Chapter 1

General Introduction

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The brain is normally protected by the blood-brain barrier (BBB) that hinders entry of unwanted compounds and cells into the brain. Dysfunction of the BBB is a prominent feature of a number of neurological disorders, including multiple sclerosis (MS). Impaired BBB function may lead to leakage of toxic compounds and migration of immune cells into the central nervous system (CNS) leading to neurological deficits. Studies described in this thesis are aimed to gain more insight into the molecular mechanisms that underlie BBB alterations and dysfunction in MS pathology in order to identify novel targets and strategies for the development of therapeutic agents to tackle MS disease progression. In the following, the background of these studies will be explained.

1. Multiple Sclerosis

MS is a chronic inflammatory demyelinating disease of the CNS, affecting over 2,5 million individuals worldwide. The onset of disease generally occurs between the age of 20 and 40 and MS is considered to be one of the most disabling neurological diseases in young adults [1]. MS incidence and prevalence is highest in western countries (Northern-Europe, North-America) where the lifetime risk of MS development is approximately one per 1000, affecting more women than men in a ratio of 3:1 [2]. During MS, immune cells infiltrate the CNS and cause damage to the protective myelin sheaths that surround the axons, which gradually leads to motor and sensory deficits. Clinical features of MS depend on number, size and location of the lesions in the CNS and are very heterogeneous. Impaired vision due to optic neuritis is a common sensory disturbance and motor disturbances include muscle weakness, tremor, paralysis and spasms. Depending on clinical features, four main subtypes of MS can be distinguished [3]. The majority (~70%) of MS cases follow a relapsing-remitting course (RRMS) characterized by clearly defined alternating episodes of neurological impairment and recovery. About half of all RRMS cases gradually develop a secondary-progressive course of MS (SPMS) within 10 years, characterized by increasing permanent neurological impairment. Two other disease courses that comprise a small part (~15%) of the total MS population are primary-progressive MS (PPMS), characterized by increasing neurologic impairment from the point of MS onset without recovery, and progressive-relapsing MS (PRMS; ~5%), characterized by a steadily increasing neurologic impairment combined with acute attacks of disability [4]. The diagnosis of MS is primarily based on clinical history and neurological examination and is supported by cerebrospinal fluid analysis and magnetic resonance imaging (MRI) of the brain and spinal cord, to determine the number and size of MS lesions and BBB leakage [5, 6].

1.1 Etiology

Although the etiology is largely unknown, MS is conventionally viewed as an autoimmune

disorder with a multifactorial background. Both genetic and environmental factors may contribute to disease susceptibility and disease outcome. Sibling and twin studies have demonstrated that the incidence of MS is higher in monozygotic twins (25-30%) compared to dizigotic twins (2-5%) [7]. Moreover, genome-wide studies have revealed that susceptibility of MS is linked to genes in the major histocompatibility complex (MHC) on chromosome 6. Alleles for certain class II genes, HLA-DR and HLA-DQ, confer the strongest risk of contracting MS [8]. Environmental factors also contribute to the risk of developing MS as the prevalence of MS generally increases with distance from the equator and is particularly high in Northern Europe and North America. This may be related to exposure to sunlight (vitamin D), diet, or (viral) infections. Numerous viruses have been associated with MS pathology, including Epstein-Barr virus [9], human herpes simplex virus [10], measles virus [11] or Chlamydia pneumonia [12], although no single infectious agent has been directly related to MS.

1.2 Pathology

MS is characterized neuropathologically by the presence of multiple focal lesions throughout the CNS [13]. MS lesions occur in all brain areas, although there are several predilection sites, like the optic nerves, periventricular white matter, brain stem, cerebellum, and spinal cord. MS white matter lesions are characterized by the destruction of myelin sheaths, oligodendrocyte cell death, axonal damage, glial scar formation and the presence of inflammatory cell infiltrates. Based on the degree of myelin loss and the presence of inflammatory cells or microglial cell activation, MS lesions can be classified as preactive, active, chronic active, and chronic inactive lesions [14]. Active MS lesions contain high numbers of immune cells, mainly T cells and monocyte-derived macrophages [15], of which the latter are mainly responsive for causing damage to the myelin sheaths surrounding axons, resulting in neuronal dysfunction. The mechanisms of CNS inflammation involve activation of autoreactive myelin specific T helper (TH) cells in the peripheral lymphoid organs, possibly by molecular mimicry, which gain entry to the CNS and form perivascular infiltrates. This process is accompanied by enhanced permeability of the blood-brain barrier (BBB) [16,17]. Local antigen-presenting cells (APCs), like microglia or perivascular macrophages, subsequently reactivate transmigrated T cells by presenting their specific target antigens. Consequently, increased amounts of proinflammatory cytokines and chemokines are locally produced, which in turn attract more monocyte-derived macrophages and lymphocytes to the site of inflammation. This inflammatory cascade finally leads to destruction of myelin sheaths and axonal loss [8]. Characteristic subsets of myeloid cells present in demyelinating lesions are foamy macrophages, which obtain their distinctive morphology by ingestion and accumulation of vast amounts of myelin-derived lipids. Glial fibrillary acidic protein (GFAP) positive reactive astrocytes with long processes are evenly distributed throughout the demyelinated areas where they inhibit remyelination, axonal sprouting and regeneration

through glial scar formation. The occurrence of gray matter MS lesions has been largely underestimated until recently. To date, evidence that extensive demyelination occurs in the cerebral cortex of patients with chronic MS is increasing [18, 19]. However, the mechanisms underlying gray matter lesion pathology remain largely unknown, as white matter lesion hallmarks like BBB dysfunction and leukocyte infiltration are not observed in gray matter lesions [20, 21].

1.3 Animal model for MS, experimental allergic encephalomyelitis

Most of our current knowledge about the pathogenesis of MS is extrapolated from an animal model called experimental allergic encephalomyelitis (EAE). EAE is a widely accepted animal model for MS, sharing its clinical, immunological and pathological characteristics [22]. Rodents or non-human primates display MS like symptoms after active immunization with myelin components or total myelin in combination with a strong adjuvant like (in)complete Freunds' adjuvant. Immunization leads to the development of autoreactive T cells in the peripheral lymphoid organs that recognize myelin proteins. These T cells finally enter the CNS where they find their antigens, which results in CNS inflammation, loss of neurological function and subsequently paralysis [22]. Transferring these autoreactive T cells into naïve animals (transfer EAE model) results in similar clinical signs as EAE induced by active immunization [23]. Depending on the immunization protocol, disease pattern may vary from monophasic type of disease with only minor myelin damage (acute EAE or transfer EAE) to the demyelinating and relapsing remitting form (chronic EAE). Generally, clinical signs manifest themselves in an ascending manner, beginning with loss of tail tonus followed by paralysis of the hind limbs, and the disease may progress to the front limbs and occasionally even to death of the animals.

Animal models like EAE have provided considerable insight into the neuroinflammatory processes that take place in the CNS in demyelinating autoimmune disorders, however, none of these EAE models are exactly similar to MS. Nevertheless, the EAE model has been proven to be very useful for our understanding of MS pathogenesis and to investigate possible therapies.

1.4 Treatment

So far, MS has not been curable and current therapies consist of lifelong disease and symptom management. They are based on the hypothesis that MS is an autoimmune disease and are therefore anti-inflammatory, immunosuppressive or immunomodulating agents. The most widely used drugs during relapses are corticosteroids like prednisone and methylprednisolone [24], which act both immunosuppressive and anti-inflammatory, reduce the duration of a relapse and accelerate recovery. RR-MS patients are often treated with interferon beta (IFN-b) or glatiramer acetate, which have both been shown to

reduce exacerbation frequency and severity and improve neurological disability [25, 26]. One of the new drugs that seems promising is natalizumab (Tysabri), which is a recombinant humanized monoclonal antibody directed against the α_4 chain of the $\alpha_4\beta_1$ integrin (or Very Late Antigen-4 (VLA-4)) that is expressed on activated lymphocytes and monocytes and is involved in transendothelial migration [27]. Natalizumab treatment significantly reduced the number of new lesions and clinical relapses by blocking leukocyte migration into the brain [28]. A novel immunosuppressant drug in phase III clinical trails for treatment of RR-MS is FTY720, which can reduce both relapse rate and the number of new lesions [29]. FTY720 targets lymphocytes, which results in the sequestering of these cells in secondary lymphoid organs [30]. Furthermore, depletion of B cells by Rituximab [31] is beneficial and reduces disease progression. Together, these results indicate that limitation of the inflammation process or blocking migration of immune cells across the BBB into the CNS during MS pathology are attractive therapeutic strategies.

2. Blood-Brain Barrier

The current understanding of lesion formation during MS pathology includes activation of $CD4^+$ or $CD8^+$ T cells specific for one or more self (myelin-associated) antigens that adhere to the surface of CNS endothelial cells and migrate into the CNS parenchyma, a process that is accompanied by severe loss of BBB integrity. The amplification of the inflammatory reaction following the primary migration of T cells and subsequent transmigration of monocytes over the blood-brain barrier (BBB) will lead to the degradation of myelin and axonal damage observed in MS lesions.

2.1 BBB morphology

The BBB is a key anatomical structure that protects the CNS microenvironment from the systemic circulation As such, it plays a crucial role in maintaining brain homeostasis by restricting transport of immune cells and molecules into the brain parenchyma. The BBB is composed of highly specialized brain endothelial cells, surrounded by a basement membrane, pericytes, perivascular macrophages and astrocytes (figure 1), of which the latter project their end feet to the BBB, thereby inducing specific BBB properties [32]. Brain endothelial cells support the barrier function and exhibit functional and morphological properties that distinguish them form peripheral endothelium. The paracellular cleft between adjacent endothelial cells (ECs) is tightly sealed due to the presence of well-developed tight junctions (TJ) and adherens junctions (AJ), thereby impeding the entrance of circulating hydrophilic molecules and immune cells into the CNS [33]. However, small gaseous molecules and a number of lipophilic agents may diffuse freely through the lipid membranes of the ECs. Other prominent features of brain endothelium are the absence of fenestrations,

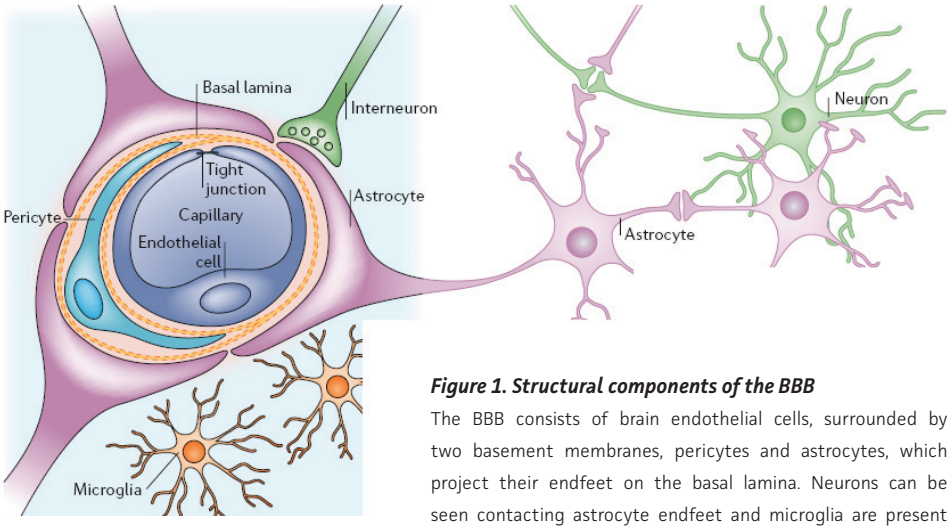

Figure 1. Structural components of the BBB

The BBB consists of brain endothelial cells, surrounded by two basement membranes, pericytes and astrocytes, which project their endfeet on the basal lamina. Neurons can be seen contacting astrocyte endfeet and microglia are present in the brain parenchyma. *Adapted from Abbott et al, Nat Review Neuroscience 2006.*

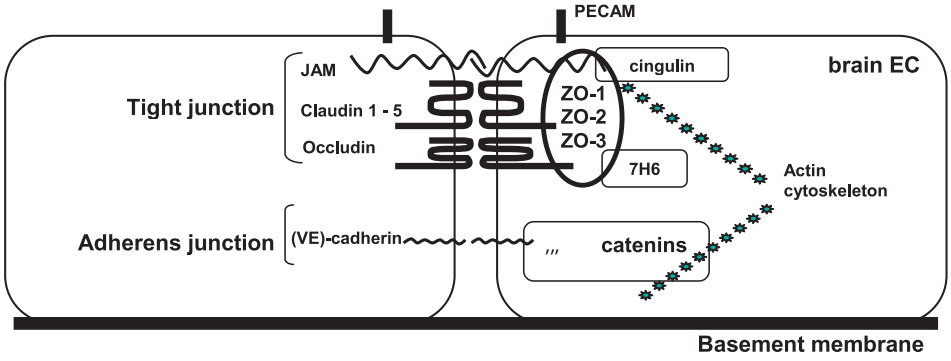

Figure 2. Tight and adherens junctions between brain EC

Schematic representation of endothelial tight junction- and adherens junction-associated proteins and their linkage to the cytoskeleton. Abbreviations: EC: endothelial cell; JAM: junctional adhesion molecule; ZO: zonula occludens; PECAM: platelet endothelial cell adhesion molecule. *Kooij et al, book chapter 2005*

low pinocytotic vesicular activity and the presence of high densities of mitochondria in the cytosol. To maintain brain homeostasis and provide the brain with essential nutrients, specific transporters and carrier molecules strictly regulate the uptake of nutrients and metabolites into the CNS. Furthermore, potential harmful compounds like drugs and toxins are excluded from the CNS by a large family of efflux pumps, which contribute to the multi-drug resistant (MDR) phenotype of the CNS [34]. Due to their specific features like TJs and efflux pumps, brain endothelial cells are crucial gatekeepers of the CNS and understanding the regulation of these structures and molecules will open avenues for the treatment of brain disorders complicated by BBB dysfunction.

2.2 Tight junctions

The main structures responsible for endothelial sealing are TJs and AJs [35, 36]. These intercellular structures are located between adjacent brain endothelial cells, consisting of transmembrane and cytoplasmic proteins that are associated with the actin cytoskeleton (figure 2). The transmembrane proteins occludin and various claudins mediate cellular interaction between brain ECs and play a major role in TJ functioning. Occludin is a phosphoprotein that spans the plasma membrane four times with intracellular location of both the amino and the carboxy termini [37, 38] and is associated with increased electrical resistance [39]. Claudins comprise a multigene family consisting of more than 20 members and contain two extracellular loops and four transmembrane domains and interact in both a homophilic and heterophilic way with claudins of adjacent cells [40]. Claudin-5 is a critical component of the BBB as it closes the BBB for small molecules up to 800 Da [41].

The carboxyterminal parts of both occludin and claudins interact with membrane-associated recruiting proteins of the zona occludens (ZO) protein family [42, 43]. ZO proteins are reported to link transmembrane proteins to the actin cytoskeleton and have signaling potential [44]. Through its interaction with TJ molecules, the actin cytoskeleton plays an active role in maintaining TJ integrity and BBB function [45]. AJ are composed of cadherins, catenins, vinculin, and actinin [46]. Although both AJ and TJ act to restrict endothelial permeability, TJ are primarily responsible for the low transendothelial permeability and high transendothelial electrical resistance (TEER) due to the limitation of ion transfer [47]. Several cytoplasmic signaling molecules, such as Rho, PI3 kinase, protein kinase C (PKC), Ca2+, heterotrimeric G proteins, cyclic adenosine monophosphate (cAMP) and phospholipase C have been localized to TJ and AJ complexes and may regulate their assembly and disassembly (for review see book chapter Kooij et al, 2005). These studies may provide novel therapeutic opportunities to prevent TJ disassembly and subsequent BBB dysfunction.

2.3 BBB transporters

To maintain brain homeostasis, 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. The brain endothelial transporters that supply the brain with nutrients include glucose transporter 1 (GLUT-1), several amino acid carriers and transporters for nucleosides, nucleobases and many other substances [34]. In contrast, potential harmful compounds are excluded from the CNS by the large family of ATP-binding cassette (ABC) efflux transporters, enabling multi-drug resistance (MDR) of the brain to xenobiotics and toxic compounds. ABC transporters consist of a variety of drug efflux pumps, including P-glycoprotein (P-gp), breast cancer resistant protein (BCRP) and multidrug resistance-associated proteins (MRPs). These efflux pumps are expressed at the luminal and abluminal membrane of brain capillary endothelial cells (figure 3) and drive cellular exclusion of a variety of exogenous compounds and drugs through the endothelial membrane against a concentration gradient at the cost of ATP hydrolysis [48].

The best-known and most widely studied representative of the ABC transporter family is P-gp (MDR1; ABCB1), a phosphorylated glycoprotein that was first identified in tumor cells, where overexpression conferred multidrug resistance [49]. Moreover, it was the first drug efflux transporter detected on BBB endothelial cells [50] where it locates at the luminal membrane. P-gp actively effluxes a wide variety of substrates and drugs, although the physiological substrates of P-gp have not been identified yet. Interestingly, P-gp is also expressed on a variety of immune cells like monocytes, DCs, T and B cells [51] and is thought to be involved in the efflux of inflammatory molecules such as steroids, prostaglandins and cytokines [51-56], thereby suggesting a potential role for this efflux pump during immune responses. At the transcriptional level these ABC transporters are under control of the orphan nuclear receptors such as steroid and xenobiotic receptor (SXR in human; or pregnane X receptor (PXR) in rodents. Expression of these ABC transporters is under the influence of a number of environmental factors like neurotransmitters, endothelial factors and inflammatory cytokines.

2.4 BBB impairment in MS and immune cell infiltration

In MS pathology, numerous changes in BBB structure and functioning have been described. These observations, derived from in vitro, in vivo and patient tissue studies, show that disruption of BBB integrity and function plays a pivotal role in MS pathology. The combined outcome of these studies has led to the notion that BBB disruption represents an early event in MS lesion formation [16]. Pathological events that may occur at the BBB include structural and spatial alterations of the TJs, enhanced permeability for blood derived components, and infiltration of inflammatory cells into the CNS (figure 3). In these processes, pro-inflammatory mediators like chemokines [57], cytokines [58], matrix metalloproteinases

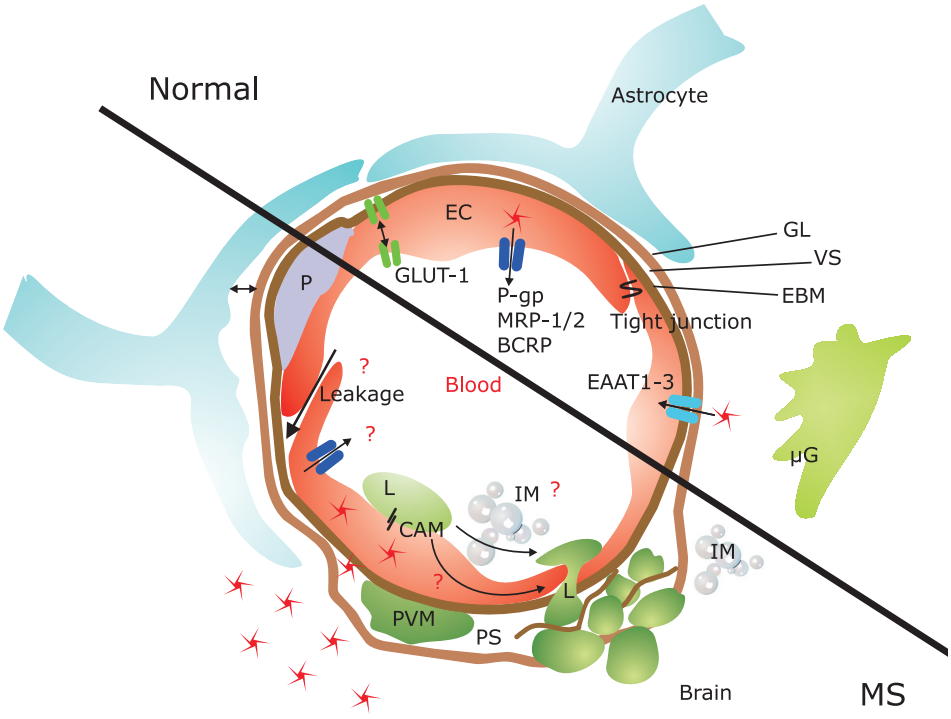

Figure 3 The BBB during health and disease.

Under healthy conditions, brain endothelial cells (EC) are interconnected by tight junction structures and have several transport systems and efflux pumps on their luminal or abluminal membranes (e.g. GLUT-1. P-gp, MRP-1, MRP-2 and BCRP). ECs are surrounded by a basement membrane (EBM), pericytes (P), perivascular macrophages (PVM) and astrocytes, which project their endfeet to brain EC, thereby forming a glia limitans (GL). During MS pathology several processes occur including BBB leakage, leukocyte (L) adhesion via cell adhesion molecules (CAM) and diapedesis, accumulation of these cells in the perivascular space (PS) and finally transmigration into the brain parenchyma. Inflammatory mediators (IM) are involved in several events but it is not known which mediators affect TJ integrity, endothelial signaling or BBB leakage. Moreover, cytotoxic molecules accumulate in the brain, which may be due to a decreased efflux capacity of the BBB.

(MMPs; [59, 60]) and reactive oxygen species (ROS) play an important role [61, 62]. The presence of various immune cells like monocyte-derived macrophages and T cells in the CNS is an important hallmark of MS pathology. It is unknown which factor initiates the infiltration of immune cells into the CNS, but the current hypothesis is that myelin specific CD4+ T cells are primed in the peripheral lymphoid organs, which encounter their target antigens during immune surveillance of the CNS, thereby triggering an immune response [8]. The general principles governing leukocyte extravasation have been thoroughly documented. It occurs according to the multistep model, which consists of rolling, tethering, firm adhesion and finally transmigration of immune cells across brain EC [63]. During the transmigration process, various molecules and proteins are involved, including selectins, chemokines, integrins and adhesion molecules [63]. Upon interaction with leukocytes, various signaling pathways are triggered in brain ECs that lead to rearrangement of the cytoskeleton and TJs, thus facilitating transendothelial migration. The active interplay between leukocytes and ECs therefore plays a crucial role during the transmigration process of leukocytes and subsequently BBB disruption during MS pathology.

Considering the importance of BBB disruption and cellular infiltration into the CNS, therapeutic strategies designed to close the barrier and prevent cellular infiltration into the brain parenchyma are interesting candidates for future treatment of MS.

3. Outline of thesis

The blood-brain barrier (BBB) controls the entry of circulating molecules and cells into the brain and plays an important role in maintaining brain homeostasis. BBB dysfunction is an important feature of MS pathology, which results in the infiltration of immune cells into the CNS, where they cause extensive damage to myelin and axons. This thesis focuses on the barrier properties of the BBB in heath and disease, as determined by its physical and efflux capacity properties.

The first part of this thesis concerns the regulation of the TJs and immune cell infiltration during MS pathology. We examined in detail tight junction dynamics during diapedesis of monocytes across brain endothelial cells, as monocyte-derived macrophages are crucial in the process of myelin degradation and subsequent tissue damage during MS. Previous studies from our group have shown that reactive oxygen species (ROS) are involved in the process of monocyte migration [61, 62], although the exact effect of ROS on BBB integrity and the mechanisms underlying ROS-mediated BBB breakdown are largely unknown. Therefore we assessed the influence of ROS on the activation of various signal transduction pathways in brain ECs in **chapter 2**. In addition, we studied pathways involved in ROS-induced

cytoskeleton and TJ rearrangements and we identified the signaling molecule protein kinase B (PKB) as a novel player in endothelial cytoskeleton and TJ dynamics. To enter the brain and exert their damage, monocytes have to pass endothelial cells that are interconnected by TJ structures. In **chapter 3**, we studied the migration process of monocytes in detail, indicating a paracellular migration route through GFP-labeled tight junction structures, and in this process we identified matrix metalloproteinases (MMPs) as crucial mediators.

In the second part of my thesis we studied the efflux properties of the BBB under neuroinflammatory conditions. In **chapter 4**, we show that P-gp expression and function was strikingly decreased in MS and EAE pathology. Loss of endothelial P-gp function was mediated by infiltrating T cells via ICAM-1 and downstream NF-κB signaling. Other ABC transporter expression patterns in MS patient material are displayed in **chapter 5**, indicating a differential expression pattern of these efflux pumps on brain endothelial cells, astrocytes and infiltrated foamy macrophages. We assessed the potential immunomodulatory role of P-gp in vivo during EAE in **chapter 6**, and showed that P-gp modulates the immune response by controlling dendritic cell (DC)-induced T cell responses and DC maturation via pro-inflammatory cytokines, thereby highlighting a novel immunomodulatory role of P-gp. Finally, in **chapter 7**, the findings described in this thesis are summarized and discussed in the context of recent developments in MS research.

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