MINI REVIEW

Immune cell trafficking across the blood-brain barrier in the absence and presence of neuroinflammation

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Abstract

To maintain the homeostatic environment required for proper function of CNS neurons the endothelial cells of CNS microvessels tightly regulate the movement of ions and molecules between the blood and the CNS. The unique properties of these blood vascular endothelial cells are termed blood-brain barrier (BBB) and extend to regulating immune cell trafficking into the immune privileged CNS during health and disease. In general, extravasation of circulating immune cells is a multi-step process regulated by the sequential interaction of adhesion and signalling molecules between the endothelial cells and the immune cells. Accounting for the unique barrier properties of CNS microvessels, immune cell migration across the BBB is distinct and characterized by several adaptations. Here we describe the mechanisms that regulate immune cell trafficking across the BBB during immune surveillance and neuroinflammation, with a focus on the current state-of-the-art *in vitro* and *in vivo* imaging observations.

Key Words

- blood-brain barrier
- immune cell migration
- life cell
- neuroinflammation
- multiple sclerosis

Multi-step immune cell migration across the vascular wall: an introduction

A glossary of immunology terms is presented in Table 1 for better understanding of this review.

Extravasation of circulating immune cells across the vascular wall is a multi-step process characterized by the sequential interaction of adhesion and signalling molecules on the vascular endothelial and immune cells. Pioneering *in vivo* and *in vitro* live cell imaging studies of the Butcher and Springer laboratories have already established in the early 1990s that immune cells as diverse as naïve lymphocytes and neutrophils use a multi-step extravasation process to leave the blood stream specifically in postcapillary venules reaching lymph nodes and inflamed tissues, respectively (1, 2). Live cell imaging has allowed to visualize that in postcapillary venules immune cells marginate and after an initial tether or capture, roll along the endothelial cell surface, a process mediated by selectins and their respective carbohydrate ligands (1). Rolling reduces the speed of the immune cells allowing for their subsequent recognition of chemokines immobilized on proteoglycans on the surface of endothelial cells with their G-protein-coupled receptors (GPCRs) (reviewed in (3)). GPCR activation triggers inside-out-activation of immune cell integrins, inducing profound conformational changes that ultimately result in a transition from low to a high affinity status of the individual integrins in addition to integrin clustering increasing integrin avidity (4). Activated integrins enable firm arrest of the immune cells on the luminal surface

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Table 1 Immunology glossary.

Terms	Explanation
Antigen presenting cells (APCs)	Innate immune cells that actively process antigens and present them on MHC-II molecules to activate CD4⁺ T cells
CD4⁺ T helper (Th) cells	Cell type of the adaptive immune system, participating and orchestrating immune responses. Upon recognition of their cognate antigen, presented by APCs on MHC-class II molecules, Th cells get activated and polarize into different Th subset, such as Th1, Th2, Th17 and others, according to the cytokines present in the surroundings
CD8⁺ T cells	Cells of the adaptive immune system mainly involved in killing of virus-infected host cells
Chemokines	Chemotactic cytokines mostly involved in immune cell trafficking by inducing chemotaxis of immune cells. Both inflammatory and homeostatic chemokines regulate immune cell trafficking across vascular walls
Cytokines	Small proteins that regulate many processes of the immune response. Proinflammatory cytokines enhance the ability of APCs to present antigen and induce expression of adhesion molecules and chemokines at the inflamed BBB
Dendritic cells	Cells of the innate immune system serving as professional antigen presenting cells
Effector/memory lymphocytes	Activated lymphocytes after antigen recognition. These cells migrate into peripheral tissue in response to inflammatory stimuli
Immune surveillance	Homeostatic immune cell trafficking process utilized by the immune system to monitor for the presence of infections in the entire body
Major histocompatibility complex class II (MHC-class II)	Molecular complex expressed by professional APCs "presenting" peptide antigens to CD4⁺ T cells on their surface
Monocytes	Cell type of the innate immune system involved once differentiated in phagocytosing and killing microbes in addition to antigen presentation and cytokine production. They can differentiate into macrophages and dendritic cells, enhancing their antigen presentation ability
Naïve lymphocytes	Mature lymphocytes that did not yet encounter their cognate antigen and constantly recirculate to secondary lymphoid organs to get exposed to antigens presented by APCs
Neutrophils	Cell type of the innate immune system involved in phagocytosing and killing microbes. Usually they are the first cells recruited into an inflamed tissue
Th1 cells	Effector CD4⁺ T cells specialized in fighting intracellular bacteria and viruses and involved in CNS autoimmunity. Their signature cytokine is IFN-γ
Th17 cells	Effector CD4⁺ T cells specialized in fighting extracellular bacteria and fungi and involved in CNS autoimmunity. Their signature cytokine is IL-17

of the endothelial cells by engagement of endothelial adhesion molecules from the immunoglobulin superfamily (IgCAMs). Subsequent polarization and crawling on the luminal side of the endothelium allows the immune cells to find the endothelial junctions, which allow for their diapedesis across the endothelial barrier (reviewed in (3)). Before reaching the tissue parenchyma, immune cells have to cross the endothelial basement membrane, a dense network of extracellular matrix proteins, which establishes an additional barrier for their passage (reviewed in (3)).

The CNS is an immune privileged organ where the endothelial, epithelial and glial brain barriers strictly control immune cell entry into the different compartments of the CNS (5). Major differences in cellular composition, vessel and barrier chacteristics between the peripheral and CNS vasculature are summarized in Table 2. Immune cells can reach the CNS via three different entry sites: via CNS parenchymal and leptomeningeal blood vessels and via the choroid plexus (6). Here we will focus on discussing our current knowledge on immune cell trafficking across CNS parenchymal and leptomeningeal microvessels, which establish the blood-brain barrier (BBB).

The blood-brain barrier

The BBB is a physical and functional barrier present at the level of the CNS microvasculature. Originally the unique biochemical characteristics of BBB endothelial cells including complex tight junctions (TJs) between the endothelial cells and polarized expression of specific transporters and efflux pumps were considered restricted to capillaries. However, recent studies have provided evidence that the unique physical and metabolic barrier characteristics extend to the endothelial cells of CNS postcapillary venules (7, 8). Therefore we and others have extended referring to this vascular segment as BBB (9), as these characteristics impact on immune cell trafficking into the CNS (7, 10, 11).

Structurally, the BBB is localized at the level of the highly specialized endothelial cells, which exert most of

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Table 2 Comparison between cellular components and vessel characteristics between peripheral and CNS capillaries and postcapillary venules.

	Periphery			CNS			
	Capillaries[a]	Postcapillary venules	Reference	Continuous capillaries	Postcapillary venules	SAS venules	Reference
Cell types							
Pericytes	+	+	(128)	+++	++	++	(7, 129)
Astrocytic endfeet	-	-		+	+ (with small gaps in between)	-	(7, 130)
Vessel characteristics							
Adhesion molecules	+	+	(131)	+/-	+ (lack of P-selectin storage in Weibel-Palade Bodies)	+ (P-selectin is stored in Weibel-Palade Bodies)	(132, 133, 134)
TJs	+	+	(135)	+ (complex and continuous)	++ (complex and continuous)	+	(10, 136)
Paracellular diffusion of water soluble molecules	+	+	(131)	Diffusion of select ions via claudin-formed pores; no diffusion of water soluble molecules	Diffusion of select ions via claudin-formed pores; no diffusion of water soluble molecules	Diffusion of select ions via claudin-formed pores; no diffusion of water soluble molecules	(137, 138)
Pinocytotic transport of water soluble molecules	+	+	(139)	- (No uncontrolled pinocytotic transport of water soluble molecules)	- (No uncontrolled pinocytotic transport of water soluble molecules)	- (No uncontrolled pinocytotic transport of water soluble molecules)	(140)
Vesicular activity	+	+	(131, 141, 142)	- (minimal)	- (minimal)	- (minimal)	(38)

[a]Depending on organ: continuous (e.g. lung), fenestrated (e.g. kidney glomeruli), sinusoid (e.g. liver).

the physical and morphological barrier characteristics of the BBB. In contrast to peripheral vascular endothelial cells, BBB endothelial cells are characterized by the presence of not only adherens junctions (AJs) but also a molecularly unique and complex as well as continuous network of TJs (12). The transmembrane vascular endothelial cadherin (VE-cadherin) mediates homophilic adhesion at the level of BBB AJs exactly as in peripheral vascular beds. VE-cadherin expression and AJs formation is prerequisite for expression of the transmembrane TJ protein claudin-5 (13) and for the maturation, maintenance and regulation of BBB TJs (13, 14). In their complexity and continuity BBB TJs rather resemble TJs of epithelial cells than of other endothelial cells (15). Claudin-5 is the most abundant transmembrane TJ protein in BBB endothelial cells and plays a crucial role in maintaining the paracellular diffusion barrier. This is shown by studies in claudin-5-deficient mice, which die perinatally (16), and by knockdown of endothelial claudin-5, which leads to cognitive impairment (17) (summarized in (18)). Other transmembrane TJ proteins expressed in BBB endothelial cells are occludin and junctional adhesion molecules (JAMs). Although occludin is not necessary for TJ formation, occludin phosphorylation contributes to TJ function (19, 20, 21) (summarized in (10)). JAMs are immunoglobulin superfamily transmembrane proteins, with JAM-A and JAM-B being the most studied in BBB endothelial cells. JAM-A contributes to the establishment of cell polarity (22) and both JAM-A and JAM-B have been described to mediate leukocyte trafficking across the BBB (23, 24, 25, 26, 27) (summarized in (10)). Tricellular contact points between BBB endothelial cells show localization of tricellulin, which otherwise has only been described in epithelial tricellular junctions. Although in every vascular bed endothelial cells form tricellular contacts, only at the BBB and the blood-retinal barrier (28, 29) endothelial cells express tricellulin, further supporting the unique barrier characteristics of BBB endothelial cells (summarized in (10)). The unique and complex TJ architecture of the BBB endothelial cells was originally thought to prohibit paracellular immune cell diapedesis as it occurs in other vascular beds (3). Early studies have provided evidence that in neuroinflammatory conditions immune cells cross the BBB or the blood-retinal barrier (BRB) preferentially through pores via the endothelial cell body (transcellular diapedesis), rather than through the brain barriers junctions (30, 31, 32).

The complex network of AJs and TJs, together with the low pinocytotic activity, the lack of fenestrae and the expression of specific sets of efflux pumps and nutrient

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transporters, restrict uncontrolled paracellular and transcellular diffusion of hydrophilic molecules across the BBB endothelium (15).

The unique barrier characteristics of BBB endothelial cells are not intrinsic but rely on the cross-talk with cellular and acellular elements at the level of CNS microvessels, commonly referred to as the neurovascular unit (NVU) (15). On their abluminal side, high numbers of pericytes are embedded in the endothelial basement membrane hereby forming a continuous, non-overlapping chain-like network (33). Brain microvessels have a higher pericyte coverage than peripheral microvessels. The ratio of pericytes to endothelial cells of the BBB ranges between 1:1 and 1:3, covering up to 50% of the endothelial abluminal surface (34, 35). In peripheral vascular beds the pericyte: endothelial ration was reported 1:100 (skeletal muscle) with an estimated abluminal endothelial coverage between 10 and 25% (36, 37). Pericytes form multiple synapse-like "peg-socket" contacts with the neighbouring endothelial cells suggesting a tight functional coupling of the high number of CNS pericytes with the BBB endothelium. Pericytes have indeed been shown to inhibit vesicular activity of BBB endothelial cells and thus limit BBB transcellular permeability (38). This may prohibit availability of vesicular stores of chemokines or other diffusible trafficking cues available in peripheral vascular beds (39) and thus contribute to the unique mechanisms involved in immune cell extravasation across the BBB.

Unlike microvessels in other tissues, parenchymal CNS microvessels are ensheathed by a second barrier, referred to as the glia limitans (Fig. 1). It is composed of polarized astrocytes, which enclose with their foot processes the abluminal aspect of parenchymal CNS microvessels and deposit a second basement membrane, the parenchymal basement membrane, thus ultimately shielding the CNS parenchyma from the vascular space (5). Astrocytes contribute to BBB maturation and maintenance via sonic hedgehog and Wnt signaling pathways (40). At the surface of the brain and spinal cord namely at the level of the leptomeninges, below the pia mater, the glia limitans perivascularis continues as glia limitans superficialis and thus covers the entire surface of the brain and spinal cord parenchyma (41) (Fig. 1). Hence, it is the glia limitans that establishes an additional border towards the CNS parenchyma, where CNS-resident cells such as microglia, oligodendrocytes and neurons are localized. At the level of the CNS capillaries, the endothelial basement membrane and the glia limitans perivascularis are in intimate association while at the level of postcapillary venules a separation between the endothelial and parenchymal basement membrane can be visualized especially in neuroinflammation (Fig. 1). This perivascular space is considered to connect to cerebrospinal fluid (CSF)-filled Virchow–Robin spaces, which harbour conventional antigen-presenting cells such as dendritic cells (42).

It is important to note that in addition to the BBB established by parenchymal CNS microvascular endothelial cells, a functional BBB can also be found at the level of the venules in the subpial and subarachnoid space (SAS) (43), despite the fact that these venules lack direct ensheathment with astrocyte endfeet. Indeed, the CSF-filled SAS is bordered by the arachnoid barrier towards the dura mater and the skull and by the glia limitans superficialis towards the CNS parenchyma (Fig. 1). Therefore, blood vessels in the SAS are not ensheathed by a second basement membrane and rather form a direct barrier between the blood and the CFS in the SAS. Nevertheless, these vessels retain BBB features and represent an important entry point for immune cells into the CNS (43) (reviewed in (9)). In addition, BBB endothelial cells in the SAS and in CNS parenchyma differ in the expression of key adhesion molecules, with important implications for immune cell trafficking into these two compartments. Resembling peripheral vascular endothelial cells, leptomeningeal endothelial cells constitutively express and store P-selectin in their Weibel-Palade bodies, which upon an inflammatory stimulus can be readily exposed on their surface and contribute to immune cell recruitment (44). In contrast, CNS parenchymal endothelial cells lack constitutive expression of P-selectin, which requires *de novo* transcription upon an inflammatory stimulus, underscoring the active role of the BBB in controlling immune cell trafficking into the CNS (45). Microvessels in the most outer layer of the meninges, the dura mater, do not form a BBB and are not addressed here as they are also separated from the CNS by the arachnoid barrier forming a meningeal blood-CSF barrier (5).

Methodological approaches to investigate immune cell trafficking across the BBB

In vitro and *in vivo* imaging approaches aiming to investigate immune cell trafficking across the BBB are confronted (i) with the challenges of the unique features of BBB endothelial cells, relying on continuous crosstalk with the elements of the NVU and (ii) the complex CNS anatomy and thus limited accessibility for imaging. Meaningful modelling of immune cell trafficking across the BBB

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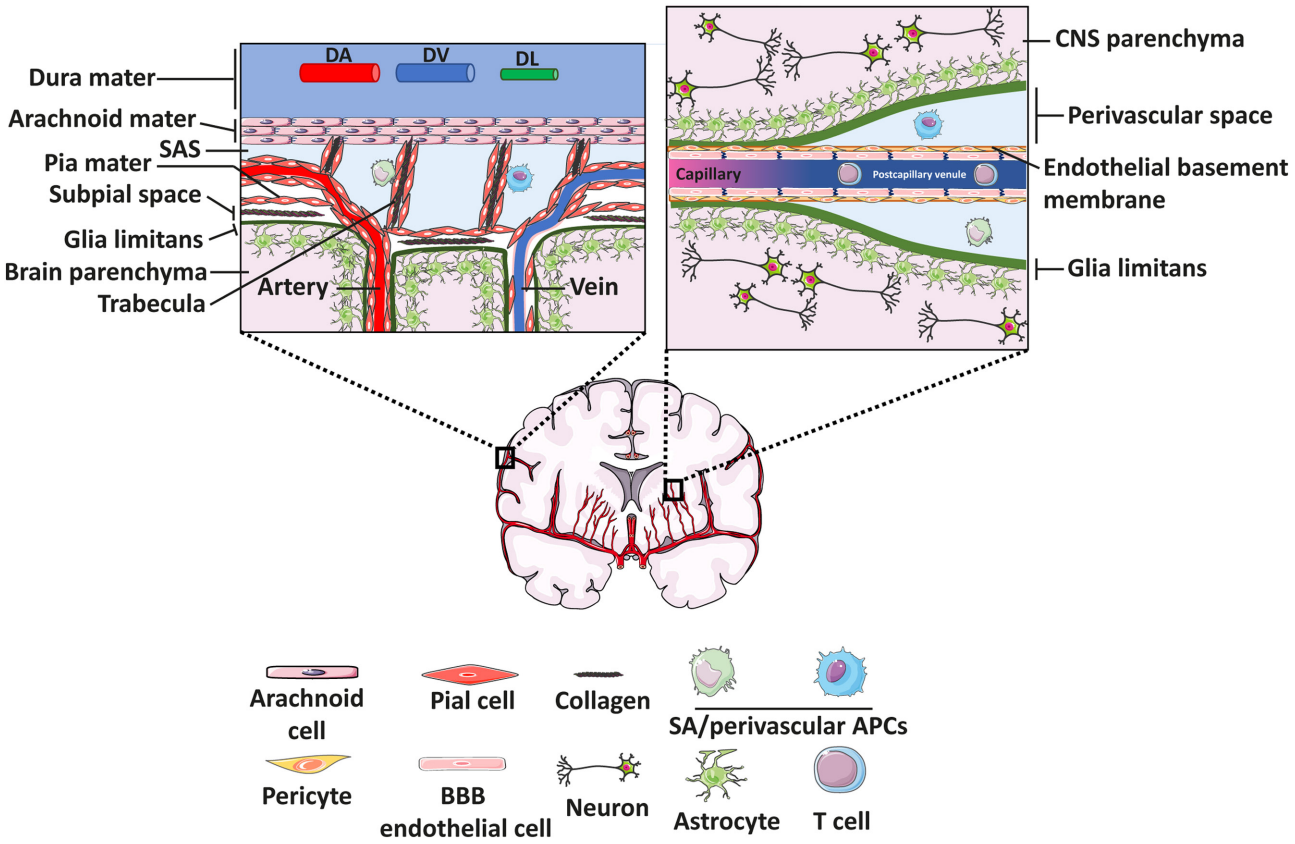

Figure 1

Leptomeningeal and parenchymal blood-brain barrier. The meninges at the surface of the brain (left) are composed by three layers, namely the dura mater, the arachnoid mater and the pia mater. In the dura mater we find dural arteries (DA) and veins (DV), as well as dural lymphatic vessels (DL). Dural blood vessels do not form a blood-brain barrier. The cells of the arachnoid mater form a blood-cerebrospinal fluid barrier (BCSFB) between the dura mater and the cerebrospinal fluid (CSF)-filled subarachnoid space (SAS). In humans the arachnoid mater is composed of several layers of arachnoid cells. The SAS harbors antigen-presenting cells (APCs), i.e. subarachnoid macrophages. Blood vessels in the SAS are ensheathed by a layer of pia mater, further connected to the arachnoid mater by trabeculae spanning the SAS. The center of the trabeculae is composed of a collagen core that is covered by cells of the pia mater. A thin layer of pia mater also covers the arteries that dive into the brain. The glia limitans is composed of the parenchymal basement membrane and astrocyte foot-processes and covers as *glia limitans superficialis* the entire surface of the CNS parenchyma and accompanies *as glia limitans perivascularis* the blood vessels in the CNS. Venules in the SAS and subpial space form a BBB albeit they lack ensheathment by astrocyte endfeet. The arachnoid and pia maters are referred to as leptomeninges. The anatomical details have been summarized in (5). The BBB at the level of CNS parenchymal vessels (right inset) is composed by highly specialized endothelial cells, held together by molecularly unique and complex tight junction strands. Pericytes are embedded in the endothelial basement membrane, while the glia limitans further ensheaths the CNS microvasculature. At the level of the capillaries, the endothelial basement membrane and glia limitans are fused. At the postcapillary venules, where immune cell trafficking takes place, the two basement membranes are separated by the CSF-filled perivascular space, which harbors rare antigen-presenting cells. Drawings of the individual cell types were adapted from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License.

in vitro requires reliable culture models that truthfully maintain BBB characteristics. Higher numbers of T cells are seen to cross a monolayer of immortalized mouse brain endothelioma bEnd5 cells, which fail to establish mature TJs, when compared to a monolayer of primary mouse brain microvascular endothelial cells, which retain excellent BBB features during 1 week in culture (46). In the presence of shear flow this *in vitro* BBB model allows to investigate extended T-cell crawling against the direction of flow, searching for rare sites permissive for diapedesis (47), a unique T cell behaviour on the BBB observed by *in vivo* imaging studies (48, 49). Thus, identification of the molecular mechanisms mediating the multi-step migration of immune cells across the BBB *in vitro* requires stringent endothelial barrier models best to be combined with sophisticated microfluidics and live cell imaging.

On the other hand, *in vivo* imaging approaches require complicated surgery preparations for cranial or spinal cord windows allowing to access the brain grey matter and spinal cord white matter tissue for the available intravital microscopy techniques (50, 51, 52). Depending on the intravital microscopy technology used,

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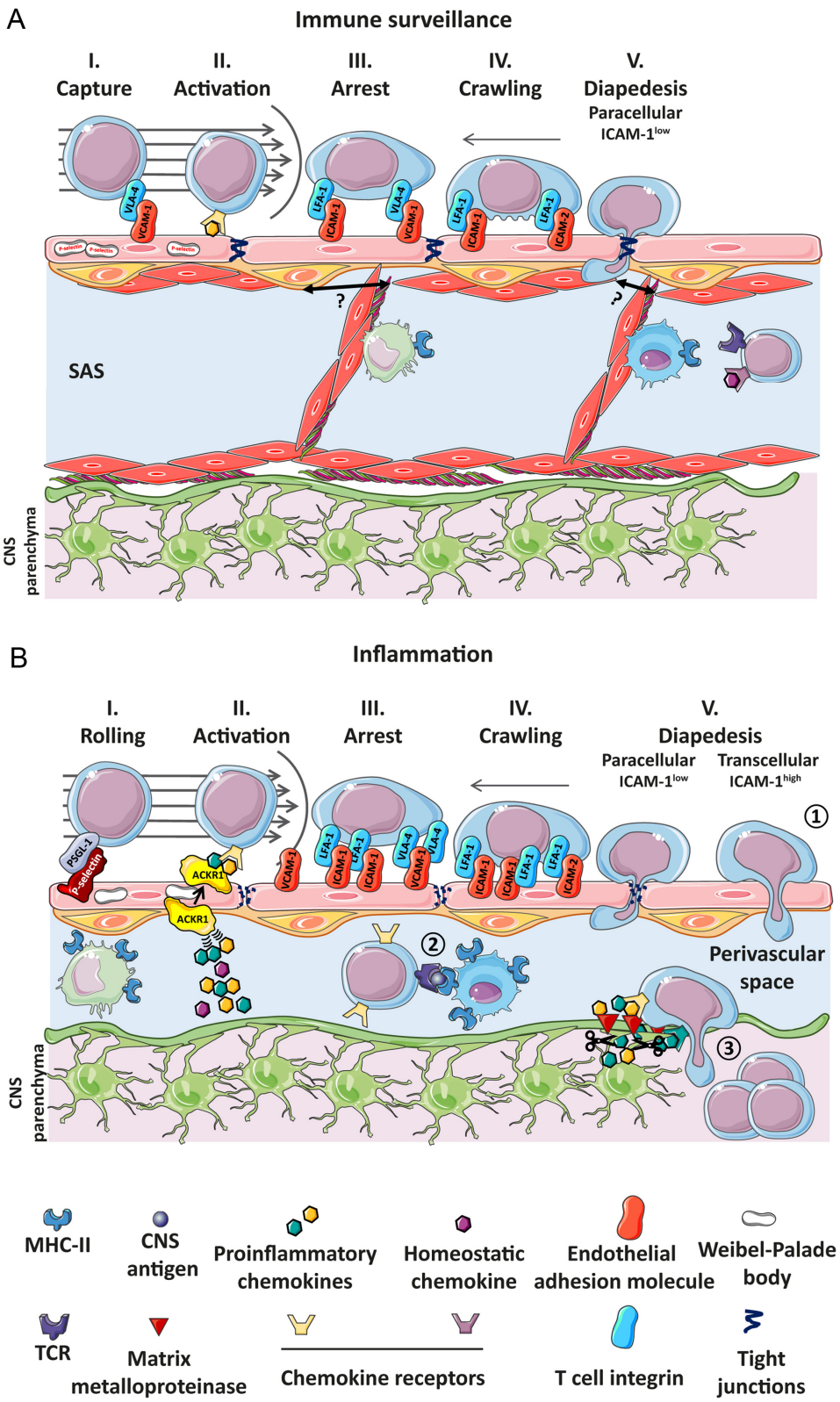

Figure 2

Multi-step T-cell extravasation across the BBB during heath and neuroinflammation. T-cell extravasation across subarachnoid venules during immune surveillance (A) or across BBB postcapillary venules during inflammation (B) is depicted. Leptomeningeal endothelial cells store P-selectin in Weibel-Palade bodies, however, in the absence of inflammation $\alpha_4\beta_1$-mediated capture is the most observed first interaction. After GPCR-mediated shear-

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imaging penetration is limited to the leptomeningeal or subpial spaces. The current lack of fluorescent reporter mice allowing to precisely identify the arachnoid barrier and glia limitans impede precise localization of superficial CNS microvessels to the subpial or SAS, especially as in the mouse the SAS spans only about 30–50 µm in healthy conditions. When performing two-photon intravital microscopy (2P-IVM), second harmonic generation signals generated by collagen type I in the dura mater and in the subpial space might provide some orientation (summarized in (10)). The lack of precise landmarks for imaging the brain barriers while following immune cell trafficking across the BBB *in vivo* presently hampers delineation of the different mechanisms mediating immune cell trafficking across the BBB in the SAS versus the CNS parenchyma.

Immune cell migration across the BBB during immune surveillance

The BBB allows for immune cell trafficking into the CNS in the absence of neuroinflammation but strictly limits CNS entry to immune cell subsets required for immune surveillance and detected in the CSF (53, 54) (Fig. 2A). The molecular mechanisms involved in the multi-step immune cell trafficking across the BBB are summarized in Table 3. The few studies that have investigated immune cell migration across the BBB during immune surveillance by intravital microscopy have mostly focused on effector/memory CD4+ T cells in the context of experimental autoimmune encephalomyelitis (EAE), a CD4+ T-cell mediated animal model of multiple sclerosis (48, 55, 56). EAE is an autoimmune disease of the CNS, which can be induced by injection of CNS myelin antigens emulsified in complete Freund's adjuvant (57), or by adoptive transfer of CNS autoantigen-specific CD4+ T cells into syngeneic naive recipients of susceptible rodent strains (58), with the former often referred as active EAE (aEAE) and the latter as transfer EAE (tEAE). The interaction of encephalitogenic CD4+ Th1 cells with the spinal cord microvasculature was found to be unique due to the lack of rolling and a prominent role of α_4 integrin - vascular cell adhesion protein-1 (VCAM-1) interaction in mediating capture and sustained arrest of these T cells to spinal cord venules (55) (Fig. 2). Th1 cell diapedesis across the non-inflamed spinal cord postcapillary venules is mediated by leukocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$ integrin) and its ligand intercellular adhesion molecule 1 (ICAM-1) (59) (Fig. 2A). GPCR signaling is prerequisite for sustained T-cell adhesion on the BBB (55); however, the chemokines or lipid mediators involved in this step remain to be determined. Mouse and human BBB endothelial cells constitutively express CCL19 (60, 61), which binds CCR7 and was therefore suggested to mediate the migration of circulating CCR7 expressing central memory T cells across the BBB as 90% of T cells in the CSF express CCR7 (62). Direct evidence for endothelial CCL19 in mediating T-cell trafficking to the CNS in the absence of neuroinflammation is however lacking.

The migration of activated CD4+ T cells across the BBB was shown in rodent animal models to be independent of antigen recognition on the CNS endothelial cells (55, 48, 51, 63, 64, 65). In contrast, antigen recognition on perivascular or leptomeningeal APCs is necessary for subsequent T-cell migration across the glia limitans and infiltration into the CNS parenchyma (48, 66, 67) (Fig. 2). CD4+ T cells localized in the SAS can crawl along not further defined scaffolds and be eventually washed away by the movement of the CSF to reach other CSF compartments in the CNS (5, 56). Interestingly, intrinsic characteristics of the leptomeningeal BBB predispose the leptomeningeal compartment as preferred site for T-cell immunosurveillance. Indeed, diapedesis of fluorescently labelled activated CD4+ T cells across parenchymal BBB endothelial cells was only observed 4–6 hours after injection (59), while accumulation in leptomeningeal spaces was already observed 2 h after injection (68).

Figure 2 Continued
resistant arrest, T cells crawl against the direction of the flow and cross the BBB endothelium preferentially via the paracellular pathway. Pial cells are reported to partially cover the venular wall in the SAS (highlighted by the question mark), but do not seem to establish a barrier for T cell extravasation. In the absence of CNS antigens presented by subarachnoid macrophages and dendric cells on MHC-II molecules, T cells will not cross the glia limitans and may rather be flushed away with the CSF. During inflammation, leptomeningeal but also parenchymal BBB endothelial cells (B) allow for activated T-cell rolling, mediated by P-selectin which is *de novo* expressed as it is not stored in Weibel-Palade bodies. Inflammatory chemokines produced by astrocytes are transported from the abluminal to the luminal side of the BBB by ACKR1. After their GPCR-dependent arrest, T cells crawl on endothelial ICAM-1 and ICAM-2 against the direction of the flow with increased levels of endothelial ICAM-1 leading to increased transcellular T cell diapedesis. Once T cells have crossed the BBB endothelium (1), CNS-antigen-specific T cells may recognize their cognate antigens on perivascular APCs (2) and become reactivated behind the BBB. Matrix metalloproteinases produced by infiltrating and perivascular-activated myeloid cells as well as astrocytes cleave the astrocytic endfeet from the parenchymal basement membrane, allowing for T-cell passage, a process guided by proinflammatory chemokines produced by astrocytes. Once in the CNS parenchyma, T cells induce CNS damage and manifestation of clinical disease symptoms (3). Drawings of the individual cell types were adapted from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License.

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Table 3 Endothelial adhesion and signaling molecules involved in multi-step immune cell trafficking across the blood-brain barrier.

Interaction step/molecule	Ligand and immune cell subset	Observation	References
Capture			
VCAM-1	α4β1-integrin⁺ encephalitogenic T cells	In vivo imaging of mouse spinal cord microvessels in the absence of neuroinflammation	(55)
Rolling			
E/P-selectin	PSGL-1⁺ encephalitogenic T cells	*In vivo* imaging of inflamed superficial mouse brain and spinal cord microvessels during EAE	(73) (71)
E/P-selectin	PSGL-1⁺ CD8 T cells from MS patients	*In vivo* imaging of superficial mouse brain microvessels in neuroinflammation	(70)
P-selectin and α4-integrin	Endogenous leukocytes	*In vivo* imaging of superficial mouse brain microvessels in neuroinflammation	(143)
Integrin activation			
ACKR1	CNS infiltrating cells	ACKR1 shuttles inflammatory chemokines from the CNS to the luminal side of the BBB - mice lacking vascular ACKR1 develop ameliorated EAE	(74)
Arrest and adhesion			
VCAM-1	Rodent encephalitogenic T cells and human T cells · α4β1-integrin (VLA-4)	*In vivo* imaging of T-cell interaction with rat and mouse spinal cord microvessels in the absence and presence of neuroinflammation; *in vitro* imaging of T-cell interaction with mouse models of the BBB under physiological flow	(47, 55, 144)
	α4-integrin on CD8 T cells	*In vitro* adhesion and transmigration assays; CD8 T cell mediated encephalitis is inhibitid by α4-integrin function blocking antibodies	(27, 145)
ICAM-1	Activated rodent CD4 and CD8 T cells	*In vitro* imaging of T cell and neutrophil interaction with mouse models of the BBB under physiological flow	(94, 106)
ICAM-2	Activated rodent CD4 and CD8 T cells	*In vitro* imaging of T cell and neutrophil interaction with mouse models of the BBB under physiological flow	(47, 94, 106)
	α4β1-integrin expressing DCs	*In vivo* imaging of mouse spinal cord microvessels and *in vivo* homing studies in the context of neurinflammation	(124, 125, 126)
Polarization			
ICAM-1		*In vitro* imaging of T cell interaction with mouse models of the BBB under physiological flow	(47)
ICAM-2		*In vitro* imaging of T cell interaction with mouse models of the BBB under physiological flow	(47)
Extended crawling against the direction of blood flow			
ICAM-1		In vitro imaging of T cell interaction with mouse models of the BBB under physiological flow	(47)
ICAM-2			(47)
Ninjurin?	Encpehalitogenic T cells · ninjurin	In vivo imaging of encephalitogenic T cells interacting with the rat spinal cord microvasculature at onset of EAE	(146)
	Encpehalitogenic T cells · a4b1-integrin VLA-4	In vivo imaging of encephalitogenic T cells interacting with the rat spinal cord microvasculature at onset of EAE – individual study showing a role for a4-integrins in T cell crawling	(146)
Diapedesis			
CD99	Probably CD99 on immune cells	Blocking CD99 affects immune cell migration across but not adhesion to human BBB models under static conditions; CD99 blockade ameliorates EAE in the mouse	(93, 147)
GPCR ligands	Pertussis toxin sensitive GPCRs on T cells	Inhibition of Gai signalling in T cells blocks diapedesis but not prior polarization or crawling on *in vitro* BBB models under flow	(94)
Caveolin-1	Encephalitogenic Th1 cells	Lack of endothelial caveolin 1 reduces transcellular diapedesis of Th1 cells into the CNS in EAE	(90)
CXCL12	CXCR4⁺ T cells, B cells and monocytes	Function blocking of CXCR4 interferes with the diapedesis of T cells, B cells and monocytes across a rodent model of the BBB under physiological flow	(119)

(Continued)

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Table 3 Continued.

Interaction step/molecule	Ligand and immune cell subset	Observation	References
Migration across the BBB – precise step not defined			
Laminin411	Mouse Th17 cells and human CD8 T cells · MCAM	Anti-MCAM antibody blocks mouse Th17 cell recruitment to the CNS and ameliorates EAE · anti MCAM antibody blocks CD8 T cell migration across the BBB *in vitro*	(148, 149)
ALCAM	Monocytes, B cells and T cells · ALCAM	ALCAM may contribute to monocyte and possibly B cell and T cell migration across the BBB based on *in vitro* studies	(118, 150) (100, 121)
	aVb3+ Th17 cells	Potential role in extracellular matrix interaction for CNS infiltration	(82)
JAM-A	CD14+CD16+ JAM-A+ monocytes	Antibody blocking of JAM-A selectively blocked migration of CD14+CD16+monocytes but not of T cells from HIV-infected people across a human *in vitro* model of the BBB	(121)
JAM-B	CNS-antigen-specific CD8 T cells	Blocking JAM-B reduces CNS infiltation of CD8 T cells and ameliorates CD8 T cell mediated neuroinflammation	(27)
JAML	Monocyte and CD8 T cells	Function blocking of JAML reduced migration of monocytes and CD8 T cells across a human *in vitro* BBB model	(151)
ICAM-1	B-cells	Migration of human B cells across a human BBB model is reduced upon blocking endothelial ICAM-1	(99)
Ninjurin	Monocytes	Peptide-mediated blocking of nijurin reduced adhesion and migration of monocytes, but not T and B cells across a human *in vitro* model of the BBB	(117)
CCL19	CCR7 on central memory T cells and activated CD8 T cells or monocytes	CCL19 is expressed at the BBB and could mediate integrin activation on rolling immune cells or their diapedesis	(60, 61, 123)
β1-integrin	β1-integrin expressing T cells	β1-integrin deficient T cells cannotenter the CNS during neuroinflammation	(127)
P-glycoprotein		Silencing of P-glycoprotein activity is shown to selectively reduce the migration of CD8+ T cells across a rodent *in vitro* model of the BBB	(8)

This table provides examples and is not exhaustive.

Immune cell trafficking across the BBB in neuroinflammation

During neuroinflammation, the BBB endothelium undergoes changes including increased expression of adhesion molecules, proinflammatory cytokines and chemokines combined with reduced expression of junctional molecules, setting the stage for increased recruitment of circulating leukocytes across the BBB (summarized in (9, 37, 40)).

CD4+ and CD8+ T cells

Once an inflammatory stimulus perturbs the CNS parenchymal microvasculature, upregulation of P-selectin allows for T-cell rolling, a process mediated by the interaction between P-selectin glycoprotein ligand 1 (PSGL-1) on the T cells and E/P-selectin on the endothelial cells (69, 70, 71) (Fig. 2B). During rolling, T cells reduce their speed, from ~1000 μm/s to 5–10 μm/s (72, 73). Paradoxically, despite their essential role in T-cell rolling on the BBB, absence of PSGL-1 and/or E/P-selectin in mice fails to reduce T-cell entry into the CNS and thus amelioration of EAE, suggesting that T-cell rolling is not required for T-cell migration across the inflamed BBB (71). In fact intravital microscopy studies have shown that a low number of T cells can eventually arrest in inflamed spinal cord vessels of E/P-selectin-deficient mice (71), subsequently allowing for their diapedesis across the BBB and initiation of EAE.

T cells do require GPCR signalling to firmly arrest on the BBB (73). However, the endothelial chemokines or lipid mediators triggering T cell arrest on the BBB are a matter of debate. In this context it is interesting to note that the atypical chemokine receptor 1 (ACKR1) is upregulated on the BBB during EAE and in MS (74). ACKR1 shuttles inflammatory chemokines produced for example, by astrocytes in neuroinflammatory conditions

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from the abluminal to the luminal side of the BBB (Fig. 2B) and lack of vascular ACKR1 ameliorates clinical signs of EAE in C57BL/6 mice (74). ACKR1-mediated chemokine shuttling may thus lead to the presence of a variety of CNS produced proinflammatory chemokines on the luminal side of the BBB.

GPCR signaling leads to inside-out-activation of integrins mediating the firm arrest of T cells on the luminal surface of the inflamed BBB endothelial cells. This crucial step is mediated by the integrins LFA-1 and very late antigen-4 (VLA-4, $\alpha_4\beta_1$ integrin) and their endothelial ligands, ICAM-1 and VCAM-1, respectively (47, 75, 76, 77, 78) (Fig. 2B). EAE studies have ruled out the involvement of integrin $\alpha_4\beta_7$ in T-cell arrest on the BBB (79, 80, 81), while recent evidence has suggested a specific role for $\alpha_v\beta_3$ integrin in Th17 cell-mediated EAE pathology in mice (82). The crucial role played by $\alpha_4\beta_1$/VCAM-1 interaction in T-cell arrest on the BBB is highlighted by the fact that blocking α_4-integrins has been translated into the most effective treatment for relapsing-remitting MS with the humanized monoclonal anti-α_4 integrin antibody natalizumab. In addition to VCAM-1, another endothelial ligand for $\alpha_4\beta_1$ integrin, namely JAM-B has been shown to be involved in CD8$^+$ (27), but not CD4$^+$ (24) T-cell migration across the BBB in mouse models.

After arrest, T cells polarize and start to crawl in search for sites permissive for diapedesis across the BBB endothelium (Fig. 2B). *In vitro* (47) and *in vivo* (48) rodent studies have shown that barrier properties of the BBB translate to post-arrest extended crawling of CD4$^+$ T cells on BBB endothelial cells, preferentially against the direction of the blood flow, searching for rare sites permissive for diapedesis (47, 83). It has been shown before that activated T cells but not neutrophils crawl against the direction of the flow on ICAM-1-coated surfaces (84). This underscores that in addition to molecular cues, shear stress impacts on directionality of T cell crawling. In fact, lack of endothelial ICAM-1 and ICAM-2 on a mouse model of the BBB, abrogates Th1 cell polarization and crawling (47, 83) supporting the notion that endothelial ICAM-1 and ICAM-2 are essential for mediating the Th1 cell crawling against the direction of flow on the BBB with no additional role of $\alpha_4\beta_1$/VCAM-1 (47, 83). Side-by-side comparison of mouse encephalitogenic Th1 and Th17 cell interaction with the BBB has shown that Th1 cells, in comparison to Th17 cells, arrest in higher numbers on the BBB *in vitro* and *in vivo,* however, both Th1 and Th17 cells rely on endothelial ICAM-1 and ICAM-2 for crawling on the BBB (49). Interestingly, genetic ablation of α_4 integrins in mouse T cells blocks Th1 cell entry into the CNS during EAE, while Th17 cells can still accumulate in the brain but not the spinal cord using LFA-1 (85, 86). These observations suggest that Natalizumab rather blocks Th1 and Th17 cell entry into the spinal cord but only Th1 cell entry into the brain in MS (9).

Barrier characteristics of the BBB endothelium not only result in extended crawling of CD4$^+$ T cells but ultimately in differences in their diapedesis. In peripheral vascular beds upon their arrest T cells crawl for short distances and promptly cross the endothelium through the endothelial junctions, a process known as paracellular diapedesis (3). Paracellular immune cell diapedesis is envisioned in a zipper-like fashion where the immune cell transiently replaces the endothelial cell adhesive connections visible as remodeling of the endothelial cell junctions (87, 88).

In accordance to the presence of complex and continuous TJs, T-cell diapedesis across the inflamed BBB has rather been observed to occur via a transcellular pathway, where the T cells form a pore through the endothelium and leave the complex TJs morphologically intact (9, 32, 89). However, a recent study found that Th1 but not Th17 cells rely on caveolin-1 for transcellular diapedesis in a mouse BBB *in vitro* model (90) suggesting that Th17 cells can cross the BBB via a paracellular pathway. The molecular mechanisms directing paracellular versus transcellular T cell diapedesis across the BBB are not yet understood.

Live cell imaging studies exploring Th1-cell diapedesis across *in vitro* mouse models of the BBB under physiological flow have shown that low versus high cell surface levels of endothelial ICAM-1 direct Th1 cells to paracellular versus transcellular sites of diapedesis across the BBB, respectively (83). These findings are in accordance to the previous observations of the increased transcellular T-cell diapedesis across the BBB during neuroinflammation, when endothelial ICAM-1 levels are high. Paracellular T-cell diapedesis across the inflamed BBB in mice was proposed to be facilitated by claudin-5$^+$ extracellular vesicles released from BBB endothelial cells that decorate the T cells, thus allowing to squeeze through BBB TJs in a zipper-like fashion (91). On the other hand BBB breakdown accompanied with impaired BBB junctional integrity does not correlate with increased paracellular T-cell diapedesis, underscoring that vascular permeability and cellular pathways of T-cell diapedesis across the BBB are regulated by different mechanisms. In fact, increased paracellular permeability of the BBB due to the lack of endothelial PECAM-1 (92) or mediated by

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pro-inflammatory cytokine stimulation does not correlate with increased paracellular but rather transcellular Th1 cell diapedesis across the mouse BBB under flow *in vitro* (83, 93). Irrespective of the cellular pathway of T-cell diapedesis across the BBB, inhibition of GPCR signalling in both, $CD4^+$ and $CD8^+$ T cells completely abrogates their diapedesis across the mouse BBB (94). These observations underscore that the BBB actively controls the cellular and molecular mechanisms of T-cell diapedesis and that intact cell-to-cell junctions are required to direct T cells to paracellular sites for diapedesis across the BBB.

T cells that have successfully crossed the BBB have not yet reached the CNS parenchyma proper but rather the CSF drained perivascular or subarachnoid space. To enter the CNS parenchyma the T cells must cross a second barrier, the glia limitans (41) (Fig. 2). Deposition of laminin α_4 and α_5 in the endothelial basement membrane allows it to be distinguished from the parenchymal basement membrane of the glia limitans, which is constituted by laminin α_1 and α_2 (95). Effector $CD4^+$ T cells do not bind to laminin α_1 and α_2 (96), and their crossing of the glia limitans in neuroinflammation is rather mediated by matrix metalloproteinases (MMPs), specifically the gelatinases MMP-2 and MMP-9 (97). MMP-2 and MMP-9 cleave β-dystroglycan, an extracellular matrix receptor of astrocyte endfeet (97) and modulate chemokine activities in the perivascular space and SAS (98), allowing for T cell crossing the glia limitans and entering the CNS parenchyma (Fig. 2B). In EAE clinical disease starts, when immune cells cross the glia limitans (97).

B-cells

Due to the difficulty of isolating and maintaining B cells in culture there are only few studies that have addressed B-cell migration across the BBB. Migration of human B cells across a human BBB model was found to be mediated by endothelial ICAM-1 but not endothelial VCAM-1 (99). As blocking $\alpha_4\beta_1$-integrins was found to decrease B-cell migration across the BBB, alternative $\alpha_4\beta_1$-integrin ligands, that is, fibronectin of JAM-B may contribute to B-cell migration across the BBB (99). Additionally, blocking chemokines produced by these BBB endothelial cells such as CCL2 and CXCL8 resulted in reduced B-cell diapedesis. Moreover, a recent study demonstrated that the activated leukocyte cell adhesion molecule (ALCAM) participates in the migration of human B cells across the inflamed BBB, and blocking ALCAM ameliorated clinical signs of a B-cell-dependent EAE model, supporting its role in B cell entry into the CNS (100).

Innate immune cells

The immune privilege of the CNS extends to innate immunity. Indeed, inflammatory responses initiated by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) caused by injection of bacterial products (101), chemokines, cytokines (102) or induced cell death in the murine CNS parenchyma (103, 104), respectively, do not elicit rapid infiltration of neutrophils or monocytes as observed during a response to such stimuli in peripheral organs (summarized in (105)). This suggests that barrier characteristics of the BBB extend to an even stricter control of innate immune cell entry into the CNS.

Neutrophils

The migration of neutrophils across the BBB has been studied in the context of acute non-sterile and sterile inflammation such as bacterial meningitis and ischemic stroke. Mimicking bacterial infection by stimulation of an *in vitro* model of the mouse BBB with LPS we have found by means of *in vitro* live cell imaging that GCPR-dependent activation of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins allows for neutrophil interaction with endothelial ICAM-1, resulting in neutrophil arrest and polarization, respectively (106). Neutrophil crawling and preferential paracellular over transcellular diapedesis across the primary mouse brain microvascular endothelial cells was dependent on ICAM-1 and ICAM-2 and both β_2 integrins. This seems to be in accordance to previous observations showing that upon αLβ2 (LFA-1)-mediated arrest neutrophils crawl using αMβ2 (Mac-1)- integrin on endothelial ICAM-1 to sites permissive for diapedesis in inflamed mouse cremaster microvessels (107). Interestingly, lack of ICAM-1 and ICAM-2 on mouse brain microvascular endothelial cells abrogated transcellular but not paracellular neutrophil diapedesis across the BBB, suggesting a role of β_2 integrins in this diapedesis pathway (106). Employing a mouse *in vitro* BBB model and a microfluidic chamber we found that the ischemic BBB does not support neutrophil migration across the barrier (106). Neutrophils do however accumulate in the brain after ischemic stroke and are considered the main cause of reperfusion injury. The molecular mechanisms involved in their potential recruitment into the CNS after ischemic stroke are a matter of debate.

Generally, neutrophil rolling on the vascular wall has been described to be dependent on P-selectin (108); however, a mouse model of cerebral ischemia excluded

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a role of the P-selectin ligand PSGL-1, in neutrophil accumulation in the CNS (109). Previous studies of ischemic stroke in ICAM-1-deficient mice, which still express soluble ICAM-1 splice variants, have proposed endothelial ICAM-1 to be involved in neutrophil interaction with the BBB (110, 111). In contrast, similar studies performed in ICAM-1^{null} mice have not confirmed this observation (112). Additional studies have proposed a role for $\alpha_4\beta_1$ integrins in neutrophil recruitment to the CNS after stroke (113), however, inhibition of α_4 integrins by natalizumab infusion failed to ameliorate acute ischemic stroke in humans in a Phase II2b trial (summarized in (105)) and blocking the endothelial $\alpha_4\beta_1$-integrin ligand VCAM-1 in experimental stroke models did not ameliorate brain damage (114). We and others have observed that after ischemic stroke in experimental mouse models but also in humans, neutrophils accumulate within the confines of the neurovascular unit and in the SAS (115, 116) rather than reaching the CNS parenchyma. This suggests that in addition to the BBB, the glia limitans provides a not yet considered barrier for neutrophil entry into the CNS.

Monocytes and dendritic cells

Monocyte migration across the BBB has been investigated in different contexts, ranging from CNS virus infections to EAE and MS. Different molecular mechanisms have been observed in monocyte migration across the BBB as compared to those for T cells and neutrophils. For instance, blocking ninjurin-1 reduced adhesion and migration of monocytes, but not T and B cells, across a human *in vitro* model of the BBB (117). Furthermore ALCAM, was identified to mediate rolling, adhesion and diapedesis of human CD14$^+$ monocytes but not of human Th1 cells on a human BBB model (118). A comparative study analyzed the role of CXCL12 by blockage of its receptor CXCR4 in human CD4$^+$, CD8$^+$, CD19$^+$ B cells and CD14$^+$ monocytes migration under flow across the human brain microvasculature. Surprisingly, monocyte but not T-cell migration across the BBB was significantly reduced by blockage of CXCR4 (119). In addition, monocytes may directly activate the BBB endothelium, as co-culture of monocytes with rat brain endothelium triggered the release of tissue-type plasminogen activator from the brain endothelial cells, leading to loss of junctional occludin and increased monocytes diapedesis across the BBB endothelium (120). Interestingly, CD14$^+$ CD16$^+$ monocytes isolated from HIV-infected patients have increased expression of JAM-A and ALCAM, and both molecules participate in CD14$^+$ CD16$^+$ monocyte migration across the BBB (121), as well as CXCR7 (122). On the other hand, Zika virus-infected monocytes depend on CCR7 and receptor for advanced glycation end (RAGE) for transmigration across the human BBB (123).

Few studies have investigated the migration of dendritic cells (DC) across the BBB. *In vivo* live cell imaging of the spinal cord microvasculature during EAE in mice demonstrated a prominent involvement of $\alpha_4\beta_1$-integrins in mediating DC arrest in inflamed spinal cord microvessels (124). A crucial role of $\alpha_4\beta_1$-integrins for CNS entry was recently also confirmed for mouse plasmacytoid DCs (125) as well as monocyte-derived DCs (126). Interestingly, steady-state migration of conventional and plasmacytoid DCs was found to be independent of α_4 integrins (126), suggesting α_4-integrin-mediated DC migration across the BBB to be a mechanism restricted to neuroinflammatory conditions. On the other hand it was shown that myeloid cells do not rely on β_1-integrins to infiltrate the CNS during EAE in mice (127), underscoring that myeloid cells may use pleiotropic mechanisms to cross the BBB.

Concluding remarks

The endothelial BBB strictly controls immune cell entry into the CNS in the absence and presence of neuroinflammation. CNS immune surveillance is ensured by restricting access of limited immune cells to the CSF drained compartments of the CNS bordered by the glia limitans. In conditions of neuroinflammation the barrier properties of the BBB are impaired and allow for increased but not entirely uncontrolled immune cell entry into the CNS. Changes in the perivascular space and SAS chemokine environment combined with impairment of the glia limitans eventually allows for immune cell infiltration into the CNS parenchyma, leading to defective CNS function and thus clinical signs of disease. A deeper understanding of the specific molecular mechanisms used by the different immune cell subsets to cross the BBB would allow for improving therapeutic targeting of immune cell subsets potentially harmful for the CNS while leaving CNS immune surveillance largely unaffected. Also, as outlined in this review, the vast majority of the studies on immune cell trafficking across the BBB have focused on inflammatory or disease conditions, while knowledge on immune cell entry into the CNS during homeostasis is still scarce and requires further investigations.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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