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ALCAM: cell adhesion molecule or tight junction?

The characterization of its role in the context of neuroinflammation

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Résumé

But : La perte de l'intégrité de la barrière hémo-encéphalique (BHE) est l'une des caractéristiques principales de la sclérose en plaques. Cette augmentation de la perméabilité est associée à une désorganisation des molécules de jonction serrée et à une augmentation de l'expression de molécules d'adhérence essentielles à l'extravasation des cellules immunitaires. Identifier de nouvelles molécules impliquées dans ce processus est donc crucial pour le développement de nouvelles thérapies contre la sclérose en plaques visant à promouvoir l'intégrité de la BHE et à diminuer la migration des leucocytes dans le système nerveux central (SNC) au cours du processus neuro-inflammatoire. Dans cette étude, le rôle spécifique de la molécule d'adhérence ALCAM, qui est exprimé à la surface des cellules endothéliales de la BHE (CE-BHE) et de certains sous-types de leucocytes, a été évalué.

Méthodologie : À l'aide d'une analyse protéomique exhaustive, notre laboratoire a identifié ALCAM comme étant une molécule d'adhérence surexprimée par les CE-BHE mises en culture dans un milieu pro-inflammatoire. Dans le but d'étudier le rôle spécifique d'ALCAM durant la diapédèse leucocytaire, nous avons induit chez des souris de type sauvages et des souris ALCAM déficientes l'encéphalite auto-immune expérimentale (EAE), le modèle animal de la sclérose en plaques. Le rôle d'ALCAM a aussi été étudié à l'aide d'un système d'adhérence sous flux laminaire. Cet appareil, qui imite un capillaire cérébral, permet de suivre en temps réel le mouvement des leucocytes, soumis à une pression physiologique, dans un tube couvert à sa base par des CE-BHE.

Résultats : En utilisant ce système d'adhérence, j'ai pu démontrer que des anticorps dirigés contre ALCAM réduisent de façon significative le roulement et l'adhérence de monocytes CD14+ humains à la surface de CE-BHE. Par ailleurs, ces anticorps préviennent de façon marquée la diminution de la vitesse moyenne des cellules au cours de l'expérience. Par le fait même, j'ai aussi observé une réduction significative de l'extravasation des monocytes traités avec de l'anti-ALCAM au travers de CE-BHE dans un modèle statique de migration. Subséquemment, j'ai démontré que ces monocytes migrent plus rapidement et en plus grand nombre au travers d'une barrière constituée de cellules endothéliales méningées à comparer à des CE-BHE. Bien que des observations similaires ont été effectuées en utilisant des

lymphocytes T CD4+ humains *ex vivo*, j'ai été incapable de reproduire ces résultats à l'aide de cellules Th1 et Th17 réactivées *in vitro*.

Par opposition à nos données *in vitro*, j'ai découvert que les souris déficientes en ALCAM développent une EAE active plus sévère que celle observée chez des souris de type sauvages. Cette EAE est par ailleurs associée à une infiltration périvasculaire de lymphocytes T pro-inflammatoires et de monocytes/macrophages de type M1 plus marqué chez les souris ALCAM déficientes. L'induction d'une EAE par transfert adoptif, dans laquelle des cellules immunitaires de type sauvage réactivées par du MOG sont injectées à des souris déficientes en ALCAM, suggère que la pathophysiologie observée durant l'EAE active serait liée à l'absence d'ALCAM au niveau de la BHE. Une caractérisation de la barrière des souris ALCAM déficientes non immunisées a par la suite révélé une réduction de l'expression de certaines molécules de jonction serrée. Une analyse plus poussée a par ailleurs démontré qu'ALCAM est lié indirectement à des molécules de jonction serrée des CE-BHE, ce qui expliquerait l'augmentation de la perméabilité de celle-ci chez les souris déficientes en ALCAM. Une analyse de la perméabilité intercellulaire de la BHE effectuée *in vitro* a d'autre part corrélé ces résultats.

Conclusion : Collectivement, nos données prouvent qu'ALCAM joue un rôle prépondérant dans la diapédèse des monocytes, mais pas des lymphocytes Th1 et Th17 au travers de la BHE. Par ailleurs, nos résultats suggèrent qu'ALCAM remplit une fonction biologique cruciale favorisant le maintien de l'intégrité de la BHE en agissant comme molécule adaptatrice intermédiaire entre les molécules de jonction serrées et le cytosquelette. De cette façon, l'absence d'ALCAM au niveau des CE-BHE promeut indirectement le recrutement de leucocytes pro-inflammatoires dans le SNC des souris atteintes de l'EAE en augmentant la perméabilité des vaisseaux sanguins de la BHE.

Mots-clés : Activated leukocyte cell adhesion molecule (ALCAM), sclérose en plaques (SEP), encéphalomyélite auto-immune expérimentale (EAE), barrière hémo-encéphalique (BHE), diapédèse leucocytaire, molécules de jonction serrée, neuroinflammation.

Abstract

Aim: The loss of blood-brain barrier (BBB) integrity is a hallmark of multiple sclerosis. It is associated with a disorganization of junctional molecules and an upregulation of cell adhesion molecules essential for immune cell transmigration. Identifying novel key players involved in this process is thus crucial for the development of MS therapies aimed at promoting BBB integrity and decreasing leukocytes trafficking into the central nervous system (CNS) during neuroinflammation. In this study, the specific role of the adhesion molecule ALCAM, found on BBB endothelial cells (BBB-ECs) and subsets of leukocytes, was assessed.

Methods: We first identified ALCAM as an important molecule upregulated during inflammation in a proteomic screen of *in vitro* cultured primary human BBB-ECs. In order to study the effects of ALCAM on leukocyte transmigration, both active and passive experimental autoimmune encephalomyelitis (EAE) was induced in ALCAM KO and WT animals. The specific role of ALCAM during leukocyte transmigration was also assessed using a modified adhesion assay under sheer-stress, in which leukocytes flow across a capillary-like channel lined with a monolayer of BBB-ECs under physiological pressure.

Results: Using the modified adhesion assay, we demonstrated that anti-ALCAM blocking antibodies significantly reduce the rolling and the adhesion of human CD14+ monocytes interacting with primary human BBB-ECs, as well as prevent their overall decrease in velocity. Concurrently, we also observed a significant reduction in the migration of *ex vivo* CD14+ monocytes, across a monolayer of human BBB-ECs. These monocytes also migrated more rapidly and in higher number across meningeal endothelial cells, as compared to BBB-ECs. While similar observations were made using *ex vivo* CD4+ T lymphocytes, we failed to reproduce these results using *in vitro* activated Th1 and Th17 cells. In opposition to our *in vitro* data, ALCAM KO mice developed a more severe active EAE associated with a significant increase in perivascular infiltration of pro-inflammatory lymphocytes (Th1/Th17) and M1 monocytes/macrophages, as compared to WT controls. In addition, EAE transfer experiments, in which ALCAM KO mice received WT MOG-reactivated splenocytes, suggested that the pathophysiology observed in active EAE was linked to the absence of ALCAM on BBB-ECs. Phenotypic characterization of un-immunized ALCAM KO mice revealed a reduced expression of BBB junctional proteins. Further analysis showed that ALCAM is indirectly associated with

tight junction molecules of the BBB-ECs, which explains the increased CNS parenchymal blood vessel *in vivo* permeability in ALCAM KO animals. Correlating with these data, primary culture of mouse brain BBB-ECs was shown to possess a lower TEER and an increased permeability coefficient.

Conclusion: Collectively, our data provide evidence of the implication of ALCAM in monocyte transmigration, but not Th1 or Th17 lymphocyte diapedesis across CNS endothelium. Our results also point to a biologically crucial function of ALCAM in maintaining BBB integrity by acting as an adaptor molecule between tight junctions and the cytoskeleton. As such, the absence of ALCAM at the level of BBB-ECs indirectly promotes the recruitment of pro-inflammatory leukocytes in the CNS of EAE animals by increasing the BBB vessels permeability.

Keywords: Activated leukocyte cell adhesion molecule (ALCAM), multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), blood-brain barrier (BBB), leukocyte transmigration, tight junction molecules, neuroinflammation.

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Liste des abréviations

AD: Alzheimer’s disease

AGT: Angiotensinogen

AHR: aryl hydrocarbon receptor

AJ: adherens junction

ALCAM: activated leukocyte cell adhesion molecule

ANG: Angiopoietin

APC: antigen presenting cell

AQP4: water channel aquaporin 4

BBB: blood-brain barrier

BCB: blood-CSF barrier

BDNFs: brain-derived neurotrophic factors

BM: basement membrane

BMB: blood-meningeal barrier

CAMs: cell adhesion molecules

CAR: coxsackie and adenoviral serotype 2/5 receptor

CCL: chemokine C-C motif ligand

CCR: chemokine C-C motif receptor

Cdc42: cell division control protein 42 homolog

CFA: complete Freund’s adjuvant

CIS: clinically isolated syndrome

CNS: central nervous system

CSF: cerebrospinal fluid

CTL: Cytotoxic T Lymphocyte

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

CXCL: chemokine C-X-C motif ligand

DC: dendritic cell

EAE: experimental autoimmune encephalomyelitis

EBV: Epstein-Barr virus

EC: endothelial cell

E-cadherin: epithelial-cadherin

EDSS: expanded disability status scale

EMMPRIN: extracellular matrix metalloproteinase inducer

ERM: ezrin/radixin/moesin

ESAM: endothelial cell-selective adhesion molecule

ESL1: E-selectin ligand 1

FGFs: fibroblast growth factors

FLAIR: fluid attenuated inversion recovery

Foxp3: forkhead box P3

GDNF: Glia-derived neurotrophic factor

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

Glut1: Glucose transporter 1

GM-CSF: granulocyte-macrophage colony-stimulating factor

GzB: granzyme B

H&E/LFB: Hematoxylin & Eosin / Luxol Fast Blue

HLA: Human Leukocyte Antigen

HMGB-1: high–mobility group box 1

i.p.: intra-peritoneal

i.v.: intravenous

ICAM-1: intercellular adhesion molecule 1

IFNγ: interferon gamma

Ig: immunoglobulin

IL: interleukin

IP-10: interferon gamma-induced protein 10

iTregs: Induced Treg

JAM: junctional adhesion molecule

JCV: John Cunningham virus

Kb: kilobases

kDa: kiloDalton

Kir4.1: inwardly rectifying potassium channel 4.1

KO: knockout

LFA-1: Lymphocyte function-associated antigen 1

LN: lymph node

LPS: lipopolysaccharide

MAG: myelin-associated glycoprotein

MAGUK: membrane-associated guanylate kinase protein family

MAPK: mitogen-activated protein kinase

MBP: myelin basic protein

MCAM: melanoma cell adhesion molecule

MCP-1: monocyte chemoattractant protein-1

MHC: major histocompatibility complex

MMP: matrix metalloproteinases

MOG: myelin oligodendrocyte glycoprotein

MRI: magnetic resonance imaging

MS: multiple sclerosis

MTR: magnetization transfer ratio

NAWM: normal appearing white matter

N-cadherin: Neural cadherin

NGF: nerve growth factors

NK: Natural Killer

NOS: Nitric oxide synthase

nTregs: natural T regulatory lymphocytes

NVU: neurovascular unit

OAPs: orthogonal arrays of particles

OCT: optical coherence tomography

OPCs: oligodendrocyte precursor cells

OSM: oncostatin M

PBMC: peripheral blood mononuclear cells

PD: proton density

PDGF-BB: Platelet Derived Growth Factor B homodimers

PDGFR-β: Platelet-derived growth factor receptors β

PECAM-1: Platelet endothelial cell adhesion molecule 1

Pgps: Permeability glycoproteins

PLP: proteolipid protein

PML: progressive multifocal leukoencephalopathy

PMVECs: pulmonary microvascular endothelial cells

PPMS: primary progressive multiple sclerosis

PRMS: progressive-relapsing multiple sclerosis

PRRs: pattern recognition receptors

PSGL1: p-selectin glycoprotein ligand 1

Ptch: Patched-1

R: receptor

RA: retinoic acid

Rac1: Ras-related C3 botulinum toxin substrate 1

RAGE: receptor for advanced glycation end-products

RANTES: regulated on activation, normal T cell expressed and secreted

RBCs: red blood cells

RhoA: Ras homolog gene family member A

ROS: reactive oxygen species

RRMS: relapsing-remitting multiple sclerosis

sALCAM: soluble ALCAM

Shh: Sonic hedgehog

Smo: Smoothened

SPMS: secondary progressive multiple sclerosis

SRCR: scavenger receptor cysteine-rich

TAMP: TJ-associated MARVEL proteins

Tc: T cytotoxic

TCR: T cell receptor

TEER: trans-endothelial electrical resistance

TGF-β: Transforming Growth Factor-β

Th: T helper

TJ: tight junction

TJaCAMs: tight junction-associated cell adhesion molecules

TLR: Toll-like receptor

TNF: Tumor necrosis factor

Treg: T regulatory lymphocyte

TYMP: thymidine phosphorylase

UV: ultraviolet

VCAM: vascular cell adhesion molecule

VE-cadherin: vascular endothelial-cadherin (cadherin 5)

VEGF: vascular endothelial growth factor

VE-PTP: vascular endothelial protein tyrosine phosphatase

VLA-4: very late antigen 4 (integrin α4β1)

ZO: Zonula occludens

À ma famille et amis qui ont toujours été là pour moi
et sans qui je n'y serais pas arrivé.

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Introduction

The central nervous system (CNS) has eluded many generations of scientists, clinicians and scholars owing to its inherent complexity and dynamic environment. While somewhat paradoxical, for generations, we could not understand the origin of our thoughts, nor the process behind it. With the rapid development of new technologies, we are slowly beginning to understand the genesis of new memories and abilities, while gradually deciphering innate capabilities both at a molecular and cellular levels. Although these scientific breakthroughs have allowed us to understand, as of now, a fraction of the CNS processes in homeostatic conditions, they have also fashioned the medical and pharmaceutical field in the context of CNS diseases.

The inflammation of the CNS can be induced by a wide variety of events, including infections, autoimmunity, traumatic brain injury and accumulation of misfolded proteins or toxic metabolites. Persistence of the inflammatory stimuli and/or failure to resolve the acute inflammatory response can lead to chronic inflammation of the CNS. Under homeostatic conditions, the CNS is widely regarded as an immunologically privileged site and its environment is well controlled and balanced by the presence of the neurovascular unit (NVU), which comprises specialized endothelial cells (ECs), pericytes, basement membranes and supporting glial cells, including astrocytes and microglia. The NVU, acting as a blood-brain barrier (BBB), restricts the ingress of peripheral leukocytes and the movement of soluble factors into the CNS. For reasons yet unknown, the immune system of some individuals can gain access to the CNS and start targeting some of its components, ultimately leading to an autoimmune disease called multiple sclerosis (MS). While MS has been studied for decades, we still haven't discovered a cure. This challenging scientific endeavour is the premise behind this thesis.

† *Parts of the introduction are based on the article "Glial influences on BBB functions and molecular players in immune cell trafficking" published in Biochimica et Biophysica Acta and written by myself.*

1. The Central Nervous System

The CNS is composed of two main groups of cells: the neurons, which transport electrical and chemical signals from one cell to the next, and the glial cells, which support the neurons and maintain an homeostatic environment in normal conditions. Astrocytes, oligodendrocytes and microglia (along with some progenitor/stem cells) are grouped under the latter category of cells. The most numerous cell types, the astrocytes, can be divided in sub-categories based on their location and morphology in the CNS, but ultimately, they all provide a physical support to the other cells, while maintaining a suitable extracellular environment for neurons (especially in neuronal-synapses). The oligodendrocytes are specialized cells that wrap around neuronal projections (the axons) of several neurons, and form lipid-rich multi-sheeted isolation membranes called myelin sheets. These sections of myelin sheets improve the rate of electrical signal propagation along the axons and are necessary to the proper functioning of the CNS [1]. Microglia are the resident macrophages of the CNS. They help maintain the neuronal network via synaptic pruning and also provide the innate immune response in case of pathogen infiltration or traumatic injury. Whereas all the previous CNS cells originate from the neuroectoderm, microglia have a mesodermal origin. Recently, they were shown to be derived from primitive erythromyeloid progenitor cells, which invade the brain from the yolk sac at a very early stage of embryonic development [2-4].

1. The CNS Vasculature

Neurons have a high requirement for oxygen and nutrients. In fact, about 20% of the oxygen and 25% of the glucose consumed by an individual are dedicated to the CNS and yet it constitutes only about 2% of the total body mass [5]. The CNS therefore requires a high volume of blood, which is carried by a dense network of highly regulated specialized vessels that provide the essential nutrients, while also restricting the entry of soluble factors. The vascular system carries freshly oxygenated blood via the two carotid arteries and the two vertebral arteries to the

circle of Willis. From there, cerebral arteries irrigate blood vessels contained within the subarachnoid space, a sub-section of the meningeal tissue surrounding the CNS (**Figure 1**).

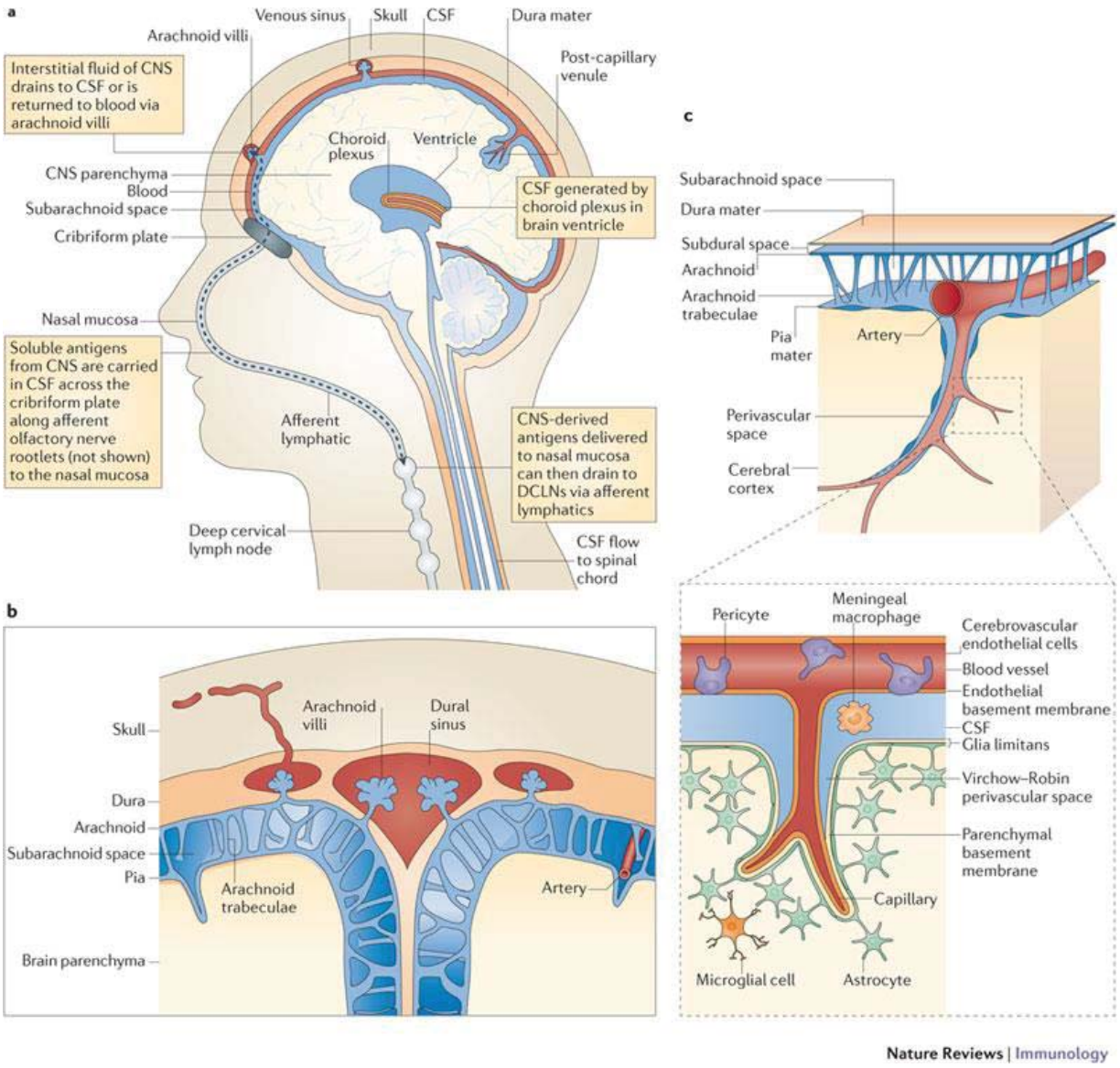

Figure 1 – The vascular system and CSF compartments of the CNS. A Image showing a transversal cut of a human head with the CSF depicted in blue. The image also outlines two areas where the CSF can drain and return to the blood: the cribriform plate and the arachnoid villi, also shown in **B**. **C** Image of a transversal cut of the different meningeal layers, also

showing the vicinity of a blood vessel penetrating the CNS. Image from Ransohoff and Engelhardt [6].

Branching blood vessels then penetrate into the CNS parenchyma. The perivascular space found between the arterioles and the invaginating pia matter is called the Virchow-Robin space. It is an area where immune cells (meningeal macrophages or peripheral leukocytes) can accumulate during CNS inflammation. Deeper into the parenchyma, arterioles and capillaries are constituted of specialized endothelial cells forming the blood-brain barrier, which will be further discussed in the following chapters. Produced by ECs on their abluminal side, the endothelial basement membrane is fused with the parenchymal basement membrane. In post-capillary venules, however, these two basement membranes can separate to form a perivascular space where infiltrating immune cells preferentially accumulate before trafficking to the parenchyma. As this region lies outside the parenchymal basement membrane, which form a continuous boundary with the glia limitans, the perivascular space is considered outside the CNS parenchyma. It can therefore be populated by meningeal macrophages under homeostatic conditions, which can sample the interstitial fluid draining toward the surface of the CNS into the cerebrospinal fluid (CSF) [6].

The CSF is carried by another network of compartments present in the CNS. This fluid occupies the subarachnoid space, the ventricles, the cisterns and sulci of the brain, as well as the central canal of the spinal cord. Under homeostatic conditions, the CSF moves within the CNS ventricular system carrying ions, glucose and some patrolling immune cells, but very few soluble proteins. The CSF also helps protect the CNS from impacts and abrasion, while adjusting to the changes in brain volume and intracranial pressure. Furthermore, it participates in the CNS environment stability by carrying away metabolic wastes and lipid-insoluble compounds. It is generated by the choroid plexus in the ventricles of the brain, where liquid from the blood permeates the fenestrated blood vessels and the ependymal cells by osmotic pressure. Since the CSF is constantly being produced, an equivalent portion is therefore continually reabsorbed. The reabsorption of the CSF by the blood can occur in the dural venous sinuses via the arachnoid villi. Besides this pathway, the CSF was demonstrated to flow through the cribriform plate, into

the nasal submucosal lymphatic channels and reaching the deep cervical lymph nodes (LN). This CSF egress is believed to be significantly important for the presentation of CNS antigens to peripheral immune cells [6-9]. While very few laboratories have studied leukocytes egress from a healthy or inflamed CNS, two recent studies have provided evidence suggesting that B lymphocyte egress from the CNS is possible [10, 11]. Using CNS tissue and draining cervical lymph nodes from the same patients, the two groups have demonstrated that B cell clones are shared between the two compartments. In addition, they demonstrated that, from the founding clones, subsequent maturation appeared to take place in the cervical lymph nodes rather than the CNS and involved a bidirectional exchange. The CSF flow towards the cervical LN and this bidirectional exchange could therefore play an important role in epitopes spreading, a phenomenon well described in animal studies whereby the antigenic targets of the CNS inflammatory attack shift over time as injury exposes additional epitopes [10-12]. Additionally, the recent rediscovery of the "CNS lymphatic system", dural lymphatic vessels, have reminded us that leukocytes and/or CNS antigens could also circulate from the subarachnoid space to the deep cervical LN via these vessels [11, 13-15]. Although this thesis will mainly focus on microvascular blood vessels, it is imperative to acknowledge the importance of the CSF and it's carrying compartments in the CNS homeostasis and during neuroinflammation.

2. Formation of the Blood-Brain Barrier

CNS blood vessel formation starts with the invagination of the perineural vascular plexus. Tip cells guide the outgrowing capillaries towards a vascular endothelial growth factor (VEGF) gradient to form vascular sprouts, in a process known as angiogenesis [16, 17]. At this early stage of neurovascular development, the primitive network of CNS vessels lacks functional barrier properties and proper organization of tight junction (TJ) complexes. Multiple signalling molecules and transcription factors, notably produced by the radial glial cells, are involved in the establishment of the anteroposterior and dorsoventral axes, which are essential during embryogenesis [18]. Many of these factors, in addition to VEFG, are involved during angiogenesis: these include Notch1, Wnt, ephrins, fibroblast growth factors (FGFs), Sonic Hedgehog and retinoic acid (RA) [19]. In conjunction, all these signalling pathways remodel and stabilize the embryonic vasculature and play a major role during the development of the BBB [20-25]. Indeed, BBB properties are not intrinsic to CNS-ECs, but emerge gradually during a maturation/specialization phase dependent on the association of ECs that line the newly formed vessels with perivascular glial cells and pericytes. This was evidenced by transplantation experiments in which peripheral vessels acquired functional and histochemical BBB features following prolonged contact with neuronal tissue [26]. In contrast, meningeal blood vessels, which express TJs but are not in direct contact with glial cells, display higher vascular permeability, as compared to BBB-ECs [23].

Pericytes are known to promote BBB properties in ECs. EC−pericyte interactions are primarily mediated by secreted Platelet Derived Growth Factor B homodimers (PDGF-BB), Notch, Transforming Growth Factor-β (TGF-β), and by N-cadherin homotypic interactions [27]. Gap junctions formed by pairs of connexin 43 hemichannels also allow direct molecular communication between the cytoplasm of pericytes and BBB-ECs. Of note, in larger blood vessels, smooth muscle cells replace pericytes and form a continuous layer surrounding ECs [27].

Although the ratio of pericytes to ECs is highest in the CNS microvasculature, the critical role of astrocytes in regulating BBB maintenance and integrity cannot be overlooked. Histological studies using fetal human brain tissue have demonstrated the presence of astrocytes in the CNS around the 9th week of gestation, and direct astrocyte-endothelial cell contact 8

weeks later [28, 29]. In the fully developed CNS, perivascular astrocytic end-feet almost completely ensheath the abluminal surface of the CNS microvasculature [30] (**Figure 2**).

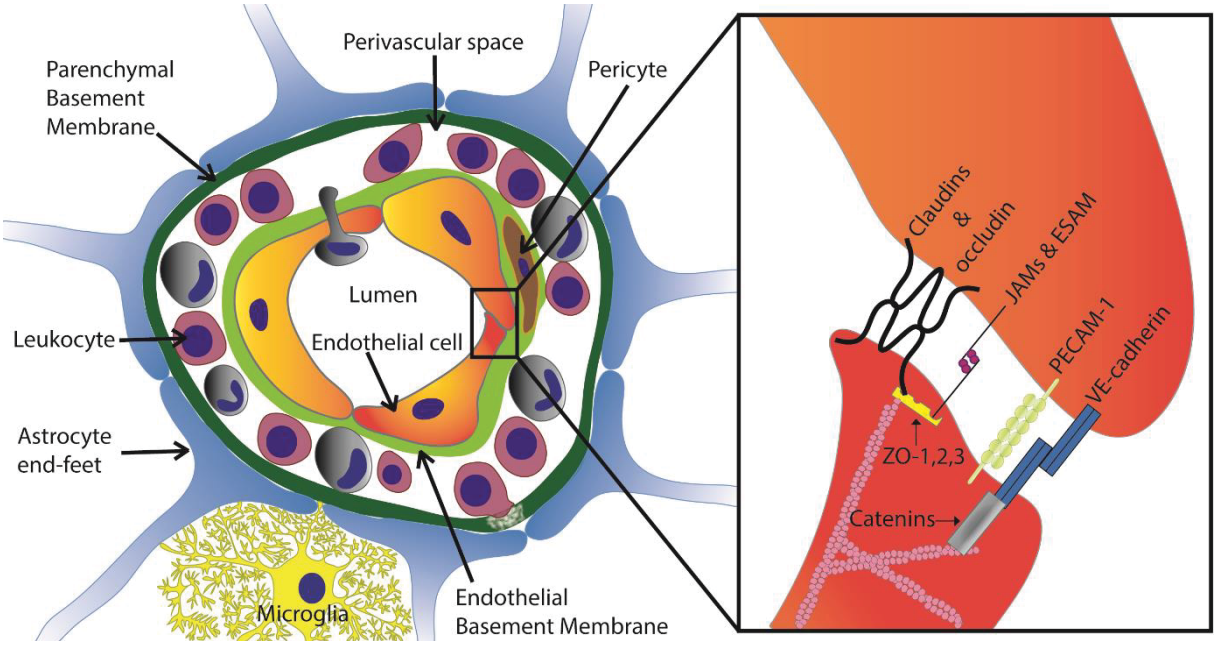

Figure 2 – The neurovascular unit (NVU). The NVU is composed of specialized endothelial cells (ECs) surrounded by two basement membranes and perivascular astrocytes and microglia. Essential in maintaining the blood-brain barrier integrity, the different tight junction and adherens junction molecules efficiently link together adjacent ECs, as shown in the magnified window. In the context of neuroinflammation, leukocytes accumulate in the perivascular space located in-between both basement membranes. Upon acquiring the capacity to secrete matrix metalloproteinases (MMPs), these leukocytes migrate into the central nervous system. *ZO-1, -2, -3: Zonula occludens / PECAM-1: Platelet endothelial cell adhesion molecule 1 / VE-cadherin: vascular endothelial-cadherin (cadherin 5).* Image from Lécuyer et al. [31].

Although astrocytes are separated from pericytes and ECs by a thin and compact double basement membrane (BM) (endothelial BM and parenchymal BM), connexins allow direct communication between these cellular constituents of the NVU [27]. In fact, astrocytes are responsible for inducing proper relative positioning of pericytes and ECs in tube-like structures *in vitro* [32] and are known to impact on BBB-ECs via different molecular pathways. Our group

has shown that astrocyte-conditioned media induces or re-establishes partial BBB properties to *in vitro* cultures of meningeal and BBB-ECs, respectively [33, 34] (and non-published data).

Finally, beyond their important role during CNS neuronal development, microglial cells also contribute to CNS angiogenesis by promoting tip cell fusion and migration [35]. Evidence brought forward by Tammela et al. [36] shows that microglia-derived VEGF-C activates VEGF receptor-3 (VEGFR-3) in tip cells and promotes angiogenesis and vascular branching. Like astrocytes, microglia are found in close proximity to the mature CNS vasculature, suggesting their involvement in regulating BBB functions in adults (**Figure 3**). But whereas numerous studies have focused on the role of microglia in BBB breakdown, few have addressed their potential contribution to maintaining BBB properties.

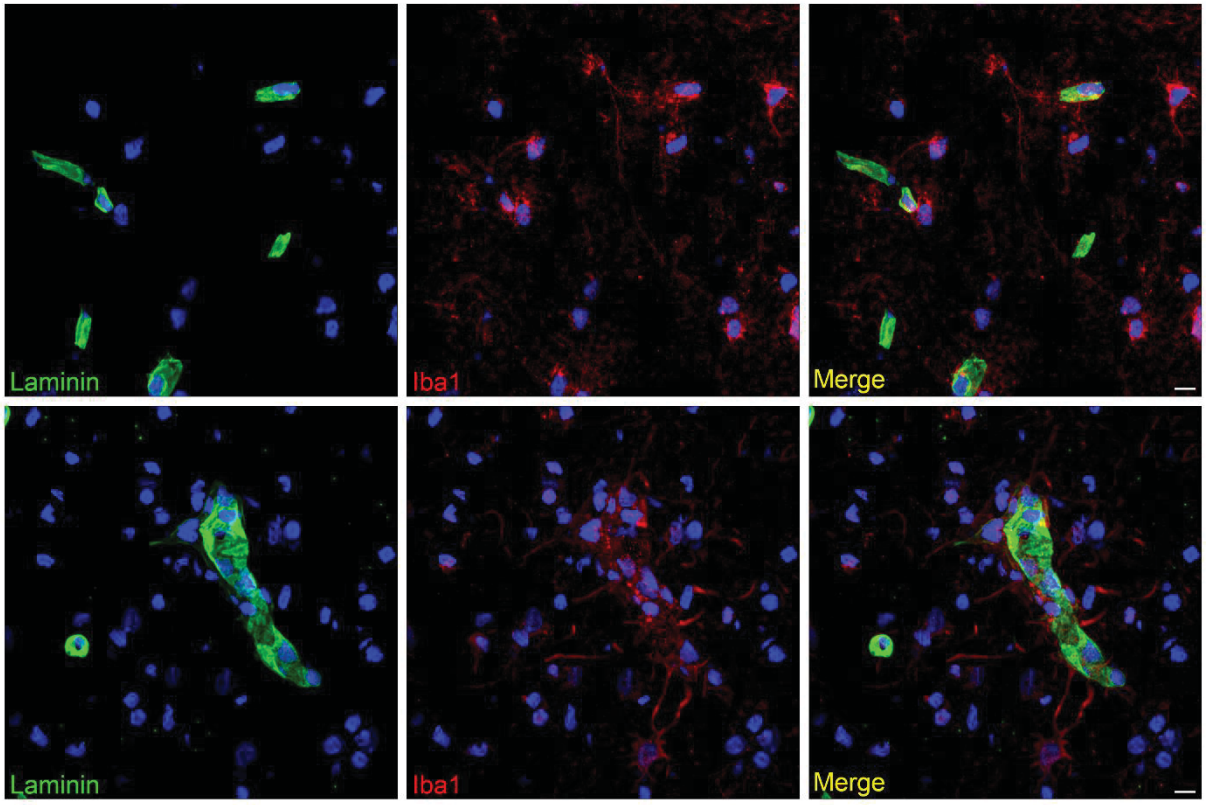

Figure 3 – Microglial interaction with the CNS vasculature. Human brain microglia (Iba1, red) is shown to interact with capillaries (Laminin, green) in both normal appearing white matter (top) and multiple sclerosis lesion (bottom) by confocal microscopy. Nuclei = blue, Topro-3. Scale bar = 10 μm. Image from Lécuyer et al. [31].

The concerted action of all these cellular components is required for the BBB to acquire and maintain its unique properties and functions. In the late stages of BBB formation, all these cellular inputs drive an increase in the organization and the complexity of TJs strands, forming inter-membranous networks of fusion points that can be visualized by freeze-fracture microscopy [37].

3. The Blood-Brain Barrier Endothelial Cells

The BBB is a highly organized multicellular complex responsible for maintaining CNS homeostasis by constantly regulating the exchange of molecules with the systemic circulation and restricting the ingress of peripheral immune cells into the CNS. The restrictive permeability of BBB-ECs is attributed in part to their inherent low pinocytic activity and their high concentration of efflux transporters. These specialized ECs also lack fenestrae and are tightly bound together by TJ and adherens junction (AJ) molecules, located in the intercellular space between adjacent ECs (**Figure 2**). TJs form the apico-lateral barrier and are composed of at least two 4-pass transmembrane protein families: the claudins [38] and TJ-associated MARVEL proteins (TAMP) (occludin [39], tricellulin [40, 41], and MARVELD3 [42]). Ig-like adhesion molecules, such as junctional adhesion molecules (JAMs), coxsackie and adenoviral serotype 2/5 receptor (CAR) [43], and endothelial cell-selective adhesion molecule (ESAM) [44] are also located at the apico-lateral barrier. Together, these proteins form large molecular aggregates located in cholesterol-rich cell membrane regions called lipid rafts [34, 45]. Along with actin filament-anchored adaptor molecules zonula occludens (ZO-1, -2 and -3) [46], cingulin [47] and membrane-associated guanylate kinase protein family (MAGUK) [48], they form a macromolecular complex capable of recruiting various protein kinases, phosphatases, and transcription factors that regulate cell polarity, proliferation and differentiation [49]. However, the contribution of TJs and TJ-associated adaptor molecules to intra- and inter-cellular signalling, gene transcription and the modulation of barrier function is still poorly understood.

AJs, which are located alongside the TJs, consist of transmembrane proteins of the cadherin family. BBB-ECs are known to express only two members of this family: vascular endothelial (VE)-cadherin and neuronal-cadherin (N-cadherin). However, N-cadherin does not play a role

at endothelial cell-cell junctions but instead establish contact between endothelial cells and pericytes [50, 51]. VE-cadherin associates via an extracellular domain with vascular endothelial protein tyrosine phosphatase (VE-PTP) [52]. A number of peripheral cytoplasmic proteins of the catenin family (α-, β-, P120) [53] also link cadherins to the cytoskeleton [54, 55]. Similarly, a third group of cell-cell junctional proteins called tight junction-associated cell adhesion molecules (TJaCAMs) are present at the basolateral side of BBB-ECs. These proteins include CD99 [56], platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31) [57, 58], intercellular adhesion molecule 1 (ICAM-1) [59], melanoma cell adhesion molecule (MCAM/CD146/S-endo-1) [60, 61], integrins and other poorly characterized proteins. TJaCAMs are known to have homophilic and heterophilic binding capacity and have been shown to mediate cell-cell or cell-basement membrane matrix adhesion [62, 63]. Together, all these junctional proteins highly restrict leukocyte transmigration under homeostatic conditions and are crucial to the molecular and cellular biology of ECs.

4. Blood-Brain Barrier Endothelial and Glial Cell Interactions in Homeostatic Conditions

Preserving neurovascular cell polarization by establishing a tightly controlled gradient between the luminal and abluminal surface of ECs is essential for maintaining the integrity and organization of TJ molecules, cell adhesion molecules, membrane receptors, ion channels, etc. The polarization of astrocytes is also critical for proper orientation of their own cellular machinery with respect to neuroglial cells and ECs, but also to establish the polarity of the BBB endothelium itself. This is exemplified in astrocyte end-feet, which are enriched in water channel aquaporin 4 (AQP4) and in inwardly rectifying potassium channel Kir4.1, both integral parts of the orthogonal arrays of particles (OAPs) [64] (**Figure 4**).

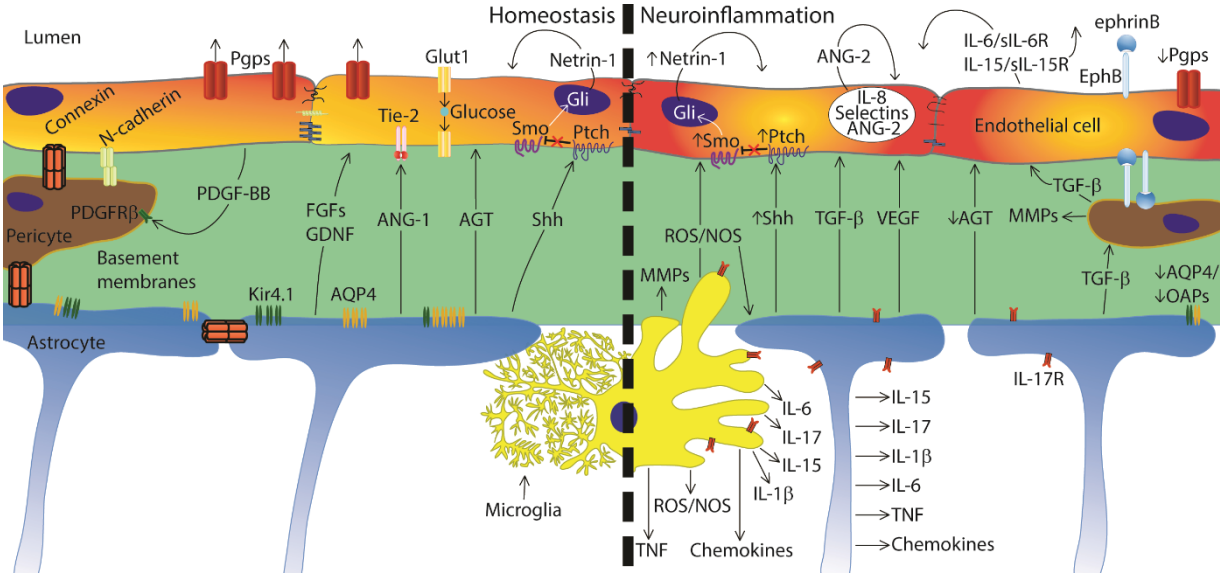

Figure 4 – Paracrine and autocrine regulation of the BBB during homeostasis and inflammation. Perivascular cells such as pericytes and glial cells have a major impact on blood-brain barrier (BBB) functions. In homeostatic conditions, these cells provide a wide variety of secreted factors which, along with contact-dependent interactions, induces barrier properties. However, upon central nervous system inflammation, every cellular constituent of the neurovascular unit can promote barrier breakdown and facilitate immune cell transmigration. *N-cadherin: Neural cadherin / Pgps: Permeability glycoproteins / Glut1: Glucose transporter 1 / Smo: Smoothened / Ptch: Patched-1 / PDGFRβ: Platelet-derived growth factor receptors β / PDGF-BB: Platelet-derived growth factor subunit B / FGFs: Fibroblast growth factors / GDNF: Glia-derived neurotrophic factor / AQP4: Aquaporin 4 / ANG1-2: Angiopoietin-1-2 / AGT: Angiotensinogen / Shh: Sonic hedgehog / MMPs: Matrix metalloproteinase / ROS: Reactive oxygen species / NOS: Nitric oxide synthase / TGF-β: Transforming growth factor β / VEGF: Vascular endothelial growth factor / OAPs: Orthogonal arrays of particles / TNF: Tumor necrosis factor.* Image from Lécuyer et al. [31].

OAPs are segregated towards the perivascular space through a specific isoform of agrin, a heparin sulphate proteoglycan located within the CNS vascular basement membranes. NtA-agrin 0, A/y0, B/z0 is the long N-terminus variant (basal laminae-binding region) that does not incorporate any amino acid at the three different possible insertion sites [65, 66]. This isoform

is secreted by both ECs and astrocytes [67, 68]. NtA-agrin forms, along with α-dystroglycan, α1-syntrophin, laminin 1 and 2, perlecan and others, a molecular scaffolding complex (dystrophin–dystroglycan complex) that binds to OAPs and links various cytoskeletal-associated proteins [69, 70]. This protein complex is required to provide optimal BBB properties, as the deficiency in one of its members, glial-α-dystrobrevin, leads to an increase in BBB permeability, water retention and can cause progressive brain oedema. This phenotype is associated with the loss of OAPs and the formation of intracellular vacuoles in astrocyte end-feet. These defects are thought to modify astrocyte-endothelial interactions and cause a deregulation of TJ molecule expression [71]. However, Haj-Yasein et al. [72] have demonstrated that the absence of AQP4 in astrocytes significantly reduces CNS water intake and consequently, brain oedema, using a stroke model in AQP4 knockout (KO) mice. It is worth pointing out that these animals have an intact BBB, unlike α-dystrobrevin-deficient mice. These studies highlight the inherent complexity of the dystrophin–dystroglycan complex in regulating BBB properties.

A growing body of evidence points towards the critical role of astrocytes in inducing BBB features and functions by promoting the proper expression and assembly of intermolecular junctions [33, 73], transporters (permeability glycoprotein 24 (Pgp24), glucose transporter 1 (GLUT1), etc.) [74], and enzymatic pathways [75, 76]. Studies have also convincingly demonstrated that astrocyte-secreted factors, including VEGF, TGF-β, glial-derived neurotrophic factor (GDNF), FGFs, and angiopoietin-1 (ANG1), are at least partly responsible for modulating BBB functions [77, 78]. Angiopoietins bind BBB-ECs via the tyrosine kinase Tie-2 receptor. Specifically, ANG1 participates in angiogenesis and vascular homeostasis by upregulating the expression of TJs, thereby reducing EC permeability [79, 80]. Similarly, FGFs promote BBB-EC tightness by regulating catenin–VE-cadherin interactions. Murakami et al. [81] have demonstrated that FGF blockade triggers VE-cadherin internalization, which leads to a loss of BBB integrity. FGFs presumably act by downregulating VEGF downstream signalling, thus preventing a destabilization of AJs. VEGF is primarily known as a pro-angiogenic factor that promotes the growth of ECs through VEGFR-1, -2 and as such, can disrupt the BBB [82]. Conversely, GDNF has been shown to decrease vascular leakage by significantly increasing the trans-endothelial electrical resistance (TEER) of BBB-ECs via an upregulation of claudin-5

expression. Although astrocytes are the main source of GNDF, pericytes have also been shown to secrete it [83, 84]. Finally, of all astrocyte-secreted factors, TGF-β is undoubtedly the most studied and paradoxically one of the least well-understood molecules. This pleiotropic cytokine is secreted by, and acts on a wide variety of cell types. It is involved in a plethora of cellular processes, including apoptosis, wound healing, embryogenesis, cell proliferation and differentiation [85-89]. Although TGF-β was originally regarded as an anti-inflammatory cytokine, mainly because of its role in promoting regulatory T cell functions, we now know that it also drives the differentiation of pro-inflammatory Th17 cells [90]. Emerging evidences highlight the dual role of TGF-β and its increased expression in several neuroinflammatory diseases, including multiple sclerosis (MS) [89], Alzheimer's disease (AD) [91] and stroke [92]. TGF-β has a neuroprotective role in the developing CNS through its effects in modulating the expression of Pgp efflux transporter on BBB-ECs and astrocyte end-feet, thereby restricting the penetration of xenobiotics into the CNS [93]. In a recent study, Pgp expression was shown to be significantly downregulated by BBB-ECs within the inferior colliculus following chemically induced focal astrocyte-microglial cell death. During the subsequent repopulation phase, a prolonged astrogliosis was observed in the affected area, while Pgp expression returned to its basal level [94]. Assessment of gadolinium and fluorescently labelled-dextran leakage also demonstrated BBB integrity disruption. This increase in permeability was maintained till the end of the regenerative phase. Altogether, these experiments highlight the role of astrocytes in maintaining Pgp and TJ expression in BBB-ECs [94-96]. Many glial factors, including the aforementioned ones, play a prominent role in regulating the physiology and functions of the BBB during homeostasis. However, they may be differentially modulated in a neuroinflammatory context, either to promote tissue repair and prevent inflammation or to increase immune cell transmigration and BBB permeability.

5. Leukocyte Diapedesis across the CNS Barrier

In neuroinflammatory disorders such as MS, pro-inflammatory encephalitogenic immune cells migrate across the BBB to gain access to the CNS and cause disease. This process has been

extensively studied over the last three decades and is thought to involve a sequential cascade of events [97-101] (**Figure 5**).

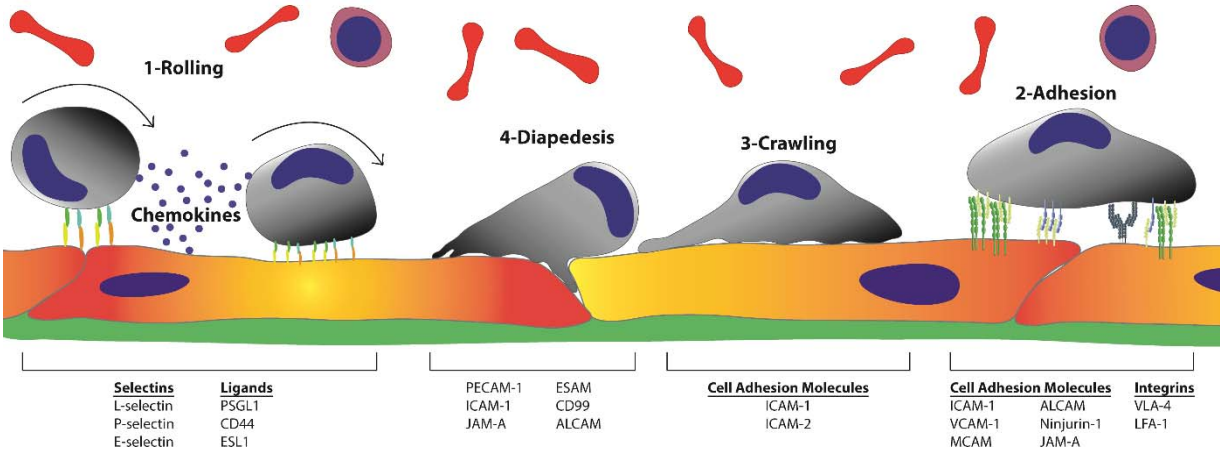

Figure 5 – The transmigration cascade. The first step in this cascade of events is termed rolling, which initiate the interaction between leukocytes and activated endothelial cells (ECs). It is mediated by various selectins and their ligands. Chemokines secreted by the ECs trigger an increase in the binding affinity and avidity of integrins for their ligands, which mediates the complete arrest of immune cells. This adhesion step is also mediated by a wide variety of cell adhesion molecules. The adherent leukocytes then undergo a process called crawling, where they move against the blood flow in order to find a suitable area to migrate across the blood-brain barrier-EC layer. *PSGL1: p-selectin glycoprotein ligand 1 / ESL1: E-selectin ligand 1 / PECAM-1: Platelet endothelial cell adhesion molecule 1 / ICAM-1,-2: Intercellular adhesion molecule 1,2 / JAM-A: Junctional adhesion molecule / ESAM: endothelial cell-selective adhesion molecule / ALCAM: activated leukocyte cell adhesion molecule / VCAM: vascular cell adhesion molecule / MCAM: Melanoma cell adhesion molecule / VLA-4: very late antigen 4 (integrin α4β1) / LFA-1: Lymphocyte function-associated antigen 1.* Image from Lécuyer et al. [31].

Initial contact between blood circulating leukocytes and the vascular endothelium is mediated by fluid dynamics. Leukocytes flowing into small capillaries and post-capillary venules tend to be off-centered, a phenomenon called margination [102]. This is caused by the

aggregation of rapidly flowing red blood cells (RBCs) in the center of blood vessels, as a result of their biconcave shape. When immune cells exit capillaries to enter post-capillary venules, RBCs push slower leukocytes on the vessel wall, at the blood-endothelium interface, where they are then free to probe the surface of BBB-ECs for potential ligands [102-104]. Not only are post-capillary venules physically favoured by fluid dynamics as the transmigration site of immune cells, but studies have also identified differences between the endothelial cells of post-capillary venules and the ones forming the rest of the vascular network. Electron microscopy studies have shown disorganization in TJ and AJ molecules in post-capillary venule ECs. These results corroborated previous data showing that post-capillary vessels cannot completely prevent the leakage of exogenous tracers into the perivascular space [105]. Additionally, recent work by the group of Dr. Sorokin has demonstrated that CNS post-capillary endothelial basement membrane lacks laminin α5, which selectively inhibits immune cell extravasation [106, 107]. These findings are substantiating experiments demonstrating the presence of different constituents in the venule basement membrane in periphery [108]. Collectively, this provides an explanation for the transmigratory tropism of leukocytes towards post-capillary vasculature in the CNS.

The short initial interaction between BBB-ECs and leukocytes is termed *capture*, *rolling* or *tethering* and is mediated by selectins and vascular cell adhesion molecule 1 (VCAM-1), expressed on the surface of BBB-ECs, and their respective ligands: carbohydrates and α4-integrins expressed on immune cells. Slow rolling along the vessels' lumen allows leukocytes to sample chemokines secreted under inflammatory conditions, such as interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1 or chemokine C-C motif ligand 2 (CCL2)), regulated on activation, normal T cell expressed and secreted (RANTES or CCL5) and interferon gamma-induced protein 10 (IP-10 or chemokine C-X-C motif ligand 10 (CXCL10)) [109, 110]. This triggers G-coupled protein signalling and initiates an intracellular cascade that leads to integrin clustering and enhances their binding affinity for their ligands, a step called *activation* [111]. Integrin clustering allows adhesion strengthening and facilitates leukocytes firm *arrest*. This step is also mediated by endothelial cell adhesion molecules (CAMs: ICAM-1, VCAM-1, MCAM, Ninjurin and activated leukocyte cell adhesion molecule (ALCAM)), the expression of which is highly upregulated upon inflammatory stimuli that originate from the

parenchyma or from the release of pro-inflammatory mediators by leukocytes [55, 112, 113]. Integrin/CAM-mediated cell adhesion is followed by *crawling* of immune cells preferentially against the direction of the blood flow to find a permissive site for transmigration [114-116]. Intraluminal crawling is dependent on ICAM-1 and ICAM-2 [115]. Of note, under certain circumstances, immune cells may adhere directly to BBB-ECs without apparent rolling [112, 117, 118]. However, it remains unclear whether prior *rolling/activation* occurred upstream of the observation site, or if it is a dispensable step of the leukocyte recruitment cascade. The final step of the cascade, *diapedesis*, is preceded by the formation of a docking structure called "transmigratory cup", whereby endothelial pseudopods reach out and surround the migrating leukocytes [119-121]. The existence of transmigratory cups, initially identified in non CNS-ECs, was confirmed using human and mouse CNS ECs [112, 113, 118]. Diapedesis can occur via two independent pathways: paracellularly, where leukocytes migrate in-between adjacent BBB-ECs or transcellularly, in which case, the immune cells extravasate directly through individual ECs by forming invasive podosomes (**Figure 6**) [122-124].

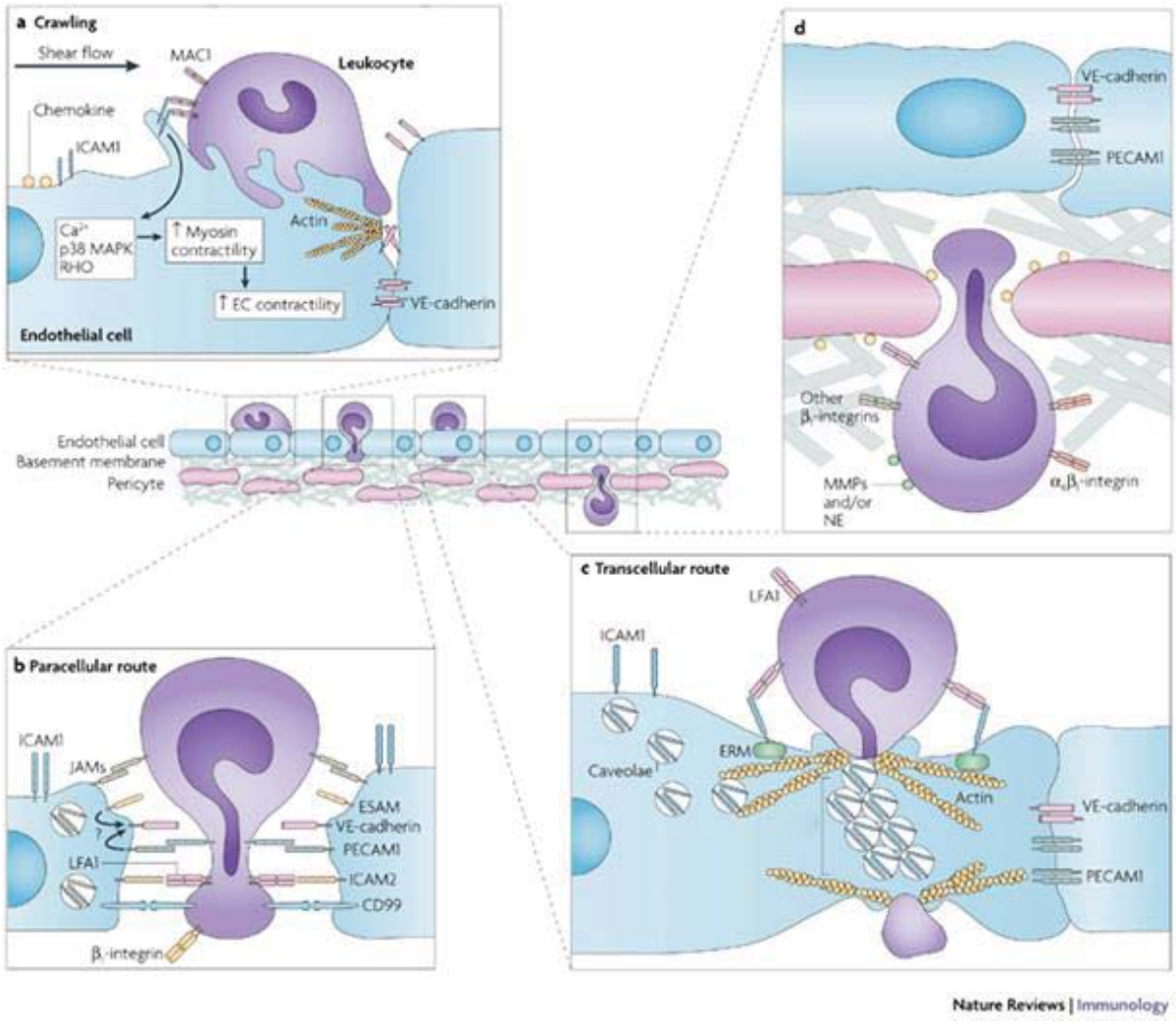

Figure 6 – Dual cellular pathway of immune cells transmigration. A Immune cell firm adhesion can trigger intracellular signalling leading to an increase in BBB-EC contractility, which in turn can facilitate diapedesis. **B** Paracellular immune cell migration pathway showing possible interactions between BBB-EC TJ, AJ, TJaCAMs and leukocyte CAMs. **C** Transcellular migration route showing the invagination of the BBB-EC around the immune cell, creating a tunnel through the cell body and the mechanisms behind this cellular process (membrane internalization and reshuffling, actin polymerization, interaction with integrins/CAMs and their intracellular binding partners ezrin/radixin/moesin (ERM)). **D** The migration of an immune cell across the basement membranes on the abluminal side of the BBB-ECs involving the secretion of MMP-2 and MMP-9 as well as different integrins and CAMs. Image adapted from Ley, K et al. [125].

In spite of the increasing knowledge on the mechanisms involved during diapedisis, the molecules that dictate whether a leukocyte extravasates via the paracellular or the transcellular routes are still ill-defined. [126]. For example, several TJaCAMs (JAM-A, CD99, PECAM-1 and MCAM) have been detected on both the apical and the basolateral surface of BBB-ECs under inflammatory conditions and therefore, could be involved in either transmigration processes [127]. One mechanism that was put forward to explain this dichotomy is the internalization of the aforementioned molecules in a complex lateral border-recycling compartment, whose functions are not fully understood yet but thoroughly discussed by Muller et al. [128, 129]. In sharp contrast, TJ and AJ molecules involved in diapedesis, such as ESAM and VE-cadherin, are present exclusively in the intercellular space between adjacent ECs and thus, could only participate in paracellular migration [129]. However, a study published by Riethmuller, C. and colleagues in 2008 has challenged this view by showing that leukocytes, in fact, migrated through ECs, albeit in close proximity to the intercellular space, without disrupting the continuity of junctions; thereby arguing against the concept of paracellular transmigration [130]. In a disease context, pro-inflammatory leukocytes can contribute to BBB disruption and increase its permeability by virtue of the cytokines they release [55, 98, 128]. In addition, transendothelial leukocyte migration can itself perpetuate the inflammatory cascade by favoring subsequent site-specific immune cell infiltration [131]. Finally, a number of yet unidentified molecules could play an important role in initiating site-specific transmigration. Therefore, this aging question stays as of yet unresolved.

While the transmigration of leukocytes across the BBB has been discussed, there are two other less well studied barriers, which can allow the passage of immune cells: the blood-meningeal barrier (BMB) and the blood-CSF barrier (BCB). The meninges are composed of three layers that surround the CNS (dura mater, arachnoid mater and pia mater) and contain the CSF located within the subarachnoid space (**Figure 1**). Though the meninges were initially considered as a simple protective tissue to the CNS, more recent studies have established that it is also the site of continual immune surveillance [132]. Leukocyte egress from meningeal blood vessels is facilitated by the increased permeability of this barrier as compared to the BBB. Further findings using intra-vital live imaging in animals have demonstrated that lymphocytes migrate across the BMB prior to the disease onset and get reactivated in the subarachnoid space

[116]. Corroborating with these results, spinal cord immune cell infiltration and lesion dissemination is usually found to originate from the meninges and progressing toward the center. Another point of entry to the CSF is the BCB or choroid plexus, which is also known to be more permeable than the BBB. There, leukocytes can migrate from the blood to the ventricles and the arachnoid space. As mentioned previously, the blood vessels forming the choroid plexus are fenestrated and the barrier is instead created by the ependymal cells surrounding the ventricles. Those cells constitutively expressed ICAM-1, VCAM-1 and P-selectins and might therefore be a point of entry for patrolling T lymphocytes in homeostatic conditions and pro-inflammatory encephalitogenic lymphocytes during neuroinflammation [6, 133].

6. Glial Influences on the Blood-Brain Barrier during Neuroinflammation

The role of gap junctions

A common denominator of many neurological diseases is the loss of BBB integrity and the presence of immune cell infiltrates in the CNS. However, it remains unclear whether BBB dysfunction precedes immune cell trafficking, or if it is a consequence of peripheral immune cell activation. While the migration process itself can modify the characteristics of leukocytes and that of BBB-ECs [134-136], astrocytes and microglia also play fundamental roles in regulating leukocyte effector function, either directly or via the modulation of BBB-EC phenotype. Our group has shown that the loss of BBB integrity associated with a dysregulation of TJ molecules occurs early during lesion formation and coincides with perivascular astrogliosis and upregulation of endothelial CAMs. These findings suggest that BBB disruption precedes any overt CNS immune cell infiltration and might be a direct consequence of glial cell activation [137]. Small molecules involved in cell signalling and metabolism can spread rapidly through the continuous astrocyte network formed by gap junctions, and also referred to as the *astrocytic syncytium* [138, 139]. This network allegedly also serves to coordinate effectively and unify the immune response to stimuli, either to maintain homeostasis or to propagate pro-inflammatory signals (**Figure 4**) [140]. As such, this mechanism might be implicated early during lesion formation. However, evidence against this hypothesis also exists: Brand-Schieber et al. demonstrated a significant decrease in expression of connexin 43 in astrocytes within

inflammatory lesions during experimental autoimmune encephalomyelitis (EAE), an animal model of MS [141]. It remains unclear whether this decreased expression translates into loss of function, and if it precedes focal inflammation. Another study has shown that the absence of astroglial connexin 43 promotes the recruitment of leukocytes via the activation of BBB-ECs (upregulation of CAM expression and chemokine secretion), effectively compromising CNS quiescence [142]. Although the absence of connexin 43 is associated with a progressive weakening of the BBB, the immune cell infiltration was not the result of an early BBB breakdown or glial cell activation in these KO animals. Unlike in other neuroinflammatory conditions, the immune cell response within the CNS of these animals was efficiently and rapidly controlled [142]. This study demonstrates an essential role for connexin-43 in maintaining CNS homeostasis, but also reveals that its absence does not, by itself, trigger a chronic autoimmune reaction. Furthermore, another study from the same group reports a significant loss of AQP4 and β-dystroglycan in astrocyte end-feet, associated with a disrupted BBB and brain oedema in mice deficient for astrocytic expression of connexin 43 [143]. These findings are in agreement with several studies which correlated CNS inflammation with impaired segregation of AQP4 at the astrocyte end-feet, as well as a disruption of dystrophin–dystroglycan complex; both involved in the regulation of brain water uptake [144]. On the other hand, another study demonstrated that the loss of both connexin 43 and 30, in double KO mice, resulted in a much worse phenotype, namely widespread white matter pathology (oedema and vacuolation of astrocytes and oligodendrocytes) accompanied with region-specific astrocytic abnormalities [145]. Yet, it is unclear whether the modulation of connexin and/or OAPs expression during neuroinflammation is beneficial or detrimental for disease resolution. In fact, reactive astrocytes can either elicit a pro- or an anti-inflammatory response.

The role of anti-inflammatory astrocyte-secreted molecules

Our group has recently uncovered the critical role of astrocyte-secreted Sonic hedgehog (Shh) in dampening CNS inflammation. We showed that Shh, which expression is upregulated upon inflammatory challenge, alleviates neuroinflammation via activation of the signal transducer Smoothened (Smo), transcription factors of the Gli family and the Shh receptor

Patched-1 (Ptch-1) expressed by BBB-ECs. BBB-ECs treated with Shh showed a reduced expression of CAMs, a decreased chemokine secretion and an increased TEER. In addition, *in vivo* permeability experiments performed on endothelial-specific Smo KO mice revealed a significant increase in BBB permeability that correlated with a deregulated expression of TJs and disrupted BMs, as compared to WT animals. [23]. We further recently demonstrated that Netrin-1 is a downstream effector of Shh that promotes BBB phenotype and functions during homeostasis and inflammation, via an autocrine signalling pathway. Netrin-1 treatment during EAE significantly reduced BBB disruption by upregulating endothelial junctional protein expression, while alleviating the clinical and pathological indices of disease. Aside from its ascribed role of promoting BBB formation, the hedgehog pathway acts with Netrin-1 as an important molecular repressor of CNS inflammation, while promoting BBB repair and integrity [146].

In order to control inflammation and promote homeostasis, astrocytes actively internalize glutamate and produce metabolizing enzymes and antioxidants, thus playing an important role in scavenging reactive oxygen species (ROS) and extracellular glutamate [147]. In a pro-inflammatory environment, activated astrocytes also selectively induce Toll-like receptor-3 (TLR3), which mediates the secretion of anti-inflammatory cytokines and neurotrophic factors [148]. Moreover, astrocytes can secrete angiotensinogen (AGT): a BBB-promoting factor that is cleaved into angiotensin-II, who then binds to type 1-angiotensin receptors expressed on BBB-ECs. Triggering this signalling cascade was shown to tighten the BBB via phosphorylation of occludin and its mobilization into intercellular lipid raft microdomains. In MS lesions, the expression of AGT by perivascular astrocytes is downregulated in a pro-inflammatory cytokine-dependent fashion, and is paralleled with a decreased expression of occludin on BBB-ECs. These findings were confirmed in AGT deficient animals, highlighting the ability of reactive astrocytes to contribute to BBB breakdown during neuroinflammation [34].

The role of VEGF

In contrast to AGT, several other astrocyte-secreted factors have been reported to promote inflammation in the CNS. Amongst them, VEGF has the capacity to disrupt the integrity of the

BBB by modulating the expression of junctional molecules, including VE-cadherin, claudin-5 and occludin; therefore, promoting immune cell migration into the CNS. The secretion of IL-1β by activated microglia can also trigger the release of VEGF and thymidine phosphorylase (TYMP) by astrocytes [149, 150]. TYMP produces 2-deoxy-D-ribose which, along with VEFG, disrupts TJ proteins and increase BBB permeability. Both factors are also driving transcriptional pathways that result in the production of angiogenic and permeability genes [151]. However, it should be noted that VEGF can trigger independent pathways depending on which side of the BBB-ECs it is being secreted. A group has shown that the presence of VEGF on the abluminal side triggers signalling from VEGFR2, which in turns increase BBB permeability via p38. On the other hand, luminal VEGFR1 is activated by circulating factors and initiates Akt signalling pathways to promote cytoprotection. This highly polarized expression of VEGF receptors was found on CNS microvasculature, but not in peripheral vessels; illustrating the importance of BBB-ECs' polarity in responding to inflammatory cues originating from the blood or parenchymal tissue [152].

Apart from its direct role on the BBB, VEGF is also known to upregulate the surface molecule ephrinB2 on different cell subsets [153]. EphB receptors and ephrinB ligands are preferentially expressed by arterial ECs, but also by smooth muscle cells and pericytes [154, 155]. EphB2 was recently shown to be essential during blood vessel assembly suggesting a possible link between astrocyte-controlled angiogenesis, VEGF and EphB2-ephrinB2 [156]. Like VEGF, ephrins are also involved in neuroinflammation, particularly in leukocyte trafficking. They do so by acting as adhesion molecules or by regulating their activity. They have also been shown to modulate directly the activity of immune cells. For example, ephrinB2 was reported to be involved in T cell co-stimulation [80]. Our group is currently working on a study demonstrating that pro-inflammatory Th17 cells express high levels of ephrinB1 and ephrinB2, which facilitate their transmigration into MS lesions. This study also provides evidence that these ephrins are essential for Th17 differentiation and pathogenicity in the context of neuroinflammation [157].

VEGF, along with FGFs, can also indirectly promote neuroinflammation by modulating the secretion of angiopoietin-2 (Ang-2) by BBB-ECs. Ang-2 is synthesized and stored, like multiple other pro-inflammatory factors (IL-8, selectins), in endothelial granules called Weibel-Palade

bodies that are involved in the rapid response of ECs to external stimuli. Recent experiments have demonstrated that Ang-2 deficient animals are unable to trigger a prompt inflammatory response following infection. An in-depth analysis of the phenotype of Ang-2 KO mice revealed that Ang-2 is involved during the adhesion process, but does not affect the rolling ability of activated leukocytes. Ang-2 might also increase BBB permeability by regulating endothelial junctional molecules and integrins that bind to the matrix [80] and potentiate endothelial responses to pro-inflammatory cytokines, thus promoting the upregulation of CAMs [158].

The role of pro-inflammatory cytokines

Aside from leukocytes, which are the main source of cytokines in the inflamed CNS, activated astrocytes and microglia are known to secrete an array of pro-inflammatory molecules, including ROS, IL-1β, IL-6, and IL-17A, to name a few. Meeuwsen et al. reported the production of IL-17A (also known as IL-17) by stimulated astrocytes, although the relevance of their findings was not clear at the time [159]. Later, Tzartos et al. detected IL-17 mRNA and protein in perivascular lymphocytes but also in astrocytes and oligodendrocytes found within active MS lesions; clearly demonstrating that IL-17A is secreted by a broad spectrum of neuroglial cells, and that it might be implicated in pathways far more complex than previously anticipated [160]. More recently, Zimmermann et al. have shown, using glial fibrillary acidic protein (GFAP)-driven IL-17A transgenic mice, that overexpression of IL-17A by astrocytes alone can activate glial cells, but is not sufficient to cause parenchymal infiltration, demyelination or neurodegeneration. However, lipopolysaccharide (LPS)-induced endotoxemia in these animals triggers enhanced microglial activation along with increased pro-inflammatory cytokine secretion, as compared to controls [161]. Another study has demonstrated that IL-17A could promote neuronal injury in a dose-dependent manner *in vitro* [162]. Furthermore, microglia, and to some degree astrocytes, are also known to express IL-17A and IL- 17C receptors (IL-17RA, IL-17RC), which upon intracellular signalling can potentiate tumor necrosis factor (TNF) effects, and trigger the release of pro-inflammatory cytokines and a wide range of chemokines involved in leukocyte trafficking, such as CXCL1 (GROα), CCL2 (MCP-1), CCL3 (macrophage inflammatory protein 1α (MIP-1α)), CCL20 (MIP-3α), CXCL2 (MIP-

2), CXCL9 (monokine induced by gamma interferon (MIG)), CXCL10 (IP-10) and CXCL11 (IP-9) [163-168]. Of note, MCP-1 was shown to increase BBB permeability by binding BBB-EC–expressed chemokine C-C motif receptor 2 (CCR2), which in turn activates small GTPase Rho and Rho kinases to trigger the reorganization of the actin cytoskeleton and redistribution of TJ proteins [169]. Conversely, IL-17A was also shown to displays CNS protective function by inducing neuronal repair via the expression of GDNF, brain-derived neurotrophic factors (BDNF) and nerve growth factors (NGF). It is worth pointing out that whereas human IL-17A can bind both IL-17RA and IL-17RC receptors, mouse IL-17A only binds IL-17RA [170]. ECs, and even neurons, also upregulate their expression of IL-17RA receptors in the inflamed CNS. We have shown that IL-17RA is expressed at high levels on blood vessels within active MS lesions and that IL-17 increases BBB permeability in a dose-dependent manner, by disrupting TJ molecules. Similar to its effect on glial cells, IL-17 induces the secretion of pro-inflammatory cytokines and chemokines by BBB-ECs (IL-6, CCL2 and CXCL8), therefore, promoting leukocyte recruitment into the CNS [135].

IL-6 is a pleiotropic cytokine produced by astrocytes [171], brain endothelial cells [109] and microglia [171, 172] that is subjected to autocrine regulation. IL-6 expression can be induced by IL-17 alone or in synergy with TNF or IL-1β. IL-6 has both neurotrophic effects (promotes neuron survival via BDNF [173]) and pro-inflammatory functions [174]. Therefore, both glial- and endothelial-produced IL-6 can influence BBB properties by modulating the expression of different CAMs, cytokines and chemokines [175]. IL-6 also induces the differentiation of naïve T cells into Th17 lymphocytes, in the presence of TGF-β. Th17 lymphocytes secrete IL-17, who acts in an autocrine feedback loop to stimulate the production of IL-6 by astrocytes. Within the CNS, Th17 lymphocytes can induce the expression of pro-inflammatory mediators by astrocytes and microglia through a contact-dependent manner, thus promoting myelin and neuronal damage [170, 176].

Another cytokine of the IL-6 family, oncostatin M (OSM) is highly produced by peripheral blood mononuclear cells (PBMC) obtained from MS patients [177]. It is also expressed by activated astrocytes and microglia surrounding MS lesions; suggesting that these cells might be an important endogenous cerebral source of OSM, in compromised CNS. OSM alone or in synergy with TNF has been shown to stimulate the expression of ICAM-1 and the production

of MCP-1 and IL-6 by BBB-ECs [178]. It can also act as a positive regulator of IL-6 expression by astrocytes [174]. More recently, OSM was also shown to decrease the TEER of rat BBB-ECs and impact on the integrity and organization of TJ molecules such as ZO-1 and claudin-5 [179]. Therefore, IL-6 family cytokines, secreted by astrocytes and microglia during neuroinflammation, contribute directly and indirectly to leukocyte trafficking across the BBB.

Reactive astrocytes and activated microglia are also known IL-15 producers. This pro-inflammatory cytokine induces the expression of hyaluronan on ECs, which in turn allows the extravasation of activated T lymphocytes into the CNS via a CD44-dependent adhesion cascade [180]. ECs themselves can secrete IL-15 and therefore, directly enhance the capacity of T lymphocytes to migrate into the CNS. IL-15 can also increase the avidity of lymphocyte function-associated antigen-1 (LFA-1) integrin to its ligand and affect the motility of immune cells [181]. Most notably, IL-15 is known to be involved in the activation of natural killer (NK) cells and can promote clonal expansion of NK-like $CD4^+$ T lymphocytes, which exert cytotoxicity towards vascular ECs [182]. Another way by which IL-15 is implicated in neuroinflammation is by virtue of its role in maintaining and activating cytotoxic $CD8^+$ T lymphocytes, known to contribute to MS pathogenesis. Saikali et al. have demonstrated that following stimulation with IL-15, $CD8^+$ T lymphocytes up-regulated lytic enzyme production, NKG2D expression and antigen-directed cytotoxicity, which increased their ability to kill glial cells and migrate across BBB-ECs [183]. Our group has recently confirmed these data in a study which identified MCAM as a marker of encephalitogenic $CD8^+$ T lymphocytes [184]. A report by Schneider et al. also shows that MS patients have elevated levels of IL-15 in their blood, as compared to healthy controls, and demonstrates that MS patient immune cells are more susceptible to the effects of this cytokine [185]. The fact that the majority of astrocytes and microglia present within demyelinating lesions express high levels of IL-15 underscores the critical role for IL-15 orchestrating multiple aspects of chronic inflammation in the CNS [183].

The role of ROS

Activated microglia produce ROS in response to NADPH oxidase activation. ROS are in part responsible for oligodendrocyte degeneration following oxidative damage during

neuroinflammation [186]. While they can cause direct damage, ROS can also increase BBB permeability by activating the PI3K/ATK pathway and by decreasing the expression of VE-cadherin, occludin and claudin-5 in BBB-ECs [187-189]. By doing so, the release of ROS facilitates leukocyte transmigration and allows leakage of plasma protein into the CNS, including fibrinogen. In a recent study, Davalos et al. have demonstrated a direct effect of fibrinogen on the activation state of microglia using two-photon live imaging. They observed an increased motility of microglia to form perivascular clusters prior to the development of CNS lesions [190]. Collectively, these data point to a feed-back/amplification mechanism between ROS production by microglia, BBB dysfunction, leakage of fibrinogen into the CNS and further activation of microglia, leading to enhanced ROS production. While we can speculate that, in experimental models of neuroinflammation, peripheral inflammation causes BBB leakage, which then induces activation of perivascular glial cells, the situation might be different in a disease context. In human neuroinflammatory diseases, it remains unclear whether glial-secreted inflammatory mediators induce the initial BBB breakdown, which causes the leakage of serum proteins that in turn promote glial cell activation in a positive feedback loop. The general consensus today is that microglia activation upregulates CAMs on the surface of brain ECs and compromises the integrity of the BBB in neurodegenerative disorders such as Alzheimer's disease and in MS [191-195].

The role of MMPs

Once they have crossed the BBB, infiltrating leukocytes accumulate in the perivascular space between the two BMs (**Figure 2**). In order to gain access to the CNS parenchyma, pro-inflammatory leukocytes need to secrete matrix metalloproteinase-2 (MMP-2) and MMP-9, as well as extracellular matrix metalloproteinase inducer (EMMPRIN), which are required to cleave fibronectin, laminin and dystroglycans that form the parenchymal BM [196, 197]. As such, the parenchymal BM and the astrocyte end-feet constitute an additional physical barrier that renders the BBB more selective and tightly controlled, especially in an inflammatory environment. Microglia can also increase BBB permeability through secretion of MMPs (MMP-1, -2, -3, -9 and -19), which significantly destabilize the parenchymal BM. Microglial-produced

MMPs have been detected within pre-active and active MS lesions, which suggest their possible involvement in early disease pathogenesis [198].

In spite of the remarkable increase of knowledge regarding the role of astrocytes and microglia during CNS inflammatory responses, further studies are needed to better understand their involvement in promoting leukocyte transmigration and BBB dysfunction, as well as their protective role in supporting cellular regeneration and CNS repair. It is becoming increasingly clear that the concomitant expression of multiple molecular and cellular effectors is required to initiate and sustain neuroinflammation, providing a mere glimpse into the complexity of the neuro-immune-glial crosstalk.

2. Leukocyte Populations

While leukocytes have been briefly mentioned in previous paragraphs, the following section will outline the different subsets of leukocytes and briefly discuss their role during an immune challenge.

1. Lymphocytes

Th1 Lymphocytes

The distinction between two different subsets of T helper cells was first made in 1986 in a now seminal study published by Mosmann, T.R. et al. [199]. It was proposed that CD4+ T lymphocytes could differentiate into a Th1 subset specialized in viral and intracellular pathogen clearance via the secretion of IFNγ or into a Th2 subset mediating humoral immune response and immune reaction against extracellular pathogens via the production of IL-4 [200]. Subsequent studies have also shown that Th1 differentiation is dependent on IL-12, which via its surface receptor, induces STAT4 regulated genes. Those genes, in turn, induce STAT1, the Th1-specific transcription factor Tbet and promote the secretion of IFNγ [201, 202]. Th1 cells are also known to secrete TNF and IL-2 following activation. Together these cytokines play a role in macrophages activation and cell cytotoxicity [203].

Th2 Lymphocytes

As previously mentioned, IL-4 is one of the major cytokines secreted by Th2 lymphocytes along with IL-5, IL-10, IL-13 and IL-25, which regulate the innate immune system. IL-4 also drives Th2 lymphocyte differentiation in a positive autocrine/paracrine feedback loop [204]. Mast cells and basophils can also produce high levels of IL-4 upon activation and are thus essential in priming Th2 cells against allergens and helminths [205]. An alternative activation pathway involves OX40-l and the Notch ligands Jagged-1 and Jagged-2 on activated dendritic cells (DC) [206]. In either pathway, the transcription factor GATA-3 is induced and promotes Th2 differentiation, while also upregulating itself in a positive feedback loop [207].

Th3 Lymphocytes

Very little is known about Th3 lymphocytes, although they appear to be involved in the protection of gut mucosal surfaces from unwanted immune responses toward ingested antigens. In this regard, they are closely related to the other regulatory cells, such as Tr1 lymphocytes (discussed later), which are responsible for oral tolerance [208]. They mediate an anti-inflammatory environment capable of inhibiting Th1 and Th2 lymphocytes by secreting primarily TGF-β but also to some extent IL-10 [209]. In this context, TGF-β is known to promote antibody class switch to IgA, which is essentially a non-inflammatory immunoglobulin. IgA does not trigger phagocytosis and it is a poor activator of the classic complement system. *In vitro* studies have also demonstrated that the differentiation of Th3 lymphocytes is enhanced by TGF-β, IL-4 and IL-10. Further findings suggest that Th3 lymphocytes can be differentiated from natural T regulatory lymphocytes (nTregs); however, it is still unclear whether Th3 lymphocytes and induced Tregs share the same lineage due to the absence of specific markers [210].

Th9 T Lymphocytes

IL-4 and TGF-β were found to promote the differentiation of naïve murine CD4+ T lymphocytes into IL-9-producing (Th9) T lymphocytes; however, no particular chemokine receptors have been linked to this potential T lymphocyte subset. On the other hand, recent studies have shown that PU.1 could be the unique transcription factor of Th9 cells [211]. Their exact function has yet to be completely determined, but they are thought to be involved in immunity against helminthic infections and they might promote inflammatory diseases via mast cells activation [212, 213]. They have also been linked to ulcerative colitis [214] and rheumatic diseases [215].

Th17 Lymphocytes

Although IL-17 (IL-17A) has been known as an important pro-inflammation molecule since the 1990s, the term Th17 has only been coined in 2005. The distinct nature of Th17 lymphocytes was established following the differentiation of naïve T lymphocytes into IL-17-secreting cells using only IL-23 in the presence of antigens and splenic feeder cells. These newly transformed cells were also not dependent on previously known Th1 or Th2 specific transcription factors [216, 217]. This major discovery was the direct consequence of previous studies that discredited Th1 lymphocytes as being solely responsible for some autoimmune diseases, such as MS. Indeed, Th1 response had been associated with autoimmunity since their discovery, as IFNγ molecules found in lesions usually correlated with the extent of the disease [218]. Further studies even showed that Th1 lymphocytes specific for cognate CNS antigens could adoptively transfer EAE into naïve host. However, the Th1 disease-driven hypothesis was challenged when mice lacking IL-12p35 [219], IL-12β2 receptor [220], IFNγ or IFNγ receptor were not protected from EAE, but rather developed a more severe disease [218]. Furthermore, at that time, a novel cytokine chain, p19, was discovered to heterodimerize with the p40 chain of IL-12. This novel cytokine was later named IL-23. Based on these new evidences, it became clear that disrupting p40 would affect not only IL-12, but also IL-23 pathways. The disease susceptibility of IL-23p19 and IL-12p35 deficient mice was therefore assessed and it was demonstrated that IL-23, but not IL-12 was necessary to induce EAE [221]. Two years later, the discovery of RORγt, a

transcription factor promoting Th17 genes, confirmed the independent nature of Th17 lymphocytes from the Th1 lineage [222].

In mice, TGF-β and IL-6 have been shown to be essential for the differentiation of naïve T cells into activated Th17 cells [223]. In humans, IL-6 and low level of TGF-β seems to be required (although debated [224, 225]), while the addition of IL-1β induces a double positive phenotype (IL-17+/IFN-γ+) [224]. Even though IL-23 was first thought to be a key Th17 differentiation cytokines, the lack of receptors on naïve T lymphocytes demonstrated otherwise. It has since been linked to the stabilization of the phenotype [226]. As a result, we know that the combined signals of TCR binding, co-stimulatory molecules and cytokine receptors induce T-bet, RORγt and RORα. In recent studies, the latter was reported to play a similar role, although not identical to RORγt, suggesting its implication in Th17 differentiation might be necessary [227]. Confirming these results, another study demonstrated that RORγt deficiency alone attenuated, but did not abolish the secretion of IL-17, while RORα and RORγt double-KO completely inhibited the generation of Th17 [228]. Together, these transcription factors induce Th17 lymphocyte-lineage genes and trigger the production of IL-17, IL-17F, IL-21, IL-22, GM-CSF and under some conditions IFNγ.

The high interest given to Th17 lymphocytes in the last few years, and especially in autoimmune diseases such as MS, is mainly due to their implication in the pathogenesis of numerous inflammatory diseases. Dr. Prat's laboratory has also previously demonstrated that IL-17 and IL-22 were involved in the disruption of junctional molecules at the level of the blood-brain barrier (BBB) during EAE. Furthermore, Th17 lymphocytes were shown to be very efficient at crossing the BBB and promoting directly brain lesions by secreting granzyme B or indirectly through the recruitment of effector lymphocytes [135]. Two years later, double positive Th17 lymphocytes were shown to preferentially cross the BBB, accumulating in the perivascular region in both EAE and MS [229]. However, adoptive EAE transfer of T lymphocytes deficient for IL-17A or IFNγ was shown to induce similar disease than the transfer of WT T lymphocytes [230]. By contrast, GM-CSF deficient T lymphocytes are incapable of inducing EAE and invading the CNS [231] and GM-CSF KO mice are resistant to EAE induction by MOG immunization [232, 233]. Corroborating these results, a recent study

demonstrated that deleting the GM-CSF receptor on $CCR2^{+}Ly6C^{hi}$ monocytes prevented the induction of EAE, whereas the deletion of the receptor on dendritic cells (DCs) or neutrophils did not modify the course of the disease [234].

Th22 T Lymphocytes

Th22 lymphocytes have only been recently described following the identification of a subset of IL-22-secreting T lymphocytes distinct from Th17 lymphocytes [235]. Analogous to Th17 lymphocytes, experiments have shown the involvement of IL-6, but also TNF in the differentiation of Th22 lymphocytes [236]. However, as expected with *in vitro* studies, other groups have induced Th22 lymphocytes using other combinations of cytokines [235]. The exact cytokine cocktail has therefore yet to be completely determined. Borrowed from the Th17 studies and further studied in a Th22 context, the aryl hydrocarbon receptor (AHR) is a ligand dependent transcription factor that translocate into the nucleus and subsequently promotes IL-22 secretion [237]. IL-22 receptors are widely distributed in tissues, but IL-22 is a cytokine that acts mainly on epithelial cells. Therefore, Th22 T lymphocytes are presumed to play an important role in skin homeostasis and in the pathogenesis of skin diseases [236].

Naturally Arising Treg (nTreg)

Tregs are the most studied regulatory T lymphocytes. They have been discovered by comparing WT mice to animals with a mutated transcription factor forkhead box P3 (Foxp3). The phenotype of the transgenic animals (Scurfy mice) is characterized by an extensive multi-organ immune infiltration and drastically elevated pro-inflammatory cytokines level [238]. nTreg cells were further characterized as expressing CD4, high levels of CD25 and Foxp3 (and low levels of CD127 in humans). During the same year, Treg lymphocytes were identified in human peripheral blood and their ability to suppress T helper and cytotoxic cell proliferation was assessed *in vitro* [239]. Like other T lymphocytes, Treg express αβ TCR and the co-stimulatory molecule CD28. They are also activated in a similar fashion via the interaction with the major histocompatibility complex class II (MHC-II) and B7 molecules, following which

they secrete anti-inflammatory cytokines (IL-10, TGF-β). They can also act via the secretion of granzymes and perforins or via the CTLA-4 inhibitory co-stimulator on their surface. nTreg lymphocytes can therefore regulate both antigen-presenting cells (APCs) and effector T lymphocytes in a contact-dependent manner [240]. Naturally arising regulatory T lymphocytes are, as their name implies, developing naturally in the thymus and reside primarily in secondary lymphoid organs, where they express CCR7 and CCR6 chemokine receptors [241].

Induced Treg (iTreg)

On the other hand, adaptive or inducible Tregs exit from the thymus as naïve single-positive CD4 T lymphocytes. Once in the periphery, they differentiate into CD45RO, CD25 high and Foxp3 positive Tregs following antigenic stimulation in the presence of self-antigens and specialized immunoregulatory cytokines, such as IL-2, TGF-β, IL-10 and IL-4 [242, 243]. It was also demonstrated that iTregs can be induced by commensal immunomodulatory molecules such as polysaccharide A from *B. fragilis*. These cells were shown to effectively produce IL-10 during bacterial colonization [244] and to possess a shorter doubling time when compared to memory T helper lymphocytes. However, the same study also demonstrated that they are more prone to apoptosis [240]. Therefore, it is thought that iTregs are the primary cells responsible for immunoregulation at the sites of inflammation. In this context, iTregs are continuously stimulated by antigen presentation and the presence of growth factors facilitates their survival [242, 243].

Tr1

Tr1 cells were first discovered while studying colitis [245]. These T regulatory cells bear resemblance to other Treg lymphocytes, however, they do not express Foxp3 and possess only low amount of CD25 molecules on their surface [246]. More recently, they were shown to express the surface markers CD49b and lymphocyte activation gene (LAG-3), which facilitate their identification *in situ* but is not unique to this T lymphocyte subset. They have been shown to require IL-10 during their differentiation process and they, in turn, secrete high quantity of

IL-10 and to some extent TGF-β [247]. However, despite the discovery of these cells at the turn of the millennium, no specific transcription factor has yet been identified. We know that Tr1 cells are mostly found in the gut mucosa and are linked to oral tolerance and T lymphocyte immunoregulation in the context of intestinal flora [248].

Cytotoxic T Lymphocyte (CTL)

CD8+ T lymphocytes are cytotoxic cells from the adaptive immune branch. Like their CD4+ T lymphocytes relative, the vast majority of CTL express αβ TCR on their surface, while the rest express γδ TCR [249]. Their activation is thus regulated in a contact-dependent manner via the MHC-I/antigen complex present on APCs and on most of the cells in the body (target cells). Th1 CD4+ T lymphocytes are also known to provide them with indirect support via the secretion of cytokines [250]. As with other T lymphocyte subsets, CD8+ T lymphocytes possess a specific transcription factor called eomesodermin (Eomes) [249, 251]. It controls genes associated with the cytotoxic role of these cells. In order to accomplish their functions, CD8+ T lymphocytes are secreting perforins, which form pores in the target cells and allow granzymes to enter the cytoplasm. These enzymes mediate the induction of caspase cascades leading ultimately to program cell death. An alternative pathway using cell surface FAS ligands can also induce target cell apoptosis following its interaction with FAS receptors. Using their cytotoxic potential, CD8+ T lymphocytes are therefore responsible for controlling viral infection and tumor cells, but in the CNS of MS patients, these cells are known to attack directly oligodendrocytes and neurons [250, 252].

B Lymphocytes

While B cells have previously been characterized as antibody-secreting lymphocytes (plasma cells), studies have demonstrated that they can also act as efficient APCs by presenting the peptide that is recognized by their B cell receptor [253]. Furthermore, B lymphocytes can modulate the local inflammatory response of both T lymphocytes and myeloid cells through the secretion of pro-inflammatory (GM-CSF, IFNγ, IL-6, TNFα and lymphotoxin-alpha) or anti-inflammatory (IL-10 and IL-35) cytokines [254]. In a disease context, the abnormal presence of

antibodies in the CNS parenchyma or in the CSF continues to represent the most consistent diagnostic feature of patients with MS (discussed in the MS chapters). Early studies have demonstrated the presence of antibodies specific for myelin peptides or for the potassium ion channel KIR4.1 in the peripheral blood of MS patients [255-257]. Corroborating with these results, others have shown the presence of anti-myelin antibodies inside phagocytic cells within MS lesions [258]. However, more recent studies have analyzed the antibodies derived from CSF-expended B lymphocyte clones of MS patients and based on those results, concluded that autoantibodies may preferentially target neurons and astrocytes [259, 260]. Another debated theory around B lymphocytes is surrounding their presence in high number inside meningeal follicular-like structures during neuroinflammation [261-266]. Notwithstanding scientists' divergent opinions on this subject, the presence of B lymphocytes in the CNS of MS patients and their contribution to disease activity is more widely accepted. In fact, this knowledge as stem the development of an anti-CD20 monoclonal antibody called Ocrelizumab, which is currently in phase III clinical trials and might soon be marketed as a therapy for MS. Anti-CD20 depletes B lymphocytes and substantially limits new disease activity [254], even though similar levels of endogenous antibodies can be found in the CSF of patients benefiting from the therapy [267]. This suggests that the therapeutic mechanisms of action by which B lymphocyte depletion limits new MS relapses are, at least in part, due to the antibody-independent role of B lymphocytes. As mentioned above, B lymphocytes can secrete pro-inflammatory cytokines and multiple studies have demonstrated that MS patients tend to have increased frequencies of those pro-inflammatory immune cells, which are also more readily induced compared to cells from healthy controls [268]. Presumably, anti-CD20 treatment reduces overall inflammatory mediators, which affects both encephalitogenic lymphocytes and myeloid cells. In fact, B lymphocyte depletion studies have demonstrated a reduction in pro-inflammatory T lymphocytes in the CNS of treated MS patients [269, 270]. Similarly, using anti-CD20 to deplete B lymphocytes after EAE onset in mice significantly reduces disease progression and the number of CNS infiltrating leukocytes. This further demonstrates that B lymphocytes contribute to EAE pathogenesis even though they do not produce pathogenic CNS targeting antibodies in most models [271]. In contrast, B lymphocyte deficient mice were shown to have no remission following the initial disease onset. This failure to resolve the ongoing CNS inflammation

correlated with the absence of IL-10-producing B lymphocytes [272]. The importance of regulatory B lymphocytes to control the immune system was further demonstrated using B lymphocyte specific IL-10 KO mice infected with murine cytomegalovirus. With this infection model, WT regulatory B lymphocytes were shown to control virus-specific CD8+ T lymphocytes responses and plasma cell expansion [273]. Although there is no consensus on the targets of IL-10-secreting B lymphocytes, dendritic cells, which are involved in the induction of T lymphocyte activation and pathogenic T lymphocytes themselves have been proposed. Nonetheless, further research will need to be conducted to identify reliably pro-inflammatory and anti-inflammatory B lymphocytes subsets or their progenitors in an effort to target more specifically the encephalitogenic immune cells.

Other Lymphocytes

While many other subsets of lymphocytes exist, they will not be discussed in detailed here (NK T lymphocytes, innate T lymphocytes, etc.). Similarly, variations of lymphocytes discussed above also exist, such as Tc17 lymphocytes (CD8+ CTL expressing IL-17) and a vast number of IL-10-producing cells, which are thought to have transient regulatory effects [274, 275]. Finally, while some scientists believe that lymphocytes can be locked in one fate, others argue that most if not all T lymphocytes are intrinsically plastic, modifying their functions based on the environment surrounding them [276-278]. In any case, it is human nature to categorise and catalogue what we discover and therefore, it is likely that the current nomenclature will be maintained.

2. Antigen-Presenting Cells

Monocytes / Macrophages

Monocytes are innate immune cells of myeloid origin identified in part by their expression of CD11b in mice and CD14 and/or CD16 in humans. These cells, found in the blood and lymphoid organs, can transform into specialized APCs called macrophages once in the tissue.

In an attempt to categorize these cells with a terminology reminiscent of the early T lymphocyte nomenclature, macrophages have been divided in two groups: the M1 or pro-inflammatory cells and the M2 or anti-inflammatory cells. Although many studies have identified "subset-specific" markers [279-281], most scientists agree that these myeloid cells are highly plastic and can adapt to their environment [282]. As such, this bi-modal taxonomy is a simple representation of what is really a complete spectrum of sub-populations and/or different stages of cell differentiation. To mitigate the inherent rigidity of this model, additional subgroups (M2a, M2b, M2c, M4, etc.) were defined by different researchers, adding to this still imperfect classification [283]. Anyhow, M1 cells have been generally identified as expressing high levels of Ly6C in mice, whereas high levels of CD14 and low levels or absence of CD16 is used to characterize this cell type in humans (classical monocytes). Once activated, these pro-inflammatory myeloid cells also express high level of co-stimulatory molecules (CD80/CD86), reactive oxygen species (ROS), nitric oxide (NO) and secrete inflammatory cytokines such as TNF, IL-12, IL-6, TNF, IL-1β and IL-23. Conversely, M2 cells, which are believed to be patrolling immune cells involved in tissue repair, angiogenesis and tissue remodeling, secrete IL-10, TGF-β, glucocorticoids and express the prototypic enzyme arginase-1. They also express high levels of CD43, CD206 and low levels of Ly6C in mice, while low levels of CD14 and high levels of CD16 have been described in humans (non-classical monocytes). Macrophages respond to tissue invasion by external pathogens through the production of pro-inflammatory cytokines but also by engulfing the inflammatory stimuli via phagocytosis. To do so, macrophages express numerous pattern recognition receptors (PRRs) on their surface, as well as MHC class I and II, which are used to present to T lymphocytes their engulfed antigens. Using a complex process combining adhesion molecules, co-stimulatory molecules and cytokines, macrophages form immune synapses with T lymphocytes and can activate/polarize them, allowing the activation of the adaptive division of the immune system [284, 285].

Dendritic Cells

Dendritic cells (DCs) carry functions similar to macrophages. They express PRRs and MHC class I and II on their surface, which makes them highly specialized APCs. As with the

monocytes/macrophages, DCs have been divided into two broad categories: conventional dendritic cells (cDCs or mDCs for myeloid dendritic cells) and plasmacytoid dendritic cells (pDCs). Whereas both mouse subset express CD11c, the prototypic mouse DC marker, the characterization of human DCs is somewhat more complex. Both human monocytes and cDCs can express CD11b, CD11c, CD13 and CD33, but DCs lack CD14/CD16. Analogous to macrophages, other sub-populations of DC have been observed. Human cDCs can be further divided into CD1c+ or CD141+ cells, which are homologous to mouse CD11b+ or CD8+ DCs, respectively [286]. On the other hand, human pDCs lack CD11b, CD11c, CD13 and CD33, but express CD123, BDCA-2 and BDCA-4 [287], whereas mouse pDCs express CD4, B220/CD45R, CD209 and Ly6C. Although cDCs and pDCs share similar precursors, the former cells are much more efficient at presenting antigens, while the latter produce massive amounts of IFN-α [288].

Additional non-lymphoid tissue APC subsets have been identified. These cells are usually referred to as tissue-resident macrophages and dendritic cells. Langerhans cells are found in the skin and mucosa, Kupffer cells in the liver, alveolar macrophages in the lungs and microglia in the CNS; each of which express different combinations of markers and perform slightly different functions adapted to their environments. Other resident-APCs without specific names, but nonetheless with highly important functions, have also been identified: meningeal macrophages, which survey the CSF, resident macrophages of the intestine, which maintain mucosal homeostasis, etc. [285, 289]. Although both of these immune cell populations are found in completely different regions of the body, they both have been implicated in the pathogenesis of MS. Indeed, a growing body of evidence suggests that the gut microbiome affects the development and the maintenance of the immune system, while being implicated in a range of disorders including inflammatory bowel diseases, autoimmune diseases, obesity, cardiovascular diseases, etc. [290-295]. The role of the gut microbiota as an MS risk factor will be further discussed in the next chapter.

3. Multiple Sclerosis

1. The History of the Disease

In 1868, Dr. Charcot, a French neurologist, described a neurological disease characterized by the presence of demyelinating plaques in the CNS. Adopting a descriptive nomenclature, he named the disease “sclérose en plaques”, which can be directly translated as plaques of sclerosis, adapted in English as multiple sclerosis [296]. He also observed that patients affected by this disease showed intermittent neurological symptoms that varied between individuals and could be recurrent. He is generally regarded as being the father of MS clinical diagnosis and the basis of his histopathological work is still valid nowadays. In 1942, Dr. Kabat, well know for his work on antibodies, demonstrated the presence of gamma immunoglobulins in the CSF of MS patients and argued that these oligoclonal bands, as observed by electrophoresis, could help diagnose MS patients [297]. More recently, the development of new imaging technology using magnetic resonance and the marketing of novel immunomodulatory drugs have facilitated both the diagnosis and the treatment of MS. However, even if our knowledge on this illness has increased tenfold in the last few years, the cause(s) of MS is still unknown and as a result, it hampers our ability to cure the disease.

2. Clinical Aspects of the Disease

MS is an idiopathic demyelinating disease of the CNS characterized by the presence of inflammatory lesions disseminated both in time and space [298]. The loss of myelin sheets surrounding neurons slows down the transmission of electrical influx along the axonal projections, which in turn causes a large spectrum of symptoms in patients. In addition, the increased metabolic demand on the affected neurons, the collateral or direct inflammatory damages and the lack of adequate support contribute to Wallerian neuronal degeneration, which is the morphological basis of permanent clinical disabilities [299]. Depending on the area affected (brain or spinal cord, and their respective subregions), patients can demonstrate sensory deficiencies such as tingling, numbness or pain, as well as visual impairment, which is often

present during the first episode of neurologic symptoms (often referred to as clinically isolated syndrome (CIS)). Motor deficiencies are also a major component of MS symptoms, especially due to their inherent impact on the patient's daily activities. Again depending on the affected region and the degree of demyelination, symptoms can vary from a loss in coordination to partial or complete paralysis of one or multiple body parts. Gaiting difficulties and spasticity of the legs are two of the most recurrent motor deficiencies observed in patients. Finally, psychological and cognitive deficits, which are often subtler disabilities and not well represented in the measurement methods of MS progression (such as the expanded disability status scale (EDSS)), include fatigue (one of the most common symptoms in MS), emotional changes and depression to name a few [300, 301].

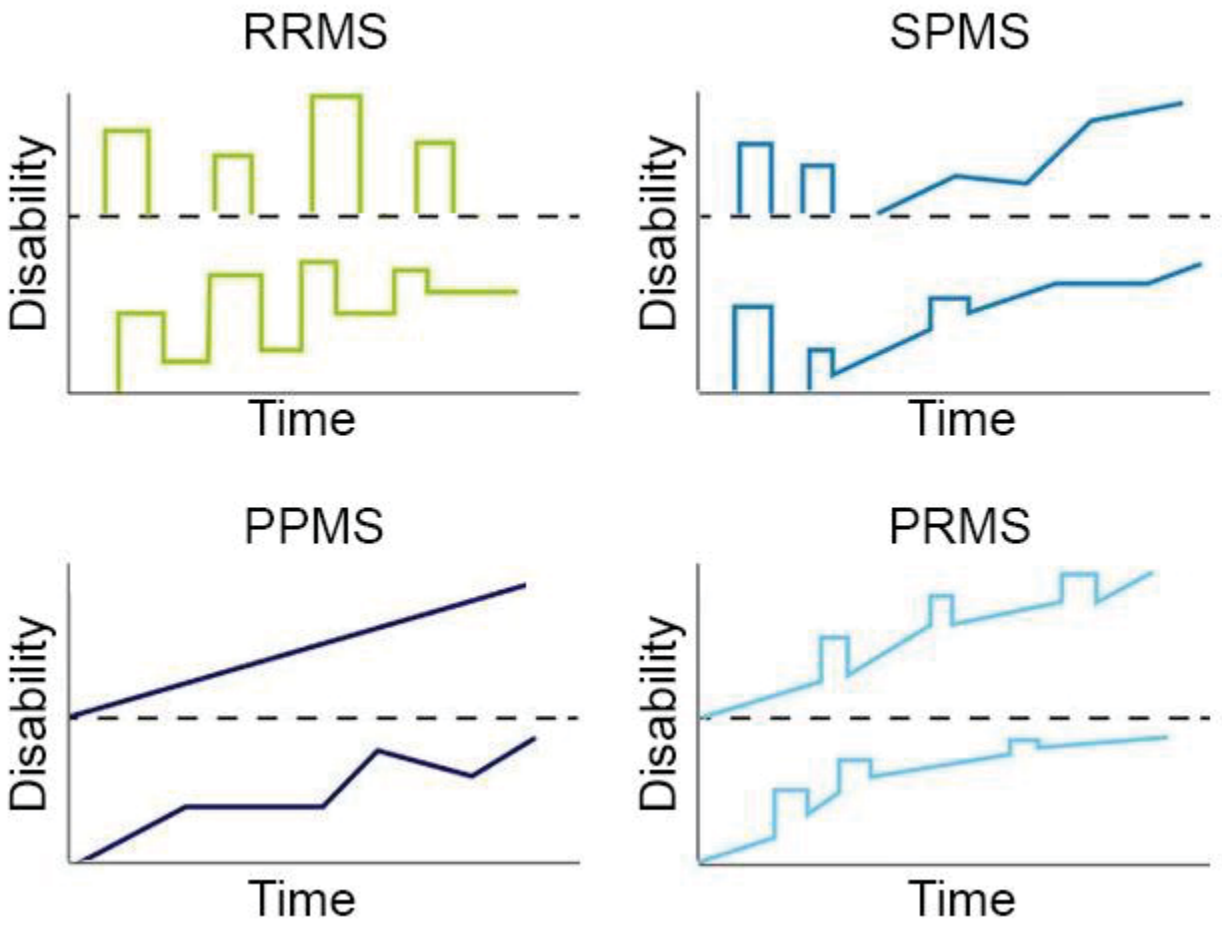

Figure 7 – Graphical representation of the different types of MS disease. Clinically measurable/perceived disabilities are shown over the dashed-line. Background/hidden disabilities and/or CNS damage are shown below the dashed-line. Copyright Marc-André Lécuyer.

Based on clinical observations, subtypes of MS disease have been described. Roughly 80 to 90% of MS patients will be affected by a relapsing-remitting form of MS (RRMS) following their CIS (**Figure 7**) [302, 303]. As the name implies, patients suffering from RRMS will have relapses (active disease with symptoms) followed by complete or incomplete remissions. A majority of these patients will later transition to a secondary progressive form of MS (SPMS), which is characterized by a progressive accumulation of disability, albeit episodic disease exacerbations may still occur. This form of the disease is believed to be the results of cumulative

hidden CNS damage and brain atrophy (**Figure 8**). A small number of patients present steady disease progression following disease onset. This form of the disease as been coined primary progressive MS (PPMS). It is the least understood form of the disease as patients present different disease courses with varying clinical and histopathological evaluations. Finally, a fourth form of the disease, progressive-relapsing MS (PRMS), was defined as having steadily worsening neurologic functions from the disease onset/diagnosis with occasional relapses [304]. In 2013, the US National Multiple Sclerosis Society Advisory Committee on Clinical Trials in Multiple Sclerosis redefined more accurately the different subtypes of MS based on the important advances in technology and clinical assessment. Patients who were previously diagnosed with PRMS are now considered primary progressive MS patients with either an active (during relapses/new lesions formation) or inactive phenotype [305].

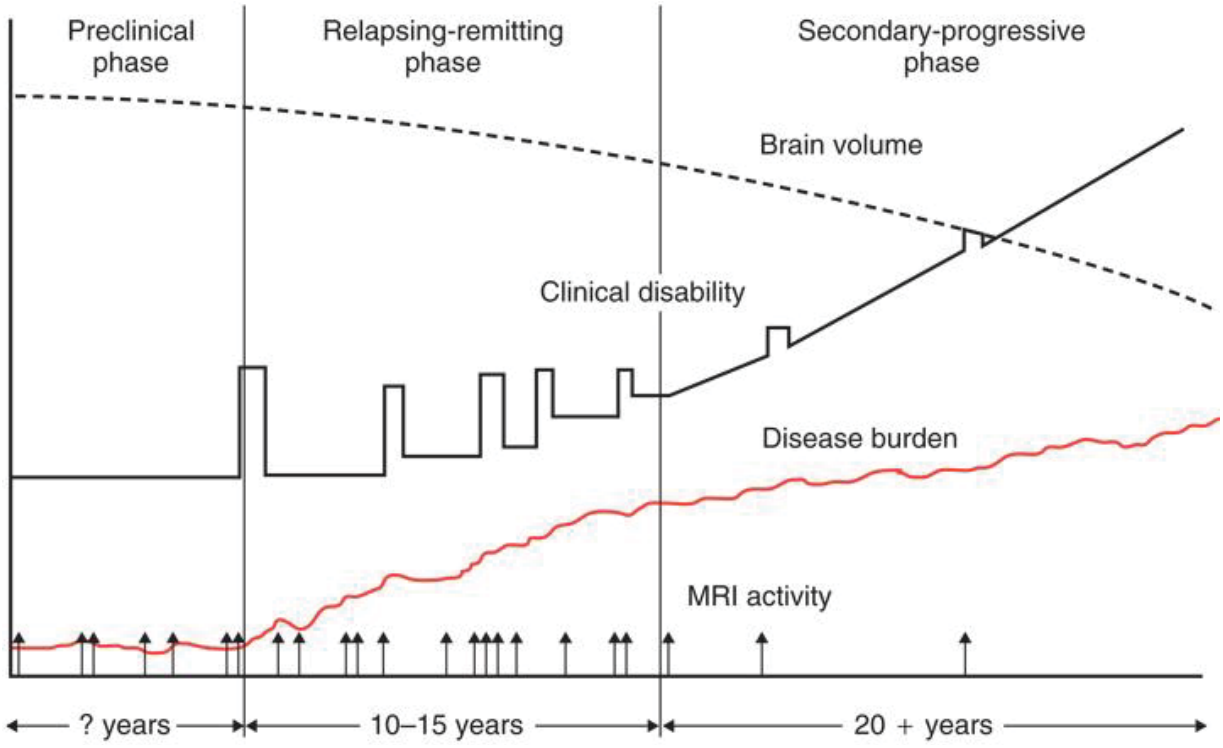

Figure 8 – Graphical representation of the clinical disabilities, disease burden, brain volume and MRI activities of a mock average RRMS patient. Image obtained from Fox, R. J. and Cohen, J. A. [306]

As eluded previously, most MS patients will develop new lesions, observed by magnetic resonance imaging (MRI), which won't translate into new clinically measurable deficiencies (**Figure 8**). In fact, the vast majority of MS patients exhibited disseminated lesions prior to their first neurologic symptoms, as revealed by MRI techniques [307]. This discordance between the lesions location/number and the clinical presentation is one of the limiting factors of MRI technology in terms of MS diagnosis. In fact, the MRI sensitivity and specificity can vary greatly depending on the location and the disease subtype. In the last few years, different imaging techniques and computer image analyses have been developed to cope with these issues. T1-weighted, T2-weighted, T2-FLAIR (fluid-attenuated inversion recovery) and proton density (PD)-weighted MRI are just a few examples of different types of imaging techniques that can be used to observe more specifically different components of the CNS [308-310]. In addition, contrast agents, such as gadolinium, can be injected intravenously to enhance MR images. With MS patients, this technique is also used to find BBB leakage, which usually also correlates with active/acute MS lesions [311, 312]. On the other hand, longitudinal measurements of magnetization transfer ratio (MTR) allows the assessment of both demyelination and remyelination of MS lesions [313]. Using this technique, recent findings suggest that demyelinated lesions found during histopathological analysis of post-mortem CNS could be the result of multiple episodes of demyelination and incomplete remyelination [314].

Another technique, the magnetic resonance (MR) spectroscopy relies on the characteristic spectral signature of biochemical compounds to measure organic compounds *in vivo*. By comparing the spectral signal of *N*-acetylaspartate, which is a relatively specific neuronal marker, with that of creatinine, MR spectroscopy can successfully assess neuronal and axonal loss [315]. Using a different approach, optical coherence tomography (OCT) also allows the measurement of neurodegeneration, neuroprotection, and potentially even neurorestoration by generating high-resolution 3D reconstructions of the retinal anatomy. While the retina is unique within the CNS, in that it contains axons and glial cells but no myelin, it is an ideal structure within which to visualize the MS disease progression. Not only the ease of access facilitates precise data acquisition, but the retina also enables scientists and clinicians to investigate the early disease processes often observed during a CIS [316-318]. Although all these techniques have allowed rapid non-invasive longitudinal examinations of MS patients, further development

is still needed to improve their efficiency and specificity in order to solely rely on them as diagnostic tools. In the meantime, the analysis of CSF for the presence of oligoclonal bands, which are found in nearly 90% of MS patients and contribute to the criteria of dissemination in space, will continue to be conducted during the evaluation of patients [319, 320].

3. MS Pathology

Although the etiology of MS is still unknown, the mechanisms behind the clinical symptoms observed have been studied for decades. Early on, scientists concentrated on the histopathological analysis of the lesions (**Figure 9**). They demonstrated that the plaques observed in the CNS are in fact areas lacking myelin sheets. In addition, these regions are often characterized by the presence of infiltrating immune cells (both myeloid cells and lymphocytes), by the presence of activated microglia, by the presence of astrogliosis/glial scaring surrounding the lesions and by an increase in BBB permeability [321]. Further studies, using more modern techniques such as flow cytometry analysis of CNS infiltrating immune cells and high resolution immunofluorescence imaging using confocal and two-photon microscopy, have allowed a deeper understanding of the different mechanisms and players involved during the pathogenesis of the disease.

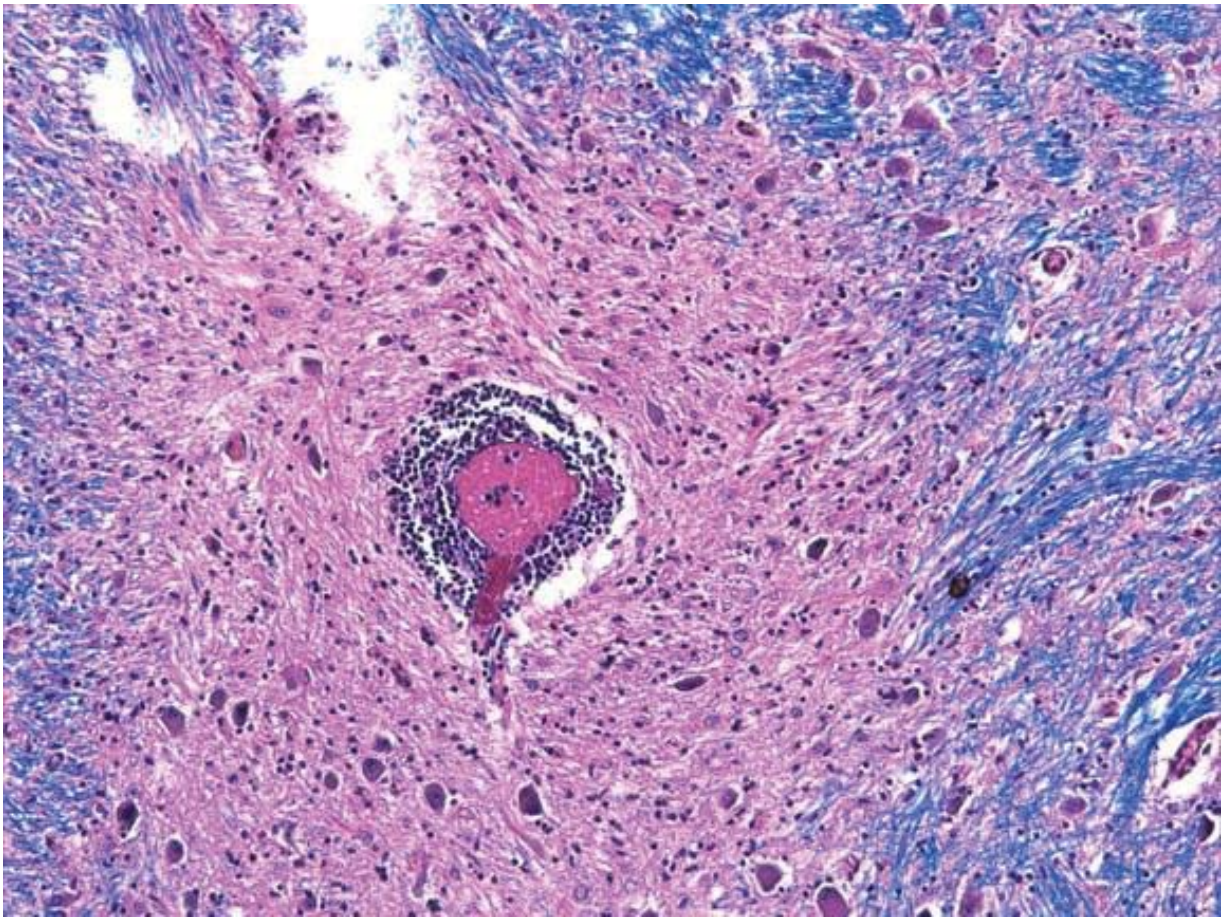

Figure 9 – Demyelinating lesion in the post-mortem brain of an MS patient. Perivascular infiltrating immune cells (dark purple) can be seen surrounding the central blood vessel in this Hematoxylin & Eosin / Luxol Fast Blue (H&E/LFB) tissue colouration. In the immediate vicinity of the vessel, the destruction of myelin sheet is denoted by the absence of blue staining. Copyright Alexandre Prat. The image was taken and stained by the group of Dr. Alexandre Prat.

T lymphocytes are one of the most important immune cell subsets involved in this process. CD8+ cytotoxic T lymphocytes can directly attack the oligodendrocytes via FAS/FAS-L-mediated cytolysis and the granzymes/perforin pathway. CD4+ T helper lymphocytes, on the other hand, favour the antibody production/maturation in B lymphocytes and promote the activation of both innate and adaptive immune cells. Both T lymphocytes subsets are found in high number in lesions and in the CSF of MS patients [322-324]. Surprisingly, studies have

demonstrated that self-specific T lymphocytes are readily found in both MS patients and healthy controls, alluding to the complexity of the disease pathogenesis [325]. B lymphocytes have also been implicated in lesion formation, especially plasma cells, by secreting anti-myelin antibodies that can trigger opsonisation via the complement factor cascade and phagocytosis via Fc-receptors [258]. Likewise, myeloid cells are highly important during disease pathogenesis. In fact, untreated MS patients were shown to have higher levels of blood monocytes secreting IL-6, TNF and IL-12 and expressing higher level of co-stimulatory molecule CD86/CD80, as compared to treated patients or healthy controls [284, 326]. Studies using animal models have also demonstrated that neutrophil and monocyte/macrophages are the first immune cell to aggregate in the meninges and the perivascular cuffs following immunization, while also encompassing the majority of immune cells present in early lesions [327-329]. Whereas activated microglia and macrophages can induce CNS damage via the secretion of pro-inflammatory factors, they can also engulf debris found in lesions and thus promote a pro-inflammatory response by presenting new CNS antigens to T lymphocytes. Indeed, both microglia and macrophages found in lesions were shown to contain a wide variety of myelin proteins (myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG)). Conversely, phagocytosis of dead cells and debris have also been demonstrated to favour remyelination by removing inhibitory molecules as well as providing trophic factors to oligodendrocyte precursor cells (OPCs) [330-332].

Following the acute inflammatory phase, immune regulatory cells, along with glial cells, can mediate the resolution of the inflammation by secreting anti-inflammatory cytokines. OPCs present in the CNS and surviving oligodendrocytes surrounding the lesions then get recruited and begin remyelinating the denuded axons. However, the thickness and complexity of the original myelin sheets is not reached and therefore, despite compensatory redistribution of ion channels, the electrical propagation often remains impaired [333, 334]. The remyelination rate can vary from one lesion to the other and in-between different individuals based on the inflammatory lesion environment, the presence of myelin debris and mitochondrial damage, the level of axonal loss/damage and the lack of trophic factors [330, 334-337]. Whereas astrocyte activation can promote inflammation, as discuss before, they are also necessary during the

remyelination phase. However, astroglial scarring can impede the remyelinating process [334, 337].

More recently discovered, the presence of diffuse lesions in the “normal appearing white matter” (NAWM), which is often used as internal control to compare a lesion to “healthy” tissue, was described as another pathological aspect of MS. These diffuse white matter lesions, probably resulting from neuronal oxidative stress, are characterized by axonal swelling and by the presence of some activated microglia and astrocytes. As patients slowly progress towards a more chronic and progressive phase of the disease, more diffuse inflammation of the NAWM and cortical demyelination are observed [338-341].

In general, MS lesions can be classified based on their inflammatory stage. An active lesion is newly formed with massive amount of infiltrating immune cells containing macrophages filled with myelin debris. Chronic active lesions are characterized by an inactive center, mostly devoid of peripheral immune cells, and surrounded by an active border, defined by the presence of infiltrating leukocytes, activated microglia and astrocytes. Variable axonal damage can be observed in these lesions. Finally, chronic inactive lesions consist in well-defined areas of demyelination lacking immune cells. Extensive astroglial scarring can be observed in these lesions [298, 336, 342]. Our group has also published recently a study demonstrating the existence of pre-active lesions. Those lesions found in both MS and spontaneous EAE display considerable BBB disruption, perivascular astrogliosis, redistribution of junctional proteins and increased expression of endothelial cell adhesion molecules, while being completely devoid of leukocytes [137].

4. Epidemiology

Prevalence

MS is a disease that affects predominantly people living between the 45th and 65th degree of the northern hemisphere, with a prevalence generally increasing with respect to the distance to the equator [343]. Canada has one of the highest prevalence rates in the world with roughly 280 cases per 100 000 inhabitants. This is probably due to a variety of confounding factors that

increase significantly the risk of developing autoimmune diseases; some of which will be discussed in further details in the following paragraphs. As comparison, Northern European countries, such as Norway, Sweden, Scotland, and Ireland, have prevalence rates ranging between 150 and 350 per 100 000 inhabitants. Conversely, Northern Asian countries are not as affected with levels lower than 70 cases per 100 000 inhabitants in Russia and between 1 and 13 cases per 100 000 inhabitants in Mongolia, Japan and China [343]. It should be noted that while these countries have lower MS prevalence rates than most north Western countries, their proportion of patients with neuromyelitis optica spectrum disorder (NMOSD), a new unifying term regrouping neuromyelitis optica (also known as Devic's disease) and what was previously described as the opticospinal form of MS, is much higher, accounting for 15 to 56 percent of MS-like cases. NMOSD is a relapsing inflammatory demyelinating disease (although a monophasic form exists) that affects the optic nerves and the spinal cord, but in some cases may also target other parts of the brain. The disease relapses tend to be more severe and often different from MS. Although the newly established nomenclature and diagnostic criteria have been left broad enough to encompass similar clinical manifestations, NMOSD is most often characterized by the presence of anti-aquaporin-4 antibodies, also known as NMO-IgG, in the serum of patients [344-346].

In northern countries, RRMS is predominantly a female disease with a ratio man to female that can reach 1:3-4. Conversely, similar number of male and female are affected by the primary progressive form of the disease [347-349]. Another striking difference between the two types of disease is the mean age of disease onset, which is between 20 and 50 years of age for RRMS and close to a decade later for PPMS [303]. However, close to half of the RRMS patients will convert to a secondary progressive form of the disease after 10 years of their initial diagnosis and this proportion keeps increasing in patients affected by the disease for longer periods of time [350].

Risk Factors

Although it is clear that MS has a geographic pattern of prevalence, other factors also contribute to the incidence of the disease. Genetic mutations, for example, have been

demonstrated to play a determining role in the etiology of MS. Nevertheless, no mutation or collection of mutations are enough to guarantee the development of MS, as demonstrated by monozygotic twin studies showing a clinical-genetic concordance rate of 20 to 30%. Dizygotic twins, on the other hand, have between 2 and 5% chances of developing the disease if their sibling has MS, which is approximately the same risk that first-degree relative shares [321, 351]. Most of the discovered polymorphisms associated with MS are immune related, with the highest relative risk associated to the Human Leukocyte Antigen (HLA)-DRB1-1501 allele. This subtype of MHC-II reportedly can present specific self-antigen to CD4+ T lymphocytes, which could lead to the activation of the immune system. Other haplotypes of the MHC class II and class I can confer either higher risk of developing MS or a protective effect [352-357]. Outside of the MHC molecules, other proteins involved in the immune system have been shown to influence MS susceptibility. Most of these genes are involved in T lymphocyte immune processes, such as activation (CTL-4, CD6), cytokine and chemokine production (IL-2) and transmigration (ALCAM) [354, 358-360].

Even if a high number of genetic mutations have been shown to contribute to MS etiology, the relatively low concordance rate of monozygotic twins demonstrates the existence of other non-genetic factors. Among them, environmental factors (often associated with socioeconomic factors) have been proposed to contribute to disease onset. One of the main supporting arguments in favour of an environmental aspect, aside the latitudinal distribution of the disease, is the dissimilarity in the prevalence rate between the Northern European descendants living closer to the equator and their ancestors. Indeed, studies have demonstrated that if an individual migrate from a region of high MS prevalence to a region with low prevalence before the age of 15, its risk of developing the disease is adjusted to the risk of the new region. The reverse being also true [361-363]. The geographical MS distribution might therefore be explained by events in someone's life that would alter significantly its immune system in a permanent way at a young age. One of the most important contributing factor to the overall quality of the environment is the socioeconomic level of a country, which, based on recent epidemiological studies, seems to be linked to the incidence rate of MS. For example, while the global MS prevalence rate is low in Asia, the highest rate is found in Japan, the most developed country [343]. Further longitudinal studies have also demonstrated that the risk of developing MS or other autoimmune

diseases increases in countries that have had major economic development in the last decades. Therefore, it is safe to assume that a portion of the environmental factor is due to the exposure to urban living conditions (pollution, chemicals, diet, smoking, stress, etc.) [364].

Industrialization has also considerably contributed to the ease of access to disease-free food and water, as well as to modern medicinal treatments. This reduced exposure to infectious agents at an early age and perhaps the overconsumption of antibiotics has given rise to the *hygiene hypothesis*, which states that exceeding cleanliness may increase the risk of allergic and autoimmune reactions by polarizing the immune system toward an immune over responsive state. In other words, the absence of regular benign infections and contacts with inflammatory triggers (pollen, dust, nuts, etc.) at an early age, which would normally shape the immune system reactivity, increases the propensity to react more aggressively to immune challenges in adulthood. Corroborating with this hypothesis, several studies concluded that parasitic infections diminish the number of relapses and the number of new demyelinating lesions in MS patients by favouring a Th2-mediated/Treg immune response [365-367]. Small clinical trials using live helminths to treat MS have been conducted and showed mitigated but encouraging results. In the future, mimicking their immuno-protective effects might lead to novel therapeutic compounds without the inconveniences [368].

The composition of the gastro-intestinal (GI) flora, a collection of thousands of different commensal organisms that covers the GI epithelium is also directly related to the consumption of antibiotics and the presence of pathogens/bacteria in the daily diet. Numerous research groups are currently studying the impact of these microorganisms on the human biology, both in homeostatic conditions and with respect to specific diseases such as MS [290, 364, 369-374]. Building up from the original studies, other groups have demonstrated that patients suffering from inflammatory bowel diseases are at greater risk of developing MS [375, 376]. Interestingly, studies have also shown that commensal gut bacteria can influence the blood-brain barrier integrity [377, 378], leukocyte polarization and even the susceptibility to EAE [379, 380], opening the way to new therapies involving faecal transplantation [381].

As opposed to the lack of infectious agents, the contact with specific viruses have been suggested as a possible cause of MS. The Epstein-Barr virus (EBV), which is present in almost

all MS patients (although also present in roughly 90% of the adult population), have been studied extensively in the last few years. EBV infection usually occurs early in life, but in industrialized countries, a late infection is possible and usually lead to the development of infectious mononucleosis. During the infection, the EBV genome is integrated in B lymphocytes DNA, where it can persist indefinitely. Studies have demonstrated that seronegative individuals are significantly less at risk of developing MS, while seropositive children with a CIS have a greater risk of being diagnosed with MS. In addition, the risk of developing MS is increased by roughly 50% in individuals that have had mononucleosis [353, 367, 382-384]. Another virus from the same family, the human herpes virus 6 has been associated with MS. Similar to EBV, it is found in the majority of the general population. Antibodies specific to the virus can be found in the CSF and the serum of MS patients in higher concentration, as compared to healthy controls. In addition, viral messenger RNA was detected surrounding MS lesions [385-389]. Based on these discoveries, several hypotheses were suggested to explain the etiology of MS. Viral antigens similar to human CNS proteins (often referred to molecular mimicry) could, once the immune system activated, divert the attack to target self-antigens. This theory is corroborated by several studies that have shown high homology between myelin peptides and proteins from the herpes virus family, the influenza virus family and some coronavirus [390-396]. The bystander cellular activation is an alternative theory put forward where a viral infection triggers the activation of the immune system (both the anti-viral and the self-specific T lymphocytes) leading to the formation of lesions, at least initially, close to infected areas of the CNS.

People in industrialized countries tend to spend more time inside, protected from the sun, than in third-world countries. Furthermore, people living closer to the poles are not exposed to as much ultraviolet (UV) radiation as people closer to the equator. This can lead to a deficiency in vitamin D, which has also been associated with a higher MS susceptibility rate [397]. Indeed, most vitamin D present in the human body is synthesized from 7-dehydrocholesterol found in the skin, which is converted to pre-vitamin D_3 following exposure to UVB rays. Next, this pre-vitamin is hydroxylated by the liver and the kidneys to become the biologically active vitamin (1,25-dihydroxyvitamin D_3). It can then bind to the vitamin D receptor on the cell surface, translocate inside the cell nucleus and regulate the transcription of genes via DNA regions called vitamin D-responsive elements. As such, although called a vitamin, this molecule acts more as

a hormone [398-400]. Vitamin D is known to play a critical role in many biological processes including the regulation of calcium in the blood, bone growth and remodeling and more importantly for MS, the regulation of the immune system [401]. While vitamin D was shown to inhibit the maturation of DCs and the proliferation/polarization of Th1/Th17 lymphocytes [398, 402-404], the efficacy of the administration of supplement to MS patients, although encouraged in the clinics, still needs to be demonstrated [301].

The fact that women with RRMS outnumber the number of men with the disease suggests that other hormonal factors are involved. This is also supported by data demonstrating a lower relapse rate during pregnancy and a disease rebound afterwards [364]. Likewise, the use of hormonal therapy to increase women's fertility have been found to significantly enhance the disease activity and clinical symptoms [405]. However, behavioural differences between the two sexes could also explain in part the risk variation. In fact, behavioural influences are the biggest unknowns in risk studies as it is difficult to account for every event in someone's life.

As briefly mentioned earlier, the diet is probably the easiest controllable and measurable behaviour, albeit with important ramifications on the overall health of an individual. Obesity, for example, is known to be responsible for many illnesses including, most notably, diabetes. Obesity has also been linked to low levels of vitamin D and an increase in MS susceptibility [406-408]. Indirectly related, a sodium rich diet has recently been associated with an increase number of pathogenic Th17 lymphocytes and a significant worsening of EAE [409, 410]. Thus, it is clear that the development of MS is a complex process involving multiple factors implicated at different levels of the human biology, and which, together, push the normally highly regulated immune system beyond its limit.

5. Experimental Autoimmune Encephalomyelitis

Similar to other autoimmune diseases, MS has not been observed in wild animals yet. Scientists have therefore relied on induced animal models to study the pathogenesis of the disease. As with many scientific and medical discoveries, the origin of EAE is the result of unrelated studies performed by Louis Pasteur at the end of the 19th century. While injecting

animals with rabies infected brain homogenates in an effort to develop a vaccine, Pasteur noticed that some recipients demonstrated similar pathological and histological features. The disorder was characterized by an ascending paralysis, which could lead to the death of the animals. A similar disease was also observed in some human patients immunized the same way following an animal bite in order to prevent a rabies infection. While some people claimed that this disorder was likely due to a bacterial contaminant, Pasteur proved otherwise by pasteurizing the homogenates prior to the injections. The disease was then called experimental allergic encephalomyelitis [411]. The cause of this lethal illness was only explained in the 1930s, when a study showed that animals immunized with brain homogenates will produce autoantibodies damaging its CNS [412]. Thirty years later, another study demonstrated that EAE could be induced in healthy animals by transfer of sensitized lymphocytes from an EAE-affected animal [413], whereas autoantibodies were insufficient to trigger the disease [321]. Adjuvants were also developed to improve the induction of the disease and ameliorate the reproducibility in research studies [414].

Nowadays, three major types of EAE are routinely used for research purposes: active, passive and spontaneous EAE. [415]. Active EAE is induced by the subcutaneous injection of a myelin peptide (MOG, PLP, MBP; depending on the strain of the animal) in an emulsion of complete Freund's adjuvant (CFA), which contain a variable quantity of inactivated *Mycobacterium tuberculosis.* Depending on the laboratory and the strain used, pertussis toxin can be injected intra-peritoneal (i.p.) at day 0 and/or day 2 post-immunization to increase leukocytes adhesion to the vasculature and favour a pro-inflammatory reaction [416-418]. Immunized animals develop a monophasic or polyphasic ascending paralysis (often followed by a chronic phase), allowing the study of different stages of the disease [419]. Passive EAE, often called adoptive EAE transfer, is induced by injecting i.p. or intravenously (i.v.) immune cells isolated from active EAE animals that have been reactivated *in vitro*. These pro-inflammatory immune cells trigger the classical ascending paralysis in the recipient animals, but atypical symptoms can sometimes be observed (ataxia, gaiting deficiency, prostration) [420, 421]. Spontaneous EAE is observed in transgenic animals. A majority of these mutations are targeted at the T cell receptor (TCR), forcing the expression of only one myelin-specific TCR. As their name implied, these animals spontaneously develop EAE after a certain period of time,

which can vary based on the transgenic modifications [422, 423]. Although not technically an EAE model, Theiler's murine encephalitis is a virus-induced demyelinating disease, which is used to study the possible viral etiology of MS. It is also useful to conduct studies on molecular mimicry and epitope spreading in the context of MS [424, 425]. Finally, many research groups are also using toxins to study different aspects of EAE pathogenesis. Allegedly the most frequently used toxin, Cuprizone is a copper chelator that causes rapid demyelination and gliosis in the CNS of the animals. It is generally used to study specifically the processes behind demyelination and remyelination, two exceedingly important aspect of MS [426].

While these models allow scientists to study different aspects of the disease both in time and space, something oftentimes difficult or impossible to accomplish in humans, they remain imperfect models which can't recapitulate completely MS pathogenesis. This was clearly demonstrated by discrepancies between human and animal results obtained following an anti-IFNα treatment. While the studies conducted on animals demonstrated a significant amelioration of EAE clinical scores, multiple studies and a clinical trial with MS patients showed an exacerbation of their symptoms. Monophasic CNS demyelination episodes and even new onset of MS disease were also described in patients with arthritis following treatment [427-432]. The lack of animal to human therapy translation can be blamed on a variety of intrinsic differences between the model and the human disease. For instance, MS is an idiopathic disease whereas EAE is artificially induced in otherwise resistant animals. These animals are also usually genetically identical and similar in age, weight, sex, etc. to facilitate the interpretation of the data by limiting the variability. It should be noted, however, that differences in disease severity, time of onset post-immunization, incidence rate, etc. do exist. This inter-laboratory variability is often caused by changes in the environment (cleanliness of the animal facility, stress level of the animals, etc.) or the genetic drifting of a colony from the source. Differences inside a colony can also be observed in a season-dependent fashion or may vary depending on the person manipulating the animals [433, 434]. Even though the animal models used in research are inherently flawed, they are still invaluable tools to study specific immunological mechanisms and biomolecular markers in an attempt to identify novel therapeutic targets.

6. Therapeutic Approaches

Relapse management therapies, which consist in steroid medications, help decrease the severity and duration of MS relapses. Their use over short periods of time is considered relatively safe with few side effects (difficulty sleeping, stomach upset, irritability, etc.). Approved MS treatments, called disease-modifying therapies, have only been demonstrated to be effective against RRMS and PRMS. However, some clinical trials are currently studying the effect of novel compounds on SPMS and PPMS patients.

The first-line treatments, IFNβ and glatiramer acetate, have a moderate efficacy in diminishing relapses, yet they are considered the safest, with few side effects (flu-like symptoms and redness/swelling at the subcutaneous injection site are the most common). They act by reducing immune cell proliferation and pro-inflammatory cytokine secretion, while also interfere with antigen presentation [298, 301, 342, 435].

Fingolimod was the first oral treatment to be approved for MS. Its mechanism of action is via the inhibition of the sphingosine-1-phosphate receptor present on T lymphocytes, which prevent the cells from leaving peripheral LNs. Fingolimod has many possible side effects including the increased risk of opportunistic infections and progressive multifocal leukoencephalopathy (PMLs), which is secondary to the uncontrolled multiplication of the John Cunningham virus (JC virus) in lymphopenic patients [301, 436, 437].

Teriflunomide is also an oral therapy that modulates the immune system. Its exact mechanism of action is still not fully understood, but studies have demonstrated that it inhibits the synthesis of pyrimidines in proliferating cells. Therefore, it is thought to reduce the number of peripheral immune cells. It has few minor side effects which include diarrhea, nausea, flu or sinus infection, upset stomach, abdominal pain, rash, abnormal liver tests and hair thinning or loss [436, 438, 439].

Dimethyl fumarate is an oral drug taken twice daily that causes T lymphocyte apoptosis and favours the production of Th2 cytokines. Dimethyl fumarate or rather its active metabolite, monomethyl fumarate, inhibits NF-κB from translocating inside the nucleus and thus suppresses NF-κB-dependent transcriptions, which impact a wide variety of pathways including the

secretion of pro-inflammatory cytokines, the expression of adhesion molecules and the modulation of oxidative mechanisms. It also favours the intra-nuclear translocation of Nrf2, which in turn exerts a neuroprotective effect by promoting the production of antioxidant enzymes. In addition, monomethyl fumarate is an agonist of the hydroxycarboxylic acid receptor 2 (HCA_2), a G-coupled protein membrane receptor, via which it reduces neutrophil adhesion and chemotaxis. HCA_2 was also demonstrated to be essential for the therapeutic effect of dimethyl fumarate during EAE, as indicated by its absence of effect in HCA_2 KO animals. As with other lymphopenia inducing drugs, one of the most serious possible side effects of this medication is the risk of developing a PML [436, 440-443].

Natalizumab was the first second-line treatment available to treat MS. It is a monoclonal antibody specific to the alpha-4 integrin subunit of VLA-4, which is implicated in the transmigration of leukocytes by binding to its ligand, VCAM-1, on activated BBB-ECs. As with all immunosuppressive treatment, Natalizumab is significantly more effective at preventing relapses in MS patients as compared to first-line therapies. As expected, however, it also has significant side effects, of which the risk of PML is the most serious. Recent studies have demonstrated that the risk of PML significantly increases following 24 months of infusions. Similarly, the risks are increased in patients that have previously been treated with another immunosuppressive agent or with patients that have anti-JCV antibodies [444-449].

Alemtuzumab has recently been approved as a second-line treatment, which consists of three to five intravenous infusion of monoclonal anti-CD52 antibodies per year. Anti-CD52 antibodies cause the depletion of some T and B lymphocytes, NK cells and monocytes that express CD52 on their surface. This treatment is significantly better at preventing relapses compared to first-line therapies. However, due to its immunosuppressive effect, some patients might develop opportunistic infections. In addition, some patients demonstrated evidence of autoimmune dysfunction (autoimmune thyroid disease and autoimmune skin disease) [450-453].

While these drugs have proven to significantly prevent relapses in RRMS patients, the most effective second-line therapies come with highly debilitating and sometime health-threatening side effects. Identifying novel therapeutic targets involved in the pathogenesis of the disease is

thus crucial for the development of new MS therapies. Whereas drugs aimed at promoting CNS repair and remyelination are cruelly missing, multiple laboratories have recently started to focus their efforts on this goal. Besides, our laboratory has been studying novel adhesion molecules expressed by encephalitogenic immune cell subsets and/or the inflamed BBB-ECs in an effort to discover new ways of promoting BBB integrity and decreasing leukocytes trafficking into the CNS during neuroinflammation.

4. Activated Leukocyte Cell Adhesion Molecule

MS is a complex disease with multiple potential therapeutic approaches. One of which consist in blocking the entry of encephalitogenic immune cells by disrupting their ability to interact with specific adhesion molecules and thus, interfere with the transmigration cascade. Following the mitigated success of Natalizumab, the alpha-4 integrin blocker with severe adverse effects, researchers are currently seeking a more elegant target instead of a broad immune-compromising agent [454]. The goal here would be to find cell-specific molecules that could be targeted and allow the sequestration of immune cell subsets outside of the CNS in a neuroinflammatory context. With this goal in mind, our group began studying activated leukocyte cell adhesion molecule. ALCAM, also known as CD166, MEMD, SB-10, HB2, KG-CAM in rat/mouse/human, neurolin in fish and DM-GRASP/SC-1/BEN in chicken, is part of the immunoglobulin superfamily. Given all its names, it is easy to presume the rich history behind this molecule. Indeed, it has been cloned independently in several organisms, hence the eclectic names found in the literature. ALCAM has been and still is heavily studied in the fields of cancer, embryology, haematopoiesis and angiogenesis. However, it was only recently that we, amongst others, have started to study this adhesion molecule with respect to leukocyte transmigration during inflammatory processes.

1. The Discovery

ALCAM, or rather its chicken homologue BEN (90% homology), was discovered in 1990 by Pourquié and colleagues. They had raised antibodies against chicken Bursal epithelium and identified a glycoprotein located in the B lymphocyte differentiation zone. Additional

immunohistochemistry stainings lead to the identification of the same protein in neurons of both the peripheral and central nervous system. Hence, they named the protein BEN for bursal epithelium and neurons [455]. Two years later, they published another article describing the presence of BEN in all three embryonic germinal layers, although differently glycosylated in the different tissues. Of outmost interest, their group also identified BEN as belonging to the immunoglobulin (Ig) superfamily with its two N-terminal variable chain-like domains, three membrane-proximal C2 domains, a transmembrane domain, and a small cytoplasmic tail composed of 33 amino acids [456]. In 1991, DM-GRASP was identified as a 95 kiloDalton (kDa) glycoprotein expressed on chicken axons and involved in neurite extension [457]. In the same year, another group independently isolated a spinal cord chick protein, which they called SC-1. Using purified SC-1, they demonstrated that SC-1+ cells could bind a SC-1 coated surface, indicating a homophilic interaction [458].

Following the discovery of other orthologues (mentioned previously), CD166 was finally characterized in human in a series of four publications in 1995. It was identified in thymic epithelial cells as a ligand for CD6, a lymphocyte costimulatory molecule extensively studied in the 90's [459, 460]. The group therefore coined the name activated leukocyte cell adhesion molecule. Furthermore, the same laboratory also identified the membrane-proximal scavenger receptor cysteine-rich (SRCR) domain (D3) of CD6 as containing the ALCAM binding site (**Figure 10**) [461, 462]. This heterotypic interaction is now known to regulate T lymphocyte development in the thymus and play a major role in the immunological synapse formation between activated APCs (ALCAM) and lymphocytes (CD6) [463].

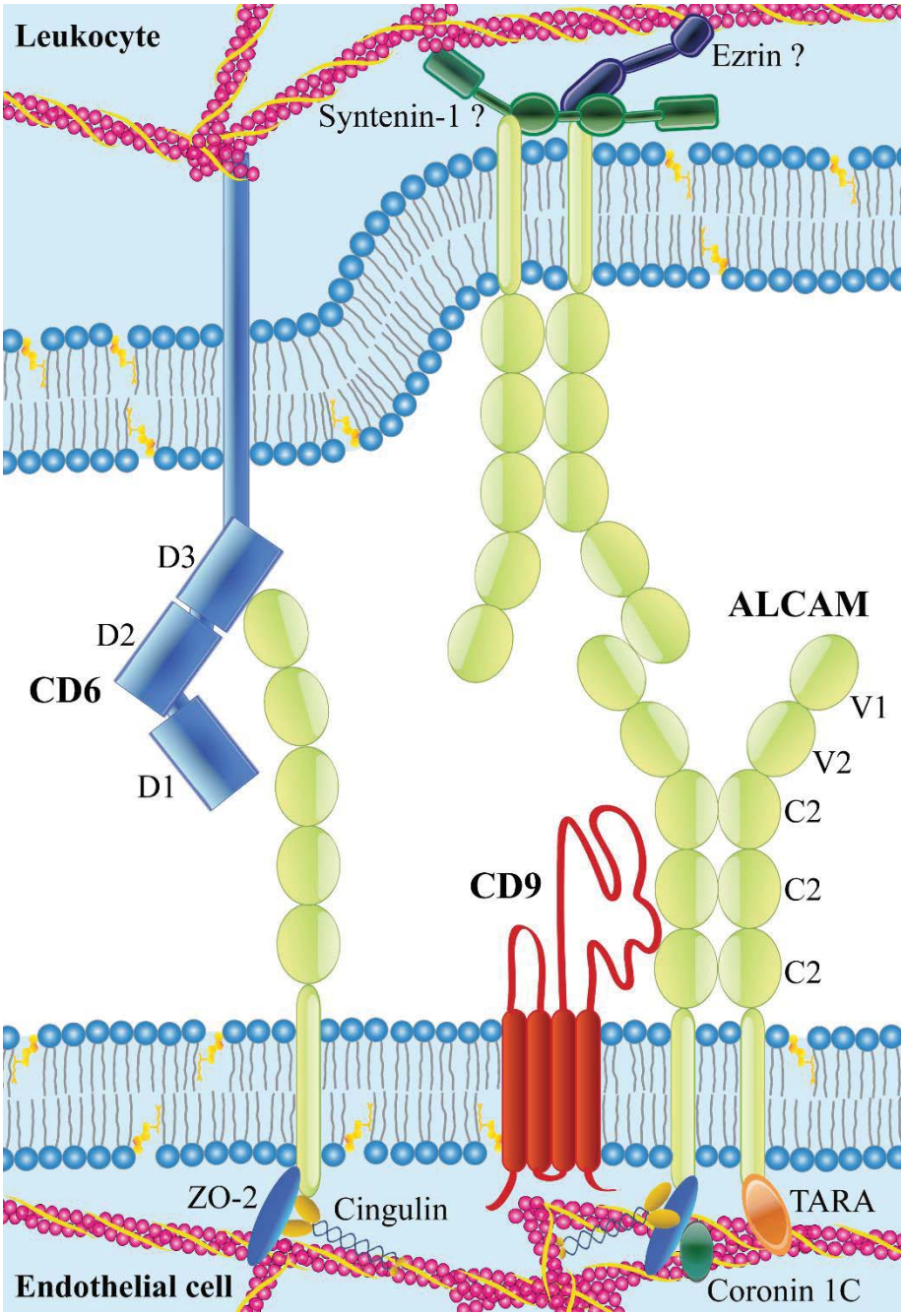

Figure 10 – Schematic representation of ALCAM homophilic and ALCAM-CD6 heterophilic interaction. ALCAM is a type 1 transmembrane cell adhesion molecule with a size of 583 amino acids (aa) and a native weight of 65 kDa (glycosylated 100-110 kDa). It has a 27 aa signal peptide, a 500 aa N-terminal extracellular domain (aa 28-527) containing three Ig-like C2-type domains and two Ig-like V-type domains, a 24 aa transmembrane domain and a

33 aa cytoplasmic domain, which is associated with β-actin cytoskeleton filaments (pink) [464]. ALCAM has a high sequence identity between species homologues especially at the N-proximal domain (V1), which mediate trans-ligand binding with CD6 and ALCAM [465]. The CD6 binding site is located in the membrane-proximal SRCR domain 3 region (D3). The membrane-proximal C2-type domains (C2) mediate lateral oligomerization and control the avidity of ALCAM binding (only groups of two ALCAM molecules are represented for simplicity, but macromolecular complexes can be formed) [466]. The intracellular binding partners of ALCAM in endothelial cells (at the bottom) are represented (ZO-2, cingulin, coronin 1C, TARA, CD9; further discussed in the results and discussion sections). The potential binding partners of ALCAM in leukocytes (Ezrin and Syntenin-1) are also depicted. Those molecules in turn bind to actin filaments (pink) and the surrounding tropomyosin fibres (yellow). Copyright Marc-André Lécuyer.

2. The Genome

In humans, the ALCAM gene is located at 3q13.1-3q13.2 and it is organized into 16 exons of nearly 150 kilobases (kb) in length [460]. As of yet, we have a limited knowledge on the different transcriptional and translational regulation of ALCAM. Studies, performed on cancerous tissues and cell lines, demonstrated that the promoter region is devoid of a canonical TATA-box, but rather has multiple GC-boxes. It also contains cis regulatory sequences such as NF-κB and GATA-1 binding sites [467-469]. Other studies provided evidences suggesting that ALCAM expression is also controlled by microRNAs. One group found that both miR-192 and miR-215 are upregulated in gastric tumors and can suppress ALCAM [470]. Similarly, miR-9 was demonstrated to inhibit ALCAM translation in different cancerous cells. Interestingly, the transcription of the miR-9 precursor RNA is induced by the P50/P65 subunits of NF-κB, which as mentioned previously, also promote the production of ALCAM, thus creating an auto-regulation [469, 471].

3. The Protein

In 2001, using a series of truncated ALCAM mutants, van Kempen and colleagues demonstrated that the homophilic ALCAM-ALCAM interaction in trans is dependent on the membrane-distal amino-terminal Ig domain and that binding avidity is controlled by ALCAM cis-clustering at the cell surface (**Figure 10**). Analogous to other members of the Ig superfamily, this mechanism was shown to be mediated by the membrane-proximal Ig domains [466]. Although the complete crystal structure of ALCAM has not been published yet, Hanka Venselaar, a PhD student at the Centre for Molecular and Biomolecular Informatics at the University of Nijmegen, has performed computer 3D model analyses of ALCAM based on its amino acid sequence and previously published crystal structure of analogous proteins (immunoglobulin light chain, immunoglobulin heavy chain and other Ig-like molecules [472]). Using her work, I completed a probable 3D model of ALCAM, where each subunit is shown with different colors (**Figure 11**).

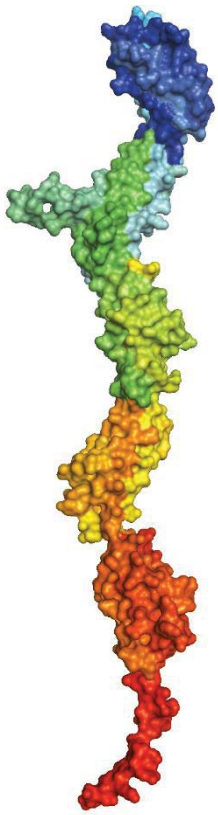

Figure 11 – Computer generated 3D model of ALCAM. This is an approximation of the 3D structure of ALCAM. The transmembrane and intra-cytoplasmic tail are shown in red. The ALCAM homotypic and heterotypic binding site is located in the dark-blue N-terminal domain. Image created by Marc-André Lécuyer with data adapted from http://www.cmbi.ru.nl/~hvensela/alcam/ALCAM_2.html.

More recently, a group has published the crystal structure of the two N-terminal domains of ALCAM along with the three SRCR domains of CD6 [473]. While this study demonstrated in detail the interaction between CD6 and ALCAM at a molecular level, this binding had been well characterized in the past by using a selective mutation approach (**Figure 10**) [459, 461, 462, 474-476]. Using single-molecule force spectroscopy, a more recent study has demonstrated that the homotypic interaction is weaker than the heterotypic ALCAM-CD6 bond, even following the disruption of the actin cytoskeleton [464]. Those results were based on several studies that had previously demonstrated a linked between ALCAM and the actin cytoskeleton

[477, 478]. One study in particular demonstrated that clustering of ALCAM is essential for cell adhesion and that it is mediated partly by actin polymerization. Their study also suggested a strengthening of the cytoskeleton linkage following ALCAM ligand binding [479].

Although publications had demonstrated a link between ALCAM and the cytoskeleton, the specific molecules involved were not known as of recently. This will be further discussed in the results and discussion sections. Moreover, the signalling pathways leading to the modulation of ALCAM adhesion still remain elusive, with only one major publication on the subject. This group first demonstrated that, in contrast to some integrins and other CAMs, small GTPases RhoA (Ras homolog gene family member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division control protein 42 homolog), which are key players in the organization of the actin cytoskeleton, are not involved in ALCAM-mediated adhesion. Instead, their results suggested that PKCα plays a dominant role in modulating the cytoskeleton-dependent avidity of ALCAM. They also concluded that ALCAM is not a direct substrate of PKCα due to a lack of phosphorylation on the molecule and the dispensable nature of the serine and threonine residues for ALCAM-mediated adhesion. Therefore, they concluded that PKCα indirectly controls ALCAM binding via cytoskeletal rearrangements [478].

4. The Functions

As mentioned previously, ALCAM is expressed during various phases of the organism development and by a wide variety of cell types. Therefore, the roles it plays can vary accordingly. ALCAM has probably been studied the most extensively in the context of cancer. It has been observed consistently in metastatic lesions of numerous cancer types, including prostate carcinoma, breast cancer, cutaneous melanoma, colorectal cancer, etc., and is thus considered a marker of cancer metastasis [480]. ALCAM has also been linked to the progression of a cancerous lesion. It was observed that as some types of tumor progress in stage and in size, ALCAM expression is proportionally increased. These results correlates with its role as a cell adhesion molecule, which consists in maintaining the cohesiveness of cellular aggregates [481]. Conversely, another study demonstrated that the lack of ALCAM or the presence of truncated mutants enhances the metastatic potential of cancerous tissues as it promotes cell motility

despite decreasing tumor growth/cell clustering [482]. Likewise, the metalloprotease ADAM17 has been demonstrated to cleave ALCAM, leading to the generation of soluble ALCAM (sALCAM) in circulation. The sALCAM molecules can compete for membrane-bound ALCAM and thus effectively block other homotypic interactions, which increases the risk of metastases [483, 484]. One hypothesis put forward to explain the importance of ALCAM in cancer proposes that the modification of a cell's genetic material to become cancerous may involve the expression of stem cell genes. This theory is based on the fact that those cells behave similar to cancerous cells (de-differentiated cells) and that ALCAM is expressed on the surface of many stem cells and embryonic tissues [480]. Finally, other groups also emphasised the importance of ALCAM in metastatic cell invasion by suggesting that it allows cancerous cells to adhere to vessel walls, similar to its role in immune cell trafficking [480].

As discussed in detail in chapter 1, ALCAM has been shown to play an important role during leukocyte transmigration. The first study to show such a role was published in 2006. It demonstrated that ALCAM is involved in the transmigration of a human monocyte cell line (THP1) across pulmonary rat microvascular endothelial cells [485]. Another study suggested a similar role for ALCAM on Tregs during their transmigration across pancreatic tumor-derived endothelial cells [486]. More recently, we have demonstrated the presence of ALCAM on human and mouse CNS microvascular endothelial cells and its high upregulation during neuroinflammation. Furthermore, ALCAM increased expression mostly co-localized with lesions present in MS and EAE tissues, which presented with disrupted TJs proteins staining and the presence of immune cells. We have also demonstrated the recruitment of ALCAM into lipid rafts upon pro-inflammatory stimuli as well as its participation in the formation of transmigratory cup upon contact with immune cells [98, 113]. A role for ALCAM in MS was further demonstrated by showing its involvement in the transmigration of *ex vivo* human CD4+ T lymphocytes, CD19+ B lymphocytes and CD14+ myeloid cells across a monolayer of BBB-ECs in an *in vitro* model of the BBB. Finally, ALCAM blocking antibodies were shown to reduce EAE clinical severity and the number of inflammatory lesions [113]. Recently, the group of Dr. Berman confirmed, in an HIV setting, the upregulation of ALCAM on infected monocytes and its crucial role during transmigration across human BBB [487, 488]. Another study also confirmed these results in HIV+ patients consuming cocaine. In addition, it demonstrated the

upregulation of ALCAM on human and mouse BBB-ECs upon the infectious immune challenge or simply following the intake of cocaine. Furthermore, the study suggested that cocaine-mediated induction of ALCAM is possible via the translocation of cocaine hijacks σ1 receptors to the plasma membrane, followed by phosphorylation of PDGF-β receptors, which in turn, activate the mitogen-activated protein kinase (MAPK), protein kinase B and NF-κB pathways leading to ALCAM expression [489]. This year, Curis and colleagues provided evidences upon the fact that ALCAM is overexpressed on the surface of HTLV-1-infected lymphocytes, resulting from the activation of the NF-κB pathway by viral proteins. Corroborating with previous results, they demonstrated that ALCAM blockade significantly reduced the infiltration of HTLV-1-infected lymphocytes into the CNS [490]. Collectively, these studies support the hypothesis that ALCAM plays an important role in the transmigration cascade and thus permits the infiltration of pathogenic peripheral blood leukocytes into the CNS. In the context of MS, targeting ALCAM would represent a viable therapeutic strategy to reduce the ongoing neuroinflammation especially considering that it does not seem to be involved in CD8+ cytotoxic T lymphocytes transmigration. Therefore, those immune cells could theoretically provide constant protection against JC viruses and thus potentially prevent PML in MS patients treated with anti-ALCAM antibodies.

Objectives and Hypothesis

While studying MS, one has to integrate its immunological expertise with the complexity of the neuronal system. In trying to understand the etiology of the disease and perhaps finding a target for a treatment, our laboratory has studied extensively in the past years the protective barrier of the CNS and the different molecules involved in leukocyte transmigration across the BBB. While therapeutic compounds restricting the capacity of pro-inflammatory leukocytes to migrate inside the CNS already exist, their lack of specificity inhibits the immune patrolling of the CNS and gives rise to multiple undesirable side effects. The identification and characterization of novel adhesion molecules implicated specifically and exclusively in the transmigration of encephalitogenic leukocytes is therefore required. The research leading to this Ph.D. thesis has been conducted in this context.

The general hypothesis of this work is that the activated BBB-ECs can modulate the inflammatory reaction, in coordination with the other cellular constituents of the NVU, via a tight control over the expression of extracellular molecules, especially cell adhesion molecules. A previous study from the laboratory has established the existence of ALCAM on human BBB-ECs using a proteomic approach. The study also demonstrated the upregulation of ALCAM on primary human BBB-EC culture upon inflammatory stimulation [45, 113, 491]. The same study has provided evidence upon the fact that ALCAM can be found in lipid rafts in the extracellular cell membrane and that it is involved in the transmigration of leukocytes into the CNS of both mice and humans. However, the characterization of the specific role of ALCAM during EAE and leukocyte transmigration was not elucidated.

The first hypothesis of this project was that ALCAM plays a role in the firm adhesion, crawling and diapedesis of encephalitogenic pro-inflammatory leukocytes across BBB-ECs. The aims were thus to study independently the cascade of events leading to monocytes, Th1 and Th17 lymphocytes diapedesis using a modified adhesion assay under shear-stress and a static migration assay.

The second hypothesis was that the absence of ALCAM on BBB-ECs and leukocytes of ALCAM KO mice significantly diminishes their EAE clinical symptoms, while reducing the absolute number of monocytes and T lymphocytes infiltrating the CNS upon immunization. The

aim was to study the role of ALCAM during neuroinflammation in mice. More specifically, the objectives were to study both *in vivo* and *in vitro* the impact of the absence of ALCAM on the immune system, the BBB and the onset of the disease, as a model to study the potential modulation of ALCAM in MS patients. All those aspects of my project will be further discussed in the following sections of this thesis.

Results:

Manuscript 1

Title: ALCAM (CD166) is involved in extravasation of monocytes rather than T cells across the blood-brain barrier

Authors: Ruth Lyck*, Marc-André Lécuyer*, Michael Abadier, Christoph B. Wyss, Christoph Matti, Maria Rosito, Gaby Enzmann, Laure Michel, Ana Garcia, Urban Deutsch, Alexandre Prat, Britta Engelhardt

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Journal: In press in The Journal of Cerebral Blood Flow & Metabolism. Manuscript ID: JCBFM-0511-16-ORIG

Contributions: The project, initiated by myself as a follow-up of Cayrol et al., 2008, consisted in studying in more detail the role of ALCAM during leukocyte transmigration across the blood-brain barrier. To conduct this study, I have developed and optimized the adhesion assay under shear stress using human cells. I have also performed the majority of the *in vitro* and *in situ* experiments using human cells and tissues. Following the publication of abstracts at international conferences, the group of Dr. Engelhardt approached us to collaborate on this project. We agreed that they would conduct the mouse experiments of the study. Therefore, Michael Abadier has performed the adhesion studies under flow using mouse cells. Christoph B. Wyss, Christoph Matti and myself have performed the Western blot on the mouse and human tissue/cells. Gaby Enzmann has performed the histology on the human CNS tissue. Maria Rosito has performed endothelial cell cultures in the laboratory of Dr. Engelhardt and helped with the adhesion assays. Laure Michel has helped with the Boyden assays performed with human cells. Ana Garcia has performed FACS analysis of the Th1 (sorted) mouse cells. Urban Deutsch has performed the mouse genotyping and managed the colony in Dr. Engelhardt's laboratory. Ruth Lyck, Britta Engelhardt, Alexandre Prat and myself have participated in the scientific

discussions, the elaboration of the different experiments and the analysis of the results. We have also constructed the figures and wrote the manuscript.

ALCAM (CD166) is involved in extravasation of monocytes rather than T cells across the blood-brain barrier

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Running title

ALCAM in immune cell extravasation across the BBB

Abstract

Activated leukocyte cell adhesion molecule (ALCAM) has been proposed to mediate leukocyte migration across the blood-brain barrier (BBB) in multiple sclerosis (MS) or experimental autoimmune encephalomyelitis (EAE). Here, we confirmed vascular ALCAM expression in human brain tissue samples *in situ* and on two different human *in vitro* BBB models. Antibody mediated inhibition of ALCAM reduced diapedesis of human $CD4^+$ Th1 but not of Th17 cells across the human BBB *in vitro*. In accordance to human Th1 cells, mouse Th1 cells showed reduced diapedesis across an $ALCAM^{-/-}$ *in vitro* BBB model under static but no longer under flow conditions. In contrast to the limited role of ALCAM in T cell extravasation across the BBB, we found a contribution of ALCAM to rolling, adhesion and diapedesis of human $CD14^+$ monocytes across the human BBB under flow and static conditions. Taken together, our study highlights potential differences in the CNS expression of ALCAM in mouse and human and supports a prominent role for ALCAM in the multi-step extravasation of monocytes across the BBB.

Keywords:

ALCAM, blood-brain barrier, immune cell extravasation, multiple sclerosis, neuroinflammation

Introduction

The blood-brain barrier (BBB) is formed by microvascular endothelial cells (ECs) of the central nervous system (CNS). It establishes a particularly tight endothelial barrier that protects the brain or spinal cord parenchyma from the changeable milieu in the blood stream while also limiting immune cell trafficking into the CNS.[1] During autoimmune neuroinflammation such as multiple sclerosis (MS) or its animal model, experimental autoimmune encephalomyelitis (EAE), high numbers of immune cells extravasate across the BBB and critically contribute to disease pathogenesis.[2] Preventing immune cell entry into the CNS has been successfully translated into the clinic with the release of the humanized anti-α4–integrin antibody natalizumab.[3] However, the low but serious concurrent risk of progressive multifocal leukoencephalopathy (PML) makes pressing the need for more detailed knowledge on the extravasation of autoagressive immune cells across the BBB.

Extravasation of immune cells across the BBB is a highly dynamic multi-step process mediated by the sequential interaction of cell adhesion and signaling molecules on the immune cell surface with their respective endothelial counter receptors.[4] *In vivo* and *in vitro* live cell imaging have revealed that upon firm adhesion, effector T cells not only resist flow, but polarize and crawl along the luminal face of the BBB endothelium until they reach a site permissive for transcellular or paracellular diapedesis.[5-9] Thereby, EC adhesion molecules and their leukocyte ligands fulfill critical roles. Previously, we have shown an essential role for intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in shear resistant arrest of encephalitogenic $CD4^+$ Th1 cells. We have also demonstrated that high level of ICAM-1 is involved during $CD4^+$ Th1 cell crawling against the flow and favors a transcellular diapedesis across the BBB.[5, 6]

Activated leukocyte cell adhesion molecule (ALCAM; CD166) on human and mouse brain ECs has also been assigned a role in the extravasation of $CD4^+$ T cells across the BBB and in the development of EAE.[10] Similar to ICAM-1, ALCAM harbors five extracellular immunoglobulin (Ig)-like domains, a single spanning transmembrane domain and a short cytoplasmic tail.[11-13] During homophilic ALCAM-ALCAM interactions, the first N-terminal Ig-domain of cis-clustered ALCAM binds to the N-terminal Ig-domain of another ALCAM in trans.[14] The stronger heterophilic ALCAM-CD6 interaction is mediated by the N-terminal Ig-domain of ALCAM and the membrane proximal scavenger receptor

cysteine-rich (SRCR) domain of CD6.[15] CD6-mediated interaction of T cells [16, 17] with ALCAM on antigen presenting cells [18] or on thymic epithelium [11] has been assigned a role in T cell activation and selection of thymocytes, respectively. In humans, the risk for MS development is associated with a distinct ALCAM polymorphism[19] and one MS risk allele, named rs17824933G, is located in intron 1 of the CD6 gene [20, 21]. Thus, ALCAM as a BBB specific ligand for neuroinflammatory cells could be an interesting candidate in the search for alternative pharmaceutical targets aiming to prevent pathological immune cell infiltration into the CNS.

We here addressed the role of endothelial ALCAM in the extravasation of immune cell subsets critically involved in EAE and MS pathogenesis across the human and mouse BBB. We revealed differences in the expression of ALCAM on the human versus mouse BBB and delineated a limited role of ALCAM in the diapedesis of $CD4^+$ Th1 or Th17 cells across the human or mouse BBB. In contrast, prominent impairment occurred at various steps of $CD14^+$ monocyte extravasation upon functional ablation of ALCAM. This suggests that ALCAM might influence MS pathogenesis by interfering with myeloid recruitment to the CNS but less so with T cells.

Materials and Methods

Mice

Wild type C57BL/6J mice were obtained from Harlan (Horst, Netherlands) and Janvier (Genest Saint Isle, France). ALCAM$^{-/-}$ mice [22] backcrossed into C57BL/6J background for 8 or more generations were kindly provided by Prof. Cornelia Halin, ETH Zürich, Switzerland. Mice were housed in individually ventilated cages under specific pathogen-free conditions. Animal procedures were performed in accordance with the Swiss legislation on the protection of animals or the guidelines of the Canadian Council on Animal Care and were approved by the Veterinary Office of the Kanton of Bern or the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) Animal Care committee (N11023APs).

In vitro models of the BBB

Human brain-like endothelial cells (HBLECs). CD34^{+} cells and pericytes were isolated and differentiated exactly as described before.[23, 24] For the collection of human umbilical cord blood, infants' parents signed an informed consent form, in compliance with the French legislation. The protocol was approved by the French Ministry of Higher Education and Research (CODE-COH Number DC2011-1321).

Human brain endothelial cells (HBECs). HBECs were isolated from non-epileptic surgical human CNS material (resection path) exactly as published. [10, 25, 26]

Human meningeal endothelial cells (HMECs). HMECs were isolated from leptomeningeal tissue removed from the CNS material. The tissue was extensively washed in PBS, cut into small pieces, and centrifuged at 1045 g for 15 min. The pellet was incubated in collagenase IV (1 mg/ml) (Sigma) at 37°C for 15 min, then washed in culture media and centrifuged at 485 g for 10 min. The pellet was resuspended in culture media and passed through a 30 µm nylon filter (Miltenyi). The fraction < 30 µm was cultured in 6-well plates pre-coated with 0.5% gelatin.

HBECs and HMECs. Informed consent and ethic approval were given prior to surgery (Centre de Recherche du Centre Hospitalier de l'Université de Montréal research ethic committee approval number HD04.046).

Primary mouse brain microvascular endothelial cells (pMBMECs). Isolation and culture of pMBMECs was performed exactly as described before.[5, 27] Cytokine stimulation of pMBMECs was done with TNF-α at 10 ng/ml, IL-1β at 20 ng/ml or TNF-α/IFN-γ at 10ng/ml and 100 U/ml for 16–20 h prior to the experiments.

Mouse brain or spinal cord microvessel

Isolation of mouse brain or spinal cord microvessels was performed as described before [28] and immediately processed for protein lysate.

Immune cell subsets

Ex vivo human $CD4^+CD45RO^+$ T cells. Venous blood samples were obtained from consenting healthy donors in accordance with institutional guidelines (Centre de Recherche du Centre Hospitalier de l'Université de Montréal research ethic committee approval number SL05.022, SL05.023 and BH07.001), and immune cells were isolated as previously published.[29]

In vitro polarized Th1 or Th17 cells. *Ex vivo* human $CD4^+CD45RO^+$ T cells were *in vitro* polarized as previously published.[30] Briefly, 0.5×10^6 $CD4^+CD45RO^+$ T cells/ml were cultured with autologous monocytes at a 1 to 0.6 ratio and soluble anti-CD3 (clone OKT3 at 2.5 µg/ml, eBioscience). For Th17 differentiation recombinant human IL-23 (25 ng/ml), anti-human IL-4 antibody (5 µg/ml) and anti-human IFN-γ antibody (5 µg/ml) were added, whereas IL-12 (10 ng/ml) in the presence of anti-IL-4 was added for Th1 differentiation (all reagents from R&D Systems). Th1 cells were harvested at day 5 and Th17 cells at day 6 of culture to be used in adhesion and transmigration experiments using HMECs or HBECs.

CD4$^+$CD45RO$^+$ Th1 cells sorted from peripheral blood. Human CD4$^+$ CD45RO$^+$ Th1 cells were directly sorted from healthy human blood donors according to differential expression of chemokine receptors (CXCR3$^+$, CCR4$^-$, CCR6$^-$) as previously described.[31, 32] Th1 cells were cultured in the presence of IL-2 (500 U/ml) for a total 20 days and then employed for adhesion assay.

Human monocytes. *Ex vivo* human CD14$^+$ monocytes were isolated from venous blood of consenting healthy donors in accordance with institutional guidelines (Centre de Recherche du Centre Hospitalier de l'Université de Montréal research ethic committee approval number SL05.022, SL05.023 and BH07.001) as previously published.[29] In brief, peripheral blood mononuclear cells were obtained using density gradient centrifugation on Ficoll-Paque™ (GE Healthcare) followed by immune-positive MACS® beads (Miltenyi) isolation and used directly (ex vivo) for monocyte/endothelial interaction under flow, adhesion and transmigration experiments with HBECs or HMECs.

Mouse CD4$^+$ Th1 cells. The encephalitogenic CD4$^+$ proteolipid protein (PLP) $_{aa139–153}$ specific Th1 cell line SJL.PLP7 (IFN-γ$^+$GM-CSF$^+$IL-4$^-$IL-17$^-$) was used for studying mouse Th1 cell interaction with pMBMECs. Activation with the cognate antigen and culture of Th1 cells was as previously published.[5, 33]

Antibodies and cytokines

Antibodies and cytokines are named in the figure legends and specified in supplementary material.

Immunohistochemistry and ALCAM quantification on human brain tissue

Human brain tissues were obtained from post-mortem autopsies supplied by the UK Multiple Sclerosis Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland (registered charity 207495, Supplementary Table 1). Immunohistochemistry on cryostat sections (12 µm) from fresh frozen tissue blocks was performed as described before.[34] ALCAM immunostaining was evaluated by

calculating the average signal intensity of all microvessels (Ø<15µm) in randomly taken pictures from control (30 pictures from 6 cases) and MS cases (NAWM: 28 pictures from 6 cases, chronic Lesion: 21 pictures from 5 cases). Average signal intensities were first calculated for single cases and tissue type and were then compared in between the different tissue types. Differences between the tissue groups were calculated using the student's t-test.

Immunofluorescence (IF)

IF staining on HBLECs. The human *in vitro* BBB was stimulated as indicated with TNF-α (10 ng/ml), IL-1β (20ng/ml) or TNF-α/IFN-γ (10 ng/ml each) for 16 h and then incubated with mouse anti-human ALCAM antibody clone 105901 (10ug/ml) or with mouse IgG1 isotype control for 30 min at 37°C. The BBB was washed, fixed in 1% PFA, unspecific binding sites were blocked in skimmed milk (5% in PBS) and incubated for 45 min with donkey anti mouse IgG-Cy3 (1:500). Images were acquired with a Nikon Eclipse E600 microscope equipped with a digital camera.

IF staining on HBECs or HMECs. HBECs or HMECs were grown to confluency in Ibidi µ-slides $VI^{0.4}$ and then treated for 24 h with TNF-α/IFN-γ (at 100 U/ml each). Cells were washed with PBS and then fixed at room temperature with 70% ethanol. Staining was performed as previously reported [35] using anti-human ALCAM antibody clone 105901 followed by donkey anti-mouse Cy3. TOPRO-3 Iodide (1:300 in Mowiol mounting medium) was used to stain nuclei. Images were acquired with a LEICA SP5 confocal microscope.

Quantitative RT-PCR

RNA was extraction, cDNA synthesis and SYBR green qPCR was exactly as described before.[6] Primers (Eurogentec S.A., Seraing, Belgium) were as follows. ALCAM (NM_009655): CTTCAGTGTTTGGGGAATGG (sense) and TTATGCCTTCAGGCTGTCCT (reverse); ICAM-1 (NM_010493): CACGCTACCTCTGCTCCTG (sense) and TCTGGGATGGATGGATACCT (reverse);

and ribosomal protein S16 (Rps16) (the endogenous control) (NM_013647): GATATTCGGGTCCGTGTGA (sense) and TTGAGATGGACTGTCGGATG (reverse).

EAE, protein lysates, Western Blot and protein quantification

Active EAE, Western Blot and protein quantification were performed according to standard methods. Details are described in supplementary material.

Adhesion assay

Adhesion of *ex vivo* sorted human $CD4^+$ Th1 cells to HBLECs. After 6 days of co-culture with pericytes on 3 µm pore size Transwell® inserts, HBLECs were treated with cytokine for 16 hours. Both, the HBLECs and human $CD4^+$ Th1 cells were pre-incubated with mouse anti-human ALCAM antibody (10µg/ml), anti-human ICAM-1 (15 µg/ml) or mouse IgG_1 control antibody (15µg/ml) for 30 min at RT. Soon after incubation 10^4 human Th1 cells labelled with CMFDA cell tracker (Life Technologies, ThermoFisher) were added on top of the human *in vitro* BBB and allowed to adhere for 30 min. Non adherent cells were then gently washed away with PBS and cells were fixed with 1% PFA in PBS, blocked in 5 % skimmed milk in PBS and incubated for 45 min with donkey anti mouse Cy3 antibody (1:500). The human *in vitro* BBB treated with mouse isotype control was incubated after fixation with mouse anti-human VE-cadherin (1:200). Assays were analyzed by fluorescence microscopy (Nikon Eclipse E600) and Th1 bound cells per pre-defined field were determined by counting five fields per filter. Assays were performed in triplicates for each value.

Diapedesis assay

Diapedesis of human immune cell subsets across HBECs or HMECs. HBECs or HMECs (3.5×10^4 cells per filter) were grown on gelatin-coated 3 µm pore size Boyden chambers (Fisher Scientific) in culture media supplemented with 40% astrocyte-conditioned media for 72 h (to confluency). When

indicated, antibodies specific for ALCAM (clone 105901, R&D Systems, 30 µg/ml), ICAM-1 (clone BBA3, R&D Systems, 10 µg/ml) or the appropriate isotype control were added to endothelial cells and immune cell subsets 1 h prior to the experiment. In presence of the blocking or control antibody, a suspension of 1x 10^6 specified leukocyte subset was added to the upper chamber and allowed to migrate for 16 - 18 h. Absolute number of cells that transmigrated to the lower chamber was then assessed. All conditions were performed in triplicate for each donor.

Diapedesis of mouse Th1 cells across pMBMECs. T cell diapedesis across pMBMECs under static conditions was assessed as described before.[5, 36]

Imaging of extravasation under flow

In vitro live cell imaging of mouse $CD4^+$ Th1 cell interaction with pMBMECs. Imaging of mouse Th1 cell dynamic interaction with pMBMECs cultured on matrigel coated cell culture surfaces (µ-$dish^{35\ mm\text{-}low}$, ibidi Vitaris, Baar, Switzerland) was performed as described before.[5, 37]

In vitro live cell imaging of human monocyte, Th1 cell or Th17 cell interaction with HBECs. Imaging of human leukocyte subset interaction with HBECs under flow was performed as previously published [29, 38].

Statistics

Statistical analysis was performed using GraphPad Prism 6.0 software (Graphpad software, La Jolla, CA, USA). Asterisks indicate significant differences (*P<0.05, **P<0.01, ***P<0.001 and ****P< 0.0001). Nonspecific differences are without label. Unless otherwise specified, data are expressed as mean ± standard error of the mean (SEM) and statistical differences of two groups were calculated by unpaired Student's *t*-test with equal SD.

Results

ALCAM expression in chronic MS lesions *in situ* and on the human BBB *in vitro*

Previously, we have described prominent ALCAM staining on brain vessels in acute MS lesions.[10] Here, we addressed expression of ALCAM in chronic MS lesions. Immunostaining of post-mortem brain tissue from MS cases confirmed ALCAM on small human brain vessels in chronic MS lesions as well as on microvessels in normal appearing white matter (NAWM) and control brain tissue samples (Fig. 1A). Quantification of staining signal intensity revealed no significant difference of microvascular ALCAM between all three types of human brain samples (Fig. 1 B). ALCAM immunostaining was also detected on large diameter brain vessels, which were however excluded from analysis because they are not involved in immune cell extravasation. Thus, unlike in acute MS lesions,[10] microvessels in chronic MS lesions do not show increased ALCAM staining.

In line with our previous observations, we here confirmed ALCAM immunostaining on primary human brain endothelial cells (HBECs) (Fig. 1C).[10] We further demonstrated ALCAM immunostaining on a second *in vitro* model of the human BBB established from $CD34^+$ cell derived human brain-like endothelial cells (HBLECs) (Fig. 1E).[23, 24] Immunofluorescence staining confirmed upregulation of endothelial ALCAM following stimulation with TNF-α, IL-1β or a combination of TNF-α and IFN-γ (TNF-α/IFN-γ) on HBECs or HBLECs (Fig.1C, E). As leptomeningeal immune cell infiltration is an important aspect in EAE and potentially in MS, we have additionally compared primary cultures of HBECs with primary cultures of human meningeal endothelial cells (HMECs) (Fig.1C). As opposed to HBECs, HMECs stained more prominently for ALCAM in resting conditions and no significant increase in staining was observed under inflammatory conditions (Fig.1C). Differential cell surface expression of ALCAM by HBECs and HMECs was confirmed by flow cytometry analysis, which also confirmed upregulation of ICAM-1 and VCAM-1 on both HBECs and HMECs upon cytokine stimulation (Fig.1D).

Limited involvement of ALCAM in the interaction of human CD4$^+$ T cells with the BBB *in vitro*

We have previously assigned a role for ALCAM in the extravasation of monocytes and CD4$^+$ T cells across the human BBB.[10] However, neither the precise effector T cell subset nor the precise step of extravasation affected by blocking ALCAM has been identified. Here, we aimed to address the role of ALCAM in the extravasation of activated CD4$^+$ Th1 and Th17 cells across the human BBB. First, we tested the adhesion of *ex vivo* human CD4$^+$CD45RO$^+$ Th1 cells sorted from human peripheral blood as described before.[39] These T cells expressed CD6 and ALCAM on their surface as determined by flow cytometry (Supplementary Fig. 1) and readily adhered to HBLECs in a static environment (Fig. 2A, B). As expected, increased numbers of Th1 cells adhered to the cytokine stimulated HBLECs when compared to unstimulated HBLECs (Fig. 2A, B). However, masking ALCAM with a function-blocking antibody did not interfere with Th1 cell adhesion to the unstimulated or stimulated HBLECs. At the same time, antibody-mediated blocking of endothelial ICAM-1 significantly reduced T cell adhesion to the unstimulated and cytokine stimulated HBLECs (Fig 2A, B). Using an *in vitro* flow system, we also tested rolling and initial arrest of human *in vitro* polarized Th1 and Th17 cells on HBECs. Th1 and Th17 cells used for this experiment were generated by *in vitro* polarization of *ex vivo* human CD4$^+$CD45RO$^+$ T cells as described.[30] These T cells expressed similar cell surface levels of CD6 but lower cell surface levels of ALCAM when compared to *ex vivo* sorted Th1 cells (Supplementary Fig. 1). The addition of function-blocking anti-ALCAM antibodies to HBECs and Th1 or Th17 cells did neither reduce their rolling nor their arrest on resting or stimulated HBECs (Fig. 2C, D and data not shown). Taken together, we concluded that ALCAM is not involved in rolling, shear resistant arrest and firm adhesion of human Th1 and Th17 cells to human *in vitro* models of the BBB.

To address the role of endothelial ALCAM in T cell diapedesis across HBECs, we used a modified Boyden chamber assay. In line with our previous observations, we found that inhibition of ALCAM reduced the diapedesis of *ex vivo* sorted CD4$^+$CD45RO$^+$ T cells across HBECs (Fig. 2E).[10] However, no effect of ALCAM blockade occurred when CD4$^+$CD45RO$^+$ T cells were allowed for diapedesis across HMECs (Fig. 2E). For refinement, we then analyzed the diapedesis of *in vitro* polarized Th1 versus Th17 cells and observed that pre-treatment of HBECs with the function-blocking anti-ALCAM antibody significantly reduced diapedesis of Th1 but not of Th17 cells (Fig. 2F, G). At the same time

antibody-mediated blocking of ICAM-1 had a more pronounced effect on the diapedesis of both T helper cell subsets across HBECs (Fig. 2F, G). Taken together, blocking ALCAM failed to reduce diapedesis of human $CD4^+$ Th17 cells across HBECs and exerted a limited effect on the diapedesis of human $CD4^+$ Th1 cells across the human BBB.

Prominent neuronal ALCAM masks endothelial ALCAM at the BBB in mice

Research using mouse *in vivo* EAE models or mouse *in vitro* BBB models has been fundamental in improving our understanding of the immune pathogenesis of MS.[40] Previously, we have demonstrated that anti-ALCAM antibody treatment ameliorates $MOG_{(aa35-55)}$-induced EAE in C57BL/6 mice.[10] To determine ALCAM expression *in vivo* in the mouse CNS during EAE, we next analyzed protein lysates from inflamed CNS tissue of C57BL/6 mice suffering from acute EAE compared to age and gender matched healthy controls. ALCAM protein levels were comparable in lysates from the brain cortex and cerebellum of both cohorts (Fig. 3A-C). In lysates from the inflamed spinal cord ALCAM protein was lowered concomitant with an increase of the ICAM-1 protein level compared to control samples (Fig. 3A, D, E). As expected, protein lysates from healthy $ALCAM^{-/-}$ C57BL/6 mice were devoid of ALCAM (Fig. 3F). To localize the cellular sources of ALCAM in the mouse brain, we performed immunofluorescence staining on frozen naïve brain or spinal cord sections and on respective tissue sections from $ALCAM^{-/-}$ mice (Supplementary Fig. 2). We found strong ALCAM immunostaining in the meninges of the mouse brain and a more moderate, widespread staining in the parenchyma of the cerebral cortex and the striatum (Supplementary Fig. 2A, C). Strong ALCAM immunoreactivity was also observed in neurons and axons of the dorsal root ganglia, the dorsal and the ventral horn (Supplementary Fig. 2D-F), and in the axons that projected to a particular dorsal lamina (Supplementary Fig. 2H). However, multi-color immunofluorescence staining failed to locate ALCAM staining on IB4-positive CNS vessels in brain and spinal cord sections of C57BL/6 mice (Supplementary Fig 2J). We therefore hypothesized that the strong signal intensity of neuronal ALCAM did not allow the detection of lower endothelial ALCAM levels *in situ.*

Therefore, we next tested the expression of ALCAM on freshly isolated CNS vessels and in primary mouse brain microvascular endothelial cells (pMBMECs) from C57BL/6 mice. ALCAM protein was readily detectable in protein lysates from brain and spinal cord vessels of wild type mice but was absent in the respective samples from ALCAM$^{-/-}$ C57BL/6 mice (Fig. 3G). Similarly, *ALCAM* mRNA was detected in unstimulated and cytokine stimulated pMBMECs from wild type C57BL/6 mice (Fig. 3H). Remarkably, the stimulation of pMBMECs with IL-1β induced a 3-fold upregulation of *ALCAM* mRNA, as compared to controls, whereas TNF-α provided no change (Fig. 3H). At the same time the levels of *ICAM-1* or *VCAM-1* mRNA were upregulated 23-fold and 8-fold upon stimulation pMBMECs with IL-1β (Fig. 3I, J). Taken together, endothelial ALCAM is expressed in mouse CNS microvascular endothelial cells but at a lower level than in human.

Endothelial ALCAM plays a limited role in mouse Th1 cell diapedesis across the BBB

We next investigated the role of ALCAM during diapedesis of Th1 cells in mouse. To specifically target the role of endothelial ALCAM, we took advantage of the ALCAM$^{-/-}$ C57BL/6 mouse as a source of pMBMECs.[22, 41] ALCAM and CD6 protein expression on mouse encephalitogenic Th1 cells [5, 33] was confirmed by flow cytometry (Supplementary Figure 3). Using the modified Boyden chamber assay, we found, in accordance with the observations made with human Th1 cells, a reduction of mouse Th1 cell diapedesis across unstimulated ALCAM$^{-/-}$ pMBMECs compared to wild type pMBMECs (Fig. 4A). However, the difference was abrogated by the stimulation of pMBMECs with TNF-α, IL-1β or TNF-α/IFN-γ (Fig. 4A). Compensatory upregulation of ICAM-1, ICAM-2 or VCAM-1 in the absence of ALCAM on stimulated ALCAM$^{-/-}$ compared to wild type pMBMECs was ruled out through side-by-side analysis of their cell surface expression (data not shown). Possibly, low levels of ALCAM on resting BBB-ECs contribute to Th1 cell migration, but ALCAM may become dispensable when other molecules involved in T cell extravasation, such as ICAM-1 or VCAM-1, are upregulated under inflammatory conditions.

To dissect the precise role of endothelial ALCAM leading to reduced Th1 cell diapedesis, we next employed *in vitro* time-lapse live cell imaging under physiological flow.[37] This approach has proven

valuable for dissecting the different roles of other CAMs during the extravasation cascade across the BBB.[5, 6, 42] First, we compared the shear resistant arrest of Th1 cells on the unstimulated or cytokine stimulated wild type or ALCAM$^{-/-}$ pMBMECs (Fig. 4B). Irrespective of the presence or absence of cytokine stimulation, we found comparable numbers of Th1 cells arresting on wild type and ALCAM$^{-/-}$ pMBMECs (Fig. 4B), demonstrating that ALCAM does not contribute to mouse Th1 cell arrest on the BBB. Next, we compared the dynamic post-arrest behavior of Th1 cells on ALCAM$^{-/-}$ and wild type pMBMECs (Fig. 4C-I). On unstimulated wild type pMBMECs, the majority of the arrested Th1 cells continuously crawled on the endothelial surface and another substantial fraction detached due to low sustained adhesive interactions, while only a minor fraction of the Th1 cells migrated across the pMBMEC monolayer (Fig. 4C). In contrast, the majority of arrested Th1 cells crossed the cytokine stimulated-endothelial monolayer and another substantial fraction of Th1 cells continuously crawled on the endothelial surface with only rare events of detachment (Fig. 4D, E). Importantly, the comparison of the dynamic Th1 cell behavior on wild type versus ALCAM$^{-/-}$ pMBMECs did not show any significant differences (Fig. 4C-E). We also assessed shorter periods of evaluation to focus on early diapedesis which, however, did not reveal any significant difference in the dynamic Th1 cell behavior on wild type versus ALCAM$^{-/-}$ pMBMECs (Fig. 4E, F). Furthermore, the speed of Th1 cell crawling (Fig. 4G), the time elapsed between Th1 cell arrest and diapedesis (Fig. 4H) or the duration of diapedesis (Fig. 4I) were comparable on wild type and ALCAM$^{-/-}$ pMBMECs. Taken together, our detailed live cell imaging analysis did not reveal any role for endothelial ALCAM in the multi-step Th1 cell migration across mouse BBB-ECs *in vitro*. Thus, the observed role for endothelial ALCAM in mediating Th1 cell diapedesis across the BBB under static conditions is abrogated under physiological flow.

ALCAM is involved in multiple steps of monocyte migration across the BBB

Considering our previous findings on ameliorated EAE upon anti-ALCAM treatment [10] but the limited role of ALCAM in Th1 cell diapedesis across the BBB, we finally aimed to delineate the role of ALCAM in monocyte migration across the BBB. After extravasation across the BBB, monocytes can differentiate into dendritic cells (DCs) and macrophages and contribute to disease pathogenesis.[43,

[44] Here, we used *ex vivo* sorted human CD14$^+$ monocytes of which 60 to 85% expressed ALCAM, as demonstrated by us and others before.[10, 45] Using the *in vitro* flow system, *ex vivo* human CD14$^+$ monocytes were allowed to interact with HBECs over a period of 30 minutes and were recorded in real time for multiple intervals of 30 seconds. Tracking of individual monocytes overtime demonstrated that blocking both, monocyte and endothelial ALCAM with a function-blocking antibody significantly reduced monocyte/HBEC interactions and interfered with an overall decrease in monocyte speed, as compared to the isotype control (Fig. 5A). More specifically, anti-ALCAM antibody treatment blocked both, rolling (Fig. 5B) and firm adhesion (Fig. 5C) of CD14$^+$ monocytes on unstimulated HBECs. The function-blocking anti-ALCAM antibody also significantly reduced firm adhesion of CD14$^+$ monocytes on TNF-α/IFN-γ stimulated HBECs (Fig. 5D). Using the modified Boyden chamber assay, we found that antibody-mediated blocking of ALCAM reduced the migration of CD14$^+$ monocytes across resting HBECs (Fig. 5E). We also correlated the diapedesis of monocytes across HMECs obtained from the same donors as the HBECs. As expected, monocytes migrated in higher numbers across the more permeable HMECs, while also being significantly blocked upon the addition of anti-ALCAM antibodies (Fig. 5E). Importantly, the effect of antibody-mediated inhibition of ALCAM on monocyte diapedesis was comparable to the extent of antibody-mediated blockage of ICAM-1 (Fig. 5E). In conclusion, ALCAM contributes to the extravasation of human CD14$^+$ monocytes across the human BBB at various individual steps, namely rolling, firm adhesion and diapedesis. Thus, we conclude that a therapeutic targeting of ALCAM in the treatment of neuroinflammation would affect the recruitment of monocytes into the CNS but exert limited effect on the recruitment of CD4$^+$ effector T cells.

Discussion

MS is an inflammatory disease of the CNS with no cure available to date. However, treatments that ameliorate and delay the progression of the disease exist. Successful therapeutic strategies targeting immune cell trafficking have been translated into the clinics such as the humanized anti-α4-integrin antibody natalizumab and the sphingosine-analogue fingolimod.[2, 3] Unfortunately, natalizumab and fingolimod harbor a low risk of developing the fatal disease PML, which is due to the complete inhibition of the CNS immune surveillance.[46, 47] To define alternative therapeutic targets aimed at inhibiting pathological immune cell entry into the CNS during MS, more detailed knowledge on the multi-step extravasation of different immune cell subsets across the BBB is required. In previous studies, we have defined the individual roles of endothelial selectins, VCAM-1, ICAM-1 and ICAM-2 in $CD4^+$ Th1 cell extravasation across the BBB during EAE.[5, 6, 49, 50] However, further endothelial cell surface proteins expressed at the BBB, such as melanoma cell adhesion molecule (MCAM, CD146),[29, 51] netrin-1,[28] Ninjurin-1,[38, 52] and ALCAM [10] were recently proposed as additional players in the migration of different immune cell subsets including Th1 and Th17 cells across the BBB and hence as potential pharmaceutical targets for the treatment of MS. However, the precise steps mediated by these additional molecules within the extravasation cascade have not been solved. To this end, we here analyzed the role of ALCAM in various steps of extravasation using human Th1 or Th17 cells and monocytes, as well as mouse Th1 cells across both human and mouse *in vitro* models of the BBB. Functional analysis was complemented by the analysis of ALCAM expression in human and mouse CNS tissue or on their respective *in vitro* models of the BBB.

In a previous study, we found increased ALCAM expression on brain vessels in acute MS lesions.[10] Similarly, inflamed vessels in the CNS of drug-abusing or HIV-infected patients [53] show increased ALCAM expression. In the present study, we confirmed the expression of ALCAM protein in human brain BBB-ECs. However, we also found that, in chronic MS lesions, vascular ALCAM is not enhanced compared to NAWM or control tissues from individuals without neurological diseases. Obviously, specific inflammatory conditions play a decisive role in the upregulation of ALCAM at the human BBB *in situ*. On the other hand, ALCAM was readily detectable on both, unstimulated stem cell- and primary cell-derived *in vitro* models of the human BBB and increased upon cytokine

stimulation in line with our previous observations.[10] Thus, our data confirm the expression of ALCAM on the human BBB *in situ* and *in vitro*.

In our previous study, we have demonstrated a role for ALCAM in the diapedesis of human $CD4^+$ but not $CD8^+$ T cells across the human BBB.[10] In apparent contrast, another study failed to find a role for endothelial ALCAM in the migration of human $CD3^+$ T cells across an *in vitro* model of the human BBB consisting of commercially available human brain microvascular endothelial cells co-cultured with astrocytes.[45] However, the latter study was based on a different experimental setup including a chemotaxis component towards CCL2 or CXCL12, longer duration of the T cell migration period and a different anti-ALCAM antibody.[45] In addition, the more heterogeneous population of $CD3^+$ T cells investigated in that study might have superseded the role of ALCAM in mediating the diapedesis of specific T cell subsets across the BBB. The role of ALCAM in the extravasation of $CD4^+$ Th1 and Th17 cells has not been addressed hitherto. Here, we confirmed the role of ALCAM in the diapedesis of mixed populations of *ex vivo* $CD4^+$ T cells across unstimulated HBECs. However, antibody-mediated inhibition of ALCAM neither blocked rolling nor flow resistant arrest of *in vitro* polarized human Th1 cells nor adhesion of *ex vivo* sorted human Th1 cells to unstimulated or stimulated HBECs or HBLECs, respectively. Nonetheless, antibody-mediated inhibition of ALCAM significantly reduced the diapedesis of *in vitro* polarized human Th1 cells across HBECs but neither adhesion nor diapedesis of human Th17 cells. Thus, our results suggest that ALCAM plays a limited role in T cell extravasation across the BBB by contributing solely to the diapedesis of human $CD4^+$ Th1 but not Th17 cells across the human BBB.

Research based on mouse species offering knock-out models for *in vivo* and *in vitro* studies for the inhibition of leukocyte trafficking has been fundamental for the clinic.[3, 40] To set the stage, we here investigated the expression of ALCAM in the mouse CNS and its role in Th1 cell extravasation across the BBB. We determined that either equal or lower amount of ALCAM protein is present in the brain or spinal cord of C57BL/6 mice during acute EAE. The inflamed condition of the samples was confirmed by the upregulation of ICAM-1 compared to control samples.[54, 55] While the expression of ALCAM on neurons has been described before,[22, 56] we here confirmed ALCAM expression *in situ* on neurons and in the meningeal layers whereas the parenchymal CNS vasculature did not visibly

stain positive for ALCAM. We concluded that in contrast to the human brain, ALCAM protein in mouse CNS vessels might be difficult to detect due to its low level compared to its high level in neurons.

To test whether the role of ALCAM in the diapedesis of Th1 cells across the BBB is conserved between human and mouse in spite of the obvious differences in ALCAM protein levels on the BBB, we used encephalitogenic mouse Th1 cells [41] and a mouse *in vitro* BBB model composed of pMBMECs.[27, 36] Isolation of pMBMECs from ALCAM$^{-/-}$ C57BL/6 mice [22] and wild type littermates in parallel enabled a direct comparison of Th1 cell interaction with the BBB in the presence or absence of ALCAM. To model an inflamed BBB with high levels of ICAM-1 and VCAM-1, pMBMECs were stimulated with pro-inflammatory cytokines.[5, 6] Under static conditions, mouse Th1 cell diapedesis was reduced across unstimulated ALCAM$^{-/-}$ compared to wild type pMBMECs. *In vitro* live cell imaging under physiological flow has proven valuable in delineating the different roles of endothelial ICAM-1, ICAM-2 and VCAM-1 during the extravasation of immune cell subsets across the BBB.[5, 6, 36, 57, 58] Interestingly, our detailed analysis revealed comparable Th1 cell shear resistant arrest, diapedesis, crawling speed or duration of diapedesis on wild type and ALCAM$^{-/-}$ pMBMECs. Upon stimulation with pro-inflammatory cytokines, the diapedesis rate of mouse Th1 cells across ALCAM$^{-/-}$ or wild type pMBMECs was also equal under static and under flow conditions. Following the strong upregulation of mouse endothelial ICAM-1 and VCAM-1 upon cytokine stimulation as compared to ALCAM and considering their essential roles in the multi-step extravasation cascade, our data suggest that ALCAM could be involved in the diapedesis of mouse Th1 cells across the BBB in unstimulated low shear stress conditions *in vivo*, but becomes dispensable during neuroinflammation.[5, 6]

To explain the prominent role of ALCAM previously observed during the development of EAE,[10] we investigated the role of monocytes, which can differentiate into DCs and macrophages after infiltrating the CNS and thus contribute to EAE and MS pathogenesis.[59] In particular, early depletion of monocytes completely abrogates clinical development of EAE [44] and depletion of monocytes after disease onset still protects from axonal loss during EAE.[60] This led us to finally analyze the role of ALCAM in various steps of monocyte extravasation across the BBB. We here confirmed a significant role of ALCAM in monocyte diapedesis across unstimulated and cytokine stimulated HBECs that previous studies have described before.[10, 45, 61] In the present study, we found additional roles for

ALCAM during monocyte rolling and shear resistant arrest on the human BBB not addressed by others hitherto. Possibly, higher levels of ALCAM on monocytes versus Th1 or Th17 cells could explain the increased effect of blocking ALCAM on the multi-step extravasation of monocytes across the BBB, as compared to the T cell subsets. In support, overexpression of ALCAM on the surface of human T-lymphotropic virus type 1 (HTLV-1)-infected lymphocytes was identified as a cause for their increased migration across the human BBB.[62]

In conclusion, we here demonstrate a higher expression of ALCAM on the human BBB, as compared to the mouse BBB. Nevertheless, in both species, ALCAM is involved in the diapedesis of Th1 cells across the uninflamed BBB, though it is not involved in shear resistant arrest, adhesion strengthening and diapedesis of Th1 cells under flow. In contrast, the prominent role of ALCAM in multiple steps of the monocyte extravasation cascade across the BBB suggests that the function blocking anti-ALCAM antibody specifically interferes with monocyte infiltration into the CNS during autoimmune neuroinflammation and thus lead to the amelioration of EAE as observed before.[10] Considering the prominent expression of ALCAM on the human BBB, ALCAM might be a suitable therapeutic target for specifically targeting myeloid cell migration into the CNS, while leaving T cell mediated CNS immune surveillance intact.

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Author Contribution statement

AG, CW, CM, FG, JW, LM, MA, MR, LM and TZ each made substantial contributions to performing experiments, acquisition and analysis of data. FS, GE, NSW and UD contributed to design of the study and provided input to the manuscript. MAL and RL substantially contributed with concept and design of the experiments, writing of the manuscript and acquisition, analysis and interpretation of data. AP and BE were involved in the overall design of the study and made substantial contributions to writing the manuscript.

Disclosure of Interest

The Authors declare that there is no conflict of interest

Supplementary material for this paper is available at:

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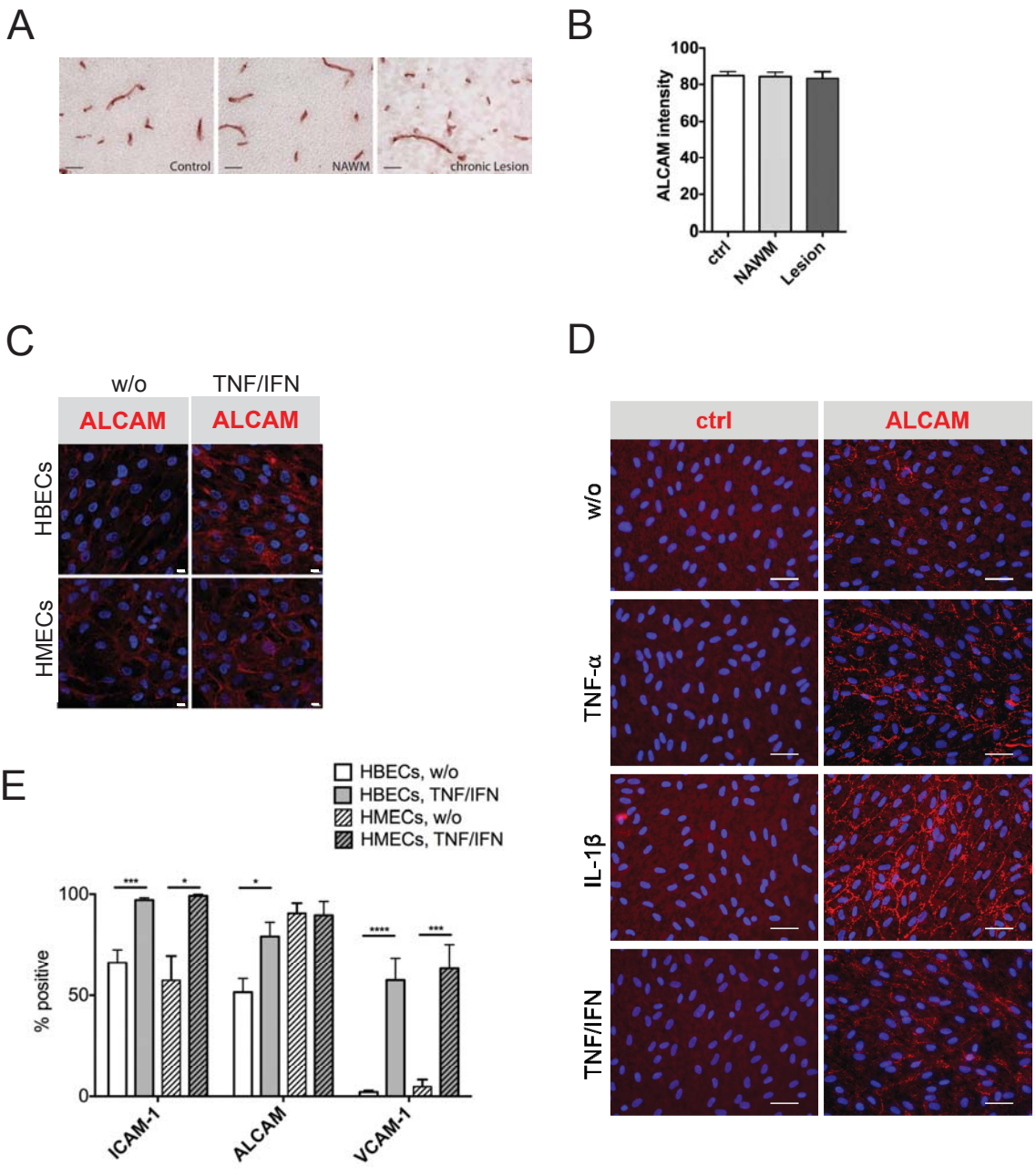
Figure 1


A
Control
NAWM
chronic Lesion
B
ALCAM intensity
100
80
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D
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ALCAM
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TNF-α
IL-1β
TNF/IFN
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HBECs, w/o
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HMECs, w/o
HMECs, TNF/IFN
% positive
100
50
0
***
*
*
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***
ICAM-1
ALCAM
VCAM-1

Figure 1:

ALCAM protein expression on the human BBB

A, ALCAM expression pattern on control, MS NAWM and chronic MS lesion tissue samples from the subcortical white matter region of post-mortem human brains as detected by immune histochemistry (polyclonal goat anti-human/mouse ALCAM antibody). Scale bar = 50 μm. B, Densitometry of ALCAM signal intensities on human brain microvessels (Ø<15μm) as shown in representative images in A. Randomly taken pictures from control (30 pictures from 6 cases) and MS cases (NAWM: 28 pictures from 6 cases, chronic Lesion: 21 pictures from 5 cases) were evaluated. ALCAM intensity is presented as arbitrary units. C, D, Anti-ALCAM immunoreactivity (clone 105901) on unstimulated or cytokine stimulated HBECs and HMECs (C) and HBLECs (D, ctrl, control antibody) is in red. Cytokine stimulation as labelled, nuclei were stained with DAPI (blue). Representative of n = 3 independent experiments. Images were acquired with a 20x objective (C, scale bar = 10 μm) and 40 x objective (D, scale bar = 20 μm). E, Quantification of ALCAM (antibody clone 3A6), ICAM-1 (antibody clone HA58) and VCAM-1 (antibody clone 51-10C9) protein expression on HBECs (n = 10) or HMECs (n = 7) as determined by flow cytometry. Values show % of positive endothelial cells.

Figure 2

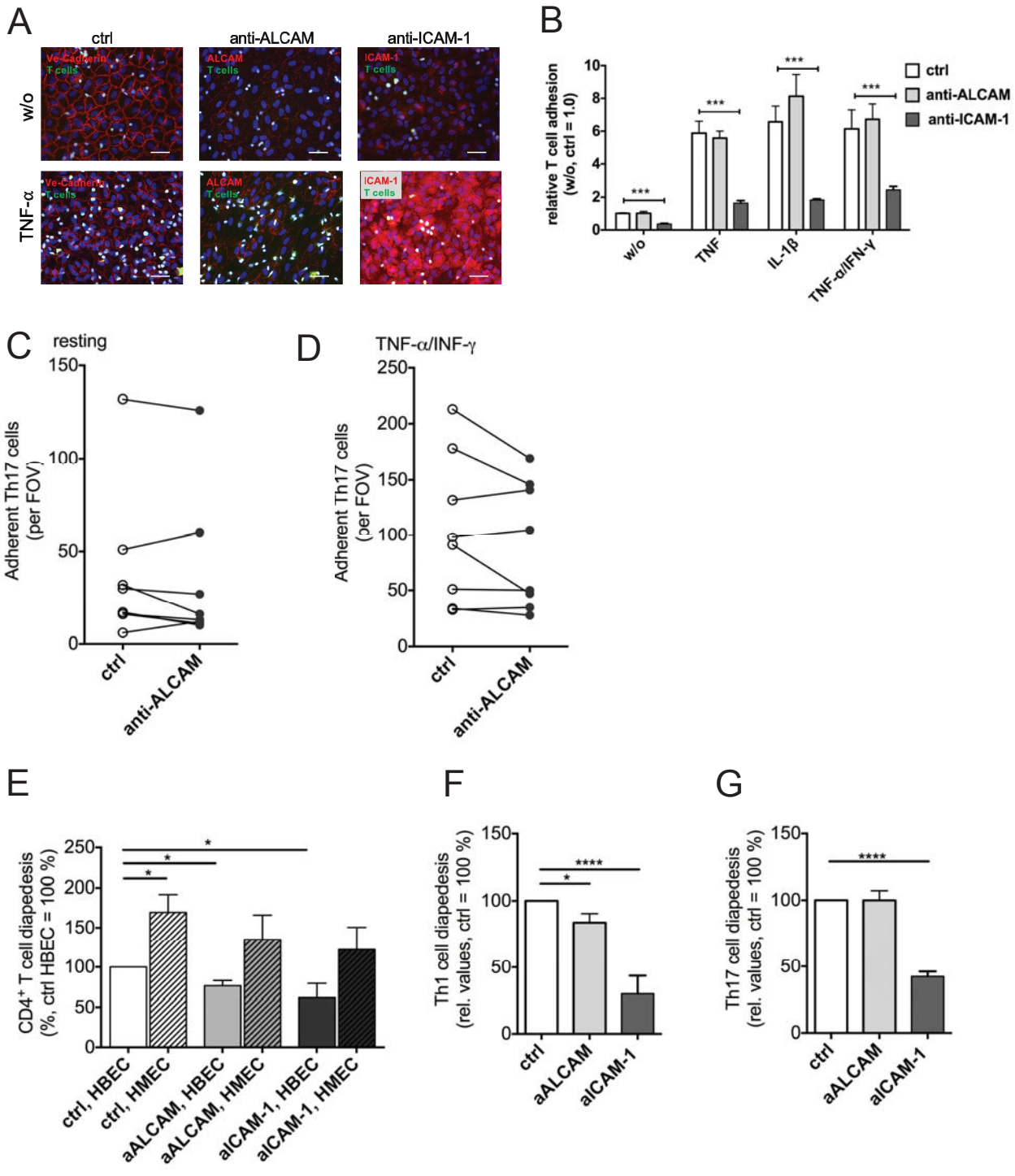
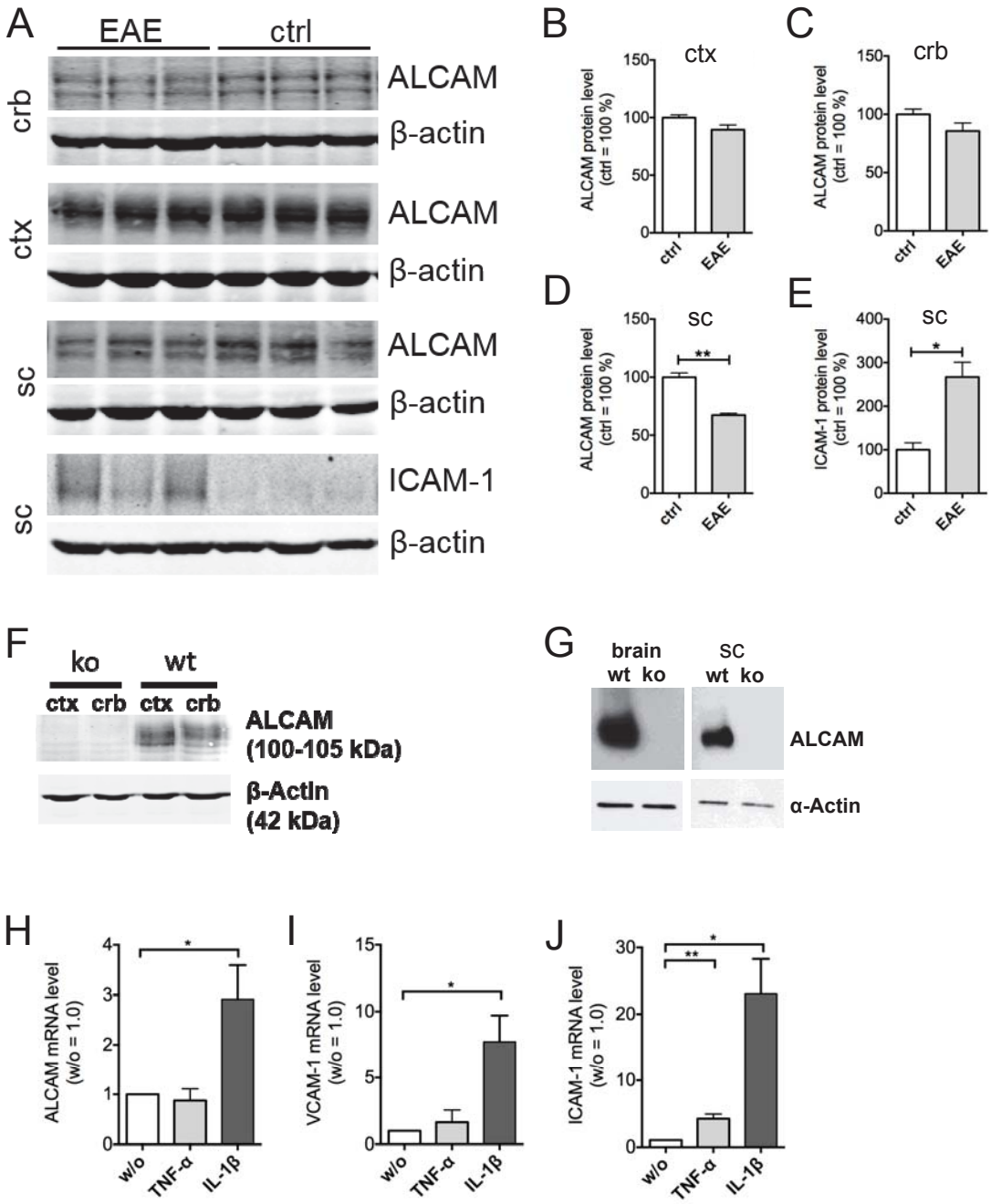

A
ctrl
anti-ALCAM
anti-ICAM-1
w/o
TNF-α
Ve-Cadherin
ALCAM
ICAM-1
T cells
B
relative T cell adhesion (w/o, ctrl = 1.0)
ctrl
anti-ALCAM
anti-ICAM-1
w/o
TNF
IL-1β
TNF-α/IFN-γ
C
resting
Adherent Th17 cells (per FOV)
D
TNF-α/INF-γ
E
CD4+ T cell diapedesis (%, ctrl HBEC = 100 %)
ctrl, HBEC
ctrl, HMEC
aALCAM, HBEC
aALCAM, HMEC
aICAM-1, HBEC
aICAM-1, HMEC
F
Th1 cell diapedesis (rel. values, ctrl = 100 %)
aALCAM
aICAM-1
G
Th17 cell diapedesis (rel. values, ctrl = 100 %)

Figure 2:

Role of ALCAM during the interaction of human Th1 or Th17 cells with HBLECs, HBECs or HMECs

A-B, Adhesion of *ex vivo* sorted human $CD4^+$ Th1 cells to HBLECs after treatment with function blocking antibodies to ALCAM or ICAM-1 or with isotype control. HBLECs were unstimulated or stimulated with TNF-α (10 ng/ml), IL-1β (20 ng/ml) or TNF-α/IFNγ (10 ng/100 U/ml). A, Representative images of $CD4^+$ Th1 cells (green, CMFDA loaded) adherent on the human BBB stained for VE-cadherin, ALCAM or ICAM-1 (red). Cell nuclei are stained with DAPI (blue). Images were acquired with a 40x objective. Scale bar, 20 µm. B, Quantification of $CD4^+$ Th1 cell adhesion to HBLECs. Values are of 3 independent experiments performed in triplicates and expressed relative to Th1 cell adhesion to isotype control treated and unstimulated HBLECs (1.0). C-D, numbers of adherent *in vitro* polarized $CD4^+$ Th17 cells on resting (C) or cytokine (TNF-α/IFN-γ) stimulated HBECs 20 minutes after Th17 cell perfusion. Th17 cells from individual healthy donors are compared side-by-side (Horizontal lines) (n = 8 experiments). E, Diapedesis of *ex vivo* human $CD4^+CD45RO^+$ T cells across HBECs or HMECs that were pre-treated with ALCAM or ICAM-1 blocking antibodies or isotype control antibody (n = 4). HBECs and HMECs from the same donors were tested side-by-side and diapedesis rates were normalized to T cell diapedesis across isotype control treated HBECs (100 %). F-G, Diapedesis of human *in vitro* polarized $CD4^+$ Th1 cells (F) or Th17 cells (G) across unstimulated HBECs that were pre-treated with ALCAM or ICAM-1 blocking antibodies or isotype control antibody (n = 4 experiments). Data are expressed relative to isotype control treated HBECs (100 %). Antibodies were anti ALCAM clone 105901 (A-G), anti ICAM-1 clone 15.2 (A, B) and clone BBA3 (E-G).

Figure 3


A
EAE
ctrl
crb
ALCAM
β-actin
ctx
ALCAM
β-actin
sc
ALCAM
β-actin
sc
ICAM-1
β-actin
B
ctx
ALCAM protein level (ctrl = 100 %)
ctrl
EAE
C
crb
ALCAM protein level (ctrl = 100 %)
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EAE
D
SC
ALCAM protein level (ctrl = 100 %)
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β-Actin (42 kDa)
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brain
SC
wt ko wt ko
ALCAM
α-Actin
H
ALCAM mRNA level (w/o = 1.0)
*
w/o
TNF-α
IL-1β
I
VCAM-1 mRNA level (w/o = 1.0)
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w/o
TNF-α
IL-1β
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ICAM-1 mRNA level (w/o = 1.0)
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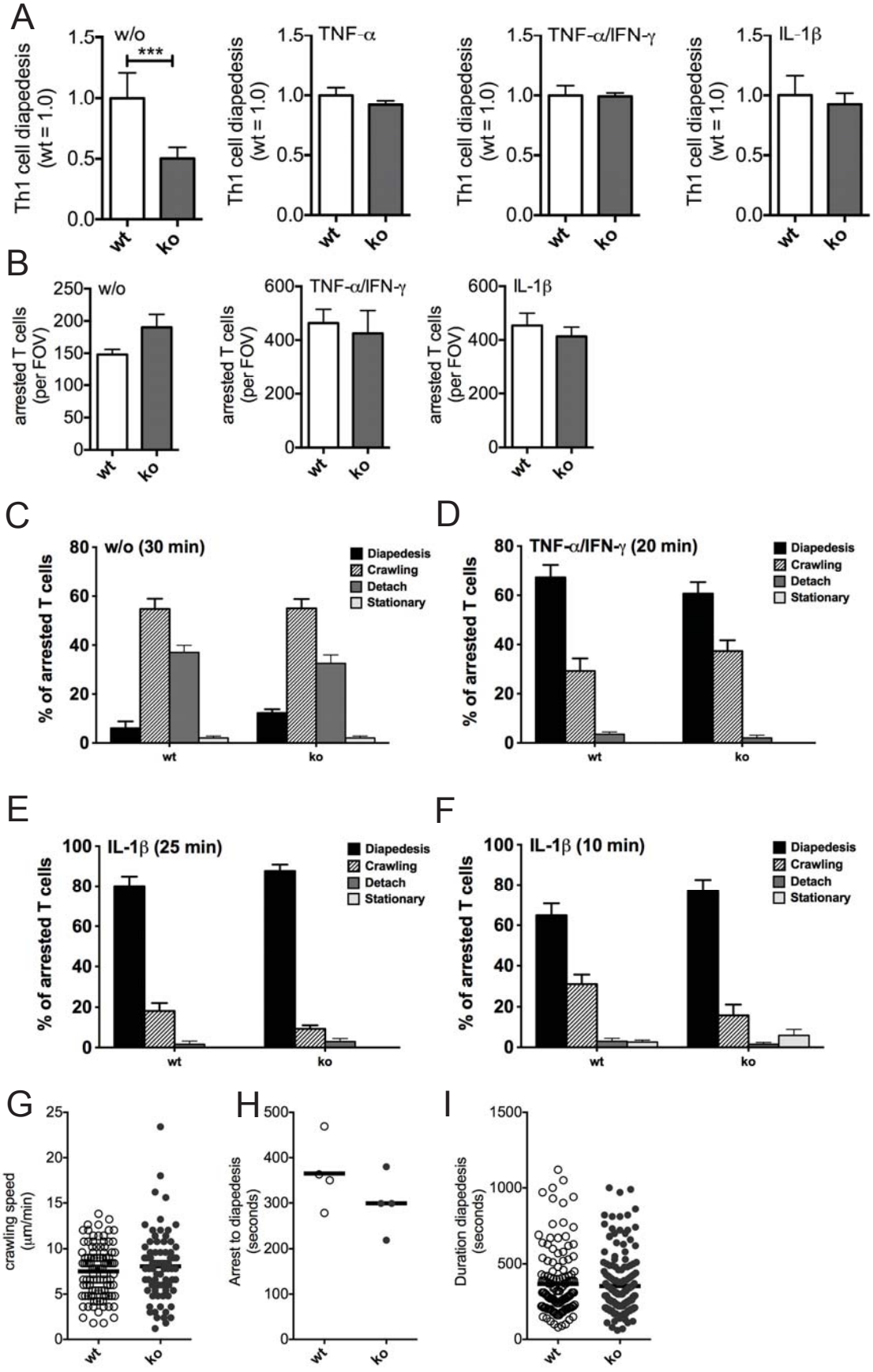
w/o
TNF-α
IL-1β

Figure 3:

ALCAM expression in the mouse CNS and on the mouse BBB

A, ALCAM or ICAM-1 protein in lysates from the brain cortex (ctx), cerebellum (crb) or spinal cord (sc) of wild type mice afflicted with acute paraplegic EAE or healthy gender and age matched control mice by Western Blot. Each lane represents protein samples from one individual mouse. Staining of β-actin proves equal loading, which was 20 µg/lane. B-E, Quantitative densitometry evaluation of the Western Blot shown in A. ALCAM or ICAM-1 signal intensities were normalized to β-actin and expressed as fractions of the healthy control set to 100 %. F, Analysis of ALCAM protein in brain cortex (ctx) or cerebellum (crb) lysates from an ALCAM$^{-/-}$ (ko) or wild type (wt) mouse by Western Blot. Equal loading of total protein amount (20 µg/lane) was confirmed through β-actin assessment. G, ALCAM protein in brain (left) or spinal cord (right, sc) microvessel lysates from healthy wild type (wt) or ALCAM$^{-/-}$ (ko) mice was assessed by Western Blot. Equal loading (20 µg/lane of brain vessels, 9 µg/lane of spinal cord vessels) was ensured by α-actin staining in parallel. A, F, G, polyclonal anti mouse/human ALCAM antibody. H-J, Relative quantification of ALCAM, ICAM-1 and VCAM-1 mRNA level in RNA lysates from wild type pMBMECs that were unstimulated (open bars) or stimulated for 4 hours with TNF-α (grey bars) or IL-1β (dark grey bars) as assessed by qPCR. Unstimulated condition was set to 1.0. Bars show the mean ± SD from five independent experiments, each performed in triplicate.

Figure 4


A
w/o
TNF-α
TNF-α/IFN-γ
IL-1β
Th1 cell diapedesis (wt = 1.0)
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wt
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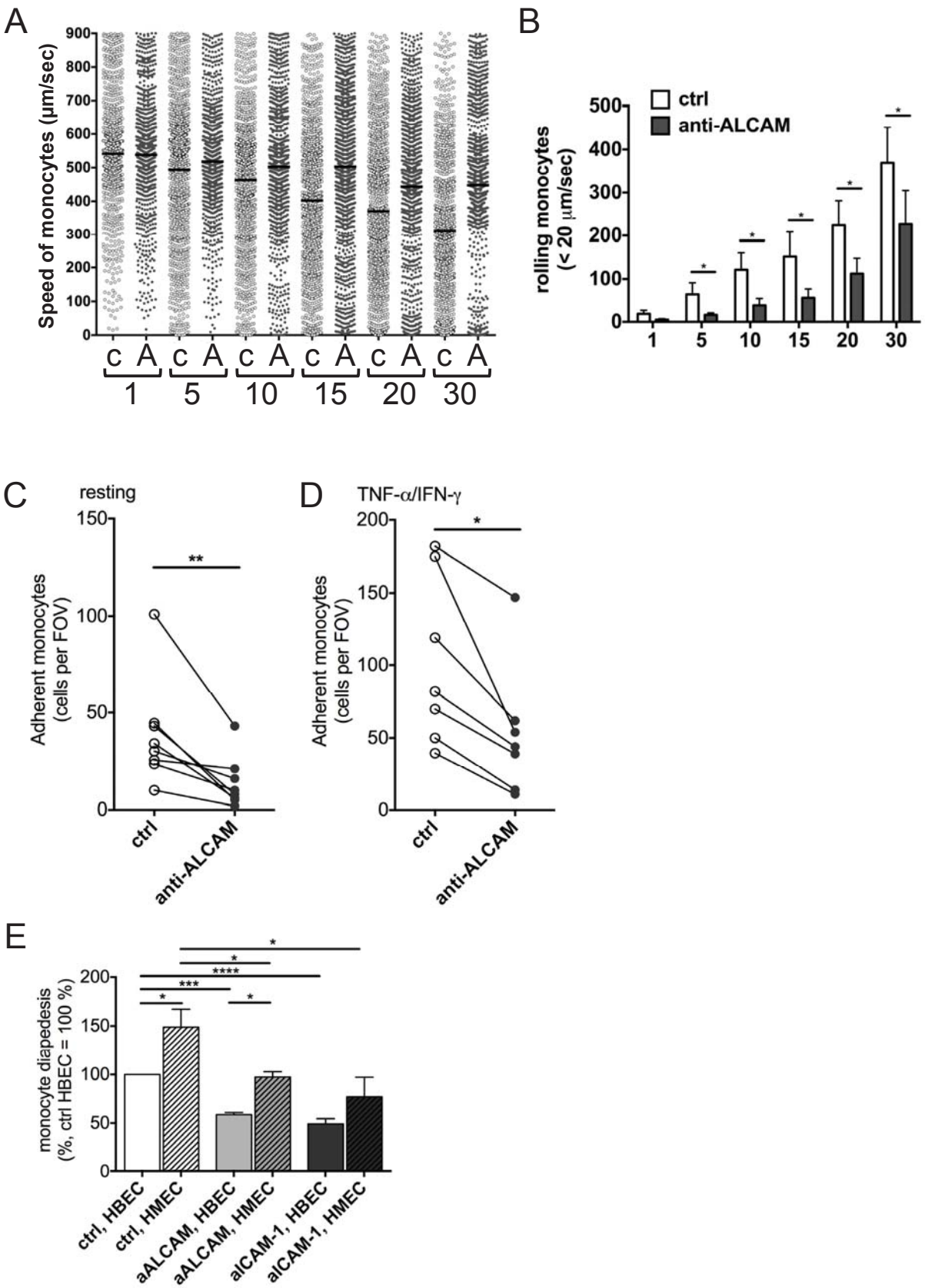
B
w/o
TNF-α/IFN-γ
IL-1β
arrested T cells (per FOV)
C
w/o (30 min)
D
TNF-α/IFN-γ (20 min)
E
IL-1β (25 min)
F
IL-1β (10 min)
% of arrested T cells
Diapedesis
Crawling
Detach
Stationary
G
crawling speed (μm/min)
H
Arrest to diapedesis (seconds)
I
Duration diapedesis (seconds)

Figure 4:

Comparison of mouse CD4$^+$ Th1 cell interaction with ALCAM$^{-/-}$ or wild type pMBMECs.

A, Diapedesis of CD4$^+$ Th1 cells across wild type (wt, black bars) or ALCAM$^{-/-}$ (ko, grey bars) pMBMECs was tested under static conditions. pMBMECs were without cytokine stimulation (w/o) or stimulated with TNF-α, TNF-α/IFN-γ or IL-1β as labelled. Diapedesis of mouse Th1 cells across ALCAM$^{-/-}$ (ko) is shown relative to Th1 cell diapedesis across wild type pMBMECs (wt) set to 1.0. Data are mean of 10 samples from three independent experiments (w/o) or mean of six samples (TNF-α, TNF-α/IFN-γ) or three samples (IL-1β) from one representative experiment. Error bars are SD. B-I, Dynamic interaction behavior of mouse Th1 cells on wild type (wt) or ALCAM$^{-/-}$ (ko) pMBMECs evaluated through *in vitro* live cell time lapse imaging under physiological flow. B-F, pMBMECs were without cytokine stimulation (w/o) or stimulated with IL-1β or TNF-α/IFN-γ as indicated. Values are the mean of 3 independent experiments. B, numbers of arrested Th1 cells per field of view (FOV). C-F, Dynamic post-arrest interaction of the Th1 cells on the pMBMECs is described in 4 behavioral categories: Diapedesis (black bars), crawling but no diapedesis (hatched bars), detachment (dark grey bars) or stationary (light grey bars). Numbers of arrested Th1 cells were set to 100 % and the behavioral categories are expressed in fractions of arrested T cells. Period of observation was 30 minutes (C, w/o), 20 minutes (D, TNF-α/IFN-γ) or 25 and 10 minutes (E, F, IL-1β). G, crawling speed of Th1 cells on the surface of IL-1β stimulated pMBMECs. Each dot represents the crawling speed of one Th1 cell. H, time elapsed between initial arrest and start of diapedesis in seconds on IL-1β stimulated pMBMECs. Each dot represents mean values from at least 50 CD4$^+$ Th1 cells in one experiment. I, duration of T cell diapedesis across IL-1β stimulated pMBMECs. Each dot represents one T cell.

Figure 5


A
Speed of monocytes (µm/sec)
900
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500
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200
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c A c A c A c A c A c A
1 5 10 15 20 30
B
ctrl
anti-ALCAM
rolling monocytes (< 20 µm/sec)
500
400
300
200
100
0
1 5 10 15 20 30
C
resting
Adherent monocytes (cells per FOV)
150
100
50
0
**
ctrl
anti-ALCAM
D
TNF-α/IFN-γ
Adherent monocytes (cells per FOV)
200
150
100
50
0
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ctrl
anti-ALCAM
E
monocyte diapedesis (%, ctrl HBEC = 100 %)
200
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ctrl, HBEC
ctrl, HMEC
aALCAM, HBEC
aALCAM, HMEC
aICAM-1, HBEC
aICAM-1, HMEC

Figure 5:

Role of ALCAM in the extravasation of human monocytes across the human BBB

A-D, Monocyte/HBEC interactions were analyzed under flow conditions. HBECs and CFSE-labeled human $CD14^+$ monocytes were treated with a function-blocking anti-ALCAM antibody or isotype control (n = 7 individual experiments). A, Monocytes were perfused over HBECs and 30-second-movies were acquired at 1 min, 5 min, 10 min, 15 min, 20 min or 30 min (x-axis: 1, 5, 10, 15, 20, 30; c, ctrl; A, anti-ALCAM). Each dot represents the mean velocity of a single $CFSE^+$ cell. The horizontal bars are representative of the mean per data set. B, Numbers of rolling monocytes (1-20 µm/sec) per FOV at 1 min, 5 min, 10 min, 15 min, 20 min or 30 min after monocyte perfusion are shown. C-D, numbers of adherent $CD14^+$ monocytes on resting (C) or cytokine (TNF-α/IFN-γ) (D) stimulated HBECs 20 minutes after monocyte perfusion. Monocytes from individual healthy donors are compared side-by-side (horizontal lines). E, Diapedesis of human monocytes across HBECs or HMECs; both cell types were pre-treated with ALCAM or ICAM-1 blocking antibodies or isotype control antibody (n = 4). HBECs and HMECs from identical donors were tested side-by-side and diapedesis rates were normalized to monocyte diapedesis across isotype control treated HBECs (100 %). Antibodies were anti ALCAM clone 105901 (A-E) and anti ICAM-1 clone BBA3 (E).

Supplementary material

Antibodies and cytokines

For immunofluorescence, flow cytometry and functional blockade of HBLECs, HBECs or HMECs or immunohistochemistry on post-mortem human brain tissue, we used monoclonal mouse anti-human ALCAM clone 105901 (R&D Systems) or clone 3A6 (BD Biosciences), VCAM-1 clone BBIG-V1 (R&D Systems) or clone 51-10C9 (BD Biosciences), ICAM-1 clone BBA3 (R&D Systems), clone HA58 (BD Biosciences) or clone 15.2 (Abcam, Cambridge, UK), mouse anti-human VE-cadherin clone F-8 (Santa Cruz Biotechnology, Heidelberg, Germany) and polyclonal goat anti-human/mouse ALCAM antibody (R&D Systems). For On-Cell Western on pMBMECs, we used rat anti-mouse monoclonal to ICAM-1 clone 29G1, VCAM-1 clone 6C7.1, ICAM-2 clone 3C4, and control anti-human monoclonal to CD44 clone 9B5 described previously.[5] For immunofluorescence on mouse tissue, we used polyclonal rabbit anti-Substance P (AB1566, 1:500, Chemicon), rat anti-mouse ALCAM clone 33.1,[22] and Isolectin B4 (IB4) from *Griffonia Simplificolia* directly labeled with FITC (1:100, Vector Laboratories). For Western Blot, we used anti-β-Actin (AC-15) from Sigma-Aldrich (Buchs, Switzerland), polyclonal goat anti ALCAM (R&D Systems) and polyclonal rabbit anti-ICAM-1 raised against mouse immune globulin domains 1 and 2 (custom made, Eurogentec, Seraing, Belgium). For FACS analysis of human or mouse T cells, we used mouse anti-human CD6 clone M-T605 (BD-Pharmingen), rat anti-mouse CD6 clone 96123 (R&D Systems), rat anti-mouse ALCAM clone eBioALC48 (eBiosciences, Vienna, Austria), polyclonal goat anti-ALCAM (R&D Systems).

Secondary antibodies used for Western Blot and On-Cell Western were goat anti-mouse IgG-IRDye® 800CW (Li-Cor, Bad Homburg, Germany) or –horse radish peroxidase (HRP), donkey anti-goat IgG-Alexa Fluor® 680 or –HRP, goat anti-rat IgG-Alexa Fluor® 680 and goat anti-rabbit IgG-Alexa Fluor® 680 (ThermoFisher Scientific). Secondary antibody used for immunohistochemistry was donkey anti-goat IgG-biotin (Jackson ImmunoResearch). Secondary antibodies used for immunofluorescence staining were donkey anti-mouse IgG-Cy3 (1:500), donkey anti-goat IgG-Cy3 (1:200) and goat anti-rat

IgG-Cy3 (1:500) (Jackson ImmunoResearch). Secondary antibodies used for FACS analysis were goat anti-rat, goat anti-mouse donkey anti-goat IgG-Phycoerythrin (PE) (Jackson ImmunoResearch).

For stimulation of pMBMECs, we used recombinant murine TNF-α from PromoKine (Vitaris AG, Baar, Switzerland), recombinant murine IL-1β and recombinant murine IFN-γ from PeproTech (Rocky Hill, NJ, USA). For stimulation of HBLECs we used recombinant human TNF-α, human IL-1β and human IFN-γ (R&D Systems, Bio-Techne AG, Zug, Switzerland). For stimulation of HBECs or HMECs we used recombinant TNF-α and IFN-γ (Gibco, Carlsbad, CA).

Cytokines used for T cell differentiation are named with the method.

Immunofluorescence staining of mouse tissue

Animals were anesthetized using isoflurane anesthesia (Abbott, Wiesbaden, Germany), and were perfused with PBS followed by 4 % formaldehyde (PFA) in PBS through the left ventricle of the heart. Tissue was removed, equilibrated in 30 % sucrose and then embedded in Tissue-Tek (OCT, Miles Inc., Vogel, Giessen, Germany), and snap-frozen in a 2-methylbutane (Merck, Darmstadt, Germany) dry ice bath at −80 °C. Cryostat sections (6-18 μm) were air dried overnight. For immunofluorescence staining, frozen tissue sections were blocked for 20 min with 2.5 % BSA, 0.1 % Triton X-100 in PBS followed by incubations with primary antibody (1 hour to overnight) and secondary antibody for 1 hour with PBS washing steps in between. After a final PBS-wash, sections were mounted in Mowiol (Calbiochem, Grogg Chemie AG, Stettlen/Deisswil, Switzerland). Images were acquired with a Leica DM5000B microscope at 20x magnification with a Leica DFC350 camera.

Flow cytometry

Human or mouse $CD4^+$ Th1 cells were washed twice with FACS buffer (PBS supplemented with 2.5% FBS and 0.1% NaN_3) and aliquoted at 0.5-1x 10^6 cells per well in a 96 round bottom well plate. HBECs at confluency prior to any passage (at passage 0) and HMECs at passage 3 - 4 were trypsinized, washed in serum containing media and then in FACS buffer. The cells were then aliquoted at 0.5x - 1x 10^5 cells per well in a 96 round bottom plate. Antibody incubation was performed for 30 min with primary

antibodies and with the PE-conjugated secondary antibodies at 4°C with washing steps in between. Cells fixed in 1% PFA/PBS or without any fixation were then analyzed by flow cytometry using a FACSCalibur and CellQuest or a BD LSR II Flow Cytometer and BD FACSDiva software (BD Biosciences, San Diego, CA) and FlowJo software (Tree Star Ashland, OR) for data analysis.

EAE, protein lysates, Western Blot and protein quantification

Active EAE was induced in 8-week-old female wild type C57BL/6J mice with 200 µg of myelin oligodendrocyte glycoprotein $(MOG)_{aa35–55}$ in complete Freund's adjuvants (CFA) (LabForce; Santa Cruz Biotechnology) supplemented with 4 mg/ml nonviable, desiccated Mycobacterium tuberculosis (H37RA; Difco/BD Biosciences/BD Clontech) subcutaneous. A total of 300 ng of pertussis toxin from Bordetella pertussis (List; LuBioScience) per mouse was administered intraperitoneal (i.p.) at days 1 and 3 postimmunization. Mice were euthanized at acute paraplegic EAE. Healthy age and gender matched C57BL/6J mice served as controls. Cortex of the cerebrum, the cerebellum and the spinal cord of PBS perfused healthy or EAE mice were harvested separately and homogenized in RIPA (50 mM Tris, pH 7.6, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1% SDS) supplemented with 2 mM EDTA and protease inhibitors (Roche Diagnostics, Rotkreuz, Switzerland). Homogenates were centrifuged at 13'800xg for 10 minutes at 4 °C. Supernatants were frozen in aliquots at -20 °C. Protein concentration was determined using the PierceTM BCA Protein Assay Kit (ThermoFisher Scientific). SDS-PAGE and Semi-Dry Western Blotting on nitrocellulose membrane (Bio-Rad Life Science, Cressier, Switzerland) was according to standard procedures. Western Blot signal detection was with the ECL system (Amersham Biosciences) combined with the Bio-Rad Gel Doc system to digitalize images or with the Odyssey® infrared reader (Li-Cor GmbH, Bad Homburg, Germany). For quantification ALCAM, ICAM-1 or β-Actin signal intensities were determined with the Odyssey® software. Then, signal intensities were normalized to β-Actin and expressed relative to the healthy control samples set to 100 %.

Supplementary Table:

Human brain tissues

Control patients

Patient	Gender	Age at death	Postmortem time	Disease Duration	MS type	Cause of death	Tissue block
C11	M	77	26			Carcinoma of the lung metastasised	C11-1
C15	M	64	18			Cardiac failure	C15-1
C20	F	60	13			Ovarian cancer	C20-2
C21	M	75	17			cva, aspiration pneumonia	C21-4
C23	F	88	20			Bronchopneumonia	C23-1
C25	M	84	5			Bladder cancer	C25-3

Multiple Sclerosis patients

Patient	Gender	Age at death	Postmortem time	Disease Duration	MS type	Cause of death	Tissue block
M01	F	56	8	31	SPMS	Breast carcinoma	M1-2
M02	F	58	16	22	PPMS	Peritonitis	M2-2
M03	F	78	18	33	SPMS	Myocardial infarction	M3-3
M05	F	74	19	26	SPMS	Septicaemia	M5-1
M07	F	20	17	17	PPMS	Pulmonary embolus	M7-1
M20	F	86	21	56	?	Pneumonia	M20-2

Statistics

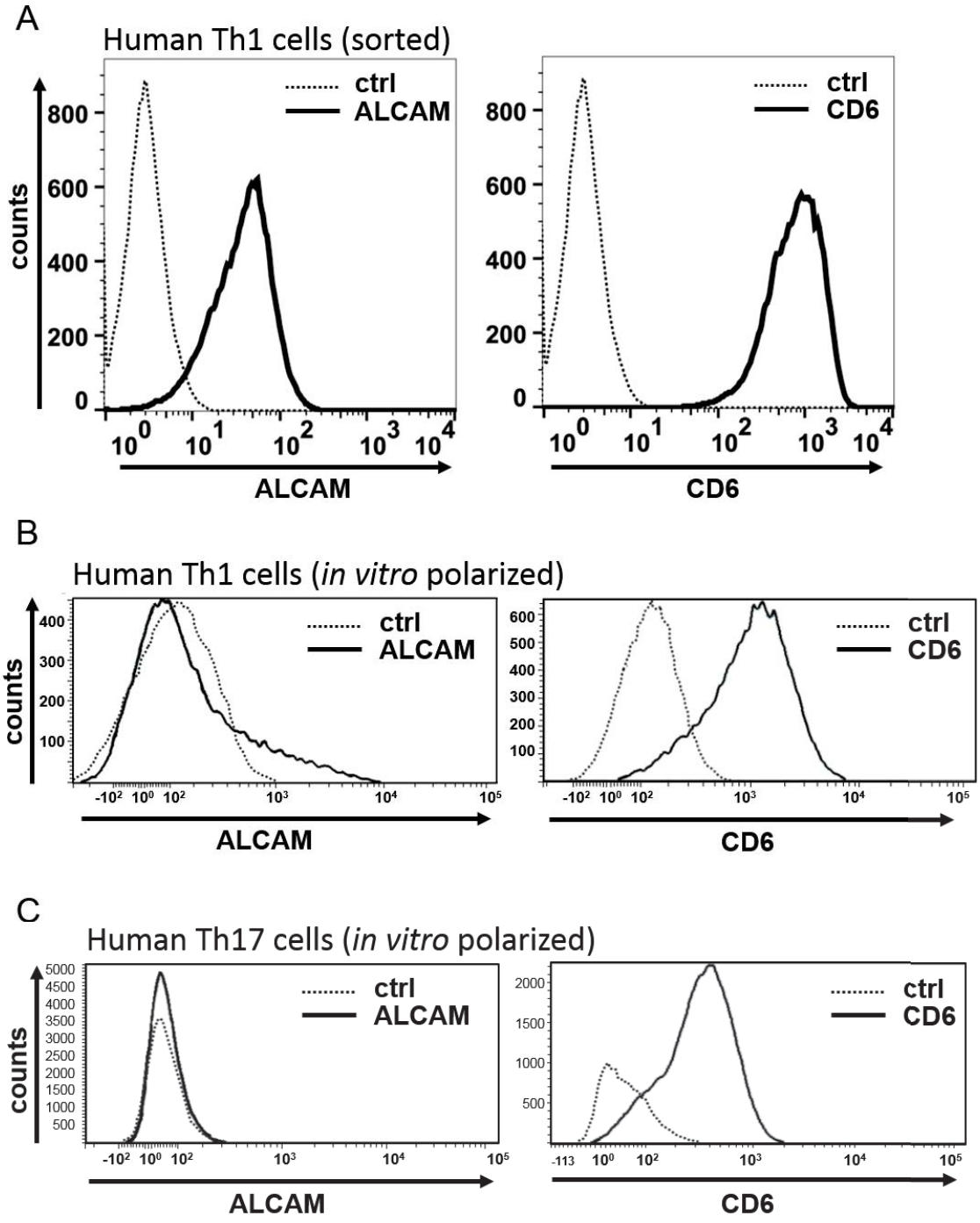
Multiple Sclerosis patients		Stdev
Average Age	62.00 years	23.63 years
Average Postmortem time	16.50 years	4.51 years
Average Disease duration	30.83 years	13.64 years
Control patients		
Average Age	74.67 years	10.95 years
Average Postmortem time	16.50 years	7.06 years
ttest age	0.2720657	
ttest postmortem time	1	

Legend to supplementary table:

Cortical tissue with subcortical white matter from six cases without any diagnosed neurological disease was taken as controls. These subjects had an average age of 74.7 years (± 10.95 SD) and an average post-mortem time of autopsy was 16.5 h (± 7.06 SD). Cortical tissues from six cases with multiple sclerosis with average disease duration of 30.8 years (± 13.6 SD) were used for this study. Their average age was 62 years (± 23.63 SD), and an average post-mortem time of autopsy was 16.5 h (± 4.51 SD).

Supplementary Figure 1:

ALCAM and CD6 expression on human Th1 or Th17 cells

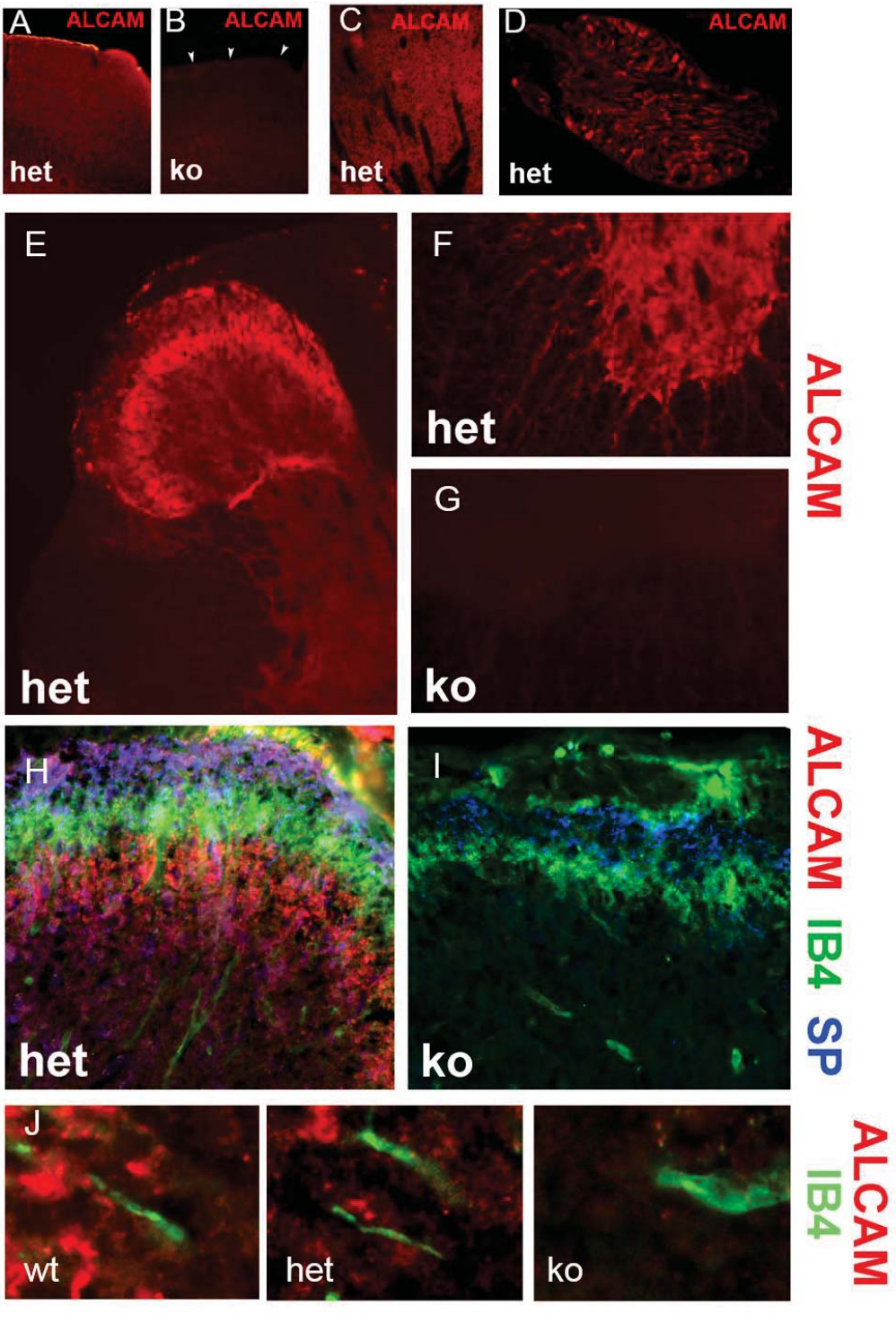


Legend to supplementary figure 1

Flow cytometry analysis of cell surface expression of ALCAM and CD6 on *ex vivo* sorted human $CD4^+$ Th1 cells (A, anti-ALCAM clone 105901, anti-CD6 clone M-T605) or on *in vitro* polarized human $CD4^+$ Th1 cells or Th17 cells (B, anti-ALCAM clone 3A6). Isotype control staining is shown as thin dotted lines and CD6 staining is shown in thick continuous lines. Y-axes show numbers of $CD4^+$ Th1 cells measured per intensity. Anti-ALCAM antibodies used were clone 105901 (A) and clone 3A6 (B). Anti-CD6 was clone M-T605 (A, B). Data are from one representative experiment out of 3 independent experiments.

Supplementary Figure 2:

ALCAM immune fluorescence on mouse CNS tissue

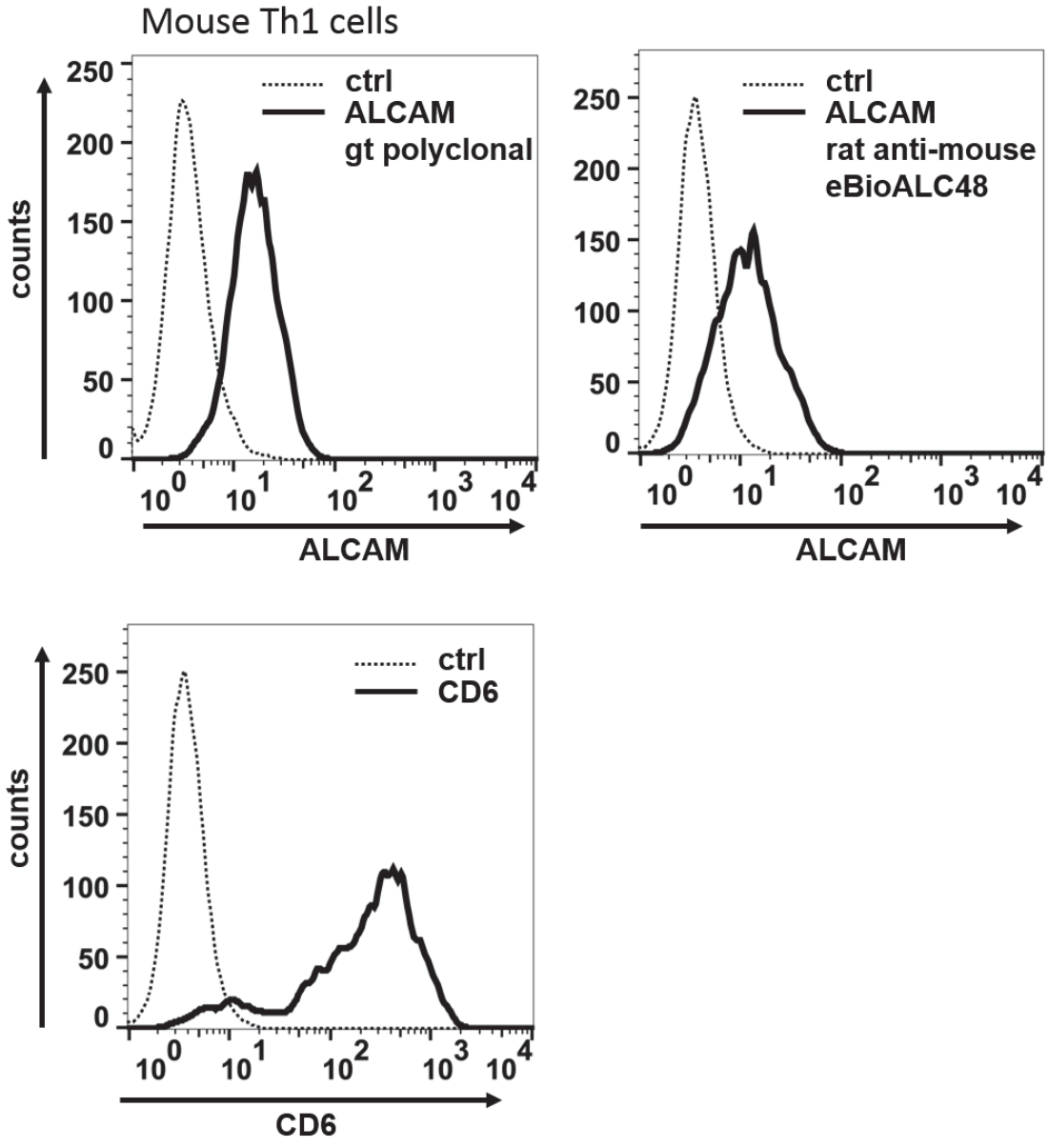


Legend to supplementary Figure 2

A-L, Representative ALCAM immunoreactivity in the CNS of an ALCAM heterozygous (cont, A, C-F, H, J), an ALCAM$^{-/-}$ (ko, B, G, I, J) and a C57BL/6J wild type mouse (wt, J). ALCAM immunoreactivity was detected with antibody clone 33.1 and is shown in red. A,C, Strong ALCAM signal in the meninges of the cerebral cortex (A) and striatum (C). D-F, strong ALCAM immunoreactivity in neurons and axons of the dorsal root ganglion (D), the dorsal (E) and the ventral horn (F). H-I, merged images of ALCAM with isolectin B4 (IB4, green) and Substance P (blue) shows ALCAM highly concentrated in the axons projected to the inner part of lamina II and to lamina III, as ALCAM immunostaining was ventral to both Substance P- and IB4-positive terminals in lamina I and the outer part of lamina II, respectively (H).[63] The laminar projections of Substance P and IB4-positive terminals did not appear to be disrupted in ALCAM$^{-/-}$ mice consistent with the lack of overt neurological phenotypes in this mouse (I).[22] J, high magnification of merged images of ALCAM with IB4 (green) of a wild type (wt, left), an ALCAM$^{+/-}$ (het) and an ALCAM$^{-/-}$ (ko, right) mouse do not reveal ALCAM immune reactivity of vessels (green). Objectives were 10x (A,B), 20x (C-I) and 63x (J).

Supplementary Figure 3:

ALCAM and CD6 expression on mouse CD4$^+$ Th1 cells



Legend to supplementary figure 3:

A-C, Flow cytometry analysis of cell surface expression of ALCAM and CD6 on mouse $CD4^+$ Th1 cells. Isotype control staining is shown as thin dotted lines, ALCAM and CD6 staining is shown in thick continuous lines. Y-axes show numbers of $CD4^+$ Th1 cells measured per intensity. Antibodies used were anti-mouse ALCAM clone eBioALC48, polyclonal anti human/mouse ALCAM 105901 and anti-mouse CD6 clone 96123. Data are from one representative experiment out of 3 independent experiments.

Manuscript 2

Title: The Dual Role of ALCAM in Neuroinflammation and Blood-Brain Barrier Homeostasis.

Authors: Marc-André Lécuyer, Olivia Saint-Laurent, Lyne Bourbonnière, Sandra Larouche, Catherine Larochelle, Laure Michel, Marc Charabati, Michael Abadier, Neda Haghayegh Jahromi, Camille Pittet, Stephanie Zandee, Ruth Lyck, Britta Engelhardt, Alexandre Prat

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Contributions: The project was initiated by myself as a follow-up of Cayrol et al., 2008. To conduct this study, I have genotyped and managed the colony of ALCAM KO mice with the help of Lyne Bourbonnière. This task was later performed entirely by Sandra Larouche. I have also performed all the EAE experiments with the help of Lyne Bourbonnière and Sandra Larouche. Additionally, I have performed all the *in vitro, ex vivo* and *in situ* experiments with, occasionally, the help of the following students. Olivia Saint-Laurent has helped with the isolation and flow cytometry analysis of mouse immune cells from both naïve and EAE animals. She has also helped with the immunofluorescence staining of tight junction and adherens junction molecules. Lyne Bourbonnière, Sandra Larouche, Catherine Larochelle, Laure Michel and Marc Charabati have also helped with the isolation of mouse leukocytes and mouse endothelial cells. Sandra Larouche has helped with the *in vivo* CNS permeability assay. Michael Abadier has performed TEER experiments in Dr. Engelhardt's laboratory. Neda Haghayegh Jahromi has performed the EAE experiment in Dr. Engelhardt's laboratory. Camille Pittet has cut the mouse CNS tissue used to perform immunofluorescence staining. Ruth Lyck, Britta Engelhardt, Alexandre Prat and myself have participated in the scientific discussions and the revision of this manuscript. Alexandre Prat and myself have elaborated the different experiments, analyzed the results, constructed the figures and wrote the manuscript.

The Dual Role of ALCAM in Neuroinflammation and Blood-Brain Barrier Homeostasis.

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Running title: ALCAM in Neuroinflammation

Keywords: ALCAM, CD166, CD6, multiple sclerosis, MS, BBB, EAE, transmigration, tight junctions, lymphocytes.

Abstract

ALCAM is a cell adhesion molecule found on blood-brain barrier endothelial cells (BBB-ECs), which was previously shown to be involved in leukocyte transmigration across the endothelium. In the present study, we found that ALCAM knock-out (KO) mice developed more severe MOG_{35-55}-induced experimental autoimmune encephalomyelitis (EAE). This was associated with a significant increase in the number of CNS-infiltrating pro-inflammatory leukocytes, as compared to WT controls. Passive EAE transfer experiments suggested that the pathophysiology observed in active EAE was linked to the absence of ALCAM on BBB-ECs. In addition, phenotypic characterization of un-immunized ALCAM KO mice revealed a reduced expression of BBB junctional proteins. Further *in vivo*, *in vitro* and molecular analysis confirmed that ALCAM is associated with tight junction molecules assembly at the BBB, explaining the increased permeability of CNS blood vessels in ALCAM KO animals. Collectively, our data point to a biologically important function of ALCAM in maintaining BBB integrity.

Introduction

The loss of blood-brain barrier (BBB) integrity is a hallmark of multiple sclerosis (MS). It is associated with the disorganization of junctional molecules which normally form complex apico-lateral molecular aggregates located in cholesterol-rich cell membrane regions called lipid rafts (1, 2). These molecules are highly regulated and are integral to the maintenance of the barrier. They can be divided in two major categories, the tight junction molecules (TJs) and located abluminally, the adherens junction molecules (AJs). The junctional adhesion molecules (JAMs), occludin and the claudins are well-characterized transcellular TJs molecules. Zonula occludens 1 and 2 (ZO-1, -2) and cingulin are adaptor molecules located intracellularly which anchor the TJs to the actin filaments and regulate them spatiotemporally (3, 4). AJs are composed of cadherin molecules, such as vascular-endothelial (VE)-cadherin and are linked to the cytoskeleton via proteins of the catenin family (α-, β-, p120) (5). Together, all these junctional proteins highly restrict leukocyte transmigration under homeostatic conditions.

Upon inflammation, cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which are two of the most studied molecules involved in immune cell transmigration and recruitment to the brain and spinal cord, are upregulated (6). Proof of concept that targeting CAMs for the treatment of autoimmune neuroinflammatory diseases, e.g. MS, was provided by the clinical success of Natalizumab, which targets the alpha4 integrin subunit of very late antigen-4 (VLA-4; binding partner to VCAM-1). However, use of Natalizumab in clinical practice is limited due to the emergence of progressive multifocal leukoencephalopathy and rebound MS activity following withdrawal (7). Identifying novel key players involved during leukocyte diapedesis is thus crucial for the development of novel therapies which aim at decreasing leukocyte trafficking into the central nervous system (CNS) during neuroinflammation.

Although the roles of ICAM-1 and VCAM-1 during leukocyte transmigration in most vascular beds have been extensively studied (8-10), additional adhesion molecules have also been shown to partake in the transmigration process of encephalitogenic immune cells, including activated leukocyte cell adhesion molecule (ALCAM/CD166) (11), MCAM (12-14), MadCAM (15, 16), VAP-1 (17, 18), Ninjurin-1 (19) and JAM-L (20). We have previously demonstrated that ALCAM is part of a group of novel adhesion molecules, found on blood-brain barrier endothelial cells (BBB-ECs), that are involved in immune cell diapedesis (11). ALCAM was initially discovered as a ligand for CD6, a co-stimulatory molecule involved in the formation of the immune synapse between lymphocytes and antigen-presenting cells (21-23). ALCAM can also bind to itself (homotypic ALCAM-ALCAM), although ALCAM-CD6 binding was later found to be the stronger of two possible interactions (24-26). Recently, both ALCAM and CD6 alleles were associated with an increased risk of developing MS (27-30).

In the current study, we found that ALCAM knock-out (KO) mice develop a more severe active experimental autoimmune encephalomyelitis (EAE) due to an increased permeability of the BBB. We also present data indicating that ALCAM indirectly links junctional molecules to the cytoskeleton, suggesting that in addition to its role in transmigration, ALCAM regulates and maintains tight junction stability by acting as an adaptor molecule.

Results

ALCAM is absent from blood-brain barrier endothelial cells and the immune compartment of ALCAM knock-out mice.

To test the hypothesis that ALCAM plays an important role during leukocyte transmigration across the BBB, we used an ALCAM knock-out mouse. We first evaluated whether ALCAM was indeed absent in the animal cells. Using Western blot, ALCAM was detected on both freshly isolated brain and spinal cord microvessel from wildtype (WT) but not from ALCAM KO animals (Fig 1A). These results were corroborated by the analysis of primary cultures of WT and ALCAM KO BBB endothelial cells (BBB-ECs) mRNA via RT-PCR. Following activation with TNF and IFNγ, *ALCAM* mRNA expression was up-regulated in EC cultures derived from WT animals, while *ALCAM* mRNA could not be detected in ALCAM KO samples (Fig 1B). Similar results were obtained by flow cytometry analysis of PECAM-1+ BBB-EC primary cultures (Fig 1C). This confirmed the absence of ALCAM on BBB-ECs, and thus allowed us to use ALCAM KO CNS blood vessels in order to study the role of ALCAM in transmigration.

Next, we assessed by flow cytometry the expression of ALCAM on *ex vivo* CD4+ T lymphocytes isolated from the CNS of early symptomatic active EAE animals, as well as CD11b+ monocytes/macrophages and CD11b+CD11c+ dendritic cells isolated from the spleen of the same animals. Whereas myeloid cells of WT animals express high levels of ALCAM even in resting state, T lymphocytes express low to intermediate levels of ALCAM only once highly activated (Fig 1D). Resting T lymphocytes do, however, express constitutively CD6, which is a co-stimulation marker and one of the ligands of ALCAM (Fig S1A-B). ALCAM KO animals had normal absolute numbers, proportion and distribution of peripheral immune cells in the spleen, lymph nodes (LNs) and thymus, as compared to their WT littermates (data not shown). Nevertheless, to assess the effect of ALCAM deficiency on lymphocyte proliferation in more details, we conducted i*n vitro*

proliferation assays using WT CD4 T lymphocytes in combination with WT or ALCAM deficient antigen-presenting cells. Both mixed leukocyte reactions and control assays showed similar percentages of cellular proliferation (50.9% ± 3.2 and 51.6% ± 2.4, respectively). Likewise, no significant difference was observed in the production of IL-17 and IFNγ by CD4 T lymphocytes (IL-17: WT= 20.9% ± 5.9 and KO= 23.4% ± 7.3; IFNγ: WT= 26.1% ± 4.7 and KO= 24.7% ± 3.2). These data confirmed results obtained beforehand using anti-ALCAM blocking antibodies (11), demonstrating that the absence of ALCAM does not significantly influence the activation of lymphocytes and *in vivo* immune homeostasis.

ALCAM KO mice develop a more severe active EAE associated with a significant increase in pro-inflammatory leukocyte infiltrating the CNS.

To assess the role of ALCAM during the pathogenesis of EAE, active EAE was induced in WT and ALCAM KO mice using MOG_{35-55} in complete Freund's adjuvant. As opposed to what we had expected based on previous publications (11, 23), ALCAM KO mice developed more severe clinical symptoms (Area under the curve (AUC) WT: 86.48 ± 6.80, ALCAM KO: 117.6 ± 7.21), showing a diminished remission and often an earlier disease onset (1 day prior to their WT littermates) (Fig 2A). Additional active EAE experiments induced with recombinant human MOG corroborated these results (data not shown). Furthermore, these observations were made in two different animal facilities using independently bred ALCAM KO mice and their WT littermates, demonstrating the certainty of the outcome. While the absolute number of immune cells found in the draining inguinal LNs of both groups were virtually identical throughout the disease course, the absolute number of splenocytes was significantly higher in ALCAM KO animals at 9 days post-induction (dpi) (Fig 2B). On the other hand, the highest absolute number of immune cells infiltrating the CNS of ALCAM KO mice occurred 3 days earlier than in their WT littermates (Fig

2C). Of those CNS infiltrating immune cells, IFNγ+, IL-17+, as well as IFNγ+/IL-17+ CD4 and CD8 T lymphocytes were found to enter the CNS of ALCAM KO animals earlier and in greater number than in WT animals, as assessed by flow cytometry (Fig 2D). In addition, the number of CD4+CD25+FOXP3+ regulatory T lymphocytes was found to be similar throughout the disease process in both ALCAM KO and WT CNS (Fig 2E), which suggests that the increased disease severity observed in ALCAM KO mice was not due to a deficiency in the immune regulatory system. Finally, and importantly, we did not observe differences in cytokine production in the periphery (data not shown), suggesting that enhanced and early recruitment of encephalitogenic lymphocytes in the CNS of ALCAM KO animals was not caused by an enhanced number of circulating activated peripheral immune cells.

While EAE is mainly a CD4+ T lymphocyte driven disease, myeloid cells also play a crucial role both in the establishment of the disease and in the remission phase. Therefore, we have assessed by flow cytometry the ratio of M1 (pro-inflammatory) versus M2 (anti-inflammatory/tissue repair) monocytes/macrophages present in the CNS. As expected from the ongoing increased clinical scores, ALCAM KO mice had a higher M1 over M2 ratio at peak of disease (12 dpi), as well as in the chronic phase of the disease (Fig 2F). Interestingly, ALCAM positive monocytes, dendritic cells and CD4 T lymphocytes were also enriched in the CNS of WT EAE animals, as compared to the periphery (data not shown); clearly suggesting the importance of ALCAM during EAE pathogenesis. Collectively, these results were corroborated by *in situ* immunofluorescence confocal microscopy of spinal cord material from ALCAM KO and WT animals harvested at 12 dpi (Score 3.0 and 2.5 respectively). Immunostainings for CD4 and F4/80 in conjunction with pan-laminin confirmed the significant increase in the number of helper T lymphocytes and macrophages in the CNS of ALCAM KO EAE mice, as compared to WT animals (Fig 2G-H).

The absence of ALCAM on BBB-ECs increase EAE disease severity.

Different cell adhesion molecules (CAMs) (i.e. ICAM-1 and VCAM-1) can compensate for the lack of specific CAMs in KO animals (31-33). We have therefore chosen to compare, by flow cytometry, the expression of other CAMs on primary cultures of WT and ALCAM KO mouse CNS ECs, under resting or stimulated conditions (TNF and IFNγ). While we confirmed that some CAMs are upregulated upon inflammation, we could not demonstrate the presence of such compensatory mechanism in ALCAM KO endothelial cells (Fig 3A). MCAM, Ninjurin-1 and CD62E, previously shown to be involved in the recruitment of specific immune cell types to the CNS (13, 14, 19, 34, 35), were also assessed and showed no significant differences between ALCAM KO and WT MBECs either in resting or stimulated conditions (data not shown).

We then performed passive (adoptive transfer) EAE by injecting WT MOG-reactivated splenocytes into both WT and ALCAM KO mice (Fig 3B). ALCAM KO recipients demonstrated the first sign of symptoms 2 days earlier than their WT littermates, and had higher clinical scores at peak of disease and subsequently during the remission phase (AUC: WT recipients: 21.43 ± 1.94, ALCAM KO recipients: 38.06 ± 5.03). The disease incidence rate was also slightly more elevated in ALCAM KO recipients as compared to WT with 100% and 95%, respectively. In addition, the absolute number of CNS infiltrating immune cells were higher in ALCAM KO recipient after 12 days following cell transfer, corroborating the clinical scores (Fig 3C). From these immune cells, the percentage of CD4+ T lymphocytes was higher in ALCAM KO, whereas the percentage of CD8+ T lymphocytes was higher in the WT recipients (Fig 3D). As expected, the percentage of ALCAM+ leukocytes was lower in ALCAM KO animals as only the donor cells express that protein. However, it demonstrates that roughly 20% of CD4+ T lymphocytes and CD11b+ monocytes present in the CNS of ALCAM KO originated from the donor cells (Fig 3D).

While the percentage of CD4 and CD8 T lymphocytes were different in the two recipient groups, the percentage of those cells positive for IFNγ or IL-17 were not that different, with the exception of IFNγ+CD4+ T lymphocytes (Fig 3E). Overall, this means that more pro-inflammatory cytokine-secreting CD44hiCD4+ T lymphocytes were present in the CNS of ALCAM KO at day 12, while the reverse was true for CD8 T lymphocytes. Ultimately, this transfer experiment allowed us to hypothesize that the absence of ALCAM on BBB-ECs might destabilize the BBB and therefore explain the increased EAE severity observed in MOG-induced active EAE.

We then performed the reverse transfer experiment, in which ALCAM KO splenocytes are injected into either ALCAM KO naïve recipients or their WT littermates. The disease onset was slightly earlier in ALCAM KO recipients and animals developed more severe EAE scores, as compared to WT recipients (AUC: ALCAM WT recipients: 60.85 ± 8.89, ALCAM KO recipients: 90.67 ± 10.69) (Fig 3F). The disease incidence was also higher in ALCAM KO mice with 83%, as compared to 72% for the WT. While the absolute number of CNS infiltrating immune cells were not significantly higher in ALCAM KO recipient after 12 dpi, a noticeable trend can be observed, albeit with a large variability (Fig 3G). Similar to the WT to KO/WT transfer experiment, a higher percentage of CD4 T lymphocytes could be observed by flow cytometry in the CNS of ALCAM KO recipients, while a smaller percentage of CD8 T lymphocytes was present (Fig 3H). Since ALCAM positive leukocytes present in the CNS can only originate from the recipient, virtually no ALCAM+ signal was detected in ALCAM KO animals as expected (Fig 3I). Finally, the percentage of CD4 cells positive for IFNγ, IL-17 or expressing high levels of CD44 were greater in ALCAM KO recipients (Fig 3J). Similar results were obtained while gating on CD8+ T lymphocytes with the exception of IFNγ, which was expressed at equally high levels in both groups (Fig 3J). Overall, this transfer experiment corroborated our hypothesis that ALCAM expression on the BBB helps maintaining its integrity.

The blood-brain barrier of ALCAM KO animals is more permeable.

We next tested the integrity of the ALCAM KO BBB *in vitro* and *in vivo*. Using a trans-endothelial electrical resistance (TEER) assay, we first demonstrated that confluent monolayers of primary mouse brain microvascular endothelial cells (pMBMECs) from ALCAM KO mouse were significantly more permeable (i.e. less resistant) than their WT counterpart (Fig 4A). Whereas TEER measurements can clearly assess the integrity of the endothelial barrier, we also decided to corroborate the result using bovine serum albumin (BSA) and 10 kDa dextran as permeability markers in a modified *in vitro* Boyden chamber assay, in which confluent monolayers of mouse BBB-ECs (MBECs) were grown on the insert. This tracer diffusion assay demonstrated that ALCAM KO MBECs have a higher permeability coefficient, as compared to WT cells (Fig 4B), when using either resting MBECs or MBECs stimulated with TNF and IFNγ. Next, we wanted to confirm these *in vitro* data with an *in vivo* approach. Using different sizes of fluorescent dextran markers injected intravenously, we measured the accumulation of these tracers in the CNS (and indirectly the permeability of the BBB) in naïve animals, as well as in EAE mice at 7 and 10 dpi. While CNS vessels of naïve ALCAM KO animals were not more permeable than those from control WT littermates, CNS vessels from pre-symptomatic ALCAM KO EAE animals were significantly more permeable to the small 3 kDa dextran marker, as compared to vessels from WT littermates (Fig 4C); the differences in vascular permeability for larger molecular weight dextran markers became apparent at 10 dpi, corresponding to the time of disease onset (Fig 4C).

Junctional molecules expression at the BBB is altered in ALCAM KO animals.

To explain the increased BBB permeability in ALCAM KO, we assessed the expression of junctional molecules. Using immunofluorescence confocal imaging, we evaluated the organization

of TJ and AJ molecules, as well as their expression levels based on the maximum fluorescence intensity. Analysis of spinal cord sections from naïve animals revealed that the expression of the tight junction protein occludin, and the adherens junction protein α-catenin, were reduced in ALCAM KO mice (Fig 4D). We further demonstrated that claudin-5, p120 and ZO-1 were also down-regulated *in situ*, at the level of the blood-spinal cord barrier in ALCAM KO naïve animals, while VE-cadherin, PECAM-1, β-catenin, ICAM-1 and VCAM-1 were similar (Fig 4E and data not shown). Similarly, we compared the expression of junctional molecules in MBECs cultures. Using the same method, α-/β-catenin, claudin-5, ZO-1 and VE-cadherin expression were shown to be reduced *in vitro*, whereas the expression of PECAM-1 and ICAM-1 were comparable (Fig 4F). Although we also analyzed the spinal cord of EAE animals, the results were much more variable depending on the clinical score (data not shown). Furthermore, the presence of massive perivascular infiltrates, the disorganization of junction molecules by the immune cells and the presence of debris in demyelinating lesions made it more challenging to analyze.

ALCAM is linked to junctional molecules

Having established that the integrity of ALCAM KO BBB is defective due to the disorganization and reduced expression of junctional molecules, we next elected to confirm a possible molecular interaction between ALCAM and junctional proteins. To do so, we performed an immunoprecipitation of ALCAM on MBECs protein lysates (Fig 5A). A proteomic analysis was performed by liquid chromatography–tandem mass spectrometry on the proteins pulled-down with ALCAM. A list of the most abundant proteins was made and from that list, probable ALCAM binding partners were identified. Eight of those were subsequently verified by Western blot. We demonstrated that an isoform of ZO-2, cingulin, TRIO and F-actin binding protein (TRIOBP, TARA), coronin 1C, β-actin and pan-tropomyosin (TPM) were all linked directly or indirectly to

ALCAM (Fig 5B). Although present on the list, both ZO-1 and occludin were not detected by Western blot in the proteins pulled-down with ALCAM (Fig 5B). Similarly, ezrin and syntenin-1, which have been recently identified as intracellular ALCAM binding partners in dendritic cells were not detected in the pulled-down protein lysate (Fig 5B) (36). These results suggest that ALCAM is present in the intercellular junctions and stabilizes the junctional molecular complexes by binding to cellular structural fibres such as actin and myosin.

Discussion

In previous studies, ALCAM has been shown to be expressed on BBB-ECs and its ligands, CD6 and ALCAM, on activated and resting leukocytes (11, 37). Furthermore, we have previously demonstrated that physical hindrance of ALCAM during active EAE, using anti-ALCAM blocking antibodies, delays the progression and reduces the severity of the disease, while also partially preventing the infiltration of leukocytes in the CNS (11). In addition, several groups have demonstrated the involvement of ALCAM in leukocyte transmigration across human or mouse BBB-endothelial cells (11, 37-40).

Using a constitutive ALCAM KO animal, we now provide evidence that, following the induction of active EAE, ALCAM deficient mice progress to a higher clinical score, with a significantly more severe ascending paralysis than their WT littermates. Correlating with this observation, a significantly higher number of leukocytes are found in the CNS of ALCAM KO animals at peak of disease. From this population of CNS infiltrating immune cells, a higher percentage of T lymphocytes are secreting the pro-inflammatory cytokines IFNγ and IL-17, as compared to those found in WT mice. Similarly, "M1" macrophages (pro-inflammatory) are found in higher number in the CNS of ALCAM deficient animals. However, the number of regulatory T lymphocytes are found in similar numbers in the CNS of ALCAM KO and WT animals, despite the fact that a previous study described the role of ALCAM in recruiting Tregs into tumour tissues (41). While these EAE results were unexpected, we also found enriched populations of ALCAM+ leukocytes infiltrating the CNS of WT EAE mice, as compared to peripheral immune compartments, suggesting that ALCAM expression on encephalitogenic immune cells is important during the transmigration process, which is in line with previous literature.

Although some groups have shown a role for the heterotypic interaction ALCAM-CD6 in the proliferation of CD4 T lymphocytes (23, 28), we have previously established that ALCAM

blockade does not influence *in vitro* proliferation and activation of human CD4 T lymphocytes (11). In the current study, we now provide *in vivo* evidence that genetic neutralization of ALCAM does not affect peripheral immune system homeostasis, nor T cell proliferation. Therefore, it is unlikely that the impeded CD6 activating signalling pathways on T lymphocytes or a compensatory mechanism are responsible for the increased clinical score of ALCAM KO mice during active EAE.

Using transfer EAE experiments, where MOG-reactive WT splenocytes are transferred to ALCAM KO animals, we confirmed that ALCAM genetic neutralization in the recipient mice lead to a more severe disease, and to an increased number of encephalitogenic leukocytes infiltrating their CNS, as compared to WT littermates. To rule out the possibility that increased immune cell infiltration could be mediated by the up-regulation of additional CAMs (ALCAM-independent pathways) in ALCAM KO animals, we assessed the expression of other CAMs on BBB-ECs and found no evidence of compensatory mechanisms. However, we could demonstrate a significant reduction of BBB integrity in ALCAM deficient animals, as supported by a reduced TEER and an increased extravasation of fluorescent molecules, both *in vitro* and *in vivo*. This altered BBB integrity was associated with a dysregulation of TJ and AJ molecules in the CNS of ALCAM KO animals, including occluding, α-, β-catenin, claudin-5, ZO-1, p120 and VE-cadherin. Differences found between *in vitro* and *in vivo* experiments might be the result of complex intrinsic glial cells contribution to the development of a tightly controlled BBB (6), which concurrently help delineate the inherent limits of *in vitro* assays. Collectively, these experiments therefore demonstrate that ALCAM deficient animals have a dysregulated BBB.

Based on previous studies demonstrating the presence of ALCAM in lipid rafts of both human and mouse BBB-ECs (11, 37) and the colocalization of human ALCAM immunostaining with BBB-EC lateral junctions (unpublished data), we hypothesized that, in homeostatic

conditions, ALCAM is located within intercellular structures and might therefore directly or indirectly bind to junctional molecules. As such, ALCAM would be similar to PECAM-1, CD99, JAMs and MCAM, which are present within BBB-EC junctions but also mediate immune cell transmigration (14, 42-45). While Masedunskas et al. (39) have previously reported the presence of ALCAM in endothelial cell junctions using pulmonary microvascular ECs transfected with rat ALCAM-GFP, the localization of endogenous ALCAM in BBB-ECs junction had still not been established. The findings presented herein, using primary cultures of MBECs, demonstrate that ALCAM is localized with intercellular junctional proteins and also bind to the TJ adaptor molecules ZO-2 and cingulin; confirming a direct link between ALCAM and intracellular TJ adaptor molecules. In contrast, transmembrane TJ molecules (occludin, the JAMs and the claudins) were not pulled-down with ALCAM, which suggests that ALCAM binds or recruits cytoplasmic adaptor molecules to junctional complexes, but does not directly interact with transmembrane TJ proteins. Using the pulled-down protein lysate, we also identified actin-binding proteins TARA and coronin 1C, which, along with the presence of β-actin and tropomyosin proteins, confirm previous studies and provide further evidence demonstrating that ALCAM is directly linked to the actin cytoskeleton (46, 47).

While no protein of the catenin or cadherin families were identified in the proteomic list, an indirect link with ALCAM via ZO-2 could still be possible in homeostatic condition. Additional proteins previously shown to bind ALCAM intracellularly, such as ezrin and syntenin-1 (36), were not pulled-down during our experiments. The discrepancy between their findings and the data presented herein could be explained by their use of the K562 cell line combined with the transfection of modified ALCAM proteins, as opposed to endogenous ALCAM obtained from primary MBECs. Similarly, Gilsanz et al. (48) have recently reported that ALCAM forms a complex with the tetraspanin molecule CD9 and the “sheddase” ADAM17, which together regulate

ALCAM expression and activity (49). While CD9 is highly expressed on endothelial cells (50), we were unable to confirm the link between ALCAM and CD9. However, the existence of such a bond in BBB-ECs is still a possibility as a different cell activation state might be necessary to observe the link. Technical differences in the protocols used could also explain the discrepancy.

Collectively, our data demonstrate that ALCAM KO mice develop a more severe active EAE, which can be explained by an increased permeability of their BBB. This loss in BBB integrity is due to a dysregulation of junctional molecules caused by the lack of ALCAM at the level of the TJ molecules. Our results also show that ALCAM indirectly and directly links junctional molecules to the actin cytoskeleton, suggesting that, in addition to its role during leukocyte extravasation, ALCAM regulates and maintains tight junction stability by acting as an adaptor molecule. Most importantly, mouse and human ALCAM molecules are 93% homologue and the binding regions, the transmembrane region and the intracytoplasmic tail are all highly conserved between most vertebrates, suggesting that the results reported herein may also be applicable to human.

Methods

ALCAM knock-out mice

The ALCAM KO C57BL/6 mice (Alcamtm1Jawe) were generously provided by Joshua A. Weiner (51). The founding animals were further backcrossed in our animal facility for 7 generations. The weight of the animals matched those of aged-matched WT C57Bl/6 and no gross abnormalities or phenotypes were apparent from the embryonic stage to the adulthood, confirming the observation made by the group of Dr. Weiner (51). Animals were kept for a maximum of 1.3 years with no health issue, which suggest a normal life expectancy. All animal procedures were approved by the CRCHUM Animal Care committee (N11023APs) and followed guidelines of the Canadian Council on Animal Care. All animal procedures executed in the Engelhardt laboratory were approved by the committee of animal experimentation of the Veterinary Department of the Kanton Bern (permit number BE42/14) and are in keeping with institutional and standard protocols for the Care and Use of Laboratory Animals in Switzerland.

Active EAE disease induction and scoring

EAE was induced in 6-9 weeks old female C57BL/6 mice as previously published (13, 14). In brief, animals were immunized subcutaneously with 200 µg of $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK; Alpha Diagnostic International) in a 100 µl emulsion of Complete Freund's Adjuvant (4 mg/ml *Mycobacterium tuberculosis*; Fisher Scientific). On day 0 and day 2, Pertussis toxin (500 ng PTX, Sigma-Aldrich) was injected intra-peritoneally (i.p.). The scoring system used was as follows: 0 = normal; 1 = limp tail; 2 = slow righting-reflex; 2.5 = difficulty walking/ataxia; 3 = paralysis of one hindlimb (monoparalysis); 3.5 = hindlimb monoparalysis and severe weakness in the other hindlimb; 4 = paralysis of both hindlimbs

(paraparalysis); 4.5 = hindlimbs paraparalysis and forelimbs weakness; 5 = moribund (requires sacrifice). Mice were scored by an investigator blinded to the transgenic group.

Active EAE in Dr. Engelhardt's laboratory was induced in 8-week-old female mice with 200 µg of $MOG_{35–55}$ in complete Freund's adjuvants (CFA) (LabForce; Santa Cruz Biotechnology) supplemented with 4 mg/ml of desiccated *Mycobacterium tuberculosis* (H37RA; Difco/BD Biosciences/BD Clontech). A total of 300 ng of PTX (List; LuBioScience) per mouse was administered i.p. at days 1 and 3 post-immunization. Weights and clinical severity were assessed twice daily and scored as: 0, healthy; 0.5, limb tail; 1, hind leg weakness; 2, hind leg paraplegia; 3, hind leg paraplegia and incontinence.

Transfer EAE

Transfer EAE was performed as previously described (13). Briefly, active EAE was induced as described above except that PTX (500 ng) was only injected on day 0. On day 7, mice were sacrificed and leukocytes were recovered from LN and spleens as previously published (52). Cells isolated were cultured for 90h in RPMI supplemented with 10% FBS, glutamine, non-essential amino acids, HEPES, sodium-pyruvate and β-mercaptoethanol. Reactivation of cells was performed in the presence of $MOG_{35–55}$, rhTGF-β, rmIL-6, rmIL-23 and rmIL-12 (R&D systems). Fresh complete medium (20% of initial volume) with rmIL-23 (500% of initial concentration) was added to all cultures on day 2. Cells were then harvested, washed in Hank's Balanced Salt Solution (HBSS) and then processed for analysis by flow cytometry. $25X10^6$ total leukocytes were injected i.p. to all the recipient female C57BL/6 animals. Recipient mice received a single dose of PTX (200 ng) i.p. on day 2 following transfer. The scoring system used was the same as described above.

Primary cultures of mouse BBB-endothelial cells (MBECs / pMBMECs)

Primary cultures of mouse brain parenchymal capillary endothelial cells were prepared from 10 to 15 wild-type or ALCAM knock-out 7-9 weeks old female C57BL/6 mice. The brains were isolated without perfusion and meninges/choroid plexuses were removed. The parenchymal tissue was minced and homogenized at low speed in a mechanical Dounce homogenizer. The homogenate was then digested in DMEM containing 0.7 mg/ml collagenase type II (Worthington Biochemical corp.) and 39 U/ml DNase I (Worthington Biochemical corp.) for 75 min at 37°C. Myelin was removed by centrifugation at 1000g for 20 min in 20% BSA-DMEM (Sigma). The remaining pellet was then shook for one hour at 37°C with a mixture of 1 mg/ml collagenase-dispase (Roche) and 39 U/ml DNase I in DMEM. The microvessels were separated from remaining glial cells and red blood cells using a 33% continuous Percoll gradient centrifuged at 1000g for 10 min. Microvessels were plated on 6-wells culture dishes coated with 5 µg/ml collagen type IV (Sigma). MBECs were cultured in DMEM supplemented with 20% (v/v) FBS (Sigma), 1 ng/ml basic fibroblast growth factor (Roche), 100 µg/ml heparin (Sigma), 1.4 µM hydrocortisone (Sigma), and 1X antibiotic-antimycotic solution (Invitrogen). The media was replaced every 24 hours during the first 3 days. Puromycin (10 µg/ml) (Sigma) was added to the media for the first 48 hours of culture. After 72 hours, 4 µg/ml of puromycin was maintained in the culture media. A confluent monolayer was formed following 4-6 days in culture. MBECs culture expressed vascular endothelial–cadherin protein. No immune reactivity for α-smooth muscle actin, glial fibrillary acidic protein, or neuronal nuclei protein could be detected, confirming the absence of contaminating smooth muscle cells, astrocytes, and neurons, respectively. To stimulate endothelial cells mouse recombinant TNF (3ng/ml) and IFNγ (60ng/ml) (R&D systems) were added to the culture media 24h prior to the experimental procedure.

The isolation and culture of primary mouse brain microvascular endothelial cells (pMBMECs) in Dr. Engelhardt's laboratory was performed as described before (9, 53).

Flow cytometry analysis

Extracellular and intracellular stainings were performed as previously described (13). Briefly, before intracellular cytokine staining (ICS), cells were activated for 5 hours with 1 μg/mL ionomycin and 20 ng/mL phorbol 12-myristate 13-acetate (PMA) in the presence of 2 μg/mL brefeldin A (all from Sigma). After staining for surface antigens, cells were then fixed and permeabilized in 4% (w/v) paraformaldehyde with 0.1% (w/v) saponin in HBSS for 10 minutes at room temperature before proceeding to intracellular staining. Mouse immune cells isolated from lymph nodes, spleen and CNS were labelled with the following antibodies against surface markers CD3, CD4, CD8, CD11b, CD11c, CD25, CD45, CD54/ICAM-1, CD62E (from BD Biosciences), CD6, CD44 from ebioscience, CD31/PECAM-1, CD34, CD102/ICAM-2, CD106/VCAM-1, CD146 from Biolegend, CD166/ALCAM (FAB1172P from R&D) and Ninjurin-1 (custom made from BD Biosciences). For intracellular staining of mouse cytokines, the following antibodies specific for mouse were used: IL-17, GM-CSF, TNF, IFN-γ (from BD Biosciences), GzB and perforin (from ebioscience). Intra-nuclear stainings were performed using eBioscience fixation/permeabilization kit in combination with anti-FOXP3 antibodies (eBioscience and BD Biosciences). Non-specific background staining was assessed using appropriate fluorochrome-matched isotype antibodies. Cells were processed on the same day for analysis on a BD LSR II and data analyzed using BD FACSDiva software (BD Bioscience).

Flow cytometry analysis strategy for M1-M2 monocytes/macrophage

Cells positive for CD45, CD11b, Ly6C^{hi}, IL-12 (all from BD Biosciences) and negative for CD11c, CD43 (BD Biosciences), NK1.1, IL-10 (BD Biosciences and Biolegend) and CD206 (Biolegend) were considered as M1 monocytes/macrophages or "classically activated" pro-inflammatory cells. Cells positive for CD45, CD11b, CD43, CD206, IL-10 and negative for CD11c, NK1.1, Ly6C^{hi}, IL-12, were considered as M2 monocytes/macrophages or "alternatively activated" anti-inflammatory cells.

***In vitro* T lymphocyte proliferation assay**

Using Miltenyi Biotec magnetic beads and columns, CD4+ T lymphocytes were isolated (negative selection) from the draining LNs of WT pre-symptomatic active EAE animals and CD11b+ monocytes were isolated (positive selection) from the spleen of both WT and ALCAM KO mice. T lymphocytes were labelled using the vital dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and were cultured in the presence of either WT or ALCAM KO monocytes for 4 days. The cytokine cocktail and media utilized during *in vitro* reactivation of immune cells to induce passive EAE was used. T lymphocyte proliferation and cytokine production were assessed by flow cytometry.

Immunostaining of CNS material and cell culture

Frozen sections of CNS specimens (brain and spinal cord) obtained from mice following rapid intracardiac perfusion were studied as previously described (54-56). First, EAE lesions, defined as areas of demyelination associated with intense perivascular immune cell infiltration, were identified by Luxol Fast Blue (LFB) and haematoxilin and eosin (H&E) staining. Sections adjacent to active lesions were selected for immunohistofluorescence. Following fixation in acetone for 10

minutes, sections were transferred to ethanol for 5 minutes, hydrated in PBS and blocked with 10% species specific serum of the secondary antibodies hosts. Primary antibodies diluted in 3% serum were incubated for 1h at room temperature or overnight at 4 degrees. Following washes with PBS-tween20 (0.05%), secondary antibodies were incubated 45 min at room temperature. Sections were then mounted using mowiol, containing Topro-3 (Invitrogen, 1:400) when indicated. Each experiment included negative controls (incubation with secondary antibodies alone). For immunocytofluorescence, MBECs were trypsinized and transferred to Ibidi µ-slides VI 0.1 coated with collagen IV. Once the cells reached confluency, they were fixed with 70% ethanol for 5 minutes then permeabilized with PBS-tween20 (0.05%) for 5 minutes. The subsequent staining procedures were the same as above. The following primary antibodies were used: rabbit anti-laminin (1:2000, Dako), rat anti-CD4 (1:70, BD Bioscience), rat anti-F4/80 (1:80, Biolegend), rabbit anti-occludin (1:50, Invitrogen), rabbit anti-α-catenin (1:40, Invitrogen), rabbit anti-claudin-5 (1:100, Invitrogen), goat anti-p120 (1:30, Santa Cruz), rabbit anti-ZO-1 (1:70, Invitrogen), rabbit anti-β-catenin (1:150, Invitrogen), rat anti-VE-cadherin (1:10, BD Bioscience), rat anti-PECAM-1 (1:300, BD Bioscience), rat anti-ICAM-1 (1:100, eBioscience). Fluorescence acquisition was performed using a Leica Confocal Microscope SP5 platform (Leica Microsystems). Image processing and analysis was done using Leica LAS AF and ImageJ (NIH) softwares.

Real Time -PCR

RT-PCR was performed as previously published (11). Briefly, total RNA was extracted from primary culture of WT and ALCAM KO mouse blood-brain barrier endothelial cells using the RNeasy Mini Kit and transcribed into complementary DNA using QuantiTect Reverse Transcription kit (both from Qiagen) according to the manufacturer's instructions. ALCAM and GAPDH specific primers were used for the PCR.

Immunoprecipitation

Mouse brain endothelial cells (MBECs) homogenates were prepared from P0 monolayers in T75 flasks. The cells were washed 3 times with room temperature PBS and then scrapped and transferred to 15 ml tubes in PBS. The cells were precipitated by centrifugation at 400g for 10 minutes at room temperature. The supernatant was removed and the cell pellets were homogenized with 200 µl of ice cold RIPA buffer (ThermoFisher scientific) per T75 flask by pipetting. Homogenates were transferred to 1.5 ml Eppendorf tubes and incubated for 20 minutes on ice, then centrifuged at 13000g for 10 min at 4°C to precipitate nuclei. The supernatants were collected in new pre-chilled 1.5 ml Eppendorf tubes. The protein concentration of homogenates was determined using the BCA protein assay kit (PIERCE) following the manufacturer instructions.

To immunoprecipitate ALCAM, 50 µl Dynabeads protein G (ThermoFisher scientific) were coupled to 10 µg of polyclonal goat anti-mouse ALCAM (R&D systems, AF1172) or to 10 µg of irrelevant goat IgG (R&D systems), following the manufacturer instructions. To avoid post-coupling elution of the immunoglobulin, they were covalently attached to the beads by BS3 (bis(sulfosuccinimidyl)suberate) cross-linking agent (ThermoFisher scientific) following the manufacturer instructions. 50 µl of the resulting anti-ALCAM Dynabeads or the irrelevant IgG control were incubated overnight with 200 µg of MBECs homogenates at 4°C with gentle rotation. The beads were then washed 4 times with PBS-tween20 0.05%. The supernatant was completely removed and 40 µl of 1X SDS loading buffer with 2.5 % β-mercaptoethanol were added to the beads and incubated 15 minutes at room temperature following resuspension. The beads were boiled at 95°C for 5 minutes and then centrifuged at 13000 RPM for 2 minutes. The supernatant

was collected and migrated in SDS-PAGE, transferred to PVDF membranes and immunostained with the corresponding antibodies.

Immunoblotting

Immunoblotting was performed as previously described (11, 57). Briefly, MBECs lysates or BBB blood vessel lysates were separated by standard SDS-PAGE and immunoblots were analyzed with the following antibodies: goat anti-ALCAM (0.25μg/ml, AF1172, R&D systems), rabbit anti-ZO-1 (1:125, Invitrogen), rabbit anti-ZO-2 (1:125, Invitrogen), rabbit anti-cingulin (1:500, Thermos), rabbit anti-TARA (1:500, Thermos), mouse anti-coronin 1C (1:500, abnova), mouse anti-β-actin (1:20000, Sigma), mouse anti-tropomyosin (1:500, Sigma), rabbit anti-occludin (1:250, Invitrogen), rabbit anti-ezrin (1 μg/ml, abcam) and rabbit anti-syntenin-1 (1:500, Bioss). Horseradish peroxidase–conjugated secondary antibodies (Dako) and the ECL system (Amersham Biosciences) were used to detect specific binding, and anti-β-actin (sigma) served as a loading control. Digital images obtained with the Bio-Rad Gel Doc system were used for band intensity analysis.

Trans-endothelial electrical resistance (TEER) measurement

Barrier properties of confluent monolayers formed by primary MBECs (53) from ALCAM KO or WT mice grown on filter inserts (0.4 μm pore size, 8.36 mm diameter; ThinCertTM, Greiner Bio-One, Vitaris AG, Baar, Switzerland) were assessed by impedance TEER measurements (CellZscope R, Nanoanalytics, Muenster, Germany) according to the manufacturer's instructions.

In vitro permeability of MBECs to tracer molecules

MBECs were isolated and cultured to 90% confluence in 6-wells plates. Then, using trypsin 0.25% diluted in PBS-EDTA (2mM) the cells were quickly detached and plated on gelatin/collagen IV-coated 3 μm pore size Boyden chambers at a density of $4x10^4$ cells per well. Cells reached confluency after 3-4 days, at which point the culture media was changed for DMEM media supplemented with 20% FBS. After 2 hours, fluorescein-isothiocyanate-labelled bovine serum albumin (FITC-BSA, 66.5kDa, Invitrogen) and Alexa 647-labelled dextran 10 kDa (Invitrogen) were added at 50 μg/ml to the upper chambers. Aliquots of 50 μl were separately harvested from each upper and lower chamber at 0 and 1 hour. Experimental conditions were prepared in triplicates. The permeability of the tracers was quantified with a fluorescence multimode plate reader (Biotek, Synergy 4) which allows to calculate the permeability coefficient of each fluorescent markers.

In Vivo BBB permeability

In vivo BBB permeability was assessed by measuring Cascade Blue-labelled dextran 3 KDa (Invitrogen) and Dextran-TRITC (20 KDa; Sigma) in the CNS of mice at different time-points. Mice were injected intravenously with saline 0.9% NaCl containing 1mg of dextran-Cascade Blue and 1mg of dextran-TRITC. Then, 15 minutes later, 200μl of blood was obtained by intracardiac puncture and placed in EDTA-coated blood tube (Sarstedt). Immediately after, the mice were perfused with ice-cold saline. The brains and spinal cords were then removed and placed in 1 ml of cold PBS protected from light. The CNS samples were weighed and then homogenized using syringes and decreasing size needles. The CNS and blood proteins were precipitated with 1 ml of 60% trichloroacetic acid. The precipitates were removed by centrifugation. Fluorescence was measured using a fluorescence multimode plate reader (Biotek, Synergy 4). Dextran-Cascade Blue

(excitation at 557 nm, emission at 575 nm) and dextran-TRITC (excitation at 390 nm, emission at 420 nm) was measured in supernatant samples using dark wall clear bottom Cornstar 96-wells plate. The quantity of dyes contained in the CNS tissue was expressed as a percentage of the fluorescence intensity found in the blood originating from the same animal, normalized based on the CNS weight.

Statistical analysis

Statistical analysis was performed using PRISM Graphpad^{TM} software and results are presented as the mean ± standard error of the mean (SEM). Paired or unpaired Students' *t*-test were performed when appropriate. Two-way Anova and Students' *t*-test were performed on the calculated area under the curve (AUC) using individual EAE scores. Only p values < 0.05 were considered statistically significant. ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$)

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The authors declare no conflict of interest related to this project.

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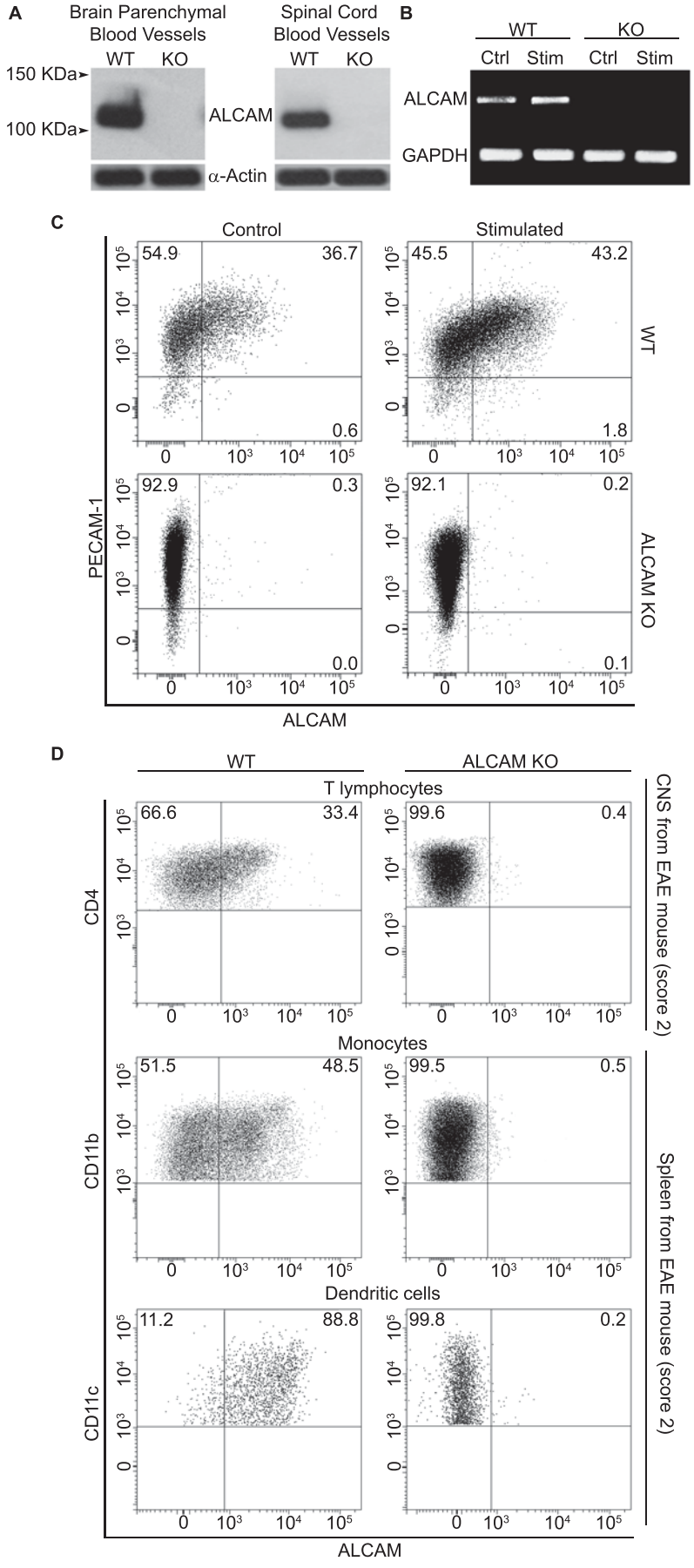

A
Brain Parenchymal Blood Vessels
Spinal Cord Blood Vessels
WT
KO
WT
KO
150 KDa
100 KDa
ALCAM
α-Actin
B
WT
KO
Ctrl
Stim
Ctrl
Stim
ALCAM
GAPDH
C
Control
Stimulated
54.9
36.7
0.6
45.5
43.2
1.8
92.9
0.3
0.0
92.1
0.2
0.1
WT
ALCAM KO
PECAM-1
ALCAM
D
WT
ALCAM KO
T lymphocytes
66.6
33.4
99.6
0.4
CD4
CNS from EAE mouse (score 2)
Monocytes
51.5
48.5
99.5
0.5
CD11b
Dendritic cells
11.2
88.8
99.8
0.2
CD11c
Spleen from EAE mouse (score 2)
ALCAM

Fig 1. Expression of ALCAM at the blood-brain barrier and on immune cells.

A Expression of ALCAM in freshly isolated blood vessels from the brain and spinal cord of WT and ALCAM KO mice, by Western blot (actin: control protein). Representative of n=5 independent experiments. **B** RT-PCR analysis of *ALCAM* mRNA obtained from primary culture of mouse blood-brain barrier endothelial cells from WT and ALCAM KO mice, either resting (Ctrl) or treated with TNF and IFNγ (Stim: Stimulated). Representative of n=2 independent experiments. **C** Expression of ALCAM and PECAM-1 on primary cultures of mouse blood-brain barrier endothelial cells obtained from WT and ALCAM KO mice, as assessed by flow cytometry. Representative of n=5 independent experiments. **D** Expression of ALCAM on *ex vivo* CD4+ T lymphocytes isolated from the central nervous system, as well as CD11b+ monocytes/macrophages and CD11b+CD11c+ dendritic cells isolated from splenocytes of ALCAM KO mice or their WT littermates, during the early symptomatic phase of active EAE. Representative of n=3 independent experiments.

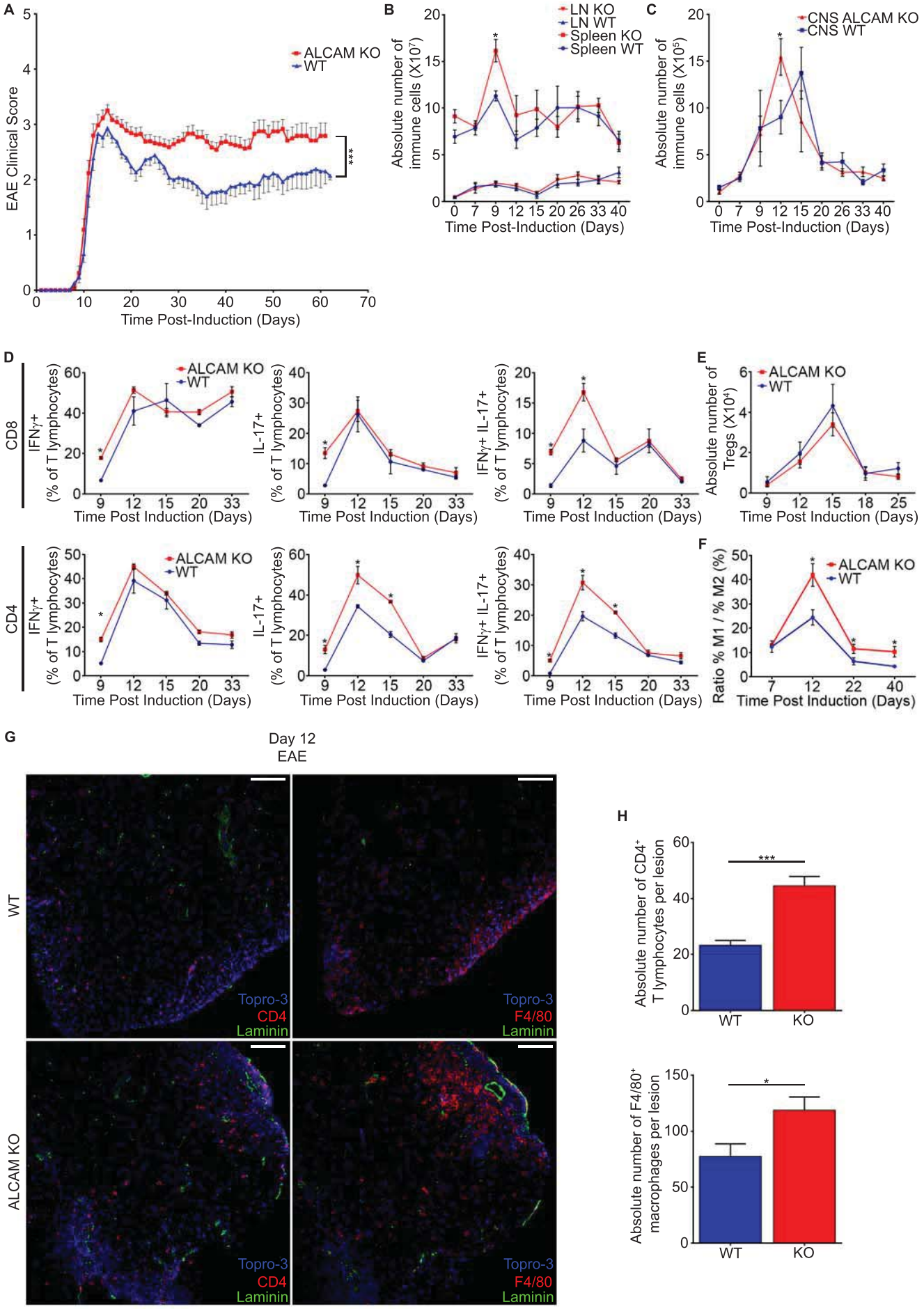

A
EAE Clinical Score
ALCAM KO
WT
***
Time Post-Induction (Days)
B
Absolute number of immune cells (X10⁷)
LN KO
LN WT
Spleen KO
Spleen WT
Time Post-Induction (Days)
C
Absolute number of immune cells (X10⁶)
CNS ALCAM KO
CNS WT
Time Post-Induction (Days)
D
CD8
CD4
IFNγ+ (% of T lymphocytes)
IL-17+ (% of T lymphocytes)
IFNγ+ IL-17+ (% of T lymphocytes)
ALCAM KO
WT
Time Post Induction (Days)
E
Absolute number of Tregs (X10⁴)
ALCAM KO
WT
Time Post Induction (Days)
F
Ratio % M1 / % M2 (%)
ALCAM KO
WT
Time Post Induction (Days)
G
Day 12 EAE
WT
ALCAM KO
Topro-3
CD4
F4/80
Laminin
H
Absolute number of CD4⁺ T lymphocytes per lesion
***
WT
KO
Absolute number of F4/80⁺ macrophages per lesion
*
WT
KO

Fig 2. ALCAM KO mice develop a more severe active EAE.

A Mean cumulative clinical EAE score from MOG_{35-55}-immunized C57BL/6 WT and ALCAM KO mice. Data shown are the mean ± SEM of 40 mice per group and representative of n=12 independent experiments. Absolute numbers of immune cells isolated from the spleens and lymph nodes (LN) **B** or from the central nervous system (CNS) **c** of WT and ALCAM KO mice at different days post induction of EAE. Data shown are the mean ± SEM of 3-12 animals per time point and representative of n=8 independent experiments. **D** Percentage of IFNγ-, IL-17- or IFNγ and IL-17-expressing CD4+ and CD8+ T lymphocytes isolated from the CNS of WT and ALCAM KO mice at different days post induction of EAE, as assessed by flow cytometry. Data shown are the mean ± SEM of 3-5 animals per time point. Representative of n=5 independent experiments. **E** Absolute numbers of CD4+CD25+FOXP3+ regulatory T lymphocytes infiltrating the CNS of ALCAM KO and WT mice at different days post induction of EAE, as assessed by flow cytometry. Data shown are the mean ± SEM of 3-6 animals per time point. Representative of n=3 independent experiments. **F** Prevalence of M1 monocyte/macrophages relative to M2 subtype isolated from the CNS of WT and ALCAM KO mice at different days post induction of EAE, as assessed by their expression of CD11b, CD43, CD206, Ly6C, IL-10, IL-12, by flow cytometry. Data shown are the mean ± SEM of 4-10 animals per time point pooled from 3 independent experiments. **G** Immunofluorescent staining of Laminin (green), TOPRO-3 (nuclei-blue) and CD4 or F4/80 (red) in spinal cord sections of ALCAM KO and WT mice at day 12 post induction of EAE. Scale bars: 100 μm. Image shown are representative of 9 sections per animal, n=4 animals per group. **H** Absolute numbers of CD4+ T lymphocytes and F4/80+ macrophages observed per lesion. n=10-15 lesions assessed from 3 animals per group. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

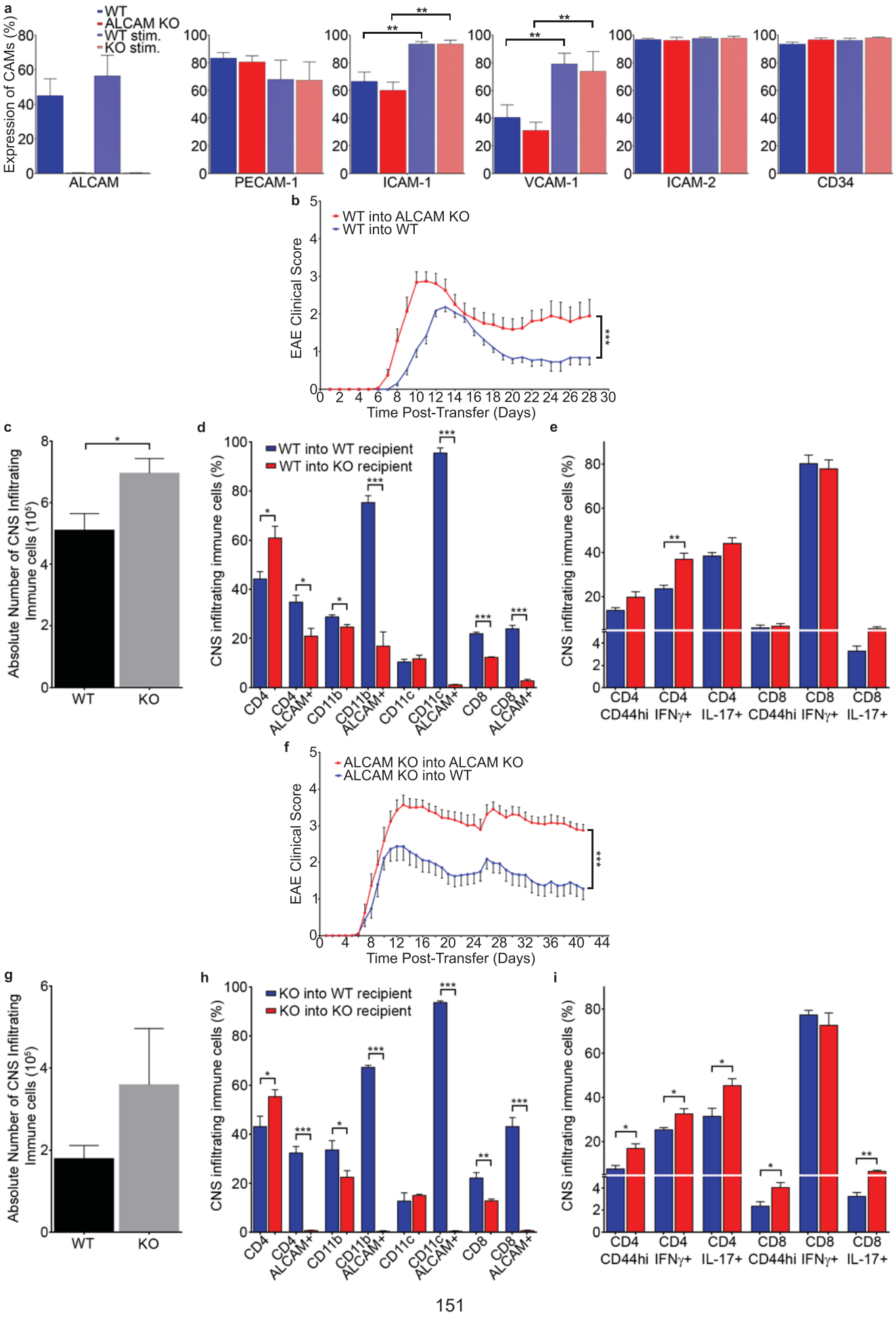

a
WT
ALCAM KO
WT stim.
KO stim.
Expression of CAMs (%)
ALCAM
PECAM-1
ICAM-1
VCAM-1
ICAM-2
CD34
b
WT into ALCAM KO
WT into WT
EAE Clinical Score
Time Post-Transfer (Days)
c
Absolute Number of CNS Infiltrating Immune cells (10⁵)
WT
KO
d
WT into WT recipient
WT into KO recipient
CNS infiltrating immune cells (%)
CD4
CD4 ALCAM+
CD11b
CD11b ALCAM+
CD11c
CD11c ALCAM+
CD8
CD8 ALCAM+
e
CNS infiltrating immune cells (%)
CD4 CD44hi
CD4 IFNγ+
CD4 IL-17+
CD8 CD44hi
CD8 IFNγ+
CD8 IL-17+
f
ALCAM KO into ALCAM KO
ALCAM KO into WT
EAE Clinical Score
Time Post-Transfer (Days)
g
Absolute Number of CNS Infiltrating Immune cells (10⁵)
WT
KO
h
KO into WT recipient
KO into KO recipient
CNS infiltrating immune cells (%)
CD4
CD4 ALCAM+
CD11b
CD11b ALCAM+
CD11c
CD11c ALCAM+
CD8
CD8 ALCAM+
i
CNS infiltrating immune cells (%)
CD4 CD44hi
CD4 IFNγ+
CD4 IL-17+
CD8 CD44hi
CD8 IFNγ+
CD8 IL-17+

Fig 3. The absence of ALCAM on BBB-ECs increases EAE disease severity.

A Expression of cell adhesion molecules (ALCAM, PECAM-1, ICAM-1, VCAM-1, ICAM-2 and CD34) on primary culture of MBECs under resting or stimulated (stim.; TNF and IFNγ) conditions, isolated from WT and ALCAM KO mice, as assessed by flow cytometry. n=4 independent experiments using 4 primary cultures. **B** Mean cumulative EAE clinical score of WT and ALCAM KO mice adoptively transferred with MOG_{35-55}-reactivated WT splenocytes. Data shown are representative of n=3 independent experiments, 20 animals per group. **C-E** Characterization of immune cells infiltrating the CNS of WT or ALCAM KO animals in the adoptive transfer EAE experiment, at 12 days post-transfer (shown in **B**). **C** Absolute numbers of immune cells isolated from the CNS of recipient mice. **D-E** Percentage of CNS infiltrating immune cells expressing the surface markers CD4, CD11b, CD11c and CD8 and percentage of ALCAM+, CD44hi, IFNγ+ and IL-17+ cells gated on the previous surface markers. Data shown are the mean ± SEM of 3-4 animals per group, and representative of 3 transfer experiments. **F** Mean cumulative EAE clinical score of WT and ALCAM KO mice adoptively transferred with MOG_{35-55}-reactivated ALCAM KO splenocytes. Data shown are representative of n=3 independent experiments, 26 WT and 20 ALCAM KO mice per group. **G-I** Characterization of immune cells infiltrating the CNS of WT or ALCAM KO animals in the adoptive transfer EAE experiment, at 12 days post-transfer (shown in **F**). **G** Absolute numbers of immune cells isolated from the CNS of recipient mice. **H and I** Percentage of CNS infiltrating immune cells expressing the surface markers CD4, CD11b, CD11c and CD8 and percentage of ALCAM+, CD44hi, IFNγ+ and IL-17+ cells gated on the previous surface markers. Data shown are the mean ± SEM of 4 animals per group, and representative of 3 transfer experiments. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

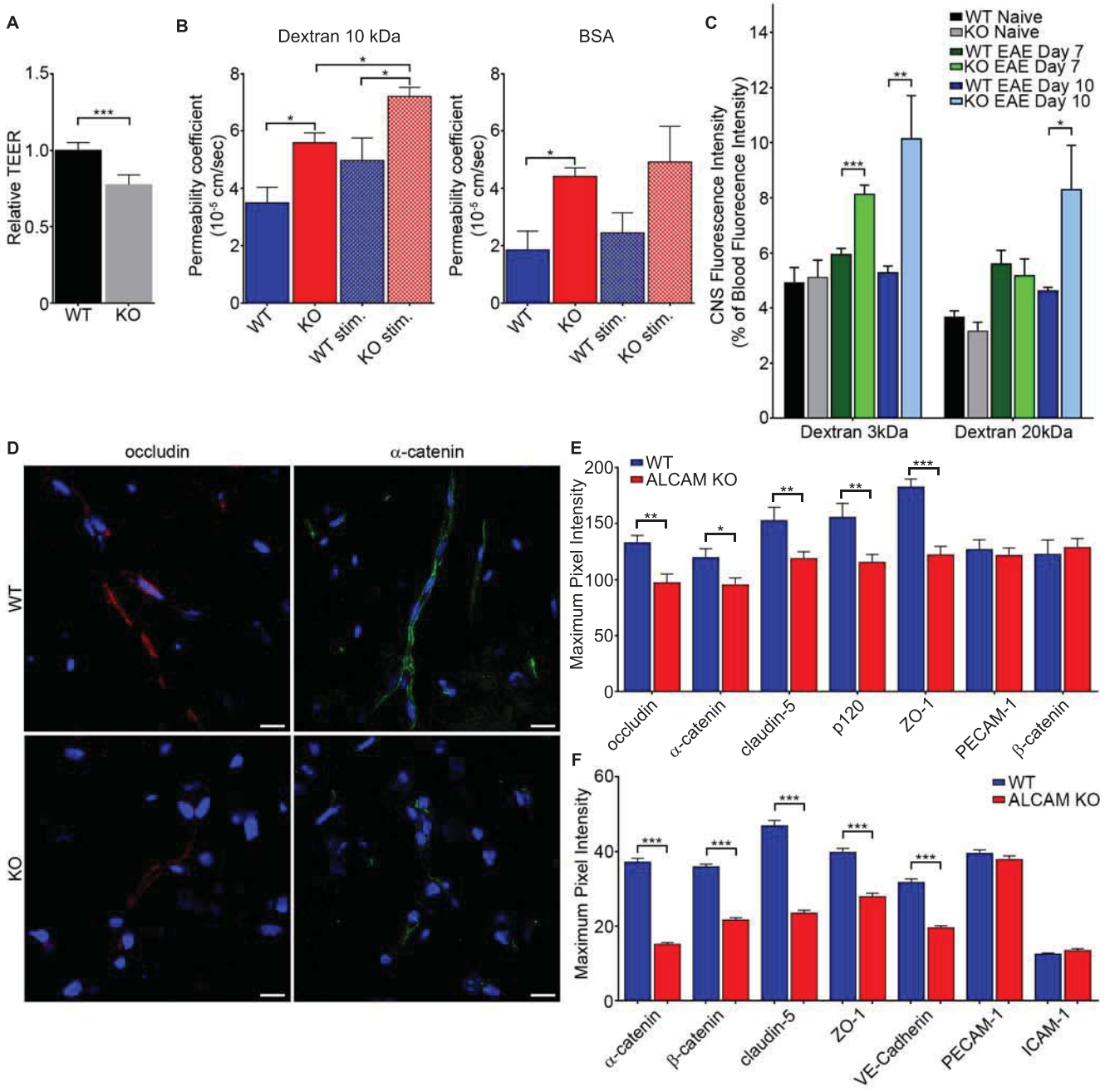

A
Relative TEER
1.5
1.0
0.5
0
***
WT
KO
B
Dextran 10 kDa
BSA
Permeability coefficient (10^{-5} cm/sec)
8
6
4
2
0
*
WT
KO
WT stim.
KO stim.
C
WT Naive
KO Naive
WT EAE Day 7
KO EAE Day 7
WT EAE Day 10
KO EAE Day 10
CNS Fluorescence Intensity (% of Blood Fluorescence Intensity)
14
12
10
8
6
4
2
0
**
***
Dextran 3kDa
Dextran 20kDa
D
occludin
α-catenin
WT
KO
E
WT
ALCAM KO
Maximum Pixel Intensity
200
150
100
50
0
occludin
α-catenin
claudin-5
p120
ZO-1
PECAM-1
β-catenin
F
WT
ALCAM KO
Maximum Pixel Intensity
60
40
20
0
α-catenin
β-catenin
claudin-5
ZO-1
VE-Cadherin
PECAM-1
ICAM-1

Fig 4. ALCAM KO mice exhibit disorganized tight junction molecules which translates into an increase in trans-endothelial cell permeability.

A TEER values of confluent monolayers of pMBMECs isolated from WT or ALCAM KO mice, expressed relative to WT values (1.0). Data shown are the mean ± SEM of 7 independent experiments performed in triplicates. **B** Permeability coefficient of 10 kDa dextran and bovine serum albumin (BSA) across monolayers of MBECs, *in vitro*, from WT or ALCAM KO mice, either untreated or treated with TNF and IFNγ (stim.). Data shown are the mean ± SEM of 3-4 replicates per conditions and representative of n=3 independent experiments. **C** *in vivo* BBB permeability using i.v. injected fluorescently-labelled 3 and 20 kDa dextran, at different time point during EAE, in WT and ALCAM KO mice. Data expressed as a percentage of blood fluorescence intensity and measured by spectrofluorometer. Data shown are the mean ± SEM of 5-15 replicates per conditions pooled from n=3 independent experiments. **D** Immunofluorescent staining of α-catenin (green) and occludin (red) in spinal cord sections of naïve ALCAM KO and WT mice. Nuclei: blue. Scale bars: 20 µm. Representative of 5 sections per animal, n=4 animals per group. **E** Maximum pixel intensity analysis of junctional molecules in naïve ALCAM KO and WT spinal cord sections, as assessed by confocal microscopy. n=25-65 blood vessels per group. **F** Maximum pixel intensity analysis of junctional molecules in primary culture of mouse BBB-ECs. n=160-260 cell junctions per group. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

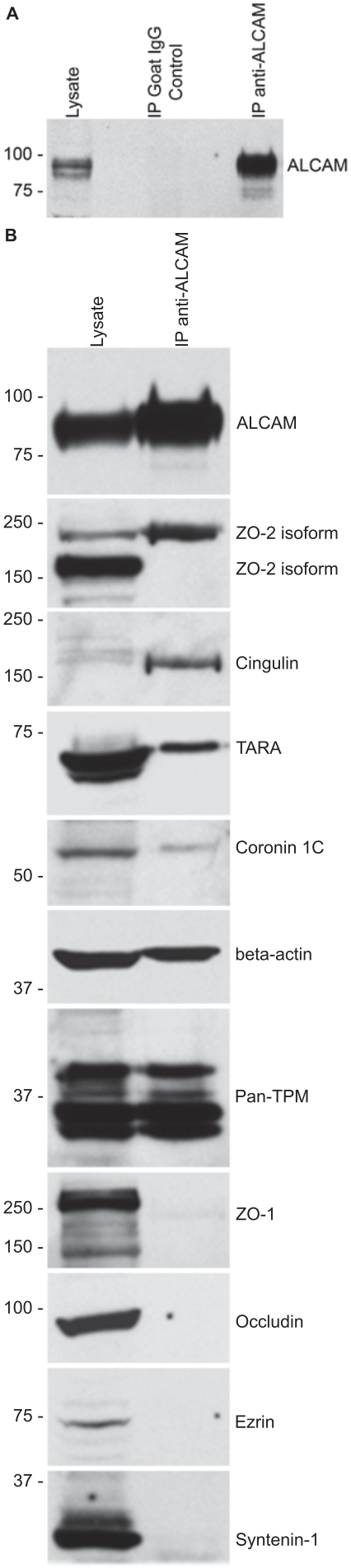
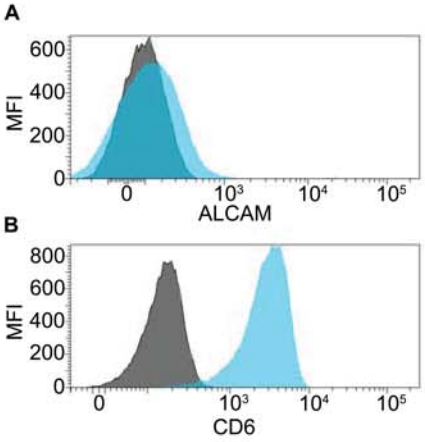

A
Lysate
IP Goat IgG Control
IP anti-ALCAM
100 -
75 -
ALCAM
B
Lysate
IP anti-ALCAM
100 -
75 -
ALCAM
250 -
150 -
ZO-2 isoform
ZO-2 isoform
250 -
150 -
Cingulin
75 -
TARA
50 -
Coronin 1C
37 -
beta-actin
37 -
Pan-TPM
250 -
150 -
ZO-1
100 -
Occludin
75 -
Ezrin
37 -
Syntenin-1

Fig 5. ALCAM binds directly and indirectly to tight junction molecules.

A Expression of ALCAM protein in whole-cell lysate from MBECs and in the ALCAM immunoprecipitation (pull down) sample, by Western blot. **B** Immunoblot for ALCAM (90-120 kDa), zonula occludens-2 (ZO-2; isoforms ± 160 kDa), cingulin (160 kDa), TRIO and F-actin binding protein (TARA; 68kDa), coronin 1C (57 kDa), β-actin (42 kDa), pan-tropomyosin (TPM; isoforms ± 33 kDa), zonula occludens-1 (ZO-1; 240-260 kDa), occludin (65-80 kDa), ezrin (69 kDa) and syntenin-1 (33 kDa) on the ALCAM pull-down lysate (right) and the corresponding MBECs total cell lysate (left).


A
600
400
200
0
MFI
0
10³
10⁴
10⁵
ALCAM
B
800
600
400
200
0
MFI
0
10³
10⁴
10⁵
CD6

Supplemental figure 1. Expression of ALCAM and CD6 on resting memory CD4+ T lymphocytes.

Mean fluorescence intensity of **A** ALCAM (blue) or **B** CD6 (blue) and their respective isotype control (grey) on CD4+ T lymphocytes isolated from naïve WT splenocytes.

Discussion

MS is a chronic debilitating disease that affects an important portion of the Canadian population. While the cause of the disease is still unknown, we know that the main pro-inflammatory agent, source of demyelination and clinical symptoms, is the immune system. Infiltrating T effector lymphocytes, pro-inflammatory myeloid cells and B lymphocytes have all been associated with neuroinflammation. In MS, the specialized vasculature forming the BBB, which normally plays a key function in regulating the CNS environment, becomes dysregulated and allows the passage of those encephalitogenic immune cells [6]. Thus, the BBB is an interesting therapeutic target to limit the migration of leukocytes and control MS relapses. In this context, this Ph.D. project aimed to study in detail the role of ALCAM in neuroinflammation and to understand the molecular mechanisms behind immune cell diapedesis.

The Role of ALCAM During Leukocytes Diapedesis

As discussed previously, therapeutic treatments targeting immune cell trafficking (Fingolimod, Natalizumab) are currently used successfully to prevent MS relapses. Unfortunately, both drugs create an immunosuppressed environment, which favours the development of PMLs. To find alternate adhesion molecules involved in immune cell transmigration, proteomic analyses of purified lymphocytes and BBB-ECs were performed [112, 113, 491]. ALCAM was one of the top candidates being flagged as highly upregulated on inflamed BBB-ECs. While the role of ALCAM as a leukocyte adhesion molecule was not known at the time, multiple studies had associated it with cell migration and adhesion during neurogenesis, angiogenesis, blastocyst implantation and tumor invasion [492, 493]. In the study published by Cayrol et al. in 2008 [113], our laboratory established that ALCAM is involved during *ex vivo* migration of monocytes, CD4 T lymphocytes and B lymphocytes, but not CD8 T lymphocytes. The goal of this work was thus to describe in more detail the function of ALCAM during leukocyte transmigration into the CNS. To this end, we analyzed the

extravasation of human and mouse Th1 and Th17 lymphocytes along with *ex vivo* CD14+ monocytes using, amongst other things, a dynamic migration assay under shear-stress. To adequately compare the results obtained following ALCAM blockade, we also conducted the experiments using anti-ICAM-1 antibodies. ICAM-1 is a well-characterized adhesion molecule involved during the adhesion, crawling and diapedesis steps of leukocytes transmigration across endothelial cells [115, 494].

In this study, we first confirmed that ALCAM is involved during static diapedesis of *ex vivo* human CD4+ T lymphocytes. We felt this confirmation was necessary as another study recently failed to block the migration of CD3+ T lymphocytes in an *in vitro* model of the human BBB [488]. However, the differences between our models might explain this discrepancy. In addition, the presence of CD8+ T lymphocytes, which are not blocked by anti-ALCAM antibodies, may have skewed their results. As opposed to *ex vivo* lymphocytes, we could not block the migration of *in vitro* polarized human Th17 lymphocytes, even if they expressed high levels of CD6. Interestingly, a small reduction in the migration of Th1 lymphocytes could be measured. One difference observed between those two immune subsets was their respective levels of ALCAM, with Th1 lymphocytes having slightly more of the adhesion molecule on their surface. The hypothesis that the homotypic ALCAM-ALCAM interaction is important during migration and less so the heterotypic ALCAM-CD6 bond, pushed us to re-evaluate the role of ALCAM during pro-inflammatory T lymphocyte trafficking. However, using sorted human Th1 lymphocytes, which expressed higher levels of ALCAM, we found similar results as with *in vitro* reactivated Th1. Therefore, we concluded that antibody-mediated ALCAM blockade only plays a limited role in human activated pro-inflammatory T lymphocytes extravasation across the BBB. Equivalent results were also obtained by comparing ALCAM KO and WT BBB-ECs during mouse Th1 lymphocyte transmigration. Our data also suggested that mouse BBB-ECs express lower levels of ALCAM, as compared to human primary culture BBB-ECs. Nonetheless, we confirmed that ALCAM plays an important role in the transmigration of monocytes. Furthermore, we discovered that it is involved specifically during rolling, shear resistant arrest and diapedesis. Finally, we demonstrated that ALCAM is over-expressed on meningeal ECs as compared to BBB-ECs and that both CD4+ T lymphocytes and monocytes migrate more rapidly and in higher number across the former CNS barrier.

Conceivably, higher expression levels of ALCAM on monocytes (which 60 to 85% of them constitutively express ALCAM) versus T lymphocytes might explain the different cell behaviours observed in ALCAM blocking experiments. Supporting this theory, a recent study demonstrated that ALCAM is overexpressed on HTLV-1-infected T lymphocytes and that as a result, it increases their migration across the human BBB [495]. Similarly, the difference observed during the migration of ex vivo naïve/inactivated CD4+ T lymphocytes and polarized Th1/Th17 lymphocytes could originate from the presence of additional highly effective adhesion molecules on the activated immune cell subsets compensating the lack of ALCAM in ALCAM KO mouse or the steric hindrance of ALCAM in human cell cultures.

Nevertheless, the fact that our results suggest that only APCs (myeloid cells and B lymphocytes; recent interesting unpublished data on B lymphocytes are corroborating the preliminary results published in Cayrol et al. [113]) are significantly relying on ALCAM to cross the BBB could provide a therapeutic advantage. These results demonstrate that an ALCAM blocking therapy could potentially specifically block myeloid cells from entering the CNS during neuroinflammation, while not compromising the immune-surveillance of the CNS by T lymphocytes. Although MS and EAE are arguably lymphocyte-driven diseases, the contribution of APCs to their pathogenesis is undeniable. For instance, myeloid cells have been shown to accumulate in abundance in demyelinating lesions, where they often represent the dominant cell type. We have also previously published a study demonstrating that Ninjurin-1, an adhesion molecule predominantly expressed on BBB-ECs and myeloid cells, contributes significantly to monocyte transmigration. When blocked, significantly fewer monocytes were found in the CNS of EAE animals, which lead to the amelioration of their clinical scores [118]. In addition, another study from the laboratory demonstrated that these peripheral monocytes are the main source of DCs in an inflamed CNS [134]. These pro-inflammatory DCs, present in both MS and EAE, have been demonstrated to be essential during lesion formation as they can polarize and reactivate CD4+ T lymphocytes, while promoting their proliferation [134, 496-498].

Further evidences demonstrating the importance of monocytes in EAE pathogenesis have been published recently. While GM-CSF KO mice are resistant to EAE, repeated injections of

recombinant GM-CSF were shown to re-establish their susceptibility via the recruitment of CD11b+Ly6C+ monocytes to the CNS [232, 233, 499]. Corroborating with these results, another study from the group of Dr. Becher demonstrated that the principal GM-CSF responders are the monocytes, while the cytokine has little effect on microglia. In addition, GM-CSF stimulation induces the production of IL-23 by monocytes, which in turn allows the polarization of CD4+ T lymphocytes into Th17 pro-inflammatory lymphocytes [231]. A follow-up study has demonstrated that deleting the GM-CSF receptor on $CCR2^{+}Ly6C^{hi}$ monocytes prevented the induction of EAE. In addition, they demonstrated that these cells produce CCL6, CCL24, and CCL17, which are known to be critical for the recruit of more myeloid cells, effectively creating an immune amplification loop during neuroinflammation. Finally, these monocytes are also known to secrete high levels of IL-1β, which modulate the BBB integrity and upregulate the expression of adhesion molecules [234, 500, 501].

Collectively, the results put forward in this first article suggest that anti-ALCAM antibodies could be an interesting therapy against MS. Besides, positive effects on EAE clinical scores have already been published in Cayrol et al. [113]. The same experiments have also demonstrated the absence of negative effect of the anti-ALCAM antibodies on the integrity of the BBB and showed no deleterious effects in the animals over multiple injections. Furthermore, based on the positive results obtained from the clinical trials of ocrelizumab, an anti-CD20 monoclonal therapy aimed at depleting B lymphocytes, for both RRMS and PPMS patients, it is conceivable that a non-T lymphocyte-related therapy be used against MS [502]. As discussed previously, although B lymphocytes play a role during disease pathogenesis by producing antibodies against CNS molecules, they also act as APCs by presenting antigens and secreting pro-inflammatory cytokines or anti-inflammatory cytokines during disease remission. While blocking disease promoting B lymphocytes is beneficial, an anti-ALCAM therapy would affect equally the regulatory B lymphocytes. Nevertheless, positive clinical outcomes of B lymphocyte depletion therapies suggest that blocking ALCAM would not impede disease remission. In fact, an increase in the percentage of CD27- B lymphocytes, which demonstrate superior capacity to produce IL-10, was observed 12 months following B lymphocyte depletion in MS patients [503]. Therefore, an ALCAM-specific therapy would in theory target all APCs (myeloid cells and B lymphocytes) and have a broad positive impact on the disease process. Especially, as

myeloid cells have been proposed to be directly and indirectly implicated in neuronal damage [299]. It would also be an important addition to the therapeutic regiment available nowadays as it would not target T lymphocytes and thus, may not be plagued by the increased risk of PMLs.

The Novel Role of ALCAM in Maintaining BBB Integrity

Although the first manuscript established precisely the role of ALCAM during leukocyte diapedesis, we were interested in seeing if those *in vitro* results would translate in an *in vivo* model of MS. Therefore, this second section of my Ph.D. project was originally aimed at understanding the role of ALCAM during neuroinflammation in the EAE model. The mechanisms underlying the complex interactions between leukocytes and the BBB would have been studied and confirmed by using transgenic animals. However, it became clear following the first few active EAE experiments that, as opposed to our initial hypothesis, ALCAM KO mice were not protected from the disease, but rather developed more severe symptoms accompanied by an increase in CNS infiltrating pro-inflammatory immune cells. Based on our published EAE results using anti-ALCAM antibodies [113] and on our new *in vitro* detailed analysis of ALCAM functions during leukocyte transmigration, we proposed that the phenotype observed was due to an upregulation of other adhesion molecules as a compensatory mechanism. Yet, we quickly refuted that theory following the analysis of the expression levels of multiple adhesion molecules using flow cytometry. We also confirmed that there was no other difference in the peripheral immune system of ALCAM KO mice (number and ratio of immune cells, antigen presentation, secretion of cytokines and chemokines, etc.), as compared to their WT littermates, that could have accounted for the results observed. Furthermore, a study conducted by the group of Dr. Weiner, the creator of the ALCAM KO strain, described the animals as appearing normal, with no apparent phenotypes or defects. The mice also bred normally and their offspring matched closely the development rate of WT pups. Upon further characterization of the animals, however, they discovered that the mice have defects in the fasciculation of retinal ganglion cells and prominent retinal dysplastic regions were observed [504]. Nevertheless, these defects do not seem to affect the animals and does not have, to the best of my knowledge, a direct influence over the course of EAE.

Using adoptive EAE transfer, in which WT leukocytes are injected into ALCAM KO or WT mice, we confirmed that the lack of ALCAM only in the recipient animals recapitulates the phenotype observed in active EAE. We thus hypothesized that the absence of ALCAM at the level of the CNS microvasculature is causing the increased EAE clinical score observed in ALCAM KO mice. Phenotypic characterization of un-immunized ALCAM KO mice revealed a reduced expression of BBB junctional proteins, which was associated with a significant increase in the permeability of the BBB of these animals. A detailed molecular analysis of ALCAM intracellular binding partners in primary cultures of mouse BBB-ECs demonstrated that the TJ adaptor molecules ZO-2 and cingulin are linked to ALCAM cytoplasmic tail. In addition, actin-binding proteins TARA and coronin 1C, along with the presence of β-actin and tropomyosin proteins in the ALCAM pulled-down protein lysate, confirmed previously published studies and provided further evidence demonstrating that ALCAM is directly linked to the actin cytoskeleton [477, 479]. Conversely, transmembrane TJ and AJ molecules, such as occludin, JAM-A, claudin-5 and VE-cadherin, were not found to be linked to ALCAM. These results suggest that ALCAM may interact with junctional molecule complexes only via TJ adaptor molecules.

A recent study published by Tudor, C. and colleagues has identified ezrin and syntenin-1 as two intracellular molecules binding to ALCAM [505]. Although our experiment did not confirm their findings, the differences observed between our results could be explained by their use of a cell line transfected with modified ALCAM proteins, as opposed to endogenous ALCAM obtained from primary mouse BBB-ECs. Interestingly, although the cytoplasmic tail of ALCAM contains clusters of positively charged amino acid residues that typically interact with members of the ezrin/radixin/moesin (ERM) family, a study performed with pulmonary microvascular endothelial cells (PMVECs) found no association between ALCAM and ERM protein family members, corroborating our results [506]. Thus, the experiments performed by Tudor, C. et al., which were designed to assess ALCAM intracellular binding partner in a cell line of human APCs, might not be relevant to mouse endothelial cells.

While this study did not support our results, other studies reported functional links between ALCAM and junctional molecules in a variety of cell types. In 2000, Tomita and colleagues

demonstrated that, in an α-catenin-deficient prostate cancer cell line, ALCAM expression is limited to the cytoplasm. However, upon transfection with α-neuronal-catenin, ALCAM is co-localized with the transfected protein at the level of the cell membrane [507]. As catenin molecules are known to interact with cadherins and the actin cytoskeleton, this study suggests that ALCAM is part of the macromolecular complexes forming cell-cell contact in prostate cancer cells. Another study published by the group of Dr. Weiner described similar protein bonds in uveal melanoma cell lines. They demonstrated that, when ALCAM is overexpressed, both N-cadherin and β-catenin localization to cell contacts is enhanced along with ALCAM, whereas silencing ALCAM results in defective adherens junctions [508].

In PMVECs, another study reported the co-localization of ALCAM with N- and VE-cadherin via confocal microscopy, while immunoprecipitation assays identified N-cadherin, VE-cadherin and β-catenin as ALCAM binding partners [506]. Strangely, ALCAM, N-cadherin and E-cadherin were isolated from the lipid rafts of PMVECs, but VE-cadherin was only detected in the bulk plasma membrane fractions, contradicting previous findings [509]. Similar results were also obtained from our laboratory using primary cultures of human BBB-ECs [113]. The localization of these proteins to lipid raft is consistent with the current literature which associates these cell membrane microdomains with the formation of complex macromolecular structures involved in immune cell transmigration (selectins, ICAM-1, VCAM-1, etc.), intercellular junction formation, cytoskeleton anchoring and intracellular signalling pathways [510-512]. Although these studies do not confirm the results obtained in the second manuscript, they do however collectively provide further evidence that ALCAM is linked to junctional molecules and the cytoskeleton.

Studies also suggest that ALCAM is linked to other cell membrane protein either in trans or in cis. Gilsanz, A. et al. [513] have recently reported that ALCAM, along with other Ig-like adhesion molecules, forms a complex with the tetraspanin molecule CD9 and the “sheddase” ADAM17, which together regulate ALCAM/sALCAM expression and activity in a TGFβ-dependent or -independent fashion [514, 515]. CD9, which acts as a membrane chaperone molecule, is also known to interact directly with claudins in a cholesterol-sensitive manner [516]. Thus, this ALCAM-CD9 bond provides a secondary indirect link between claudins and

ALCAM. Though CD9 is highly expressed on endothelial cells [517], we did not detect it in the ALCAM immunoprecipitation assay. Technical differences in the protocols used (detergent, cell type, antibody used, etc.) and different stimulation states of endothelial cells could explain this discrepancy. Indeed, since CD9 is known to enhance ALCAM-mediated adhesion by promoting its clustering on the cell surface, perhaps a CD9-ALCAM binding would have been detected in stimulated BBB-ECs [513].

Although the identified deficiency in the BBB integrity of ALCAM KO mice is a viable explanation to the worsening of EAE clinical score, EAE remains a complex disease affected by multiple biomolecular mechanisms. As such, other factors overlooked could be modified in ALCAM KO mice and participate on different levels to the disease pathogenesis. RAGE (receptor for advanced glycation end-products) could be one of those molecules. It is a close structural and functional homolog of ALCAM and it was recently identified by von Bauer and colleagues to be upregulated in ALCAM deficient animals, potentially as a compensatory mechanism [518]. RAGE also shares common features with TLRs as it has been described to bind to danger-associated molecular patterns (DAMPs), such as S100 proteins and HMGB-1, released by cells upon cellular stress and necrosis. As such, RAGE, which can be upregulated in many tissues by inflammatory mediators, is involved in propagating acute and chronic inflammation and leads to perpetuated cell activation [519-522]. The recent study by von Bauer et al. also identified S100B as a ligand of both RAGE and ALCAM, which induces a dose- and time-dependent expression of members of the NF-κB family [518]. Moreover, other studies demonstrated that in the CNS of EAE animals, RAGE is upregulated on infiltrating macrophages and CD4+ T lymphocytes, as well as on BBB-ECs, while S100 proteins are over-expressed in the spinal cord tissue. Furthermore, the administration of soluble RAGE, acting as a molecular decoy, or anti-RAGE antibody significantly reduces EAE symptoms and leukocyte infiltration in the CNS [523, 524]. Expression of dominant negative RAGE (a mutant form of RAGE without the cytosolic domain and lacking the capacity of signal transduction) selectively in CD4+ T lymphocytes also reduced the inflammatory reaction in EAE [523]. Corroborating with these results, EAE symptoms were shown to be significantly increased in mice overexpressing RAGE in a Tie2-dependent manner [525]. Another study suggesting that RAGE is expressed by oligodendrocytes in response to oxidative stress provides further evidence of the

implication of RAGE in EAE and MS [526]. Taken together, these studies link RAGE and ALCAM to leukocyte transmigration across the BBB and reveal important mechanisms contributing to neuroinflammation. Overall, the results described in the second manuscript demonstrate that ALCAM is associated with junctional molecules and thus participate in the modulation of intercellular connexions between BBB-ECs.

Future Perspective

Multiple genetic studies have identified ALCAM and CD6 gene polymorphisms as risk factors in MS, increasing the odds of developing the disease by 1.18 to 2.34 [358-360, 527]. Thus, these studies provide further evidences of the implication of ALCAM and CD6 in MS pathogenesis. On the other hand, since ALCAM is expressed in multiple cell types (endothelial cells, immune cells, epithelial cells, neurons, tumours, stem cells, etc.), it would be essential to characterize and understand the different physiological functions it partakes in both homeostatic and pathological conditions before considering therapeutic ALCAM modulation in MS patients. Case in point, the deleterious effects of ALCAM deletion in the pathogenesis of EAE. While the two manuscripts of this thesis clarify the specific role of ALCAM during immune cell trafficking and identify novel intracellular binding partners, the exact signaling pathways downstream of ALCAM are still unknown. Therefore, additional biomolecular studies would be needed to identify all molecules interacting with ALCAM in BBB-ECs and leukocytes. These studies would also potentially help define the players involved in paracellular and transcellular diapedesis and decipher their respective mechanisms. Likewise, it would be important to understand specifically why blocking ALCAM doesn't affect more the transmigration of T lymphocytes even if they express CD6 and ALCAM. Again this would require a thorough understanding of the binding partners of ALCAM as multiple molecules both intracellular and extracellular might be involved in regulating its avidity. As previously mentioned, other adhesion molecules and integrins could also overshadow the role ALCAM is playing during T lymphocyte diapedesis. A recent study from the group of Dr. Wiendl has provided further evidences for this theory by concluding that VLA-4-blockade alone did not affect significantly the adhesion of MCAM+ Th17 T lymphocytes, but the combination of anti-MCAM and anti-

VLA-4 did [528]. One could therefore argue for the existence of inherent redundancies in the molecular players involved during immune cell transmigration. As multiple important cellular systems are known to have redundant pathways, it would make sense for a mechanism involved in the protection of the organism from external pathogens to possess fail-safes. Mapping the macromolecular cell surface complexes would be essential not only in MS but also in other pathologies where ALCAM has been described. This is the case for most cancers, where understanding the exact role of ALCAM in tumour progression and metastasis would be necessary before modulating ALCAM in an effort to diminish the infiltration of deleterious Tregs and increase pro-inflammatory anti-tumor immune cell recruitment. Likewise, therapeutic modulation of ALCAM in HIV+ patients could prevent the infection of the CNS by the virus or help control the inflammatory reaction in HTLV-1 infected patients.

Conclusion

Although the etiology and the physiopathology of MS is still not fully understood, evidences point to a critical role fulfilled by CNS infiltrating pro-inflammatory leukocytes during lesion formation. The research experiments conducted as part of this thesis have provided new evidences of the implication of ALCAM during this process. The first hypothesis of this project was that ALCAM plays a role in the firm adhesion, crawling and diapedesis of encephalitogenic pro-inflammatory leukocytes across BBB-ECs. Studies presented in this thesis have confirmed the hypothesis on *ex vivo* human CD14+ monocytes. However, experiments using a conventional static migration assay or the modified adhesion assay under shear-stress have demonstrated the relative absence of effect of anti-ALCAM antibodies on the T lymphocyte transmigration cascade. The second hypothesis was that the absence of ALCAM on BBB-ECs and leukocytes significantly diminishes the severity of EAE symptoms, while reducing the absolute number of leukocytes infiltrating the CNS following immunization. Our initial active EAE experiments rapidly disproved this theory. Collectively, our data have demonstrated that ALCAM KO mice develop a more severe active EAE, due at least in part to an increase in the permeability of their BBB. This loss in BBB integrity is the result of a dysregulation of junctional molecules triggered by the absence of ALCAM, which was shown to directly and indirectly associate with TJ molecules and the cell cytoskeleton. Thus, ALCAM regulates and maintains tight junction stability by acting as an adaptor molecule. Overall, these results confirm the importance of the BBB in preserving the environment of the CNS and suggest that ALCAM could be targeted by disease-modifying therapies to limit peripheral monocytes from participating in lesion formation in MS patients.

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