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The Role of A20 in Blood Brain Barrier Maintenance
Die Rolle von A20 in der Aufrechterhaltung der Blut-Hirn-Schranke

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Für Mama und Papa

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List of Abbreviations

Abbreviation	Full name
AJ	Adherent junction
ALCAM	Activated leukocyte cell adhesion molecule
APC	Antigen presenting cell
ApoE	Apolipoprotein E
AQP4	Aquaporin 4
AR	Clematichinenoside
AUC	Area under the curve
BBB	Blood brain barrier
BCA	Bichinol-4-Carbonacid
BEC	Brain endothelial cell
BHS	Blut-Hirn-Schranke
BM	Basement membrane
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
Cezanne	Cellular zinc finger anti NFκB
CFA	Complete Freud’s adjuvant
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cu	Copper
CYLD	Cylindromatosis tumor suppressor protein
DC	Dendritic cell
DIS	Disseminated in space
DIT	Disseminated in time
DMT	Disease-modifying therapy
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cells
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GFAP	Glial fibrillary acidic protein

HA20	A20 haploinsufficiency
HLA	Human leukocyte antigen
Hprt	Hypoxanthin-Guanin-Phosphoribosyltransferase
HSC	Hematopoietic stem cell
Iba1	Ionized calcium-binding adapter molecule 1
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IFN-β	Interferon-beta
IFN- γ	Interferon-gamma
IRAK	IL-1β-associated kinase
i.p.	Intraperitoneal
i.v.	Intravenous
JAM	Junctional adhesion molecules
LEC	Lung epithelial cell
LFA	Leukocyte function-associated antigen
LPS	Lipopolysaccharide
Lys	Lysine
MHC	Major histocompatibility comples
MBP	Myelin basic protein
MMP	Metalloproteinase
MOG_{35-55}	myelin oligodendrocyte glycoprotein 35-55
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
MSC	Myeloid stem cell
NF-κB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells
NIK	NF-κB-inducing kinase
NK	Natural killer cell
NPSLE	Neuropsychiatric SLE
NVU	Neurovascular unit

OSE	Opticospinal EAE
OTU	Ovarian tumor
O_2	Oxygen
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PP	Primary-progressive
PTx	Pertussis toxin
qPCR	Quantitative real time PCR
rBMEC	Rat microvessel endothelial cells
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RP	Relapsing-progressive
RR	Relapse-remitting
Shh	Sonic hedgehog
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SP	Secondary-progressive
TAB	Transforming growth factor-β-activated kinase 1 binding protein
TAK	Transforming growth factor-β-activated kinase
TEER	Transepithelial electrical resistance
Teff	Effector T cell
TEM	Transendothelial migration
TGFβ	Transforming growth factor beta
Th	T-helper type
TJ	Tight junction
TLR	Toll-like-receptor
TNF	Tumor necrosis factor
TNFAIP	Tumor necrosis factor α-induced protein
TRADD	TNF-receptor-associated protein with a death domain
TRAF	TNF-receptor-associated factor
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1

VLA	Very late antigen
ZNS	Zentrales Nervensystem
Zn^{2+}	Zinc
ZO	Zonula occludens

Table 1: List of Abbreviations

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1 Zusammenfassung

Das Ubiquitin-modifizierende Protein A20 ist von entscheidender Bedeutung für die Regulation von Entzündungen, da es die kanonische NF-κB Signalübertragung negativ reguliert und somit als entzündungshemmender Mediator fungiert. Es wurde gezeigt, dass Einzelnukleotid-Polymorphismen im A20/TNFAIP3-Gen das Risiko erhöhen, verschiedene Autoimmunerkrankungen wie Multiple Sclerose (MS) zu entwickeln. In dieser Studie untersuchen wir die Rolle von A20 in Endothelzellen (EC) der Blut-Hirn-Schranke (BHS) bei der Aufrechterhaltung der BHS-Integrität und die Infiltration von Immunzellen bei fehlender Entzündung, sowie die Konsequenzen einer EC-spezifischen Deletion von A20 auf neuroinflammatorische Prozesse in der Homöostase sowie dessen Auswirkungen auf den Verlauf von experimenteller autoimmun Enzephalomyelitis (EAE), einem Mausmodell der MS. Wir konnten zeigen, dass die BHS-Integrität durch das Fehlen von A20 in ECs des zentralen Nervensystems (ZNS) nicht verändert wurde und dass es keinen Unterschied in der Expression von Verbindungsproteinen gab. Zudem konnten wir auch keine Diskrepanz im Krankheitsverlauf der aktiven EAE bei A20-defizienten Mäusen im Vergleich zu Kontrollmäusen feststellen. Jedoch konnten wir eine dramatisch erhöhte Expression der Adhäsionsmoleküle ICAM-1 und VCAM-1 bei fehlender Entzündung beobachten, was auf eine starke Aktivierung des Endothels der BHS hinweist. Wir konnten weiterhin zeigen, dass ein A20-Mangel die Infiltration von Immunzellen in das ZNS in der Homöostase antreibt. Unsere Daten belegen ferner, dass ein A20-Mangel in ZNS-ECs die Entwicklung einer Astrogliose begünstigt und somit zur Entstehung einer Neuroinflammation beiträgt.

Zusammenfassend schlagen wir vor, dass ein A20-Mangel in BHS-ECs bei fehlender Entzündung zu einer verlängerten NF-κB-Signalübertragung führt, was wiederum zu einer entzündungsfördernden Genexpression führt, einschließlich der Gene für ICAM-1 und VCAM-1. Dies trägt wahrscheinlich zu einer festen Adhäsion von Immunzellen an der BHS bei, was anschließend eine erhöhte T-Zell-Transmigration in das ZNS fördert. Darüber hinaus können A20-defiziente BHS-ECs eine spontane Neuroinflammation mit Veränderungen der Astrozytenreaktivität vorantreiben. Zusammen resultiert das in einem entzündlichen Phänotyp, welcher möglicherweise die Entstehung von neurologischen Erkrankungen vorantreiben könnte.

2 Introduction / Goal of the dissertation

The control of gene expression is an inevitable mechanism for most organisms to adapt to changes in their environment. Nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB) influences gene expression that has an impact on cell survival, differentiation and proliferation, as well as the regulation of the immune system (Hayden and Ghosh, 2012). During inflammation NF-κB is activated by proinflammatory cytokines which leads to an activation of gene expression, triggering an increased production of cytokines and chemokines and therefore leading to an immune response (Lawrence, 2009).

Since NF-κB activation is central to many cell processes, tight regulation is absolutely necessary to ensure tissue homeostasis (Renner and Schmitz, 2009). A20, also known as tumor necrosis factor α-induced protein (TNFAIP) 3, plays a key role in the regulation of NF-κB signaling. It has been shown that A20 has two distinct catalytic domains, both of which cooperate to downregulate NF-κB signaling (Wertz *et al.*, 2004). Mutations in the TNFAIP3 gene are linked to autoimmune diseases such as systemic lupus erythematosus (SLE), psoriasis, and multiple sclerosis (MS) (Musone *et al.*, 2008; Nair *et al.*, 2009; Jager *et al.*, 2009). In MS, an abnormal response of the body's immune system is directed against neurons of the central nervous system (CNS), leading to inflammation and causing damage to the myelin-sheaths which isolate and protect neurons in the healthy CNS. This process manifests itself in clinical symptoms such as visual and sensory impairment, neuropathic pain, and movement disorders (Compston and Coles, 2008). Moreover, MS is associated with a breakdown of the blood brain barrier (BBB), which usually protects the brain against circulating toxins and pathogens while allowing nutrients to enter the brain. In MS, a disorganization of junctional molecules, which restrict leukocyte infiltration under physiological conditions, and an upregulation of adhesion molecules leads to an increase of infiltrating immune cells into the CNS and therefore to a more severe progression of inflammation (Lécuyer *et al.*, 2017).

In recent years, new findings in the neuroinflammatory research field have contributed substantially to our understanding of the underlying pathophysiological mechanisms of MS, many of which are based on the widely available rodent models of MS, amongst them the experimental autoimmune encephalomyelitis (EAE) model system

(Constantinescu *et al.*, 2011). With the help of this model the role of A20 on the progression of neuroinflammation has been investigated by different work groups. In 2013, Wang *et al.* examined the function of A20 in EAE in order to answer the unresolved question of how it regulates MS pathogenesis. Therefore, they first deleted A20 in neuroectodermal cells (astrocytes, neurons, and oligodendrocytes; $A20^{\Delta Nestin}$ mice), resulting in a significantly stronger EAE together with an increased T cell infiltration into the CNS, an enhanced production of cytokines and chemokines, and a higher expression of inflammatory genes. Next, they concentrated on astrocytes since they are a major source of chemokines. Wang *et al.* generated $A20^{\Delta GFAP}$ mice which lack A20 selectively in astrocytes. Again, mice showed more severe clinical symptoms to EAE with an increased number of infiltrating cells (Wang *et al.*, 2013). In 2018, Voet *et al.* created an inducible $A20^{\Delta Cx3Cr1}$ mouse lacking A20 specifically in microglia. These mice were hypersensitive to EAE, showing extensive demyelination, axonal damage, inflammation, and immune cell infiltration. Moreover, they could show that genes linked to cell activation, microglia polarization, major histocompatibility complex (MHC) class 1, type one interferon signaling, and inflammatory signaling such as NF-κB were upregulated. These results underline the important role of A20 in securing CNS homeostasis and preventing the development of inflammatory CNS pathology (Voet *et al.*, 2018).

Even though it is well known that defects in A20-dependant regulation of NF-κB contributes to several different inflammatory and autoimmune diseases, its role in endothelial cells is largely unknown (Chu *et al.*, 2011). The aims of the present study are to identify the role of A20 in endothelial cells on the maintenance of BBB integrity in steady state and to evaluate its impact on autoimmune diseases.

3 Literature review

3.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that often affects young adults, typically starting between 20 and 40 years of age (Nicholas and Rashid, 2013). In the developed world, it is the leading cause of disability in young and middle-aged people (Koch-Henriksen and Sørensen, 2010). First symptoms of MS include visual and sensory impairment, neuropathic pain, as well as movement disorders (Compston and Coles, 2008). Even though the etiology of MS is not fully understood yet, it is most likely linked to a genetic predisposition (Hafler *et al.*, 2007).

3.1.1 Epidemiology and etiology

Approximately 2.8 million people suffer from MS worldwide, among them more than 280.000 in Germany alone and prevalence has risen steadily over the last years (Deutsche Multiple Sklerose Gesellschaft Bundesverband e.V., 2023; Holstiege *et al.*, 2017). In general, women have a higher risk of developing MS with an increasing female to male ratio of 1.4 in 1955 to 2.3 in 2000 (Kamm *et al.*, 2014). Until today, the female to male ratio has reached more than 3:1 (Compston and Coles, 2008). Moreover, studies have shown that the risk of getting MS increases with greater distance from the equator. Regions of high risk include Northern USA, Canada, Australia, New Zealand, and Israel, with the greatest risk among Northern Europeans (Browning *et al.*, 2012; Kurtzke, 2000; Compston and Coles, 2008). Regions with low prevalence can be found in South America and Asia (Kurtzke, 2000). Moreover, it seems that the risk for MS correlates with the place of residence in childhood. Studies of migration patterns have shown that moving from a high- to a low-risk region in childhood is associated with a decreased risk of MS, whereas a child migrating from a low- to a high-risk region takes on the higher risk level of the new region (Compston and Coles, 2008).

Even though MS is not considered a hereditary disease, genetic factors are known to contribute to the risk of developing MS. Many years ago studies have found a correlation between genetic variations in the human leukocyte antigen (HLA), which primarily encodes immune-related antigen recognition molecules within the major

histocompatibility complex (MHC), and MS (Jersild *et al.*, 1973). Moreover, genome-wide association studies identified more than 100 single nucleotide polymorphisms (SNP) connected to MS, including SNPs within the interleukin-2 receptor α gene, the interleukin-7 receptor α gene or the TNFAIP3 gene (Hoffjan *et al.*, 2015; Hafler *et al.*, 2007). Interestingly, most SNPs identified occurred in gene loci related to T cell differentiation and activation, providing evidence that the critical disease mechanisms involve immune dysregulation (Sawcer *et al.*, 2011).

Not only genetic factors seem to play a role in the risk of developing MS, but also environmental and behavioral factors, including infections with Eppstein-Barr virus, smoking, obesity, as well as low levels of vitamin D (Bjornevik *et al.*, 2022; Abdelrahman *et al.*, 2014; Heydarpour *et al.*, 2018; Novo and Batista, 2017; Simpson *et al.*, 2018).

3.1.2 **Clinical Subtypes**

In 1996, the Advisory Committee on Clinical Trials of new Agents in MS of the United States National Multiple Sclerosis Society defined four standardized clinical subtypes of MS in order to ensure accurate communication, design and recruitment of clinical trials and treatment decisions. These categories were divided according to the clinical course of the disease: relapsing-remitting (RR) MS, primary-progressive (PP) MS, secondary-progressive (SP) MS, and relapsing-progressive (RP) MS (Lublin and Reingold, 1996). Since these categories did not include imaging and biological correlate, the categories were re-examined by the International Advisory Committee on Clinical Trials of MS in 2013 (Lublin *et al.*, 2014). As a result, the RPMS category was eliminated and the clinically isolated syndrome (CIS) was introduced as a new category to describe MS (Lublin *et al.*, 2014).

Until today the different MS subtypes are defined as following:

3.1.2.1 Clinically Isolated Syndrome

CIS is defined as the first clinical presentation of a disease showing characteristics of an inflammatory demyelinating progress that could be MS, typically involving the optic nerve, brainstem, spinal cord, or cerebral hemispheres (Miller *et al.*, 2005). When CIS is accompanied by brain lesions that are similar to those seen in MS and that can be detected by magnetic resonance imaging (MRI), the patient has a 60 to 80 percent

chance of being diagnosed with MS in the following years (National Multiple Sclerosis Society, 2019). In order to diagnose MS in CIS patients, a clinical or MRI demonstration of dissemination of cortical lesions to other regions can be used (Thompson *et al.*, 2018).

3.1.2.2 Relapsing-remitting MS

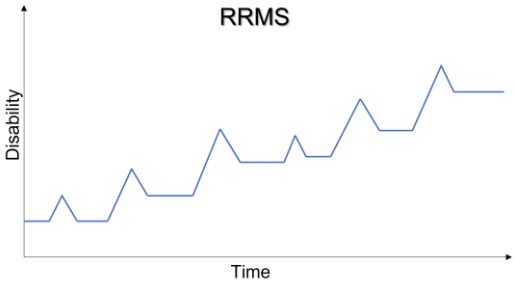

Figure 1: RRMS disease activity over time

After a relaps, symptoms may disappear or partially disappear.

Patients suffering from RRMS experience periods of neurological deficits, called relapses, followed by episodes of partial to complete recovery, called remission. The episodes occur sporadically and usually do not exceed 1 to 5 relapses per year (Compston and Coles, 2008). A relapse is defined as an episode of neurological symptoms in the absence of fever or infection, which lasts at least 24 hours and is accompanied by a demyelinating event in the CNS (Polman *et al.*, 2011). This form of MS is present in 85% to 90% of patients at diagnosis (Browning *et al.*, 2012), and over 95% of pediatric MS patients initially show a relapsing-remitting disease course (Polman *et al.*, 2011).

3.1.2.3 Secondary-progressive MS

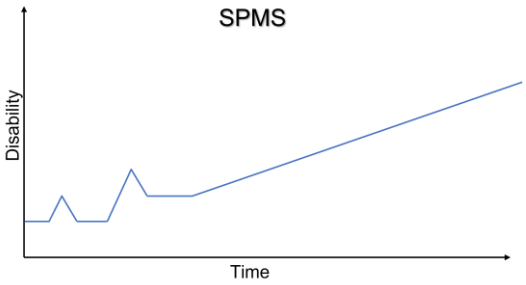

Figure 2: SPMS disease activity over time

SPMS may occur after several years of RRMS, with increasing disability over time.

Within three decades, approximately 65% of RRMS patients transition into a secondary progressive phase (Scalfari *et al.*, 2014). SPMS patients experience continuous neurocognitive decline without symptom-free remissions, that usually manifest as motor and long tract-symptoms (Browning *et al.*, 2012). There are no criteria determining the exact point when RRMS transitions to SPMS (Lublin *et al.*, 2014). Men often experience a more rapid progression to SPMS than women, and the older the patient is at first presentation of symptoms, the faster the progression occurs (Browning *et al.*, 2012; Scalfari *et al.*, 2014).

3.1.2.4 Primary-progressive MS

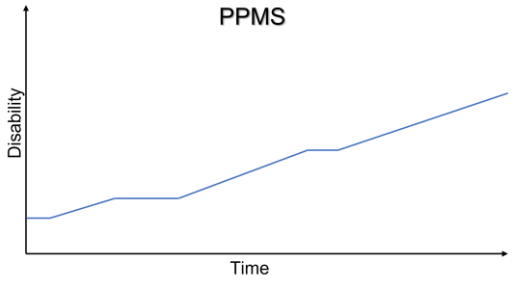

Figure 3: PPMS disease activity over time

In PPMS, symptoms worsen steadily with no sign of relapse or remission.

In contrast to SPMS patients, primary-progressive patients show no sign of relapse or remission in the course of disease, with symptoms steadily increasing from diagnosis (Thompson *et al.*, 1991; Lublin and Reingold, 1996). Unlike in RRMS, men and women have almost the same risk of PPMS (1.1-1.3 to1 compared to the 3:1 ratio of MS overall), and the mean age of onset is greater. Moreover, PPMS is almost never seen in children (Rice *et al.*, 2013).

3.1.3 **Diagnosis**

In order to diagnose MS, clinical symptoms, MRI findings, and analysis of cerebrospinal fluid (CSF) are required. In the past, MS was diagnosed with the help of the Poser's criteria, which define MS as the occurrence of at least two demyelinating attacks lasting at least 24 hours and involving two or more different parts of the CNS (Poser *et al.*, 1983). With the introduction of the McDonald criteria in 2000, the usage of MRI was integrated in the diagnostic scheme for MS (McDonald *et al.*, 2001). Moreover, examination of CFS was then used in order to maintain information about inflammation and immunological disturbances. The McDonald criteria were modified in 2005 (Polman *et al.*, 2005) and again in 2010 (Polman *et al.*, 2011). Accordingly, the presence of T2-weighted lesions by MRI which are disseminated in space (DIS) and time (DIT) are predictive for MS. DIS is defined as at least one T2 lesion in two or more of the four multiple sclerosis-typical regions, including juxtacortically, periventricularily, in the posterior fossa, and the spinal cord. To fulfill the DIT criterion, asymptomatic gadolinium enhancing and non-enhancing lesions have to be simultaneously present in one MRI scan, or a new T2 lesion has to be detected in a follow-up MRI (Polman *et al.*, 2011). Moreover, the presence of oligoclonal bands in the CSF, which can be found in about 90% of MS patients, helps to support the diagnosis of MS (Deisenhammer *et al.*, 2019).

3.1.4 **Immunopathogenesis**

Based on findings from immunological, histopathological, and genetic studies, as well as experiences from clinical trials, it is widely accepted that MS pathogenesis has a major immunological component. Two opposing hypotheses on the initiation of MS have been suggested. The most accepted hypothesis claims that a CNS antigen-specific immune activation takes place in the periphery and leads to an immune response in the CNS. The second hypothesis proposes an initiating event in the CNS leading to an activation of microglia and an immune reaction, followed by the recruitment of immune cells to the CNS (Hemmer *et al.*, 2015).

The first theory proposes, that autoreactive T cells are primed by antigen presenting cells (APC) such as dendritic cells (DC) in peripheral lymphoid organs through mechanisms of molecular mimicry, cross reactivity, and bystander activation (Sospedra and Martin, 2005; Koch *et al.*, 2013). After activation, these autoreactive T-helper type (Th) 1 and Th17 cells migrate towards the blood brain barrier (BBB) and into the CNS (Yadav *et al.*, 2015). The migration of T cells is mediated by the upregulation of different adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which are primarily expressed on endothelial cells (EC) (Ortiz *et al.*, 2014). They interact with the leukocyte function-associated antigen (LFA)-1, or the very late antigen (VLA)-4 on Th 1 cells (Engelhardt and Ransohoff, 2012). The CC-chemokine receptor (CCR)-6 expression on Th17 cells allows an interaction with its ligand CC-chemokine ligand (CCL)-20 on EC, promoting T cell migration (Goverman, 2009). Once T cells crossed the BBB, they are reactivated by local APCs, leading to the secretion of proinflammatory cytokines like interferon-gamma (IFN-γ) or interleukin (IL)-17 (Legroux and Arbour, 2015). These cytokines recruit more inflammatory cells, stimulate plasma cells to produce antibodies which target myelin sheaths, and activate microglia and astrocytes (Kamm *et al.*, 2014). They themselves release more cytokines, leading to further recruitment of inflammatory cells, for example monocytes, and continuing breakdown of the BBB (Cannella and Raine, 1995). In the parenchyma, monocyte-derived macrophages and microglia promote demyelination, leading to neurodegeneration (Bogie *et al.*, 2014). Furthermore, activation of astrocytes inhibits oligodendrocyte differentiation and therefore reduces remyelination, intensifying neurodegeneration (Racke, 2009). Moreover, B cell activation is considered to contribute to CNS damage in MS as they

can function as APCs, secrete cytokines, and release pathogenic antibodies (Wekerle, 2017). Additionally, B cells can differentiate into antibody-secreting plasma cells. Plasma cells release immunoglobulin G (IgG) which causes demyelination and axonal damage (Claes *et al.*, 2015).

The alternative hypothesis proposes that the forming of MS lesions starts with the loss of oligodendrocytes in the presence of reactive microglia but barely any other inflammatory cells (Henderson *et al.*, 2009). The exact reason for the death of oligodendrocytes is still unknown but it has been proposed that the loss of oligodendrocytes might occur due to a genetic mutation, a metabolic disturbance, or oxidative stress with microglia as a primary source of reactive oxygen species (Luo *et al.*, 2017). Due to local tissue damage, antigens are released and drain out of the CNS either along perivascular pathways or via APCs in the CSF. They drain toward deep cervical lymph nodes where they are presented by APCs and induce a secondary immune response (Laman and Weller, 2013). Finally, the activation of antigen specific T cells in the draining lymph nodes results in the targeting of myelin and oligodendrocytes, leading to demyelination and neurodegeneration (Hemmer *et al.*, 2015). However, there is a lot of criticism directed at this hypothesis. For instance, neurodegenerative diseases affecting oligodendrocytes or myelin sheaths, for example in white matter disease, do not necessarily lead to a destructive reaction of the adaptive immune system (Lin *et al.*, 2017). Furthermore, genetic studies done in patients do not support the theory of primary damage to oligodendrocytes in MS patients (Hemmer *et al.*, 2015).

Although for many years research for autoimmunity in the CNS has been focused on cluster of differentiation (CD) 4^+ T cells, the importance of $CD8^+$ T cells has recently emerged. One reason for this is that $CD4^+$ T cells are outnumbered by $CD8^+$ T cells in brain lesions of MS patients (Salou *et al.*, 2015). Studies showed that the number of MHC class I expressing astrocytes, oligodendrocytes, microglia, and macrophages, which are recognized by $CD8^+$ T cells, is elevated in MS lesions (Höftberger *et al.*, 2004).

Regulatory T (Treg) cells seem to play a role in the pathogenesis of MS as well. The function of Treg cells is to maintain tolerance against self-antigens and to prevent inflammation in the CNS by secreting soluble mediators such as IL-10 and

transforming growth factor beta (TGFβ) (Goverman, 2009). Usually, Tregs inhibit the production of pro-inflammatory cytokines through the suppression of effector T (Teff) cells. Moreover, they seem to play a role in eliminating antigen-presenting and autoantibody-producing B cells by secreting perforin and granzyme (Danikowski *et al.*, 2017). In MS, there seems to be an impairment of Treg function, causing an improper silencing of autoreactive T cells specific for myelin antigens (Mastorodemos *et al.*, 2015).

3.1.5 **Therapies for MS**

Up to date, there is no cure for MS. The therapies available aim at improving symptoms and reducing the relapse rate and MRI disease activity (disease-modifying therapies, DMT) (Kamm *et al.*, 2014). The therapy of MS is based on three therapeutic principles: treatment of exacerbations, slowing disease progression, and symptomatic therapies. In order to manage an acute relapse in MS, high-dose intravenous (i.v.) administration of corticosteroids such as methylprednisolone is recommended, as it reduces symptoms, shortens recovery time, and improves motor function (Hart and Bainbridge, 2016). For long-term therapy of RRMS, DMTs comprising interferon-beta (IFN-β) and glatiramer acetate are used, delaying disease progression and severity, but not showing a curative effect (Tsareva *et al.*, 2016). As second-line treatment, mitoxantrone, an anti-inflammatory medication, natalizumab, which inhibits the transmigration of inflammatory cells into the CNS by blocking the interaction of T-cell integrin with its ligand VCAM-1, and other drugs (including methotrexate, dalfampridine, and fingolimod) are used (Khoy *et al.*, 2020; Browning *et al.*, 2012). The first line therapy of PPMS is ocrelizumab, a type 1 anti-CD20 IgG monoclonal antibody which depletes pre-B cells, immature B cells, mature B cells, and memory B cells (Bigaut *et al.*, 2019). In order to fight balance and mobility impairments, muscle weakness, spasticity, and ataxia, physiotherapy is most commonly prescribed for all MS patients (Feinstein *et al.*, 2015). As an alternative therapeutic option stem cell therapy has gained interest over the last years (Rahim *et al.*, 2018). Stem cells can be divided into two groups: hematopoietic stem cells (HSC) and myeloid stem cells (MSC). As an attempt to restart the immune system, HSCs are transplanted, which then migrate to the bone marrow, where they produce new immune cells that do not attack myelin or other brain tissues any longer (Paula A Sousa *et al.*, 2015). The transplantation of MSCs leads to the suppression of T cells, B cells, and natural killer

cells (NK), making it a valuable therapy for MS patients (Sherbet, 2016). In general, the transplantation of stem cells has the potential to become a treatment option for MS. However, it should only be carried out as a part of studies (Hemmer et al., 2021).

3.1.6 **Experimental Autoimmune Encephalomyelitis**

In recent years new findings in the neuroinflammatory research field have contributed substantially to our understanding of the underlying pathophysiological mechanisms of the disease. This has been made possible by the establishment of several animal models, the most commonly used among them being the rodent experimental autoimmune encephalomyelitis (EAE) model. EAE can be induced actively by injecting a water-oil emulsion of complete Freund's adjuvant (CFA; supplemented with *Mycobacterium tuberculosis*) mixed with myelin derived protein and peptide, followed by the injection of pertussis toxin (PTx) the same day and two days later (Kurschus, 2015). Encephalitogenic peptides that can be used include myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}), myelin basic protein (MBP), proteolipid protein (PLP), and others (Robinson *et al.*, 2014). Alternatively, passive activation of EAE can be achieved by transferring pre-activated myelin-specific $CD4^+$ T cells into naïve mice (Ben-Nun *et al.*, 1981; McCarthy *et al.*, 2012). In the case of active immunization myelin-specific $CD4^+$ T cells circulating in the periphery of naïve mice are primed in secondary lymphoid organs and subsequently migrate towards the CNS where they infiltrate brain and spinal cord (Stromnes and Goverman, 2006; Waisman and Johann, 2018). Once the T cells crossed the BBB, they are reactivated by local APCs, starting an inflammatory cascade which leads to the recruitment of an exceeding number of immune cells into the CNS, eventually causing tissue damage (Constantinescu *et al.*, 2011).

The course of EAE in mice is generally characterized by paralysis beginning at the tail and ascending to the limbs and forelimbs, but the pathological features can differ depending on the animal species, strain, induction method, and auto-antigen used (Gold *et al.*, 2006; Procaccini *et al.*, 2015). For example, while the immunization of C57BL/6 mice with MOG_{35-55} peptide results in a monophasic disease course, SJL/J mice display a relapsing-remitting disease course upon immunization with PLP peptide (Terry *et al.*, 2016).

From the pathogenesis point of view, EAE is a good model to study the mechanisms of MS. Nevertheless, there are essential differences between EAE and MS. One of the major differences is that the induction of EAE requires an external immunization step, whereas the pathology in MS patients including the generation of autoantibodies is not artificially induced and therefore the pathogenic mechanisms can be different (Hart *et al.*, 2011). Moreover, for EAE to work myelin peptides are diluted in adjuvants containing bacteria and mice are injected with PTx, triggering a response of the innate immune system (Darabi *et al.*, 2004). In order to reduce the discrepancies between EAE and MS, new and optimized models are continuously created, or species more closely related to humans (e.g. marmosets) are used (Hart *et al.*, 2011). For example, a T cell receptor transgenic mouse specific for MOG_{35-55}, also referred to as 2D2 mouse, has been generated. These mice develop spontaneous optic neuritis without any clinical or histological evidence of EAE, a situation which is often described in MS patients at the onset of RRMS and before the involvement of brain and spinal cord (Bettelli *et al.*, 2003). Later, 2D2 mice were crossed to MOG-specific Ig heavy-chain knock-in mice containing B lymphocytes that produce the heavy chain of a demyelinating MOG-specific antibody. The offspring, termed OSE (opticospinal EAE) mice, develop spontaneous inflammatory demyelination and the lesions resemble those of neuromylitis optica, a disease closely related to human MS (Krishnamoorthy *et al.*, 2006).

3.2 **Blood Brain Barrier**

As early as 1885, first studies to show the existence of a selective barrier between the blood and the brain were done by Paul Ehrlich. For this purpose, he injected Evan's blue dye intravenously into a rat and could observe that all organs except the brain were stained. In 1913, his graduate student injected dye into the CSF of dogs and found that only the brain and spinal cord tissue stained, concluding that there was a barrier between the blood and the brain (Saunders *et al.*, 2014). Nowadays it is well established that there is a total of three interfaces that function as barriers between the blood and the CNS: the blood-CSF barrier, the arachnoid barrier, and the BBB (Abbott *et al.*, 2010). In the following passage, focus will be put on the latter.

3.2.1 **Function**

Blood vessels play an important role in the transportation of oxygen (O_2) and nutrients to all the organs of the body, including the brain. In the CNS, blood vessels differ from those in the rest of the body. A unique barrier is formed – called the blood-brain barrier (BBB) – that allows a tight regulation of the movement of ions, molecules, and cells from the blood to the brain, providing a stable environment for neuronal function. (Ballabh *et al.*, 2004). Moreover, the BBB helps to keep the central and peripheral transmitter pools separated, as the CNS and the peripheral nervous system use many of the same neurotransmitters. Furthermore, many plasma proteins including albumin and pro-thrombin are damaging to brain tissue and thus have to be kept outside the CNS (Abbott *et al.*, 2010). The blood vessels of the BBB are of a continuous non-fenestrated type, protecting the CNS from toxins and pathogens circulating in the blood, thus preventing inflammation and disease (Daneman and Prat, 2015). Larger molecules including insulin and iron transferrin enter the CNS via receptor-mediated endocytosis, whereas nutrients such as glucose and amino acids cross the BBB with the help of selective transporters (Ballabh *et al.*, 2004).

3.2.2 **Anatomy of the Blood Brain Barrier**

The BBB is made up of cellular and non-cellular components that together form the neurovascular unit (NVU). At the level of capillaries, the NVU comprises a basement membrane (BM) and three different cell types: endothelial cells (EC), pericytes, and astrocytes (Abbott *et al.*, 2006; Daneman, 2012).

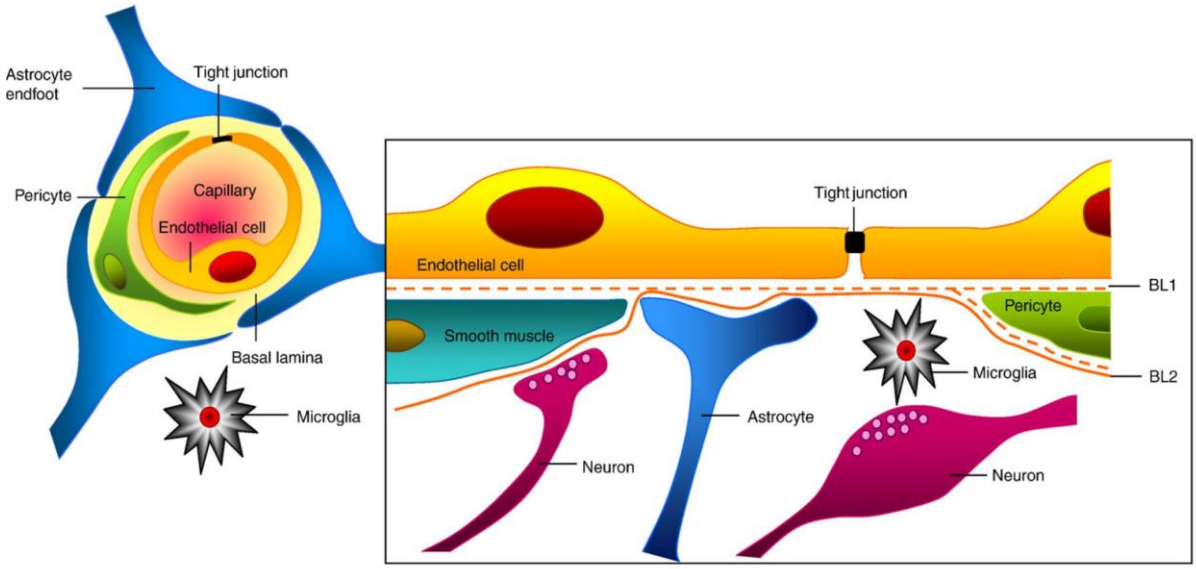

Figure 4: Anatomy of the BBB (Abbott et al., 2010).

The blood brain barrier (BBB) consists of endothelial cells (EC), a basement membrane, pericytes, and astrocytes. The ECs are closely connected by tight junctions, sealing the paracellular diffusion path. ECs and pericytes contribute to the basement membrane surrounding the abluminal surface of blood vessels. Astrocytes encircle the abluminal side of CNS capillaries with their end feet projections, maintaining the barrier properties. Neuronal projections onto smooth muscle cells regulate local cerebral blood flow. Microglia are the immunocompetent cells of the CNS

3.2.2.1 Endothelial Cells

A single specialized EC layer makes up the innermost luminal part of the BBB (Serlin *et al.*, 2015). ECs are closely connected by junctional complexes made up of tight junctions (TJ) and adherens junctions (AJ) (Abbott *et al.*, 2010). TJs consist of different transmembrane proteins, including occludin, junctional adhesion molecules (JAM), and proteins from the claudin family (Bauer and Traweger, 2016). They are linked to the cytoskeleton by different cytoplasmic TJ proteins for example members of the zonula occludens (ZO) family (Daneman, 2012). With the help of numerous different transporters, ECs can tightly regulate the movement of ions and molecules between the blood and the brain, amongst them the glucose transporter 1, high affinity cationic amino acid transporter 1, and several efflux transporters to remove toxins and metabolic waste products from the CNS, including the P-glycoprotein multidrug transporter and the breast cancer resistance protein (Chow and Gu, 2015). One of the key functions of ECs is to control leukocyte infiltration across the BBB. Under physiological conditions, ECs show a low expression of leukocyte adhesion molecules, including E-selectin, P-selectin, ICAM-1 and VCAM-1, restricting immune cell infiltration into the CNS (Daneman, 2012). However, this changes under pathological

circumstances. Upon inflammation, the expression of ICAM-1, VCAM-1, activated leukocyte cell adhesion molecule (ALCAM), and other adhesion molecules that are involved in the process of diapedesis is upregulated, resulting in an increased recruitment of immune cells to the CNS (Lécuyer *et al.*, 2017).

3.2.2.2 Pericytes

Pericytes completely surround the abluminal surface of the endothelium, with a higher pericyte coverage of the vessel of the CNS compared to the peripheral vasculature (Keaney and Campbell, 2015). They play an important role in angiogenesis, regulating capillary diameter, controlling cerebral blood flow, and maintaining BBB integrity (Obermeier *et al.*, 2013). Pericytes closely interact with ECs – they even share one basement membrane – and they are connected to each other by so-called *peg-socket contacts* which contain tight-, gap-, and adherens junctions (Armulik *et al.*, 2005). Together with ECs, they play an important role in preventing leukocyte transmigration into the CNS. It has been shown, that leukocytes preferably cross the BBB at regions with low coverage of laminin-511 and type IV collagen, called low expression regions, which are associated with gaps between pericytes (Voisin *et al.*, 2010).

3.2.2.3 Astrocytes

The third cellular component of the BBB are astrocytes. With their cell body astrocytes surround neurons to help them maintain their homeostatic balance and at the same time they can encircle the abluminal side of CNS capillaries with their end feet projections (Keaney and Campbell, 2015). This way, astrocytes can regulate blood vessel contraction and O_2 supply of the brain based on neuronal activity (Attwell *et al.*, 2010). Furthermore, astrocytes are a key player in the regulation of water homeostasis in the brain as they express the water channel aquaporin 4 (AQP4) in their end feet (Haj-Yasein *et al.*, 2011). They are also involved in upregulating BBB features, such as tight junctions, the localization of transporters, and specialized enzyme systems (Wolburg and Lippoldt, 2002; Abbott *et al.*, 2006). For the regulation of BBB integrity, several different mechanisms have been proposed. Astrocytes can increase the expression of occludin and claudins and decrease the secretion of chemokines and ICAM-1 expression by releasing Sonic hedgehog (Shh) which can bind to hedgehog receptors on ECs (Alvarez *et al.*, 2011b). Another study showed that astrocytes can regulate the BBB by the release of apolipoprotein E (ApoE) (Hafezi-Moghadam *et al.*, 2007; Nishitsuji *et al.*, 2011). In fact, ApoE deficient mice show an increased Evans

blue permeability at the BBB which worsens with age (Hafezi-Moghadam *et al.*, 2007). It has been proposed that astrocytic ApoE regulates TJ integrity by activation of protein kinase C and phosphorylation of occludin (Nishitsuji *et al.*, 2011).

3.2.2.4 Basement Membrane

The basement membrane (BM) is a unique form of extracellular matrix that surrounds the abluminal surface of blood vessels (Xu *et al.*, 2019). The CNS' BM is 20-200 nm thick and consists of laminins, collagen type IV, heparan sulfate proteoglycans, nidogens, and several other glycoproteins (Engelhardt and Sorokin, 2009; Daneman, 2012). It can be divided into two parts: the inner vascular BM which is secreted by ECs and pericytes, and the outer parenchymal BM which is secreted by astrocytes (Daneman and Prat, 2015). Astrocytic-derived laminin of the cerebral BM can bind to the integrin α2 receptor on pericytes and regulate their differentiation, for instance a disruption of the BM converts them to a contractile phenotype, which decreases TJ expression in ECs and reduces AQP4 levels in astrocytes (Keaney and Campbell, 2015).

3.2.3 **The BBB in Disease**

Under normal conditions, the blood vessels of the CNS form a strong barrier, regulating the movement of molecules and cells. However, this barrier function is comprised under pathological conditions. Disruption of the BBB has been observed in many different diseases, including stroke, epilepsy, Alzheimer's disease, and MS (Kassner and Merali, 2015; van Vliet *et al.*, 2015; van de Haar *et al.*, 2016; Minagar and Alexander, 2003). Up to a certain extent, this is necessary to allow immune cells to enter the CNS to clear debris and repair injuries and damage (Daneman, 2012). Nevertheless, an excessive amount of active immune cells in the CNS can lead to neurodegeneration as seen in MS (Goverman, 2009). At the same time, a loss in BBB integrity allows plasma proteins and water to pass, leading to an increased intracranial pressure (Nag *et al.*, 2009).

The BBB plays a crucial role in the development of MS as it deteriorates and loses its protective function. As mentioned above, two distinct changes to the BBB have been described in MS: an increase in BBB permeability through the disruption of TJ, and the recruitment and activation of immune cells into the CNS. The loss of TJ proteins can be mediated by the release of cytokines from infiltrating leukocytes as well as

astrocytes, pericytes, and microglia. For instance, tumor necrosis factor (TNF) α and IFN-γ are released by leukocytes and can interact with their respective receptors on ECs. This results in a change of the cellular TJ and AJ protein distribution, including JAM-A and claudin-5 (Wong *et al.*, 1999; Alvarez *et al.*, 2011a). Moreover, it has been shown that stimulation of ECs with IFN-γ promotes redistribution and endocytosis of occludin, claudin-1, claudin-4, and JAM-A (Bruewer *et al.*, 2003). TNF-α and IFN-γ have also been shown to stimulate chemokine secretion and ICAM-1 and VCAM-1 expression on ECs, leading to transmigration of leukocytes through the BBB (Lombardi *et al.*, 2008). In addition, matrix metalloproteinases (MMP) may play a role in the disruption of the BBB. MMP serum levels have been reported to be higher in MS patients compared to healthy controls, especially those of MMP-2 and MMP-9 (Boziki and Grigoriadis, 2018). MMP-2 and MMP-9 activity plays an important role in leucocyte transmigration across the parenchymal BM as they degrade dystroglycanes which connect astrocytes to the BM (Agrawal *et al.*, 2006).

3.2.3.1 Transendothelial migration

The infiltration of leukocytes into the CNS is considered an early event in the development of MS, which is favored by BBB breakdown. The transendothelial migration (TEM) of leukocytes into the CNS, also called diapedesis, requires different steps: leukocyte rolling, activation, adhesion, and locomotion. The rolling phase is induced by the first contact of leukocytes with adhesion molecules (L-, E-, or P-Selectin) on the luminal side of the endothelium, or the binding of α4-integrins on leukocytes to VCAM-1 on ECs (Engelhardt and Ransohoff, 2012). This leads to a subsequent slowing-down of leukocytes. As a result of intracellular signaling, integrins on leukocytes change from a low to a high affinity state and bind to their endothelial ligands, including ICAM-1 and -2 and VCAM-1 (Chigaev *et al.*, 2003; Filippi, 2016). Once the leukocytes are firmly attached, they flatten and polarize to enable directional migration across the BBB. They crawl across the endothelial apical surface to find a permissive site of transmigration (Phillipson *et al.*, 2006). Leukocyte motility is highly dependent on asymmetric rearrangement of their cytoskeleton, where filamentous actin polymerizes to form the cell's leading edge whereas actomyosin assembles along the cell's rear end (Filippi, 2019). It has been shown that leukocytes generate numerous ICAM-dependent finger-like protrusions. These protrusions create deep invaginations through endothelial junctions as well as on ECs away from the junctions,

acting as mechanosensors on the endothelial cell surface in order to find a site which allows TEM (Carman, 2009; Carman *et al.*, 2007).

In general, there are two different routes for cells to cross the BBB: the transcellular and the paracellular diapedesis routes. Most of the time, TEM takes place at the endothelial cell borders, called paracellular TEM (Winger *et al.*, 2014). For paracellular TEM, it is necessary for TJ as well as AJ to remodel. Studies have shown, that upon interactions of ICAM-1 on ECs and β2-integrin on immune cells VE-Cadherin is phosphorylated, leading to a loosening of the AJ (Filippi, 2019; Winger *et al.*, 2014). Moreover, claudin-5 is removed from the site of transmigration at the cell-cell junctions, forming a gap for TEM (Winger *et al.*, 2014). In contrast to paracellular TEM, it has been observed that leukocytes can also transmigrate through the endothelial cell bodies (transcellular), leaving the TJ's and AJ's morphology intact (Engelhardt and Ransohoff, 2012). While crawling on the endothelial surface, leukocytes use their protrusions to identify a site for transmigration. Once the permissive site has been identified, leukocyte - EC contacts fuse, forming a transcellular channel between the apical and basal membrane (Carman *et al.*, 2007).

After passing the endothelial cell layer, leukocytes migrate through pericytes lining the blood vessels and the vascular basement membrane to reach the interstitial space (Filippi, 2019).

3.3 **NF-κB Pathway**

The essential function of the immune system is to sense pathogens and initiate an immune response against infections. As the immune system is an interactive network of immune cells, humoral factors, and cytokines, its function is highly dependent on complex signaling pathways and strict regulation (Parkin and Cohen, 2001).

A precise inflammatory response is based on a coordinated activation of several signaling pathways that regulate the expression of pro- and anti-inflammatory mediators and the recruitment of leukocytes from the blood. The transcription factor Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) plays a crucial role in the regulation of this process. The contribution of NF-κB to diseases can easily be observed in the context of autoimmune diseases and chronic inflammation, where

proinflammatory cytokines lead to an activation of NF-κB, which then promotes the production of even more proinflammatory cytokines (Lawrence, 2009). Moreover, it has been shown that NF-κB plays an important role in the development and maintenance of cancer, such as glioblastoma and colorectal cancer (Cahill *et al.*, 2016; Hassanzadeh, 2011).

3.3.1 **Signaling**

There are two separate signaling pathways leading to the activation of NF-κB: the canonical (classical) and non-canonical (alternative) pathway (Jarosz *et al.*, 2017).

The canonical NF-κB signaling pathway is triggered by proinflammatory cytokines, such as IL-1, TNF-α, and bacterial lipopolysaccharides (LPS) as shown in Figure 5 (Lawrence, 2009). For instance, the binding of TNF-α to TNF receptors triggers the recruitment of TNF-receptor-associated protein with a death domain (TRADD). TRADD further binds to TNF-receptor-associated factor (TRAF) 2 and receptor-interacting protein (RIP) 1 (Hirata *et al.*, 2017). TRAF 2 mediates the polyubiquitination of RIP1 which can then recruit transforming growth factor-β-activated kinase (TAK) 1 binding proteins (TAB) 1, TAB2, and TAB3 (Xu and Lei, 2021). TAB2 and TAB3 form a signaling complex with the protein kinase TAK1, leading to its activation (Blonska *et al.*, 2005). Subsequently, TAK1 stimulates phosphorylation-dependent activation of the IκB kinase (IKK) complex (Ridder *et al.*, 2015). This complex consists of a regulatory subunit IKKγ, also called NEMO, and two catalytic subunits IKKα, and IKKβ. To be activated, IKKβ has to be phosphorylated (Jarosz *et al.*, 2017). Hereupon, the IKK complex binds to and phosphorylates IκBα, an inhibitory protein of the NF-κB complex. This leads to ubiquitination of IκBα, targeting it for proteasomal degradation (Mitchell *et al.*, 2016). The released NF-κB complex, a homo- or heterodimer of Rel family proteins, including p65, also known as RelA, RelB, c-Rel, p50, and p52, accumulates in the cell and translocates into the nucleus. The subunits share an N-terminal Rel homology domain, enabling to bind to consensual regions in the DNA. Hereby, they regulate the expression of genes involved in inflammation, cell survival, and apoptosis (Renner and Schmitz, 2009; Cahill *et al.*, 2016). However, IL-1 signaling leads to the recruitment of IL-1β-associated kinase (IRAK) 1 and 4 to the receptor. IRAK 1 subsequently binds to TRAF 6, leading to the activation of the IKK complex (Iwai, 2012).

Unlike the canonical NF-κB pathway, the non-canonical signals are transduced in a NEMO-independent manner. Instead, the binding of lymphotoxin-β, CD40 ligand, and B-cell activating factor to their related receptors results in an activation of the NF-κB-inducing kinase (NIK) (Jarosz *et al.*, 2017). NIK plays a dual role in the non-canonical NF-κB pathway. On the one hand, active NIK phosphorylates p100, which is then processed into p52. Together with RelB, p52 can form a dimer that translocates into the nucleus and binds DNA in order to activate transcription. On the other hand, activated NIK phosphorylates p100 within IκBδ, This results in a release of NF-κB dimers for the activation of gene expression (Mitchell *et al.*, 2016; Lawrence, 2009). In general, activation of the non-canonical NF-κB pathway regulates genes required for B-cell activation as well as lymphoid organogenesis (Jarosz *et al.*, 2017).

3.3.2 **Negative regulation**

Inflammation is an important reaction of the immune system to fight infections. Nevertheless, immune response can become harmful when it occurs in an excessive manner or when it is not adequately terminated, causing chronic inflammation, oncogenesis, and autoimmune diseases (Renner and Schmitz, 2009). Thus, several cellular and molecular mechanisms tightly regulate NF-κB signaling to manage an adequate termination of inflammation and maintain homeostasis (Yu *et al.*, 2020a).

One primary component of the regulation of NF-κB are self-regulating feedback loops. Within less than an hour after the stimulation with TNF-α, IκBα completely disappears in the cytoplasm and the binding activity of NF-κB reaches its peak. Newly synthesized IκBα reappears in the cytoplasm two hours after stimulation. IκBα subsequently enters the nucleus and removes NF-κB from the DNA, ensuring the termination of the initial NF-κB response (Sun *et al.*, 1993).

Furthermore, there are different target structures for the feedback regulation of the NF-κB pathway, one of them being TAK1. During inflammation, TNF-α binds to the TNF receptor 1. This activates a cascade that results in the autophosphorylation-dependent activation of TAK1 (Hirata *et al.*, 2017). This is negatively regulated by the dual specificity phosphatase DUSP14, as it dephosphorylates TAK1 within its kinase loop (Zheng *et al.*, 2012). Moreover, protein phosphatase 6 associates with and deactivates TAK1 by dephosphorylation, suggesting that protein phosphatase 6 is a negative regulator of TAK1 (Kajino *et al.*, 2006).

In addition, the IKK complex represents another target structure for the regulation of NF-κB signaling. The recruitment of protein phosphatase 1 by an adaptor protein contributes to the dephosphorylation and subsequent inactivation of the IKK complex. (Li *et al.*, 2008). Another feedback mechanism for the direct inhibition of IKK is controlled by the uptake of zinc (Zn^{2+}). As a transcriptional target of NF-κB, zinc transporter ZIP8 is upregulated during inflammation. This leads to an increased Zn^{2+} uptake, inducing NF-κB inhibition by blocking the IKK complex (Jarosz *et al.*, 2017).

Moreover, NF-κB signaling is regulated by ubiquitination, a post-translational modification of proteins. The conjugation of ubiquitin, a 76 amino acid protein, to the targeted proteins is catalyzed by an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-protein ligase (Iwai, 2012). Ubiquitin chains are conjugated to their target structure through one of their lysine (Lys) resides, typically Lys48 and Lys63 (Renner and Schmitz, 2009). The type of ubiquitin chain plays an essential role in determining the substrate's fate. Whereas Lys48-linked polyubiquitin targets a structure for proteasomal degradation, Lys63-linked polyubiquitin chains are participating in signal transduction and deoxyribonucleic acid (DNA) repair (Iwai, 2012). Some of the most well studied deubiquitinases that play a role in the inhibition of NF-κB are cylindromatosis tumor suppressor protein (CYLD), cellular zinc finger anti- NF-κB (Cezanne), and A20 (Mooney and Sahingur, 2021; Mathis *et al.*, 2015; Hu *et al.*, 2013). A20 is a protein, which is synthesized upon NF-κB stimulation. It functions both as a ubiquitinating and a de-ubiquinating enzyme targeting RIP1 and NEMO (Yu *et al.*, 2020a). For once, A20 removes K63-linked ubiquitin from target substrates, thus negatively regulating NF-κB signaling. At the same time, A20 functions as a ubiquitin ligase, labeling substrates for proteasomal degradation (Renner and Schmitz, 2009).

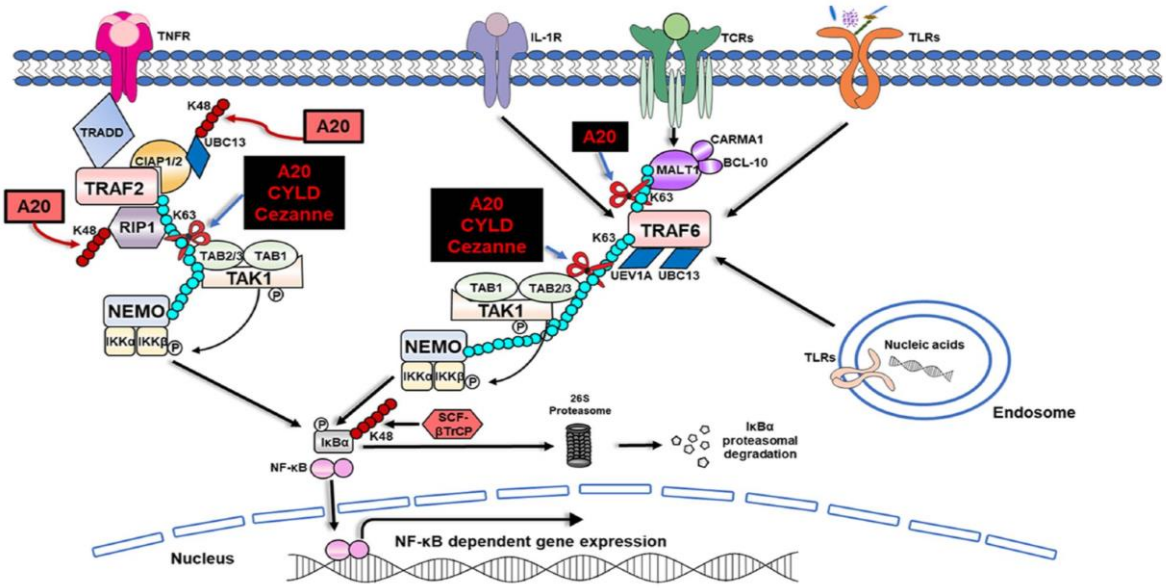

Figure 5: Regulation of NF-κB by ubiquitination and the deubiquitinases: A20, CYLD, and Cezanne (Mooney and Sahingur, 2021)

Stimulation with TNF, IL-1, MCH-presented antigen or LPS leads to TAK1 and subsequent IKK activation, leading to the degradation of IκB. This eventually results in nuclear translocation of NF-κB and targeting of gene transcription. These signaling pathways can be inhibited by the deubiquitinases A20, CYLD, and Cezanne.

3.4 **A20**

A20 was originally identified in human umbilical vein endothelial cells upon stimulation with TNF-α, giving it it's gene name tumor necrosis factor α-induced protein 3 (TNFAIP3) (Dixit *et al.*, 1990).

3.4.1 **Structure and function**

A20 is a protein of 90 kDa consisting of 790 amino acids. It comprises an amino-terminal ovarian tumor (OTU) domain and seven A20-like zinc finger domains in the carboxy terminus (Komander and Barford, 2008). The zinc finger 4 domain contains an E3 ubiquitin ligase which can interact with an E2 ubiquitin conjugating enzyme catalyzing Lys48-linked polyubiquitination (Wertz *et al.*, 2004). Simultaneously, the OTU domain operates as a deubiquitinase. With its cysteine protease catalytic triade, it hydrolyses Lys48- and Lys63-linked ubiquitin chains and removes them from the substrate (Evans *et al.*, 2004).

Originally, A20 was characterized as an inhibitor of TNF-α-induced apoptosis (Opipari *et al.*, 1992). In addition to being an anti-apoptotic protein, A20 functions as a potent regulator of NF-κB-dependent gene expression. This was first indicated by the observation that A20 can inhibit its own NF-κB-dependent expression (Krikos *et al.*, 1992). Moreover, it was shown that expression of A20 was not only induced by TNF-α, but also by other stimuli such as IL-1, LPS, activation of the B cell surface receptor CD-40, and Epstein-Barr virus latent membrane protein (Jäättelä *et al.*, 1996; Eliopoulos *et al.*, 1997). All these stimuli are involved in the activation of NF-κB signaling, further emphasizing the role of A20 in the reduction of pro-inflammatory signaling via NF-κB. This was eventually confirmed by the generation of A20-deficient mice. They developed massive inflammation and severe cachexia and showed sustained NF-κB response (Lee *et al.*, 2000).

The first mechanism of A20 to restrict NF-κB signaling was identified as deubiquitination and subsequent ubiquitin-mediated degradation of RIP1. Upon stimulation with TNF-α, the OTU domain removes Lys63-linked ubiquitin chains from RIP1, preventing its interaction with NEMO. Hereupon, A20's zinc finger 4 polyubiquitinates RIP1 with Lys48-linked ubiquitin chains, targeting it for proteasomal degradation (Wertz *et al.*, 2004).

A20 is also involved in NF-κB signaling in response to other receptors than TNF receptors. For instance, A20 inhibits polyubiquitination and activation of TRAF 6 in the toll-like receptor 4 and IL-1 receptor pathways (Heyninck and Beyaert, 1999; Boone *et al.*, 2004). Together with TRAF-6, the E2 enzyme Ubc13 catalyzes the Lys63-linked polyubiquitination of TAK1 which then activates IKK. In addition, TRAF-6 interacts with the E2 ubiquitin conjugating enzyme UbcH5c to synthesize polyubiquitin chains that activate IKK (Xia *et al.*, 2009). Ubc13 also acts as an E2 enzyme for other E3 ubiquitin ligases, such as TRAF-2 in TNF-α-induced signaling. It was shown, that A20 inhibits the E3 ligase activity of TRAF-6 and TRAF-2 by antagonizing its interactions with Ubc13 and UbcH5c. Furthermore, A20 triggers Lys 48-linked ubiquitination of Ubc13 and UbcH5c, thus initializing their proteasomal degradation (Shembade *et al.*, 2010).

There are many more signaling pathways controlled by A20 (see table 2).

Pathway	Substrate	Action A20	Consequence
	A20	Homodimerization of A20 proteins (independent of OTU and ZnF4)	High-order oligomerization for efficient inhibition of NF-κB signaling
	RIP1	Recognizes RIP1-K63Ub (ZnF4) Removes K63Ub from RIP1 (OTU) Adds K48Ub on RIP1 leading to degradation (ZnF4)	Inhibition of TNF-induced NF-κB signaling
TNF	LUBAC–NEMO	Binding to linear Ub (ZnF7) Prevents LUBAC–NEMO interaction	Inhibition of TNF-induced NF-κB signaling
	Ubc13	Prevents interaction of Ubc13 with TRAF2/ cIAPs (OTU) Adds K48Ub on Ubc13 leading to degradation (ZnF4)	Inhibition of TNF-induced NF-κB signaling
	ASK1	Binding to and polyUb of ASK1 leading to degradation (ZnF4)	Inhibition of JNK-mediated apoptosis
IL-1	Ubc13/UbcH5c	Prevents interaction of Ubc13/UbcH5c with TRAF6 (OTU) Adds K48Ub on Ubc13/UbcH5c leading to degradation (ZnF4)	Inhibition of IL1-induced NF-κB signaling
	TRAF6	Interacts with TRAF6	Inhibition of IL1-induced NF-κB signaling
IL-17	TRAF6 and CBAD domain of IL17RA	Binds to IL17RA-CBAD and prevents polyUb of TRAF6 (OTU+ZnF4/ZnF5)	Inhibition of IL17-induced NF-κB and MAPK signaling
TCR/CD28	MALT1	Removes K63Ub from MALT1 (OTU)	Inhibition of TCR-induced NF-κB signaling
	TRAF6	Removes K63Ub from TRAF6 (OTU)	Inhibition of LPS-induced NF-κB signaling
TLR	Ubc13/UbcH5	Prevents interaction of Ubc13/UbcH5c with TRAF6 (OTU) Adds K48Ub on Ubc13/UbcH5c leading to degradation (ZnF4)	Inhibition of LPS-induced NF-κB signaling
	Beclin1	Removes K63Ub from Beclin1	Inhibition of LPS-induced autophagy
DR4/5	Caspase-8 (?)	Removes polyubiquitin from caspase-8 (OTU)	Inhibition of DR-induced apoptosis
NOD1/2	RIP2	Removes K63Ub from RIP2 (OTU)	Inhibition of MDP-induced NF-κB signaling
RIG-I	TRAF3–TBK1/IKKi	Disrupts interaction between TRAF3 and TBK1/IKKi, thereby disrupting K63Ub of TBK1/ IKKi	Inhibition of virus-induced IRF signaling
Wnt	Axin	Binds to the β-catenin destruction complex and adds K48Ub to Axin, leading to degradation (OTU)	Inhibition of Wnt signaling
Tight junction	Occludin	Deubiquitinates non-K48Ub occludin	Regulation of tight junction dynamics promoting intestinal barrier function

Table 2: Signaling pathways controlled by A20 through interaction with specific substrates (Catrysse et al., 2014)

3.4.2 **A20 in disease**

Over the past few years, multiple studies mainly based on genome-wide association studies of genetic material from large groups of patients have detected A20/TNFAIP3 as a suspectability locus for the development of inflammatory and autoimmune disease. For instance, three independent SNPs in the TNFAIP3 region were identified that are associated with the development of systemic lupus erythematosus (SLE) (Musone *et al.*, 2008). Moreover, A20 SNPs were associated with psoriasis, rheumatoid arthritis, Crohn's disease, MS, and many more (Nair *et al.*, 2009; Plenge *et al.*, 2007; Wellcome Trust Case Control Consortium, 2007; Hoffjan *et al.*, 2015). A20 is not only associated with autoimmune diseases. Genome-wide association studies also revealed that mutations and deletions in A20 also have an impact on B cell lymphomas and Hodgkin's lymphoma (Schmitz *et al.*, 2009). In addition, insufficient A20 expression has been assumed to contribute to the incidence and prognosis of

several other malignant tumors, including hepatocellular carcinoma and breast cancer (Chen *et al.*, 2015; Vendrell *et al.*, 2007).

In order to further study cell type specific A20 deficiency and its consequences for autoimmune disease pathology several conditional A20 knockout mouse lines were generated. That way, it could be shown that mice lacking A20 specifically in intestinal epithelial cells manifested an increased response to experimental colitis and mice are more prone to develop colon tumors (Vereecke *et al.*, 2010). Epidermis-specific A20-knockout mice were generated to study the role of A20 in skin homeostasis and psoriasis. However, these mice did not show spontaneous skin inflammation. Instead, they developed keratinocyte hyperproliferation and ectodermal organ abnormalities such as unkempt hair, longer nails, and sebocyte hyperplasia (Lippens *et al.*, 2011). In addition, A20 deficient mice in dendritic cells, myeloid cells, and B cells have been generated to further study the role of A20 in disease pathology (Catrysse *et al.*, 2014).

With the help of EAE, a rodent model of MS, the role of A20 in the progression of neuroinflammation has been investigated thoroughly. At first, mice lacking A20 specifically in neuroectodermal cells (astrocytes, neurons, and oligodendrocytes) were generated, resulting in a significantly stronger EAE, increased T cell infiltration into the CNS, an enhanced production of cytokines and chemokines, and a higher expression of proinflammatory genes. Next, mice which lack A20 specifically in astrocytes were generated. Once again, mice showed more severe clinical symptoms to EAE together with an increased number of infiltrating cells. Interestingly, when deleting A20 specifically in neurons, mice developed symptoms comparable to wild type mice (Wang *et al.*, 2013). A few years later, A20 was deleted exclusively in microglia. These mice were hypersensitive to EAE, showing extensive demyelination, axonal damage, inflammation, and immune cell infiltration. Moreover, genes linked to cell activation, microglia polarization, MHC class 1, type one interferon signaling, and inflammatory NF-κB signaling were upregulated (Voet *et al.*, 2018). Moreover, it was shown that A20 deletion in microglia leads to spontaneous $CD8^+$ T cell infiltration to the CNS where they obtain a viral response signature. This suggests that microglial A20 functions as a sensor for viral infections (Mohebiany *et al.*, 2020).

Even though A20 is highly expressed in endothelial cells and it is well known that defects in A20-dependent regulation of NF-κB signaling contributes to several different

inflammatory and autoimmune diseases, its role in CNS endothelial cells is largely unknown.

The aims of the present study are to identify the role of A20 in CNS endothelial cells on the maintenance of BBB integrity in the steady state and to investigate whether the deletion of A20 in endothelial cells influences the outcome of EAE.

4 Material and Methods

4.1 Materials

4.1.1 Chemicals

Name of Chemical/ Reagent	Supplier
Acetone	Merck, Darmstadt
Agarose	AppliChem, Darmstadt
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Steinheim
β-Mercaptoethanol	Sigma-Aldrich, Steinheim
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim
Brefeldin A	Sigma-Aldrich, Steinheim
Collagenase II	AppliChem, Darmstadt
CXR Reference Dye	Promega, USA
DNAse I	Roche, Switzerland
ECL Western blotting substrate	Thermo Scientific, Langenselbold
Ethanol	AppliChem, Darmstadt
Ethidium bromide	Sigma-Aldrich, Steinheim
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Steinheim
Fetal calf serum (FCS)	Boehringer Mannheim GmbH, Mannheim
4 kDa FITC Dextran	Sigma-Aldrich, Steinheim
Gene ruler 100 bp Plus DNA ladder	Thermo Scientific, Langenselbold
GoTaq® qPCR Master Mix	Promega, USA
Hank's buffered saline solution (HBSS)	Sigma-Aldrich, Steinheim
HEPES	Gibco Life Technologies GmbH, Karlsruhe
Hydrogen Peroxide	Merck, Darmstadt
Isoflurane	Abbvie, Ludwigshafen
Isopropanol	AppliChem, Darmstadt
Methanol	Roth, Karlsruhe
Milk powder	Sigma-Aldrich, Steinheim
My Taq Red DNA- polymerase	Sigma-Aldrich, Steinheim
Olive oil	Sigma-Aldrich, Steinheim

Papain from papaya latex	Sigma-Aldrich, Steinheim
Percoll	Sigma-Aldrich, Steinheim
Pertussis toxin	Biotrend, Köln
Proteinase K	Roche, Switzerland
REDTaq® Ready Mix™ PCR Reaction Mix	Sigma-Aldrich, Steinheim
Roti-Histofix 4%	Roth, Karlsruhe
RPMI-1640 medium	Sigma-Aldrich, Steinheim
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	AppliChem, Darmstadt
Tamoxifen	Sigma-Aldrich, Steinheim
TissueTek® O.C.T™ compound	Sakura Finetek, Staufen
Trichloroacetic acid solution	Sigma-Aldrich, Steinheim
Tris	Roth, Karlsruhe
Triton X-100	Sigma-Aldrich, Steinheim
Tween 20	Sigma-Aldrich, Steinheim
Vectashield® Antifade Mounting Medium with DAPI	Vector Laboratories, USA

Table 3: Chemicals and reagents

4.1.2 **General buffers, solutions, and media**

Buffer	**Components**	
D1 staining buffer		PBS
	1 %	BSA
	0.3 %	Triton X-100
FCS buffer	500 ml	PBS
	2 %	FCS
20x MOPS buffer	1 M	Tris-HCl (pH 7.5)
	1 M	MOPS
	20 mM	EDTA
	2 %	SDS
1x RIPA buffer	50 mM	Tris
	150 mM	NaCl

	1 % 0.5 % 1 % 2 mM	IGEPAL Sodium deoxycholate SDS EDTA (pH 8.0)
T-cell medium	 10 % 100 units/ml 2 nM 50 µM 1 mM 1 %	RPMI-1640 FCS P/S L-Glutamine β-Mercaptoethanlo HEPES Non-essential amino acid cell culture supplement
Tail lysis buffer	10 mM 5 mM 0.2 % 200 mM	Tris-HCl EDTA (pH 8.0) SDS NaCl
TBS-T	50 mM 150 mM 0.1 %	Tris NaCl (pH 7.4) Tween-20
1x Transfer buffer	192 mM 25 mM 10 %	Glycine Tris Methanol
50x Tris-acetate-EDTA (TAE) buffer	2 M 1 M 50 mM	Tris Acetic acid EDTA (pH 8.0)

Table 4: General buffers, solutions, and media

4.1.3 **Kits**

Kit	**Supplier**
BD Cytofix/Cytoperm Fixation/ Permebabilization Solution Kit	BD Bioscience
GE Healthcare AmershamTM ECL Prime Western-Blot Reagent	Thermo Fisher Scientific

PierceTM BCA Protein Assay Kit	Thermo Fisher Scientific

Table 5: Kits

4.1.4 **Laboratory Equipment**

Equipment	**Supplier**
Airing cupboard, Incubator	Binder GmbH, Tuttlingen
Aspiration system, Vacusafe	Integra Biosciences, Fernwald
Branson Ultrasonics™ Sonifier Modell 250	Fisher Scientific, Schwerte
Centrifuge 5417R	Eppendorf, Wesseling-Berzdorf
Centrifuge Megafuge 16R	Thermo Scientific, Langenselbold
Dounce homogenizer	DWK Life Science, USA
FACSCanto™II	BD Biosciences, Heidelberg
FastPrep-24™ Sample Preparation System	MP Biomedicals, Eschwege
Gel chamber, Compact L/XL	Biometra Analytik, Jena
Gel-Imager: Gel Doc XR	Biorad, München
GentleMACS™ Dissociator/ OctoMACS	Miltenyi Biotec, Bergisch Gladbach
High-current power supply PowerPac™ HC	Biorad, München
Infinite ® M200Pro NanoQuant, Tecan	Tecan Trading AG, Switzerland
Microcentrifuge Galaxy MiniStar	VWR International, USA
Mixing Block MB-102	Hangzhou Bioer Technology Co., Ltd, China
MyCycler™ Thermal cycler	Biorad, München
Sprout-Mini-centrifuge	Technolab GmbH, Herne
StepOnePlus™Real-Time PCR system	Applied Biosystem, USA
T3000 Thermocycler	Biometra, Goettingen
TCS SP8 inverse confocal fluorescence microscope	Leica, Wetzlar
Thermo Scientific™ Arktik™ Thermocycler	Fisher Scientific, Schwerte

Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad, USA
Vortex mixer	VWR International, USA
Waterbath, WNE 7	Memmert, Schwabach
XCell Sure Lock™ Electrophoresis Cell	Thermo Fisher Scientific, USA

Table 6: Laboratory Equipment

4.1.5 **Materials**

Equipment	**Supplier**
1,5 ml Eppendorf tube	Sarstedt, Nümbrecht
15 mlFalcon tube	Greiner-bio One, Frickenhausen
50 ml Falcon tube	Greiner-bio One, Frickenhausen
6- Well plate	Thermo Scientific, Langenselbold
96- Well plate	Greiner-bio One, Frickenhausen
NativePAGE™ 4-16 % Bis-Tris Gel	Thermo Fisher Scientific, USA
Metal bead lysing matrix tubes	MP Biomedicals, Eschwege
PCR- Eppendorf tube	Biozym Scientific GmbH, Oldendorf
Pipette tips	Starlab, Hamburg
Pipettes	Greiner-bio One, Frickenhausen

Table 7: Materials

4.2 **Molecular Biology**

4.2.1 **DNA extraction**

Tail biopsies were collected from SLC-Cre x A20$^{fl/fl}$ mice at the age of 5 days. For the extraction of genomic DNA, tails were lysed overnight in tail lysis buffer containing 2.5 % (v/v) proteinase K using a shaker (56 °C, 400 rpm). The next day, samples were centrifuged at 13500 rpm for 10 min at 16 °C. To precipitate the DNA, the supernatant was mixed with an equal volume of isopropyl alcohol and subsequently vortexed. Samples were centrifuged at 13500 rpm for 15 min at 16 °C to pellet the DNA and supernatant was discarded. Next, DNA was washed with 70 % (v/v) ethanol and it was centrifuged one last time at 13500 rpm for 10 min at 16 °C. Supernatant was discarded

and DNA was dried upside down for 30 min at 37 °C. Finally, DNA was resuspended in 200 µl distilled water, and shaken for 2 hours at 56 °C to dissolve.

4.2.2 **Polymerase chain reaction (PCR)**

PCR was performed to genotype mice for the presence of targeted alleles or transgenes using specific primers. The total volume used for PCR was 20 µl, containing 10 µl RED Taq Ready Mix, 10 pmol of each primer, and 100 ng of template DNA. Reactions were performed either in the T3000 Thermocycler or MyCycler thermal cycler. Reactions were run out following the principal program: denaturation at 94 °C for 4 min, followed by 30-35 cycles of 94 °C for 30 sec, 58 or 60 °C (annealing) for 30 sec, and 72 °C for 30 sec, and a final extension step at 72 °C for 10 min. The primers used are listed in table 8.

Name of primer	Oligonucleotide sequence	T_{ANN} °C	Direction
A20 fw	agtctgggactggatgtagc	60	sense
A20 rev	ctggctaaggccttgatacc	60	anti-sense
SLC-Cre fw	gctattcatgtcttggaagc	58	sense
SLC-Cre rev	caggttcttcctgacttcat	58	anti-sense
1260 fw	gagactctggctactcat	58	sense
1260 rev	ccttcagcaagagctggggac	58	anti-sense

Table 8: Primers for PCR

4.2.3 **Agarose gel electrophoresis**

DNA fragments were separated by size with the help of agarose gel electrophoresis. Separation was performed in 2 % (w/v) agarose gel in 1x TAE buffer supplemented with 0.5 mg/ ml of the DNA intercalating substance GelRed. Electrophoresis was carried out in 1x TEA buffer with a constant voltage of 120 V. Finally, separated DNA fragments were visualized under UV light using the Gel Doc imaging system and were compared to the GeneRuler 100bp Plus DNA Ladder.

4.2.4 **RNA isolation**

Ribonucleic acid (RNA) was isolated from whole tissue spinal cords of perfused mice. Tissue samples and 800 µl TRIzol reagent were pipetted into metal bead lysing matrix

tubes. The tissue was incubated for 5 min at room temperature and was homogenized using the FastPrep-24 homogenizer (speed: 6.0; time: 40 sec). The lysate was then centrifuged for 10 min at 12000 g at 4 °C and the clear supernatant was transferred to a new tube. Next, 200 µl of trichloromethane were added and mixed my shaking. Samples were incubated for 2 min at room temperature and then centrifuged for 15 min at 12000 g at 4 °C. The mixture separated into a lower red phase, an interphase, and an upper aqueous phase containing the RNA. The aqueous phase was transferred into a new tube and 400 µl of isopropanol was added. Samples were centrifuged for 10 min at 12000 g at 4 °C. The RNA precipitate formed a white gel-like pellet at the bottom of the tube. The supernatant was discarded. The pellet was washed twice by resuspending it in 500 µl of 70 % (v/v) ethanol, briefly vortexing, and centrifuging for 5 min at 7500 g at 4 °C. The supernatant was discarded, and the pellet was airdried for 10 min. Finally, RNA was dissolved and incubated in 50 µl of RNase-free water for 10 min at 55 °C. The concentration of RNA was determined by measuring the absorption at 260 nm and 280 nm using Tecan microplate reader and the program i-control.

4.2.5 **Quantitative real time PCR (qPCR) of spinal cord**

Complementary DNA (cDNA) was synthesized out of 1 µg of RNA using the superscript II reverse transcriptase (Invitrogen). Subsequently, cDNA was diluted with Nuclease free water (1:5). Master mix was prepared by mixing 5 µl Sybr Green, 0.1 µl RefDye, and 3 µL Nuclease free water per sample. One part of diluted cDNA was further mixed with eight parts of master mix. Thereof, 9 µl were pipetted on 1 µl of primer. Finally, qPCR was performed in the StepOnePlus™ Real-Time PCR "Thermocylcer" system and fold enrichment was calculated using Delta-Delta CT method normalized to Hypoxanthin-Guanin-Phosphoribosyltransferase (Hprt) as reference housekeeping gene. Primers for *Il-1a, Il-1b, Il-6, Tnf, Nos2, Cxcl1, C1qa, CD3e, Gfap* and *Aif1* (Iba1) were ordered as QuantiTect Primer Assays (Table 9). Primers for *Cd68, Lgals3, Tap1, Stat1*, *Igtp,* and *Hprt* were designed with the primer-BLAST tool from the National Center for Biotechnology Information (NCBI). Sequences for the self-designed primers are given in table 10.

Name of primer	
Aif1	NM_019467
CD3e	NM_007648

Cxcl1	NM_008176
C1qa	NM_007572
Gfap	NM_001131020
Il-1a	NM_010554
Il-1b	NM_008361
Il-6	NM_031168
Nos2	NM_010927
Tnf	NM_013693

Table 9: Primers from Qiagen QuantiTect Primer Assays

Name of primer	**Primer fw**	**Primer rev**
Cd68	AGCTGCCTGACAAGGGACAC	GGTTGATTGTCGTCTGCGGG
Hprt	CGTGGTGATTAGCGATGATG	TCCAAATCCTCGGCATAATG
Igtp	GAGCCTGGATTGCAGCTTTGT	AAGGTCTATGTCTGTGGGCCT
Lgals3	AGCGGCACAGAGAGCACTAC	GGTAGGCCCCAGGATAAGCA
Stat1	GGCGCTGCTTGGCTCTCTTA	GCTGCTGAAGCTCGAACCAC
Tap1	GGAGCTTTGCCAACGAGGAG	CCGCCCAGGTACAGAATTCCC

Table 10: Primers ordered at Metabion

4.2.6 **Protein extraction of brain**

4.2.6.1 Cell lysis

For cell lysis, 10 ml RIPA buffer were mixed with 1 tablet PhosphoSTOP and 100 µl protease inhibitor and pipetted into a glass dounce homogenizer. ½ brain of A20$^{\Delta BEC}$ or control mice were added and mechanically homogenized in 1000 µl of pre-assembled solution. Samples were transferred into 1.5 ml Eppendorf tubes and boiled for 5 min at 100 °C. Afterwards, samples were centrifuged for 15 min at full speed at 4 °C and supernatant was carefully transferred into new Eppendorf tubes. Lysate was stored at -20 °C.

4.2.6.2 BCA Protein Assay

Protein concentration of sample lysate was determined using the BCA™ Protein *Assay Kit*. Principle of this method is the Biuret reaction, which means the reduction of Copper $(Cu)^{2+}$ to Cu^{+} through protein peptide bindings in an alkaline milieu. Reduced Cu^{+} forms a purple complex with Bichinol-4-Carbonacid (BCA), which is measured by photometry at 562 nm.

As reference, a standard curve of BSA was prepared by diluting 1:2 in PBS (Table 11). Samples were diluted 1:50 in PBS. To a 96 well plate 10 µl sample or standard were added in duplicates. BCA solution was prepared by mixing 1 volume Reagent A with 50 volumes Reagent B. Subsequently, 200 µl BCA solution were added to each well. Samples and standard were incubated for 30 min at 37 °C in the dark and concentrations were determined by measuring the absorption at 562 nm using Tecan microplate reader i-control.

Well	Concentration BSA
A1/2	2 mg/ml
B1/2	1 mg/ml
C1/2	0.5 mg/ml
D1/2	0.25 mg/ml
E1/2	0.125 mg/ml
F1/2	0.063 mg/ml
G1/2	0.031 mg/ml

Table 11: BSA Standard

4.2.7 **Western Blot**

To an Eppendorf tube, 5 µl 4x Loading Dye, 1 µl DTT, 100 µg protein sample and water were added to reach a final volume of 20 µl. Samples were briefly centrifuged and heated to 95 °C for 10 min, leading to denaturation of the samples. In the meantime, gel chambers were prepared and 1x MOPS buffer was added. Samples were

centrifuged again, and gel was loaded with 18 µl per sample and 8 µl pre stained protein ladder. Gel chamber was connected to a power unit and gel was run for 20 min at 80V, followed by 80 min at 120V, until the blue stain of the protein ladder reached the bottom of the gel. Whatman® Cellulose Filter Paper was cut into 6 x 8 cm pieces and primed in 1x transfer buffer. Membrane was inactivated in methanol and primed in 1x transfer buffer. Whatman paper, membrane, and gel were assembled in a semi-dry blotting chamber (Figure 6).

Figure 6: Blotting Chamber

Assembly of Whatman paper, membrane and gel in blotting chamber: Three layers of Whatman paper primed in 1x transfer buffer, followed by membrane which is inactivated in methanol and primed in 1x transfer buffer, followed by gel, topped by three layers primed Whatman paper

1-2 ml 1x transfer buffer were added on the top paper, and liquid around the paper stock was removed. Gel was plotted for 1 hr at 25 V, 0.2 A per gel. Next, membrane was rinsed in 1x TBS-T and blocked for 1 hr at room temperature in TBS-T containing 5 % (m/v) milk to block unspecific binding sides. Primary antibodies (Table 12) were added in milk/ TBS-T over night at 4 °C. On the next day, the membrane was washed three times for 5 min in TBS-T and secondary antibodies (Table 13) were added in milk/ TBS-T for 1 hr at room temperature. Membrane was washed three times in TBS-T and image was developed. For that, membrane was placed on foil and 1 ml ECL 1 and 1 ml ECL 2 were evenly distributed on the membrane. Membrane was incubated in the dark for 3 min and was transferred to new foil. Image was developed using the Gel Doc XR+ gel documentation system. Next, membrane was washed and blocked with 5 % milk for 1 hr at room temperature. As house keeping gene, β-actin was added 1/20000 in 5 % milk for 30 min at room temperature. Membrane was washed and image was developed. Bands were quantified by densitometric analysis using the ImageLab v6 software and proteins of interest were normalized to actin levels.

Primary antibody	Clone	Host	Dilution	Company
β-actin	AC15	mouse	1/20000	Merck
GFAP	GA5	mouse	1/1000	eBioscience
Occludin	EPR2099 2	rabbit	1/2500	Abcam
VE-Cadherin	VECD1	rat	1/100	BioLegend

Table 12: Primary antibodies used for western blot

Secondary antibody	Host	Dilution	Company
α-rabbit	mouse	1/5000	Santa Cruz
α-rat	goat	1/5000	Santa Cruz
α-mouse	goat	1/2500	Santa Cruz

Table 13: Secondary antibodies used for western blot

4.3 Cell Biology

4.3.1 Endothelial cell isolation from CNS tissue

For CNS EC-isolation, mice were deeply anesthetized with isoflurane and perfused with 0.9 % NaCl. Brain and spinal cord were isolated and digested in 2 mg/ ml papain solution containing 40 µg/ ml DNase I for 30 min at 37 °C. In between, tissue was mechanically homogenized using Milteny gentle MACS™ dissociator. The reaction was stopped with 10 ml PBS/FCS and cell suspension was meshed through a 70 µm cell strainer. Samples were centrifuged for 7 min at 300 g at 4 °C. The pellet was dissolved and centrifuged for 30 min at 400 g at 15 °C (acceleration 1, no break) with a 22 % Percoll gradient. The pellet, containing a mix of CNS cells, was used for Flow Cytometry staining.

4.3.2 Immune cell isolation from CNS tissue

For CNS immune cell isolation, brains and spinal cords of steady state mice or mice 30 days post immunization were isolated from NaCl perfused mice and digested with 2 mg/ml collagenase II and 2 µg/ml DNase I for 20 min at 37 °C. Tissues were homogenized with syringe and needle and cell suspensions were centrifuged with a 70 % - 37 % - 30 % Percoll gradient for 40 min at 500 g at 16 °C (acceleration 1, no

break). Cell ring between the 70 % and the 37 % phase was transferred to new Falcon and washed in PBS/FCS. Finally, cells were used for Flow Cytometry staining.

4.3.3 MOG recall of spinal cord infiltrates

Immune cells isolated from spinal cords were restimulated with 20 µg MOG_{35-55} peptide and Brefeldin A for 6 h at 37 °C in T cell medium. Afterwards, cells were harvested and washed in PBS/FCS. In the end, they were used for Flow Cytometry staining.

4.3.4 Flow Cytometry

Prior to fluorescence labeling, FC receptors were blocked using FC-block (BioXCell) to prevent unspecific antibody binding. Single cell suspensions isolated from CNS EC were stained against the surface markers ICAM-1, VCAM-1, CD31, Ly6C, CD45, CD11b, and viability dye (table 14). Isolated immune cells from CNS were stained on the surface with antibodies against CD11b, CD45, CD4, CD8, TCRβ, Ly6G, CD69, and viability dye. Restimulated immune cells isolated from spinal cords were stained against the surface markers CD4, CD8, CD90.2, and viability dye (table 14). For intracellular staining, cells were subsequently fixed and permeabilized using Cytofix/ Cytoperm kit (BD) and stained against IL-17A, IFN-γ, CD40L. For T_{reg} staining cells were fixed and permeabilized using the PE Anti-Mouse/Rat Foxp3 Staining Set (eBiosience) and stained against FoxP3 (table 14). All samples were acquired with FACS Canto II and analyzed with Flow Jo software.

Antigen	Fluorochrome	Dilution	Clone	Company
FC-Block (CD16)	/	1/100	2.4G2	BioXCell
CD4	PerCP PE	1/200 1/200	GK1.5 GK1.5	BioLegend BioLegend
CD8	PE-Cy7 V500	1/1000 1/200	53-6.7 53-7.3	BioLegend BioLegend
CD11b eBioscience™	eFluor 450	1/200	M1/70	Thermo Fisher Scientific
CD31	PE	1/75	MEC 13.3	BioLegend
CD40L	APC	1/200	MR1	BioLegend
CD45	BV510	1/200	30-F11	BioLegend

CD69 eBioscience™	FITC	1/100	H1.2F3	Thermo Fisher Scientific
CD90.2	PerCP	1/1000	53-2.1	BioLegend
FoxP3 eBioscience™	FITC	1/200	FJK-16s	Thermo Fisher Scientific
ICAM-1	APC	1/200	YN1/1.7.4	BioLegend
VCAM-1	Pe-Cy7	1/200	429 (MVCAM.A)	BioLegend
Ly6C	PerCP	1/200	HK1.4	BD Bioscience
Ly6G	PE	1/1000	1A8	BioLegend
TCRβ	APC	1/1000	H57-597	BioLegend
Viability dye eBioscience™	APC-eFluor 780	1/1000	/	Thermo Fisher Scientific

Table 14: Flow Cytometry antibodies and reagents

4.3.5 **Immunohistochemistry**

Mice were perfused with 0.9 % NaCl and brains and spinal cords were isolated and fixed in 4 % PFA overnight at 4 °C. Afterwards, tissue was incubated at 4 °C in 30 % sucrose for 3 days to dehydrate. Tissue was embedded in TissueTek® O.C.T™ compound and cut into 10µm slices. For staining, slices were blocked in 1x Roti immunoblock in D1 buffer for 1 h at room temperature and were then incubated with primary antibody in D1 buffer at 4 °C overnight. On the next day, slices were washed with D1 buffer diluted 1:3 in PBS for 10 minutes twice. Then, secondary antibody was added for 1 h at room temperature. Afterwards, sections were washed once with D1 diluted 1:3 in PBS and twice with PBS for 10 min each. Slices were mounted with Vectashield® Antifade Mounting Medium with DAPI and images were acquired at a TCS SP8 inverse confocal fluorescence microscope.

Antibody	Fluorophore	Clone	Host	Dilution	Company	Cat. Number
CD31		polyclonal	Rabbit	1/100	Abcam	Ab28364
GFAP		GA5	Mouse	1/500	eBioscience	53-9892-82
Iba1		Polyclonal	Rabbit	1/1000	Fisher Scientific	NC9288364

Anti-rabbit	CF488A		Goat	1/800	Sigma-Aldrich	SAB4600044
Anti-mouse	Cy555		[NR1]Goat	1/800	Sigma-Aldrich	SAB4600229

Table 15: Antibodies for immunohistochemistry

4.4 Mouse experiments

4.4.1 Mouse strain

The following mouse strain was used for experiments: $A20^{fl/fl}$ mice (Hövelmeyer *et al.*, 2011) were crossed to tamoxifen-inducible SLCo1c1-$CreER^{T2+/-}$ mice (Ridder *et al.*, 2011), $Cre^{+/-}$ offspring were used as experimental mice, herein called $A20^{\Delta BEC}$. $Cre^{-/-}$ littermates were used as wildtype controls. In order to activate Cre recombinase, experimental mice were treated with tamoxifen at the age of 6-7 weeks. To avoid unwanted effects of tamoxifen treatment, control animals were treated equally.

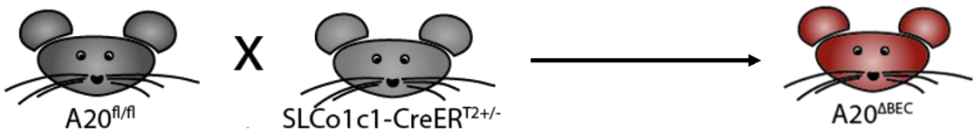

Figure 7: Generation of $A20^{\Delta BEC}$ mice

Mice were house bred under specific pathogen-free conditions. All mice were on the C57BL/6 background and experiments were performed with mice of 10-14 weeks of age according to guidelines of the central animal facility institution (TARC, University Mainz).

4.4.2 Tamoxifen Treatment

Tamoxifen was dissolved in 1 ml of 96 % (v/v) ethanol and 19 ml of olive oil by rotating over night at 4 °C to a final concentration of 2 mg/ ml. Mice at the age of 6-7 weeks were intraperitoneally (i.p.) injected once daily with 0.2 mg tamoxifen for five consecutive days.

4.4.3 **Active EAE induction**

Mice were immunized subcutaneously with 50µg MOG_{35-55} peptide (Gene Script, amino acid sequence: MEVGWYRSPFSRVVHLYRNGK) emulsified in complete Freund's adjuvant complemented with 8 mg/ ml of heat-inactivated *Mycobacterium tuberculosis* at the base of their tail. Additionally, mice were i.p. injected with 150-200 ng of pertussis toxin (PTx) in PBS on day 0 of immunization and day 2 post immunization (Figure 8). Mice were weighed daily, and clinical symptoms were assessed and documented as a score ranging from 0 to 6 in order to monitor the course of disease (Table 16). Mice that reached a score of 4.25 or higher were sacrificed according to the animal allowances.

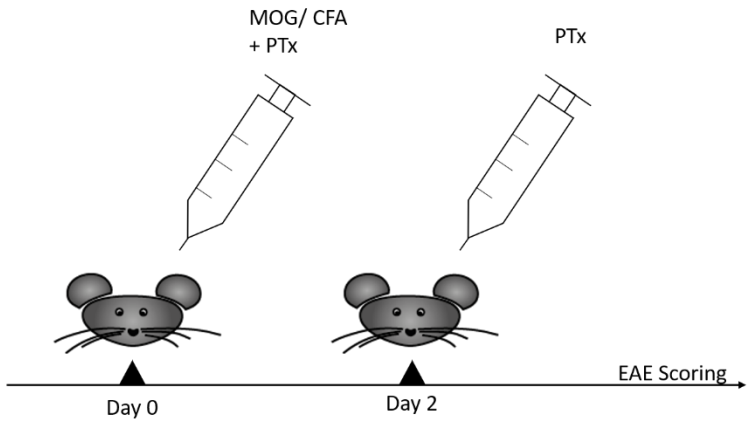

Figure 8: EAE induction

EAE is induced with 50 µg MOG_{35-55} and CFA. PTx is injected on day 0 and day 2. Mice are observed and scored for clinical symptoms daily.

Score	Symptoms and Behavior
0	no symptoms, normal behavior
0.5	decreased tail tone / tail partially paralyzed
1	tail completely paralyzed
1.25	mouse can be turned to the dorsal side but turns back immediately
1.5	mouse can be turned to the dorsal side but can turn back rather easily

1.75	mouse can be turned to the dorsal side but needs more effort to turn back
2	mouse can be turned to the dorsal side and stays at least 1 sec in this position
2.25	mouse walks with lowered buttocks
2.5	mouse walks with lowered buttocks and shows a waddling gait
2.75	gait sorely afflicted, but walking movements are still recognizable
3	legs are weak but still move forward
3.25	partial paralysis of the hind legs
3.5	paralysis of one of the hind legs
3.75	paralysis of both hind legs but mouse still moves forward effortlessly
4	mouse shows difficulties with moving forward
4.25	mouse stays in its position and moves forward only with supreme effort
4.5	mouse does not move forward and bends to the side
4.75	mouse lies on one side even if it is turned to the other side
5.0	mouse lies apathetically on the belly or side, breathing slowly, eyes (almost) closed
6.0	mouse is dead

Table 16: Scoring system for EAE

4.4.4 **FITC-Dextran analysis**

Mice were intravenously (i.v.) injected with 200 µl of 2 mM solution of 3-5 kDa FITC-Dextran in sterile PBS. After 10 minutes, perfused spinal cords were weighed and homogenized in 1 ml 60 % (v/v) trichloroacetic acid using a dounce homogenizer. Samples were centrifuged for 30 min at 10000 g at 4 °C and supernatant was collected. Fluorescence was measured using Tecan fluorescence plate reader with excitation at 470 nm and 520 nm. Measurements were quantified according to standard curve and per mg of tissue.

4.5 Bioinformatic Analysis

4.5.1 Statistics

Statistical analysis was performed with Prism Graph Pad (Version 8). Differences were evaluated by unpaired two-tailed students t-test for comparison of two groups, or one-way ANOVA (analysis of variance) with Bonferroni's post hoc test for comparison of more than two groups. All values are represented as mean ± SEM and P values were considered as significant with $p<0.05$ *; $p<0.1$ **; $p<0.001$ ***; $p<0.0001$ ****.

5 Results

Part of the following data has already been published in The Journal of Clinical Investigation (JCI) on October 19th 2023 (Johann *et al.*, 2023).

5.1 The BBB integrity of A20$^{\Delta EBC}$ mice is not impaired

A20 dysfunction had been suspected to play a crucial role in the development of autoimmune and neuroinflammatory diseases for many years. One of the factors contributing to this assumption is its influence on the stabilization of BBB integrity. *In vitro* studies with rat microvessel endothelial cells (rBMEC) have shown that endothelial A20 increases the expression of occludin and ZO-1 while decreasing the expression of ICAM-1, indicating an essential role in the maintenance of BBB integrity and its role in alleviating cerebral inflammatory injury (Han *et al.*, 2016). Interestingly, Ridder *et al.* proposed that endothelial TAK1 and NEMO play an important role in protecting the BBB through NF-κB signaling. Using NEMO^{KO} mice and TAK-1^{KO} mice they were able to show that occludin expression was lower in both strains compared to control mice. At the same time, they observed decreased levels of A20 expression. Therefore, they conclude that TAK1 and NEMO prevent the degradation of occludin through an induction of A20 and suggest that A20 stabilizes occludin through deubiquitination, therefore preventing it from proteasomal degradation (Ridder *et al.*, 2015).

Even though these studies imply an important role of CNS A20 on the integrity of the BBB, it has not yet been studied *in vivo*. Therefore, we aimed to investigate whether removal of A20 in CNS endothelial cells affected the permeability of the BBB. To do so, we aimed to assess the condition of intercellular junctions, precisely tight junctions and adherent junctions, by analyzing protein levels of occludin and VE-cadherin with western blot. As shown in Figure 9 A and B, no significant difference could be seen. However, we observed a tendency of decreasing occludin levels. We therefore further investigated BBB integrity using a tracer. We injected 4 kDa FITC-Dextran to A20$^{\Delta BEC}$ and control mice and compared the fluorescence signaling of their spinal cord tissue. As positive control, EAE mice with a score of 2,5 were used, and for negative control, mice were injected with PBS (Sham). Again, no significant difference was observed, indicating that there was no change in BBB integrity (Figure 9 C).

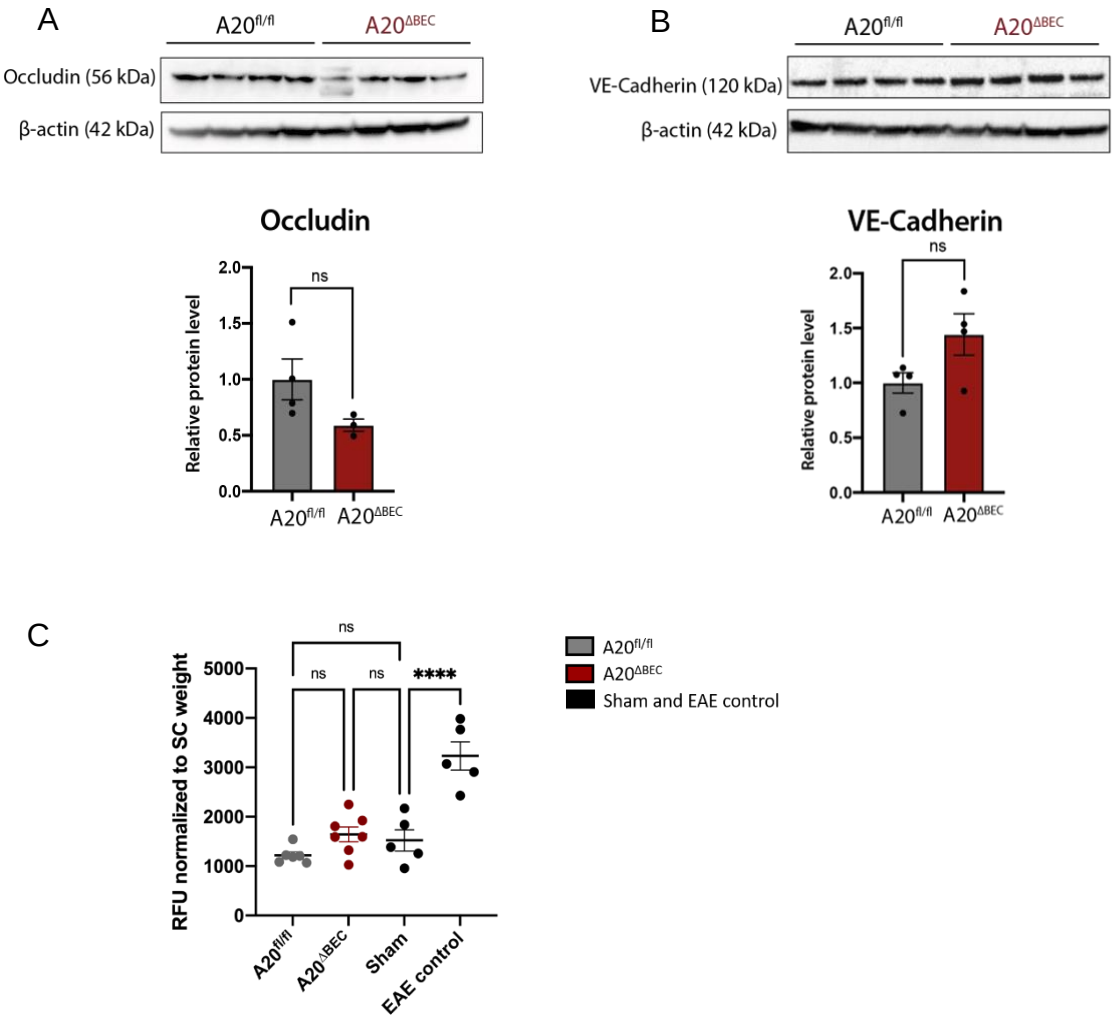

Figure 9: The BBB integrity of A20$^{\Delta BEC}$ is not impaired

Analysis of steady state A20$^{\Delta BEC}$ and control mice. **(A and B)** Representative image of western blot and quantification for occludin (A) and VE-Cadherin (B) normalized to β-actin. **(C)** Quantification of FITC-labeled Dextran (4 kDa) in spinal cord, 10 min after i.v. injection. Date are shown as mean ± SEM and analyzed using two tailed unpaired student's t-test, ****p<0.0001.

5.2 **A20$^{\Delta EBC}$ mice show increased adhesion molecule expression during steady state**

Inflammation does not only affect the BBB by changing tight junction and adherens junction molecules thus leading to a breakdown of BBB integrity. It can also lead to a change in adhesion molecule levels promoting transendothelial migration of immune cells into the CNS. Therefore, we were curious whether A20 signaling had any impact on the expression of adhesion molecules in steady state. In order to study the expression of ICAM-1 and VCAM-1 on BBB ECs, we analyzed brain and spinal cord of A20$^{\Delta BEC}$ mice and compared them to naïve littermate controls. Interestingly, flow cytometric analysis of CNS ECs (gated on CD11b^{-}, CD45^{-}, CD31^{+}, Ly6C^{+}) from A20$^{\Delta BEC}$ mice revealed highly upregulated expression of ICAM-1 and VCAM-1 in steady state, compared to control ECs (Figure 10). This shows, that A20 signaling is crucial for the downregulation of ICAM-1 and VCAM-1 on BBB ECs.

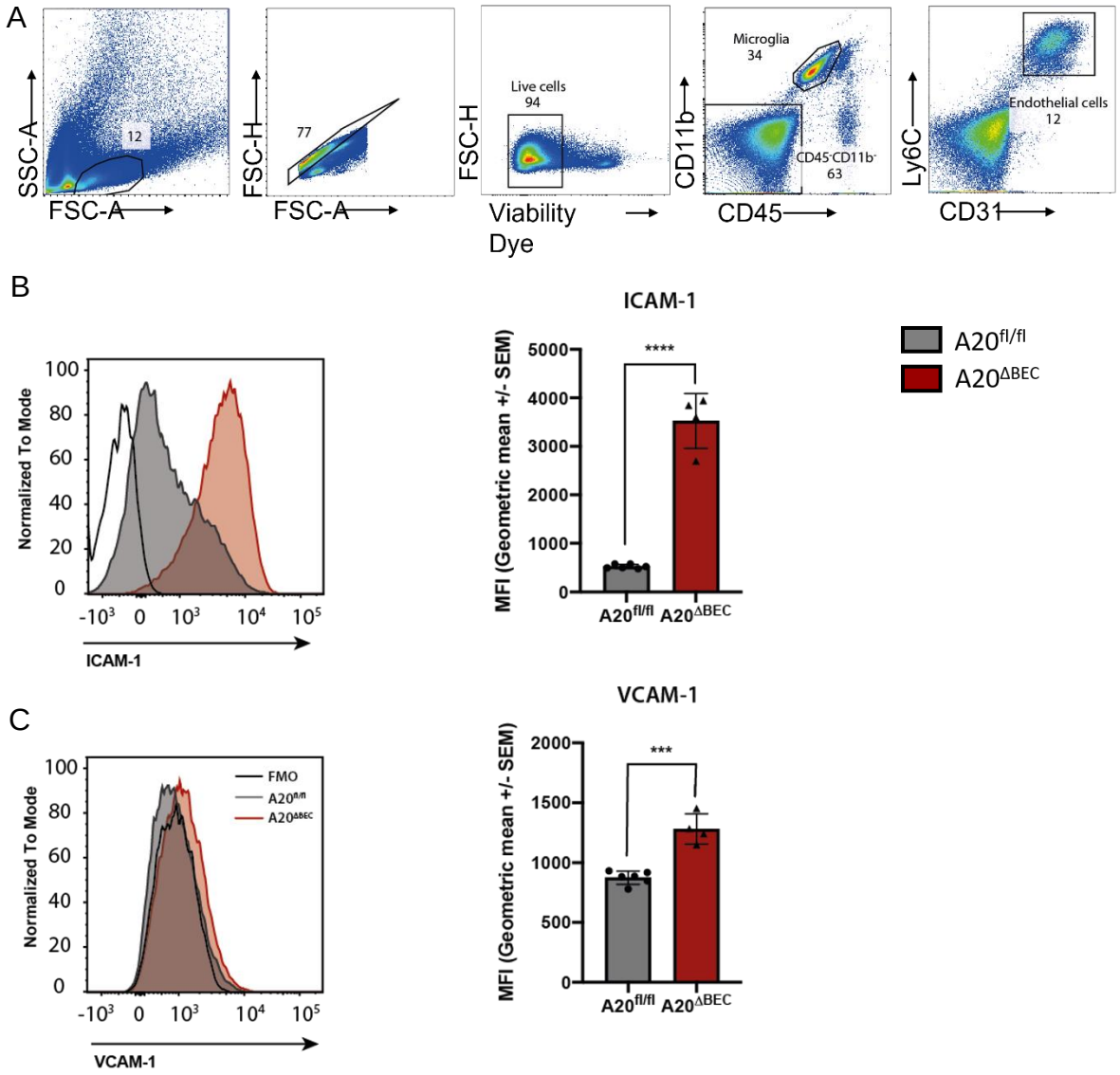

Figure 10: A20$^{\Delta BEC}$ mice show increased adhesion molecule expression during steady state

Flow cytometry analysis of CNS ECs isolated from brain and spinal cord of steady state A20$^{\Delta BEC}$ mice **(A)** Representative gating strategy to identify living single cells - CD11^{+}CD45^{+}CE31^{+}Ly6C^{+} ECs. **(B and C)** Flow cytometric analysis of mean fluorescence intensity (MFI) of ICAM-1^{+} (B) and VCAM-1^{+} (C) CNS ECs of steady state mice. Data in B and C are shown as mean ± SEM and analyzed using two tailed unpaired student's t-test, ***p<0.001, ****p<0.0001.

5.2.1 A20-deficiency in BBB ECs is associated with immune cell infiltration in steady state

Given that ICAM-1 and VCAM-1 play a major role in the transendothelial migration of leukocytes into the CNS, we investigated whether A20-deficiency and subsequent higher expression of adhesion molecules was associated with an increased infiltration of leukocytes into the CNS. For that, we isolated brain and spinal cord of $A20^{\Delta BEC}$ mice and control animals and analyzed the amount of infiltrating T cells and infiltrating myeloid cells, defined as $CD45^+TCR\beta^+CD4^+/CD8^+$ and $CD45^+CD11b^{high}TCR\beta^-$, respectively. As can be seen in Figure 11 A and B, we noted a significant increase in the total amount of infiltrating cells. More specifically, we saw a higher number of infiltrating myeloid cells, $CD4^+$ T cells, and $CD8^+$ T cells. Furthermore, the number of resident microglia was also elevated in $A20^{\Delta BEC}$ mice compared to Cre-negative littermate controls. We then took a closer look at the $TCR\beta^+$ T cells and wanted to see whether there was a difference in the proportion of infiltrating $CD4^+$ and $CD8^+$ T cells. As shown in Figure 11 C, no difference could be seen.

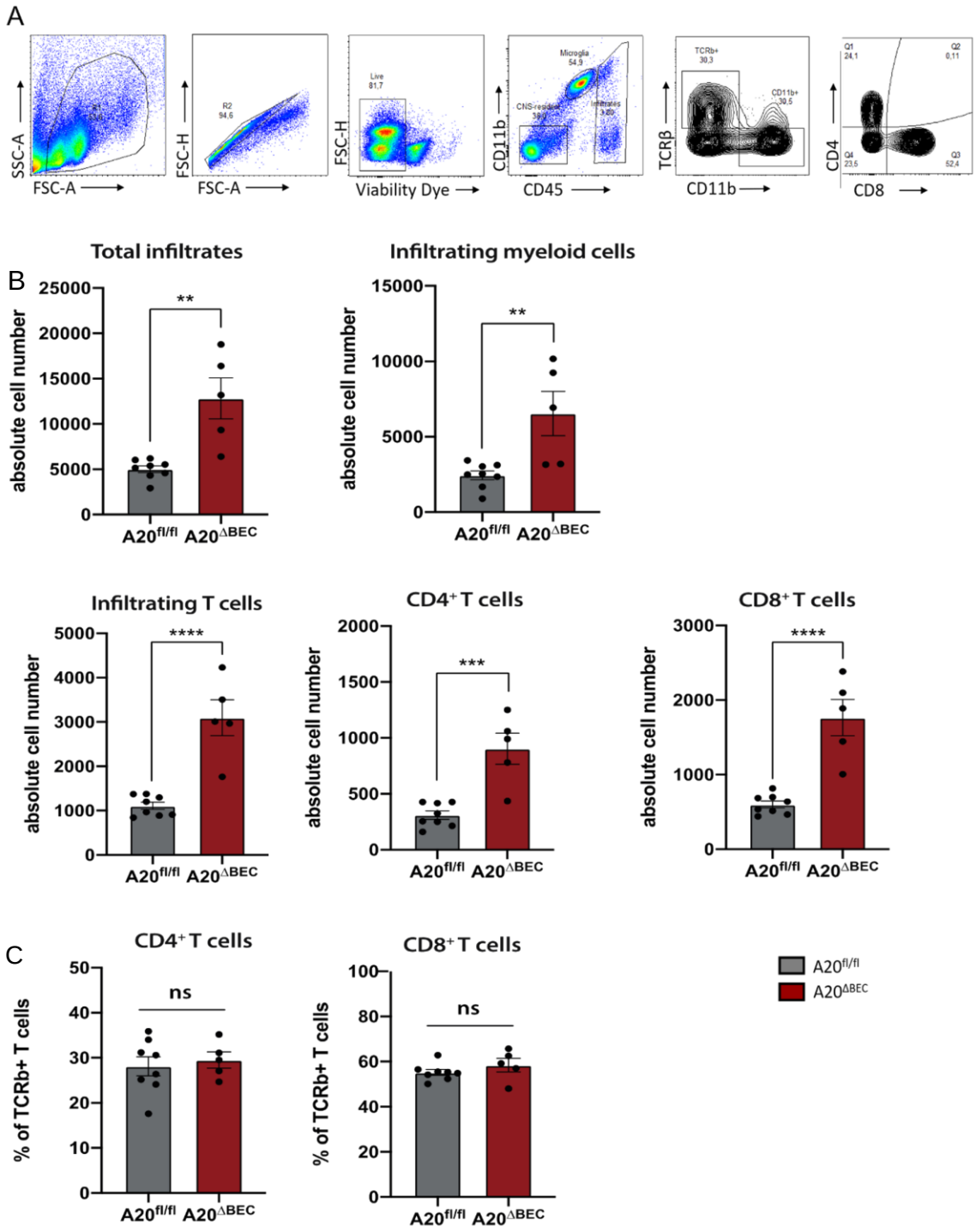

Figure 11: A20-deficiency in BBB ECs is associated with immune cell infiltration in steady state

Flow cytometry analysis of immune cell infiltration from brain and spinal cord of steady state mice **(A)** representative gating strategy to identify living single cells - $CD45^+TCR\beta^+CD4^+/CD8^+$ T cells and $CD45^+CD11b^{high}TCR\beta^-$ infiltrating myeloid cells. **(B-C)** Flow cytometric analysis of the absolute cell number of total infiltrates, infiltrating myeloid cells, and infiltrating T cells, further subdivided into $CD4^+$ and $CD8^+$ T cells **(B)**, and relative amount of $TCR\beta^+$ T cells **(C)**. Data in B and C are shown as mean ± SEM and analyzed using two tailed unpaired student's t-test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

We could further confirm these findings with the help of qPCR of A20$^{\Delta BEC}$ spinal cord tissue. CD3e was used as a marker for T cells and values were normalized to Gapdh and control mice. We observed equally increased expression levels of CD3e in A20$^{\Delta BEC}$ compared to naïve animals as we saw in terms of cellular infiltrates with flow cytometry (Figure 12).

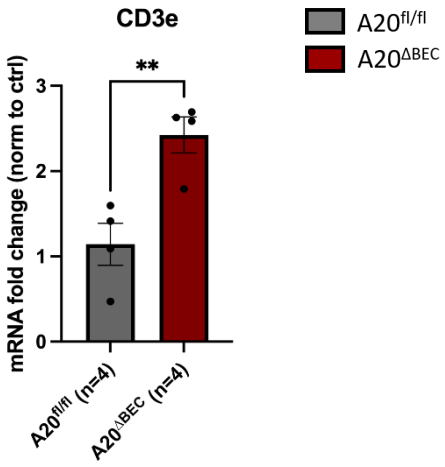

Figure 12: A20-deficiency promotes T cell migration into the CNS

Quantitative analysis of real-time qPCR of steady state spinal cord. Data is shown as mean ± SEM and analyzed by using two tailed unpaired student's t-test **p<0.01.

5.2.2 **A20$^{\Delta BEC}$ mice develop signs of reactive astrogliosis in the brain**

Due to the increased numbers of peripherally-derived immune cells in the CNS of steady state A20$^{\Delta BEC}$ mice as well as the highly activated signature of CNS ECs, we next attempted to assess the overall neuroinflammatory status of naïve A20$^{\Delta BEC}$ mice. In mice with A20-deficiency in microglia, the increased numbers of CD8^{+} T cells were found to cause dramatic changes in microglia cell numbers and morphology, likely through the release of IFN-γ (Mohebiany *et al.*, 2020). In addition, we speculated that deficiency of A20 in BECs might trigger the release of pro-inflammatory cytokines from these cells, that could induce reactive astrogliosis and/or microgliosis. As A20$^{-/-}$ mice show dramatically increased levels of the pro-inflammatory cytokines IL-6, TNF, IL1β and monocyte chemoattractant protein (MCP)-1 as well as higher levels of iNOS, the inducible nitric oxide synthase, which is induced under inflammatory conditions in the

brain (Guedes *et al.*, 2014), we probed mRNA isolated from whole brain tissue of A20$^{\Delta BEC}$ mice and littermate controls with primers against these and other pro-inflammatory cytokines and chemokines. We did not observe elevated levels of *Il1a*, *Il1b*, *Il6* or *Nos2*, yet we observed increased expression levels of *Tnf* and *Cxcl1* (Figure 13 A). Since TNF and CXCL1 have been shown to trigger microglial cells and astrocytes towards a reactive phenotype, we next wanted to see if these cell types showed altered activation markers in A20$^{\Delta BEC}$ mice (Liu *et al.*, 2020; Liddelow *et al.*, 2017). By analyzing mRNA levels of the microglia marker *Aif1* (IBA1) as well as the microglia activation markers *Cd68*, *C1qa* and *Lgals3* we could not observe any alterations in terms of microglia reactivity between A20$^{\Delta BEC}$ mice and littermate controls (Figure 13 B).

Furthermore, we did not observe any differences when looking at microglia by IBA1 immunohistochemistry in the cortex of A20$^{\Delta BEC}$ mice (Figure 13 C). However, we detected elevated levels of *Gfap* mRNA encoding for glial fibrillary acidic protein (GFAP) in the brains of A20$^{\Delta BEC}$ mice (Figure 14 A). We could confirm the elevation of this marker for reactive astrocytes also on protein level by western blot analysis (Figure 14 B and C) as well as immunohistochemistry (Figure 14 D and E). Interestingly, we not only observed signs of reactive astrogliosis at the brain border regions (Figure 14 D), but also in astrocytes closely associated with vessels (Figure 14 E). It has recently been shown that factors secreted by activated ECs are able to induce a specific reactive astrocyte subtype that differs from the subtype resulting from activation through microglial secreted factors (Taylor *et al.*, 2022). In addition, single cell sequencing data from LPS-treated mice have identified 10 clusters of different neuroinflammatory astrocyte subtypes that reside in different anatomic locations, of which one cluster (cluster 8) is closely associated with vessels and the brain surface (Hasel *et al.*, 2021). We sought to determine whether the marker genes for this cluster 8 reactive astrocytes are also upregulated in the brains of our A20$^{\Delta BEC}$ mice and indeed, we found high levels of *Tap1*, *Stat1* and *Igtp* in our mutant mice (Figure 14 F).

Taken together, our data indicates that A20 expressed by brain ECs protects from neuroinflammation. We found signs of astrogliosis at the surface of the brain and in the vicinity of blood vessels in A20$^{\Delta BEC}$ mice.

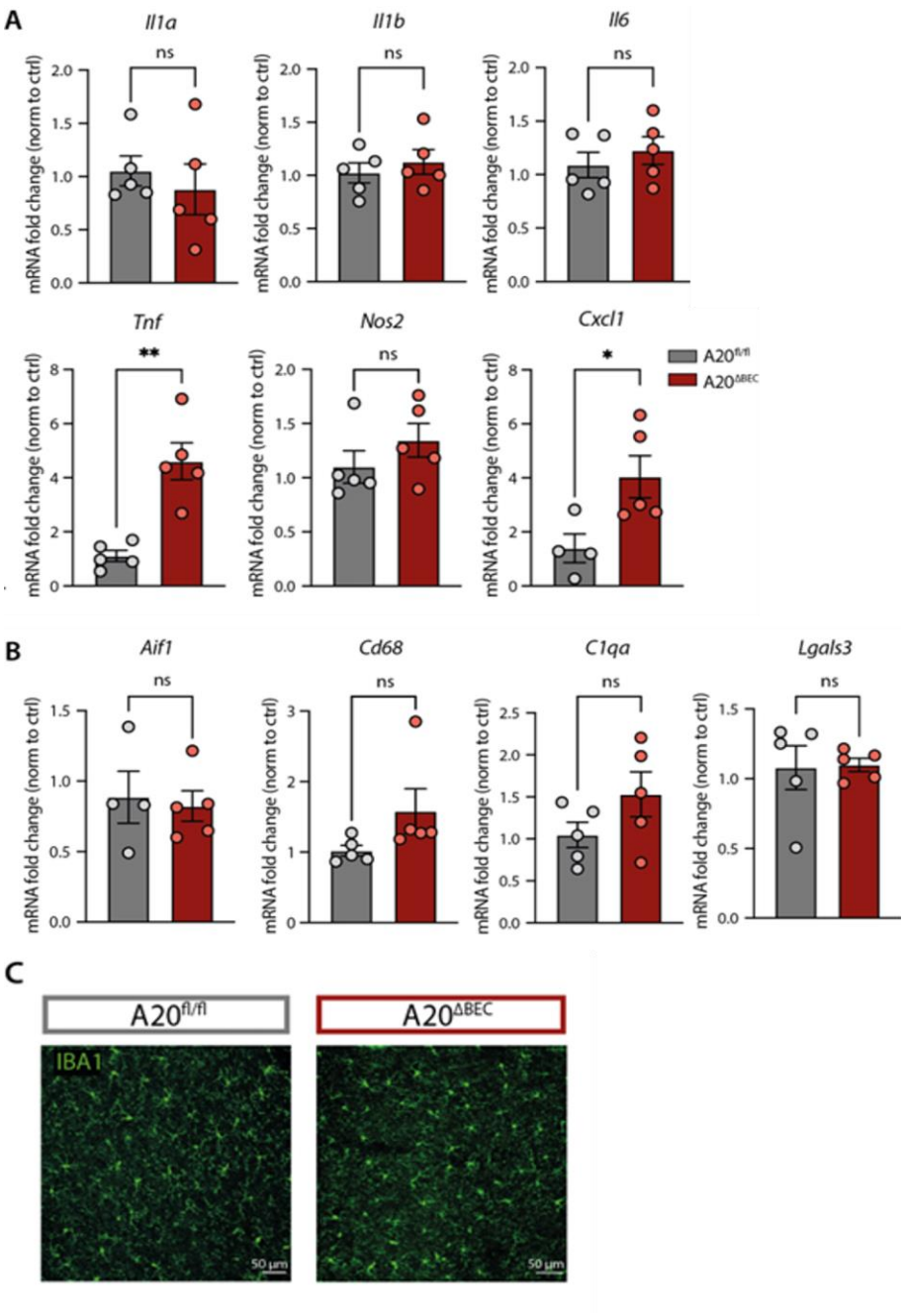

Figure 13: A20$^{\Delta BEC}$ mice show no sign of microgliosis in the steady state

(A) RT-PCR analysis of whole brain tissue from A20$^{\Delta BEC}$ and littermate control mice. Shown are mRNA levels normalized to Hprt and controls of the proinflammatory cytokines Il1a, Il1b, Il6, Tnf as well as Nos2 (encoding for iNOS) and the chemokine Cxcl1. **(B)** RT-PCR analysis of whole brain tissue from A20$^{\Delta BEC}$ and littermate control mice for microglia activation markers Aif1 (encoding for IBA1), Cd68, C1qa and Lgals3. **(C)** Representative confocal images of brain tissue from A20$^{\Delta BEC}$ and littermate control mice stained for IBA1. Data is shown as mean ± SEM and was analyzed using two-tailed unpaired student's t-test with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns = not significant. All scale bars = 50 µm.

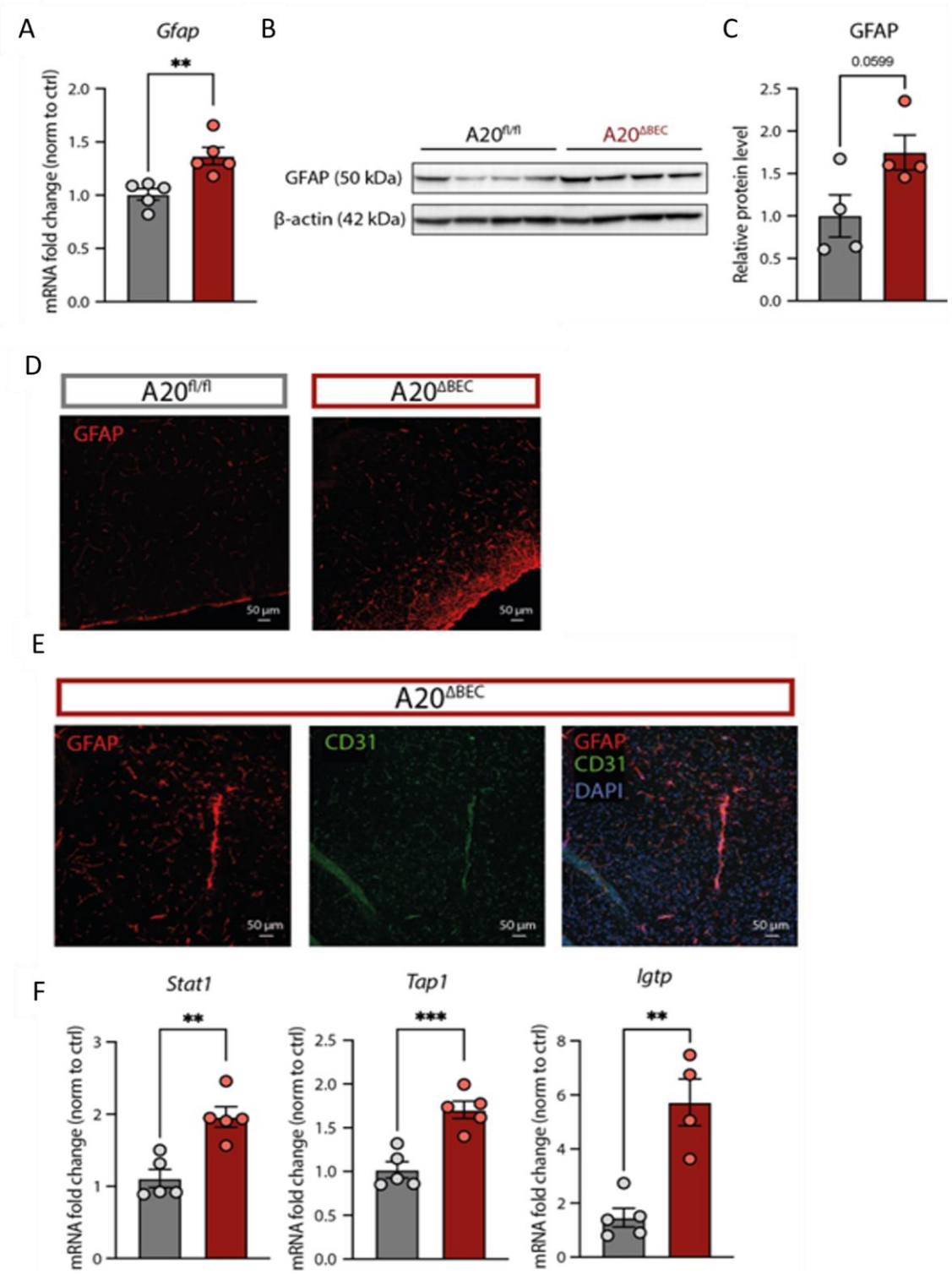

Figure 14: A20$^{\Delta BEC}$ mice show signs of astrogliosis in the steady state

(A) RT-PCR analysis of the reactive astrocyte marker Gfap in whole brain tissue. **(B)** Western blot analysis of GFAP with **(C)** quantification of relative GFAP levels normalized to β-actin levels and controls. **(D)** Representative confocal images of brain tissue from A20$^{\Delta BEC}$ and littermate control mice stained for GFAP. **(E)** Representative confocal images of brain tissue from A20$^{\Delta BEC}$ for GFAP (red), CD31 (green) and nuclear staining (DAPI, blue) showing close connection between highly reactive astrocytes and endothelial cells. **(F)** RT-PCR analysis of cluster 8 reactive astrocyte markers Stat1, Tap1 and Igtp according to Hasel et al. (Hasel *et al.*, 2021). Data is shown as mean ± SEM and was analyzed using two-tailed unpaired student's t-test with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns = not significant. All scale bars = 50 µm.

5.2.3 **Loss of A20 in BBB ECs does not alter active EAE disease outcome**

Cell-type specific A20 deficiency in the CNS and its consequences for autoimmune disease pathology has been closely investigated with the help of EAE. For example, it was shown that A20-deficient microglia showed stronger EAE and an increased T cell infiltration into the CNS (Voet *et al.*, 2018). Moreover, mice lacking A20 specifically in astrocytes also presented more severe clinical symptoms to EAE together with enhanced immune cell infiltration (Wang *et al.*, 2013). However, up to date it has not been investigated whether the deletion of A20 in BBB ECs influences the outcome of EAE. To assess the role of A20 particularly in BBB ECs during neuroinflammation, we induced active EAE in $A20^{\Delta BEC}$ and naïve mice by immunization with MOG_{35-55}/CFA and PTx four weeks after the last tamoxifen injection (Figure 15 A). Deletion of A20 in CNS ECs did not alter onset or EAE severity compared to Cre-negative littermate controls (Figure 15 B and C).

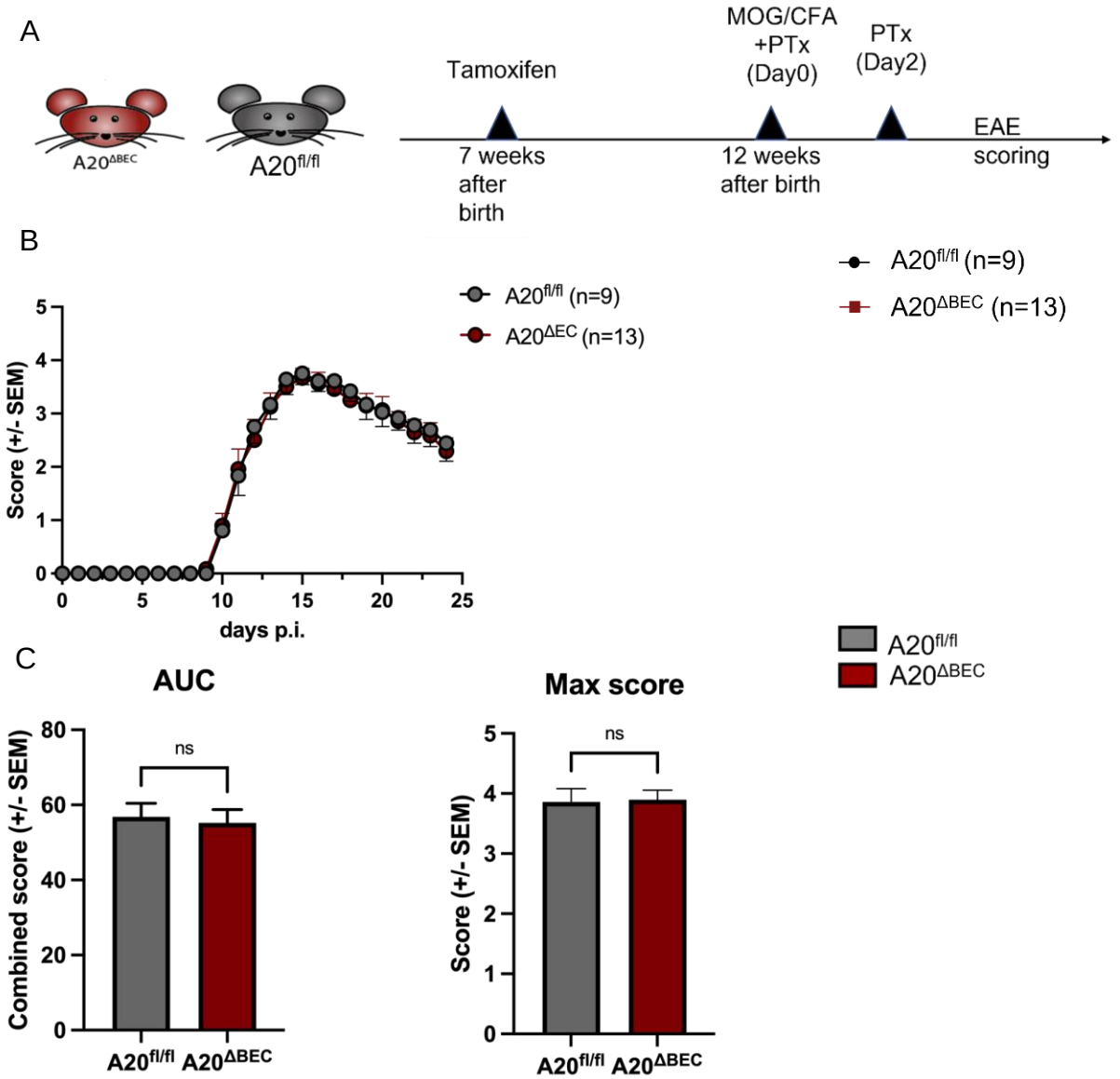

Figure 15: Loss of A20 in BBB ECs does not alter active EAE disease outcome

(A) Schematic representation of tamoxifen injection and induction of active EAE by immunization with MOG_{35-55}/CFA and PTx injection. **(B and C)** Disease course was monitored (B) and area under the curve and mean maximum scores were analysed (C). Data in B and C are shown as mean ± SEM and were analyzed using One-way ANOVA (B) and two tailed unpaired student's t-test (C)

5.2.4 **EC-A20 does not drive leukocyte infiltration during active EAE**

Even though the course of active EAE was not affected by the deletion of A20 in CNS ECs, we aimed to investigate whether there is a difference in leukocyte infiltration to the CNS. To do so, we induced active EAE in $A20^{\Delta BEC}$ and control mice. 30 days post immunization we isolated spinal cords and analyzed the infiltration of different immune cells. For this purpose, we used a T cell panel and a myeloid panel. For the T cell panel, we activated the isolated CNS cell infiltrates for 6 hr *in vitro* with the MOG_{35-55} peptide before staining to analyze the infiltration of MOG antigen specific T cells. The expression of CD40L served as an indicator for recently activated T cells. The myeloid panel was stained straightaway. As shown in Figure 16, we could not see a significant increase of antigen-specific T cell infiltrates into the CNS of $A20^{\Delta BEC}$ mice. Moreover, we analyzed the absolute cell numbers of regulatory T cells using FoxP3 as a marker, monocytes, and dendritic cells. Again, no difference was observed (Figure 16).

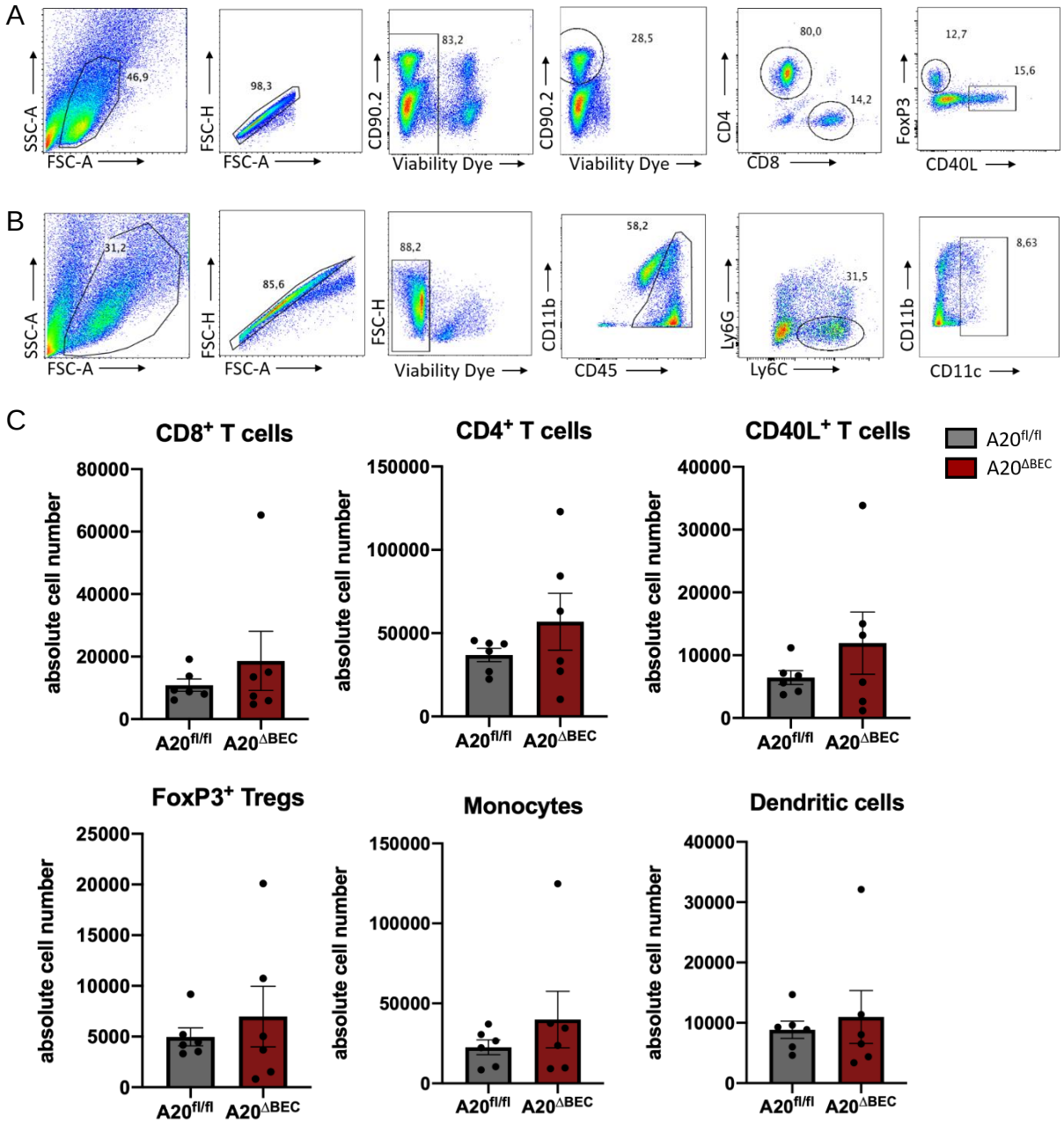

Figure 16: EC-A20 does not drive leukocyte infiltration during active EAE

(A-D) Flow cytometry analysis of immune cells isolated from spinal cord of immunized mice with MOG_{35-55}/CFA and PTx injection on day 30 post immunization. **(A)** Representative gating strategy to identify living single cells - $CD90.2^+CD4^+CD40L^+$ MOG-specific T cells and $CD90.2^+CD4^+FoxP3^+$ T_{reg}. **(B)** Representative gating strategy to identifypre-gated living single cells - $CD11b^{high}Ly6C^{high}$ monocytes and $CD11b^{high}CD11c^{high}$ - dendritic cells. **(C)** Flow cytometry analysis of the absolute cell number of CD8+ T-cells, CD4+ T-cells, CD40L+ MOG-specific T cells, Treg, monocytes, and dendritic cells in spinal cord. Data in C are shown as mean ± SEM and analyzed using two tailed unpaired student's t-test.

6 Discussion

A20 is considered a central gatekeeper in the regulation of inflammation through the inhibition of NF-κB signaling. Polymorphisms in the TNFAIP3 gene have been shown to play a crucial role in MS pathogenesis (Hoffjan *et al.*, 2015). Moreover, inappropriate functioning of the anti-inflammatory protein A20 is described to play an important role in the course of EAE pathogenesis. For instance, deletion of A20 in astrocytes and microglia leads to the development of an increased EAE severity and without microglial A20, $CD8^+$ T cells spontaneously migrate into the CNS in steady state and cause neuronal modificatiosn (Wang *et al.*, 2013; Voet *et al.*, 2018; Mohebiany *et al.*, 2020). Although studies indicate that the full knockout of A20 also leads to changes in the BBB ECs by causing upregulation of ICAM-1 and VCAM-1 (Guedes *et al.*, 2014), the role of A20 specifically in brain endothelial cells (BEC) on the maintenance of BBB integrity and its influence on the progression of EAE is not well understood.

We here highlight that the absence of A20 in BECs does not alter BBB integrity. We additionally could not observe a discrepancy in EAE disease outcome in mice lacking A20 in BECs compared to control mice. However, we could see an increased expression of the adhesion molecules ICAM-1 and VCAM-1. Furthermore, we show that A20-deficiency in BECs drives immune cell infiltration in the steady state but that this effect is compensated in the course of EAE and that astrocytes showed an increased reactivity profile. We indicate that inflammatory mediators either released by ECs or infiltrating immune cells drive spontaneous neuroinflammation in these mice. The clinical course of EAE disease was not affected by the BEC-specific A20 deletion and also the increased immune cell infiltration seen in the steady state was compensated in the course of EAE.

We propose that the increase in immune cell infiltrates into the CNS in the steady state could be caused by an increase in transmigration over the BBB due to the dramatic upregulation of the adhesion molecule expression. We further conclude that inflammatory mediators released either by activated ECs themselves or by infiltrating immune cells drive a reactive phenotype in the CNS-resident astrocytes. We hypothesize that these effects cannot be seen during active EAE in $A20^{\Delta BEC}$ mice, as A20 deletion is covered by a general inflammatory response in this situation.

6.1 A20-deficiency in BBB ECs in steady state

6.1.1 A20$^{\Delta BEC}$ mice do not show a loss of BBB integrity

During inflammation of the CNS, one of the major changes is a structural reorganization of junctional molecules leading to a loss of BBB integrity (Daneman, 2012). Surprisingly, we could neither observe an increase in fluorescence in the spinal cord tissue after injection of a 4 kDa FITC-Dextran tracer into A20$^{\Delta BEC}$ mice, nor could we show a significant difference in protein levels of occludin and VE-cadherin with western blot, suggesting no loss of function of tight junctions and adherent junctions. However, we did observe a tendency towards reduced occludin protein levels, yet even if additional experiments will confirm a downregulation of occludin when A20 is lacking in BECs, our results emphasize, that CNS endothelial A20 expression does not have an impact on BBB integrity in the steady state.

These observations seem to contradict the results of Han et al., who showed that Clematichinenoside (AR), a triterpene saponin made from the root of *Clematis chinensis*, restores the integrity of the BBB by upregulating A20 and subsequent suppression of NF-κB signaling (Han *et al.*, 2016). In this study, the authors investigated the effect of AR on BBB integrity by evaluating transepithelial electrical resistance (TEER) values as well as tight junction protein expression in response to hypoxia/reoxygenation by using rat brain microvascular endothelial cells. Interestingly, they could show that AR and consecutively higher levels of A20 sustained TEER values at a high level and that this treatment further caused increased expression of occludin and ZO-1 (Han *et al.*, 2016). Unlike Han *et al*., we used an *in vivo* model of BEC-specific A20-deficiency for our experiments. Therefore, it is inevitable to keep in mind that the BBB is not only made up of ECs but also of a basement membrane, pericytes, and astrocytes, all together forming the NVU (Daneman, 2012). The different components of the BBB closely interact with each other. For instance, it has been shown that astrocytes play an important role in the regulation of BBB integrity through adjusting the expression of occludin and claudin (Alvarez *et al.*, 2011b). Additionally, it has been shown that crosstalk between ECs and pericytes enhances TJ formation in the developing BBB (Obermeier *et al.*, 2013). The influence of astrocytes and pericytes on BBB integrity may compensate for changes in ECs, thus maintaining the functional BBB even in the absence of A20.

The specific role of A20 in barrier function has been investigated in many different organs using conditional A20-knockout mice also *in vivo*. The deletion of A20 exclusively in intestinal epithelial cells resulted in the spontaneous development of inflammation of the intestine. These mice showed characteristics of epithelial tight junction disruption, including the loss of occludin, hence resulting in greater intestinal permeability (Kolodziej *et al.*, 2011). Furthermore, the role of A20 in lung epithelial cells (LEC) was investigated using a mouse model of LEC-restricted A20 deletion. It could be shown that A20 is crucial for maintaining and restoring endothelial barrier integrity after inflammatory lung vascular injury through stabilizing VE-cadherin expression at adherent junctions (Soni *et al.*, 2018). Even though several studies have investigated the consequences of impaired NF-κB signaling, only few focused on BBB ECs. Ridder *et al*. showed that deletion of NF-κB essential modulator (NEMO) or the kinase TAK1, both key components of the NF-κB pathway, in BBB ECs lead to a disruption of the BBB and endothelial cell death. Hereupon, they propose that TAK1-NEMO signaling plays a crucial role in the protection of the brain endothelium and the maintenance of CNS homeostasis. Ridder et al. further demonstrated that TAK1 and NEMO protect the BBB by stabilizing occludin, an essential tight junction protein, through reducing its proteasomal degradation. They suggest a mechanism in which TAK1 and NEMO induce an IKK2 and p65 NF-κB-mediated pathway that induces A20 expression, which is then able to deubiquitinate and stabilize occludin, thus protecting the integrity of the BBB (Ridder *et al.*, 2015). Although our data shows a tendency towards reduced occludin levels, we provide evidence that this does not necessarily translate into loss of BBB integrity, as we do not see a difference in FITC-Dextran CNS leakage in $A20^{\Delta BEC}$ mice. This emphasizes that A20 does not play a decisive role in the maintenance of BBB integrity.

Nonetheless, BBB integrity might be impaired to a minor extent that we were unable to visualize with the used method. It has been described that an intact BBB cannot be crossed by compounds with a molecular weight higher than 180 Da. In order to determine BBB integrity, different methods using various markers have been established, the most commonly used method being the injection of dyes. These markers vary in size from a molecular weight of 376 Da (sodium fluorescein) to 961 Da (Evan's Blue) or even 70 kDa (FITC-Dextran). However, Evan's Blue reversibly binds to albumin, forming a bigger complex with a molecular weight of 6,8 kDa (Kozler and Pokorný, 2003). For our experiments, we used a FITC-Dextran with a molecular weight

of 4 kDa, a tracer that is sensitive enough to detect gross changes such as the ones occurring during severe neuroinflammation like EAE.

6.1.2 **A20-deficiency in CNS ECs drives adhesion molecule expression**

Under pathological conditions, a disruption of the BBB is usually accompanied by an increased secretion of inflammatory cytokines as well as higher ICAM-1 and VCAM-1 expression on ECs (Daneman, 2012). The latter play an important role in the transmigration of leukocytes through the BBB as they guide the firm adhesion of immune cells to the endothelium (Carman, 2009). In MS pathology, this process has also been observed. Activated Th1 and Th17 cells migrate towards the BBB and into the CNS, a process which is mediated by the upregulation of adhesion molecules on ECs (Engelhardt and Sorokin, 2009; Minagar and Alexander, 2003; Ortiz *et al.*, 2014). This also matches the study by Canella and Raine who found high levels of ICAM-1 and VCAM-1 in MS lesions (Cannella and Raine, 1995). In our experiments we aimed to investigate whether A20-deficiency in CNS ECs would lead to alterations in adhesion molecules which could explain the increase of immune cell infiltrates in the CNS.

Our present findings are in agreement with the previously published literature. We could demonstrate that mice lacking A20 in CNS ECs show a dramatically upregulated expression of the adhesion molecules ICAM-1 and VCAM-1 in the steady state, suggesting pro-inflammatory signaling within BBB ECs even in the absence of an external stimulus. These observations also correlate with those of Han *et al*. In an *in vitro* experiment, they found that AR blocked the expression of nucleus NF-κB and decreased the levels of ICAM-1 via upregulating A20 (Han *et al.*, 2016). Furthermore, Guedes *et al*., could show that A20-knockout mice present a baseline activation of the brain endothelium with an increased immunostaining for ICAM-1 and VCAM-1(Guedes *et al.*, 2014). While our data is in line with previous studies, we were able to quantify the extend of adhesion molecule upregulation by using flow cytometry. Strikingly, we found that almost 100% of BECs now express ICAM-1, which led us to further investigate immune cell infiltration into the CNS.

6.1.3 **CNS immune cell infiltration is enhanced in naïve A20$^{\Delta BEC}$ mice**

Under physiological conditions, low expression of adhesion molecules as well as the tight BBB structure prevents infiltration of peripherally-derived immune cells into the CNS, making it an immune privileged organ (Engelhardt and Sorokin, 2009). Under pathological circumstances, however, immune cell migration into the CNS is inevitable in order to clear debris and repair damage. However, excessive numbers of activated immune cells can also result in neurodegeneration and axonal damage as generally seen in MS (Racke, 2009). Interestingly, A20-deficient mice reveal massive inflammation in multiple organs, such as the liver, kidneys, intestine, joints and bone marrow, accompanied by increased numbers of active lymphocytes, granulocytes, and macrophages (Lee *et al.*, 2000). In addition, Mohebiany *et al*. could show an increase in CD8$^+$ T cell infiltration into the CNS in mice lacking A20 specifically in microglia (A20$^{\Delta MG}$) even in the absence of an external stimulus (Mohebiany *et al.*, 2020).

Of note, we also detected increased numbers of myeloid cells, CD4$^+$ T cells, and CD8$^+$ T cells in the steady state of A20$^{\Delta BEC}$ mice. Yet, the ratio of CD4 and CD8-expressing cells was unaltered, suggesting a different underlying mechanism that drives immune cell infiltration when compared to A20$^{\Delta MG}$ mice. A20-deficient microglia were found to display a viral gene expression signature, which, through the expression of type-I interferons, cause massive infiltration of CD8$^+$ T cells as a defense mechanism (Mohebiany *et al.*, 2020). Our data rather hints towards a mechanism that promotes immune cell infiltration regardless of their cell type.

It has been shown that T cell diapedesis can not only take place through an impaired BBB, but also through an intact barrier with T cells infiltrating through endothelial cell bodies, leaving TJ molecules intact (Wolburg *et al.*, 2005). Interestingly, this process, called transcellular diapedesis, has been shown to be the preferred route for T cells to enter the CNS during EAE (Wolburg *et al.*, 2005). Furthermore, studies have demonstrated that ICAM-1 and VCAM-1 play a major role in transcellular diapedesis as they function as adhesion molecules for immune cells (Carman, 2009). In our experiments, A20$^{\Delta BEC}$ mice manifested a dramatic upregulation of ICAM-1 and VCAM-1 levels in steady state while barrier properties were not impaired. We therefore suggest that the upregulation of these adhesion molecules could trigger transcellular diapedesis of immune cells, leading to an increased count of immune cells within the CNS as observed in these mice.

In addition, we found high levels of CXCL1 in the CNS of naïve A20$^{\Delta BEC}$ mice. CXCL1 is well-known to be involved in the recruitment of immune cells to sides of inflammation. Moreover, CXCL1 was shown to be secreted by peripheral endothelial cells upon TNF stimulation and to promote the adhesion of neutrophils to the luminal endothelial layer, which is essential for neutrophil transmigration (Girbl *et al.*, 2018). The high levels of CXCL1 expressed in A20$^{\Delta BEC}$ mice could thus contribute to the increased numbers of myeloid cells observed in the CNS of these mice.

Taken together, this data confirms that A20 expressed by CNS ECs is a central gatekeeper to secure CNS homeostasis and prevent potentially dangerous immune cell infiltration by controlling the expression of adhesion molecules and other pro-inflammatory mediators.

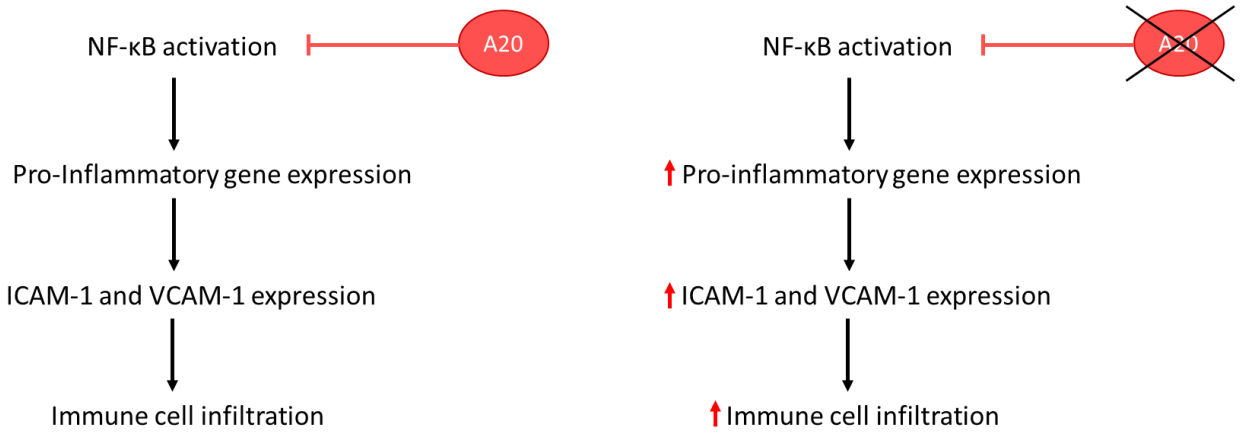

Figure 17: A20-deficiency drives immune cell infiltration through upregulating ICAM-1 and VCAM-1 expression

A20-deficiency in BBB ECs leads to prolonged NF-κB signaling. This results in pro-inflammatory gene expression, including ICAM-1 and VCAM-1 and subsequent immune cell infiltration into the CNS.

6.1.4 A20-deficiency in BBB ECs drives neuroinflammation: Potential impact on neurological manifestations

The complete loss of A20 in mice results in dramatic microglial and astrocytic activation and in increased expression levels of the cytokines *IL1b*, *IL6* and *Tnf* in the hippocampus and cortex (Guedes *et al.*, 2014). Up to now, the origin of this neuroinflammatory phenotype is not fully understood. Guedes *et al.* suggest two different scenarios, where in one scenario LPS derived from commensal bacteria might cross an impaired BBB, causing Toll-like receptor (TLR)-mediated microglial activation. In the second scenario, CNS-resident cell activation due to intrinsic A20-deficiency was proposed to result in increased pro-inflammatory cytokine production which activates other CNS-resident cells, creating a self-sustained inflammatory environment (Guedes *et al.*, 2014). Since we could confirm that the BBB integrity is not impaired, the second scenario is most feasible. Yet, which of the CNS-resident cells function as initiators of the self-sustained inflammatory environment is still not entirely clear. Astrocytes, neurons and oligodendrocytes are considered unlikely to be the driving forces in this scenario, as A20-deficiencyin these cells does not cause spontaneous neuroinflammation (Wang *et al.*, 2013). In contrast, A20-deficient microglia do result in spontaneous neuroinflammation with alterations in microglia and astrocyte reactivity, however, EC activation has not been investigated in $A20^{\Delta MG}$ mice (Mohebiany *et al.*, 2020).

In a recent study it was proposed that factors released by LPS-activated endothelial cells can induce a reactive astrocyte phenotype that differs from a microglia-driven phenotype, yet the mediators secreted from endothelial cells are still unknown (Taylor *et al.*, 2022). We here aimed at investigating whether A20-deficient endothelial cells can also drive spontaneous neuroinflammation with alterations in microglia and astrocyte reactivity. While we did not observe any alterations in the reactivity state of microglia, we indeed observed signs of reactive astrogliosis in our $A20^{\Delta BEC}$ mice. Levels of GFAP were increased both in mRNA and protein in the brains of $A20^{\Delta BEC}$ mice and $GFAP^{+}$ astrocytes were dramatically increased in the cortex of these mice. Interestingly, we also found a strong upregulation of GFAP in astrocytes surrounding big endothelial vessels in the cortex, implying that A20-deficient endothelial cells could release modulators that have a direct effect on astrocytes in the vicinity of vessels. Interestingly, expression analyses of pro-inflammatory cytokines and chemokines in

the brains of A20$^{\Delta BEC}$ mice revealed dramatically increased levels of *Tnf* and *Cxcl1*, potentially being secreted by A20-deficient ECs in the steady state. Further investigation using sorting of ECs of A20$^{\Delta BEC}$ mice will be needed in order to prove that these mediators are in fact produced by A20-deficient ECs themselves and not by other CNS-resident or infiltrating cells.

In the past, crosstalk between different components of the BBB and other cells of the CNS including neurons and microglia have been discussed controversially and the mediators and mechanisms of this crosstalk are largely unknown (Banks *et al.*, 2018). Nevertheless, it is unambiguous that intercellular communication plays an essential role in the regulation of BBB integrity and CNS homeostasis. It has been shown that astrocytes play a major role in promoting BBB permeability by driving the expression of chemokines and adhesion molecules (Abbott *et al.*, 2006). The existing literature supports an inductive influence of ECs on astrocytes. Ballabh *et al.* described that astrocytes cocultured with ECs changed their morphology from confluent monolayers to multicellular columns (Ballabh *et al.*, 2004). In general, under pathological conditions such as infection or neurodegeneration, microglia and astrocytes respond with morphological and transcriptional changes. Reactive microglia are characterized by swollen cell bodies and fewer processes as well as upregulation of activation markers such as CD68, MHC-II and Lgals3 (Li and Zhang, 2016). However, our data implies that strong activation of ECs is not involved in the induction of reactive microgliosis, indicating that other factors are responsible for the development of a reactive microglia phenotype.

During infection or neurodegeneration, also astrocytes respond with a reactive astrogliosis, involving morphological changes as well as alterations in gene expression (Zamanian *et al.*, 2012). Reactive astrogliosis can be beneficial as it improves recovery after CNS trauma and in MS, but it can also be harmful as it can inhibit axonal regeneration after CNS injury (Zamanian *et al.*, 2012; Voskuhl *et al.*, 2009). In MS and EAE, reactive astrogliosis is commonly observed starting from the early stages of lesion formation. One of the best-known characteristics of reactive astrocytes is the upregulation of GFAP, which can be found throughout the CNS in MS (Brambilla, 2019) and as shown in our data, can be driven by activated ECs *in vivo.*

In recent years, single-cell RNA sequencing has allowed for a more detailed view on reactive astrocyte subpopulations. In a study published by Hasel *et al.,* nine different reactive astrocyte subpopulations were defined to evolve upon inflammatory exposure to LPS, occupying distinct locations in the mouse brain (Hasel *et al.*, 2021). Interestingly, one of these subpopulations (cluster 8) was predominantly found in layer one of the cortex next to the brain surface and often associated with brain vessels, closely resembling the locations of reactive astrocytes found in our A20$^{\Delta BEC}$ brains. We thus probed our A20$^{\Delta BEC}$ brain tissues for expression of cluster 8 astrocyte markers, which include the master regulator of IFN-dependent transcription *Stat1*, the transporter associated with antigen processing 1 (*Tap1*) and the IFN-γ-inducible GTP-binding protein *Igtp.* Indeed, we found a dramatic upregulation of all three markers in our brain samples from A20$^{\Delta BEC}$ mice. In the study of Hasel *et al.* it was proposed that cluster 8 astrocytes can evolve upon IFN-stimulation (Hasel *et al.*, 2021). Whether interferons are absolutely required or whether other inflammatory mediators such as CXCL1 or TNF can also contribute to the generation of this subpopulation, requires further investigations. Also, it needs to be proven that the upregulation of these markers indeed derived from astrocytes, as other cellular sources like infiltrating immune cells could also be responsible for increased levels of these genes.

Interestingly, endothelial-derived CXCL1 was recently reported to mediate activation of astrocytes. Endothelial cells lacking the cyclin-dependent kinase 5 were found to express excessive amounts of CXCL1, which led to increased levels of GFAP in the hippocampus of Cdh5-Cre;Cdk5$^{fl/fl}$ mice. This CXCL1-driven reactive astrogliosis was furthermore associated with impaired glutamate reuptake through glutamate transporter 1, eventually leading to increased extracellular levels of the excitatory neurotransmitter glutamate and triggering the development of seizures (Liu *et al.*, 2020). Proper astrocytic function is well-known to be important to safeguard neuronal activity and astrogliosis is highly associated with the development of neurological symptoms such as seizures (Blanco-Suárez *et al.*, 2017). Interestingly, seizures are a common clinical manifestation of neuropsychiatric SLE (NPSLE), a disease that was recently associated with reduced A20 levels. Other symptoms of NPSLE range from headache and cognitive impairment to memory loss and stroke. Different factors have been identified to contribute to the pathogenesis of NPSLE, including cytokine-mediated inflammation, disruption of the BBB, brain-reactive autoantibodies and immune-cell mediated inflammation (Schwartz *et al.*, 2019). In a recent study, mice

heterozygous for A20 were found to develop mild neuroinflammation and signs of NPSLE (Daems *et al.*, 2020). These mice showed increased expression levels of pro-inflammatory cytokine and chemokine genes like *IL1b* and *Tnf*, *Ccl2* and *Cxcl10* as well as increased expression of the reactive astrocyte marker *Gfap* in hippocampus tissue. Behavioral analyses furthermore revealed mild cognitive impairments in female A20 heterozygous mice at the age of 6 months which was increased upon LPS treatment (Daems *et al.*, 2020). Using a mouse model of SLE, molecular mechanisms for the recruitment of leukocytes have been studied thoroughly. It was shown that the interaction of α4-integrin and VCAM-1 plays a crucial role in mediating the migration of immune cells to the brain (James *et al.*, 2003). A20 also plays an important role in the pathogenesis of the recently described autoinflammatory disease A20 haploinsufficiency (HA20). HA20 is caused by heterozygous loss-of-function mutations in TNFAIP3, leading to insufficient deubiquitinating activity of A20 and subsequently increased NF-κB signaling (Zhou *et al.*, 2016). Symptoms are diverse even across patients with the same genetic variant and vary from recurrent oral, gastrointestinal and genital ulcers to polyarthritis, periodic fever, skin involvement, and even CNS vasculitis (Yu *et al.*, 2020b; Aeschlimann *et al.*, 2018). The development of CNS vasculitis is characterized by expression of adhesion molecules and subsequent infiltration of leukocytes into the arterial walls (Kelley, 2004). Interestingly, we also observed an increased expression of the adhesion molecules ICAM-1 and VCAM-1 in our $A20^{\Delta BEC}$ mice. Our data therefore indicates that the development of NPSLE and CNS vasculitis could be promoted by the absence of EC-specific A20 and that SNPs in the TNFAIP3 locus of ECs might be connected to the progression of these diseases.

Together, our data hints towards a potential functional involvement of BBB-endothelial-mediated astrogliosis through factors released from A20-deficient BBB ECs, which could lead to improper neuronal function. However, experimental data to support this idea, including electrophysiological analyses of neurons in $A20^{\Delta BEC}$ mice, as well as behavioral analyses focusing on cognitive functions and seizure development, are required to prove this theory.

6.2 A20-deficiency in BBB ECs does not drive EAE disease

Activated NF-κB signaling has been described in multiple cell types in the CNS of MS patients, including T cells, microglia, and astrocytes (Yue *et al.*, 2018). Several studies using different A20 knockout mice have suggested that A20 signaling attenuates the course of EAE by inhibiting NF-κB signaling. These studies further showed that A20 plays a critical role in mediating immune cell infiltration into the CNS during neuroinflammation (Wang *et al.*, 2013; Voet *et al.*, 2018; Mohebiany *et al.*, 2020). However, only few studies have investigated the effect of A20 signaling in BBB ECs and CNS endothelial A20 has not yet been studied *in vivo*. By using a transgenic mouse model, we aimed to selectively investigate the influence of A20 signaling in ECs in EAE developement.

The induction of active EAE leads to an activation of myelin-specific T cells in the periphery which then migrate across the BBB into the CNS (Bittner *et al.*, 2014). As we already observed an increased infiltration of immune cells into the CNS in steady state, we consequently expected a more severe course of EAE in our $A20^{\Delta BEC}$ mice. To our surprise, we could not see that deletion of A20 in CNS ECs alters the onset or severity of active EAE. However, this data does not provide definite evidence that A20 deficiency in ECs does not drive EAE. Nonspecific effects caused by complete Freud's adjuvant (CFA) and pertussis toxin (PTx) must be considered to possibly conceal any effects caused by the deletion of A20 in CNS ECs. EAE is generally induced by peripheral injection of MOG_{35-55} peptide emulsified in CFA which contains heat-inactivated *Mycobacterium tuberculosis*. This is necessary in order to achieve a strong adjuvant-driven immune activation since immunization with MOG_{35-55} peptide alone is not sufficient to induce the disease (Bittner *et al.*, 2014). However, studies have indicated that immunization with CFA alone leads to an increased BBB permeability to serum proteins, indicating that CFA provokes BBB breakdown (Namer *et al.*, 1994; Rabchevsky *et al.*, 1999). A similar effect has been described for PTx which is applied on day 0 of immunization and day 2 post immunization to enable proper EAE induction (Bittner *et al.*, 2014). Studies suggested that PTx promotes EAE by opening up the BBB, making it easier for T cells to migrate to the CNS (Linthicum, 1982; Munoz *et al.*, 1984). Further studies indicate that PTx pushes the expansion of neuroantigen-specific Th1 and Th2 cells (Hofstetter *et al.*, 2002). Interestingly, Hauptmann *et al*. recently showed that ICAM-1 expression by BBB ECs is already induced through the

immunization with CFA without MOG_{35-55} peptide (Hauptmann *et al.*, 2020). We presume that this effect covers up any impact of missing A20 of BECs on the course of active EAE. Of note, the disease course in the active EAE model used in this study was comparably high, with the majority of mice reaching a clinical scale of 4 or above, which is close to the upper limit of disease severity. Thus, it is also possible that the strength of the disease prohibits the detection of differences between the groups. This should be addressed in future experiments by dialing down the concentrations of MOG/PTx to achieve a milder disease course.

To circumvent the CFA-induced peripheral immune activation, passive EAE provides an alternative approach to induce EAE by skipping the peripheral priming phase. In passive EAE, pre-activated myelin-reactive T cells are transferred into naïve host mice. Therefore, no additional immunization of the host mice is needed and this model bypasses any nonspecific effects caused by CFA (McPherson *et al.*, 2014). Additional studies using a passive transfer model of EAE will be necessary to further investigate the role of A20 in EAE pathology, especially in regard to facilitated entry of encephalitogenic immune cells into the CNS due to the increased adhesion molecule expression already in the steady state.

7 Summary

The ubiquitin-modifying protein A20 is crucially important for the regulation of central nervous system (CNS) inflammation as it negatively regulates canonical NF-κB signaling, thus functioning as an anti-inflammatory mediator. Single nucleotide polymorphisms (SNP) in the A20/TNFAIP3 gene have been shown to increase the risk of developing different autoimmune diseases, such as multiple sclerosis (MS). In this study, we investigate the role of A20 in blood brain barrier (BBB) endothelial cells (EC) on the maintenance of BBB integrity and the infiltration of immune cells in steady state, as well as the consequences of EC-specific deletion of A20 on neuroinflammatory processes in brain homeostasis and its influence on the course of experimental autoimmune encephalitis (EAE), a mouse model of MS. We could show that BBB integrity was not altered by the lack of A20 in ECs of the CNS and that there was no difference in the expression of junctional proteins. We could not see a discrepancy in active EAE disease outcome in A20-deficient mice compared to control mice, either. However, we were able to observe dramatically increased expression of the adhesion molecules ICAM-1 and VCAM-1 in the steady state, indicating a severe activation of the endothelium of the BBB. We could further show that A20-deficiency drives immune cell infiltration into the CNS in the steady state. Our data further provides evidence that A20-deficency of CNS ECs promotes the progression of astrogliosis and therefore leads to the development of neuroinflammation.

In summary, we suggest that A20-deficiency in BBB ECs in steady state leads to increased NF-κB signaling, resulting in pro-inflammatory gene expression, including ICAM-1 and VCAM-1. This likely promotes a tight adhesion of immune cells to the BBB, subsequently causing increased T cell transmigration into the CNS even in the absence of pathology. Moreover, A20-deficient BBB ECs can drive spontaneous neuroinflammation with alterations in astrocyte reactivity. Together, this results in a neuroinflammatory phenotype with impaired CNS homeostasis, which could potentially drive the development of neurological diseases.

8 References

Abbott, N.J., Patabendige, A.A.K., Dolman, D.E.M., Yusof, S.R. and Begley, D.J. (2010), “Structure and function of the blood-brain barrier”, *Neurobiology of disease*, Vol. 37 No. 1, pp. 13–25.

Abbott, N.J., Rönnbäck, L. and Hansson, E. (2006), “Astrocyte-endothelial interactions at the blood-brain barrier”, *Nature reviews. Neuroscience*, Vol. 7 No. 1, pp. 41–53.

Abdelrahman, H.S., Selim, H.S., Hashish, M.H. and Sultan, L.I. (2014), “Epstein-Barr virus in multiple sclerosis”, *The Journal of the Egyptian Public Health Association*, Vol. 89 No. 2, pp. 90–95.

Aeschlimann, F.A., Batu, E.D., Canna, S.W., Go, E., Gül, A., Hoffmann, P., Leavis, H.L., Ozen, S., Schwartz, D.M., Stone, D.L., van Royen-Kerkof, A., Kastner, D.L., Aksentijevich, I. and Laxer, R.M. (2018), “A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease”, *Annals of the rheumatic diseases*, Vol. 77 No. 5, pp. 728–735.

Agrawal, S., Anderson, P., Durbeej, M., van Rooijen, N., Ivars, F., Opdenakker, G. and Sorokin, L.M. (2006), “Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis”, *The Journal of experimental medicine*, Vol. 203 No. 4, pp. 1007–1019.

Alvarez, J.I., Cayrol, R. and Prat, A. (2011a), “Disruption of central nervous system barriers in multiple sclerosis”, *Biochimica et biophysica acta*, Vol. 1812 No. 2, pp. 252–264.

Alvarez, J.I., Dodelet-Devillers, A., Kebir, H., Ifergan, I., Fabre, P.J., Terouz, S., Sabbagh, M., Wosik, K., Bourbonnière, L., Bernard, M., van Horssen, J., Vries, H.E. de, Charron, F. and Prat, A. (2011b), “The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence”, *Science (New York, N.Y.)*, Vol. 334 No. 6063, pp. 1727–1731.

Armulik, A., Abramsson, A. and Betsholtz, C. (2005), “Endothelial/pericyte interactions”, *Circulation research*, Vol. 97 No. 6, pp. 512–523.

Attwell, D., Buchan, A.M., Charpak, S., Lauritzen, M., Macvicar, B.A. and Newman, E.A. (2010), “Glial and neuronal control of brain blood flow”, *Nature*, Vol. 468 No. 7321, pp. 232–243.

Ballabh, P., Braun, A. and Nedergaard, M. (2004), “The blood-brain barrier: an overview: structure, regulation, and clinical implications”, *Neurobiology of disease*, Vol. 16 No. 1, pp. 1–13.

Banks, W.A., Kovac, A. and Morofuji, Y. (2018), “Neurovascular unit crosstalk: Pericytes and astrocytes modify cytokine secretion patterns of brain endothelial cells”, *Journal of cerebral blood flow and metabolism official journal of the International Society of Cerebral Blood Flow and Metabolism*, Vol. 38 No. 6, pp. 1104–1118.

Bauer, H. and Traweger, A. (2016), “Tight Junctions of the Blood-Brain Barrier - A Molecular Gatekeeper”, *CNS & Neurological Disorders - Drug Targets*, Vol. 15 No. 9, pp. 1016–1029.

Ben-Nun, A., Wekerle, H. and Cohen, I.R. (1981), “Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein”, *Nature*, Vol. 292 No. 5818, pp. 60–61.

Bettelli, E., Pagany, M., Weiner, H.L., Linington, C., Sobel, R.A. and Kuchroo, V.K. (2003), “Myelin Oligodendrocyte Glycoprotein–specific T Cell Receptor Transgenic Mice Develop Spontaneous Autoimmune Optic Neuritis”, *The Journal of experimental medicine*, Vol. 197 No. 9, pp. 1073–1081.

Bigaut, K., Seze, J. de and Collongues, N. (2019), “Ocrelizumab for the treatment of multiple sclerosis”, *Expert review of neurotherapeutics*, Vol. 19 No. 2, pp. 97–108.

Bittner, S., Afzali, A.M., Wiendl, H. and Meuth, S.G. (2014), “Myelin Oligodendrocyte Glycoprotein (MOG35-55) Induced Experimental Autoimmune Encephalomyelitis (EAE) in C57BL/6 Mice”, *Journal of Visualized Experiments JoVE*, No. 86.

Bjornevik, K., Cortese, M., Healy, B.C., Kuhle, J., Mina, M.J., Leng, Y., Elledge, S.J., Niebuhr, D.W., Scher, A.I., Munger, K.L. and Ascherio, A. (2022), “Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis”, *Science (New York, N.Y.)*, Vol. 375 No. 6578, pp. 296–301.

Blanco-Suárez, E., Caldwell, A.L.M. and Allen, N.J. (2017), “Role of astrocyte-synapse interactions in CNS disorders”, *The Journal of physiology*, Vol. 595 No. 6, pp. 1903–1916.

Blonska, M., Shambharkar, P.B., Kobayashi, M., Zhang, D., Sakurai, H., Su, B. and Lin, X. (2005), “TAK1 is recruited to the tumor necrosis factor-alpha (TNF-alpha) receptor 1 complex in a receptor-interacting protein (RIP)-dependent manner and cooperates with MEKK3 leading to NF-kappaB activation”, *The Journal of biological chemistry*, Vol. 280 No. 52, pp. 43056–43063.

Bogie, J.F.J., Stinissen, P. and Hendriks, J.J.A. (2014), “Macrophage subsets and microglia in multiple sclerosis”, *Acta neuropathologica*, Vol. 128 No. 2, pp. 191–213.

Boone, D.L., Turer, E.E., Lee, E.G., Ahmad, R.-C., Wheeler, M.T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O., McNally, E., Pickart, C. and Ma, A. (2004), “The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses”, *Nature immunology*, Vol. 5 No. 10, pp. 1052–1060.

Boziki, M. and Grigoriadis, N. (2018), “An Update on the Role of Matrix Metalloproteinases in the Pathogenesis of Multiple Sclerosis”, *Medicinal chemistry (Shariqah (United Arab Emirates))*, Vol. 14 No. 2, pp. 155–169.

Brambilla, R. (2019), “The contribution of astrocytes to the neuroinflammatory response in multiple sclerosis and experimental autoimmune encephalomyelitis”, *Acta neuropathologica*, Vol. 137 No. 5, pp. 757–783.

Browning, V., Joseph, M. and Sedrak, M. (2012), “Multiple sclerosis: a comprehensive review for the physician assistant”, *JAAPA official journal of the American Academy of Physician Assistants*, Vol. 25 No. 8, pp. 24–29.

Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C.A., Madara, J.L., Hopkins, A.M. and Nusrat, A. (2003), “Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms”, *Journal of immunology (Baltimore, Md. 1950)*, Vol. 171 No. 11, pp. 6164–6172.

Cahill, K.E., Morshed, R.A. and Yamini, B. (2016), “Nuclear factor-κB in glioblastoma: insights into regulators and targeted therapy”, *Neuro-oncology*, Vol. 18 No. 3, pp. 329–339.

Cannella, B. and Raine, C.S. (1995), “The adhesion molecule and cytokine profile of multiple sclerosis lesions”, *Annals of neurology*, Vol. 37 No. 4, pp. 424–435.

Carman, C.V. (2009), “Mechanisms for transcellular diapedesis: probing and pathfinding by 'invadosome-like protrusions'”, *Journal of cell science*, Vol. 122 No. Pt 17, pp. 3025–3035.

Carman, C.V., Sage, P.T., Sciuto, T.E., La Fuente, M.A. de, Geha, R.S., Ochs, H.D., Dvorak, H.F., Dvorak, A.M. and Springer, T.A. (2007), “Transcellular diapedesis is initiated by invasive podosomes”, *Immunity*, Vol. 26 No. 6, pp. 784–797.

Catrysse, L., Vereecke, L., Beyaert, R. and van Loo, G. (2014), “A20 in inflammation and autoimmunity”, *Trends in immunology*, Vol. 35 No. 1, pp. 22–31.

Chen, H., Hu, L., Luo, Z., Zhang, J., Zhang, C., Qiu, B., Dong, L., Tan, Y., Ding, J., Tang, S., Shen, F., Li, Z. and Wang, H. (2015), “A20 suppresses hepatocellular carcinoma proliferation and metastasis through inhibition of Twist1 expression”, *Molecular cancer*, Vol. 14, p. 186.

Chigaev, A., Zwartz, G., Graves, S.W., Dwyer, D.C., Tsuji, H., Foutz, T.D., Edwards, B.S., Prossnitz, E.R., Larson, R.S. and Sklar, L.A. (2003), “Alpha4beta1 integrin affinity changes govern cell adhesion”, *The Journal of biological chemistry*, Vol. 278 No. 40, pp. 38174–38182.

Chow, B.W. and Gu, C. (2015), “The molecular constituents of the blood-brain barrier”, *Trends in neurosciences*, Vol. 38 No. 10, pp. 598–608.

Chu, Y., Vahl, J.C., Kumar, D., Heger, K., Bertossi, A., Wójtowicz, E., Soberon, V., Schenten, D., Mack, B., Reutelshöfer, M., Beyaert, R., Amann, K., van Loo, G. and Schmidt-Supprian, M. (2011), “B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice”, *Blood*, Vol. 117 No. 7, pp. 2227–2236.

Claes, N., Fraussen, J., Stinissen, P., Hupperts, R. and Somers, V. (2015), “B Cells Are Multifunctional Players in Multiple Sclerosis Pathogenesis: Insights from Therapeutic Interventions”, *Frontiers in immunology*, Vol. 6, p. 642.

Compston, A. and Coles, A. (2008), “Multiple sclerosis”, *Lancet (London, England)*, Vol. 372 No. 9648, pp. 1502–1517.

Constantinescu, C.S., Farooqi, N., O'Brien, K. and Gran, B. (2011), “Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS)”, *British journal of pharmacology*, Vol. 164 No. 4, pp. 1079–1106.

Daems, C., Sékulic, M., Vulsteke, V., van Loo, G., D’Hooge, R., Callaerts-Végh, Z. and Callaerts, P. (2020), “A20/TNFAIP3 heterozygosity predisposes to behavioral symptoms in a mouse model for neuropsychiatric lupus”, *Brain, Behavior, & Immunity - Health*, Vol. 2, p. 100018.

Daneman, R. (2012), “The blood-brain barrier in health and disease”, *Annals of neurology*, Vol. 72 No. 5, pp. 648–672.

Daneman, R. and Prat, A. (2015), “The blood-brain barrier”, *Cold Spring Harbor perspectives in biology*, Vol. 7 No. 1, a020412.

Danikowski, K.M., Jayaraman, S. and Prabhakar, B.S. (2017), “Regulatory T cells in multiple sclerosis and myasthenia gravis”, *Journal of neuroinflammation*, Vol. 14 No. 1, p. 117.

Darabi, K., Karulin, A.Y., Boehm, B.O., Hofstetter, H.H., Fabry, Z., LaManna, J.C., Chavez, J.C., Tary-Lehmann, M. and Lehmann, P.V. (2004), “The third signal in T cell-mediated autoimmune disease?”, *Journal of immunology (Baltimore, Md. 1950)*, Vol. 173 No. 1, pp. 92–99.

Deisenhammer, F., Zetterberg, H., Fitzner, B. and Zettl, U.K. (2019), “The Cerebrospinal Fluid in Multiple Sclerosis”, *Frontiers in immunology*, Vol. 10, p. 726.

Deutsche Multiple Sklerose Gesellschaft Bundesverband e.V. (2023), “Was ist MS?”, available at: https://register.awmf.org/assets/guidelines/030-050l_S2e_Diagnose-Therapie-Multiplen-Sklerose-Neuromyelitis-Optica-Spektrum-MOG-IgG-assoziierte_Erkrankungen_2021-05_1-verlaengert_01.pdf (accessed 26 March 2023).

Dixit, V.M., Green, S., Sarma, V., Holzman, L.B., Wolf, F.W., O'Rourke, K., Ward, P.A., Prochownik, E.V. and Marks, R.M. (1990), “Tumor necrosis factor-alpha induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin”, *Journal of Biological Chemistry*, Vol. 265 No. 5, pp. 2973–2978.

Eliopoulos, A.G., Stack, M., Dawson, C.W., Kaye, K.M., Hodgkin, L., Sihota, S., Rowe, M. and Young, L.S. (1997), “Epstein-Barr virus-encoded LMP1 and CD40 mediate IL-6 production in epithelial cells via an NF-kappaB pathway involving TNF receptor-associated factors”, *Oncogene*, Vol. 14 No. 24, pp. 2899–2916.

Engelhardt, B. and Ransohoff, R.M. (2012), “Capture, crawl, cross: the T cell code to breach the blood-brain barriers”, *Trends in immunology*, Vol. 33 No. 12, pp. 579–589.

Engelhardt, B. and Sorokin, L. (2009), “The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction”, *Seminars in immunopathology*, Vol. 31 No. 4, pp. 497–511.

Evans, P.C., Ovaa, H., Hamon, M., Kilshaw, P.J., Hamm, S., Bauer, S., Ploegh, H.L. and Smith, T.S. (2004), “Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity”, *The Biochemical journal*, Vol. 378 No. Pt 3, pp. 727–734.

Feinstein, A., Freeman, J. and Lo, A.C. (2015), “Treatment of progressive multiple sclerosis: what works, what does not, and what is needed”, *The Lancet Neurology*, Vol. 14 No. 2, pp. 194–207.

Filippi, M.-D. (2016), “Mechanism of Diapedesis: Importance of the Transcellular Route”, *Advances in immunology*, Vol. 129, pp. 25–53.

Filippi, M.-D. (2019), “Neutrophil transendothelial migration: updates and new perspectives”, *Blood*, Vol. 133 No. 20, pp. 2149–2158.

Girbl, T., Lenn, T., Perez, L., Rolas, L., Barkaway, A., Thiriot, A., Del Fresno, C., Lynam, E., Hub, E., Thelen, M., Graham, G., Alon, R., Sancho, D., Andrian, U.H. von, Voisin, M.-B., Rot, A. and Nourshargh, S. (2018), “Distinct Compartmentalization of the Chemokines CXCL1 and CXCL2 and the Atypical Receptor ACKR1 Determine Discrete Stages of Neutrophil Diapedesis”, *Immunity*, Vol. 49 No. 6, 1062-1076.e6.

Gold, R., Linington, C. and Lassmann, H. (2006), “Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research”, *Brain a journal of neurology*, Vol. 129 No. Pt 8, pp. 1953–1971.

Goverman, J. (2009), “Autoimmune T cell responses in the central nervous system”, *Nature reviews. Immunology*, Vol. 9 No. 6, pp. 393–407.

Guedes, R.P., Csizmadia, E., Moll, H.P., Ma, A., Ferran, C. and da Silva, C.G. (2014), “A20 deficiency causes spontaneous neuroinflammation in mice”, *Journal of neuroinflammation*, Vol. 11, p. 122.

Hafezi-Moghadam, A., Thomas, K.L. and Wagner, D.D. (2007), “ApoE deficiency leads to a progressive age-dependent blood-brain barrier leakage”, *American journal of physiology. Cell physiology*, Vol. 292 No. 4, C1256-62.

Hafler, D.A., Compston, A., Sawcer, S., Lander, E.S., Daly, M.J., Jager, P.L. de, Bakker, P.I.W. de, Gabriel, S.B., Mirel, D.B., Ivinson, A.J., Pericak-Vance, M.A., Gregory, S.G., Rioux, J.D., McCauley, J.L., Haines, J.L., Barcellos, L.F., Cree, B., Oksenberg, J.R. and Hauser, S.L. (2007), “Risk alleles for multiple sclerosis identified by a genomewide study”, *The New England journal of medicine*, Vol. 357 No. 9, pp. 851–862.

Haj-Yasein, N.N., Vindedal, G.F., Eilert-Olsen, M., Gundersen, G.A., Skare, Ø., Laake, P., Klungland, A., Thorén, A.E., Burkhardt, J.M., Ottersen, O.P. and Nagelhus, E.A. (2011), “Glial-conditional deletion of aquaporin-4 (Aqp4) reduces blood-brain water uptake and confers barrier function on perivascular astrocyte endfeet”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 108 No. 43, pp. 17815–17820.

Han, D., Fang, W., Zhang, R., Wei, J., Kodithuwakku, N.D., Sha, L., Ma, W., Liu, L., Li, F. and Li, Y. (2016), “Clematichinenoside protects blood brain barrier against ischemic stroke superimposed on systemic inflammatory challenges through up-regulating A20”, *Brain, behavior, and immunity*, Vol. 51, pp. 56–69.

Hart, B.A. 't, Gran, B. and Weissert, R. (2011), “EAE: imperfect but useful models of multiple sclerosis”, *Trends in molecular medicine*, Vol. 17 No. 3, pp. 119–125.

Hart, F.M. and Bainbridge, J. (2016), “Current and emerging treatment of multiple sclerosis”, *The American journal of managed care*, Vol. 22 No. 6 Suppl, s159-70.

Hasel, P., Rose, I.V.L., Sadick, J.S., Kim, R.D. and Liddelow, S.A. (2021), “Neuroinflammatory astrocyte subtypes in the mouse brain”, *Nature neuroscience*, Vol. 24 No. 10, pp. 1475–1487.

Hassanzadeh, P. (2011), “Colorectal cancer and NF-κB signaling pathway”, *Gastroenterology and Hepatology From Bed to Bench*, Vol. 4 No. 3, pp. 127–132.

Hauptmann, J., Johann, L., Marini, F., Kitic, M., Colombo, E., Mufazalov, I.A., Krueger, M., Karram, K., Moos, S., Wanke, F., Kurschus, F.C., Klein, M., Cardoso, S., Strauß, J., Bolisetty, S., Lühder, F., Schwaninger, M., Binder, H., Bechman, I., Bopp, T., Agarwal, A., Soares, M.P., Regen, T. and Waisman, A. (2020), “Interleukin-1 promotes autoimmune neuroinflammation by suppressing endothelial heme oxygenase-1 at the blood-brain barrier”, *Acta neuropathologica*, Vol. 140 No. 4, pp. 549–567.

Hayden, M.S. and Ghosh, S. (2012), “NF-κB, the first quarter-century: remarkable progress and outstanding questions”, *Genes & development*, Vol. 26 No. 3, pp. 203–234.

Hemmer, B., Kerschensteiner, M. and Korn, T. (2015), “Role of the innate and adaptive immune responses in the course of multiple sclerosis”, *The Lancet Neurology*, Vol. 14 No. 4, pp. 406–419.

Hemmer B. et al. (2021), “Diagnose und Therapie der Multiplen Sklerose, Neuromyelitis-optica-SpektrumErkrankungen und MOG-IgG-assoziierten Erkrankungen, S2k-Leitlinie”, S2k-Leitlinie, available at: www.dgn.org/leitlinien (accessed 26 March 2023).

Henderson, A.P.D., Barnett, M.H., Parratt, J.D.E. and Prineas, J.W. (2009), “Multiple sclerosis: distribution of inflammatory cells in newly forming lesions”, *Annals of neurology*, Vol. 66 No. 6, pp. 739–753.

Heydarpour, P., Manouchehrinia, A., Beiki, O., Mousavi, S.E., Abdolalizadeh, A., -Lakeh, M.M. and Sahraian, M.A. (2018), “Smoking and worsening disability in multiple sclerosis: A meta-analysis”, *Acta neurologica Scandinavica*, Vol. 138 No. 1, pp. 62–69.

Heyninck, K. and Beyaert, R. (1999), “The cytokine-inducible zinc finger protein A20 inhibits IL-1-induced NF-κB activation at the level of TRAF6”, *FEBS letters*, Vol. 442 No. 2-3, pp. 147–150.

Hirata, Y., Takahashi, M., Morishita, T., Noguchi, T. and Matsuzawa, A. (2017), “Post-Translational Modifications of the TAK1-TAB Complex”, *International journal of molecular sciences*, Vol. 18 No. 1.

Hoffjan, S., Okur, A., Epplen, J.T., Wieczorek, S., Chan, A. and Akkad, D.A. (2015), “Association of TNFAIP3 and TNFRSF1A variation with multiple sclerosis in a German case-control cohort”, *International journal of immunogenetics*, Vol. 42 No. 2, pp. 106–110.

Hofstetter, H.H., Shive, C.L. and Forsthuber, T.G. (2002), “Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells”, *Journal of immunology (Baltimore, Md. 1950)*, Vol. 169 No. 1, pp. 117–125.

Höftberger, R., Aboul-Enein, F., Brueck, W., Lucchinetti, C., Rodriguez, M., Schmidbauer, M., Jellinger, K. and Lassmann, H. (2004), “Expression of Major Histocompatibility Complex class I Molecules on the Different Cell Types in Multiple Sclerosis Lesions”, *Brain Pathology*, Vol. 14 No. 1, pp. 43–50.

Holstiege, J., Steffen, A., Goffrier, B. and Bätzing, J. (2017), “Epidemiologie der Multiplen Sklerose – Eine populationsbasierte deutschlandweite Studie”.

Hövelmeyer, N., Reissig, S., Xuan, N.T., Adams-Quack, P., Lukas, D., Nikolaev, A., Schlüter, D. and Waisman, A. (2011), “A20 deficiency in B cells enhances B-cell proliferation and results in the development of autoantibodies”, *European journal of immunology*, Vol. 41 No. 3, pp. 595–601.

Hu, H., Brittain, G.C., Chang, J.-H., Puebla-Osorio, N., Jin, J., Zal, A., Xiao, Y., Cheng, X., Chang, M., Fu, Y.-X., Zal, T., Zhu, C. and Sun, S.-C. (2013), “Otud7b controls noncanonical NF-κB activation via deubiquitination of TRAF3”, *Nature*, Vol. 494 No. 7437, pp. 371–374.

Iwai, K. (2012), “Diverse ubiquitin signaling in NF-κB activation”, *Trends in cell biology*, Vol. 22 No. 7, pp. 355–364.

Jäättelä, M., Mouritzen, H., Elling, F. and Bastholm, L. (1996), “A20 zinc finger protein inhibits TNF and IL-1 signaling”, *Journal of immunology (Baltimore, Md. 1950)*, Vol. 156 No. 3, pp. 1166–1173.

Jager, P.L. de, Jia, X., Wang, J., de Bakker, Paul I W, Ottoboni, L., Aggarwal, N.T., Piccio, L., Raychaudhuri, S., Tran, D., Aubin, C., Briskin, R., Romano, S., International MS Genetics Consortium, Baranzini, S.E., McCauley, J.L., Pericak-Vance, M.A., Haines, J.L., Gibson, R.A., Naeglin, Y., Uitdehaag, B., Matthews,

P.M., Kappos, L., Polman, C., McArdle, W.L., Strachan, D.P., Evans, D., Cross, A.H., Daly, M.J., Compston, A., Sawcer, S.J., Weiner, H.L., Hauser, S.L., Hafler, D.A. and Oksenberg, J.R. (2009), “Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci”, *Nature Genetics*, Vol. 41, 776 EP -.

James, W.G., Bullard, D.C. and Hickey, M.J. (2003), “Critical role of the alpha 4 integrin/VCAM-1 pathway in cerebral leukocyte trafficking in lupus-prone MRL/fas(lpr) mice”, *Journal of immunology (Baltimore, Md. 1950)*, Vol. 170 No. 1, pp. 520–527.

Jarosz, M., Olbert, M., Wyszogrodzka, G., Młyniec, K. and Librowski, T. (2017), “Antioxidant and anti-inflammatory effects of zinc. Zinc-dependent NF-κB signaling”, *Inflammopharmacology*, Vol. 25 No. 1, pp. 11–24.

Jersild, C., Hansen, G., Svejgaard, A., Fog, T., Thomsen, M. and Dupont, B. (1973), “Histocompatibility determinants in Multiple Sclerosis, with special reference to clinical course”, *The Lancet*, Vol. 302 No. 7840, pp. 1221–1225.

Johann, L., Soldati, S., Müller, K., Lampe, J., Marini, F., Klein, M., Schramm, E., Ries, N., Schelmbauer, C., Palagi, I., Karram, K., Assmann, J.C., Khan, M.A., Wenzel, J., Schmidt, M.H., Körbelin, J., Schlüter, D., van Loo, G., Bopp, T., Engelhardt, B., Schwaninger, M. and Waisman, A. (2023), “A20 regulates lymphocyte adhesion in murine neuroinflammation by restricting endothelial ICOSL expression in the CNS”, *Journal of Clinical Investigation*.

Kajino, T., Ren, H., Iemura, S.-I., Natsume, T., Stefansson, B., Brautigan, D.L., Matsumoto, K. and Ninomiya-Tsuji, J. (2006), “Protein phosphatase 6 down-regulates TAK1 kinase activation in the IL-1 signaling pathway”, *The Journal of biological chemistry*, Vol. 281 No. 52, pp. 39891–39896.

Kamm, C.P., Uitdehaag, B.M. and Polman, C.H. (2014), “Multiple sclerosis: current knowledge and future outlook”, *European neurology*, Vol. 72 No. 3-4, pp. 132–141.

Kassner, A. and Merali, Z. (2015), “Assessment of Blood-Brain Barrier Disruption in Stroke”, *Stroke*, Vol. 46 No. 11, pp. 3310–3315.

Keaney, J. and Campbell, M. (2015), “The dynamic blood-brain barrier”, *The FEBS journal*, Vol. 282 No. 21, pp. 4067–4079.

Kelley, R.E. (2004), “CNS vasculitis”, *Frontiers in bioscience a journal and virtual library*, Vol. 9, pp. 946–955.

Khoy, K., Mariotte, D., Defer, G., Petit, G., Toutirais, O. and Le Mauff, B. (2020), “Natalizumab in Multiple Sclerosis Treatment: From Biological Effects to Immune Monitoring”, *Frontiers in immunology*, Vol. 11.

Koch, M.W., Metz, L.M., Agrawal, S.M. and Yong, V.W. (2013), “Environmental factors and their regulation of immunity in multiple sclerosis”, *Journal of the neurological sciences*, Vol. 324 No. 1-2, pp. 10–16.

Koch-Henriksen, N. and Sørensen, P.S. (2010), “The changing demographic pattern of multiple sclerosis epidemiology”, *The Lancet Neurology*, Vol. 9 No. 5, pp. 520–532.

Kolodziej, L.E., Lodolce, J.P., Chang, J.E., Schneider, J.R., Grimm, W.A., Bartulis, S.J., Zhu, X., Messer, J.S., Murphy, S.F., Reddy, N., Turner, J.R. and Boone, D.L. (2011), “TNFAIP3 maintains intestinal barrier function and supports epithelial cell tight junctions”, *PloS one*, Vol. 6 No. 10, e26352.

Komander, D. and Barford, D. (2008), “Structure of the A20 OTU domain and mechanistic insights into deubiquitination”, *The Biochemical journal*, Vol. 409 No. 1, pp. 77–85.

Kozler, P. and Pokorný, J. (2003), “Altered blood-brain barrier permeability and its effect on the distribution of Evans blue and sodium fluorescein in the rat brain applied by intracarotid injection”, *Physiological research*, Vol. 52 No. 5, pp. 607–614.

Krikos, A., Laherty, C.D. and Dixit, V.M. (1992), “Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements”, *Journal of Biological Chemistry*, Vol. 267 No. 25, pp. 17971–17976.

Krishnamoorthy, G., Lassmann, H., Wekerle, H. and Holz, A. (2006), “Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation”, *Journal of Clinical Investigation*, Vol. 116 No. 9, pp. 2385–2392.

Kurschus, F.C. (2015), “T cell mediated pathogenesis in EAE: Molecular mechanisms”, *Biomedical Journal*, Vol. 38 No. 3, pp. 183–193.

Kurtzke, J.F. (2000), “Epidemiology of multiple sclerosis. Does this really point toward an etiology? Lectio Doctoralis”, *Neurological sciences official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology*, Vol. 21 No. 6, pp. 383–403.

Laman, J.D. and Weller, R.O. (2013), “Drainage of cells and soluble antigen from the CNS to regional lymph nodes”, *Journal of neuroimmune pharmacology the official journal of the Society on NeuroImmune Pharmacology*, Vol. 8 No. 4, pp. 840–856.

Lawrence, T. (2009), “The nuclear factor NF-kappaB pathway in inflammation”, *Cold Spring Harbor perspectives in biology*, Vol. 1 No. 6, a001651.

Lécuyer, M.-A., Saint-Laurent, O., Bourbonnière, L., Larouche, S., Larochelle, C., Michel, L., Charabati, M., Abadier, M., Zandee, S., Haghayegh Jahromi, N., Gowing, E., Pittet, C., Lyck, R., Engelhardt, B. and Prat, A. (2017), “Dual role of ALCAM in neuroinflammation and blood-brain barrier homeostasis”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 114 No. 4, E524-E533.

Lee, E.G., Boone, D.L., Chai, S., Libby, S.L., Chien, M., Lodolce, J.P. and Ma, A. (2000), “Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice”, *Science (New York, N.Y.)*, Vol. 289 No. 5488, pp. 2350–2354.

Legroux, L. and Arbour, N. (2015), “Multiple Sclerosis and T Lymphocytes: An Entangled Story”, *Journal of neuroimmune pharmacology the official journal of the Society on NeuroImmune Pharmacology*, Vol. 10 No. 4, pp. 528–546.

Li, H.-Y., Liu, H., Wang, C.-H., Zhang, J.-Y., Man, J.-H., Gao, Y.-F., Zhang, P.-J., Li, W.-H., Zhao, J., Pan, X., Zhou, T., Gong, W.-L., Li, A.-L. and Zhang, X.-M. (2008), “Deactivation of the kinase IKK by CUEDC2 through recruitment of the phosphatase PP1”, *Nature immunology*, Vol. 9 No. 5, pp. 533–541.

Li, T. and Zhang, S. (2016), “Microgliosis in the Injured Brain: Infiltrating Cells and Reactive Microglia Both Play a Role”, *The Neuroscientist a review journal bringing neurobiology, neurology and psychiatry*, Vol. 22 No. 2, pp. 165–170.

Liddelow, S.A., Guttenplan, K.A., Clarke, L.E., Bennett, F.C., Bohlen, C.J., Schirmer, L., Bennett, M.L., Münch, A.E., Chung, W.-S., Peterson, T.C., Wilton, D.K., Frouin, A., Napier, B.A., Panicker, N., Kumar, M., Buckwalter, M.S., Rowitch, D.H., Dawson, V.L., Dawson, T.M., Stevens, B. and Barres, B.A. (2017), “Neurotoxic reactive astrocytes are induced by activated microglia”, *Nature*, Vol. 541 No. 7638, pp. 481–487.

Lin, J., Wang, D., Lan, L. and Fan, Y. (2017), “Multiple Factors Involved in the Pathogenesis of White Matter Lesions”, *BioMed research international*, Vol. 2017, p. 9372050.

Linthicum, D.S. (1982), “Development of acute autoimmune encephalomyelitis in mice: Factors regulating the effector phase of the disease”, *Immunobiology*, Vol. 162 No. 3, pp. 211–220.

Lippens, S., Lefebvre, S., Gilbert, B., Sze, M., Devos, M., Verhelst, K., Vereecke, L., Mc Guire, C., Guérin, C., Vandenabeele, P., Pasparakis, M., Mikkola, M.L., Beyaert, R., Declercq, W. and van Loo, G. (2011), “Keratinocyte-specific ablation of the NF-κB regulatory protein A20 (TNFAIP3) reveals a role in the control of epidermal homeostasis”, *Cell death and differentiation*, Vol. 18 No. 12, pp. 1845–1853.

Liu, X.-X., Yang, L., Shao, L.-X., He, Y., Wu, G., Bao, Y.-H., Lu, N.-N., Gong, D.-M., Lu, Y.-P., Cui, T.-T., Sun, N.-H., Chen, D.-Y., Shi, W.-X., Fukunaga, K., Chen, H.-S., Chen, Z., Han, F. and Lu, Y.-M. (2020), “Endothelial Cdk5 deficit leads to the development of spontaneous epilepsy through CXCL1/CXCR2-mediated reactive astrogliosis”, *The Journal of experimental medicine*, Vol. 217 No. 1.

Lombardi, A., Cantini, G., Piscitelli, E., Gelmini, S., Francalanci, M., Mello, T., Ceni, E., Varano, G., Forti, G., Rotondi, M., Galli, A., Serio, M. and Luconi, M. (2008), “A new mechanism involving ERK contributes to rosiglitazone inhibition of tumor necrosis factor-alpha and interferon-gamma inflammatory effects in human endothelial cells”, *Arteriosclerosis, thrombosis, and vascular biology*, Vol. 28 No. 4, pp. 718–724.

Lublin, F.D. and Reingold, S.C. (1996), “MS categories 1996 // Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis”, *Neurology*, Vol. 46 No. 4, pp. 907–911.

Lublin, F.D., Reingold, S.C., Cohen, J.A., Cutter, G.R., Sørensen, P.S., Thompson, A.J., Wolinsky, J.S., Balcer, L.J., Banwell, B., Barkhof, F., Bebo, B., Calabresi, P.A., Clanet, M., Comi, G., Fox, R.J., Freedman, M.S., Goodman, A.D., Inglese, M., Kappos, L., Kieseier, B.C., Lincoln, J.A., Lubetzki, C., Miller, A.E., Montalban, X., O'Connor, P.W., Petkau, J., Pozzilli, C., Rudick, R.A., Sormani, M.P., Stüve, O., Waubant, E. and Polman, C.H. (2014), “Defining the clinical course of multiple sclerosis: the 2013 revisions”, *Neurology*, Vol. 83 No. 3, pp. 278–286.

Luo, C., Jian, C., Liao, Y., Huang, Q., Wu, Y., Liu, X., Zou, D. and Wu, Y. (2017), “The role of microglia in multiple sclerosis”, *Neuropsychiatric disease and treatment*, Vol. 13, pp. 1661–1667.

Mastorodemos, V., Ioannou, M. and Verginis, P. (2015), “Cell-based modulation of autoimmune responses in multiple sclerosis and experimental autoimmmune encephalomyelitis: therapeutic implications”, *Neuroimmunomodulation*, Vol. 22 No. 3, pp. 181–195.

Mathis, B.J., Lai, Y., Qu, C., Janicki, J.S. and Cui, T. (2015), “CYLD-mediated signaling and diseases”, *Current drug targets*, Vol. 16 No. 4, pp. 284–294.

McCarthy, D.P., Richards, M.H. and Miller, S.D. (2012), “Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease”, *Methods in molecular biology (Clifton, N.J.)*, Vol. 900, pp. 381–401.

McDonald, W.I., Compston, A., Edan, G., Goodkin, D., Hartung, H.-P., Lublin, F.D., McFarland, H.F., Paty, D.W., Polman, C.H., Reingold, S.C., Sandberg-Wollheim, M., Sibley, W., Thompson, A., van den Noort, S., Weinshenker, B.Y. and Wolinsky, J.S. (2001), “Recommended diagnostic criteria for multiple sclerosis: Guidelines from the international panel on the diagnosis of multiple sclerosis”, *Annals of neurology*, Vol. 50 No. 1, pp. 121–127.

McPherson, R.C., Cambrook, H.E., O'Connor, R.A. and Anderton, S.M. (2014), “Induction of passive EAE using myelin-reactive CD4+ T cells”, *Methods in molecular biology (Clifton, N.J.)*, Vol. 1193, pp. 187–198.

Miller, D., Barkhof, F., Montalban, X., Thompson, A. and Filippi, M. (2005), “Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis”, *The Lancet Neurology*, Vol. 4 No. 5, pp. 281–288.

Minagar, A. and Alexander, J.S. (2003), “Blood-brain barrier disruption in multiple sclerosis”, *Multiple sclerosis (Houndmills, Basingstoke, England)*, Vol. 9 No. 6, pp. 540–549.

Mitchell, S., Vargas, J. and Hoffmann, A. (2016), “Signaling via the NFκB system”, *Wiley interdisciplinary reviews. Systems biology and medicine*, Vol. 8 No. 3, pp. 227–241.

Mohebiany, A.N., Ramphal, N.S., Karram, K., Di Liberto, G., Novkovic, T., Klein, M., Marini, F., Kreutzfeldt, M., Härtner, F., Lacher, S.M., Bopp, T., Mittmann, T.,

Merkler, D. and Waisman, A. (2020), “Microglial A20 Protects the Brain from CD8 T-Cell-Mediated Immunopathology”, *Cell reports*, Vol. 30 No. 5, 1585-1597.e6.

Mooney, E.C. and Sahingur, S.E. (2021), “The Ubiquitin System and A20: Implications in Health and Disease”, *Journal of dental research*, Vol. 100 No. 1, pp. 10–20.

Munoz, J.J., Bernard, C.C.A. and Mackay, I.R. (1984), “Elicitation of experimental allergic encephalomyelitis (EAE) in mice with the aid of pertussigen”, *Cellular immunology*, Vol. 83 No. 1, pp. 92–100.

Musone, S.L., Taylor, K.E., Lu, T.T., Nititham, J., Ferreira, R.C., Ortmann, W., Shifrin, N., Petri, M.A., Ilyas Kamboh, M., Manzi, S., Seldin, M.F., Gregersen, P.K., Behrens, T.W., Ma, A., Kwok, P.-Y. and Criswell, L.A. (2008), “Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus”, *Nature Genetics*, Vol. 40, 1062 EP -.

Nag, S., Manias, J.L. and Stewart, D.J. (2009), “Pathology and new players in the pathogenesis of brain edema”, *Acta neuropathologica*, Vol. 118 No. 2, pp. 197–217.

Nair, R.P., Duffin, K.C., Helms, C., Ding, J., Stuart, P.E., Goldgar, D., Gudjonsson, J.E., Li, Y., Tejasvi, T., Feng, B.-J., Ruether, A., Schreiber, S., Weichenthal, M., Gladman, D., Rahman, P., Schrodi, S.J., Prahalad, S., Guthery, S.L., Fischer, J., Liao, W., Kwok, P.-Y., Menter, A., Lathrop, G.M., Wise, C.A., Begovich, A.B., Voorhees, J.J., Elder, J.T., Krueger, G.G., Bowcock, A.M., Abecasis, G.R., for the Collaborative Association Study of Psoriasis, Paschall, J., Malloy, M.J., Pullinger, C.R., Kane, J.P., Gardner, J., Perlmutter, A., Miner, A., Hiremagalore, R., Ike, R.W., Lim, H.W., Christophers, E., Henseler, T., Franke, A. and Wise, C. (2009), “Genome-wide scan reveals association of psoriasis with IL-23 and NF-κB pathways”, *Nature Genetics*, Vol. 41, 199 EP -.

Namer, I.J., Steibel, J., Poulet, P., Mauss, Y., Mohr, M. and Chambron, J. (1994), “The role of Mycobacterium tuberculosis in experimental allergic encephalomyelitis”, *European neurology*, Vol. 34 No. 4, pp. 224–227.

National Multiple Sclerosis Society, “Clinically Isolated Syndrome (CIS)”, available at: https://www.nationalmssociety.org/What-is-MS/Types-of-MS/Clinically-Isolated-Syndrome-(CIS) (accessed 18 June 2019).

Nicholas, R. and Rashid, W. (2013), “Multiple sclerosis”, *American family physician*, Vol. 87 No. 10, pp. 712–714.

Nishitsuji, K., Hosono, T., Nakamura, T., Bu, G. and Michikawa, M. (2011), “Apolipoprotein E regulates the integrity of tight junctions in an isoform-dependent manner in an in vitro blood-brain barrier model”, *The Journal of biological chemistry*, Vol. 286 No. 20, pp. 17536–17542.

Novo, A.M. and Batista, S. (2017), “Multiple Sclerosis: Implications of Obesity in Neuroinflammation”, *Advances in neurobiology*, Vol. 19, pp. 191–210.

Obermeier, B., Daneman, R. and Ransohoff, R.M. (2013), “Development, maintenance and disruption of the blood-brain barrier”, *Nature medicine*, Vol. 19 No. 12, pp. 1584–1596.

Opipari, A.W., Hu, H.M., Yabkowitz, R. and Dixit, V.M. (1992), “The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity”, *Journal of Biological Chemistry*, Vol. 267 No. 18, pp. 12424–12427.

Ortiz, G.G., Pacheco-Moisés, F.P., Macías-Islas, M.Á., Flores-Alvarado, L.J., Mireles-Ramírez, M.A., González-Renovato, E.D., Hernández-Navarro, V.E., Sánchez-López, A.L. and Alatorre-Jiménez, M.A. (2014), “Role of the blood-brain barrier in multiple sclerosis”, *Archives of medical research*, Vol. 45 No. 8, pp. 687–697.

Parkin, J. and Cohen, B. (2001), “An overview of the immune system”, *The Lancet*, Vol. 357 No. 9270, pp. 1777–1789.

Paula A Sousa, A. de, Malmegrim, K.C.R., Panepucci, R.A., Brum, D.S., Barreira, A.A., Carlos Dos Santos, A., Araújo, A.G., Covas, D.T., Oliveira, M.C., Moraes, D.A., Pieroni, F., Barros, G.M., Simões, B.P., Nicholas, R., Burt, R.K., Voltarelli, J.C. and Muraro, P.A. (2015), “Autologous haematopoietic stem cell transplantation reduces

abnormalities in the expression of immune genes in multiple sclerosis", *Clinical science (London, England 1979)*, Vol. 128 No. 2, pp. 111–120.

Phillipson, M., Heit, B., Colarusso, P., Liu, L., Ballantyne, C.M. and Kubes, P. (2006), "Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade", *The Journal of experimental medicine*, Vol. 203 No. 12, pp. 2569–2575.

Plenge, R.M., Cotsapas, C., Davies, L., Price, A.L., Bakker, P.I.W. de, Maller, J., Pe'er, I., Burtt, N.P., Blumenstiel, B., DeFelice, M., Parkin, M., Barry, R., Winslow, W., Healy, C., Graham, R.R., Neale, B.M., Izmailova, E., Roubenoff, R., Parker, A.N., Glass, R., Karlson, E.W., Maher, N., Hafler, D.A., Lee, D.M., Seldin, M.F., Remmers, E.F., Lee, A.T., Padyukov, L., Alfredsson, L., Coblyn, J., Weinblatt, M.E., Gabriel, S.B., Purcell, S., Klareskog, L., Gregersen, P.K., Shadick, N.A., Daly, M.J. and Altshuler, D. (2007), "Two independent alleles at 6q23 associated with risk of rheumatoid arthritis", *Nature Genetics*, Vol. 39 No. 12, pp. 1477–1482.

Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L., Lublin, F.D., Montalban, X., O'Connor, P., Sandberg-Wollheim, M., Thompson, A.J., Waubant, E., Weinshenker, B. and Wolinsky, J.S. (2011), "Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria", *Annals of neurology*, Vol. 69 No. 2, pp. 292–302.

Polman, C.H., Reingold, S.C., Edan, G., Filippi, M., Hartung, H.-P., Kappos, L., Lublin, F.D., Metz, L.M., McFarland, H.F., O'Connor, P.W., Sandberg-Wollheim, M., Thompson, A.J., Weinshenker, B.G. and Wolinsky, J.S. (2005), "Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria"", *Annals of neurology*, Vol. 58 No. 6, pp. 840–846.

Poser, C.M., Paty, D.W., Scheinberg, L., McDonald, W.I., Davis, F.A., Ebers, G.C., Johnson, K.P., Sibley, W.A., Silberberg, D.H. and Tourtellotte, W.W. (1983), "New diagnostic criteria for multiple sclerosis: guidelines for research protocols", *Annals of neurology*, Vol. 13 No. 3, pp. 227–231.

Procaccini, C., Rosa, V. de, Pucino, V., Formisano, L. and Matarese, G. (2015), “Animal models of Multiple Sclerosis”, *European journal of pharmacology*, Vol. 759, pp. 182–191.

Rabchevsky, A.G., Degos, J.-D. and Dreyfus, P.A. (1999), “Peripheral injections of Freund's adjuvant in mice provoke leakage of serum proteins through the blood–brain barrier without inducing reactive gliosis”, *Brain Research*, Vol. 832 No. 1-2, pp. 84–96.

Racke, M.K. (2009), “Immunopathogenesis of multiple sclerosis”, *Annals of Indian Academy of Neurology*, Vol. 12 No. 4, pp. 215–220.

Rahim, F., Arjmand, B., Tirdad, R. and Saki Malehi, A. (2018), “Stem cell therapy for multiple sclerosis”, *Cochrane Database of Systematic Reviews*, Vol. 3 No. 9, e3145.

Renner, F. and Schmitz, M.L. (2009), “Autoregulatory feedback loops terminating the NF-kappaB response”, *Trends in biochemical sciences*, Vol. 34 No. 3, pp. 128–135.

Rice, C.M., Cottrell, D., Wilkins, A. and Scolding, N.J. (2013), “Primary progressive multiple sclerosis: progress and challenges”, *Journal of neurology, neurosurgery, and psychiatry*, Vol. 84 No. 10, pp. 1100–1106.

Ridder, D.A., Lang, M.-F., Salinin, S., Röderer, J.-P., Struss, M., Maser-Gluth, C. and Schwaninger, M. (2011), “TAK1 in brain endothelial cells mediates fever and lethargy”, *The Journal of experimental medicine*, Vol. 208 No. 13, pp. 2615–2623.

Ridder, D.A., Wenzel, J., Müller, K., Töllner, K., Tong, X.-K., Assmann, J.C., Stroobants, S., Weber, T., Niturad, C., Fischer, L., Lembrich, B., Wolburg, H., Grand'Maison, M., Papadopoulos, P., Korpos, E., Truchetet, F., Rades, D., Sorokin, L.M., Schmidt-Supprian, M., Bedell, B.J., Pasparakis, M., Balschun, D., D'Hooge, R., Löscher, W., Hamel, E. and Schwaninger, M. (2015), “Brain endothelial TAK1 and NEMO safeguard the neurovascular unit”, *The Journal of experimental medicine*, Vol. 212 No. 10, pp. 1529–1549.

Robinson, A.P., Harp, C.T., Noronha, A. and Miller, S.D. (2014), “The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment”, *Handbook of clinical neurology*, Vol. 122, pp. 173–189.

Salou, M., Nicol, B., Garcia, A. and Laplaud, D.-A. (2015), “Involvement of CD8(+) T Cells in Multiple Sclerosis”, *Frontiers in immunology*, Vol. 6, p. 604.

Saunders, N.R., Dreifuss, J.-J., Dziegielewska, K.M., Johansson, P.A., Habgood, M.D., Møllgård, K. and Bauer, H.-C. (2014), “The rights and wrongs of blood-brain barrier permeability studies: a walk through 100 years of history”, *Frontiers in neuroscience*, Vol. 8, p. 404.

Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C.C.A., Patsopoulos, N.A., Moutsianas, L., Dilthey, A., Su, Z., Freeman, C., Hunt, S.E., Edkins, S., Gray, E., Booth, D.R., Potter, S.C., Goris, A., Band, G., Oturai, A.B., Strange, A., Saarela, J., Bellenguez, C., Fontaine, B., Gillman, M., Hemmer, B., Gwilliam, R., Zipp, F., Jayakumar, A., Martin, R., Leslie, S., Hawkins, S., Giannoulatou, E., D'alfonso, S., Blackburn, H., Martinelli Boneschi, F., Liddle, J., Harbo, H.F., Perez, M.L., Spurkland, A., Waller, M.J., Mycko, M.P., Ricketts, M., Comabella, M., Hammond, N., Kockum, I., McCann, O.T., Ban, M., Whittaker, P., Kemppinen, A., Weston, P., Hawkins, C., Widaa, S., Zajicek, J., Dronov, S., Robertson, N., Bumpstead, S.J., Barcellos, L.F., Ravindrarajah, R., Abraham, R., Alfredsson, L., Ardlie, K., Aubin, C., Baker, A., Baker, K., Baranzini, S.E., Bergamaschi, L., Bergamaschi, R., Bernstein, A., Berthele, A., Boggild, M., Bradfield, J.P., Brassat, D., Broadley, S.A., Buck, D., Butzkueven, H., Capra, R., Carroll, W.M., Cavalla, P., Celius, E.G., Cepok, S., Chiavacci, R., Clerget-Darpoux, F., Clysters, K., Comi, G., Cossburn, M., Cournu-Rebeix, I., Cox, M.B., Cozen, W., Cree, B.A.C., Cross, A.H., Cusi, D., Daly, M.J., Davis, E., Bakker, P.I.W. de, Debouverie, M., D'hooghe, M.B., Dixon, K., Dobosi, R., Dubois, B., Ellinghaus, D., Elovaara, I., Esposito, F., Fontenille, C., Foote, S., Franke, A., Galimberti, D., Ghezzi, A., Glessner, J., Gomez, R., Gout, O., Graham, C., Grant, S.F.A., Guerini, F.R., Hakonarson, H., Hall, P., Hamsten, A., Hartung, H.-P., Heard, R.N., Heath, S., Hobart, J., Hoshi, M., Infante-Duarte, C., Ingram, G., Ingram, W., Islam, T., Jagodic, M., Kabesch, M., Kermode, A.G., Kilpatrick, T.J., Kim, C., Klopp, N., Koivisto, K., Larsson, M., Lathrop, M., Lechner-

Scott, J.S., Leone, M.A., Leppä, V., Liljedahl, U., Bomfim, I.L., Lincoln, R.R., Link, J., Liu, J., Lorentzen, A.R., Lupoli, S., Macciardi, F., Mack, T., Marriott, M., Martinelli, V., Mason, D., McCauley, J.L., Mentch, F., Mero, I.-L., Mihalova, T., Montalban, X., Mottershead, J., Myhr, K.-M., Naldi, P., Ollier, W., Page, A., Palotie, A., Pelletier, J., Piccio, L., Pickersgill, T., Piehl, F., Pobywajlo, S., Quach, H.L., Ramsay, P.P., Reunanen, M., Reynolds, R., Rioux, J.D., Rodegher, M., Roesner, S., Rubio, J.P., Rückert, I.-M., Salvetti, M., Salvi, E., Santaniello, A., Schaefer, C.A., Schreiber, S., Schulze, C., Scott, R.J., Sellebjerg, F., Selmaj, K.W., Sexton, D., Shen, L., Simms-Acuna, B., Skidmore, S., Sleiman, P.M.A., Smestad, C., Sørensen, P.S., Søndergaard, H.B., Stankovich, J., Strange, R.C., Sulonen, A.-M., Sundqvist, E., Syvänen, A.-C., Taddeo, F., Taylor, B., Blackwell, J.M., Tienari, P., Bramon, E., Tourbah, A., Brown, M.A., Tronczynska, E., Casas, J.P., Tubridy, N., Corvin, A., Vickery, J., Jankowski, J., Villoslada, P., Markus, H.S., Wang, K., Mathew, C.G., Wason, J., Palmer, C.N.A., Wichmann, H.-E., Plomin, R., Willoughby, E., Rautanen, A., Winkelmann, J., Wittig, M., Trembath, R.C., Yaouanq, J., Viswanathan, A.C., Zhang, H., Wood, N.W., Zuvich, R., Deloukas, P., Langford, C., Duncanson, A., Oksenberg, J.R., Pericak-Vance, M.A., Haines, J.L., Olsson, T., Hillert, J., Ivinson, A.J., Jager, P.L. de, Peltonen, L., Stewart, G.J., Hafler, D.A., Hauser, S.L., McVean, G., Donnelly, P. and Compston, A. (2011), “Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis”, *Nature*, Vol. 476 No. 7359, pp. 214–219.

Scalfari, A., Neuhaus, A., Daumer, M., Muraro, P.A. and Ebers, G.C. (2014), “Onset of secondary progressive phase and long-term evolution of multiple sclerosis”, *Journal of neurology, neurosurgery, and psychiatry*, Vol. 85 No. 1, pp. 67–75.

Schmitz, R., Hansmann, M.-L., Bohle, V., Martin-Subero, J.I., Hartmann, S., Mechtersheimer, G., Klapper, W., Vater, I., Giefing, M., Gesk, S., Stanelle, J., Siebert, R. and Küppers, R. (2009), “TNFAIP3 (A20) is a tumor suppressor gene in Hodgkin lymphoma and primary mediastinal B cell lymphoma”, *The Journal of experimental medicine*, Vol. 206 No. 5, pp. 981–989.

Schwartz, N., Stock, A.D. and Putterman, C. (2019), “Neuropsychiatric lupus: new mechanistic insights and future treatment directions”, *Nature reviews. Rheumatology*, Vol. 15 No. 3, pp. 137–152.

Serlin, Y., Shelef, I., Knyazer, B. and Friedman, A. (2015), “Anatomy and physiology of the blood-brain barrier”, *Seminars in cell & developmental biology*, Vol. 38, pp. 2–6.

Shembade, N., Ma, A. and Harhaj, E.W. (2010), “Inhibition of NF-kappaB signaling by A20 through disruption of ubiquitin enzyme complexes”, *Science (New York, N.Y.)*, Vol. 327 No. 5969, pp. 1135–1139.

Simpson, S., van der Mei, I. and Taylor, B. (2018), “The Role of Vitamin D in Multiple Sclerosis: Biology and Biochemistry, Epidemiology and Potential Roles in Treatment”, *Medicinal chemistry (Shariqah (United Arab Emirates))*, Vol. 14 No. 2, pp. 129–143.

Soni, D., Wang, D.-M., Regmi, S.C., Mittal, M., Vogel, S.M., Schlüter, D. and Tiruppathi, C. (2018), “Deubiquitinase function of A20 maintains and repairs endothelial barrier after lung vascular injury”, *Cell death discovery*, Vol. 4, p. 60.

Sospedra, M. and Martin, R. (2005), “Immunology of multiple sclerosis”, *Annual review of immunology*, Vol. 23, pp. 683–747.

Stromnes, I.M. and Goverman, J.M. (2006), “Active induction of experimental allergic encephalomyelitis”, *Nature protocols*, Vol. 1 No. 4, pp. 1810–1819.

Sun, S.C., Ganchi, P.A., Ballard, D.W. and Greene, W.C. (1993), “NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway”, *Science (New York, N.Y.)*, Vol. 259 No. 5103, pp. 1912–1915.

Taylor, X., Cisternas, P., Jury, N., Martinez, P., Huang, X., You, Y., Redding-Ochoa, J., Vidal, R., Zhang, J., Troncoso, J. and Lasagna-Reeves, C.A. (2022), “Activated endothelial cells induce a distinct type of astrocytic reactivity”, *Communications biology*, Vol. 5 No. 1, p. 282.

Terry, R.L., Ifergan, I. and Miller, S.D. (2016), “Experimental Autoimmune Encephalomyelitis in Mice”, *Methods in molecular biology (Clifton, N.J.)*, Vol. 1304, pp. 145–160.

Thompson, A.J., Banwell, B.L., Barkhof, F., Carroll, W.M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M.S., Fujihara, K., Galetta, S.L., Hartung, H.P., Kappos, L., Lublin, F.D., Marrie, R.A., Miller, A.E., Miller, D.H., Montalban, X., Mowry, E.M., Sorensen, P.S., Tintoré, M., Traboulsee, A.L., Trojano, M., Uitdehaag, B.M.J., Vukusic, S., Waubant, E., Weinshenker, B.G., Reingold, S.C. and Cohen, J.A. (2018), "Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria", *The Lancet Neurology*, Vol. 17 No. 2, pp. 162–173.

Thompson, A.J., Kermode, A.G., Wicks, D., MacManus, D.G., Kendall, B.E., Kingsley, D.P. and McDonald, W.I. (1991), "Major differences in the dynamics of primary and secondary progressive multiple sclerosis", *Annals of neurology*, Vol. 29 No. 1, pp. 53–62.

Tsareva, E., Kulakova, O., Boyko, A. and Favorova, O. (2016), "Pharmacogenetics of multiple sclerosis: personalized therapy with immunomodulatory drugs", *Pharmacogenetics and genomics*, Vol. 26 No. 3, pp. 103–115.

V. Sherbet, G. (2016), "Molecular Approach to Targeted Therapy for Multiple Sclerosis", *CNS & Neurological Disorders - Drug Targets*, Vol. 15 No. 1, pp. 20–34.

van de Haar, H.J., Burgmans, S., Jansen, J.F.A., van Osch, M.J.P., van Buchem, M.A., Muller, M., Hofman, P.A.M., Verhey, F.R.J. and Backes, W.H. (2016), "Blood-Brain Barrier Leakage in Patients with Early Alzheimer Disease", *Radiology*, Vol. 281 No. 2, pp. 527–535.

van Vliet, E.A., Aronica, E. and Gorter, J.A. (2015), "Blood-brain barrier dysfunction, seizures and epilepsy", *Seminars in cell & developmental biology*, Vol. 38, pp. 26–34.

Vendrell, J.A., Ghayad, S., Ben-Larbi, S., Dumontet, C., Mechti, N. and Cohen, P.A. (2007), "A20/TNFAIP3, a new estrogen-regulated gene that confers tamoxifen resistance in breast cancer cells", *Oncogene*, Vol. 26 No. 32, pp. 4656–4667.

Vereecke, L., Sze, M., Mc Guire, C., Rogiers, B., Chu, Y., Schmidt-Supprian, M., Pasparakis, M., Beyaert, R. and van Loo, G. (2010), "Enterocyte-specific A20

deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis", *The Journal of experimental medicine*, Vol. 207 No. 7, pp. 1513–1523.

Voet, S., Mc Guire, C., Hagemeyer, N., Martens, A., Schroeder, A., Wieghofer, P., Daems, C., Staszewski, O., Vande Walle, L., Jordao, M.J.C., Sze, M., Vikkula, H.-K., Demeestere, D., van Imschoot, G., Scott, C.L., Hoste, E., Gonçalves, A., Guilliams, M., Lippens, S., Libert, C., Vandenbroucke, R.E., Kim, K.-W., Jung, S., Callaerts-Vegh, Z., Callaerts, P., Wit, J. de, Lamkanfi, M., Prinz, M. and van Loo, G. (2018), "A20 critically controls microglia activation and inhibits inflammasome-dependent neuroinflammation", *Nature communications*, Vol. 9 No. 1, p. 2036.

Voisin, M.-B., Pröbstl, D. and Nourshargh, S. (2010), "Venular basement membranes ubiquitously express matrix protein low-expression regions: characterization in multiple tissues and remodeling during inflammation", *The American journal of pathology*, Vol. 176 No. 1, pp. 482–495.

Voskuhl, R.R., Peterson, R.S., Song, B., Ao, Y., Morales, L.B.J., Tiwari-Woodruff, S. and Sofroniew, M.V. (2009), "Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS", *The Journal of neuroscience the official journal of the Society for Neuroscience*, Vol. 29 No. 37, pp. 11511–11522.

Waisman, A. and Johann, L. (2018), "Antigen-presenting cell diversity for T cell reactivation in central nervous system autoimmunity", *Journal of molecular medicine (Berlin, Germany)*, Vol. 96 No. 12, pp. 1279–1292.

Wang, X., Deckert, M., Xuan, N.T., Nishanth, G., Just, S., Waisman, A., Naumann, M. and Schlüter, D. (2013), "Astrocytic A20 ameliorates experimental autoimmune encephalomyelitis by inhibiting NF-κB- and STAT1-dependent chemokine production in astrocytes", *Acta neuropathologica*, Vol. 126 No. 5, pp. 711–724.

Wekerle, H. (2017), "B cells in multiple sclerosis", *Autoimmunity*, Vol. 50 No. 1, pp. 57–60.

Wellcome Trust Case Control Consortium (2007), “Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls”, *Nature*, Vol. 447 No. 7145, pp. 661–678.

Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L., Ma, A., Koonin, E.V. and Dixit, V.M. (2004), “De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling”, *Nature*, Vol. 430 No. 7000, pp. 694–699.

Winger, R.C., Koblinski, J.E., Kanda, T., Ransohoff, R.M. and Muller, W.A. (2014), “Rapid remodeling of tight junctions during paracellular diapedesis in a human model of the blood-brain barrier”, *Journal of immunology (Baltimore, Md. 1950)*, Vol. 193 No. 5, pp. 2427–2437.

Wolburg, H. and Lippoldt, A. (2002), “Tight junctions of the blood–brain barrier”, *Vascular Pharmacology*, Vol. 38 No. 6, pp. 323–337.

Wolburg, H., Wolburg-Buchholz, K. and Engelhardt, B. (2005), “Diapedesis of mononuclear cells across cerebral venules during experimental autoimmune encephalomyelitis leaves tight junctions intact”, *Acta neuropathologica*, Vol. 109 No. 2, pp. 181–190.

Wong, R.K., Baldwin, A.L. and Heimark, R.L. (1999), “Cadherin-5 redistribution at sites of TNF-alpha and IFN-gamma-induced permeability in mesenteric venules”, *The American journal of physiology*, Vol. 276 No. 2, H736-48.

Xia, Z.-P., Sun, L., Chen, X., Pineda, G., Jiang, X., Adhikari, A., Zeng, W. and Chen, Z.J. (2009), “Direct activation of protein kinases by unanchored polyubiquitin chains”, *Nature*, Vol. 461 No. 7260, pp. 114–119.

Xu, L., Nirwane, A. and Yao, Y. (2019), “Basement membrane and blood-brain barrier”, *Stroke and vascular neurology*, Vol. 4 No. 2, pp. 78–82.

Xu, Y.-R. and Lei, C.-Q. (2021), “TAK1-TABs Complex: A Central Signalosome in Inflammatory Responses”, *Frontiers in immunology*, Vol. 11.

Yadav, S.K., Mindur, J.E., Ito, K. and Dhib-Jalbut, S. (2015), “Advances in the immunopathogenesis of multiple sclerosis”, *Current opinion in neurology*, Vol. 28 No. 3, pp. 206–219.

Yu, H., Lin, L., Zhang, Z., Zhang, H. and Hu, H. (2020a), “Targeting NF-κB pathway for the therapy of diseases: mechanism and clinical study”, *Signal transduction and targeted therapy*, Vol. 5 No. 1, p. 209.

Yu, M.-P., Xu, X.-S., Zhou, Q., Deuitch, N. and Lu, M.-P. (2020b), “Haploinsufficiency of A20 (HA20): updates on the genetics, phenotype, pathogenesis and treatment”, *World journal of pediatrics WJP*, Vol. 16 No. 6, pp. 575–584.

Yue, Y., Stone, S. and Lin, W. (2018), “Role of nuclear factor κB in multiple sclerosis and experimental autoimmune encephalomyelitis”, *Neural regeneration research*, Vol. 13 No. 9, pp. 1507–1515.

Zamanian, J.L., Xu, L., Foo, L.C., Nouri, N., Zhou, L., Giffard, R.G. and Barres, B.A. (2012), “Genomic analysis of reactive astrogliosis”, *The Journal of neuroscience the official journal of the Society for Neuroscience*, Vol. 32 No. 18, pp. 6391–6410.

Zheng, H., Li, Q., Chen, R., Zhang, J., Ran, Y., He, X., Li, S. and Shu, H.-B. (2012), “The Dual-specificity Phosphatase DUSP14 Negatively Regulates Tumor Necrosis Factor- and Interleukin-1-induced Nuclear Factor-κB Activation by Dephosphorylating the Protein Kinase TAK1*”, *The Journal of biological chemistry*, Vol. 288 No. 2, pp. 819–825.

Zhou, Q., Wang, H., Schwartz, D.M., Stoffels, M., Park, Y.H., Zhang, Y., Yang, D., Demirkaya, E., Takeuchi, M., Tsai, W.L., Lyons, J.J., Yu, X., Ouyang, C., Chen, C., Chin, D.T., Zaal, K., Chandrasekharappa, S.C., Hanson, E.P., Yu, Z., Mullikin, J.C., Hasni, S.A., Wertz, I.E., Ombrello, A.K., Stone, D.L., Hoffmann, P., Jones, A., Barham, B.K., Leavis, H.L., van Royen-Kerkof, A., Sibley, C., Batu, E.D., Gül, A., Siegel, R.M., Boehm, M., Milner, J.D., Ozen, S., Gadina, M., Chae, J., Laxer, R.M., Kastner, D.L. and Aksentijevich, I. (2016), “Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease”, *Nature Genetics*, Vol. 48 No. 1, pp. 67–73.

9 Publications

Johann, L., Soldati, S., Müller, K., Lampe, J., Marini, F., Klein, M., Schramm, E., Ries, N., Schelmbauer, C., Palagi, I., Karram, K., Assmann, J.C., Khan, M.A., Wenzel, J., Schmidt, M.H., Körbelin, J., Schlüter, D., van Loo, G., Bopp, T., Engelhardt, B., Schwaninger, M. and Waisman, A. (2023), “A20 regulates lymphocyte adhesion in murine neuroinflammation by restricting endothelial ICOSL expression in the CNS”, *Journal of Clinical Investigation*.

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11 Curriculum vitae

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