

What is the blood–brain barrier (not)?

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In 1900, summarizing his experiments with toxins and Ehrlich's earlier observations with intravital dyes, the Berlin physician Lewandowski concluded that 'brain capillaries must hold back certain molecules'. Illustrating this phenomenon with persuasive beauty, the subsequently evolving metaphor of a 'Bluthirnschranke' (blood–brain barrier, BBB) gained wide acceptance, but the extension of its meaning into the context of inhibiting leukocyte recruitment into the brain is imprecise. On the basis of the original work by Ehrlich, Lewandowski and Goldmann we re-define the BBB as a capillary barrier for solutes, and clarify that leukocyte recruitment requires two differentially regulated steps: (i) passage across postcapillary venules into Virchow–Robin spaces, and (ii) subsequent progression across the glia limitans into the neuropil. We propose that the second step frequently involves perivascular antigen-recognition and the induction of ectoenzymes, for example matrix metalloproteinases (MMPs).

'The metaphor is often smarter than the poet' (Heiner Müller).

Anatomical misconceptions have been sources of confusion

Leukocyte recruitment into the brain is an important topic in neuroimmunology. A deeper understanding of its regulation might provide clues for treating diseases such as multiple sclerosis (MS) [1]. However, numerous studies in the field suffer from an inappropriate view or understanding of the blood–brain barrier (BBB). Terms including 'leukocytes cross the blood–brain barrier' often leave open the question of whether these cells passed the vascular wall to reside in perivascular (Virchow–Robin) spaces or progressed across the glia limitans into the neuropil, although this distinction is of evident clinical importance.

The simple and common misconception that the diffusion of soluble molecules and cell recruitment are regulated by the same mechanisms at the same site of the vascular tree has caused much confusion and imprecise evaluation of data. It is often ignored that solute diffusion is controlled at the capillary level by the BBB, while cell recruitment occurs at postcapillary venules. Often, the cell populations of the vascular wall, the perivascular space and the juxtavascular neuropil are not properly distinguished (Box 1).

The aim of this article is to review the original observation leading to the concept of a 'Bluthirnschranke', and to distinguish its morphological and functional correlates from those involved in leukocyte recruitment. The unique topography of the perivascular space surrounding pre- and post-capillary brain vessels, and its evolving importance as a decisive checkpoint in neuroinflammation, will also be explained.

The original observations

Ehrlich's unwanted finding

For the following, it is helpful to distinguish between the phenomenon and its interpretation(s). The phenomenon was first noted by Ehrlich [2] in a series of experiments designed to compare the oxygen consumptions of different organs. To this end, Ehrlich used 'intravital dyes', the colours of which changed with their redox state. His incidental, but seminal, observation (which, in fact, was unwanted as it spoiled his studies) was that some of the dyes, following injection into veins, arteries or subcutaneously, stained various organs strongly, but the brain weakly or not at all. This remarkable peculiarity of the brain required a proper explanation, and countless neuroscientists since then have performed similar experiments to work out what blocks the access of hydrophilic molecules from blood to brain. For good reasons, three studies are cited as key papers: Lewandowski's work 'On the cerebrospinal fluid' [3], Goldmann's 'Intravital labelling of the central nervous system' [4], and the 'Fine structural localization of a blood–brain barrier to exogenous peroxidase' by Reese and Karnowski [5]. These papers provided trendsetting interpretations of the phenomenon in that they zoomed in progressively on the structural correlates of the BBB, from a special 'surface' (Ehrlich, [2]) to the molecular machinery forming tight junctions, membrane channels and transport systems.

Lewandowski's identification of the capillary wall as a barrier

Lewandowski is often cited for having coined the term 'Bluthirnschranke' in his 1900 paper [3], but the term cannot be found therein. By the time of his study, spinal punctures had been introduced in humans to inject drugs such as cocaine directly into the cerebrospinal fluid (CSF). Lewandowski's primary aim was to compare the effects of peripheral versus intrathecal strychnine and ferrocyanate to predict proper dosage for therapeutic purposes. In dogs, sheep and rabbits, he found that lethal effects after intrathecal application were achieved with only 1% of

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Box 1. Sources of confusion – nomenclature of vessel associated cells

For the correct localization of cells that have left the bloodstream in the brain, three compartments must be distinguished: the vessel wall, the perivascular (Virchow–Robin) space, and the neuropil. The neuropil adjacent to the blood vessel is often referred to as the juxtavascular site.

The vessel wall consists of:

- Endothelium
- An inner vascular basement membrane surrounding the abluminal side of endothelial cells
- Smooth muscle cells ('media') and pericytes (pre- and postcapillary vessels)
- Pericytes (capillaries)
- Outer vascular basement membranes covering smooth muscle cells and pericytes

The perivascular compartment consists of:

- Perivascular fluid (lymphatic drainage from the neuropil)
- Perivascular cells. This heterogeneous population includes:
 - leptomeningeal mesothelial cells
 - perivascular macrophages at various activation states

Under inflammatory conditions, a wide range of leukocytes enter the perivascular spaces.

The neuropil (brain parenchyma) is delineated by the glia limitans consisting of:

- A basement membrane that differs from the vascular basement membranes
- Astrocytic endfeet
- Fewer juxtavascular microglial endfeet compared with astrocytic endfeet

The neuropil and perivascular space are separated by the glia limitans. At capillaries, which do not possess perivascular spaces, the basement membrane of the vessel wall and the basement membrane of the glia limitans fuse ('fused gliovascular membrane'), enabling intimate contact of astrocytes with pericytes and endothelial cells (Figure 1).

At the light microscopic level, it is often difficult to distinguish between pericytes, perivascular cells and juxtavascular microglia, but under inflammatory conditions, it is important to determine whether infiltrating leukocytes are located in perivascular spaces or invade the neuropil. Immunolabelling astrocytes and basement membranes or ultrastructural analysis are suitable methods to overcome this problem.

the dose required using the subcutaneous route. His pioneering conclusion was that the capillary wall can apparently block the access of certain molecules.* Clearly, this concept substantiated Ehrlich's interpretation that 'surfaces relevant for diffusion must have smaller pores in brain compared to liver and kidney'† and focussed

* Original statement: 'Es lässt sich dieser Widerspruch kaum anders erklären, als durch die Hypothese, dass die Capillarwand den Uebertritt bestimmter Stoffe, wie etwa des Natriumferrocyanats, verhindere...'. [This contradiction cannot be explained in any other way than by the hypothesis that the capillary wall blocks the entrance of certain substances, for example ferrocyanate...].

† Original statement: 'Weiterhin ergibt sich unter diesen Voraussetzungen, dass die die Aufnahme beherrschenden Flächen am Gehirn ... feinstporöse Beschaffenheit besitzen, während im Gegenteil Leber und Nierenzellen grobkörniges Material durchlassen.'. [Furthermore, it can be concluded from these observations that the surfaces controlling penetration into the brain are hardly porous, while those in the liver and the kidney allow larger substances to pass.].

correctly on the microvasculature of the brain as the site of control.

Goldmann's study and the role of phagocytosis

Goldmann's 1913 study [4] was inspired by his notion that not only the brain but also the foetus remained 'white as snow' after the injection of dyes such as cyanosin. He found that the cell populations storing fat and glycogen were also active in retaining injected dyes. Therefore, he proposed the existence of a 'physiologische Grenzmembran' (physiological bordering membrane) shielding the placenta and brain through avid phagocytosis. Indeed, his comparison of the two organs grasped their shared feature: their location 'behind' what has been subsequently addressed as a 'tissue barrier'. This commonality is still emphasized when the brain and placenta are referred to as 'immunologically privileged' sites [6,7].

The 'dirty little secret' of the neuroanatomists

Another intriguing observation is the first, and since then rare [8], description of what one could call 'the dirty little secret of the neuroanatomists' (analogous to the one formulated for immunologists [9]). Goldmann noted that, at first glance, tracers injected into the blood do not cross brain vessels [4]. A closer look revealed that the neuropil (i.e. the parenchyma proper, bordered by the glia limitans) remained unstained, but the dyes accumulated in cells of the choroid plexus, the leptomeninges and along the perivascular spaces, which he and his contemporaries correctly regarded as lymphatic clefts [3,10]. The label was stored in granules of cells which Goldmann recognized as 'susceptible for chemotactic signals, capable of migration, and phagocytic'. The perivascular phagocytes have been described several times since then [11–13], and are currently the focus of attention because they might include a population of antigen-presenting cells, the presence or recruitment of which is crucial for the onset of neuroinflammation ([14–17]; see later).

Reese and Karnovsky's work

The observation of dyes in the vessel wall led Goldmann [4] to argue explicitly against the concept of a barrier at the endothelial level, which later gained wide acceptance. It was the technically brilliant paper by Reese and Karnovsky [5] that localized an important structural correlate underlying Ehrlich's observation at the endothelial-cell level. Using injections of horseradish peroxidase (HRP), the authors wanted to define what hinders the penetration of this enzyme from blood to neuropil using high quality electron microscopy. They detected characteristic overlaps between brain endothelial cells in capillaries and stated: 'The failure of peroxidase to penetrate these intercellular spaces was attributed to tight junctions that were present in every region of overlap' and 'it was concluded that they form continuous belts or zonulae occludentes.' Beyond doubt, these tight junctions in capillaries are crucial elements in proper BBB function [18], and much has been learned about the underlying molecular substrate (for reviews, see Refs [19–22]).

In the same paper [5], Reese and Karnovsky reported a second influential observation: they noted the relative

'paucity' of transport vesicles in capillaries and regarded this feature as another 'manifestation of a blood–brain barrier'. In addition, the authors stated that 'metabolic pumps are necessary to maintain a blood–brain concentration gradient. Such a mechanism has been postulated for maintaining ion gradients between blood and brain. These pumps may well be located at the endothelium, which is structurally organized as a continuous surface across which the pumps could act.' Thus, this key paper not only localized the site of barrier function to subcellular endothelial structures, but also changed the image of the BBB from a static physical wall to a more dynamic entity.

An integrated view

It is noteworthy that the presence of specialized tight junctions in capillaries and their blockade of solute diffusion do not contradict Goldmann's observation of leakage and phagocytosis in the vessel wall and beyond. The 'dirty little secret' can be explained easily by the endothelium in pre- and post-capillary vessels having less pronounced BBB characteristics when compared with that of capillaries. Postcapillary venules are equipped with P-face tight junctions, but their density apparently does not completely inhibit the penetration of injected tracers [22,23]. This is unsurprising when we consider the current understanding that BBB-typical differentiation of endothelial cells requires intimate interaction with astrocytes [24], which is best provided in the capillary segment. In pre- and post-capillary vessels, the astrocytic endfeet of the glia limitans

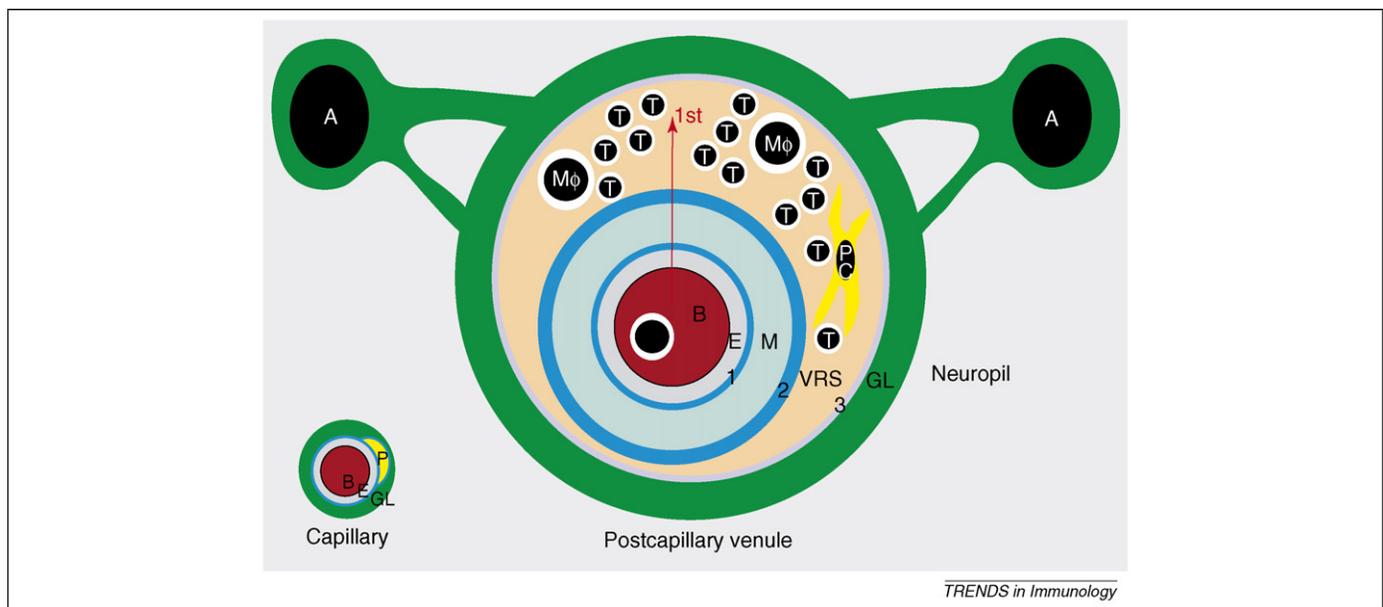
Table 1. The cellular composition and differentiation between blood and brain vary from arteriole to capillary to venule in the CNS^{a,b}

Cells and features	Arteriole	Capillary	Venule
Smooth muscle cells	+	–	+
Pericytes	+	+	+
Endothelial tight junctions	N.d.	Belts of tight junctions [5]	Non-specialized
Permeability for BBB markers	N.d.	No	Yes
Intimate contact between astrocytic endfeet and the vascular wall/presence of perivascular spaces	No/yes	Yes/no	No/yes
Perivascular macrophages	+	+	+

^aAbbreviations: +, present; –, absent, n.d., not determined.

^bFor further details see Ref. [22].

are separated from the endothelial layer by pericytes and smooth muscle cells forming the media and by the Virchow–Robin space (Table 1, Figure 1). Therefore, it should no longer be taboo to mention that classical markers of BBB function might well penetrate the endothelium around larger vessels and are then phagocytosed within the vascular wall and perivascular spaces [5]. Instead of being a contradiction, this observation should be interpreted as showing that perivascular phagocytes contribute to the BBB function by acting as scavengers in perivascular spaces. It is also important to keep in mind that the surface area of the capillary endothelium outnumbers that of pre- and post-capillary vessels [25]. Irrespective of the



TRENDS in Immunology

Figure 1. The first step in neuroinflammation. Topography of capillaries and postcapillary venules in relation to the neuropil (grey background), showing a cross-section through a postcapillary venule and a capillary in the brain. Astrocytes have processes with endfeet that form the glia limitans isolating the vascular compartment (vessel wall and perivascular space) from the brain parenchyma. In postcapillary venules, three compartments with at least seven layers can be distinguished between the blood (red) and the neuropil: endothelium (grey ring), media (light blue), Virchow–Robin space (orange), glia limitans (green), inner and outer vascular basement membranes (1 and 2, mid-blue), and the basement membrane on top of the glia limitans (3, light purple). The different colours of the vascular and the astroglial basement membranes indicate that they differ in their content of laminin isoforms [55]. The differences in the biochemical composition of the vascular and the glia basement membranes might explain how leukocytes under normal conditions can pass the former but not the latter. In capillaries (bottom left), there is only one basement membrane (blue) between endothelial cells and the astrocytic endfeet of the glia limitans; this is termed the 'fused gliovascular membrane'. This intimate contact, which is absent in venules, might drive the BBB-typical specialization of endothelium. The inner and outer vascular membranes are interconnected. The role of pericytes is ill-defined. The sizes of the structures have been adopted from electron microscopic analysis. The first step in neuroinflammation (1st) involves the passage of T cells and macrophages across the vascular wall and is not necessarily related to pathology. The population of perivascular cells is heterogeneous and includes leptomeningeal mesothelial cells, and macrophages which can function as antigen-presenting cells. The population of macrophages is replaced regularly by blood-borne mononuclear cells [14,38,40–41]. Abbreviations: A, astrocyte; B, blood; E, endothelium; GL, glia limitans; M, media; Mφ, macrophage; P, pericyte; PC, perivascular cell; T, T cell; VRS, Virchow–Robin space.

degree to which tight junctions, phagocytosis, enzyme and transporter systems operate in different areas of the vascular tree, the observation that many hydrophilic dyes do not label the neuropil is real and this is what the term 'Bluthirnschranke' intended to illustrate (Box 2).

How the concept of a BBB was taken over by neuroimmunologists

Medawar [26] observed that immune tolerance to grafts in the brain breaks down when a second graft of the same tissue is inserted under the skin. He concluded that 'skin homografts transplanted to brain submit to, but cannot elicit, an immune state' and proposed that this 'is consistent with the view that a lymphatic drainage system is required to create a state of immunity, but not necessary to enforce a response to it.' It is noteworthy that functional drainage through the cribriform plate was described in the 19th century [27], but these studies were not accessible to Medawar because they were written in German. Later, the concept of drainage from the brain to cervical lymph nodes was re-established [7,28,29].

A linkage between the BBB and the immune privileged state of the brain was suggested by Barker and Billingham [6]: 'One property is the uniqueness of the cerebral circulation characterized by the blood-brain barrier, which has long been known to prevent the escape of blood-borne dyes into all but few areas of the brain. We now know that this is due to the presence of occluding junctions between the plasma membranes of adjacent endothelial cells and

the paucity of transport vesicles in these cells in brain capillaries. It is conceivable either that this anatomic barrier, and/or a layer formed by the end processes of neuroglial cells on the capillary walls, restricts diapedesis and so helps restrain lymphocytic infiltration and also the development of inflammatory responses in the brain, which would surely prejudice the integrity of functional connections between neurons.'

As a great example of scientific gossip, this careful consideration transformed into more-rigid statements, for example, 'the brain is an immune privileged site due to the BBB', which are still used in the neuroimmunology literature. Indeed, the concept seemed to fit perfectly as an explanation for the old notion that leukocytes are rare in the brain (an 'uncongenial environment for lymphoid cells' [30,31]). Most importantly, cellular infiltration and BBB dysfunction are not necessarily related [32].

As an initial consideration, the tight-junctional barrier is at its tightest in the capillary endothelium [22,23]), whereas postcapillary venules are the preferred site of leukocyte recruitment [33]. Moreover, there is evidence that leukocytes traverse the endothelium through a transcellular route (as opposed to a paracellular one), at a distance from, and irrespective of, the presence of tight junctions [34,35]. Studies in experimental autoimmune encephalomyelitis (EAE) revealed that areas of enhanced BBB permeability do not correlate with sites of infiltration [36]. Furthermore it is clear that the endothelium does not provide an insurmountable barrier for T and B cells under certain (experimental) conditions [37–39], and the same applies to monocytes that regularly reach perivascular spaces [14,40,41]. We have found that blood monocytes infiltrate layers of anterograde axonal degeneration, where they transform into microglia-like elements, whereas leakage of the classical BBB markers Evans Blue and HRP is not observed in the same area [42,43].

By impeding the entrance of blood molecules including antibodies or complement factors, the BBB might impact on the immune privileged status of the brain, but it should be noted that the BBB is not an absolute barrier to immunoglobulins [44]. It is currently unknown at which part of the vascular tree they enter. However, in terms of inflammatory cells, we have gained a more dynamic picture involving the ongoing dialogue between neural cells and infiltrating leukocytes [7,45,46]. This evolving body of knowledge was predicted by Barker and Billingham, who followed their consideration, cited earlier in this article, on the role of the BBB in immune privilege by saying: 'The other property is that brain tissue may contain chemical ingredients that discourage even normal leukocytic trafficking through its parenchyma.' [6].

The two steps in neuroinflammation

Pre- and post-capillary vessels are accompanied by leptomeningeal protrusions from the surface of the brain forming the perivascular spaces. Thus, the vessel wall of postcapillary venules is separated from the neuropil by an additional compartment, which is connected to the subpial and subarachnoid space and partly filled with cerebrospinal fluid (Figure 1). Therefore, cells crossing the vessel wall do not enter the neuropil directly, but have performed

Box 2. Sources of confusion – what is the BBB?

Proposed definition

The 'blood-brain barrier' is a metaphor which was used originally to explain why the brain – in contrast to most organs – remains 'white as snow' [4] following the injection of hydrophilic dyes.

Interpretation of the observation

The lack of dye diffusion into the neuropil is attributed mostly to tight junctions and characteristic expression patterns of transport systems in capillary endothelial cells ('manifestations of a BBB').

In pre- and post-capillary segments, where endothelial BBB characteristics are less pronounced than in capillaries, there is also a functional 'post-endothelial barrier' provided by phagocytes in the vessel wall (pericytes) and the perivascular spaces.

Statements including 'leukocytes cross the BBB' are confusing for three reasons

- (i) They imply that the same mechanisms underlying the barrier for solutes also hinder the entrance of cells.
- (ii) Solute diffusion is regulated at the capillary level, whereas leukocyte recruitment occurs preferentially in post-capillary venules. There is a gradual transition along the vascular tree, from capillaries to post-capillary venules, in terms of reduced endothelial BBB marker expression, greater densities of perivascular macrophages, the presence of pericytes and smooth muscle cells, and the proximity to the glia limitans. In the post-capillary segment, the vessel wall and glia limitans are separated by an additional compartment, the Virchow–Robin space.
- (iii) They do not indicate whether cells which have passed the vessel wall of post-capillary venules are trapped in Virchow–Robin spaces or progress across the glia limitans into the neuropil. This is of evident clinical importance.

Passage across the vessel wall and progression across the glia limitans are two differentially regulated steps (Figure 1, Figure 2).

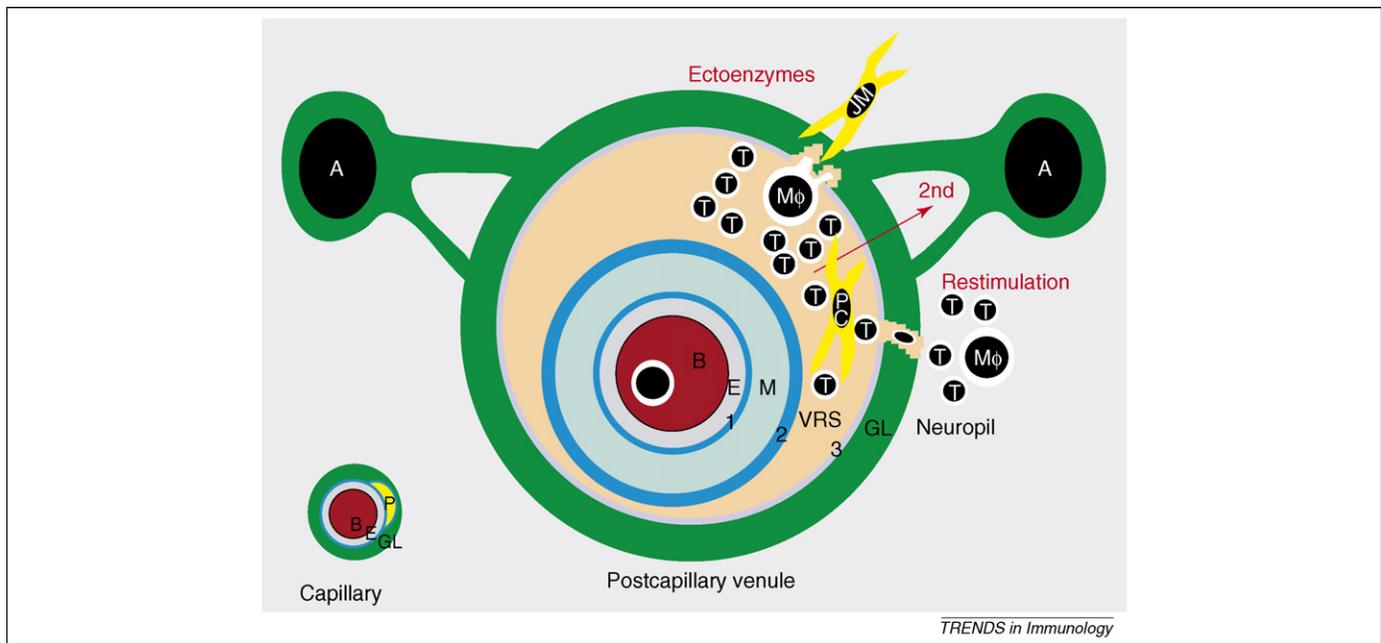


Figure 2. The second step in neuroinflammation. This step (2nd) is much more restricted and depends on the presence of ‘vessel-associated’ antigen-presenting cells [14–16], which are indispensable for the onset of EAE, the animal model of MS. It is currently unclear whether they are located in the perivascular space or represent juxtavascular microglia (JM). It is also unclear whether co-infiltrating DCs or differentiating monocytes, as opposed to resident perivascular phagocytes, present antigen in the perivascular space. Recent data indicate that ectoenzymes including MMP-2 and MMP-9, which can cleave components of the third basement membrane, are required for the induction of autoimmune neuroinflammation [56]. This basement membrane differs from the vascular membrane in its expression pattern of laminin isoforms [55]. Perivascular restimulation of T cells might be required for the induction of such ectoenzymes either by the T cells themselves or neighbouring macrophages. It is currently unknown whether the astrocytic endfeet of the glia limitans are destroyed or actively retracted during parenchymal infiltration. Abbreviations: A, astrocyte; B, blood; E, endothelium; GL, glia limitans; JM, juxtavascular microglia; M, media; Mφ, macrophage; P, pericyte; PC, perivascular cell; T, T cell; VRS, Virchow–Robin space.

the first step of migration from the blood into the brain. As described, monocytes reach perivascular spaces readily during maintenance of the perivascular phagocyte population, but the vast majority are retained in this compartment and do not progress into the neuropil. In fact, perivascular accumulation of inflammatory cells is a histopathological hallmark of several neuroimmune diseases, including MS.

The second step involves crossing the glia limitans (Figure 2), which seems to provide a strict additional barrier in at least two ways: mechanically and functionally. There is constitutive expression of the death ligand CD95L on the astrocytic endfeet [47], which might partly underlie a prominent hallmark of neuroinflammation, namely perivascular apoptosis [48]. In fact, during EAE, mice deficient for CD95 or its ligand exhibit much lower numbers of apoptotic, perivascular T cells [49,50]. Interestingly, these mice are resistant to clinical EAE, anticipating the later finding that CD95 and CD95L are also involved in the effector mechanisms of the disease [51].

Astrocytes express the receptor CD95, but a highly pro-inflammatory environment is required to confer their susceptibility to CD95L-mediated degeneration [52]. Under these conditions, large numbers of T cells could ‘overcome’ the astrocytes and, thus, conquer the glia limitans. However, it is currently unknown whether astrocytes of the glia limitans are partly eliminated or actively retract their processes during the course of intraparenchymal infiltration. There is also a considerable number of microglial processes within the glia limitans [53]; the role of these is ill-defined. Addressing the function of these

juxtavascular microglial cells [54] (Box 1, Figure 1) is certainly an important issue.

Another clue towards understanding what enables or drives cells to pass the glia limitans derives from studies demonstrating that the presence of macrophages in perivascular spaces is required for T cells to progress. Tran *et al.* [15] were the first to demonstrate that macrophage depletion does not interfere with the first step, but completely blocks the second step, thereby causing massive T-cell accumulation in Virchow–Robin spaces. Further studies suggested a role for a discrete population of CD11c⁺ cells in the perivascular area [16], although whether these cells are perivascular-resident or newly incoming (i.e. co-infiltrating) cells has yet to be determined. Importantly, the basement membranes of the vessel and the basement membrane of the glia limitans differ in their content of laminin isoforms, with only the basement membrane of the glia limitans exhibiting laminin 1 and laminin 2 [55]. This observation might help to explain why cells capable of performing step 1 cannot necessarily proceed. A recent study demonstrates that matrix metalloproteinase (MMP)-2 and MMP-9 with high affinity for dystroglycan, which anchors laminins 1 and 2, are required for inducing intraparenchymal neuroinflammation [56]. These observations provide a scenario of the interaction between T cells and macrophages following perivascular re-stimulation and/or signals from the neuropil (chemokines such as monocyte chemoattractant protein [MCP]-1, also known as CCL2, [57]) inducing the expression of MMPs and other ectoenzymes enabling penetration of the glia limitans [58]. Owens and co-workers have now demonstrated that mice overexpressing CCL2 exhibit perivascular, but

not intraparenchymal, infiltrates. Following injection of pertussis toxin (PTx), the leukocytes progressed into the neuropil, which was accompanied by clinical symptoms. Remarkably, treatment of PTx-injected mice with the broad-spectrum MMP inhibitor BB-94 (also known as Batimastat) diminished both step 2 and clinical symptoms [59].

Although much work has yet to be done to understand fully these aspects of neuroinflammation, one conclusion is clear: whatever initiates step 2, it is almost certain that the underlying mechanisms have little or nothing to do with the observation that tracers injected into the blood do not label the brain.

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