

# The meningeal and choroidal infiltration routes for leukocytes in stroke

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**Abstract:** Stroke is a major health burden as it is a leading cause of morbidity and mortality worldwide. Blood flow restoration, through thrombolysis or endovascular thrombectomy, is the only effective treatment but is restricted to a limited proportion of patients due to time window constraint and accessibility to technology. Over the past two decades, research has investigated the basic mechanisms that lead to neuronal death following cerebral ischemia. However, the use of neuroprotective paradigms in stroke has been marked by failure in translation from experimental research to clinical practice. In the past few years, much attention has focused on the immune response to acute cerebral ischemia as a major factor to the development of brain lesions and neurological deficits. Key inflammatory processes after stroke include the activation of resident glial cells as well as the invasion of circulating leukocytes. Recent research on anti-inflammatory strategies for stroke has focused on limiting the transendothelial migration of peripheral immune cells from the compromised vasculature into the brain parenchyma. However, recent trials testing the blockage of cerebral leukocyte infiltration in patients reported inconsistent results. This emphasizes the need to better scrutinize how immune cells are regulated at the blood–brain interface and enter the brain parenchyma, and particularly to also consider alternative cerebral infiltration routes for leukocytes, including the meninges and the choroid plexus. Understanding how immune cells migrate to the brain *via* these alternative pathways has the potential to develop more effective approaches for anti-inflammatory stroke therapies.

**Keywords:** choroid plexus, leukocyte infiltration, meninges, neuroinflammation, stroke

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## Introduction

The inflammatory response to cerebral ischemia is an important element in the onset and progression of stroke. Necrotic cells and cell debris induce neuroinflammation by activating resident microglia and astrocytes.<sup>1,2</sup> After this initial glial cell activation, there is an increase in cerebral cytokine and adhesion molecule expression leading to the recruitment of peripheral leukocytes to the lesion site.<sup>3–5</sup> Monocytes/macrophages and neutrophils infiltrate the ischemic tissue in large numbers within the first days, whereas lymphocytes appear in the parenchyma later on.<sup>1,6</sup> Importantly, previous studies have reported a critical role of T cells in secondary neuroinflammation after brain ischemia.<sup>7,8</sup> We and others have shown that blockage of lymphocyte trafficking diminished the

infarct volume in different cerebral ischemia models, resulting in improvement of stroke outcome and suggesting a possible therapeutic target.<sup>9–11</sup> Several clinical trials have been initiated since then to test pharmacological approaches blocking lymphocyte migration using fingolimod (FTY720), natalizumab (anti- $\alpha$ 4-integrin immunoglobulin G4) and enlimomab Intercellular Adhesion Molecule-1 (anti-ICAM-1 antibody) with controversial results. While two trials testing fingolimod in stroke have shown promising results with reduction of infarct volume progression,<sup>12,13</sup> both studies testing natalizumab and enlimomab have not been able to demonstrate a significant effect on the respective primary endpoint.<sup>14,15</sup> Failure to induce consistent neuroprotection by anti-inflammatory therapies in patients with stroke has been discussed

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elsewhere.<sup>16,17</sup> While several aspects of study design, statistical rigor and translational aspects from mouse to men need to be considered in this regard, the inefficient targeting of lymphocytes, at the predominantly investigated transendothelial migration route, might also account for the lack of efficacy of these anti-inflammatory treatments. A better understanding of how cells of the peripheral immune system access the central nervous system (CNS) and interact with the brain microvasculature would likely lead to the development of more efficient immune interventions for individuals with stroke.

In the stroke field, the transendothelial migration of leukocytes into the ischemic brain, across the compromised blood–brain barrier (BBB), has been investigated most extensively.<sup>11,18,19</sup> Interestingly, recent studies from stroke and other brain diseases point to an important role of the alternative cerebral infiltration route, the meningeal compartment and the choroid plexus (ChP).<sup>20–22</sup> Specifically, we have demonstrated that a subtype of proinflammatory T cells accumulate early after stroke in the meninges<sup>20</sup> and that cerebral ischemia induces the recruitment of lymphocytes from the ChP to the peri-infarct brain tissue.<sup>21</sup> However, mechanisms of brain infiltration of leukocytes from these alternative routes and how they may contribute to the infarct development are still unknown.

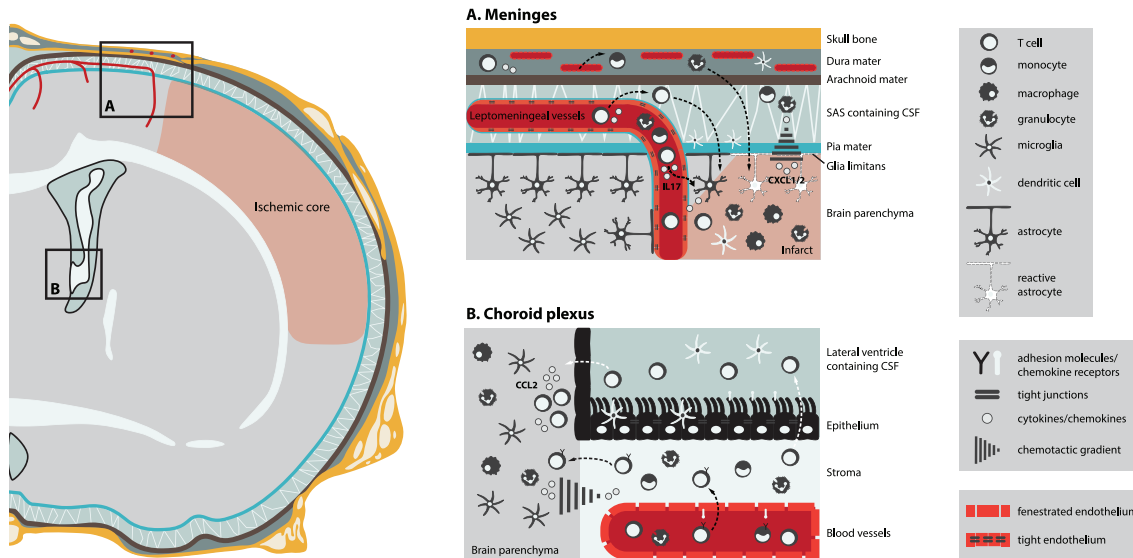
In this review, we examine the immune mechanisms occurring at the meninges and the ChP in brain diseases, with special attention paid to stroke. In addition to the BBB, the meninges and ChP act as potent regulators for immune cell activation and migration routes into the injured brain. Importantly, their distinct locations, structural differences, different composition of resident immune cells and differential pattern of surface molecule expression in comparison to the BBB may guide leukocytes to utilize preferentially one or the other meningeal or choroidal infiltration routes. Here, we first summarize the anatomical structure of the meningeal and choroidal compartments, which immune cells populate these tissues and how they become activated and migrate into the brain parenchyma. We next discuss how these compartments may regulate the inflammatory response to stroke and influence disease progression, making assumptions for similar mechanisms in humans in the light of current information from postmortem human specimens

and patient imaging. Elucidation of the role of the meninges and the ChP in ischemic stroke will advance our understanding of how peripheral immune cells influence stroke pathobiology. Future studies should explore the function of these alternative cerebral routes for leukocytes that could lead to novel pharmacologic interventions involving both meningeal and choroidal compartments in stroke.

### **The alternative CNS barriers for leukocyte trafficking in stroke**

Complex endothelial or epithelial barriers separate the brain from blood-derived circulating molecules and effector immune cells. Maintenance of the integrity of these barriers is essential for proper brain function.<sup>23</sup> Stroke is associated with a leakage of the BBB which was previously associated with propagating the influx of leukocytes into the brain.<sup>24,25</sup> However, to date three distinct routes for leukocyte migration from the blood into the brain parenchyma have been described: through the parenchymal vessels of the BBB, *via* the meningeal blood circulation and across the epithelial cells of the choroid plexus.<sup>26,27</sup>

To better interpret the results published in the field, we first aim to clarify certain aspects of the terminology used to describe the different blood–CNS barriers. Specifically, we refer to ‘interface’ as a structure which upon injury allows the passage of immune cells from the intracranial vasculature to the extracellular compartments. Thus, the blood–CNS interface systems refer to the following: the BBB which encloses the parenchymal microvessels and the glial limitans of the brain parenchyma; the blood meningeal interfaces formed between either the pia-arachnoid blood vessels and the cerebrospinal fluid (CSF) of the subarachnoid space (SAS), the pial blood vessels of the pial basement membrane and the glial limitans, and across the dural blood vessels; and the ChP interfaces between the fenestrated capillaries and the stromal side of the ChP epithelial cells, and the apical choroidal region and the CSF of the brain ventricles.<sup>28,29</sup> In contrast to this terminology of ‘interface’ which denotes structure on an organ level as entry sites for circulating leukocytes, the term ‘barrier’ refers to specific endothelial or epithelial cell layers which demonstrate a selective permeability for soluble particles, such as the BBB and the blood–CSF barrier (BCSFB), which have been reviewed in detail elsewhere.<sup>27,30–32</sup>



**Figure 1.** Meningeal and choroidal cerebral infiltration routes for leukocytes in poststroke neuroinflammation. Cerebral ischemia induces the release of chemotactic cues, such as CXCL1/2 and CCL2, from activated parenchymal glial cells, leading to the recruitment of leukocytes from the meninges and from the choroid plexus into the infarct area. The upregulation of adhesion molecules in the meningeal and choroidal vasculatures after stroke may possibly allow the transmigration of leukocytes from the blood vessel lumen to the brain parenchyma. Arrows indicate routes of possible migration for leukocytes from (A) the meninges: dura mater blood vessels and leptomeningeal vessels (black arrows) and (B) the choroid plexus: ChP stroma (black arrows), possibly the preferred route after stroke, and cerebrospinal fluid (CSF) circulation (white arrows) into the ischemic brain parenchyma.

Due to distinct structural characteristics (endothelial *versus* epithelial cells) and anatomical location (superficial cortical space *versus* deep ventricular space) as well as different molecule expression (adhesion molecules expression and chemotactic cues) between the blood barrier systems, entry into the CNS might be limited to immune cell subsets that hold the specific molecular keys required to cross these interfaces. Thus, one can hypothesize that targeting a specific route for leukocyte infiltration will have a greater impact on stroke outcome. Here we first describe the structure of the alternative routes for leukocytes to enter the CNS: the meningeal and choroidal paths (Figure 1).

### *Structure and function of the blood–CNS interfaces*

**The meninges.** The meninges consist of three layers of connective tissue which enclose the brain and the spinal cord: the dura mater, the arachnoid mater and the pia mater. All components of the meninges show considerable structural and functional heterogeneity. The dura

mater is described as two epithelial layers of dense fibrous tissue.<sup>33</sup> The outermost periosteal layer is tightly adherent to the calvarium (or skull cap) and plays an important role in the skull bone development.<sup>34</sup> The inner dura mater layer is defined as the meningeal dural layer and is firmly attached to the periosteal layer except at distinct regions to form large venous channels known as the dural venous sinuses, which serve as a draining system from the CNS.<sup>35</sup> The major blood vessels that supply the dura run in its outer periosteal surface and vary according to different areas of the skull, among them the internal carotid artery which supplies the more anterior part of the skull.<sup>35</sup> Directly attached to the meningeal dural layer is the arachnoid and pia mater which build the SAS between the dura mater and the CNS.<sup>29,36</sup> The two structures also known as the leptomeninges are attached by strands of connective tissue, the arachnoid trabeculae. The CSF circulates within the SAS between the arachnoid, composed of tight intercellular junctions, and the pia mater, which is a semipermeable membrane.<sup>29</sup> Outflow of CSF from the SAS takes place through arachnoid villi or

granulations that evert into the dural sinuses. While this system is efficient for water and small compound clearance, macromolecules and immune cells are preferentially drained through the meningeal lymphatic vessels.<sup>37–39</sup> Numerous blood vessels (arteries and veins) are associated with the leptomeninges. They are composed of a monolayer of nonfenestrated endothelial cells attached by intercellular tight junctions and in contrast to vessels in the subpial space they are not covered by astrocyte end feet.<sup>40</sup> As leptomeningeal blood vessels enter the brain parenchyma they carry with them arachnoid and pial tissues and together with glial end feet form a cuff known as the perivascular space in which antigen-presenting cells can be found<sup>41,42</sup> as well as a concomitant accumulation of lymphocytes.<sup>43</sup> Indeed, in disease settings, the meningeal blood vessels appear to be impermeable to defined cell components, such as red blood cells, but permeable to migrating inflammatory cells. The failure of erythrocytes to enter the perivascular spaces in patients with subarachnoid hemorrhage suggests that the pia mater does form an effective barrier to large molecules and nonmigratory cells. However, in inflammatory conditions affecting the meninges, such as infective meningitis, polymorphonuclear leukocytes and macrophages are seen throughout the subarachnoid space as well as in the subpial and perivascular spaces.<sup>44</sup>

As mentioned above, meningeal blood vessels run along the dura mater, in the SAS and at the pia basement membrane, and eventually penetrate the brain parenchyma. Previous studies investigating lymphocyte egress from the vasculature in these various meningeal compartments have studied the contribution of the dura mater as well as the leptomeninges (arachnoid and pia mater), which results in some discrepancy on information about the blood–meningeal interface at the different meningeal levels across various brain diseases. This particular focus on substructures of the meninges could also be attributed to the use of specific techniques investigating meningeal lymphocyte transmigration and need to be kept in mind when drawing conclusions about the role of the ‘meninges’ in a specific disease setting as the described findings most often reflect only events at a particular meningeal compartment. In particular, multiphoton *in vivo* imaging studies focus mainly on the leptomeningeal compartment,<sup>45</sup> flow cytometric analysis of cell composition is widely performed on the dura mater and arachnoid mater,<sup>46</sup>

while histological studies might investigate the various meningeal structures.<sup>47</sup>

*The choroid plexus.* The ChP is not only involved in CSF production but also in homeostasis of the brain parenchyma by supplying the brain with nutrients, by clearing toxic compounds and by surveying the immunological status of the brain.<sup>48</sup> The ChP is found in all four cerebral ventricles and potentially allows cell trafficking from the choroid blood vessels and the CSF to the brain parenchyma.<sup>31</sup> Structurally, the ChP is composed of a single layer of epithelial cells continuous with the ependymal cells that line the ventricles. At the apical side, the ChP epithelium has numerous villi increasing the surface area for a substantial flux of solutes and water from the blood through the ChP epithelium to the ventricles. Reduction in size of microvilli due to aging has been related to reduced ability to produce CSF, which may affect brain function.<sup>49,50</sup> The basal side of the ChP epithelium faces the ChP stroma, which is a highly vascularized tissue, in which capillaries have a fenestrated endothelium facilitating non-restrictive substance secretion and absorption in contrast to the tightly sealed ChP epithelium. Tight junction and adhesion molecules are found between adjacent choroidal cells restricting the passage of solutes from the blood circulation into the CSF.<sup>51,52</sup> This highly polarized ChP structure, defined by the fenestrated vasculature and tight epithelium, forms the ChP interfaces which are possible gates allowing cell migration in disease setting.<sup>48,53</sup> Both blood flow and CSF production by the ChP epithelium are regulated by sympathetic and parasympathetic innervation, probably *via* the glossopharyngeal and vagus nerves.<sup>54</sup> Vagal nerve stimulation inhibits the release of proinflammatory cytokines<sup>55,56</sup> and improves stroke outcome.<sup>57</sup> One can assume that activation of the vagal nerve after stroke may have a direct impact on the ability of ChP to produce cytokines/chemokines<sup>58</sup> and further influence cell migration, although further investigations are needed.

The ChP receives its blood supply from the anterior and posterior circulation. The anterior choroidal artery, which branches from the internal carotid or middle cerebral artery, supplies the ChP from the lateral ventricle. The posterior choroidal arteries, which branch from the posterior cerebral artery, supply the lateral ventricle as well as the third ventricle, whereas the ChP of the



fourth ventricle is supplied by the anterior and posterior inferior cerebellar arteries.<sup>59</sup> Ischemic stroke has been reported in patients in the territory of the anterior choroidal artery and more rarely in the lateral and medial posterior choroidal arteries.<sup>60,61</sup> Whether changes in the function of the ChP supplied by these choroidal arteries contribute to the neurological deficits is unclear. Several studies reported that cerebral ischemia induced in rodents led to necrosis of the ChP. Likewise, proximal middle cerebral artery occlusion induced ischemic lesions of the ipsilateral ChP<sup>21</sup> and permanent global ischemia induces choroidal cell death resulting in ChP atrophy and preceding neuronal cell death in the hippocampus.<sup>62–64</sup> Apart from direct injury to the ChP, both transient global ischemia and common carotid artery occlusion models caused ChP tissue disruption with intraventricular leakage of high molecular weight blood molecules.<sup>65</sup> Additionally, pathogenic processes in Alzheimer's disease contribute to the low efficacy of the ChP to clear amyloid  $\beta$  (A $\beta$ ) peptides, resulting in an accumulation of A $\beta$  in the brain. This, in turn, enhances the progression of the disease.<sup>66</sup> These studies indicate that damage to choroidal cells may result in disruption of the ChP selective barrier, leading to the entry of neurotoxic substances, inflammatory mediators or migration of immune cells to the CSF.

### Immune cell populations of the meninges and ChP

Under physiological conditions, brain resident microglia are present in large numbers, whereas fewer immune cells from the innate and adaptive immune system can be found in the brain parenchyma.<sup>67</sup> Immune surveillance of several regions of the CNS occurs at steady state by patrolling T cells and dendritic cells (DCs).<sup>27</sup> The CSF of healthy individuals contains between 1000 and 3000 leukocytes per ml, and is enriched in memory T cells compared with blood and secondary lymphatic organs.<sup>68</sup> Interestingly, several reports have shown that at steady state immune cells of the innate and adaptive system are present in the meninges and choroid plexus.<sup>69</sup> They are located in the CSF and lymphatics and may act either as patrolling cells detecting subtle changes in brain homeostasis or as a drainage system to signal a peripheral immune response during injury.<sup>70,71</sup> Also, similarly to parenchymal blood vessels, cells circulating in the meningeal and choroidal vessels

may bridge the impaired or inflamed vasculature and infiltrate the brain parenchyma upon injury (Figure 1). Moreover, resident immune cells of the dural compartment or the subpial structure of the meninges as well as in the ChP stroma may sense cytokines/chemokines released from the injured brain tissue. Such resident immune cells at the borders of the brain might either invade the parenchyma themselves after activation by brain-released stimuli or influence neighboring cells towards either a pro- or anti-inflammatory phenotype.<sup>27,53</sup> These different aspects are discussed in more detail in the following sections.

### T lymphocytes

Recently, we have demonstrated the importance of the meninges as a site of T-cell accumulation and the ChP as an alternative route for lymphocyte brain infiltration after stroke.<sup>20,21</sup>  $\gamma\delta$ T cells are nonconventional lymphocytes which display several innate cell-like features allowing them to become rapidly activated following ischemic injury.<sup>72</sup> Using the transgenic mouse model expressing a Kikume Green-Red photoconvertible fluorescent protein in all cell types,<sup>73</sup> we showed that intestinal T cells are mobilized from the gut and were exclusively located in the meningeal compartment after stroke.<sup>20</sup> Specifically, immunohistochemistry and flow cytometry analysis revealed that interleukin (IL)-17-producing  $\gamma\delta$ T cells increased in the meninges early after stroke onset<sup>20</sup> and preceded their accumulation in the ischemic area.<sup>8,74</sup> This was associated with an upregulation of chemotactic genes *CXCL1/CXCL2* contributing to brain injury, possibly through the promotion of neutrophil infiltration.<sup>20</sup> Together, the meninges could function as a checkpoint in postischemic inflammation and orchestrate leukocyte infiltration into the brain parenchyma *via* chemotactic cues.

In a second study, we investigated the ChP as a possible route for T-cell entry into the brain parenchyma by distal occlusion of the middle cerebral artery in mice. This model of cortical stroke has the advantage of not affecting the blood flow in the anterior choroidal artery, which provides blood supply to the ChP in the lateral ventricle. We have reported that T cells were the main population in the ipsilateral ChP of the lateral ventricle after an ischemic stroke compared with other myeloid cells. In the same study, we showed a cluster of T cells in the peri-infarcted cortex

between the lateral ventricle and the lesion core. To assess the migration of T cells from the ChP to the brain parenchyma after stroke, we specifically photo labelled T cells in the ipsilateral ChP using a T-cell photoactivated mouse model. We detected that approximately two thirds of all perilesional T cells in the peri-infarcted cortex 24 h after stroke have been photoactivated, indicating their choroidal invasion pathways in contrast to only one third of T cells migrating *via* other invasion routes (e.g. transendothelial or meningeal).<sup>21</sup> Interestingly, blocking the CSF circulation by intraventricular Matrigel injection did not affect T-cell invasion after stroke, suggesting that choroidal T cells translocate preferentially from the ChP stroma to the brain parenchyma but not transmigrating to the CSF compartment. These data support the hypothesis that the ChP acts as a predominant route of lymphocyte infiltration after an ischemic stroke. In contrast to previous findings, the reduced number of invading CD3<sup>+</sup> T cells did not affect the volume of the infarct.<sup>21</sup> It is plausible that compensatory mechanisms, such as the involvement of other peripheral immune cells or resident microglia, tampered with the reduced infiltration of T cells and thus failed to significantly contribute to inflammatory lesion expansion.

Previous studies in animal models of primary autoimmune CNS disease have guided current studies on the role of the ChP in stroke. Experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis, was induced by intravenous transfer of CD4<sup>+</sup> effector T cells reactive against the myelin component, myelin basic protein (MBP). The fate of these T cells was followed across the leptomeninges of the spinal cord by two-photon imaging.<sup>75,76</sup> T cells appear crawling intraluminally in the meningeal vessels of the subarachnoid space, followed by their transendothelial migration to the perivascular space of the pia mater. There, MBP T-cell receptor-restricted CD4<sup>+</sup> cells encounter perivascular macrophages or DCs with antigen presentation capabilities. Upon contact with these antigen-presenting cells, effector T cells increased the expression of proinflammatory mediators and this expression profile remained stable after T-cell infiltration into the CNS parenchyma. In light of these findings in EAE, it would be of great interest to investigate the meningeal route for T-cell transmigration after stroke. It is conceivable that the preferred route of infiltration

for T-cell subpopulations would be disease dependent. In a recent study, Schläger and colleagues found that the meningeal route for brain infiltration of CD4<sup>+</sup> T cells in EAE was preferentially used as very few cells were observed in the ChP.<sup>77</sup> In contrast, the ChP route was mainly involved in T-cell brain accumulation after stroke. Indeed, we have demonstrated that the experimental infarction of the ChP significantly decreased T-cell migration to the ischemic area.<sup>21</sup>

The recruitment of circulating T cells in the meningeal compartments and the ChP is mediated by the sequential interaction of different adhesion and chemokine/cytokine cues, leading to the interaction between circulating immune cells and endothelial or epithelial cells.<sup>78</sup> In particular, the cellular and molecular events leading to lymphocyte cerebral infiltration across the meningeal<sup>75,77</sup> and the ChP<sup>79,80</sup> interfaces have been described in EAE, and involved the disruption of the endothelial tight junctions and the upregulation of different adhesion molecules on the outer cell membrane of vascular endothelial cells: selectins (P, E and L selectins), immunoglobulins and integrins Vascular Cell Adhesion Molecule-1 (VCAM-1), Very Late Antigen-4 (VLA-4), (ICAM-1), which lead to leukocyte rolling on the surface of endothelial cells, adhesion to the endothelial wall and paracellular or transcellular migration.<sup>32,81</sup> This sequence of events shares similarities with some features of lymphocyte recruitment across the BBB.<sup>32</sup> The differential regulation of specific adhesion molecules or chemotactic cues involved in T-cell migration at the distinct interfaces of the parenchymal vessels, the meninges and the ChP has been discussed in detail before<sup>32,82,83</sup> and is beyond the scope of this review. Nevertheless, it would be of interest to investigate the molecular basis of leukocyte infiltration through the meningeal vessels and ChP tissue in a stroke context. For instance, it has been reported that the C-C chemokine ligand-20 (CCL20), the ligand for the C-C chemokine receptor-6 (CCR6), is constitutively expressed in the ChP but not on the endothelium of CNS parenchymal capillaries.<sup>84</sup> Interestingly, infiltration of CCR6<sup>+</sup> T helper (Th)-17 *via* the choroid plexus was shown to be essential for the initial phase of EAE.<sup>84</sup> Although little is known about how leukocytes are recruited into the brain after stroke, we have shown that intracerebral T-cell migration from the ChP to the peri-infarcted cortex is driven by a CCL2 chemokine gradient between these two compart-

ments, mainly produced by parenchymal macrophages and microglia.<sup>21</sup>

### Dendritic cells

DCs have been located in the meninges and ChP of the CNS.<sup>85,86</sup> Specifically, DCs were identified on the internal region of the dura mater and on the surface of the pia mater facing the SAS. DCs thereby have ready access to CSF-circulating antigens.<sup>86</sup> Similarly, the ChP stroma contains high numbers of major histocompatibility complex class II expressing macrophages and DCs.<sup>42,87,88</sup> Immature DCs reside in between the ChP epithelial cells and extend their dendrites into the CSF<sup>88</sup> where they have a sentinel function by sampling the CSF micromilieu for antigens. DCs can further present antigens to T cells entering the ChP stroma.<sup>87</sup> This continuous immunosurveillance by choroidal DCs allows specific responses to disease and injury. In EAE an increase of CD11c<sup>+</sup> DCs was observed in the ChP as well as in the leptomeninges of the spinal cord prior to the onset of EAE symptoms.<sup>76,89</sup> Specifically, meningeal DCs were shown to activate CD4<sup>+</sup> T cells before EAE disease manifestation was observed.<sup>76</sup> These findings suggest that the meninges represent an important site for primary interaction of DCs with antigen-specific T cells. However, it remains to be defined whether meningeal and choroidal DCs upon activation are actively recruited to the brain parenchyma after EAE and sustain the autoreactive immune response during the progression of the disease. Stroke involves DCs either as antigen-presenting cells<sup>90</sup> or independently to their antigen-presenting function,<sup>91</sup> thus one can speculate a similar role for meningeal and choroidal DCs in stroke, as previously demonstrated in autoimmune CNS models. These findings on functional DC–T-cell interaction in the meninges indicate that the meninges are a potent polarizing compartment for T cells *via* antigen presentation and costimulation by DCs activity before their entry into the injured brain. Alternatively, activation of DCs in these compartments due to an injury may participate in the peripheral immune system response after antigen presentation to T cells of the cervical lymph nodes,<sup>92</sup> which still remains to be conclusively investigated.

### Macrophages

Monocytes/Macrophages have been observed under physiological conditions in all layers of the

meninges<sup>85,93</sup> and in the ChP.<sup>42,86</sup> In contrast to DCs, macrophages, by their location within the connective tissue of the meningeal compartment and ChP, are less exposed to the CSF, suggesting a tissue macrophage function which is distinct from that of DCs. The phenotypes of macrophages are dictated by tissue-specific signals and the health or disease state.<sup>93</sup> Particularly, when mice were tested for spatial memory, they showed an accumulation of IL-4-producing T cells in the meninges over time.<sup>22</sup> Importantly, acute depletion of T cells from the meningeal space or the deletion of IL-4 from T cells resulted in impairment of spatial memory and led to a proinflammatory skew in meningeal macrophages, pointing to an important role for meningeal macrophages in learning and memory. The spatial memory performance could be rescued by using IL-4-primed anti-inflammatory macrophages.<sup>22</sup> Likewise, depletion of meningeal and perivascular macrophages using clodronate liposomes increased clinical symptoms and bacterial load in a mouse model of pneumococcal meningitis.<sup>94</sup> Interestingly, the meninges were associated with neuroprotection following an ischemic stroke *via* preconditioned monocytes.<sup>95</sup> Low doses of the proinflammatory mediator lipopolysaccharide (LPS) before an ischemic injury induced protection after transient occlusion of the middle cerebral artery, a phenomenon known as preconditioning.<sup>95,96</sup> This effect was recapitulated by adoptive transfer of monocytes isolated from LPS-preconditioned mice. Specifically, the neuroprotective monocytes were recruited to meningeal vessels, where they dampened the expression of proinflammatory genes involved in the activation of neutrophils and chemotactic factors such as Csf3,<sup>97</sup> indicating an essential role of the meningeal compartment as a key immunoregulator in ischemic brain injury. Moreover, a recent study reported an increase in monocytes/macrophages in the CSF and ChP after stroke.<sup>98</sup> The study found that GFP<sup>+</sup>CD11b<sup>+</sup> macrophages expressing preferentially an M2-like phenotype were located between the lateral ventricle and the ischemic core. To address the role of M2-like macrophages after cerebral ischemia, *in vitro* M2-polarized cells were directly injected into the lateral ventricle. Both motor and cognitive functions were improved post stroke in animals treated with M2-polarized macrophages, although the infarct volume remained unaffected.<sup>98</sup> Importantly, Ge and colleagues demonstrated an upregulation of several adhesion molecule and chemokine genes in the ChP after stroke, which are associated with the homing and

migration of monocytes.<sup>98</sup> Similarly, traumatic brain injury led to a release of the chemokine CCL2 from the ChP in a polarized manner, leading to the accumulation of neutrophils and monocytes in the ChP stroma and their transmigration into the injured cortex.<sup>58,99</sup>

#### Neutrophil granulocytes

Experimental stroke induced an increase in neutrophil granulocyte counts in the infarct region between 1 and 3 days after stroke onset, depending on the investigated stroke model.<sup>1,100</sup> Interestingly, neutrophils were detected in the leptomeninges as well as in the cortical basal lamina and perivascular space a few hours after ischemia induction prior to brain infiltration.<sup>20,101</sup> In addition, ischemic tissue from fatal cases of human stroke revealed positive immunostaining for neutrophils in the leptomeninges and perivascular spaces.<sup>101</sup> Traumatic brain injury (TBI) induces leakage of blood vessels of the subarachnoid space as well as in the perivascular space, leading to cell death of meningeal cells and disruption of the glia limitans.<sup>102</sup> Within an hour after injury, myeloid cells, mainly neutrophil granulocytes, were highly motile and interacted with dead cells in the meningeal compartment.<sup>102</sup> Interestingly, in this study, application of the reactive oxygen species scavenger glutathione on the surface of the skull, allowing its penetration through the porous structure of the skull bone, resulted in survival of meningeal myeloid cells as well as glia limitans preservation, a process associated with a reduction in meningeal cell death.<sup>102</sup> Another study reported that neutrophils were found both in the leptomeninges and ChP of the nondamaged area as well as the injured side. However, neutrophils remained within the leptomeninges and ChP of the nontraumatized hemisphere, whereas neutrophils accumulated in the ipsilateral hemisphere. These results suggest that the leptomeninges and ChP are possible gates for neutrophil infiltration into the injured area after brain injury, similar to the parenchymal vasculature.<sup>103</sup> Interestingly, invasion of neutrophils after TBI was regulated by an upregulation of tumor necrosis factor  $\alpha$  and IL-1 $\beta$  in the ipsilateral ChP, causing the production of neutrophil chemoattractants in the ChP, such as cytokine-induced neutrophil chemoattractant (CINC). CINC upregulation leads to a temporary neutrophil recruitment to the ipsilateral ChP 24 h after

TBI, since no neutrophils could be noted at 48 h post lesion.<sup>58</sup>

#### Mast cells

Mast cells are resident immune cells of the meninges, located in the perivascular space and in the dura mater, where they act as early regulators of barrier integrity. Mast cells are activated early after cerebral ischemia and contribute to the BBB breakdown by their gelatinase activity.<sup>104</sup> Also depletion of the mast cell population has been shown to dampen granulocyte and macrophage infiltration after stroke, whereas T cells and microglia were not affected.<sup>105</sup> Taken together, meningeal mast cells can exacerbate stroke outcome in mice, highlighting a critical function for the meningeal compartment after stroke while the specific interaction of mast cells with other immunocompetent cells, neurons and glial cells after stroke still needs to be elucidated.

#### Conclusion and perspectives

Although numerous preclinical trials showed an improvement of the stroke outcome when circulating lymphocytes were reduced, the first clinical trials testing anti-inflammatory therapies in stroke have generated conflicting results. While fingolimod (FTY720) could reduce the infarct volume, natalizumab and enlimomab failed to show an effect on their primary endpoints.<sup>12–15</sup> One of the reasons for these discrepancies might be due to the incomplete concept that lymphocytes infiltrate into the brain mainly *via* the transendothelial route of parenchymal capillaries, without considering the meninges and ChP. Natalizumab (anti-CD49d) and enlimomab (anti-ICAM-1) aimed to block the lymphocyte entrance to the CNS by blocking specific adhesion molecules required for transendothelial migration across parenchymal vessels. However, CD49d as well as ICAM-1 are both expressed at the apical side of the ChP epithelium but not on ChP endothelial cells.<sup>79</sup> Hence, stromal lymphocytes in the ChP vasculature have no access to these adhesion molecules and blocking of these will not affect the ChP infiltration route. In contrast, fingolimod reduces the number of circulating lymphocytes independent of adhesion molecule expression at the various migration routes, which might explain why currently the only positive results on treatment efficacy are obtained with this drug in patients with stroke compared with natalizumab and enlimomab.



Our increasing understanding of the highly complex and anatomically as well as molecularly diverse structures of the three main invasion routes to the postischemic brain, the parenchymal vasculature, the meninges and the choroid plexus, reveals the previously unrecognized diversity of selective invasion routes for leukocyte subpopulations and poses a problem on efficiently targeting proinflammatory, neurotoxic leukocyte entry. Therefore, the results of current clinical trials aiming at blocking poststroke leukocyte migration need to be interpreted in light of the uncertainty regarding the involved invasion routes and their particular selectivity for specific subpopulations. Designing more efficient drugs targeting cerebral leukocyte invasion after stroke will require a much more detailed understanding of the contribution of the different invasion routes, their specifications in expression of chemotactic cues and adhesion molecules, and identification of site-specific pharmacological targets.

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The authors declare that there is no conflict of interest.

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