FACULTY OF SCIENCES

THE EFFECT OF THE ADAPTIVE IMMUNE SYSTEM ON THE BRAIN BARRIERS IN HEALTH AND DISEASE

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List of Abbreviations

A	
ACK	Ammonium-chloride-potassium
AD	Alzheimer's disease
AJ	Adherens junction
APC	Antigen presenting cell
APP	Amyloid precursor protein
AQP4	Aquaporin-4
Aβ	Amyloid-beta
α-SMA	Alpha-smooth muscle actin
B	
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BLMB	Blood-leptomeningeal barrier
BM	Basement membrane
BSA	Bovine serum albumin
C	
CCL20	C-C motif chemokine ligand 20
CFA	Complete Freud's adjuvant
ChP	Choroid plexus
CNS	Central nervous system
CPE	Choroid plexus epithelial cells
CSF	Cerebrospinal fluid
CSF-1R	Colony-stimulating factor-1 receptor
CVO	Circumventricular organ
D	
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
dCLN	Deep cervical lymph node
DMEM	Dulbecco's Modified Eagle Medium
D-PBS	Dulbecco's phosphate-buffered saline
DSS	Dextran sulfate sodium

DTR	Diphtheria toxin receptor
E	
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F	
FACS	Fluorescence-activated cell sorting
FAD	Familial Alzheimer's disease
FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
G	
GFAP	Glial fibrillary acidic protein
GWAS	Genome-wide association studies
H	
HBSS	Hank's balanced salt solution
HIBCPP	Human malignant choroid plexus papilloma cells
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
I	
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon-gamma
IL	Interleukin
IL2-Rα	Interleukin-2 receptor alpha
IL2-Rγ	Interleukin-2 receptor gamma
ImmCPE	Murine immortalized choroid plexus epithelial cells
ISF	Interstitial fluid
K	
kDa	Kilodalton
KO	Knockout

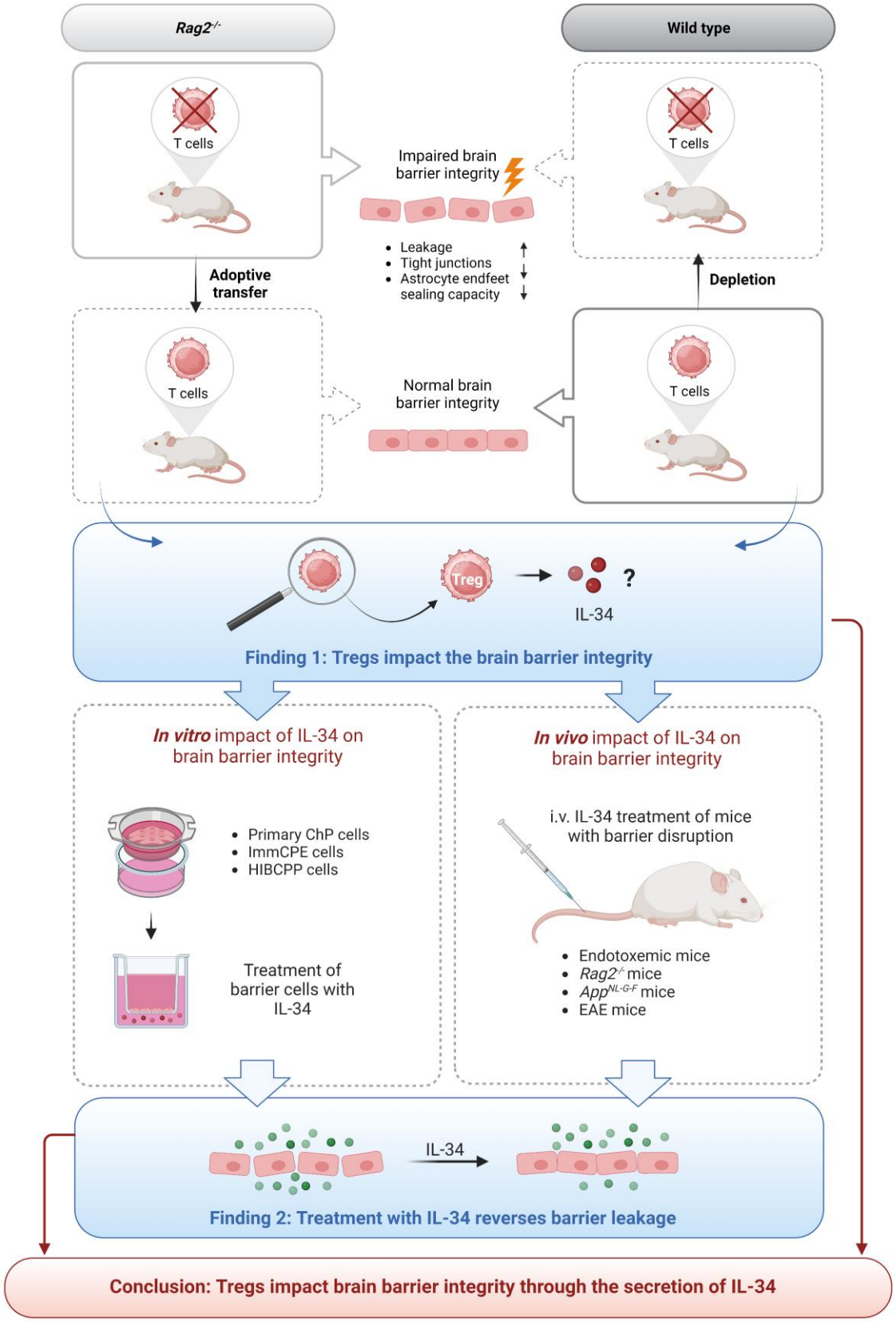
L	
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharides
LPV	Lymphotropic papovavirus
M	
MA	Meningeal arteries
MAGUK	Membrane-associated guanylate kinase-like protein
MAPT	Microtubule-associated protein Tau
MBP	Myelin basic protein
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
ML	Meningeal lymphatics
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MV	Meningeal veins
N	
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor-κ-gene binding
NGS	Normal goat serum
NK cell	Natural killer cell
NO	Nitric oxide
NVU	Neurovascular unit
P	
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PLP	Proteolipid protein
PLX	Pexidartinib
PPMS	Primary progressive multiple sclerosis
PSEN	Presenilin
p-Tau	Hyperphosphorylated Tau
PTP-ζ	Receptor-type protein-tyrosine phosphate-zeta
PVA	Polyvinyl alcohol

PTX	Pertussis toxin
R	
RAG2	Recombination activating gene 2
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
S	
SAS	Subarachnoid space
SLYM	Subarachnoidal lymphatic-like membrane
SPF	Specific pathogen-free
SPMS	Secondary progressive multiple sclerosis
T	
TCR	T cell receptor
TEER	Trans-epithelial/endothelial electrical resistance
TGF-β	Transforming growth factor-beta
TJ	Tight junction
TLR4	Toll-like receptor 4
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TREM2	Triggering receptor expressed on myeloid cells 2
U	
UVB	Ultraviolet B
V	
VCAM-1	Vascular adhesion molecule-1
VE-cadherin	Vascular endothelial-cadherin
VLA-4	Very late antigen-4
Z	
ZO	Zonula occludens

English summary

Much like firewalls that protect computer networks from attacks, the central nervous system (CNS) barriers safeguard the brain from harmful substances and invading immune cells. Specifically, the blood-brain barrier (BBB) and the blood-CSF barrier (BCSFB) protect the brain from the fluctuating environment of the blood to maintain homeostasis within the CNS, which is required for proper neuronal functioning. Just as a compromised firewall leaves a computer system vulnerable to crashes, the disruption of the brain barriers fuels disease progression in many neurological disorders. This 'crash' of the neurological network is present in diseases like multiple sclerosis (MS) and Alzheimer's disease (AD). Consequently, the development of a therapy that aims at restoring the integrity and function of these barriers can limit immune cell infiltration, reduce neuroinflammation and potentially slow the progression of these neurological disorders.

Considering the emerging role of the immune system in influencing CNS components, we focused on the impact of adaptive immune cells on brain barrier integrity. To accomplish this, we used a mouse model deficient in *recombination activating gene 2* (*Rag2*$^{-/-}$), which lacks mature T and B cells. Through adoptive transfer experiments in *Rag2*$^{-/-}$ mice, we demonstrated that the absence of T cells, but not B cells, compromised brain barrier integrity. This finding was further supported by T and/or B cell depletion experiments in wild type mice. Furthermore, we specifically identified regulatory T cells (Tregs) as important T cell subset for the observed effects on the brain barrier integrity. As Tregs are known to secrete interleukin (IL)-34, we investigated the potential involvement of this cytokine in the modulation of brain barrier integrity. Our results revealed that IL-34 treatment improved brain barrier integrity both *in vitro* and *in vivo*. Moreover, we showed reduced levels of IL-34 in the plasma of mouse models for AD and MS. Remarkably, replenishment of IL-34 alleviated the disruption of brain barriers observed in these AD and MS mouse models. In conclusion, we propose that Tregs play a significant role in maintaining brain barrier integrity in both healthy and diseased conditions through the expression of IL-34. These findings contribute to a better understanding of the intricate relationship between Tregs and brain barrier function, opening up new possibilities for therapeutic interventions in neurological disorders.



Graphical summary *Rag2, recombination activating gene 2*; Treg, regulatory T cell; IL-34, interleukin-34; ChP, choroid plexus; ImmCPE, murine immortalized ChP epithelial cells; HIBCPP, human malignant ChP papilloma cells; i.v., intravenous; LPS, lipopolysaccharides; *App*, amyloid precursor protein; EAE, experimental autoimmune encephalomyelitis; endotoxemic mice: wild type mice that received an LPS-trigger.

Part 1: Introduction

1.1. The brain barriers: the firewall of our neurological network

The central nervous system (CNS) can be compared to a computer network that controls the body's functions. It receives input from various sensory organs, processes this information, and then sends output to control movement and behaviour. Much like a firewall that protects a computer network from external threats, tightly sealed barriers safeguard the brain from neurotoxic substances and pathogens. The three main barriers, the **blood-brain barrier (BBB)**, the **blood-cerebrospinal fluid (CSF) barrier (BCSFB)** and the **blood-arachnoid barrier**, are crucial in separating the CNS parenchyma from the fluctuating environment of the blood (Fig. 1) (Engelhardt *et al*, 2017). Next to providing a physical barrier, they regulate the selective passage of molecules in and out of the brain (Cousins *et al*, 2022; Mastorakos & McGavern, 2019). As a result, the brain is able to maintain a strictly regulated **homeostatic environment** required for optimal neuronal functioning (Engelhardt & Sorokin, 2009). Besides the three main barriers, the CNS compartments of the spinal cord and retina also have specialized barrier systems, respectively, the blood-spinal cord barrier and the blood-retina barrier. However, these share a lot of the properties with the main barriers (De Bock *et al*, 2014).

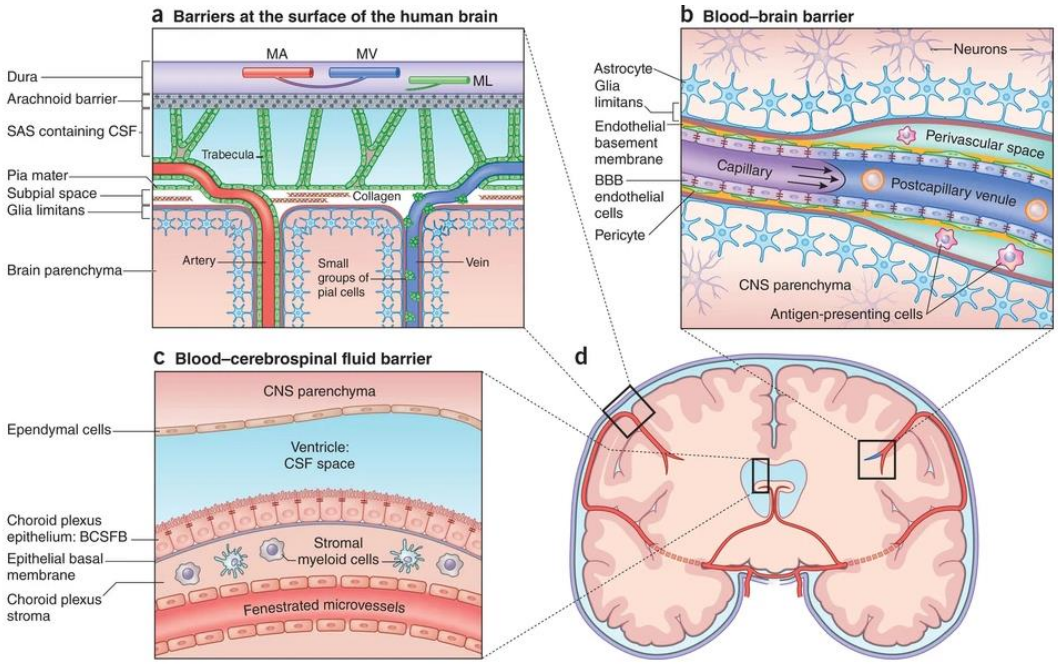


Figure 1: Overview of the barriers in the central nervous system (CNS). (A) The meninges are built up of three membranes: the dura, arachnoid, and pia mater. Recently, a fourth layer has been described between the arachnoid and pia mater, the so-called subarachnoidal lymphatic-like membrane (SLYM), but this is still under debate (Møllgård et al, 2023). Sheet-like trabeculae span the cerebrospinal fluid (CSF)-filled subarachnoid space (SAS) that is located in between the arachnoid and pia mater. The arachnoid mater provides a barrier between the fenestrated blood vessels in the dura mater and the CSF. **(B)** The blood-brain barrier (BBB) restricts passage of blood-borne molecules into the CNS parenchyma and consists of specialized endothelial cells, connected via tight junctions, an endothelial basement membrane (BM), and a glia limitans. The latter is made up of astrocyte endfeet and a parenchymal BM. At postcapillary venules, the endothelial BM and glia limitans are not fused, creating a perivascular space. **(C)** The blood-CSF barrier (BCSFB) restricts the diffusion of molecules from the blood into the CSF and is located at the choroid plexus (ChP). The barrier consists of tightly connected epithelial cells. **(D)** Coronal section of the human brain. MA, meningeal arteries; MV, meningeal veins; ML, meningeal lymphatics. Figure adapted from (Engelhardt *et al*, 2017).

1.1.1. The blood-brain barrier

The brain is a highly metabolically active organ that requires a continuous supply of oxygen and glucose (Raichle & Gusnard, 2002). Unlike some other organs, the brain does not have its own energy reserves to meet these high demands. Therefore, energy is provided through an extensive network of brain vasculature. The BBB is found within this extensive vascular network and is composed of **specialized endothelial cells** that line the lumen of the brain capillaries (Fig. 1B). These endothelial cells are unique in that they are tightly sealed by **tight junctions (TJs)** and **adherens junctions (AJs)**, creating a physical barrier that limits the paracellular permeability (Kadry *et al*, 2020). Additionally, the transcellular movement of molecules across the endothelial cells of the BBB is limited due to the absence of fenestration and the extremely low degree of pinocytosis (Daneman, 2012). However, several **transport mechanisms** exist to ensure the transport of essential molecules and metabolites in and out of the CNS. These mechanisms include passive diffusion, facilitated diffusion and active transport. In addition, some substances have the potential to reach the brain but are prevented to do so by the action of efflux transporters actively pumping the molecules out of the CNS parenchyma (Begley & Brightman, 2003). Moreover, endothelial cells of the BBB contain enzymes that allow for the metabolization and inactivation of various compounds (Daneman, 2012). Together, the **'physical barrier'**, the **'transport barrier'**, and the **'metabolic barrier'** protect the brain from harmful substances, but on the flip side of the coin, they also limit drug entry into the brain (Pardridge, 2005).

TJs form fusion sites, or so-called 'kissing points', in the plasma membranes of two adjacent endothelial cells (Fig. 2) (Wolburg & Lippoldt, 2002). The main components of TJs are **claudins**, **occludins** and **zonula occludens** (ZOs) proteins (Gloor *et al*, 2001). Both occludins and claudins are integral membrane proteins. Even though their sequence homology is minimal, they are structurally very similar. They have four transmembrane domains, two extracellular loops and a cytoplasmic carboxy-terminal tail (Wolburg & Lippoldt, 2002). Claudins are sufficient for the formation of TJ strands, whereas occludins do not appear to be essential in this process. This is demonstrated by a study where the deletion of claudin-5, the most highly expressed claudin in adult brain endothelium, results in early death in mice due to a leaky BBB (Nitta *et al*, 2003). In contrast, mice with a deleted occludin gene are viable, indicating that these proteins are not critical for BBB formation (Saitou *et al*, 2000). However, occludins mainly appear to be important in the regulation of TJs (Yu *et al*, 2005). Both occludins and claudins are **bridged to the cytoskeleton** via ZO scaffolding

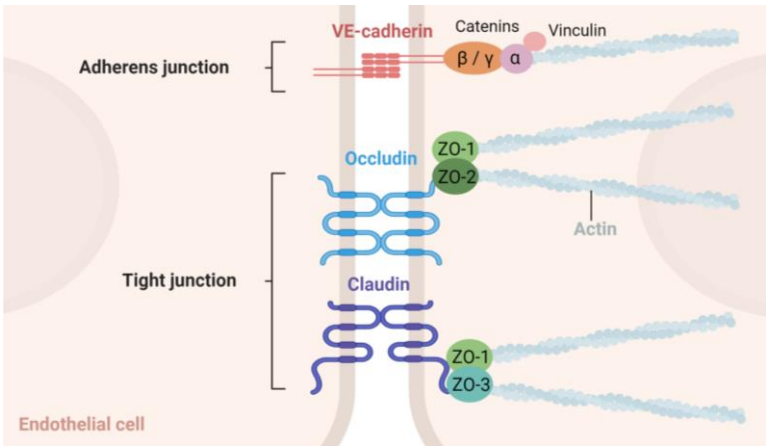


Figure 2: Tight junctions (TJs) and adherens junctions (AJs) in the blood-brain barrier. TJs consist of a complex of transmembrane proteins, including claudins and occludins, that are connected to the actin cytoskeleton via zonula occludens (ZO)-1, -2, and -3 proteins. AJs consist of vascular endothelial (VE)-cadherin linked to the actin cytoskeleton via catenins (α/β/γ) and vinculin. Image created with BioRender.

proteins, including ZO-1, -2, and -3. The ZO proteins are peripheral membrane proteins of the membrane-associated guanylate kinase-like protein (MAGUK) family (Kadry *et al*, 2020). They bind to occludins and claudins through PDZ interaction motifs and, on the other hand, to actin via their COOH-terminus, thereby contributing to the stabilization of the TJ complex (Wolburg & Lippoldt, 2002; Kadry *et al*, 2020). TJs not only prevent the paracellular passage of molecules between EC but also separate the membrane of the endothelial cells in an apical and basal domain. This polarization, typically a feature of epithelia, restricts the movement of lipids and proteins within the membrane from the apical to the basal part (Mandel *et al*, 1993).

Next to TJs, AJs also play an important role in cell-cell adhesion (Fig. 2) (Engelhardt & Sorokin, 2009). The main component of AJs in the BBB is a transmembrane glycoprotein known as **vascular endothelial (VE)-cadherin**. This Ca^{2+}-dependent adhesion protein couples adjacent endothelial cells through homophilic interactions. Intracellularly, catenins bridge VE-cadherin to the actin cytoskeleton, stabilizing the AJ complex (Dejana *et al*, 2008). More specifically, the cytoplasmic domain of VE-cadherin binds to β- and γ-catenin, which subsequently bind to the actin cytoskeleton via α-catenin and vinculin (Meng & Takeichi, 2009).

The BBB is not solely composed of the core EC monolayer. In fact, it is closely associated with **perivascular astrocytic endfeet, perivascular neurons, pericytes** and **microglia** (Fig 1B). In addition to its cellular components, the BBB is also encompassed by two basement membranes (BMs): the inner **capillary BM**, secreted by endothelial cells and pericytes, and the outer **parenchymal BM**, mainly secreted by astrocytic endfeet (Daneman & Prat, 2015; Engelhardt *et al*, 2017). Combined, the parenchymal BM and astrocytic endfeet form the glia limitans. At the level of capillaries, the capillary BM, and the glia limitans are fused. However, at postcapillary venules, the adjacent BMs create a perivascular space that contains antigen-presenting cells (APCs), such as perivascular macrophages, and fluid (Engelhardt *et al*, 2016). Together, these cellular and acellular components of the BBB establish a functional and structural unit known as **the neurovascular unit (NVU)** (Engelhardt *et al*, 2017).

Pericytes are embedded in the capillary BM and send finger-like projections to endothelial cells, attaching to them via **N-cadherin in peg-and-socket junctions** (Brown *et al*, 2019). They play a key role in maintaining BBB integrity, promoting angiogenesis, and microvascular stability (Armulik *et al*, 2010; Brown *et al*, 2019). Furthermore, pericytes exhibit contractile properties through the expression of alpha-smooth muscle actin (α-SMA), tropomyosin, and myosin. This allows them to modulate the diameter of capillaries, thereby regulating the cerebral blood flow in response to neuronal activity (Brown *et al*, 2019; Armulik *et al*, 2011). **Astrocytes**, which can be distinguished by the expression of vimentin and glial fibrillary acidic protein (GFAP), are the most abundant type of glial cells in the CNS. These cells are involved in a range of physiological and biochemical processes. For example, their endfeet contribute to the homeostasis of ions, amino acids, neurotransmitters, and water in the brain (Abbott *et al*, 2006). Additionally, astrocytes extend processes to neurons and contact blood vessels with their endfeet, enabling **communication between neurons and the microvasculature**. In response to synaptic activity, intracellular Ca^{2+} levels rise and vasodilatory factors are secreted from the endfeet to regulate cerebral blood flow (Anderson & Nedergaard, 2003). Moreover, *in vitro* studies have shown that astrocytes can enhance BBB integrity by reinforcing TJs and regulating the transport of molecules across the brain endothelium (McAllister *et al*, 2001; Dehouck *et al*, 1990; Abbott *et al*, 2006).

1.1.2. The blood-cerebrospinal fluid barrier

While most blood vessels in the CNS are protected by a BBB, two specialized structures lack tightly packed endothelial cells: the **circumventricular organs (CVOs)** and the **choroid plexus (ChP)** (Mastorakos & McGavern, 2019). Both structures lie within the interconnected ventricles of the brain, which are filled with CSF. The CVOs are attached to the third and fourth ventricles and serve as a direct connection between the brain and peripheral blood. The fenestrated blood vessels allow the CVOs to monitor blood-borne molecules (sensory CVOs) and to secrete neuroendocrine hormones into the bloodstream (secretory CVOs) (Morita *et al*, 2016). The ChP is a cauliflower-like structure which is present in all four ventricles. This structure has an extensive capillary network, of which the endothelial cells lack TJs and a glia limitans, consequently allowing molecules to diffuse freely from the blood into the ChP stroma. However, a layer of **epithelial cells covers the fenestrated capillaries** providing a barrier between the blood and the CSF in the ventricles, known as the blood-CSF barrier (BCSFB) (Fig. 1C) (Mastorakos & McGavern, 2019; Cousins *et al*, 2022). In contrast to the endothelial cells of the blood vessels in the ChP and the ependymal cells lining the ventricles, the ChP epithelial cells are tightly connected by TJs, similar to those found at the BBB. Nonetheless, they express different isoforms of claudins and varying levels of occludins and ZOs, leading to a 10-15 times lower resistance compared to the BBB (Kratzer *et al*, 2012; Saito & Wright, 1984). Additionally, similar transport mechanisms as in the BBB regulate the influx and efflux of molecules in and out of the CSF. The epithelial cells also provide an enzymatic barrier with antioxidant and metabolizing enzymes (Strazielle & Ghersi-Egea, 2013).

Next to limiting the movement of molecules from the ChP stroma to the CSF, the epithelial cells of the ChP are also responsible for **the production of the CSF**. They do this at a remarkable rate of 350 µl per min, producing approximately 500 ml of CSF per day in an average adult human (Cserr, 1971). CSF is composed of water, ions, neurotransmitter, and a low concentration of proteins that are transported from the plasma across the BCSFB. Additionally, it contains components produced by the ChP itself, such as transthyretin (Wichmann *et al*, 2022). The fluid is of key importance in the proper functioning of the CNS. Its main function is **the maintenance of CNS homeostasis by clearing waste products from the brain parenchyma** (Wichmann *et al*, 2022). This works via the recently rediscovered **glymphatic system**, which describes the astrocyte-mediated fluid exchange between the CSF and the interstitial fluid (ISF) of the brain parenchyma (Fig. 3) (Jessen *et al*, 2015). The CSF in the brain ventricles can freely move to the subarachnoid

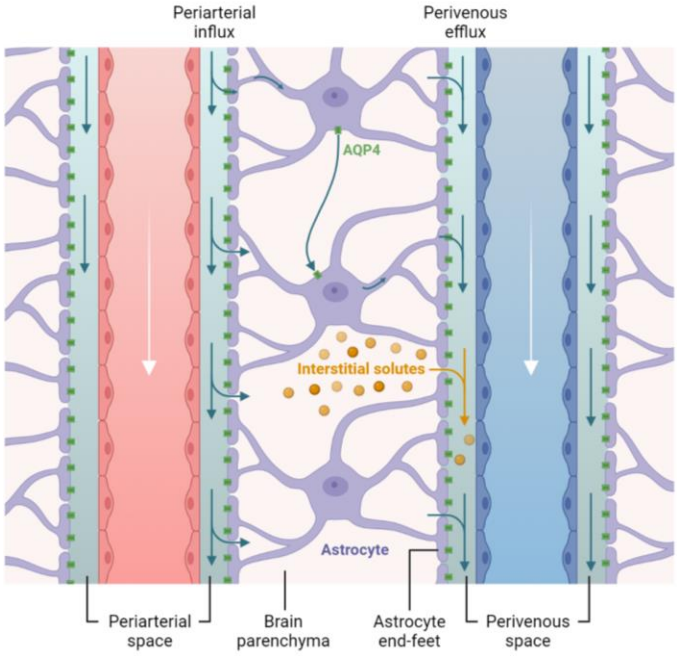


Figure 3: The glymphatic system. Cerebrospinal fluid (CSF) moves from the periarterial space of penetrating arteries into the brain parenchyma. The flux of CSF removes interstitial solutes and drains to the perivenous spaces. AQP4, aquaporin 4. Image created with BioRender based on (Ray *et al*, 2019).

space (SAS), which surrounds the CNS. From there, CSF can flow into the perivascular spaces of the arteries that penetrate the brain parenchyma. Next, CSF can leave this periarterial space and enter the brain parenchyma, where it exchanges with ISF (Jessen *et al*, 2015). Therefore, the fluid needs to cross the glia limitans, established by astrocyte endfeet. In this process, the water channel aquaporin 4 (AQP4), expressed by these endfeet, is believed to play an important role (Trillo-Contreras *et al*, 2019). CSF movement clears away the interstitial solutes to perivenous drainage pathways. Researchers were able to visualize this process via injection of fluorescent tracers in the cisterna magna of mice, a region where CSF is accessible, and subsequent two-photon imaging (Iliff *et al*, 2012). Further drainage happens either directly through the veins or via the SAS to the cervical lymphatic system (Johnston *et al*, 2004). This mechanism that operates during our sleep promotes a healthy and functional brain as toxic substances that have accumulated during the day are cleared from the brain parenchyma. During ageing, the glymphatic activity is strongly decreased which might contribute to the progression of neurodegenerative diseases, such as Alzheimer's disease (AD), where clearance of accumulated Aβ peptides is disturbed (Jessen *et al*, 2015).

1.1.3. Barriers at the surface of the brain

The blood-arachnoid barrier is the least explored and structurally most intricate among all brain barriers. The barrier is part of the **meninges**; a three-layered structure located at the surface of the brain and spinal cord. From the outmost to the innermost, the meninges is made up of the **dura, arachnoid and pia mater** (Fig. 1A) (Mastorakos & McGavern, 2019). The latter two, together referred to as the leptomeninges, are separated by the CSF-filled SAS. Sheet-like trabeculae divide the SAS into different CSF compartments and connect the pia to the arachnoid mater (Weller, 2005). The arachnoid mater consists of two layers of epithelial cells that are linked with TJs and express efflux transporter. This barrier separates the dura mater from the CSF in the SAS. In the dura mater, blood vessels are fenestrated, and thus, open for diffusion of water and solutes from the periphery. Consequently, the arachnoid barrier can be seen as **a blood-CSF barrier at the surface of the brain** (Mastorakos & McGavern, 2019; Engelhardt *et al*, 2017). The pia mater is a single-cell layer that covers the surface of the brain. Arteries that penetrate the CNS from the SAS are also ensheathed by a layer of pial cells, while veins are only covered by groups of pial cells (Engelhardt *et al*, 2017). The cells of the pia mater are not joined together by TJs, but they are connected through gap junctions and desmosomes. As a result, the pia mater allows for the passage of water, solutes, and immune cells while preventing the crossing of erythrocytes (Hutchings & Weller, 1986; Alcolado *et al*, 1988; Weller, 2005). Of note, a recent study proposes a fourth meningeal layer, called the subarachnoid lymphatic-like membrane (SLYM) (Møllgård *et al*, 2023). Møllgård and colleagues suggest that the SLYM subdivides the SAS into two different compartments and serves as a tight barrier for solutes larger than 3 kilodaltons (kDa). However, the validity of these findings remains a topic of debate and many researchers disagree with the presence of the SLYM.

1.1.4. The brain: not an immune-privileged site anymore

For a long time, the brain was believed to be completely sealed off from the immune system. This immune-privileged status of the brain took root a century ago when it was observed that, only in the brain, heterologous tumours or development of embryonic tissue could survive (Murphy & Sturm, 1923; Medawar, 1948). Moreover, some anatomical features of the CNS further fed this concept, such as the presence of the tight brain barriers and the lack of lymphatic vasculature that enables immunosurveillance (Fabry *et al*, 2008; Wekerle, 2006). **All of this led to the prevailing view that the brain and the immune system live largely separate** and only clash when immune cells start attacking the body's own cells in diseases such as multiple sclerosis (MS). This concept of CNS immune privilege has been extensively reviewed, debated and reinterpreted (Engelhardt *et al*, 2017; Forrester *et al*, 2018; Louveau *et al*, 2015a). Meanwhile, it is becoming increasingly clear that the brain has its own resident immune cells and that immune cells circulate at the fluid-filled brain borders (Fig. 4).

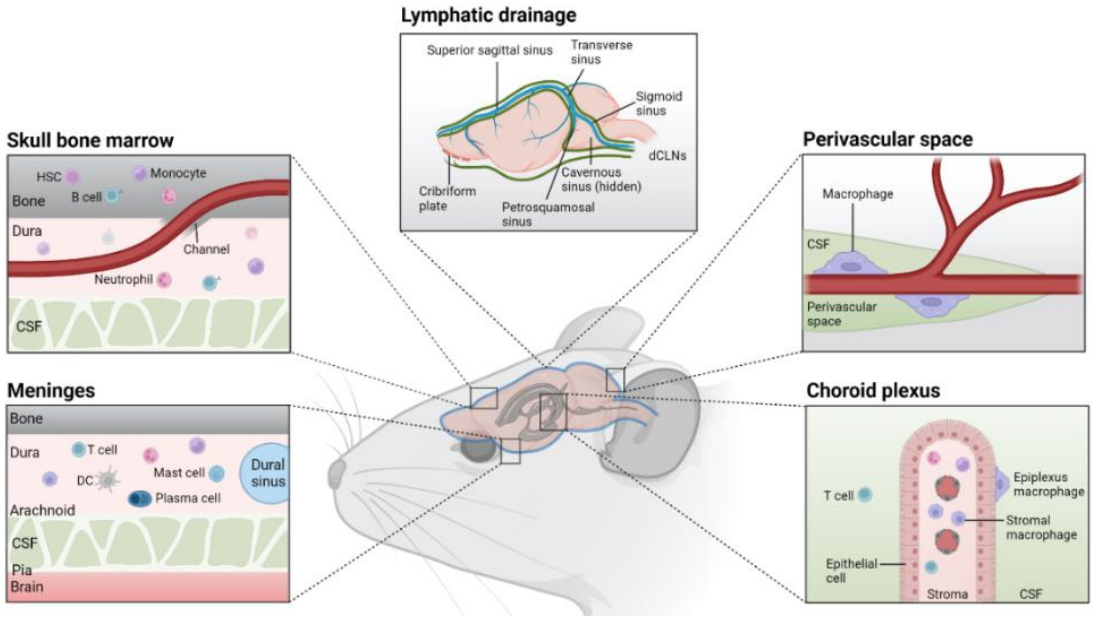


Figure 4: The brain borders: an anatomical site for neuro-immune interactions. The triple-layered membranes of the meninges surround the brain. The dura mater serves as an important immune centre containing abundant immune cells, including T cells, B cells, mast cells, dendritic cells (DCs), monocytes, and plasma cells. Overlying the meninges, the skull contains bone marrow that is functionally connected to the meninges and underlying brain and serves as a local source of immune cells. Another brain border, the choroid plexus (ChP), is situated within the ventricles of the brain and produces cerebrospinal fluid (CSF), which contains T cells. The stroma of the ChP contains stromal myeloid cells, such as macrophages and DCs. Another specific type of tissue-resident macrophages, the epiplexus macrophages, are located at the ventricular side of the ChP epithelium. Numerous lymphatic efflux routes exist for the drainage of brain interstitial fluid containing proteins and immune cells via the CSF. Lastly, perivascular spaces exist surrounding penetrating brain vasculature that contains CSF and resident immune cells, such as the perivascular macrophages. dCLN, deep cervical lymph node; HSC, hematopoietic stem cell. Image created via Biorender.

Microglia, the primary **resident immune cells** of the brain, provide the tissue with innate immune sensing, inflammatory effector functions and tissue repair services. Under homeostatic conditions, most of the CNS compartments are inaccessible for **circulating myeloid cells** while upon certain perturbations circulating myeloid cells can be recruited to the parenchyma. In addition to microglia, the healthy brain contains dendritic cells (DCs) and

to a lower extent also T, B, and natural killer (NK) cells, although whether these latter populations are truly brain-resident remains controversial (Mrdjen *et al*, 2018; Korin *et al*, 2017). Interestingly, Pasciuto *et al*. recently identified a population of **brain resident CD4$^+$ T cells** present in both the healthy mouse and human brain. Brain resident CD4$^+$ T cells are distinct from circulating CD4$^+$ T cells and regulate microglia maturation (Pasciuto *et al*, 2020). Moreover, mice that develop without CD4$^+$ T cells present a defect in pruning immature neuronal connections during development, leading to excessive numbers of synapses and abnormal behaviour (Pasciuto *et al*, 2020).

Next to parenchymal immune cells, also immune cells at the different brain borders, including the meninges, choroid plexus (ChP), CNS-draining lymph nodes and the perivascular spaces, play a crucial role in immune surveillance (Fig. 4). In these compartments, various immune cells can sample CNS antigens (Rustenhoven *et al*, 2021; Mazzitelli *et al*, 2022; Pulous *et al*, 2022), protect the brain from pathogenic insults (Fitzpatrick *et al*, 2020), manipulate behavioural paradigms (Ribeiro *et al*, 2019; Filiano *et al*, 2016) and provide immune cells to the brain during health and in response to diverse insults (Herisson *et al*, 2018; Brioschi *et al*, 2021; Cugurra *et al*, 2021). Recently, a local source of immune cells at the brain borders, namely the bone marrow of the skull, was identified that responds to signals carried in the CSF, prompting it to produce and release immune cells (Cugurra *et al*, 2021). Altogether, these recent findings push us away from the concept that the CNS is a strict immune-privileged site to a more nuanced and complex view in **which the brain barriers divide the CNS into compartments that differ with respect to their accessibility to the immune system** (Engelhardt *et al*, 2017).

1.1.5. The role of CNS brain barriers in neurological diseases

As mentioned above, the brain barriers are fundamental components that ensure proper functioning of the CNS. Consequently**, the breakdown of these barriers is involved in various neurological disorders**, such as MS, stroke, brain trauma, and AD (Profaci *et al*, 2020; Cai *et al*, 2018; Balusu *et al*, 2016; Engelhardt *et al*, 2022). The loss of barrier integrity results in infiltration of immune cells and impaired transport and clearance of molecules, thereby contributing to disease progression (Engelhardt *et al*, 2017). Notably, the entry of immune cells can be both beneficial and detrimental. On one hand, **immune cells can cause neuronal dysfunction, injury, and neurodegeneration**. On the other hand, they can enter the CNS to clear debris and repair injuries.

In this thesis, we will focus on the relationship between the brain barriers and two neurological diseases, namely AD and MS. Therefore, a comprehensive overview of these diseases and their implications on the brain barriers will be given below. In addition, we will use lipopolysaccharides (LPS) as inflammatory trigger to induce brain barrier leakage both *in vitro* and *in vivo*. LPS is a major component of the outer membrane of Gram-negative bacteria that is often used for studying inflammation-induced barrier disruption. LPS is shown to directly increase brain barrier permeability by decreasing the expression and altering the distribution of TJs, including ZO-1 and claudin-5 (Veszelka *et al*, 2007; Seok *et al*, 2013; Cheng *et al*, 2018). LPS binding to the Toll-like receptor 4 (TLR4) on brain barrier cells, leading to expression of the nuclear factor-κ-gene binding (NF-κB), plays an important role in this process of TJ damage (Tiruppathi *et al*, 2008; Cheng *et al*, 2018).

1.2. Alzheimer's disease

1.2.1. Current facts and figures of AD

Alzheimer's disease (AD) is an age-related **neurodegenerative disorder** of the CNS underlying 60-80% of all **dementia** cases (Burns & Iliffe, 2009; Jellinger, 1998). Worldwide, over 55 million people are affected by dementia and this number is expected to rise to 139 million in 2050 (World Alzheimer Report 2022: Life after diagnosis: Navigating treatment, care and support, 2022). As **the world's population is ageing**, both the death toll and the costs are accelerating. Globally, the annual cost of dementia is estimated at US$ 1 trillion, which is set to double by 2030 (Cummings *et al*, 2022). In addition to this economic burden, the number of AD-related deaths has more than doubled, whereas deaths from heart disease, the leading cause of death, decreased by 7.3% (2022 Alzheimer's disease facts and figures, 2022). Moreover, in Belgium, dementia has become the number one cause of death (Sciensano). The rise in AD deaths likely results from two factors: a real increase in the number of deaths attributed to AD, and secondly, improved reporting of AD-related deaths over time (Tejada-Vera, 2013). Altogether, the increasing prevalence and mortality of AD impose a major public health crisis. This highlights the urgency for developing therapies to prevent, delay the onset and slow the progression of the disease.

1.2.2. Symptoms and pathological hallmarks of AD

Patients suffering from AD are characterized by progressive and disabling deficits in cognitive function. **Memory loss** is one of the earliest and most prominent symptoms of AD, manifested in difficulties with recalling recently acquired information, names, and faces, problems with word-finding and repeatedly asking the same questions. As the disease progresses, patients may experience a decline in their learning, abstract thinking, and problem-solving abilities. **Speech disturbances** and problems with **visuospatial orientation** further impair daily-life activities and result in confusion and social withdrawal. Moreover, cognitive decline may lead to **alterations in personality and behaviour** such as increased agitation, apathy, and depression. In later stages, **motor functions deteriorate** and patients become completely bed-bound and dependent on others for their care (2022 Alzheimer's disease facts and figures, 2022). AD ultimately becomes fatal, often due to acute conditions such as aspiration pneumonia (Brunnström & Englund, 2009). The overall duration of the disease is subjective to individual variability, for instance, age and biological sex, and varies between 15 and 24 years (Vermunt *et al*, 2019). Taken together, the burdensome symptoms and the long duration of illness greatly impact the daily life of the patients, their families, caregivers, and the healthcare system.

Pathologically, AD brains are distinguished by atrophy of the medial temporal lobe, including the hippocampus and the frontal lobe. Additionally, due to the loss of both white and grey matter, the brain ventricles become enlarged (Halliday *et al*, 2003). The main molecular hallmarks of AD are the accumulation of **amyloid-beta (Aβ) peptides into senile plaques** in the extracellular matrix between neurons and the formation of **neurofibrillary tangles (NFT) composed of hyperphosphorylated Tau (p-Tau)** proteins inside neurons (Fig. 5). These

pathological changes result in synaptic and neuronal loss, causing neurodegeneration and, ultimately, symptoms associated with AD (Jellinger, 1998).

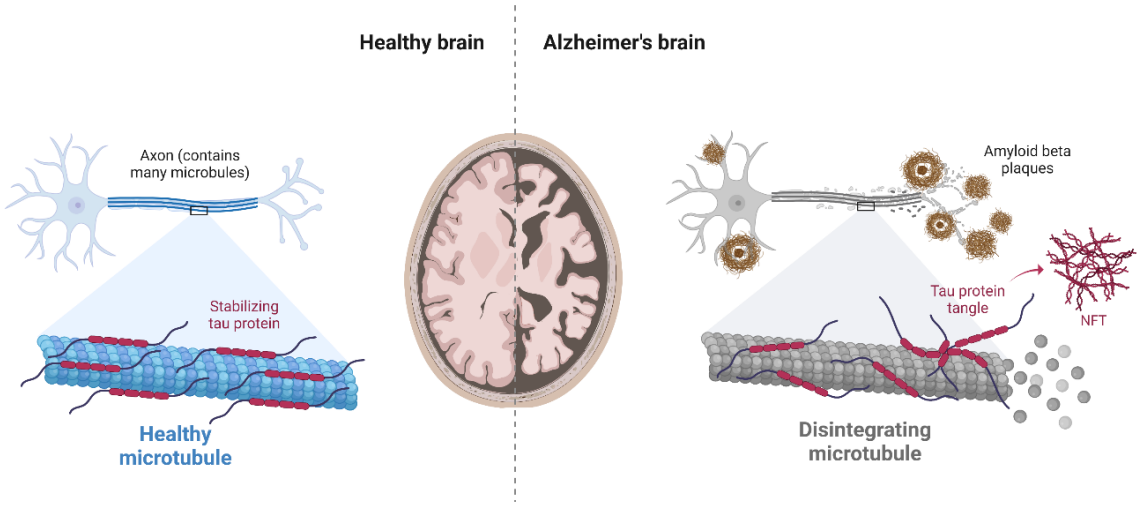


Figure 5: The pathological hallmarks of Alzheimer's disease (AD). The AD brain is characterized by atrophy of the medial temporal and frontal lobe, leading to enlarged ventricles. Molecularly, there is deposition of amyloid-beta (Aβ) protein in senile plaques outside the neurons and formation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated Tau protein inside the neurons. This results in the loss of synapses which ultimately leads to neurodegeneration and cognitive decline. Image created with BioRender.

Aβ peptides are formed as by-products of the proteolytic cleavage of the **amyloid precursor protein (APP)**, a transmembrane protein abundantly expressed in the brain and primarily localized in the synapses of neurons (O'Brien & Wong, 2011). While the physiological function of soluble APP remains largely unclear, it is thought to contribute to various processes such as neurite outgrowth, synaptic plasticity, and synaptogenesis (Pearson & Peers, 2006). There are at least two ways in which APP can be processed: the **non-amyloidogenic and the amyloidogenic pathway**. The first pathway involves cleavage by the enzyme α-secretase, which hinders Aβ formation and generates the neuroprotective sAPPα fragment. Alternatively, sequential cleavage by **β-secretase** and then **γ-secretase** produces Aβ peptides (Fig. 6A). Depending on the exact site of cleavage, different isoforms of Aβ can be produced with $A\beta_{40}$ and $A\beta_{42}$, consisting of respectively 40 and 42 amino acids, being the most abundant (LaFerla *et al*, 2007). During disease, these Aβ peptides can assemble into oligomers, fibrils and eventually amyloidogenic plaques (Fig. 6B) (Serrano-Pozo *et al*, 2011). While $A\beta_{40}$ is produced at higher levels, $A\beta_{42}$ is typically more prevalent in the extracellular plaques (Iwatsubo *et al*, 1994). The $A\beta_{42}$ variant is thought to be more neurotoxic due to its greater hydrophobicity and higher tendency to form aggregates (Jarrett *et al*, 1993). Therefore, the ratio between these Aβ species is an important factor for the rate of amyloidogenesis (Jan *et al*, 2008).

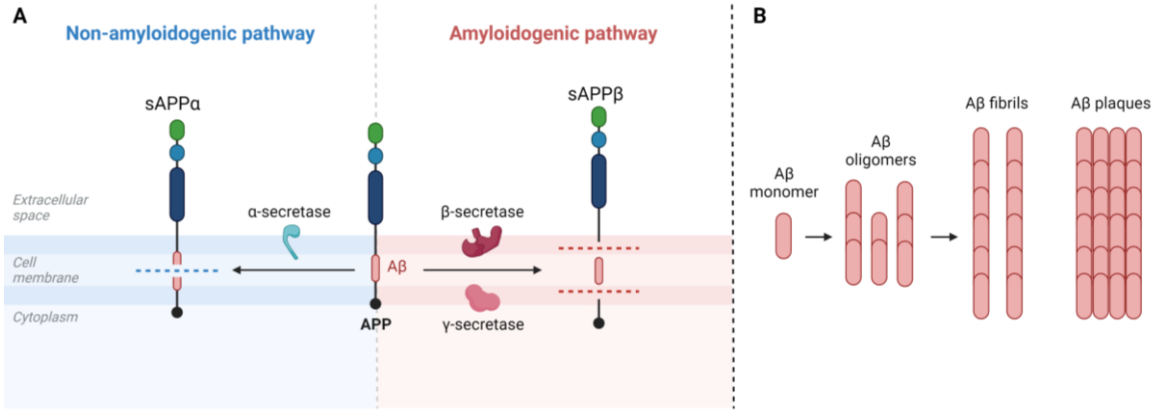


Figure 6: Schematic overview of amyloid precursor protein (APP) processing and Aβ aggregation. (A) The processing of APP occurs through two pathways. In the non-amyloidogenic pathway, APP is sequentially cleaved by α- and γ-secretase, producing neuroprotective sAPPα fragments. In the amyloidogenic pathway, APP is cleaved by β- and γ-secretase, forming Aβ peptides. **(B)** The Aβ monomers are the building blocks of Aβ oligomers, which aggregate to form fibrils, and ultimately amyloid plaques. Image created with BioRender.

Tau is a **microtubule-associated protein** that plays a vital role in maintaining the structural integrity of neurons. It is encoded by the *microtubule-associated protein Tau (MAPT)* gene and is predominantly found in the axons, where it binds with tubulin to regulate microtubule stability, dynamics and axonal transport (Barbier *et al*, 2019; Kent *et al*, 2020). Besides its role as a microtubule-binding protein, the physiological function of Tau remains unclear, as Tau knockout (KO) mice do not show major developmental abnormalities compared to wild type mice (Hummel *et al*, 2016). However, Tau is reported to play a role in a wide range of physiological processes, including neuronal maturation, glucose metabolism, motor function and myelination (Kent *et al*, 2020). The functioning of Tau is tightly controlled by its phosphorylation status, with a delicate balance between kinases and phosphatases ensuring its normal activity (Iqbal *et al*, 2005). In AD, this balance between kinases and phosphatases is disrupted, leading to the **hyperphosphorylation of Tau** (p-Tau) and its inability to bind to microtubules. Consequently, there is a breakdown in the microtubule structure, the **accumulation of p-Tau in NFTs**, and ultimately neuronal degeneration (Fig. 5) (Iqbal *et al*, 2005). In early AD stages, Tau seeding typically starts in the entorhinal cortex and further propagates to the hippocampus and neocortex, as categorized by the Braak staging (Braak & Braak, 1995). Remarkably, brain atrophy and associated cognitive decline correlate strongly with this pattern of Tau accumulation, not with Aβ deposition (Giannakopoulos *et al*, 2003).

In addition to the deposition of Aβ plaques and the formation of NFTs, **neuroinflammation has emerged as a third important hallmark of AD**. Neuroinflammation is defined as the innate immune response to CNS tissue damage (Heneka et al, 2015). During this response, brain resident cells, such as microglia and astrocytes, become activated and show elevated production of various immune molecules and inflammatory mediators, including inflammatory cytokines, factors of the complement pathway, nitric oxide (NO), and reactive oxygen species (ROS) (Dujardin et al, 2022; McAlpine & Tansey, 2008; Wyss-Coray & Rogers, 2012; Maier et al, 2008; Guillot-Sestier & Town, 2013). Upon chronic activation, the detrimental effects predominate the beneficial roles of these immune cells, leading to neurotoxicity and, eventually, AD pathogenesis (Heneka et al, 2015; Frost et al, 2019). In addition, studies have shown that neuroinflammation occurs early during AD pathogenesis

and is not simply a response to Aβ and p-Tau plaques. For instance, it is shown that astrocytic and microglial activation precedes Aβ deposition and that inflammatory changes can already be detected in the CSF of patients with mild cognitive impairment (Heneka & O'Banion, 2007; Heppner et al, 2015). This is further supported by genome-wide association studies (GWAS) showing that **genes associated with innate immunity and inflammation are linked with AD development** (Bis et al, 2020; Kamboh et al, 2012). For instance, immune receptors, such as CD33 and triggering receptor expressed on myeloid cells 2 (TREM2), and other genes involved in the regulation of the immune function in and outside the CNS are identified as risk genes (Kamboh et al, 2012; Sierksma et al, 2020). Moreover, Xie *et al*. recently demonstrated that **low-grade peripheral inflammation exacerbates AD pathology in AD mice**, reflected by increased microglial activation, Aβ pathology, and neuronal dysfunction (Xie *et al*, 2021).

1.2.3. The $App^{NL\text{-}G\text{-}F}$ mouse model for preclinical AD research

Transgenic mouse models are powerful resources to gain a better understanding of the pathogenesis of AD in humans and to evaluate potential therapeutic approaches. Most of these mouse models are based on the genetic mutations present in familial AD, namely in the *APP* gene and in the γ-secretase subunit genes *presenilin 1 (PSEN1)* and *presenilin 2 (PSEN2)*. The first-generation mouse models often used a transgenic *APP* overexpression strategy. An excessive amount of APP results in increased Aβ aggregation, and consequently, mice that exhibit AD pathology. However, overexpression may affect the physiological functions of APP, resulting in side effects unrelated to AD (Saito *et al*, 2014). Therefore, Saito *et al*. developed a second-generation transgenic mouse model, called the $App^{NL\text{-}G\text{-}F}$ mice, using a **knock-in approach of the *APP* gene**. These transgenic mice overcome the undesired side effects associated with the first-generation model, as APP is expressed at wild type levels. The APP gene has a **humanized Aβ region along with three mutations linked to familial AD**: the Swedish "NL", the Iberian "F", and the Arctic "G" mutations (Nilsson *et al*, 2014). The Swedish mutation causes increased production of total Aβ, while the Iberian mutation only elevates neurotoxic $Aβ_{42}$ production (Saito *et al*, 2014). The Arctic mutation promotes Aβ aggregation by facilitating oligomerization and reducing proteolytic degradation (Nilsson *et al*, 2014). Of note, multiple mutations within the same *APP* gene are typically not present in human patients. This raises the concern that these mutations could interact with one another, which may limit the accuracy of this model in representing the human disease (Yshii *et al*, 2022). However, a significant advantage of the $App^{NL\text{-}G\text{-}F}$ model compared to first-generation models is the absence of mutations in the *PSEN1* gene. This is particularly relevant when investigating the interplay between the immune system and AD, as *PSEN1* expression has been identified in splenocytes, including T cells. The possibility of off-target PSEN1 processing in T cells could lead to aberrant physiological processes unrelated to AD (Yshii *et al*, 2022).

1.2.4. The latest developments in AD therapy

Currently, the US Food and Drug Administration (FDA) has approved seven drugs for AD, of which five are only symptomatic treatments (Pardo-Moreno *et al*, 2022; Reardon, 2023a). Symptomatic medication does not change the underlying pathology of AD but rather **alleviates the neurological symptoms**. They work either by inhibiting the breakdown of the neurotransmitter acetylcholine (e.g. donepezil, galantamine, rivastigmine) or by blocking the binding of the neurotransmitter glutamate to the N-methyl-D-aspartate receptor (e.g.

memantine), thereby enhancing cognitive performance (Pardo-Moreno *et al*, 2022). More recently, the FDA approved two disease-modifying therapies: aducanumab and lecanemab. These are **monoclonal antibodies that target the Aβ plaques** in the brain. Aducanumab showed a reduction in Aβ aggregates in patients with mild cognitive impairment or mild AD. However, the clinical benefits were shown to be inconclusive (Tampi *et al*, 2021). In contrast, data from the phase III clinical trials of lecanemab suggested significant cognitive benefits for early AD patients (van Dyck *et al*, 2023). However, the effects on cognition are small and there are concerns about the safety of the drug (Reardon, 2023a). Additionally, although not yet FDA-approved, donanemab was shown to significantly slow cognitive and functional deterioration in AD patients. This monoclonal antibody also specifically targets the Aβ plaques and has demonstrated a remarkable 40% reduction in cognitive decline compared to the placebo group (Reardon, 2023b).

The lack of effective disease-modifying drugs highlights the need to change gears and rethink the strategies for therapy development. The current focus lies on targeting pathogenic Aβ or p-Tau aggregates. However, AD is a complex disease that should be tackled on multiple fronts. In recent years, it became clear that the immune system is a key player in AD, leading to the idea that **immune-regulatory therapies could hold great potential.**

1.2.5. The brain barriers in AD

In AD, the leakiness of the brain barriers is increased (Balusu *et al*, 2016; Cai *et al*, 2018). As described above, AD is characterized by the presence of Aβ plaques, NFTs and neuroinflammation, leading to neurodegeneration and cognitive decline (Burns & Iliffe, 2009). In addition, evidence shows that **early cerebrovascular dysfunction contributes to AD onset and progression** (Starr *et al*, 2009; Montagne *et al*, 2015; van de Haar *et al*, 2016). The dysfunction of the BBB results in aberrant transport, and subsequently, the **failure of Aβ clearance** from the CNS, leading to the accumulation of Aβ in the brain instead of being transported to the peripheral circulation (Tarasoff-Conway *et al*, 2015). Additionally, structural components of the neurovascular unit, including pericytes, astrocytes, endothelial cells and TJs are affected in AD, thereby contributing to impaired clearance. Moreover, BBB dysfunction has been shown to accelerate Aβ generation and promote neuroinflammation (Cai *et al*, 2018). Conversely**, Aβ aggregates can induce barrier disruption**, setting off a vicious cycle that exacerbates Aβ accumulation and fuels the progression of AD (Song *et al*, 2017). In addition, Aβ also upregulates the expression of vascular adhesion molecules, promoting the entry of leukocytes into the brain that contribute to neuroinflammation (Pietronigro *et al*, 2016). At the BCSFB, Aβ accumulation is associated with morphological and functional changes of the ChP epithelial (CPE) cells. These changes lead to decreased CSF production, increased permeability, impaired Aβ clearance, and recruitment of immune cells into the CNS, thereby amplifying AD progression (Balusu *et al*, 2016; Solár *et al*, 2020). Interestingly, the Vandenbroucke lab found that intracerebroventricular injection of $A\beta_{1\text{-}42}$ oligomers induces morphological alterations of CPE cells, loss of BCSFB integrity, and increased ChP inflammation. These effects were linked to **increased activity of matrix metalloproteinases** (MMP) (Brkic *et al*, 2015). Moreover, they showed that **tumour necrosis factor receptor-1 (TNFR1) signaling** is involved in these detrimental events in the ChP. Specifically, TNFR1 deficiency resulted in preservation of BCSFB integrity and no upregulation of MMP expression in AD mouse models, providing an interesting therapeutic target (Steeland *et al*, 2018).

1.2.6. The adaptive immune system in AD

The formation of Aβ plaques outside neurons and NFTs inside neurons have been well-known AD hallmarks for decades. More recently, while shifting away from the notion of the brain as an immune-privileged site, it became clear that both peripheral and central inflammation also contribute to AD pathogenesis and progression (Heneka *et al*, 2015; Walker *et al*, 2019; Xie *et al*, 2021). While the interaction between the innate immune system and AD pathology is extensively studied, the role of the adaptive immune system remains largely unknown. The hypothesis that the adaptive immune system contributes to AD pathogenesis is supported by a study in which an immune-deficient AD mouse model was generated, namely the ***Rag-5xFAD* model**. To generate this model, *recombination activating gene 2 (Rag2)/IL-2 receptor gamma (Il2-rγ)* double-knockout mice were crossed with the AD mouse model *5xFAD* (Marsh *et al*, 2016). This *Rag-5xFAD* model lacks T and B cells and thus lacks an adaptive immune response. The model exhibits dramatically **increased Aβ pathology** and greatly **exacerbated neuroinflammation**. Additionally, they showed that repopulation of the missing adaptive immune cells via bone marrow transplantation diminished Aβ pathology (Marsh *et al*, 2016). In contrast, a study by Späni *et al* showed reduced Aβ pathology in a different AD mouse model (*APP/PSEN1*) crossed with *Rag2*-deficient (*Rag2*$^{-/-}$) mice. Moreover, the replenishment of adaptive immune cells enhanced microgliosis and increased phagocytosis of Aβ aggregates in 8-month-old mice. Although these results are contrasting, they suggest an important role for adaptive immune cells in AD pathology. More evidence comes from a recent study on **multiple large cohorts** of patients with AD that showed an adaptive immune response in the blood and CSF of these individuals (Gate *et al*, 2020). However, it remains unclear whether, and to what extent adaptive immune cells enter the brain and contribute to neuroinflammation in AD patients.

One important part of the adaptive immune system is the **T cell response**. During AD, peripheral immune cells travel from the CSF to the deep cervical lymph nodes of the lymphatic system, where they can react to brain antigens (Louveau *et al*, 2015b). Moreover, evidence shows that besides the role of **peripheral T cells** in AD pathology, T cells can also **infiltrate the brain**. As a response to inflammatory AD pathology, **CD8$^+$ T cells** can enter the brain, interact with microglia near the plaques, and induce cytotoxic effects (Unger *et al*, 2020; Rogers *et al*, 1988; Gate *et al*, 2020; Togo *et al*, 2002). **CD4$^+$ T cells** can also infiltrate the brain and act destructively, analogous to CD8$^+$ T cells. However, some CD4$^+$ subtypes can reduce innate inflammation and limit neuronal damage and degradation (Bryson & Lynch, 2016). A third type of T cells known to invade the brain are **regulatory T cells.** Multiple groups have provided evidence for an overall **protective role** of Tregs in restoring memory deficits, reducing plaque load, and decreasing microglial activation in AD models (Baek *et al*, 2016; Dansokho *et al*, 2016; Alves *et al*, 2017). Taken together, the **interplay between the adaptive immune system and AD** remains unclear. Nonetheless, interfering with the adaptive immune system to alter AD development might hold **therapeutic potential**.

1.3. Multiple sclerosis

1.3.1. Current facts and figures of MS

Multiple sclerosis (MS) is a **chronic demyelinating disease** of the CNS that impacts around 2,8 million individuals worldwide. The prevalence of the disease has risen by 30% since 2013, which is accompanied by a growing economic burden (Walton *et al*, 2020). Of note, the increased prevalence can be partially attributed to improved reporting and diagnosis. MS is the most common **non-traumatic disabling condition of young adults** that is present in both developed and developing countries (Dobson & Giovannoni, 2019). The exact causes and mechanisms behind the disease are not well-known. Nonetheless, the risk of MS increases with the distance from the equator, which may be attributed to factors such as vitamin D insufficiency and ultraviolet B (UVB) exposure. Additionally, women are twice as likely to have the disease (Walton *et al*, 2020).

1.3.2. Symptoms and pathological hallmarks of MS

In MS, the immune system attacks the insulating covers of the brain, spinal cord, and optic nerves, which results in impaired signal transduction between neurons, and eventually axonal degeneration and neurological deficits. The disease has a highly variable and unpredictable course, with different clinical forms existing. The most common form is **relapsing-remitting MS (RRMS)**, which is characterized by periods of symptoms lasting around 1-3 weeks (relapse), followed by a recovery phase (remission). Often, patients transition to **secondary progressive MS (SPMS)**, in which the relapses occur more frequently (Weiner, 2008). Around 10-15% of patients experience progressive MS from the onset, called **primary progressive MS (PPMS)** (Charabati *et al*, 2023). This MS subtype relates to higher degenerative processes and reduced inflammatory response. The symptoms experienced during MS attacks can vary widely among patients and within the same individual over different attacks. Common symptoms include muscle weakness, visual disturbances, difficulty with walking, numbness and dysfunction of the urinary and intestinal systems (Ghasemi *et al*, 2017). Although the exact cause of MS remains elusive, it is widely believed that the disease is caused by a complex **interplay between genetic and environmental factors**. Epstein-Barr virus infection is the most extensively studied non-genetic factor, but also vitamin D insufficiency, obesity and smoking may be involved in the susceptibility to MS (Ascherio, 2013). Additionally, several genes are shown to be associated with MS, with one of the strongest risk genes being the major histocompatibility complex (MHC) human leukocyte antigen (HLA) gene, specifically the DR2 haplotype. The protein encoded by this gene is present on the surface of T cells, where it plays an important role in the recognition of antigens (Weiner, 2008; Dyment *et al*, 2004). This gene, along with other risk genes associated with the immune system, strongly suggests that MS is not a disease originating in the brain, but rather a disorder of the immune system.

Hence, MS is considered to be an **autoimmune disease** characterized by inflammatory plaques in the white and grey matter of the CNS, infiltration of leukocytes, disruption of the BBB and neurodegeneration (Frohman *et al*, 2006). The formation of the demyelinating lesions is initiated by the **infiltration of myelin specific $CD4^+$ T helper cells, namely Th1 and Th17 cells,** into the CNS (Fig. 7). Remarkably, both healthy individuals and MS patients have these autoreactive T cells circulating in the peripheral blood (Pette *et al*, 1990). However, in healthy people, these T cells remain in a naïve state and are kept under control by regulatory T cells

(Tregs), whereas in MS patients, these T cells are activated by an unknown trigger. The activated T cells can cross the damaged BBB and migrate into the CNS, where they can interact again with APCs, such as macrophages and B cells, that present a myelin antigen. Alternatively, T cells can also invade the CNS through the BCSFB and blood-leptomeningeal barrier (BLMB), which provide access to the CSF and subpial space, respectively (Fig. 7) (Charabati *et al*, 2023). Once reactivated, the encephalitogenic T cells release pro-inflammatory cytokines, resulting in further **disruption of the BBB** and subsequent **infiltration and/or activation of macrophages, microglia and astrocytes** (Weiner, 2008; Frohman *et al*, 2006; Compston & Coles, 2002). Consequently, macrophages and microglia initiate the inflammatory attack on myelin sheaths and oligodendrocytes, which are the cells that produce myelin in the CNS. This triggers axonal degeneration and eventually results in the neurological symptoms associated with MS. More recently, this view was adapted by studies indicating that not only $CD4^+$ T cells but also **$CD8^+$ T cells and B cells** play a role in the development of inflammatory lesions in MS (Wagner *et al*, 2020; Machado-Santos *et al*, 2018). Notably, B cells seem to be important as APCs, generating activated myelin-reactive T cells, rather than producing antibodies (Zamvil & Hauser, 2021).

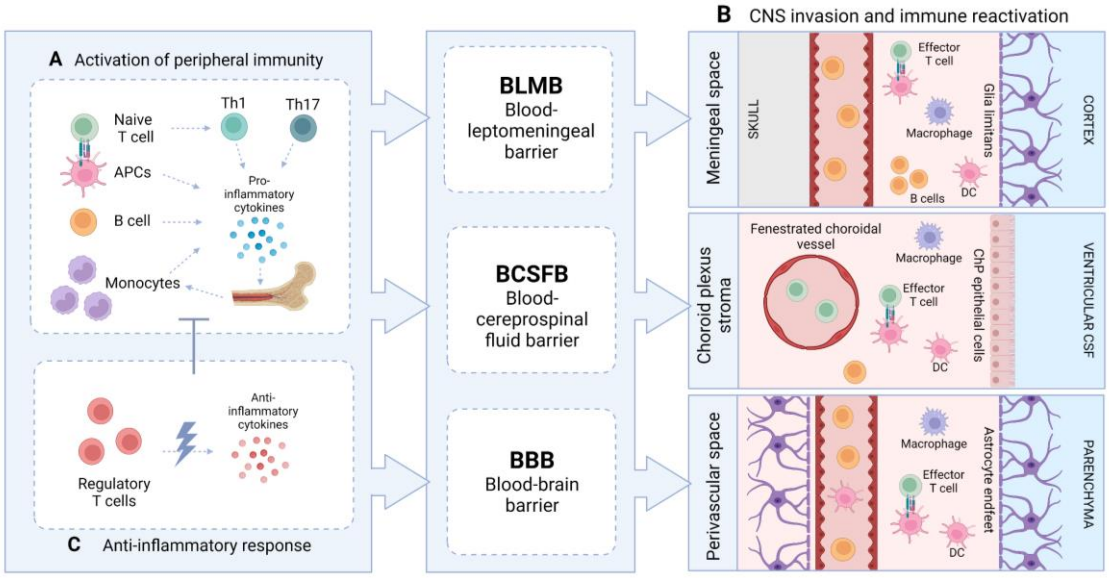


Figure 7: Current perspective of multiple sclerosis (MS) pathogenesis. **(A)** T cells become activated in the periphery by antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells. The secretion of pro-inflammatory cytokines causes further activation of macrophages and disruption of the blood-brain barrier (BBB). **(B)** Self-reactive T cells infiltrate the CNS through the BBB, the blood-leptomeningeal barrier (BLMB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB provides direct access to the brain, while the BCSFB provides access to the CSF, and thus, indirectly to the parenchyma. The BLMB provides access to the subpial space and, to some extent, the CSF. Once in the CNS, the T cells become reactivated by local APCs. **(C)** Impairment of anti-inflammatory responses, managed by regulatory T cells, contributes to MS pathogenesis. Image created with BioRender based on (Charabati *et al*, 2023).

In healthy individuals, Tregs produced in the thymus are essential for **maintaining immune tolerance and preventing autoimmune diseases** (Vignali *et al*, 2008). Interestingly, in MS, defects in the number and/or function of Tregs have been reported, such as reduced suppressive capacity, stability and migratory potential (Dominguez-Villar & Hafler, 2018; Li *et al*, 2019; Venken *et al*, 2008). In MS mouse models, adoptive transfer of functional Tregs suppressed self-reactive T cell-induced inflammation and boosted remyelination (Curotto de Lafaille *et al*, 2008; Dombrowski *et al*, 2017). These findings imply that a cell-based therapy centred on Tregs might be an interesting approach for treating MS.

Intriguingly, researchers have challenged the view of MS as an autoimmune disorder and propose that **MS might be a primary degenerative disorder**, such as AD and Parkinson's disease (Stys *et al*, 2012). This is supported by a study where oligodendrocyte death and microglia activation preceded the infiltration of lymphocytes in early MS lesions of RRMS patients (Barnett & Prineas, 2004). These findings indicate that loss of oligodendrocytes might be the root cause of MS that, on one hand, activates the innate immune response to clear the debris and, on the other hand, triggers a secondary immune cell infiltration in response to myelin shedding (Stys *et al*, 2012). Further evidence comes from the fact that most anti-inflammatory drugs are effective in RRMS patients, but not in PPMS patients (Charabati *et al*, 2023). Altogether, the **adaptive immune system plays a prominent role in MS pathogenesis**, however, the exact mechanisms underlying MS remain uncertain.

1.3.3. The EAE mouse model for preclinical MS research

MS pathogenesis and potential therapeutic strategies have been extensively studied using the **experimental autoimmune encephalomyelitis (EAE) mouse model** (Robinson *et al*, 2014, 8). EAE in mice is either induced by **immunization with myelin antigens** in adjuvant, which leads to the activation of autoreactive T cells, or by the **adoptive transfer** of these activated auto-antigen T cells (Croxford *et al*, 2011). Their target antigens are mostly myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). This process leads to infiltration of T cells and monocytes in the brain, and demyelination of axons, which are features like those observed in MS patients. However, it is essential to view the results obtained from the EAE model with a critical lens. The model is based on inflammation induced by self-reactive $CD4^+$ T cells against one or a few myelin antigens, which does not take into account the complex responses of $CD8^+$ T cells and B cells (Lassmann & Bradl, 2017).

1.3.4. Latest developments in MS therapy

In contrast to the unmet clinical need in AD therapy, many disease-modifying therapies for MS have received FDA approval. MS drugs act on the peripheral immune system and work through three main mechanisms: **immunomodulation**, **inhibition of immune cell trafficking** and **immunodepletion** (Charabati *et al*, 2023). Most of these therapies target the relapsing stages of MS that are characterized by an inflammatory attack. Unfortunately, they are often not effective for patients suffering from PPMS. Immunomodulatory drugs, such as IFN-β treatment, can limit the pro-inflammatory phenotype of $CD4^+$ and $CD8^+$ T cells and boost anti-inflammatory Treg cell production (Baecher-Allan *et al*, 2018). Anti-trafficking drugs limit the entry of immune cells into the CNS. For example, natalizumab does this by blocking very late antigen-4 (VLA-4), which is a surface receptor used by circulating leukocytes to migrate into the CNS (Brandstadter & Katz Sand, 2017). Immunodepletion can be done for both T and B lymphocytes. For instance, ocrelizumab is an anti-CD20 monoclonal antibody that effectively depletes B cells (Greenfield & Hauser, 2018). Interestingly, this treatment is also shown to be effective for PPMS patients (Montalban *et al*, 2017). Taken together, many advancements have been made in the development of disease-modifying therapies for MS, however, a lot of these drugs are still associated with notable side effects. Additionally, effectively treating progressive MS continues to be a major hurdle that requires a deeper understanding of the disease and its subtypes.

1.3.5. The brain barriers in MS

In MS, a chronic inflammatory and demyelinating disease of the CNS, the breakdown of the BBB is a key hallmark (Charabati *et al*, 2023). Increased permeability of the BBB is believed to be an early event in MS that allows immune cells, such as T cells and monocytes, to infiltrate the CNS. Once in the CNS, they attack the myelin sheath and contribute to neuroinflammation, resulting in demyelination, axonal loss and ultimately neurodegeneration (Engelhardt *et al*, 2022). Typically, immune cells can cross endothelial cells by rolling, adhesion and diapedesis. Cell-cell adhesion is facilitated through the interaction between integrins expressed on leukocytes and their ligands expressed on endothelial cells. Notably, integrins such as **lymphocyte function-associated antigen-1 (LFA-1)** and **VLA-4** engage with **intercellular adhesion molecules-1 (ICAM-1) and vascular adhesion molecules-1 (VCAM-1)** situated on the surface of endothelial cells (Charabati *et al*, 2023). Normally, endothelial cells of the BBB have low expression of VCAM-1 and ICAM-1. However, inflammatory conditions upregulate the expression of these surface adhesion molecules, and additionally, reduce junctional integrity (Engelhardt *et al*, 2022). Thus, a **first wave** of immune cell infiltration can increase the permeability of the BBB by releasing pro-inflammatory cytokines, leading to a massive **second wave** of self-reactive T cell infiltration (Balasa *et al*, 2021). This results in a self-feeding loop eventually causing neurodegeneration and symptoms associated with MS. The first wave of immune cells is believed to enter through the BCSFB (Reboldi *et al*, 2009) and/or the blood-leptomeningeal barrier (Bartholomäus *et al*, 2009), as shown in rodent models. Notably, a role for C-C motif chemokine ligand 20 (CCL20), a chemokine constitutively expressed on ChP epithelial cells, was shown for T cell entry across the BCSFB (Reboldi *et al*, 2009).

While the mechanisms of immune infiltration described above are mainly based on $CD4^+$ T cells, self-reactive $CD8^+$ T cells employ additional mechanisms suggested to rely on antigen presentation in MHC-I by BBB endothelial cells (Galea *et al*, 2007). This is supported by a recent study of Aydin *et al.* that shows that BBB endothelial cells can present exogenous antigens to $CD8^+$ T cells both *in vitro* and *in vivo* (Aydin *et al*, 2023). Specifically, these endothelial cells can take up and process self-antigens from both the luminal (i.e., blood side) and abluminal side, and subsequently **present these antigens in MHC-I to $CD8^+$ T cells that circulate in the bloodstream**. During inflammation, adhesion molecules are upregulated allowing $CD8^+$ T cells to adhere and recognize their antigen. It was previously proposed that antigen recognition on brain endothelial cells results in crawling of $CD8^+$ T cells over the BBB followed by diapedesis across the BBB. However, the authors demonstrated that upon antigen recognition, these autoantigen $CD8^+$ T cells receive a stop signal and induce **endothelial apoptosis and breakdown of the inflamed BBB**. Notably, besides antigen recognition, the activation of naïve $CD8^+$ T cells requires a second signal. This antigen-independent signal is mediated by the interaction between co-receptors CD80 and CD86 on the APC and CD28 on T cells. However, there is ongoing debate regarding the presence of these co-stimulatory molecules on endothelial cells. Interestingly, studies have revealed that adhesion molecules such as ICAM-1 and VCAM-1 can serve as alternative co-stimulatory signals for T cell activation in the absence of CD80 and CD86, providing an alternative mechanism for $CD8^+$ T cell activation at the BBB (Pober *et al*, 2017). Limiting immune cell trafficking across the BBB has been successful in treating RRMS. For example, natalizumab targets VLA-4 on leukocytes and significantly reduces the formation of new lesions (Baecher-Allan *et al*, 2018). Interestingly, besides preventing T cell infiltration, these therapies might also play an important role in inhibiting $CD8^+$ T cell-mediated BBB breakdown (Aydin *et al*, 2023).

1.3.6. The adaptive immune system in MS

In contrast to AD, the involvement of the adaptive immune response in MS onset and progression is more evident. As mentioned above, particularly T cells, and to a lesser extent B cells, play a crucial role in the pathogenesis of MS. Among **$CD4^+$ T cells**, the Th1 and Th17 subsets are primarily implicated in the disease process, targeting myelin proteins in the CNS (Charabati *et al*, 2023). These T cell subsets secrete pro-inflammatory cytokines such as interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα), interleukin (IL)-6, IL-12, and IL-23. Amongst other things, these cytokines activate microglia and monocytes, facilitate inflammatory cell infiltration, disrupt the brain barriers, and contribute to neurodegeneration (Charabati *et al*, 2023). Additionally, accumulating evidence shows a role for **$CD8^+$ T cells** in MS. $CD8^+$ T cells are more abundant in active MS lesions compared to $CD4^+$ T cells and their expansion is shown in the blood and CSF of MS patients (Lassmann, 2019). However, it remains unclear whether these T cells are detrimental or beneficial (Wagner *et al*, 2020). Some studies show a suppressive effect of $CD8^+$ T cells on myelin-specific $CD4^+$ T cell activity (Baughman *et al*, 2011), while other studies reveal that $CD8^+$ T cells also contribute to the damaging of the myelin sheaths and oligodendrocytes, leading to demyelination and disease progression. The latter is supported by the close proximity of $CD8^+$ T cells to oligodendrocytes and their polarization of cytolytic granules towards these cells (Saxena *et al*, 2008). In contrast, **Tregs** exert a protective role in MS by suppressing immune responses and secreting anti-inflammatory cytokines (Dominguez-Villar & Hafler, 2018). Lastly, **B cells** mainly act as APCs, presenting myelin antigens to T cells and promoting their activation (Zamvil & Hauser, 2021). Moreover, B cells can interact with other immune cells and secrete pro-inflammatory cytokines, further amplifying the inflammatory response within the CNS (Li *et al*, 2018).

Part 2: Aim of Research Project

The blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) play a critical role in protecting the brain parenchyma from harmful substances and immune cells and maintaining a stable milieu for neuronal function. Amongst other factors, the presence of these tight barriers funded the idea of the brain as an immune-privileged site. However, more recent findings have indicated a more complex interplay between the immune system and the brain than previously assumed, thereby changing this perspective. It is now widely accepted that the brain barriers divide the central nervous system (CNS) into compartments that differ with respect to their accessibility to the immune system. Intriguingly, disruption of the brain barriers is a common feature in many neurological disorders, including multiple sclerosis (MS) and Alzheimer's disease (AD), allowing infiltration of innate and adaptive immune cells that contribute to the disease. The precise mechanisms involved are not yet fully understood, however, **reversing or halting this barrier breakdown might be a promising avenue for treating these diseases**. To understand the dysfunction of the brain barriers in disease states, it is important to have a more comprehensive understanding of how they function in non-inflammatory conditions. In addition, emerging evidence shows that T cells home to the brain and shape different CNS components. For instance, T cells patrol brain borders during immune surveillance (Engelhardt *et al*, 2017), and recently identified brain-resident $CD4^+$ T cells were shown to regulate microglia maturation (Pasciuto *et al*, 2020).

Consequently, we hypothesized that the adaptive immune system, particularly T cells, could potentially shape the integrity of the CNS barriers. Therefore, in this thesis, we aim to **investigate whether a lack of adaptive immunity impacts the integrity of the BBB and the BCSFB**. To achieve this, we make use of *Rag2*$^{-/-}$ mice, a mouse model that lacks adaptive immunity (Shinkai *et al*, 1992), and reconstitution via adoptive transfer of T and/or B cells. Additionally, to verify findings in this model, we make use of antibody-mediated depletion of T and B cells in wild type mice.

Secondly, if interesting differences occur, we will dive deeper into T and/or B cell subsets and try **to pinpoint a specific lymphocyte subset important in shaping and/or maintaining the brain barriers**. Moreover, we aim to unravel the mechanisms by which this specific lymphocyte subset ensures proper brain barrier functionality in **healthy conditions** and in **mouse models of AD and MS**. For the latter, we make use of transgenic *App*$^{NL-G-F}$ mice and experimental autoimmune encephalomyelitis (EAE) mice to model AD and MS, respectively.

Overall, the goal of this thesis is to contribute to a more complete understanding of the **effect of the adaptive immune system on the brain barriers in health and disease**, and consequently, improve the ability to treat and manage neurological disorders.

Part 3: Results

3.1. Characterization of the *Rag2*$^{-/-}$ mouse model

3.1.1. The permeability of the brain barriers is increased in *Rag2*$^{-/-}$ mice

The adaptive immune response comprises the humoral and cell-mediated response, respectively mediated by B and T lymphocytes. As described before, the role of the adaptive immune system is evident in diseases like MS and is becoming increasingly prominent in diseases like AD. However, since the brain was long considered to be sealed off from the immune system, the impact of the adaptive immune cells on CNS components in non-pathological conditions is still an area of intensive research. To investigate the effect of the adaptive immune system on the CNS barriers, the *Rag2*$^{-/-}$ mouse model was used. These mice fail to generate mature T or B lymphocytes and thus lack an adaptive immune response. To confirm adaptive immune deficiency in this model, flow cytometry was performed on splenocytes extracted from *Rag2*$^{-/-}$ mice. Analysis confirmed a lack of $CD3^+$ T cells and $CD19^+$ B cells (Fig. 8A). Next, the leakiness of the BCSFB and the BBB of mice with and without a functional adaptive immune system, respectively wild type and *Rag2*$^{-/-}$ mice, were compared. For this, 4 kilodalton (kDa) fluorescein isothiocyanate (FITC)-dextran was intravenously (i.v.) injected and systemically circulated for 15 min (Fig. 8B). Subsequently, BBB and BCSFB leakage was assessed. For the BBB, different regions were separately analysed, namely the hippocampus, cortex, and cerebellum. Mice were perfused with PBS to remove all the blood present in the brain. To look at the BCSFB leakage, CSF was taken by cisterna magna puncture. Next, fluorescence was quantified as a measure of the leakage of FITC-dextran across the BBB and BCSFB. Measurement of fluorescence showed significantly higher leakage of FITC-dextran from the blood into the CSF and the brain parenchyma of the hippocampus, cortex, and cerebellum in *Rag2*$^{-/-}$ mice (Fig. 8C-D).

3.1.2. Tight junctions are impaired in the brain barriers of *Rag2*$^{-/-}$ mice

Besides determining the permeability of the BCSFB and the BBB, tight junctional integrity was evaluated. The BCSFB and BBB have differences in tight junctional proteins, however, ZO-1 is important in both barriers. Confocal imaging revealed a more diffuse distribution of ZO-1 protein in *Rag2*$^{-/-}$ mice compared to wild type mice (Fig. 9). At the level of the BCSFB, total ZO-1 expression did not show a statistically significant difference, although a noticeable trend is visible. However, when the maximal length of continuous ZO-1 expression was measured, a significant difference was observed between wild type and *Rag2*$^{-/-}$ mice (Fig. 9A). Additionally, the distribution of continuous ZO-1 length was analysed, revealing a significant reduction in ZO-1 fragments longer than 20 µm in *Rag2*$^{-/-}$ mice compared to wild type mice, while shorter fragments did not show a difference (Fig. 9A). Among the different regions where the BBB is present, only at the level of the cerebellum a significant reduction in ZO-1 expression in *Rag2*$^{-/-}$ mice compared to wild type mice was observed (Fig. 9B).

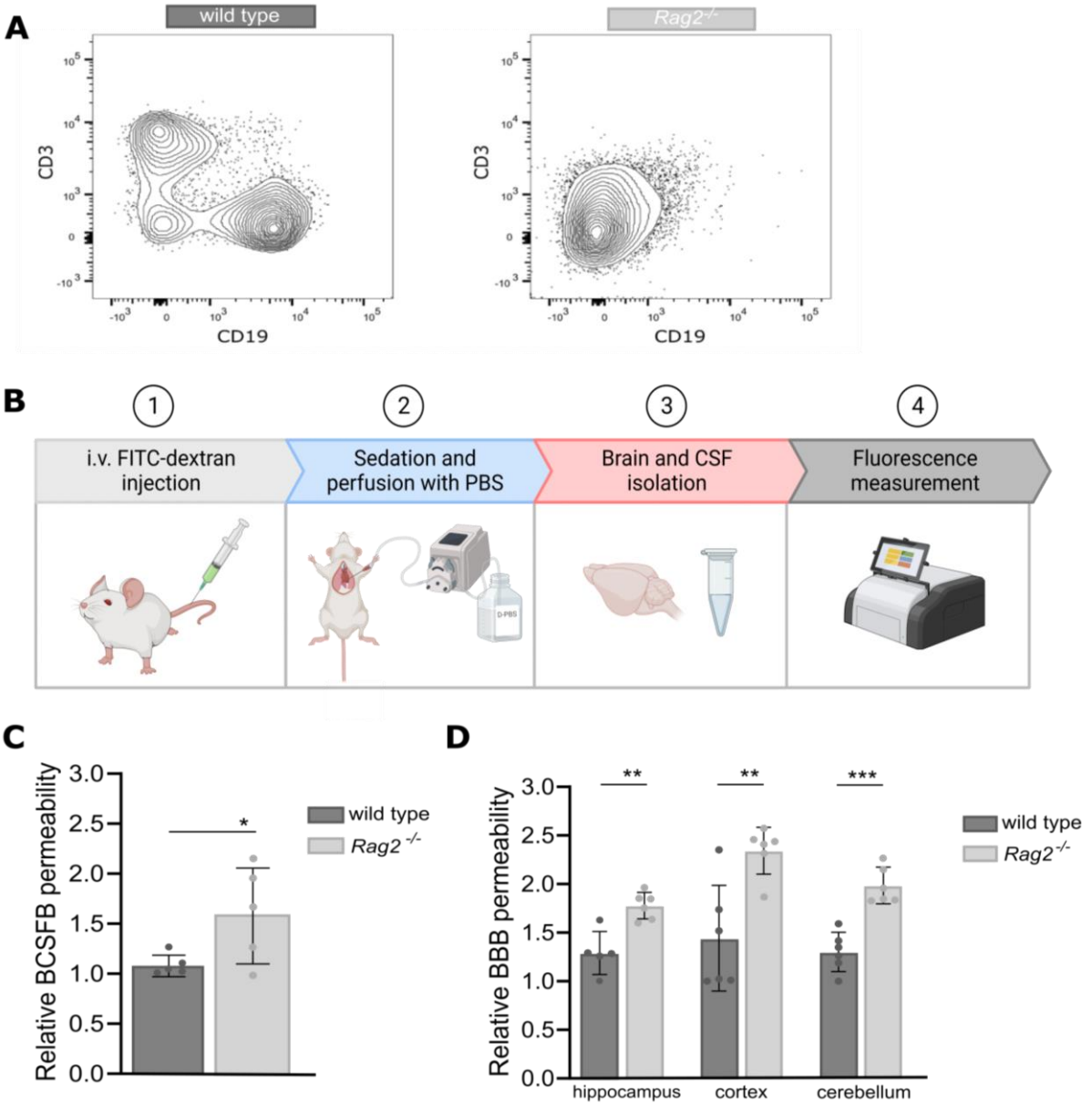


Figure 8: Increased permeability of the blood-cerebrospinal fluid barrier (BCSFB) and the blood-brain barrier (BBB) in *Rag2*$^{-/-}$ mice. (A) Flow cytometry was performed on splenocytes from wild type and *Rag2*$^{-/-}$ mice to confirm a lack of CD3^{+} T cells and CD19^{+} B cells in *Rag2*$^{-/-}$ mice. **(B)** The relative brain barrier permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran that leaked into the CSF and brain parenchyma of the hippocampus, cortex, and cerebellum after intravenous (i.v.) injection. Mice were sedated with ketamine/xylazine and perfused with D-PBS to remove FITC-dextran-containing blood from the circulation. Next, CSF was isolated via cisterna magna puncture and the brain regions were isolated. Lastly, fluorescence was measured in the CSF and, after overnight formamide extraction, in the brain regions. **(C)** Relative BCSFB permeability was determined in the CSF 15 min after i.v. injection of 4 kDa FITC-dextran. **(D)** Relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=5/6 mice per group). Statistical significance was determined by two-tailed Student's t-test; *p < 0.05, ** p < 0.01, *** p < 0.001.

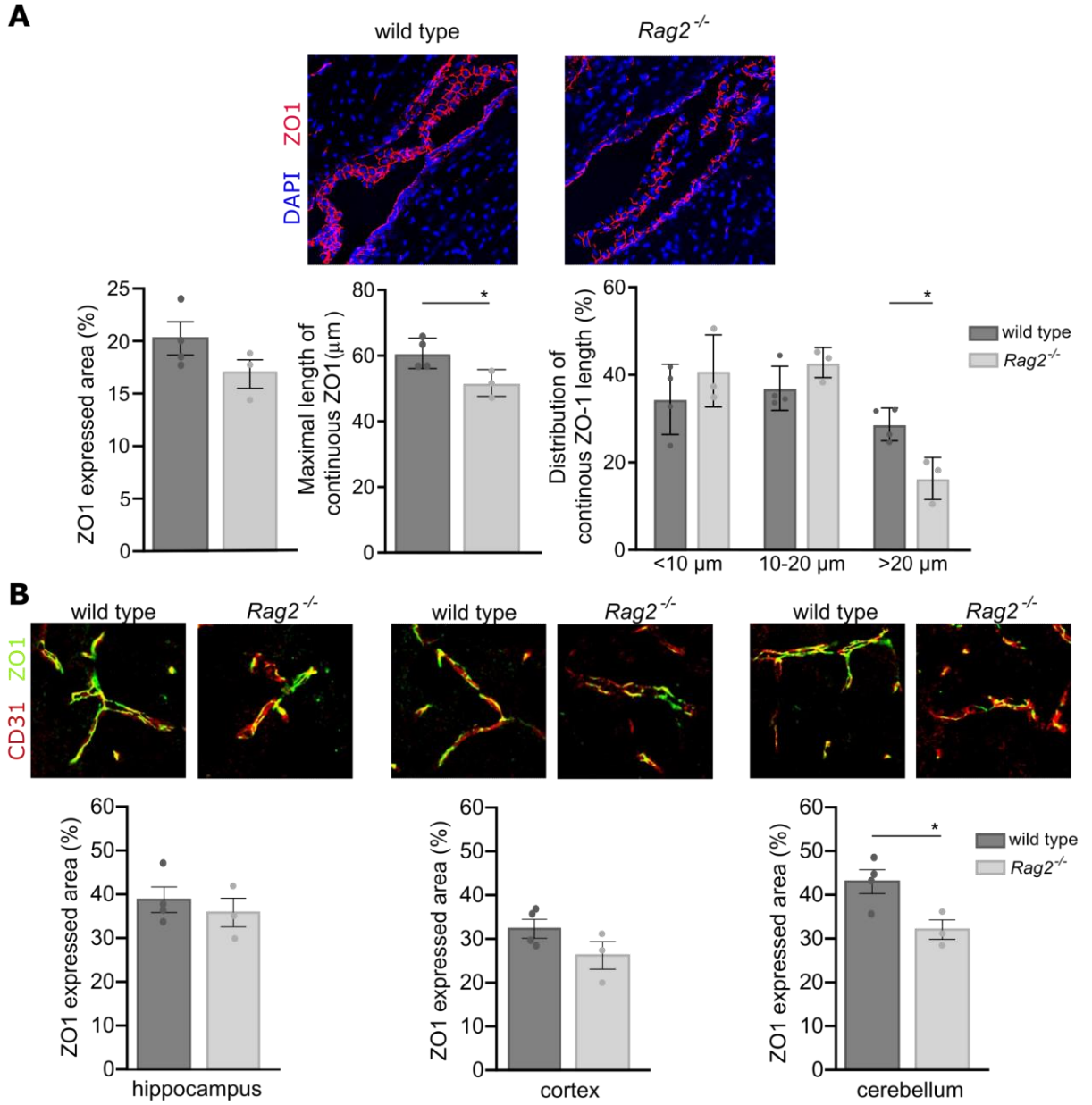


Figure 9: Impaired zonula occludens-1 (ZO-1) protein expression in the blood-cerebrospinal fluid barrier (BCSFB) and the blood-brain barrier (BBB) of *Rag2*$^{-/-}$ mice. (A) On top: representative confocal images of ZO-1 (red) and DAPI (blue) immunostaining in the choroid plexus of wild type and *Rag2*$^{-/-}$ mice. Below: a comparison of total ZO-1 signal, the maximal length of continuous ZO-1 (µm), and the distribution of the lengths of continuous ZO-1 (µm) in wild type and *Rag2*$^{-/-}$ mice. **(B)** On top: representative confocal images of ZO-1 (green) and CD31 (red) immunostaining at the vasculature of the hippocampus, cortex, and cerebellum of wild type and *Rag2*$^{-/-}$ mice. Below: a comparison of total ZO-1 signal in hippocampus, cortex, and cerebellum of wild type and *Rag2*$^{-/-}$ mice. Data are shown as mean ± SEM (n=3/4 mice per group). Quantification was done by Junhua Xie. Statistical significance was determined by two-tailed Student's t-test; *p < 0.05.

3.1.3. The sealing capacity of astrocyte endfeet is reduced in *Rag2*$^{-/-}$ mice

The BBB is a complex and organized structure with both cellular and acellular components, wherein astrocytes play a pivotal role in ensuring barrier integrity (Engelhardt *et al*, 2017). This critical function is attributed to the endfeet of astrocytes, which function as sealing structures around blood vessels. To evaluate whether the sealing capacity of these endfeet is compromised in *Rag2*$^{-/-}$ mice, potentially contributing to increased BBB permeability, immunostaining with aquaporin 4 (AQP4) antibodies was done, followed by confocal microscopy imaging. Subsequently, a line was drawn through the blood vessels, and the AQP4 signal intensity along this axis was quantified (Fig. 10A). The maximum intensity signals of several blood vessels were determined and compared between wild type and *Rag2*$^{-/-}$ mice. The results revealed a significant reduction in the sealing capacity of astrocytes in *the Rag2*$^{-/-}$ mice (Fig. 10B).

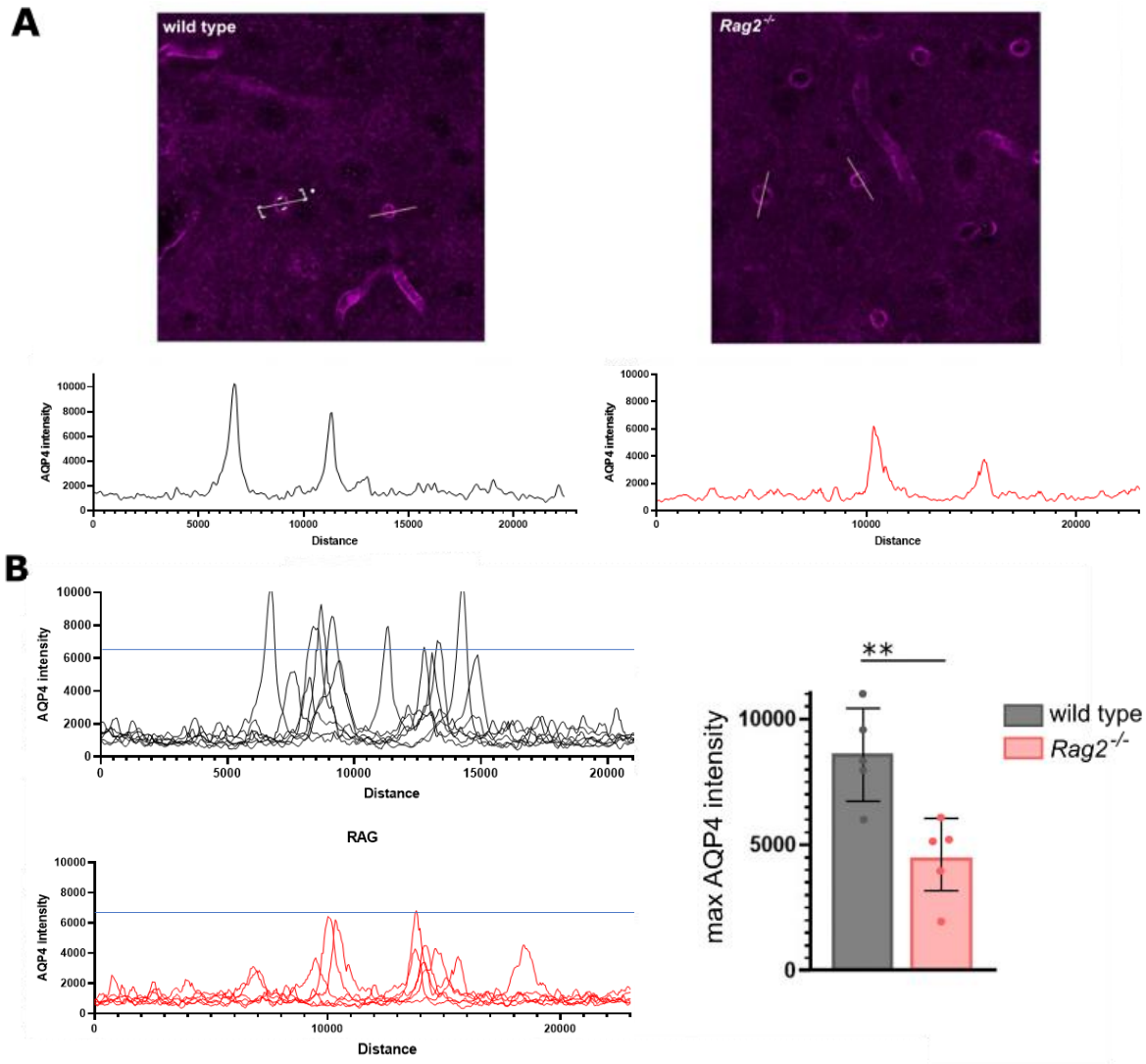


Figure 10: Less sealing capacity of astrocyte endfeet in *Rag2*$^{-/-}$ mice. (A) Astrocyte endfeet, surrounding brain vessels, were stained on brain sections of wild type and *Rag2*$^{-/-}$ mice with anti-aquaporin 4 (AQP4) antibody and visualized by confocal microscopy. On top: representative confocal images of AQP4 staining. Below: the intensity of the staining was determined along a line through each blood vessel and plotted. **(B)** Left: measurements of multiple blood vessels were merged. Right: the maximum AQP4 intensities were compared between wild type and *Rag2*$^{-/-}$ mice. Data are shown as mean ± SEM (n=5 per group). Quantification was done by Junhua Xie. Statistical significance was determined by two-tailed Student's t-test; ** $p < 0.01$.

3.2. Brain barrier integrity in presence and absence of T and B cells

3.2.1. T cell adoptive transfer in *Rag2*$^{-/-}$ mice rescues brain barrier integrity

To determine whether replacing the missing adaptive immune cell populations in the periphery could reverse the effects of immune deficiency on brain barrier integrity in *Rag2*$^{-/-}$ mice, adoptive transfer of mature T and/or B cells was performed. CD3^{+} T cells and CD19^{+} B cells were isolated from the spleen of a wild type donor and i.v. injected into *Rag2*$^{-/-}$ acceptor mice (Fig. 11A). One week after adoptive transfer, leakage of the barriers was assessed similarly as described above (Fig. 11B). The results demonstrated that adoptive transfer of T cells leads to a reduction in permeability of both the BBB and BCSFB in *Rag2*$^{-/-}$ mice (Fig. 11B-C). Transfer of B cells alone did not restore barrier integrity, while the combined transfer of T and B cells did, indicating a main role of T cells in this process.

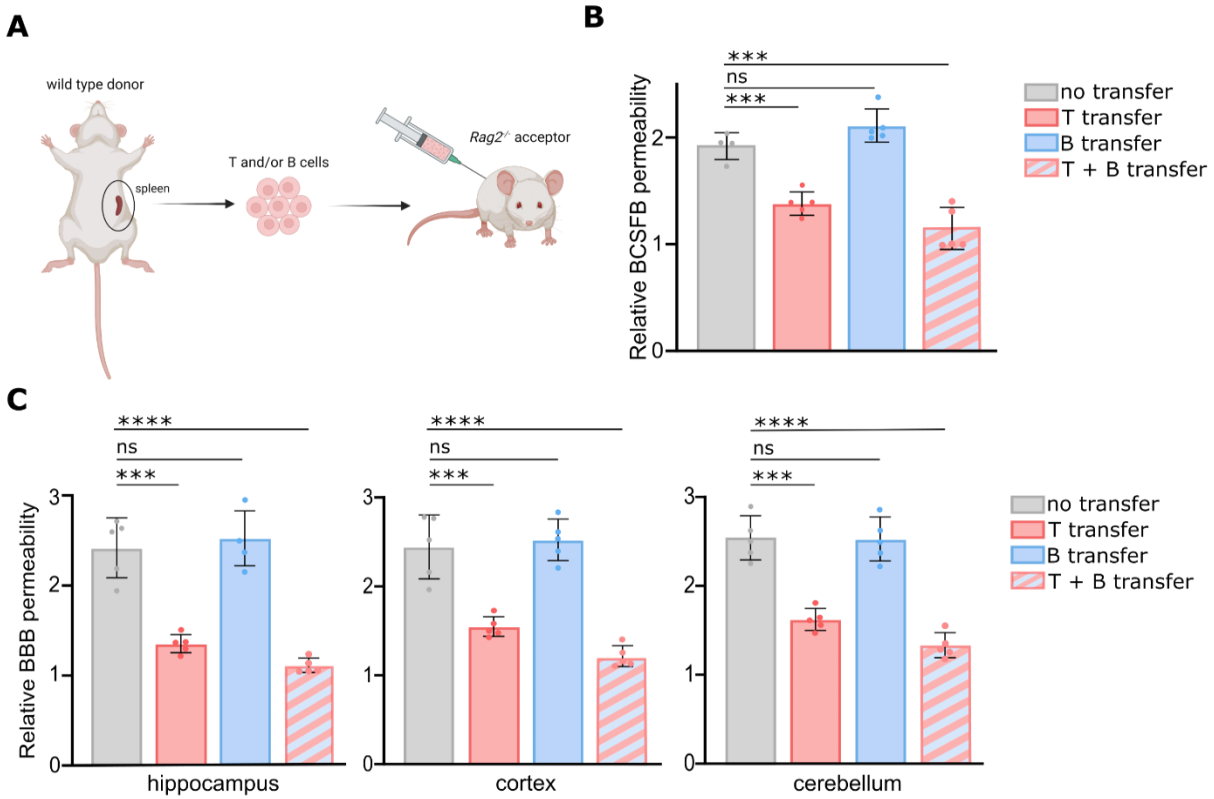


Figure 11: T and B cell adoptive transfer improves the blood-CSF barrier (BCSFB) and blood-brain barrier (BBB) integrity in *Rag2*$^{-/-}$ mice. (A) Spleens were isolated from wild type donor mice. T and/or B cells were isolated and subsequently adoptively transferred to *Rag2*$^{-/-}$ acceptor mice. **(B)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after intravenous (i.v.) injection. **(C)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=4/5 mice per group). Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparisons test; *** $p < 0.001$, **** $p < 0.0001$, ns: non-significant.

3.2.2. T cell depletion in wild type mice promotes brain barrier disruption

To validate the effects of T cells on barrier integrity observed in *Rag2*$^{-/-}$ mice, antibody-mediated depletion of T and/or B cells in wild type mice was performed (Fig. 12A). CD3ε polypeptide chain antibody against the T cell receptor (TCR) complex was used for T cell depletion, while an antibody against B220, an isoform of CD45 expressed on B cells, was used for B cell depletion. Non-reactive isotype antibodies were used as a control. The depletion of T and B cells was verified by flow cytometry (data not shown). Consistent with the findings above, T cell depletion significantly increased the permeability of the BCSFB and BBB, while B cell depletion had no significant impact on barrier integrity (Fig. 12B-C). Furthermore, the depletion of both T and B cells resulted in a comparable increase in barrier permeability as T cell depletion alone.

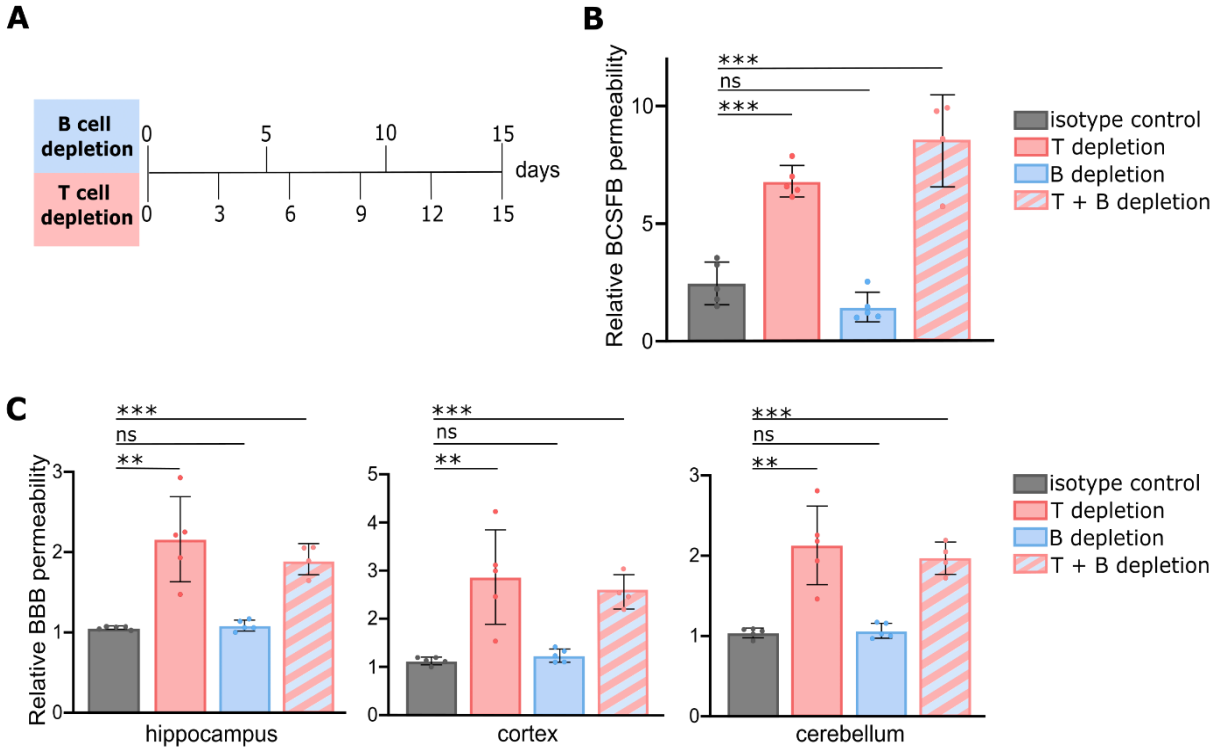


Figure 12: T and B cell depletion in wild type mice impairs the blood-CSF barrier (BCSFB) and blood-brain barrier (BBB). (A) In wild type mice, antibodies against T cells were intraperitoneally (i.p.) injected every 3 days for 15 days. Antibodies against B cells were i.p. injected every 5 days for 15 days. Wild type mice injected with non-reactive isotype antibodies were used as control. **(B)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after intravenous (i.v.) injection. **(C)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=4/5 mice per group). Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparisons test; **p < 0.01, *** p < 0.001, ns: non-significant.

3.2.3. The presence of Tregs is essential for brain barrier integrity

The previous findings identified the impact of T cells, but not B cells, on barrier integrity in non-inflammatory conditions. Consequently, it is important to pinpoint these effects to a specific T cell subtype. Interestingly, a study in mouse models of ischemic stroke indicated that adoptive Treg treatment protects against BBB disruption (Li *et al*, 2013). Tregs are immunosuppressive $CD4^+$ T cells that maintain immune homeostasis and prevent autoimmunity (Vignali *et al*, 2008). Besides their role in cerebral ischemia, a reduction of the functionality and/or number of Tregs is common in many other neurological disorders, such as in AD and MS. Remarkably, disruption of the brain barriers is a key factor in the pathogenesis of MS and is increasingly being recognized as a prominent factor in the progression of AD. However, it remains unclear whether there is a direct link between the dysfunction and reduction of Tregs and the compromised integrity of the brain barriers in these diseases. Therefore, the capacity of Tregs to directly impact the brain barriers was investigated under normal physiological conditions. To this end, $CD4^+CD25^+$ Tregs were isolated from wild type donors and adoptively transferred into *Rag2*$^{-/-}$ mice to determine whether they could restore the impaired barrier function that was previously observed in this mouse model (Fig. 13A). Brain barrier leakage was assessed after i.v. injection of FITC-dextran, as described before. Results showed a significant decrease in BCSFB and BBB permeability upon transfer of Tregs (Fig. 13B-C).

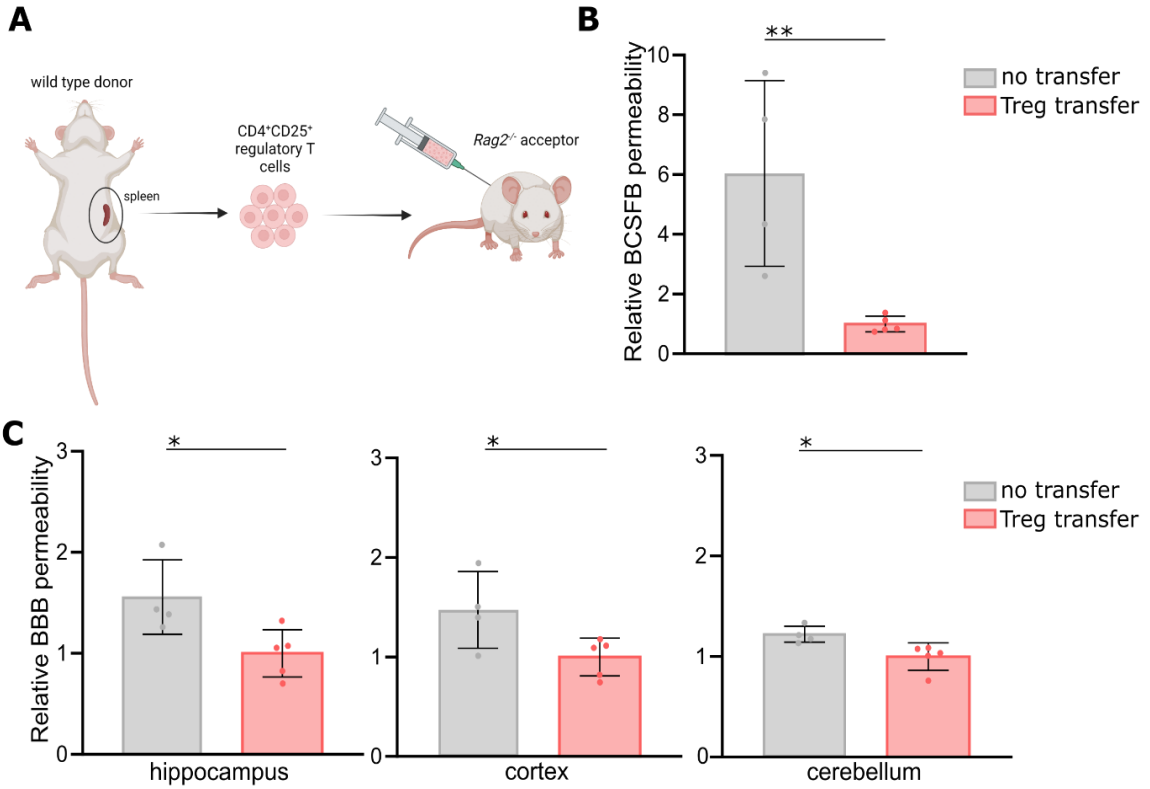


Figure 13: Regulatory T cells (Treg) adoptive transfer improves blood-CSF barrier (BCSFB) and blood-brain barrier (BBB) integrity in *Rag2*$^{-/-}$ mice. (A) Spleens were isolated from wild type donor mice. $CD4^+CD25^+$ cells were isolated and subsequently adoptively transferred to *Rag2*$^{-/-}$ acceptor mice. **(B)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after intravenous (i.v.) injection. **(C)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=4/5 mice per group). Statistical significance was determined by two-tailed Student's t-test; *$p < 0.05$, **$p < 0.01$.

Additionally, antibody-mediated depletion of Tregs was performed to validate the effects seen in *Rag2*$^{-/-}$ mice (Fig. 14A). Depletion of the Tregs was done with anti-CD25 antibody and was verified by flow cytometry analysis (data not shown). Subsequently, brain barrier leakage was assessed as described before. The results revealed that the leakiness of the BCSFB and BBB was significantly increased in wild type mice without Tregs (Fig. 14B-C). These findings are consistent with the results observed in *Rag2*$^{-/-}$ mice and point to an important role for Tregs in maintaining brain barrier integrity.

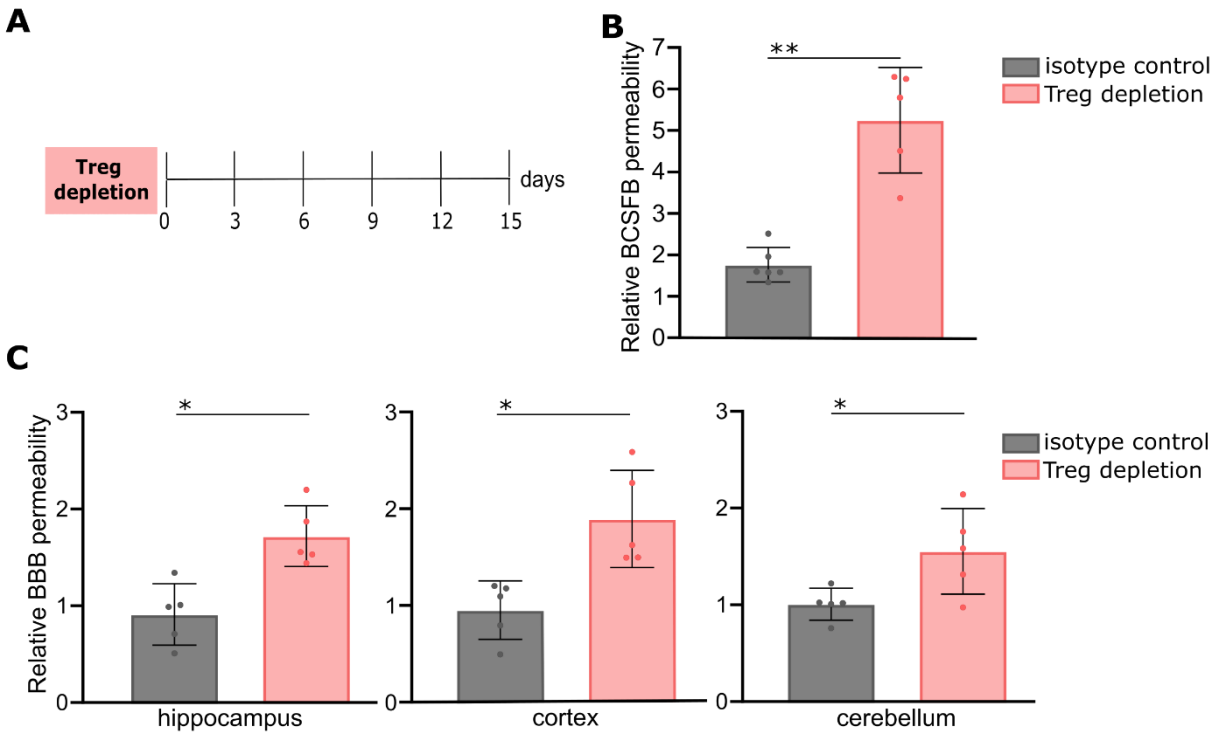


Figure 14: Regulatory T (Treg) depletion impairs the blood-CSF barrier (BCSFB) and blood-brain barrier (BBB) in wild type mice. (A) In wild type mice, antibodies against Treg cells were intraperitoneally (i.p.) injected every 3 days for 15 days. Wild type mice injected with non-reactive isotype antibodies were used as control. **(B)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after intravenous (i.v.) injection. **(C)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=5/6 mice per group). Statistical significance was determined by two-tailed Student's t-test; *p<0.05, **p < 0.01.

3.3. The mechanisms behind the effect of Tregs on brain barrier integrity

3.3.1. Tregs possibly exert their effect on the brain barriers through IL-34

Interestingly, in the lab of Prof. Bieke Broux (UHasselt), they recently discovered that in untreated RRMS patients, the percentage of IL-34 expressing Tregs is decreased compared to those of healthy individuals (Fig. 15A, data not published). In addition, Jin *et al.* showed that IL-34 restored *in vitro* BBB disruption induced by pro-inflammatory cytokines through the upregulation of TJ proteins (Jin *et al*, 2014). Altogether, these data suggest that IL-34 plays a crucial role in Treg function. Lack of IL-34 might negatively impact disease progression and possibly affect the integrity of the brain barriers. Therefore, there is a need to clarify if IL-34 expressed by Tregs has an impact on the brain barriers in neuroinflammatory conditions, and if so, how this process works.

IL-34 acts through binding of three receptors: colony-stimulating factor 1 receptor (CSF-1R), receptor-type protein-tyrosine phosphatase-zeta (PTP-ζ) and syndecan-1 (Lin *et al*, 2008; Nandi *et al*, 2013; Segaliny *et al*, 2015). RNA-sequencing databases suggest that mainly CSF-1R is highly expressed on the ChP and brain microvessels (Fig. 15C). Moreover, the study by Jin *et al.* showed that CSF-1R is expressed on endothelial cells of the BBB and that inhibition of the receptor ablates the positive effect IL-34 has on pro-inflammatory cytokine-induced BBB disruption *in vitro* (Jin et al, 2014). Additionally, the lab of Prof. Bieke Broux showed the presence of the CSF-1R on the human brain microvascular endothelial cell line hCMEC/D3 (Fig. 15B). This has led to the hypothesis that IL-34 might exert the protective effect on the brain barriers through binding of the CSF-1R.

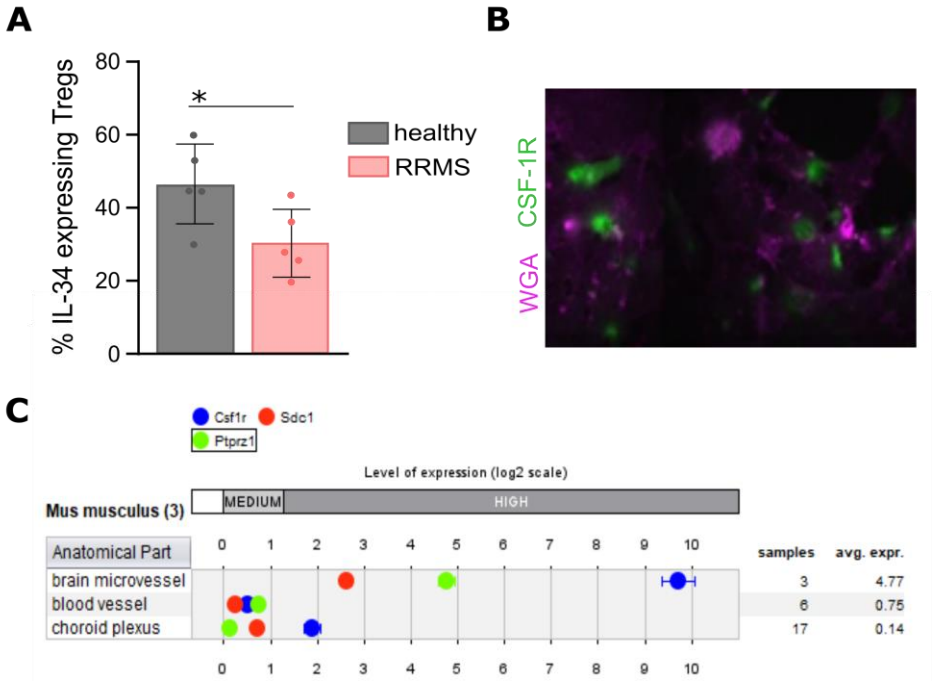


Figure 15: Regulatory T cells (Tregs) secrete interleukin (IL)-34 that possibly impacts brain barriers through colony-stimulating factor 1 receptor (CSF-1R) binding. (A) Data from the lab of Prof. Bieke Broux (UHasselt) shows that peripheral Tregs from healthy controls produce IL-34, which is significantly reduced in Tregs from relapsing-remitting multiple sclerosis (RRMS) patients, as measured by flow cytometry (data not published). **(B)** In the lab of Prof. Bieke Broux, they showed that CSF-1R is expressed on human brain microvascular endothelial cell line hCMEC/D3 (data not published). **(C)** RNA-sequencing data shows mRNA expression levels of the CSF-1R, receptor-type protein-tyrosine phosphatase-zeta (PTP-ζ) and syndecan-1 in brain microvessels and the choroid plexus (ChP). Created with GENEVESTIGATOR. WGA, Wheat germ agglutinin.

3.3.2. *In vitro* application of IL-34 restores brain barrier disruption

To investigate whether IL-34 impacts the integrity of the brain barriers, *in vitro* transwell experiments were performed. First, ChP epithelial (CPE) cells were isolated from wild type and *Rag2*$^{-/-}$ mice, and subsequently seeded onto a semi-permeable filter within the upper chamber of a transwell insert. Over time, the cells formed a tightly interconnected monolayer, which was assessed by monitoring the trans-epithelial electrical resistance (TEER), and experiments were started when both wild type and *Rag2*$^{-/-}$ CPE cells reached a similar TEER (Fig. S1). These *in vitro* barrier systems closely mimic the *in vivo* situation, wherein the apical section of the transwell insert represents the CSF side, while the basal compartment represents the blood side of the CPE cells. Consequently, the permeability of the barriers was assessed by introducing 4 kDa FITC-dextran to the blood side, followed by the measurement of fluorescent leakage into the apical section, and thus the CSF. CPE cells of both wild type and *Rag2*$^{-/-}$ mice were treated with IL-34 for 8 hours to assess whether this improves *in vitro* BCSFB integrity. Cells treated with PBS were used as a control. At the 6-hour timepoint, FITC-dextran was added, and 2 hours later, leakage was measured (Fig. 16A). Analysis revealed a statistically significant reduction in BCSFB permeability of CPE cells derived from *Rag2*$^{-/-}$ mice with the addition of IL-34, as compared to PBS treatment (Fig. 16A). In wild type primary CPE cells, no difference in permeability was observed. Additionally, in the lab of Prof. Bieke Broux, they performed similar transwell experiments on primary brain capillary endothelial cells isolated from wild type and *Rag2*$^{-/-}$ mice. Consistent with our findings for the BCSFB, results revealed a significant decrease in leakage when brain capillary endothelial cells from *Rag2*$^{-/-}$ mice were treated with IL-34 (Fig. S2).

Furthermore, the human malignant ChP papilloma cell line (HIBCPP) and a murine immortalized CPE cell line (ImmCPE) were evaluated in a similar transwell experiment. HIBCPP cells, derived from a malignant ChP papilloma of a Japanese woman, exhibit key features of a functional BCSFB, such as the formation of TJs, development of a high TEER and low permeability for macromolecules (Ishiwata *et al*, 2005). Additionally, ImmCPE cells were created in the host lab by transgenic overexpression of an SV40 large T-antigen fragment under the control of a lymphotropic papovavirus (LPV) promoter (Pauwels *et al*, 2022). Both cell lines were seeded onto transwell inserts until a tight single-cell layer was formed, which was assessed by monitoring the TEER (Fig. S1). For these cell lines, an LPS trigger was used to induce barrier leakage. Accordingly, treatment with LPS resulted in increased leakage following FITC-dextran administration, as compared to PBS treatment. Stimulation of HIBCPP and ImmCPE cells with IL-34 showed significant restoration of LPS-induced leakage, but not completely to wild type levels (Fig. 16B-C). Additionally, when IL-34 was co-administered with FITC-dextran at the 6-hour timepoint, a significant reduction in barrier permeability was observed, equivalent to the condition where IL-34 was present from the beginning (Fig. 16B-C).

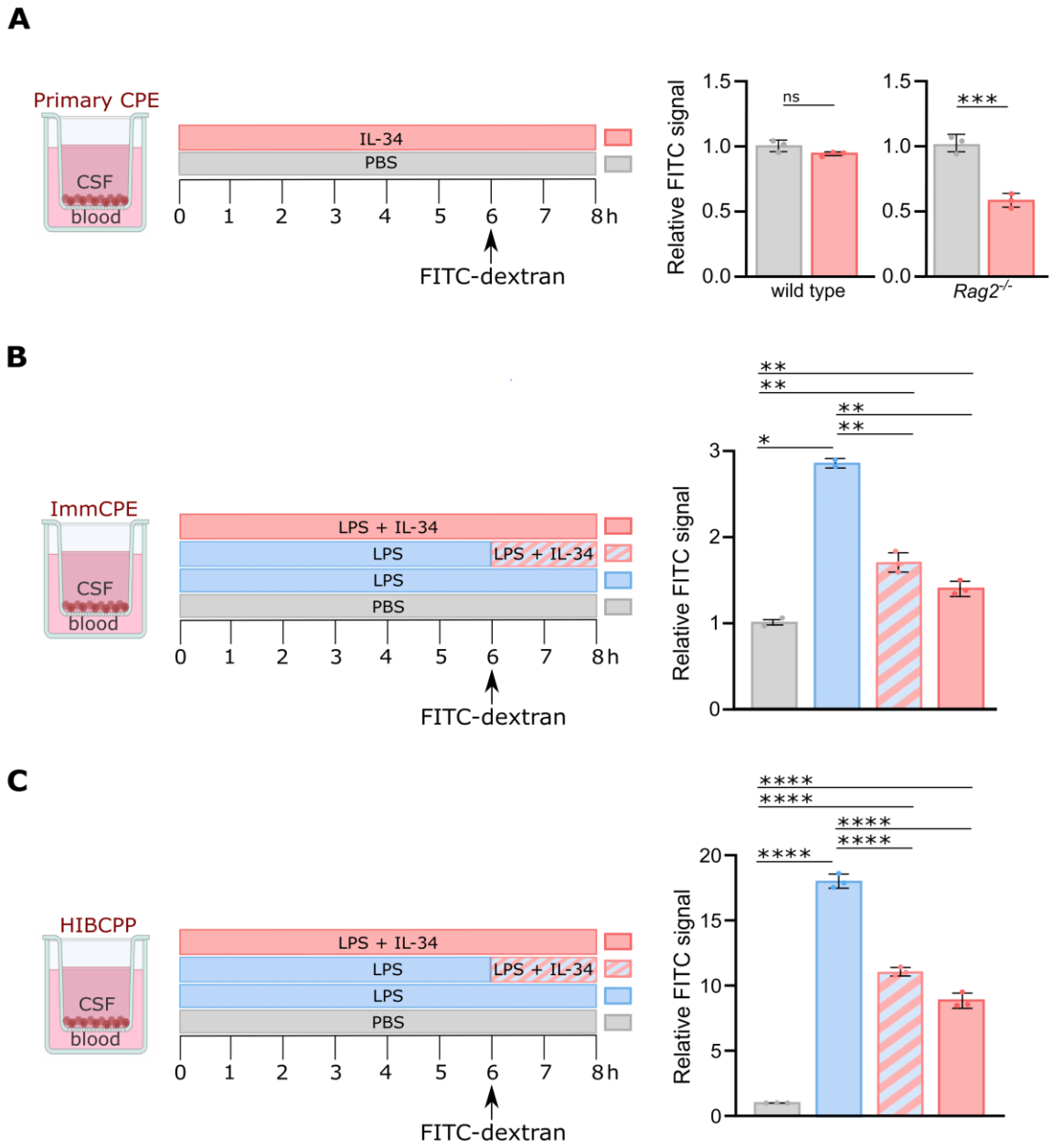


Figure 16: *In vitro* administration of interleukin (IL)-34 rescues leakage of primary choroid plexus epithelial (CPE) cells from *Rag2*$^{-/-}$ mice and LPS-treated human and mouse BCSFB cell lines. (A) Primary choroid plexus epithelial (CPE) cells were isolated from wild type and *Rag2*$^{-/-}$ mice and seeded in the apical compartment of a transwell system. Cells were stimulated for 8 hours with IL-34. PBS treatment was used as control. At the 6-hour timepoint, 4 kDa FITC-dextran was added to the basal compartment (blood side), and 2 hours later, leakage was determined by measuring fluorescence in the apical compartment (CSF side). **(B)** Murine immortalized CPE cells (ImmCPE) and **(C)** human malignant ChP papilloma (HIBCPP) cells were seeded in the apical compartment of a transwell system. Leakage was induced by an LPS trigger. PBS treatment was used as control. Cells were treated for 8 hours or 2 hours with IL-34. Leakage was determined as described above. Data are shown as mean ± SEM (n=2/3 per group). Statistical significance was determined by two-tailed Student's t-test for (A) and one-way ANOVA and Tukey's multiple comparisons test for (B) and (C); *p < 0.05, **p < 0.01, *** p < 0.001, **** p <

3.3.3. *In vivo* administration of IL-34 restores brain barrier disruption

Previous findings showed an impact of IL-34 on brain barrier integrity *in vitro*. To validate whether these effects can be translated to an *in vivo* context, mice that exhibit brain barrier leakage were treated with IL-34. More specifically, IL-34 was administered at regular intervals of 2 hours over a 24-hour period to *Rag2*$^{-/-}$ mice (Fig. 17A). The dosage of recombinant mouse IL-34 was calculated to obtain levels consistent with those observed under physiological conditions in the blood (41,2 pg/mL). At the 24-hour timepoint, leakage was determined by measuring fluorescence in the CSF and brain parenchyma after i.v. injection of 4 kDa FITC-dextran. Results showed that administration of IL-34 significantly decreased BBB and BCSFB leakage in *Rag2*$^{-/-}$ mice (Fig. 17B-C).

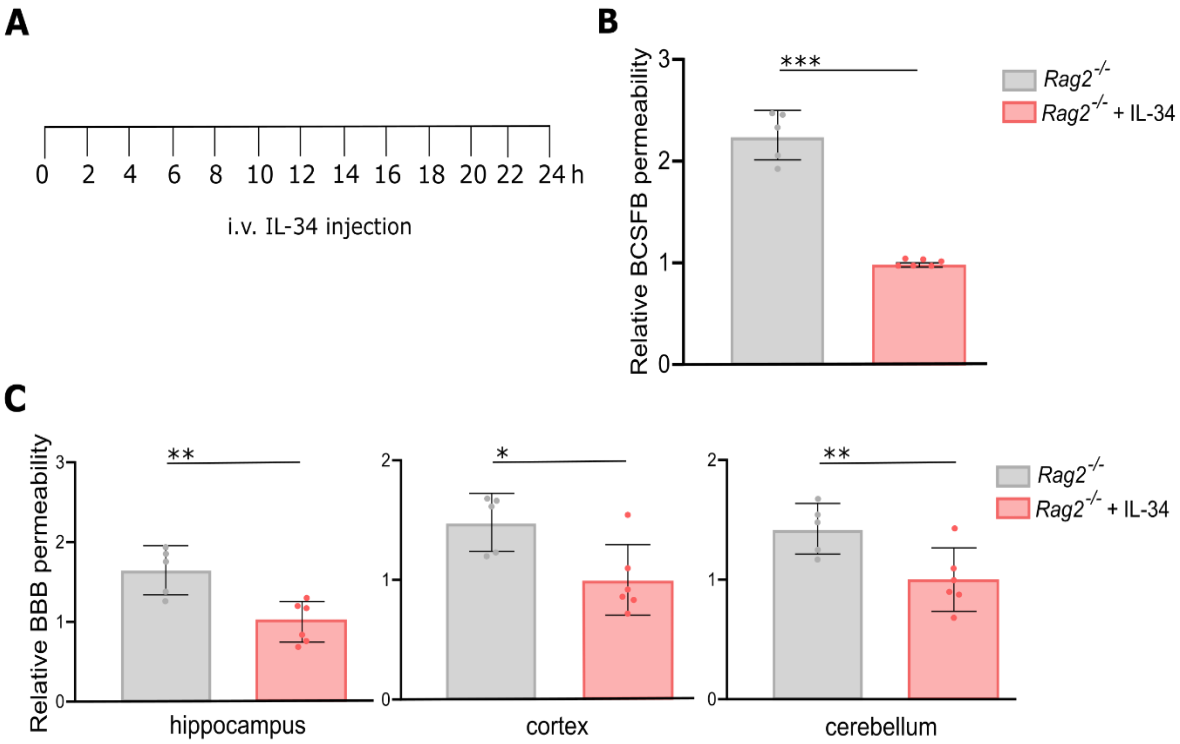


Figure 17: *In vivo* administration of interleukin (IL)-34 rescues blood-CSF barrier (BCSFB) and blood-brain barrier (BBB) leakage in *Rag2*$^{-/-}$. (A) *Rag2*$^{-/-}$ mice were treated with intravenous (i.v.) IL-34 injections every 2 hours for a period of 24 hours. **(B)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after i.v. injection. **(C)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=5/6 mice per group). Statistical significance was determined by two-tailed Student's t-test; *p < 0.05, **p < 0.01, ***p < 0.001.

The findings above provide insights into the impact of IL-34 on brain barrier integrity in a non-inflammatory context. To further explore the effects of IL-34 on the brain barriers in a different context, brain barrier leakiness was evaluated in wild type mice where systemic inflammation was mimicked by an LPS trigger (i.e., endotoxemic mice). A preliminary experiment was performed to determine the minimal dosage of LPS required to induce brain barrier leakage. Based on previous experiments in the host lab, it was known that a dosage of 20 µg LPS was insufficient to induce leakage. Therefore, doses of 50 µg, 150 µg, and 300 µg LPS were evaluated in wild type mice via i.p. injection, followed by i.v. injection of 4 kDa FITC-dextran to assess leakage in the prefrontal cortex (for the BBB) and CSF (for the BCSFB) (Fig. S3). The results indicated leakage at the 50 µg LPS dose, although statistical significance was not achieved for the BCSFB due to the small number of mice used and the death of some mice. Subsequently, wild type mice were administered the 50 µg LPS dose via i.p. injection, and IL-34 was administered at regular 2-hour intervals over a 24-hour period (Fig. 18A). Fluorescence measurements revealed a significant decrease in permeability of the BCSFB and BBB following IL-34 treatment in *Rag2*$^{-/-}$ mice (Fig. 18B-C). These *in vivo* results suggest that IL-34 has the ability to restore brain barrier integrity, which is consistent with the *in vitro* findings.

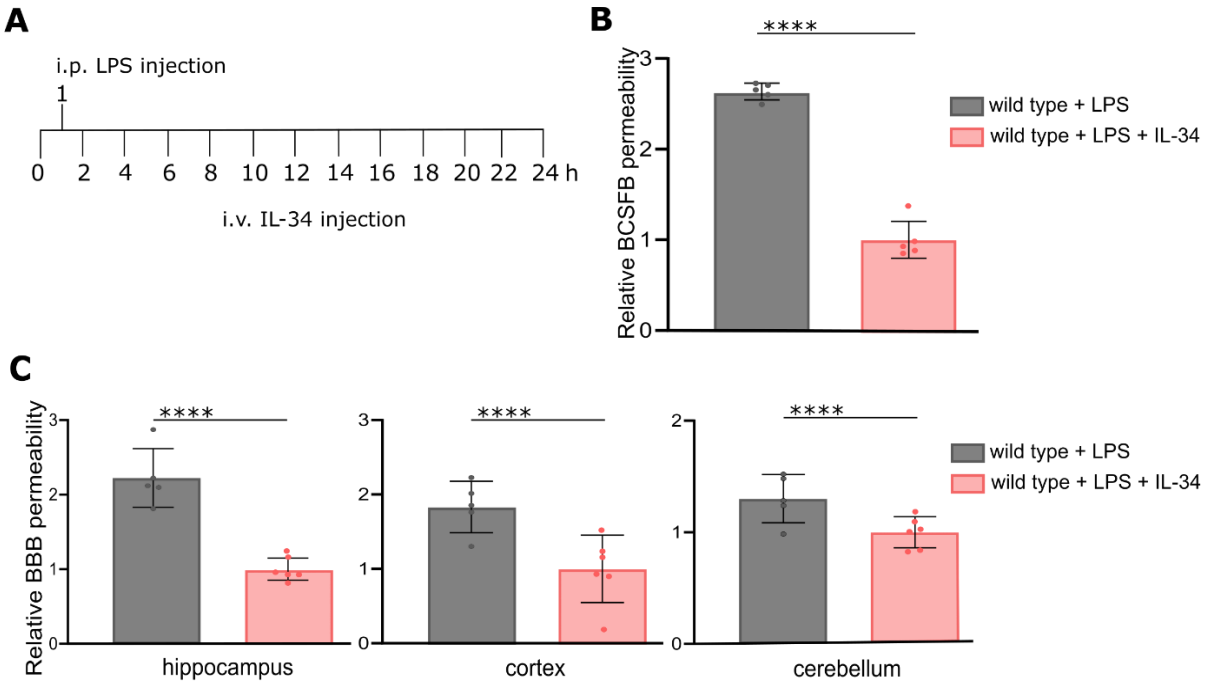


Figure 18: *In vivo* administration of interleukin (IL)-34 rescues blood-CSF barrier (BCSFB) and blood-brain barrier (BBB) leakage in wild type mice with LPS-induced peripheral inflammation. (A) Wild type mice were treated with intravenous (i.v.) IL-34 injections every 2 hours for a period of 24 hours. At the 1-hour timepoint, mice received an intraperitoneal (i.p.) LPS trigger (50 µg) to induce brain barrier leakage. **(B)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after i.v. injection. **(C)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=5/6 mice per group). Statistical significance was determined by two-tailed Student's t-test; ****$p < 0.0001$.

3.3.4. Blockade of CSF-1R has minimal impact on the brain barriers

To assess whether IL-34 exerts its effects on brain barrier integrity through binding of the CSF-1R, mice were treated with a low dose (75 mg/kg) of CSF-1R small molecule inhibitor pexidartinib (PLX3397). Wild type mice were provided with PLX3397-supplemented food for a duration of 9 days, while control mice received food with the same composition but without PLX3397 (Fig. 19A). The food was weighed before and after the PLX3397 treatment period to evaluate the average food intake per mice. No significant difference in food intake was observed between the two treatment groups (Fig. 19B). Subsequently, brain barrier integrity was determined after i.v. injection of 4 kDa FITC-dextran. Results showed a minor increase in BCSFB leakage, but no significant differences in BBB leakage were observed after CSF-1R inhibition (Fig. 19C-D).

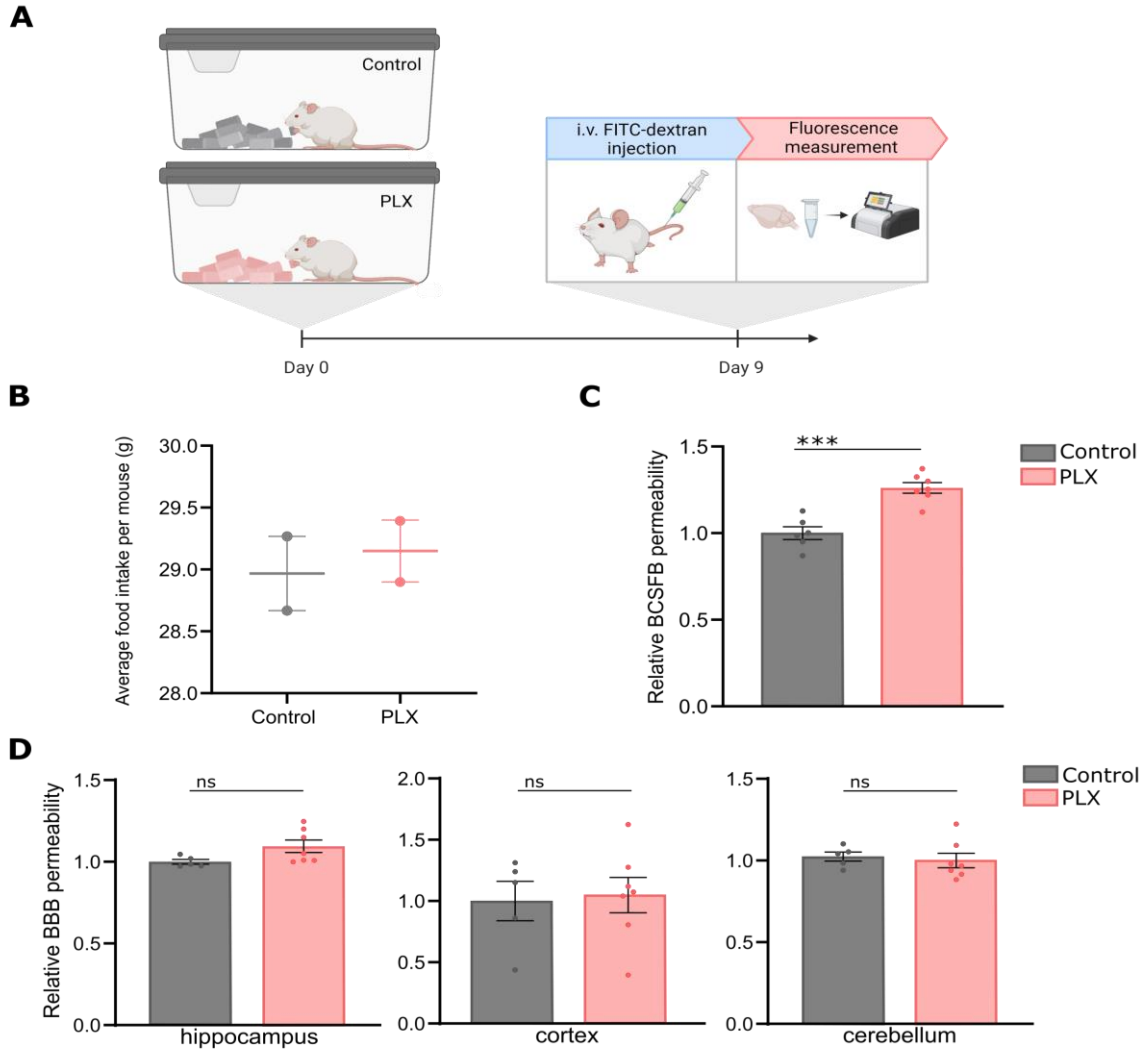


Figure 19: Colony-stimulating factor 1 receptor (CSF-1R) inhibition has minimal impact on the blood-CSF barrier (BCSFB) and no impact on the blood-brain barrier (BBB) integrity in wild type mice. (A) Wild type mice were treated with food supplemented with PLX3397 (PLX) or control food. At day 9, mice were sacrificed, and BBB leakage was determined. **(B)** The food was weighed before and after PLX treatment per cage and divided by the number of mice in the cage to determine average food uptake per mouse (in g). Two cages per treatment group were used. **(C)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after i.v. injection. **(D)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=5/7 mice per group). Statistical significance was determined by two-tailed Student's t-test; ***p < 0.001, ns: non-significant.

3.4. The impact of IL-34 in neurological disease models

3.4.1. IL-34 is reduced in mouse models for AD and MS

Brain barrier disruption is a common feature in many neurological disorders, such as AD and MS (Cai *et al*, 2018; Balusu *et al*, 2016; Engelhardt *et al*, 2022). In MS, brain barrier disruption results in the influx of pro-inflammatory cells that contribute to the disease, while in AD, impaired barrier integrity hinders clearance of Aβ plaques. In its turn, these inflammatory cells and Aβ plaques can cause further disruption of the brain barriers, contributing to a vicious cycle that progressively worsens AD and MS. Consequently, restoring barrier integrity holds promising therapeutic potential for these and other neurological disorders. Interestingly, the lab of Prof. Bieke Broux showed a reduced number of IL-34 expressing Tregs in untreated RRMS patients (Fig. 15A). Therefore, we determined the concentration of IL-34 present in the plasma of mouse models of AD and MS, namely the *App*$^{NL\text{-}G\text{-}F}$ and EAE models, by enzyme-linked immunosorbent assay (ELISA). In 35-week-old *App*$^{NL\text{-}G\text{-}F}$ mice, the IL-34 concentration in the plasma was significantly lower compared to age-matched wild type control mice (Fig. 20A). Similarly, this decrease was seen in EAE mice 16 and 26 days after disease induction (Fig. 20B). The results showed no significant difference in IL-34 concentration in the plasma of EAE mice 7 days after induction compared to naïve EAE mice. This can be attributed to the fact that these mice have a clinical score of zero, and thus, do not yet have leakage of the brain barriers (Fig. 20B).

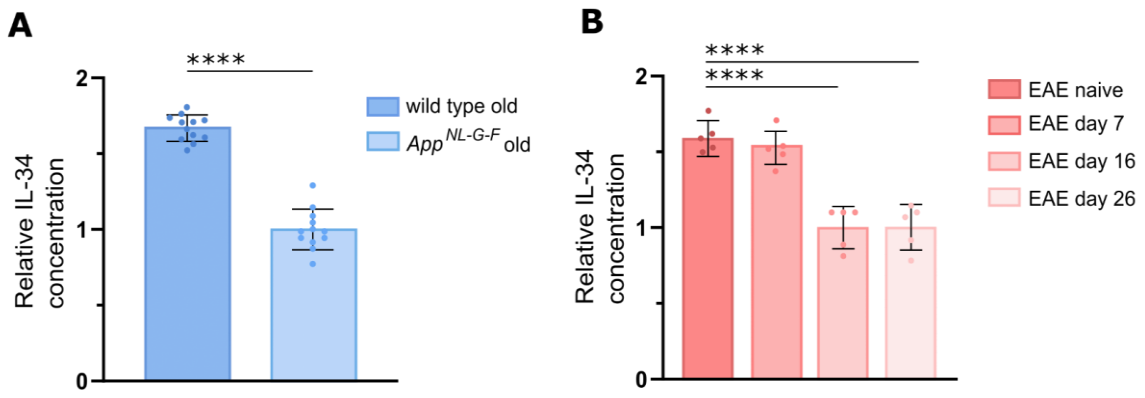


Figure 20: The interleukin (IL)-34 concentration in the plasma of experimental autoimmune encephalomyelitis (EAE) and *App*$^{NL\text{-}G\text{-}F}$ mice is lower compared to wild type mice. (A) IL-34 concentration was determined in the plasma of 35-week-old *App*$^{NL\text{-}G\text{-}F}$ mice and age-matched wild type controls by enzyme-linked immunosorbent assay (ELISA). **(B)** IL-34 concentration was determined in the plasma of EAE mice 7 days, 16 days, and 26 days after immunization with $MOG_{33\text{-}35}$ peptide by ELISA. Naïve EAE mice were used as control. Statistical significance was determined by two-tailed Student's t-test (A) and one-way ANOVA (B); ****$p < 0.0001$.

3.4.2. IL-34 treatment partially rescues brain barrier disruption in EAE and *App*$^{NL-G-F}$ mice

Previous findings showed a reduced concentration of IL-34 in the plasma of EAE and *App*$^{NL-F-G}$ mice. Additionally, we have demonstrated that *in vivo* administration of IL-34 restored brain barrier integrity in *Rag2*$^{-/-}$ mice and LPS-induced barrier leakage in wild type mice. As brain barriers are disrupted in EAE and *App*$^{NL-F-G}$ mice, we hypothesized that IL-34 administration might improve barrier integrity in these disease models. Therefore, IL-34 was administered to EAE and *App*$^{NL-F-G}$ mice 3 times a day for 3 days in a row (Fig. 21A, D). Non-treated wild type, EAE and *App*$^{NL-F-G}$ mice were used as control. At the day 3, brain barrier leakage was determined after i.v. injection of 4 kDa FITC-dextran. The results showed a significant decrease in the BCSFB permeability of IL-34-treated *App*$^{NL-G-F}$ and EAE mice, as compared to control mice (Fig. 21B, E). The BBB leakage observed in *App*$^{NL-G-F}$ mice was completely restored by IL-34 treatment in the hippocampus and cerebellum (Fig. 21C). However, results showed no significant reduction in BBB permeability in the cortex of these mice, although a noticeable trend was observed. In IL-34-treated EAE mice, BBB permeability was significantly lowered in the cortex and cerebellum, but not in the hippocampus (Fig. 21F). However, in the hippocampus, non-treated EAE mice did not show an increased barrier leakage compared to wild type mice, therefore, IL-34 does not have any beneficial impact.

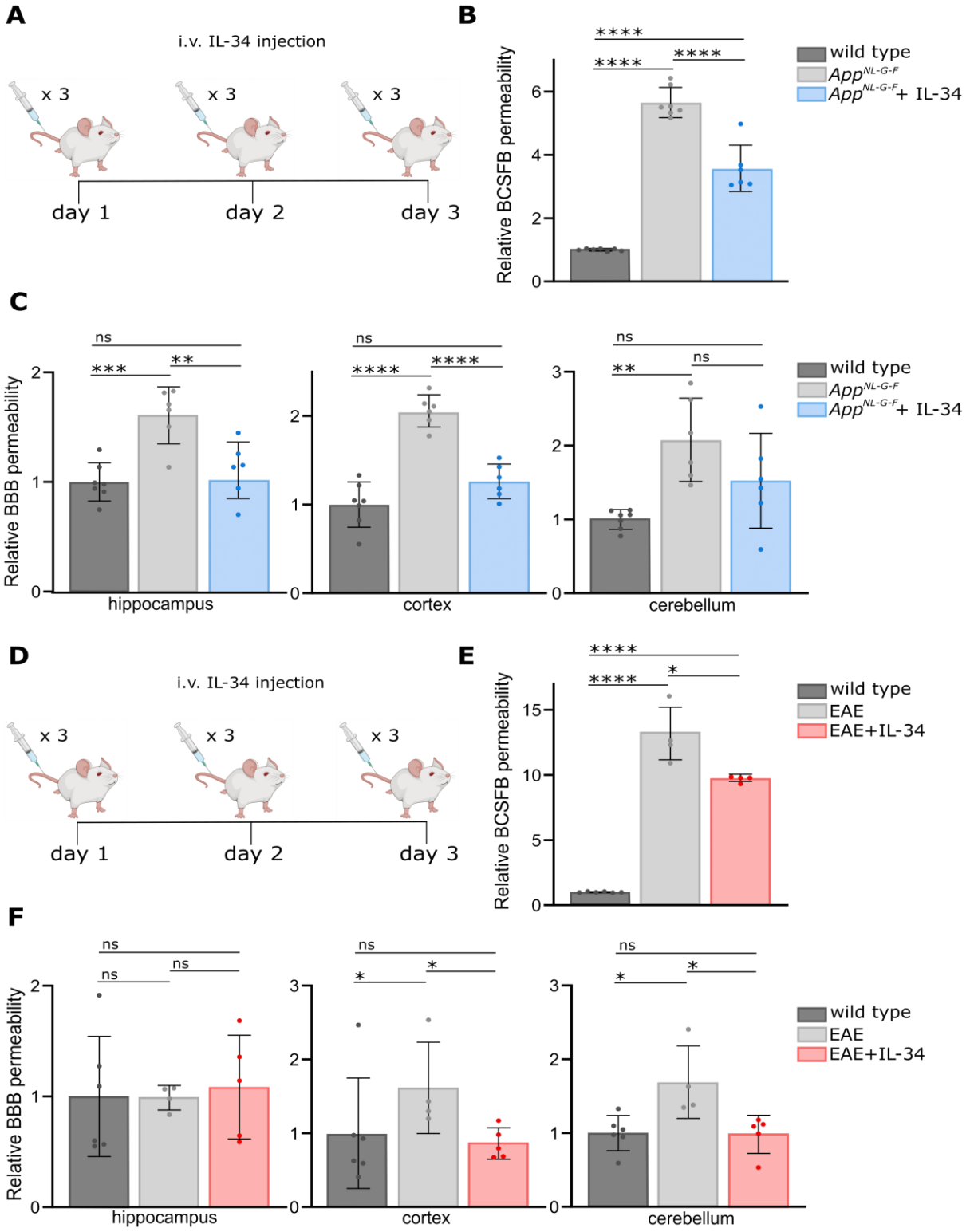


Figure 21: Interleukin (IL)-34 partially restores blood-brain barrier (BBB) and blood-CSF barrier (BCSFB) disruption in experimental autoimmune encephalomyelitis (EAE) and *App*$^{NL-G-F}$ mice. (A/D) *App*$^{NL-G-F}$ / EAE mice were treated with intravenous (i.v.) IL-34 injections 3 times a day for 3 days in a row. Non-treated *App*$^{NL-G-F}$ / EAE and wild type mice were used as control. **(B/E)** The relative BCSFB permeability was determined by measuring the level of 4 kDa fluorescein isothiocyanate (FITC)-dextran in the CSF 15 min after i.v. injection. **(C/F)** The relative BBB permeability was determined in the hippocampus, cortex, and cerebellum 15 min after i.v. injection of 4 kDa FITC-dextran. Data are shown as mean ± SEM (n=3/6 mice per group). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; *p < 0.05, **p < 0.01, ****p < 0.0001, ns: non-significant.

Part 4: Discussion

In the CNS, protective barriers shield the brain from the constantly changing environment of the blood. These brain barriers, including the blood-brain barrier (BBB) and the blood-CSF barrier (BCSFB), maintain a precisely controlled milieu within the CNS necessary for optimal neuronal function. Besides providing physical protection, they also play a critical role in shaping immune responses within the CNS. Disruption of these barriers results in impaired brain function, as observed in neurological disorders such as Alzheimer's disease (AD) and multiple sclerosis (MS). In AD, impaired barriers hinder the clearance of Aβ peptides from the brain, leading to their accumulation and the formation of Aβ plaques, a hallmark feature of AD. Furthermore, the presence of these Aβ plaques, in turn, induces barrier disruption, thereby amplifying AD pathogenesis. In addition, impaired brain barriers might allow infiltration of pro-inflammatory immune cells, which contribute to neuroinflammation seen in AD. In MS, brain barrier disruption is a prominent feature that results in the infiltration of immune cells. Infiltrating self-reactive T cells mount an inflammatory attack against the insulating covers of the brain, spinal cord, and optic nerves, leading to impaired communication between neurons. This triggers axonal degeneration and neurological deficits associated with MS. Moreover, the pro-inflammatory T cells cause further disruption of the brain barriers, worsening disease progression. Consequently, tightening the brain barriers holds therapeutic potential for these neurological disorders.

T cells improve brain barrier integrity

In contrast to the long-held belief of the brain as an immune-privileged site, accumulating evidence highlights a dynamic interaction between T cells and the CNS. For instance, T cells carry out immune surveillance at the borders of the CNS and are important in modulating CNS components, such as microglia (Engelhardt *et al*, 2017; Pasciuto *et al*, 2020). Based on these findings, we aimed to explore the role of adaptive immune cells in shaping and/or maintaining brain barriers. Therefore, we compared the brain barrier integrity in *Rag2*$^{-/-}$ mice, a mouse model that lacks mature T and B lymphocytes, with wild type mice. The *Rag2*$^{-/-}$ mice exhibited increased brain barrier leakage, both at the BBB and BCSFB, demonstrating the impact of adaptive immune cells on the permeability of the barriers. In addition, these mice showed an impaired distribution of ZO-1 in the CPE cells that form the BCSFB and reduced sealing capacity of astrocyte endfeet in the BBB. These findings provide possible mechanisms underlying the observed increase in barrier leakage. On top of that, replacing the missing T cells, effectively reversed the effects of immune deficiency on brain barrier integrity. Despite these effects, *Rag2*$^{-/-}$ mice are not embryonic lethal, however, the absence of T and B cells might affect brain development and homeostasis. For instance, Pasciuto *et al*. showed that microglia require CD4^{+} T cells for their maturation and synaptic pruning function (Pasciuto *et al*, 2020). Additionally, several studies show behavioural abnormalities in *Rag*-deficient mice (Cushman *et al*, 2003; Rattazzi *et al*, 2013, 2015). Thus, in *Rag2*$^{-/-}$ mice, the lack of T and B cells might show an impact on both the formation and the maintenance of the brain barriers. To avoid the interference of these abnormalities, we investigated the effect of acquired immune deficiency on the maintenance of the brain barriers by depleting adaptive immune cells in wild type mice. This revealed that the depletion of T cells, but not B cells, increased brain barrier

permeability. Altogether, these data proof that T cells modulate the integrity of the brain barriers.

The effects on the brain barrier integrity can be attributed to Tregs

Tregs are immunosuppressive $CD4^+$ T lymphocytes marked by the expression of the transcription factor Forkhead box P3 (FoxP3) and the interleukin 2 (IL-2) receptor α-chain (CD25) (Vignali et al, 2008). Their importance in maintaining immune homeostasis and preventing autoimmunity is achieved through various mechanisms, including secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β, direct cell-cell contact, and metabolic disruption of effector T cells (Vignali et al, 2008). Besides their immunosuppressive role, healthy Tregs were shown to have regenerative capacity. For instance, Tregs can induce remyelination by promoting oligodendrocyte precursor cell differentiation and potentiate the recovery of neurological injury (Dombrowski *et al*, 2017; Ito *et al*, 2019). Additionally, depletion of Tregs has been shown to worsen the pathophysiology of cerebral ischemia, a disease characterized by the influx of innate and adaptive immune cells and a compromised BBB (Iadecola & Anrather, 2011; Li *et al*, 2013). Consequently, we postulated that the previously observed effects on brain barrier integrity by T cells might be attributed to Tregs specifically. Next to ischemic stroke, many other neurological disorders, such as AD and MS, exhibit decreased functionality and/or abundance of Tregs (Ciccocioppo *et al*, 2019). Moreover, disruption of the brain barriers is a common characteristic of AD and MS, implying a potential association between Treg dysfunction and impairment of brain barrier integrity. Consequently, we investigated whether there is a direct link between the absence of Tregs and the impaired brain barrier integrity in the *Rag2*$^{-/-}$ mouse model. Our findings revealed that reconstitution of Tregs in *Rag2*$^{-/-}$ mice improved brain barrier leakage. In contrast, Treg depletion in wild type mice increased barrier permeability. Conclusively, this showed that Tregs positively impact brain barrier integrity.

Of note, Tregs for adoptive transfer were isolated from wild type donor mice by positive selection of $CD4^+$ pre-enriched T cells labelled with CD25 antibody, which recognizes the α chain of the IL-2 receptor (IL2-Rα). However, CD25 is also expressed on activated T cells, B cells and to a lower extent on DCs. Thus, the $CD4^+CD25^+$ fraction may contain activated $CD4^+$ T cells in addition to Tregs. To address this issue, fluorescent-assisted cell sorting (FACS) could be performed based on the expression of FoxP3. However, since FoxP3 is an intracellular transcription factor, its staining is not suitable for isolating viable Tregs. A possible solution is to make use of $FoxP3^{GFP}$ knock-in mice and perform FACS sorting for $CD4^+GFP^+$ cells. Similarly, depletion with anti-CD25 antibody is not exclusive for Tregs. Alternatively, mice that express diphtheria toxin receptor (DTR) in Tregs can be used for transient Treg depletion. Consequently, the potential impact of activated $CD4^+$ T cells on brain barrier permeability cannot be entirely excluded. Nevertheless, in SPF wild type mice the population of activated $CD4^+$ T cells is relatively low (Pasciuto *et al*, 2020).

IL-34 restores brain barrier disruption

As mentioned above, Li and colleagues demonstrated a protective effect of Tregs in a mouse model of cerebral ischemia, including protection against BBB disruption. Interestingly, these effects were observed prior to Treg infiltration into the brain, suggesting that their neuroprotective activity is peripheral in nature. This could be facilitated by releasing protective mediators into the peripheral blood or by targeting other peripheral cells that

subsequently impact the site of brain injury (Li *et al*, 2013). Tregs are known to produce the cytokine IL-34, which is a potential candidate protective mediator. Indeed, evidence shows that IL-34 has suppressive functions in allogeneic immune responses and mediates transplant tolerance (Bézie *et al*, 2015). Additionally, in a recent study, rodent models deficient in IL-34 (*Il-34*$^{-/-}$) were generated to investigate its role in Treg development and function (Freuchet *et al*, 2022). The results revealed that the absence of IL-34 increased the severity of dextran sulfate sodium (DSS)-induced colitis in comparison to control animals. Furthermore, the authors evaluated the suppressive function of CD4^{+} Tregs *in vivo* using a rat model of wasting disease induced by i.v. transfer of T CD4^{+}CD45RC^{high} effector cells in *Il2rg*$^{-/-}$ animals. The findings indicated that *Il34*$^{-/-}$ CD4^{+} Tregs were unable to control the development of wasting disease, while CD4^{+} Tregs from *IL-34*$^{+/+}$ rats efficiently controlled the progression of the disease. Moreover, *Il-34*$^{-/-}$ mice exhibited slightly worsened EAE progression, while adenoviral-mediated IL-34 overexpression resulted in delayed development of EAE, as compared to control mice. Interestingly, the lab of Prof. Bieke Broux (UHasselt) discovered a decreased percentage of IL-34 expressing Tregs in RRMS patients. Hence, these data provide evidence that IL-34 is an important player in the suppressive functions of Tregs.

The main receptor for IL-34 is the CSF-1R, which is a class III receptor tyrosine kinase (Lin *et al*, 2008). The cytokine shares this receptor with the macrophage colony-stimulating factor (M-CSF), however, they have low sequence homology and are different in biological activity and signal activation (Chihara et al, 2010). The CSF-1R is mainly expressed in myeloid lineage cells, such as microglia, in which IL-34 is required for survival and proliferation (Wang *et al*, 2012). However, RNA-sequencing data shows that the CSF-1R is also highly expressed in the ChP and on vascular endothelial cells in the brain. Additionally, a study by Jin *et al*. provided evidence for the presence of the CSF-1R on brain vascular endothelial cells by immunostaining. Moreover, they showed that *in vitro* induced BBB disruption by the pro-inflammatory cytokines IL-1β and TNF-α could be rescued by IL-34. More specifically, IL-34 upregulated TJ proteins such as claudin-5 and occludin, while the addition of a CSF-1R inhibitor prevented this IL-34 effect (Jin *et al*, 2014). Based on these findings, we hypothesized that Treg-derived IL-34 directly impacts brain barrier integrity through binding of the CSF-1R. To address this, we performed *in vitro* transwell assays with primary CPE cells from *Rag2*$^{-/-}$ mice, murine ImmCPE cells, and human HIBCPP cells. These experiments demonstrated that direct application of IL-34 restored BCSFB integrity in *Rag2*$^{-/-}$ primary CPE cells and rescued LPS-induced BCSFB disruption in both ImmCPE and HIBCPP cells. Interestingly, similar transwell assays were performed for primary BBB cells in the lab of Prof. Bieke Broux (UHasselt). Consistent with our findings for the BCSFB, their results also demonstrated a direct effect of IL-34 on the BBB integrity in primary brain microvascular endothelial cells from *Rag2*$^{-/-}$ mice. Currently, transwell experiments on human brain microvascular endothelial cells (hCMEC/D3) and murine brain endothelial cells (bEnd.3) are ongoing. These findings support that IL-34 has the capability to directly enhance the integrity of the brain barriers *in vitro*. In a future step, the mechanisms behind this improvement of the brain barriers can be investigated by looking into the effect of IL-34 on TJ expression, with a main emphasis on ZO-1 expression. As mentioned above, we observed a reduced sealing capacity of astrocyte endfeet in the *Rag2*$^{-/-}$ mouse model. Consequently, it could be of interest to explore the potential effect of IL-34 on this aspect, e.g., via co-culture experiments with brain vascular endothelial cells and

astrocytes. Additionally, the *in vivo* effect on astrocyte endfeet can be analysed in IL-34 treated endotoxemic mice.

Based on these *in vitro* studies, we aimed to validate the observed effect of IL-34 in an *in vivo* context. To this end, IL-34 was administered at regular intervals to *Rag2*$^{-/-}$ mice at a dose equivalent to physiological levels of IL-34 detected in the blood. *Rag2*$^{-/-}$ mice are characterized by impaired brain development and early brain barrier leakage in non-inflammatory conditions. In addition, wild type mice that received an LPS trigger were included to investigate inflammation-induced brain barrier leakage. Our findings demonstrated that IL-34 treatment effectively improved brain barrier integrity in both *Rag2*$^{-/-}$ mice and LPS-treated wild type mice. However, it should be noted that the CSF-1R is highly expressed in macrophages, including microglia. In the CNS, IL-34 produced by neurons binds to the CSF-1R on microglia, promoting their differentiation and proliferation (Wang *et al*, 2012). Given that microglia are part of the neurovascular unit of the BBB, these cells possibly contribute to the observed *in vivo* effects of IL-34 on the brain barrier integrity. However, several studies show that the depletion of microglia does not impact the BBB integrity (Parkhurst et al, 2013; Elmore et al, 2014). Interestingly, a recent paper by Drieu and colleagues demonstrated that leptomeningeal and perivascular macrophages regulate CSF flow dynamics under homeostatic conditions (Drieu et al, 2022). These macrophages, which reside along the vasculature, exert this function by regulating the arterial motion. Depletion of these macrophages results in the accumulation of extracellular matrix proteins and obstruction of the perivascular spaces, possibly impairing brain barrier function. Given that these cells express the CSF-1R, treatment with IL-34 might impact their function. Consequently, they might also play a role in the observed *in vivo* IL-34 effects on the brain barrier integrity. Additionally, macrophage populations are also residing at the BCSFB, namely the epiplexus macrophages at the apical epithelial surface of the ChP and the stromal macrophages in the stromal space between epithelial and endothelial layers of the ChP. While the role of these macrophages in BCSFB maintenance is unknown, IL-34 stimulation of these macrophages might also contribute to improved brain barrier integrity. Nevertheless, our *in vitro* experiments provide evidence of a direct effect of IL-34 on the integrity of the brain barriers, which means that the impact of microglia and macrophages would be additive to the direct effects of IL-34. To investigate this, *in vitro* co-culture experiments of brain barrier cells together with microglia and macrophages can be performed to study their impact on brain barrier integrity.

IL-34 possibly works through other mechanisms than CSF-1R

IL-34 mainly acts through binding of the CSF-1R. Additionally, Jin *et al.* showed that inhibition of the CSF-1R depletes the protective effect of IL-34 on the BBB *in vitro* (Jin *et al*, 2014). To verify the mechanism of action of IL-34, a selective small molecule inhibitor of the CSF-1R, namely PLX3397, was orally administered to wild type mice. Our findings indicated that PLX3397-mediated inhibition of the CSF-1R had no significant impact on BBB integrity and only minimal effects on BCSFB integrity. Given the high expression of the CSF-1R in myeloid lineage cells, completely blocking the CSF-1R results in depletion of microglia in the brain parenchyma (Elmore *et al*, 2015, 2014). Therefore, a low dose (75 mg/kg) of PLX3397 was used to avoid microglia depletion (Han *et al*, 2020). Nonetheless, it is possible that this low dose is not sufficient for blocking the CSF-1R on the brain vascular endothelial cells and CPE cells, which might explain why we see only minimal effect on brain barrier integrity.

Furthermore, in line with previous findings from the lab of Prof. Bieke Broux (UHasselt) and others demonstrating the presence of CSF-1R on endothelial cells of the BBB, we aimed to determine whether the CSF-1R is also expressed on CPE cells. Therefore, immunostainings were performed on brain cryosections, but we were unable to show the presence of the receptor on CPE cells. To overcome potential limitations in our staining protocol, we implemented several optimization steps. For example, antigen retrieval, which involves the use of citrate buffer and heat treatment to enhance the accessibility of the target antigen for antibody binding. Additionally, we used brain sections with different durations of tissue fixation, including overnight and 4-hours fixation. Despite these efforts, we were unable to obtain successful staining of the CSF-1R on CPE cells. Nevertheless, various strategies can be employed to achieve successful staining in the future. For instance, validating different antibodies for the CSF-1R and alternative antigen retrieval methods, such as enzymatic antigen retrieval using proteinase K instead of heat-induced antigen retrieval. Additionally, cutting thicker sections using a vibratome or utilizing non-fixed samples are also options that can be explored.

Taken together, these findings suggest that IL-34 possibly works through alternative mechanisms rather than CSF-1R binding. Besides CSF-1R, IL-34 binds to two other receptors: receptor-type protein-tyrosine phosphatase-zeta (PTP-ζ) and syndecan-1 (Nandi *et al*, 2013; Segaliny *et al*, 2015). Up to now, PTP-ζ is shown to be expressed on neuronal progenitors and glial cells. IL-34 binding to the receptor results in the activation of pathways that inhibit the proliferation, clonogenicity and motility of the target cells. Syndecan-1, on the other hand, is mainly found on myeloid cells, and to a lower extent on epithelial and endothelial cells (Bernfield *et al*, 1992). Syndecan-1 does not have intrinsic signalling activity, but rather serves as a cell adhesion molecule and co-receptor. More specifically, the receptor modulates the interaction between IL-34 and CSF-1R and controls the bioavailability of IL-34 by acting as a sponge. Additionally, IL-34 action on syndecan-1 was found to induce cellular migration of myeloid cells. In follow-up experiments, the presence of the CSF-1R, PTP-ζ and/or syndecan-1 can be verified in CPE cells and brain vascular endothelial cells by immunostainings and Western blot. Additionally, the impact of IL-34 binding to PTP-ζ and syndecan-1 on brain barrier integrity can be investigated. *In vitro* transwell assays can be performed with the addition of selective blockers for CSF-1R, PTP-ζ or syndecan-1, and combinations of these blockers. If the observed effect of IL-34 on the brain barrier integrity diminishes in the presence of these blockers, it would indicate that these receptors are involved in mediating the IL-34 effect. Moreover, considering that IL-34 is a cytokine that has been recently identified, there may exist additional receptors that have yet to be discovered, which could contribute to the modulation of brain barrier integrity.

IL-34 improves brain barrier integrity in neurological disease models

As mentioned before, impaired brain barriers are common in many neurological diseases, including AD and MS, and, the disruption of the brain barrier leads to the infiltration of pro-inflammatory cells, exacerbating the disease progression. Additionally, in AD, compromised barrier integrity impairs the clearance of Aβ peptides. These inflammatory cells and Aβ plaques contribute to a detrimental cycle, further compromising brain barrier function and worsening the conditions of AD and MS. Consequently, sealing these barriers is a promising therapeutic avenue for AD and MS. Interestingly, a decrease in Treg number and/or

functionality is also a characteristic feature of AD and MS. In MS patients, a decrease in the number of Tregs has been demonstrated in the peripheral blood, accompanied by a diminished immunosuppressive capacity compared to healthy individuals (Li et al, 2013; Venken et al, 2008). This reduction in Treg function can contribute to the development of autoreactivity and inflammation within the CNS, which are typical hallmarks of MS. Similarly, in AD patients, a reduction of immunosuppressive properties of Tregs has been shown (Ciccocioppo et al, 2019; Faridar et al, 2020). Moreover, Tregs were demonstrated to have neuroprotective effects in rodent models of both AD and MS (Baek et al, 2016; Koutrolos et al, 2014). On top of that, the lab of Prof. Bieke Broux (UHasselt) revealed a reduced number of IL-34-expressing Tregs in the blood of RRMS patients. Based on these findings, we hypothesized that the dysregulation in the number or activity of Tregs in individuals with AD and MS possibly results in reduced secretion of IL-34. This, in turn, could have detrimental effects on the integrity of the brain barriers. Therefore, we determined the levels of IL-34 present in the plasma of mouse models of AD and MS, respectively the *App*$^{NL\text{-}G\text{-}F}$ and EAE models. The results revealed a lower IL-34 concentration in the plasma of *App*$^{NL\text{-}G\text{-}F}$ and EAE mice, as compared to age-matched controls.

Our previous data revealed a protective effect of IL-34 on the brain barriers both *in vitro* and *in vivo*, under non-inflammatory conditions as well as in the presence of LPS-induced peripheral inflammation. Together with the finding that IL-34 is reduced in the plasma of *App*$^{NL\text{-}G\text{-}F}$ and EAE mice, we hypothesized that replenishing IL-34 to its normal physiological levels could potentially restore the observed brain barrier disruption in these mice. To test this hypothesis, mice were treated with IL-34 at regular intervals using a concentration equivalent to physiological levels in the blood. While the precise mechanisms of IL-34 action remain elusive, we demonstrated that IL-34 treatment rescued brain barrier disruption in both *App*$^{NL\text{-}G\text{-}F}$ and EAE mice.

Interestingly, the most pronounced impact of IL-34 was observed on the BCSFB of *App*$^{NL\text{-}G\text{-}F}$ and EAE mice. Both the BBB and BCSFB are composed of polarized brain barrier cells characterized by TJs and AJs. As a result, their apical and basal regions exhibit distinct characteristics, including potentially differing expression profiles of membrane receptors. In our *in vitro* and *in vivo* experiments, we propose that IL-34 exerts its effects from the blood side of the brain barriers, rather than from the CSF or brain parenchymal side. Therefore, we added IL-34 to the blood side in our transwell experiments and administered IL-34 through i.v. injections in our *in vivo* studies. Notably, accumulating evidence suggests that T cells tend to accumulate predominantly in the perivascular spaces during inflammation, rather than infiltrating the brain parenchyma (McCandless *et al*, 2006; Engelhardt *et al*, 2017). This observation raises the possibility that Tregs secrete IL-34 within the perivascular space, where the cytokine can exert its effects on the BBB from the parenchymal side, rather than the blood side. Conclusively, a future objective is to explore whether Treg-derived IL-34 also impacts the BBB from the parenchymal side.

Future objectives

While our data revealed an impact of IL-34 on the brain barrier integrity in *App*$^{NL\text{-}G\text{-}F}$ and EAE mice, it remains unclear whether there is a direct link between the dysfunction of Tregs and the impaired brain barriers in these mouse models. Therefore, future research is required to elucidate whether it is Treg-derived IL-34 specifically that impacts the brain barrier integrity.

One possible strategy is the generation of Treg-specific IL-34 knockout (KO) mice. This can be obtained through crossbreeding of a mouse model that has FoxP3 promoter-driven Cre recombinase with a mouse line containing the IL-34 gene flanked by LoxP sites. In these mice, EAE can be induced to model MS or *App*$^{NL-G-F}$ mice can be used to model AD. By comparing the brain barrier integrity of these mouse models with full IL-34 KO mice and wild type controls, the contribution of Treg-derived IL-34 to brain barrier function can be determined. Nevertheless, our data from *Rag2*$^{-/-}$ mice showed that brain barrier integrity is already impaired in baseline conditions even in the absence of inflammation. This suggests that mice lacking Treg-specific IL-34 may exhibit impaired development of the brain barriers prior to the onset of AD or MS. To address this issue, an inducible knockout system could be generated. An alternative approach involves the development of a Treg-specific IL-34 overexpression model. This can be achieved by introducing the IL-34 gene downstream of a strong, constitutive promoter, but interrupted by a stop codon flanked by LoxP sites. Through crossbreeding this mouse model with FoxP3-Cre mice, a Treg-specific IL-34 overexpression model can be generated. This mouse model has a proper formation of the brain barriers and can be used to investigate whether IL-34 overexpression can rescue the brain barrier disruption in *App*$^{NL-G-F}$ and EAE mice.

Additionally, the exact signalling pathways induced by IL-34 in brain vascular endothelial cells and CPE cells remain unclear. To gain insights into these pathways, bulk RNA sequencing can be performed on these cells after stimulation with IL-34. More specifically, human and mouse cell lines can be used for the BCSFB (HIBCPP and ImmCPE) and the BBB (hCMEC/D3 and bEnd.3). Similar to our transwell experiments, inflammation-related brain barrier disruption can be induced by an LPS-trigger. Alternatively, brain vascular endothelial cells and CPE cells can be isolated directly from *App*$^{NL-G-F}$ and EAE mice. Subsequently, these primary cells can be cultured and treated with IL-34, comparing them to non-treated cells. In this way, differentially expressed genes and related pathways can be identified in these different conditions. Moreover, the effect of IL-34 on TJ expression markers can be verified in these *in vitro* models.

Conclusion

The results presented in this thesis reveal that *Rag2*$^{-/-}$ mice have compromised brain barriers, indicating a role of the adaptive immune system in shaping brain barriers. Through adoptive transfer and depletion experiments in *Rag2*$^{-/-}$ and wild type mice, we demonstrated that this effect on the brain barriers can be pinpointed to Tregs specifically. From the literature, it is known that Tregs secrete IL-34, and in addition, we showed that IL-34 rescues brain barrier disruption both *in vitro* and *in vivo*. Based on these findings, we propose that Tregs play a key role in maintaining brain barrier integrity through the secretion of IL-34. However, the precise mechanisms by which IL-34 exerts its effects on brain barriers remain elusive, as inhibition of CSF-1R showed only minimal impact. This indicates the involvement of alternative pathways or receptors through which IL-34 might operate, which will need further research. Additionally, brain barrier disruption is observed in neurological diseases, such as AD and MS. Remarkably, our data revealed that IL-34 treatment effectively restores brain barrier integrity in mouse models of AD and MS. Taken together, these findings hold great promise for the development of Treg-based therapy aimed at restoring brain barrier function in these neurological disorders.

Part 5: Materials and Methods

Housing and ethics statement Mice were housed in individually ventilated cages with 14h light/10h dark cycles and *ad libitum* food and water in specific pathogen-free (SPF) conditions. In all experiments age- and gender-matched wild type littermates were used. All animal studies were conducted in compliance with governmental and EU guidelines for the care and use of laboratory animals and were approved by the ethical committee of the Faculty of Sciences, Ghent University, Belgium.

Mouse models *Rag2*$^{-/-}$ mice are deficient in the *RAG2* gene and fail to generate mature T and B lymphocytes (RRID:IMSR_JAX:008449, Jackson Laboratory). *App*$^{NL-G-F}$ mice carry the Arctic, Swedish, and Iberian mutations as described in (Saito *et al*, 2014) . *Rag2*$^{-/-}$ and *App*$^{NL-G-F}$ mice were maintained in our animal house at the VIB Center for Inflammation Research. EAE mice were generated by immunization of 8 to 15-week-old C57BL/6 wild type mice with the MOG_{35-55}/CFA Emulsion PTX Hooke Kit (EK-2110; Hooke Laboratories) following the manufacturer's guidelines. Briefly, mice were subcutaneously injected with an emulsion of MOG_{35-55} in complete Freund's adjuvant (CFA) two times (total of 200 µg/mouse), followed by administration of pertussis toxin (PTX, 121 ng/mouse) in Dulbecco's phosphate buffered saline (D-PBS, 14190-094; Gibco), first on the day of immunization and then again 24 h later. Clinical signs of disease were scored on a scale of 0–5 following the scoring guide of Hooke Laboratories. For all experiments, C57BL/6 J mice were used as a control.

Quantification of BBB and BCSFB permeability The BCSFB and BBB permeability was evaluated according to the methods described before (Vandenbroucke *et al*, 2012). Shortly, 4 kDa FITC-labelled dextran (FD4-1G; Sigma-Aldrich) was injected i.v. 15 min before CSF collection. CSF was obtained from the fourth ventricle via cisterna magna puncture using needles made from borosilicate glass capillary tubes (B100-75-15; Sutter Instruments) (Balusu *et al*, 2016; Vandenbroucke *et al*, 2012). Next, mice were anaesthetized with a lethal dose of ketamine/xylazine (100 mg/kg ketamine; 20 mg/kg xylazine) and perfused with D-PBS/heparin (0.2% heparin, H-3125; Sigma-Aldrich), afterwards, brain regions were isolated and snap frozen in liquid nitrogen. CSF was diluted 100-fold in sterile D-PBS and the BCSFB leakage was measured by quantifying fluorescence at $\lambda_{ex}/\lambda_{em}$ = 485/520 nm on the FLUOstar Omega (BMG Labtech). Brain samples were finely cut and incubated overnight at 37°C in formamide (47671; Sigma-Aldrich) while shaking and in the dark. The next day, the supernatant was collected after centrifugation at 400 g-forces (g) for 7 min. The degree of BBB leakage was determined by measuring fluorescence at $\lambda_{ex}/\lambda_{em}$ = 485/520 nm on the FLUOstar Omega.

Cell lines and cell culture For BCSFB-related *in vitro* assays, the human malignant ChP papilloma cell line HIBCPP (Ishiwata *et al*, 2005) and the murine immortalized ChP epithelium cell line immCPE (Pauwels *et al*, 2022) were used. The HIBCPP cells and immCPE cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (11320033; Gibco) supplemented with respectively 10% or 20% fetal calf serum (FCS), in laminin (L2020; Sigma-Aldrich)-coated flasks. Media were further supplemented with non-essential amino acids (M-7145; 100x solution Sigma), L-glutamine (BE17-605 F; Lonza), Na-pyruvate (S-8636; Sigma-Aldrich), penicillin-streptomycin (P4333; 100x solution Sigma-Aldrich). All cells were cultured at 37 °C and 5% CO_2.

Immunohistochemistry For ZO-1, CSF-1R and IL-34 immunostainings on brain sections, mice were transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in D-PBS. After overnight fixation of the brains in 4% PFA at 4°C, 18 µm cryosections were cut using a cryostat (Micron HM500) and mounted on slides. After air drying, sections were again fixated with 4% PFA for 8 min and antigen retrieval was performed in 1/100 citrate buffer (S2031; Dako). After two washing steps with PBS, samples were blocked for 1 h at room temperature (RT) with 5% normal goat serum (NGS) and 0.3% Triton X-100 (10789704001; Roche) in PBT (0.5% bovine serum albumin (BSA, 001-000-162; Jackson ImmunoResearch), 0.02% Triton X-100 in 1X PBS), and then incubated with anti-ZO-1 primary antibody (1/500, 617300; Invitrogen) and anti-CD31 primary antibody (1/100, DIA-310; Dianova) or anti-CSF-1R primary antibody (1/100, 14-1152-81; Invitrogen) and anti-IL-34 primary antibody (1/200, AF5195; R&D systems) diluted in blocking buffer. After incubation overnight at 4 °C, samples were washed with PBS and incubated with goat anti-rabbit DyLight633 secondary antibody (1/400, 35562; ThermoFisher Scientific) for ZO-1, goat anti-rat DyLight488 secondary antibody (1/400, SA5-10018; ThermoFisher Scientific) for CD31, goat anti-rat DyLight488 secondary antibody (1/400, SA5-10018; ThermoFisher Scientific) for CSF-1R and donkey anti-sheep DyLight647 secondary antibody (1/400, 713-605-147; Jackon ImunnoResearch) for IL-34 diluted in PBT with 0.1% Triton X-100 for 90 min at RT. Finally, a 4',6-Diamidino-2-Phenylindole (DAPI, 1/1000, D1306; Invitrogen) staining was performed to counterstain the nuclei and the slides were mounted with polyvinyl alcohol (PVA) mounting medium with DABCO (Merck). Images were acquired using a confocal laser scanning microscope (Zeiss LSM780) and were analysed with Zen software.

For AQP4 immunostainings on brain sections, mice were transcardially perfused with ice-cold 4% PFA in D-PBS. After overnight fixation of the brains in 4% PFA at 4°C, the brains were embedded in low-gelling agarose (A4018-50G; Sigma-Aldrich) and 50 µm sections were cut using the Leica VT1200 S Vibratome. After washing the sections with PBS, samples were blocked for 45 min at RT with 5% NGS and 0.5% Triton X-100 in PBS. Next, samples were incubated with anti-AQP4 primary antibody (1/1000, 429 006; Synaptic systems) diluted in blocking buffer ON at 4°C. After washing with PBS, samples were incubated with goat anti-chicken Alexa Fluor 568 (1/1000, A-11041; Invitrogen) secondary antibody diluted in blocking buffer. Finally, DAPI (1/1000, D1306; Invitrogen) was performed to counterstain nuclei for 5 min, and samples were mounted on slides with PVA mounting medium with DABCO. Images were acquired using a confocal laser scanning microscope (Zeiss LSM780) and were analysed with Zen software.

Adoptive transfer The adoptive transfer of T and B cells was conducted on a 1:1 donor-to-acceptor mouse basis according to sex. Spleens were isolated from wild type mice and transferred to fluorescence-activated cell sorting (FACS) buffer (PBS, 0.5% BSA). Next, spleens were passed through a cell strainer (70 µm) (734-0003; BD Falcon) to make a single-cell suspension. The cells were precipitated by centrifugation for 6 min at 300 g and 4°C. After discarding the supernatant, red blood cells were removed using Ammonium-Chloride-Potassium (ACK) lysing buffer (10-548E; Lonza Bioscience). The cell pellet was resuspended in separation buffer (PBS, pH 7.2, 0.5% BSA, 2 mM ethylenediaminetetraacetic acid (EDTA)). Dead cells were stained with Trypan Blue (0.4%, 15250061; Gibco) and live cells were counted using a hematocytometer. T, B and Treg cells were further isolated by negative selection using the mouse Pan T cell isolation kit II (130-095-130; Miltenyi Biotec), the mouse Pan B cell

isolation kit II (130-104-443; Miltenyi Biotec) and the mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (130-091-041; Miltenyi Biotec) following the manufacturer's guidelines. *Rag2*$^{-/-}$ mice were reconstituted with 8 million of T, B cells in a total volume of 200 µl via tail vein injections. Control mice were injected with 200 µl of PBS. After 7 days, barrier integrity was determined as described above.

Antibody-mediated depletion In wild type mice, T cells were depleted by intraperitoneal (i.p.) injections of 300 µg/mouse of InVivoPlus anti-mouse CD3ε antibody (BP0001-1; BioXcell) dissolved in PBS every 3 days for 15 days. B cells were depleted by i.p. injections of 300 µg/mouse of InVivoMAb anti-mouse B220 (BE0067; BioXcell) dissolved in PBS every 5 days for 15 days. Treg cells were depleted by i.p. injections of 300 µg/mouse of InVivoMAb anti-mouse CD25 (BE0012; BioXcell) dissolved in PBS every 3 days for 15 days. Control animals received i.p. injections of 300 µg/mouse InVivoMAb rat IgG2a isotype control (BE0089; BioXcell), InVivoMAb polyclonal rat IgG (BE0094; BioXcell) and InVivoMAb rat IgG1 isotype control, anti-horseradish peroxidase (BE0088; BioXcell) dissolved in PBS for respectively T, B and Treg cells. On day 16, barrier integrity was determined as described above. Depletion was verified by staining of blood lymphocytes with fixable viability dye, CD45-BUV810; CD3-APC; CD19-BV605.

Transwell assay To identify the *in vitro* effect of IL-34 on brain barrier integrity, hCMEC/D3, bEnd.3, ImmCPE and HIBCPP cell lines were grown on transwell collagen-coated membrane 0.4um pore size inserts (CLS3495-24EA; Sigma-Aldrich) in a 24-well plate. Before transferring the cells to the inserts, dead cells were stained with Trypan Blue (0.4%, 15250061; Gibco) and live cells were counted using a hematocytometer. To each insert, $1{,}5 \times 10^8$ cells in 300 µl were added, and 500 µl of medium was added to the basal compartment. Confluency was determined by measuring the transepithelial/transendothelial electrical resistance (TEER) using the EVOM2 epithelial volt/ohm meter (World Precision Instruments). When the cells reached confluency, the following conditions were applied: PBS for 6h (control), LPS (1 µg/mL, tlrl-pelps; InvivoGen) for 6h, LPS (1 µg/mL) together with IL-34 (50 ng/mL, 5195-ML-010; R&D systems) for 6h, and LPS (1 µg/mL) for 6h with the addition of IL-34 (50 ng/mL) after 4h. At the 6h timepoint, 4 kDa FITC-dextran (FD4-1G; Sigma-Aldrich) was added to the basal compartments of the 24-well plate. After 2h, samples were taken from the apical compartment, followed by measuring the fluorescence at $\lambda_{ex}/\lambda_{em}$ = 485/520 nm on the FLUOstar Omega (BMG Labtech).

Choroid plexus isolation and primary transwell assay Brains were isolated from wild type and *Rag2*$^{-/-}$ mice and excess blood was washed off. ChP was dissected from the lateral and third ventricles, immersed in DMEM/F12 medium (11320033; Gibco) and centrifuged at 1000 rpm for 5 min. The cell pellet was washed with Hanks' Balanced Salt Solution (HBSS, 14175053; Gibco) and incubated in HBSS supplemented with 0.2% pronase (537088; Calbiochem) for 7 min at 37°C while shaking. After the digestion reaction was stopped by adding DMEM/F12, cells were centrifuged at 1000 rpm for 5 min, cells were resuspended in DMEM/F12 medium and 300 µl was added to transwell collagen-coated membrane 0.4um pore size inserts (CLS3495-24EA; Sigma-Aldrich). To the basal compartment, 500 µl of medium was added. Confluency was determined by measuring the TEER using the EVOM2 epithelial volt/ohm meter (World Precision Instruments). When the cells reached confluency, 4 kDa FITC-dextran (FD4-1G; Sigma-Aldrich) was added to the basal compartments of the 24-well plate. After 2h,

samples were taken from the apical compartment, followed by measuring the fluorescence at $\lambda_{ex}/\lambda_{em}$ = 485/520 nm on the FLUOstar Omega (BMG Labtech).

***In vivo* administration of IL-34** Recombinant mouse IL-34 (74,16 pg in 200 µl per mouse, 5195-ML-010; R&D systems) was i.v. injected every 2h for 24h in *Rag2*$^{-/-}$ mice and wild type mice. Wild type mice additionally received an LPS injection (50 µg in 200 µl per mouse) at the 1h timepoint. At the 24h timepoint, the mice were sedated, and the barrier integrity was determined as described above. EAE and *App*$^{NL-G-F}$ mice were i.v. injected 3 times a day for 3 days in a row with recombinant mouse IL-34 (74,16 pg in 200 µl per mouse, 5195-ML-010; R&D systems) or with sterile D-PBS as control. Six hours after the last injection, mice were sedated, and the barrier integrity was determined as described above.

Plasma isolation Blood was taken from EAE and *App*$^{NL-G-F}$ mice by heart punction and centrifuged at 1300 g and 4°C for 10 min. Supernatant was taken and centrifuged again at 2400 g and 4°C for 15 min.

Measurement of IL-34 by ELISA To determine the level of IL-34 in the plasma *App*$^{NL-G-F}$ mice, 35- week-old mice and age-matched wild type controls were used. For EAE, mice of 7 days, 16 days, and 26 days after immunization with MOG_{35-55} were used. Naïve EAE mice were used as control. The level of IL-34 in the plasma of EAE and *App*$^{NL-G-F}$ mice was determined using the LEGEND MAX™ Mouse IL-34 ELISA kit (439107; BioLegend) following the manufacturer's guidelines.

Flow cytometry Mice were sedated with a lethal dose of ketamine/xylazine (100 mg/kg ketamine; 20 mg/kg xylazine) and blood was collected from the right ventricle. Next, mice perfused with D-PBS/heparin (0.2% heparin, H-3125; Sigma-Aldrich) and brains were collected in serum-free RPMI medium (27016021; Gibco) supplemented with 3% FCS on ice. Brain samples were cut into small pieces and incubated at 37°C for 30 min, with pipetting up and down every 10 min. The single-cell mixture was passed through a cell strainer (70 µm) (734-0003; BD Falcon) and the cell suspension was centrifugated at 400 g and 4°C for 7 min. The cell pellet was resuspended in 30% percoll (17-0891-02; GE Healthcare) in HBSS (14175053; Gibco). After centrifugation at 600 g and 4°C for 10 min, the cell pellet was resuspended in staining buffer with fixable viability dye, CD45-BUV810; CD3-APC; CD19-BV605.

Oral PLX-3397 treatment Inhibition of the CSF-1R in wild type mice was done via oral administration of pexidartinib (PLX-3397). PLX-3397 (HY-16749; MedChemExpress) was formulated into the AIN-76A rodent diet (Research Diets Inc., New Brunswick NJ USA) at a dose of 75 mg/kg. Control mice received AIN-76A rodent diet without PLX-3397. After 9 days of *ad libitum* administration, the mice were sedated, and the barrier integrity was determined as described above.

Statistics Data are shown as mean ± SEM. Two-tailed Student's t-test for parametric data was used to compare two groups. One-way ANOVA with Dunnett's or Tukey's multiple comparisons test was used to compare 3 or more groups. GraphPad Prism 9.0 was used for statistical analysis. Differences were considered significant at $p < 0.05$.

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Attachments

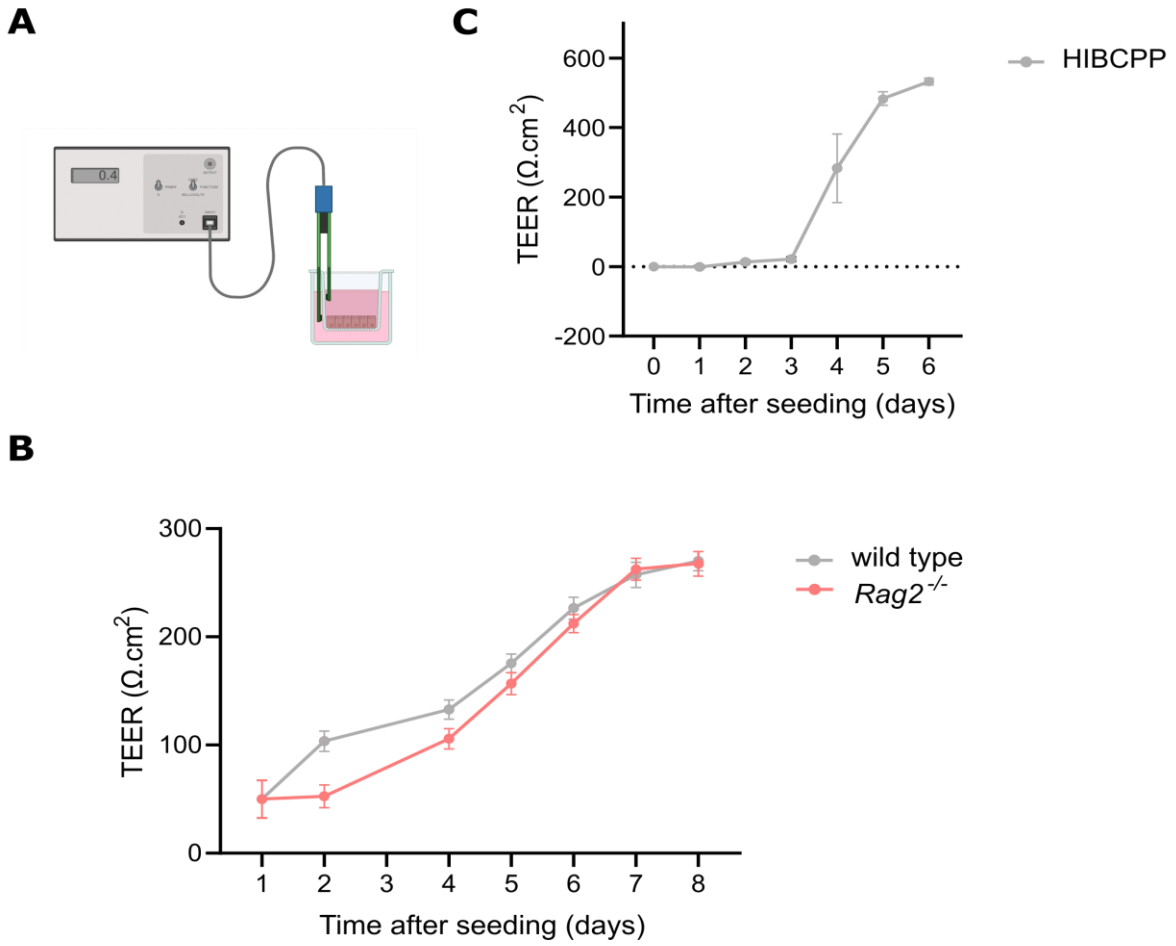

Figure S1: Trans-epithelial electrical resistance (TEER) shows when confluency is reached by cells grown on transwell inserts. (A) The resistance is measured with the EVOM2 Epithelial Volt/Ohm meter by placing two electrodes in the basal compartment and the transwell insert. TEER is calculated by multiplying the surface area of the transwell (in cm^2) with the resistance (in Ohm, Ω) measured. Each transwell insert has a surface area of 0.33 cm^2. **(B)** TEER of wild type and *Rag2*$^{-/-}$ mice in days after seeding the cells on the transwell insert. **(C)** TEER of the human malignant choroid plexus papilloma cell line (HIBCPP) in days after seeding the cells on the transwell insert. Of note, the TEER of murine immortalized choroid plexus epithelial cells (ImmCPE) was not measured.

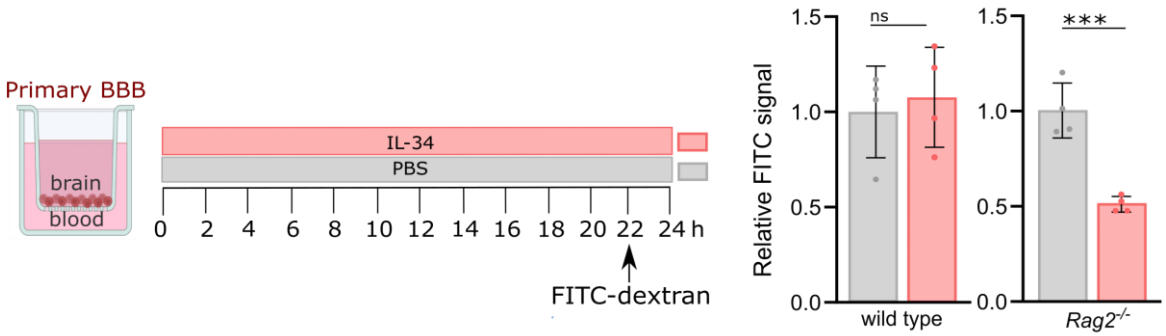

Figure S2: *In vitro* administration of interleukin (IL)-34 rescues leakage of primary brain microvascular endothelial cells from *Rag2*$^{-/-}$ mice. Primary brain microvascular endothelial cells were isolated from wild type and *Rag2*$^{-/-}$ mice and seeded in the apical compartment of a transwell system. Cells were stimulated for 24 hours with IL-34. PBS treatment was used as control. At the 22-hour timepoint, 3 kDa FITC-dextran was added to the basal compartment (blood side), and 2 hours later, leakage was determined by measuring fluorescence in the apical compartment (brain parenchymal side). Wild type results are not shown. Data are shown as mean ± SEM (n= 3 per group). Statistical significance was determined by two-tailed Student's t-test; ***$p < 0.001$, ns: non-significant. Results were obtained in the lab of Prof. Bieke Broux (UHasselt).

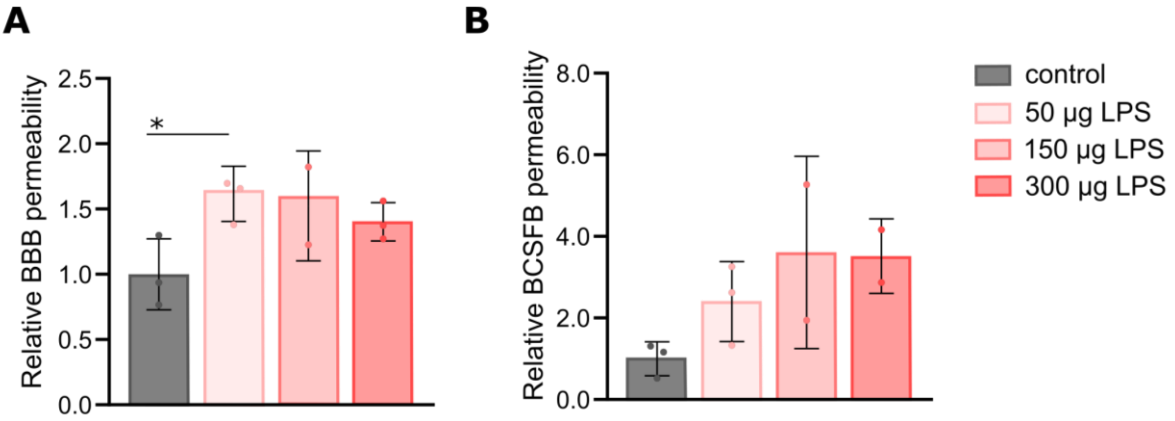

Figure S3: Wild type mice show brain barrier leakage at a dosage of 50 µg lipopolysaccharides (LPS). Three dosages of LPS; 50 µg, 150 µg, and 300 µg, were administered to wild type mice by intraperitoneal injections. PBS-injected mice were used as control. Brain barrier leakage was determined in the prefrontal cortex for the blood-brain barrier (BBB) and in the cerebrospinal fluid (CSF) for the blood-CSF barrier (BCSFB) 15 min after intravenous injection of 4 kDa fluorescein isothiocyanate (FITC)-dextran. Data are shown as mean ± SEM (n=2/3 mice per group). Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparisons test; *$p < 0.05$.