Chapter 7

Summarizing discussion

MS is a chronic inflammatory demyelinating disease of the CNS. One of the early hallmarks of MS lesion development is blood-brain barrier (BBB) dysfunction, which is associated with infiltration of immune cells into the CNS and subsequent tissue damage. Understanding of molecular mechanisms that regulate BBB function and integrity is essential to identify targets to prevent BBB dysfunction in neuroinflammatory diseases and to limit neurological deficits. Studies described in this thesis aimed to gain more insight into the pathogenetic mechanisms that underlie BBB alterations, thereby providing new therapeutic avenues for the treatment of MS. Here, the results of my thesis will be summarized and discussed.

Tight junction dynamics during immune cell migration into the CNS

The BBB controls the entry of circulating molecules and cells into the brain and is characterized by the presence of specialized brain endothelial cells (ECs) that are connected by tight junctions (TJs). An early event in MS lesion formation is the migration of T cells and monocytes from the circulation into the CNS. Monocyte-derived macrophages subsequently cause damage to the myelin sheaths surrounding axons, which results in neuronal dysfunction. In general, transendothelial migration of leukocytes occurs in several consecutive steps that involve various sets of adhesion molecules and inflammatory mediators like chemokines [1-3]. The migration of leukocytes across the BBB requires active participation of brain ECs to rearrange their cytoskeleton and TJs, thereby facilitating the entry of immune cells into the CNS. Previous studies from our group have shown that reactive oxygen species (ROS) are crucial mediators during the migration process, since ROS enhance monocyte adhesion and migration [4, 5], although mechanisms underlying ROS-mediated breakdown of the BBB remained unknown. In chapter 2 we therefore assessed the influence of ROS on BBB integrity and underlying signaling events that in turn may facilitate monocyte transmigration. In our studies, extracellularly administered ROS directly affect the integrity of brain endothelial cell monolayers, reduce transendothelial electrical resistance (TEER) and increase permeability. ROS-induced alterations in endothelial integrity are preceded by cytoskeletal rearrangements and redistribution and disappearance of the TJ proteins claudin-5 and occludin. Recently, it was shown that hydrogen peroxide exposure enhances BBB permeability and reduces TEER [6-8] as well as facilitates the formation of actin stress fibers in bovine brain endothelium [8]. Our results provide new data showing that superoxide, which is the most predominant ROS produced during inflammation, affects the integrity of brain endothelial cell monolayers. The organization of TJ proteins occludin and ZO-1 is altered by exogenous ROS in both epithelial cells [9-11] and brain ECs [8, 12]. Since claudin-5 is one of the most important TJ proteins in barrier formation [13], our results highlight an important role for ROS in the regulation of claudin-5 at the cellular junction. Moreover, regulation of the actin cytoskeleton and TJ complexes in brain endothelial is mediated by various intracellular signaling events (see book chapter Kooij G. et al., 2005), like Rho GTPases [14, 15]. In chapter

2 we identified specific signaling pathways activated through ROS administration, that include RhoA and PI3 kinase. Moreover, we identified PKB as a novel player in cytoskeleton and TJ dynamics that acted downstream of RhoA and PI3 kinase. ROS have been previously shown to negatively regulate peripheral endothelial cell-cell adhesion, in which another Rho GTPase (Rac-1) appeared to play an important role [16, 17]. These results may support the view of an important regulatory role for ROS and Rho GTPase signaling during TJ dynamics. By disturbing TJ assembly, ROS contribute to decreased BBB integrity, which facilitates the influx of leukocytes and supports an inflammatory response. Furthermore, PKB phosphorylation and RhoA activation may be early markers of BBB dysfunction. Agents that selectively inhibit these effects like antioxidants or signaling inhibitors for RhoA, PI3 kinase or PKB may be used therapeutically to modulate neuroinflammatory diseases complicated by BBB dysfunction. Indeed, specific inhibitors of these signaling pathways prevent ROS-induced monocyte migration across an in vitro model of the BBB (chapter 2), which was previously observed for antioxidants like lipoic acid and luteolin both in vitro and in vivo during EAE [5, 18] and Rho GTPase inhibitors [19]. Together, our results highlight a pivotal role for ROS during monocyte migration into the CNS.

Besides ROS, other molecules are involved in transendothelial migration of immune cells, including pro-inflammatory mediators like chemokines [20], cytokines [21] and matrix metalloproteinases (MMPs) [22, 23]. These molecules regulate BBB integrity and subsequent TJ dynamics. As described above, occludin and claudin-5 are important TJ molecules and are connected to the actin cytoskeleton, which together results in an active role in maintaining TJ integrity and BBB function [24]. Infiltrating leukocytes and brain ECs secrete MMPs that degrade and remodel extracellular matrix components, such as fibronectin, collagen and laminin [25] and play a crucial role during leukocyte migration into the brain parenchyma. In chapter 3, we studied the TJ dynamics of occludin during monocyte diapedesis. Live cell imaging studies on GFP-tagged occludin in brain EC demonstrate that monocytes scroll towards cell-cell contacts, induce endothelial gap formation associated with local disappearance and degradation of occludin and subsequently transmigrate through the junction. MMP inhibitors prevented all these processes, showing an important role for MMPs in immune cell diapedesis and TJ dynamics. Moreover, in vivo MMP inhibitors have been shown to reduce the severity of EAE, which suggests that inhibition of MMP activity may be an interesting therapeutic tool to prevent MS lesion formation. Besides the known effects of MMPs on extracellular matrix remodeling, it can possibly directly degrade occludin, as occludin contains a putative MMP cleavage site in its first extracellular loop [26]. These findings indicate that cellular infiltration may directly contribute to loss of tight junction proteins in neuro-inflammation. Interestingly, in brains of patients with multiple sclerosis an abnormal expression of occludin and ZO-1 was observed in inflamed cerebral tissue, which contains numerous cellular infiltrates [27, 28]. Moreover, vascular loss of TJ proteins was observed in

animal models for neuroinflammation, which coincides with leukocyte extravasation [29, 30]. Together, these data implicate that endothelial TJs are involved in leukocyte trafficking across the BBB.

Although the process of transendothelial migration of immune cells has been investigated intensively for decades, the pathway by which inflammatory cells cross endothelium has become a matter of debate. Leukocytes can migrate across the endothelial layer either through endothelial cell junctions (the paracellular route) or through the endothelial cell body (transcellular route). Both in vitro [31] and in vivo [32, 33] experiments have provided evidence for both pathways. However, our studies using brain endothelial cells in an in vitro system did not provide evidence of transcellular migration. It could very well be that different vascular beds or different stimulations of EC or monocytes increase the incidence of transendothelial traversal via a transcellular route. Our paracellular migration experiments were performed in the absence of cytokines or chemokines, whereas previous reported transcellular migration experiments were dependent on TNF-α-stimulated endothelial cells and treatment with monocyte chemoattractant protein-1, platelet activating factor, and stromal cell-derived factor-1, respectively [31]. Preliminary results from our group indeed indicate that endothelial activation by cytokines leads to enhanced transcellular migration of immune cells, indicating that both pathways are involved in transendothelial migration.

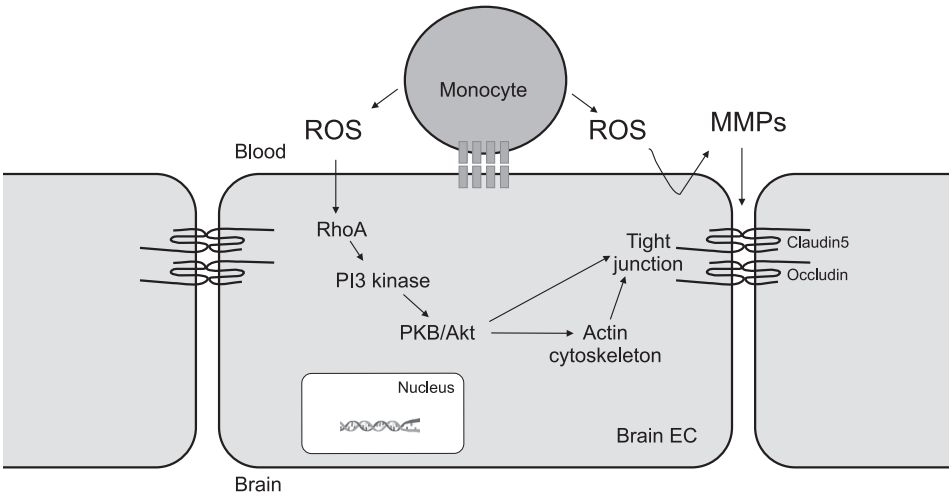

Figure 1. TJ dynamics during monocyte migration

Monocytes adhere to activated endothelial cells, which triggers ROS production. Next, ROS activate signal transduction pathways in brain EC, such as RhoA and PI3 kinase, which induce cytoskeleton alterations and tight junction rearrangements, thus facilitating monocyte transendothelial migration. In addition, ROS can activate endothelial cell-associated MMPs, which in turn can degrade TJ proteins, thereby facilitating transcellular monocyte diapedesis.

Transmigration of immune cells into the CNS is an important hallmark of MS lesion formation. The transmigration process requires active participation of brain ECs to rearrange their cytoskeleton and TJs, thereby facilitating the entry of immune cells into the CNS. We have identified several critical players that regulate TJ and cytoskeleton dynamics during monocyte diapedesis like ROS and MMPs (summarized in figure 1). Interestingly, it has been described that ROS can activate endothelial cell-associated MMPs, which in turn facilitates transendothelial migration of leukocytes across peripheral endothelial cells [34]. Preliminary data from our group indicate that both leukocytes and extracellular ROS induce MMPs in brain EC (unpublished results), thereby strengthening a direct link between ROS and MMPs. Moreover, endothelial-signaling pathways like RhoA, PI3 kinase and PKB appeared to be important regulators of BBB integrity. Our results illustrate that therapeutics aimed at the stabilization of the tight junction or targeting inflammatory mediators like ROS by anti-oxidants (e.g. fumarate [35]) or MMPs by specific inhibitors may be beneficial in neurological disorders to reduce cellular infiltration, thereby preventing the neuroinflammatory attack as seen in the pathology of MS.

BBB efflux transporters in MS

Disruption of the blood-brain barrier (BBB) is suggested to play a key role in MS pathology [36]. As described above, we and others previously focused on defects in the integrity of the BBB during MS, including permeability, tight junction and basement membrane alterations, that lead to cellular influx [27, 28, 36-39]. However, BBB dysfunction can also occur at the level of the molecular transporter properties of the BBB. Normally, the BBB strictly regulates influx and efflux of a variety of proteins and molecules by different transporters and carrier molecules, making the BBB a selective transport barrier. Moreover, the BBB has a key role in protecting the brain from unwanted compounds by a large family of ATP-binding cassette (ABC) transporters [40], creating multi-drug resistance (MDR) of the brain. Since it is suggested that ABC transporters are involved in the removal of inflammatory mediators from cells, potential alterations in their expression and function at the BBB may contribute to pathogenesis of neuro-inflammatory disorders such as MS. We therefore investigated the expression pattern of various MDR proteins like P-gp, MRP-1, MRP-2 and BCRP in various well-characterized human MS lesions in chapter 4 and 5. We observed a striking decreased expression of cerebrovascular P-gp in active inflammatory MS lesions. Loss of P-gp expression coincided with decreased in vivo P-gp function during EAE, indicating P-gp malfunction at the level of the BBB during neuroinflammation. Since P-gp is involved in cellular extrusion of inflammatory mediators [41-43], loss of expression and function will likely result in the entry of detrimental compounds, like prostaglandins and cytokines, into the CNS and may therefore enhance tissue damage. In view of these findings, locally restoring endothelial P-gp expression and function should be considered as a novel neuroprotective strategy. At

the transcriptional level P-gp is under the control of the orphan nuclear receptors such as steroid and xenobiotic receptor (SXR in human; or pregnane X receptor (PXR) in rodents) [44]. Corticosteroids, such as dexamethasone, are widely used to reduce inflammation, and are an accepted treatment for MS [45]. Interestingly, it is known that dexamethasone can improve the barrier function not only by increasing of the tightness of brain endothelial cell tight junctions [46], but also through the induction of P-gp expression at the BBB [47], thereby restoring specific protection mechanisms of the brain endothelium. Interestingly, a novel drug for the treatment of MS is FTY720 [48], which acts as an immunosuppressive drug but may also increase P-gp activity as it is an shingosine-1 phosphate analogue [49, 50]. Conversely, since a number of substrates for P-gp such as statins [51], corticosteroids [45, 52] and cannabinoids [53, 54] are currently used in the treatment of MS, loss of P-gp transporter function at the BBB has the advantage to specifically deliver drugs to affected areas, thereby locally reducing tissue damage.

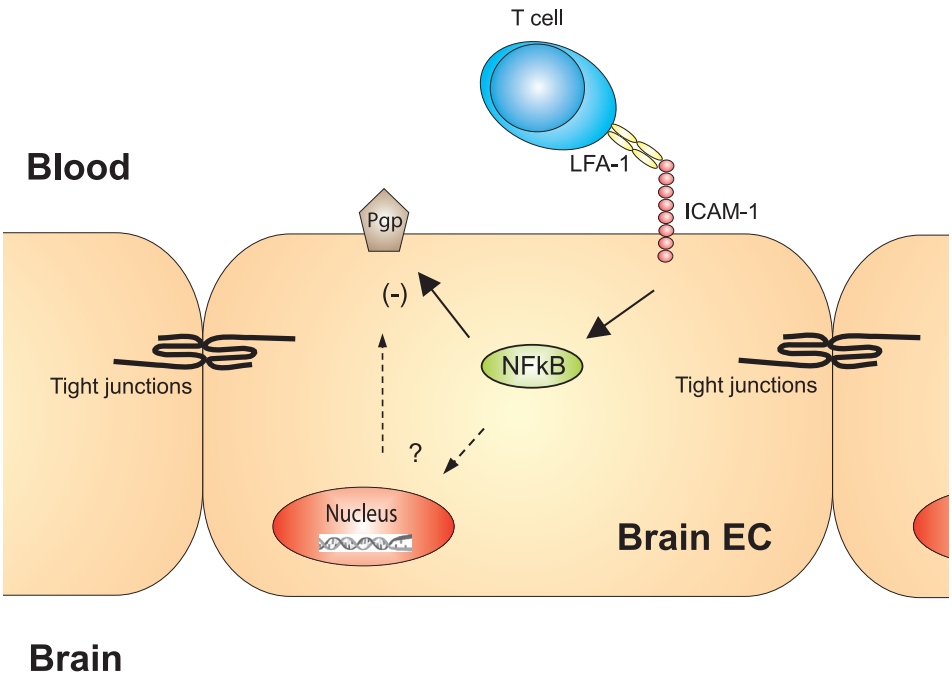

Figure 2. T lymphocytes regulate endothelial P-gp expression and function

T cells adhere to activated endothelial cells via LFA-1/ICAM-1 interactions. Upon adhesion, the NF-κB signal transduction pathway is activated which in turn negatively regulates P-gp function, either directly or indirectly via transcriptional regulation of P-gp expression.

Active MS lesions contain numerous inflammatory cells, predominantly T cells and monocyte-derived macrophages. In chapter 4 we demonstrate that the interaction of predominantly $CD4^+$ T-cells and endothelium leads to a reduction of endothelial P-glycoprotein function in vitro. Under pathological conditions, immune cells gain access to the brain through various well-defined molecular processes and the adhesion and subsequent diapedesis of T-cells through brain endothelial cells has been shown to predominantly depend on LFA-1 / ICAM-1 interactions [55]. ICAM-1 is capable of activating signaling pathways in brain endothelial cells, which results in re-arrangement of the endothelial cytoskeleton and tight junctions, allowing leukocyte migration [55]. In chapter 4 we demonstrate that reduced endothelial P-gp function is controlled by ICAM-1 activation. Interestingly, this process is dependent on NF-κB activation, which is yet an unknown pathway to operate down-stream of ICAM-1 (summarized in figure 2). NF-κB has previously been linked to be involved in regulation of P-gp expression and activity in brain capillaries under inflammatory conditions [56]. However, it remains to be determined whether NF-κB affects P-gp function via P-gp expression or via downstream signaling molecules. ICAM-1 activation and the NF-κB signaling cascade are associated with the pathogenesis of autoimmune demyelinating diseases and neurodegenerative disorders [55, 57, 58]. Therefore, intervention in LFA-1 / ICAM-1 interactions by blocking antibodies or disruption of NF-κB signaling by specific peptides [58] may represent attractive therapeutic approaches to prevent P-gp malfunction and subsequently limit neuroinflammation.

In contrast to P-gp, the expression pattern of other BBB specific ABC transporters is not affected on endothelial cells during MS pathology (chapter 5), which suggests a differential regulation of ABC transporters during pathological conditions, for example an NF-κB independent pathway. However, endothelial ABC transporter activity may still be affected during neuroinflammation, as we showed for P-gp in vivo during EAE (chapter 4). Indeed, in vitro functional MRP-1 assays on brain EC have revealed that TNF-α or immune cells are able to decrease MRP-1 activity, whereas its expression remained unaffected (unpublished results). Further research is needed to determine the role of inflammatory mediators on ABC transporter activity and their contribution to neuroinflammation. Other cell types present in MS lesions display an altered expression pattern of MRP-1, MRP-2, P-gp and BCRP. In chapter 5 we demonstrate that hypertrophic astrocytes have enhanced expression of P-gp, MRP-1 and MRP-2, which coincides with increased function of P-gp and MRP-1 in astrocytes isolated from MS lesions compared to astrocytes derived from control white matter. Activation of astrocytes has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer's disease, inflammatory demyelinating diseases and human immunodeficiency virus (HIV)-associated dementia [59]. Upon activation, reactive astrocytes can secrete different neurotrophic factors for neuronal survival. However, it is believed that severe activation augments an inflammatory response,

leading to neuronal death and brain injury [60]. Reactive astrocytes are important producers of inflammatory mediators in the CNS and may therefore amplify a neuroinflammatory response [61]. As endogenous substrates for ABC transporters may include inflammatory mediators, such as steroids, prostaglandins, leukotrienes and cytokines [41-43, 62-65], increased ABC transporter expression and function on reactive astrocytes may result in local efflux of inflammatory mediators in MS lesions, enhancing the inflammatory response. Alternatively, loss of endothelial P-gp expression and function (chapter 4) may be compensated by increased expression of MDR efflux transporters on reactive astrocytes in the immediate vicinity of MS lesions. As a result, astrocytes attempt to function as a second barrier when the BBB is compromised, which has been previously suggested for P-gp [66]. Nevertheless, further research is needed to determine the composition of inflammatory mediators that can be secreted by ABC transporters, which will provide opportunities to interfere in the neuroinflammatory process.

In inflammatory demyelinating MS lesions foamy macrophages are abundantly present, which acquire their distinctive morphology by ingestion and accumulation of vast amounts of myelin-derived lipids. Foamy macrophages originate from both resident microglia and infiltrating monocytes [67] and are thought to display an anti-inflammatory phenotype [68]. In chapter 5 we describe an enhanced expression of BCRP, MRP-1 and MRP-2 on infiltrated foamy macrophages. Notably, enhanced function of BCRP and MRP-1 of myelin-laden macrophages could be mimicked in vitro. Although the exact role of BCRP- and MRP-1 function on macrophages is unknown, it has been described that macrophages rely on cholesterol efflux mechanisms to maintain cellular cholesterol homeostasis by means of ABC transporters ABCA1 and ABCG1 [69]. Since MRP-1 and BCRP participate in cellular detoxification [70, 71], we suggest that these ABC transporters are involved in the removal of phagocytosed myelin products like cholesterol from foamy macrophages and thereby control cellular homeostasis. Interestingly, inhibition of MRP-1 on macrophages prevented myelin phagocytosis in vitro (unpublished results), which suggests a new functional role of MRP-1 on macrophages during pathological conditions.

ABC transporters are highly expressed at the BBB and can effectively remove a remarkably wide variety of substrates out of the brain, possibly including inflammatory agents. Proper transporter function is therefore critical in the maintenance of brain homeostasis and to limit neuroinflammation. Our results show an important role for ABC transporters during MS pathology as their expression and function is differentially regulated on various cell types present in MS lesions. The exact role of MRP-1, MRP-2 and BCRP on astrocytes and foamy macrophages needs to be determined, however our data suggest that these ABC transporters contribute to pathology by secreting inflammatory mediators or mediate myelin phagocytosis on macrophages, highlighting their therapeutical potential. Moreover,

we defined a crucial role for cerebrovascular P-gp during neuroinflammation. As we shown previously in chapter 2 and 3, immune cells appeared to be capable of affecting specific brain endothelial properties, thereby inducing BBB dysfunction and subsequently aggravating neuroinflammation. Blocking immune cell interactions with brain endothelial cells during neuroinflammation as reported for instance for Tysabri [72, 73] or agents that directly restore P-gp function beside their immunosuppressive effects (dexamethasone or FTY720) provide additive neuroprotective strategies at the level of the brain capillaries to fight neuro-inflammatory disorders.

P-gp as a novel immunomodulator

The mechanisms of CNS inflammation in MS and EAE involve generation of autoreactive, myelin specific T helper cells in the peripheral lymphoid organs, which subsequently enter the brain, initiate an immune response and eventually cause destruction of myelin sheaths and axonal loss [74]. Antigen-presenting cells like dendritic cells (DCs) are important regulators of immune responses by presenting their captured antigens to specific T cells [75]. In general, the maturation status of DCs is a key determinant of the immune response [76]. The molecules or proteins that regulate DC maturation and thereby control immune responses are currently under extensive investigation, since they may provide targets for immune modulation. As P-gp is expressed on a variety of immune cells like monocytes, DCs, T and B cells [63] and is involved in the efflux of inflammatory molecules such as steroids, prostaglandins and cytokines [41-43, 63, 64], we hypothesized that P-gp has an immunomodulatory function. In chapter 6 we identified P-gp as a regulator of immune responses by controlling DC maturation and subsequent specific T cell responses. Moreover, P-gp knock-out mice (mdr1a/1b-/- mice) displayed decreased clinical signs of EAE, which coincided with decreased inflammation in the brain and an overall reduced T cell response. In chapter 6 we defined the mechanism showing that P-gp mediated DC maturation and DC-induced T cell responses by influencing the excretion of proinflammatory cytokines like TNF-α and IFN-γ. A controversial issue remains whether P-gp itself is capable to transport cytokines as suggested by some groups [41, 63, 77] or that P-gp is involved in the secretion of other relevant physiological substrates like platelet activating factor [64] that in turn may affect cytokine secretion [78] as a secondary effect. Nevertheless, addition of TNF-α and IFN-γ to P-gp deficient DCs restored their maturation capacity, highlighting an important role for P-gp and these cytokines during DC maturation and subsequent immune responses. DCs are ideal targets for immunotherapeutic strategies due to their intrinsic capacity to efficiently present antigens to T cells and regulate T cell responses. Interestingly, two compounds, IFN-β and glatiramer acid (GA) that are widely used in the clinic to treat MS patients have DCs as one of their main targets [79, 80]. We here postulate that targeting P-gp on DCs can prevent T cell specific immune responses to interfere in inflammatory diseases.

Beside its well-known role in MDR, evidence is accumulating that ABC transporters may also be involved in immune related processes based on their expression on immune cells and their potential capacity to mediate the efflux of inflammatory mediators. Our results highlight a novel role for P-gp as a key regulator of immune responses by controlling DC maturation and subsequent DC-induced T cell responses in vitro and in vivo. Previously, MRP-1 activity has been shown to be involved in DC differentiation [81], which strengthens the link between ABC transporters and immune responses. However, a controversial issue remains about the exact role of P-gp on the BBB during neuroinflammation. In chapter 4 we observed a loss of cerebrovascular P-gp expression in MS patient material, which coincided with a loss of functional P-gp in vivo during EAE. We postulated that the loss of P-gp was a prerequisite for disease pathogenesis. Surprisingly, P-gp knockout mice revealed reduced clinical signs of EAE, which appeared to be mediated by a dominant peripheral role of P-gp during DC-induced T cell responses. Further research is needed to determine the contribution of P-gp on the BBB during neuroinflammation, for example by using bone marrow chimeric mice that lack BBB specific P-gp. In that way, also the role of other ABC transporters during neuroinflammatory diseases can be determined in more detail.

Concluding remarks and future directions

The BBB controls the entry of circulating molecules and cells into the brain and plays an important role in maintaining brain homeostasis. One of the early hallmarks of MS lesion development is BBB dysfunction, which results in the infiltration of immune cells into the CNS, where they cause extensive damage to myelin and axons. Understanding of the regulation of the function and integrity of the BBB is essential to identify agents that can prevent BBB dysfunction in neuroinflammatory diseases thus limiting neurological deficits. In this thesis we provide more insight into the molecular mechanisms that underlie BBB alterations and dysfunction (summarized in figure 3). Our results illustrate that therapeutics aimed at stabilization of tight junctions or targeting inflammatory mediators like ROS or MMPs may be beneficial in neurological disorders to reduce cellular infiltration of immune cells into the brain. Previous studies have shown that antioxidant therapy is beneficial in vitro and in vivo in animal models for MS [82]. However, the use of exogenous antioxidants for MS treatment has drawbacks, as large amounts of antioxidants are required to achieve functional antioxidant levels in the CNS. Therefore, the induction of endogenous antioxidant enzymes by activators of the nuclear factor-E2-related factor (Nrf2) and antioxidant response elements (ARE) pathway may be an interesting approach to obtain sufficient levels of antioxidants to interfere with pathological processes underlying MS lesion formation. Future studies should provide insight into the value of Nrf2/ARE enzyme inducers for the treatment of neuroinflammatory diseases, such as MS.

Importantly, in this thesis we show that immune cells themselves substantially contribute

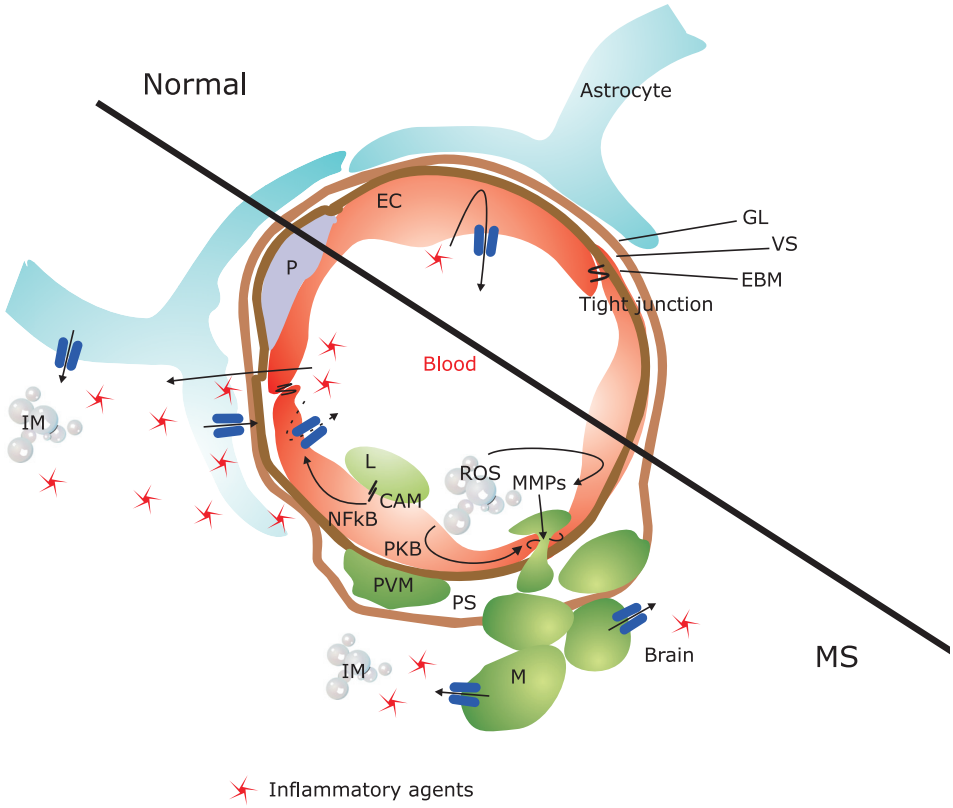

Figure 3. The BBB during health and disease

Lymphocytes adhere to activated brain endothelium, which triggers ROS production. In turn, ROS activate endothelial MMP production and signal transduction via PKB that both results in TJ rearrangements and subsequently leukocyte transmigration. Leukocyte binding to brain EC via LFA-1 / ICAM-1 interaction triggers signal transduction via NF-κB, which in turn decreases efflux mechanisms like P-gp (dotted arrow), thereby enhancing brain influx of inflammatory agents. Astrocytes upregulate efflux mechanisms as a secondary barrier but can also secrete inflammatory agents into the brain parenchyma. Finally, transmigrated monocyte-derived macrophages phagocytose myelin and upregulate ABC transporter systems, thereby enhancing brain inflammation. Blocking ROS, MMPs, signaling molecules or specific ABC transporter function at the BBB may be beneficial for the treatment of MS to prevent lesion formation and progression.

to BBB dysfunction, as they are able to affect specific brain endothelial properties, like TJ integrity or efflux capacity (figure 1 and 2). In turn, this may facilitate the cellular influx into the CNS and aggravate the inflammatory response. Blocking immune cell interactions with brain ECs during neuroinflammation like LFA-1 / ICAM-1 or as described for VLA-4 / VCAM-1 [72, 73] may therefore be the most attractive strategy at the level of the brain capillaries to

combat neuro-inflammatory disorders. Moreover, we have identified a novel role for ABC transporters in MS pathology and in particular for P-gp as an immunomodulatory molecule. As P-gp and other ABC transporters are expressed on cells of the immune system and are suggested to be involved in the secretion of inflammatory mediators, our results provide tools for immune modulation, thereby halting inflammatory attack during (neuro) inflammation. Further research is warranted to elucidate which inflammatory molecules are excreted via ABC transporters, and whether this happens directly or indirectly via other relevant physiological substrates. Moreover, it needs to be determined what the role of other ABC transporters is on astrocytes and foamy macrophages in MS lesions and the potential combined effect of ABC transporters during immune responses. We have highlighted a novel immunomodulatory role of P-gp in this thesis, whereas other ABC transporters may act in a similar fashion. In conclusion, our findings have contributed to the understanding of BBB dysfunction and immunomodulation under neuropathological conditions. Our results may provide novel avenues for the treatment of MS to prevent lesion formation and progression.

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