Chapter 1

General introduction

Adapted from

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Chapter 1

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1. Multiple sclerosis (MS)

1.1. Clinical feature and diagnosis

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) with an autoimmune component in which reactive immune cells recognize and attack myelin antigens. It has been estimated that approximately 2.5 million people suffer from MS worldwide with a typical age of onset between 20 and 50 years and a female:male ratio of 3:1\(^1\). Because the neurological symptoms are highly dependent on the location of the lesions within the CNS, MS is seen as a heterogeneous disease. Based on the course of the disease, MS can be divided into three main clinical forms: relapsing-remitting MS (RR-MS), secondary progressive MS (SP-MS) and primary progressive MS (PP-MS)\(^3\). Furthermore, clinically isolated syndrome (CIS) was not included in the initial MS clinical descriptors but it is now recognized as the first clinical episode of neurologic symptoms caused by inflammation and demyelination in the CNS. Nevertheless, a CIS episode does not meet the criteria for a diagnosis of MS\(^3,4\). RR-MS is the predominant form of MS as it affects approximately 85% of MS patients. It is characterized by acute attacks (relapses) that last for days to weeks, followed by a period of partial or full recovery (remission) of the clinical symptoms. A great majority of RR-MS patients will in time develop SP-MS, which is characterized by initial relapses followed by a more progressive phase with gradual deterioration of neurological function. PP-MS patients experience a steady progression of the disease from disease onset, with no clear relapses or remissions.

A method commonly used by clinicians to quantify disability in MS patients and to monitor changes in the level of disability over time is the Expanded Disability Status Scale (EDSS). Briefly, EDSS scale ranges from 0 to 10 based on an examination by a neurologist\(^5\). Due to the heterogeneity of the demyelinated lesions, there is no single test or specific clinical diagnostic tool for MS. MS is often clinically diagnosed by neurological and cognitive evaluation, clinical history of the patients combined with magnetic resonance imaging (MRI) and/or on the presence of oligoclonal bands within the cerebral spinal fluid (CSF)\(^6\).

1.2. Etiology

Despite considerable research efforts, the underlying causal factors for MS still remain unknown. Studies have shown that MS arises from a combination of genetic and environmental causes, and have identified a number of risk genes predisposing to MS\(^7\). Indeed, family members of MS patients have an increased risk of developing the disease compared to non-related individuals\(^8\). Moreover, monozygotic twins have a higher risk of developing MS compared to dizygotic twins\(^9\). Although there has been a large number of genome wide association studies (GWAS), only a few genes that influence disease susceptibility have been consistently identified, with the alleles of the major histocompatibility complex class II (MHC-II) showing the strongest link to MS\(^10\). However, the impact of these factors is modest. Besides a potential genetic background or intrinsic causes, environmental factors
may also contribute to the development of MS\textsuperscript{11}. Interestingly, the geographic distribution of the incidence of MS varies, with a sparse distribution around the equator that increases with increasing latitude, suggesting that MS is a disease of more temperate climates. Accordingly, high sun exposure and vitamin D levels were shown to correlate with a lower susceptibility for MS\textsuperscript{12,13}. In addition, bacterial and viral infections have also been shown to be associated with MS. Particularly, infection with Epstein-Barr virus appears to increase the risk of subsequently developing MS\textsuperscript{14-16}. Human endogenous retroviruses (HERV) form another group of viruses that has been linked to MS pathogenesis. Indeed, HERV-derived viral particles have been detected in both MS brains and CSF and correlate with clinical progression and prognosis\textsuperscript{17}. Furthermore, lifestyle factors such as smoking and diet are also thought to increase the risk of developing MS\textsuperscript{18-20}.

Finally, the majority of the studies conducted over the past decades has focused on the discovery of the immunological and genetic causes of the disease, supporting the ‘outside-in’ model of MS, according to which a systemic abnormality of the immune system targets the CNS\textsuperscript{21}. However, questions have been raised to what extent inflammation and/or autoimmunity are the major triggers of the disease. These questions have led to the development of an alternative hypothesis referred to as the ‘inside-out model’. According to this model, and opposite to the ‘outside-in’ model, the initial event in MS pathophysiology might be a primary cytodegeneration (possibly affecting oligodendrocytes and myelin) which promotes a secondary autoimmune and inflammatory response\textsuperscript{21}. Nonetheless, the initial trigger(s) of MS still need to be defined and which of the two proposed models is the most likely one is still being discussed.

### 1.3. Pathology

The neuropathological process of MS includes multifocal and chronic neuro-inflammation, breakdown of the BBB immune cell infiltration, demyelination, oligodendrocyte loss, reactive gliosis and axonal degeneration\textsuperscript{22}. In MS, lesion formation is a local phenomenon that occurs predominantly in the white matter (WM) of the CNS, mostly in the spinal cord (SC), brain stem, optic nerve and periventricular areas\textsuperscript{23}. Although MS has historically been considered a WM demyelinating disease, the WM is not the only area affected as demyelination of the cortical and deep grey matter (GM) has also been detected\textsuperscript{24}. Interestingly, GM lesion pathology differs from that of WM lesions, since GM lesions are characterized by demyelination and neurodegeneration, but infiltrating leukocytes are scarcely detected. Also, BBB failure in GM lesions is less prominent, as compared to WM lesions, although subtle but persistent BBB malfunctioning has been demonstrated\textsuperscript{25}. WM lesions can be classified as pre-active, active (demyelinated or non-demyelinating), chronic active, and chronic inactive lesions\textsuperscript{26}. Pre-active lesions do not show profound demyelination, but are characterized by modest WM abnormalities including clusters of activated microglial cells and few perivascular leukocytes.
In contrast, active demyelinating lesions are characterized by loss of myelin and presence of abundant monocyte-derived foamy-macrophages, containing myelin degradation products. A chronic active MS lesion is a demyelinated lesion characterized by a hypocellular center and hypercellular rim of hypertrophic astrocytes, microglia, and macrophages. In addition, parenchymal and perivascular macrophages and lymphocytes as well as reactive astrocytes are observed in chronic active lesions. Finally, chronic inactive lesions are demyelinated and hypocellular with moderate expression of MHC-II and few lipid-phagocytosing macrophages present.

1.4. Treatments

The armamentarium for the treatment of MS is rapidly increasing and nowadays over 10 different disease-modifying therapies (DMTs) are approved by the Food and Drug Administration (FDA) and available in the clinic (Table 1). Because of the autoimmune component of MS, the majority of currently developed DMTs target the immune system and aim to reduce relapse frequency and accumulated disability in RR-MS. This approach is moderately effective in RR-MS, but has relatively little benefit for patients suffering from progressive forms of MS and may lead to serious side effects. Therefore, there is a high and unmet need for the discovery of more specific drugs aimed at modulating disease progression. The ideal DMT to effectively treat progressive MS will most likely depend on a combination of therapies that aims to resolve inflammation, target CNS innate immune processes and fight neurodegeneration. Furthermore, as the BBB plays a prominent role

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<th>Generic name</th>
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<th>Mechanism of action</th>
<th>Efficacy</th>
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<td>(IFN)-β</td>
<td>Betaseron</td>
<td>Inhibits T cell division, MMP</td>
<td>RR-MS</td>
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<td>Extavia</td>
<td>Inhibits migration across the BBB</td>
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<td>Avonex</td>
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<td>Rebif</td>
<td>Decreases Th17 cells</td>
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<td>Glatramer acetate</td>
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<td>Blocks MHC-II, Increase IL-10 and IL-4</td>
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<td>Glatopa</td>
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<td>Mitoxantrone</td>
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<td>Reduces Th1 cytokine, inhibits monocyte migration</td>
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<td>Natalizumab</td>
<td>Tysabri</td>
<td>Monoclonal antibody against VLA-4 (α4β1 integrin)</td>
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<td>Blocks T and B cell migration</td>
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<td>Fingolimod</td>
<td>Gilenya</td>
<td>Sphingosine–1–phosphate (S1P) receptor agonist</td>
<td>RR-MS</td>
<td>Oral</td>
<td>Bradycardia, infection, PML</td>
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<td>Sequesters lymphocytes in lymph nodes</td>
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<td>Inhibits migration of DC to secondary lymphoid organs</td>
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<td>Teriflunomide</td>
<td>Aubago</td>
<td>Inhibits pyrimidine synthesis</td>
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<td>Tecfidera</td>
<td>Activates the Nrf2 pathway</td>
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<td>Anti-inflammatory and anti-oxidative stress</td>
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<td>Dimethyl fumarate</td>
<td>Lemtrada</td>
<td>Depletes T and B cells</td>
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<td>Ocrelizumab</td>
<td>Ocrevus</td>
<td>Depletes CD20+ B cells</td>
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<td>PP-MS</td>
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<td>Cladribine</td>
<td>Leustatin</td>
<td>Induce lymphocyte apoptosis</td>
<td>RR-MS</td>
<td>Oral</td>
<td>Infection, lymphopenia</td>
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<td>Reduction in CD4 and CD8 T</td>
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in MS pathophysiology, preventing BBB failure or accelerating its recovery by restoring inflammation-induced molecular changes may well provide a novel way of modulating disease progression\(^3\). Finally, the development of novel biomarkers will be crucial in order to improve disease diagnosis, predict disease progression and improve clinical outcomes.

1.5. Animal model for MS

Experimental autoimmune encephalomyelitis (EAE) was first described in non-human primates that were immunized with a CNS homogenate in 1933\(^3\). Since then a number of different EAE models have been developed and contributed in unraveling the molecular mechanisms involved in acute and chronic inflammation, lymphocyte trafficking and the role of BBB during CNS inflammation\(^3\). Neuropathological features of EAE include mononuclear cell infiltration into the CNS, BBB dysfunction and, depending on the type of EAE model used, demyelination\(^4\). EAE can be induced in a multitude of species and its pathology varies depending on the species and strains as well as on the immunizing antigen used\(^5\). There are two commonly used methods to induce either the ‘active’ or the ‘passive’ form of EAE. The active form of EAE is induced by immunization of animals with myelin antigens, such as myelin basic protein (MBP) or proteolipid protein (PLP) in complete or incomplete Freund’s adjuvant (CFA or IFA, respectively)\(^6\). Instead, the passive EAE, also known as adoptive-transfer EAE, can be induced in naïve/recipient animals by transferring pathogenic CD4\(^+\) T cells generated in donor EAE animals by active immunization\(^7\). Upon immunization, dendritic cells (DCs) mature in the lymph nodes where they present myelin-derived peptides to naïve T cells\(^8\). During this process, upregulation of co-stimulatory molecules as CD80, CD86 and CD40, which interact with CD28 and CD40 ligands, as well as secretion of pro-inflammatory cytokines mediate the activation and differentiation of T cells. In mice, differentiation of CD4\(^+\) T helper cells into pro-inflammatory interferon (IFN)-\(\gamma\) or IL17-producing Th1 and Th17 cells, respectively, has been shown essential for EAE induction. Depending on the immunization protocol, EAE can be either acute, characterized by a monophasic disease course followed by partial recovery, or chronic with a more relapsing-remitting course\(^9\). The disease onset typically occurs around day 9-14 post-immunization, reaching the peak of disease 3 to 5 days after onset. The severity of EAE is evaluated by measuring the motor dysfunction and the severity of paralysis. Specifically, score 0.5 is associated with limp tail, score 1 with hind leg weakness, score 2 with hind leg paraparesis, and score 3 with hind leg paraparesis and incontinence\(^10\). Importantly, during EAE, like in MS, the WM demyelinating lesions are associated with the presence of T cells, monocyte-derived macrophages, myelin loaded foamy macrophages, and B cells\(^11,12\). Furthermore, several molecular mechanisms leading to MS pathogenesis are also reflected in the EAE model. Therefore, from this perspective, EAE represents a solid model to study and elucidate disease-associated mechanisms in order to test and develop novel drugs. Indeed, research employing EAE as model for MS has directly contribute to the development of several approved therapies for MS,
including glatiramer acetate (Copaxone), Azathioprine, Mitoxantrone and Natalizumab. Nonetheless, like all animal models, particularly when applied for translation to the clinic, EAE also has its limitations and to which extent it resembles the full pathology as seen in MS is still widely debated. EAE is very heterogeneous in terms of induction methods, immunological and pathological features, and despite a chronic rat and mouse EAE model, most of the EAE models fail to mimic the progressive phase of MS, mostly characterized by axonal and neuronal loss. Interestingly, the usage of different species more closely related to humans, such as the common marmoset, have helped in closing the gap between EAE and MS. Nevertheless, similar to the rodent mouse model, the non-human primates are not exempt from disadvantages. Indeed, the high costs and ethical considerations make them difficult species for experimental animal studies, especially for the routine screening of large numbers of compounds.

2. The blood-brain barrier (BBB) and the neurovascular unit (NVU)
   2.1. Structure and function of the BBB
A major hallmark of MS pathology is represented by the inflammation and impairment of the BBB, leading to disruption of global brain homeostasis and promoting immune cell infiltration into the CNS. The BBB is a selective barrier composed by specialized brain endothelial cells (BECs) tightly connected through specific proteins present in the tight junctions (TJs) and adherens junctions (AJs) (Figure 1). This close interconnection between the BECs provides a ‘physical barrier’ to solutes from the blood to the brain. The BBB has a major role in maintaining brain homeostasis: it supplies nutrients and excludes waste.

Figure 1. Simplified scheme showing AJs and TJs of the BBB. Adapted from Derada Troletti C. et al., Biochim Biophys Acta, 2016.
products from the CNS, it limits both transcellular and paracellular passage of cells and molecules from the systemic circulation into the CNS and vice versa, thereby controlling the critical microenvironment necessary for proper neuronal function. Transcellular diffusion of hydrophilic molecules is limited due to a low rate of transcytotic vesicles, an extremely low pinocytotic activity and expression of active efflux membrane pumps of the ATP-binding cassette family such as P-glycoprotein (Pgp), which drive cellular exclusion of more lipophilic compounds. Moreover, transcytosis in CNS endothelial cells (ECs) is regulated by the major facilitator super family domain containing 2a (Mfsd2a). Indeed, genetic ablation of Mfsd2a results in a leaky BBB from embryonic stages through to adulthood.

In order to regulate the crucial influx of components needed within the CNS, BECs possess specific transporters that actively transport nutrients into the CNS, for instance glucose transporters (GLUT-1-3). Paracellular diffusion of hydrophilic molecules and immune cells trafficking is restricted by a complex network of TJ proteins which seal the inter-endothelial space.

The junction complexes that provide the characteristic phenotype of the BBB are dynamic structures that respond to the local microenvironment of the brain endothelium. TJ complexes in itself can activate intracellular signaling pathways directly by engaging signaling proteins or growth factor receptors, or indirectly by capturing transcription factors (TFs) at the plasma membrane. Moreover, the transmembrane junctional proteins are connected to the cytoskeleton through interaction with intracellular adaptor proteins.

Important proteins regulating TJ complex formation include occludin, which was the first of the TJ proteins to be discovered, claudins (in particular claudin-1, -3, -5 and -12) and junctional adhesion molecules (JAM-A, JAM-B and JAM-C). The most important adaptors include proteins from the zonula occludens family (ZO-1, ZO-2 and ZO-3), which are scaffolding proteins that bind several effector proteins. Vascular endothelial cadherin (VE-cadherin) plays a major role in cell-cell contacts and function of the AJ by binding to β-catenin and γ-catenin which anchor the complex to actin cytoskeleton. In addition to VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1) mediates homophilic adhesion.

### 2.2. The NVU

It is currently accepted that the functionality of the BBB is only possible through close contact between BECs and other cell types like astrocytes, pericytes, and neighboring CNS cells, such as microglia and neurons, creating a dynamic structure named the neurovascular unit (NVU). In particular, astrocytes enclose the BECs with their astrocytic (end-feet) processes and closely regulate ion flow, blood volume and cerebral blood flow. Moreover, astrocytes modulate synaptic transmission, thereby contributing to neuronal communication, firing thresholds and plasticity.

Due to the high metabolic needs of nervous tissue and the dynamic pattern of neural activity, the microcirculation of the brain must be highly responsive to the tissue it
supplies. Indeed, “metabolic coupling” of brain activity and CNS blood flow is crucial for normal neuronal functioning. Because disruption of the BBB is often associated with pathological changes in cerebral blood flow, it was suggested that BBB permeability changes were due to an active involvement of neurons in BBB integrity. Evidence has been found for direct innervation of the microvasculature and/or associated astrocytic processes by noradrenergic, serotonergic, cholinergic, and GABA-ergic neurons. Whether neurons are critical in the development of the BBB phenotype is not fully understood. However, it is apparent that they can regulate several critical aspects of BBB function, from CNS blood flow to BBB permeability.

Pericytes are specialized, contractile cells located at the abluminal surface of capillary blood vessels and have recently emerged as a major contributor to the development and maintenance of the BBB. Particularly, pericytes play a functional role in blood vessel stabilization, blood flow regulation, and in the formation of the blood-brain/retina barrier. Their morphology differs with their position along the vascular bed, reflecting the existence of subpopulations with diverse functions. Reduction in the number of CNS pericytes has been linked to neurovascular disruption in both Alzheimer’s disease (AD) and amyotrophic lateral sclerosis. Nevertheless, the molecular mechanisms by which pericytes mediate vascular stability and their role in chronic neurodegenerative disorders still remain elusive.

Perivascular macrophages are also part of the NVU and represent a distinct population of resident brain macrophages characterized by a close association with the cerebral vasculature. It has been shown that perivascular macrophages are able to alter the function of the BBB by decreasing its paracellular permeability in vitro.

The last components of the NVU that should be mentioned here are the microglia cells, a type of innate-immune mononuclear phagocytes that are located in the parenchyma of the CNS. Microglia form one of the first defense mechanisms against pathogens and harmful compounds that cross the BBB. Additionally, several studies also imply that microglia, once activated, can directly alter BBB function through secretion of matrix metalloproteinases (MMPs), enzymes that may degrade the extracellular matrix (ECM) and thereby increase BBB permeability.

In addition to the NVU, the ECM also interacts with the cerebral microvascular endothelium. The ECM is a dynamic, physiologically active component of all living tissues and it is made up of water, proteoglycans, minerals and fibrous proteins. The main function of the ECM is to provide structural and biochemical support to the BBB. Furthermore, the ECM may also contribute to functionality of the BBB since the expression of matrix proteins can influence the expression of endothelial TJs. Since the disruption of the ECM is strongly associated with increased BBB permeability in pathological states, an intact structure of the ECM is required for the correct functioning of the BBB. Overall, the permeability of the BBB is dynamically regulated by the concerted action of the different cell types and proteins to maintain its proper functioning and thus maintain optimal neuronal function.
2.3. Dysfunction of the BBB during MS

Importantly, integrity and proper function of the BBB is of pivotal importance in order to maintain brain homeostasis and functionality. BBB dysfunction has often been reported to be a major hallmark of MS pathogenesis as it represents an early event during the course of disease. It is known that activated leukocytes and CNS-resident cells secrete pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IFN-γ which promote an activated inflammatory phenotype of BECs. Inflamed BECs show increased expression of cell adhesion molecules (CAM), like intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and PECAM-1, which are essential for leukocytes migration into the CNS during MS lesion formation. Finally, inflamed BECs can directly enhance both leukocyte adhesion and migration by producing several chemokines like the chemokine (C-C motif) ligands 2 and 5 (CCL2 and CCL5, respectively), and the chemokine (C-X-C motif) ligands 8 and 10 (CXCL8 and CXCL10, respectively). As a consequence, numerous blood-derived lymphocytes and monocytes can enter the brain by crossing the damaged BBB. Moreover, an inflammatory environment also alters the structural architecture of the junctions of the BBB, thereby increasing BBB permeability. Due to inflammation, the BBB loses its phenotypic characteristics and protective function both during the relapsing-remitting and the progressive phases of the disease. Particularly, altered localization of ZO-1 expression in blood vessels was found in active MS lesions in post-mortem material of patients, and, to a lesser extent, in NAWM. BBB leakage and irregularities in the TJs persist also in inactive lesions, suggesting that BBB dysfunction occurs even upon low grades of inflammation. Furthermore, cell-specific ectopic expression of claudin-1 in ECs reduces BBB permeability and ameliorates the chronic phase of EAE. Next, using a murine model of MS, which makes use of a virus to cause inflammation in the CNS, it was found that CD8+ T cells may activate astrocytes which in turn leads to a decreased level of claudin-5 and occludin in ECs, thereby aggravating disease. Finally, CNS inflammation also affects the expression and function of several efflux pumps of the BBB, such as Pgp, illustrating that multiple aspects of proper BBB function are impaired.

2.4. Immune cells trafficking into the CNS

In active MS lesions, large numbers of leukocytes migrate across the inflammatory-activated BBB, mainly at the level of post capillary venules. Leukocyte migration through the BBB is a complex multi-step process which is tightly regulated by the interplay of various CAM, integrins, cytokines and chemokines (Figure 2). Lymphocyte transendothelial migration consists of a sequential set of events involving adhesion and rolling, activation of chemokine receptors on the immune cells through contact with chemokines, arrest and, finally, crawling of the lymphocyte to the endothelial surface to determine a permissive site for diapedesis. At first, to overcome the shear forces induced by blood flow,
lymphocytes get into contact with the ECs via the interaction between the lymphocytes surface selectin PSGL-1 with E- and P-selectins, expressed by the inflamed ECs. Secondly, the binding between the G-protein-coupled receptors (GPCRs), expressed on the lymphocyte surface, with the chemokines presented on the endothelial luminal surface, allows firm adhesion to occur. The next step is the arrest of the lymphocytes, which is mediated by the interaction between the α4β1 (VLA-4) and αLβ2 (LFA-1) integrins and the endothelial adhesion molecules ICAM-1 and VCAM-1. Once a lymphocyte has been arrested, it may polarize and begin to crawl. Binding to ICAM-1 triggers an intracellular signaling cascade in the endothelium and docking structure formation, preparing the cell for the last step of the process, the actual diapedesis99,100. Diapedesis may occur via two pathways; paracellular, in which the leukocyte passes between adjacent ECs or transcellular, in which the leukocyte travels through the ECs body, leaving the junctions intact98. By in vitro studies, it was shown that leukocyte diapedesis negatively influences BBB integrity, thereby aggravating BBB failure101. This observation is supported by studies using Natalizumab or Alemtuzumab which demonstrated that blocking T cell infiltration into the brain not only reduces the inflammation but also reduces the number of gadolinium-enhanced lesions dramatically102.

3. The Notch signaling pathway

3.1. Notch signaling in vascular development

An important pathway involved in cell-cell communication, particularly during vascular development, is the highly conserved Notch signaling pathway103. Notch signaling is initiated by binding of Notch receptors (in mammals Notch 1-4) on a signal-receiving cell to a Notch
ligand of the Delta-like family (DLL1, -3 and -4) or the Jagged family (JAG1 and JAG2) on a signal-sending cell. Upon ligand binding, the Notch receptor protein is cleaved and the resulting Notch-intracellular domain (NICD) is released into the cytoplasm and translocates to the nucleus where it induces transcription of downstream genes, like HES-1 and HES-5. The Notch receptor family is known to regulate differentiation of various cell types and tissues during embryogenesis, but also postnatally. During embryogenesis, Notch signaling is involved in vascular development and angiogenesis. Indeed, impairment in the Notch signaling pathway results in cardiovascular abnormalities and severe vascular defects in mice, leading to lethality at early stages of development. Postnatally, Notch signaling regulates the formation of tip cells, thereby controlling vessel sprouting and branching. Moreover, the Notch signaling pathway also regulates the maturation of oligodendrocyte precursor cells into mature oligodendrocytes, as well as myelination of axons and activation of microglia upon inflammation. Although the importance of Notch signaling in vascular formation is well established, it is unknown whether the Notch pathway is involved in the specialized barrier function of BECs and whether this is affected by inflammatory processes.

3.2. Regulation of Notch signaling by glycosylation
Post-translational modification, such as glycosylation, of Notch receptors is an important regulatory mechanism serving to fine-tune the receptor signaling properties. Interestingly, defects in the glycosylation machinery in mice results in a lethal phenotype in mid-gestation with severe defects in somitogenesis, vasculogenesis, cardiogenesis and neurogenesis, recapitulating some of the features observed in Notch knockout mice. Glycosylation of the receptor’s extracellular EGF repeats occurs in the endoplasmic reticulum and Golgi apparatus where the glycosylation machinery adds glycans in a progressive manner. Particularly, different types of glycans exist, including asparagine-linked N-glycans and several serine- or threonine-linked O-glycans. In the endoplasmic reticulum the enzyme Protein O-fucosyltransferase 1 (POFUT1) adds an O-fucose to the EGF domain of the receptors. In the Golgi apparatus, this O-fucose moiety is extended with GlcNAc by Fringe glycosyltransferases.

In mammals, three different Fringes exist: Radical Fringe (RFNG), Manic Fringe (MFNG) and Lunatic Fringe (LFNG). It has been shown that Notch glycosylation by LFNG inhibits the binding and signaling mediated via JAG1 while potentiating the binding and signaling via DLL1. Nevertheless, the function of glycosylation in the adult vasculature is not clear and whether this regulatory mechanism also plays a role during BBB dysfunction has not been determined.

4. Endothelial cells plasticity
4.1. Endothelial to mesenchymal transition (EndoMT)
ECs have the capability to de-differentiate and to acquire mesenchymal and stem cell-like
characteristics in a process called endothelial to mesenchymal transition (EndoMT). EndoMT is a phenomenon similar to the better understood epithelial to mesenchymal transition (EMT) and like EMT, was first thought to be a purely developmental process, with the majority of the studies focusing on heart development, where ECs de-differentiate to form both the valves and septa of the adult heart. However, more recent work indicated that EndoMT may also occur in adult tissues during several pathological disorders including brain diseases like cerebral cavernous malformation, bacterial meningitis and brain tumors. On a molecular level, EndoMT is characterized by the degradation of endothelial vascular basement membrane, cell-to-cell junction rearrangements and reduced expression of endothelial markers, such as the AJs and TJs proteins. In contrast, ECs undergoing EndoMT acquire mesenchymal and stem cells-like properties, including gain of migratory capacity, increased expression of fibroblast and mesenchymal-specific markers, such as fibroblast specific protein 1 (FSP1), fibronectin and N-cadherin. These phenotypic and functional changes require the interplay between different signaling pathways, with inflammatory and transforming growth factor (TGF)-β signaling playing a dominant role in activation of EndoMT key TFs, such as SNAIL, ZEB1, ZEB2 and TWIST1 (Figure 3).

Interestingly, EndoMT has been shown to be responsible for EC de-differentiation in different vascular beds, thus attracting attention of the filed brain pathology research. Nevertheless, the potential contribution of EndoMT to MS pathophysiology has not been investigated yet.

4.2. Transforming growth factor beta (TGF-β)
The TGF-β family, which includes the three different isoforms TGF-β1, β2, and β3, is a family of growth factors and cytokines produced by different cell types and controls several

Figure 3. Simplified scheme of the most important pathways and players in EndoMT. Adapted from Derada Troletti C. et al., Biochim Biophys Acta, 2016.
processes such as cell proliferation and differentiation. Interestingly, TGF-β is one of the major signaling molecules that can induce EndoMT\textsuperscript{140,141}, since it is able to induce the expression of SNAI1 and SNAI2 family members\textsuperscript{142-144}. Since MS is considered to be an autoimmune disorder, it is important to highlight the role of TGF-β in the immune system. The major function of TGF-β in this context is to maintain immune tolerance since it can regulate lymphocyte proliferation, differentiation and survival\textsuperscript{145}. However, TGF-β also plays an important role during inflammatory conditions. In the presence of IL-6, TGF-β drives the differentiation of T helper 17 cells, a T cell subpopulation involved in MS and EAE pathogenesis\textsuperscript{146,147}. Moreover, local expression of TGF-β1 in the CNS parenchyma can enhance immune cell infiltration and amplify CNS impairment during EAE\textsuperscript{148}. Importantly, TGF-β can also be secreted by different cells of the NVU. Particularly, pericytes and astrocytes are known to release TGF-β which contributes to BBB development\textsuperscript{51,62,109}. Changes in the correct communication between BECs and cells from the NVU or deficiency in the TGF-β signaling results in an abnormal distribution of junctional proteins of BECs and may lead to increased vascular permeability\textsuperscript{109,149-151}. Furthermore, the cellular localization and distribution of three different isoforms of TGF-β were analyzed on frozen MS brain tissue sections with different stages of lesion activity\textsuperscript{152}. In active and chronic active demyelinated lesions, perivascular foamy macrophages as well as hypertrophic reactive astrocytes show abundant TGF-β immunoreactivity compared to the normal control brain tissue sections where TGF-β immunoreactivity was only present on resting microglia cells\textsuperscript{152}. Finally, TGF-β1 stimulation of both bovine retinal ECs and human BECs increase BBB permeability via tyrosine-phosphorylation of both claudin-5 and VE-cadherin\textsuperscript{153}. Nevertheless, the interplay between inflammation, TGF-β regulation and BBB integrity during MS still need to be elucidated.

5. The resolution of inflammation

5.1. General aspects of inflammation and resolution

Importantly, a proper orchestrated inflammatory response plays a beneficial role in the body’s intrinsic response against damaged cells, pathogens and external compounds. However, as observed in many chronic diseases, including neurological disorders\textsuperscript{154-156}, failure to resolve inflammation leads to chronic inflammation\textsuperscript{156}. An acute inflammatory response is mediated by numerous pro-inflammatory cytokines, chemokines as well as pro-inflammatory lipid mediators (LM), such as prostaglandins (PG) and leukotrienes (LT) produced from the essential fatty acid arachidonic acid (AA). The prostaglandins PGE\textsubscript{2} and PGD\textsubscript{2} and the leukotriene LTB\textsubscript{4} stimulate the migration of neutrophils, the first cells that enter the affected tissue (Figure 4). Next, the protective acute inflammatory response evolves to ensure the repair of injured tissues, elimination of pathogens and finally restore tissue homeostasis. Upon the onset of inflammation, the process known as resolution of
inflammation is immediately activated\textsuperscript{157,158} in order to successfully return to homeostasis and prevent chronic inflammation. Initially, the resolution phase was thought to be a passive process, but is now recognized as an active event\textsuperscript{159}. Similar to Virchow’s cardinal signs of inflammation, like rubor (redness), calor (heat), tumor (swelling), dolor (pain) and functio laesa (loss of function), there are five cardinal signs of resolution\textsuperscript{157,160}. One of the key cardinal signs of resolution is cell clearance\textsuperscript{158}, in which neutrophil apoptosis occurs and, as a consequence, efferocytosis through recruited monocyte-derived macrophages\textsuperscript{158,161}. The other four cardinal signs are cessation of leukocyte recruitment, counter regulation of pro-inflammatory mediators, transition from classical activated macrophages to a more alternative phenotype, restoration of vascular integrity and re-entering of leukocytes in the vasculature and lymphatics\textsuperscript{157,158,160-163} (Figure 4).

The resolution phase is mediated by specialized pro-resolving lipid mediators (SPMs) which are synthetized from the ω-3 or ω-6 polyunsaturated fatty acids (PUFA). Currently, four different chemical families have been classified: lipoxins (LXs), resolvins (RVs), protectins (PDs) and maresins (MaRs)\textsuperscript{164,165}. Whereas RVs, PDs and MaRs are originated from the ω-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the starting point for synthesis of the LXs is the AA, a ω-6 PUFA generated from linoleic acid\textsuperscript{156,166,167} (Figure 5).

During resolution of inflammation, the very same cells recruited to the inflammatory milieu producing inflammatory mediators, undergo a temporal lipid mediator class switch, whereby they stop producing classical eicosanoids from ω-6 AA and start to biosynthesize SPMs\textsuperscript{154,156}. This is possible through the stereoselective and concerted action of the same enzymes engaged in the classical eicosanoids production, namely cyclooxygenase (COX) COX-2, lipoxygenases (LOX) LOX-5, LOX-12 and LOX-15 as well as cytochrome P450 (CYP450) and several epoxide hydrolases\textsuperscript{154,156}.

Figure 4. Lipid mediators in the acute inflammatory response, resolution and other outcomes. Reprinted by permission from Springer Nature Terms and Conditions for RightsLink Permissions Springer Nature Customer Service Centre GmbH: [Springer Nature] [Nature] [Pro-resolving lipid mediators are leads for resolution physiology, Charles N. Serhan], [Copyright © 2018 Copyright Clearance Center, Inc. All Rights Reserved] (2014).
SPMs are potent mediators that extinguish the eicosanoid-induced fire of inflammation by activating local resolution programs\(^{168}\), also by directly modulating T cell responses\(^{169}\) via five separate G protein-coupled receptors (e.g. ALX/FPR2, GPR32/DRV1, ChemR23/ERV, BLT1 and GPR18/DRV2)\(^{170}\). Generally, SPMs promote resolution of inflammation via reducing leukocyte recruitment and transmigration to the inflammatory site and enhancing macrophage phagocytosis to clear apoptotic cells and tissue debris. Furthermore, SPMs have been shown to decrease and regulate the secretion of pro-inflammatory cytokines, chemokines and mediators in order to suppress inflammatory pathways\(^{156,171}\).

Beside the protective role of different SPMs in various inflammatory disease models\(^{160,172-177}\), evidence that SPMs exert potent neuroprotective and pro-resolution actions have recently been shown in the context of AD\(^{178-180}\), stroke\(^{181-183}\), traumatic brain and spinal cord injury\(^{184-186}\), neuropathic pain\(^{186,187}\) and recently in EAE\(^{188}\). Nevertheless, whether MS patients display an impaired resolution of neuro-inflammation, thereby aggravating disease, still remains largely unknown. Importantly, direct knowledge on the resolution pathway in MS patients may led to the development of novel potential biomarkers for MS diagnosis and to novel tools to ultimately limit MS pathogenesis in several disease clinical stages.

### 5.2. Lipoxins (LXs)

LXs were the first SPMs to be identified and exhibit potent anti-inflammatory and resolution actions\(^{189,190}\). Similarly to PGs, LXs are also metabolites from AA. However, PG biosynthesis requires the action of the COXs, instead LXs are generated by the action of LOXs\(^{190}\). Three main pathways of LXs synthesis have been identified. The first one involves a two step pathway in which the enzymes 15-LOX first oxygenize AA into 15S-HpETE and subsequently, 5-LOX is needed for the conversion of 15S-HpETE into LXs. The second pathway is initiated by 5-LOX to catalyze AA to leukotriene LTA\(_4\), which will be further converted by 12/15-LOX into LXs. Finally, LXs synthesis can be triggered also by aspirin, which promotes the acetylation of COX-2 leading to a change in COX2 activity and

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**Figure 5. Simplify scheme illustrating the biosynthesis of different SPMs.**
the chirality of the products, which are termed aspirin-triggered (AT) lipoxins. LXA₄ regulates cellular functions through its specific receptor (ALX/FPR2 and GPR32) activation expressed in a variety of cells. At first, it was shown that topically applied LXA₄ is able to reduce leukocyte infiltration and plasma leakage in a LTB₄-induced skin inflammation model in the hamster cheek. Afterwards, the pro-resolving role of LXs was demonstrated in several in vitro and in vivo models, where it was shown that LXA₄ can reduce inflammatory cell infiltration, attenuate inflammation-induced tissue damage, downregulate pro-inflammatory cytokines and reactive oxygen species (ROS) and finally promote anti-inflammatory signals. Importantly, the beneficial/pro-resolving effects of LXs were also shown in different CNS-related disorders. For example, LXA₄ was shown to attenuate inflammation-induced pain, decrease the infarct volume, suppress inflammatory mediators production, ameliorate BBB dysfunction and promote neurological recovery. Finally, AT-LXA₄ treatment reduced AD-like pathologies, improved synaptic density and modulated glial cell functions, in AD transgenic mice.

5.3. Resolvins (RVs)

RVs are SPMs that are biosynthesized from the precursor essential ω-3 PUFAs. Two major groups of the RVs exist, the E and D-series derived from the EPA and the DHA, respectively. In human, six types of RvDs have been identified (RvD1-6), with RvD1 being the most characterized one. Briefly, the biosynthesis of RvD1 starts with the action of the enzyme 15-LOX-1 which catalyzes the conversion of DHA to 17S-HpDHA, subsequently oxygenized by 5-LOX and converted to RvD1. The E-series are synthesized by the CYP450, or aspirin modified COX-2 enzymes, converting EPA into 18R-HpEPE. Subsequently, 18R-HpEPE is reduced via a peroxidase to 18R-HEPE and finally converted into the different types of RvEs. Different receptors for the RVs have been identified, including the CMKLR1 (also known as ChemR23), GPR32, ALX/FPR2 and BLT1. Similarly to LXA₄, RvD1 exerts potent pro-resolving functions and it is able to modulate class-switching of the AA cascade, thereby inhibiting the production of LTB₄ and enhancing LXA₄ synthesis. Similar to LXA₄, also RvD1 and RvE1 have been shown to attenuate inflammation-induced pain.

Furthermore, a recent study revealed profound alterations in the ω-3 and ω-6 PUFA pathways in plasma samples derived from EAE mice. Particularly, it was shown that RvD1 treatment ameliorates EAE clinical symptoms in EAE mice via a reduction in the number of CNS infiltrating cells and alternatively activated macrophages (M2) polarization of the CNS infiltrating monocytes/macrophages and microglia compared with the vehicle-treated EAE group.

5.4. Protectins (PDs) and maresins (MaRs)

PDs are the first SPMs discovered in the CNS and are generated by the conversion of DHA to 17S-HpDHA via the action of 15-LOX-1. Subsequently, the product is converted to PD1 (also known as neuroprotectin D1 (NPD1)). Furthermore, there is also an aspirin-triggered
epimer for PD1, AT-PD1. Functionally, PD1 was found to reduce brain ischemia volume, suppress CNS leukocytes infiltration and inhibit NF-κB activation and COX-2 expression in a mouse stroke-reperfusion model. Moreover, PD1 was found to be significantly reduced in the hippocampus of AD patients compared to age-matched controls. Importantly, DHA-derived PD1 provided neuroprotection and anti-apoptotic functions that promotes survival in stressed neuronal and glial cells. Being DHA-derivatives, maresins (MaRs) are the most recent SPMs being discovered and were originally identified in macrophages.

Both MaR1 and PD1 have been shown to protect against neuropathic pain in mice after nerve trauma. As other SPMs, MaRs display potent anti-inflammatory and pro-resolving actions inhibiting neutrophil infiltration and microglia activation and promote macrophages switching from a classically activate/pro-inflammatory phenotype (M1) to an alternatively activated/anti-inflammatory and more phagocytic phenotype (M2).

### 6. Thesis aim and outline

Multiple sclerosis (MS) is a neuro-inflammatory disease of the central nervous system (CNS) characterized by chronic neuro-inflammation, demyelination and ultimately neurodegeneration. The underlying pathogenesis involves an initial alteration of peripheral and central immune responses, as well as a disruption and inflammation of the blood-brain barrier (BBB), leading to a substantial infiltration of leukocytes, contributing to disease onset and progression. Despite the progress in the field, the underlying mechanisms for the loss of function of the BBB during acute and chronic neuro-inflammation still remain unclear. Therefore, a better understanding of such mechanisms may ultimately lead to new therapeutic strategies to restore brain homeostasis and BBB function, potentially fighting disease progression.

In this regard, processes that resolve inflammation have been better understood in recent years. While the initial acute inflammatory response is host protective, efficient resolution of inflammation is required to avoid tissue damage and restore tissue homeostasis. Recent studies suggest that chronic inflammation and autoimmunity may be a consequence of failure to resolve inflammation, a process mediated by newly discovered metabolites termed specialized pro-resolving lipid mediators (SPMs). Importantly, a typical feature observed during MS pathology is chronic inflammation, which suggests impairment in the natural process to resolve inflammation. Nevertheless, data on the role of resolution and SPMs in MS remain largely unknown.

In light of this scenario, in this thesis we aimed to:

1. Explore different molecular mechanisms and signaling pathways involved in BBB dysfunction upon acute and chronic inflammation
2. Identify potential alterations of the resolution process in the peripheral blood of MS patients and assess the therapeutic potential of one SPM (Lipoxin A4, LXA4) in a mouse model for MS
The first aim is addressed in chapters 2 and 3. Particularly, in chapter 2 we demonstrate a critical role of the Notch signaling pathway in the function of the BBB under resting and inflammatory conditions. Indeed, inflammation was shown to inhibit the Notch signaling pathway, indicated by a reduced level of its downstream target HES-1, leading to BBB dysfunction in vitro, altered localization and loss of endothelial junctions. Furthermore, we provide evidence on the role of Lunatic Fringe protein in the regulation of Notch glycosylation and signaling. In chapter 3, we further investigate a novel concept in the field of brain endothelial cell (BEC) dysfunction by showing for the first time the presence of endothelial to mesenchymal transition (EndoMT) during MS pathology, pointing towards a de-differentiation of BECs upon neuro-inflammation, as potential mechanism of BBB dysfunction. These findings are of particular importance in order to develop new therapeutic strategies that specifically aim to restore BBB quiescence.

The second aim of the thesis is the focus of chapters 4 and 5. Particularly, in chapter 4, we shed new lights on the resolution process in MS, providing evidence of alterations of the resolution pathway in the blood of MS patients, thereby highlighting the potential to use SPMs as novel biomarkers for MS diagnosis as well as new therapeutic tools to limit MS pathogenesis. Moreover, we show that different SPMs potently inhibited inflammation-induced BBB dysfunction and monocyte transendothelial migration.

In chapter 5 we explore the therapeutic potential of LXA₄ during experimental autoimmune encephalomyelitis (EAE), a well-established mouse model for MS. Notably, we showed substantial changes in the spinal cord (SC) lipidome in mice suffering from EAE. Importantly, LXA₄ treatment normalizes such alterations, ameliorates neuro-inflammation in vivo and suppresses pro-inflammatory T cell responses in patient-derived human T cells. Overall, these findings indicate that boosting resolution of inflammation can be regarded as a novel approach to hamper neuro-inflammation.

Finally, in chapter 6 the above chapters are discussed together with their implications and future perspective.
Chapter 1

References


General introduction


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Chapter 1


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Chapter 1


