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Master of Biomedical Sciences

Master's thesis

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Janne Verreycken

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization

Molecular Mechanisms in Health and Disease

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Blood-brain barrier protection by IL-34: a novel, non-canonical function of regulatory T cells*

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Running title: BBB protection by Treg-derived IL-34

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ABSTRACT

Multiple sclerosis (MS) is a demyelinating autoimmune disease mainly affecting young adults. The relapsing-remitting MS (RR-MS) type is correlated with extensive blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) disruption. This disruption is related to an influx of inflammatory cells in the central nervous system (CNS) resulting in neuronal damage. We hypothesize that IL-34 derived from regulatory T cells (Tregs) has a protective role in preserving the integrity of these brain barriers during neuroinflammation. In human and murine *in vitro* models, IL-34 appears to shield BBB endothelial cells and BCSFB epithelial cells from inflammatory stimuli by reducing the barrier permeability. Moreover, we observed that *in vitro*, brain barrier cells derived from RAG-deficient mice exhibit dysfunctional barrier properties to large molecules, which was partially rescued when treated with IL-34. Our findings also suggest that IL-34 induces oligodendrocyte precursor cell differentiation. Furthermore, Tregs derived from RR-MS patients showed reduced IL-34 expression compared to healthy controls, while overall lymphocyte production of IL-34 was unaffected. Lastly, in a preclinical model of MS, experimental autoimmune encephalomyelitis (EAE), *Il-34* mRNA levels remained unaltered in the CNS. In contrast, the expression of its receptor, *Csf1r,* was significantly upregulated in EAE compared to naïve animals. In conclusion, it appears that Treg-derived IL-34 is involved in preserving brain barrier integrity in an inflammatory environment. Treg cell therapy might be a valuable therapeutic approach for targeting brain barriers in individuals with MS, particularly due to the combination with their other tissue regenerative effects, including remyelination.

INTRODUCTION

2.8 million people worldwide suffer from the autoimmune disease **multiple sclerosis (MS)** ([1](#)). MS primarily affects young adults and is the most frequent cause of non-traumatic disability amongst this group of people ([2](#)). The disease is characterized by demyelinated focal lesions and neurodegeneration in both white and grey matter of the central nervous system (CNS) ([3](#), [4](#)). Clinical manifestations of MS include cognitive impairment, vision loss, fatigue, and other neurological deficits ([5](#)). Patients are diagnosed based on the combination of symptoms, imaging of lesions (e.g. magnetic resonance imaging (MRI)), and laboratory results (e.g. presence of oligoclonal bands in the cerebrospinal fluid (CSF)) ([6](#)). Thus far, the etiology of the disease remains unknown. However, most experts agree that onset is induced by a combination of genetic predisposition and environmental triggers ([7](#)). Over 200 loci are linked to MS susceptibility, for example single nucleotide polymorphisms (SNPs) in the human leukocyte antigen (HLA) class II alleles ([8](#), [9](#)). In addition, non-genetic factors, mainly related to lifestyle, are of major importance and interact with genetic factors,

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collectively enhancing the risk for MS in a synergistic manner. Examples of non-genetic aspects are viral infections (Epstein-Barr virus (EBV) ([10](#)) and cytomegalovirus (CMV) ([11](#))), smoking, diet, adolescent obesity, stress, latitude and sun exposure ([12](#)). Most patients present with recurrent acute episodes, undergoing full recovery in between. This type is called relapsing-remitting MS (RR-MS) and is mostly associated with brain barrier disruption ([2](#)). Consequently, an excessive immune response occurs in the CNS targeting the myelin and nerve fibers ([5](#)). Around 65% of these RR-MS patients evolve into the secondary progressive type (SP-MS), indicated by the accumulation of disability. On the other hand, 20% of patients experience progressive disease from onset, called primary progressive MS (PP-MS), predominantly affecting the nerves of the spinal cord ([7](#)). Both progressive types of MS show prominent degenerative processes while lacking extensive brain barrier disruption and an uncontrolled inflammatory response ([2](#), [5](#)). Nonetheless, progression is linked to compartmentalized inflammation located at the leptomeninges and lesion sites ([13](#)). Lastly, only accounting for 5%, progressive-relapsing MS patients suffer relapses while remissions are not complete, leading to continuously present symptoms that worsen between relapses ([7](#), [14](#)). The treatment of MS

often requires a multidisciplinary approach, including disease-modifying therapies (DMTs), symptom management, psychological help and rehabilitation ([6](#)). Current DMTs primarily target RR-MS due to the highly inflammatory nature of this disease type ([15](#)). Only two DMTs are currently available for PP- and SP-MS patients, ocrelizumab and siponimod, respectively ([16](#), [17](#)). Despite decreasing the number and duration of relapses, these therapies cannot prevent progression of the disease and patients still deteriorate ([15](#)).

An early hallmark in the disease course of RR-MS is **disruption of the blood-brain barrier (BBB) (Figure 1)** ([2](#))**.** The BBB, consisting of endothelial cells, pericytes, and astrocytic endfeet, along with neurons forms the neurovascular unit (NVU). Endothelial cells of the BBB (BBB-ECs) are tightly connected by tight junctions (TJs) and adherens junctions (AJs). These TJs include transmembrane proteins (e.g. occludin and claudin-5) and cytoplasmatic proteins (e.g. ZO-1, -2, and -3), while VE-cadherin and catenins are the main AJs ([2](#)). Junctional proteins limit the movement of molecules across the BBB. For the influx of nutrients and efflux of waste molecules, BBB-ECs express solute carrier proteins and selective efflux transporters, respectively ([18](#)). Furthermore, lack of expression of cell adhesion
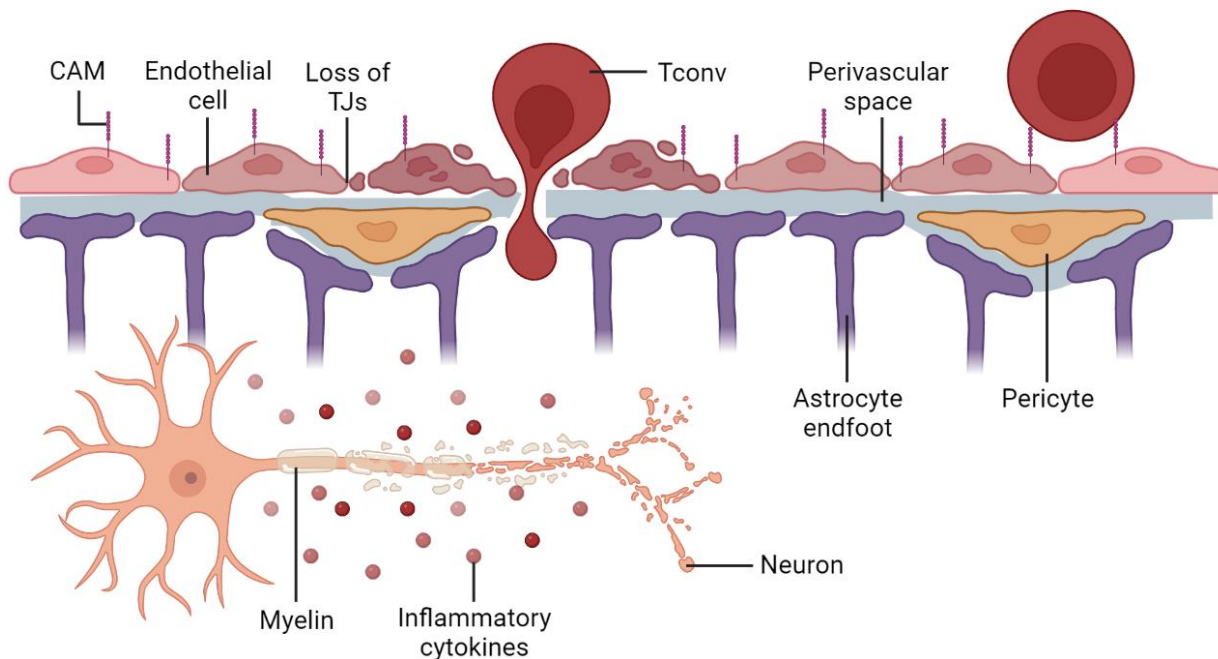

Figure 1: Summary of blood-brain barrier (BBB) disruption in multiple sclerosis (MS). MS is characterized by a leaky BBB. Endothelial cells lose tight junctions (TJs), and upregulate the expression of cell adhesion molecules (CAMs). These changes lead to an increase in adhesion and migration of pro-inflammatory T conventional cells (Tconv), resulting in an inflammatory environment in the central nervous system and damage to the myelin sheath surrounding axons. Figure was created using Biorender.com.

molecules (CAMs) prevents the entry of most immune cells into the CNS ([19]). For a long time, the brain was considered to be 'immune privileged'. However, this theory has now been revised as evidence arose for a baseline CNS immune surveillance ([18], [19]).

In MS, many autoreactive peripheral blood mononuclear cells (PBMCs) are present. These immune cells will produce many cytokines; e.g. tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were found to be upregulated in the serum of MS patients ([20]). The presence of these pro-inflammatory cytokines in MS alters the architecture of TJs on BBB-ECs, and increases the expression of CAMs ([21-23]). These molecular changes induce BBB leakiness and consequently increase the migration of autoreactive immune cells ([24]). This event occurs early in the disease, suggesting an important role in disease development. In contrast, during MS progression, the BBB is partially restored ([13]). In a recent study, it was demonstrated that BBB-ECs are able to present antigens to CD8+ T cells. This interaction prevented the migration of CD8+ T cells but resulted in apoptosis of the endothelial cells ([25]). Conversely, the view of immune-mediated BBB disruption was recently challenged by Nishihara *et al.* ([26]). These researchers reprogrammed and expanded human induced pluripotent stem cells (hiPSCs) and differentiated them into brain microvascular endothelial cell (BMEC)-like cells to assess the intrinsic barrier characteristics of MS-derived BMECs. An increase in the permeability of MS-derived BMECs was observed compared to healthy BMECs, and the increased expression of ICAM-1 indicated an intrinsic inflammatory phenotype in these cells ([26]).

Alterations in BBB permeability consequently lead to immune cell infiltration ([2]). Adhesion receptors on the surface of activated T cells interact with CAMs, leading to the rolling and tethering of the cells. Ultimately, T cells are able to migrate through the cell layer. This complete process is called diapedesis ([2]). These immune cells, primarily encephalitogenic T cells, release chemokines and cytokines in the CNS, thereby initiating a self-sustained inflammatory cascade ([27]). T cell migration and subsequent neuroinflammation induce demyelination and neurodegeneration, eventually resulting in the aforementioned symptoms of MS ([27]). In the CNS, myelin sheaths are axon-wrapping membranes established by oligodendrocytes. These lipid-rich structures are essential for

improving the conduction of neural impulses ([28]). Thus, demyelination as a consequence of inflammatory damage to oligodendrocytes and myelin, results in a conduction block. Fortunately, studies show that around 20% of MS patients demonstrate extensive remyelination ([29], [30]). This occurs due to the generation of new mature oligodendrocytes from the activation, migration, proliferation and differentiation of oligodendrocyte precursor cells (OPCs) into myelinating oligodendrocytes ([28]). However, in many patients this process is limited or completely absent despite the presence of sufficient numbers of OPCs, suggesting an inability of OPCs to differentiate and mature ([28]). Myelin is not only essential in nerve conduction, it also protects the axon from degeneration by excreting neurotrophic factors ([31]). Therefore, unresolved remyelination, in addition to acute inflammation, leads to axonal and neuronal injury.

Another important barrier in the development of MS is the **blood-cerebrospinal fluid barrier (BCSFB)**. This barrier is mainly located at the choroid plexus (CP), a vascularized structure protruding the ventricles ([32]). The CP consists of a fenestrated endothelium next to an epithelium connected by TJs. Besides its main function of CSF production, the CP also regulates physiological immune cell infiltration and secretes neurotrophic factors ([33], [34]). Most research on the role of the CP in MS has been conducted in the EAE animal model. Here, it was established that EAE induction resulted in loss of TJ protein ZO-1 and increased the extravasation of leukocytes and IgG into the CSF and CNS across CP epithelium ([35]). Recently, it was confirmed that the CP acts as an immune reservoir in physiological conditions. During EAE, however, this structure was identified as an entry site to the CNS ([36]). Even more, one research group found that the entry of Th17 cells through the CP is required for the initiation of the disease ([37]). During this process, Th17 cells are reactivated, resulting in the release of pro-inflammatory cytokines that subsequently activate the BBB. This research led to a current hypothesis that leukocyte infiltration across the CP precedes the second, extensive wave of infiltration across the BBB. Hence, it is accepted that both barriers are heavily involved in MS pathology.

Regulatory T cells (Tregs) are CD4+CD25hi lymphocytes expressing forkhead box P3 (FoxP3) that retain the immune homeostasis in

healthy individuals. Deriving from CD4+CD8- thymocytes, the majority of Tregs differentiate in the thymus (tTregs) due to moderately high-affinity T cell receptor (TCR) signaling of autoreactive nature ([38](#)). The presence of interleukin (IL)-2 and transforming growth factor beta (TGF-β), and signaling through CD28 are required for this differentiation process ([39](#)). IL-2 signaling through the IL-2Rα (CD25) on Tregs activates the transcription factor STAT5, which in turn induces expression of the *Foxp3* gene, the master transcription factor of Treg development and suppressive function ([40](#)). In addition, Tregs can develop from naïve CD4+FoxP3- T cells in the periphery (pTregs). These cells are induced by contact with non-self-antigens in the presence of IL-2 and TGF-β, resulting in unstable FoxP3 expression ([41-43](#)). While tTregs suppress immune reactions against self-antigens, pTregs are suggested to play a role in maintaining tolerance against commensal antigens ([39](#)). Next to the heterogeneity of Treg subpopulations, they also demonstrate extensive plasticity influenced by the local microenvironment ([44](#)). IL-12 and IL-2 induce IFN-γ production by Tregs, related to the adaptation to a Th1-like phenotype ([44](#)). Conversion to an IL-17 producing Th17 phenotype can be induced by IL-1β, or IL-6 in the absence of TGF-β ([45](#)).

Tregs can induce suppression directly via cell-cell contact, or indirectly via the secretion of soluble factors. Direct suppression involves, for example, cytotoxic T-lymphocyte associated protein 4 (CTLA-4) which downregulates the expression of costimulatory molecules (e.g. CD80 and CD86) on antigen-presenting cells (APCs) ([46](#)). Lymphocyte Activation Gene-3 (LAG-3) on the Treg cell surface also interacts with the major histocompatibility complex class II (MHC-II) on immature dendritic cells (DCs), thereby suppressing DC maturation ([46](#)). On the other hand, Tregs can act through the modulation of cytokines and molecules in the environment. Firstly, they secrete anti-inflammatory cytokines (e.g. IL-10, TGF-β, IL-35) which induce inhibitory molecules on T cells and downregulate the expression of MHC-II molecules on DCs ([39](#)). Secondly, they are able to induce programmed cell death via the secretion of cytotoxic molecules such as granzyme B ([46](#), [47](#)). Lastly, Tregs can deprive the immune microenvironment from IL-2, thereby inducing T cell apoptosis ([48](#)).

The main goal of Treg-induced suppression is preventing the development of autoimmunity.

Adoptive transfer of Tregs in an animal model for MS, experimental autoimmune encephalomyelitis (EAE), demonstrates protection against disease development, whereas depletion of Tregs enhanced the disease severity ([49](#), [50](#)). In MS, Tregs were shown to be less suppressive and fail to suppress activated, self-antigen targeted T cells, thereby being unable to restore the immune homeostasis ([51](#)). One explanation for this dysfunction is the reduced output of newly generated Tregs, called recent thymic emigrants (RTE), in RR-MS patients ([52](#), [53](#)). Due to an increased number of memory Tregs, the amount of Tregs in MS patients is stable, yet the suppressive functionality of the Treg population is lower ([52](#)). Additionally, both mRNA and protein FoxP3 expression in RR-MS-derived Tregs was significantly reduced compared to healthy and SP-MS patients ([54](#)). In contrast, Tregs of SP-MS patients have a normal suppressive capacity and FoxP3 expression ([55](#)). Interestingly, Tregs are capable of migrating towards the inflamed CNS, and they show an increased frequency in the CSF of RR-MS patients ([56](#), [57](#)). RR-MS patients also display an increased frequency of Th1-like IFN-γ secreting FoxP3 T cells ([58](#)). This was also observed in EAE, where the inflammatory setting of the CNS induced FoxP3 instability leading to IFN-γ production of myelin-reactive Tregs ([59](#)). After EAE resolution, FoxP3 expression recovered. It was also shown that Tregs lose their suppressive function and adapt a Th17-like phenotype when in contact with the inflamed BBB endothelial cell layer ([60](#)).

Treg cell therapy has been considered as the ultimate treatment for (auto)immune diseases. The first clinical trial was conducted in patients with graft-versus-host disease (GvHD) ([61](#)). Here, CD4+CD25+CD127- Tregs were expanded *ex vivo* and administered to patients, alleviating symptoms and resulting in a reduction of steroid use in chronic GvHD patients. Additional studies in GvHD indicated safety and efficacy of this treatment ([62](#), [63](#)). Treg cell therapy is being tested in many diseases, with numerous of them currently being in early clinical trials (listed by Amini *et al.* ([64](#))). In the majority of the studies, adoptive transfer of Tregs was safe and well tolerated, with varying results concerning efficacy ([64](#)). A limited phase 1 clinical trial has been conducted using autologous Tregs as a treatment for RR-MS ([65](#)). Here, Tregs were administered either intravenously or intrathecally. No relapses and no deterioration of

the disease were observed in the latter group, suggesting an advantage of bypassing the BBB. These results align with the observation that the interaction between Tregs and an inflamed BBB results in loss of FoxP3 expression by Tregs, consequently diminishing its suppressive function (60). Nonetheless, these results emphasize the capacity of Tregs to modulate disease activity. Due to Treg dysfunction in many autoimmune diseases, the transfer of autologous Tregs might not be sufficient to halt disease progression. Therefore, the safety and efficacy of *ex vivo* Treg manipulation is currently the topic of extensive research.

Besides immune suppression, healthy Tregs have non-canonical, regenerative functions. Tregs are able to directly induce OPC differentiation mediated by CCN3, resulting in myelin regeneration (66). In addition, Tregs show neuroprotective effects. They induce neural stem cell proliferation and suppress neurotoxic astrogliosis (67, 68). Studies in ischemic stroke also indicated indirect BBB-protective effects of healthy Tregs (69, 70). Numerous other organs have been shown to benefit from these non-canonical functions of Tregs (71).

Tregs express the **cytokine IL-34**, which was shown to be involved in immune tolerance after organ transplantation (72). Its main receptor is the colony-stimulating factor 1 receptor (CSF1R), which is shared with the growth factor CSF-1 (73). The active region of CSF-1 and IL-34 is similar, yet they showcase different biological activities. This might be due to the binding nature of both ligands as CSF-1:CSF1R interactions are rather hydrophilic, whereas IL-34:CSF1R interactions are more hydrophobic (73, 74). Binding of noncovalently linked IL-34 dimers to 2 copies of CSF1R results in autophosphorylation of tyrosine residues and receptor homodimerization, thereby activating several signaling pathways (74). Alternatively, IL-34 undergoes interactions and exerts biological activity through the receptor-type protein tyrosine phosphatase zeta (PTP-ζ) and syndecan-1 (75, 76). The highest, yet not exclusive, expression of IL-34 was observed in the brain and the skin, deriving from neurons and keratinocytes, respectively (77, 78). IL-34 stimulates monocyte growth, survival and differentiation into M2-like macrophages (79). In the brain, the main function of IL-34 is maintenance of microglia, the brain resident macrophages (77, 78). During pathological conditions, IL-34 adapts a dual role. In CNS pathology, IL-34 shows neuroprotective

effects by regulating microglial function (80). One example is the attenuated neurotoxicity induced by TGF-β, which is derived from IL-34-stimulated microglia in neuron-microglia cocultures (81). In addition, IL34$^{-/-}$ mice experience more severe EAE compared to wild-type (WT) mice, whereas administration of an adeno-associated virus (AAV) encoding for IL-34 delayed EAE development (82). Additionally, it was shown that IL-34 improves BBB integrity after inflammation by increasing the expression of TJs in the mouse brain endothelial cell line MBEC4 (83). This effect is most likely due to interactions with the CSF1R on CNS capillary endothelial cells (83).

We hypothesize that Treg-derived IL-34 protects the brain barriers during neuroinflammation. Our findings suggest that IL-34 partially protects brain barriers against damaging mediators. Besides, it appears that IL-34 boosts OPC differentiation. RR-MS patients show a decreased IL-34 expression by Tregs, whereas the expression by total lymphocytes remains unaltered. Knowledge on this topic is important for future research aimed at developing a novel Treg cell therapy for the treatment of MS.

EXPERIMENTAL PROCEDURES

Human endothelial cell line – Human cerebral microvascular endothelial cells (hCMEC/D3 cell line) were cultured using the EGM-2 MV medium (Lonza) at 37°C and 5% CO_2. Cells (± 24.500/cm²) are grown on 75 µg/ml collagen type I (Merck) coating in plates or inserts. When confluency was almost reached, medium was changed to experimental medium (basal EBM-2 medium (Lonza) supplemented with 5 ng/ml fibroblast growth factor (FGF) (Merck), 1.4 µM hydrocortisone (Sigma), 10 mg/ml Gentamicin (Sigma), 1 mg/ml amphotericin (Merck) and 2.5% fetal bovine serum (FBS; Gibco)). Six days after seeding, cells were treated for 8 or 24 h with 50 ng/ml human recombinant IL-34 (R&D systems) in serum-reduced (0.25% FBS) experimental medium without hydrocortisone. Inflammation was induced using 10 ng/ml IFN-γ and 100 ng/ml TNF-α (Peprotech) or 10 µg/ml lipopolysaccharide (LPS; Merck). Using these cells, diffusion assay, flow cytometry and real-time transendothelial electrical resistance (TEER) experiments were performed.

Primary mouse brain microvascular endothelial cells – Primary mouse brain

microvascular endothelial cells (mBMECs) were isolated from four to six week old mice with a C57BL/6 background. Brains were dissected followed by removal of the meninges, mincing and homogenization. The tissue was digested with 1.05 mg/ml collagenase type II (Gibco, Thermo Fisher Scientific) and 58.5 U/ml DNase I in Dulbecco's Modified Eagle Medium (DMEM) while shaking (150 rpm) at 37°C for 75 min. DMEM containing 20% bovine serum albumin (BSA; Sigma) was added for the removal of myelin by 20 min centrifugation at 1000 g. Further digestion was initiated by shaking (150 rpm) for 1 h at 37°C with 1 mg/ml collagenase/dispase (Roche Diagnostics GmbH) and 39 U/ml DNase I in DMEM. Microvascular endothelial cells were collected by using a 33% continuous Percoll gradient and centrifuging at 1000 g for 10 min. The obtained microvascular endothelial cells were cultured in DMEM containing 20% FBS (Biowest), 1 ng/ml FGF, 100 µg/ml heparin, 1.4 µM hydrocortisone (all Merck) and 0.5% penicillin/streptomycin (pen/strep; Sigma), and cells were plated on 10 µg/ml collagen type IV (Merck)-coated inserts. To purify the endothelial cell culture, medium was supplemented with 10 µg/ml puromycin (Sigma-Aldrich) for 48 h, 4 µg/ml puromycin for 24 h and no puromycin for the remaining culturing time. Cells were treated with 50 ng/ml recombinant mouse IL-34 (R&D systems). 10 ng/ml IFN-γ and 100 ng/ml TNF-α (Peprotech), or 10 µg/ml LPS (Merck) were used to induce inflammation and barrier disruption.

Primary mouse choroid plexus epithelial cells – Choroid plexus epithelial (CPE) cells were isolated from two to four week old mouse pups. The CP was isolated from both lateral and fourth ventricles. Tissue was digested using 0.2% pronase (Sigma-Aldrich) for seven minutes. Afterwards, cells were cultured until confluent on 20 µg/ml laminin coating (Sigma-Aldrich) in DMEM/F12 medium supplemented with 10% FBS, 2 mM L-Glutamine (Gibco) and 1% pen/strep at 37 °C and 5% CO_2.

Murine oligodendrocyte precursor cells – Primary mouse OPCs were isolated from newborn pups, following the previously described shake off method ([84]). Cells were plated on glass coverslips and cultured in SATO differentiation medium at 8.5% CO_2. OPCs were treated with recombinant IL-34 (25, 50 and 100 ng/ml, R&D systems) at day 0, 2 and 4 of differentiation. At day 6, cells were fixed with 4% PFA.

Diffusion assay – hCMEC/D3, mBMEC or CPE cells were cultured in transwell inserts (3 µm Thincerts, translucent, Greiner bio-one for hCMEC/D3 and mBMECs; 0.4 µm, Corning for CPE cells). Confluency of the monolayer was determined by manual TEER measurements every 24 h using the $EVOM^2$ resistance meter (World Precision Instruments, Florida, USA). When reaching confluency, the cells were treated with vehicle (PBS) or 50 ng/ml IL-34 (R&D systems) with or without pro-inflammatory cytokines IFN-γ and TNF-α (Peprotech) or LPS (Merck) for 8 h. For the last 2 h of treatment, 3 kDa dextran-fluorescein (excitation 498 nm, emission 517 nm), 10 kDa dextran-cascade blue (excitation 401 nm, emission 419 nm) and/or 66,5 kDa BSA-AF594 (excitation 590 nm, emission 618 nm) (all Invitrogen) were added at a concentration of 100 µg/ml. After 2 h, fluorescence in the bottom well was measured using a microplate reader (CLARIOstar Plus, BMG Labtech). Values were normalized to vehicle or IL-34 condition of each experiment.

Real-time transendothelial electrical resistance – hCMEC/D3 cells were grown on collagen-coated 16-well RTCA E-Plates (Agilent, Santa Clara, CA, USA). A PalmSens 4 impedance analyser, controlled by PSTrace software (PalmSens BV, Houten, The Netherlands), measures the TEER (in Ω) in gold microelectrode-containing wells every 20 min from 1 Hz to 1000 kHz at five frequencies per decade. Cells were grown until confluent, and treated for 24 h. Using a custom-made Python script, data analysis was performed using TEER values at a frequency of 6309.57 Hz, which reflect the intercellular junctions. Data are depicted as Ω.

Immunofluorescence – Cover glasses with OPCs are blocked with 1% BSA (USBiological) for 30 min at room temperature. Next, cells are incubated for 4h at room temperature with rat anti-MBP (1:500; Merck) and mouse anti-O4 (1:1000; R&D). After washing, cells are incubated with Alexa fluor 488- or 555- conjugated secondary antibodies (1:600; Invitrogen). Cell nuclei were stained using 4,6'-diamidino-2-phenylindole (DAPI; Invitrogen). Lastly, cells were mounted using Fluoromount-G (Invitrogen) and imaged using the Leica DM2000 LED microscope.

ELISA – PBMCs were derived from human blood using the ficoll density gradient method (Stemcell). Next, Tregs were isolated by positive CD25 selection using magnetic activated cell

sorting (MACS) (Miltenyi Biotec) and fluorescence-activated cell sorting (FACS) using the FACSAria Fusion (BD Biosciences) with CD25 kiravia Blue 520, CD127 BV421 and CD4 AF700 (all Biolegend). Tregs were stimulated for 6 days using anti-CD3 (10 µg/ml; Invitrogen), anti-CD28 (1 µg/ml; BD Biosciences) and recombinant IL-2 (300 U/ml; Peprotech). After 6 days of stimulation, Treg supernatant was frozen at -20 °C and later used for IL-34 analysis using the ELISA MAX Deluxe Set Human IL-34 (BioLegend) following the manufacturer's instructions.

Flow cytometry – PBMC freezings from MS patients and healthy controls were derived from the University Biobank Limburg (UBiLim). Patient information is summarized in **Table S1**. Prior to freezing, these PBMCs were isolated from whole blood by the ficoll density gradient method. After thawing, the cells were stimulated with 200 ng/ml phorbol 12-myristate 13-acetate (PMA; Merck), 100 ng/ml calcium ionomycin (CaI; Merck) and Golgiplug and Golgistop (Invitrogen) for 4 h. Cells are stained with the fixable viability dye eFluor 506 (eBioscience) followed by a surface staining with following antibodies: CD25 BB515 (BD Biosciences), CD3 AF700, CD4 BV421, CXCR3 Brilliant Violet 786, CCR6 PE-Cy7, CCR4 PE/Dazzle 594 and CD8 PerCP (all Biolegend). Next, the cells are fixed and permeabilized using the FOXP3/Transcription factor staining buffer kit (Thermo Fisher Scientific) following manufacturer's instructions. This was followed by intranuclear staining with AF647 FoxP3 (Biolegend) and PE IL-34 (R&D systems) antibodies.

hCMEC/d3 cells were treated with 10 ng/ml IFN-γ and 100 ng/ml TNF-α or 50 ng/ml IL-34 (R&D systems, 5265-IL-010) for 24 h. A surface staining was performed using the following antibodies: ICAM-1 PE/Dazzle 594, VCAM-1 APC (both Biolegend) and VE-cadherin FITC (BD Biosciences). All samples were acquired on BD LSRFortessa and analysed using FlowJo (version 10.8.1) (BD Biosciences).

Quantitative PCR – CNS tissue was collected from C57BL/6 mice in which EAE was actively induced (MOG$_{35-55}$ kit by Hooke laboratories). Tissues were collected from naïve mice and mice at EAE peak and RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen). Using the NanoDrop 2000/2000c spectrophotometer, RNA concentration was determined and cDNA was made using qScript cDNA SuperMix (Quanta Biosciences). Next, a mix was prepared containing SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific), RNase free water, 10 µM forward and reverse primer (Integrated DNA Technologies) (**Table S2**) and 12.5 ng cDNA. Quantitative PCR was performed using the StepOnePlus Real-Time PCR detection system (Life technologies) at following cycle conditions: 20 s at 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Ct values were normalized against housekeeping genes YWHAZ and HPRT.

Statistics – Analyses were performed using Graphpad Prism 9.5.0. Outliers were detected using the Grubbs' test, followed by the exclusion of these data points. Normality of the data and equality of variances were tested, followed by unpaired t-test, one-way ANOVA (post-hoc Tukey test) or Kruskal-Wallis test (post hoc Dunn's test). Data are shown as mean ± standard error of the mean (SEM) and considered statistically significant when p<0.05.

RESULTS

IL-34 tends to increase BBB integrity in vitro – To determine whether IL-34 protects the BBB *in vitro,* hCMEC/D3 endothelial cells were treated for 8h with IL-34 in the presence or absence of pro-inflammatory cytokines (IFN-γ and TNF-α). The permeability of the cell layer was measured using a diffusion assay, whereas the integrity was determined by measuring TEER in real-time. Treatment with IL-34 alone did not alter the permeability of hCMEC/D3 cells (**Figure 2a-c**). IFN-γ and TNF-α were used as an inflammatory stimulus to the cells, thereby increasing the permeability by approximately 1.5 compared to vehicle (**Figure 2a-c**). Co-treatment with IL-34 decreased the permeability for all molecular weight constituents, yet this effect is not statistically significant from either inflammation or vehicle (**Figure 2a-c**). The inflammatory environment also decreased the integrity of the cell layer, as depicted by the TEER (**Figure 2d**). IL-34 seemed to partially protect against these damaging mediators.

Furthermore, we investigated the effect of IL-34 *in vitro* on primary mBMECs. Treatment was induced when the cell layer reached confluency (**Figure 2e**). IL-34 alone already seemed to reduce the permeability compared to vehicle-treated cells (**Figure 2f-h**). The treatment of the cells with inflammatory cytokines increased the permeability of the layer for 3 kDa and 66,5 kDa, but not for 10 kDa constituents
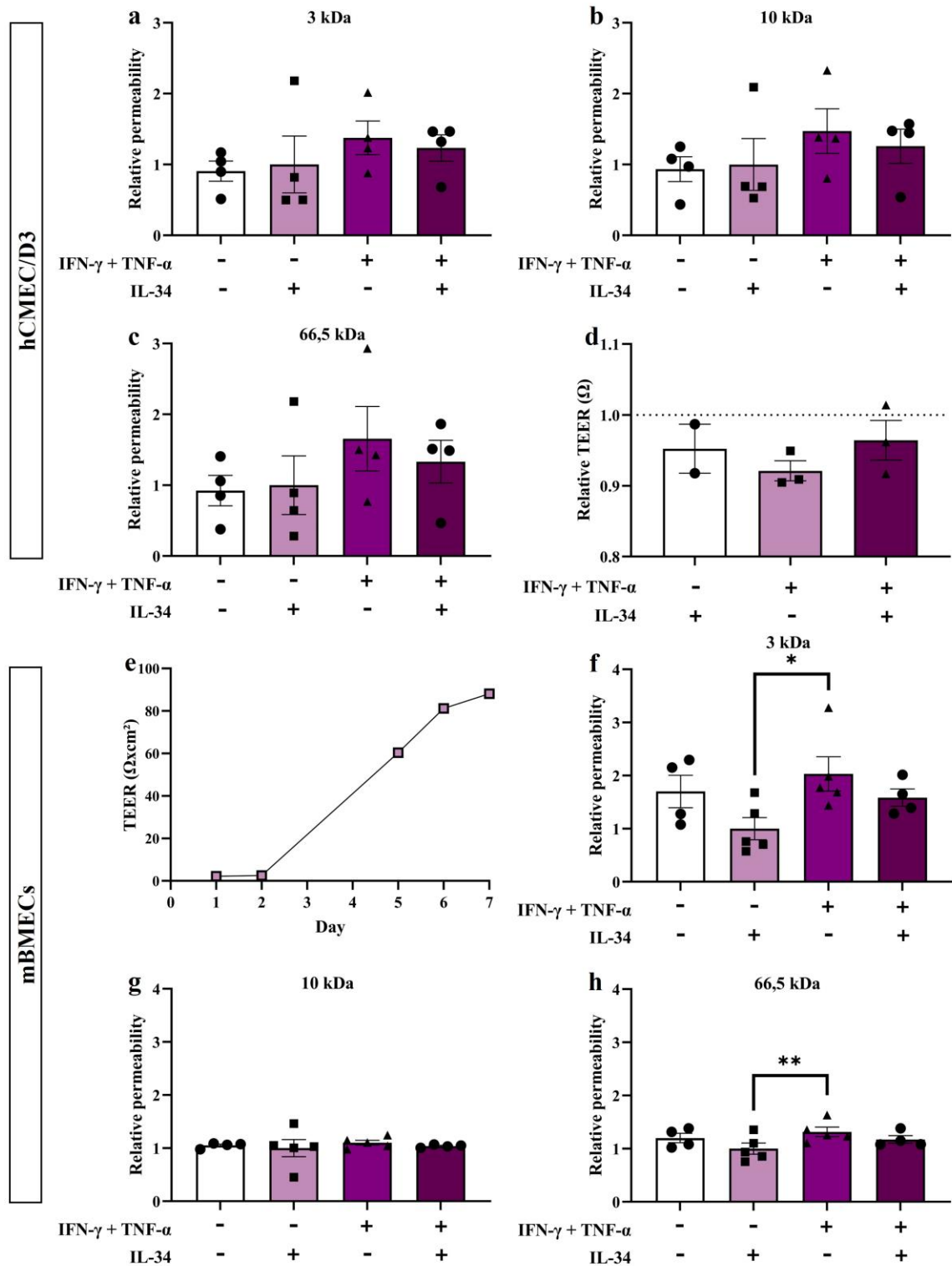
Figure 2: IL-34 tends to protect against inflammation-induced BBB-EC disruption *in vitro*. a-c. Permeability of human endothelial cells (hCMEC/D3) to components of different molecular weights after 8h treatment with IFN-γ (10 ng/ml) and TNF-α (100 ng/ml), IL-34 (50 ng/ml) or a combination. Values are normalized to IL-34 condition. (n = 4). d. TEER of hCMEC/D3 cells after 24h treatment with IFN-γ (10 ng/ml) and TNF-α (100 ng/ml), IL-34 (50 ng/ml) or a combination. Cells were treated when confluency was reached. Values are normalized to vehicle condition (dotted line). (n = 3). e. Representative figure of TEER values derived from primary mouse brain endothelial cells (mBMECs) reaching confluency. f-h. Permeability of mBMECs *in vitro* to components of different molecular weights after 8h of treatment with IFN-γ (10 ng/ml) and TNF-α (100 ng/ml), IL-34 (50 ng/ml) or a combination. Values are normalized to IL-34 condition. (n = 5). Kruskal-Wallis test with post-hoc Dunn's test. *p<0.05. **p<0.01. Data are represented as mean ± SEM.

(**Figure 2f-h**). Here, addition of IL-34 partially protected the endothelial cell layer, characterized by a suggested decrease in inflammatory-induced permeability (**Figure 2f-h**). Altogether, these data indicate that IL-34 partially protects the endothelial cell layer of the BBB against inflammatory-induced disruption.

In addition to inflammatory cytokines, LPS was used to compromise barrier integrity *in vitro*. Compared to treatment with IL-34 alone, LPS seemed to increase the permeability of the endothelial cell layer (**Figure 3a-c**). Co-treatment of LPS and IL-34 tended to partially protect the cell layer against LPS-induced damage (**Figure 3a-c**). In addition, this harmful stimulus was used to disrupt the *in vitro* barrier of CPE cells, representing the BCSFB. LPS seemed to increase the permeability of the layer threefold, while the addition of IL-34 tended to decrease the permeability back to vehicle levels (**Figure 3d**).

Brain barrier cells of RAG-deficient mice have an intrinsic leakiness in vitro – Our previous data have indicated that RAG-deficient (RAG$^{-/-}$) mice, characterized by a lack of mature lymphocytes, show decreased barrier integrity *in vivo* (unpublished data). Therefore, we sought to investigate whether this effect is inherent,

potentially leading to altered barrier permeability in an *in vitro* setting. Primary mBMECs derived from RAG$^{-/-}$ mice showed a similar permeability to small molecules (3 and 10 kDa, **Figure 4a,b**), but an increased permeability to large molecules (66,5 kDa, **Figure 4c**) compared to WT-derived mBMECs. Our findings suggest that *in vitro,* IL-34 treatment of RAG$^{-/-}$ cells partially repaired the integrity of this layer. Furthermore, CPE cells isolated from RAG$^{-/-}$ mice showed a suggested increase in permeability (**Figure 4d**). IL-34 treatment reversed this effect completely.

IL-34 does not alter CAM or junction expression on endothelial cells – To elucidate the underlying molecular mechanisms of the suggested BBB-protective effect of IL-34, we sought to examine any alterations of CAM expression on treated hCMEC/D3 cells using flow cytometry (**Figure 6a,b**). IL-34 alone did not cause alterations in ICAM-1 and VCAM-1 expression. Conversely, inflammatory cytokines IFN-γ and TNF-α upregulated VCAM-1, but not ICAM-1 expression. Co-treatment of the inflammatory cytokines with IL-34 did not alter the CAM expression. Next, the effect of IL-34 on AJ molecule VE-cadherin was investigated (**Figure 6c**). IFN-γ and TNF-α decreased the
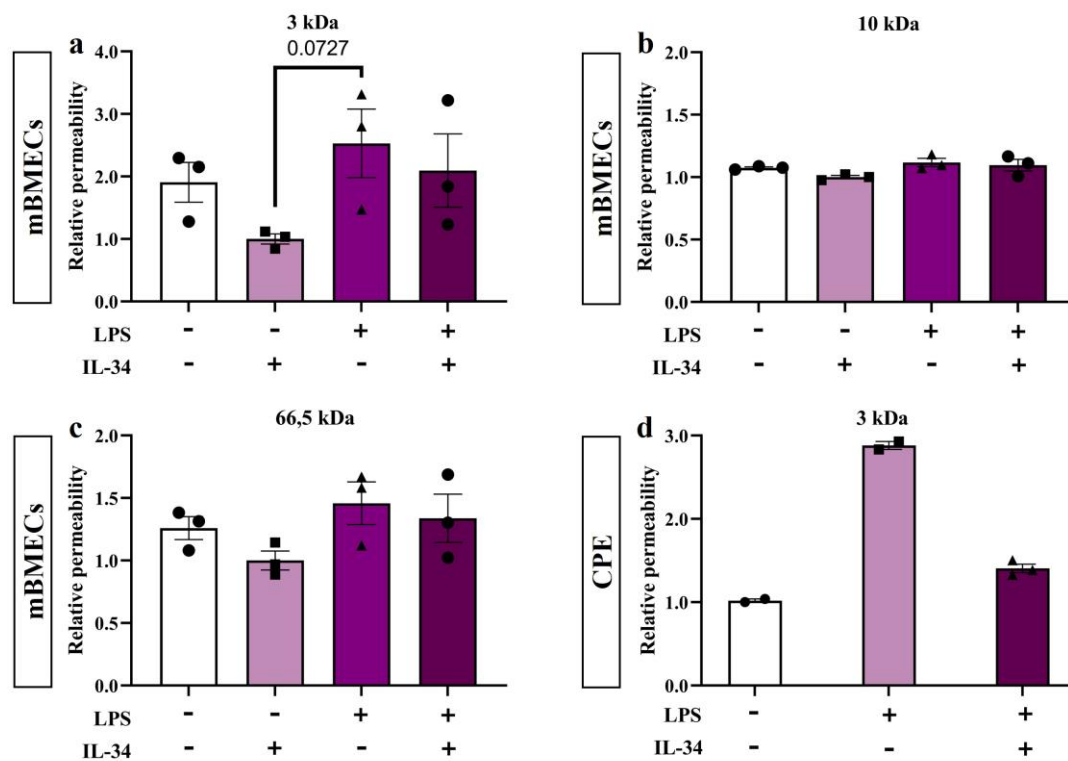

Figure 3: IL-34 is likely to protect against LPS-induced brain barrier disruption *in vitro*. a-c. Permeability of the *in vitro* barrier consisting of primary mouse brain microvascular endothelial cells (mBMECs) to different molecular weight components after 8h treatment with LPS (10 µg/ml) and/or IL-34 (50 ng/ml). Values are normalized to IL-34 condition. (n = 3). d. Permeability of choroid plexus epithelial (CPE) cells *in vitro* after LPS (1 µg/ml) and/or IL-34 (50 ng/ml) treatment. Values are normalized to the vehicle condition. (n = 2-3). Kruskal-Wallis test with post-hoc Dunn's test. Data are presented as mean ± SEM.

VE-cadherin expression, whereas co-treatment with IL-34 did not protect against the lost expression. In summary, IL-34 does not protect BBB-ECs against inflammation-induced upregulation of CAMs and downregulation of VE-cadherin.

IL-34 tends to mediate other regenerative functions – Tissue regeneration, and remyelination specifically, by Tregs has come forward in the past years ([66](#)). Using the tool GENEVESTIGATOR, we observed that murine and human OPCs express *Csf1r* mRNA ([85](#)). Therefore, the effect of recombinant IL-34 on OPC differentiation was investigated. 25 ng/ml of IL-34 tended to increase the expression of the early differentiation marker O4, while 50 ng/ml IL-34 additionally increased the expression of the late differentiation marker myelin basic protein (MBP) (**Figure 5**). 100 ng/ml IL-34 predominantly induced O4 expression. In addition, all IL-34 concentrations seemed to enhance the morphological complexity of the cells as depicted by increased branching of the cells, an indication of advanced differentiation (**Figure 5**).

Tregs produce IL-34, which is hampered in RR-MS – To investigate whether $CD4^+CD25^+CD127^{low}$ Tregs express IL-34 after 6 days of stimulation with anti-CD3, anti-CD28 and IL-2, an ELISA was performed on healthy Treg supernatant. Indeed, Treg supernatant contained IL-34 after 6 days of stimulation. However, a high donor variability was observed (0 pg/ml – 63,74 pg/ml) (**Figure 7a**). Next, alterations in IL-34 expression by various T cell subtypes were assessed using flow cytometry (gating strategy is illustrated in **Figure S1**). To start, the general IL-34 expression by lymphocytes was determined. No alterations in the percentage of $IL-34^+$ lymphocytes were observed (**Figure 7b**). It has been recognized that in the autoimmune disease MS, Tregs are disturbed in their functionality ([55](#)). Hence, we investigated whether this included an altered IL-34 response as well. A significant decrease in the percentage of $IL-34^+$ Tregs, and a suggestive decrease in expression of IL-34 in Tregs were observed in RR-MS patients compared to healthy controls (**Figure 7c,d**). Th17 ($CD4^+CCR6^+$), Th1 ($CD4^+CXCR3^+$) and Th2 ($CD4^+CCR4^+$) subsets
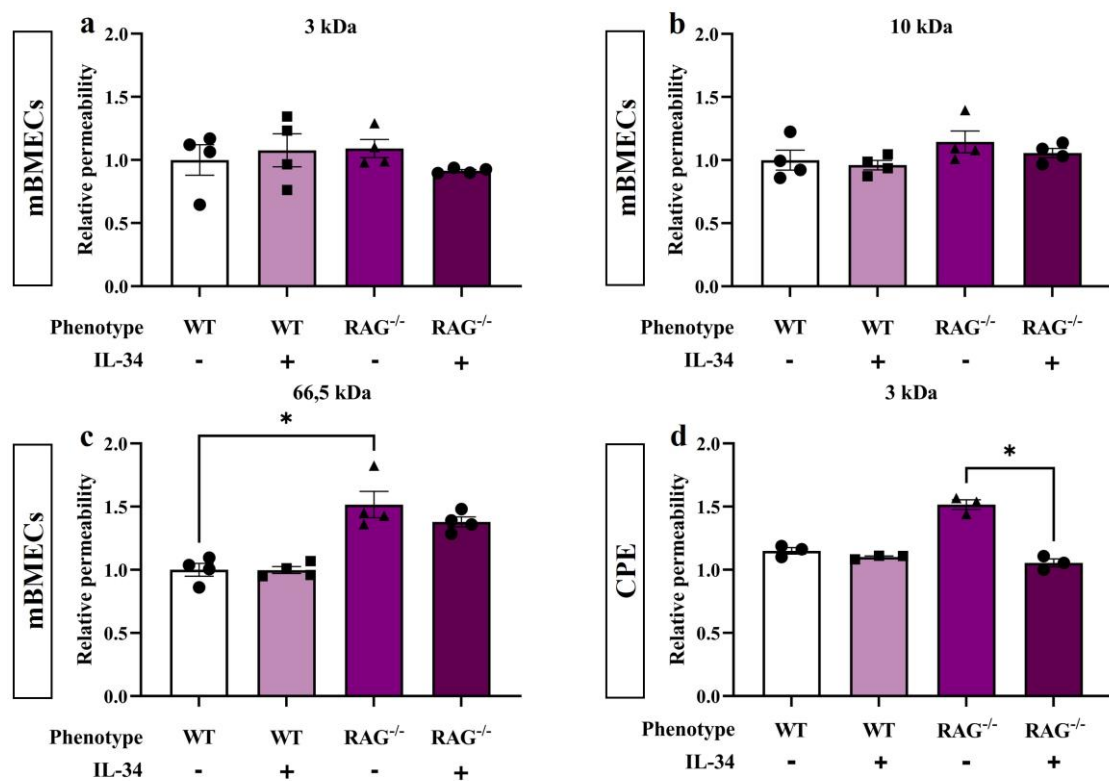

Figure 4: RAG$^{-/-}$-derived brain barrier cells show increased leakiness, which tends to be partially reversed by IL-34. a-c. Permeability of primary brain microvascular endothelial cells (mBMECs) derived from wild-type (WT) or RAG-deficient (RAG$^{-/-}$) mice, treated for 8h with vehicle or IL-34 (50 ng/ml). Values are relative to vehicle condition in WT cells. (n = 4). d. Permeability of choroid plexus epithelial (CPE) cells derived from WT or RAG$^{-/-}$ mice. (n = 3). Kruskal-Wallis test (Dunn's post-hoc test). *p<0.05. Data are represented as mean ± SEM.
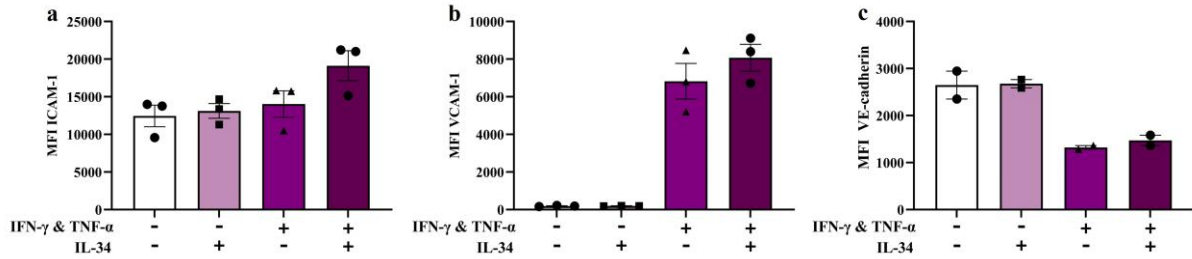
Figure 6: IL-34 does not alter the expression of CAMs or junction proteins. a,b. Mean fluorescence intensity (MFI) of cell adhesion molecules ICAM-1 (a) and VCAM-1 (b) on hCMEC/D3 cells was measured using flow cytometry after 24h treatment with either IFN-γ (10 ng/ml) and TNF-α (100 ng/ml), IL-34 (50 ng/ml) or the combination. (n = 3). c. MFI of the adherens junction (AJ) VE-cadherin on hCMEC/D3 cells measured by flow cytometry after 24h treatment. (n = 2). Data are represented as mean ± SEM. Kruskal Wallis test with Dunn's post-hoc test. Data are represented as mean ± SEM.
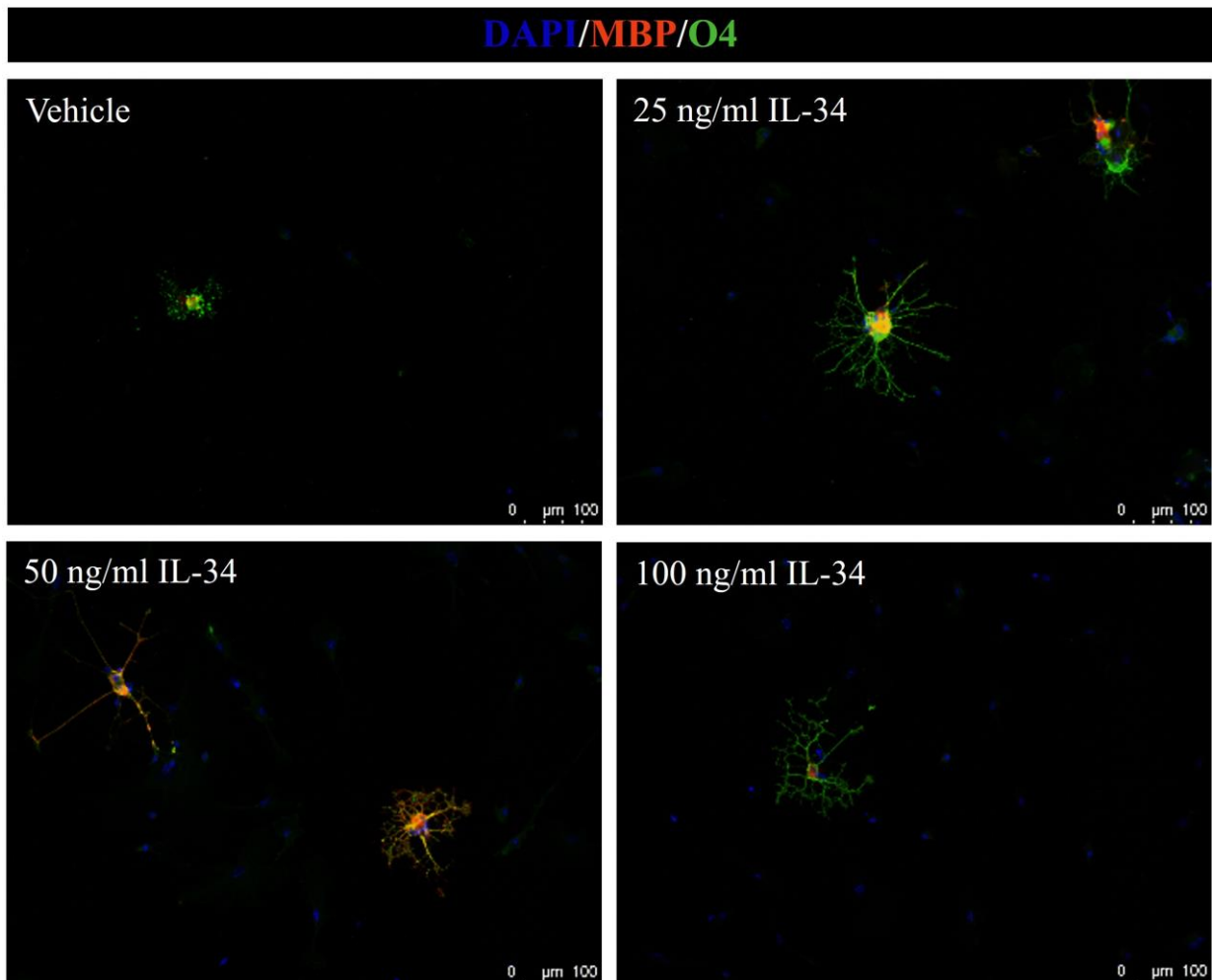

Figure 5: IL-34 seems to induce oligodendrocyte precursor cell (OPC) differentiation. Representative images of early (O4, green) and late (MBP, red) OPC differentiation markers after treatment with different concentrations of IL-34. Pictures were taken at 20x magnification, scale bars represent 100 µm.

showed no altered IL-34 expression in RR-MS (**Figure 7e-g**). In addition, IL-34 expression in $CD8^+$ T cells and monocytes was unchanged (**Figure S2**).

Il-34 *downregulation correlates with EAE severity while* Csf1r *is increased at EAE peak* – Next, it was determined whether *Il-34* and *Csf1r* mRNA levels were altered in active EAE, an animal model for MS. For this purpose, qPCR was performed on spinal cord tissue of naïve mice and mice at the peak of disease. *Il-34* expression in the spinal cord was unaltered in EAE compared to naïve mice (**Figure 8a**). However, mice with a milder disease course (score 0.5-1.5) showed a noticeable trend of higher *Il-34* expression compared to naïve mice, and a significant increase compared to EAE mice with a more severe disease (score 2-3) (**Figure 8b**). Thus, disease severity seems to negatively correlate with *Il-34* expression. On the other hand, expression of the main receptor for IL-34, the *Csf1r*, is significantly increased during the peak phase of EAE (**Figure 8c**). *Csf1r* expression is comparable between animals with low and high EAE scores (**Figure S3**). However, as this was

measured in whole spinal cord tissues, it is unclear whether the elevated *Csf1r* expression is derived from the brain barrier cells.

DISCUSSION

Brain barrier disruption is one of the key hallmarks in the initiation of MS pathology, as myelin-reactive immune cells are able to reach the vulnerable CNS tissue ([2]). Repair of these barriers is therefore a therapeutic target for halting MS in an early disease stage. Here, we aimed to determine whether Tregs have the capacity to protect the brain barriers in a neuro-inflammatory environment by producing IL-34. In short, we show that Tregs produce IL-34, and its expression by Tregs is decreased in RR-MS patients compared to healthy controls. IL-34 expression in other immune cell subsets and all lymphocytes combined is unaltered in RR-MS. In addition, our results suggest that IL-34 can partially restore barrier integrity (both BBB and BCSFB) during an inflammatory challenge. Lastly, IL-34 tends to boost OPC differentiation, an essential part of the remyelination process.
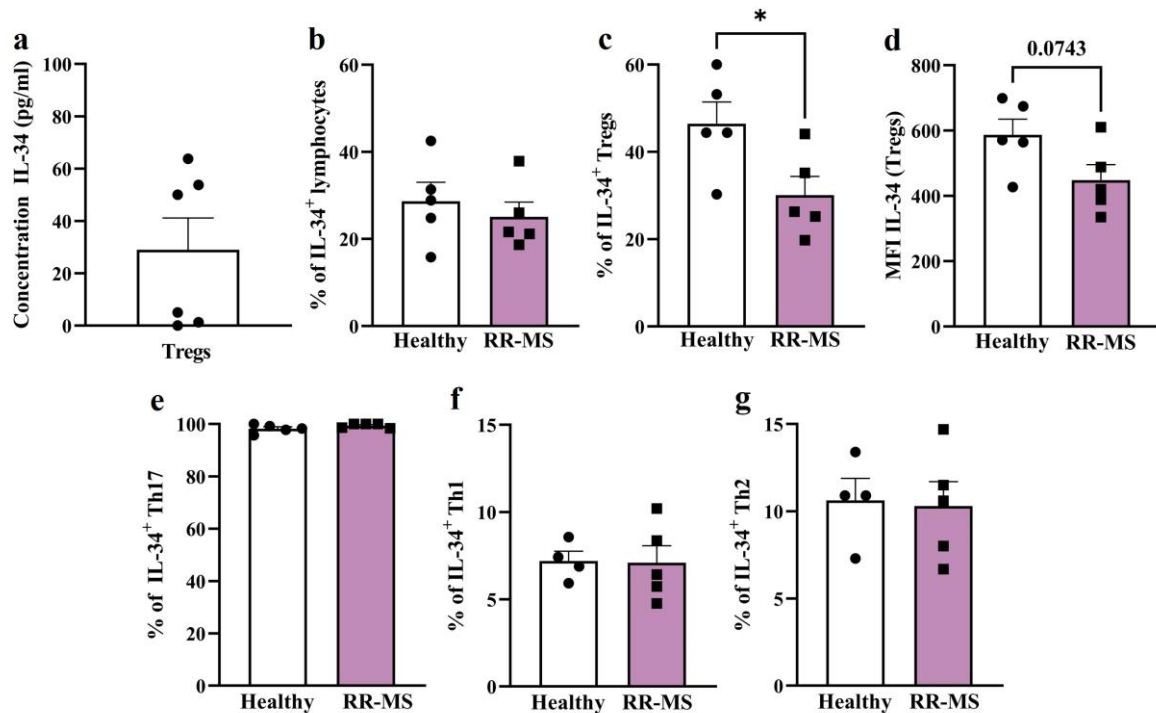

Figure 7: Human Tregs express IL-34, a feature hampered in RR-MS-derived Tregs compared to healthy controls. a. Concentration of IL-34 in human Treg supernatant after 6 days of stimulation with anti-CD3, anti-CD28 and IL-2, as measured by ELISA (n = 6, healthy donors). b-g. IL-34 expression measured by flow cytometry. b. The proportion of IL-34 expressing lymphocytes in healthy donors and RR-MS patients. c-d. The percentage of IL-34 expressing Tregs (c) and mean fluorescence intensity (MFI) of IL-34 by Tregs (d) in healthy donors and RR-MS patients. e-f. Proportion of IL-34 expressing $CCR6^+$ Th17 (e), $CXCR3^+$ Th1 (f) and $CCR4^+$ Th2 (g) subsets in healthy donors and RR-MS patients. n = 5. Unpaired t-test,*p<0.05. Data are represented as mean ± SEM. Gating strategy is displayed in **Figure S1**.
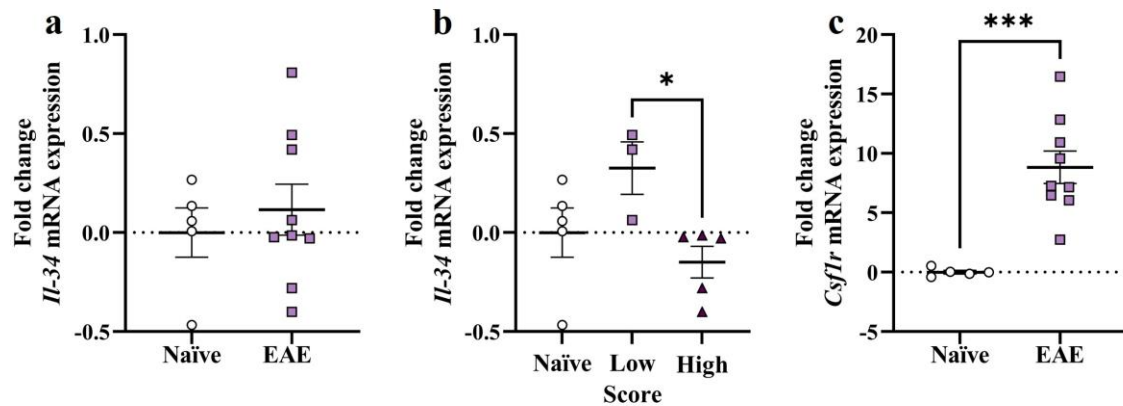
Figure 8: Disease severity negatively correlates with *Il-34* expression while *Csf1r* expression is increased during EAE peak. Naïve mice are compared to EAE mice at peak of disease. a. Fold change of *Il-34* mRNA expression in spinal cord tissue of EAE mice, (a) combined or (b) subdivided in low (0.5-1.5) vs. high (2-3) EAE score. n = 5 (naïve), n = 3 (low score), n = 5 (high score). c. Fold change of *Csf1r* mRNA expression in spinal cord of EAE mice (all scores; subdivision in **Figure S3**). n = 5 (naïve), n = 9 (EAE). Unpaired t-test (a,c) and Kruskal-Wallis test with post-hoc Dunn's test (b). *$p<0.05$, ***$p<0.001$. Data are represented as mean ± SEM.

Bézie *et al*. were the first, and only, to describe IL-34 expression by FOXP3+CD45RCloCD8+ and CD4+ Tregs, and define the cytokine as Treg-specific ([72]). Our results are consistent with theirs regarding the proportion of IL-34 expressing Tregs in healthy conditions (approximately 45%). In contrast, we found that IL-34 is also expressed by other leukocytes and is, therefore, not a Treg-specific cytokine. Only a few previous studies have focused on IL-34 levels in MS. In serum, no differences were found between RR-MS and control groups ([86]). This is in line with our findings, where no difference was observed in IL-34 expression of all lymphocytes. In the CSF, studies report opposing results concerning IL-34 levels. One study showed a downregulation in MS patients compared to healthy controls ([87]), a second study found no difference ([88]), whereas a third study indicated an increase in the MS group ([89]). In the latter study, however, the control group consisted of pseudotumor cerebri patients. This is an unsuitable control group, given that one of the characteristics of this disease is elevated CSF volume ([90]). In the autoimmune diseases systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), IL-34 levels in serum and synovial fluid (RA) were increased ([91-93]). Higher IL-34 serum levels in RA were even considered a risk factor for disease progression ([94]). In contrast, in vascular dementia, a disease also associated with BBB dysfunction ([95]), serum IL-34 levels were reduced, which was correlated with cognitive impairment ([96]). These results illustrate the complexity of IL-34's roles

in physiological and disease conditions. No published reports have investigated the effect of a specific disease state on the IL-34 expression by Tregs. Our findings indicate that in the case of RR-MS, the expression of IL-34 by Tregs is reduced. Moreover, this reduction in IL-34 expression may be further intensified by the decreased frequency of Tregs in individuals with MS ([97]).

A prior study showed that mice lacking IL-34 exhibited an accelerated EAE development but not a significant increase in disease severity ([82]). The administration of an IL-34 coding adenovirus combined with a suboptimal rapamycin dose ameliorated EAE. In contrast, our findings indicated that disease severity in EAE was correlated to a decrease in *Il-34* mRNA expression levels in CNS tissue of EAE mice. The authors hypothesized that this effect was attributed to either disturbed microglia or Treg function in IL-34-/- animals. However, this study was unable to demonstrate this hypothesis and did not investigate any differences in brain barrier disruption, as EAE is an MS disease model related to BBB disruption ([98]). Given that only the timing of EAE onset differed and not the disease score, it is possible that the absence of IL-34's protective effect resulted in a more rapid breakdown of the barriers. This would align with our *in vitro* data on the brain-barrier protective effect of IL-34.

Previous research also showed that impaired CSF1R signaling results in BBB disruption during cerebrovascular pathology ([99]). Here, mutations in the CSF1R were discovered in

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patients with the rare condition adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP). Post-mortem analysis of patients carrying this mutation displayed a disruption in claudin-5 linearity and IgG leakage, indicating BBB disruption. The authors dedicated this effect mainly to microglia-endothelial crosstalk. Nevertheless, when endothelial cells alone were treated with a CSF1R inhibitor, a decrease was seen in ZO-1 and occludin. This indicates direct effects of CSF1R signaling on BBB integrity next to the indirect effects through microglia. On the other hand, these findings are in contrast with ours, as we found that CSF1R is strongly upregulated in CNS tissue during EAE peak. Regardless, the cellular source of this increase is not clear and might not be brain barrier cells. For example, mural neurons upregulate the CSF1R after injury ([100](#)). Additionally, in NOD-EAE mice, a model for progressive MS, the CSF1R was increasingly expressed during peak and chronic stages ([88](#)), consistent with our findings in the C57BL/6-EAE model. This study correlated the increased CSF1R expression to disease progression and microglial activation. Thus, from our findings, we cannot conclude whether BBB-ECs or CPE cells also undergo this upregulated CSF1R expression during neuroinflammation. Despite a potential upregulation of the receptor in brain barrier cells, IL-34 might be sequestered by CSF1R-expressing microglia, still resulting in a loss of effect on brain barriers.

In the CP, syndecan-1, an alternative receptor for IL-34 signaling, reduces leukocyte recruitment ([101](#)). Here, they showed that syndecan-1 is expressed on the basolateral side of CP epithelium, but not on BBB or CP endothelium. EAE induction led to a loss of syndecan-1, and syndecan knock-out mice suffered more severe EAE than WT mice. With these findings in mind, we must consider the possibility that IL-34 can exert its effects through different receptors on the different brain barriers.

Furthermore, our findings imply that IL-34 has the potential to protect brain barriers against various harmful stimuli. This corresponds to previous results, which indicate a protective effect by IL-34 on a mouse brain capillary endothelial cell line MBEC4 ([83](#)). Here, IL-34 increased the TEER value in a concentration-dependent manner after the barrier was disrupted with pro-inflammatory cytokines TNF-α and IL-1β. This particular cell line, however, was shown to exhibit notable modifications in

paracellular brain barrier properties compared to primary cells, including loss of TJ expression ([102](#)). In addition, no information is available regarding the role of IL-34 in BBB or BCSFB protection in humans. Therefore, this research provides substantial value in elucidating the brain barrier-protective effect of IL-34. The underlying mechanism of this effect is yet to be unraveled. The study mentioned earlier attributed their findings of BBB protection by IL-34 to an increase in claudin-5 and occludin, but not ZO-1 ([83](#)). Our findings indicate that IL-34 could not rescue the loss of AJ VE-cadherin by pro-inflammatory stimuli. In addition, IL-34 did not reduce CAM expression on inflamed hCMEC/D3 cells, and accordingly will most likely not influence lymphocyte migration.

We have also investigated the effect of a RAG-deficient phenotype on brain barrier formation. Especially the effect on the permeability of CPE cells was noteworthy, although the permeability of BBB-ECs to large molecules was also affected. These results indicate an intrinsic barrier dysfunction due to a lack of B and T cells, including Tregs, during development. In addition, it was previously shown that the absence of lymphocytes can alter the transcriptome of oligodendrocytes ([103](#)). This implies that the RAG-deficient phenotype has implications on non-immune tissues, extending beyond its known effects associated with the immune system. Here, our results suggest that IL-34 has the capacity to restore brain barrier integrity and thus can compensate for the lack of B and T cells. Hence, this could suggest a role for Treg-derived IL-34 in pre- or postnatal development of brain barriers.

The used stimuli are well-known and validated molecules to induce barrier disruption. TNF-α and IFN-γ were shown to reduce TEER values and corresponding TJ molecules occludin (TNF-α and IFN-γ), claudin-5 (only TNF-α) and AJ molecule VE-cadherin (only IFN-γ) ([104](#), [105](#)). LPS similarly causes a downregulation of TJ molecules occludin and ZO-1 ([106](#)). Nonetheless, the barrier characteristics of the hCMEC/D3 cell line did not seem flawless at baseline, given that the permeability to dextrans of these cells was tenfold the permeability of the primary mBMECs. This is, however, in contrast with previous reports that state that this cell line has restricted permeability ([107](#)). This aspect made it more challenging to induce any notable increase in permeability, and thus is a limitation of this study. Another shortcoming is the

contradiction around the source of CSF1R upregulation in EAE CNS tissue. To elucidate the contribution of brain barrier cells in this effect, other techniques must be consulted, e.g. performing a co-staining of CD31 and CSF1R using immunohistofluorescence or flow cytometry on EAE spinal cord tissue. Valuable information would also derive from IL-34 levels in the serum from these EAE mice, given that this is the main point of contact with the brain barrier cells.

BBB protection is currently the only known tissue regenerative function of IL-34, besides its indirect effects through the modulation of immune cells. Our findings suggest an additional regenerative function, as OPC differentiation possibly leads to remyelination. We show that IL-34 treatment increased MBP and O4 intensity in OPCs, along with enhanced morphological complexity. Nevertheless, further steps must be undertaken before drawing a definitive conclusion, including determining the effect of IL-34 on OPC proliferation and migration. Previous reports indicate a lack of CSF1R mRNA and protein expression in cells of the oligodendrocyte lineage ([108], [109]). In contrast, other studies showed mRNA expression of the receptor in both oligodendrocytes and OPCs, as is depicted in the gene expression database GENEVESTIGATOR ([85]).

Altogether, this study contributes to the current knowledge of Treg regenerative functions, especially brain barrier repair. These findings could have implications for the future development of a Treg cell therapy for MS, and by extension other neurodegenerative diseases. Current therapies inhibiting lymphocyte migration to the CNS, such as sphingosine-1-phosphate (S1P) receptor modulators and natalizumab, effectively prevent relapses ([110]). This indicates that repair of the BBB might be a valuable therapeutic target. Considering their additional capacities in tissue regeneration such as remyelination ([66]), the utilization of Tregs as a cellular therapy could be the holy grail for treating MS patients.

CONCLUSION

Brain barrier integrity is crucial for brain homeostasis. In MS, this integrity is compromised leading to immune cell infiltration, demyelination and neurodegeneration. We sought to investigate whether Treg-derived IL-34 could improve brain barrier integrity. *In vitro*, our results suggest that IL-34 partially protects the BBB and BCSFB against damaging mediators such as pro-inflammatory cytokines, LPS, and intrinsic dysfunction by the RAG-deficient phenotype. The underlying mechanism is yet to be elucidated, but our results do not indicate any effects of IL-34 treatment on CAMs or VE-cadherin expression. Furthermore, our findings imply that IL-34 could induce OPC differentiation, a crucial component of remyelination. In addition, we show that Tregs express IL-34, and its expression is reduced in RR-MS patients. In CNS tissue of EAE mice, *Il-34* levels seem to inversely correlate with disease severity, whereas a significant increase in *Csf1r* was detected compared to naïve animals. The source of this upregulation, however, remains unknown. This knowledge can lead to future research on the capacity of Tregs to ameliorate MS, possibly resulting in a Treg cell therapy for MS and other neurodegenerative or autoimmune diseases.

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Author contributions – BB and PB conceived and designed the research. JV performed experiments and data analysis under the supervision of PB. JV wrote the paper and PB carefully edited the manuscript.
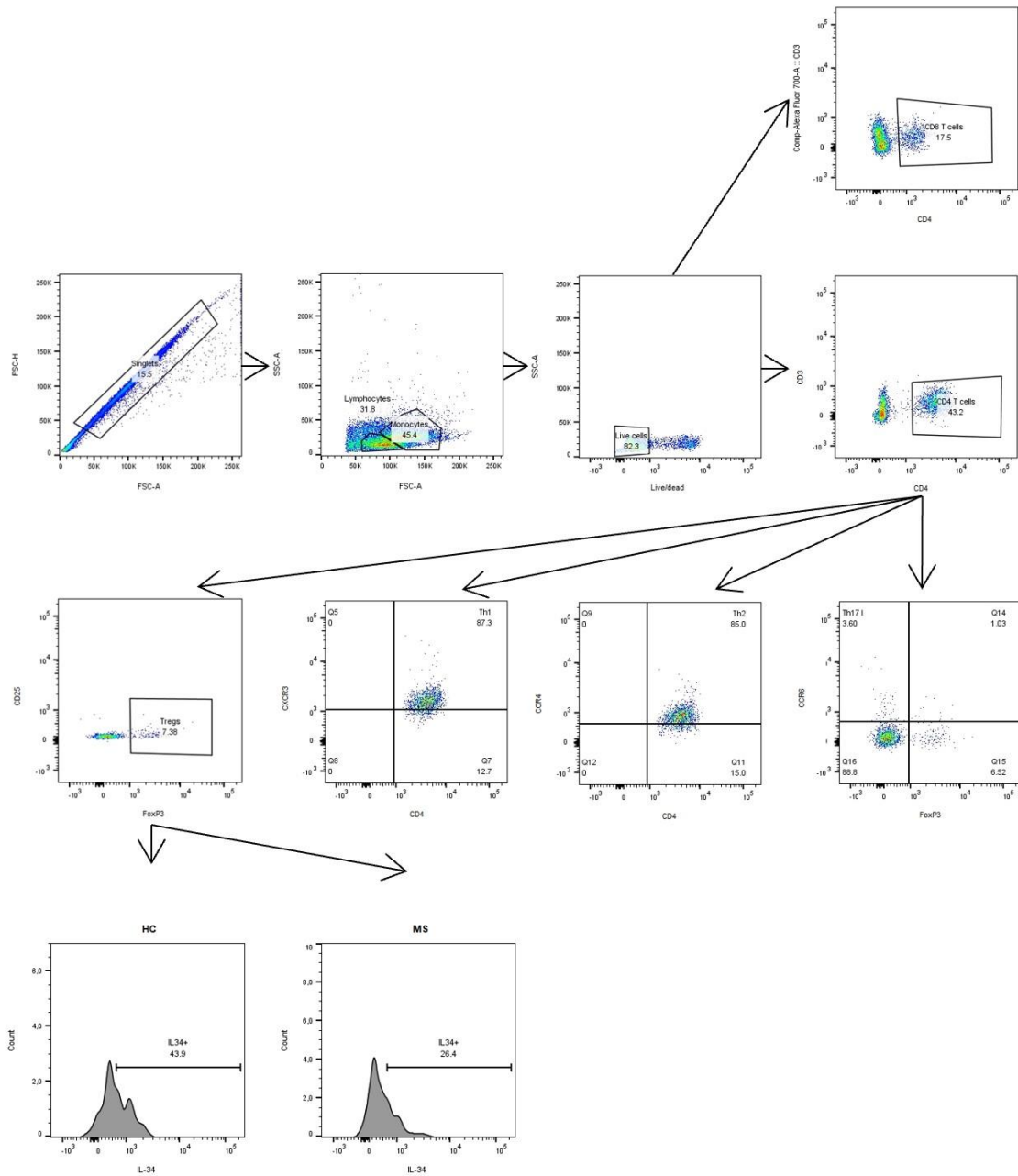
SUPPLEMENTARY FIGURES AND TABLES

Figure S1: Gating strategy for different immune cell subsets and representative histograms of IL-34 expression in Tregs. Flow cytometry was performed on PBMCs derived from healthy controls and RR-MS patients. The illustrated gating strategy was implemented to analyze IL-34 expression in different immune cell subsets. Representative plots are depicted for IL-34 gating in the Treg population.
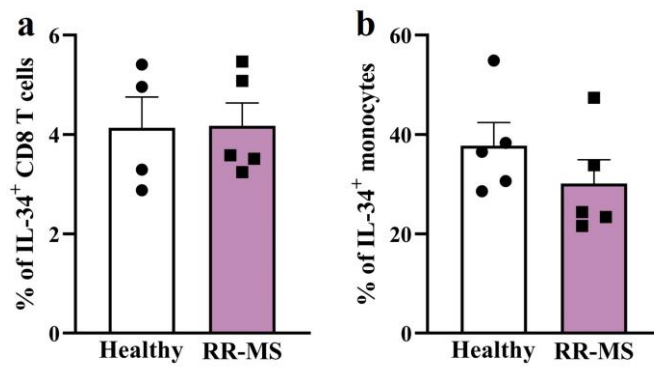
Figure S2: CD8 T cells and monocytes do not show altered IL-34 expression in RR-MS patients. IL-34 expression of CD8 T cells (a) and monocytes (b) in healthy donors and RR-MS patients as measured by flow cytometry. n = 5. Unpaired t-test. Gating strategy is shown in **Figure S1**.
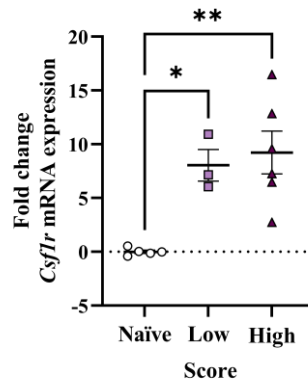

Figure S3: *Csf1r* expression is unaltered between low and high score EAE. Fold change of *Csf1r* mRNA expression in spinal cord tissue of EAE mice, subdivided in low (0.5-1.5) vs. high (2-3) EAE score. n = 5 (naïve), n = 3 (low score), n = 6 (high score). One-way ANOVA with post-hoc Tukey test. *$p<0.05$, **$p<0.01$

Table S2: Primer sequences for qPCR.

Gene	Forward primer	Reverse primer
Il-34	5'-CTTTGGGAAACGAGAATTTGGAGA-3'	5'-GCAATCCTGTAGTTGATGGGGAAG-3'
Csf1r	5'-GCAGTACCACCATCCACTTGTA-3'	5'-GTGAGACACTGTCCTTCAGTGC-3'
Ywhaz	5'-GCAACGATGTACTGTCTCTTTTGG-3'	5'-GTCCACAATTCCTTTCTTGTCATC-3'
Hprt	5'-CTCATGGACTGATTATGGACAGGAC-3'	5'-GCAGGTCAGCAAAGAACTTATAGCC-3'

Table S1: Donor characteristics.

	Healthy	**RR-MS**
Number	5	5
Gender (M/F)	2/3	2/3
Age ± SEM	32.3 ± 2.267	32.6 ± 3.187
EDSS ± SEM	NA	1.375 ± 0.375

RR-MS, relapsing-remitting MS; M, male; F, female; SEM, standard error of the mean; EDSS, expanded disability status scale; NA, not applicable.