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Going Against the Tide – How Encephalitogenic T Cells Breach the Blood-Brain Barrier

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Key Words
T cell extravasation · Blood-brain barrier · Crawling · Diapedesis · Inflammation

Abstract
During multiple sclerosis or its animal model, experimental autoimmune encephalomyelitis, circulating immune cells enter the central nervous system (CNS) causing neuroinflammation. Extravasation from the blood circulation across the vessel wall occurs through a multistep process regulated by adhesion and signal transducing molecules on the immune cells and on the endothelium. Since the CNS is shielded by the highly specialized blood-brain barrier (BBB), immune cell extravasation into the CNS requires breaching this particularly tight endothelial border. Consequently, travelling into the CNS demands unique adaptations which account for the extreme tightness of the BBB. Modern imaging tools have shown that after arresting on BBB endothelium, in vivo or in vitro encephalitogenic effector/memory T cells crawl for long distances, possibly exceeding 150 μm along the surface of the BBB endothelium before rapidly crossing the BBB. Interestingly, in addition to the distance of crawling, the preferred direction of crawling against the flow is unique for T cell crawling on the luminal surface of CNS microvessels. In this review, we will summarize the cellular and molecular mechanisms involved in the unique T cell behavior that is obviously required for finding a site permissive for diapedesis across the unique vascular bed of the BBB.

Introduction

Barriers of the central nervous system (CNS) are cellular interfaces shielding the vulnerable physiology of the brain or spinal cord parenchyma from the changeable milieu in the periphery. The largest brain barrier surface area is formed by the endothelial cells of the parenchymal CNS capillaries referred to as the blood-brain barrier (BBB). Further endothelial CNS barriers are formed by the pial vessels of the meninges and the inner retinal vasculature; the latter also named the blood-retina barrier [1]. BBB endothelial cells are the barrier-forming constituents of the neurovascular unit, which is further composed of the endothelial basement membrane containing a high number of pericytes, the parenchymal basement membrane and antigen-presenting cells residing in the perivascular space bordered by both basement membranes and astrocytic end-feet covering the entire ablu-

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minal face of the BBB [2, 3]. The endothelial BBB establishes a physical barrier by inhibiting paracellular diffusion of water-soluble molecules via complex tight junctions interconnecting brain microvascular endothelial cells and by limiting transcellular diffusion due to a low pinocytotic activity of the brain microvascular endothelium. At the same time, specific transport and efflux systems enable BBB endothelial cells to build a metabolic interface between the CNS and the blood ensuring the import of nutrients into and export of toxic metabolites out of the CNS. In contrast to the endothelial BBB, the blood-CNS barrier at the choroid plexus is formed by plexus epithelial cells secreting the cerebrospinal fluid, while the adjacent microvessels are permeable due to fenestration of their endothelial cells [3, 4].

Extravasation of encephalitogenic effector/memory T cells from the blood circulation across the BBB into the healthy CNS is a critical event for the development of autoimmune neuroinflammation such as experimental autoimmune encephalomyelitis (EAE), which is considered an animal model for multiple sclerosis [5]. Nevertheless, the BBB was regarded as a strong barrier for immune cells until seminal studies by Wekerle et al. [6] and Hickey et al. [7] demonstrated T cell extravasation across the BBB. Their experiments revealed that low numbers of encephalitogenic effector/memory T cells were able to reach perivascular spaces of CNS parenchymal blood vessels hours after intravenous injection into healthy rats [8]. Hence, those T cells must have crossed the parenchymal BBB. Twenty years later, based on in vivo imaging experiments performed in laboratory mice and rats, it is now established that initial contact formation between T cells and the healthy BBB of the spinal cord [9] or the noninflamed blood-retina barrier in the eye [10] does occur. Furthermore, the choroid plexus has been shown to be a potential entry site for encephalitogenic Th17 cells into the CNS [11, 12]. However, the vast majority of T cells which enter the CNS and contribute to the pathology of EAE extravasate at later stages of the disease across the already inflamed BBB or across pial microvessels to reach the CNS parenchyma [13].

Extravasation of T cells across the inflamed BBB during EAE is a multistep process mediated by the sequential interaction of cell adhesion molecules and chemokine receptors on the T cell surface with their respective counter receptors on the luminal surface of the CNS microvascular endothelial cells [14]. Decades ago, mononuclear cells adherent to BBB endothelium had already been visualized in fixed in situ samples by means of transmission electron microscopy [15]. However, only in the past few years has in vivo and in vitro live cell imaging of T cell interaction with BBB endothelium revealed that T cell adhesion does not simply translate into stationary T cell immobilization on the microvascular luminal surface. Rather, adherent T cells undergo a dynamic process of interactions with the inflamed BBB endothelium before diapedesis [16–18]. This dynamic process involves adhesion strengthening of the T cells after their arrest accompanied by T cell spreading and polarization. Immediately upon polarization the T cells start to crawl along the endothelial surface, a phenomenon which was also observed on non-BBB microvessels [19, 20]. However, specifically at the BBB T cells crawl for long distances, potentially exceeding 150 μm before diapedesis, and intriguingly their direction of crawling is preferentially against the direction of blood flow [16–18]. During crawling, T cells probe the endothelium with invasive cell protrusions to find a site permissive for diapedesis which can occur via two different routes: the paracellular route, which is between two endothelial cells through the inter-endothelial junctions, or the transcellular route, which requires formation of a pore through the body of an endothelial cell [15, 21]. A specific adaptation of T cell extravasation across the BBB appears to be the predominant diapedesis along the transcellular route [22]. After diapedesis across the endothelial BBB, T cells accumulate in the perivascular space where they build inflammatory cuffs. Here, encephalitogenic T cells can interact with antigen-presenting cells presenting myelin antigens leading to antigen-specific T cell reactivation. Finally, reactivated T cells breach the outer basement membrane to infiltrate the brain or spinal cord parenchyma.

Cell Adhesion Molecules Involved in T Cell Trafficking across the BBB

Three distinct protein families of adhesion molecules fulfill prime roles in the process of T cell extravasation across an endothelial monolayer, namely the selectins, integrins and the cell adhesion molecules of the Ig superfamily (IgCAMs).

The selectin family consists of three type I transmembrane glycoproteins with a distal lectin-like domain that can specifically bind to sialyl-LewisX-like carbohydrate structures exposed on their glycoprotein counter receptors such as P-selectin glycoprotein ligand-1 (PSGL-1) [23]. L-selectin is expressed by most leukocytes but – due to shedding – absent from effector/memory T cells [24]. The endothelial P- and E-selectin are likewise not readily avail-

able for extravasation of T cells across the BBB. Endothelial P-selectin is constitutively expressed in CNS meningeal but not parenchymal endothelial cells and in endothelial cells of the fenestrated capillaries of the choroid plexus where it is stored as a preformed protein in Weibel-Palade bodies. In contrast, E-selectin is not constitutively expressed in endothelial cells, its expression rather depends on de novo synthesis, which is readily induced in all endothelial cells including the BBB during inflammatory conditions [12, 25, 26]. During EAE, expression of E-selectin cannot be detected [26–28]. However, systemic injection of LPS can induce E-selectin expression in endothelial cells of leptomeningeal microvessels [29].

Integrins are heterodimers of two single transmembrane-spanning cell surface molecules, the α- and the β-integrin subunit. The integrin dimer occurs in three different functional conformations: a bent low affinity conformation, a straight intermediate affinity conformation and a high affinity conformation [30]. Change of conformation and thus affinity maturation are subject to signaling events in the T cell induced via chemokine receptor signaling [14] or via engagement of T cell adhesion molecules under shear flow conditions [31, 32].

The most prominent endothelial IgCAM members for T cell trafficking across the BBB identified to date are intercellular CAM (ICAM)-1, ICAM-2 and vascular CAM (VCAM)-1 [33]. In addition, platelet/endothelial CAM (PECAM)-1 and activated leukocyte CAM (ALCAM) are IgCAM members with implications for T cell extravasation across brain endothelium [34, 35]. All IgCAMs share a common molecular structure with multiple extracellular Ig-like domains at their N-terminus, a single spanning transmembrane domain and a short cytoplasmic tail. ICAM-1 has 5 Ig-like domains. ICAM-2 is smaller with only 2 Ig-like domains which are homologues to the very first two domains of ICAM-1 [36]. The integrin LFA-1 (αLβ2-integrin, CD11aCD18) expressed on T cells binds to the first Ig-like domain of ICAM-1 and ICAM-2, whereas the integrin Mac-1 (αMβ2, CD11bCD18) expressed on myeloid cells only interacts with ICAM-1 since its binding site localizes to the third domain of ICAM-1 not present in ICAM-2 [37]. Endothelial VCAM-1 exists in 2 alternative splice isoforms with 6 or 7 Ig-like domains and can interact with the T cell expressed α4 integrins VLA4 (α4β1) and α4β7 [14]. While ICAM-2 is constitutively expressed by all vascular endothelial cells irrespective of the inflammatory conditions, ICAM-1 and VCAM-1 are expressed at basal levels under noninflamed conditions. They are strongly upregulated during EAE and by proinflammatory cytokines on meningeal [29] and CNS parenchymal microvascular endothelial cells [38, 39], as well as on the choroid plexus epithelium [40].

Initial Contact of Circulating Effector/Memory T Cells with Inflamed CNS Microvessels

To extravasate from the blood stream into the CNS parenchyma T cells must get into intimate contact with the BBB. Adhesive interactions between the T cells and the luminal surface of the blood vessels must be strong enough to resist shear forces exerted by the blood flow which in mice are in the range of 4–6 dyn/cm^2 in noninflamed or inflamed spinal cord postcapillary venules with diameters ranging from 20 to 60 μm [9, 41], and of 15 dyn/cm^2 in central retinal veins with a diameter of 65–75 μm during experimental autoimmune uveitis [10]. Initial contact of T cells with the inflamed BBB occurs through two mechanistically distinct principles – the tethering and subsequent rolling or the transient capture. Rolling T cells are in close contact with the luminal surface of the microvessels and move at a speed between 5 and 10 μm/s [29], which is much slower than blood flow velocity of at least 1,000 μm/s [29, 41–43]). Transient capture to retinal or spinal cord microvascular endothelial cells occurs instead of rolling as an abrupt but transient stop of the T cells to the vessel wall mostly in the absence of neuroinflammation [9, 10, 41]. Capture of T cells to the vascular wall seems to be unique for activated T cells interacting with CNS microvessels as it has not been observed elsewhere.

Molecular mechanisms of initial contact formation of T cells with the inflamed BBB are different from those at the noninflamed BBB since, in addition to T cell capture, T cell rolling is specifically observed under inflammatory conditions of the CNS. Whereas T cell capture to the BBB in the absence of neuroinflammation is mediated by α4-integrin/VCAM-1 interactions, α4-integrins are no longer required for the initial contact of T cells mediated by T cell rolling and capture with the inflamed BBB during EAE [41, 44]. Interestingly though, α4-integrin-mediated T cell rolling in brain leptomeningeal vessels was observed in mice after systemic injection of TNF-α [29]. EAE studies in C57BL/6 mice with conditional α4-integrin deleted T cells revealed a selective ability of Th17 but not Th1 cells to enter the brain parenchyma independent of α4-integrins [45, 46]. Thus, it is coherent that upon α4-integrin deficiency only encephalitogenic Th17 and not encephalitogenic Th1 cells can transfer EAE [46]. However, only in vivo imaging experiments will reveal the

different molecular requirements of individual encephalitogenic T cell subsets for initial contact formation with the inflamed BBB.

In addition, endothelial P-selectin interacting with PSGL-1 on the T cells mediates T cell rolling in inflamed leptomeningeal brain microvessels of SJL/J and C57BL/6 mice during EAE [28]. Functional absence of P-selectin almost completely abolished initial contact formation of T cells in this vascular bed supporting the predominant role for P-selectin/PSGL-1 in T cell rolling [28]. Interestingly, development of EAE is independent of P-selectin or PSGL-1, as mice lacking these molecules develop EAE indistinguishable from wild-type animals [26, 28, 47–50]. However, in SJL/J mice the dual blockade of α4-integrin with P-selectin inhibited disease progression more effectively than treatment with an anti-α4 integrin antibody alone [28]. Therefore, a redundant role of the interaction partners P-selectin/PSGL-1 and VCAM-1/α4-integrin in the process of T cell rolling and capture on the inflamed BBB or inflamed leptomeningeal microvessels can be assumed [2, 28].

Chemokines Activate Integrins on the Surface of T Cells

Once the T cell has performed an initial contact with the endothelial surface, its interaction strength with the endothelial surface must be increased for sustained blood flow shear resistance. This is achieved in a very fast process in the range of milliseconds by changing the binding affinity of T cell-expressed integrins to their respective endothelial IgCAM ligands from a low-affinity to a high-affinity conformation [30, 51, 52]. This rapid signaling is induced by the binding of chemokines, displayed on the luminal surface of the endothelial cells, to chemokine receptors present on the T cell. Amongst the approximately 50 different chemokines only CCL2, CCL19, CCL21 and CXCL12 were related to EAE pathology and are expressed by BBB endothelial cells of mice or human [53–55]. The luminal surface of the endothelial cells is decorated by the endothelial glycocalyx, which is a network of negatively charged membrane-bound proteoglycans and glycoproteins [56, 57]. Proper functioning of chemokines in mediating the triggering of leukocyte arrest has been shown to be dependent on chemokine immobilization on heparan sulfate glycosaminoglycans of the endothelial glycocalyx [58–61].

Receptors for chemokines belong to the protein family of G-protein-coupled receptors. Once a chemokine engages its respective chemokine receptor on the T cell this induces a G-protein-dependent signaling cascade in the T cell affecting cytoskeletal dynamics and integrin conformation [30, 51]. Involvement of chemokine receptor signaling in the process of T cell extravasation can be tested by treatment of the T cells with pertussis toxin, which modifies the intracellular Gαi protein through ADP-ribosylation such that it cannot bind to G-protein-coupled receptors and in consequence Gαi-specific signal transmission is interrupted [62]. Pertussis toxin treatment of encephalitogenic T cells prior to their transfer into mice revealed that the transition of T cell capture to T cell firm arrest to the healthy [9] or to the inflamed CNS microvasculature [29] critically depends on the activation of integrins via Gαi signaling. Nevertheless, those chemokines luminally displayed on the BBB endothelium and accounting for integrin activation in the process of T cell arrest to this specific vascular bed still need to be identified.

Dynamic Behavior of Adherent T Cells is an Essential Prerequisite for Diapedesis across the BBB

Multiple in vitro studies carried out under static conditions documented essential roles for endothelial ICAM-1 and VCAM-1 as ligands for T cell-expressed LFA-1 and α4-integrins, respectively, for firm adhesion of encephalitogenic T cells to brain endothelium [38, 63–66]. But, due to the lack of imaging and the absence of flow conditions, those studies neither analyzed the dynamic behavior of the T cell on the brain endothelium during adhesion nor did these assays account for shear resistant adhesiveness of the T cell on the brain endothelium. To obtain a more detailed view of the spatiotemporal behavior of the adherent T cell at the inflamed BBB and to account for physiological shear of blood flow, we recently analyzed the dynamic behavior of encephalitogenic T cells interacting with TNF-α-stimulated primary mouse brain microvascular endothelial cells (pMBMECs) in an in vitro imaging approach under flow conditions [18]. We observed that immediately upon arrest T cells underwent spreading of their cell body together with a cell shape change toward a polarized morphology displaying a broad lamellipodium at the front and a projected uropod at the rear (fig. 1) [18]. Spreading and polarization of the encephalitogenic T cells was a rapid process that was completed within only 1–3 min [18]. Strikingly, we noticed an extensive crawling behavior of the adherent T cell on pMBMECs prior to their diapedesis at a mean velocity of 4 μm/min (fig. 2). During an observation period of 30 min less than 5% of

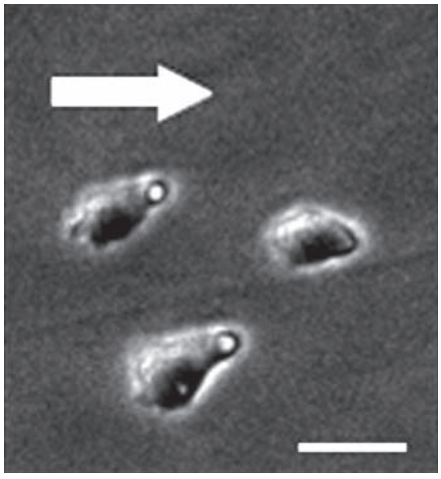

Fig. 1. Effector/memory T cells polarize and crawl on ICAM-1. Crawling T cells retain their polarized cell morphology with a broad lamellipodium at the front and a projected uropod at the rear. Effector/memory CD4+ Th1 cells crawling on immobilized recombinant ICAM-1 are shown. The arrow shows the direction of flow. Scale bar = 20 μm. Image represents one image of a time-lapse movie and was taken with the AxioObserver Z1 (Zeiss, Feldbach, Switzerland).

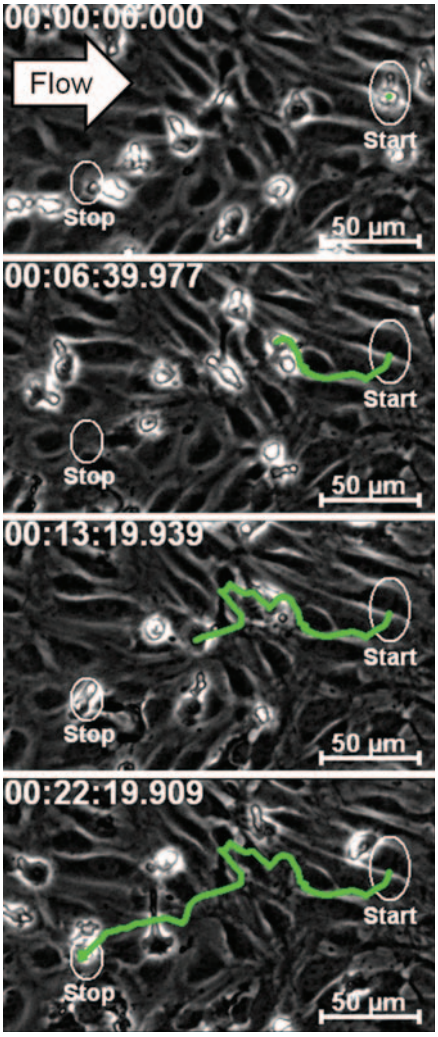

Fig. 2. Crawling path of an effector/memory T cell on BBB endothelial cells. T cells crawl long distances on BBB endothelial cells before they find a site permissive for diapedesis. Four images of a time-lapse movie are shown (relative time points are indicated in the upper left corner of each image). Effector/memory CD4+ Th1 cells were perfused over pMBMECs at low shear conditions. Then, shear was enhanced to physiological 1.5 dyn/cm^2 and T cell crawling was visualized by automated time-lapse image recording as described previously [17, 18]. One T cell is tracked from its site of attachment (start) until the site of diapedesis (stop). During a period of 22 min, this T cell crawled 160 μm on BBB endothelial cells with a net direction against flow. The arrow in the first image depicts the direction of flow. Scale bar = 50 μm. Images were taken with an inverted microscope, the AxioObserver Z1 (Zeiss).

arrested T cells remained stationary, whereas almost 95% of the T cells either continuously crawled on the surface of the BBB endothelial monolayer or underwent diapedesis after crawling [18]. Most interestingly, the crawling of T cells was directed against blood flow – a phenomenon never observed on non-BBB endothelial cells in vitro or in the peripheral vasculature in vivo. Taken together, it is now obvious that the formerly assigned 'T cell adhesion' step of the multi-step T cell extravasation cascade – at least at the BBB – combines 3 sequential events, namely shear resistant arrest of T cells, T cell spreading/polarization and T cell crawling against shear (fig. 3). These distinct steps might be individually regulated by different adhesion and signaling molecules.

In our in vitro approach, we analyzed the individual roles of endothelial ICAM-1, ICAM-2 and VCAM-1 for shear resistant T cell arrest, T cell spreading/polarization and directed T cell crawling against the flow. We found that ICAM-1 and VCAM-1 have redundant roles in mediating shear resistant arrest of encephalitogenic T cells to the BBB endothelial cells [18]. Thus, only in the functional absence of both, ICAM-1 and VCAM-1, complete abrogation of T cell arrest on the BBB occurred [18]. Interestingly, spreading, polarization and subsequent crawling of encephalitogenic effector/memory T cells on stimulated pMBMECs required endothelial ICAM-1 or ICAM-2. In the absence of ICAM-1 and ICAM-2 on pMBMECs T cells were found to completely lose their ability to crawl against the flow, demonstrating at the same time that endothelial VCAM-1 is not able to support T cell crawling against shear on the BBB [18].

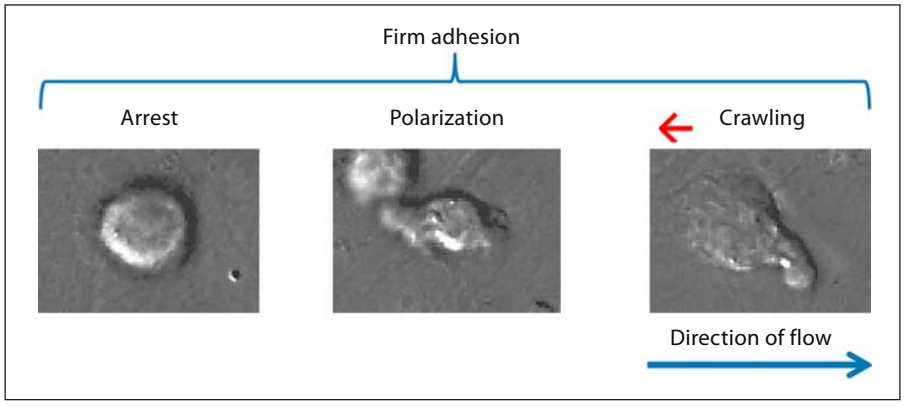

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Fig. 3. Dynamic behavior of firmly adherent T cells. Adherent T cells do not remain stationary. Instead T cells adherent to the BBB reveal a dynamic interaction pattern with the endothelial surface: shortly after shear-resistant arrest (left image) T cells polarize (middle image) and then start crawling (right image). On pMBMECs, T cell crawling is preferentially against the direction of shear and covers extraordinarily long distances before diapedesis. Images are extracted from images of a time-lapse movie in which effector/memory CD4+ T helper cells were interacting with pMBMECs as described previously [17, 18]. Microscopic images were acquired with the AxioObserver Z1 microscope and a 63× glycerol immersion objective (Zeiss).

In vivo, dynamic behavior of adherent encephalitogenic T cells during their interaction with inflamed leptomeningeal microvessels of the spinal cord has recently been visualized using 2-photon IVM [16]. Bartholomäus et al. [16] induced EAE by intravenous injection of GFP-expressing encephalitogenic T cells into healthy Lewis rats. The interaction of these GFP-positive T cells with the spinal cord microvasculature was then followed microscopically on day 1, 2 and 3 post-transfer into the animal. During the observation period the number of intraluminal GFP-positive T cells increased over time with a maximal number of adherent T cells on day 3 post-transfer, coinciding with clinical onset of EAE. Strikingly, adherent encephalitogenic T cells did not remain stationary but were extremely motile and crawled at a mean velocity of 12.5 μm/min for a mean duration of 15 min along the luminal surface of the leptomeningeal microvessels before they transmigrated across the endothelial barrier. Obviously, the crawling velocity of T cells observed in this in vivo imaging study was 3-fold increased compared to our in vitro study (table 1). This apparent discrepancy might be due to differences in the crawling velocities between the in vitro and in vivo situation with the in vitro situation not mimicking in full the in vivo cytokine and blood environment. Similar to the in vitro system, a substantial fraction of T cells crawled for the complete observation period of 30 min on the luminal surface of the vessels. A hitherto unique observation was the luminal crawling of the encephalitogenic T cells with a preferential direction against the blood flow. This finding is in perfect concordance to the results of our in vitro experiments (table 1). Apparently, in vivo directionality of crawling was induced by the shear of blood flow, because after extravasation the crawling behavior of T cells along the abluminal surface of the vessel wall no longer showed any strict directionality. On the molecular level dynamic adherence of the T cells was dependent on α4-integrin/VCAM-1 and LFA-1/ICAM-1 as function-blocking antibodies targeting the respective integrins, leading to an instantaneous detachment of intraluminally adherent T cells [16].

In vitro, we confirmed that sensation of the direction of shear is an intrinsic feature of encephalitogenic T cells, because T cells efficiently crawled against the direction of flow on purified recombinant ICAM-1 (fig. 1) or ICAM-2, but not VCAM-1 in the absence of any endothelial cell [18]. One possible reason for the prime role of LFA-1 compared to α4-integrins for the crawling of T cells on CNS endothelium against flow could be a higher affinity of the LFA-1/ICAM-1 interaction as compared to α4-integrin/VCAM-1 interaction. However, a direct comparison of binding affinities from both interaction pairs is not available to date. An alternative or additional reason for the prime role of LFA-1 for T cell crawling against flow could be based on different downstream signaling cascades triggered through LFA-1 or α4-integrin engagement, which then induce differential T cell dynamic behavior on ICAM-1 or VCAM-1, respectively [67].

Whereas mechanosensing of flow occurs at the level of the T cells, the remarkably long distances of up to several hundred micrometers which T cells have to crawl on the

Table 1. Comparison of T cell crawling on different brain endothelial cells

	bEnd5 [17]	pMBMECs [18]	Leptomeningeal microvessels [16]
Experimental setting	In vitro live cell imaging Model: immortalized brain endothelial cell line	In vitro live cell imaging Model: primary mouse brain microvascular endothelial cells	In vivo live cell imaging. Model: two photon in vivo imaging in rat and mouse spinal cord
Stimulation of endothelium	TNFα	TNFα	Clinical onset of transfer EAE
T cells	Encephalitogenic effector/ memory proteolipid protein-specific $CD4^+$ TH1 cells	Encephalitogenic effector/ memory proteolipid protein-specific $CD4^+$ TH1 cells	Encephalitogenic myelin basic protein-specific CD4+ effector T cells, expressing GFP under the ubiquitin C promoter
Mean crawling velocity, μm/min	2.9	4.0	12.5
Mean crawling distance before diapedesis, μm	25.3	55.2	187.5[a]
Mean crawling distance within 30 min of continuous crawling, μm	105	137	375[b]
Predominant crawling against the direction of flow	Yes	Yes	Yes
Role of endothelial ICAM-1/ VCAM-1 or T cell LFA-1/α4 integrin for T cell shear-resistant arrest on BBB endothelium	n.d.	Shear-resistant T cell arrest requires endothelial VCAM-1 and ICAM-1	Anti-LFA-1 pretreated T cells detach instantaneously from the walls of microvessels upon systemic application of an anti α4-integrin antibody

n.d. = Not determined.

[a] Value was not indicated in this research article, but calculated from the mean velocity multiplied by the mean crawling duration time before diapedesis.

[b] Value was not indicated in this research article, but calculated from the mean velocity and the observation period of 30 min.

BBB before finding an appropriate spot for diapedesis (fig. 2) is a unique characteristic defined by the highly specialized BBB endothelium [16, 18]. When comparing immortalized brain endothelioma cell lines (bEnd5) to pMBMECs with regard to the crawling distances of T cells before diapedesis we observed important differences. It became evident that T cells crawled significantly longer distances on pMBMECs as compared to bEnd5 [17]. In contrast to pMBMECs, bEnd5 fail to establish a tight permeability barrier due to an immature molecular composition of their cellular junctions [17, 68] and therefore do not reflect BBB-specific characteristics as closely as pMBMECs [68]. Thus, the unique tight junction architecture and low permeability of pMBMECs significantly influenced T cell interaction with these unique barrier-forming endothelial cells (table 1) [17]. It therefore seems that molecular cues for T cell diapedesis are sparse on the BBB leading to T cell polarization and subsequent crawling against the flow in search of sites permissive for migrating across the brain endothelium. This assumption is further substantiated by the extremely long crawling distances documented in the in vivo imaging study (table 1) [16].

In general, a dynamic behavior of adherent T cells is not unique to BBB endothelial cells but has also been observed on endothelial cells from other tissues [69, 70]. Also, similar morphological transitions – polarization and crawling – have been described for adherent monocytes and neutrophils in peripheral vascular beds [14, 71, 72]. Therefore, similar mechanisms might account for the dynamic behavior of different immune cell subsets in different vascular beds. Initially, post-arrest crawling prior to diapedesis has been observed in peripheral microvessels for immune cells of both the innate and adaptive immune systems in vivo. Auffray et al. [73] described extensive crawling of monocytes along the healthy microvasculature of the dermis; a behavior that appeared like safeguard patrolling for signs of infection or tissue damage. Upon inflammatory stimuli the distance of mono-

cyte crawling before diapedesis was dramatically shortened compared to the noninflamed situation [73]. Similarly, neutrophils were found to start crawling immediately upon their arrest to chemokine-stimulated postcapillary venules of the cremaster muscle in vivo [43, 74]. In addition, crawling of T cells was observed on peripheral-derived endothelial cells such as human umbilical vein endothelial cells (HUVECs) or human dermal microvascular endothelial cells (HDMVECs) under physiological flow in vitro [20, 69, 70, 75]. As a general observation, interfering with leukocyte or T cell crawling resulted in a reduced efficiency of extravasation and thus demonstrates that crawling is required for efficient diapedesis of various types of immune cells [43, 75, 76].

During crawling, T cells retain their polarized cell morphology with a broad and flat lamellipodium at the leading edge and a projected uropod at the trailing edge as shown for encephalitogenic effector/memory T cells (fig. 1) and similarly also for IL-2 stimulated CD4+ T cells [18, 70, 77]. While crawling on endothelial cells, T cells form and retract invasive cell protrusions on their ventral side predominantly behind their leading edge [75, 77]. The identity of these invasive cell protrusions has not been clarified to date, although a number of names, including podosomes, invadopodia and invasive filopodia, were assigned to them in various research articles [16, 67, 70, 78, 79]. Podosomes and invadopodia are actin-based dynamic cell protrusions that function for adhesion to and degradation of extracellular matrix components. Notably, podosomes and invadopodia have a well-defined molecular architecture characterized by an actin-rich core that is surrounded by regulatory and scaffolding proteins to tightly regulate actin filament turnover [80]. Filopodia are thin, finger-like cell protrusions that are filled with tight parallel bundles of filamentous actin but lack pericellular proteolytic activity [81]. To express the invasive character of T cell protrusions and likewise to denote the uncertain identity of these structures the term invadosome-like protrusions (ILP) was coined in a recent review [21]. Due to the yet unclear nature of invasive T cell protrusions we will therefore adopt the term of ILPs throughout this review.

Since the crawling T cell has to resist shear forces exerted by the blood flow, ILPs could be supportive for shear-resistant adhesive contacts during crawling on the endothelial surface. Importantly, memory T cells might be better adapted to crawling against shear because the density of ILPs underneath the crawling memory T cell is increased 2- to 3-fold compared to the crawling naïve T cell [70]. Moreover, the number of ILPs on peripheral blood human T cells crawling on TNFα-activated and CXCL12-bearing HUVECs or crawling on recombinant ICAM-1 overlaid with CXCL12 was found to be increased under shear flow compared to shear-free conditions [70].Together with the observations that T cell crawling is required for efficient diapedesis and that the presence of flow in vitro significantly enhanced the rate of T cell diapedesis when compared to T cell diapedesis across endothelial cells under shear-free conditions, the conclusion can be drawn that physiological flow is an important supportive factor for successful T cell crawling and diapedesis [70, 82].

At the tip of each ILP high affinity LFA-1 engages endothelial ICAM-1 [70], which is then organized in ring-like structures with a diameter of 0.2–1.0 μm as visualized by ICAM-1-GFP-expressing endothelial cells [75]. Taken together, these findings of a dynamic interaction of high affinity LFA-1 on cell protrusions invading the endothelial cell body with endothelial ICAM-1 could explain our findings on the exceptional role of endothelial ICAMs in supporting shear-resistant T cell crawling against the direction of flow on BBB endothelial cells [18]. However, proof for the immediate relation between formation of ILP with high affinity LFA-1 by the T cells that interact with ring-like ICAM-1 structures on the endothelium in directing T cell crawling against shear is lacking.

In summary, post-arrest T cell behavior on the BBB is unique due to the predominant crawling of the T cells against the direction of flow on the luminal surface on the CNS microvasculature and the exceptionally long distance T cells need to cover by crawling on the BBB before finding a site permissive for diapedesis (fig. 2, 4). As predominant crawling against shear of encephalitogenic effector/memory T cells is also observed on recombinant ICAM-1 and ICAM-2 but not on VCAM-1, mechanosensing is achieved by the T cells and is probably mediated by β2-integrin interactions with ICAM-1 and ICAM-2. In contrast, the necessity to crawl over long distances to find the rare sites available for diapedesis is a unique characteristic of the BBB as this cannot be observed during the interaction of encephalitogenic effector/memory T cell with non-barrier-forming microvascular endothelial cells which provide multiple sites for T cell diapedesis.

Route of T Cell Diapedesis

Diapedesis is the last step of the multistep extravasation cascade of T cells across the endothelial layer of the microvessel, where the T cells move from the luminal surface to the abluminal surface of the endothelial monolayer. In

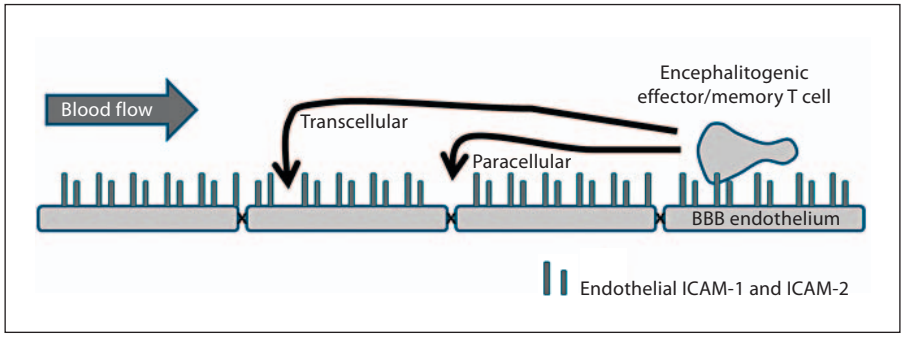

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Fig. 4. Extravasation of effector/memory T cells requires long-distance crawling against shear. Before diapedesis across the BBB encephalitogenic effector/memory T cells crawl long distances, which can exceed 150 μm. The predominant direction of T cell crawling on the luminal face of the BBB endothelium is against shear. The major endothelial ligand for T cell polarization and crawling against shear is ICAM-1. In the absence of ICAM-1, endothelial ICAM-2 functionally co-opts endothelial ICAM-1. The precise pathway of diapedesis across the BBB (para- or transcellular) is not resolved yet. Details for BBB determinants or on the molecular mechanism of T cell diapedesis are omitted due to excellent recent reviews in the field [3, 15, 21, 83–85].

general, for diapedesis two alternative pathways across the endothelium exist: the paracellular route through the endothelial cell-cell junctions and the transcellular route directly through a pore through the endothelial cell body (fig. 4) [21, 83]. Until recently, the paracellular route was regarded as the major pathway of diapedesis because functional blockage of diverse junctional molecules of the endothelium resulted in the reduced extravasation of neutrophils and myeloid cells in vivo and in vitro [83]. At the BBB the high complexity of endothelial tight junctions would require a highly coordinated opening and resealing of the complex BBB tight junctions for diapedesis of T cells along the paracellular route. Because such a process seems difficult to achieve, the transcellular pathway of T cell diapedesis across the BBB might be the preferred route [84]. In fact, a number of ultrastructural studies providing serial section electron micrographs have documented transcellular diapedesis of inflammatory cells across the BBB during EAE leaving tight junctions morphologically intact [15, 22, 85]. In vitro proof of transcellular diapedesis of IL-2-activated T cells [77, 86] highlights the existence of a transcellular pathway of T cell diapedesis across an endothelial monolayer [87]. In particular, specific characteristics of the endothelial cells within different tissues seem to influence the route of diapedesis taken by T cells. A direct comparison of the diapedesis pathway between HUVECs and HDMVECs demonstrated that the transcellular route is used by 10% of T cells for migration across HUVECs whereas 30% of T cells used the transcellular route to cross HDMVECs [75, 77]. Although a systematic quantitative analysis of the precise pathway for the diapedesis of T cells across BBB endothelium is not available to date, it is tempting to speculate that the complex tight junction architecture of BBB endothelium favors transcellular over paracellular T cell diapedesis.

Transcellular diapedesis of T cells across an endothelial monolayer is a direct consequence of the dynamic formation and retraction of ILPs during crawling. In vitro live cell imaging studies have demonstrated that IL-2-stimulated human T cells, which cross the endothelium via the transcellular route, use an endothelial pore with a diameter of 4–5 μm [75, 77]. This transendothelial pore obviously forms by fusion of multiple small pores induced in the endothelial cells by ILPs of the crawling T cells as elegantly shown by observing T cell migration across endothelium expressing ICAM-1-GFP or palmitoylated YFP-labeled cell membranes [75]. The essential requirement of ILPs for transcellular diapedesis was demonstrated by biochemical inhibition of the formation of ILPs by the crawling T cell through an Src kinase inhibitor, which resulted in selective inhibition of transcellular diapedesis [75, 79]. Accordingly, T cells derived from patients with Wiskott-Aldrich syndrome or X-linked thrombocytopenia, who both carry genetic defects affecting a regulatory factor for actin cytoskeletal dynamics (WASP) and, thus, exhibit a defect in cell protrusion formation, were found to have significantly reduced diapedesis rates via the transcellular but not via the paracellular pathway compared to T cells from healthy individuals [75].

The inflamed endothelial cell actively contributes to pore formation by attracting ILP of IL-2-activated effector/memory T cells through intracellular chemokine vesicles and through membrane delivery and fusion mechanisms required for pore formation [75, 88]. Ultra-

structural analysis of TNFα-stimulated HUVECs or HDMVECs demonstrated the localization of vesicles close to the luminal surface or enriched in close proximity to ILPs [75, 88]. Endothelial chemokines were shown to be released from the vesicles at the contact point of crawling effector/memory T cells [88]. The essential role of these endothelial chemokines for formation of ILPs and T cell diapedesis was demonstrated through pertussis toxin treatment of the T cells prior to their interaction with TNFα-stimulated HUVECs, which did not alter crawling of effector CD4+ Th1 cells and cytotoxic CD8+ type 1 T cells, but significantly reduced the number of ILPs [88]. However, diapedesis of T cells across TNFα-stimulated HUVECs was completely blocked in the absence of G-protein-coupled receptor signaling in T cells [88].

Fusion of cell membranes as required for the formation of a transcellular pore is mediated via fusogenic proteins. Endothelial VAMP2 and VAMP3 are two fusogenic proteins specifically enriched around ILPs of the T cell and their biochemical inhibition specifically blocked transcellular but not paracellular diapedesis across TNFα stimulated HDMVECs in vitro [75]. In addition, caveolin-1, which is a scaffolding protein of a specific type of vesicles, the caveolae, has been described to significantly contribute to transcellular but not to paracellular T cell diapedesis across TNFα-stimulated HUVECs in vitro [77]. However, the increased concentration of caveolin-1 specifically at the site of transcellular diapedesis of T cells in TNFα-stimulated HUVECs or HDMVECs [77] could not be reproduced in a second study [75]. Thus, further studies, especially in vivo, are necessary to delineate the role of caveolin-1 in favoring transcellular over paracellular diapedesis.

Finally, it is noteworthy to mention that transcellular diapedesis of T cells across the endothelium might still involve junctional molecules. For example, blocking the endothelial cell-cell adhesion molecule PECAM-1 interfered not only with paracellular but likewise with transcellular diapedesis, as demonstrated during analysis of neutrophil diapedesis across HUVECs [83, 89]. As endothelial junction molecules such as CD99, PECAM-1 and JAM-A localize around transcellularly diapedesing monocytes, neutrophils or T cells, a functional role of these molecules in transcellular diapedesis is conceivable [75, 83, 89, 90]. However, complete absence of PECAM-1 in PECAM-1$^{-/-}$ mice was associated with an earlier onset of EAE concurrent with an earlier CNS infiltration of mononuclear cells when compared to wild-type animals. In vitro, absence of endothelial PECAM-1 increased the diapedesis rate of encephalitogenic T cells across immortalized PECAM-1 deficient brain endothelioma cells compared to wild-type bEnd5 in a static diapedesis experiment [34]. Thus, diapedesis of effector/memory T cells across brain endothelium seems to require molecular mechanisms that are distinct from those involved in neutrophil diapedesis across the endothelium.

Besides restricting paracellular diffusion of water-soluble molecules through complex tight junctions, unspecific transcellular transport of blood-borne molecules across the BBB is hampered by its low pinocytotic activity [3]. This seems to be in apparent contrast to proposing that the BBB favors transcellular over paracellular T cell diapedesis. However, the BBB has the unique ability to control a vast amount of specific and directed vesicle fusion and transport mechanisms involved in the receptor-mediated or adsorptive-mediated transcytosis of nutrients, ensuring the transport of nutrients into the high energy-consuming CNS tissue [3]. Moreover, during inflammatory conditions endothelial cells of the BBB have an increased number of intracellular vesicles, which are indeed able to form elongated transendothelial channel-like structures [85]. Thus, BBB endothelium does harbor the molecular players required for transcellular diapedesis of T cells. The signals that might favor T cell diapedesis across the BBB via transendothelial pores over the paracellular pathway through the complex BBB tight junctions require further study.

Outlook

Due to the unique characteristics of the endothelial cells forming the BBB, specific mechanisms of T cell trafficking into the CNS can be expected at each step of the multistep cascade. Having crossed the BBB endothelium, T cells encounter two basement membranes: the endothelial basement membrane containing α4- and α5-laminins and the CNS-specific parenchymal basement membrane containing α1- and α2-laminins [91]. Due to their different laminin isoforms, breaching of the basement membranes requires mechanistically two distinct processes. Within the endothelial basement membrane laminin α4 is the essential binding partner for α6β1 integrin on encephalitogenic T cells, whereas a high content of α5 laminin selectively inhibits α6β1 integrin-mediated T lymphocyte migration across the endothelial basement membrane [92]. The α1 and α2 laminin isoforms present in the parenchymal basement membrane cannot be bound by encephalitogenic effector/memory T cells, and thus lead to a perivascular accumulation of T cells having breached the endothelial basement membrane [91, 93]. In

order to cross the parenchymal basement membrane and to infiltrate the CNS parenchyma and elicit EAE, encephalitogenic T cells critically depend on reactivation by perivascular or leptomeningeal antigen presenting cells [94]. Finally, for crossing the glia limitans encephalitogenic T cells rely on the activity of the TNF-α-induced activity of the metalloproteinases MMP2 and MMP9 provided by infiltrating macrophages, which cleave the dystroglycan anchor of the astrocyte foot processes to the parenchymal basement membrane and thus open gates for T cell invasion into the CNS parenchyma [95].

Taken together, the process of T cell extravasation across the highly specialized BBB endothelium requires specific adaptations enabling the T cells to find sites permissive for diapedesis. While some of these specific adaptations are quite well understood to date, others remain to be defined. The BBB-specific phenomenon reviewed here is the extensive crawling of adherent T cells against the direction of blood flow prior to diapedesis (fig. 4). That behavior might be fundamental for T cells to find a site permissive for diapedesis across the BBB. Apparently, LFA-1 mediated-interaction with endothelial ICAM-1 fulfills a prime role in this process. Combined with the most recent findings on the exceptional role of endothelial chemokines for the formation of ILPs by the crawling-activated effector/memory T cells [88] one might speculate that the identification of respective chemokines in BBB endothelial cells could provide key information on the molecular players for the concerted mechanism of T cell extravasation across the BBB. In the end, knowledge of trafficking mechanisms unique for the BBB will allow the development of novel therapeutic approaches specifically targeting individual steps of T cell extravasation into the CNS.

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