Review

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Feature Review

Capture, crawl, cross: the T cell code to breach the blood–brain barriers

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The central nervous system (CNS) is an immunologically privileged site to which access of circulating immune cells is tightly controlled by the endothelial blood–brain barrier (BBB; see Glossary) localized in CNS microvessels, and the epithelial blood–cerebrospinal fluid barrier (BCSFB) within the choroid plexus. As a result of the specialized structure of the CNS barriers, immune cell entry into the CNS parenchyma involves two differently regulated steps: migration of immune cells across the BBB or BCSFB into the cerebrospinal fluid (CSF)-drained spaces of the CNS, followed by progression across the glia limitans into the CNS parenchyma. With a focus on multiple sclerosis (MS) and its animal models, this review summarizes the distinct molecular mechanisms required for immune cell migration across the different CNS barriers.

Immune privilege of the CNS

CNS homeostasis is the prerequisite for proper electrical activity of neural networks. The endothelial BBB in CNS parenchymal microvessels (Figure 1), the blood–leptomeningeal barrier (BLMB) in meningeal microvessels on the surface of the brain and spinal cord, as well as the epithelial BCSFB in the choroid plexus, protect the CNS from the constantly changing milieu in the blood stream by strictly controlling the movement of molecules across their interfaces (Box 1). This protective barrier between the CNS and periphery also establishes an interface between the immune system and the CNS. Immune cell trafficking to the CNS is therefore rigorously controlled by mechanisms that operate via these barriers. Passage into the CNS is limited to immune cell subsets that hold the specific molecular keys required to breach these barriers.

For immunosurveillance of the CNS, immune cells cross the outer protective barriers but are retained in the CSF-drained perivascular, leptomeningeal or ventricular spaces and thus remain separated from the CNS parenchyma by the glia limitans (Box 1). Within the CSF-drained spaces, immune cells interact with antigen presenting cells (APCs), which are strategically localized immediately behind these barriers, in search for their specific antigen. During neuroinflammation, however, immune cells breach the glia limitans and enter the CNS parenchyma proper. This is exemplified by the observation that, in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, induction of clinical signs only occurs once immune cells penetrate the glia limitans and reach the CNS parenchyma, whereas accumulation of inflammatory cells within the perivascular or leptomeningeal CSF-drained spaces does not translate into clinical disease [1–3]. These observations highlight the significance of the glia limitans as the effective border for immune cell entry into the CNS parenchyma. Interestingly, in ancestral vertebrates, astroglial cells established the BBB, but during evolution, the endothelial barrier has arisen independently several times implying a strong selective advantage for vertebrates [4]. Establishing a two-walled compartment around the CNS resembling that of a medieval castle [5] (http://www.tki.unibe.ch/britta.htm) might have been one of the evolutionary

Glossary

Astrocytes: a subtype of ectodermal derived glial cells in the CNS characterized by many foot-like processes interacting with neurons and embracing the abluminal aspect of CNS microvessels.
Blood–brain barrier (BBB): a diffusion barrier formed by the unique cellular and molecular characteristics of endothelial cells and the glia limitans perivascularis of the microvessels in the CNS parenchyma. At the level of CNS capillaries, the BBB forms a direct barrier.
Blood–cerebrospinal fluid barrier (BCSFB): a diffusion barrier formed by the epithelial cells of the choroid plexus localized in the ventricles of the brain.
Blood–leptomeningeal barrier (BLMB): a diffusion barrier formed by the unique cellular and molecular characteristics of endothelial cells of the leptomeningeal microvessels at the surface of the brain and spinal cord. Strictly speaking, the BLMB forms a blood–CSF barrier.
Choroid plexus: a highly vascularized and branched villous structure developing as a protuberance from the ependymal cells lining the brain ventricles. On the outside, the choroid plexus is lined by epithelial cells involved in the production of CSF.
Cribriform plate: a sieve like structure within the ethmoid bone which separates the nasal cavity from the skull cavity.
Glia limitans: composed of the parenchymal basement membrane and astrocyte foot processes and covers the entire surface of the brain and spinal cord on external surfaces towards the leptomeningeal/subarachnoid space (glia limitans superficialis) and internally towards the perivascular spaces (glia limitans perivascularis).
Leptomeningeal/subarachnoidal space: CSF-drained space at the surface of the CNS that protects and cushions the brain and spinal cord. This space is bordered on the outside by the arachnoid mater and towards the CNS parenchyma by the pia mater. Blood vessels at the surface of the CNS are localized in this space.
Paracellular diapedesis: migration of cells in between endothelial or epithelial cells including transient opening of their cellular junctions.
Pericyte: vascular mural cell embedded within the endothelial basement membrane of blood microvessels.
Transcellular diapedesis: migration of cells through endothelial or epithelial cells forming a pore through the cell body.

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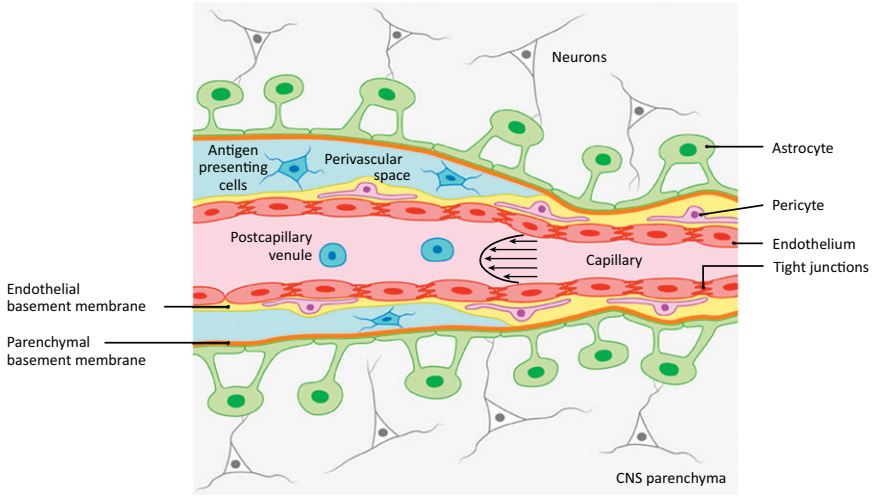

Figure 1. Neuroanatomy of the vascular blood–brain barrier (BBB). The BBB is localized to central nervous system (CNS) microvessels, the capillaries, and post-capillary venules. The highly specialized endothelial cells (red) establish the barrier proper by their low pinocytotic activity and by sealing the paracellular space with complex tight junctions. They deposit an endothelial basement membrane (yellow), in which many embedded pericytes (pink) can be found. The endothelial basement membrane is molecularly distinct from the parenchymal basement membrane (orange), which is deposited by astrocytes and establishes together with the astrocytic endfeet (green) the glia limitans perivascularis. At the vascular segment of CNS capillaries, the endothelial and parenchymal basement membranes merge and cannot be ultrastructurally distinguished. However, at the post-capillary venules, these basement membranes separate to provide a cerebrospinal fluid (CSF)-drained perivascular space (blue), in which antigen-presenting cells can be found. Neurons (gray) are localized in the CNS parenchyma and thus protected from the periphery by this complex vascular structure.

strategies to counteract selection pressure for an effective immunosurveillance of the CNS by the adaptive immune system without endangering CNS homeostasis.

In this review, we summarize how the outer BBB, BLMB, and BCSFB control the migration of immune cell subsets across and their dwelling right behind these barriers during immunosurveillance. Furthermore, we propose that migration of immune cells from the blood stream into the CNS parenchyma is a process only occurring during neuroinflammation and requires penetration of a second barrier, the glia limitans. This a fundamentally different process that, in addition to the multistep interaction of circulating cells with the vascular wall, requires penetration of a second barrier lacking in all other organs, and therefore involves mechanisms that are unique to the CNS.

Principles of the multistep extravasation of immune cells

The recruitment of circulating immune cells into any given tissue is mediated by the sequential interaction of different

Box 1. Morphology of the brain barriers

The BBB is formed by highly specialized endothelial cells in CNS microvessels, which inhibit uncontrolled transcellular passage of molecules by an extremely low pinocytotic activity and restrict the paracellular diffusion of hydrophilic molecules by an elaborate network of complex tight junctions between the endothelial cells (reviewed in [76]). Barrier properties are not intrinsic to CNS microvascular endothelial cells but rather rely on the continuous interaction with the underlying endothelial basement membrane and its embedded pericytes, as well as on the interaction with astrocytes that produce a second basement membrane known as the parenchymal basement membrane and several growth factors and morphogens relevant for barrier maturation and maintenance [77,78]. Astrocytes ensheath the abluminal aspect of the CNS microvessels with their endfeet, which together with the parenchymal basement membrane define the glia limitans that marks the border to the CNS parenchyma [79]. With the exception of CNS capillaries, where the endothelial and parenchymal basement membranes fuse to form one composite basement membrane, in all brain parenchymal vessels, the endothelial and parenchymal basement membranes are structurally and biochemically distinct entities, which define the inner and outer limits of a CSF-filled perivascular space, in which rare APCs can be found (reviewed in [79,80]). CSF perivascular spaces open towards the leptomeningeal/subarachnoidal space on the surface of the brain and spinal cord, facilitating drainage of extracellular fluids to the CNS surface and finally into the venous blood flow [81]. Alternatively, CSF drainage pathways connect directly via the cribriform plate to nasal lymphatics and deep cervical lymph nodes (summarized in [82]). Both pathways therefore compensate for the lack of lymph vessels in the CNS [83]. On the surface of the brain and spinal cord, the leptomeningeal blood vessels lack the direct ensheathment by astrocytic endfeet and thus the glia limitans perivascularis [84]. Instead, the entire CSF-drained leptomeningeal space is sealed off towards the CNS parenchyma by the glia limitans superficialis, resembling the glia limitans perivascularis and surrounding the entire surface of the brain and spinal cord (reviewed in [79,80]). Despite these morphological differences, meningeal CNS microvessels also establish a functional barrier [85]. Meningeal microvessels have therefore also been referred to as BBB, although strictly speaking, leptomeningeal vessels rather establish a barrier between the blood stream and the CSF-drained leptomeningeal space. Therefore, we refer to this vascular barrier here as the BLMB. In addition to the endothelial BBB and BLMB, another major barrier sealing off the CNS from the changing milieu in the periphery is established by the epithelial cells of the choroid plexus. Unique claudin-11-induced parallel oriented tight junction strands around the choroid plexus epithelium inhibit paracellular diffusion of water-soluble molecules and thus set up the BCSFB, [86]. The choroid plexus plays a central role in the formation and regulation of CSF. It extends as a villous structure from the ventricular walls into the lumen of the brain ventricles where a single layer of epithelial cells surrounds the choroid plexus parenchyma harboring an extensive network of microvessels, allowing free diffusion of molecules across the endothelial cells through fenestrations and intercellular gaps (reviewed in [86]).

adhesion and/or signaling molecules on immune cells and endothelial cells lining the vessel wall (summarized in [6,7]). The multistep interaction starts with an initial transient contact of the circulating immune cell with the vascular endothelium, mediated by adhesion molecules of the selectin family (L-, E-, or P-selectin) and their respective glycosylated ligands [e.g., P-selectin glycoprotein ligand (PSGL-1)], or alternatively by α4-integrins and their ligands of the immunoglobulin (Ig) superfamily [vascular cell adhesion molecule (VCAM)-1 and mucosal addressin cell adhesion molecule (MAdCAM)-1]. After the initial tether, the immune cell rolls along the vascular wall with greatly reduced velocity. The rolling immune cell can then bind chemotactic factors from the family of chemokines presented on the endothelial surface. Chemokines bind to G-protein-coupled receptors (GPCRs) on the immune cell surface delivering a GPCR inside-out signal to integrins (α4- and β2-integrins) present on the immune cell surface that increases their affinity and avidity by inducing both conformational changes and clustering. Only integrins with increased affinity/avidity are able to mediate the arrest of the immune cells to the vascular endothelium by binding to their endothelial ligands of the Ig-superfamily [intercellular adhesion molecule (ICAM)-1, ICAM-2, VCAM-1, and MAdCAM-1]. This ultimately leads to integrin-mediated post-arrest immune cell adhesion strengthening and polarization, followed by immune cell crawling on the endothelium. During the crawling process that is predominantly regulated by high affinity leukocyte function-associated antigen (LFA)-1 and its endothelial ligands ICAM-1 and ICAM-2, immune cells probe the endothelium with invadosome-like protrusions [8] in search of a site permissive for diapedesis. The endothelium might form docking structures or transmigratory cups by embracing the immune cell with endothelium-derived membrane protrusions [8]. Two distinct migration pathways for immune cells across the endothelium have been observed: paracellular diapedesis through the endothelial junctions and transcellular diapedesis by inducing the formation of pore-like structures in the endothelium. Platelet endothelial cell adhesion molecule (PECAM)-1 and likely also CD99 and junctional adhesion molecule (JAM)-A are recruited to sites of diapedesis from a vesicular compartment, called lateral border recycling compartment (LBRC), in a process depending on microtubules and kinesin leading to paracellular immune cell diapedesis. The molecular mechanisms of transcellular diapedesis are less well defined.

Towards understanding immunosurveillance of the CNS

Immunosurveillance of the CNS requires the migration of circulating immune cells either across the endothelial BBB or BLMB or across the epithelial BCSFB in the absence of neuroinflammation. By tracing intravenously injected radioactively labeled encephalitogenic T cells in Lewis rats, it was first observed in the 1980s that freshly activated T cell blasts, but not resting T cells, can cross the BBB in the spinal cord [9,10]. Underlining the unique barrier characteristics of the BBB, however, T cell extravasation was not very efficient, and very few T cells were observed in perivascular locations [9]. More than 10 years later, these observations were confirmed by following the interaction of encephalitogenic T helper 1 (Th1) cells with the spinal cord microvasculature, using epifluorescence intravital microscopy (IVM) in SJL/J mice [11]. The T cell blasts failed to roll but instead abruptly stopped in the spinal cord microvessels; an event referred to as capture [11]. α4-integrins were involved in mediating both the initial Th1 cell capture and the subsequent G-protein-dependent Th1 cell arrest in response to low amounts of constitutively expressed VCAM-1 on the BBB endothelium. Th1 cell extravasation was first observed at 3 h after infusion and involved additional molecules such as LFA-1 [11,12]. The lack of rolling, the predominant involvement of α4-integrins and the extended time period required for diapedesis underline the unique characteristics of the multistep T cell extravasation across the BBB [11]. This resembles data from live cell imaging of the interaction of activated T cells with the retinal microvasculature that establishes the blood–retina barrier and closely resembles the BBB. In those studies, the prolonged time for T cell extravasation across noninflamed retinal microvessels was shown to depend on the local induction of endothelial VCAM-1 and ICAM-1 around adhering T cells as a prerequisite for their subsequent extravasation [13]. All these experiments focused on studying the migration of highly activated effector/memory T cells across the BBB, thus modeling immunosurveillance of the CNS after peripheral activation of the adaptive immune system.

T cell migration across the BLMB seems more efficient as intravenously injected fluorescently labeled T cells can be detected in the brain leptomeningeal spaces at 2 h after infusion [14]. Although T cell diapedesis across the BLMB seems faster when compared to the BBB, its efficiency still seems strictly limited by the BLMB, because live observations of T cell interactions with leptomeningeal microvessels through the skull of young mice failed to detect any T cell interaction with the BLMB [15]. T cell migration across the BLMB was shown to depend on endothelial P-selectin [14]. In contrast to the endothelial cells of the BBB, BLMB endothelial cells constitutively express P-selectin, which is stored in Weibel–Palade bodies and can readily be released to the endothelial surface [16]. In addition, the molecular architecture of BLMB endothelial tight junctions was recently described to differ from that of BBB endothelial cells [17]. By providing trafficking cues that are distinct from those of the BBB, the BLMB might thus preferentially allow for T cell extravasation during immunosurveillance. Little is known about the chemokines involved in this process. The lymphoid chemokine CCL19 is constitutively expressed in CNS endothelial cells in mouse and human brains [18,19]. Chemokine CC receptor (CCR7)-dependent brain metastasis of leukemic T cells into brain leptomeningeal spaces [20] suggests luminal availability of CCL19 at the BLMB, where it could trigger the extravasation of $CCR7^+$ T cell subpopulations, such as central memory T (TCM) cells. By contrast, the excellent diffusion characteristics of CCL19 make it a less likely candidate for mediating arrest during T cell extravasation.

Intravenously injected fluorescently labeled T cells are observed within the choroid plexus parenchyma with similar kinetics as in the leptomeningeal spaces [14]. P-selectin, which is constitutively expressed by the endothelial cells of the fenestrated choroid plexus microvessels

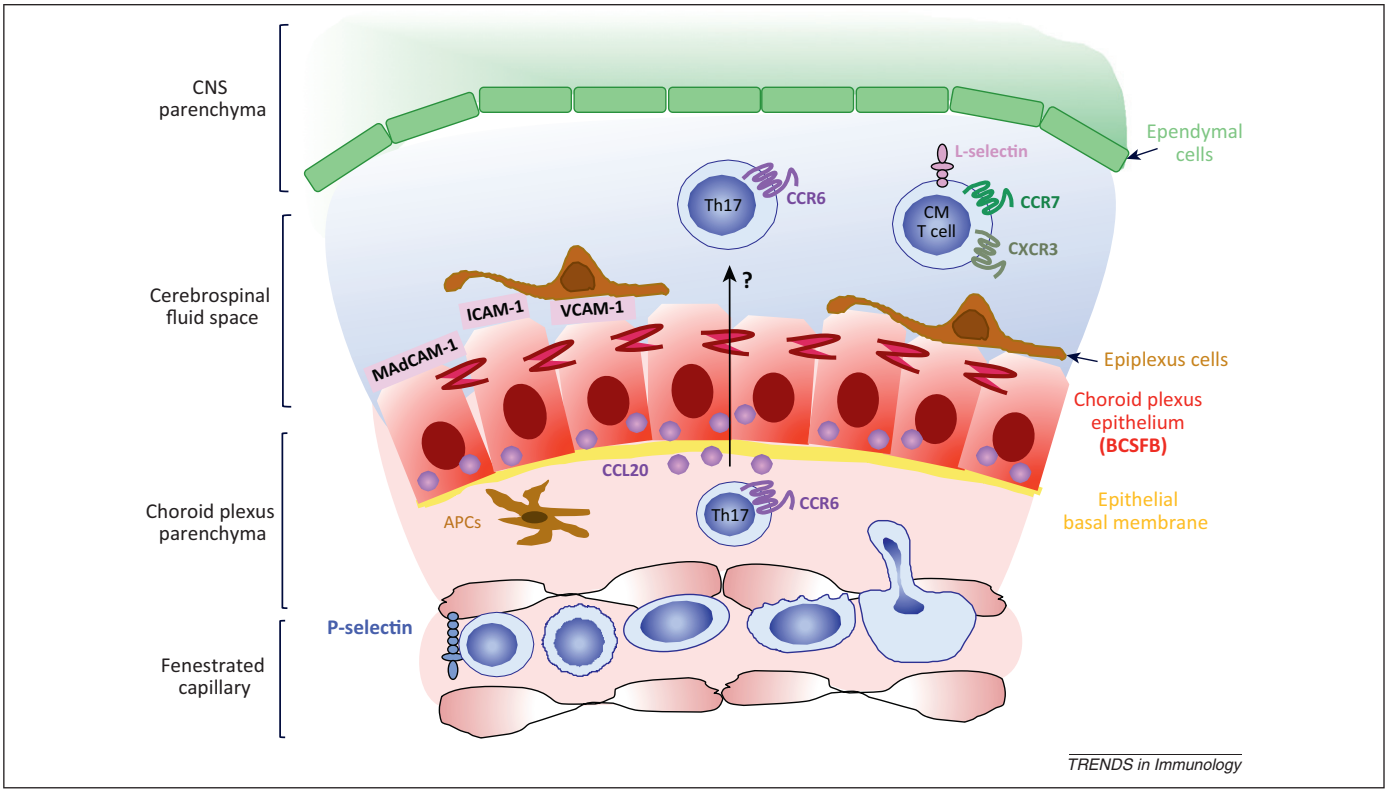

Figure 2. Molecular mechanisms involved in T cell migration across the epithelial blood–cerebrospinal fluid barrier (BCSFB). The choroid plexus might be a preferential T cell entry site into the central nervous system (CNS) during immunosurveillance, that is, in the absence of neuroinflammation. Circulating T cells extravasate in a P-selectin-dependent manner across fenestrated capillaries to reach the choroid plexus parenchyma, which is outside the CNS. To reach the CSF-filled ventricles, T cells need to breach the BCSFB established by choroid plexus epithelial cells. The paracellular pathway is sealed by unique tight junctions between the choroid plexus epithelial cells. $CCR6^+$ T helper (Th)17 cells may use chemokine CC ligand (CCL)20 expressed by choroid plexus epithelium as a guidance cue to migrate across the BCSFB into the CSF-filled ventricular space. The high number of central memory T (TCM) cells found in the CSF of humans suggests that this T cell subset preferentially crosses the BCSFB. The molecular mechanisms used by TCM cells to migrate across the BCSFB are unknown. Functional expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and under inflammatory conditions, mucosal addressin cell adhesion molecule (MAdCAM)-1 is restricted to the apical surface of choroid plexus epithelial cells, and is thus not available for the basolateral to apical migration of immune cells across the BCSFB. The choroid plexus is in constant movement and these adhesion molecules might instead allow T cells to crawl along the surface of the choroid plexus epithelium or alternatively might mediate the adhesion of antigen-presenting cells, so called epiplexus cells, ensuring their strategic localization behind the BCSFB to present CNS antigens to the T cells during immunosurveillance.

[21], mediates T cell extravasation into this site [14]. From the choroid plexus parenchyma, which is outside the CNS, T cells could reach the CSF-filled ventricles by breaching the BCSFB (Figure 2). Indirect evidence that T cells use the BCSFB as entry site into the CNS was provided by the observation that in healthy individuals T cells found in the ventricular and lumbar CSF are mainly TCM cells and thus distinct from T cell subpopulations present in the circulation or in the inflamed brain parenchyma during EAE or MS [22,23]. This suggests that, in healthy individuals, $CD4^+$ TCM cells enter the CSF in a regulated manner via the BCSFB to execute immune surveillance of the CNS. Although constitutive expression of functional ICAM-1 and VCAM-1 has been described on choroid plexus epithelial cells [24], their strict localization to the apical aspect of the choroid plexus epithelial cells [25] make them unlikely candidates for mediating T cell migration across the BCSFB from the basolateral to the apical side. The first molecular mechanism for T cell migration into the CNS via the choroid plexus was provided by a recent study in the model of myelin oligodendrocyte glycoprotein (MOG)-peptide induced EAE in C57BL/6 mice, demonstrating that encephalitogenic Th17 cells may penetrate the BCSFB via CCR6 binding to CCL20, which is produced by choroid plexus epithelial cells, but not by endothelial cells of the BBB and BLMB in rodents and humans [26]. $CCR6^+$ EGFP-expressing encephalitogenic Th17 cells were found to accumulate and remain in the leptomeningeal spaces [26], suggesting that they might trigger neuroinflammation from this site. These observations suggest that autoagressive T cells orchestrate the subsequent influx of inflammatory cells during EAE or MS exclusively from CSF-drained leptomeningeal or perivascular locations. In fact, this was already suggested more than 20 years ago in a study that followed intravenously injected radioactively labeled encephalitogenic T cells into the CNS and found them to remain restricted to perivascular localizations during the initiation and first clinical episode of EAE [27]. Chemokine CXC ligand (CXCL)12 is constitutively expressed by CNS endothelial cells and was recently found to be released at the basolateral site in murine and human brain tissues [28,29], where it mediates the retention of CXCR4-expressing encephalitogenic T cells within the CSF-drained perivascular and leptomeningeal spaces [28]. CXCR4 therefore inhibits immune cell entry into the CNS parenchyma during immunosurveillance in the absence of neuroinflammation.

Breaching the inflamed brain barriers

APCs are strategically localized behind the BBB, the BLMB, and the BCSFB. Perivascular spaces behind the

BBB harbor rare dendritic cells (DCs) [30], whereas the leptomeningeal spaces harbor a significant number of leptomeningeal macrophages [3]. Finally, MHC class II-expressing macrophages referred to as Kolmer or epiplexus cells adhere to the apical aspect of the epithelial cells forming the BCSFB [31]. The CSF produced by the choroid plexus drains from the ventricles towards the leptomeningeal spaces, which are connected to the perivascular spaces and interstitial fluid of the CNS parenchyma drains into the CSF. Thus, APCs localized to these compartments will continuously be exposed to all CNS antigens and thus ensure immunosurveillance of the CNS. If autoaggressive neuroantigen-specific T cells therefore cross the BBB, BLMB, or the BCSFB and subsequently recognize their antigen on any of those APCs, they will trigger the inflammatory events leading to the expression of additional trafficking molecules on these outer brain barriers, allowing for the recruitment of additional immune cells from the bloodstream into the CSF-drained perivascular and subarachnoid spaces and finally across the glia limitans into the CNS parenchyma. Direct evidence for this scenario has recently been provided by a two-photon intravital time-lapse imaging study in a Lewis rat model of EAE [3]. Intravenously transferred GFP^+ encephalitogenic T cells accumulated within leptomeningeal spinal cord vessels 1–2.5 days after transfer, where they extravasated and remained restricted to the leptomeningeal space until onset of clinical EAE at 3 days after transfer, which coincided with the migration of inflammatory cells across the glia limitans into the CNS parenchyma [3]. Antigen recognition was not involved in T cell penetration across the BLMB as intravenously infused GFP^+ ovalbumin-specific T cells readily accumulated in the leptomeningeal space. Ovalbumin-specific T cells, however, failed to leave the leptomeningeal space unless ovalbumin-pulsed APCs were injected into the leptomeningeal space, which allowed the T cells to enter the CNS parenchyma. This suggests antigen recognition to induce the molecular mechanisms required for immune cell infiltration into the CNS parenchyma. The finding that $CCR6^+$ encephalitogenic Th17 cells, after entering the CNS through the BCSFB, accumulate in leptomeningeal spaces in mice [26], together with recent evidence for meningeal inflammation early in MS that is related to cortical subpial demyelination, similar to that observed in EAE [32], argues that neuroinflammation might be preferentially initiated within the leptomeningeal spaces. From here inflammatory cells could invade the CNS parenchyma by crossing the glia limitans superficialis of the surface of the brain and spinal cord.

Rolling and capture

Upon recognition of antigen and the initiation of autoimmune inflammatory events, endothelial cells of leptomeningeal brain microvessels, as well as spinal cord microvessels, upregulate additional signals to allow T cell rolling. Live cell imaging of T cell interactions with the inflamed spinal cord microvessels during ongoing EAE in mice has shown that α4β1-integrin is no longer required for the initial T cell capture or rolling but is still needed to mediate T cell adhesion [33,34]. Instead, T cell tethering and rolling are mediated by PSGL-1 interacting with its endothelial ligand P-selectin, as observed in inflamed brain leptomeningeal microvessels [35,36] (Figure 3). Increased numbers of circulating $CD4^+$ T cells expressing high levels of PSGL-1 are found in MS patients and these cells show enhanced migration across human brain endothelial cells *in vitro* [37]. PSGL-1 only functions as a selectin ligand when decorated by specific carbohydrate side chains [38], which was not addressed by this study. Another study of humans and mice has shown that $CD8^+$ rather than $CD4^+$ T cells from MS patients can engage PSGL-1 to roll on P-selectin *in vitro* and in inflamed leptomeningeal vessels in an EAE model [36]. By contrast, studies in mice lacking functional PSGL-1 or E- and P-selectin have not found a role for these molecules in EAE pathogenesis (summarized in [39]). Further studies are therefore needed to understand the contribution of PSGL-1 and its selectin ligands in T cell migration across the brain barriers. Supporting this, a specific polymorphism in *PSGL1* associates with primary-progressive MS [40].

Chemokine induced integrin activation

Stable adhesion of encephalitogenic T cells within brain meningeal microvessels during EAE requires signaling via GPCRs present on the surface of the circulating immune cells [15]. This suggests that chemokines or eicosanoids displayed on the luminal side of the brain endothelium are involved in T cell recruitment across the endothelial brain barriers during EAE. Expression of the lymphoid chemokines CCL19 and CCL21 is upregulated in brain endothelial cells in a mouse model of EAE, and induces firm adhesion of encephalitogenic Th1 cells to inflamed brain microvessels in frozen brain sections *in situ* [18]. These T cells expressed $CCR7^+$ [18], however, Th1 cells also express CXCR3 that interacts with CCL21, and thus the chemokine receptors involved in integrin-mediated T cell arrest on the inflamed BBB and BLMB remain to be defined. Increased expression of CXCL12 observed in the CNS during EAE and MS is accompanied by loss of polarized expression of this chemokine in CNS microvascular endothelial cells [28,29]. Luminally available CXCL12 might therefore mediate T cell arrest to the inflamed BBB or BLMB in EAE and MS [28,29,41]. Alternatively, effector T cells might bypass the necessity for G-protein-mediated integrin activation for arrest and crawling as recently demonstrated for effector T cell interaction with non-CNS endothelium *in vitro* [42]. In this scenario, effector T cells were instead found to require GPCR signaling for the contact-guided consumption of intraendothelially stored chemokines to diapedese across inflamed endothelium [42].

Arrest–polarization–crawling

Integrins in their activated conformation are required to mediate arrest of T cells within CNS microvessels at the level of postcapillary venules. Upregulation of the integrin ligands ICAM-1 and VCAM-1 is observed on endothelial cells of the BBB and the BLMB, as well as on choroid plexus epithelium of the BCSFB in EAE (summarized in [5], Figures 3 and 4). The inflammatory cells localized around ICAM-1 and VCAM-1-positive CNS venules stain

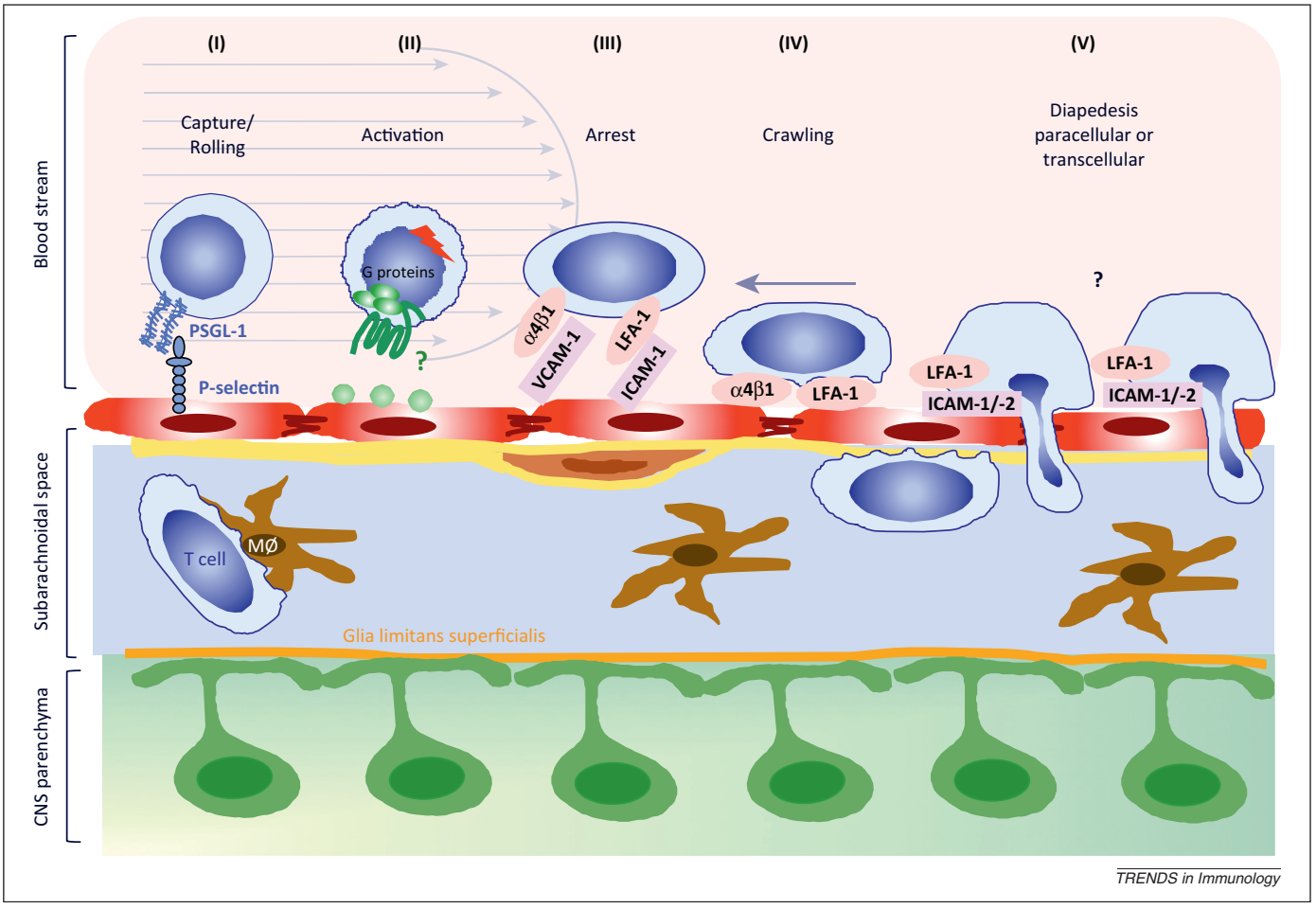

Figure 3. Molecular mechanisms involved in T cell migration across the endothelial blood–leptomeningeal barrier (BLMB). Recognition of antigen on leptomeningeal macrophages by T cells in the subarachnoidal space triggers inflammatory events leading to the upregulation of adhesion molecules on endothelial cells of leptomeningeal vessels (red cells). P-selectin that is stored in Weibel–Palade bodies of meningeal blood vessel endothelial cells is rapidly released on the endothelial cell surface allowing P-selectin glycoprotein ligand (PSGL)-1-mediated rolling of T cells and the reduction of their velocity. The chemokines that trigger G-protein-dependent integrin activation are not yet characterized. Leukocyte function-associated antigen (LFA)-1–intercellular adhesion molecule (ICAM)-1- and α4β1-integrin–vascular cell adhesion molecule (VCAM)-1-dependent T cell arrest is followed by T cell crawling at velocities of 10–12 μm/min (calculated from [3]) preferentially against the direction of flow on the luminal surface of the inflamed leptomeningeal microvascular endothelial cells. After transcellular or paracellular diapedesis, T cells crawl in random directions along the abluminal side of the leptomeningeal vessels and eventually interact with leptomeningeal macrophages.

positive for LFA-1, the ligand for ICAM-1, and for α4β1-integrin but not for α4β7-integrin, the ligand for VCAM-1, respectively. Similarly, in MS lesions, LFA-1$^+$ inflammatory cells accumulate around venules with high endothelial expression of ICAM-1 [43,44]. In contrast to EAE, VCAM-1 expression has been described on CNS microvessels in some [45] but not in other [46] MS lesions. However, in human CNS microvessels, the CS1 domain of a spliced variant of fibronectin (FN-CS1) could serve as an alternative ligand for α4-integrins as recently observed *in vitro* [47].

The functional involvement of LFA-1–ICAM-1- and α4β1-integrin–VCAM-1-mediated T cell adhesion to inflamed CNS endothelium *in vitro* has been proven by multiple assays investigating T cell adhesion to inflamed cerebral vessels [48,49] or to cultured brain microvascular endothelial cells in adhesion assays [47,50–53]. However, these studies were performed under static conditions omitting physiological flow, and the precise involvement of these molecules in T cell arrest versus post-arrest T cell behavior on the brain endothelium such as T cell polarization or T cell crawling to sites of diapedesis could not be determined. *In vitro* time lapse imaging techniques have more recently shown that 3 min after cytokine-stimulated arrest on primary brain endothelial cells, encephalitogenic Th1 cells polarize and begin to crawl on the surface of the brain endothelial cells, preferentially against the direction of flow, to find sites permissive for diapedesis across the endothelial barrier [54]. Although T cell arrest on the brain endothelial cells involves α4-integrin–VCAM-1 and LFA-1–ICAM-1 interactions, T cell polarization and crawling are exclusively mediated by LFA-1 binding to endothelial ICAM-1 and ICAM-2 [54]. In the absence of endothelial ICAM-1 and ICAM-2, T cells lose their ability to polarize and crawl and the few that remain attached to the brain endothelium seem repeatedly to undergo α4-integrin–VCAM-1-mediated arrest [54]. Mechanosensing of the flow is a characteristic of the T cells, which could also crawl against the flow in the absence of endothelial cells on purified ICAM-1 and ICAM-2, but not on VCAM-1 [54]. By contrast, the necessity for the T cells to crawl long distances to find a site for diapedesis is a unique characteristic of the highly specialized barrier forming CNS endothelial cells: T cells crawl significantly longer

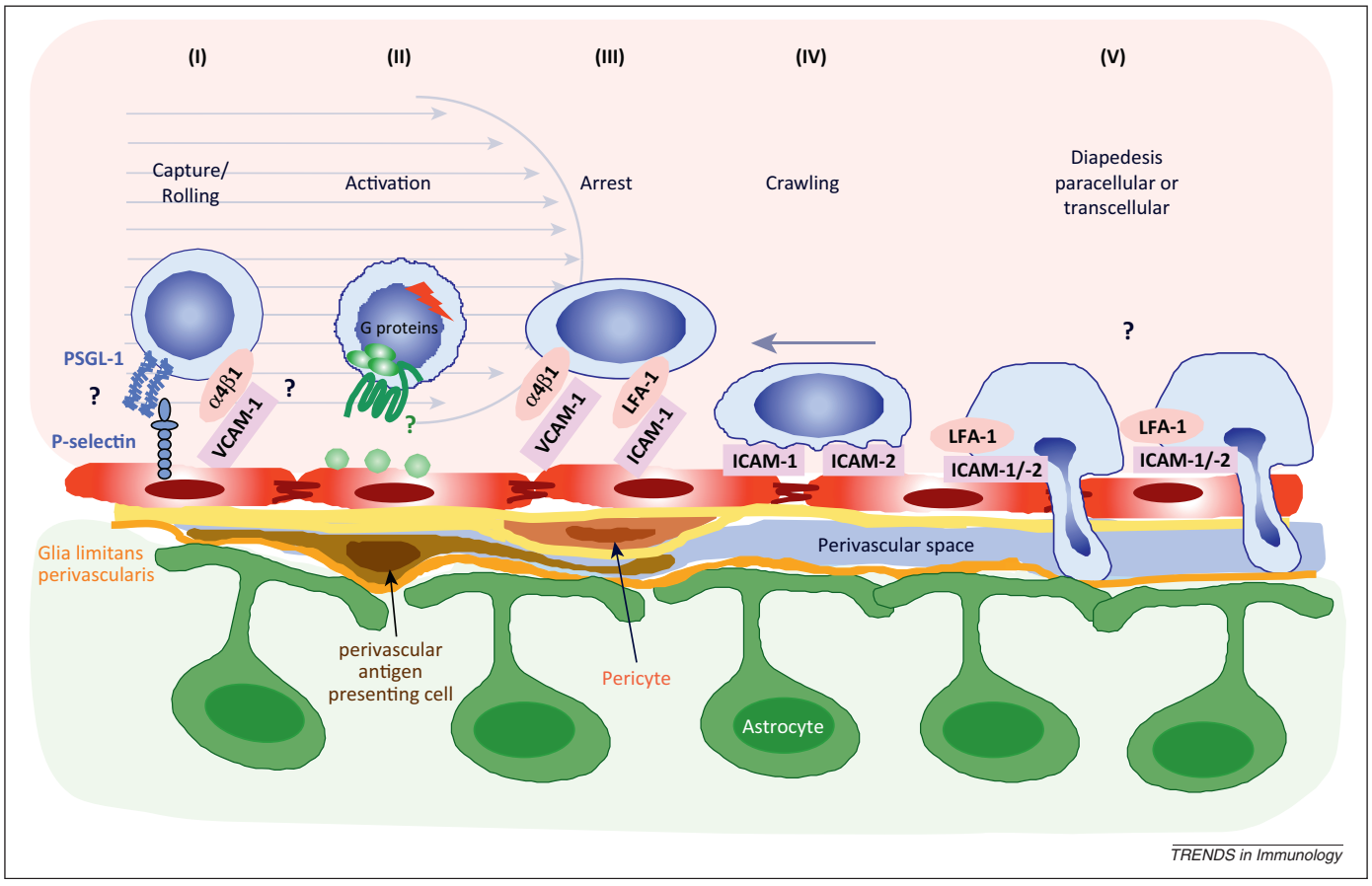

Figure 4. Molecular mechanisms involved in T cell migration across endothelial blood–brain barrier (BBB). As a result of the different neuroanatomy and expression of adhesion and tight junction proteins in parenchymal versus leptomeningeal endothelial cells, the molecular mechanisms of T cell migration across the BBB might be distinct from those controlling T cell migration across the blood–leptomeningeal barrier (BLMB). A predominant role of α4β1-integrins in mediating T cell capture and subsequent G-protein-dependent T cell arrest might be unique to the BBB in the spinal cord. P-selectin can be upregulated during neuroinflammation, providing a cue for P-selectin glycoprotein ligand (PSGL-1)-mediated T cell rolling during ongoing neuroinflammation. G-protein-dependent integrin activation on T cells triggers their arrest on the BBB in a Leukocyte function-associated antigen (LFA)-1–intercellular adhesion molecule (ICAM)-1- and α4β1-integrin–vascular cell adhesion molecule (VCAM)-1-dependent manner. At least in an *in vitro* BBB model, T cells polarize and crawl against the direction of flow with a mean velocity of 4 μm/min in an ICAM-1- and ICAM-2-dependent manner. Diapedesis across the BBB is observed to occur preferentially through the endothelium, leaving the tight junctions molecularly intact.

distances on CNS endothelial cells as compared to non-barrier-forming endothelium [55]. T cells also crawl against the direction of blood flow *in vivo* and do so for extended distances in leptomeningeal spinal cord microvessels during the onset of EAE, before finding a site permissive for diapedesis across the BLMB [3]. Dynamic adherence of the T cells depends on α4-integrin–VCAM-1 and LFA-1–ICAM-1, because infusion of function-blocking antibodies targeting T cell integrins leads to instantaneous detachment of the intraluminally adherent T cells [3].

The development of EAE in many models including Lewis rats, guinea pigs and SJL/J mice can be abrogated by blocking α4-integrins, β1-integrins or VCAM-1 (summarized in [5]). By contrast, blocking the α4β7-integin heterodimer fails to inhibit EAE development [56] and ectopic expression of the α4β7-integin ligand MAdCAM-1 in CNS endothelial cells fails to trigger CNS recruitment of α4β7-integrin$^+$ T cells and aggravation of EAE [57]. Thus α4β1-integrin rather than α4β7-integrin mediates T cell interaction with CNS endothelium.

Therapeutic targeting of α4-integrins has been translated into the clinic, where the humanized monoclonal anti-α4-integrin antibody natalizumab has proven beneficial in the treatment of relapsing–remitting MS. These observations underline the important role for α4β1-integrin in mediating shear resistant arrest of encephalitogenic T cells to the inflamed CNS endothelium or that α4-integrin contributes to other immune mechanisms relevant in EAE and MS pathogenesis. In this context, it is interesting to note that until recently [57], not much attention has been paid to the previous observation that EAE, when actively induced in C57BL/6 mice by immunization with myelin oligodendrocyte glykoprotein (MOG) in CFA, cannot efficiently be blocked by targeting α4-integrins [35]. An explanation for this finding might be the recent observation that T cell specific deletion of α4-integrins in C57BL/6 mice inhibits the migration of encephalitogenic Th1 but not of encephalitogenic Th17 cells into the CNS parenchyma [58,59]. Thus, efficient inhibition of EAE or MS might occur in Th1- rather than in Th17-driven disease. α4-integrin-deficient Th17 cells are able to enter the brain but not the spinal cord and this migration requires LFA-1. Thus, in accordance with previous observations showing a predominant role for α4-integrins in the capture and arrest of encephalitogenic Th1 cells in spinal cord microvessels [11], Th1 cells seem preferentially to cross spinal cord microvessels in an α4β1-integrin-dependent manner, whereas Th17 cells might enter the brain via the BCSFB in a CCR6/CCL20-dependent manner. Th17 cell

entry into the brain occurs in the absence of α4-integrins but is dependent on LFA-1 [58]. Despite the proven contribution of LFA-1 and its endothelial ligands ICAM-1 and ICAM-2 in T cell arrest, polarization, and crawling on the inflamed brain endothelium *in vitro* [54], their functional inhibition or absence in a variety of EAE models has produced contradictory results, ranging from inhibiting EAE to increasing severity of EAE or having no effect at all [60–62]. These differences might be due to a varied contribution of Th1 versus Th17 cells in the different EAE models investigated. Furthermore, a recent study has described the presence of alternatively spliced functional ICAM-1 isoforms that influence EAE pathogenesis in several ICAM-1 mutant mice [63]. Therefore, further investigations will be necessary to delineate fully the role of LFA-1 and its ligands ICAM-1 and ICAM-2 in the trafficking of different T cell subsets across the brain barriers.

Transcellular versus paracellular diapedesis

Although endothelial ICAM-1 and ICAM-2 are essential for T cell crawling on the endothelium, passage of low numbers of T cells across brain endothelial monolayers can still be observed in the absence of ICAM-1 and ICAM-2 [54], suggesting different options for T cell diapedesis across the endothelial brain barriers. In principle, T cells can choose two alternative pathways to cross endothelial barriers. Extravasation of immune cells across vascular beds in peripheral tissues usually occurs through the endothelial junctions via the paracellular pathway. Investigations of immune cell diapedesis across the BBB into the inflamed CNS by means of transmission electron microscopy have invariably observed immune cell diapedesis to occur through the endothelial cells, that is, via a transcellular pathway, leaving the tight junctions morphologically intact (summarized in [64]). Only recently, transcellular diapedesis of immune cells through pores formed by the endothelial cells has received more attention and has been repeatedly observed in several studies (summarized in [8]). The complexity of the CNS endothelial tight junctions might favor transcellular over paracellular diapedesis because migration of T cells though BBB or BLMB tight junctions requires their coordinated opening and resealing. Furthermore, extended T cell crawling in search of a site permissive for diapedesis is only observed on barrier-forming CNS endothelium *in vitro* and *in vivo*, but not on brain endothelial cell monolayers that fail to establish a

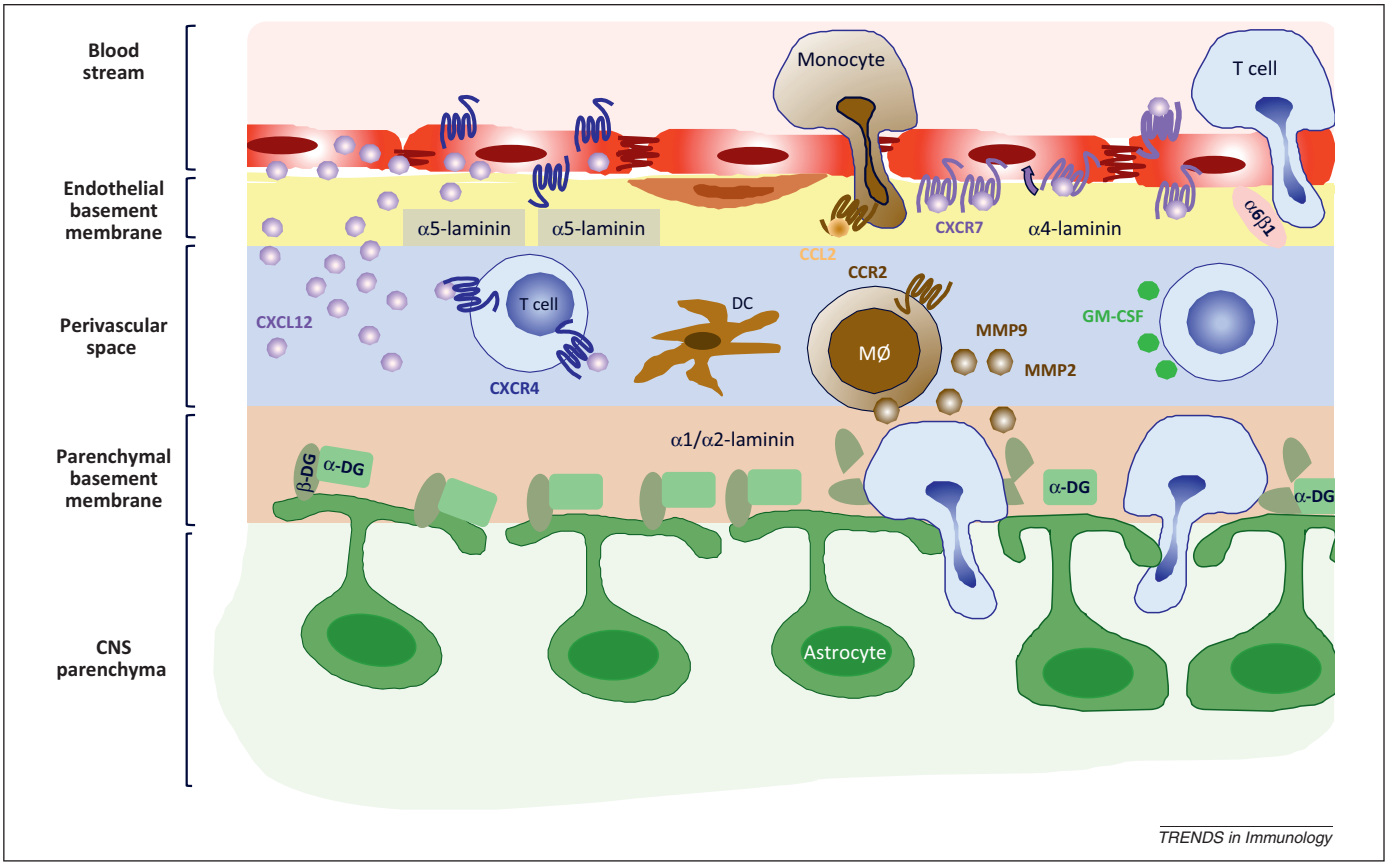

Figure 5. Molecular mechanism involved in T cell migration across the glia limitans. Upon breaching the endothelial cell layer, T cells have to penetrate the endothelial basement membrane (yellow) characterized by the localization of prevalent α4-laminin and patchy deposits of α5-laminin [66]. Although α4-laminin promotes T cell migration, α5-laminin inhibits T cell diapedesis. In the perivascular or leptomeningeal space (blue), high amounts of chemokine CXC ligand (CXCL)12 produced by endothelial cells and astrocytes are required to retain chemokine CXC receptor (CXCR)4^{+} immune cells in this space (shown on the left). Upregulation of CXCR7 during experimental autoimmune encephalomyelitis (EAE) on the abluminal side of the brain endothelial cells leads to rapid internalization or luminal translocation of CXCL12, allowing the release of CXCR4^{+} immune cells from the perivascular compartment (shown on the right). Granulocyte–macrophage colony-stimulating factor (GM-CSF) secreted by encephalitogenic T cells recruits myeloid cells such as monocytes or dendritic cells into the cerebrospinal fluid (CSF)-drained perivascular/leptomeningeal spaces; most likely in a CCL2/CCR2-dependent manner. Leptomeningeal macrophages are required to trigger T cell diapedesis across the glia limitans because they are the source of active matrix metalloproteinases MMP-2 and MMP-9 [87]. MMP-2 and MMP-9 cleave the extracellular matrix receptor β-dystroglycan from the astrocyte end-feed (green cells, shown on the right), which allows T cell penetration across the glia limitans into the central nervous system (CNS) parenchyma. Inhibition of CXCR7 or lack of MMP-2 and MMP-9 activity leads to immune cell accumulation in the perivascular space and amelioration of the clinical signs of EAE, supporting the notion that immune cell infiltration of the CNS parenchyma is necessary to trigger disease.

tight barrier [55]. On these leaky brain endothelial monolayers, T cells arrest and rapidly crawl to the next cell–cell junction for diapedesis. Thus, CNS endothelial cells might strictly control T cell diapedesis by directing them to rare sites, preferentially allowing for transcellular diapedesis. This pathway requires significant membrane trafficking, which has been found to be increased under inflammatory conditions in which CNS endothelial cells show increased numbers of intracellular vesicles able to form elongated transendothelial channel-like structures [65]. Future studies using live cell imaging will allow us to determine the signals that favor transcellular or paracellular diapedesis of T cells across the BBB or BLMB.

Breaching the endothelial basement membrane and the glia limitans

Upon penetration of the BBB or BLMB, T cells face the endothelial basement membrane where T cells preferentially migrate at sites containing the laminin isoform α4 but little or no laminin α5 in an α6β1-integrin-dependent manner [66,67] (Figure 5). Mice lacking α4-laminin are less susceptible to EAE due to the inhibitory role of laminin α5 on T cell migration across endothelial cell basement membranes of the BBB and BLMB [66]. Thus, the observation that β1-integrins are essential for T cell entry into the CNS parenchyma during EAE [34] might result from T cells using α4β1-integrin to cross the endothelial cell layer and subsequently α6β1-integrin to cross the endothelial basement membrane.

Although expression of endothelial CXCL12 is increased during EAE [28], upregulated expression of the chemokine receptor CXCR7 on inflamed endothelial cells of the BBB and BLMB leads to rapid translocation of CXCL12 by the inflamed endothelial cells, and thus, release of inflammatory cells from the basolateral side of the BBB and BLMB (Figure 5) [68].

To enter the CNS parenchyma, immune cells must finally traverse the glia limitans. The parenchymal basement membrane at the glia limitans is biochemically distinct from the endothelial cell basement membrane, with expression of laminin α1 and α2 rather than the endothelial laminins α4 and α5. Encephalitogenic $CD4^+$ T cells cannot interact with laminin α1 and α2 [67]. Rather, T lymphocyte penetration of the parenchymal basement membrane during EAE correlates with sites of focal activity of matrix metalloproteinases (MMPs), namely the gelatinases MMP-2 and MMP-9 [1]. In MOG-induced EAE in C57BL/6 mice, MMP-2 and MMP-9 cleave β-dystroglycan, a cell surface receptor anchoring astrocyte endfeet to the parenchymal basement membrane, suggesting a specific regulatory role for proteases in leukocyte extravasation across the glia limitans [1]. The regulatory role of MMPs might extend to modification of inflammatory chemokines released into the perivascular spaces during neuroinflammation because chemokines are physiological substrates for MMPs [69]. Supporting this, transgenic overexpression of CCL2 in the CNS parenchyma induces recruitment of immune cells across the BBB and BLMB into the perivascular and leptomeningeal spaces [70], but induction of MMP-2 and MMP-9 activity is required to release immune cell migration out of the perivascular cuff, across the parenchymal basement membrane and glia limitans into the CNS parenchyma [2].

Concluding remarks

Here, we discussed how evolutionary selection pressure towards allowing immunosurveillance of the CNS without endangering its homeostasis might have led to the development of a two-walled compartment around the CNS. The outer wall, constituted by the BBB, BLMB or BCSFB can be breached by distinct T cell subsets. Specific signals released from the highly specialized outer barrier cells ensure that patrolling T cells remain in the CSF-drained perivascular or leptomeningeal compartments right behind these barriers where they remain separated from the CNS parenchyma by the inner wall, the glia limitans. Recognition of their specific antigen on the APCs strategically localized between these barriers initiates neuroinflammation, characterized by the expression of additional traffic signals on the BBB, BLMB, and BCSFB, and the migration of immune cells from the blood stream into the CNS parenchyma. Neuroinflammation therefore requires penetration of the glia limitans, which is a second barrier that is unique to the CNS. Immune cell trafficking to the CNS is therefore unique and an attractive target for therapeutic intervention of neuroinflammatory disorders.

Therapeutic targeting of α4-integrins with the humanized anti-α4-integrin antibody natalizumab provides an effective treatment of relapsing–remitting MS. Unfortunately, natalizumab is associated with an increased risk of developing progressive multifocal leukoencephalopathy (PML); an often fatal disease of the CNS caused by JC virus infection of oligodendrocytes [71]. Because $CD8^+$ T cells are in charge of controlling virus infections, development of PML is thought to be due to natalizumab-mediated inhibition of CNS immunosurveillance by virus-specific $CD8^+$ T cells. A recent study has suggested a role of α4-integrins in $CD8^+$ T cell migration across the BBB *in vitro* [72], and studies addressing molecular mechanisms involved in migration of $CD8^+$ T cells into the CNS *in vivo* have supported this [34,73]. Because most PML cases occur in idiopathic or AIDS-related CD4 deficiency, natalizumab may additionally or alternatively interfere with the replacement of perivascular and leptomeningeal APCs by blocking the recruitment of circulating APCs or their precursors from the blood stream across the brain barriers. The latter view is supported by the finding that radiosensitive dendritic cells reside in the meninges and the choroid plexus [74], and that immature DCs rely on α4β1-integrins to migrate across the BBB and BLMB [75].

Future work should compare the relevance of the BBB, BLMB, and the BCSFB as entry sites for different immune cell subsets into the CNS in health and disease, and aim to define the role of the glia limitans during neuroinflammation to identify the molecular cues required for breaching the respective barriers by the individual immune cell subsets. This will improve understanding of the benefits and risks associated with targeting α4-integrins therapeutically and may help identify novel molecular targets to block specifically CNS recruitment of destructive immune cells while leaving the migration of protective immune cell subsets into the CNS unaffected.

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