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Molecular mechanisms involved in T cell migration across the blood–brain barrier

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Summary. In the healthy individuum lymphocyte traffic into the central nervous system (CNS) is very low and tightly controlled by the highly specialized blood–brain barrier (BBB). In contrast, under inflammatory conditions of the CNS such as in multiple sclerosis or in its animal model experimental autoimmune encephalomyelitis (EAE) circulating lymphocytes and monocytes/macrophages readily cross the BBB and gain access to the CNS leading to edema, inflammation and demyelination. Interaction of circulating leukocytes with the endothelium of the blood–spinal cord and blood–brain barrier therefore is a critical step in the pathogenesis of inflammatory diseases of the CNS. Leukocyte/endothelial interactions are mediated by adhesion molecules and chemokines and their respective chemokine receptors. We have developed a novel spinal cord window preparation, which enables us to directly visualize CNS white matter microcirculation by intravital fluorescence videomicroscopy. Applying this technique of intravital fluorescence videomicroscopy we could provide direct *in vivo* evidence that encephalitogenic T cell blasts interact with the spinal cord white matter microvasculature without rolling and that α4-integrin mediates the G-protein independent capture and subsequently the G-protein dependent adhesion strengthening of T cell blasts to microvascular VCAM-1. LFA-1 was found to neither mediate the G-protein independent capture nor the G-protein dependent initial adhesion strengthening of encephalitogenic T cell blasts within spinal cord microvessel, but was rather involved in T cell extravasation across the vascular wall into the spinal cord parenchyme. Our observation that G-protein mediated signalling is required to promote adhesion strengthening of encephalitogenic T cells on BBB endothelium *in vivo* suggested the involvement of chemokines in this process. We found functional expression of the lymphoid chemokines CCL19/ELC and CCL21/SLC in CNS venules surrounded by inflammatory cells in brain and spinal cord sections of mice afflicted with EAE suggesting that the lymphoid chemokines CCL19 and CCL21 besides regulating lymphocyte homing to secondary lymphoid tissue might be involved in T lymphocyte migration into the immune-privileged CNS during immunosurveillance and chronic inflammation. Here, I summarize our current knowledge on the sequence of traffic signals involved in T lymphocyte recruitment across the healthy and inflamed

blood–brain and blood–spinal cord barrier based on our *in vitro* and *in vivo* investigations.

Keywords: Blood–brain barrier, lymphocyte trafficking, adhesion molecule.

Introduction

Homeostasis of the central nervous system (CNS) microenvironment is essential for its normal function and is maintained by the blood–brain barrier (BBB). The BBB is formed by highly specialized capillary endothelial cells, which inhibit transcellular passage of molecules across the barrier by an extremely low pinocytotic activity. The lack of fenestrae and an elaborate network of complex tight junctions (TJ) between the endothelial cells restrict the paracellular diffusion of hydrophilic molecules. On the other hand, in order to meet the high metabolic needs of the CNS tissue, specific transport systems selectively expressed in the capillary brain endothelial cell membranes mediate the directed transport of nutrients into the CNS or of toxic metabolites out of the CNS.

Because of the presence of the BBB, the lack of lymphatic vessels and the absence of classical major histocompatibility complex (MHC)-positive antigen presenting cells the CNS is considered an immunologically privileged site. In fact, under physiological conditions lymphocyte entry into the healthy CNS across the BBB is kept at a low level. During inflammatory diseases of the CNS such as in multiple sclerosis (MS) or its prototype animal model experimental autoimmune encephalomyelitis (EAE), however, circulating immunocompetent cells readily get access to the CNS. Employing the animal model of EAE it was first shown that $CD4^+$ autoaggressive T cells, when freshly activated *in vitro*, i.e. outside the CNS, can migrate across the healthy BBB into the CNS and start the molecular events leading to inflammation, loss of barrier properties and subsequently edema formation and finally demyelination (Hickey et al., 1991; Wekerle et al., 1986). Resting T cells with the same antigen specificity where shown to be incapable of transferring the disease and it was argued that they cannot penetrate the endothelial BBB. Thus, it was concluded that immunosurveillance of the CNS does take place and freshly activated but not resting T cells can enter the CNS across the healthy BBB. This indicates an active role for the endothelial BBB in regulating lymphocyte entry into the CNS by limiting it to freshly activated lymphocytes. During inflammatory demyelinating diseases the active role of the BBB in regulating inflammatory cell entry into the CNS continues as the altered phenotype of BBB endothelium during EAE, characterized by enhanced expression of traffic signals like adhesion molecules and chemokines, leads to enhanced leukocyte traffic into the CNS and consecutively chronic inflammation, however T lymphocyte entry is limited to T cells with an activated/memory and a characteristic adhesion molecule phenotype (Engelhardt et al., 1998b). Thus, understanding the molecular mechanisms involved in T lymphocyte migration across the blood–brain barrier in health and disease will help to understand immunosurveillance and chronic inflammatory of the CNS.

The multi-step model of lymphocyte endothelial interaction

In search of their specific antigen naive lymphocytes continuously recirculate through the body migrating from one lymphoid organ to another via the blood stream and the lymphatics. Lymphocytes exit the blood stream within the lymph nodes via specialized postcapillary venules, the high endothelial venules (HEVs). As the blood-borne lymphocytes flow with a remarkable speed in relation to their size specialized mechanisms for their interaction with the HEV endothelium are required. These mechanisms embody a multistep process, in which different adhesion or signalling molecules on lymphocytes and

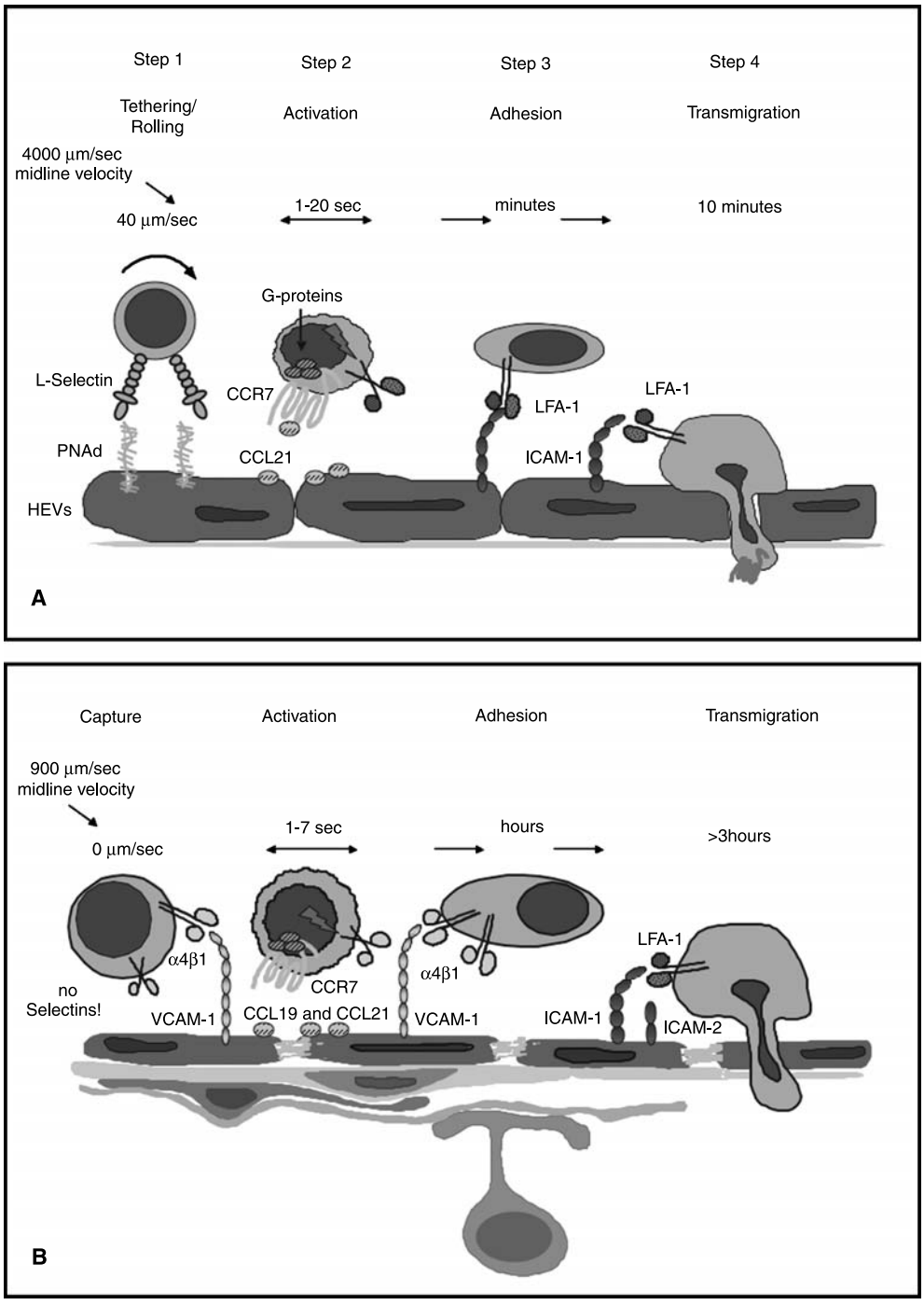

Fig. 1. The mutistep model of lymphocyte/endothelial recognition and recruitment from the blood. **A** Molecular mechanisms appyling for the recruitment of naive lymphocytes across HEV endothelium in peripheral lymph nodes. The velocities of free flowing and rolling lymphocytes and the time intervals observed for each individual step are derived from in situ microscopic observations as reviewed in Butcher et al. (1999). **B** Molecular mechanisms applying for the recruitment of T lymphoblasts across BBB endothelium in the spinal cord white matter. The velocities of free flowing lymphocytes and the time intervals observed for each individual step are derived from our intravital microscopic observations as described above

endothelial cells of the HEVs participate in a sequential manner (Butcher et al., 1999). In a first step lymphocytes make a transient contact with the vascular endothelium, generally mediated by adhesion molecules of the selectin-family and their respective carbohydrate ligands initiating rolling of the lymphocyte along the endothelial cell surface (step 1). Rolling greatly reduces the velocity of the travelling lymphocyte, which can now ''sample'' the local endothelial cell surface for activating factors, i.e. chemokines, that mostly act through serpentine receptors of the Gαi protein-linked chemoattractant receptor subfamily on the lymphocyte (step 2). Binding of a chemokine to its receptor results in a pertussis toxin sensitive rapid intracellular signal in the lymphocyte leading to the ''activation'' (i.e. conformational change and clustering) of integrins constitutively expressed on the lymphocyte surface, which then mediate firm arrest of the lymphocyte to the vessel wall, resistant to continuing blood shear forces (step 3). Only ''activated'' integrins mediate the firm adhesion of the lymphocyte to the vascular endothelium by binding to their endothelial ligands, which belong to the immunoglobulin (Ig)-superfamily. Firm lymphocyte arrest is followed by transendothelial migration of the lymphocytes either in between the endothelial cells or through the endothelial cell proper (step 4). Thus successful recruitment of circulating lymphocytes into the lymphatic tissues depends on the productive lymphocyte/endothelial interaction during each of these sequential steps (Fig. 1). The molecular players for lymphocyte homing to peripheral lymph nodes is depicted in Fig. 1. The specific regulation of homing of naive lymphocytes into the lymph nodes, spleen and Peyer's Patches significantly enhances the efficiency of the adaptive immune system, guiding naive lymphocytes directly into the secondary lymphatic tissues, where they scan antigen presenting cells for their specific antigens, eventually respond to their specific antigens and differentiate into memory and effector cells. In contrast to their precursors these previously stimulated memory/effector lymphocytes will then recirculate through extralymphoid tissues, especially during inflammation. Until recently the brain was considered to be ignored by patroulling effector/memory T cells due to the lack of traffic signals provided by the blood–brain barrier.

Traffic signals for circulating T lymphoblasts on blood–brain barrier endothelium

The observations that upon adoptive transfer of EAE into syngeneic naive recipients by intravenous injection of freshly activated myelin protein specific $CD4^+$ T cells lead to their immigration into the CNS across the healthy BBB changed the concept of the immunepriviledge of the CNS. It became clear that the BBB would not inhibit but rather actively restrict lymphocyte entry into the CNS by allowing freshly activated but not resting T cells to cross the BBB (Wekerle et al., 1986; Hickey et al., 1991). Thus activated but not resting T cells must have the receptors to bind to the respective ligands on the BBB.

In order to define the molecular nature of such ''brain homing-receptors'' on the T lymphocytes and to analyze the entire multistep decision cascade provided by the highly specialized endothelial cells of the BBB allowing T cell entry into the CNS we have investigated the expression of adhesion molecules on CNS endothelium in healthy mice and mice afflicted with EAE by means of *in situ* hybridization and immunohistology. Besides PECAM-1 and ICAM-2, which are expressed on vascular endothelial cells elsewhere, we found consitutive expression of VCAM-1 and to a lower degree of ICAM-1 on a subpopulation of microvessels in the healthy brain and spinal cord of SJL/N mice (Engelhardt et al., 2003; Steffen et al., 1994). During EAE upregulated expression of both, ICAM-1 and VCAM-1, but not of E- and

P-selectin could be observed on CNS endothelium (Steffen et al., 1994; Engelhardt et al., 1997). We and others could show that ICAM-1 and VCAM-1 mediate adhesion of lymphocytes to inflamed cerebral vessels on frozen brain sections *in vitro* (Steffen et al., 1994; Yednock et al., 1992). *In vivo* monoclonal antibody inhibition studies confirmed the involvement of VCAM-1 and its ligand α4-integrin (Engelhardt et al., 1998a) but not E- and P-selectin (Engelhardt et al., 1997) in the pathogenesis of EAE as antibodies directed against VCAM-1 and its ligand α4-integrin but not against E- and P-selectin successfully blocked the development of clinical EAE in the SJL/N mouse. In accordance to these findings, we recently found that neither antibody inhibition studies blocking the E- and P-selectin ligand PSGL-1 nor lack of PSGL-1 alters the development of EAE in SJL and C57Bl/6 mice (Engelhardt et al., 2005).

In order to define the exact role of α4-integrin and VCAM-1 in T cell recruitment across the BBB endothelium we next set up *in vitro* studies, where we investigated the interaction of freshly activated autoaggressive T cell blasts with the brain endothelial cell line bEnd5 (Laschinger and Engelhardt, 2000). Whereas antibodies blocking α4-integrins or endothelial VCAM-1 significantly reduced adhesion of encephalitogenic T cell blasts to brain endothelium, the very same antibodies did not influence transendothelial migration of autoaggressive T cell blasts across a bEnd5 monolayer. Our *in vitro* observations therefore suggested that *in vivo* α4/VCAM-1 interactions are not involved in transendothelial migration of encephalitogenic T cells across the BBB but rather mediate earlier steps of T cell/BBB-interaction such as firm adhesion.

Due to the lack of involvement of selectins and the selectin ligand PSGL-1 in EAE we hypothesized that α4-integrin might be involved in mediating rolling of encephalitogenic T lymphocytes along the vascular wall within the CNS. In order to investigate T cell interaction with the vascular wall under physiological shear observation of the CNS microcirculation by intravital fluorescence microscopy was necessary. As in EAE the CNS white matter is the battle field of the inflammatory attack with preference of the spinal cord, we have developed a novel spinal cord window preparation in collaboration with P. Vajkoczy (Mannheim, Germany), which enables us to directly visualize CNS white matter microcirculation by intravital fluorescence videomicroscopy (Vajkoczy et al., 2001). Using this novel technique we gained direct evidence that T lymphoblast interaction with the BBB in the spinal cord white matter is in fact unique due to the lack of rolling of the T cells along the vascular wall. Rather, constitutively expressed VCAM-1 mediates the G-protein independent prompt arrest (capture) of circulating encephalitogenic T cell blasts via α4-integrin to the endothelium of the healthy BBB (Vajkoczy et al., 2001). Transient capture is followed by G-protein dependent α4-integrin/VCAM-1 mediated adhesion strengthening (Vajkoczy et al., 2001) (Fig. 1).

A crucial role for chemokines in successful recruitment of encephalitogenic T cells across the BBB was therefore suggested by the requirement for signalling via G-protein coupled, PTX-sensitive receptors on encephalitogenic T cells to firmly arrest on BBB endothelium *in vivo*. By performing *in situ* hybridization studies we found expression of the lymphoid chemokine CCL19 at the level of the BBB endothelium on a subpopulation of CNS venules and induced CCL21 in inflamed CNS venules during EAE (Alt et al., 2002). These chemokines had previously been reported to be solely expressed in secondary lymphatic tissue and to be involved in the recruitment of naïve lymphocytes across the HEVs into secondary lymphatic tissue. Expression of their common receptor CCR7 was detected on encephalitogenic T cells, which could chemotax spe-

cifically towards both, CCL19 or CCL21 in a concentration dependent and pertussis toxin-sensitive manner, comparable to naive lymphocytes *in vitro*. Additionally, in frozen section assays functional ablation of CCR7 or blocking CCL19 and CCL21 reduced binding of encephalitogenic T lymphocytes to inflamed venules in the brain. Thus, the lymphoid chemokines CCL19 and CCL21 seem to be involved in the recruitment of encephalitogenic T lmphocytes into the immune-priviledged CNS.

Besides α4-integrin the β2-integrin LFA-1 also present on the surface of encephalitogenic T cells has been suggested to be involved in the pathogenesis of EAE. Using the spinal cord window model and intravital fluorescence video microscopy, we investigated the possible involvement of LFA-1 on circulating encephalitogenic T cells in their multi-step interaction with the BBB endothelium *in vivo.* LFA-1 was found to neither mediate the G-protein independent capture nor the G-protein dependent adhesion strengthening of encephalitogenic T cell blasts within spinal cord microvessel of healthy mice (Laschinger et al., 2002). In contrast, blocking LFA-1 on encephalitogenic T lymphoblasts resulted in a significantly reduced number of T cells migrating across the vascular wall into the spinal cord parenchyme. Thus, encephalitogenic T cells use LFA-1 for transendothelial migration but not for capture and adhesion in spinal cord microvessels *in vivo*. These findings were further supported by our *in vitro* findings that endothelioma cells lacking both ligands for LFA-1, namely ICAM-1 and ICAM-2 can still support adhesion of T cells to activated endothelium but do no longer support transendothelial migration of T cells *in vitro* (Lyck et al., 2003). Re-expression of ICAM-1 and/or ICAM-2 in the ICAM-$1^{-/-}$ICAM-$2^{-/-}$ endotheliomas was found to reconstitute transendothelial migration of T lymphocytes. Thus, engagement of T cells via LFA-1 on endothelial ICAM-1 and/or ICAM-2 is required for successful migration across the endothelial vessel wall of CNS microvessels.

Interestingly, although we observed T cell interaction with the vascular wall in both, CNS capillaries and post-capillary venules, extravasation of encephalitogenic T cells was only observed at the level of post-capillary venules. This is consistent with the observations of the localization of inflammatory cuffs, which characteristically form around the post-capillary venules but not around capillaries, indicating that extravasation of autoagressive T cells and inflammatory cells preferentially occurs at this level. In contrast to peripheral organs, where extravasation of leukocytes takes several minutes, we observed that extravasation of encephalitogenic T cells across the BBB takes several hours (Laschinger et al., 2002; Vajkoczy et al., 2001), supporting the notion that transendothelial migration across the BBB follows unique mechanisms. It should be noted that initial T cell recruitment across the healthy BBB is not accompanied by BBB leakiness as observed by intravital microscopy (Vajkoczy et al., 2001). Although the general believe is that lymphocytes migrate across the interendothelial junctions, the passageway of lymphocytes in transendothelial migration across the BBB is still a matter of debate. In order to investigate whether the complex tight junctions of the BBB are involved in transendothelial migration of T lymphocytes across the BBB we performed immunofluorescence stainings for tight junction molecules in the mouse model of EAE. In tight junctions of healthy CNS vessels in the mouse we detected occludin, ZO-1, claudin-5 and claudin-3. In EAE brain and spinal cord sections we observed the selective loss of claudin-3 immunostaining from tight junctions of venules surrounded by inflammatory cuffs, whereas the localization of the other tight junction proteins remained unchanged (Wolburg et al., 2003). Although these data apparently point to an involvement of endothelial tight junctions in T cell recruitment across the

BBB, when performing transmission electron microscopy on ultrathin sections derived from brains of mice afflicted with EAE lymphocyte extravasation across the BBB could solely be observed at parajunctional areas rather than through the tight junctions (Wolburg et al., 2005). The highly specialized BBB with its unique tight junctions might therefore trigger BBB specifc passageways for lymphocytes across the BBB during immunosurveillance and inflammatory disease.

Interestingly, the interaction of activated T cells with microvessels forming the blood–retina barrier (BRB) in the eye were found to be similar (Xu et al., 2003). Performing intravital microscopy with subsequent analysis by whole-mount of the total population of migrated fluorescent it was found that only activated T cells crossed the BRB. T lymphoblasts were found to be captured within the retinal microvessels by a mechanism closely resembling our observations for T cell interaction within spinal cord microvessels. Pre-treatment of transferred cells with anti-LFA-1 antibodies were found to suppress the recruitment of T cells across the BRB, but the relative contributions of LFA-1 and α4-integrins to capture as compared to diapedesis remains to be investigated. In further resemblance to T cell recruitment across the BBB in the CNS white matter, in the eye T lymphoblast cells failed to cross retinal venules until 8 hrs after injection supporting a unique mechanisms of T cell diapedesis across the BBB and BRB.

As the microvascular architecture differs in CNS grey matter versus CNS white matter, we have additionally started to compare T lymphocyte recuritment across the BBB in these different compartments using intravital microscopy. In order to gain intravital microscopic access to the CNS grey matter microcirculation, an acute cranial window preparation has been developed, which allows observation of the pial and cortical, i.e. CNS grey matter, microcirculation respectively (Vajkoczy et al., 2000). Similar to the CNS white matter, in healthy mice T lymphoblast interaction with the vascular wall could be observed in capillaries of the CNS grey matter and pial capillaries and post-capillary venules. A striking difference was observed when comparing the mean velocity of T lymphocytes traveling through cerebral vessels. Within the CNS grey matter and within pial vessles we measured velocities significantly above 2 mm/s compared to mean velocities of around 900 m/s observed within the spinal cord white matter vessels. Interestingly, within a given time the number of T lymphoblasts having extravasated into CNS grey matter verus CNS white matter was not significantly different. Thus, differences in microhemodynamics within the CNS grey and white matter might not have a direct impact in T cell recuritment across the BBB, however, the molecular mechanisms involved at the different sites will have to be exploited in greater detail. This is supported by findings of others who observed when performing intravital microscopy of the cerebral cortex that T lymphocytes roll within the superficial brain microvessels and that P-selectin and PSGL-1 as well as α4-integrin are involved in this process (Piccio et al., 2002; Kerfoot and Kubes, 2002). The observation that blocking of α4-integrin but not of P-selectin and PSGL-1 interferes with the development of EAE suggests that selectin mediated leukocyte recruitment across the BBB is not required for T cell migration across the BBB.

New developments and conclusion

The absence of rolling, the possible involvement of the lymphoid chemokines CCL19 and CCL21 and the predominant involvement of α4-integrin and VCAM-1 make T lymphoblast interaction with the BBB unique and suggest that these specific mechanisms

operate to limit lymphocyte entry into this immunoprivileged site (Fig. 1). Although due to more limited methods the information on the molecular mechanisms involved in immune cell recruitment across the BBB in humans are less well described. To date there is, however, no reason to believe that the trafficking mechanisms discovered in the animal models do not hold true in humans. The findings based on EAE experiments that α4-integrin is a central molecule in mediating leukocyte migration across the BBB was confirmed by clinical trials treating multiple sclerosis patients with a humanized antibody directed against α4-integrin (Miller et al., 2003). The humanized anti-α4 integrin antibody natalizumab, marketed as Tysabri, produced an impressive reduction of the disease activity in multiple sclerosis patients and was recently approved for use in patients. After several thousands of patients have been treated with natalizumab without apparent adverse events, unexpectedly 3 patients developed progressive multifocal leukoencephalopathy (PML), a fatal reactivation of JC virus within CNS glial cells, which usually exclusively occurs in patients with generalized immunosuppression (Kleinschmidt-Demasters and Tyler, 2005; Langer-Gould et al., 2005; Van Assche et al., 2005). Thus, PML must be considered as treatment-related in these cases and the ongoing natalizumab trials are presently suspended. It remains to be shown, whether natalizumab by itself suffices for the development of PML, or whether rather a combination of features, i.e. multiple scerosis, immunsuoppressive therapy plus natalizumab are required for the development of PML. In any case, these tragic occurrences strongly support the urgent need for continued research to understand not only the molecular mechanisms involved in leukocyte trafficking across the BBB during chronic CNS inflammation but also those mechanisms involved in leukocyte migration across the BBB maintaining CNS immunosurveillance.

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