Mannose receptor expression specifically reveals perivascular macrophages in normal, injured and diseased mouse brain

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ABSTRACT

Perivascular macrophages are believed to have a significant role in inflammation in the central nervous system (CNS). They express a number of different receptors which point towards functions in both innate immunity, through pattern-associated antigen recognition, phagocytosis and cytokine responsiveness, and acquired immunity, through antigen presentation and co-stimulation. We are interested in the receptors which are differentially expressed by perivascular macrophages and microglia in both the normal CNS as well as in neuroinflammation and neurodegeneration. In this paper we report the use of a well characterized monoclonal antibody 5D3 to localize the expression of the mannose receptor to perivascular macrophages in both the normal CNS and in various models of brain pathology. Mannose receptor expression was limited to perivascular, meningeal and choroid plexus macrophages in normal, inflamed, injured and diseased CNS. In particular activated microglia and invading haematogenous leucocytes were mannose receptor negative while expressing the F4/80 antigen, macrosialin (CD68), FcRII (CD32), scavenger receptor (CD204) and CR3 (CD11b/CD18). Since the perivascular macrophages expressing the mannose receptor are known to be the only constitutively phagocytic cells in the normal CNS, we injected clodronate-loaded liposomes intracerebroventricularly in control mice to deplete these cells. In these mice there was no detectable mannose receptor expression in perivascular spaces after immunocytochemistry with the 5D3 monoclonal antibody. This underlines the value of the monoclonal antibody 5D3 as a tool to selectively study murine

perivascular macrophages. Mannose receptor expression by macrophages located at blood-brain (perivascular), brain-CSF (meningeal) and CSF-blood (choroid plexus) interfaces supports a functional role of these cells in responding to external stimuli such as infection.

INTRODUCTION

Cerebral perivascular macrophages (PVMs) are interesting cells by virtue of their location at the blood-brain barrier (BBB). They are situated between the endothelial basement membrane and the glia limitans (Graeber et al., 1992). They are the only cells in the brain parenchyma which display constitutive phagocytic potential (Kida et al., 1993) and immunophenotypical markers of activation such as MHC class II, B7, CD40 and FcR (Williams et al., 2001). They are the first cells to respond to inflammation within the brain in various experimental settings such as EAE (Hickey et al., 1987), neuronal damage (Streit et al., 1989) and intracerebroventricular injection of IFN-γ or TNF-α (Lassmann et al., 1991). They also readily respond to peripheral immune activation after systemic lipopolysaccharide administration (Breder et al., 1994; Elmquist et al., 1997; Herkenham et al., 1998; Schiltz and Sawchenko, 2002). Moreover, they have been shown to be capable of effective antigen presentation (Ford et al., 1995; Hickey and Kimura, 1988). Thus they are ideally situated to respond to pro-inflammatory stimuli arising from both within the brain and from the periphery.

PVMs have mostly been studied in the rat because of the availability of the monoclonal antibody ED2, which recognizes CD163. CD163 is a member of the scavenger receptor Type B family and has several functions: haemoglobin scavenging (Kristiansen et al., 2001), cell adhesion (Barbe et al., 1996; Wenzel et al., 1996), and perhaps an anti-inflammatory role (Hogger and Sorg, 2001). In the rat central nervous system (CNS), ED2 selectively labels perivascular, meningeal and

choroid plexus macrophages in both normal and inflamed CNS (Dijkstra et al., 1985). Recently a monoclonal antibody (EDHu-1) recognizing human CD163 and thus perivascular macrophages, has become available (Kristiansen et al., 2001). Studying PVM biology in the mouse has been hampered by the lack of availability of such a selective marker. Although murine CD163 has been cloned (Schaer et al., 2001), an anti-mouse CD163 antibody is still lacking. The monoclonal antibody 2F8 recognizes the murine scavenger receptor A which is limited to PVMs in the normal brain (Mato et al., 1996). However this receptor is expressed by microglia during different forms of injury (Bell et al., 1994), making it hard to study PVMs selectively in pathological conditions.

We have systematically studied a number of monoclonal antibodies to mouse macrophage antigens in order to further characterize the murine PVM's expression profile and thus gain more insight into its function. One of these antibodies is the monoclonal antibody 5D3 which recognizes the mannose receptor (MR). The MR is a Type I transmembrane C-type lectin which best recognizes branched mannose-containing carbohydrate structures on both microbial and host proteins (Linehan et al., 2000a). Its extracellular portion includes 8 carbohydrate recognition domains (CRDs) and an N-terminal cysteine-rich domain (CR), which display separate lectin activities. It is expressed by tissue macrophages, hepatic and lymphatic endothelium, glomerular mesangial cells and "perivascular microglia" in the normal brain (Linehan et al., 1999). Its functions include receptor-mediated endocytosis and phagocytosis, modulation of microbicidal potential, cell adhesion, stimulation of cytokine secretion,

targeting of antigen to MHC Class II pathway and antigen trafficking in lymphoid organs (Apostolopoulos and McKenzie, 2001).

We have investigated MR expression in the normal CNS as well as in several models of pathology. We were especially interested to determine whether the MR is still selectively expressed on PVMs in these models. Our study clearly shows that the MR is exclusively expressed by perivascular macrophages (PVMs), meningeal macrophages (MMs) and choroid plexus macrophages (CPMs) in the normal CNS, as is the case with ED2 in the rat. Moreover its expression remains limited to these macrophages even under conditions of acute and chronic neurodegeneration and following an inflammatory challenge. Mannose receptor expression by macrophages situated at blood-brain (perivascular), brain-CSF (meningeal) and CSF-blood (choroid plexus) interfaces supports a functional role of these cells in responding to external stimuli such as infection.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, obtained from Harlan-Orlac (Bicester, UK), were 4-6 weeks old on arrival and were group-housed under a 12h light:12h dark schedule. Mice were maintained under controlled environmental conditions with pelleted food and water *ad libitum*. The experiments were carried out under Home Office Licence and in accordance with the Animals (Scientific Procedures) Act, 1986. All efforts were made to minimize both the suffering and the number of animals used.

Preparation of multilamellar liposomes

Multilamellar mannosylated liposomes were prepared as described before (van Rooijen and Sanders, 1994). Briefly, 178mg phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and 27mg cholesterol (Sigma Chemicals) were dissolved in 8ml chloroform which was added to 9.25mg *p*-amino-phenyl-α-D-mannopyranoside (Sigma Chemicals) dissolved in 5ml methanol in a 500ml round-bottom flask. This was dried *in vacuo* on a rotary evaporator to form a film. The molar ratio of phosphatidylcholine / cholesterol / mannoside was 7:2:1. The lipid film was dispersed in 10ml of phosphate buffered saline (PBS, 0.15M NaCl in 10mM phosphate buffer, pH 7.4) for the preparation of PBS-containing mannosylated liposomes. To enclose the clodronate, 2.5g (a gift of Roche Diagnostics, Mannheim, Germany) was dissolved in 10ml milliQ (set at pH 7.3 with NaOH) in which the lipid film was

dispersed and the preparations were kept for 2h at room temperature, sonicated for 3min, washed and resuspended in 10ml of PBS. The size of the liposomes ranged from 0.2 to 3μm.

Intraventricular liposome infusion

Mice were deeply anaesthetized with isoflurane in O_2 and mounted on a stereotaxic frame. In a first experiment the published protocol for PVM depletion in the rat (Polfliet et al., 2001a) was adapted to the mouse on a per brain weight basis. Thus 8μl of clodronate or PBS-containing mannosylated liposomes were infused into the 4^{th} ventricle of 4 mice over 25min using a Hamilton syringe fitted with a 27G needle. This preliminary study resulted in suboptimal depletion despite correct localization of the injection being confirmed by serial sectioning of the brain around the injection site. The protocol was therefore modified. The liposome dose was doubled and injections were made rostrally in the lateral ventricles in order to facilitate access of the liposomes to the whole brain parenchyma. Thus, in a second experiment, 8μl of clodronate or PBS-containing mannosylated liposomes were infused into *each* lateral ventricle of 3 mice slowly over 12.5min using a pulled glass capillary (co-ordinates: -0.22mm, lateral 1mm and depth 2mm). On each side, the capillary was left *in situ* for 2.5min, withdrawn partially and left for a further 2.5min before being removed completely, to avoid reflux of liposomes along the injection tract. The operated mice were sacrificed 5 days later.

Controls

Three naïve mice were used as controls for the brain pathology models, except in the case of the Wallerian degeneration model, where the contralateral left unoperated optic nerve was used as control.

Brain pathology models

Experiments were carried out as described previously (Andersson et al., 1991; Andersson et al., 1992; Betmouni et al., 1996; Lawson et al., 1994). All surgery was performed on anaesthetised animals with Avertin (2,2,2-tribromoethanol in tertiary amyl alcohol) at a dose of 0.1ml/5g body weight. With the exception of the Wallerian degeneration model, all the mice were injected stereotaxically in the dorsal hippocampus (co-ordinates: bregma –1.94mm, lateral 1.5mm and depth -1.37mm). A pulled glass micropipette was used for lipopolysaccharide (LPS) and kainic acid (KA) injection, and a Hamilton syringe with a blunt 26S needle for the ME7 prion brain homogenate injection. For all animal models, the mice were allowed to recover under a warm lamp and then housed in IVC racks (Techniplast UK, Kettering, UK) following surgery. All the mice were sacrificed by deep anaesthesia with 20% sodium pentobarbital (Sagatal, Rhone Merieux Ltd., Harlow, Essex, UK).

Acute inflammatory model. Three animals were injected with 1µl of LPS (2µg/µl) (*Salmonella abortus equi*, Lot No 69F4003, Sigma Chemicals, Poole, UK) in the right dorsal hippocampus and sacrificed 24h later (Andersson et al., 1992).

Acute neurodegeneration model. Three mice were injected with 1µl of KA (1nM) (Sigma Chemicals, Poole, UK) in the right dorsal hippocampus and sacrificed three days later (Andersson et al., 1991).

Chronic neurodegeneration model. Mice were bilaterally injected in the dorsal hippocampus with 1µl of ME7 prion brain homogenate using a Hamilton syringe fitted with a blunt 26S needle (Betmouni et al., 1996). The syringe was left *in situ* for an additional 1min to prevent reflux along the injection tract. The ME7 prion brain homogenate was derived from the brains of C57BL/6J mice at 22 weeks old post-inoculation showing clinical signs of disease (10% w/v in sterile PBS). Mice were sacrificed at the terminal stage of the disease (21 weeks, n=3).

Wallerian degeneration model. The mouse right optic nerve was crushed intraorbitally under an operating microscope with jeweller forceps for 10sec (ONC) (Lawson et al., 1994). Mice were killed at 7 and 28 days after the injury (n=3 at each time point).

Tissue processing and immunohistochemistry

Animals were transcardially perfused with 0.9% w/v heparinized saline under terminal anaesthesia with 20% sodium pentobarbital. Brain tissue was rapidly dissected, frozen in Tissue-Tek OCT embedding compound (Sakura Finetek Europe B.V, Zoeterwoude, NL) and stored at -20ºC until use. Coronal sections of 10µm thickness were cut on a cryostat, dried, fixed in absolute alcohol for 10min at 4ºC and

processed for indirect immunohistochemistry. All incubations were carried out at room temperature. The sections were first pre-adsorbed with 10% normal rabbit serum for 30min and then incubated for 2h with rat monoclonal anti-mouse antibodies against the following antigens: mannose receptor (clone 5D3), CD68 (clone FA11), CD11b (clone 5C6), CD204 (clone 2F8), CD3 (clone KT3) or MHC Class II (clone TIB120). After washing in 0.1M phosphate-buffered saline, sections were incubated with biotinylated rabbit anti-rat IgG secondary antibody for 30min, washed again and then incubated with avidin-biotin-peroxidase complex (Vectastain Elite ABC) for 30min. After another wash, the peroxidase was visualized using 0.05% 3,3'-diaminobenzidine (DAB) as chromogen and 0.05% hydrogen peroxide as substrate. All the sections were counterstained with haematoxylin and dehydrated before mounting in DePeX (BDH Laboratory supplies, Poole, UK). Negative control sections were incubated in the absence of the primary antibody. Normal rabbit serum, biotinylated secondary antibody, and avidin-biotin-peroxidase complex were purchased from Vector Laboratories (Peterborough, UK). The antibodies from clones 5D3, 2F8, 5C6 and TIB120 were kindly provided by Professor S Gordon (Sir William Dunn School of Pathology, Oxford, UK); FA11 and KT3 was purchased from Serotec Ltd (Kidlington, UK). The immunocytochemistry protocol for the anti-MR antibody (5D3) was also investigated on brains fixed in 10% neutral buffered formalin, Bouin's solution, 4% paraformaldehyde and periodate-lysine-paraformaldehyde (PLP). Positive staining was achieved on formalin-fixed sections only if pepsin (pepsin 0.04% in 0.1M HCl, 10min) or microwave (3min in citrate buffer, cool for 5min, re-microwave 3min in citrate buffer) antigen retrieval steps

were included, the former giving the better results. No positive staining was seen in Bouins' solution, 4% paraformaldehyde or PLP-fixed tissue.

For double immunofluorescence, brain sections were first blocked with 10% rabbit serum and then incubated overnight with mannose receptor antibody (1:50) followed by biotinylated rabbit anti-rat IgG for 45min and streptavidin-conjugated AF546 (Molecular Probes, Cambridge Bioscience, Cambridge, UK) for 30min. During the second round, sections were blocked with 10% goat serum, incubated with a rat monoclonal antibody against the γ1 chain of mouse laminin (Neomarkers, Freemont, CA, USA) (1:250) for 90min, followed by AF488-conjugated goat anti-rat IgG (Molecular Probes) for 30min. Sections were mounted in Mowiol (Harlow Chemical, Harlow, UK) and visualized with a LSM 510 Meta confocal laser scanning microscope (Carl Zeiss Ltd, Germany). Images were examined with the Zeiss LSM 5 Image Examiner software.

RESULTS

Control animals

Sections from the brains of three naïve C57BL/6J mice and from the intact contralateral optic nerves of mice with a right ONC were incubated with the anti-MR antibody. Three populations of macrophages were clearly stained: PVMs, meningeal macrophages (MMs) and choroid plexus macrophages (CPMs), reminiscent of ED2 staining in the rat. PVMs were seen exclusively around vessels and located abluminal to endothelial cells and separate from the surrounding brain parenchyma (Figure 1). Double immunofluorescence showed MR-positive cells sandwiched between the γ1-laminin-chain-positive endothelial and glial basement membranes (Figure 2). The cells had stout cell bodies and bore elongated flattened processes which both encircled and ran longitudinally along the vessels. They were particularly noticeable at bifurcations/trifurcations. Few cells in the white matter tracts such as the corpus callosum and the normal optic nerve were revealed by the anti-MR antibody (data not shown).

Perivascular and meningeal macrophages, which were considered to express the MR in the above experiment, are known to be the only constitutively phagocytic cells in the normal CNS (Kida et al., 1993). Indeed this is in keeping with MR expression since one of the MR's described functions is receptor-mediated endocytosis (Apostolopoulos and McKenzie, 2001). Moreover the MR-positive cells were located at sites (perivascular spaces and meninges) which are readily accessible

to intracerebroventricularly injected substances. This is reminiscent of ED2 positive macrophages in the rat brain, which have been shown to be selectively depleted after intracerebroventricular injection of clodronate-loaded liposomes (Polfliet et al., 2001a). Liposomes are phagocytosed and intracellular accumulation of clodronate is toxic by forming a non-hydrolyzable analogue of ATP (Russell and Rogers, 1999) and triggering apoptosis (van Rooijen et al., 1996). We therefore injected clodronate-loaded liposomes intracerebroventricularly in control mice to interrogate the function of MR-positive cells and verify their identity as PVMs. In these mice, no mannose receptor could be detected in perivascular spaces after immunocytochemistry with the 5D3 monoclonal antibody (Figure 1). Sections of brains from naïve or PBS-containing liposome injected mice revealed MR positivity during the same immunohistochemistry experiment. This showed that the MR expressing cells were constitutively phagocytic which confirms their identity as PVMs.

Meningeal macrophages were also depleted (Figure 1) though not completely. This is in sharp contrast with the rat, where complete depletion of meningeal macrophages is achievable with 5-fold lower doses (on a per brain weight basis) of intracerebroventricular clodronate-loaded liposomes (Polfliet et al., 2001a). This might be due to a smaller subarachnoid space in the mouse and thus a more restricted flow of CSF over the brain surface, effecting liposomal delivery to the meninges. Alternatively it could mean that the mannose receptor is expressed by other cells in the meninges besides macrophages.

Choroid plexus macrophages were the least affected by the intracerebroventricular injection, similar to the rat. This is because tight junctions are

expressed in the ependyma of the choroid plexus (Oldfield and McKinley, 1995). This impedes the access of intracerebroventricularly injected liposomes to choroid plexus macrophages. Indeed DiI-labelled liposomes also fail to access circumventricular organ tissue when injected intracerebroventricularly in the rat (data not shown).

The immunocytochemistry protocol used in this study revealed the MR equally well in C57BL/6J and BALB/c mice.

Acute inflammatory model

To model an acute inflammatory injury without cell death, an intracerebral injection of 2μg of LPS was made into the right dorsal hippocampus of 3 mice (Andersson et al., 1992). The animals were sacrificed 24 hours later. At this time point, neutrophil margination to cerebral endothelium and microglial activation is observed in the brain (Andersson et al., 1992). The sections were stained with the anti-MR antibody. Only the PVMs were revealed by the antibody, in both the injected and uninjected hemispheres (Figure 3). While widespread upregulation of scavenger receptor, CD68, CR3 and MHC Class II on microglia was seen in this model (data not shown) in agreement with previous results (Bell et al., 1994), MR expression was confined to PVMs, MMs and CPMs; it was not upregulated on microglia or other cells.

Excitotoxic model

Acute cerebral inflammation associated with neuronal death was modeled by intracerebral injection of the excitotoxin KA (1nM) in the right dorsal hippocampus. Mice were sacrificed 3 days later. Little or no neutrophil recruitment is usually observed in these pathological conditions whereas microglia are activated. (Andersson et al., 1991). The MR antibody still selectively and exclusively labeled PVMs around the blood vessels (Figure 3), MMs and CPMs, with more PVMs in the ipsilateral side compared to the contralateral one. There was no detectable microglial expression of the MR, but microglia did express scavenger receptor, CD68, CR3, and MHC Class II (data not shown) in agreement with previous results (Andersson et al., 1991; Bell et al., 1994).

Chronic neurodegeneration model

Bilateral injection of ME7 prion brain homogenate was performed in the mouse dorsal hippocampus. This induces chronic neurodegeneration accompanied by an atypical inflammation which contrasts with the acute inflammation accompanying the above-mentioned two models (Perry et al., 2002). Three animals were killed during the late stage of the disease, at 21 weeks. At this time point, marked microglial and astrocytic activation as well as T cell infiltration has been described (Betmouni et al., 1996) and indeed upregulation of CR3 and CD68 was seen (Figure 4). Once again, MR expression was limited to PVMs (Figure 4), MMs and CPMs: it was upregulated on PVMs and they appeared to be more numerous. The strict perivascular distribution

of the MR was incompatible with expression by CD68-positive microglia or CD3-positive T cells which were found in the parenchyma (Figure 4).

Wallerian degeneration model

The mouse right optic nerve was crushed intraorbitally. The animals were killed at 7 and 28 days. It has been shown that microglia in the degenerating distal optic nerve segment are activated as evidenced by F4/80 glycoprotein and CD68 expression, with a maximum at day 7 (Lawson et al., 1994). This was confirmed in this study since CR3 and CD68 were upregulated. However the MR antibody still only revealed PVMs and MMs; the former were few in number (Figure 3).

DISCUSSION

Our results are in agreement with a previous study showing restricted perivascular expression of the MR in the healthy adult CNS using immunocytochemistry and *in situ* hydridization (Linehan et al., 1999). The only other study of MR expression in the brain (Burudi and Regnier-Vigouroux, 2001) found a more diverse expression of the MR including astrocytes and neurons using immunocytochemistry. However this group used a different antibody (a rabbit polyclonal raised against a 22-mer from the cytoplasmic domain of MR) while 5D3, used in this study, is a mouse monoclonal antibody raised against a much larger fragment of the MR spanning carbohydrate recognition domains 4-7 (Zamze et al., 2002). The antibody 5D3 has been thoroughly characterized using immunocytochemistry, immunoprecipitation, Western blot, enzyme-liked immunosorbent assay and fluorescence-activated cell sorting (Martinez-Pomares et al., 2003; Zamze et al., 2002).

We have set out to further explore the phagocytic capacity of MR-positive cells in the brain by injecting clodronate-loaded liposomes intracerebroventricularly – these are lethal to cells which ingest them. We show that MR-positive cells possess constitutive phagocytic potential since they are depleted. This also confirms their perivascular location since the intraventricularly injected liposomes could reach these cells. Their precise location within the perivascular space was established using confocal microscopy – they lie between the endothelial and glial basement membranes. This establishes the identity of the MR-positive cells as perivascular macrophages.

Our liposomes were mannosylated and one may argue that they would therefore target any cell which expresses mannose-binding molecules, including the MR. However it is quite clear that constitutive robust phagocytic ability is a prerequisite for ingestion of these multilamellar liposomes: they are large (size ranges from 0.2 to 3 μm), they have been shown to be only taken up by perivascular macrophages in the rat (Polfliet et al., 2001a), and the non-phagocytic ependymal epithelium, which expresses cerebellar soluble lectin (CSL), a mannose binding lectin (Kuchler et al., 1994), is not effected by this treatment (data not shown).

In the brain parenchyma it was noted that PVMs occur predominantly in grey matter: the corpus callosum had very few PVMs for instance. It is thus no surprise that no MR staining was observed in naïve optic nerve. The same has been noted with regard to ED2-positive PVMs in the rat (data not shown). This might be related to capillary density which is *circa* 4-fold higher in grey matter as opposed to white matter (Cavaglia et al., 2001).

We have used a spectrum of models to study several types of brain inflammation: acute inflammation accompanying excitotoxicity (kainic acid) (Andersson et al., 1991), acute inflammation associated with the presence of endotoxin (lipopolysaccharide) (Andersson et al., 1992), chronic neurodegeneration (ME7 prion disease) (Betmouni et al., 1996) and chronic inflammation following Wallerian degeneration (optic nerve crush) (Lawson et al., 1994). These models are characterized by different cytokine profiles (Blond et al., 2002; Boche et al., 2003;

Cunningham et al., 2002; Palin et al., 2004) yet this was not reflected in the distribution of MR expression. In all models, MR expression was restricted to PVMs (as well as meningeal and choriod plexus macrophages), with no staining of either microglia or infiltrating haematogenous leucocytes in the parenchyma. Thus the identification of the MR as a PVM marker supports the notion that PVMs do not migrate into the parenchyma during inflammation, an issue which has been brought into question previously (Angelov et al., 1996). The possibility still remains that they migrate but lose MR expression. Also, the availability of such a marker in the mouse which retains its selectivity during brain pathology provides the opportunity to study this macrophage population in a species which is more amenable to genetic manipulation.

The mannose receptor has various functions which are of interest with regard to its expression by PVMs. The first is the recognition of various microbial ligands (from *Mycobacterium tuberculosis, Candida albicans, Pneumocystis carinii, Klebsiella pneumoniae, Streptococcus pneumoniae*) resulting in their receptor-mediated phagocytosis and enhanced microbicidal activity (Linehan et al., 2000a). Interestingly most of these pathogens are known to infect the CNS. Perivascular and meningeal macrophages, with the MR as a member of their broad armamentarium, are ideally situated to prevent infectious organisms from gaining access to the brain parenchyma. Indeed their depletion using liposomes resulted in a worsening of disease in a *Streptococcus pneumoniae* model of meningitis in the rat (Polfliet et al., 2001b).

The second identified function of the MR is recognition and clearance of endogenous ligands, of which several have been identified (Linehan et al., 2000b). One of these is tissue plasminogen activator (tPA): the MR serves in its clearance by receptor-mediated endocytosis (Smedsrod et al., 1988). This function would be expected to be protective in acute inflammation accompanying excitotoxicity or focal ischaemia, since tPA is a key player in tissue destruction in this setting via activation of plasmin (Tsirka et al., 1997; Wang et al., 1998).

The third role of the MR is adhesion. Binding between the MR on lymphatic endothelium and L-selectin on T cells was found to be important in the adherence of T cells to the endothelium (Irjala et al., 2001). The MR is not expressed on circulating rodent monocytes, so it must be expressed once the monocyte enters the perivascular space. It is tempting to suggest that it is important in tethering PVMs in the perivascular space. Interestingly brain microvessels display binding sites for the carbohydrate recognition domains 4-7 on the extracellular portion of the MR (Linehan et al., 2001). The MR knockout mouse should help in identifying any such role in adhesion (Lee et al., 2002).

A fourth hypothesized role of the MR is antigen transport. Ligand binding sites for the cysteine-rich domain of the MR have been detected in developing germinal centers in spleen and have been followed on dendritic-like cells migrating from the subcapsular area of lymph nodes into follicular areas (Martinez-Pomares et al., 1996). It has thus been suggested that the MR directs antigen (bound by the carbohydrate recognition domains) towards sites of developing clonal immune responses (by the cysteine-rich domain). It would be interesting to see whether such

migration is seen in cervical lymph nodes following brain inflammation. A fully functional soluble form of MR is generated by shedding of cellular MR by metalloprotease activiy (Martinez-Pomares et al., 1998) and a similar antigen transport potential has been suggested for soluble MR. Such a scenario is possible during CNS inflammation since interstitial fluid from the perivascular space is known to drain directly into nasal lymphatics and thus cervical lymph nodes through channels in the cribriform plate (Weller et al., 1996).

CD163, a member of the scavenger receptor Type B superfamily, is a selective PVM marker in rat (Dijkstra et al., 1985) and human CNS (personal communication by Babs Fabriek). Several similarities in function between CD163 and the MR are thus apparent. CD163 is similarly restricted to the perivascular macrophage in both normal and diseased brain parenchyma, plays a role in cell adhesion (Barbe et al., 1996; Wenzel et al., 1996) and is shed from the cell surface in a soluble form by metalloprotease activity (Hintz et al., 2002).

In summary, we have shown that murine PVMs express the mannose receptor, which is involved in receptor-mediated phagocytosis, and that they are constitutively phagocytic. The mannose receptor is also expressed by MMs and CPMs, but not by microglia. Mannose receptor expression by macrophages located at blood-brain (perivascular), brain-CSF (meningeal) and CSF-blood (choroid plexus) interfaces supports a functional role of these cells in immunosurveillance of the brain's external environment. We have further shown that mannose receptor expression remains restricted to these subsets of macrophages in various models of brain pathology.

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LEGENDS

Figure 1: Mannose receptor expression in naïve brain

Representative brain sections from mice injected intracerebroventricularly with clodronate-loaded (C, D) or PBS-loaded liposomes (A, B), showing complete depletion of meningeal (C) and perivascular (D) macrophages. The sections were stained with the anti-mannose receptor antibody 5D3 and counterstained with haematoxylin. (A) and (C) show the meninges overlying the cortex; (B) and (D) show the cerebral parenchyma. Scale bar = 20μm

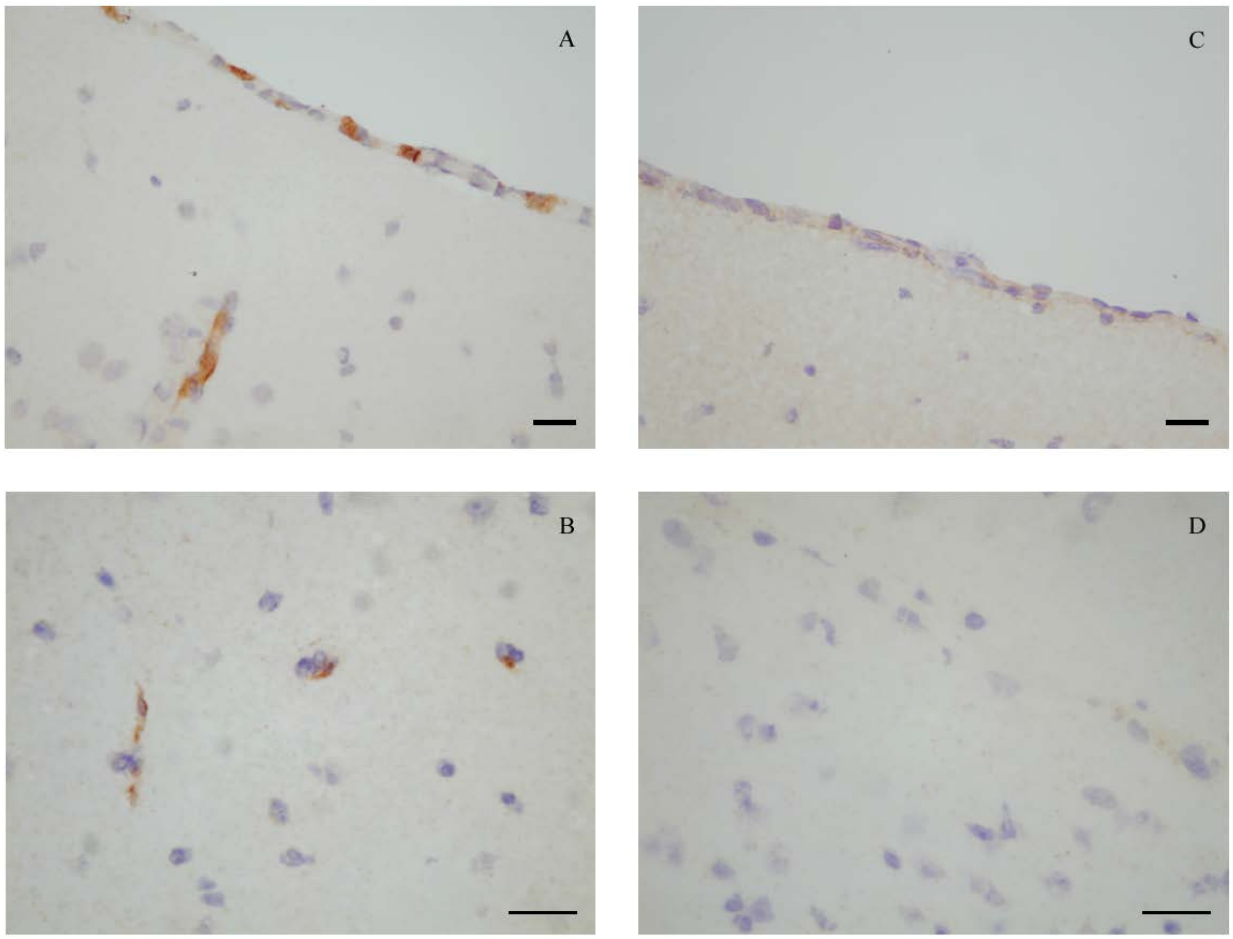


Figure 2: Localization of the PVM in the perivascular space by confocal microscopy. Confocal image from a naïve brain section double stained for the MR (red) and the γ1-laminin chain (green), which labels both glial and endothelial basement membranes. The image, 0.28μm in thickness, shows a vessel trifurcation with a PVM (red) characteristically located in the angle between two of the vessels (green) (A). The image was studied using the Zeiss LSM V Image Examiner software, which shows the PVM to be situated between the endothelial (*) and glial (o) basement membranes (B-C). Image B is an orthogonal view of the PVM in image A, taken at the level of the green line. Images C-D are two other examples. Yellow colour represents areas of adhesion between the PVM and adjacent basement membrane. L=lumen.

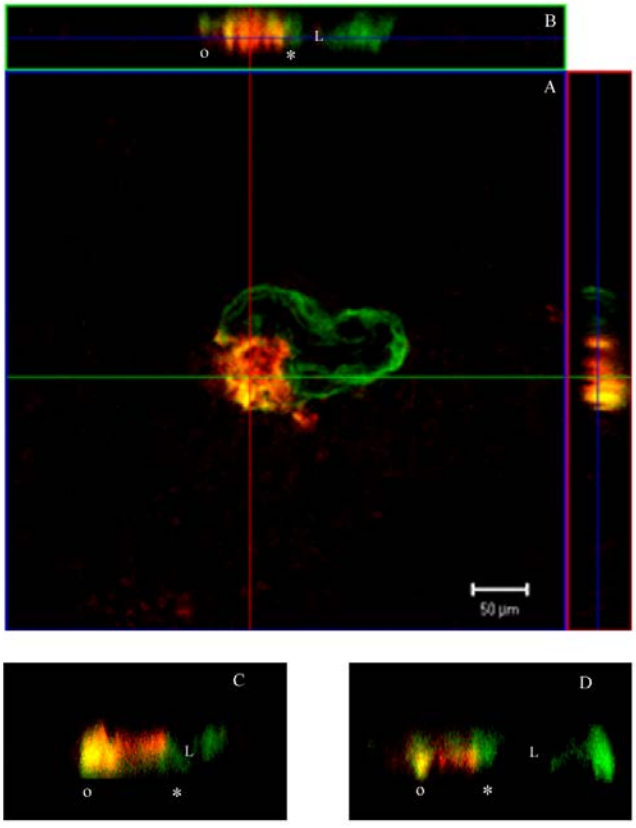


Figure 3: Perivascular macrophages in different pathologies

Representative brain sections at the level of the dorsal hippocampus from mice injected with LPS (A) and KA (B) and 7 days after optic nerve crush (C). They were stained with the anti-mannose receptor antibody 5D3 and counterstained with haematoxylin. MR expression remains restricted to the perivascular and meningeal compartments in these different pathologies. Scale bars A,B = 20μm; C = 40μm

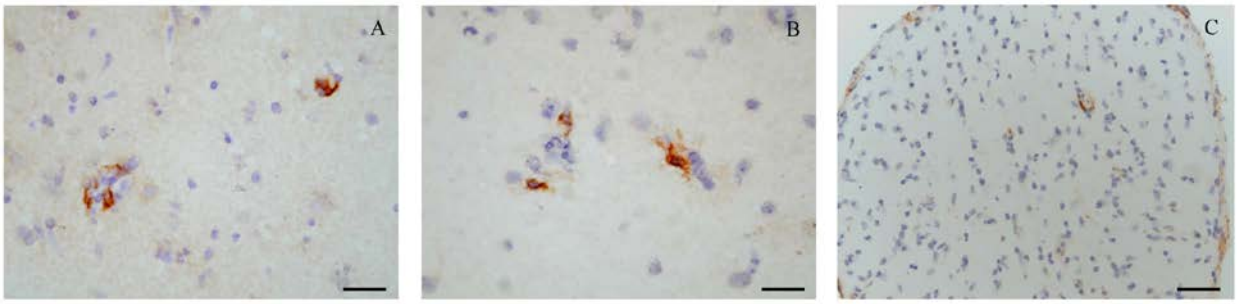
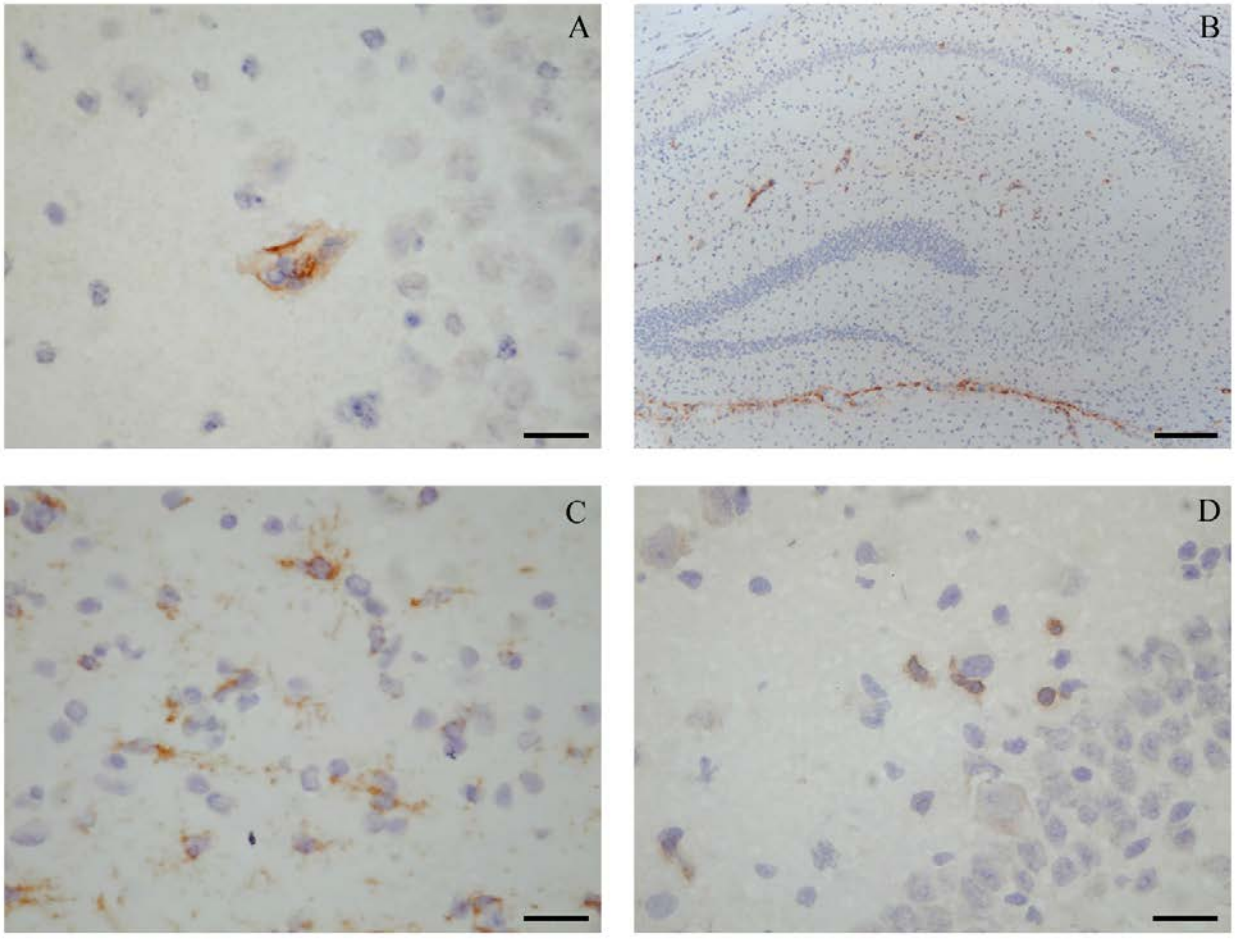


Figure 4: Perivascular macrophages in chronic neurodegenerative disease

Representative brain sections at the level of the dorsal hippocampus from mice injected with ME7 prion. They were stained with the anti-mannose receptor antibody 5D3 (A, B), the anti-CD68 antibody FA11 (C) and the anti-CD3 antibody KT3 (D). The slides were counterstained with haematoxylin. MR expression has a strict perivascular distribution (A,B) incompatible with expression by microglia (C) and T cells (D) which are situated in the parenchyma proper. Scale bars: A, C, D = 20 μm; B = 100 μm



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