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Review

How do immune cells overcome the blood–brain barrier in multiple sclerosis?

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ABSTRACT

The presence of the blood–brain barrier (BBB) restricts the movement of soluble mediators and leukocytes from the periphery to the central nervous system (CNS). Leukocyte entry into the CNS is nonetheless an early event in multiple sclerosis (MS), an inflammatory disorder of the CNS. Whether BBB dysfunction precedes immune cell infiltration or is the consequence of perivascular leukocyte accumulation remains enigmatic, but leukocyte migration modifies BBB permeability. Immune cells of MS subjects express inflammatory cytokines, reactive oxygen species (ROS) and enzymes that can facilitate their migration to the CNS by influencing BBB function, either directly or indirectly. In this review, we describe how immune cells from the peripheral blood overcome the BBB and promote CNS inflammation in MS through BBB disruption.

1. Introduction

The central nervous system (CNS) compartment, along with testis and eye, is considered an immunoprivileged site [1]. The presence of a blood–brain barrier (BBB) restricts the movement of soluble mediators and leukocytes from the periphery to the CNS. Despite the presence of this tightly regulated BBB, leukocyte entry into the CNS is an early event in multiple sclerosis (MS), an inflammatory disorder characterized by the formation of multifocal lesions in the brain and spinal cord. Acute MS lesions, featuring areas of demyelination, axonal loss and immune cell infiltrates, display BBB disruption as evidenced by *in vivo* gadolinium uptake on magnetic resonance imaging (MRI) and post-mortem evidence of focal micro-vascular leakage. Whether BBB dysfunction precedes immune cell infiltration or is the consequence of perivascular leukocyte accumulation remains to be established. While it has been suggested that BBB dysfunction can precede immune infiltration and demyelination in MS, leukocyte migration, both directly and indirectly, modifies BBB permeability. This is best exemplified by the clinical use of Natalizumab, an anti-$\alpha 4\beta 1$ integrin antibody known to restrict leukocyte migration to the CNS, which decreases lesion genesis and the number of lesions showing gadolinium enhancement [2]. Moreover, the contribution of the peripheral immune system to BBB dysfunction has become evident with the introduction of treatments mainly acting on leukocyte priming, activation and availability in the periphery (such as Mitoxantrone, Cladribine and Fingolimod). These therapeutic approaches also result in a decreased number of new gadolinium-enhancing lesions [3–6], supporting the concept that the overall state of peripheral immune cell activation leads to BBB dysfunction. The subject of this review is to describe how immune cells from the peripheral blood overcome the BBB and promote CNS inflammation in MS.

2. The blood–brain barrier (BBB)

In the CNS, large cerebral arteries entering the brain branch into smaller arteries and arterioles consisting of ECs surrounded by pericytes and variable layers of smooth muscle cells. Early anatomical studies performed by Jones [7] indicate that «(vessels entering the cortex carry) a small protrusion of the sub-arachnoid space before it, then pierces the attenuated pia matter, but still remains separated from the cortex by a small gap which is finally obliterated by neuroglial processes (...). As the vessel penetrates deeper into the cortex, the surrounding depression becomes tubular and is filled by (amorphous) dense material (...). The level at which the fusion occurs (of parenchymal and endothelial basement) is variable (...):it may extend as far as cortical layer VI, but in most

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cases it is complete by the time the vessel reaches the upper part of layer III». This space, now called the Virchow–Robin space, therefore does not extend into the white matter [7,8]. It is merely visible in young healthy individuals, and more apparent with ageing and disease. In capillaries, fused endothelial and parenchymal BMs in which pericytes are embedded come into direct contact with the astrocytic end-feet. This is the exact level at which the features of a fully competent BBB are present [9].

Leukocytes can access the CNS compartment (brain, spinal cord and CSF) either via the choroid plexus and the leptomeningeal vessels, to enter the CSF, or by the parenchymal capillaries and post-capillary venules, to enter the perivascular space [10]. E- and P-selectins are expressed in leptomeningeal and choroid plexus vessels but not in the parenchymal capillary/post-capillary microvessels [11]. Blocking these two selectins restrict cell rolling, adhesion and trafficking to the CSF, but does not impact on the development of EAE lesions [10]. This suggests that CSF accumulation of immune cells is not necessary for lesion development. Therefore, only BBB-related events occurring at the level of parenchymal capillaries and post-capillary venules have been considered for this review.

In CNS capillaries and post-capillary venules, where most leukocyte trafficking takes place [9,12], the CNS microvasculature is composed of specialized endothelial cells, often referred as BBB-endothelial cells (BBB-ECs). BBB-ECs lack fenestrations, exhibit low pinocytic but high efflux transporter activity and thus limit transcellular diffusion [13–15]. They also express tight junction (TJ) and adherens junction (AJ) proteins that reduce paracellular permeability [1,16–19]. TJs are zipper-like structures that link two adjacent cells and are formed by the close interaction and the assembly into macromolecular complexes of at least three families of transmembrane proteins: claudins, occludin and junctional adhesion molecules (JAMs). These macromolecular complexes, located into cholesterol-enriched membrane microdomains called lipids rafts, are anchored to actin filaments via adaptor molecules that include zona occludens 1 (ZO1), ZO2, ZO3, cingulin and Ca^{2+}-dependent serine protein kinase (CASK). AJs are formed by transmembrane proteins such as vascular-endothelial (VE)-cadherin and are linked to the cytoskeleton by catenins (α, β and p120) [16,20]. This specialized BBB endothelium is lined by embedded pericytes, which provide support, guidance and barrier properties during embryogenesis and before astrocyte generation [21,22], and by a vascular basement membrane (BM) formed by specialized extracellular matrix (ECM) molecules such as laminin 8 and 10, collagen type IV, perlecan and others [1,23]. A second BBB BM, called the parenchymal BM, is formed by proteins such as laminin 1, 2 and dystroglycan, and the ensheathing astrocytic endfeet which cover over 90–99% of the abluminal surface of CNS microvessels [16,24]. The small anatomic area found between the vascular and the parenchymal BMs is called the perivascular space, and it is an area where initial reactivation of lymphocytes takes place following entry across BBB-ECs [24].

Astrocytes exert a critical influence on the BBB phenotype due to their close apposition to the cerebral microvasculature. They help to maintain BBB integrity and immune quiescence through contact-dependent mechanisms and by releasing essential soluble factors such as basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), glial-derived neurotrophic factor (GDNF), angiotensinogen, angiopoietin I, src-suppressed C-kinase substrate (SSeCKS) and more recently members of the Hedgehog family [16,20,25,26]. Conversely, astrocytes can release inflammatory cytokines under neuropathological conditions such as MS, leading to ECs activation and BBB dysfunction [16,27]. There is also a prominent correlation between astrocyte differentiation and BBB development as astrocyte polarization parallels endothelial differentiation and BBB maturation [28–30]. In addition, neurons and microglia are known to modulate the barrier phenotype by secreting factors that influence BBB maintenance and by inducing the expression of immune related molecules such as chemokines and cell adhesion molecules (CAMs) by BBB-ECs [16,20,29].

3. The basics of leukocyte migration across the BBB

Leukocytes have to perform various actions before being entitled to move across the endothelial layer (into the perivascular space) and to subsequently find a way through the parenchymal BM (into the brain parenchyma). The classic steps of capture-rolling-tethering, activation, arrest-crawling and transmigration/diapedesis have been well and extensively described [1,12,24,31]. Each step involves interaction of BBB-ECs and leukocytes via expression of CAMs by BBB-ECs (such as intercellular CAM-1 (ICAM-1) and VCAM-1) and via expression or activation of their cognate ligands on leukocytes (such as αLβ2 (LFA-1) and α4β1 (VLA-4)). In addition, recent studies have demonstrated the important role of ECM components in the migration of leukocytes across the BBB, as the laminin composition of the vascular and possibly parenchymal BMs determine accessibility to the CNS [32]. Resting T cells have a limited ability to enter the CNS parenchyma, but it has been previously shown that freshly activated T cells can migrate into the CNS regardless of their antigen specificity [33,34]. Moreover, migration of leukocytes through the BBB increases permeability, which favors subsequent leukocyte infiltration [35,36]. The entry of pro-inflammatory leukocytes into the CNS is thus considered an early phenomenon that can trigger the events leading to neuroinflammation, BBB disruption and MS plaque formation [12,37]. Immune cells of MS subjects express inflammatory cytokines, reactive oxygen species (ROS) and enzymes that can facilitate their migration to the CNS by influencing BBB function, either directly or indirectly.

4. Direct influence of immune cells on the BBB

This section refers to the effect of cytokines, ROS and matrix metalloproteinases (MMPs) produced by peripheral blood mononuclear cells (PBMCs) that can directly disrupt components of the BBB or act on receptors expressed by BBB-ECs (Fig. 1).

4.1. Cytokines and soluble factors

4.1.1. TNF-α and IFN-γ

Tumor necrosis factor alpha (TNF-α) is elevated in PBMCs as well as in serum of MS patients and its level correlates with disease activity and secondary progression [38–42]. Moreover, a polymorphism of TNF-α -308 is associated with reduced MS risk [43]. Interferon-gamma (IFN-γ) level is often elevated in PBMCs and in the serum of MS subjects [38,39], especially during relapses [40,44], although this remains controversial [42,45,46]. BBB-ECs express TNF receptor 1 (TNFR1) [47,48] and IFN-γ receptor (IFN-γR) [47,49], and TNF-α itself is also reported to increase IFN-γR expression in microvascular ECs [50]. TNF-α and IFN-γ act synergistically to modulate the expression of a wide array of chemokines, cytokines and CAMs. This synergy is partly attributed to a different subcellular recruitment of phosphorylated extracellular signal-regulated kinase (Erk1/2) in human microvascular ECs, leading to different but converging downstream signalling [50,51].

In the periphery, TNF-α and IFN-γ affect the cellular distribution of TJs and AJs proteins [20]. Intravenous administration of TNF-α to mice results in an increased BBB permeability [52], and high levels of TNF-α in serum downregulate occludin expression by BBB-ECs in a mouse model of liver failure [53]. However, TNF-α is reported to have no direct influence on occludin or ZO1 expression by human

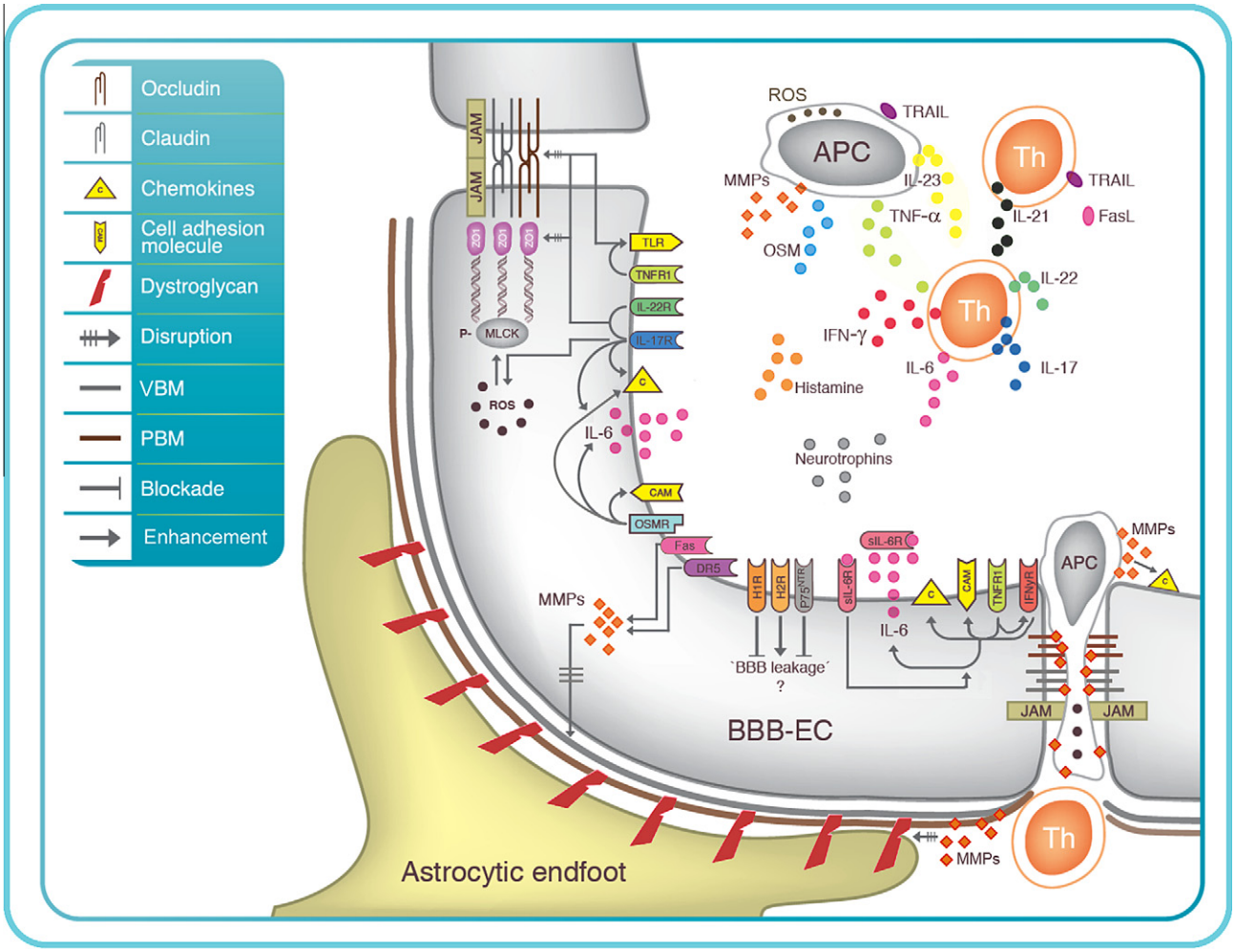

Fig. 1. Effect of cytokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) produced by peripheral blood mononuclear cells (PBMCs) that can directly disrupt components of the BBB or act on receptors expressed by BBB-ECs. *Abbreviations:* APC, antigen-presenting cell; BBB-EC, blood–brain barrier endothelial cell; CAM, cell adhesion molecule; C, chemokine; DR5, death receptor 5; FasL, Fas ligand; H1R, histamine receptor 1; H2R, histamine receptor 2; IFNγR, interferon-γ receptor; IL, interleukin; JAM, junctional adhesion molecules; MMPs, matrix metalloproteinases; MLCK, myosin light chain kinase; NTR, neurotrophin receptor; OSMR, oncostatin M receptor; PBM, parenchymal basement membrane; P, phosporylated; R, receptor; ROS, reactive oxygen species; sIL-6R, soluble IL-6 receptor;Th, T helper lymphocyte;TLR, Toll-like receptor; TNFR1, tumor necrosis factor receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; VBM, vascular basement membrane; ZO1, zona occludens 1.

brain microcapillary ECs [54]. In our experience, *in vitro* TNF-α and IFN-γ stimulation alters the architecture of junction proteins on primary cultures of BBB-ECs without an apparent change in their levels of expression [20]. More recently, TNF-α treatment of human BBB-ECs was reported to cause a strong upregulation of Toll-like receptors (TLRs)-2 and -3, and stimulation of TLRs with appropriate ligands was shown to downregulate TJ proteins expression [55]. Although appealing, the notion that TNF-α affects TJ architecture indirectly through TLRs remains to be confirmed.

Both TNF-α and IFN-γ influence the expression and secretion of numerous chemokines by BBB-ECs. TNF-α increases CCL2, CXCL8 and CCL5 [48,56] and IFN-γ induces CXCL10 [56,57]. Combination of TNF-α and IFN-γ synergistically induce expression of CXCL10, CXCL9, CX3CL1, CCL3, CCL4 and CCL5, and causes a redistribution of CCL2 to the basal surface and CCL3 to the apical surface of BBB-ECs [50,51,58]. These chemokines promote both adhesion of leukocytes to ECs (indirectly through avidity-maturation of integrins) and migration of leukocytes across BBB-ECs [58–61].

Stimulation of BBB-ECs with both TNF-α and IFN-γ increases expression levels of ICAM-1 [48,62,63], VCAM-1 [48,62,64], ALCAM [65], MCAM [66], E- and P-selectins [67,68] and various cytokine receptors by CNS vessels (our unpublished data). The interaction of VCAM-1-VLA-4 is implicated in capture and strong adhesion of CD4 T cells to CNS microvessels [12,69] and ICAM-1-LFA-1 is responsible for the firm adhesion, crawling, polarization and extravasation of T cells across the BBB [12]. Through the expression of LFA-1, VLA-4 and other CAM ligands, activated leukocytes can thus interact efficiently with inflamed BBB-ECs. Interestingly, while ICAM-1 expression is associated with infiltration of inflammatory T cells during early lesion formation in experimental autoimmune encephalitis (EAE), it is also involved in the migration of anti-inflammatory and protective regulatory T lymphocytes into the CNS. ICAM-1 early blockade therefore improves clinical and pathological indices of EAE, but a late blockade worsens it, outlining the complexity of leukocyte-BBB interactions and the potential beneficial effects of the so-called *pro-inflammatory cytokines* [70].

4.1.2. TRAIL and FasL

Another member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL), is expressed by activated monocytes and lymphocytes both as a membrane-bound protein and in a soluble form [71]. TRAIL polymorphism is associated with a higher risk for MS in the Japanese population [72]. TRAIL levels are reduced in its soluble form in serum from MS patients, with a possible inverse correlation with the number of lymphocytes positive for TRAIL [73]. Levels of soluble Fas ligand (FasL), a death receptor

ligand which can reduce apoptosis of encephalitogenic T cells when present in its soluble form, has been reported as elevated in blood of active MS [74]. BBB-ECs express both Fas and the TRAIL receptor DR5 [48]. TRAIL direct effect on the BBB remains controversial. Our group has demonstrated that FasL and to a lesser extent TRAIL induce Erk1/2 activation in BBB-ECs, triggering the release of MMP-9, a molecule known to favour immune cell entry into the CNS (see Section 4.3) [48]. On the other hand, TRAIL has been described as an inhibitor of activation and proliferation of encephalitogenic T cells in periphery and a mediator of parenchymal cell death in the CNS, but without affecting BBB-ECs or leukocyte migration to the CNS [75].

4.1.3. IL-6 and family

Interleukin (IL)-6 is a pleiotropic cytokine that displays both pro- and anti-inflammatory properties. Increased levels of IL-6 and soluble IL-6 receptor (sIL-6R) in serum of MS patients have been reported when compared to healthy controls (HC) [76,77]. These were however not different when compared to other non-inflammatory neurological diseases [42]. In human microvascular ECs, TNF-α and IFN-γ induce IL-6 production, and IFN-γ induces SOCS-3 expression, which in turn interferes with IL-6-induced STAT-3 activation, thus leading to a switch towards IL-6 pro-inflammatory properties [78]. In EAE, IL-6 plays a critical role [79] and has been implicated in generation of Th17 lymphocytes [80]. More recently, it has been reported that IL-6 trans-signalling indirectly modulates VCAM-1 expression by BBB-ECs and leukocyte recruitment in the spinal cord [81]. However, IL-6 can be produced by numerous cells of the CNS compartment and whether IL-6 produced by activated PBMCs affects BBB integrity remains to be demonstrated [82].

Oncostatin M (OSM) is a member of the IL-6 family. OSM is produced by monocytes and macrophages [83], with higher expression in PBMCs from MS patients [38,62]. Moreover, in MS lesions, OSM is found in infiltrating leukocytes, reactive astrocytes and microglia. The OSM receptor components are expressed by human brain ECs (gp130 and OSMRβ) and OSM stimulation induces expression of ICAM-1, IL-6 and CCL2 by brain ECs, especially when ECs are synergistically treated with TNF-α [62]. OSM also directly decreases transendothelial electric resistance of rat brain capillary ECs and induces a structural disorganization of ZO1 and claudin-5 [83]. Thus, OSM could play both a direct and indirect role in favouring leukocyte migration through the BBB.

4.1.4. IL-23, IL-17, IL-21 and IL-22

IL-23 is a cytokine produced by myeloid antigen-presenting cells. IL-23 was shown to be significantly more abundant in the serum and PBMCs of MS subjects [84,85]. IL-23 is critical in EAE as it maintains an expanded pool of pro-inflammatory IL-17-expressing (Th17) lymphocytes [86–88], a cell subset found in both active MS and EAE lesions [89,90]. Furthermore, IL-17 and IFN-γ production by neuroantigen-specific T cells in the peripheral blood predicts EAE disease outcome [91]. IL-17A and -17F protein and mRNA are elevated in PBMCs from clinically isolated syndrome (CIS) and in RRMS [44,92], especially during relapses [46,93]. Serum levels of IL-17 demonstrate a trend towards higher levels in MS patients [84]. Th17 lymphocytes express IL-17 but also IL-21, IL-22 and Granzyme B. In MS patients BBB-ECs express IL-17R and IL-22R, with their ligands IL-17 and IL-22 promoting BBB disruption and immune cell migration [89]. IL-17 increases BBB permeability through a time-dependent down-regulation of occludin and disturbances in ZO1 expression and organization [89,94]. IL-17 appears to promote lymphocyte and monocyte migration also by increasing CCL2, IL-6 and CXCL8 secretion by BBB-ECs [89], and through an increase in ROS formation in ECs that could affect their contractile machinery via phosphorylation of the myosin light chain [94].

IL-21, another Th17 cytokine, was reported to be an alternative pathway to maintain and expand the Th17 pool [95,96], and IL-21R polymorphism has been recently linked to EAE and MS susceptibility [97]. Most infiltrating $CD4^+$ T cells are positive for IL-21 and most $CD4^+$, $CD8^+$ and $CD19^+$ cells express IL-21R in active MS lesions. Cortical neurons also express significantly more IL-21R in MS than in controls [98]. Peripheral (non-CNS) ECs express IL-21R and IL-21 is reported to inhibit angiogenesis [99], enhance lymphocyte migration and induce expression of CCL20 by gut epithelial cells [100]. Whether similar findings can be reproduced in brain ECs remains to be determined. Nevertheless, IL-21 manipulations in EAE yielded conflicting data [101]: Vollmer et al. initially reported an increased disease severity following IL-21 treatment [102], Nurieva et al. showed a decreased disease severity and a lower production of IL-17 by CNS-infiltrating $CD4^+$ T cells in IL-21 KO mice [103] and Korn T et al. and Nurieva et al. [95,103] demonstrated a defective Th17 generation in IL-21R deficient mice and in IL-21 KO mice, respectively. On the other hand, Sonderegger et al. reported no improvement of EAE course in IL-21R KO or IL-21 KO mice [104], Piao et al. reported increased EAE severity following administration of IL-21R Fc [105] and Liu et al. reported an earlier onset and more severe deficits but a faster recovery in IL-21R KO mice, which they attributed to a transitory alteration of Treg response and NK cells distribution [106]. Overall, taking these important discrepancies into consideration, it is difficult to firmly establish that IL-21 plays a critical role in EAE or on BBB function or dysfunction.

4.1.5. Histamine

The most important source of histamine is mast cells (peripheral or central) although basophils, macrophages, lymphocytes and neurons can also produce histamine (for review see [107]). In early MS, histamine levels in peripheral blood are increased [108]. BBB-ECs express histamine receptors, and histamine can increase BBB permeability [107], when administered peripherally [109] and centrally [110]. EAE results are however conflicting as there are numerous peripheral and central sources and targets of histamine. Moreover, histamine can exhibit neuroinflammatory or neuroprotective properties depending on the receptor implicated (H1R, H2R, H3R and H4R) [107]. A small open-label trial has shown beneficial effects of a H1R receptor antagonist in MS [111] and a case-control study suggested that use of H1R blockers was associated with a decreased MS risk [112]. Whether these effects occur through leukocyte proliferation and activation, through BBB permeability or through neurotransmission modulation is still unknown. Earlier studies had reported no effect of H1R antagonists but a protective effect of H2R antagonists on histamine-induced BBB leakage, and an increase in permeability following treatment with H2R agonists [109,113]. In contrast, a recent study describes that, even if H1RKO mice are less susceptible to EAE, they display an increased basal BBB permeability. Furthermore, overexpression of H1R by ECs in otherwise H1R-deficient mice (H1RKO-vonWillebrandFactor^{H1R}) is protective in EAE, resulting in a decreased BBB permeability without changes in ICAM-1 expression [114]. Moreover, histamine and H1R or H2R agonists decrease activation, proliferation and adhesion of autoreactive T cells to BBB-ECs [115], outlining the potential of histamine as a pleiotrophic amine, with both pro- or anti-inflammatory functions.

4.1.6. Neurotrophins

mRNA levels of brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerve growth factor (NGF) are significantly lower in PBMCs of untreated MS patients as compared to HC, especially in monocytes [116–118]. Moreover, neurotrophins levels are increased in PBMCs from HC but not from MS following activation

with either anti-CD40 monoclonal antibody (mAb), TNF-α, IFN-γ, IL-10 or IL-17, and the reverse is true for stimulation with anti-CD3/CD28 mAb [118]. In EAE, anti-NGF antibodies increase disease severity [119,120]. BBB-ECs express NGF receptor mRNA [47] and glial-derived neurotrophic factor (GDNF) receptor alpha 1 and 3 [47,121]. $P75^{NTR}$, a low affinity pan-neurotrophin receptor [120], is expressed by brain ECs in inflammatory conditions [122,123]. $P75^{NTR}$ KO mice suffer a more severe EAE course, a possible increased BBB permeability and an increased immune infiltration especially of T lymphocytes, suggesting that $p75^{NTR}$ is involved in leukocyte-BBB interaction [122,124]. Moreover, NGF interferes with monocyte migration through BBB-ECs in vitro [125] and increases brain capillary ECs proliferation [123]. In one study, BDNF administration attenuated blood-spinal cord barrier permeability in a spinal cord injury model [126], although this effect was not seen in a previous study [127]. GDNF, another neurotrophic factor, has been shown to enhance the BBB properties of brain capillary ECs in vitro [121]. Therefore, a reduction in neurotrophins expression, especially in monocytes, could result in an increased immune infiltration, although the exact mechanisms underlying the impact of neurotrophins on immune cell infiltration and neuroinflammation such as seen in MS remains to be studied, especially as neurotrophins and their receptors are expressed by PBMCs, BBB-ECs, neurons and glial cells.

4.2. Reactive oxygen species (ROS)

ROS exist in various forms with hydrogen peroxide (H_2O_2), the superoxide (O_2^-) and its derivatives being the most abundant in eukaryotic cells [128]. Superoxide can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a very powerful oxidant able to modify protein function [129,130]. The oxidative stress balance is reported to be shifted towards a prevalence of ROS over antioxidants in MS patients [131,132]. ROS are known to play a pivotal role in MS pathogenesis as they are produced by activated microglia and macrophages during the process of myelin phagocytosis and subsequently through the development of demyelinating lesions [133,134]. In the chronic phase of the disease, non-inflammatory mechanisms such as mitochondrial dysfunction support the formation of ROS and lead to oligodendrocyte damage and axonal degeneration [135]. However, high levels of free radicals can also damage brain endothelium and affect BBB permeability [136,137]. ROS can affect the CNS endothelium by disrupting the junctional proteins. In this regard, $ONOO^-$ is known to decrease the expression of claudin-5 [138], and H_2O_2 induces aberrant expression of occludin and ZO1 associated with increase in BBB permeability [139]. ROS can also change the phosphorylation state of junctional proteins, which results in alterations in the BBB phenotype [138], and they can affect the BBB by promoting transendothelial immune cell migration across the BBB [140]. The production of ROS by migrating leukocytes and particularly by monocytes is thought to result from activation following their interaction with ECs [140]. In addition, ROS can also activate redox signalling pathways such as the JAK-STAT pathway and thus trigger an inflammatory response known to lead to TNF-α production in myeloid cells and expression of CAMs such as ICAM-1, VCAM-1 and PECAM-1 in BBB-ECs [141–144]. IL-17 production (see Section 4.1) also affects BBB function [89] and this seems to occur in part via induction of ROS production by ECs via the NADPH oxydase and xanthine-oxydase enzymes [94]. After crossing the BBB, infiltrating macrophages continue with their extensive oxidative damage in active demyelinating lesions where they mostly affect foamy macrophages and perivascular astrocytes and thus further destabilizes the BBB [145]. The importance of ROS at the level of the BBB has been confirmed in studies using antioxidant therapies that result in reduction of monocyte migration across BBB-ECs and suppression of clinical symptoms in EAE [140,146,147], although limited data is currently available in MS.

4.3. Matrix metalloproteinases (MMPs)

MMPs are endopeptidases that serve as effectors of cell migration, cytotoxicity, inflammation and tissue remodeling via degradation of ECM components [148]. MMPs can be secreted by activated T cells and macrophages [149,150]. MMPs are expressed as inactive zymogens and their activation process is regulated by tissue inhibitors of MMPs (TIMPs). MMPs are secreted in response to both exogenous insults and inflammatory cytokines such as tumour necrosis factor (TNF)-α [151] and IL-1β [152]. MMP-2, MMP-3, MMP-7 and MMP-14 mRNAs are elevated in RRMS [153–155], MMP-8 serum levels are increased in MS, and MMP-9 mRNA and serum levels are increased in MS. TIMPs levels are reported as similar to controls, although there are some conflicting data [84,153,155–157]. High MMP-9 and low TIMP-1 levels are predictive for development of new gadolinium-enhancing lesions [157] and disease activity in MS patients correlates with the potential of their PBMCs to degrade ECM components like laminin [158]. In EAE it has been shown that the expression of active MMP-2 and -9 by T cells, monocytes and dendritic cells is required for their migration across the BBB and their subsequent invasion of the CNS compartment [60,149,159,160]. Active expression of these and other MMPs is known to mediate BBB disruption by degrading junctional complex proteins [161–163]. In terms of ECMs, macrophage derived MMP-2 and -9 are known to cleave β-dystroglycan, a transmembrane receptor playing a crucial role in the barrier phenotype as it anchors the astrocytic endfeet to the parenchymal BM [149]. In contrast, overexpression of TIMP-1, one of the MMPs negative regulators, in the CNS results in reduced leukocyte transmigration and diffusion into the parenchyma of EAE animals. Such decrease was associated with low MMP activity in the perivascular and parenchymal areas [164]. These data correlate with the high EAE incidence observed in mice lacking TIMP-1 [165]. In addition, MMPs also modulate immune cell activation and migration across the BBB endothelium by regulating the activation of important modulators of cell transmigration such as chemokines, cytokines and CAMs [166–171].

Extracellular MMP inducer (EMMPRIN) is a factor expressed by PBMCs as a membrane-bound or a soluble form, with both forms inducing MMP production [172]. EMMPRIN is expressed by infiltrating leukocytes and CNS resident cells in MS lesions. In EAE, there is a higher proportion of EMMPRIN-positive lymphocytes and monocytes/macrophages, which colocalize with areas of MMP-9 expression and MMP-2/9 activity. Moreover, anti-EMMPRIN antibody reduced EAE severity by decreasing the number of perivascular infiltrates and the activity of MMP-2 and 9, without affecting peripheral activation [173].

4.3.1. E-Indirect influence of immune cells on the BBB

This section refers to the effect of cytokines produced by migrating PBMCs that act on microglia, astrocytes or neurons, which in turn release factors acting on BBB-ECs or disrupting components of the BBB, and to the effect of ROS and MMPs secreted by glial cells in response to ambient neuroinflammation, and which mediate BBB disruption.

4.4. Cytokines and soluble factors

4.4.1. Granzyme B and perforin

Granzyme B produced by T cells shows cytolytic activity against neurons and cleaves oligodendrocytes transaldolase, thus participating in CNS inflammation [89,174–176]. Moreover, the perforin/granzyme pathway is thought to play a major role in mediating lysis of ECs by natural killer (NK) cells [177] and perforin is implicated in

apoptosis of cerebral ECs in a cerebral malaria model [178]. It has recently been demonstrated in a variant model of Theiler's murine encephalomyelitis virus that activated CD8 T cells can contribute to BBB disruption via perforin-dependent astrocyte activation and TJ alteration [179]. Perforin was later shown to increase neuronal VEGF expression that in turn would decrease occludin in CNS endothelium, resulting in a leaky BBB [180].

4.4.2. IL-1beta and vascular endothelial growth factor (VEGF)

Activated macrophages can secrete proIL-1β, that will then be processed to its active form by caspase-1, which is reported as elevated in MS [181]. In EAE, astrocytes seem to constitute the major source of VEGF and its expression is induced upon IL-1β stimulation [182]. Therefore, through IL-1β activation of astrocytes, and activation of immune cells and neurons, there is increased VEGF release during relapses [182–184]. As VEGF promotes angiogenesis and EC sprouting, and decreases occludin and claudin-5 expression [180,182], the final endpoint of IL-1β-VEGF axis is a significant increase in BBB permeability, with serum protein deposition in CNS tissue and edema.

4.4.3. TNF-α and IFN-γ

On pericytes, IFN-γ induces expression of ICAM-1 and MHC class I and II. TNF-α on the other hand induces expression of VCAM-1 by pericytes [185], and could induce apoptosis of retinal pericytes *in vitro* [186]. While infiltrating macrophages secrete TNF-α, they also induce production of TNF-α by microglia [187], which then increases BBB permeability [188]. Astrocytes express IFN-γR *in vivo* [189]. Under IFN-γ activation, astrocytes can become neurotoxic, express ICAM-1 and secrete CXCL11 [190]. They can also express CXCL9, CXCL10 and CXCL11 when stimulated with both IFN-γ and TNF-α [191]. On the other hand, a recent study has demonstrated that IFN-γ-treated astrocytes can induce apoptosis of autoreactive T cells through astrocyte-derived immune suppressor factor (AdIF), a novel protein which can prevent EAE by rendering encephalitogenic T cells susceptible to apoptosis [192]. IFN-γ also upregulates IL-18 binding protein (endogenous inhibitor of IL-18) expression by microglia, and overexpression of IL-18bp reduces Th17 responses and therefore alleviates EAE pathology [193]. Neurons also express IFN-γR, and so far the effect seems to be mostly neuroprotective, as observed with IL-18 [194].

4.4.4. Oncostatin M

As mentioned previously, OSM is found in MS lesions in infiltrating leukocytes, reactive astrocytes and microglia [62]. OSM can induce the secretion of TNF-α and iNOS by microglia via activation of the NF-κB pathway. Activation of this pathway results in neurotoxicity [195] and BBB hyper-permeability [188]. OSM has also been demonstrated to inhibit adult neural precursor cell proliferation [196], induce apoptotic death in neurons [197] and stimulate IL-6 production by astrocytes [198], although a neuroprotective role and a pro-remyelination role via astrocytes and ECs stimulation has also been suggested [199–201]. Although, one study showed improved clinical and pathological indices of EAE using exogenous OSM [202], it is not clear whether this occured through BBB-related mechanisms.

4.4.5. IL-23 and IL-17

Upon activation, microglia and infiltrating macrophages and DCs are an important source of IL-23 in MS lesions [203]. IL-23 secretion within the CNS promotes IL-17-expressing lymphocytes expansion, and IL-17 is reported to induce the production of IL-6 and CXCL2 by microglia [204,205], suggesting a microglia-dependant amplification loop between IL-23, IL-17 and IL-6 in neuroinflammation. Microglia was also shown to express functional IL-23R [206] and could thus express IL-17, although this remains to be confirmed.

IL-17R was also shown to be expressed by astrocytes [204]. IL-17 was previously reported to induce IL-6 and IL-1β secretion by rodent astrocytes, as well as synergizing with IFN-γ and IL-1β or TNF-α to induce NO production in astrocytes [207]. IL-17 displays a synergistic effect with IL-6/sIL-6R on expression of IL-6, IL-12, CCL2, CXCL10 and CCL20 by astrocytes [208]. Moreover, IL-17 displays a synergistic effect with TNF-α on expression of CXCL1, CXCL2 and CCL20 by astrocytes, and abrogation of IL-17-induced signalling in astrocytes results in milder EAE and lesser CNS immune infiltration [191,209]. Taken together, IL-17 can lead to a significant disruption of the BBB, either directly by acting on BBB-ECs, or indirectly by acting on astrocytes and microglia.

4.5. Reactive oxygen species

As a result of the inflammatory process driven by CNS-infiltrating immune cells, ROS are also being produced by glial cells in close apposition to the BBB, such as astrocytes and microglia. In active demyelinating MS lesions, high oxidative damage accumulates in macrophages and hypertrophic astrocytes, represented by the production of 4-hydroxy-2-nonenal (damaged cellular membranes), nitrotyrosine residues (altered protein conformation) and 8-hydroxy-2'-deoxyguanosine (oxidative damage to DNA and RNA) [145,210,211]. Microglia can generate great amounts of superoxide, hydroxyl radicals, hydrogen peroxide and nitric oxide [212]. This production is enhanced in MS as activated microglia are known to express higher levels of myeloperoxidase, NADPH oxidase, xanthine oxidase and iNOs, all known as ROS-generating enzymes [128,133]. These multiple changes are known to induce demyelination and oligodendrocyte death, but can also affect the BBB phenotype and lead to BBB disruption, through a severely compromised communication between neurovascular unit members.

4.6. Matrix metalloproteinases

In the normal CNS, the expression of MMP-2, -7 and -9 by astrocytes and microglia is thought to control physiological processes such as cell migration, differentiation and survival via ECM remodelling. In acute and chronic MS lesions, astrocytes express moderate levels of MMP-2-, -3 and -9 [213]. In contrast, higher levels of TIMP-1 are found in astrocytes surrounding perivascular infiltrated areas and microglial nodules, and as TIMP-1 is a negative regulator of MMPs, this pattern of expression is thought to be a mechanism to counteract the inflammatory process and subsequent damage occurring at the BBB [165,214]. Expression of MMP-1, -2, -3 and -9 has been found in microglial nodules of MS patients [213] and MMP-19 expression is detected in microglial-like cells associated with preactive and active MS lesions [215]. In addition, membrane type-MMPs (MT-MMPs), which are considered to be regulatory due to their ability to cleave substrates in the vicinity of cell membranes, are also expressed by microglia. In EAE, most MMPs are upregulated, while MT-MMPs MMP-15, -16, -17 and -24 are downregulated [216,217]. Interestingly, their pattern of regulation is independent of the proinflammatory environment provided by cytokines such as IFN-γ, TNF-α and IL-1β [216]. Thus, unlike astrocytes, microglia seem to be contributing to the inflammatory process by upregulating the expression of pro-inflammatory MMPs that in conjunction with those produced by infiltrating leukocytes further destabilize the BBB.

5. Concluding remarks

In summary, activated leukocytes from MS patients can enhance BBB permeability by expression and secretion of inflammatory cytokines, soluble factors, ROS and MMPs that can, directly or

indirectly (via neuroglial cells) disrupt TJ architecture, alter basement membrane proteins and increase expression of chemokines and CAMs by BBB-ECs. This leads to an increase in BBB permeability and leukocyte migration across the BBB into the CNS, which in turn leads to lesion development in MS. However, clinical trials have demonstrated that molecules previously labelled as *pro-inflammatory*, such as TNF-α, can also have beneficial effects in MS [218] and that CNS infiltration by leukocyte is essential for viral immunosurveillance [3,219]. Immune cells overcoming the BBB are also needed to promote migration of anti-inflammatory Th2 lymphocytes and of regulatory T cells [70], which are classically held responsible for CNS repair. These dual and opposite roles of the so-called *pro-inflammatory* cytokines constitute the most significant limitations of our ability to identify the exact molecular and cellular pathways involved in lesion formation in MS.

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