, dermis and liver and saphenous vein endothelium [19]. Our results are in agreement with

previous reports demonstrating production of CCL2 and CXCL8 by HBEC isolated from temporal lobe of epileptic patients under resting conditions [37] and following stimulation with cytokines [38] or with supernatants derived from allogeneic or myelin basic protein reactive TH1 cells [39]. In this study, the chemokine profiles from primary brain endothelium were similar, regardless of whether the cells came from MS patients or temporal lobe resection, and were broadly similar to the results with the cell line hCMEC/D3, suggesting that, in MS, HBEC do not show increased chemokine production *per se* but rather respond normally to the inflammatory environment they are exposed to by releasing chemokines. However, the level of production of CXCL8 and CXCL10 by brain endothelium is high by comparison with non-brain endothelia [19]. The finding with CXCL10 is notable, since this chemokine acts on CXCR3, which is strongly expressed on activated TH1 cells, precisely the population that is thought to drive the neuropathology of MS.

In contrast to other chemokines, CXCL8 secretion by HBEC was more variable either in resting or cytokine-stimulated cells. However, both EP and MS HBEC appeared to secrete variable levels of CXCL8 depending on the individual donor rather than on tissue type. This variability in CXCL8 expression has been observed by others in response to the HIV-1 protein tat [40] and in endothelial cells from non-CNS tissues in response to cytokines [19]. It is possible that the number of cell divisions is a critical factor regulating the storage of CXCL8 in non-brain endothelial ~~Weibel-Palade bodies~~cells, as previously demonstrated [41]. ~~The number of Weibel-palade bodies of cultured endothelial cells decrease as the number of passages increases [42].~~ In primary cultures, the number of cell divisions at the time of the assay could have varied between donors as the yield of capillary fragments and hence the number of cell divisions to attain confluence is dependent on a number of factors (post-mortem time, quantity of tissue, cause of death, among others). Another possibility involves inter-individual variation amongst different donors. Indeed, CXCL8 plasma levels of healthy blood donors varied greatly in a recent study [4342] and variability has also been demonstrated between different ethnic groups [4443].

The downregulatory effect of IFN γ and TGF β on the TNF α -induced upregulation of CXCL8 secretion merits attention. Indeed, TGF β is considered to be an anti-inflammatory cytokine and its expression has been detected in active MS lesions [4544]. However, CXCL8 inhibition by TGF β was only partial suggesting that it may not exert its anti-inflammatory effects in the presence of high levels of pro-inflammatory cytokines. The inhibitory effect of IFN γ on TNF α -induced CXCL8 production has been reported in other cell types such as monocytes [4645]. The activation of NF κ B and AP-1 by TNF α , required for CXCL8 transcription, is inhibited in the presence of IFN γ in endothelial cells [4746]. The suppression of CXCL8 production by IFN γ may thus be considered a protective effect against CXCL-8 mediated neutrophil infiltration. Another important finding from the present study is that brain endothelium does not produce CCL3 *in vitro*. This is consistent with the observations *in situ*, which suggest that astrocytes, rather than endothelium are the main source of CCL3. These results stress the importance of correlating *in vitro* and *in situ* studies when investigating MS pathogenesis.

How does chemokine expression by HBEC relate to leukocyte infiltration? Apical release of chemokines by brain endothelium *in vitro* may rather reflect the rapid dilution of this chemokine by the blood stream *in vivo*. Indeed, chemokines released in the circulation would be rapidly degraded by proteases or their actions neutralized by decoy chemokine receptors such as DARC expressed by erythrocytes [4847]. Alternatively, chemokines released by endothelial cells may bind to the endothelial glycocalyx either on the secretory cell itself or on other endothelial cells further along the capillary wall trapping immune cells within the inflamed area either at the lumen or within the perivascular space. This may apply to CXCL10, as high levels of its receptor, which might serve to bind this chemokine, are detected on the abluminal side of brain endothelial cells *in situ*. Indeed, previous studies stress the importance of chemokines in directing leukocyte trafficking into the CNS. In chronic

relapsing EAE in mice, disease severity correlated with CCL3 production during the initial acute phase, but more closely with CCL2 levels during relapse [4948]. These observations and the fact that CCL2-null mice do not develop EAE indicate a potential role for CCL2 in the development of neuroinflammation [18]. Whether CCL2 is essential in EAE has however been questioned by other studies which show that EAE can develop in a number of CCR2-deficient mice [5049]. In this model neuropathological examination showed a higher proportion of neutrophils and fewer macrophages than in normal animals, which suggests that macrophages can be partly replaced by neutrophils in producing the EAE pathology although it still implies that CCL2/CCR2 are important in monocyte migration into the CNS. In multiple sclerosis the levels of CCL3 in the CSF have a weak positive relationship to the level of cells present [5150] but this may merely reflect the fact that inflammation will activate astrocytes to produce CCL3, and does not necessarily imply that CCL3 is required to drive leukocyte transmigration. Indeed other evidence suggests that CCL2 is also important in controlling monocyte migration in MS and that migrating cells lose their CCR2 receptor as they transmigrate [10].

There is also considerable evidence that at least some endothelial-derived chemokines regulate leukocyte trafficking into the CNS, specifically of TH1 cells. Supernatants from TH1, but not TH2, cells induce production of CXCL10, CXCL8 and CCL2 by HBEC [39]. Using an *in vitro* human BBB model, Prat et al. [37] demonstrated that antibody neutralization of CCL2 considerably reduced migration of T lymphocytes isolated from MS patients across HBEC. Indeed, CCL2 has been shown to be crucial for the TH1 immune response in EAE [17]. These studies were expanded to show, using intravital microscopy, that treatment with anti-CCL2 or anti-CCL5 antibodies prevent leukocyte adhesion, but not rolling, in EAE [5251]. Our results have shown that brain endothelium itself is a major source of CCL2 as well as CXCL10, both of which have been implicated in the development of TH1-type inflammatory reactions in MS.

The expression of chemokine receptors on brain endothelium as determined by fluorescence microscopy and FACS analysis shows some similarity to other endothelia, with high expression of CXCR1 and CXCR3. Our findings are generally in agreement with Berger et al [28] who showed expression of CXCR1-3 by HBEC, although the expression of CXCR2 was low in the present study. In addition, we confirmed a low expression of CCR5 in agreement with other *in vitro* [28, 5352] and *in situ* [5453] studies. By contrast, although CCR2 expression has previously been reported in HBEC [28], and more recently also in mouse BEC at the protein and mRNA level [5554], we could not demonstrate CCR2 expression either at the protein or transcript level in our study. Species differences in CCR2 expression cannot be ruled out at present. In the study of Berger et al [28], HBEC were positive by immunofluorescence using goat polyclonal antibodies to CCR2A but not with those to CCR2B, whereas we used a more specific CCR2 monoclonal antibody. In addition, Andjelkovic et al [5352] showed binding sites to CCL2 in isolated human brain capillaries, although this may either be due to other cell types expressing CCR2 such as perivascular cells/pericytes, or to other endothelial chemokine receptors (i.e. DARC).

The function of endothelial chemokine receptors has not been defined. Interestingly, CXCR1 and CXCR3 bind to CXCL8 and CXCL10 respectively, which would allow secreted chemokines to act in an autocrine fashion. Previous studies have suggested that signalling via CXCR1 is angiogenic, whereas signalling via CXCR3 is angiostatic [27]. Another potential function for endothelial chemokine receptors is to clear the plasma of free chemokines, so that leukocytes do not become activated unless they are triggered by chemokines held on the endothelial glycocalyx. Finally, it has been proposed that chemokine receptors could be involved in transport of chemokines across endothelium. Our observation that the subcellular localization of CXCR3 is altered in MS lesions compared to NAWM is suggestive of this hypothesis. Indeed, there appears to be an increase in the intracellular pool of CXCR3, and a decrease in the abluminal membrane in MS lesions. The fact that CXCL10 induces the internalisation of its receptor [5655] might account for the observed increase in the

intracellular pool of CXCR3 as CXCL10 is increased in MS lesions and could explain the decrease of the receptor on the abluminal side. CCR2 has been suggested to act as a means of transport for its chemokine ligand, CCL2 across the BBB [5756]. It is possible that the same case applies for CXCR3 and its ligand, as they are greatly increased in the lesions, mainly in the active lesions. Indeed, we have shown that CXCR3 is upregulated by cytokine treatment in a human brain endothelial cell line. However, our *in vitro* results should be approached with caution as hCMEC/D3 cells do not retain all characteristics of the BBB phenotype, such as high transendothelial resistance [20], and the pattern of chemokine expression was somewhat different between primary HBEC and the immortalised cell line. Whether ~~this is the case for~~ CXCR3 transports CXCL10 across human brain endothelium remains to be determined.

This study has shown that human brain endothelium cultured *in vitro* expresses a similar pattern of chemokines and chemokine receptors to that seen *in situ*. It has shown that brain endothelium responds to cytokine stimulation by secreting chemokines, and does so with the same response pattern as other microvascular endothelium. However it also highlights the high secretion of CXCL8 and CXCL10, in comparison with other endothelia, which may explain the distinctive TH1-pattern of chronic inflammation in the brain, seen in diseases such as MS.

Acknowledgements

We are grateful to Dr R. Selway and staff at King's College Hospital, London, the UK Multiple Sclerosis Tissue Bank, and the MRC London Brain Bank for Neurodegenerative Diseases (Dr. Safa Al-Sarraj, Clinical Director) for provision of human tissues for use in our investigations. This work was supported by The Multiple Sclerosis Society of Great Britain and Northern Ireland.

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Figure legends

Fig. 1: Chemokine expression in multiple sclerosis brain tissue

Representative photographs taken from sections of the frontal lobe of cases with MS (Table 1) immunolabelled to detect CCL2, CCL5, CXCL8 or CXCL10. [A, A', A'', H]: paraffin-embedded sections at 3µm thickness. [B-G, I]: frozen sections cut at 20µm thickness [A, A', A'', B, H] are taken from non-demyelinated areas. [C-G, I] are taken from demyelinating plaque areas. [A'] and [A''] represent high power photographs of endothelial cells expressing CCL2 from the same case as shown in [A]. [A' and A''] show higher power photographs of endothelial cells expressing CCL2. Polarised expression is indicated by an arrowhead in [A']. The (unlabelled) nucleus of an endothelial cell is indicated by the adjacent (*e*) in [A'']. The asterisk in [B] denotes a perivascular cell expressing CCL2, associated with the external (parenchymal) blood vessel wall. The scale bar represents approx. 115µm in [A], 20µm in [A', A''], 195µm in [B], 410µm in [C], 150µm in [D], 95µm in [E,F], 85µm in [G], 100µm in [H] and 335µm in [I]. Nuclei have not been counterstained.

Fig. 2: Chemokine release into culture medium by primary HBEC and the hCMEC/D3 cell line under basal conditions and following stimulation by cytokines for 48 h.

Supernatants from confluent monolayers of HBEC or hCMEC/D3 cells were collected after 48 h stimulation with TNF α (50 ng/ml), IFN γ (100 ng/ml) and TGF β (25 ng/ml) or a combination of them and assayed by sandwich ELISA for (a) CXCL8 (b) CXCL10 (c) CCL2 and (d) CCL5. Results presented are means \pm S.E.M. of 3-5 different experiments for HBEC isolated from MS brain tissue (white), 3 for HBEC isolated from temporal lobe resections from epileptic patients (grey) and 3 for the hCMEC/D3 cell line (black) with duplicate wells. Significant differences were determined by ANOVA followed by a post hoc t-test for CCL2, CCL5 and CXCL10 and by a Kruskal-Wallis and Mann-Whitney test for CXCL8. * significant difference ($p < 0.05$) versus the control; Δ significant difference ($p < 0.05$) of sample

treated with a combination of cytokines versus TNF- α treated sample; • significant difference ($p < 0.05$) of sample treated with a combination of cytokines versus IFN γ treated sample.

Fig 3: Chemokine release into the apical and baso-lateral culture medium by primary HBEC grown on filters under basal conditions and following stimulation by cytokines.

Supernatants from confluent monolayers of human brain endothelial cells grown on filters were collected at 48 h from both the apical (white) and the basolateral sides (grey) and assayed by sandwich ELISA for CXCL8, CXCL10, CCL2, and CCL5. Results presented are means \pm S.D. from two different experiments with duplicate wells. Significant differences were determined by ANOVA followed by a post hoc t-test. * significant difference ($p < 0.05$) versus the control; • significant difference ($p < 0.05$) between concentration at the apical chamber and the basal chamber.

Fig. 4. Chemokine receptor expression by primary HBEC detected by (a) semi-quantitative RT-PCR and (b) immunocytochemistry. cDNA for CCR1, CCR5, CXCR1, CXCR2 and CXCR3 was detected in primary HBEC but not that of CCR2 (+). Cyclophilin was used as positive control and to ensure equal cDNA loading for each sample. Negative controls (-) of samples incubated in the absence of RT were included. Results are representative of 2 experiments. (b) Immunofluorescence staining of primary HBEC for CCR5, CXCR1, CXCR2 and CXCR3 (Texas red; bottom). CXCR1 and CXCR3 showed a predominant surface staining extending over the cell body. CXCR2 and CCR5 showed predominantly vesicle staining. Scalebar = 20 μ m.

Fig. 5. Chemokine receptor expression by hCMEC/D3 cells under basal conditions and following stimulation by cytokines for 24 h. (a) Representative FACScan histograms show the expression of unstimulated hCMEC/D3 cells (black line) and hCMEC/D3 cells stimulated with 500ng/ml TNF α and 10ng/ml IFN γ for 24 h (grey line). hCMEC/D3 cells were fixed

with 4% PAF, permeabilised with triton X-100, labelled with FITC- or PE- labelled antibodies specific for CXCR1, CXCR2, CXCR3, CCR1, CCR2 and CCR5, and analysed by FAScan. Cells labelled with FITC- or PE- labelled isotope-matched antibodies are represented as grey filled histograms. The histograms are of one experiment representative of three. **(b)** Results are expressed as the mean fluorescence for chemokine receptor expression by unstimulated hCMEC/D3 cells (white) and hCMEC/D3 cells stimulated with 500ng/ml TNF α and 10ng/ml IFN γ (grey) for 24 h. Control values have been subtracted. Significance was tested on normalised values by ANOVA with a post hoc t-test (* p<0.05). Results presented are the mean \pm S.E.M. of three different experiments.

Fig. 6. Immunofluorescent detection of CXCR1 and CXCR3 in human NAWM and MS lesions. Fixed 50 μ m thick sections cut with a vibratome were permeabilised with triton X-100 and then processed for detection of either CXCR1 (b) or CXCR3 (a, c) (in red) and either von Willebrand factor (a), an endothelial cell marker, or GFAP (b,c), an astrocyte marker, (in green) by immunohistochemistry in NAWM (a, b) and in a chronic active MS lesion (c). Merged images show the localisation of CXCR1 and CXCR3 within the neurovascular unit. Results are representative of experiments from six donors. Scale bar= 40 μ m

Fig. 7. Electron micrographs of CXCR1 and CXCR3-labelled MS brain tissue. Fixed 50 μ m thick sections were processed for detection of CXCR1 and CXCR3 using silver-enhanced gold particles and visualized by electron microscopy. (A) Brain capillary endothelial cells and the perivascular end-feet of astrocytes show CXCR1-antigenic sites positively labelled with gold particles in NAWM. (B) In a chronic active lesion (MHC class II +), both brain capillary endothelial cells and pericytes are labelled for CXCR1. Inset represents dashed area at higher magnification showing positive immunogold labelling for CXCR1 on the luminal surface of brain endothelial cells. (C) Leukocytes interacting with brain endothelial cells within the lumen (asterisk) are highly positive for CXCR1 in a chronic active lesion (MHC class II +).

(D) In a silent lesion, CXCR3 is distributed mainly within the cytoplasm of endothelial cells (some within endosomes) but also on the luminal (asterisk) and abluminal membranes.

Abbreviations: AE=astrocytic end-feet; EC= endothelial cell; E= erythrocyte; L=leukocyte;

P=pericyte. Scale bar= 1 μ m

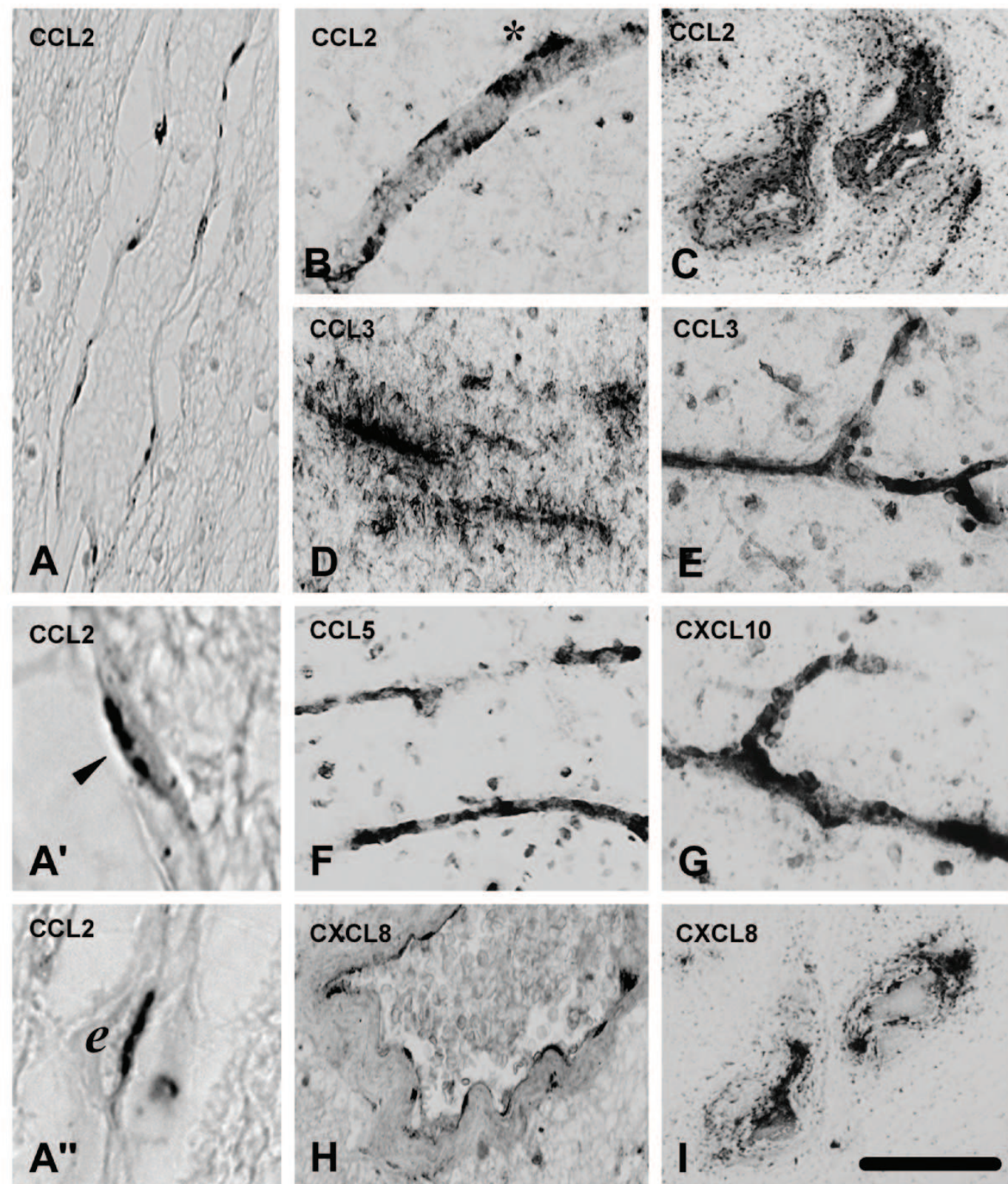
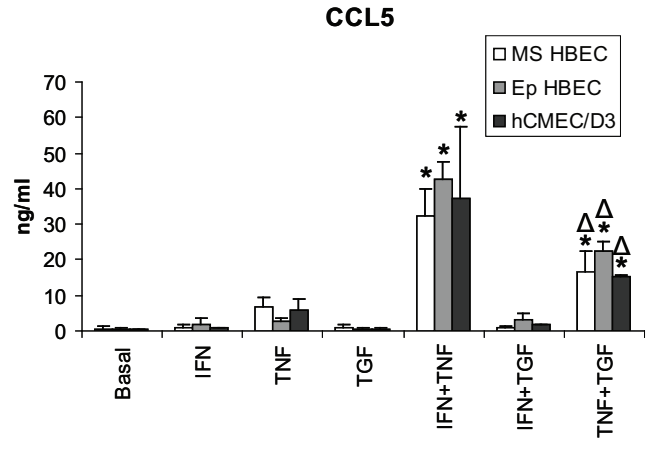
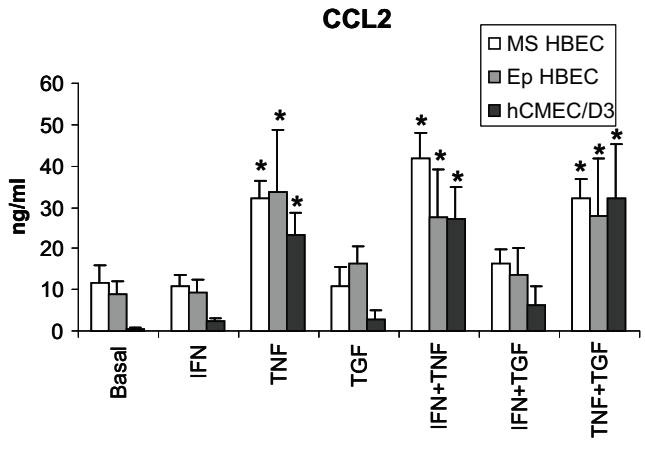
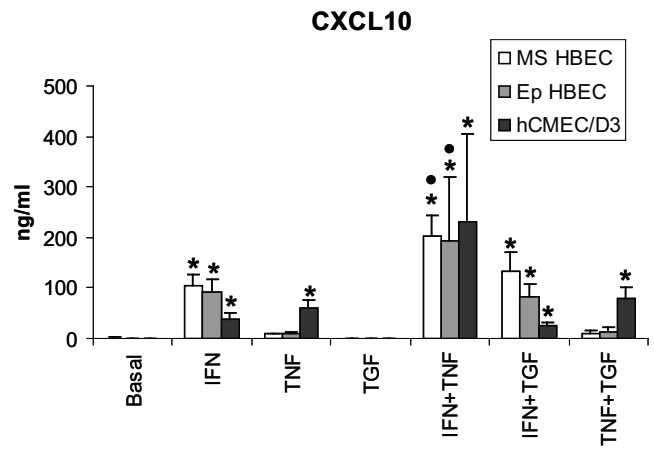
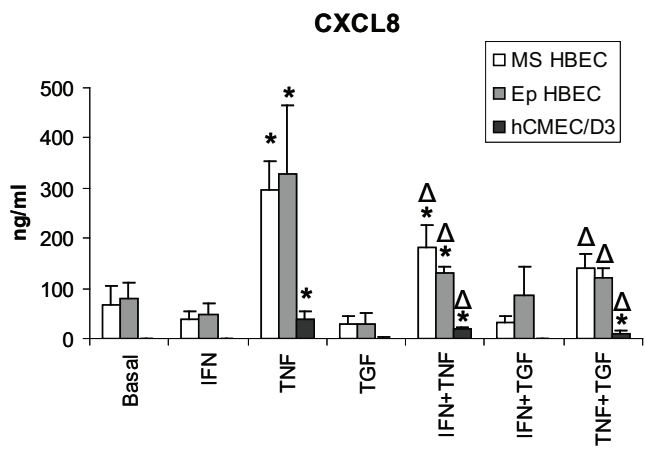
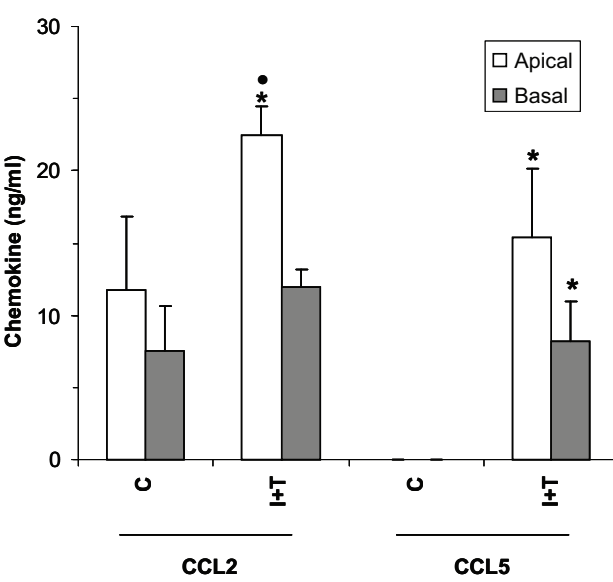
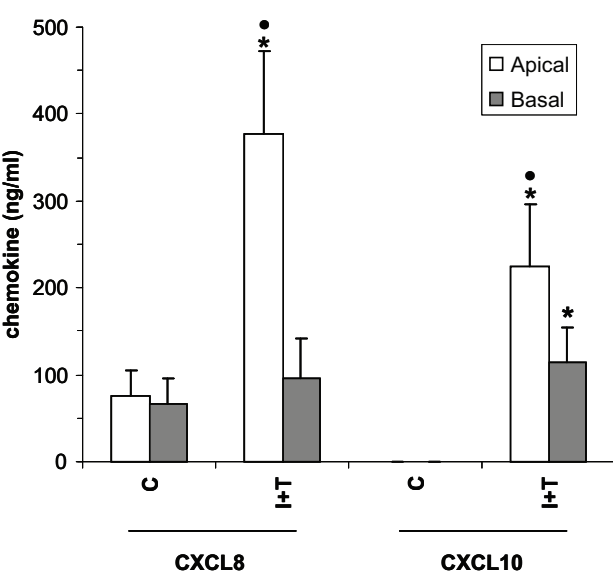
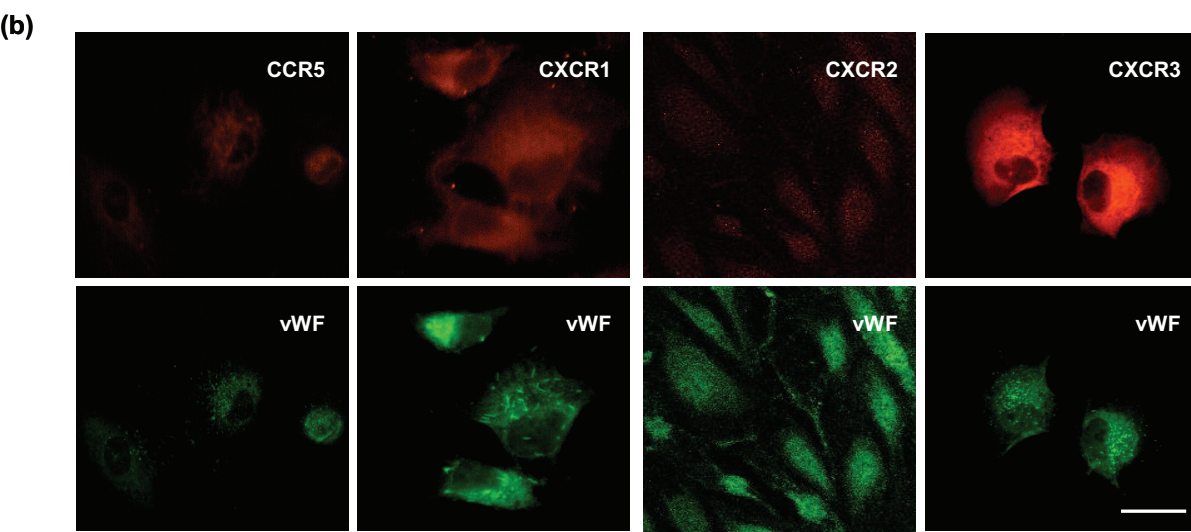
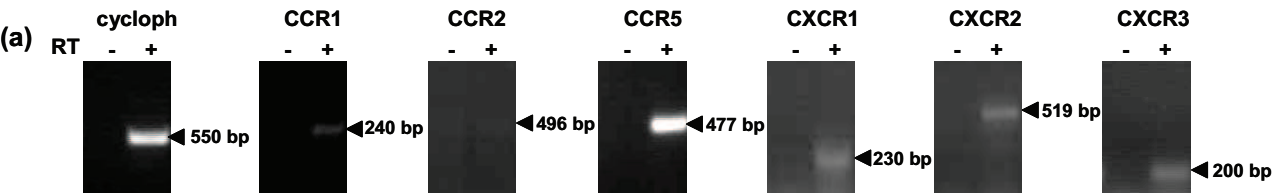


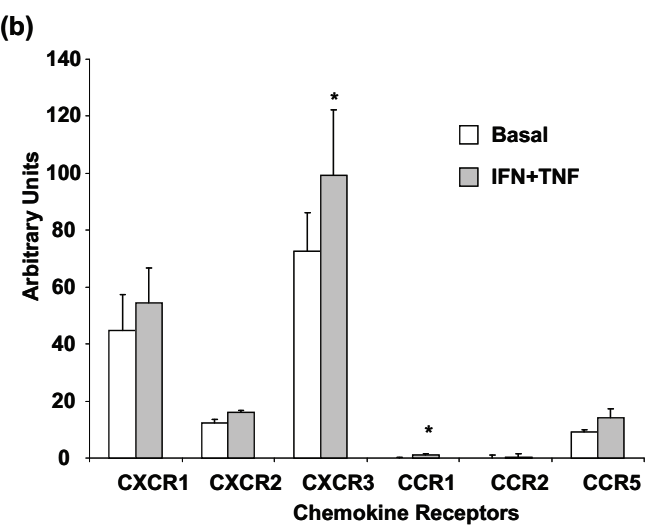
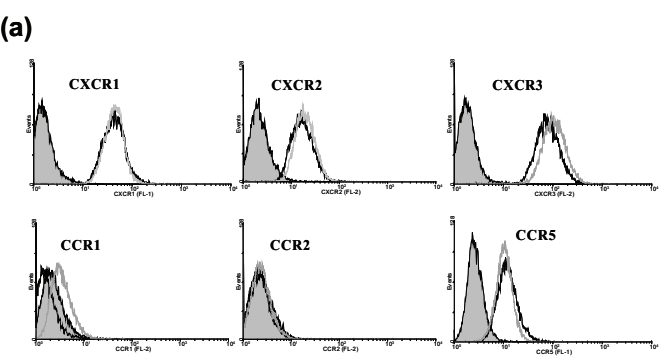
Figure 1

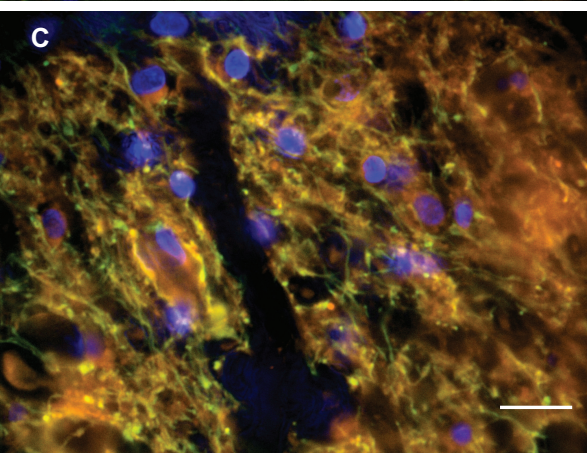
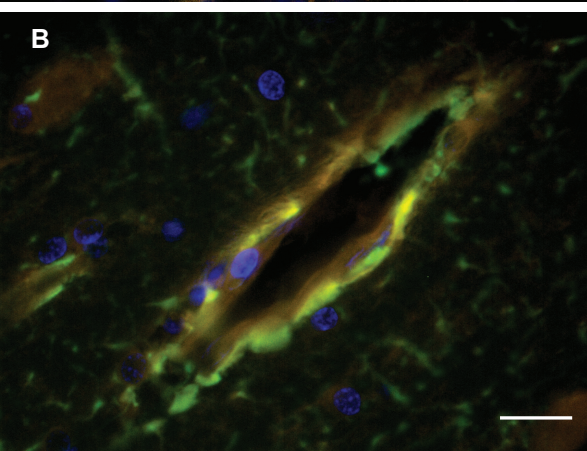
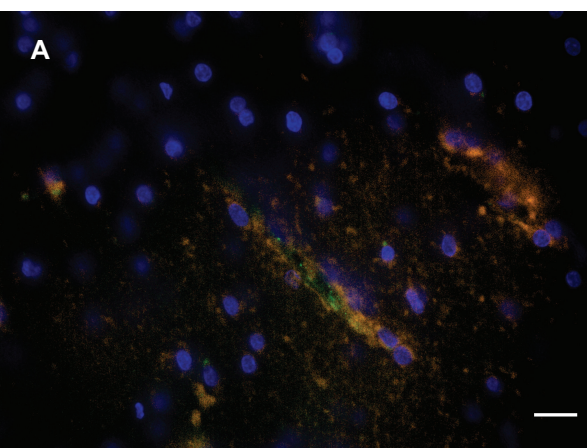
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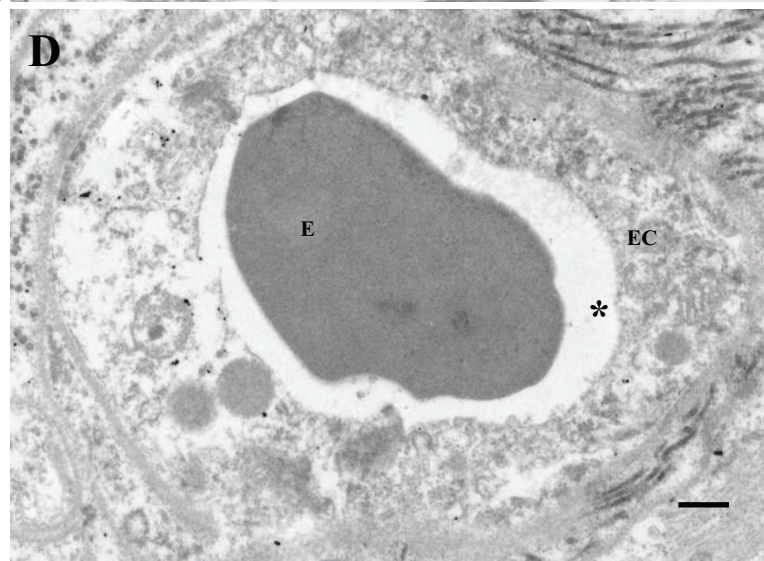
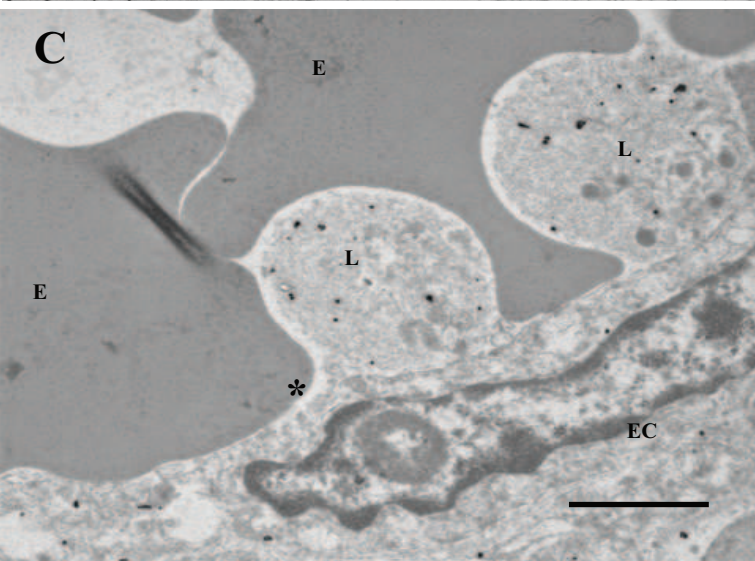
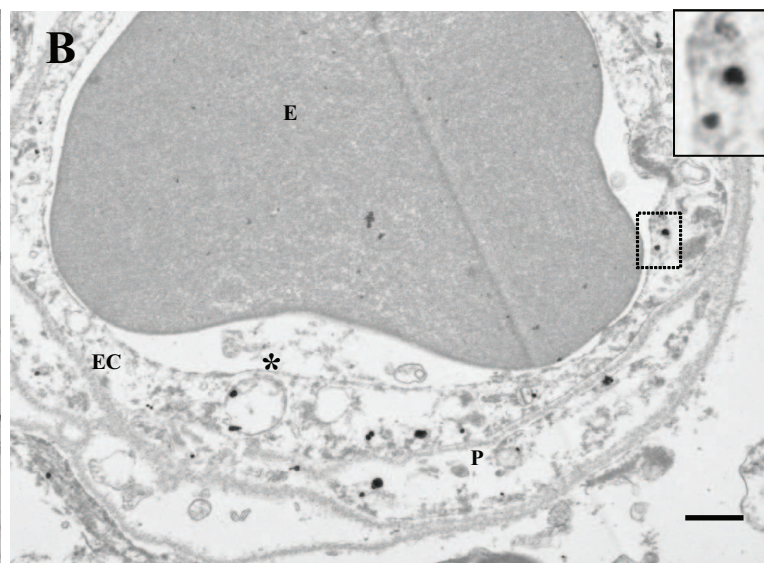
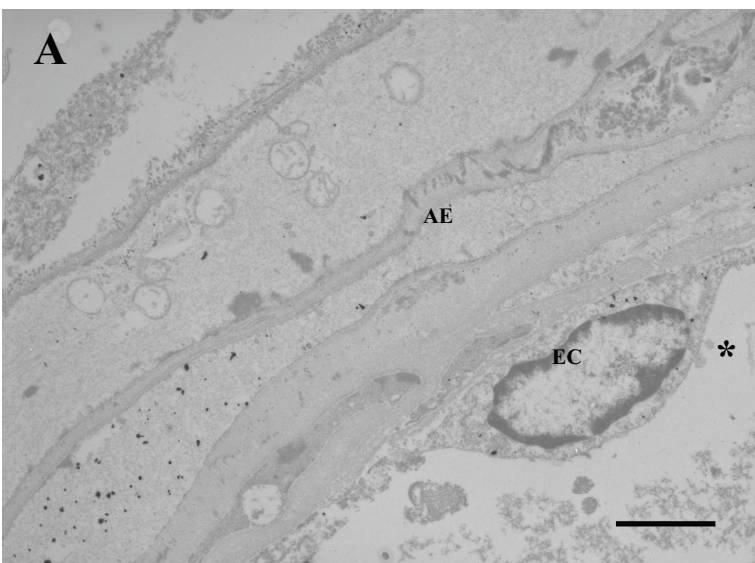












Case	Age (years)	Gender	Postmortem delay (h)	Cause of death	Lesion activity	Source
1†‡	34	Female	15	Pneumonia	Active and silent lesions	MSTB
2†‡	44	Female	36	Multiple sclerosis	Active and silent lesions	MSTB
3†‡	50	Female	13	Multiple sclerosis	Chronic active lesions	MSTB
4*	53	Male	<30	Septicaemia due to upper <u>respiratory</u> tract infection, multiple sclerosis	n.d.	MSTB
5*†	59	Female	32	Respiratory failure, cardiac arrest	NAWM	MSTB
6*	59	Female	<30	Pneumonia	n.d.	MSTB
7*†	70	Female	21	Aspiration pneumonia, sepsis, multiple sclerosis	Silent lesions	MSTB
8†	71	Female	36	Bronchopneumonia, multiple sclerosis	NAWM	MSTB
9*	92	Male	<30	“Old age”	n.d.	MSTB
10†	34	Female	32	Paracetamol overdose	Silent lesions	LBBND
11†	47	Male	7	Bilateral bronchopneumonia	Chronic active lesions	LBBND
12†	54	Female	32	Pulmonary embolus	Active and silent lesions	LBBND
13†	56	Female	6	Chronic MS	Silent lesions	LBBND

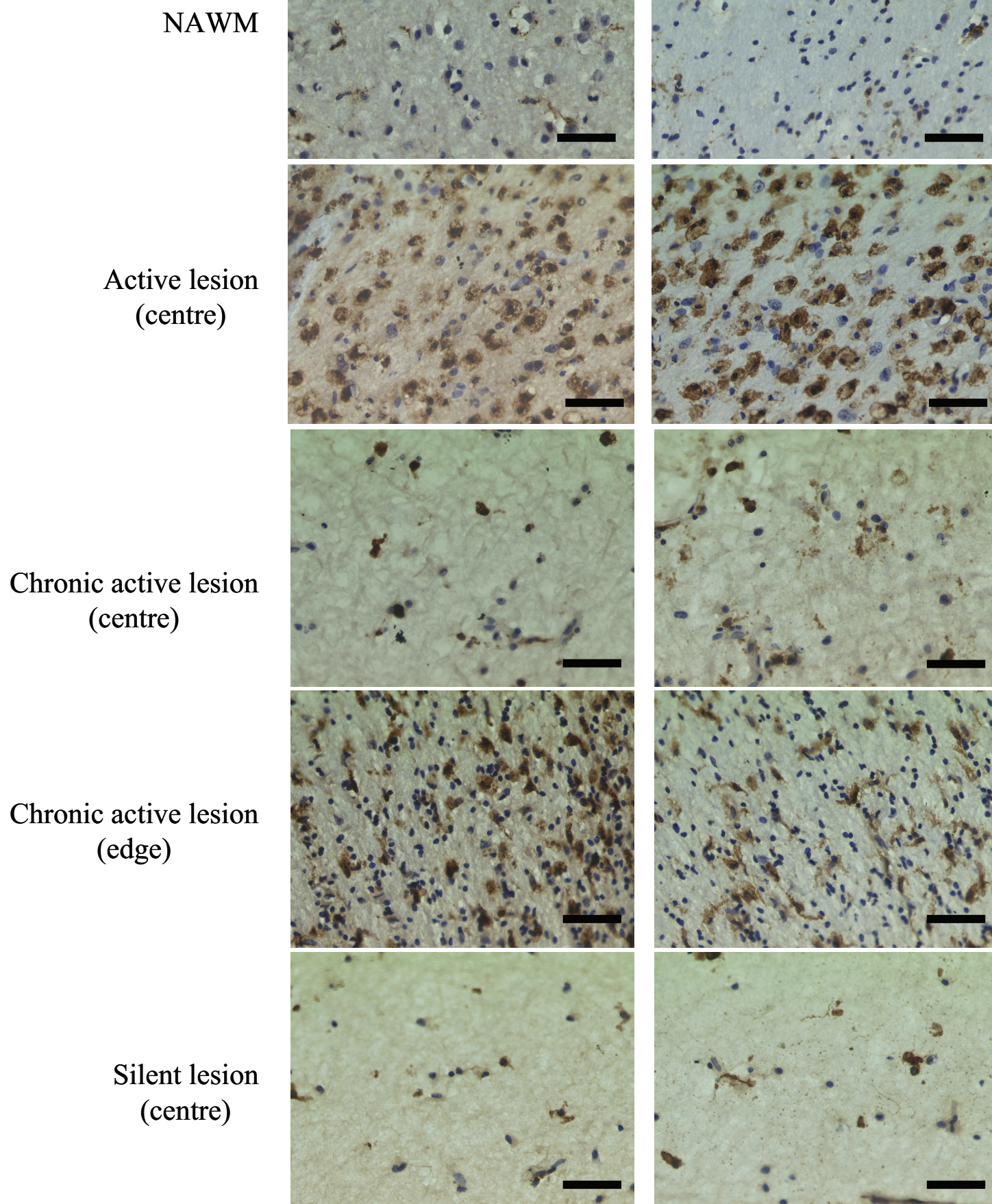
Table 1. Demographic details of cases diagnosed with multiple sclerosis, from which brain tissue was obtained. (MSTB) UK Multiple Sclerosis Tissue Bank; (LBBND) MRC London Brain Bank for Neurodegenerative Diseases; (n.d.) indicates ‘not determined’; (NAWM) indicates ‘normal appearing white matter’; (*) denotes cases from which tissue was used for primary culture of HBEC; (†) denotes cases from which tissue was used for immunohistochemical and immunofluorescence studies; (‡) denotes cases from which tissue was used for immuno-EM studies.

Table 2. Distribution of CXCR1 and CXCR3 antigenic sites in human brain capillary endothelial cells *in situ*[#]

	NAWM		Active Lesion (MHC class II +)		Silent Lesion (MHC class II -)	
	CXCR1	CXCR3	CXCR1	CXCR3	CXCR1	CXCR3
No. gold particles/ μm^2	0.63 \pm 0.13	0.79 \pm 0.22	0.54 \pm 0.13	0.84 \pm 0.22	0.56 \pm 0.14	0.32 \pm 0.06*
Luminal membrane (%)	8.9 \pm 1.9	14.5 \pm 4.1	9.8 \pm 2.7	16.5 \pm 6.8	19.7 \pm 7.9	12.3 \pm 4.3
Abluminal membrane (%)	3.6 \pm 1.9	20.6 \pm 6.4	3.6 \pm 1.7	4.5 \pm 1.9*	6.6 \pm 2.4	17.8 \pm 6.9
Cytoplasm (%)	87.4 \pm 2.8	64.9 \pm 7.4	86.6 \pm 3.8	79.0 \pm 6.9*	73.7 \pm 7.9	69.9 \pm 7.3

[#]corresponding to 16-19 capillaries from three cases for each tissue type.

* P <0.05 compared to NAWM. Results represent the mean \pm S.E.M.



Suppl. Fig. 1: Immunohistochemical detection of CD68 or MHC class II in NAWM and in MS active, chronic active and silent lesions counter stained with hematoxylin.

Fixed 50 mm thick sections cut with a vibratome were permeabilised with triton X-100 and then processed for detection of CD68 or MHC class II by immunohistochemistry. Sections were then counterstained with haematoxylin to visualise cell nuclei in NAWM and in MS lesions. Scale bar=50 mm