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Review

Disruption of central nervous system barriers in multiple sclerosis

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ABSTRACT

The delicate microenvironment of the central nervous system (CNS) is protected by the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCB). These barriers function in distinct CNS compartments and their anatomical basis lay on the junctional proteins present in endothelial cells for the BBB and in the choroidal epithelium for the BCB. During neuroinflammatory conditions like multiple sclerosis (MS) and its murine model experimental autoimmune encephalomyelitis (EAE), activation or damage of the various cellular components of these barriers facilitate leukocyte infiltration leading to oligodendrocyte death, axonal damage, demyelination and lesion development. This manuscript will review in detail the features of these barriers under physiological and pathological conditions, particularly when focal immune activation promotes the loss of the BBB and BCB phenotype, the upregulation of cell adhesion molecules (CAMs) and the recruitment of immune cells.

1. Introduction

The blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCB) are a complex vasculature network that forms a continuous cellular barrier between the central nervous system (CNS) and the systemic circulation [2]. Most of the important metabolic exchanges that are critical to CNS homeostasis occur through this tightly regulated network. The BBB and to some extend the BCB are thus generally considered to be the structures responsible for providing nutrients, oxygen, ions, vitamins and co-factors as well as peptides and proteins to the CNS [33]. By extension, they have been described as the limiting factor to prevent the entry of xenobiotics, toxic metabolites and immune cells into the CNS. The functionality of the BBB is achieved through intricate interactions between BBB endothelial cells (BBB-ECs), perivascular astrocytes, pericytes and neighbouring CNS cells, creating a dynamic and multi-cellular component named the neurovascular unit (NVU) [82]. As for the BCB the anatomical basis of the barrier is on the epithelial cells making up the choroid plexus (CP) [56], with the soluble factors and/or contact-mediated mechanisms responsible of the barrier function to be established. The maintenance of a precisely regulated biochemical and immunological microenvironment is essential for proper CNS function and changes in its delicate balance have been associated with CNS pathologies, such as multiple sclerosis (MS) [66].

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MS is a chronic idiopathic demyelinating inflammatory disease of the CNS. While its aetiology remains unknown, currently available data suggest that a combination of factors (environment, viruses and dietary conditions), in conjunction with genetic susceptibility might drive the inflammatory response against antigenic determinants that are specifically expressed in the CNS [66,130,156]. Critical aspects of MS lesion formation include (i) influx of activated and CNS-specific immune cells from the peripheral vascular compartment to the CNS tissue and (ii) nervous tissue damage, as best exemplified by the presence of demyelinating lesions (called plaques) located in the white and to some extend in the cortical grey matter. Despite recent debate on the presence of a relative importance of white vs. grey matter lesions, a typical and universal feature of MS remains that lesions are scattered throughout the CNS parenchyma and are characterized by extensive immune cell infiltration (lymphocytes, monocytes, dendritic cells, etc.) in the perivascular space of small and medium caliber vessels [112,113]. These infiltrating cells may further traffic into the CNS parenchyma, causing demyelination, oligodendrocyte death and axonal damage [55,130]. Activated leukocytes locally release inflammatory cytokines and chemokines leading to focal immune activation of ECs, loss of the BBB and BCB phenotype, upregulation of cell adhesion molecules (CAMs) and the recruitment of additional immune cells, often called bystander leukocytes [21]. The infiltrated cells subsequently secrete additional cytokines and chemokines that may either enhance or dampen the inflammatory process. Although peripheral blood leukocyte infiltration plays an essential role in MS lesion development, there is also evidence suggesting that BBB dysfunction precedes immune cell infiltration. This may only reflect CNS innate immune cell (microglia) activation and/or astrocyte dysfunction and does not necessarily means that primary BBB dysfunction is the leading cause of MS lesion formation. Nevertheless and while a primary BBB or BCB dysfunction is not considered to be the cause of MS, recent evidence suggest that immune-mediated activation (or damage) of the various BBB cellular components significantly contributes to MS lesion development and expansion [132]. In this manuscript we will review some of the key observations that associate loss of BBB phenotype with CNS inflammatory events and MS lesion formation. The mechanisms of inflammation-induced BBB disruption, CAM upregulation and immune cell trafficking to the CNS will be discussed in detail. These observations will be expanded to review the changes in the BCB during MS and its role in immune cell infiltration to the CNS.

2. BBB under physiological conditions

CNS vessels associated with the BBB are formed by highly specialized ECs that inhibit the transcellular and paracellular diffusion of macromolecules and cells due to their distinctive structural and functional features [71,104]. These unique characteristics are in part due to interactions with pericytes, astrocytes and neurons in what is now known as the NVU. In contrast to vessels of peripheral organs, the CNS-ECs have specialized transport systems, low pinocytic activity, higher mitochondrial volume fraction and lack transendothelial fenestrations [19]. Due to the very sparse pinocytic activity, the paracellular flux is limited. Moreover, the uptake of essential molecules is mediated through specific carrier and transport systems [71,104]. In addition, the paracellular cleft between adjacent lateral endothelial membranes is almost completely sealed because of the presence of continuous strands of junctional complex proteins that include tight junctions (TJs) [79,104,200,207] and adherens junctions (AJs) [174].

3. Cellular composition of the BBB

Glial cells of the NVU are key contributors to the BBB phenotype. Unique communication between BBB-ECs and perivascular cells allows for complex signaling events between CNS glial cells and the CNS endothelium. These intricate cellular interactions control and maintain BBB function (TJ maintenance, metabolic and specialized transporter expression). Such signaling events allow for a rapid regulation and remodeling of the BBB, a phenomena crucial to maintain CNS homeostasis in response to physiological and pathological stimuli [2,82,156,158]. Three features of the NVU are essential for proper BBB functioning: the pericytes, the astrocytes and the basement membrane (BM).

Pericytes provide physical support to ECs and also play a role in the maturation and maintenance of the BBB by secreting growth factors and extracellular matrix components. The number of pericytes associated with blood vessels varies among different types of vessels, with those in the CNS exhibiting the highest pericyte coverage [13]. In addition, *in vitro* models of the BBB normally used co-cultures of BBB-ECs and astrocytes, but when pericytes are added to the system, the triculture system reorganize into stable capillary-like structures, demonstrating the important role of pericytes in the formation and maintenance of the vasculature and the BBB [84,109]. Pericytes are known to participate in these process by secreting transforming growth factor (TGF)-β [50], angiopoietins [87], platelet-derived growth factor (PDGF)-B [119] and sphingosine-1-phosphate (S1P) [13]. Pericytes, through N-cadherin-dependent binding to ECs [69], promote vascular maturation/integrity [136] and participate in reducing BBB permeability. Pericytes also induce angiogenesis and are known to support cerebral blood flow in experimental models of ischemia and brain tumors [83,108]. Although pericytes have multifunctional roles in BBB development and maintenance, their exact functions remain under intense investigation [17,136]. Finally, it has been suggested that pericytes participate in immune reactions as they performed antigen presenting cell-like activities. In this regard, CNS pericytes are known to express major histocompatibility complex-II (MHC-II), possess phagocytic activities and absorb soluble molecules delivered into the blood or CSF [17,18]. However, this particular function still in debate, as studies clearly demonstrating that this phenotype is exclusively due to pericytes and no perivascular macrophages are lacking.

Astrocytes are crucial constituents of the NVU and are important inducers of BBB properties. Astrocytic endfeet ensheathe 99% of the surface of the brain microvessels and are separated only by a thin but compact BM [82]. Perivascular astrocytic processes are a specific feature of CNS microvessels and their formation coincides with the development of barrier properties [152]. Astrocyte–EC interactions regulate ECs proliferation, angiogenesis, transporter protein expression, TJs protein expression, TJ morphology and are known to modulate inflammatory responses in the CNS [1,2,138,156,158,219]. Primary BBB-ECs isolated and cultured *in vitro* rapidly lose some of their BBB-features, such as P-glycoprotein and TfR expression [35,114]. Interestingly, BBB function can be reinstated by co-culturing BBB-ECs in the presence of astrocytes or astrocyte conditioned media (ACM) [158,191,212]. These observations underscore the importance of signals supplied by astrocytes and provide evidence that factors needed for reliable BBB function are at least partly soluble and secreted [138]. However, the exact identity of the soluble and contact-dependent factors provided by astrocytes still remains largely unknown. Potential candidates include members of the fibroblast growth factor (FGF) family [165], glia-derived neurotropic factor (GDNF) [91], TGF-β [50], Src-suppressed C kinase substrate (SSeCKS) [116], Meteorin [150], angiotensinogen [212] and more recently members of the Hedgehog family [7]. The current view is that astrocytes regulate various aspects of BBB physiology using a multitude of factors with redundant functions and that understanding the complex astrocyte–BBB-EC interactions under physiological and pathological conditions might lead to the development of novel therapeutic strategies to either enhance or conversely restrict BBB permeability.

The BM, found between the BBB-ECs and perivascular cells, is composed of several extracellular matrix (ECM) components, including laminins [96] (Fig. 1 lower left panel), fibronectin [195], dystroglycans [47], collagen type IV and heparin sulfate proteoglycans [194,195]. Ultrastructural and immunofluorescent studies have shown that this structure is made of two membranes closely associated with the brain microvasculature: these are referred to as the endothelial and the parenchymal BM [82]. The BM functions as a tissue (anatomical) scaffold on which NVU cells are attached and form a substrate for cellular differentiation and gene expression. Endothelial-, astrocyte- and to a lesser degree pericyte-derived extracellular matrix proteins enhanced BBB integrity, demonstrating the importance of glial matrix constituents [81]. Soluble factors secreted by glial cells can be captured by ECM proteins depending on their charge, and thus the ECM is regarded as a structure which increases local concentration and paracrine potency of biologically active molecules. BBB-ECs constitutively express a number of integrins important in EC adhesion to the basal lamina and alterations in the expression of BBB basal lamina or integrins correlates with BBB property breakdown [47,49].

4. Adherent and tight junctions: molecular fences and dynamic signaling regulators of BBB integrity

The intercellular spaces of tight and impermeable biological barriers are sealed by large molecular complexes called TJs. In the BBB and in other biological barriers, TJs are composed of large multiprotein complexes that mediate tight intercellular contacts between adjacent cells and act as molecular fences [149,219]. TJs consist of at least three known types of transmembrane proteins including occludin, claudins and junctional adhesion molecules (JAMs) [200]. The extracellular loops of these proteins form a paracellular barrier that selectively excludes most blood-borne substances from the CNS. While occludin is not required for the structural integrity of the TJs, as mice deficient in occludin can still form proper functioning TJs [167], claudins are essential components of BBB maintenance [127,140]. Claudins and occludin create an impermeable seal between cells through homophilic and heterophilic binding of their extracellular loops [196]. Claudin-3 and -5 are preferentially expressed in brain ECs and play an important role in vessel permeability and angiogenesis [140]. JAM-A localizes to the intercellular TJs of murine [9,15,200] and human CNS-ECs [200] where it promotes cell to cell contact in a homophilic manner. Although its role in restricting BBB permeability remains unclear, JAM-A is known to be implicated in leukocyte transmigration [124], angiogenesis [135] and as a receptor for viruses [22]. Although JAM-B and -C have been detected in murine [15] and porcine [134] CNS-ECs, their expression and role at the human BBB remain to be fully elucidated.

The cytoplasmic region underlying the TJ transmembrane proteins contains a vast number of adaptor proteins that mediate the link between the TJ proteins and the actin microfilaments. Claudins, occludin and JAM-A interact with a variety of structural proteins and therefore regulate a wide array of signaling pathways essential for EC survival and TJ assembly, maintenance and regulation [19]. Zona occludens (ZO-1, -2 and -3) and the membrane-associated guanylate kinase inverted proteins (MAGI-1 and -3) are important adaptor proteins of the membrane-associated guanylate kinase (MAGUK) protein family [78]. They are known to bridge and immobilize membrane-anchored TJ proteins with actin filaments in EC and epithelial barriers. These adaptor proteins contain numerous protein-protein interaction domains, such as SH3 and PDZ [78]. These domains interact with the cytoplasmic tail of integral membrane protein and bind to other adaptor proteins, leading to the formation of intricate signaling scaffolds. Several other intracellular proteins have been shown to be involved in the biogenesis and the maintenance of TJs and they include: AF-6, cingulin, Ca^{2+}-dependent serine protein kinase (CASK) and 7H6 antigen as well as various signaling molecules like RhoA and Rac [77,125,206,218].

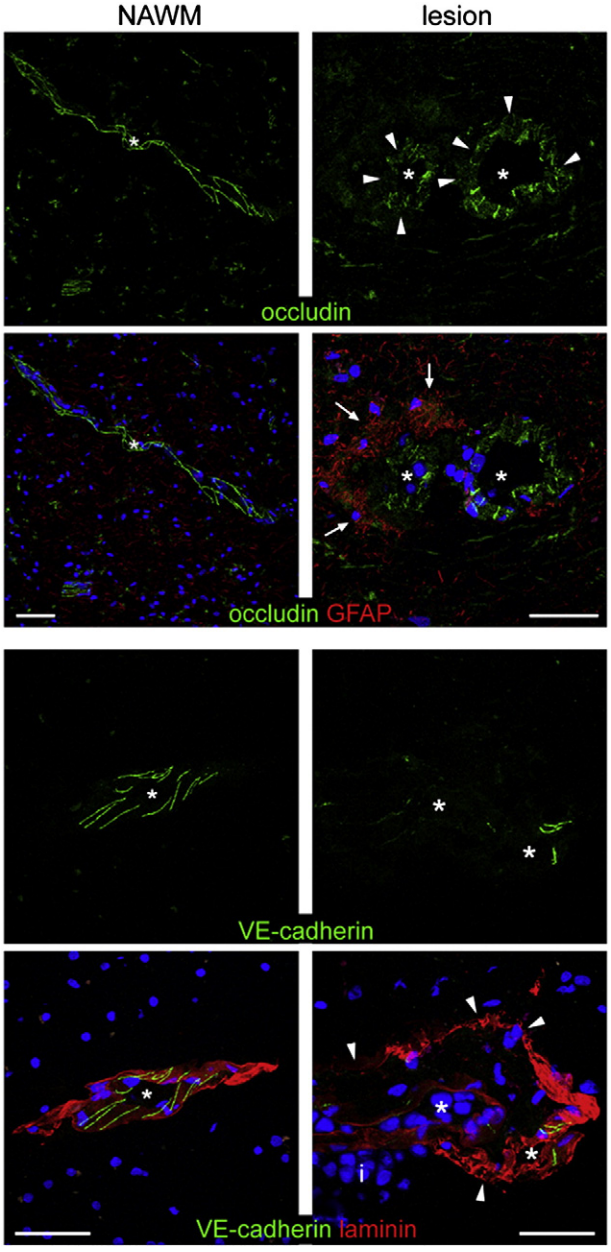

Fig. 1. BBB changes in MS. Occludin and VE-cadherin stainings are shown in green, GFAP in red (upper panels), laminin in red (lower panels) and the TOPRO-3-stained nuclei in blue. Scale bars indicate 50 μm, vessels are marked by asterisks and i denotes immune infiltrates. Zipper like structures of intact junctions are seen in normal-appearing white matter (NAWM) vessels (left panels). Decreased expression and focal degradation (arrowheads) of TJ (occludin) and AJ (VE-cadherin) proteins is detected in vessels associated with lesions (right panels). Astrogliosis is found as increase numbers of $GFAP^+$ endfeet (arrows) in the periphery of lesional vessels (upper right panel). Laminin expression in NAWM vessels is seen as a smooth and continuous layer labeling the basal lamina (lower left panel). In active lesions, laminin expression decreases in the endothelial and parenchymal basement membranes (arrowheads) (lower right panel).

AJs also contribute to the low permeability of the BBB and BCB by mediating the cell to cell adhesion in endothelium and epithelium [46]. They partially regulate paracellular permeability and consist of the transmembrane proteins cadherins and the cytoplasmic proteins catenins. The adhesive properties of AJs are due to the homophilic interactions between the extracellular domains of calcium-dependent cadherins on the surface of adjacent cells, while catenins are the linkers with the actin cytoskeleton and support the formation of

adhesive contacts between cells [19,111]. ECs express high levels of vascular endothelial (VE)-cadherin, also known as cadherin-5 and the neuronal cadherin (N-cadherin) [25]. VE-cadherin, as most of the members of this family, is linked to the catenins p120, β-catenin and plakoglobin through its cytoplasmic tail [45]. This AJ complex binds to α-catenin and interacts with the actin-binding proteins α-actinin, ajuba, zonula occludens-1 (ZO-1) among others to link the cytoskeleton with the cellular membrane [111,159,204]. Clustering of VE-cadherin at cell–cell contacts promotes the formation of multimolecular complexes that comprise signaling, regulatory and scaffold proteins [46]. In addition, VE-cadherin is involved in vascular permeability via the induced phosphorylation and internalization induced by vascular endothelial growth factor (VEGF) and by its association with vascular endothelial tyrosine phosphatase [68,141]. The levels of N-cadherin expression in ECs are comparable to VE-cadherin, but it has a diffuse distribution on the cellular membrane with poor clustering in the junctional complexes [46]. Thus, it has been suggested that N-cadherin does not play a role at the level of the junctional complex and rather acts at heterotypic cell–cell contacts between ECs and pericytes [69,148]. Other atypical members of the cadherin family, such as T-cadherin (cadherin-13) and P-cadherin, are variably expressed in different types of ECs [92]. T-cadherin lacks the transmembrane and intracellular domains and it is not enriched in AJs [92]. T-cadherin primarily functions as a signaling molecule that regulates barrier function through a complex signaling network [10] and also suppresses angiogenesis by inhibiting migration of ECs [166]. Regarding catenins, their exact mechanism of action to promote cadherin-cytoskeleton contact is not fully understood, but it is proposed that cadherin clustering promotes the recruitment of monomeric α-catenin when cells establish cell–cell contacts [137,155]. As α-catenin is bound to cadherin through β-catenin or plakoglobin, molecules of α-catenin would detach from cadherins and associate to form homodimers that promote actin bundling. Thus, the association of VE-cadherin with catenins is certainly required for full cellular control of endothelial permeability and junctional complex stabilization [46]. In addition, there is interplay between TJs and AJs as assembly of TJs is influenced by the interaction between ZO1 and catenins [127].

5. The blood–cerebrospinal fluid barrier

An intact BBB and BCB are essential for maintenance of a healthy CNS. Upon inflammatory conditions leukocyte infiltration into the CNS also occurs in the ventricles via the CPs [62,173]. These structures are extensions of the ependymal lining into the ventricles and are made up of highly specialized cuboidal and ciliated epithelial cells surrounding a stroma with blood vessels lacking BBB properties. The main function of CPs is to secrete cerebrospinal fluid (CSF) which is important for mechanical protection of the brain, regulation of the intracranial volume, distribution of neuroendocrine factors and buffering of extracellular fluid ions and other solutes [30,175,208]. The BCB is the cellular boundary between choroid epithelial cells that separates the blood from the CSF and its anatomical basis is due to the junctional proteins located between these cells. TJs of the BCB are found directly underneath the microvilli of choroid cells forming parallel strands that are sparsely interconnected according to freeze-fracture studies [62]. Studies in humans and rodents have shown that choroid cells express the TJ proteins ZO1, occludin, claudin-2, -3, -11 and JAM-A [8,120,207]. The presence of AJ proteins has been examined and cadherin, α-catenin and β-catenin are also found in choroid epithelial cells [8].

6. BBB changes during MS lesion formation

Changes at the level of the BBB during inflammation are related to two main processes: (i) BBB disruption, which implies leakage and alteration of junctional components, and (ii) BBB activation, which relates to the capacity of ECs, astrocytes and possibly pericytes to express and secrete immune factors able to influence the recruitment, the effector functions and the survival of leukocytes entering the brain.

Under physiological conditions, the assembly and maintenance of TJs are tightly regulated events. Although the function of TJs and AJs as molecular fences between adjacent cells is well understood, the role of TJs in immune cell migration between adjacent ECs and epithelial cells at the BBB and the BCB remains to be defined. Under neuroinflammatory conditions, as is the case in MS, TJ and AJ deregulation is associated with loss of BBB permeability and increase solute and immune cell infiltration in the CNS [151]. Alterations in the BBB can be identified through histological analysis in post-mortem samples or clinically by using imaging techniques such as MRI (magnetic resonance imaging). Such changes in the BBB are thought to occur at the early phase of lesion formation, as immune cell infiltration originates in the surroundings of parenchymal microvessels [66,219]. During the early stages of MS, the inflammatory state and the leakiness of the blood vessels are thought to facilitate the movement of leukocytes and immune soluble mediators exacerbating the inflammatory process and leading to multifocal perivascular infiltrates. Such infiltrates are rich in antigen presenting cells and lymphocytes, predominantly cytotoxic T cells and T helper (Th)1 and Th17 T cells, which are known to play an important role in demyelinating perivascular areas [5,64,89,97,98,131]. In addition, alterations in the BBB and infiltration of immunopathogenic leukocytes are accompanied by edema, axonal loss and gliosis [55,65,66]. All these features potentially lead to reversible neuronal dysfunction early in the disease process, whereas permanent neurological deficits are currently seen as the result of a long-term immune-dependant neurodegenerative process [26,192].

In MS, the breakdown of the BBB is thought to be transient, although recurrence may be observed at the same or different locations within interval of weeks, months or even years [80]. The subsequent progress and lesion development is irregular and involves additional phases of BBB leakage, immunologically mediated demyelination and various degrees of axonal transection [55,203]. It is well recognized that the expression and organization of junctional proteins are known to change during neuroinflammatory and infectious processes [9,79]. To assess the state of the BBB and particularly its anatomical components has been a difficult task in MS patients due to the scarcity of frozen post-mortem samples and the technical difficulties in determining expression of various junctional complex proteins in CNS tissue. Thus, these constraints have limited the analysis of BBB pathophysiology in the different type of lesions. Analysis of CNS vessels in MS has shown alterations in the expression of TJ and AJ proteins within lesions when compared with the vasculature in the normal-appearing white matter (NAWM) [101,147,154]. Some recent images from our lab depicting TJ and AJ alterations in MS lesions are found in Fig. 1. Abnormalities in the expression of junctional proteins coincide with perivascular astrogliosis (Fig. 1 upper right panel), serum protein leakage and it has been proposed that dysfunctional TJs and subsequently impaired endothelial function allow more cells and immunologically active molecules to access the CNS, enhancing demyelination, axonopathy and tissue injury [147]. In addition, the expression pattern of junctional proteins in active and inactive white matter lesions, and in NAWM within acute MS, PPMS (primary progressive MS) and SPMS (secondary progressive MS) indicates a higher degree of endothelial abnormalities in active (40%) vs. inactive lesions (23% in PPMS and 37% in SPMS). Surprisingly, 13% of the vasculature in NAWM displayed abnormalities associated with BBB permeability, although to a lesser extent when compare to lesions [117]. Thus, abnormalities in the NAWM and presumably in the normal-appearing grey matter (NAGM) may represent the subtle

changes in BBB permeability seen in non-enhancing focal and diffuse lesions, and such disturbances could correspond to the largely uncharacterized first steps of lesion formation, called either nascent or pre-active lesions [85,197].

7. Role of immune soluble mediators in TJ alterations

MS and EAE are immune CNS mediated disorders characterized by immune cell infiltration and upregulation of pro-inflammatory cytokines and chemokines such as IL-1β, IL-17, IL-22, IFN-γ and CCL2 among others [16,97,98,122]. Most of these immune mediators are released by leukocytes during transmigration, and within the CNS and aside of promoting and expanding immune cell activation they can also affect the integrity of the BBB. Elevated levels of TNF-α and IL-1β in EAE are associated with changes in the BBB permeability [70] and such increase has been also demonstrated in the CSF of MS patients [177,178]. *In vivo* and *in vitro* studies have shown that IL-1β indirectly destabilizes the BBB by inducing expression of matrix metalloproteinase-9 (MMP-9) [28,184,201], an enzyme known to cleave occludin, ZO-1, claudin-5 and other junctional complex associated molecules [14,24,215]. Degradation/downregulation of TJ proteins correlate with the elevated levels of MMP-9 expression reported in MS patients. IL-17 and IL-22 have also been associated with changes in the BBB. In this regard, treatment of primary cultures of human BBB-ECs with both cytokines induced alterations in the BBB permeability that coincided with decrease expression of occludin and ZO-1. These findings correlated with the reduction of both proteins in CNS blood vessels highly infiltrated in EAE and MS sections [98] a finding that was recently confirmed by Huppert [88]. In ECs, TNF-α is able to induce intercellular gaps that resulted from loss of VE-cadherin in AJs [205]. TNF-α can also affect barrier permeability by upregulating the expression of NF-κB, which induces the transcription of myosin light chain kinase (MLCK), a factor known to induce internalization of TJ proteins [143]. Co-stimulation with IFN-γ and TNF-α are known to affect EC permeability by affecting the cellular distribution of JAM-A, claudin-5 and by inducing focal loss of VE-cadherin [146,211]. In addition, IFN-γ induces actin restructuration and decreases the protein levels and the subcellular localization of ZO-1 [27,217]. In epithelial cells IFN-γ stimulation promotes endocytosis of occludin, claudin-1 and JAM-A but not ZO-1 or AJ proteins [31]. Conversely, removal of IFN-γ results in repositioning of occludin, JAM-A and claudin-1 in the cell membrane, from intracellular pools [32].

Cytokines, oxidative stress and various growth factors can also affect the stability of the BBB, by modifying the phosphorylation state of the TJs. For instance, VEGF affects the barrier permeability by increasing the phosphorylation of occludin and ZO-1 [11], an effect reversed by treatment with dexamethasone [12]. The chemokine CCL2 (formerly known as MCP-1) is also known to affect the permeability of the BBB and as VEGF, it induces phosphorylation of occludin, ZO-1, ZO-2 and claudin-5, at least in murine systems [185]. CCL2 also induces loss of occludin, claudin-5, ZO-1, ZO-2 through endocytosis mediated events [186,187]. Monocytes/macrophages also participate in the CCL2 induced changes in BBB permeability as interactions between monocytes and BBB-ECs also result in phosphorylation of TJ proteins [151,187]. Reactive oxygen species, in particular H2O2, has been shown to promote BBB disruption [63,88,115,169] and to favor immune cell attachment to BBB-EC [172]. This oxidant-mediated disruption and activation of the BBB was found to be detrimental in animal models of MS, as elegantly proven by Schreibelt [169,170] and extensively reviewed in 2007 [171].

In contrast to the detrimental effect of pro-inflammatory cytokines, other immune soluble mediators such as IFN-β and IL-25 promote BBB integrity and impermeability. IFN-β is a type I IFN with immunodulatory effects that has been used for the treatment of MS for over 15 years [86,216]. *In vitro* and *in vivo* studies have shown that IFN-β increases the transendothelial resistance, reduces permeability, stabilizes the barrier function [106,107] and induces cell surface shedding of adhesion molecules on CNS-ECs [34,73]. On the other hand, IL-25 is a member of the IL-17 family and is expressed in CNS-ECs. Stimulation of ECs with inflammatory cytokines such as TNF-α, IL-17, IFN-γ and IL-1β reduces IL-25 expression, a pattern also observed in active MS lesions and in EAE [183]. IL-25 seems protective for the BBB, as the reduced expression of claudin-5, JAM-A and occludin induced by TNF-α can be re-established upon treatment with IL-25 [183]. Taken together, these studies provide strong evidence that numerous cytokines play important role in regulating multiple aspects of TJ proteins and ultimately BBB permeability. In the following sections, we will explore additional roles of cytokines at the BBB/NVU, especially regarding expression of adhesion molecules and the migration of immune cells.

8. Alterations in the basal lamina during MS and EAE

In addition to the junctional complexes between ECs, the basal lamina surrounding the brain vasculature adds another layer of complexity to the BBB. Therefore, alterations in the structure and organization of this layer may affect the permeability of the barrier and subsequently the movement of leukocytes and immune mediators into the brain. Following migration through the CNS endothelium, leukocytes accumulate within the perivascular space and cross the parenchymal BM to enter the brain [180]. To accomplish this, immune cells produce MMPs and other enzymes involved in the degradation and remodeling of the BM [5]. In the murine model of MS, experimental autoimmune encephalomyelitis (EAE), as well as in human MS it has been shown that T cell and macrophage derived MMP-2 and -9 support leukocyte entry into the CNS parenchyma by specifically cleaving the endothelial BM and dystroglycan, a receptor that anchors astrocyte endfeet to the parenchymal BM via high affinity interactions with ECM [4,20,74]. As a result of the neuroinflammatory environment in CNS vessels and the expression of MMPs, MS lesions are characterized by the presence of irregular and discontinuous BMs and deposition of ECM components within the perivascular infiltrates [195] (Fig. 1 lower right panel and Fig. 2B and D). Regarding their function during the course of MS, it is has been shown in EAE that expression of laminins 411 and 511, which are found in the endothelial BM, is influenced by proinflammatory cytokines and their pattern of expression is associated with areas of T cell infiltration [180]. Thus, infiltrates are found in vessels expressing laminin 411 and low levels of 511, while in the absence of 411, laminin 511 is ubiquitously expressed in the CNS vasculature and this phenotype is associated with low T cell infiltration and milder disease [213]. These data indicate that the BM is not only a physical component of the BBB, as it can actively support the transmigration of T cells into the CNS through the interaction laminin α4-integrin α6β1 [213] and by acting as a reservoir of chemotactic agents, such as chemokines [123].

9. BCB disruption in MS and EAE

In contrast to parenchymal microvessels, which possessed BBB properties, the basis of the barrier properties in the CP lays on the TJs of the choroidal cells. The choroidal vessels are fenestrated and lack barrier properties, thus migration of immune cells within the CPs should be easily achieved when compare with their counterparts in parenchyma [208]. But, the few evidence available regarding leukocyte migration in the CPs indicates that a small number of cells move through this barrier and in fact, the number of leukocytes accumulating in the choroidal stroma is very low when compare to the perivascular infiltrates of parenchymal lesions. Studies in EAE have shown aberrant cellular morphology characterized by electron-dense and electron-light choroid cells. In these animals the junctions seemed to be structurally intact, although the distribution and the ratio of different junctional proteins

were altered under these inflammatory conditions [56,62,208]. To date, there are no studies addressing the mechanisms involved in the moderate changes seen in the anatomical components (junctional proteins) of the BCB during EAE. Damage of the BCB in MS has been also poorly explored and most studies on this topic are focused to understand the migration of leukocytes through the CP and the mechanisms associated with this process [164].

10. Leukocyte transmigration through the CNS barriers

Under physiological conditions, a low number of immune cells continuously access the CNS in a process called immune surveillance [60]. Immune cells can enter the CNS through 3 distinct routes; the first two involve the BBB via pial vessels to the subarachnoid space or via brain microvessels to the parenchyma. In the third route immune cells can gain access to the CNS in ventricular areas through the non-BBB vessels of the choroidal stroma and then through the choroid cells forming the BCB [56,60,162]. A fourth route has been suggested and involves migration from subependymal vessels in innervating leptomeninges and through the ependyma into the ventricles [8]. During physiological immunosurveillance, leukocytes migrate into the CNS via the meninges and the BCB at the CPs; while all the known routes of migration are effective during inflammatory conditions [8,102,162]. Transendothelial leukocyte migration through the BBB is a multi-step process characterized by (i) leukocyte

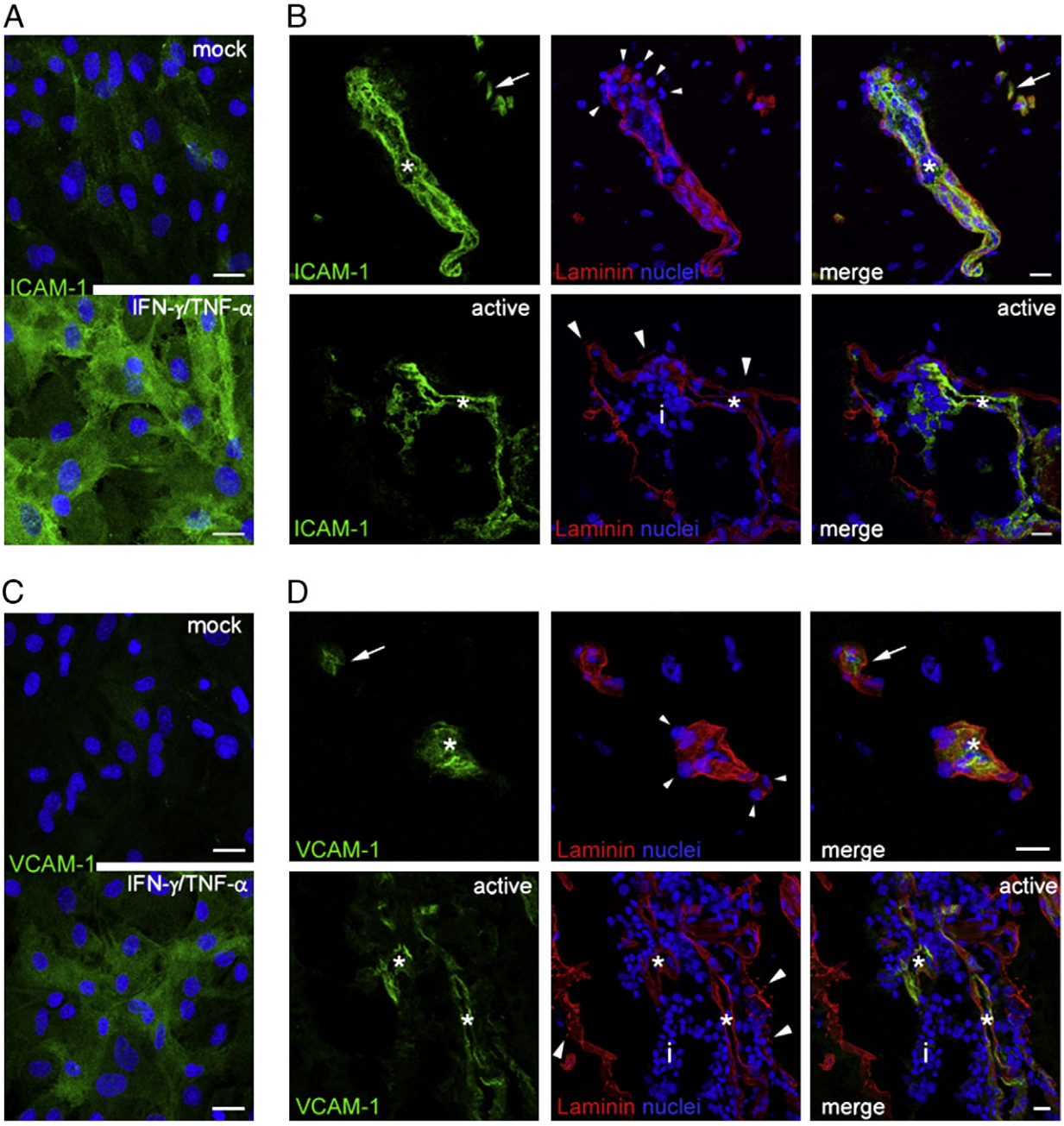

Fig. 2. Differential expression of cell adhesion molecules (CAMs) in inflamed ECs. The expression of the CAMs ICAM-1 and VCAM-1 is shown in cultures of human primary BBB-ECs (A and C) and in MS tissues (B and D). The CAMs are depicted in green, the ECM component laminin in red and the nuclei in blue. Scale bars indicate 10 μm. ICAM-1 (A) and VCAM-1 (C) expression is upregulated on the surface of BBB-ECs when stimulated with the cytokines IFN-γ and TNF-α during 16 h. ICAM-1 (B) and VCAM-1 (D) are expressed in NAWM vessels (arrow) and they are upregulated in vessels associated with pre-active lesions displaying infiltration of leukocytes (arrowheads) and partial loss of laminin expression (upper panels). ICAM-1 and VCAM-1 expression is high in vessels (asterisk) associated with active MS lesions and it is even detected in some infiltrating cells (i) (lower panels). Laminin expression decreases in the endothelial and parenchymal basement membranes with laminin^{+} fragments (large arrowheads) found in perivascular areas (lower panels).

tethering and rolling (selectin and α4-integrin dependent), (ii) activation of leukocytes and integrin avidity changes by chemokine stimulation of G-protein-linked receptors, (iii) arrest and adhesion strengthening mediated by endothelial CAMs and leukocyte integrins and (iv) diapedesis [118].

11. Involvement of selectins, CAMs and integrins in leukocyte tethering and rolling in the BBB during EAE and MS

The short and initial interaction between the brain endothelium and the leukocyte is mediated by selectins and their carbohydrate ligands or by α4-integrins [58,99,100,103]. Although P-selectin is preferentially expressed in meningeal and choroidal vessels under normal conditions [40,57,59] and it is upregulated in the whole CNS vasculature upon neuroinflammation, its role in supporting leukocyte extravasation remains controversial as recent studies have shown that despite the blockade or absence of P-selectin, mice developed clinical EAE indistinguishable from controls [58,100]. In contrast, E-selectin expression is only observed in meningeal vessels, but not in parenchymal vessels under inflammatory conditions [72]. In EAE it has been demonstrated that E-selectin-deficient animals displayed no differences in symptomatology when compared to their WT counterparts [53]. Studies in humans correlate with the mouse findings as immunostaining analyses have shown expression of E- and P-selectin in meningeal vessels of normal individuals and suggest that immunosurveillance is mediated by these molecules [103]. In MS, E-selectin is upregulated in microvessels isolated from MS autopsy material [202] and studies analyzing the soluble forms of P- and E-selectin have demonstrated higher levels of these molecules in the blood and CSF of MS patients, although a clear association between the levels found and the type of MS still remain controversial [51,54,128,129]. It has been also shown that $CD8^+$ but not $CD4^+$ T cells from MS patients show increased rolling on endothelium expressing P-selectin *in vitro* and that antibodies directed against P-selectin glycoprotein ligand-1 (PSGL-1) block the recruitment of $CD8^+$ T cells through murine brain vessels [23]. On the other hand, early immune cell infiltration in EAE mostly occurs through pial vessels and most of the $CD4^+$ T cells involved in this process also express α4β1 [179]. It was then shown that α4-integrins actively participate in the rolling and tethering of immune cells by mediating the initial capture and G-protein dependent arrest of encephalitogenic T cell blasts crossing the white matter microvasculature [193] as well as endogenous leukocytes and encephalitogenic T cells in all the CNS vasculature [99,100,153]. The EAE data correlate with MS, as analyses of human brain sections have shown expression of α4β1 integrin in T cells located in perivascular cuffs and brain parenchyma, while ECs expressed its receptor, vascular cell adhesion molecule 1 (VCAM-1) [37,52,198]. These observations are supported by experiments showing that human $CD4^+$ T cells preferentially roll using α4 integrins, whereas $CD8^+$ T cells do it through the P-selectin–PSGL1 interaction [23]. Thus, leukocyte tethering and rolling in meningeal microvessels appears to be E- and P-selectin dependant and their expression can be upregulated during neuroinflammatory conditions like MS and EAE. On the other hand, while ECs of parenchymal vessels express E- and P-selectin only under neuroinflammatory conditions, neutralization of selectins has no effect in the development of EAE. Conversely, blockade of α4-integrins inhibit immune cell rolling in white matter microvessels, reducing the clinical severity of EAE and MS. Overall, the crucial role played by α4-integrins in the binding of inflammatory leukocytes to the CNS vasculature has resulted in the generation of therapies currently used in the treatment of MS and in the proof of concept that migration-targeting therapies are effective in controlling lesion formation in EAE and human MS.

12. Leukocytes arrest and binding are mediated by integrin activation and CAMs

CNS-ECs actively participate in neuroinflammatory reactions and can directly influence immune cell migration by regulating the expression of cell adhesion molecules (CAMs), cytokines and the secretion and presentation of chemokines [39,121]. Chemokines are small heparin-binding proteins involved in various stages of leukocyte transendothelial migration into different organs, including the brain [163]. They are particularly recognized for their role in activating leukocyte tethering and rolling on endothelial surfaces. In this regard, chemokines immobilized on endothelial surfaces interact with their receptor located on immune cells; this G-protein receptor activation triggers Gαi-dependent intracellular signaling [153,193]. The activation of this signaling pathway stimulates conformational changes in the integrins expressed by immune cells that result in a transition from a low to high affinity/avidity state facilitating the arrest of leukocytes to the endothelium [43,176]. The redundancy of these molecular activation signals of integrin avidity is apparent as more than 10 different chemokines are known to affect the integrin dependant arrest of various immune cells [42,160].

The enhanced binding properties of integrins and the upregulation of their counterparts in the endothelium under inflammatory conditions provide the ideal environment to increase the local transmigration of immune cells across BBB-ECs. While unactivated (resting) human and mouse BBB-ECs express low levels of the CAMs, inflammatory conditions (IFN-γ/TNF-α) dramatically upregulate the expression of intercellular adhesion molecule 1 (ICAM-1) (Fig. 2A), VCAM-1 (Fig. 2C) and the recently described [41], activated leukocyte cell adhesion molecule (ALCAM) (Fig. 3A) [76,156,209,210]. Such phenotype is associated with changes in the anatomical components of the BBB as the expression and morphology of TJ proteins and cytoskeletal components are disturbed (Fig. 3A). Likewise, the binding and migration of leukocytes increases through this inflamed endothelium [3,41,75,210]. Regarding *in situ* expression, ICAM-1 expression in ECs and its counter-ligand lymphocyte function-associate molecule 1 (LFA-1) in leukocytes were first described within MS lesions 20 years ago [181]. Likewise, ICAM-1 is known to be upregulated in CNS-ECs during EAE relapses. During subsequent remission ICAM-1 expression was however downregulated; each subsequent relapse was then characterized by corresponding upregulation [36]. High numbers of perivascular infiltrating cells positive for LFA-1 [126] and α4β1-integrin, the ligands for ICAM-1 and VCAM-1, but not for L-selectin or α4β7-integrin have been also detected in MS lesions [57,61]. Our own analysis of MS tissues confirms previous findings and correlates with *in vitro* data, as ICAM-1 and VCAM-1 are upregulated in blood vessels of MS lesions displaying both low and high levels of immune cell infiltration (Fig. 2B and D) when compared with counterparts in NAWM. Interestingly, upregulation of ALCAM in BBB-ECs was found to be localized at the areas of contact with immune cells and coincided with abnormal expression of TJ proteins (Fig. 3B), suggesting that ALCAM-mediated leukocyte migration was associated with a breach in the BBB. Moreover, in pre-active lesions where blood vessels display small perivascular infiltrates, leukocytes are mostly enclosed between the vessel and the parenchymal BM (Fig. 2B and D upper panels), whereas in active lesions fragments of ECM components can be detected in the periphery of the vessel, indicating extensive vascular reorganization (Fig. 2B and D lower panels).

13. Additional CAMs involve in leukocyte migration to the CNS

While leukocyte migration across CNS endothelium has long been thought to depend on prototypic BBB-associated endothelial CAMs (such as ICAM-1, VCAM and PECAM-1), recent studies have identified additional CAMs that regulate leukocyte transmigration across ECs during neuroinflammation. JAM-A has been implicated in the transmigration of different leukocyte subsets. The role of JAM-A depends on the model studied and is highly dependent on the nature of the neuroinflammatory stimulus [142]. CD44 is the hyaluronic receptor expressed on leukocytes and ECs and antibodies directed against this molecule limit migration of T cells to the CNS in EAE [29]. In MS CD44 expression is upregulated on T cells during relapses [182]. CD47 is an adhesion receptor expressed in brain ECs. Upon interaction with its ligands, the

signal regulatory proteins (SIRP)-alpha and SIRPgamma, CD47 modulates the migration of macrophages, dendritic cells and T cells into the inflamed CNS [44,188]. In addition, CD47 also inhibits phagocytosis and cytokine production in APCs [48]. Due to these inhibitory functions, CD47 has been considered as a "don't eat me signal" [145,214] that is downregulated in active MS lesions [105] and contrast with its role promoting leukocyte infiltration and neuroinflammation.

Molecules with adhesive and enzymatic functions such as CD73 and vascular adhesion protein-1 (VAP1) are also involved in leukocyte migration [133]. CD73 is an ectoenzyme that metabolizes adenosine monophosphate (AMP) precursor into adenosine, a molecule known to be anti-inflammatory and neuroprotective. IFN-β treatment in MS patients upregulates CD73 expression in CNS-ECs [139] and this correlated with reduce transmigration of CD4 T cells, rendering CD73 expression protective in MS [139]. Studies in EAE correlate with the findings made in human, but surprisingly, CD73 in murine CNS is mainly expressed in choroid vessels and not in their parenchymal counterparts [133]. Another polyfunctional adhesion molecule is VAP1, a semicarbazide-sensitive amine oxidase first detected in adhesion assays to inhibit lymphocyte binding to high endothelial venules [168]. Upon inflammatory conditions VAP-1 is rapidly translocated to the luminal surface of ECs [93], where it functions as an adhesion molecule and also contribute to leukocyte-EC interaction by inducing expression of adhesion molecules (P- and E-selectin and ICAM-1) and chemokines (CXCL8) [94,95,110]. Finally, VAP-1 relevance to neuroinflammation was recently demonstrated as (i) inhibitors of its enzymatic activity

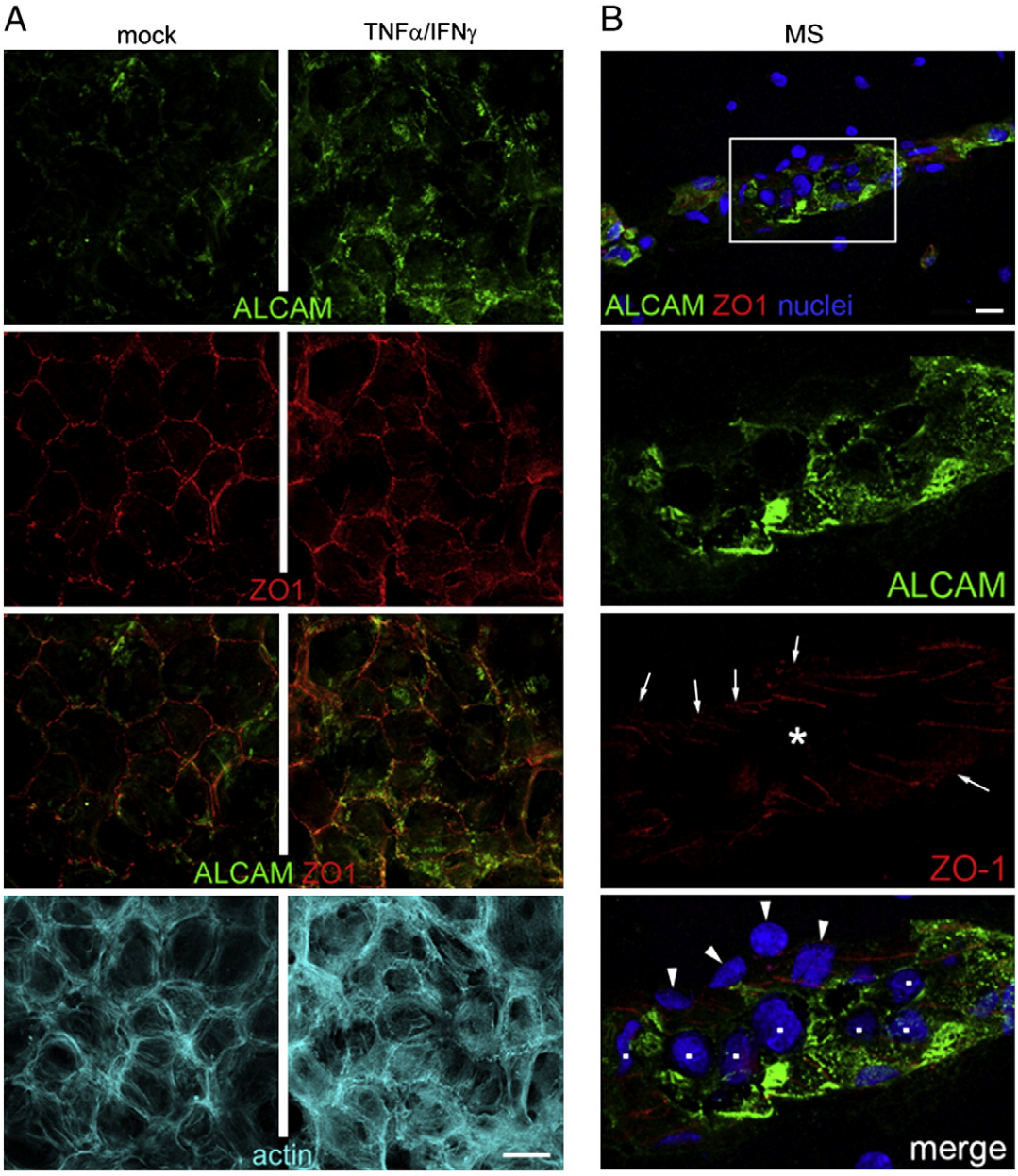

Fig. 3. ALCAM expression is upregulated in ECs under neuroinflammatory conditions. The expression of ALCAM is shown in cultures of human primary BBB-ECs (A) and in a MS lesion (B). ALCAM is depicted in green, the TJ protein ZO1 in red, actin (stained with phalloidin) in light blue (in A) and the nuclei in blue (in B). Scale bars indicate 10 μm. (A) ALCAM expression on the surface of BBB-ECs untreated (mock) or inflamed for 16 h with TNF-α and IFN-γ. The pattern expression of the TJ protein ZO1 and the cytoskeleton (F-actin) is altered in inflamed BBB-ECs (right panels). (B) ALCAM is upregulated in ECs of vessels associated with MS lesions. High power views of the inset in the upper panel show a vessel (asterisk), containing leukocytes (white dots) surrounded of $ALCAM^{+}$ endothelial membranes and extravasating cells (arrowheads). Areas of immune cell extravasation are associated with disrupted architecture and partial loss of intercellular junctions (arrows).

ameliorate inflammation in EAE [144] and (ii) high levels of VAP-1 have been detected in serum of patients with active MS [6].

14. Chemokine production by BBB-ECs

Even though BBB-ECs are able to express MHC class II and B7 co-stimulation molecules under inflammatory conditions they are considered to be poor or non-professional antigen presenting cells [157], unless this antigen presentation is supported by the presence of IL-2 or by fully competent APCs. Nevertheless, BBB-ECs and astrocytes of the NVU are potent producers of cytokines and chemokines. Inflammatory cytokines (IL-1, TNF-alpha, IFN-gamma, IL-17, Il-22) produce by CNS resident cells or by migrating leukocytes are known to induce cytokine and chemokine secretion by CNS-ECs. In the context of CNS inflammation, BBB-ECs produce and secrete pro-inflammatory cytokines and chemokines and have the capacity to immobilize chemokines on the luminal vascular surface [38,97,98]. BBB-ECs produce the cytokines IL-6, IL1α/β, GM-CSF and the chemokines MCP-1/CCL2, MIP-1α/CCL3, CCL5/RANTES, CXCL8/IL-8, CXCL10/IP-10 in response to inflammatory challenge [38,42,90,98,123]. Chemokines and cytokines are locally secreted and responsible for the recruitment and activation of immune cell. This is the basis for the inflammatory cascade that leads to the focal inflammation of BBB-ECs and the recruitment of bystander immune cells. The infiltrated bystander cells secrete additional cytokines and chemokines that may further enhance CNS inflammation or promote a gradual resolution of the inflammation. Although immune cell infiltration plays an essential role in MS lesion development, there are experimental evidences that suggest that BBB dysfunctions precede cell infiltration and that BBB activation actively participate in the initiation of lesion formation.

15. Leukocyte migration through the BCB in neuroinflammation

Aside from the morphological changes previously described in the CP during neuroinflammation, the low number of immune cells detected in this structure have made difficult to determine the role played by the BCB in EAE and MS. In this regard, leukocyte entry through the CP first requires binding and migration through the fenestrated endothelium. P-selectin has been the only adhesion molecule described in choroidal vessels under normal conditions and it appears to play role in facilitating migration of CD4 T cells [103]. The CAMs, ICAM-1 and VCAM-1 have not been detected in this endothelium under normal conditions [61,103,189], while in MS, but not in EAE, VCAM-1 seems to play a role in leukocyte transmigration as upregulated expression was detected in the choroidal endothelium of MS patients [199]. Once in the choroidal stroma, leukocytes migrate to the abluminal side of the choroid cells where a basal lamina presents as a barrier for the infiltrating cells. To date the mechanisms to cross this extracellular matrix have not been elucidated, but it can be speculated that immune cells used MMPs and other ECM degrading enzymes to transverse the basal lamina. It remains equally undetermined how leukocytes transmigrate from the abluminal side to the ventricle. In this sense, two possible mechanisms could be taking place, migration in between choroidal cells and affecting the junctional complex or transcellularly through the choroidal epithelium. Early studies in EAE have shown that the CAMs, ICAM-1 and VCAM-1 are upregulated in the choroid epithelial cells, but the expression is mostly limited to the apical side of the cells [62], suggesting that these CAMs may promote immune cell binding in the apical side of the choroid cells. Recent findings in MS contradict the EAE data as VCAM-1 was only upregulated in the choroidal endothelium, but not in the choroidal cells. VCAM-1 may be an important player in promoting leukocyte entry into the ventricular system as therapy with natalizumab considerably reduces the number of lymphocytes in the CSF [67]. Although other routes of immune cell migration into the CNS, such as the leptomeninges, the ependyma and the circumventricular organs cannot be discounted [8,103,173]. Thus, the role played by CAMs in the migration of leukocytes through the BCB requires further investigation and better correlation between MS and its murine model. The expression of non-classical adhesion molecules has been also studied in the CP. CD73 is a cell surface enzyme that catalyzes the breakdown of AMP to adenosine. CD73 expression is not detected on brain ECs of EAE mice, but high expression is found in the CP epithelium where it seems to regulate lymphocyte immunosurveillance between the blood and the CSF [133].

In addition to CAMs, the chemokine system also plays a fundamental role in leukocyte migration through the BCB. Recently, evidence for a chemokine receptor 6-CCL20-driven influx of pathogenic Th17 lymphocytes in EAE has been reported [164]. These important findings suggest that BCB might be a place of migration in EAE, in addition to BBB-related events involved in lesion formation. Confirmation of this novel mechanism for immune cell recruitment to the CNS in additional models of EAE and in human MS is however both awaited and needed.

16. Current and future BBB-related treatments

Current treatments of neuroinflammatory diseases aim at dampening the inflammatory cascade in the CNS and BBB-ECs are primary targets to limit the infiltration of CNS-specific leukocytes and to limit local inflammatory reactions. Most of the current therapies for MS have a rather broad mechanism of action and are anti-inflammatory, immunosuppressive or immunomodulating agents. Of interest to us is the integrin blocking antibody natalizumab. Natalizumab is a humanized monoclonal antibody against the leukocyte VLA-4 (VCAM-1 ligand). Treatment with this antibody (natalizumab) blocks the VLA-4 dependent migration of immune cell across the BBB-ECs and reduces the amount of lesions detected by MRI, but because of adverse secondary effects this drug is now highly controlled. This antibody is a very potent agent to limit leukocyte transmigration and indicate that endothelial CAM association with leukocyte integrins is a valid and effective method to limit neuroinflammatory reactions [58,161,190]. Despite the efficacy of Natalizumab to control signs and symptoms of MS, its use is unfortunately limited by the appearance of clinically important viral CNS infection, such as progressive multifocal leukoencephalopathy (PML). Emergence of PML in Natalizumab-treated MS patients is associated with a relative but persistent cellular immunosupression of the CNS compartment, related to VLA-4 neutralization.

However, based on the idea that immune cell exclusion from tissues should preclude inflammation, tissue entry of leukocytes is a particularly interesting target to modulate CNS-specific immune reactions, especially if more specific adhesion molecules can be identified on subsets of immune cells. Important research efforts are underway to identify novel adhesion molecules of the BBB and their counter-ligand on leukocytes using large scale, high-throughput methods (genomics and proteomics). Identification of adhesion molecules that would control the transmigration of selected leukocyte subsets would certainly be beneficial in organ-targeted inflammatory diseases and would probably demonstrate better long-term safety profiles, especially as regards the control of CNS inflammation and the progression of solid and metastatic tumors.

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