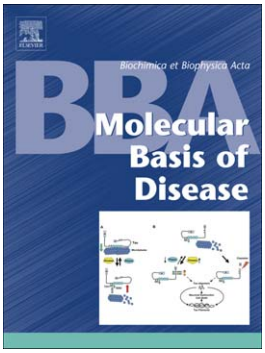

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Glial influences on BBB functions and molecular players in immune cell trafficking

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Summary

The blood-brain barrier (BBB) constitutes an elaborate structure formed by specialized capillary endothelial cells, which together with pericytes and perivascular glial cells regulate the exchanges between the central nervous system (CNS) and the periphery. Intricate interactions between the different cellular constituents of the BBB are crucial in establishing a functional BBB and maintaining the delicate homeostasis of the CNS microenvironment. In this review, we discuss the role of astrocytes and microglia in inducing and maintaining barrier properties under physiological conditions as well as their involvement during neuroinflammatory pathologies.

Keywords

Blood-brain barrier (BBB), Neurovascular unit (NVU), Neuroinflammation, Glial cells, Astrocytes, Endothelial cells, Microglia, Multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), Tight junction molecules, IL-17.

Inflammation of the central nervous system (CNS) can be induced by a wide variety of events, including infections, autoimmunity, traumatic brain injury and accumulation of misfolded proteins or toxic metabolites. Persistence of the inflammatory stimuli and/or failure to resolve the acute inflammatory response can lead to chronic inflammation of the CNS. Under homeostatic conditions, the CNS is widely regarded as an immunologically privileged site and its environment is well controlled and balanced due to the presence of the neurovascular unit (NVU), which comprises specialized endothelial cells (ECs), pericytes, basement membranes and supporting glial cells, including astrocytes and microglia. The NVU, acting as a blood-brain barrier (BBB), restricts the ingress of peripheral leukocytes and the movement of soluble factors into the CNS. Complex interactions between glial cells and BBB-ECs are essential to the barrier functions and its formation.

A common denominator of many neurological diseases is the loss of BBB integrity and the presence of immune cell infiltrates in the CNS. However, it remains unclear whether BBB dysfunction precedes immune cell trafficking, or if it is a consequence of peripheral immune cell activation. While the truth might lie somewhere in-between, one needs to keep in mind that underlying disease-dependent factors and patient's variability based on population heterogeneity make answering this question a challenging endeavour. Although this falls outside the scope of this review, we will, on the other hand, outline the influence of glial cells on BBB functions and discuss the various players involved in immune cell trafficking into the CNS.

Formation of the blood-brain barrier

CNS blood vessel formation starts with the invagination of the perineural vascular plexus. Tip cells guide the outgrowing capillaries towards a vascular endothelial growth factor (VEGF) gradient to form vascular sprouts, in a process known as angiogenesis [1, 2]. At this early stage of neurovascular development, the primitive network of CNS vessels lacks functional barrier properties and proper organization of tight junction (TJ) complexes. Multiple signaling molecules and transcription factors, notably produced by the radial glial cells, are involved in the establishment of the anteroposterior and dorsoventral axes, which are essential during embryogenesis [3]. Many of these factors, in addition to VEFG, are involved during angiogenesis: these include Notch1, Wnt, ephrins, fibroblast growth factors (FGFs), Sonic Hedgehog and retinoic acid (RA) [4]. In conjunction, all these signaling pathways remodel and stabilize the embryonic vasculature and play a major role during the development of the BBB [5-10]. Indeed, BBB properties are not intrinsic to CNS-ECs, but emerge gradually during a maturation/specialization phase dependent on the association of ECs that line the newly formed vessels with perivascular glial cells and pericytes. This was evidenced by transplantation experiments in which peripheral vessels acquired functional and histochemical BBB features following prolonged contact with neuronal tissue [11]. In contrast, meningeal blood vessels, which express TJs but are not in direct contact with glial cells, display higher vascular permeability, as compared to BBB-ECs [8].

Pericytes are known to help impart BBB properties to ECs. EC−pericyte interactions are primarily mediated by secreted Platelet Derived Growth Factor B homodimers (PDGF-BB), Notch, Transforming Growth Factor-β (TGF-β), and by N-

cadherin homotypic interactions [12]. Gap junctions formed by pairs of connexin 43 hemichannels also allow direct molecular communication between the cytoplasm of pericytes and BBB-ECs. Of note, in larger blood vessels, smooth muscle cells replace pericytes and form a continuous layer surrounding ECs [12].

Although the ratio of pericytes to ECs is highest in the CNS microvasculature, the critical role of astrocytes in regulating BBB maintenance and integrity cannot be overlooked. Histological studies using fetal human brain tissue have demonstrated the presence of astrocytes in the CNS around the 9th week of gestation, and direct astrocyte-endothelial cell contact 8 weeks later [13, 14]. In the fully developed CNS, perivascular astrocytic end-feet almost completely ensheath the abluminal surface of the CNS microvasculature [15] (Figure 1). Although astrocytes are separated from pericytes and ECs by a thin and compact double basement membrane (BM) (endothelial BM and parenchymal BM), connexins allow direct communication between these cellular constituents of the NVU [12]. In fact, astrocytes are responsible for inducing proper relative positioning of pericytes and ECs in tube-like structures *in vitro* [16] and are known to impact on BBB-ECs via different molecular pathways. Our group has shown that astrocyte-conditioned media induces or re-establishes partial BBB properties to *in vitro* cultures of meningeal and BBB-ECs, respectively [17, 18] (and non-published data).

Finally, beyond their important role during CNS neuronal development, microglial cells also contribute to CNS angiogenesis by promoting tip cell fusion and migration [19]. Evidence brought forward by Tammela et al. [20] shows that microglia-derived VEGF-C activates VEGF receptor-3 (VEGFR-3) in tip cells and promotes angiogenesis and vascular branching. Like astrocytes, microglia are found in close

proximity to the mature CNS vasculature, suggesting their involvement in regulating BBB functions in adults (Figure 2). But whereas numerous studies have focused on the role of microglia in BBB breakdown, few have addressed their potential contribution to maintaining BBB properties.

The concerted action of all these cellular components is required for the BBB to acquire and maintain its unique properties and functions. In the late stages of BBB formation, all these cellular inputs drive an increase in the organization and the complexity of TJs strands, forming inter-membranous networks of fusion points that can be visualized by freeze-fracture microscopy [21].

The blood-brain barrier endothelial cells

The BBB is a highly organised multicellular complex responsible for maintaining CNS homeostasis by constantly regulating the exchange of molecules with the systemic circulation and restricting the ingress of peripheral immune cells into the CNS. The restrictive permeability of BBB-ECs is attributed in part to their inherent low pinocytic activity and their high concentration of efflux transporters. These specialized ECs also lack fenestrae and are tightly bound together by TJ and adherens junction (AJ) molecules, located in the intercellular space between adjacent ECs (Figure 1). TJs form the apico-lateral barrier and are composed of at least two 4-pass transmembrane protein families: the claudins [22] and TJ-associated MARVEL proteins (TAMP) (occludin [23], tricellulin [24, 25], and MARVELD3 [26]). Ig-like adhesion molecules, such as junctional adhesion molecules (JAMs), coxsackie and adenoviral serotype 2/5 receptor (CAR) [27], and endothelial cell-selective adhesion molecule (ESAM) [28] are also

localized at the apico-lateral barrier. Together, these proteins form large molecular aggregates located in cholesterol-rich cell membrane regions called lipid rafts [18, 29]. Along with actin filament-anchored adaptor molecules zonula occludens (ZO-1, -2 and -3) [30], cingulin [31] and membrane-associated guanylate kinase protein family (MAGUK) [32], they form a macromolecular complex capable of recruiting various protein kinases, phosphatases, and transcription factors that regulate cell polarity, proliferation and differentiation [33]. However, the contribution of TJs and TJ-associated adaptor molecules to intra- and inter-cellular signalling, gene transcription and modulation of barrier function is still poorly understood.

AJs, which are located on the basal side of TJs, consist of transmembrane proteins of the cadherin family. BBB-ECs are known to express only two members of this family: vascular endothelial (VE)-cadherin and epithelial-cadherin (E-cadherin) [34, 35]. VE-cadherin associates via an extracellular domain with vascular endothelial protein tyrosine phosphatase (VE-PTP) [36]. A number of peripheral cytoplasmic proteins of the catenin family (α-, β-, P120) [37] also link cadherins to the cytoskeleton [38, 39]. Similarly, a third group of cell-cell junctional proteins called tight junction-associated cell adhesion molecules (TJaCAMs) are present at the basolateral side of BBB-ECs but are independent of either TJs or AJs. These proteins include CD99 [40], platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31) [41, 42], intercellular adhesion molecule 1 (ICAM-1) [43], melanoma cell adhesion molecule (MCAM/CD146/S-endo-1) [44, 45], integrins and other poorly-characterized proteins. TJaCAMs are known to have homophilic and heterophilic binding capacity and have been shown to mediate cell-cell or cell-basement membrane matrix adhesion [46, 47]. Together, all these junctional proteins

highly restrict leukocyte transmigration under homeostatic conditions and are crucial to the molecular and cellular biology of ECs.

Blood-brain barrier endothelial and glial cell interactions during homeostasis

Preserving neurovascular cell polarization by establishing a tightly controlled gradient between the luminal and abluminal surface of ECs is essential for maintaining the integrity and organization of TJ molecules, cell adhesion molecules, membrane receptors, ion channels, etc. The polarization of astrocytes is also critical for proper orientation of their own cellular machinery with respect to neuroglial cells and ECs, but also to establish the polarity of the BBB endothelium itself. This is exemplified in astrocyte end-feet, which are enriched in water channel aquaporin 4 (AQP4) and in inwardly rectifying potassium channel Kir4.1, both integral parts of the orthogonal arrays of particles (OAPs) [48] (Figure 3). OAPs are segregated towards the perivascular space through a specific isoform of agrin, a heparin sulphate proteoglycan located within the CNS vascular basement membranes. NtA-agrin 0, A/y0, B/z0 is the long N-terminus variant (basal laminae-binding region) that does not incorporate any amino acid at the three different possible insertion sites [49, 50]. This isoform is secreted by both ECs and astrocytes [51, 52]. NtA-agrin forms, along with α-dystroglycan, α1-syntrophin, laminin 1 and 2, perlecan and others, a molecular scaffolding complex (dystrophin–dystroglycan complex) that binds to OAPs and links various cytoskeletal-associated proteins [53, 54]. This protein complex is required to provide optimal BBB properties, as the deficiency in one of its members, glial-α-dystrobrevin, leads to an increase in BBB permeability, water retention and can cause progressive brain oedema. This phenotype is associated with the

loss of OAPs and the formation of intracellular vacuoles in astrocyte end-feet. These defects are thought to modify astrocyte-endothelial interactions and cause a dysregulation of TJ molecule expression [55]. However, Haj-Yasein et al. [56] have demonstrated that the absence of AQP4 in astrocytes significantly reduces CNS water intake and consequently, brain oedema, using a stroke model in AQP4 knockout (KO) mice. It is worth pointing out that these animals have an intact BBB, unlike α-dystrobrevin-deficient mice. These studies highlight the inherent complexity of the dystrophin–dystroglycan complex in regulating BBB properties.

A growing body of evidence points towards the critical role of astrocytes in inducing BBB features and functions by promoting proper expression and assembly of intermolecular junctions [17, 57], transporters (permeability glycoprotein 24 (Pgp24), glucose transporter 1 (GLUT1), etc.) [58], and enzymatic pathways [59, 60]. Studies have also convincingly demonstrated that astrocyte-secreted factors, including VEGF, TGF-β, glial-derived neurotrophic factor (GDNF), FGFs, and angiopoietin-1 (ANG1), are at least partly responsible for modulating BBB functions [61, 62]. Angiopoietins bind BBB-ECs via the tyrosine kinase Tie-2 receptor. Specifically, ANG1 participates in angiogenesis and vascular homeostasis by upregulating the expression of TJs, thereby reducing EC permeability [63, 64]. Similarly, FGFs promote BBB-EC tightness by regulating catenin–VE-cadherin interactions. Murakami et al. [65] have demonstrated that FGF blockade triggers VE-cadherin internalization, which leads to a loss of BBB integrity. FGFs presumably act by downregulating VEGF downstream signalling, thus preventing a destabilization of AJs. VEGF is primarily known as a pro-angiogenic factor that promotes the growth of ECs through VEGFR-1,-2 and as such, can disrupt the BBB [66].

Conversely, GDNF has been shown to decrease vascular leakage by significantly increasing the trans-endothelial electrical resistance (TEER) of BBB-ECs via an upregulation of claudin-5 expression. Although astrocytes are the main source of GNDF, pericytes have also been shown to secrete it [67, 68]. Finally, of all astrocyte-secreted factors, TGF-β is undoubtedly the most studied and paradoxically one of the least well understood molecules. This pleiotropic cytokine is secreted by, and acts on a wide variety of cell types. It is involved in a plethora of cellular processes, including apoptosis, wound healing, embryogenesis, cell proliferation and differentiation [69-73]. Although TGF-β was originally regarded as an anti-inflammatory cytokine, mainly because of its role in promoting regulatory T cell functions, we now know that it also drives the differentiation of pro-inflammatory Th17 cells [74]. Emerging evidences highlight the dual role of TGF-β and its increased expression in several neuroinflammatory diseases, including multiple sclerosis (MS) [73], Alzheimer's disease (AD) [75] and stroke [76]. TGF-β has a neuroprotective role in the developing CNS through its effects in modulating the expression of Pgp efflux transporter on BBB-ECs and astrocyte end-feet, thereby restricting the penetration of xenobiotics into the CNS [77]. In a recent study, Pgp expression was shown to be significantly downregulated by BBB-ECs within the inferior colliculus following chemically induced focal astrocyte-microglial cell death. During the subsequent repopulation phase, a prolonged astrogliosis was observed in the affected area, while Pgp expression returned to its basal level [78]. Assessment of gadolinium and fluorescently labelled-dextran leakage also demonstrated BBB integrity disruption. This increase in permeability was maintained till the end of the regenerative phase. Altogether, these experiments highlight the role of astrocytes in maintaining Pgp and TJ expression

in BBB-ECs [78-80]. Many glial factors, including the aforementioned ones, play a prominent role in regulating the physiology and functions of the BBB during homeostasis. However, they may be differentially modulated in a neuroinflammatory context, either to promote tissue repair and prevent inflammation or to increase immune cell transmigration and BBB permeability.

Leukocyte transmigration across the CNS barrier

In neuroinflammatory disorders such as MS, pro-inflammatory encephalitogenic immune cells migrate across the BBB to gain access to the CNS and cause disease. This process has been extensively studied over the last three decades and is thought to involve a sequential cascade of events [81-85] (Figure 4). Initial contact between blood circulating leukocytes and the vascular endothelium is mediated by fluid dynamics. Leukocytes flowing into small capillaries and post-capillary venules tend to be off-centered, a phenomenon called margination [86]. This is caused by the aggregation of rapidly flowing red blood cells (RBCs) in the center of blood vessels, as a result of their biconcave shape. When immune cells exit capillaries to enter post-capillary venules, RBCs push slower leukocytes on the vessel wall, at the blood-endothelium interface, where they are then free to probe the surface of BBB-ECs for potential ligands [86-88]. Not only are post-capillary venules physically favoured by fluid dynamics as the transmigration site of immune cells, but studies have also identified differences between the endothelial cells of post-capillary venules and the ones forming the rest of the vascular network. Electron microscopy studies have shown disorganization in TJ and AJ molecules in post-capillary venule ECs. These results corroborated previous data showing that post-capillary vessels cannot completely prevent the leakage of exogenous

tracers into the perivascular space [89]. Additionally, recent work by the group of Dr. Sorokin has demonstrated that CNS post-capillary endothelial basement membrane lacks laminin α5, which selectively inhibits immune cell extravasation [90, 91]. These findings are substantiating experiments demonstrating the presence of different constituents in the venule basement membrane in periphery [92]. Collectively, this provides an explanation for the transmigratory tropism of leukocytes towards post-capillary vasculature in the CNS.

The short initial interaction between BBB-ECs and leukocytes is termed *capture*, *rolling* or *tethering* and is mediated by selectins and vascular cell adhesion molecule 1 (VCAM-1), expressed on the surface of BBB-ECs, and their respective ligands: carbohydrates and α4-integrins expressed on immune cells. Slow rolling along the vessels' lumen allows leukocytes to sample chemokines secreted under inflammatory conditions, such as interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1 or chemokine C-C motif ligand 2 (CCL2)), regulated on activation, normal T cell expressed and secreted (RANTES or CCL5) and interferon gamma-induced protein 10 (IP-10 or chemokine C-X-C motif ligand 10 (CXCL10)) [93, 94]. This triggers G-couple protein signalling and initiates an intracellular cascade that leads to integrin clustering and enhances their binding affinity for their ligands, a step called *activation* [95]. Integrin clustering allows adhesion strengthening and facilitates leukocytes firm *arrest*. This step is also mediated by endothelial cell adhesion molecules (CAMs: ICAM-1, VCAM-1, MCAM, Ninjurin and activated leukocyte cell adhesion molecule (ALCAM)), the expression of which is highly upregulated upon inflammatory stimuli that originate from the parenchyma or from the release of pro-inflammatory mediators by leukocytes [39, 96,

97]. Integrin/CAM-mediated cell adhesion is followed by *crawling* of immune cells preferentially against the direction of blood flow to find a permissive site for transmigration. Intraluminal crawling is dependent on ICAM-1 and ICAM-2 [98]. Of note, under certain circumstances, immune cells may adhere directly to BBB-ECs without apparent rolling [96, 99, 100]. However, it remains unclear whether prior *rolling/activation* occurred upstream of the observation site, or if it is a dispensable step of the leukocyte recruitment cascade. The final step of the cascade, *diapedisis*, is preceded by the formation of a docking structure called "transmigratory cup", whereby endothelial pseudopods reach out and surround the migrating leukocytes [101-103]. The existence of transmigratory cups, initially identified in non CNS-ECs, was confirmed using human and mouse CNS ECs [96, 97, 100]. Diapedisis can occur via two independent pathways: paracellularly, where leukocytes migrate in-between adjacent BBB-ECs or transcellularly, in which case, the immune cells extravasate directly through individual ECs by forming invasive podosomes [104-106].

In spite of the increasing knowledge on the mechanisms involved during diapedisis, the molecules that dictate whether a leukocyte extravasates via the paracellular or the transcellular routes are still ill-defined. [107]. For example, several TJaCAMs (Junctional adhesion molecule-A (JAM-A), CD99, PECAM-1 and MCAM) have been detected on both the apical and the basolateral surface of BBB-ECs under inflammatory conditions and therefore, could be involved in either transmigration processes [108]. One mechanism that was put forward to explain this dichotomy is the internalization of the aforementioned molecules in a complex lateral border-recycling compartment, whose functions are not fully understood yet but thoroughly discussed by

Muller et al. [109, 110]. In sharp contrast, TJ and AJ molecules involved in diapedesis, such as ESAM and VE-cadherin, are present exclusively in the intercellular space between adjacent ECs and thus, could only participate in paracellular migration [110]. However, a 2008 study has challenged this view by showing that leukocytes, in fact, migrated through ECs, albeit in close proximity to the intercellular space, without disrupting the continuity of junctions; thereby arguing against the concept of paracellular transmigration [111]. In a disease context, pro-inflammatory leukocytes can contribute to BBB disruption and increase its permeability by virtue of the cytokines they release [39, 82, 109]. In addition, transendothelial leukocyte migration can itself perpetuate the inflammatory cascade by favoring subsequent site-specific immune cell infiltration [112]. Finally, a number of yet unidentified molecules could play an important role in initiating site-specific transmigration. Therefore, this aging conundrum stays as of yet unresolved.

Glial influences on the blood-brain barrier during neuroinflammation

While the migration process itself can modify the characteristics of leukocytes and that of BBB-ECs [113-115], astrocytes and microglia also play fundamental roles in regulating leukocyte effector function, either directly or via the modulation of BBB-EC phenotype. Our group has shown that the loss of BBB integrity associated with a dysregulation of TJ molecules occurs early during lesion formation and coincides with perivascular astrogliosis and upregulation of endothelial CAMs. These findings suggest that BBB disruption precedes any overt CNS immune cell infiltration and might be a direct consequence of glial cell activation [116]. Small molecules involved in cell signalling and metabolism can spread rapidly through the continuous astrocyte network

formed by gap junctions, and also referred to as the *astrocytic syncytium* [117, 118]. This network allegedly also serves to coordinate effectively and unify the immune response to stimuli, either to maintain homeostasis or to propagate pro-inflammatory signals (Figure 3) [119]. As such, this mechanism might be implicated early during lesion formation. However, evidence against this hypothesis also exists: Brand-Schieber et al. demonstrated a significant decrease in connexin 43 expression in astrocytes within inflammatory lesions during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS [120]. It remains unclear whether this decreased expression translates into loss of function, and if it precedes focal inflammation. Another study has shown that the absence of astroglial connexin 43 promotes the recruitment of leukocytes via the activation of BBB-ECs (upregulation of CAM expression and chemokine secretion), effectively compromising CNS quiescence [121]. Although the absence of connexin 43 is associated with a progressive weakening of the BBB, the immune cell infiltration was not the result of an early BBB breakdown or glial cell activation in these KO animals. Unlike in other neuroinflammatory conditions, the immune cell response within the CNS of these animals was efficiently and rapidly controlled [121]. This study demonstrates an essential role for connexin 43 in maintaining CNS homeostasis, but also reveals that its absence does not, by itself, trigger a chronic autoimmune reaction. Furthermore, another study from the same group reports a significant loss of AQP4 and β-dystroglycan in astrocyte end-feet, associated with a disrupted BBB and brain oedema in mice deficient for astrocytic expression of connexin 43 [122]. These findings are in agreement with several studies which correlated CNS inflammation with impaired segregation of AQP4 at the astrocyte end-feet, as well as a disruption of dystrophin–

dystroglycan complex; both involved in the regulation of brain water uptake [123]. On the other hand, another study demonstrated that the loss of both connexin 43 and 30, in double KO mice, resulted in a much worse phenotype, namely widespread white matter pathology (oedema and vacuolation of astrocytes and oligodendrocytes) accompanied with region-specific astrocytic abnormalities [124]. Yet, it is unclear whether modulation of connexin and/or OAPs expression during neuroinflammation is beneficial or detrimental for disease resolution. In fact, reactive astrocytes can either elicit a pro- or an anti-inflammatory response.

Our group has recently uncovered the critical role of astrocyte-secreted Sonic hedgehog (Shh) in dampening CNS inflammation. We showed that Shh, which expression is upregulated upon inflammatory challenge, alleviates neuroinflammation via activation of the signal transducer Smoothened (Smo), transcription factors of the Gli family and the Shh receptor Patched-1 (Ptch-1) expressed by BBB-ECs. BBB-ECs treated with Shh showed a reduced expression of CAMs, a decreased chemokine secretion and an increased TEER. In addition, *in vivo* permeability experiments performed on endothelial-specific Smo KO mice revealed a significant increase in BBB permeability that correlated with a dysregulated expression of TJs and disrupted BMs, as compared to WT animals. [8]. We further recently demonstrated that Netrin-1 is a downstream effector of Shh that promotes BBB phenotype and functions during homeostasis and inflammation, via an autocrine signalling pathway. Netrin-1 treatment during EAE significantly reduced BBB disruption by upregulating endothelial junctional protein expression, while alleviating the clinical and pathological indices of disease. Aside from its ascribed role of promoting BBB formation, the hedgehog pathway acts

with Netrin-1 as an important molecular repressor of CNS inflammation, while promoting BBB repair and integrity [125].

In order to control inflammation and promote homeostasis, astrocytes actively internalize glutamate and produce metabolizing enzymes and antioxidants, thus playing an important role in scavenging reactive oxygen species (ROS) and extracellular glutamate [126]. In a pro-inflammatory environment, activated astrocytes also selectively induce Toll-like receptor-3 (TLR3), which mediates the secretion of anti-inflammatory cytokines and neurotrophic factors [127]. Moreover, astrocytes can secrete angiotensinogen (AGT): a BBB-promoting factor that is cleaved into angiotensin-II, who then binds to type 1-angiotensin receptors expressed on BBB-ECs. Triggering this signalling cascade was shown to tighten the BBB via phosphorylation of occludin and its mobilization into intercellular lipid raft microdomains. In MS lesions, the expression of AGT by perivascular astrocytes is downregulated in a pro-inflammatory cytokine-dependent fashion, and is paralleled with a decreased expression of occludin on BBB-ECs. These findings were confirmed in AGT deficient animals, highlighting the ability of reactive astrocytes to contribute to BBB breakdown during neuroinflammation [18].

In contrast to AGT, several other astrocyte-secreted factors have been reported to promote inflammation in the CNS. Amongst them, VEGF has the capacity to disrupt the integrity of the BBB by modulating the expression of junctional molecules, including VE-cadherin, claudin-5 and occludin; therefore, promoting immune cell migration into the CNS. The secretion of IL-1β by activated microglia can also trigger the release of VEGF and thymidine phosphorylase (TYMP) by astrocytes [128, 129]. TYMP produces 2-deoxy-D-ribose which, along with VEFG, disrupts TJ proteins and increase BBB

permeability. Both factors are also driving transcriptional pathways that result in the production of angiogenic and permeability genes [130]. However, it should be noted that VEGF can trigger independent pathways depending on which side of the BBB-ECs it is being secreted. A group has shown that the presence of VEGF on the abluminal side triggers signaling from VEGFR2, which in turns increase BBB permeability via p38. On the other hand, luminal VEGFR1 is activated by circulating factors and initiates Akt signaling pathway to promote cytoprotection. This highly polarized expression of VEGF receptors was found on CNS microvasculature, but not in peripheral vessels; illustrating the importance of BBB-ECs' polarity in responding to inflammatory cues originating from the blood or parenchymal tissue [131].

Apart from its direct role on the BBB, VEGF is also known to upregulate the surface molecule ephrinB2 on different cell subsets [132]. EphB receptors and ephrinB ligands are preferentially expressed by arterial ECs, but also by smooth muscle cells and pericytes [133, 134]. EphB2 was recently shown to be essential during blood vessel assembly suggesting a possible link between astrocyte-controlled angiogenesis, VEGF and EphB2-ephrinB2 [135]. Like VEGF, ephrins are also involved in neuroinflammation, particularly in leukocyte trafficking. They do so by acting as adhesion molecules or by regulating their activity. They have also been shown to modulate directly the activity of immune cells. For example, ephrinB2 was reported to be involved in T cell co-stimulation [64]. Our group is currently working on a study demonstrating that pro-inflammatory Th17 cells express high levels of ephrinB1 and ephrinB2, which facilitate their transmigration into MS lesions. This study also provides evidence that these ephrins

are essential for Th17 differentiation and pathogenicity in the context of neuroinflammation [136].

VEGF, along with FGFs, can also indirectly promote neuroinflammation by modulating the secretion of angiopoietin-2 (Ang-2) by BBB-ECs. Ang-2 is synthesized and stored, like multiple other pro-inflammatory factors (IL-8, selectins), in endothelial granules called Weibel-Palade bodies that are involved in the rapid response of ECs to external stimuli. Recent experiments have demonstrated that Ang-2 deficient animals are unable to trigger a prompt inflammatory response following infection. An in-depth analysis of the phenotype of Ang-2 KO mice revealed that Ang-2 is involved during the adhesion process, but does not affect the rolling ability of activated leukocytes. Ang-2 might also increase BBB permeability by regulating endothelial junctional molecules and integrins that bind to the matrix [64] and potentiate endothelial responses to pro-inflammatory cytokines, thus promoting the upregulation of CAMs [137].

Aside from leukocytes, which are the main source of cytokines in the inflamed CNS, activated astrocytes and microglia are known to secrete an array of pro-inflammatory molecules, including ROS, IL-1β, IL-6, and IL-17A, to name a few. Meeuwsen et al. reported the production of IL-17A (also known as IL-17) by stimulated astrocytes, although the relevance of their findings was not clear at the time [138]. Later, Tzartos et al. detected IL-17 mRNA and protein in perivascular lymphocytes but also in astrocytes and oligodendrocytes found within active MS lesions; clearly demonstrating that IL-17A is secreted by a broad spectrum of neuroglial cells, and that it might be implicated in pathways far more complex than previously anticipated [139]. More recently, Zimmermann et al. have shown, using glial fibrillary acidic protein (GFAP)-

driven IL-17A transgenic mice, that overexpression of IL-17A by astrocytes alone can activate glial cells, but is not sufficient to cause parenchymal infiltration, demyelination or neurodegeneration. However, lipopolysaccharide (LPS)-induced endotoxemia in these animals triggers enhanced microglial activation along with increased pro-inflammatory cytokine secretion, as compared to controls [140]. Another study has demonstrated that IL-17A could promote neuronal injury in a dose-dependent manner *in vitro* [141]. Furthermore, microglia, and to some degree astrocytes, are also known to express IL-17A and IL- 17C receptors (IL-17RA, IL-17RC), which upon intracellular signalling can potentiate tumor necrosis factor (TNF) effects, and trigger the release of pro-inflammatory cytokines and a wide range of chemokines involved in leukocyte trafficking, such as CXCL1 (GROα), CCL2 (MCP-1), CCL3 (macrophage inflammatory protein 1α (MIP-1α)), CCL20 (MIP-3α), CXCL2 (MIP-2), CXCL9 (monokine induced by gamma interferon (MIG)), CXCL10 (IP-10) and CXCL11 (IP-9) [142-147]. Of note, MCP-1 was shown to increase BBB permeability by binding BBB-EC–expressed chemokine C-C motif receptor 2 (CCR2), which in turn activates small GTPase Rho and Rho kinases to trigger the reorganization of the actin cytoskeleton and redistribution of TJ proteins [148]. Conversely, IL-17A was also shown to displays CNS protective function by inducing neuronal repair via the expression of GDNF, brain-derived neurotrophic factors (BDNF) and nerve growth factors (NGF). It is worth pointing out that whereas human IL-17A can bind both IL-17RA and IL-17RC receptors, mouse IL-17A only binds IL-17RA [149]. ECs, and even neurons, also upregulate their expression of IL-17RA receptor in the inflamed CNS. We have shown that IL-17RA is expressed at high levels on blood vessels within active MS lesions and that IL-17 increases BBB

permeability in a dose-dependent manner, by disrupting TJ molecules. Similar to its effect on glial cells, IL-17 induces the secretion of pro-inflammatory cytokines and chemokines by BBB-ECs (IL-6, CCL2 and CXCL8), therefore, promoting leukocyte recruitment into the CNS [114].

IL-6 is a pleiotropic cytokine produced by astrocytes [150], brain endothelial cells [93] and microglia [150, 151] that is subjected to autocrine regulation. IL-6 expression can be induced by IL-17 alone or in synergy with TNF or IL-1β. IL-6 has both neurotrophic effects (promotes neuron survival via BDNF [152]) and pro-inflammatory functions [153]. Therefore, both glial- and endothelial-produced IL-6 can influence BBB properties by modulating the expression of different CAMs, cytokines and chemokines [154]. IL-6 also induces the differentiation of naïve T cells into Th17 lymphocytes, in the presence of TGF-β. Th17 lymphocytes secrete IL-17, who acts in an autocrine feedback loop to stimulate the production of IL-6 by astrocytes. Within the CNS, Th17 lymphocytes can induce the expression of pro-inflammatory mediators by astrocytes and microglia through a contact-dependent manner, thus promoting myelin and neuronal damage [149, 155].

Another cytokine of the IL-6 family, oncostatin M (OSM) is highly produced by peripheral blood mononuclear cells (PBMC) obtained from MS patients [156]. It is also expressed by activated astrocytes and microglia surrounding MS lesions; suggesting that these cells might be an important endogenous cerebral source of OSM, in compromised CNS. OSM alone or in synergy with TNF has been shown to stimulate the expression of ICAM-1 and the production of MCP-1 and IL-6 by BBB-ECs [157]. It can also act as a positive regulator of IL-6 expression by astrocytes [153]. More recently, OSM was also

shown to decrease the TEER of rat BBB-ECs and impact on the integrity and organization of TJ molecules such as ZO-1 and claudin-5 [158]. Therefore, IL-6 family cytokines, secreted by astrocytes and microglia during neuroinflammation, contribute directly and indirectly to leukocyte trafficking across the BBB.

Reactive astrocytes and activated microglia are also known IL-15 producers. This pro-inflammatory cytokine induces the expression of hyaluronan on ECs, which in turn allows the extravasation of activated T lymphocytes into the CNS via a CD44-dependent adhesion cascade [159]. ECs themselves can secrete IL-15 and therefore, directly enhance the capacity of T lymphocytes to migrate into the CNS. IL-15 can also increase the avidity of lymphocyte function-associated antigen-1 (LFA-1) integrin to its ligand and affect the motility of immune cells [160]. Most notably, IL-15 is known to be involved in the activation of natural killer (NK) cells and can promote clonal expansion of NK-like $CD4^+$ T lymphocytes, who exert cytotoxicity towards vascular ECs [161]. Another way by which IL-15 is implicated in neuroinflammation is by virtue of its role in maintaining and activating cytotoxic $CD8^+$ T lymphocytes, known to contribute to MS pathogenesis. Saikali et al. have demonstrated that following stimulation with IL-15, $CD8^+$ T lymphocytes up-regulated lytic enzyme production, NKG2D expression and antigen-directed cytotoxicity, which increased their ability to kill glial cells and migrate across BBB-ECs [162]. Our group has recently confirmed these data in a study which identified MCAM as a marker of encephalitogenic $CD8^+$ T lymphocytes [163]. A report by Schneider et al. also shows that MS patients have elevated levels of IL-15 in their blood, as compared to healthy controls, and demonstrates that MS patient immune cells are more susceptible to the effects of this cytokine [164]. The fact that the majority of

astrocytes and microglia present within demyelinating lesions express high levels of IL-15 underscores the critical role for IL-15 orchestrating multiple aspects of chronic inflammation in the CNS [162].

Activated microglia produce ROS in response to NADPH oxidase activation. ROS are in part responsible for oligodendrocyte degeneration following oxidative damage during neuroinflammation [165]. While they can cause direct damage, ROS can also increase BBB permeability by activating the PI3K/ATK pathway and by decreasing the expression of VE-cadherin, occludin and claudin-5 in BBB-ECs [166-168]. By doing so, the release of ROS facilitates leukocyte transmigration and allows leakage of plasma protein into the CNS, including fibrinogen. In a recent study, Davalos et al. have demonstrated a direct effect of fibrinogen on the activation state of microglia using two-photon live imaging. They observed an increased motility of microglia to form perivascular clusters prior to the development of CNS lesions [169]. Collectively, these data point to a feed-back/amplification mechanism between ROS production by microglia, BBB dysfunction, leakage of fibrinogen into the CNS and further activation of microglia, leading to enhanced ROS production. While we can speculate that, in experimental models of neuroinflammation, peripheral inflammation causes BBB leakage, which then induces activation of perivascular glial cells, the situation might be different in a disease context. In human neuroinflammatory diseases, it remains unclear whether glial-secreted inflammatory mediators induce the initial BBB breakdown, which causes the leakage of serum proteins that in turn promote glial cell activation in a positive feedback loop. The general consensus today is that microglia activation upregulates

CAMs on the surface of brain ECs and compromises the integrity of the BBB in neurodegenerative disorders such as Alzheimer's disease and in MS [170-174].

Once they have crossed the BBB, infiltrating leukocytes accumulate in the perivascular space between the two BMs (Figure 1). In order to gain access to the CNS parenchyma, pro-inflammatory leukocytes need to secrete matrix metalloproteinase-2 (MMP-2) and MMP-9, as well as extracellular matrix metalloproteinase inducer (EMMPRIN), which are required to cleave fibronectin, laminin and dystroglycans that form the parenchymal BM [175, 176]. As such, the parenchymal BM and the astrocyte end-feet constitute an additional physical barrier that renders the BBB more selective and tightly controlled, especially in an inflammatory environment. Microglia can also increase BBB permeability through secretion of MMPs (MMP-1, -2, -3, -9 and -19), which significantly destabilize the parenchymal BM. Microglial-produced MMPs have been detected within pre-active and active MS lesions, which suggest their possible involvement in early disease pathogenesis [177].

In spite of the remarkable increase of knowledge regarding the role of astrocytes and microglia during CNS inflammatory responses, further studies are needed to better understand their involvement in promoting leukocyte transmigration and BBB dysfunction, as well as their protective role in supporting cellular regeneration and CNS repair. It is becoming increasingly clear that the concomitant expression of multiple molecular and cellular effectors is required to initiate and sustain neuroinflammation, providing a mere glimpse into the complexity of the neuro-immune-glial crosstalk.

Conclusion

In this review, emphasis was placed on the influence of glial cells on BBB properties and functions. Nevertheless, it should be kept in mind that the NVU is a complex multicellular structure whose members constantly interact to modulate each other's functions, under both physiological and pathological conditions. As such, the BBB not only protects and maintains homeostasis of the CNS, but it also allows tightly-controlled communication with the periphery. As discussed in this review, glial cells are able to influence BBB physiology/phenotype by secreting a number of factors. However, our understanding of the cellular sources and the molecular signals involved in regulating BBB properties is undoubtedly far from complete. The existence of glial cell heterogeneity within the CNS and the redundancy of molecular pathways that regulate the unique properties of the BBB add to the complexity of understanding the intricate interactions between the cellular constituents of the NVU and how they drive neuroprotective/neurotoxic responses.

BBB breakdown is a hallmark of several neuropathologies. Therefore, therapeutics aimed at repairing the BBB or restoring its steady state could undoubtedly provide significant clinical benefits, especially if administered early in the disease course. Currently, the only available and widely used therapeutic approach to improve BBB integrity is glucocorticosteroid treatment. However, their use can lead to an overt suppression of the immune response in the periphery. Thus, further understanding the cellular and molecular interactions at the NVU will help in designing novel targeted therapies against neuroinflammatory disorders.

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Figure legends

Figure 1. The neurovascular unit (NVU). The NVU is composed of specialised endothelial cells (ECs) surrounded by two basement membranes and perivascular astrocytes and microglia. Essential in maintaining the blood-brain barrier integrity, the different tight junction and adherens junction molecules efficiently link together adjacent ECs, as shown in the magnified window. In the context of neuroinflammation, leukocytes accumulate in the perivascular space localized in-between both basement membranes. Upon acquiring the capacity to secrete matrix metalloproteinases (MMPs), these leukocytes migrate into the central nervous system. *ZO-1, -2, -3: Zonula occludens / PECAM-1: Platelet endothelial cell adhesion molecule 1 / VE-cadherin: vascular endothelial-cadherin (cadherin 5)*

Figure 2. Microglial interaction with the CNS vasculature. Human brain microglia (Iba1, red) is shown to interact with capillaries (Laminin, green) in both normal appearing white matter (top) and multiple sclerosis lesion (bottom) by confocal microscopy. Nuclei = blue, Topro-3. Scale bar = 10 μm.

Figure 3. Paracrine and autocrine regulation of the BBB during homeostasis and inflammation. Perivascular cells such as pericytes and glial cells have a major impact on blood-brain barrier (BBB) functions. In homeostatic conditions, these cells provide a wide variety of secreted factors which, along with contact-dependent interactions, induces barrier properties. However, upon central nervous system inflammation, every cellular constituent of the neurovascular unit can promote barrier breakdown and

facilitate immune cell transmigration. *N-cadherin: Neural cadherin / Pgps: Permeability glycoproteins / Glut1: Glucose transporter 1 / Smo: Smoothened / Ptch: Patched-1 / PDGFRβ: Platelet-derived growth factor receptors β / PDGF-BB: Platelet-derived growth factor subunit B / FGFs: Fibroblast growth factors / GDNF: Glia-derived neurotrophic factor / AQP4: Aquaporin 4 / ANG1-2: Angiopoietin-1-2 / AGT: Angiotensinogen / Shh: Sonic hedgehog / MMPs: Matrix metalloproteinase / ROS: Reactive oxygen species / NOS: Nitric oxide synthase / TGF-β: Transforming growth factor β / VEGF: Vascular endothelial growth factor / OAPs: Orthogonal arrays of particles / TNF: Tumor necrosis factor*

Figure 4. The transmigration cascade. The first step in this cascade of events is termed rolling, which initiate the interaction between leukocytes and activated endothelial cells (ECs). It is mediated by various selectins and their ligands. Chemokines secreted by the ECs trigger an increase in the binding affinity and avidity of integrins for their ligands, which mediates the complete arrest of immune cells. This adhesion step is also mediated by a wide variety of cell adhesion molecules. The adherent leukocytes then undergo a process called crawling, where they move against the blood flow in order to find a suitable area to migrate across the blood-brain barrier-EC layer. *PSGL1: p-selectin glycoprotein ligand 1 / ESL1: E-selectin ligand 1 / PECAM-1: Platelet endothelial cell adhesion molecule 1 / ICAM-1,-2: Intercellular adhesion molecule 1,2 / JAM-A: Junctional adhesion molecule / ESAM: endothelial cell-selective adhesion molecule / ALCAM: activated leukocyte cell adhesion molecule / VCAM: vascular cell adhesion*

molecule / MCAM: Melanoma cell adhesion molecule / VLA-4: very late antigen 4 (integrin $\alpha 4\beta 1$) / LFA-1: Lymphocyte function-associated antigen 1

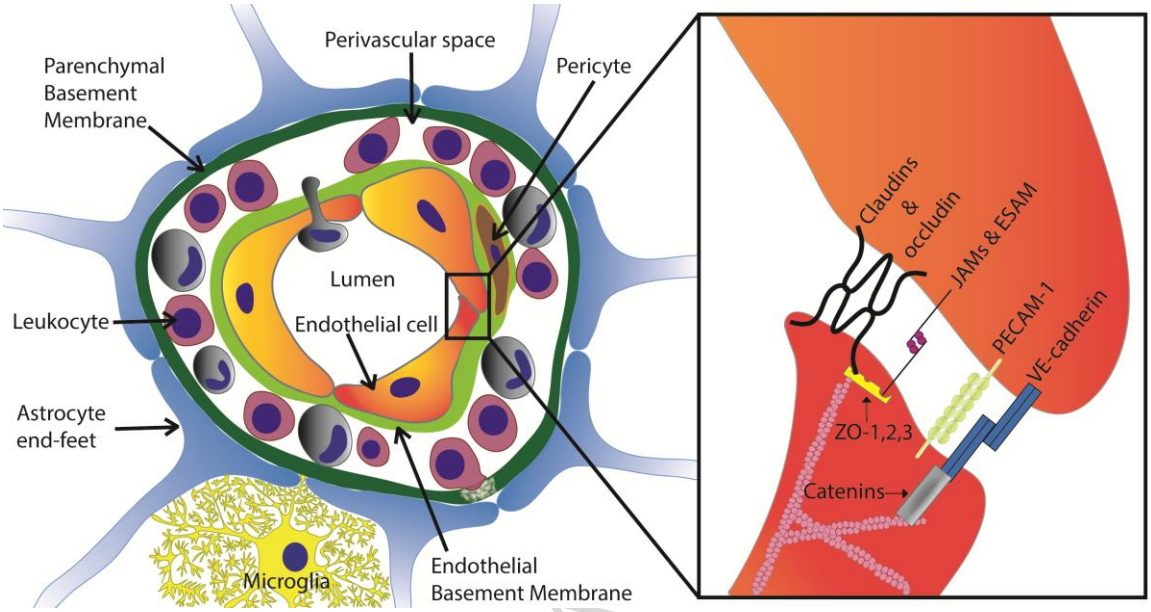

Figure 1

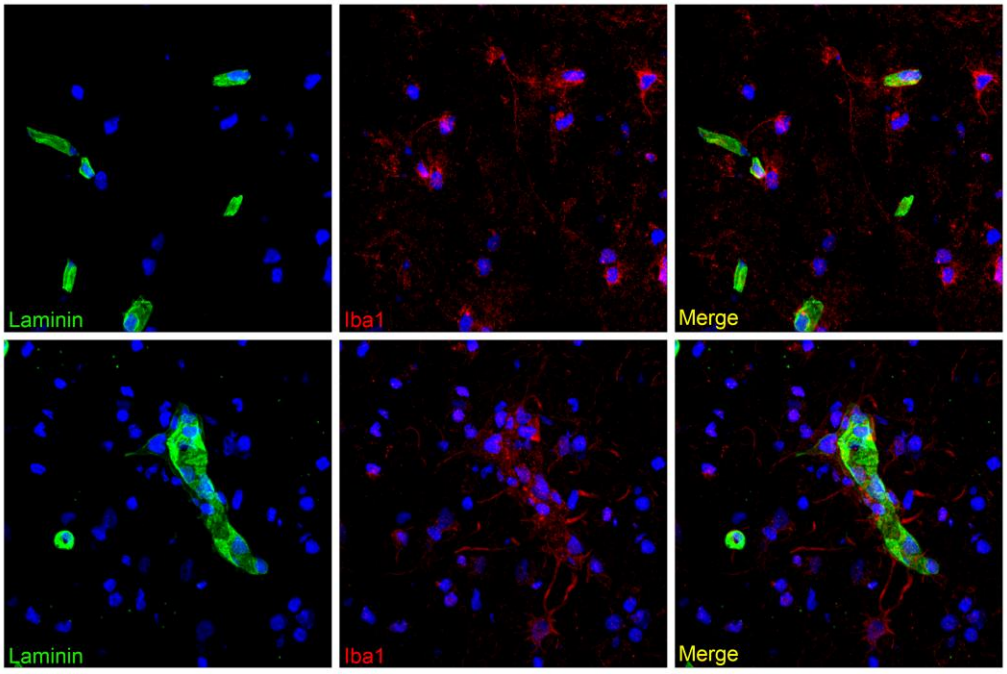

Figure 2

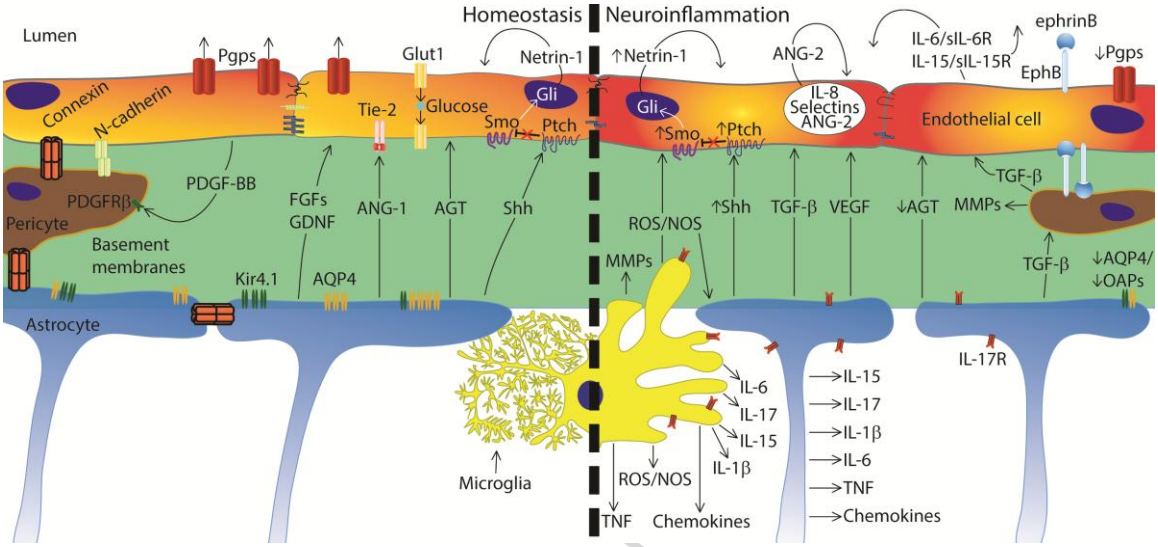

Figure 3

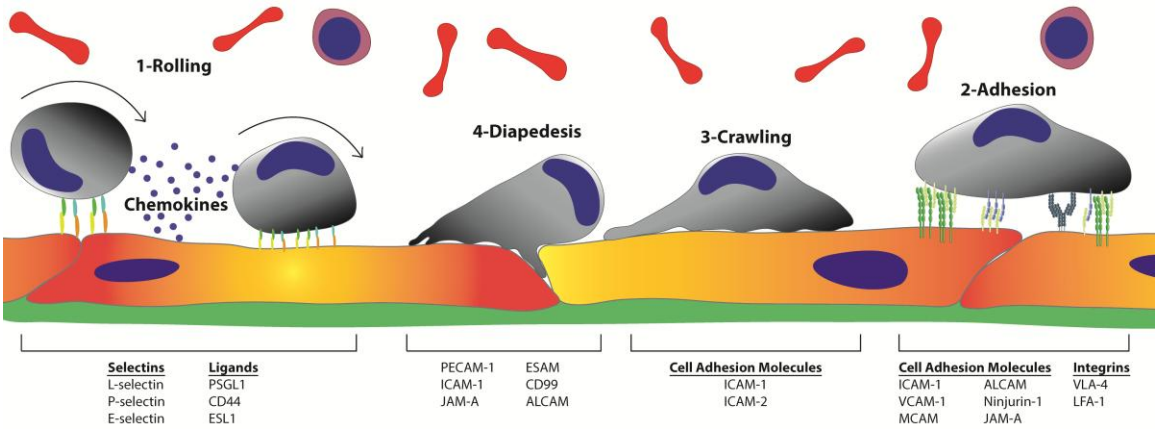

Figure 4

Highlights

- The neurovascular unit acts as a physical and functional blood-brain barrier (BBB)
- The BBB regulates the exchange between the central nervous system and the periphery
- The BBB is formed by endothelial cells interacting with glial cells and pericytes
- These perivascular cells are involved in the formation and maintenance of the BBB
- These cells can have both a beneficial and detrimental role during neuroinflammation