Université de Montréal

Régulation moléculaire de la barrière hémo-encéphalique

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Résumé

La Sclérose en plaques (SEP) est une maladie auto-immune inflammatoire démyélinisante du système nerveux central (SNC), lors de laquelle des cellules inflammatoires du sang périphérique infiltrent le SNC pour y causer des dommages cellulaires. Dans ces réactions neuroinflammatoires, les cellules immunitaires traversent le système vasculaire du SNC, la barrière hémo-encéphalique (BHE), pour avoir accès au SNC et s'y accumuler. La BHE est donc la première entité que rencontrent les cellules inflammatoires du sang lors de leur migration au cerveau. Ceci lui confère un potentiel thérapeutique important pour influencer l'infiltration de cellules du sang vers le cerveau, et ainsi limiter les réactions neuroinflammatoires. En effet, les interactions entre les cellules immunitaires et les parois vasculaires sont encore mal comprises, car elles sont nombreuses et complexes. Différents mécanismes pouvant influencer la perméabilité de la BHE aux cellules immunitaires ont été décrits, et représentent aujourd'hui des cibles potentielles pour le contrôle des réactions neuro-immunes. Cette thèse a pour objectif de décrire de nouveaux mécanismes moléculaires opérant au niveau de la BHE qui interviennent dans les réactions neuroinflammatoires et qui ont un potentiel thérapeutique pour influencer les interactions neuro-immunologiques.

Ce travail de doctorat est séparé en trois sections. La première section décrit la caractérisation du rôle de l'angiotensine II dans la régulation de la perméabilité de la BHE. La seconde section identifie et caractérise la fonction d'une nouvelle molécule d'adhérence de la BHE, ALCAM, dans la transmigration de cellules inflammatoires du sang vers le SNC. La troisième section traite des propriétés sécrétoires de la BHE et du rôle de la chimiokine MCP-1 dans les interactions entre la BHE et les cellules souches.

Dans un premier temps, nous démontrons l'importance de l'angiotensinogène (AGT) dans la régulation de la perméabilité de la BHE. L'AGT est sécrété par les astrocytes et métabolisé en angiotensine II pour pouvoir agir au niveau des CE de la BHE à travers le

récepteur à l'angiotensine II, AT1 et AT2. Au niveau de la BHE, l'angiotensine II entraîne la phosphorylation et l'enrichissement de l'occludine au sein de radeaux lipidiques, un phénomène associé à l'augmentation de l'étanchéité de la BHE. De plus, dans les lésions de SEP, on retrouve une diminution de l'expression de l'AGT et de l'occludine. Ceci est relié à nos observations *in vitro,* qui démontrent que des cytokines pro-inflammatoires limitent la sécrétion de l'AGT. Cette étude élucide un nouveau mécanisme par lequel les astrocytes influencent et augmentent l'étanchéité de la BHE, et implique une dysfonction de ce mécanisme dans les lésions de la SEP où s'accumulent les cellules inflammatoires.

Dans un deuxième temps, les techniques établies dans la première section ont été utilisées afin d'identifier les protéines de la BHE qui s'accumulent dans les radeaux lipidiques. En utilisant une technique de protéomique nous avons identifié ALCAM (Activated Leukocyte Cell Adhesion Molecule) comme une protéine membranaire exprimée par les CE de la BHE. ALCAM se comporte comme une molécule d'adhérence typique. En effet, ALCAM permet la liaison entre les cellules du sang et la paroi vasculaire, via des interactions homotypiques (ALCAM-ALCAM pour les monocytes) ou hétérotypiques (ALCAM-CD6 pour les lymphocytes). Les cytokines inflammatoires augmentent le niveau d'expression d'ALCAM par la BHE, ce qui permet un recrutement local de cellules inflammatoires. Enfin, l'inhibition des interactions ALCAM-ALCAM et ALCAM-CD6 limite la transmigration des cellules inflammatoires (monocytes et cellules T CD4+) à travers la BHE *in vitro* et *in vivo* dans un modèle murin de la SEP. Cette deuxième partie identifie ALCAM comme une cible potentielle pour influencer la transmigration de cellules inflammatoires vers le cerveau.

Dans un troisième temps, nous avons pu démontrer l'importance des propriétés sécrétoires spécifiques à la BHE dans les interactions avec les cellules souches neurales (CSN). Les CSN représentent un potentiel thérapeutique unique pour les maladies du SNC dans lesquelles la régénération cellulaire est limitée, comme dans la SEP. Des facteurs qui limitent l'utilisation thérapeutique des CSN sont le mode d'administration et leur maturation en cellules neurales ou gliales. Bien que la route d'administration préférée pour les CSN soit la

voie intrathécale, l'injection intraveineuse représente la voie d'administration la plus facile et la moins invasive. Dans ce contexte, il est important de comprendre les interactions possibles entre les cellules souches et la paroi vasculaire du SNC qui sera responsable de leur recrutement dans le parenchyme cérébral. En collaborant avec des chercheurs de la Belgique spécialisés en CSN, nos travaux nous ont permis de confirmer, *in vitro*, que les cellules souches neurales humaines migrent à travers les CE humaines de la BHE avant d'entamer leur différenciation en cellules du SNC. Suite à la migration à travers les cellules de la BHE les CSN se différencient spontanément en neurones, en astrocytes et en oligodendrocytes. Ces effets sont notés préférentiellement avec les cellules de la BHE par rapport aux CE non cérébrales. Ces propriétés spécifiques aux cellules de la BHE dépendent de la chimiokine MCP-1/CCL2 sécrétée par ces dernières. Ainsi, cette dernière partie suggère que la BHE n'est pas un obstacle à la migration de CSN vers le SNC. De plus, la chimiokine MCP-1 est identifiée comme un facteur sécrété par la BHE qui permet l'accumulation et la différentiation préférentielle de cellules souches neurales dans l'espace sous-endothélial.

Ces trois études démontrent l'importance de la BHE dans la migration des cellules inflammatoires et des CSN vers le SNC et indiquent que de multiples mécanismes moléculaires contribuent au dérèglement de l'homéostasie du SNC dans les réactions neuro-immunes. En utilisant des modèles *in vitro*, *in situ* et *in vivo,* nous avons identifié trois nouveaux mécanismes qui permettent d'influencer les interactions entre les cellules du sang et la BHE. L'identification de ces mécanismes permet non seulement une meilleure compréhension de la pathophysiologie des réactions neuroinflammatoires du SNC et des maladies qui y sont associées, mais suggère également des cibles thérapeutiques potentielles pour influencer l'infiltration des cellules du sang vers le cerveau.

Mots-clés : Système nerveux central, barrière hémo-encéphalique, cellules endothéliales, neuroinflammation, sclérose en plaques, transmigration, diapédèse, astrocyte, cellules souches neurales, protéomique

Abstract

Multiple Sclerosis is an inflammatory demyelinating disease in which immune cells from the peripheral blood infiltrate the central nervous system (CNS) to cause a pathologic neuroinflammatory reaction. Blood borne leucocytes cross the restrictive cerebral endothelium, the blood brain barrier (BBB), to gain access to the CNS parenchyma and cause cellular damage leading to the characteristic demyelinating lesions. The BBB is the interface between the blood and the CNS and as such is a critical mediator of neuro-immune reactions and an important therapeutic target to modulate neuroinflammation. It is essential to have a better understanding of the molecular mechanisms that regulate the BBB properties to elaborate new therapeutic strategies to modulate the BBB and thus the local neuroinflammation reaction.

This Ph.D. thesis describes three distinct molecular mechanisms which regulate key BBB properties. The first section describes a novel role for the renin-angiotensin system (RAS) in the neuro-vascular unit (NVU) as a regulator of paracellular permeability. The second part of this thesis characterises the role of a novel adhesion molecule of the BBB, ALCAM. The third part of this work studies the interactions between neural stem cells (NSC) and the BBB and identifies MCP-1 as a critical factor involved in NSC recruitment to the CNS.

In the first experimental section we provide evidence that angiotensinogen (AGT) produced and secreted by astrocytes, is cleaved into angiotensin II (AngII) and acts on type 1 angiotensin receptors (AT_1) expressed by BBB endothelial cells (ECs). Activation of AT_1 restricts the passage of molecular tracers across human BBB-derived ECs through threonine-phosphorylation of the tight junction protein occludin and its mobilization to lipid raft membrane microdomains. We also show that AGT knockout animals have disorganized occludin strands at the level of the BBB and a diffuse accumulation of the endogenous serum protein plasminogen in the CNS, as compared to wild type animals. Finally, we demonstrate a

reduction in the number of AGT-immunopositive perivascular astrocytes in multiple sclerosis (MS) lesions, which correlates with a reduced expression of occludin similarly seen in the CNS of AGT knockout animals. Such a reduction in astrocyte-expressed AGT and AngII is dependent, *in vitro*, on the pro-inflammatory cytokines tumor necrosis factor-α and interferon-γ. Our study defines a novel physiological role for AngII in the CNS and suggests that inflammation-induced downregulation of AngII production by astrocytes is involved in BBB dysfunction in MS lesions.

In the second experimental part we focus on adhesion molecules of the BBB. Using a lipid raft-based proteomic approach, we identified ALCAM (*Activated leukocyte cell adhesion molecule*) as an adhesion molecule involved in leukocyte migration across the BBB. ALCAM expressed on BBB endothelium co-localized with CD6 expressed on leukocytes and with BBB endothelium transmigratory cups. ALCAM expression on BBB cells was up-regulated in active multiple sclerosis and experimental auto-immune encephalomyelitis (EAE) lesions. Moreover, ALCAM blockade restricted transmigration of $CD4^+$ lymphocytes and monocytes across BBB endothelium *in vitro* and *in vivo*, and reduced the severity and time of onset of EAE. Our findings point to an important role for ALCAM in leukocyte recruitment into the brain and identify ALCAM as a potential therapeutic target to dampen neuroinflammation.

The third experimental part of this thesis studies the interactions between NCS and BBB. NCS represent an attractive source for cell transplantation and neural tissue repair. After systemic injection, NCS are confronted with the specialized BBB endothelial cells before they can enter the brain parenchyma. We investigated the interactions of human fetal neural precursor cells with human brain endothelial cells in an *in vitro* model using primary cultures. We demonstrated that human fetal neural precursor cells efficiently and specifically migrate to sub-endothelial space of human BBB-endothelium, but not pulmonary artery endothelial cells. When migrated across BBB-endothelial cells, fetal neural precursor cells spontaneously differentiate to neurons, astrocytes and oligodendrocytes. Effective migration and subsequent differentiation was found to be dependant on the chemokine CCL2/MCP-1, but not CXCL8/IL-8. Our findings suggest that an intact blood-brain barrier is not an intrinsic obstacle

to neural stem cell migration into the brain and that differentiation of neural precursor cells occur in a sub-endothelial niche, under the influence of the chemokine CCL2/MCP-1.

These three experimental sections demonstrate the crucial roles that the BBB plays in regulating the CNS homeostasis. Under pathological conditions, such as during neuro-immune reactions, the BBB is altered and becomes an important local player. The three different molecular mechanisms described in this thesis, contribute to our understanding of the BBB and may allow for the development of novel therapeutic strategies to limit neuroinflammation.

Keywords : Central nervous system, blood brain barrier, neuroinflammation, multiple sclerosis, transmigration, diapedesis, astrocyte, neural stem cell, proteomic

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Liste des abréviations

aa:	acides aminés
ACM:	*astrocyte conditionned media*
ACE-1:	*angiotensin convertng enzyme -1*
AJ:	jonctions adhérentes
AGT:	angiotensinogène
ALCAM:	*activated leukocyte cell adhesion molecule*
AngII	Angiotensine II
AT :	recepteur a l'agiotensine
BCA:	acide bicinchoninique
BDNF:	*brain-derived neurotrophic factor*
BHE / BBB:	barrière hémo-encéphalique
BSA:	*bovine albumin serum*
CCL:	ligand des chimiokines C-C
CCR:	récepteur de chimiokines C-C
CD:	*cluster of differentiation*
CE:	cellules endothéliales
CE-BHE:	cellules endothéliales de la barrière hémo-encéphalique
CFA:	*Complete Freund's adjuvant*
CFSE:	*carboxyfluorescein succinimidyl ester*
CMH:	complexe majeur d'histocompatibilité
CPA:	cellules présentatrices d'antigène
C-terminale:	*carboxyl-terminal*
DC:	*dendritic cells*
DMEM:	*Dulbecco's modified eagle's medium*

DRM: *detergent resistant membrane*

EAE: encéphalomyélite auto-immune expérimentale

EBV: virus Epstein-Barr

ECGS: *endothelial cell growth supplement*

ERM: *ezrin, radixin, moesin*

FITC*:* *fluorescein isothiocyanate*

GDNF: *glial cell line-derived neurotrophic factor*

GFAP: *glial fibrillary acidic protein*

GLUT-1: *glucose transporter-1*

GM-CSF: *granulocyte macrophage colony-stimulating factor*

HLA: *human leukocyte antigen*

ICAM-1: *intercellular adhesion molecule-1*

IFN-β: interféron bêta

IFN-γ: interféron gamma

IgG: immunoglobuline G

IL: interleukine

JAM: *junctional adhesion molecule*

TJ: jonctions serrées

kDa: *kiloDalton*

LAT-1: *large neutral amino acid transporter*

LCR: liquide céphalorachidien

LFA-1: *lymphocyte function-associated antigen-1*

LEMP: leucoencéphalopathie multifocale progressive

LPS: lipopolysaccharide

MAC-1: *macrophage-1 antigen*

MAdCAM: *mucosal addressin cell adhesion molecule*

MB: membrane basale

MBP: *myelin basic protein*

MCA: milieu conditionné par les astrocytes

MCAM: *melanoma cell adhesion molecule*

MCP-1: *monocyte chemoattractant protein-1*

M-CSF: *macrophage colony-stimulating factor*

MEC: matrice extracellulaire

MFI: *mean fluorescence intensity*

MIP-1α: *macrophage inflammatory protein-1alpha*

mM: millimolaire

MMP: *matrix metalloproteinases*

MOG *:* *myelin oligodendrocyte glycoprotein*

NAWM: *normal appearing white matter*

NK: *natural Killer cells*

N-terminale: *amino-terminal*

O.C.T.™*:* *optimal cutting temperature*

PAGE: *polyacrylamide gel electrophoresis*

PBMC: *peripheral blood mononuclear cells*

PBS: *phosphate buffered saline*

PB: *sang périphérique*

PECAM-1: *platelet endothelial cell adhesion molecule-1*

PLP: *proteolipid protein*

PVDF: *polyvinylidene fluoride*

Rpm: rotations par minute

RAS: *renin-angiotensin system*

SDF-1: *stromal cell-derived factor-1*

SDS: *sodium dodecyl sulfate*

SEM: *standard error of mean*

SEP: sclérose en plaques

SNC / CNS: système nerveux central

SRA: système rénine-angiotensine

TBS: *tris-buffered saline*

TGF-β: *transforming growth factor-beta*

T_H: *T helper lymphocyte* (lymphocytes T auxiliaires: $CD4^+$)

TNF: *tumor necrosis factor*

T_{reg}: lymphocyte T régulateur

UEA-I: *Ulex europaeus agglutinin-I*

VCAM-1: *vascular cell adhesion molecule-1*

VE-cadherin: *vascular endothelial-cadherin*

VEGF: *vascular endothelial growth factor*

VIH: virus humain d'immunodéficience

VHH-6: virus de l'herpès humain -6

VLA-4: *very late antigen-4*

vWF: *von Willebrand factor*

ZO: *zona occludens*

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SECTION 1 INTRODUCTION

Objectifs généraux et structure de la thèse

Ce travail de doctorat visait l'identification de nouveaux mécanismes moléculaires de la barrière hémo-encéphalique (BHE) impliqués dans les réactions neuro-immunes, plus particulièrement dans le cadre de la sclérose en plaques (SEP). Trois études distinctes présentées dans cette thèse décrivent des mécanismes moléculaires de la BHE qui contribuent à la pathophysiologie de la SEP et qui sont susceptibles de devenir des cibles thérapeutiques intéressantes pour limiter les réactions neuroinflammatoires. Ces trois volets de la thèse sont liés ensemble par une des expertises du laboratoire du Dr Alexandre Prat, c'est à dire l'étude *in vitro*, *in situ* et *in viv*o de la BHE dans la SEP.

La SECTION 1 est une revue de la littérature sur la SEP et plus spécifiquement sur les rôles de la BHE dans cette maladie. Les mécanismes moléculaires qui régissent les fonctions de la BHE, à l'état physiologique et dans les réactions neuroinflammatoires, seront exposés. La SECTION 2 qui est présentée sous la forme d'un manuscrit décrit le rôle local du système rénine-angiotensine (SRA) au niveau de l'unité neuro-vasculaire (UNV) et identifie l'angiotensinogène (AGT) comme un médiateur, sécrété par les astrocytes, qui régule des fonctions importantes de la BHE. La SECTION 3 présente l'identification et la caractérisation de la molécule d'adhérence ALCAM (*Activated Leukocyte Cell Adhesion Molecule/CD166*) dans la migration de leucocytes à travers la BHE dans un contexte de réactions neuroinflammatoires. La SECTION 4 est une étude *in vitro* des interactions entre les cellules endothéliales (CE) de la BHE et les cellules souches neurales (CSN). Cette étude décrit le rôle de la chimiokine MCP-1/CCL2 (*monocyte chemotactic protein-1*) dans la transmigration et la différentiation des CSN.

1.1. La Sclérose en Plaques

1.1.1 Description sommaire

La SEP est la maladie chronique, idiopathique et inflammatoire du système nerveux central (SNC) la plus fréquente. Elle constitue la première cause de déficits neurologiques non traumatiques et de perte de qualité de vie chez les jeunes adultes dans les pays occidentaux[1, 2]. Dans la SEP, les leucocytes du sang périphérique (PB) infiltrent le SNC et s'y accumulent pour former des plaques (ou lésions) de démyélinisation[3, 4]. Ces lésions focales de démyélinisation, décrites dans les années 1860 par le Dr Jean-Martin Charcot[5], sont à l'origine des symptômes cliniques hétérogènes de la maladie. Les lésions diffèrent dans leur taille et leur localisation d'un patient à l'autre. Le diagnostic de la SEP est un diagnostic clinique qui se fait à l'aide des critères révisés de Mac Donald[6] pour documenter la présence de déficits neurologiques et de lésions disséminées dans le temps et l'espace. L'hétérogénéité des manifestations cliniques, ainsi que l'évolution de la maladie, peuvent être, en partie, expliquées par les interactions multiples et complexes entre l'environnement et la génétique qui influencent la susceptibilité à la SEP.

1.1.2 Épidémiologie et hypothèses étiologiques

La SEP affecte les hommes et les femmes partout dans le monde, mais la maladie se retrouve 2 à 3 fois plus fréquemment chez les femmes que chez les hommes[7]. Au Canada la prévalence de la SEP est une des plus importantes dans le monde, avec 240 individus atteints sur 100 000.

L'importance de l'environnement dans le développement de la SEP est démontré par la variation géographique de la prévalence de la maladie dans le monde, puisque la prévalence de la SEP diminue à mesure que l'on se rapproche de l'équateur[2, 8, 9]. L'épidémiologie environnementale de la SEP est encore mal comprise et plusieurs hypothèses sont

actuellement à l'étude comme l'exposition au soleil et à la vitamine D[10-13], l'exposition à certains virus (EBV, VHH-6)[14-19], l'alimentation, les contaminants et le tabagisme[7, 9].

La SEP a aussi des facteurs de susceptibilité génétique comme le démontrent les études génétiques. Le risque de développer la SEP est plus important chez la femme que chez l'homme. Il en est de même lorsqu'un membre de la fratrie est atteint, le rapport des cotes est de 16.8 chez ces individus par rapport à la population générale[20]. Le risque qu'un jumeau monozygotique développe la maladie quand l'autre en est atteint est de 25-30 %, tandis qu'il est beaucoup plus faible chez les jumeaux dizygotiques (3-5 %)[20]. Plusieurs variantes de différents gènes influencent le risque de SEP. Cependant, la SEP est une maladie à traits complexes et les variations d'un seul gène ne sont pas suffisantes pour expliquer la maladie. Des études ne cessent de trouver des polymorphismes génétiques associés à la SEP, notamment en ce qui concerne les gènes HLA-DRB1, CTLA-4, CD45, APOE, CD6, ALCAM, IL-7R et IL-2[21-26]. Par contre ces gènes expliquent actuellement de 20-25% de l'hérédité de la SEP. La majorité des gènes impliqués sont des gènes qui ont un rôle dans le système immunitaire, ce qui suggère fortement que la SEP est associée à un dérèglement de l'immunité. Les premières variantes identifiées sont les plus fortement associées au risque de développer la SEP, des gènes du complexe majeur d'histocompatibilité (HLA-DRB1-1501 avec un rapport des cotes de 3.08)[22] et semblent faciliter la présentation d'antigène du soi qui pourrait mener à l'activation aberrante du système immunitaire contre des antigènes du SNC[27]. Les autres gènes impliqués ont des effets modestes sur le risque de développer la SEP avec des rapports des côtes de l'ordre de 1.1 à 2.4[28]. La génétique de la SEP et les effets biologiques des différents polymorphismes associés à la SEP restent encore mal compris. La caractérisation des divers gènes impliqués a un impact important sur notre compréhension des mécanismes biologiques qui agissent sur le risque et la progression de la maladie.

La SEP est donc une maladie complexe multigénique où les interactions entre l'environnement et la génétique jouent un rôle critique qui facilite la réaction neuroinflammatoire pathologique.

1.1.3 Symptômes et diagnostic

La SEP est caractérisée par des symptômes et une évolution variable et hétérogènes de la maladie [17]. Plusieurs symptômes neurologiques peuvent être associés à la SEP; ils incluent des paresthésies aux membres, des atteintes visuelles, de la diplopie, des déficits moteurs, des déficits sensitifs, des troubles de la démarche, de l'incontinence, de l'asthénie et des dysfonctions cognitives. La SEP peut également prendre plusieurs formes cliniques, la forme la plus fréquente (90 % des cas) étant cyclique, avec des symptômes qui s'aggravent (poussées) et s'améliorent (rémission) de manière spontanée et variable dans le temps (RR-MS). Ces patients évoluent souvent vers une forme progressive de la maladie, la maladie secondairement progressive (SP-MS) où l'on observe une diminution ou une disparition des épisodes de poussées et de remissions et une progression stable et irréversible des déficits neurologiques. Certains patients (10%) présentent une forme primaire progressive de la SEP, dans laquelle la maladie est définie par une progression constante des déficits neurologiques sans présence de poussées ou remission[29].

La variabilité des signes et symptômes cliniques peut être corrélée à l'hétérogénéité spatiale, temporelle et pathologique des lésions de SEP[30-32]. Le diagnostic de la SEP est un diagnostic clinique et se fait à l'aide des critères qui documentent la présence de déficits neurologiques et de lésions à l'imagerie par résonance magnétique (IRM) disséminées dans le temps et l'espace. La confirmation d'une réaction immunitaire au niveau du SNC (réaction neuroinflammatoire), mise en évidence cliniquement par la présence d'anticorps dans le liquide céphalo-rachidien (bandes oligoclonales), n'est nécessaire que pour les cas cliniques

où le diagnostic est difficile. Les critères révisés de Mac Donald[33-36] qui permettent le diagnostic de la SEP sont exposés dans le tableau I.

Tableau I Critères révisés de McDonald pour le diagnostic de la SEP

Présentation clinique	Données supplémentaires nécessaires au diagnostic de SEP
Deux attaques ou plus, preuves objectives cliniques de deux lésions ou plus	Aucune
Deux attaques ou plus, preuves objectives cliniques d'une lésion	Preuve de dissémination dans l'espace: -IRM ou -deux lésions ou plus par IRM consistantes avec la SEP et LCR positif pour des banques oligoclonales ou -attendre une autre attaque clinique qui implique un différent site
Une attaque, preuves objectives cliniques de deux lésions ou plus	Preuve de dissémination dans le temps: - IRM - Deuxième attaque clinique
Une attaque, preuves objectives cliniques d'une lésion (mono symptomatique ; syndrome clinique isolé)	Preuve de dissémination dans l'espace: -IRM ou -deux lésions ou plus par IRM consistantes avec la SEP et LCR positif pour des bandes oligoclonales et -dissémination dans le temps : -IRM -deuxième attaque
Progression neurologique insidieuse qui suggère une SEP	Progression de la maladie pour un an et Deux des suivants : -IRM cérébrale positive (9 lésions en T2 ou 4 lésions en T2 et PEV positifs) -IRM spinale positive

	-LCR positif

Adaptation de Polman CH et al, 2011[36].

SEP : sclérose en plaque, IRM : imagerie à résonance magnétique, LCR : liquide céphalo-rachidien, PEV : potentiels évoqués visuels

Si les critères sont respectés et qu'il n'y a pas de meilleure explication pour la présentation clinique, alors le diagnostic de SEP est posé. S'il y a une suspicion mais que les critères ne sont pas tous respectés, on évoque un diagnostic de SEP possible.

LCR positif pour les bandes oligoclonales détectées par des méthodes établies.

IRM positive selon les critères radiologiques de Barkhof et al et Tintore et al.[37, 38]

1.1.4 Physiopathologie de la SEP

Les événements qui déclenchent la SEP sont encore mal compris, mais il est généralement accepté que la séquence d'événements pathologiques qui mène aux déficits neurologiques est caractérisée par une phase auto-immune inflammatoire démyélinisante, survenant à la suite d'une infiltration focale du SNC par des cellules immunitaires[3]. Dans la SEP, la réaction immunitaire pathologique semble être dirigée, entre autres, contre les antigènes de la myéline[39] et possiblement contre des épitopes neuronaux[40]. Cette réaction neuroinflammatoire entraîne la formation de plaques focales (ou lésions) de démyélinisation, dans lesquelles on peut observer une inflammation focale chronique et des dommages cellulaires irréversibles. Le nombre de plaques, leur localisation, leur taille et leur forme varient d'un patient à l'autre. La présence de lésions corticales dans la matière grise est aussi notée, en plus des lésions typiques situées dans la matière blanche, un phénomène méconnu jusqu'à tout récemment[41-43]. Notre compréhension de la maladie est basée sur les observations des données épidémiologiques, génétiques, pathologiques, radiologiques et par analogie aux modèles animaux de la SEP[4, 30, 30, 44-47]. Tôt dans la SEP, on constate une dysfonction de la BHE qui est associée à des signes inflammatoires. Ces observations se traduisent en clinique par la visualisation à l'IRM cérébrale de lésions réhaussantes après l'infusion de gadolinium, un agent de contraste[48-52]. Les études pathologiques d'échantillons de cerveau et de moelle épinière de patients de SEP démontrent la présence de lésions ovales macroscopiques disséminées dans tout le SNC.

Bien que la caractérisation histopathologique des lésions de SEP reste encore ambiguë, la classification pathomorphologique de Lucchinetti *et al.* garde beaucoup d'importance[31, 32, 53, 54]. Cette classification décrit 4 types de lésions basés sur l'aspect histologique de la démyélinisation. La présence d'infiltrats périvasculaires et parenchymateux de lymphocytes T CD4+, CD8+, de lymphocytes B et de macrophages a été documentée dans tous les types de lésions, mais prédomine surtout dans les lésions de type I. Au sein des plaques de

démyélinisation, on retrouve des différences dans les niveaux d'expression de divers médiateurs inflammatoires, dans la quantité de dommage cellulaire aux oligodendrocytes et dans le nombre de lésions axonales[32, 42, 43, 55]. Plus la phase progressive de la maladie est avancée, plus on retrouve des dommages cellulaires aux oligodendrocytes et aux neurones. Dernièrement, cette classification a été remise en cause et une classification selon l'âge présumé des lésions est maintenant privilégiée[44, 56-59]. En effet, plusieurs études pathologiques récentes qui ont tenté de classifier les différents types de lésions ont soulevé l'hypothèse que l'hétérogénéité des lésions reflète des lésions à différents stades de développement plutôt que différentes formes de la maladie[60-62]. Les lésions pré-actives et les lésions actives sont caractérisées par une démyélinisation partielle, une prolifération oligodendrocytaire et des infiltrats leucocytaires périvasculaires focaux. Les lésions plus âgées, ou chroniques, seraient caractérisées par une perte complète de la myéline et des oligodendrocytes, des dommages axonaux, une fibrose astrocytaire (astrogliose) et une présence variable de cellules immunitaires. Même s'il n'y a pas de consensus actuellement[63], il est clair qu'au sein des lésions pathologiques de SEP on retrouve la présence de leucocytes, de médiateurs inflammatoires, de démyélinisation, d'astrogliose et de dommages axonaux. De plus, de subtils dérèglements moléculaires dans toutes les cellules impliquées dans les lésions sont notées : au niveau de l'endothélium, au niveau des leucocytes infiltrant et au niveau des cellules du SNC (astrocytes, microglies, oligodendrocytes et neurones) [3, 55, 64]. Actuellement, il est difficile de déterminer si ces multiples dérèglements cellulaires sont la cause, ou plutôt la conséquence, des réactions neuroinflammatoires chroniques décrites dans les plaques SEP. Par contre, ces différents événements pathologiques contribuent certainement à la formation des lésions caractéristiques de la maladie. Ainsi, la SEP est associée à des plaques de démyélinisation associées à une infiltration et une accumulation leucocytaire périvasculaire et focale dans le SNC, ainsi que de nombreux dérèglements cellulaires pathologiques.

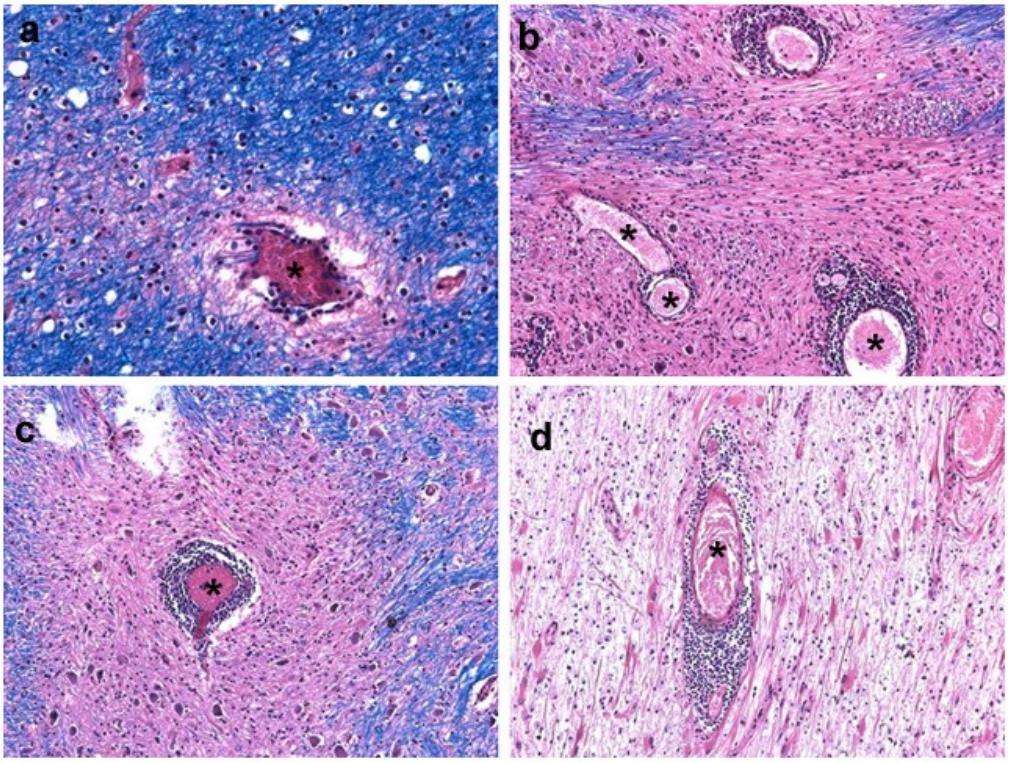

Figure 1. Représentation pathologique des plaques de SEP.

Sections de lésions de SEP colorées avec l'éosine, l'hématoxyline et la coloration bleue spécifique à la myéline (*Luxol fast blue*). Ces coupes histologiques proviennent de tissu post-mortem de lésions de démyélinisation sous-corticale du SNC de deux patients atteint de SEP (patient A-a, b, c et patient B-d). Cette figure montre quatre différentes plaques de SEP qui démontrent l'hétérogénéité des manifestations pathologiques. Il n'est pas clair si cela représente l'hétérogénéité de la maladie ou simplement des plaques à différents stades d'évolution. Une infiltration pathologique de leucocytes, représentée par les petites cellules à noyaux denses qui s'accumulent dans l'espace périvasculaire (l'espace intravasculaire est marqué par un *) est observés dans les lésions. De plus, une perte focale de myéline caractéristique des plaques de SEP est démontrée par la perte de la coloration bleue. Selon l'hypothèse la plus récente (a) représente une plaque jeune, (b) et (c) représentent des plaques

actives et (d) représente une plaque chronique avec la perte des oligodendrocytes, des lésions axonales et de l'astrogliose.

1.1.5 L'encéphalomyélite auto-immune expérimentale comme modèle animal de la SEP

L'encéphalomyélite auto-immune expérimentale (EAE), qui est le modèle animal de la SEP le plus utilisé, a permis une meilleure compréhension des mécanismes moléculaires impliqués dans les réactions neuro-immunes. L'EAE démontre l'importance du système immunitaire, car la maladie y est induite en immunisant l'animal contre les protéines de la myéline, ce qui cause une réaction neuroinflammatoire pathologique et amène des déficits neurologiques chez l'animal. Historiquement, l'EAE a été découverte autour de 1885 chez l'homme, alors que le vaccin contre la rage, fait à partir de moelle épinière de lapin, pouvait causer des symptômes neurologiques. Ces cas d'encéphalomyélites post-vaccinales montraient une pathologie semblable à celle décrite par Charcot pour les patients de SEP, soit une infiltration leucocytaire focale et périvasculaire accompagnée de plaques de démyélinisation dans le cerveau et la moelle épiniere[45, 65-67].

Plusieurs études effectuées entre 1900 à 1970 ont étudié l'EAE dans différentes espèces animales et différentes lignées d'une même d'espèce. L'EAE présente des signes cliniques qui diffèrent selon l'espèce étudiée et le protocole expérimental utilisé[45, 68, 69]. L'EAE peut être induite chez l'hôte sous la forme active ou passive. L'EAE active implique un protocole d'immunisation de l'animal contre un peptide de la myéline, ce qui amène à une pathologie auto-immune neuroinflammatoire démyélinisante. La forme passive est induite par un transfert de leucocytes activés, d'un animal chez qui l'EAE active a été induite dans un animal hôte sain. Le transfert exclusif de cellules immunitaires activées étant suffisant pour induire une réaction neuroinflammatoire pathologique, ceci démontre l'importance du système immunitaire dans les maladies inflammatoires démyélinisantes. Actuellement plusieurs

modèles animaux d'EAE sont utilisés en recherche. Les modèles murins sont les plus courants (souris et rat), bien que d'autres modèles, surtout les modèles simiens, gagnent en popularité[70-72]. Chez la souris, les lignées C57BL/6 et les SJL sont fréquemment utilisées pour l'EAE, car la maladie y est facile à induire et que ces souris sont aisément modifiables génétiquement[73, 74].

Dans les modèles murins d'EAE, l'immunisation se fait avec des peptides de myéline en présence d'adjuvant complet de Freund et de toxine pertussique. L'adjuvant et la toxine augmentent l'immunogénicité du peptide de la myéline et potentialisent la réaction immunitaire. De plus la toxine pertussique affecte directement la BHE et facilite l'infiltration de cellules immunitaires au SNC. Dans ces modèles l'EAE atteint principalement la moelle épinière et, dans une moindre mesure, le cerveau. Suite à l'induction de la maladie, il faut compter 5 à 15 jours avant l'apparition des premiers symptômes neurologiques. Ceux-ci apparaissent comme une paralysie progressive ascendante, et sont associés à un score clinique entre 1 et 5. Le système de classification se détaille comme suit : 1 – queue flasque; 2 – ataxie; 3 – paralysie des membres inférieurs; 4 – paralysie des membres supérieurs; 5 – moribond.

Bien que ce modèle animal récapitule partiellement les dommages inflammatoires au SNC suite à une réaction neuroinflammatoire, plusieurs lacunes importantes existent et limitent l'extrapolation des résultats provenant de l'EAE au patient atteint de SEP. La variabilité entre les différents modèles, qui dépendent en grande partie du protocole d'induction et de l'espèce à l'étude[45, 68, 69, 74-77], limite les applications de l'EAE[73, 75, 76, 78-80]. Par exemple, les modèles d'EAE chez la souris diffèrent selon les lignées et le protocole d'induction. Dans la lignée C57BL/6, la maladie peut être monophasique et chronique, et l'induction se fait avec un épitope de la protéine de la myéline MOG (*Myelin Oligodendrocyte Glycoprotein*). Dans la lignée SJL, la maladie est typiquement multiphasique, et le peptide utilisé provient d'une autre protéine de la myéline, le PLP (*proteolipid protein*) [65]. La différence dans l'épitope utilisé pour l'induction de la maladie s'explique par les différents

haplotypes de CMH propre à la lignée de sourie étudiée. La lignée C57BL/6 reste la plus populaire, une des raisons étant la facilité de la manipulation génétiquedans cette lignée de sourie susceptible.

Plusieurs différences existent entre l'EAE et la SEP. La SEP est une maladie complexe qui présente des manifestations cliniques, pathologiques et immunologiques variables. Les modèles animaux ne récapitulent pas cette complexité. Une des différences les plus importantes est que l'EAE est induite activement ou passivement chez l'animal alors que la SEP se développe spontanément chez l'humain. Des avancées récentes ont permis de développer des modèles spontanés dans des souris génétiquement modifiées[40, 81]. Les souris généralement utilisées sont des lignées génétiquement homogènes et par rapport à la population humaine qui est génétiquement hétérogène. L'évolution de la maladie est différente entre l'EAE et la SEP. Ces différences se traduisent par une réponse au traitement qui diffère entre l'EAE et la SEP. En effet, un traitement qui limite efficacement les signes cliniques de l'EAE n'aura pas nécessairement le même effet chez les patients atteint de SEP. C'est le cas de plusieurs thérapies comme les peptides ligands altérés et les anti-TNF, qui limitent l'EAE mais n'ont pas démontré d'efficacité en SEP[45, 65, 69, 74-76, 79, 80, 82-85]. Malgré les différences entre la SEP et l'EAE, on note aussi plusieurs similarités, notamment au niveau histologique. Les lésions d'EAE présentent des plaques focales de démyélinisation avec d'importantes infiltrations leucocytaires dans l'espace périvasculaire et des dommages axonaux. Certaines différences importantes sont tout de même notées en ce qui concerne le type de cellules immunitaires infiltrantes[45].

Malgré ces difficultés, l'EAE reste un outil essentiel à la recherche. Il permet l'étude de la surveillance immunitaire, des réactions neuroinflammatoires et des mécanismes pathogéniques des réactions neuroinflammatoires. Bien que seulement une minorité des traitements identifiés grâce à l'EAE ait démontré une efficacité chez l'humain, l'EAE reste une plateforme expérimentale importante pour évaluer des stratégies thérapeutiques *in vivo*[74-

[76, 79, 80]. L'EAE a permis d'élaborer au moins trois stratégies thérapeutiques actuellement utilisées pour la SEP : l'acétate de glatiramer, la mitoxantrone et le natalizumab[86-88].

1.1.6 Les traitements de la SEP

L'étiologie de la maladie étant mal comprise, le traitement actuel de la SEP vise à limiter les symptômes de la maladie, à prévenir les exacerbations et à ralentir la progression de la maladie, mais n'offre pas d'option curative. Comme dans plusieurs autres maladies inflammatoires, les agents immuno-modulateurs, comme les glucocorticoïdes, sont importants pour le contrôle des exacerbations de la maladie[87, 89-92]. Selon la forme de la maladie, le traitement d'entretien diffère d'un patient à l'autre.

Les agents immunosuppresseurs, comme le mitoxantrone, ont une bonne efficacité clinique. Cependant, leur utilité est limitée par l'importance des effets secondaires (cardiotoxicité et néoplasie), surtout chez de jeunes patients, en âge de reproduction[86]. Les cliniciens privilégient ainsi les agents immuno-modulateurs qui sont mieux tolérés[89, 93]. L'interféron bêta et l'acétate de glatiramer (GA) sont des stratégies thérapeutiques de choix en SEP. Ces agents ont des propriétés immuno-modulatrices importantes et agissent sur tout l'organisme et le système immunitaire, pouvant modifier l'expression de plusieurs centaines de gènes[91]. Ces traitements ont des effets multiples et complexes, et les mécanismes moléculaires sous-jacents des agents immuno-modulateurs sont encore mal compris et toujours à l'étude[88, 89, 91, 94]. Par contre, ces deux agents ont une efficacité clinique significative et permettent de diminuer l'apparition de lésions à l'IRM et de diminuer la sévérité des signes cliniques. Ils sont actuellement utilisés chez les patients atteints de RR-MS. Les traitements de la forme progressive de SEP sont beaucoup plus limités, probablement à cause de la prédominance des dommages axonaux par rapport aux événements inflammatoires dans cette forme de la maladie.

Dernièrement, plusieurs nouveaux traitements immuno-modulateurs sont arrivés sur le marché. Le fingolimod est la première thérapie orale approuvée en avril 2011. Cet analogue de la sphingosine favorise la séquestration des leucocytes dans les organes lymphoïdes, et diminue le nombre de leucocytes dans le sang périphérique. Ce phénomène est associé à une diminution des exacerbations et de la progression de la SEP[95-99]. Les effets secondaires principaux concernent le rythme cardiaque et la susceptibilité aux infections. Deux cas de pneumonies mortelles au virus *varicella zoster* ont aussi été rapportés. Cette thérapie a été très attendue chez les patients atteints de SEP, car elle représente une thérapie efficace, bien tolérée, mais surtout facilement administrable. Deux autres thérapies orales ont ensuite été approuvées. Le tériflunomide est la deuxième thérapie orale pour la SEP cyclique, approuvée en septembre 2012. Le tériflunomide est un agent immuno-modulateur bien toléré qui a des effets cytostatique à travers l'inhibition la voie de synthèse de novo des pyrimidines. [100, 101]. Le diméthyle fumarate est la troisième thérapie orale approuvée en mars 2013 pour la SEP cyclique. Cette molécule a longtemps été utilisée pour le traitement du psoriasis et a des propriétés immuno-modulatrices reconnues. Le mécanisme d'action exacte reste mystérieux mais ce composé agit comme immuno-modulateur entre autre en diminuant le glutathion (GSH) et en induisant des protéines anti-inflammatoires[102, 103]. Plusieurs autres stratégies thérapeutiques restent à l'étude, certaines agissent de façon non spécifique (statine) [95, 96, 104-107], tandis que certaines autres stratégies sont plus spécifiques (anticorps anti-CD20, rituximab) [91, 108] ou plus agressives (transplantation de moelle osseuse) [91, 92, 108].

Une des stratégies thérapeutiques à l'étude en SEP est d'un intérêt direct pour le laboratoire et pour cette thèse, le natalizumab[87, 109, 110]. Le natalizumab est un nouveau traitement qui a été temporairement retiré du marché, à cause de ses effets secondaires trop importants[111-113]. Cet agent est un anticorps monoclonal humanisé qui bloque spécifiquement l'intégrine VLA-4 (*Very Late Antigen-4*) exprimée par la majorité des leucocytes. La protéine VLA-4 joue un rôle crucial dans l'adhésion et les interactions entre un leucocyte du sang et les CE, ce qui mène à la transmigration de la cellule immunitaire à travers l'endothélium vers

l'organe cible[87, 109, 110]. Le natalizumab limite donc spécifiquement les interactions entre les cellules immunitaires et l'endothélium cérébral, et peut ainsi réduire l'infiltration des cellules immunitaires dans le SNC chez les patients atteints de SEP. Cet antagoniste du VLA-4, qui a été élaboré à partir d'études dans le modèle d'EAE, a une efficacité clinique démontrée dans la SEP, puisqu'il réduit les lésions à l'IRM et diminue les symptômes cliniques de façon significative[87, 109, 110]. Par contre, le natalizumab limite aussi la surveillance immunitaire physiologique du SNC, ce qui peut entraîner la réactivation du virus JC chez l'homme, un virus latent au sein du SNC. La réactivation du virus JC associée à un traitement immuno-modulateur peut se traduire cliniquement par une leucoencéphalite progressive multifocale (LEMP) pouvant être mortelle[111-113]. Le natalizumab reste un traitement efficace de la SEP et est approuvé dans certaines conditions pour les cas réfractaires aux thérapies moins risquées. Ce traitement démontre l'importance de l'infiltration immunitaire au sein du SNC dans la SEP et identifie les interactions entre l'endothélium cérébral et le système immunitaire comme une cible thérapeutique efficace pour limiter les réactions neuroinflammatoires. Un effet immuno-modulateur plus modéré serait souhaitable pour ne pas agir sur la surveillance immunitaire.

Dans la SEP le système immunitaire infiltre le SNC et y cause des plaques de démyélinisation qui entraînent des déficits neurologiques. Bien que l'étiologie et la pathophysiologie de la maladie restent à clarifier, de nombreuses nouvelles stratégies thérapeutiques sont en développement. Dans le laboratoire du Dr Alexandre Prat, le rôle de la BHE dans les réactions neuroinflammatoires est un thème de recherche important, dans l'espoir d'identifier de nouvelles stratégies thérapeutiques qui impliquent la BHE pour limiter les réactions neuro-immunes.

1.2 La barrière hémo-encéphalique et l'unité neuro-vasculaire

1.2.1 Le système nerveux central, un organe privilégié

Le SNC est un organe extrêmement complexe, qui nécessite un microenvironnement délicatement régulé pour permettre le fonctionnement optimal des neurones et des cellules gliales. Le cerveau reçoit 20 % du débit cardiaque; ce volume sanguin est réparti dans tout le cerveau via une micro-vasculature spécialisée, la barrière hémo-encéphalique (BHE). On estime que chaque neurone a son propre capillaire sanguin, ce qui totaliserait plus de 400 km de capillaires, occupant plus de 20 m^2 de surface[114]. Cette vasculature permet un contrôle strict de la composition du milieu extracellulaire du SNC et garantit ainsi le fonctionnement optimal du SNC, qui dépend des neurones, entourés de myéline, pour la transmission des influx nerveux. Les oligodendrocytes sont les cellules myélinisantes qui entourent et isolent les axones des neurones et permettent une transmission efficace et rapide de l'influx nerveux. La myéline est composée de membranes plasmiques spécialisées provenant des oligodendrocytes. On y retrouve plusieurs protéines spécifiques (comme MOG, *Myelin oligodendrocyte glycoprotein,* et PLP, *Proteo lipid protein*), enrichies dans des membranes lipidiques enroulées autour de la gaine de l'axone qui débute et se termine aux nœuds de Ranvier. La myéline permet ainsi une conduction saltatoire et rapide de l'influx nerveux et est essentielle au fonctionnement du SNC[29]. Les cellules gliales du SNC sont composées des astrocytes et des microglies, et jouent un rôle important dans la structure du cerveau ainsi que dans la régulation de l'homéostasie du SNC. Les cellules microgliales sont les macrophages résidents du SNC. En règle générale, les cellules du SNC, et plus particulièrement les neurones et les oligodendrocytes, sont très sensibles aux variations du microenvironnement cérébral. Comme les neurones sont des cellules qui ne se renouvellent pas, les dommages cellulaires au SNC peuvent entraîner des déficits neurologiques permanents.

Pour assurer l'homéostasie du SNC, les échanges entre le SNC et le compartiment sanguin, essentiels à l'apport énergétique et nutritif, à l'évacuation des déchets et toxines et à la surveillance immunitaire, sont finement régulés[115-119]. La BHE, qui représente l'interface entre le sang et le SNC, contrôle les échanges avec le SNC à trois niveaux : elle présente une diffusion paracellulaire diminuée, elle exerce un contrôle strict des échanges entre le sang et le parenchyme cérébral à travers des systèmes de transport spécialisés et elle limite la transmigration des leucocytes vers le cerveau.

L'endothélium spécialisé de la BHE se retrouve principalement dans les capillaires et les microvaisseaux du SNC. Historiquement, les expériences du Dr Paul Ehrlich et de son étudiant le Dr Éric Goldmann au début des années 1900 ont démontré la séparation du sang et du SNC. C'est cette compartimentalisation entre le sang et le parenchyme du SNC qui est à l'origine du terme BHE[120-122]. Ensuite, des études de microscopie électronique avec la ferritine et la peroxydase ont démontré que les jonctions intercellulaires de la vasculature du SNC sont plus étanches qu'ailleurs dans le corps[123, 124]. Depuis ces expériences, le concept de BHE a beaucoup évolué et on reconnaît la présence d'une séparation similaire entre le sang et le parenchyme tissulaire dans deux autres organes : les organes reproducteurs (testicules ou ovaires) et la rétine[125-129].

Aujourd'hui, le concept de BHE est mieux décrit par le terme d'unité neuro-vasculaire (UNV), car ce terme reflète l'importance des cellules non-endothéliales qui contribuent à l'étanchéité de cette barrière. En effet, de nombreuses données récentes soulignent l'importance des interactions complexes entre les CE de la BHE (CE-BHE) et les cellules gliales du SNC, pour réguler les propriétés spécifiques de l'endothélium cérébral. L'UNV comprend les CE-BHE, la membrane basale vasculaire, les péricytes de la BHE, l'espace périvasculaire et les cellules qu'il contient (macrophages et cellules mésothéliales), la *glia limitans* qui constitue une seconde membrane basale et les terminaisons d'astrocytes, de microglies et de neurones en contact intime avec les CE-BHE (Figure 2)[114, 116, 130-132]. À l'état

physiologique, la membrane apicale des CE cérébrales est la seule partie du SNC directement en contact avec le compartiment sanguin. La séparation entre le sang et le SNC, qui maintient l'homéostasie du SNC et régule l'accès des ions, des molécules, des protéines sériques, des toxines et des cellules immunitaires du PB vers le SNC, est donc directement dépendante de ces CE.

Pour conserver l'homéostasie du SNC et limiter les réactions inflammatoires néfastes, seul un petit nombre de cellules immunitaires patrouillent le SNC pour la surveillance immunitaire à l'état physiologique. Associé à la partition sang-SNC, l'accès réduit des leucocytes au SNC confère une immuno-protection relative, appelée privilège immunitaire[115, 133-135]. Le concept de privilège immunitaire du SNC existe depuis les années 1900, lorsque certains antigènes[136], des virus[137], des bactéries[138] et des tumeurs[139] sont cachés du système immunitaire périphérique lorsqu'ils sont transplantés dans le SNC[139]. Ce concept a co-évolué avec le concept de BHE puisque ces deux concepts sont intimement liés. La partition entre le SNC et le sang est une des propriétés les plus importantes de la BHE et de l'UNV, et contribue au privilège immunitaire du SNC. En réalité, plusieurs propriétés intrinsèques au SNC sont aussi importantes pour le privilège immunitaire, soit l'absence de cellules présentatrices d'antigènes (CPA) professionnelles capables d'activer des lymphocytes dans le SNC non inflammé et l'absence de système lymphatique conventionnel dans le SNC (le drainage 'lymphatique' se fait par les espaces périvasculaires). Ces propriétés intrinsèques limitent l'activation du système immunitaire ainsi que le drainage d'antigènes et de CPA du cerveau jusqu'aux organes lymphatiques. De plus, l'expression du complexe majeur d'histocompatibilité (CMH) est minimale dans le SNC. Les cellules du SNC limitent également l'activation immunitaire par l'expression de facteurs qui inhibent la prolifération lymphocytaire et favorisent le développement de cellules immunitaires régulatrices (PD-1, IDO, TGF-beta, TRAIL) [133-135]. Par contre, la présence de médiateurs inflammatoires dans le SNC peut compromettre le privilège immunitaire par de multiples mécanismes, tels que la disruption des propriétés restrictives de la BHE, la perte des propriétés immuno-modulatrices

intrinsèques du SNC, la présence de CPA professionnelles dans le SNC, la facilitation du drainage du SNC, l'induction de l'expression du CMH par les cellules du SNC et l'activation des cellules microgliales[133, 135].

Pour compliquer le tableau, certaines études récentes indiquent que les propriétés de l'endothélium diffèrent selon l'emplacement anatomique au sein du SNC[140-142]. L'endothélium restrictif qui se retrouve dans le parenchyme du SNC se distingue de la vasculature des méninges, des ventricules et des plexus choroïdiens[114, 132]. De plus, les artères, plus contractiles, sont surtout associées au contrôle du flot sanguin, les capillaires semblent plutôt responsables du transport de nutriments, alors que les veinules post-capillaires sont les sites de prédilection pour l'infiltration cellulaire[127, 140-144].

Afin de permettre l'homéostasie du microenvironnement du SNC et de limiter les dommages cellulaires lors de réactions neuroinflammatoires, le privilège immunitaire du SNC dépend non seulement de l'UNV, mais aussi de la région anatomique et d'une régulation complexe du système immunitaire.

1.2.2 L'unité neuro-vasculaire

Comme mentionné plus haut, le concept d'UNV prend de plus en plus d'importance pour expliquer les propriétés de la BHE. La BHE réfère particulièrement aux CE du SNC et à leurs propriétés spécifiques, tandis que l'UNV englobe les CE de la BHE, la matrice extracellulaire sous-endothéliale et gliale, les péricytes et les contacts avec les extensions astrocytaires et neuronales du SNC (Figure 2). En effet, toutes ces structures et ces types cellulaires contribuent et modulent les propriétés spécifiques de la BHE médiées par les CE. La localisation anatomique unique des CE-BHE entre le sang et les cellules du SNC, ainsi que les contacts intimes entre les CE, les astrocytes et les neurones, contribuent à l'acquisition et au maintien des propriétés spécifiques à l'endothélium de la BHE[130, 132, 145, 146]. Plusieurs mécanismes moléculaires ont été découverts qui expliquent en partie ces propriétés, et les

prochains paragraphes décriront l'état des connaissances concernant la BHE et son rôle dans les réactions neuroinflammatoires.

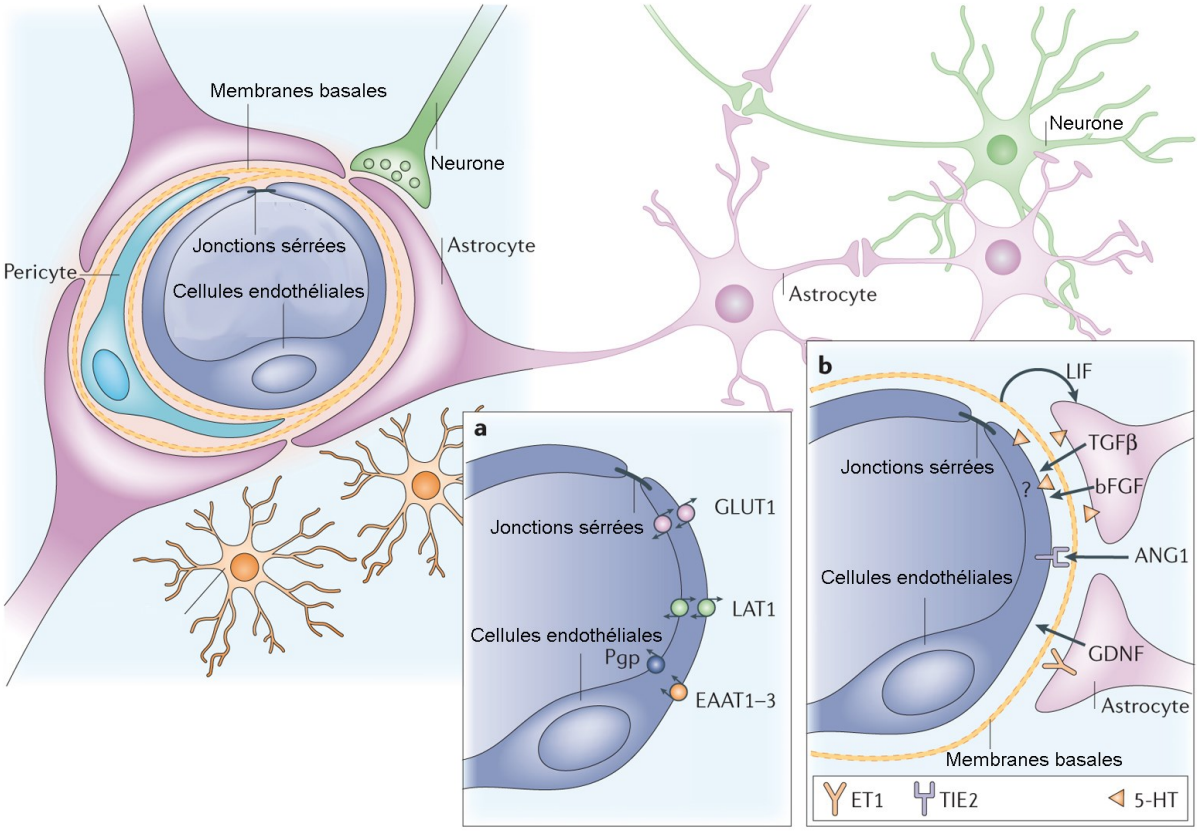

Figure 2. Organisation de la BHE et de l'UNV.

L'UNV est composée de CE spécialisées, de deux membranes basales, de péricytes, d'astrocytes ainsi que des neurones qui entourent la BHE. (**a**) Les CE-BHE ont plusieurs propriétés spécifiques telles que l'expression de jonctions serrées et de transporteurs comme GLUT-1, LAT-1 et l'EAAT1-3. (b) Les propriétés spécifiques de la BHE sont influencées et modulées par des facteurs astrocytaires, tel que le TGF-b, le b-FGF, le GDNF, la sérotonine (5-HT) et l'angiopoétine (ANG1). Les CE peuvent à leur tour agir sur les astrocytes en sécrétant différents médiateurs, tels que le LIF. Ce schéma est adapté de Abbott *et al*[114].

1.2.3 Les cellules endothéliales de la barrière hémo-encéphalique

Les CE des microvaisseaux de la BHE forment le premier contact et la première barrière entre le sang et le SNC, participent à la régulation du microenvironnement du SNC et contribuent activement au recrutement de leucocytes dans les réactions immunitaires au sein du SNC. Les CE-BHE possèdent plusieurs mécanismes anatomiques et moléculaires qui expliquent, au moins en partie, leurs propriétés restrictives.

Au niveau anatomique, les CE-BHE sont les seules cellules vasculaires qui ont des contacts extensifs avec les cellules gliales[116, 132, 147]. Certaines études laissent entendre que plus de 90 % de la surface basale des CE-BHE est en contact intime avec des cellules gliales, un phénomène qui ne se retrouve nulle part ailleurs dans l'organisme[130, 148].

Au niveau moléculaire, les CE-BHE sont des cellules polarisées qui ont peu d'activité de pinocytose et qui présentent une absence de fenestration, ce qui limite la diffusion transcellulaire du sang vers le SNC[116, 132, 147]. Les CE cérébrales ont un plus grand nombre de mitochondries, ce qui reflète leur importante activité métabolique et leur utilisation de nombreux systèmes de transport spécialisés[116, 132, 147]. Les CE-BHE ont également des complexes macromoléculaires étanches aux jonctions intercellulaires, les jonctions adhérentes (JA) et les jonctions serrées (JS). Ces complexes moléculaires sont essentiels pour limiter la diffusion paracellulaire. Ces fonctions propres aux CE-BHE contribuent à l'imperméabilité de l'endothélium cérébral.

1.2.3.1 Transporteurs

L'expression sur la membrane des CE-BHE de transporteurs spécifiques permet la régulation du transport d'ions, d'acides aminés, de peptides, de métabolites et de nutriments du PB vers le SNC (Figure 2). Ces transporteurs incluent le transporteur de glucose-1 (GLUT-1), des co-transporteurs glucose-sodium, le récepteur à la transferrine, des transporteurs

d'acides aminés (EAAT1-3, *excitatory amino acid transporter* et LAT-1, *large amino acid transporter*), de vitamines, de minéraux, de nucléosides et de nombreux autres composés. Certaines substances peuvent être acheminées au SNC par endocytose, ce qui permet le transport de molécules de grande taille et de protéines membranaires complexes. Tous ces systèmes de transport sont finement régulés, afin de contrôler les échanges entre le sang et le SNC, et ainsi de conserver l'homéostasie nécessaire au bon fonctionnement cérébral[149-151]. Les CE cérébrales expriment aussi des protéines de la famille des transporteurs ABC, comme les p-glycoprotéines (P-gp1, P-gp3, MRP), au niveau de leur membrane apicale. Ces protéines sont de véritables pompes moléculaires qui excrètent de nombreuses substances du SNC jusqu'au sang, notamment les toxines du sang, mais aussi de nombreux médicaments qui ont donc un accès limité au SNC. Les CE-BHE expriment aussi de nombreuses enzymes sur leur membrane apicale qui métabolisent les médicaments et les nutriments. Cette barrière enzymatique dépend, entre autres, de la gamma-glutamyl transpeptidase et de l'alkaline phosphatase[114, 130, 152-154].

1.2.3.2 Jonctions intercellulaires et perméabilité

Les JS sont les structures principales impliquées dans la restriction de la diffusion paracellulaire au niveau de l'endothélium cérébral. Ces complexes macromoléculaires relient les CE adjacentes, et agissent donc comme une barrière intercellulaire permettant de diminuer la perméabilité (Figure 2 et 3).

Les CE-BHE expriment des JS analogues à celles qui se retrouvent sur les barrières épithéliales. Ces JS sont particulièrement imperméables quand on les compare aux structures similaires exprimées par les autres endothéliums du corps humain[155]. La perméabilité *in vitro* d'un endothélium peut être évaluée de plusieurs manières, les méthodes courantes évaluant la résistance électrique d'une monocouche de CE ainsi que la perméabilité à différentes tailles de molécules[156, 157]. Dans ces expériences, les CE-BHE démontrent une

perméabilité réduite par rapport à l'endothélium provenant d'un autre organe. Les JS matures, qui se retrouvent principalement au niveau des jonctions intercellulaires des cellules qui forment une barrière imperméable (comme c'est le cas dans les épithéliums[155, 158, 159]), sont des complexes macromoléculaires qui lient les CE adjacentes entre elles. Les JS consistent d'au moins trois familles différentes de protéines transmembranaires[160], soit la protéine occludine, les protéines claudines et les protéines JAM (*junction associated molecule*).

L'occludine, une des composantes principales des JS, est exprimée aux jonctions intercellulaires et se retrouve surreprésentée dans les CE cérébrales[152]. Les données provenant des modèles animaux qui n'expriment pas cette protéine démontrent que l'occludine ne joue pas un rôle structural, mais qu'elle module plutôt les JS par des mécanismes de signalisation intracellulaire encore peu connus[161]. Par contre, les travaux *in vitro* indiquent que la surexpression de l'occludine limite la perméabilité de monocouches épithéliales[162-165]. L'occludine comprend 4 domaines transmembranaires et les terminaisons carboxyle et aminée se retrouvent dans l'espace intracellulaire. Ces domaines intracellulaires de l'occludine peuvent interagir avec de nombreuses protéines adaptatrices intracellulaires, et contiennent de multiples sites de phosphorylation qui régulent son association avec les JS et son rôle dans la perméabilité paracellulaire[163, 166-170].

Les claudines font partie d'une famille d'au moins 24 protéines différentes (claudines 1 à 24). Ces protéines jouent un rôle structural important dans les JS et permettent la formation d'un sceau imperméable entre les cellules adjacentes, à travers des liaisons homo- et hétérophiliques. La composition exacte des JS en claudines influence directement l'imperméabilité de l'endothélium[171, 172]. Les claudines-1, -3, -5 et -12 sont les claudines préférentiellement exprimées au niveau des CE cérébrales. La claudine-5 a été particulièrement étudiée au niveau de la BHE, et les animaux qui n'expriment pas cette protéine présentent une réduction de l'intégrité de la BHE et une perte de l'imperméabilité aux petites molécules[173]. Le consensus actuel est que les claudines forment la base des JS et que

les autres protéines, telles que l'occludine, augmentent l'imperméabilité de la monocouche sans y avoir de rôle structural.

Il existe trois protéines JAM (JAM-1 à 3). JAM-1 est localisée au niveau des JS[127, 174] et semble jouer un rôle dans la perméabilité paracellulaire et dans les interactions avec les leucocytes lors de la transmigration de ces derniers à travers l'endothélium[175-178].

Les protéines transmembranaires des JS interagissent avec de nombreuses protéines intracellulaires qui régulent leur expression, leur localisation et leur fonction, et qui contrôlent l'association des JS avec les microfilaments d'actine du cytosquelette[179-187]. Les protéines Zona Occludens (ZO-1, -2 et -3) sont des protéines adaptatrices intracellulaires de la famille des MAGUK (*Membrane Associated Guanylate Kinase)* qui s'associent aux protéines membranaires des JS. Les ZO forment des plateformes de protéines intracellulaires qui stabilisent les JS, s'associent à d'autres molécules intracellulaires pour ancrer les JS à l'actine et participent à la signalisation intracellulaire qui contrôle et régule la fonction des JS[188-191]. Par contre, l'expression de ces protéines se retrouve dans de nombreux types cellulaires qui ne possèdent pas de JS. Ces protéines remplissent donc des fonctions variées dans les différents types cellulaires. Au niveau de la BHE, la perte de ZO-1 dans les JS est associée à la disruption des JS et à la perte de l'imperméabilité de cet endothélium[192, 193]. Plusieurs autres molécules intracellulaires participent également à la régulation des JS, telles que MAGI-1 et -3[194], MUPP-1[195], ZONAB[146, 155, 196-198], la cinguline[199-201] et RhoA[202-206].

Des nouvelles protéines qui interagissent et participent aux JS sont constamment identifiées, comme ESAM[207], LYRIC[208], CAR[195], la tricelluline[209, 210], AF-6[211] et CASK[212, 213]. Les JS forment des complexes macromoléculaires dynamiques qui limitent la diffusion paracellulaire et sont exprimés au niveau des barrières cellulaires comme les épithéliums et les endothéliums restrictifs (incluant la BHE). La composition exacte des JS est toujours à l'étude, et semble différer avec le type cellulaire et l'emplacement anatomique. Au niveau de la BHE, les molécules les mieux étudiées des JS sont l'occludine, les claudines-3 et

-5, JAM-1 et ZO-1[132, 152]. Les JS limitent la perméabilité des monocouches de CE aux ions, aux molécules et aux cellules immunitaires. Les fonctions des JS ont été démontrées de plusieurs manières *in vitro*. Tout d'abord, l'expression des JS entraîne une augmentation de la résistance électrique d'une monocouche de CE de 50 à 500 fois [156]. De plus, leur expression est également liée à la diminution de la perméabilité à différentes molécules (dextrans de différentes tailles, sucrose, BSA, etc.)[156]. Enfin, les JS séparent les membranes des CE en membrane apicale et en membrane basale. C'est ainsi que les CE-BHE peuvent exprimer différentes molécules de façon polarisée, ce qui est important pour plusieurs systèmes de transporteurs.

Les CE-BHE expriment aussi un autre type de complexe intercellulaire qui participe à l'imperméabilité de l'endothélium cérébral, les jonctions adhérentes (JA) [214]. Ces complexes forment des liaisons entre les CE et régulent en partie la perméabilité paracellulaire[215-218]. Les JA sont formées des protéines transmembranaires cadhérines et PECAM-1/CD31 (*platelet derived cell adhesion molecule*) qui, à travers des interactions homophiliques, lient les CE entre elles. Les CE-BHE expriment les protéines VE-cadhérine (*Vascular endothelium cadherin*)[215], associée aux jonctions intercellulaires, et N-cadhérine (*neuronal cadherin*)[219], qui lie les CE aux péricytes. Ces cadhérines interagissent avec les protéines intracellulaires de la famille des caténines (alpha, bêta et p120-caténine)[220, 221], VE-PTP (*Vascular Endothelial Protein Tyrosine Phosphatase*)[222, 223], ZO-1[198, 224], vinculine[225], alpha-actinine[212] et plakoglobine[222] pour se lier aux fibres d'actine et moduler la perméabilité vasculaire. Les caténines sont aussi des protéines de signalisation intracellulaire, et elles peuvent agir en tant que facteur de transcription. Ainsi, ces protéines participent activement à l'expression et à la régulation des AJ. Les JA semblent également interagir avec les TJ, et pourraient jouer un rôle clé dans la formation et dans la régulation de ces dernières[226].

Les JS et les JA jouent un rôle dans la perméabilité aux molécules de petites et grandes tailles en agissant comme des barrières intercellulaires. Elles peuvent ainsi influencer la

transmigration des cellules immunitaires par la voie paracellulaire, et forment des pôles de signalisation pour réguler l'intégrité de la BHE [181, 215, 216, 227]. De plus, il existe de subtiles différences dans la composition des JS et des JA au niveau d'un même tissu[125]. En effet, la composition de ces jonctions diffère même dans les différentes structures du SNC. Actuellement, il n'y a que peu de données sur les propriétés de la BHE en fonction du site anatomique. Néanmoins, certaines différences ont été décrites entre les CE-BHE du parenchyme de la matière blanche, de la matière grise et celles de la moelle épinière[143]. D'autres études sont nécessaires pour mieux comprendre le rôle de la composition des jonctions intercellulaires selon le site anatomique et les conséquences de ces différences sur la fonction de la BHE.

Ces jonctions intercellulaires peuvent interférer avec la transmigration de cellules immunitaires à travers la BHE lorsque celles-ci empruntent le trajet paracellulaire. En effet, certaines études démontrent une perte transitoire des JS et des JA lors de la diapédèse des leucocytes[196, 228, 228, 229]. Les mécanismes exacts menant à la disruption des complexes intercellulaires sont toujours à l'étude. Les évènements de signalisation intracellulaire, la phosphorylation des protéines des JS et de multiples interactions avec des GTPases permettent de moduler les jonctions intercellulaires de la BHE[130, 145]. Lors de la diapédèse, la sécrétion de protéases par les leucocytes peut aussi contribuer au remodelage des jonctions intercellulaires[229].

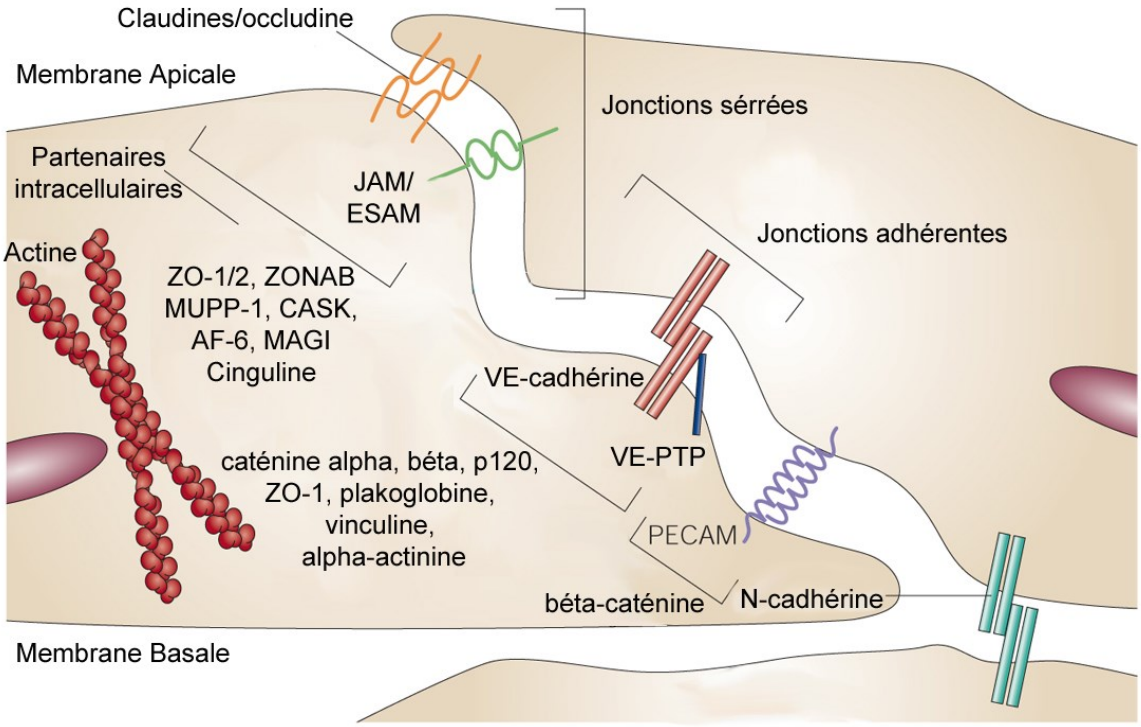

Figure 3. Jonctions intercellulaires de la BHE.

Les CE-BHE expriment plusieurs types de complexes macromoléculaires qui lient les CE ensemble et assurent l'étanchéité de l'endothélium. Les JS sont formées des claudines et de l'occludine. Ces protéines sont associées à plusieurs molécules de signalisation et de liaison à l'actine, telles que ZO-1/2, ZONAB, CASK, AF-6, MAGI et la cinguline. Les JA sont composées des protéines PECAM et VE-cadhérine, et sont associées au cytosquelette par les protéines caténines, ZO-1, plakoglobine, vinculine et alpha-actinine. N-cadhérine est exprimée par les CE et les péricytes et stabilise les interactions de ces deux types cellulaires. Schéma adapté de Dejana E *et al.*[198].

1.2.3.3 Molécules d'adhérence et diapédèse

Les jonctions intercellulaires spécialisées des CE-BHE contribuent à l'étanchéité de la BHE et sont un des mécanismes moléculaires importants qui participent au privilège immunitaire du SNC. Cependant, les interactions entre les leucocytes et les CE, qui illustrent la capacité transmigratoire, dépendent également de plusieurs autres facteurs. Dans les années 1990, un modèle en quatre étapes a été établi pour illustrer les interactions entre les parois vasculaires et les leucocytes, et expliquer la circulation des leucocytes du sang vers différents organes à travers l'endothélium vasculaire[230, 231]. Ce processus transmigratoire (diapédèse) permettant de traverser les CE est un moment critique et irréversible de toute réaction inflammatoire (Figure 4).

Dans un premier temps, les leucocytes du sang se mettent à rouler et à ralentir sur la paroi vasculaire. Ce phénomène dépend des sélectines exprimées sur les CE et de leurs ligands sur les leucocytes. Ces protéines, P- et E-sélectine sur les CE et L-sélectine sur les leucocytes, forment des interactions de faibles forces avec leurs ligands PSGL-1, ESL-1 et GLYCAM-1/MadCAM-1, respectivement. Ces interactions permettent aux leucocytes de ramper sur l'endothélium, sans toutefois être arrêtés [232-235]. Ce rapprochement donne ainsi aux leucocytes l'occasion de sonder la paroi vasculaire.

Dans un second temps, ces interactions favorisent la sécrétion et la présentation de chimiokines par les CE[236-238]. Ces agents sécrétés ont des propriétés chimiotactiques qui facilitent le recrutement local des leucocytes. Les chimiokines présentées à la surface apicale des CE activent les intégrines sur les leucocytes du sang. Cette activation augmente l'affinité des intégrines sur les leucocytes pour leurs ligands exprimés par les CE. Les chimiokines augmentent aussi l'expression de nombreuses molécules par les CE, telles que les sélectines et les molécules d'adhérence. Ces phénomènes favorisent le recrutement et l'attachement local de leucocyte sur la paroi vasculaire.

La troisième étape permet l'immobilisation du leucocyte sur la paroi vasculaire. Cette étape dépend des molécules d'adhérence des CE et de leurs ligands sur les leucocytes, les intégrines[239, 240]. En présence de facteurs inflammatoires (cytokines et chimiokines), l'expression des molécules d'adhérence sur les CE augmente de façon concomitante à l'augmentation de l'affinité des intégrines sur les leucocytes, ce qui amène à des interactions de haute affinité entre le leucocyte et l'endothélium, causant ultimement l'arrêt complet du leucocyte. Les molécules d'adhérence classiques des CE sont ICAM-1 (*intercellular cell adhesion molecule-1*)[241] et VCAM-1 (*vascular cell adhesion molecule-1*)[240, 242], qui se lient respectivement aux hétéro-dimères d'intégrines LFA-1 (*leucocyte function associated-1*, alphaL beta2) et VLA-4 *(very late antigen-4*, alpha4 beta1) sur les leucocytes.

La dernière étape est la transmigration, ou diapédèse, du leucocyte à travers l'endothélium. La transmigration est la seule étape de ce modèle qui est irréversible. La diapédèse peut se faire par la voie transcellulaire (à travers la CE) ou paracellulaire (entre deux CE). Dans cette étape, les molécules d'adhérence, les intégrines et les jonctions intercellulaires jouent un rôle important[230, 231]. À l'état physiologique, les CE-BHE sont quiescentes et n'expriment que très faiblement les molécules qui favorisent les interactions avec les leucocytes. C'est ce qui limite l'accès des cellules du sang au SNC[243].

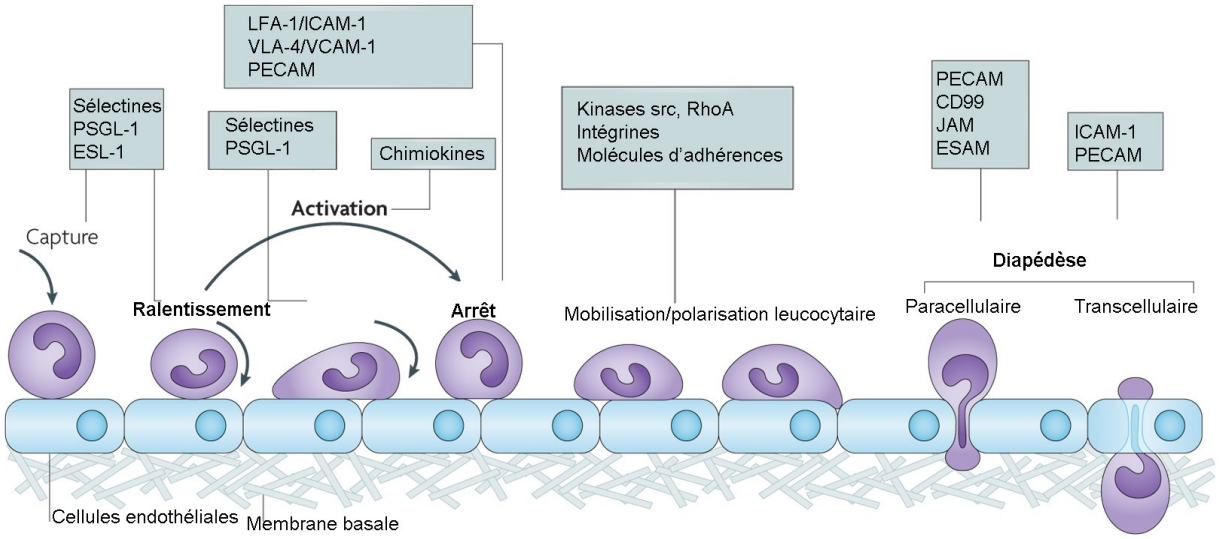

Figure 4. Modèle de la transmigration d'un leucocyte à travers un endothélium.

Les quatre étapes originales sont indiquées en caractères gras, soit: 1) le **ralentissement** du leucocyte qui dépend des sélectines et de leurs ligands; 2)**l'activation** du leucocyte qui est faite par les chimiokines présentées localement; 3)**l'arrêt** du leucocyte sur la paroi vasculaire qui dépend des molécules d'adhérence et des intégrines; 4)la **diapédèse** du leucocyte à travers la CE, par la route transcellulaire ou paracellulaire, qui dépend, entre autres des molécules d'adhérence. La figure présentée ici inclut certains raffinements au modèle des années 1990, comme les étapes de capture, de mobilisation et de polarisation, ainsi que certaines des nouvelles protéines impliquées dans ces processus (PECAM, ESAM, CD99). Schéma adapté de Ley K *et al*.[244].

1.2.3.4 Nouveaux développements

Depuis les années 90 le modèle de la diapédèse c'est raffiné. Chacune des étapes de ce modèle se révèle être beaucoup plus complexe et dynamique. Tout d'abord, de nouvelles protéines ont été identifiées à chacune de ces étapes, mais on observe aussi des variations qui dépendent des types cellulaires étudiés (provenance des CE et type de leucocytes à l'étude) ou des conditions expérimentales (infection, inflammation ou surveillance immunitaire) [244-246]. Par exemple, il existe de nombreuses cytokines et chimiokines qui sont sécrétées à différentes concentrations selon le type cellulaire et les conditions d'études. Chacun de ces facteurs a des effets sensiblement différents, parfois redondants ou antagonistes. On peut donc concevoir que les différentes combinaisons de facteurs sécrétés peuvent influencer grandement (faciliter ou limiter) l'activation locale du leucocyte, la diapédèse et l'infiltration tissulaire.

Les contacts entre les CE et les leucocytes seraient plutôt similaires aux interactions dynamiques entre deux velcros et impliqueraient une multitude de différentes protéines. Ces contacts intimes permettraient la transmission directe des messages en provenance de la CE au leucocyte et vice-versa, à travers les interactions de protéines membranaires et la sécrétion/présentation locale de médiateurs solubles, comme les chimiokines[247, 248]. Au niveau des CE, on commence à comprendre certains des évènements clés de ce processus. Les molécules d'adhérence servent d'attache pour le leucocyte, mais transmettent aussi des signaux intracellulaires essentiels à la diapédèse[249, 250]. Dans le cas d'ICAM-1, une des molécules d'adhérence prototypiques liée au cytosquelette par des protéines adaptatrices, la liaison et l'agrégation de cette protéine active de nombreuses protéines intracellulaires (comme la kinase p60 *src*), mobilise le Ca^{2+} intracellulaire, active la GTPase Rho, altère la phosphorylation de VE-cadhérine et affecte de nombreux procédés cellulaires comme la contractilité cellulaire et la force des jonctions intercellulaires[251-255]. D'autres molécules d'adhérences, et les protéines intercellulaires auxquelles elles sont

associées, peuvent aussi participer aux signaux intracellulaires. Il s'agit entre autres de PECAM-1/CD31[177, 256], VCAM-1[253, 257] et JAM-1[174, 177, 178]. Ces signaux sont essentiels à la diapédèse, puisque l'inhibition de ces voies de signalisation intracellulaire limite la transmigration. Plusieurs études tentent actuellement d'élucider les différents signaux intracellulaires initiés par les molécules d'adhérence, ainsi que leurs rôles dans la diapédèse. Les contacts entre les leucocytes et les CE sont très complexes et peuvent être comparés aux contacts entre une cellule T et une CPA dans le système immunitaire (ce qu'on appelle la synapse immunitaire) [258, 259]. Des études récentes démontrent le dynamisme de ces contacts, pendant lesquels des extensions leucocytaires sondent activement la paroi vasculaire afin de coordonner la diapédèse à travers la CE[260, 261]. Le leucocyte peut ainsi se positionner avant de se polariser, pour finalement entamer la diapédèse. Les interactions entre les CE et les leucocytes influencent énormément et peuvent moduler le degré d'activation et de maturation des leucocytes lors de la diapédèse. C'est le cas des monocytes, qui maturent pour devenir des CPA professionnelles lors du passage à travers les CE-BHE[262].

La diapédèse est un processus très complexe qui requiert une coordination très précise entre le leucocyte et la CE. Ce n'est que récemment que l'on a reconnu le rôle proactif de la CE dans la diapédèse, qui subit une réorganisation radicale de sa morphologie pour faciliter le passage du leucocyte[263]. Des études *in vitro* caractérisent actuellement une structure macromoléculaire, la coupe transmigratoire (*transmigratory cup*). Cette structure membranaire qui entoure le leucocyte présente un enrichissement local de nombreuses molécules d'adhérence, de signalisation et du cytosquelette[244, 247, 248, 253, 263, 264]. Les interactions entre le leucocyte et la CE facilitent le rassemblement tridimensionnel (*3-D clustering*) des molécules d'adhérence dans la coupe transmigratoire, ce qui optimise la propagation des signaux inter- et intra-cellulaires nécessaires à la diapédèse. Ces agrégats de molécules d'adhérence sont associés à l'actine et à de multiples protéines de signalisation telles que *RhoA* et les protéines ERM-*ezrin/radixin/moesin*[253]. Les signaux intracellulaires contribuent, entre autres, à la réorganisation du cytosquelette des CE, à la dissolution des jonctions intercellulaires et à leur

réassemblage ultérieur. Le changement de contractilité et de morphologie de la CE dépend de la protéine myosine et facilite la transmigration. Quant à la dissolution et au réassemblage des jonctions intercellulaires, des mécanismes complexes d'internalisation et de recyclage de la membrane des CE sont impliqués lors de la diapédèse[265, 266].

Durant la diapédèse, le leucocyte peut emprunter deux routes: la route paracellulaire ou la route transcellulaire[248, 260, 267, 268]. Dans la route paracellulaire, la route prototypique, les jonctions intercellulaires sont complètement dissoutes, puis réassemblées après le passage de la cellule immunitaire[269]. Les leucocytes peuvent aussi prendre la voie transcellulaire, par laquelle ils traversent le cytoplasme de la CE par la formation d'un trou ou pore transcellulaire[267]. Ces deux routes semblent employer une coupe transmigratoire, mais les protéines impliquées et les mécanismes moléculaires sous-jacents diffèrent sensiblement. Les rôles des protrusions leucocytaires, des protéines ERM, des cavéolines, de l'internalisation et du recyclage des membranes de la CE paraissent plus critiques dans la diapédèse transcellulaire que dans le processus paracellulaire[265, 269-271]. L'implication de ces nouvelles observations n'est pas encore claire, et la route de diapédèse reste un sujet de recherche très actif. *In vitro,* dans les CE micro-vasculaires comme les CE-BHE, on retrouve 70 % de transmigration paracellulaire et 30 % de transmigration transcellulaire[261, 267]. Le leucocyte doit ensuite franchir la MB des vaisseaux, un processus qui est étudié actuellement, et qui semble dépendre de la présence de facteurs chimiotactiques, de constituants de la MB, d'intégrines et d'enzymes de dégradation leucocytaire[244, 272]. Certaines de ces enzymes de dégradation comme la MMP2 et la MMP-9 (*Matrix Metalloproteinase*) sont essentielles pour l'infiltration leucocytaire dans le SNC. Ces enzymes permettent la dégradation des protéines des jonctions intercellulaires et de la membrane basale pour ainsi permettre l'infiltration tissulaire[229, 273, 274].

On identifie régulièrement de nouvelles protéines impliquées dans les interactions entre les leucocytes et les CE et le modèle de la transmigration cellulaire est constamment raffiné (Figure 5). D'intérêt pour le traitement des maladies où une réaction

inflammatoire est préjudiciable pour l'hôte, l'inhibition d'une des étapes qui mène à la diapédèse permet de limiter l'infiltration des cellules inflammatoires dans l'organe cible et donc les dommages qui en dépendent. Une telle stratégie a démontré son efficacité dans la SEP avec le natalizumab. Plusieurs études utilisant des anticorps bloquants ou la délétion génétique ciblent de nouvelles protéines des CE, qui s'ajoutent à ICAM-1 et à VCAM-1 comme des molécules importantes dans la diapédèse et des cibles thérapeutiques potentielles[275, 276]. Ces nouvelles molécules d'adhérence incluent ICAM-2[277], JAM-1[174, 177, 178], ESAM[278], PECAM[177, 256, 279], CD99[280], CD44[281], CD47[282], CD73[283], VAP-1[283].

Comment se fait-il que l'on découvre autant de différentes molécules transmembranaires impliquées dans la diapédèse? Certaines hypothèses suggèrent que chaque molécule est nécessaire de façon séquentielle, d'autres s'en tiennent au concept du velcro et certaines proposent que chaque type cellulaire et chaque organe cible utilise sa propre combinaison de protéines[247, 248]. Par contre, il est aussi possible que toutes ces molécules participent dynamiquement pour former un complexe multimoléculaire, ou une série de complexes, qui permet la formation d'une plateforme de diapédèse[248]. Cette dernière hypothèse est très élégante car elle se rapproche des mécanismes que l'on retrouve dans les contacts intercellulaires rapprochés, où l'on observe fréquemment la présence de complexes macromoléculaires dynamiques (jonctions intercellulaires, synapse immunologique et synapse nerveuse). Ces idées sont toutes intéressantes, et seules des études futures pourront répondre à ces hypothèses. Par contre, la découverte de nouvelles molécules impliquées dans la diapédèse reste clé: non seulement elle conduit à une meilleure compréhension des évènements moléculaires, mais elle propose surtout de multiples cibles potentielles pour limiter l'accumulation de cellules inflammatoires dans un organe cible.

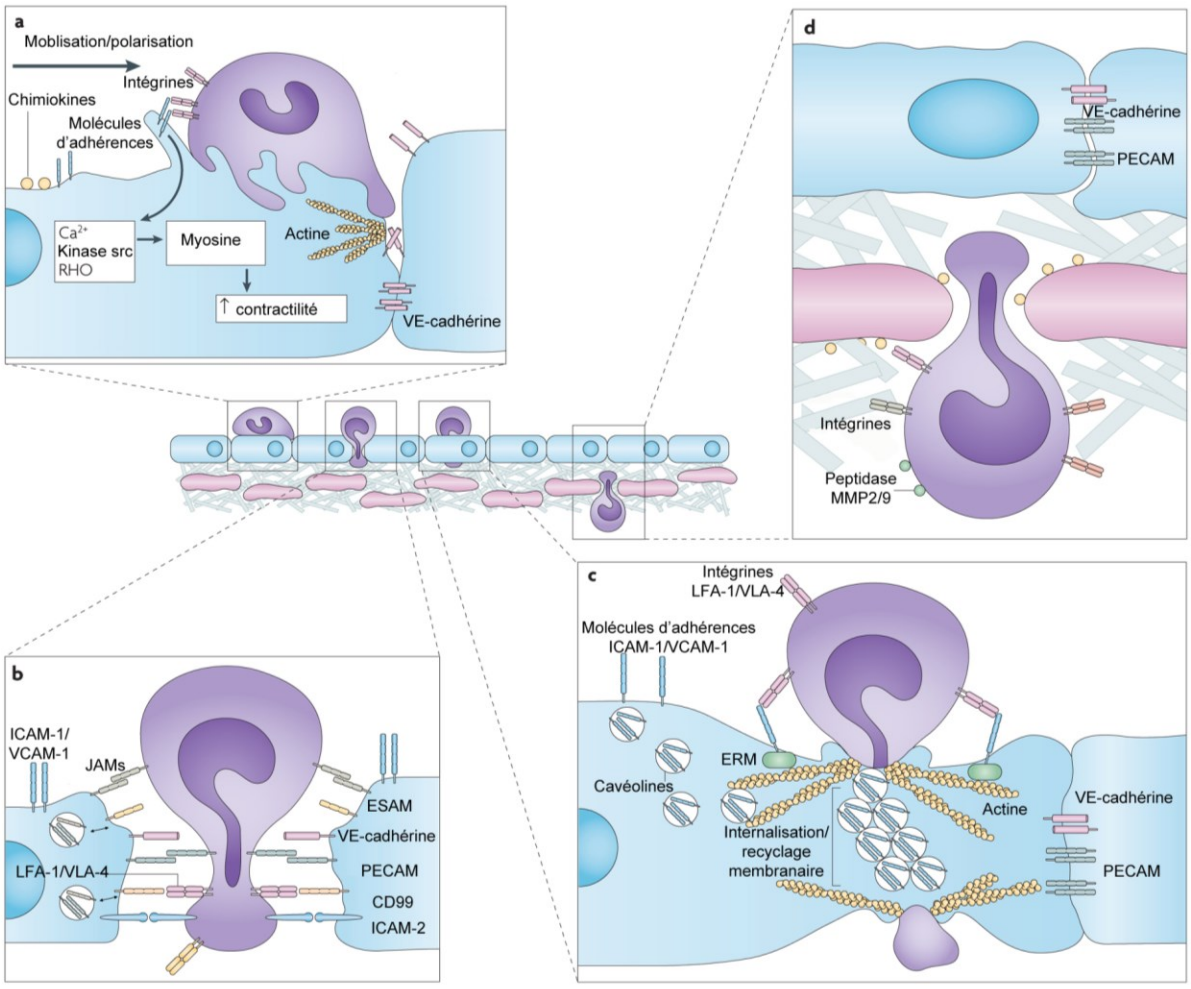

Figure 5. Nouveaux développements concernant la diapédèse.

La diapédèse est un évènement très complexe. (**a**) Le leucocyte interagit avec la CE à travers des contacts intimes et évanescents. Ces contacts, où les molécules d'adhérence se lient aux intégrines, permettent la transmission de signaux intracellulaires qui affectent le Ca2+, la kinase *src* et la protéine *Rho* pour jouer sur la contractilité cellulaire et préparer la

diapédèse. (**b**) La route classique, paracellulaire, implique la dissolution des complexes intercellulaires et de multiples interactions entre la CE et le leucocyte. Les protéines PECAM, ESAM, CD99, JAM-1, ICAM-2 sont parmi les protéines impliquées. (**c**) La route transcellulaire utilise certaines molécules d'adhérence pour se frayer un chemin à travers le cytoplasme de la CE. Des phénomènes d'internalisation et de recyclage membranaires participent activement lors de la transmigration transcellulaire pour former un canal pour le leucocyte. Les protéines ERM sont des candidates qui semblent être impliquées dans la liaison des molécules d'adhérence au cytosquelette. (d) La migration à travers la MB est une étape importante de l'infiltration de l'organe sous-jacent. Les leucocytes migrent entre les péricytes, interagissent avec la matrice extracellulaire et avec les intégrines et facilitent le passage au travers de la MB par l'expression de protéases, telles que MMP-2 et MMP-9. Schéma adapté de Ley K *et al.*[244].

1.2.4 Le rôle des cellules gliales dans l'UNV

Bien que les propriétés fonctionnelles de la BHE se traduisent au niveau des CE, le microenvironnement maintenu au sein du SNC joue également un rôle dans la modulation et l'induction des propriétés de la BHE. Les capillaires de la BHE forment le seul endothélium en contact rapproché avec les cellules gliales, les péricytes et les neurones. Dans les vaisseaux de plus grande taille (artères et veines), une couche de cellules musculaires limite les contacts entre l'endothélium et le tissu sous-jacent. Dans les capillaires et les microvaisseaux du SNC, plus de 90 % de la surface basale des CE est en contact avec le parenchyme cérébral[130]. Ces contacts privilégiés, surtout ceux qui existent entre les pieds astrocytaires et les CE, permettent des interactions locales bidirectionnelles entre les cellules du SNC et la vasculature, et ainsi une modulation fine des propriétés de la BHE[114, 116, 130, 132, 145, 153]. Ces études ont démontré l'importance de nombreux facteurs membranaires et sécrétés, provenant des péricytes et des cellules gliales, qui modulent la BHE. Ces facteurs influencent l'expression de transporteurs membranaires, modulent les jonctions intercellulaire et gardent les CE-BHE dans un état basal, quiescentes, en limitant l'expression des sélectines, des molécules d'adhérence et des cytokines et chimiokines. Ainsi, ces facteurs agissent localement et peuvent expliquer en partie les propriétés spécifiques de la vasculature cérébrale.

Les péricytes sont des cellules adjacentes aux microvaisseaux qui partagent la membrane basale avec les CE. Dans le SNC on estime qu'il y a un péricyte pour trois CE, ce qui représente un enrichissement important par rapport aux autres organes, tels que les muscles où le ratio est de 1:100[145]. Les péricytes contribuent à la stabilité mécanique des microvaisseaux par le dépôt de matrice extracellulaire[284]. En plus, les péricytes sécrètent de nombreux facteurs qui influencent l'angiogenèse et qui gardent les CE matures quiescentes[285-287]. Les mécanismes moléculaires par lesquels ces cellules influencent l'endothélium cérébral restent mal compris. Cependant, les péricytes ont la capacité de sécréter de nombreux facteurs de croissance et facteurs angiogéniques susceptibles d'influencer la BHE. Les agents tels que

le TGF-bêta (*tumor growth factor-beta*), l'angiopoétine, le PDGF-bêta (*platelet derived growth factor-beta*) et le Wnt peuvent moduler les propriétés de la BHE et influencent l'étanchéité de cette dernière[285, 287-291]. Quoique les interactions CE-péricytes soient essentielles au développement de la BHE[292], elles sont pour l'instant mal décrites, surtout en ce qui concerne leur rôle dans les réactions neuroinflammatoires.

Les astrocytes sont les cellules gliales qui forment le plus de contacts avec l'endothélium cérébral, à travers des interactions très complexes et finement régulées entre ces deux types cellulaires. Les astrocytes peuvent moduler plusieurs des propriétés de la BHE, et peuvent ainsi influencer l'expression des jonctions intercellulaires (imperméabilité, des transporteurs (transport de l'eau, des ions et des nutriments), les systèmes enzymatiques (barrière métabolique) et la quiescence des CE (barrière immunologique) [116, 293-297]. Les astrocyte jouent aussi un rôle dans la régulation de la prolifération, du contrôle du flot sanguin et de l'angiogenèse[298, 299]. Les CE isolées à partir de spécimens du SNC perdent les propriétés restrictives de la BHE après plusieurs passages en culture *in vitro,* à moins qu'on ajoute à la culture un milieu conditionné par les astrocytes qui permet de récupérer ces propriétés (imperméabilité, expression de P-gp et autres transporteurs) [295-297, 300]. De même, des CE qui proviennent d'un autre organe peuvent acquérir des propriétés restrictives similaires à celles de la BHE lorsqu'elles sont mises en culture en présence de milieu conditionné par les astrocytes[296, 301]. Ainsi, à l'état physiologique, les facteurs astrocytaires sont clés dans la potentialisation des propriétés restrictives des CE-BHE. Les contacts entre les astrocytes et les CE se font à travers les pieds astrocytaires, une structure cellulaire spécialisée où plusieurs protéines sont enrichies, telle qu'aquaporine-4 (AQP-4), Kir4.1, dystroglycane et agrine[114, 132]. À travers ces structures, les astrocytes peuvent sécréter de nombreux facteurs susceptibles d'influencer les CE-BHE. Ces facteurs incluent l'endothéline (ET-1), la sérotonine (5-HT), le TGF-bêta, le GDNF (*glial derived growth factor*), le b-FGF (*beta-fibroblast growth factor*) et l'angiopoétine (ANG1 et son récepteur TIE-1)[114, 116, 132, 293, 302-306]. Les CE peuvent elles aussi sécréter des facteurs, comme le LIF (*leukemia inhibitory factor*) qui agit sur les astrocytes. Il

peut y avoir ainsi des boucles de rétroaction complexes qui permettent aux deux types cellulaires de s'influencer mutuellement (Figure 2)[307]. Les effets des CE sur les astrocytes restent peu connus. Les astrocytes participent aussi aux synapses neuronales, contribuent aux nœuds de Ranvier, modulent les influx nerveux et peuvent coupler le flot sanguin à l'activité neuronale[114]. Dans l'UNV, l'astrocyte semble donc être un type cellulaire qui module les propriétés de la BHE à travers une multitude de mécanismes moléculaires possiblement redondants. De nouvelles molécules qui participent aux échanges entre les CE et les astrocytes, actuellement à l'étude, semblent influencer la BHE, entre autres à travers les JS. Il s'agit de SSeCKS (*Scr-suppressed C kinase substrate*) [299], meteorin[308] et les membres de la famille Hedgehog[309]. Dans le laboratoire, la BHE est étudiée en utilisant des modèles *in vitro* et *in vivo*. Pour reproduire le mieux possible les conditions physiologiques *in vivo*, les CE cérébrales humaines sont mises en culture en présence de milieu conditionné par les astrocytes. Ainsi, les monocouches de CE-BHE étudiées *in vitro* sont en présence de facteurs astrocytaires, ce qui augmente leurs propriétés restrictives et reproduit les propriétés de la BHE (imperméabilité, transporteurs, jonctions intercellulaire, état quiescent).

Les neurones du cerveau sont des cellules très actives qui requièrent une régulation fine de leur apport métabolique et de leur microenvironnement. Cette régulation se fait à travers l'UNV et la BHE. Les neurones peuvent influencer le flot sanguin et la perfusion sanguine, qui sont augmentés avec l'activité neuronale[310]. L'UNV et la BHE sont potentiellement influencées par un grand nombre de substances sécrétées par les neurones, tels les neurotransmetteurs adrénergiques, sérotoninergiques, cholinergiques et GABA-ergiques[152]. Bien que les mécanismes moléculaires des interactions entre les CE-BHE et les neurones restent mystérieux, il est clair que ces derniers peuvent moduler les propriétés de l'UNV.

La matrice extracellulaire des membranes basales (MB) de l'UNV (MB endothéliale interne et gliale externe) interagit avec les CE-BHE et contribue à la structure de la vasculature et à son imperméabilité. En effet, la matrice extracellulaire forme une surface

d'ancrage pour les CE qui interagissent avec les protéines de la matrice (collagène IV, laminine, protéoglycanes) à travers les intégrines exprimées sur la membrane basale des CE[311]. Ces interactions, qui ont une importance structurale, modulent plusieurs processus intracellulaires et peuvent même influencer l'expression des jonctions intercellulaires[312]. Les deux MB de l'UNV ont des compositions sensiblement différentes. la MB endothéliale est riche en laminine alpha 4 et 5, alors que la membrane parenchymateuse est enrichie en laminine alpha 1 et 2[313, 314]. De plus, la matrice extracellulaire peut présenter les facteurs solubles localement sécrétés pour potentialiser leur efficacité paracrine en augmentant leur concentration locale. Le rôle de ces MB et l'importance de leurs différents constituants sont des sujets de recherche intéressants, surtout en ce qui concernent leur influence sur l'infiltration leucocytaire[274, 315, 316].

En résumé, les cellules du SNC qui forment l'UNV supportent et contribuent activement et dynamiquement aux propriétés spécifiques des CE-BHE à travers, entre autres, la régulation des complexes intercellulaires et de la quiescence des CE.

1.3. La dérégulation des propriétés de la BHE lors des réactions neuroinflammatoires

1.3.1. La BHE et les réactions neuro-immunes

Bien que le terme de BHE suggère des propriétés statiques, la plupart des propriétés de la BHE sont dynamiques et sont modulées dans de nombreuses situations, particulièrement lors des réactions neuroinflammatoires[145, 309, 317]. Lors de réactions aiguës, la BHE devient localement plus perméable et facilite transitoirement la transmigration des leucocytes au cerveau et la surveillance immunitaire. Les réactions immunitaires aiguës dans le SNC sont

généralement bénéfiques et autolimitées. Par contre, dans des réactions inflammatoires chroniques, le processus inflammatoire est exacerbé et affecte toutes les cellules environnantes du SNC, qui sont très sensibles aux changements du microenvironnement.

C'est le cas dans la SEP, où l'on retrouve des leucocytes, de multiples altérations cellulaires incluant une dérégulation de l'UNV, une démyélinisation ainsi que des dommages aux axones des neurones au sein des plaques de démyélinisation [318, 319]. La séquence exacte des étapes de la formation des plaques de SEP n'est pas encore établie mais les changements de la BHE semblent prendre place très tôt dans la formation de ces lésions. Les CE-BHE participent à l'exacerbation locale de la réaction inflammatoire, et favorisent ainsi la création de la plaque de SEP[44, 320]. En effet, on observe au sein des plaques de SEP une diminution de l'étanchéité de la BHE, une altération de l'expression des transporteurs et une perte de la quiescence des CE[321]. Ces changements se traduisent par une altération des jonctions intercellulaires et des transporteurs, par une fuite de protéines plasmiques dans le parenchyme tissulaire et par l'infiltration de cellules inflammatoires dans l'espace périvasculaire et dans le SNC[48, 52, 117, 317, 322-324]. Les leucocytes infiltrants sécrètent des médiateurs inflammatoires et d'autres facteurs qui endommagent les oligodendrocytes et les cellules gliales, permettent la présentation d'antigènes de la myéline et activent localement les leucocytes. Les cellules gliales endommagées se mettent également à sécréter des médiateurs inflammatoires et perpétuent la réaction inflammatoire locale. On suppose qu'un cercle vicieux prend place, dans lequel les changements des jonctions intercellulaires et les perturbations des fonctions de la BHE permettent une accumulation de cellules inflammatoires, qui à leur tour affectent le parenchyme cérébral et l'UNV. Ces phénomènes entraînent la démyélinisation, limitent la remyélinisation, favorisent les dommages aux neurones et augmentent l'astrogliose[309, 317, 318, 325, 326]. Les mécanismes exacts qui régissent les dommages cellulaires sont très complexes et dépendent entre autres des types de leucocytes qui infiltrent le SNC. Récemment, on a noté l'importance des cellules Th17, un sous-type de lymphocytes T, qui jouent un rôle important dans les réactions neuroinflammatoire[78, 327-329]. Il faut également souligner que les CPA et les

différents sous-types de lymphocytes T et de lymphocytes B sont également présents dans les plaques de SEP[245, 262, 320, 330-333].

Dans la SEP les atteintes de la BHE visualisée *in vivo* par l'IRM semble plutôt transitoire, parfois récurrente et peuvent prendre place plusieurs fois au même endroit ou à différents endroits[41]. Étant donné qu'il est très ardu de lier les observations cliniques aux observations pathologiques chez l'humain, la plupart des hypothèses sur la physiopathologie des plaques de SEP sont inférées à partir d'études sur des échantillons post-mortem et à partir d'extrapolation des modèles animaux[42].

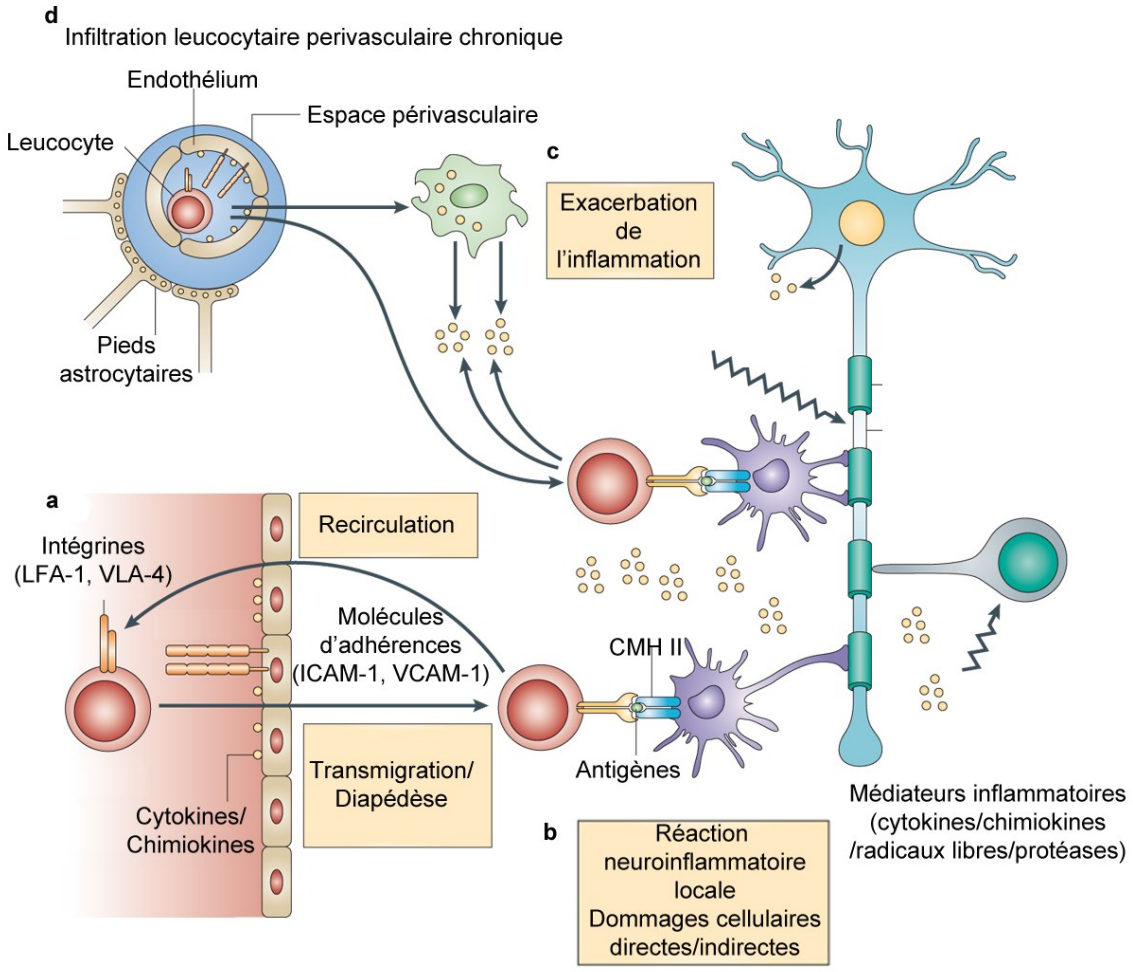

Figure 6. Modèle simplifié de la physiopathologie des plaques de SEP.

(**a**) Des leucocytes traversent la BHE et plutôt que de re-circuler dans la périphérie, infiltrent le SNC, où ils reconnaissent des peptides de la myéline et des neurones à travers le CMH de classe II. C'est ce qui cause une réaction neuro-immune inflammatoire locale. (**b**) Cette réaction immunitaire est caractérisée par l'activation des leucocytes infiltrant, qui sécrètent localement des médiateurs inflammatoires. Les médiateurs et les leucocytes affectent et endommagent, directement et indirectement, les cellules gliales et neuronales qui, à leur tour,

se mettent à sécréter des facteurs inflammatoires. (**c**) Les dommages cellulaires et la sécrétion importante de facteurs inflammatoires par les leucocytes et les cellules gliales exacerbent et perpétuent la réaction inflammatoire locale, ce qui affecte les cellules du SNC et de l'UNV. (**d**) Ceci amène à l'accumulation pathologique de leucocytes dans l'espace périvasculaire, à des dommages aux oligodendrocytes et aux axones de neurones. Ces changements se traduisent macroscopiquement par des plaques focales de démyélinisation et cliniquement par des déficits neurologiques. Schéma adapté de Steinman L *et al.*[326].

1.2.6 Altération de la perméabilité de la BHE dans les lésions de SEP

Dans les lésions de SEP et d'EAE, on note une perte locale de l'imperméabilité de la BHE, qui se manifeste par une accumulation de protéines sériques dans le SNC et de multiples altérations au niveau de l'UNV et de la BHE[334]. Dans les plaques de SEP, on retrouve une accumulation pathologique de protéines sériques dans le parenchyme cérébral (albumine, IgG, fibrinogène). Il en va de même dans les lésions d'EAE[335, 336]. De plus, on peut démontrer dans l'EAE une infiltration de différents colorants et traceurs moléculaires injectés dans le sang dans le parenchyme des lésions (seulement lors des réactions neuroinflammatoires et pas à l'état physiologique). Cette augmentation de la perméabilité de la BHE semble dépendre d'une altération des jonctions intercellulaires et de la membrane basale.

Bien que tous les mécanismes moléculaires qui modifient la perméabilité de l'endothélium ne soient pas bien compris, on observe systématiquement des altérations au niveau des JA et des JS de la BHE dans les plaques de SEP. Le laboratoire du Dr Alexandre Prat, ainsi que d'autres groupes, ont démontré une perte de la cohésion et de la continuité des brins de JS dans les lésions de SEP par rapport à la matière blanche d'apparence normale chez les patients de SEP ou à la BHE de cerveaux normaux. On observe, entre autres, des altérations de l'expression d'occludine, de claudine-5, de VE-cadhérine, des caténines et de ZO-1[48, 48, 307, 309, 324, 334, 337-340].

Dans les plaques de SEP, la MB qui entoure les micro-vaisseaux de la BHE est altérée. On peut en effet observer une altération de la structure et de la composition de cette couche de matrice extracellulaire, qui est fréquemment irrégulière et présente une altération de ses composantes (surtout des laminines 411 et 511)[274, 311, 313-315]. Les conséquences de ces modifications de la MB sont à l'étude. L'hypothèse actuelle est que cette membrane n'a pas seulement un rôle de support structurel, mais qu'elle peut aussi activement influencer la perméabilité vasculaire et la transmigration leucocytaire au SNC.

1.2.7 Médiateurs inflammatoires et altérations de l'UNV

Lors de réactions neuroinflammatoires, l'expression des cytokines et des chimiokines est localement augmentée dans le tissu lésé, ce qui entraîne l'infiltration et l'accumulation de leucocytes dans l'espace péri-vasculaire. Les cellules inflammatoires de l'espace péri-vasculaire sont formées, entre autres, de CPA, de lymphocytes B, de lymphocytes cytotoxiques et de lymphocytes Th1 et Th17[76, 78, 331]. Ces nombreux types cellulaires interagissent entre eux et sécrètent à leur tour de nombreux médiateurs inflammatoires. On retrouve ainsi une sécrétion et un enrichissement local de différentes cytokines et chimiokines dont l'IL-1-bêta, l'IL-17, l'IL-22, l'IFN-gamma, le GM-CSF, le TNF-alpha et le MCP-1. Ces médiateurs s'accumulent localement dans la matrice extracellulaire et à la surface des cellules environnantes, et permettent de perpétuer et de potentialiser l'activation des leucocytes. En réponse à ces médiateurs inflammatoires, les différents sous-types cellulaires de l'UNV subissent des dommages et sécrètent des facteurs inflammatoires ou chimiotactiques. C'est le cas des astrocytes et des CE-BHE, qui augmentent leur sécrétion de différents médiateurs suite au contact des facteurs inflammatoires leucocytaires comme l'IL-1, le TNF-a, l'IFN-g et l'IL-17. Les cellules gliales et les CE-BHE peuvent donc devenir des sources importantes d'IL-6, d'IL-1, de GM-CSF, de MCP-1/CCL-2, de MIP-1a/CCL3, de RANTES/CCL5 et d'IL-8/CXCL8[238, 341-346]. Ces facteurs affectent à leur tour les leucocytes avoisinants, accentuant davantage leur activation et leur maturation. De plus, cette sécrétion par les cellules gliales est associée à une diminution des facteurs qui entretiennent une BHE étanche et quiescente[307, 317, 347]. Une boucle de rétroaction positive prend donc place au sein des plaques de SEP, ce qui est la cascade de base de l'exacerbation de la réaction immunitaire dans l'inflammation chronique.

Les leucocytes sécrètent aussi d'autres facteurs solubles et de nombreuses enzymes de dégradation, susceptibles d'influencer la perméabilité vasculaire en agissant sur les jonctions intercellulaires. Ces facteurs incluent le VEGF qui agit sur la phosphorylation des protéines

des TJ[222, 278, 348]. Les leucocytes expriment de nombreuses protéases, comme MMP-2 et MMP-9, qui peuvent dégrader les jonctions intercellulaires et les MB pour ainsi faciliter l'infiltration cellulaire au SNC[229, 349]. Par contre, certains médiateurs solubles qui ont des effets anti-inflammatoires favorisent la maturation des jonctions intercellulaires et la quiescence des CE-BHE. C'est le cas de l'IFN-bêta[350] et de l'IL-25[351]. C'est donc la combinaison et la concentration locale de ces différents facteurs qui modifient et influencent les propriétés de la BHE et de l'UNV pendant les réactions neuro-immunes.

1.2.8 Perte de la quiescence des CE-BHE

Comme mentionné plus haut, les CE sont affectées par le microenvironnement inflammatoire des plaques de SEP, soit via des effets directs sur les CE-BHE ou par l'entremise d'autres composantes de l'UNV. Les différents facteurs inflammatoires localement sécrétés agissent à plusieurs niveaux sur les CE-BHE pour favoriser la transmigration cellulaire. Les CE-BHE augmentent l'expression membranaire des molécules d'adhérence et sécrètent différents facteurs pro-inflammatoires, ce qui les prédispose à la diapédèse. Les médiateurs inflammatoires, surtout les chimiokines, s'accumulent sur la membrane des CE et dans la matrice extracellulaire avoisinante, ce qui potentialise leur effet localement.

Bien que différentes études remettent en question le rôle des sélectines dans l'infiltration des leucocytes au sein du SNC, ces protéines sont localement surexprimées dans les microvaisseaux de la BHE dans les lésions de SEP et sur les leucocytes des patients atteints de SEP[235, 352-356]. Les interactions entre sélectines semblent surtout importantes dans l'infiltration leucocytaire au niveau des microvaisseaux méningés. Actuellement, il n'existe pas de thérapie efficace qui cible les sélectines[325, 352]. Les chimiokines présentées sur la membrane apicale des CE recrutent localement les leucocytes et activent les récepteurs transmembranaires sur les leucocytes qui sondent la paroi vasculaire inflammée. L'interaction des chimiokines avec leurs récepteurs amène à une signalisation intracellulaire à travers les protéines Galphai, ce qui se

traduit par un changement de conformation au niveau des intégrines leucocytaires et une augmentation de leur affinité pour leurs ligands[357]. Au moins une dizaine de chimiokines différentes, dont le MCP-1/CCL2[358, 359], peuvent ainsi affecter les leucocytes. En même temps, les médiateurs inflammatoires influencent l'expression de multiples protéines des CE, ce qui amène à une surexpression des molécules d'adhérence sur la membrane apicale (comme c'est le cas pour les molécules d'adhérence prototypiques ICAM-1 et VCAM-1). Dans un tel contexte inflammatoire, les conditions sont idéales pour promouvoir les interactions entre les leucocytes du sang et les CE, et permettre ainsi l'infiltration leucocytaire. Par opposition à un environnement pro-inflammatoire l'UNV favorise la quiescence des CE-BHE en limitant l'expression de chimiokines et des molécules d'adhérence, ce qui n'encourage pas l'infiltration leucocytaire. Aujourd'hui, de nombreuses molécules participant à la diapédèse ont été identifiées. Il est intéressant de constater que certaines de ces protéines sont aussi surexprimées dans les lésions de SEP et incluent CD44, CD47, CD73 et VAP-1[282, 360-363].

Pour résumer, le processus inflammatoire local amène à des interactions complexes et dynamiques entre les leucocytes infiltrants et les cellules du SNC dans les lésions de SEP. Ceci entraîne la perte locale des propriétés restrictives de la BHE en agissant sur l'étanchéité et la quiescence de cette dernière. De plus, l'inflammation locale modifie le comportement des astrocytes et des autres cellules de l'UNV. Ces phénomènes facilitent le recrutement et l'infiltration locale de cellules immunitaires du sang vers les régions du SNC où le processus inflammatoire prend place. Ils favorisent également l'exacerbation et la chronicisation de la réaction neuro-immune. Les différents médiateurs inflammatoires sécrétés par les leucocytes et les cellules gliales elles-mêmes peuvent ensuite potentialiser davantage le processus inflammatoire local, chroniciser l'inflammation et causer des dommages aux oligodendrocytes et aux neurones.

1.3 La BHE comme cible thérapeutique pour limiter les réactions neuroinflammatoires

L'UNV est donc centrale dans les procédés neuroinflammatoires et les CE-BHE sont un des sous-types cellulaires clés qui semblent avoir la capacité de limiter ou de promouvoir localement l'inflammation. Il y a un intérêt grandissant à décrire les mécanismes moléculaires qui régissent les propriétés de la BHE, afin de tenter ensuite de les modifier pour influencer les réactions immunitaires au sein du SNC[69, 80, 105, 108, 110, 318, 325, 364-366].

1.3.1 Les thérapies actuelles et la BHE

Plusieurs des stratégies thérapeutiques utilisées en SEP ont des effets non-spécifiques sur la BHE. Dans les cas d'exacerbations aiguës de SEP, les stratégies se limitent souvent à la corticothérapie. Les corticostéroïdes modulent et limitent l'activation immunitaire, mais agissent aussi au niveau des CE-BHE en jouant sur divers mécanismes moléculaires, entre autres en favorisant la maturation des JS[367, 368]. L'IFN-bêta, l'un des traitements d'entretien de la SEP, possède plusieurs propriétés immuno-modulatrices. Il semble qu'un des mécanismes sous-jacents à l'IFN-bêta soit la stabilisation de la BHE[350, 363]. Les mécanismes moléculaires impliqués sont encore à l'étude et incluent la stabilisation des jonctions intercellulaires et la diminution de l'expression des molécules d'adhérence[369]. Plusieurs nouveaux médicaments oraux sont sur le marché depuis les deux dernières années. Ces composés immuno-modulateurs influencent le système immunitaire pour limiter les épisodes de poussées et la progression de la SEP. Les effets de ces médicaments oraux sur la BHE, s'il y en a, n'ont pas encore été étudiés. Enfin, le natalizumab est la seule stratégie qui cible directement et spécifiquement les interactions entre les leucocytes et l'endothélium cérébral. Tel que discuté plus tôt, c'est une stratégie thérapeutique efficace

qui présente des effets secondaires importants mais qui justifie, en quelque sorte, la BHE comme cible thérapeutique valable[87, 109-113, 370].

1.3.2 Les directions de la recherche actuelle

Plusieurs stratégies sont à l'étude pour identifier, cibler et moduler les fonctions de la BHE dans les réactions neuroinflammatoires. Les techniques de génomique et de protéomique permettent de trouver des groupes de protéines sur- et sous-exprimés dans ce contexte[366, 371-378]. Ainsi, lors d'un contact avec des médiateurs inflammatoires, on note dans les CE l'expression différentielle de protéines qui participent à plusieurs procédés cellulaires, incluant des protéines de signalisation intracellulaire, des protéines impliquées dans le réarrangement du cytosquelette, des protéines impliquées dans les jonctions intercellulaires et leur régulation et des protéines impliquées dans les interactions CE-leucocytes. Ces groupes de protéines sont donc d'intérêt pour influencer les fonctions de la BHE et identifier de nouvelles cibles thérapeutiques potentielles pour moduler les réactions neuroinflammatoires.

Objectifs du projet de doctorat

Les traitements actuels de la SEP sont imparfaits, ce qui explique l'intérêt grandissant pour la caractérisation de nouvelles stratégies et cibles thérapeutiques. Étant donné que l'étiologie et la physiopathologie de la maladie sont très complexes et encore mal comprises, beaucoup de projets de recherche sont consacrés à mieux comprendre les dérèglements moléculaires sous-jacents. Ce travail de doctorat s'inscrit dans ce contexte.

L'hypothèse générale de ce projet de doctorat est que la BHE influence et module les réactions neuroinflammatoires à travers plusieurs mécanismes moléculaires différents. La caractérisation de ces différents mécanismes moléculaires aidera à comprendre

le rôle de la BHE dans la physiopathologie des réactions neuro-immunes, confirmera l'attrait des CE-BHE comme cible thérapeutique valable et permettra d'éclaircir un peu le tableau complexe qui se dresse dans les réactions neuro-immunes où de multiples altérations prennent place, tant au niveau intracellulaire qu'entre les différentes cellules impliquées (UNV, leucocytes, neurones). Ce travail est d'autant plus pertinent qu'il ouvre de nouvelles avenues potentielles de recherche thérapeutique pour limiter les réactions neuroinflammatoires et réparer les dommages cellulaires au sein du SNC. Spécifiquement, ce travail étudie trois aspects distincts de la BHE : les interactions entre astrocytes et les CE-BHE dans la modulation des jonctions intercellulaires, les molécules d'adhérence de la BHE et les propriétés sécrétrices des CE-BHE.

En premier, un nouveau mécanisme de régulation est identifié, dans lequel l'angiotensinogène (AGT) sécrété par les astrocytes est métabolisé localement en angiotensine II (AGTII) qui agit localement sur l'endothélium cérébral pour y moduler les jonctions intercellulaires. L'AGT se révèle comme un facteur astrocytaire qui maintient l'étanchéité de la BHE en agissant sur la régulation des protéines des TJ. À travers les récepteurs AT1 des CE-BHE, l'AGT modifie la phosphorylation de l'occludine et sa distribution au sein de domaines membranaires spécialisés, les radeaux lipidiques. Ces effets se traduisent par un endothélium plus étanche. Par contre, dans les plaques de SEP et dans les souris qui n'ont pas d'AGT, on observe une diminution de l'AGT et une altération concomitante des JS. Ces résultats identifient un rôle local du système rénine-angiotensine dans l'UNV pour promouvoir une BHE imperméable, et démontrent une altération de ce système lors de réactions neuroinflammatoires. La modulation de cette boucle signalétique pourrait s'avérer d'intérêt pour moduler la perméabilité de l'endothélium cérébral. De plus ces travaux démontrent l'importance des radeaux lipidiques dans la régulation des propriétés de la BHE.

Dans un second temps, à partir d'une étude de protéomique des radeaux lipidiques des CE-BHE, une nouvelle molécule d'adhérence de la BHE est décrite. Il s'agit

d'ALCAM, une molécule d'adhérence exprimée par l'endothélium cérébral. Les ligands de cette molécule, CD6 et ALCAM elle-même, se retrouvent sur les lymphocytes et les CPA, respectivement. Les travaux présentés dans la deuxième section expérimentale démontrent que l'inhibition des interactions entre ALCAM et ses ligands limite la transmigration des lymphocytes T CD4+ et des monocytes à travers les CE-BHE *in vitro*. L'effet de l'anticorps bloquant contre ALCAM est aussi démontré dans l'EAE, où l'injection intra-péritonéale de cet agent limite l'infiltration des leucocytes au cerveau, diminue la sévérité de la maladie et limite la formation de lésions démyélinisantes. Ce deuxième volet décrit le rôle des molécules d'adhérence de la BHE, et identifie ALCAM comme une nouvelle molécule d'adhérence avec un potentiel thérapeutique pour limiter l'infiltration leucocytaire au SNC.

Le troisième volet traite des propriétés sécrétrices des CE et des nouvelles thérapies potentielles pour les maladies neuro-dégénératives. L'utilisation de cellules souches neurales (CSN) a un potentiel énorme dans le traitement de maladies où il y a des dommages axonaux et des déficits neurologiques irréversibles, comme dans la SEP. Dans un tel contexte, la méthode privilégiée d'injection sera certainement la voie intraveineuse plutôt que l'injection locale dans le SNC. Les mécanismes qui régissent les interactions entre les CSN et les CE-BHE auront donc une importance capitale dans une telle stratégie thérapeutique. Ce travail *in vitro* démontre que les CSN ont la capacité de transmigrer à travers une monocouche de CE-BHE. Cette étude démontre aussi que le MCP-1/CCL2 sécrété par les CE-BHE est un facteur important dans la transmigration et dans la différenciation des CSN dans l'espace sous-endothélial. Cette troisième partie identifie la capacité préférentielle des CE-BHE à sécréter du MCP-1/CCL2, par rapport aux CE d'origine non-cérébrale, comme une propriété qui module les interactions entre les CSN et les CE-BHE.

Ce travail de doctorat précise donc trois des mécanismes moléculaires qui régissent certaines des propriétés fondamentales de la BHE. L'AGT est un facteur astrocytaire qui régule les JS et donc l'étanchéité de la BHE. ALCAM fait partie des molécules

d’adhérence de la BHE et contribue à la diapédèse de certains sous-types de leucocytes à travers cette dernière. Finalement la capacité de sécrétion de MCP-1/CCL2 des CE-BHE est déterminante dans les interactions entre les CSN et les CE. Cette thèse identifie aussi les radeaux lipidiques comme des structures membranaires importantes pour la régulation des fonctions de la BHE.

SECTION 2 MANUSCRIT 1

Angiotensin II controls occludin function and is required for blood-brain barrier maintenance: relevance to Multiple Sclerosis

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*Karolina Wosik et Romain Cayrol ont contribué également au travail.

Contributions

Le projet a été élaboré et initié par la Dre Karolina Wosik et le Dr Alexandre Prat. J'ai participé aux expériences *in vitro* et à l'étude *in situ*. La Dre France Berthelet nous a donné accès aux spécimens de patients de SEP. Monique Bernard a participé aux expériences *in vitro*. Le Dr Robert Moumdjian et le Dr Alain Bouthillier nous approvisionnent en biopsies cérébrales à partir desquelles les CE-BHE sont isolées. Le Dr Timothy L. Reudelhuber nous a donné accès aux spécimens de souris déficientes en AGT. Le manuscrit a été rédigé par le Dr Alexandre Prat, la Dre Karoline Wosik et moi-même.

Angiotensin II controls occludin function and is required for blood-brain barrier maintenance: relevance to Multiple Sclerosis

Short title: Angiotensin II promotes maintenance of the BBB

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Abstract

The Blood-Brain Barrier (BBB) restricts molecular and cellular trafficking between the blood and the central nervous system (CNS). Although astrocytes are known to control BBB permeability, the molecular determinants of this effect remain unknown. We show that angiotensinogen (AGT) produced and secreted by astrocytes is cleaved into angiotensin II (AngII) and acts on type 1 angiotensin receptors (AT_1) expressed by BBB endothelial cells (ECs). Activation of AT_1 restricts the passage of molecular tracers across human BBB-derived ECs through threonine-phosphorylation of the tight junction protein occludin and its mobilization to lipid raft membrane microdomains. We also show that AGT knockout animals have disorganized occludin strands at the level of the BBB and a diffuse accumulation of the endogenous serum protein plasminogen in the CNS, as compared to wild type animals. Finally, we demonstrate a reduction in the number of AGT-immunopositive perivascular astrocytes in multiple sclerosis (MS) lesions, which correlates with a reduced expression of occludin similarly seen in the CNS of AGT knockout animals. Such a reduction in astrocyte-expressed AGT and AngII is dependent, *in vitro*, on the pro-inflammatory cytokines tumor necrosis factor-α and interferon-γ. Our study defines a novel physiological role for AngII in the CNS and suggests that inflammation-induced downregulation of AngII production by astrocytes is involved in BBB dysfunction in MS lesions.

Introduction

The blood-brain barrier (BBB) regulates central nervous system (CNS) homeostasis by controlling the passage of blood-borne molecules and cells from the periphery to the CNS. Compromise of BBB integrity is a hallmark of several neurological disorders, including brain and spinal cord trauma, brain neoplasms and multiple sclerosis (MS). In MS, BBB disruption contributes to the multifocal infiltration of auto-reactive inflammatory immune cells, leading to tissue destruction and neurological impairment.

The BBB consists of specialized endothelial cells (ECs) strongly anastomosed to one another through multiprotein tight junction (TJ) complexes. This cerebral microvasculature is in close apposition to a dense network of astrocytic endfeet, which promote the formation and maintenance of the BBB through contact-dependent, and soluble factor-induced mechanisms (Abbott, 2002;Abbott et al., 2006;Nedergaard et al., 2003). Tao-Cheng and Brigthman first demonstrated that soluble astrocytic factors re-induced a BBB phenotype in rodent brain-derived ECs grown *in vitro* (Tao-Cheng and Brightman, 1988), a phenomenon confirmed in other systems (Neuhaus et al., 1991;Prat et al., 2001;Stanimirovic et al., 1995). The identity of the astrocytic molecule(s) responsible for this effect remains unclear.

The blood-borne octapeptide angiotensin II (AngII) is generated from angiotensinogen (AGT) via cleavage by renin and angiotensin converting enzyme-1 (ACE1), an enzymatic cascade defined as the renin-angiotensin system (RAS). While the peripheral blood endocrine RAS regulates blood volume, arterial pressure and kidney filtration rate and is a critical systemic

player in fluid homeostasis (de Gasparo et al., 2000), the biological functions of the CNS RAS range from control of cerebral blood flow to memory retention and neuronal regeneration (Morimoto and Sigmund, 2002;Saavedra, 2005). Furthermore, in a seminal observation, Kakinuma suggested that in rodents, AngII and one of its metabolites in the brain, AngIV, are also involved in CNS vascular repair following injury (Kakinuma et al., 1998;Yanai et al., 2000). In the rodent CNS, AGT immunopositive astroglial cells are found in the hippocampus, thalamus, hypothalamus, anterior and posterior cingulate cortex, basal and lateral amygdala, caudate and globus pallidus, as well as in the brainstem. In the cortex, AGT expression seems restricted to neurons (Intebi et al., 1990;Mungall et al., 1995;Stornetta et al., 1988).

In this study, we hypothesized that AGT metabolites are secreted by human astrocytes and act on angiotensin receptors (AT) on brain ECs to promote BBB maintenance through post-translational TJ protein modifications.

Results

Human astrocytes produce AngII; brain endothelium expresses cognate receptors

RT-PCR was performed using primers specific for human AGT (Juillerat-Jeanneret et al., 2004) and ACE1. As seen in figure 1a, primary cultures of human astrocytes express AGT as well as ACE1 mRNA. Supernatants were also collected after one and three days in culture and levels of AngII were assessed by ELISA: the active AngII metabolite is secreted by human astrocytes (12,9 ± 1,6 pg/ml at 24h) and while human BBB derived ECs do not produce AngII, they do produce ACE1 (data not shown). GFAP-expressing astrocytes grown in primary culture *in vitro* were also subjected to intra-cellular immunostainings for AGT and analyzed by flow cytometry (figure 1b). As shown, the majority (80.6%) of $GFAP^+$ astrocytes were also positive for AGT, confirming that the source of AGT in these cultures was astrocytes and not contaminating neurons.

Immunostainings for AGT (low magnification histochemsitry-figure 1c and higher magnification histofluorescence-figure 1d) were further performed on normal human brain sections using antibodies specific for AGT and S100, a marker of perivascular astrocyte (Ghandour et al., 1981) (Kacem et al., 1998). Low magnification immunochemistry staining for AGT revealed an abundance of astrocyte-like cells in the hemispheric and cerebellar white matter of human CNS material (figure 1c), and confirmed high numbers of AGT+ astrocytes in hypothalamus, thalamus, hippocampus, corpus callosum and in several brainstem nuclei (including solitary tract nuclei) (data not shown). Additional AGT^+ cells, likely pyramidal

neurons (by morphology) were also seen in the hemispheric cortical grey matter and in cerebellar neurons (data not shown). S100 (red) positive parenchymal and perivascular astrocytes express AGT (green), as seen in the overlay pictures (yellow, arrows; vessel lumen indicated with *, figure 1d), confirming that in the white matter perivascular astrocytes express AGT. From these results we conclude that human astrocytes thus express AGT, *in vitro* and *in situ* in the brain, which can subsequently be cleaved into AngII through the action of ACE1 expressed by astrocytes and brain ECs.

To ascertain that human brain ECs have the capacity to respond to astrocyte-derived AGT products, AT_1 and AT_2 receptor expression on BBB-ECs was assessed by western blot. BBB-ECs express both AT_1 and AT_2 when grown in primary cultures (figure 1e) and *in situ*, as shown by immunohistofluorescence staining on human brain capillaries, discernible by caveolin-1 co-expression (figure 1f).

Astrocyte-secreted AngII decreases EC permeability via the AT_1 receptor

Confluent BBB-EC monolayers *in vitro* take on a cobblestone appearance and form a semi-permeable barrier which restricts diffusion of soluble tracers. We and others have previously demonstrated that astrocyte-derived soluble factors in ACM decrease BBB-EC permeability to soluble tracers (Prat et al., 2001;Stanimirovic et al., 1995). In a modified Boyden chamber permeability assay using human BBB-ECs, 40% ACM (v/v) induced an important decrease in permeability to the large molecular weight tracer FITC-BSA (figure 2a). This effect was long lasting, as evidenced by the important difference in tracer diffusion up to 72h (n=3 independent experiments, in duplicate, $p<0.0001$ at 72h). Addition of AngII (10^{-8} M, 72h) to

BBB-ECs paralleled the effect of ACM (figure 2b). Similar results were obtained using the small molecular weight tracer ^{14}C-sucrose, a marker of paracellular transport. The permeability of untreated BBB-EC monolayers to ^{14}C-sucrose was 21,5% at 3h, ACM and AngII reduced the permeability of ECs to 13.1 and 8,7%, respectively ($p<0.05$, n=2 in duplicate). At 6h, ^{14}C-sucrose permeability was further reduced from 41,6% (untreated) to 21,2% (AngII) and 24,4% (ACM, $p<0.01$, data not shown).

To assess whether astrocyte-produced AngII was responsible for the BBB-EC barrier promoting effects of ACM, BBB-ECs were cultured in ACM, in conjunction with AT_1 or AT_2 non-peptide receptor antagonists Losartan or PD123,319 (de Gasparo et al., 2000), respectively (10^{-7}M). AT_1 antagonist Losartan reversed the barrier promoting effects of ACM, increasing the permeability of BBB-ECs to levels of untreated ECs (figure 2c, n=3, in duplicate, $p<0.005$). In contrast, AT_2 antagonist PD123,319 did not abrogate the effect of ACM on permeability (figure 2d). Antagonists alone had no effect (data not shown). In addition, media from astrocytes treated with Captopril (10^{-7}M), an ACE1 inhibitor, was unable to reproduce the effects of ACM (figure 2e, n=3, in duplicate, $p<0.005$). In separate experiments, astrocytes were grown on the reverse side of the transwell and allowed to contact BBB-ECs in a co-culture assay. Using this system, we could demonstrate an even greater effect of astrocytes on the permeability of BBB-ECs ($p<0.01$, as compared to ACM; figure 2f), supporting the notion that astrocyte-EC proximity is important in the regulation of BBB permeability.

To confirm that AngII decreases BBB permeability through the AT_1 receptor, the effects of the selective synthetic AT_1 and AT_2 receptor non-peptide agonists L-162,313 and CGP-42112A (respectively) (Vianello et al., 1998) were assessed. Treatment of BBB-EC monolayers with L-162,313 (10^{-8}M), but not with CGP-42112A (10^{-8}M), resulted in a significant reduction of permeability, similar to both ACM and AngII (figure 2b, n=3, in duplicate, $p<0.005$ as compared to non treated cells). Our data demonstrates that astrocyte-produced AngII promotes barrier properties in BBB-ECs through ligation of the AT_1 receptor.

Astrocytic factors and AngII promote occludin concentration into lipid rafts

Nusrat and colleagues (Nusrat et al., 2000) have shown that in the T84 gut epithelial cell line, hyperphosphorylated forms of the TJ protein occludin localized to detergent insoluble lipid rafts. We tested whether endothelial TJs of the BBB were concentrated in lipid rafts and whether this concentration is necessary for barrier function. Lipid rafts from BBB-ECs were isolated by sucrose flotation following solubilization with Brij58. Following centrifugation, twelve fractions were collected from top (fraction 1) to bottom (fraction 12). High phospholipid and cholesterol concentration, as well as enrichment of the lipid raft markers GM1-ganglioside and CD59 demonstrate the presence of lipid rafts in fractions 4 and 5 (figure 3a). To determine if BBB TJs localized to lipid rafts, rafts were isolated from BBB-ECs and blotted for TJ protein junctional adhesion molecule-1 (JAM-1) and occludin. JAM-1 was strongly concentrated in lipid rafts of BBB-ECs grown under basal conditions (figure 3b). In contrast, occludin expression was low in BBB-EC lipid rafts when cells were not supplemented. Upon treatment of BBB-ECs with ACM or AngII (10^{-8}M, figure 3b),

expression of occludin shifted to lipid raft fractions: densitometry analysis of western blots revealed that 23% (with ACM) and 28% (with AngII) of total occludin were found in lipid rafts, as compared to 11% in the untreated BBB-ECs ($n=4$, $p<0.05$). In contrast, no change in JAM-1 localization was seen, as 20, 23, and 21% of total JAM-1 were found in the rafts of untreated, ACM and AngII treated BBB-ECs, respectively ($n=4$). Protein determination in gradient fractions (3a, white squares) showed that protein levels are below the detection threshold in lipid rafts, confirming that occludin expression relative to total protein content was clearly enriched in BBB-EC rafts and suggesting convergence of TJ proteins to rafts.

To assess the functional significance of TJ protein assembly in lipid rafts, BBB-ECs were grown in Boyden chambers in the presence of ACM and treated for 1h with lipid raft disrupting agents methyl-β-cyclodextrin (MβCD, 5mM), filipin (2 μg/ml), or nystatin (50 μg/ml) (Pike, 2004) prior to addition of FITC-BSA. A marked decrease in cholesterol and the absence of occludin was evident in fractions 4 and 5 of MβCD, filipin or nystatin-treated BBB-ECs (figure 3c, MβCD shown), confirming lipid raft disruption. Seventy-two hours post treatment, MβCD, filipin or nystatin in conjunction with ACM, induced a marked increase in permeability of BBB-ECs to FITC-BSA (figure 3d), as compared to cells grown in ACM alone. When compared to non treated cells (showing ~50% permeability at 72h, as before), BBB-ECs grown in ACM plus lipid raft disrupting agents showed a 17.8 ± 5.3% increase in permeability, as compared to a decrease of 12 ± 2.9% with ACM alone. Our data suggest that AngII secreted by astrocytes induces, at the level of the human BBB, the functional

rearrangement of specific TJ proteins to lipid rafts, a phenomenon necessary to promote BBB integrity.

Astrocytic factors and AngII influence occludin phosphorylation in lipid rafts

Occludin phosphorylation is an essential determinant of its localization and function. To assess the effects of ACM and AngII on occludin phosphorylation, cell lysates from BBB-ECs were immunoprecipitated using anti-phosphorylated-tyrosine (anti-P-Tyr) or anti-phosphorylated-threonine (anti-P-Thr) antibodies and blotted for occludin (figure 3e). ACM and AngII induced a down-regulation of P-Tyr occludin (37 and 11% of untreated, respectively) and an upregulation of P-Thr occludin (162 and 228% of untreated, respectively) when compared to untreated BBB-ECs. No change in total occludin could be detected. To determine whether these changes occurred at the level of membrane microdomain-associated occludin, lipid rafts from BBB-ECs were isolated as before, and anti-P-Tyr or anti-P-Thr immunoprecipitations were performed on lipid raft (fractions 4-5) and soluble fractions (fractions 11- 12). Immunoprecipitated lipid raft-derived proteins enriched for phosphorylated-Tyr and -Thr were subsequently immunoblotted, as shown in figure 3f. BBB-EC treatment with ACM or AngII induced a downregulation of P-Tyr occludin in lipid rafts only (R lanes, 6 and 7% of untreated, respectively) and an upregulation of P-Thr occludin in lipid rafts (254 and 351% of untreated, respectively), and to a lesser extent in the soluble occludin pool (S lanes) (figure 3f). In addition, a second occludin band of higher molecular weight appears in AngII treated cells, likely representing the hyper-phosphorylated form of the protein, as previously described (Nusrat et al., 2000).

Pro-inflammatory cytokines disrupt AngII production by human astrocytes *in vitro*

As neuroinflammatory conditions are associated with a BBB breakdown, we further evaluated the effects of the pro-inflammatory cytokines IFN and TNFα on the production of AGT/AngII by astrocytes. Figure 4a shows a substantial decrease of AGT mRNA in astrocytes treated with IFN, TNFα (100 U/ml, 24h) or both. This downregulation was further confirmed by ELISA, which shows an almost complete absence of AngII peptide in the media of astrocytes following 24h treatment with either inflammatory cytokine (figure 4b). Moreover, a decrease in ACE1 mRNA was seen after treatment with IFN(100 U/ml, 24h) as well as with the combined treatment IFN and TNFα (100 U/ml, 24h, figure 4a), suggesting a reduction in the cleavage of AGT to AngII.

Decreased number of AGT immunopositive astrocytes in MS lesions

Infiltration of inflammatory cells into the brain parenchyma is a hallmark of MS. To test whether such inflammation can affect AGT expression *in situ*, we assessed AGT expression by perivascular S100 positive astrocytes in four MS brains. Areas counted were categorized in 3 groups with respect to demyelination and cellular infiltration as assessed by LFB (myelin) and H&E staining: active plaques (white matter showing demyelination and cellular infiltration – n=14 sections from 6 MS patients; figure 5g-l), inactive plaques (demyelination without cellular infiltration – n=10 sections from 6 MS patients; figure 5 d-f) and normal appearing white matter (NAWM – no demyelination nor infiltration – n=11 sections from 6 MS patients; figure 5a-c). LFB/H&E, as well as AGT (green) and S100 (red) stains are shown for each category of MS lesions in figures 5a-l, and demonstrate an important reduction in

perivascular astrocyte expression of AGT (green) in active lesions (panels i and l) and in chronic lesions (panel f), as compared to NAWM (panel c) or control human CNS (not shown). The corner inserts of panels b-c display a greater magnification of a S100 positive astrocyte immunopositive for AGT (arrowhead). In parallel, the corner inserts of panels k-l display S100 positive astrocytes negative for AGT (arrowhead).

The percentage of S100 astrocytes immunopositive for AGT was 77.8 ± 3.6% in white matter in normal brain, 87.6 ± 5.3% in NAWM in MS, and 85.8 ± 4.01% in NIN brain (figure 5m). In contrast, in both active (50 ± 4.8%) and inactive (51.8 ± 6.9%) MS lesions, a marked decrease in the number of AGT positive astrocytes was evident ($p<0.01$ for active and inactive plaques as compared to normal brain, $p<0.001$ for active and inactive plaque as compared to NAWM or NIN, figure 5m). This effect was not due to an increase in the absolute number of astrocytes in MS plaques (as a result of reactive astrogliosis), as the percentage of nuclei that are S100 positive was similar between groups, with the exception of active plaques, showing a decrease in the percentage of S100 positive nuclei, most likely due to the presence of infiltrating immune cells. Immunostainings for AGT and S100 in control normal brain (2 patients, n=13 sections) and tissue from non-inflammatory neurological disease controls (NIN – 4 samples from Parkinson's disease, n=12 sections) were similar to those performed on NAWM (data not shown).

<u>Decreased occludin expression in MS lesions</u>

In MS patients, brain capillaries found in active lesions also showed a marked reduction in peak occludin strand fluorescence intensity (111 ± 21; n=55 strands in 12 capillaries) as

compared to brain capillaries from NAWM (205 ± 16 n=50 strands in 15 capillaries; $p<0.0001$) (figure 5n). MS lesions additionally presented a significant reduction in occludin strand thickness around brain capillaries (1.87 ± 0.48μm, n=55 strands, 12 capillaries; $p<0.01$), as compared to NAWM tissue (3.62 ± 0.87μm, n=50 strands, 15 capillaries).

Decreased occludin expression in brain capillaries of AGT deficient mice

Brain sections from wild type (wt) and AGT knockout (AGT-/-) mice were stained for the TJ protein occludin. Distinct strands of occludin were visible in brain capillaries. Stacks of 70-100 images were acquired per vessel using the Leica SP5 confocal microscope and collapsed onto a one-dimensional x-y-z projection. Figure 6a shows brain capillaries from wt (upper panels) and AGT-/- brains (lower panels). The peak fluorescence intensity (in arbitrary units) of each strand as well as its thickness (in μm) was assessed using the LAS AF Leica software. Occludin strands from AGT-/- brains displayed a significantly reduced peak strand fluorescence intensity (122.4 ± 5.3; n=54 strands in 8 capillaries) as compared to brain capillaries from wt mice (215.6 ± 2.8; n=41 strands in 7 capillaries; $p<0.0001$) (figure 6b). AGT-/- brains also presented a significant reduction in occludin strand thickness (0.99 ± 0.03μm, n=59 strands, 8 capillaries; $p<0.0001$), as compared to wt mice (1.78 ± 0.07μm, n=40 strands, 7 capillaries). Morphologically, occludin-positive strands in AGT-/- animals were also more frequently discontinued and displayed a beaded appearance, suggestive of TJ strand disorganization. Expression of additional TJ proteins, such as claudin-5, ZO-1 and JAM-1 were not affected in the CNS of AGT-/- animals, when compared to wt mice (figure 6c).

Taken together, our results demonstrate that in AGT deficient mice, as well as in MS lesions, occludin expression is diminished at TJ strands.

Leakage of serum proteins into the CNS of AGT-/- animals

In order to confirm the functional *in vivo* significance of AGT disruption in the CNS, adult mice CNS sections from AGT-/- and wt animals were immunostained for the serum proteins plasminogen and albumin, proteins normally not found in the CNS. Plasminogen immunostainings in AGT-/- animals revealed a discrete and diffuse perivascular and parenchymal signal, confirming chronic accumulation of high molecular weight peripheral blood proteins in the CNS (figure 6d and figure 7). The distribution of serum protein leakage in the CNS of AGT-/- animals correlated with previously reported sites of AGT expression in the CNS of rodents (Mungall et al., 1995). The percentage of vessels which showed perivascular accumulation of plasminogen was assessed for each CNS region and revealed important accumulation in the hippocampus (48%), thalamus (43%) motor and sensory cortex (27%). Interestingly, the highest proportion of vessels in which plasminogen accumulation was detected in AGT-/- animals was found in the corpus callosum (50%), a region universally affected in MS. Such staining was not observed in wt animals and could not be found in AGT-/- mice when the anti-plasminogen Ab was pre-absorbed with fresh serum. Accumulation of the endogenous serum protein albumin was also detected in the CNS parenchyma of AGT-/- animals, although albumin immunostaining was less intense than that of plasminogen and seemed to be concentrated around large meningeal vessels (figure 6d).

CNS sections from AGT-/- and wt animal were also immunostained for the presence of immune cells, to evaluate if AGT deficient animals had greater immune surveillance of the CNS. Anti-CD3 immunostainings revealed the presence of occasional but rare lymphocytes in the CNS of either wt or AGT-/- animals (less than one immunopositive cell per field, $n>30$ fields studied), suggesting that AGT metabolites are not involved in the control of immune cell migration to the CNS (data not shown).

Discussion

In this study, we demonstrate that astrocyte-derived AngII acts via the AT_1 receptor on human brain ECs to decrease the permeability of the BBB. This effect is driven by the mobilization of the TJ protein occludin into brain EC lipid rafts and the change in its phosphorylation status within these membrane microdomains. We further demonstrate that in MS plaques, the number of perivascular AGT-expressing astrocytes, as well as occludin immunoreactivity on microvessels, is decreased in comparison to non-infiltrated white matter from MS patients. Such a decrease in AGT production could be reproduced *in vitro* when human astrocytes were cultured in the presence of the TNFα and IFN□. Finally, we show that in AGT deficient mice, occludin-positive immunofluorescence is diminished and disorganized at TJ strands, while claudin-5, JAM-1 and ZO-1 remain unaffected.

As shown in experimental BBB models, astrocyte-derived soluble factors are essential for the development and maintenance of an impermeable BBB. Immediately *ex vivo* brain microvascular ECs lose their trans-endothelial electrical resistance (TEER) (Rubin et al., 1991) and become permeable to soluble tracers (Prat et al., 2001). Strong TEER and better barrier properties can be re-induced when cells are grown in the presence of ACM (Abbott, 2002;Arthur et al., 1987;Neuhaus et al., 1991). Our data support previous reports showing AGT expression by rodent astrocytes (Clemens et al., 1986;Deschepper et al., 1986;Lynch et al., 1987;Stornetta et al., 1988) and demonstrate AT_1 and AT_2 receptor expression by human microvascular brain ECs *in situ.* Our study provides evidence that astrocyte-produced AngII,

acting on human BBB-ECs via the AT_1 receptor, is an important effector of astrocytic re-induction of BBB integrity *in vitro*, a situation analogous to vascular repair of the BBB following injury. Kakinuma et al. (Kakinuma et al., 1998;Yanai et al., 2000) found that in AGT deficient mice, BBB reconstruction was incomplete following cold injury. We demonstrate that astrocyte-derived AngII drives the mobilization of the TJ protein occludin into specialized membrane microdomains in BBB-ECs, leading to the induction of a better barrier. In fact, AGT-deficient mice exhibit a significantly lower expression of occludin at BBB-TJ strands *in vivo*, confirming the validity of the *in vitro* findings. This observation is associated with an increased permeability to the endogenous serum proteins plasminogen and albumin, but not with an increased immune surveillance by lymphocytes, suggesting that although astrocyte-secreted AngII influence the ability of BBB-ECs to restrict the passage of soluble molecules from the periphery to the CNS, additional signals are required to induce immune privilege. Whether other TJ proteins, such as claudins or JAMs, are involved in the ability of the BBB to protect the CNS against immune cell invasion remains to be established.

The phosphorylation status of occludin also dictates protein targeting to the membrane and is crucial for TJ formation (Farshori and Kachar, 1999;Hirase et al., 2001;Huber et al., 2001;Petty and Lo, 2002). *In vitro*, Tyr phosphorylation of occludin increases barrier permeability in peripheral and umbilical vein ECs (Lampugnani et al., 1997;Wachtel et al., 1999). *In vivo*, BBB disruption secondary to cerebral ischemia correlates with enhanced Tyr phosphorylation of occludin on brain capillaries (Kago et al., 2006). Our data using human BBB-ECs further demonstrates that astrocyte-secreted AGT metabolites are required to induce

Tyr dephosphorylation and Thr phosphorylation of occludin, a phenomenon associated with occludin concentration within cholesterol-enriched membrane microdomains (rafts) and needed to promote a more competent BBB.

MS is the prototypical neuroinflammatory disease in which BBB breach is observed, leading to infiltration of blood borne molecules and cells into the brain. We show that human astrocytes exposed to IFN□/TNFα not only down-regulate AGT/AngII expression, but also ACE1, thereby interfering with occludin function and BBB impermeability to soluble molecules. This is paralleled *in situ* by the decrease in AGT-expressing astrocytes around blood vessels and occludin–expressing ECs in MS plaques, when compared to normal or non-infiltrated and non-demyelinated white matter of MS patients. Since AGT-/- animals do not display increased numbers of immune cells patrolling the CNS and because astrocytes (*in vitro*) down-regulate AGT production when treated with pro-inflammatory cytokines, we must conclude that AGT and occludin disruption in MS lesions are likely to be the reflection of local inflammatory mediators and thus are a consequence, not a cause, of inflammation. However, the fact that both active (infiltrated) and chronic (demyelinated but without immune cells) MS plaques display similar levels of AGT-expressing astrocytes, tends to signify that reactive astrocytes fail to re-initiate AGT expression, once the infiltrating immune cells leave the CNS. In this specific case, we postulate that either astrocytes acquire compensatory molecular signals which can functionally replace AngII, or that BBB injury and leakage occur in chronic inactive lesions, but are below the threshold of detection by standard magnetic resonance imaging with gadolinium injection.

Our data could provide a biological explanation for the higher prevalence of CNS and vascular malformations seen in newborns of women treated with inhibitors of the renin-angiotensin system (Cooper et al., 2006). One could speculate that AGT metabolites are critical for BBB and peripheral vascular formation during the prenatal period, a phenomenon which closely resembles vascular repair. Many studies have described a detrimental role of the RAS with regards to brain edema in experimental animal models of stroke (Blezer et al., 2001;Ito et al., 2001;Kucuk et al., 2002;Lou et al., 2004), observations seemingly divergent from ours. Such differences can be explained by the fact that most studies were conducted in hypertensive animals, a model in which sub-clinical BBB dysfunction is certainly present, and during which CNS compensatory mechanisms probably occur. One could also argue that in humans, diuretics and calcium channel blockers which stimulate the RAS and increase AngII production, are just as effective as ACE inhibitors in decreasing cerebral edema during stroke, suggesting that the exact role of the RAS during ischemia is still not resolved (Hansson, 2000;Nissen et al., 2004;Ovbiagele et al., 2005).

Our study defines a new physiological role for AngII at the level of the human BBB and provides a novel molecular mechanism to explain BBB disruption in MS lesions. We can postulate that RAS-related agents may be seen as novel candidate molecules to either allow greater access of molecules across the BBB, as would be beneficial for the treatment of CNS tumors and neurodegenerative conditions, or conversely to restrict the movement of soluble inflammatory molecules from the blood to the CNS, as would be beneficial in MS.

Materials and Methods

Primary cell isolation & culture

BBB-ECs were isolated from CNS tissue specimens from temporal lobe resections from young adults undergoing surgery for the treatment of intractable epilepsy, as previously described (Prat et al., 2000;Stanimirovic et al., 1997). Informed consent and ethic approval was given prior to surgery (ethics approval number HD04.046). Although cultures express endothelial markers factor VIII, Ulex Agglutenens Europaensis-1 binding sites, and antigen HT-7 until passages (P) 7-8, only early passages (P1-P3) were used in this study to prevent variability in the expression of occludin at later passages. No immunoreactivity for β-tubulin, α-myosin or glial fibrillary acidic protein (GFAP) could be detected, confirming the absence of contaminating smooth muscle cells or astrocytes. Occasional CD68 positive cells (microglia) were seen (less than 1% at P2) and their number decreased along passages, due to their strong adherence to plastic and their non-proliferative nature.

Astrocyte conditioned media (ACM) was harvested once a week from confluent flasks of human fetal astrocyte cultures. To obtain astrocyte cultures, fetal CNS tissue (cerebral hemispheres) was obtained at 17-23 weeks of gestation following CIHR-approved guidelines. Astrocyte isolation has previously been described (D'Souza et al., 1995;Jack et al., 2005;Wosik et al., 2001). Human fetal astrocytes were used between P2 and P4 and cultures were determined to be >90% pure, as determined by GFAP immunostaining.

Tissue & Immunohistochemistry

Formaldehyde-fixed and paraffin-embedded archival CNS tissue from control and MS patients was obtained from the Neuropathology Department at Notre-Dame Hospital. Six brain specimens from MS patients were examined, three male (51, 42 and 42 years of age) and three female (49, 58 and 68 years of age); evolution of disease was between 2 and 20 years. Four brain specimens from Parkinson's patients (all male, 65-72 years of age) and two brains from control patients (traumatic death, 51 years of age and unknown age, male) were investigated. In all control cases, cause of death was non-neurological and autopsies were performed within 6-24 hours from time of death. The generation of AGT deficient mice has been described previously (Lochard et al., 2003;Methot et al., 2001). Paraffin-embedded whole mouse brains were used from wt and AGT-/- null animals.

For immunohistochemistry and fluorescence staining, 3µm thick tissue sections were deparaffinized in 3 successive changes of toluene and rehydrated in 100% and 95% ethanol, water and phosphate buffered saline (PBS). For luxol fast blue (LFB) and haematoxylin and eosin (H&E), slides were incubated at 60˚C overnight in LFB solution (0.95% Solvent Blue 38, 90% ethanol, 0.5% acetic acid), rinsed with 95% ethanol and water. Differentiation was done with repeated 15 second (s) washes in 0.05% lithium carbonate, 20s in 70% ethanol and water. Slides were subsequently incubated in haematoxylin solution for 8 minutes (min), rinsed in water and differentiated in 1% lithium carbonate (15s). A 1min incubation in eosin solution (20% eosin stock solution, 10% acetic acid, 50% ethanol in water) preceded dehydration with two washes of ethanol (95% and 100%, 1min) and a toluene wash (2min).

Slides were mounted with Permount. All reagents are from Sigma (Oakville, Ontario, Canada).

For AGT and S100 stains, enzymatic antigen retrieval was performed with trypsin (0.1% trypsin, 0.1% $CaCl_2$ in 20mM Tris pH 7.6) for 10min at 37˚C. For occludin and zonula occludens-1 (ZO-1), enzymatic antigen retrieval with proteinase K was done (20µg/ml in TE buffer pH8) for 15min at 37˚C. For GFAP and junctional adhesion molecule (JAM-1), sodium citrate (0.3% sodium citrate in 0.004M HCl solution) heat antigen retrieval was required whereas an EDTA (1mM) heat antigen retrieval was necessary for the claudin-5 stain. For albumin, AT_1, AT_2, caveolin-1 (cav-1) and plasminogen, no antigen retrieval was necessary. GFAP and AGT immunostainings could not be performed concurrently, because they require different antigen retrieval protocols. Sections were subsequently permeabilized with 1% triton X-100 (5min, room temperature (RT)) and blocked by incubation in HHG (1mM HEPES, 2% horse serum, 10% goat serum in HBSS – Sigma) plus 0.5% triton X-100 for 1h at RT. Primary antibodies against AT_1, AT_2, cav-1, plasminogen (all 1/50, Santa Cruz Biotechnology, Santa Cruz, CA), S100 (1/100), albumin (1/1000, Abcam, Cambridge, MA), occludin (1/50), ZO-1 (1/50), Claudin-5,(1/100) (all from Zymed, South San Francisco, CA), GFAP (1/500 DakoCytomation, Glostrup, Denmark) and JAM-1 (1/50, BD biosciences, Franklin Lakes, NJ) were incubated overnight at 4˚C. Anti-AGT (1/200, USBiologicals, Swampscott, MA) was incubated for 1h at RT. Following washing, AT_1, AT_2, AGT, Claudin-5, GFAP, JAM-1, plasminogen, occludin and ZO-1 stains were amplified using biotin conjugated anti-rabbit (1/400) and anti-mouse (1/200) antibodies followed by streptavidin-fluorescein isothiocyanate

(FITC, 1/1000; DakoCytomation) and streptavidin-Cy3 (1/400, Jackson ImmunoResearch Laboratories, West Grove, PA). Albumin, Cav-1 and S100 staining were revealed with Cy3 conjugated anti-rabbit (1/400) and anti-mouse antibody (1/400, Jackson ImmunoResearch). Nuclei were stained with Hoechst 33258 dye (1/2000, 10min, RT, Invitrogen, Carlsbad, CA) or with TO-PRO3 (1/300 in PBS 15min, pre-treatment with RNase A 100µg/ml, 30min; Molecular Probes, Eugene, OR). For immunohistochemistry, the AGT signal was visualized using anti-rabbit biotin followed by horseradish peroxidase conjugated-avidin complex and revealed using chromogen diaminobenzidine (Vector Laboratories, Burlingame, CA) enhanced with nickel ammonium sulfate. In all cases, control stainings were performed omitting the primary antibody and no immunopositive cells could be detected. Stainings were visualized using either a Leica DM6000 microscope with OpenLab software or the Leica SP5 confocal microscope and analyzed using the Leica LAS AF software.

Semi-quantitative analysis of occludin signal intensity and strand thickness were performed as follow: 30 x-y planar images (0.1 µm thickness) of occludin immunostainings were recorded sequentially and reconstructed (collapsed) as a 3 µm x-y-z image. Occludin strand immunostaining intensity and thickness were calculated using the Leica LAS AF software. All microscopy data was recovered by two investigators blinded to the disease/treatment group (n>40 for each CNS condition studied).

Flow Cytometry

Human astrocytes were permeabilized with paraformaldehyde (PFA) 4% and Saponin 0.1% (Sigma), incubated with recombinant mouse-anti-GFAP conjugated to alexa 488 (1/500, BD biosciences) and rabbit-anti-AGT (1/200) for 1h at 4^{o}C, washed three times with1%FCS/PBS, incubated for 30 min mouse anti-rabbit PE secondary antibody (1/500, BD biosciences) and then visualized on a BD LSRII flow cytometer. Analysis was performed using the BD-FACSDiva software.

Permeability assays

BBB-ECs grown in primary cultures were used to generate an *in vitro* model of the human BBB, as previously reported (Alter et al., 2003;Biernacki et al., 2004;Prat et al., 2002). On day 0, BBB-ECs were plated on gelatin-coated 3µm pore size Boyden chambers (BD biosciences) at a density of 2.10^4 cells per well in EC culture media and were supplemented, where applicable, with 40% (v/v) ACM or ACM+ACE1 inhibitor (ACM treated with an ACE1 inhibitor, Captopril (10^{-7}M, Sigma) every 24h for a period of 5 days) and were allowed to grow for 72h to reach confluency. During this period, cells were treated every 24h with AngII (10^{-8}M, Sigma), Losartan (10^{-7}M, a generous gift from Dr J. Chan, CHUM Hôtel-Dieu), PD 123,319 (10^{-7}M, Sigma), CGP-42112A (10^{-8}M, Sigma) or L-162,313 (10^{-8}M, Sigma). On day 3, 1h following the last treatment, FITC-labeled bovine serum albumin (FITC-BSA, Invitrogen) (50µg/ml) or ^{14}C-sucrose (250nCi/ml, MP Biomedicals, Aurora, OH) were added to the upper chambers and their diffusion across the EC monolayer was assessed by sampling upper and lower chambers at specific time points. Fluorescence intensity and radioactivity in

the media were monitored using a FL600 microplate fluorescent reader (Biotek, Winooski, VT) and liquid scintillation analyzer (A2100, Packard Instruments, Downers Grove, IL). Molecular tracer diffusion representing BBB permeability is calculated using the formula [BSA lower chamber] X 100 / [BSA upper chamber], as previously published (Ifergan et al., 2006). When applicable, confluent Boyden chambers were treated for 1h with raft disrupting agents MβCD (5mM), filipin (2μg/ml) or nystatin (50 μg/ml) (all from Sigma) and washed prior to the addition of FITC-BSA.

In the co-culture experiments, BBB-ECs (2.10^4 cells) and astrocytes (2.10^4 cells) were plated on the luminal and abluminal sides of the transwell respectively, allowing the astrocytes to directly contact the BBB-ECs through the pores of the transwell. Permeability experiments were performed using FITC-labeled BSA as described above.

Lipid raft isolation and analysis

For the isolation of lipid rafts, 6 T75 flasks of confluent BBB-ECs per condition were washed with ice cold PBS and scraped on ice. Following centrifugation, the pellet was resuspended in 1ml 1% Brij58 (Sigma) in separation buffer (150mM NaCl, 25mMTris HCl pH7.4) with protease inhibitors (BD Biosciences) and cells were solubilized for 30min on ice. The cell lysate was transferred to a glass homogenizer, dounced with 10 strokes of a loose fitting pestle and mixed with 1ml of 85% sucrose (Sigma) in separation buffer (w/v). The resulting 42.5% sucrose cell lysate was overlaid with 6ml 35% sucrose and 4ml 5% sucrose (w/v in separation buffer) and centrifuged for 24h at 39 000rpm at 4˚C in a Beckman SW41 rotor. Twelve 1ml

fractions were collected at the meniscus, top to bottom. Cholesterol, phospholipid and protein concentrations in each fraction were assayed using commercially available kits: Amplex red cholesterol assay kit (Molecular Probes), Phospholipids B Enzymatic colorimetric method kit (Wako, Richmond, VA) and BCA Protein assay kit (Pierce, Rockford, IL).

Reverse transcription - polymerase chain reaction, ELISA

All reagents used were from Invitrogen. RNA was isolated using the TRizol reagent. Three µg of RNA per reaction were used in a reverse transcription - polymerase chain reaction (RT-PCR) reaction with 3.3µM random hexamer primers, 3mM dNTPs, RNase-OUT recombinant ribonuclease inhibitor, 3mM DTT and 400U Maloney murine leukemia virus (MMLV)-RT for 1h at 42˚C and 10min at 75˚C to produce cDNA. Two µl of cDNA were then amplified using 5U Taq DNA polymerase, 1.5mM $MgCl_2$, 0.2mM dNTPs and 50pmol forward (F) and reverse (R) primers. AGT primers have previously been published (Juillerat-Jeanneret et al., 2004). Primers used were: AGT F: 5'-tcc acc tcg tca tcc aca-3'; R: 5'-ggc tcc cag ata gag aga-3'; ACE1 F: 5'-acc tca acc tgc atg cct ac-3'; R: 5'-cag cat cga ctt gtt cca ga-3'; GAPDH F: 5'-caa agt tgt cat gga tga cc-3'; R: 5'-cca tgg aga agg ctg ggg-3'. The reaction mixture was placed in an Eppendorf 'mastercycler' thermal cycler for 10min at 94˚C followed by 30 cycles of 1min at 94˚C, 45sec at 60˚C and 1min at 72˚C. After amplification, 20µl of each sample was resolved on a 1.5% agarose gel with ethidium bromide.

AngII enzyme Immunoassays were performed on undiluted culture supernatants according to manufacturer's instructions (SPI bio, Massy, France). When applicable, cells were treated for

24h with human recombinant IFNγ and/or TNFα (R&D Systems, Minneapolis, MN) prior to harvesting of media.

Immunoprecipitation (IP) and western blot

For AT_1 and AT_2 western blots, BBB-ECs were lysed in NP-40 buffer (10mM Tris-HCl, 10mM NaCl, 3mM $MgCl_2$ and 0.5% NP-40) supplemented with protease inhibitors. Thirty µg of total protein were electrophoresed on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) and blocked for 1h at RT in 5% milk. Membranes were incubated overnight with rabbit anti-human AT_1 or AT_2 antibody (1/250 in milk; Santa Cruz) followed by a 1h incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (DakoCytomation). Specific binding was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ).

To assess for GM1 localization in sucrose gradient fractions, 5µl of each fraction was run on a 12% gel, transferred and blocked as before and incubated with HRP-cholera toxin B subunit (Molecular Probes, 1/10 000 in milk, 1h at RT). Other western blots on sucrose fractions were performed by running 10-35µl of each fraction on a 6-12% gel under reducing conditions, transfer and blocking as before and overnight incubation with the primary antibody: rabbit anti-occludin (1/100), mouse anti-JAM-1 (1/500), goat anti-CD59 (1/200, R&D Systems), mouse anti-transferrin receptor (1/1000, BD Biosciences). HRP-conjugated secondary antibody was incubated for 1h at RT. All signals were revealed using the ECL reagents.

For IPs, whole cell lysates or sucrose fractions were incubated overnight at 4˚C with agarose bead-conjugated monoclonal anti-P-Thr or anti-P-Tyr antibodies on a rotary shaker (8µl of compact beads per reaction, Santa Cruz). Agarose beads were spun down, washed three times in PBS, resuspended in loading buffer and run on a 12% SDS-polyacrylamide gel under reducing conditions. Western blots for occludin were performed as above.

Densitometric analyses were performed on a Bio-Rad Gel Doc System using the Quantity One software (Bio-Rad) and are expressed as percentage of fraction 12 or as percentage of untreated, when applicable.

Statistical analysis

Permeability curves following different treatments were evaluated using repeated measures two-way ANOVA followed by a Bonferroni post-test comparing all pairs of data points. All data points are expressed as mean ± standard error of the mean (SEM) from at least n=3 experiments, each conducted in duplicate. Permeability 72h post raft-disrupting drug treatment was evaluated by one-way ANOVA. Results of AGT+/S100+ cell counts were evaluated using one way-ANOVA, followed by Bonferroni's Multiple Comparison Test; scatter plots show individual data points and mean. Differences of occludin peak strand fluorescence intensity and strand thickness were evaluated using an unpaired Student’s t-test.

Figures

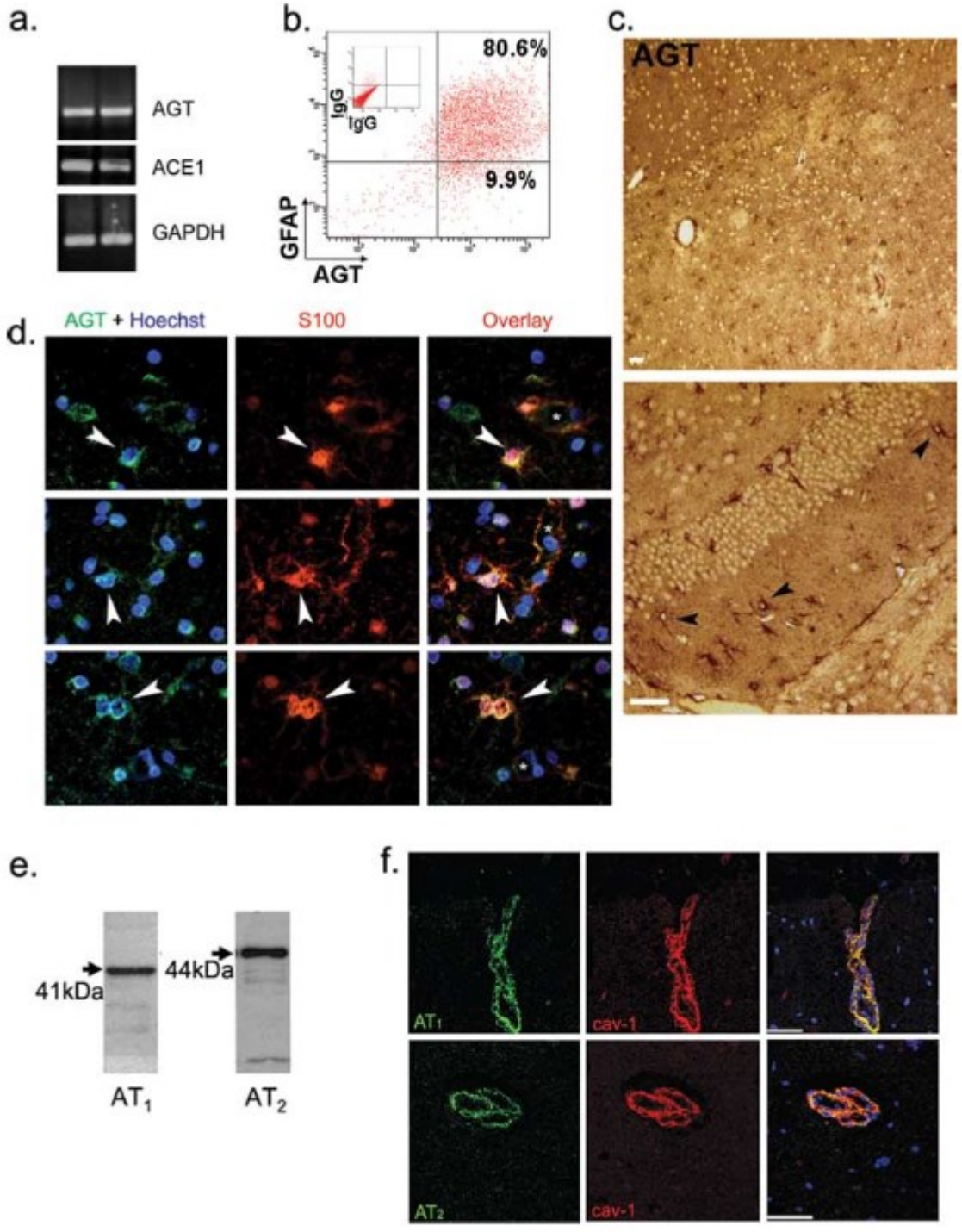
a.
AGT
ACE1
GAPDH
b.
80.6%
9.9%
IgG
IgG
GFAP
AGT
c.
AGT
d.
AGT + Hoechst
S100
Overlay
e.
41kDa
44kDa
AT1
AT2
f.
AT1
cav-1
AT2
cav-1

Figure 1: Angiotensinogen, Angiotensin II and angiotensin receptor expression in human brain. **(a)** PCR of human astrocytes in culture for angiotensinogen (AGT) and angiotensinogen converting enzyme-1 (ACE1), two astrocyte preparations are shown. **(b)** Flow cytometry analysis of human fetal astrocytes in culture immunostained for GFAP and AGT (n=3). **(c)** Immunohistochemistry on normal human CNS sections (hemispheric and cerebellar) reveals the presence of numerous AGT-positive cells (n=8 sections, 2 CNS samples). White scale bars are 20 μm. **(d)** Immunohistofluorescence on normal human sub-cortical white matter shows S100 positive astrocytes (red) are immunopositive for AGT (in green), as evidenced by the yellow color in the overlay pictures. Vessel lumen is shown with *. Nuclei were stained with Hoechst (blue). **(e)** Western blots for angiotensin receptors AT_1 and AT_2 on human BBB-ECs. **(f)** Immunohistofluorescence of normal human sub-cortical white matter shows brain capillaries are immunopositive for both caveolin-1 (cav-1; red) as well as for AT_1 and AT_2 (green); overlays are shown on the right panels; TO-PRO3-stained nuclei are blue. White scale bars in fluorescence pictures are 50μm.

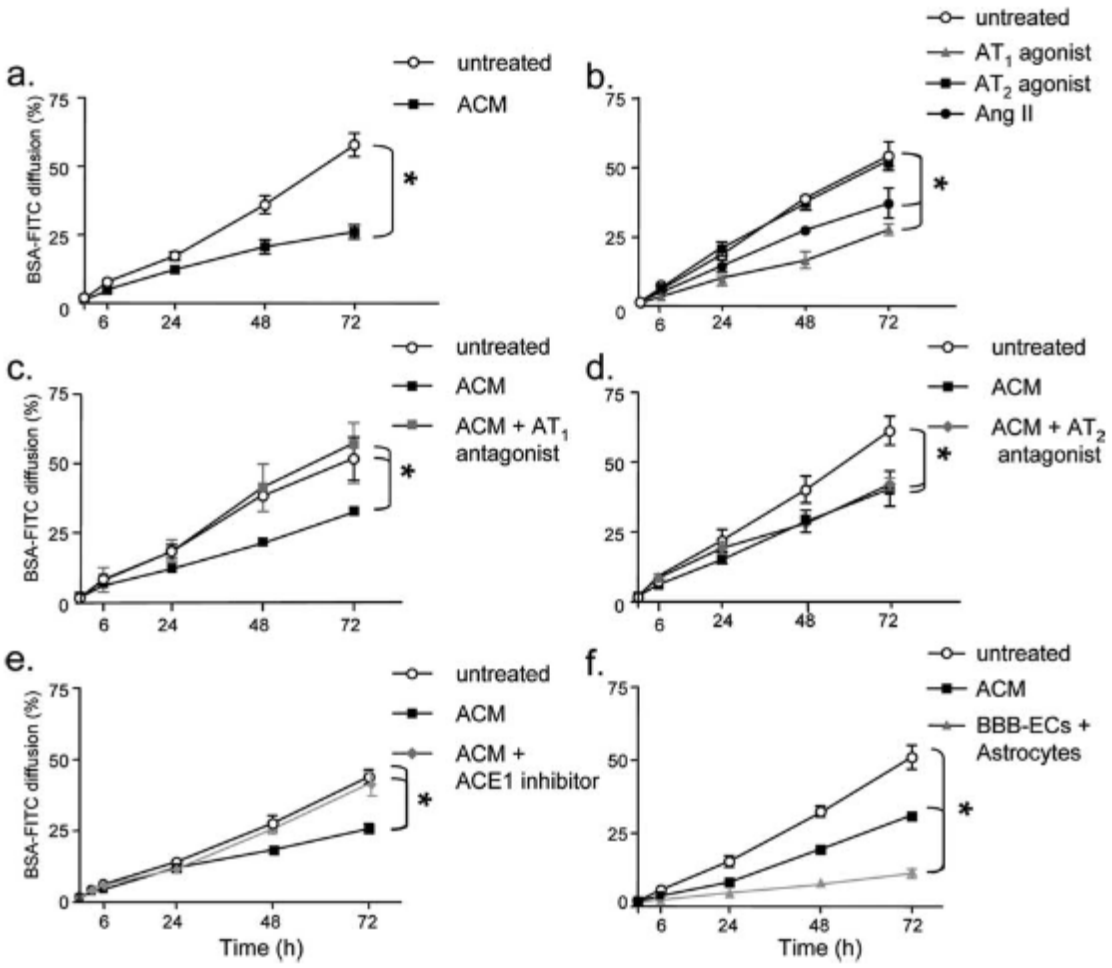

Figure 2: Astrocyte-secreted angiotensin II decreases endothelial cell permeability via the AT1 receptor. **(a)** Permeability of BBB-EC monolayers grown in the modified Boyden chamber assay to FITC-BSA is substantially decreased when cells are grown in the presence of astrocyte conditioned media (■, ACM 40% v/v), when compared to cells grown under basal conditions (○, untreated) (n=3, in duplicate, *$p<0.005$, $p<0.0001$ Bonferroni post-test at 48 and 72h, respectively). **(b)** A similar decrease in permeability is seen when BBB-ECs are grown in the presence of AngII (●,10^{-8} M , AngII) or the angiotensin receptor AT_1 agonist L-162,313 (▲, 10^{-8}M n=3, in duplicate, *$p<0.005$ as compared to non treated cells). AT_2 agonist CGP-42112A (■,10^{-8} M) did not affect permeability. **(c** and **d)**

BBB-EC monolayers were grown in the presence of ACM with or without the AT_1 receptor antagonist Losartan **(c)** (■, 10^{-7}M) or the AT_2 receptor antagonist PD123,319 **(d)** (◆, 10^{-7}M). The AT_1 receptor antagonist blocks the permeability decreasing effect of ACM; the AT_2 antagonist does not (n=3, in duplicate, *p<0.005 between indicated curves). **(e)** Astrocyte cultures were treated with ACE1 inhibitor (◆, Captopril, 5 days at 10^{-7} M) to reduce AngII levels in ACM. Captopril treated ACM did not promote BBB integrity, as compared to untreated ACM (n=3, in duplicate, *p<0.005 between indicated curves). **(f)** BBB-ECs were co-cultured with astrocytes (▲), with ACM (■) or alone (○) for 72h to demonstrate that astrocyte-EC proximity is important for barrier properties (n=2, in duplicate, p<0.01). All data are expressed as mean ± SEM.

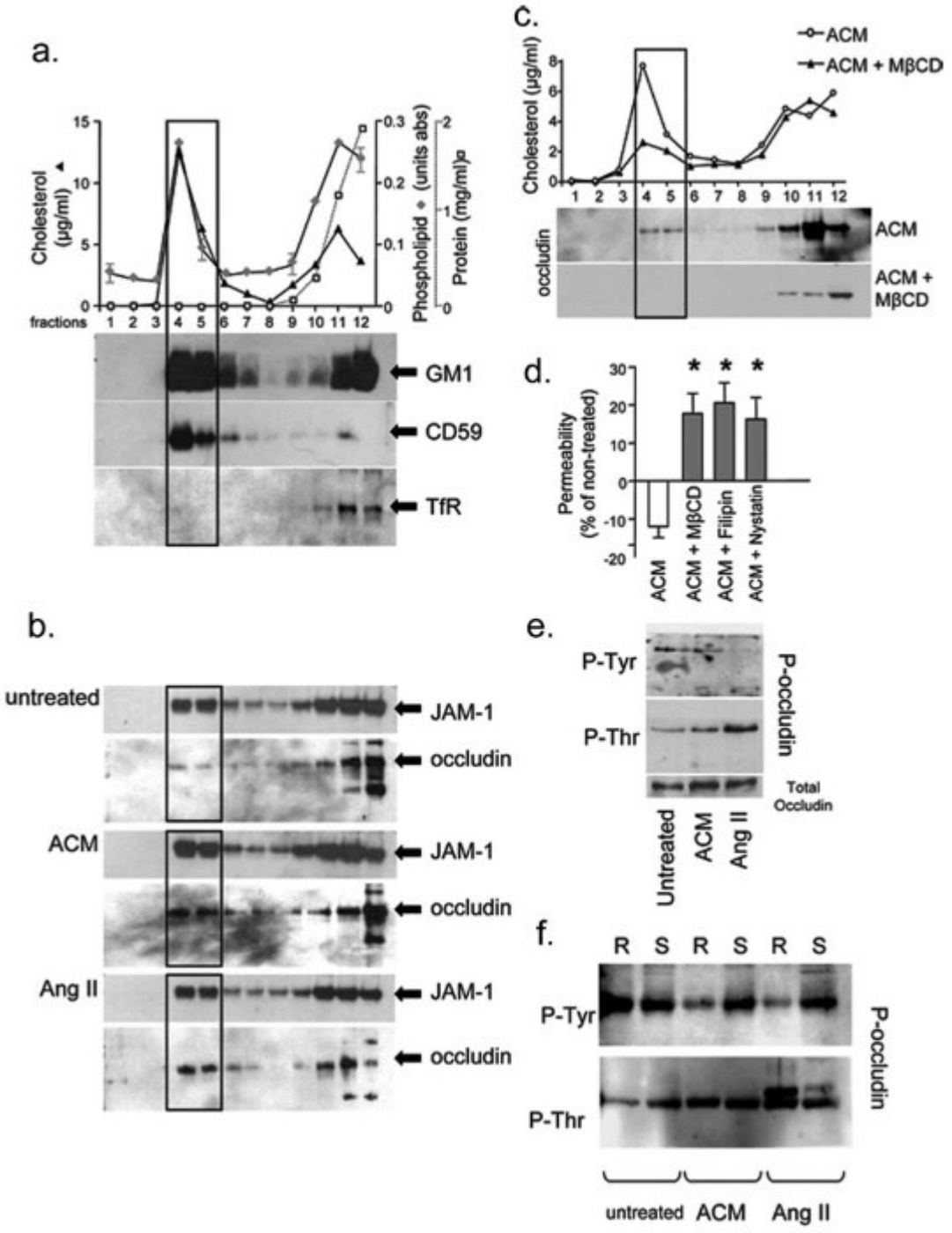
a.
Cholesterol (μg/ml)
Phospholipid (units abs)
Protein (mg/ml)
fractions 1 2 3 4 5 6 7 8 9 10 11 12
GM1
CD59
TfR
b.
untreated
JAM-1
occludin
ACM
JAM-1
occludin
Ang II
JAM-1
occludin
c.
ACM
ACM + MβCD
Cholesterol (μg/ml)
occludin
ACM
ACM + MβCD
d.
Permeability (% of non-treated)
ACM
ACM + MβCD
ACM + Filipin
ACM + Nystatin
e.
P-Tyr
P-Thr
P-occludin
Total Occludin
Untreated
ACM
Ang II
f.
R S R S R S
P-Tyr
P-Thr
P-occludin
untreated ACM Ang II

Figure 3: Lipid raft profiles of blood-brain barrier endothelial cells. **(a)** Characterization of BBB-EC sucrose density fractions with regards to cholesterol concentration (▲, left axis, in µg/ml), phospholipid content (◆, right axis, in values of absorbance) and protein concentration (□, far right axis in mg/ml). Lipid rafts are concentrated in fractions 4 and 5 (rectangular outline) where peak values of cholesterol and phospholipids are seen. Fractions 4 and 5 also show a high concentration of lipid raft markers GM1 ganglioside and CD59, but no evidence of transferrin receptor (TfR), a marker of non-lipid raft membrane. **(b)** Western blots for the tight junction (TJ) protein junctional adhesion molecule 1 (JAM-1) in sucrose density fractions show a similar concentration of JAM-1 in lipid raft fractions under all conditions tested (untreated: 20%, ACM: 23%, AngII: 21% of total occludin). The TJ molecule occludin is weakly expressed in lipid rafts when cells are grown under basal conditions (untreated) but is enriched in fractions 4 and 5 when BBB-ECs are grown in the presence of astrocyte conditioned media (ACM) or angiotensin II (10nM, AngII) (untreated: 12%, ACM: 23%, AngII: 28% of total occludin). **(c)** When BBB-ECs grown in the presence of ACM are treated for 1h prior to the isolation of lipid rafts with the raft disrupting agent methyl-β-cyclodextrin (MβCD), lipid rafts are dissolved as evidenced by the sharp decrease in cholesterol concentration in fractions 4 and 5, as well as the absence of occludin. Similar data was obtained with filipin and nystatin (data not shown) **(d)** Permeability of BBB-EC monolayers grown in ACM, 72h following a 1h treatment with the raft disrupting drug MβCD (17.79 ± 5.31%), filipin (20.05 ± 5.2%) or nystatin (17.03 ± 4.9%) is strongly increased when compared to cells grown in ACM alone (-11.99 ± 2.91%). Percentage values are expressed in comparison to permeability of cells grown under basal conditions, ($p<0.05$, n=3, in duplicate). **(e)** Immunoprecipitation experiments using antibodies specific to phosphorylated forms of threonine (P-Thr) or tyrosine (P-Tyr): whole cell lysates of BBB-ECs grown in the presence of ACM or AngII show an up-regulation of P-Thr occludin (169 and 228% of untreated, respectively) and a down-regulation of P-Tyr occludin (37 and 11% of untreated, respectively) as compared to cells grown under basal conditions. **(f)** When raft (R,

fractions 4 and 5) and soluble (S, fractions 11 and 12) fractions are immunoprecipitated separately, only the raft fractions display a decrease in P-Tyr occludin (6 and 7% of occludin, respectively) while P-Thr occludin upregulation (254 and 351% of untreated, respectively) is evident in both raft and soluble fractions, particularly upon AngII treatment.

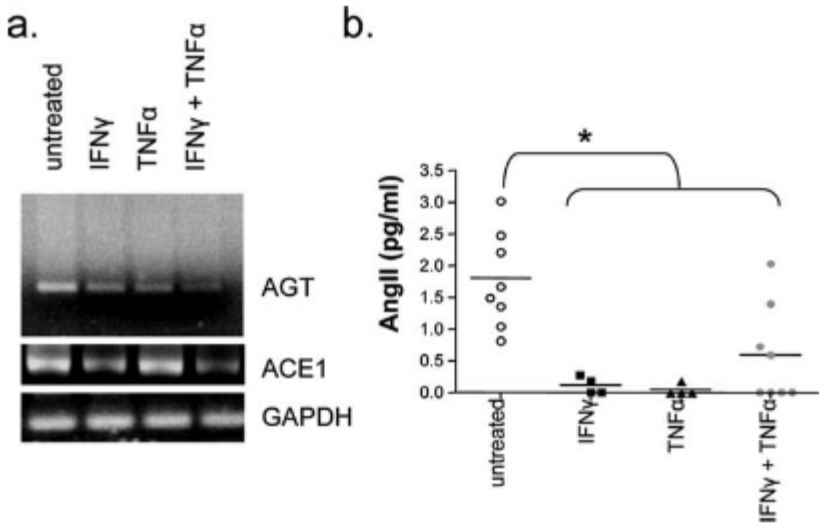

Figure 4: Pro-inflammatory cytokines disrupt angiotensin production by astrocytes in vitro. **(a)** PCR analysis of angiotensinogen (AGT) expression by human astrocytes grown *in vitro* shows a marked decrease in AGT expression when astrocytes are cultured in the presence of pro-inflammatory cytokines IFN-□ and/or TNF-α (100U/ml, 24h). Astrocytes also show a decrease expression in angiotensin converting enzyme (ACE) 1 when treated with IFN□ or both IFN□□TNFα (100U/ml, 24h) **(b)** 24h supernatants of human astrocytes grown in the presence of IFN□ and/or TNFα show decreased levels of angiotensin II as assessed by ELISA (n=4 cytokines alone, n=8 untreated and both cytokines, *p<0.01, IFN□, TNFα, IFN□+TNFα when compared to untreated).

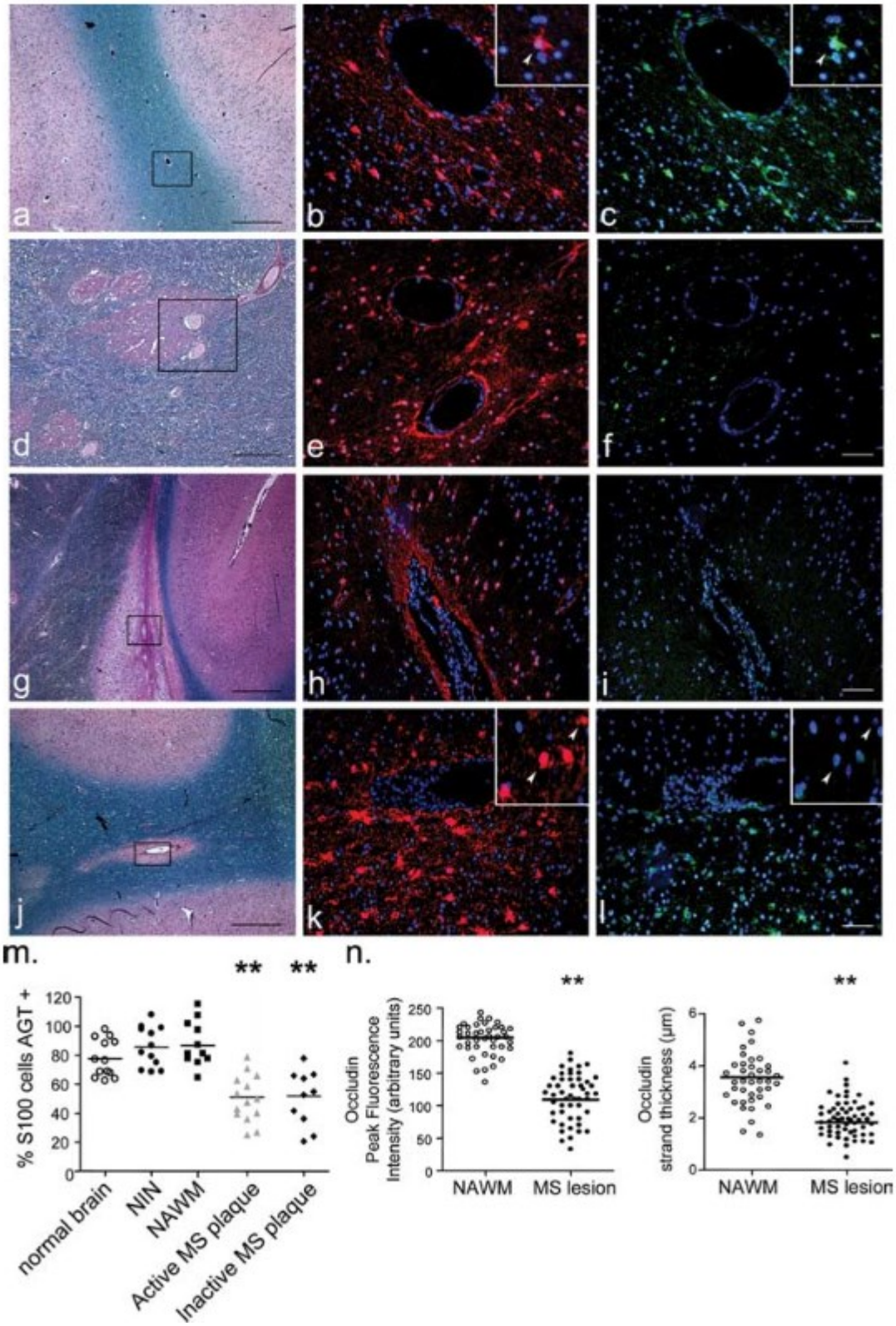
a
b
c
d
e
f
g
h
i
j
k
l
m.
% S100 cells AGT +
120
100
80
60
40
20
0
**
**
normal brain
NIN
NAWM
Active MS plaque
Inactive MS plaque
n.
Occludin
Peak Fluorescence
Intensity (arbitrary units)
250
200
150
100
50
0
**
NAWM
MS lesion
Occludin
strand thickness (µm)
6
4
2
0
**
NAWM
MS lesion

Figure 5: Staining of multiple sclerosis brain for angiotensinogen and S100. LFB and H&E stains; Angiotensinogen (AGT, in green) and S100 (in red) immunohistofluorescence of brain specimens from multiple sclerosis (MS) patients. **(a-c)** normal appearing white matter of MS brain; **(d-f)** inactive plaque; **(g-i)** and **(j-l)** active plaque. GFAP and AGT immunostainings could not be performed simultaneously because they require different antigen retrieval protocols and therefore S100 was used as the perivascular astrocyte marker. Rectangular outlines in LFB/H&E show area analyzed with regards to AGT/S100 expression. Greater magnification of S100 astrocyte immunopostive for AGT is shown in normal appearing white matter (NAWM) (corner inserts of panels b-c, arrowheads) as well as S100 astrocytes negative for AGT in active lesions (corner inserts of panels k-l, arrowheads). Black scale bars in LFB and H&E are 250µm, white scale bars in fluorescence pictures are 50µm. **(m)** Percentage of S100 astrocytes immunopositive for AGT in human was determined to be 77.78 ± 3.58% in normal brain (n=13, 2 patients), 87.64 ± 5.32% in normal-appearing white matter (n=11 sections; NAWM) of 6 MS brain, and 85.85 ± 4.01% in brain samples from 4 non-inflammatory neurological conditions (NIN, n=14 sections from 4 patients). Active (50.96 ± 4.76%; n=14 sections) and inactive MS lesions (51.79 ± 6.89%; n=10 sections, both from 6 MS patients) show a significant decrease in the number of AGT expressing astrocytes (**$p<0.01$ for active and inactive plaque as compared to normal brain, $p<0.001$ as compared to NAWM or NIN; all numbers expressed as mean ± SEM). **(n)** Quantification of occludin strand peak fluorescence intensity and thickness in NAWM and active MS lesions reveals a marked decrease of occludin immunosignal in active MS lesions (**$p<0.01$, mean ± SEM, from >50 vessels per group).

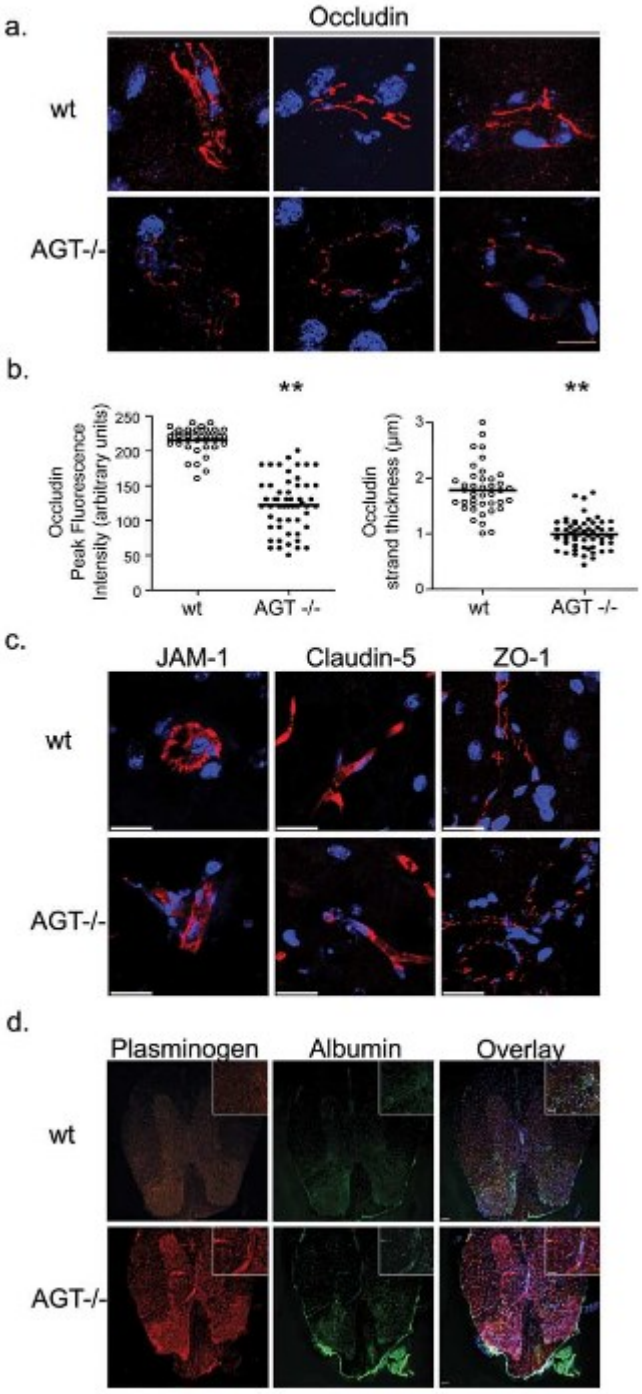
a.
Occludin
wt
AGT-/-
b.
**
**
Occludin Peak Fluorescence Intensity (arbitrary units)
250
200
150
100
50
0
wt
AGT -/-
Occludin strand thickness (µm)
3
2
1
0
wt
AGT -/-
c.
JAM-1
Claudin-5
ZO-1
wt
AGT-/-
d.
Plasminogen
Albumin
Overlay
wt
AGT-/-

Figure 6: Tight junction protein expression and BBB dysfunction in the CNS of angiotensinogen null animals. **(a)** x-y-z projection of occludin staining in the brain capillary of wild type (wt) and AGT null (AGT-/-) animals; occludin staining (in red) of wt mouse brain (upper panels, 3 different microvessels shown) is more intense and continuous than occludin staining of AGT-/- (lower 3 panels, scale bar=20 μm). **(b)** Quantification of occludin strand peak fluorescence intensity and thickness in wt and AGT-/- brain reveals a marked decrease in AGT -/- animals (**p<0.0001, mean ± SEM). **(c)** x-y-z projection of JAM-1, claudin-5 and ZO-1 staining (all in red) in brain capillaries of wild type (wt) and AGT null (AGT-/-) animals. x-y-z projections were reconstructed from 70-100 individual images acquired by confocal microscopy (scale bar=20 μm). **(d)** x-y planar images of plasminogen and albumin immunostainings in the CNS of wt and AGT-/- animals (scale bar=50 μm, in insert 25 μm). TO-PRO3 stained nuclei appear in blue.

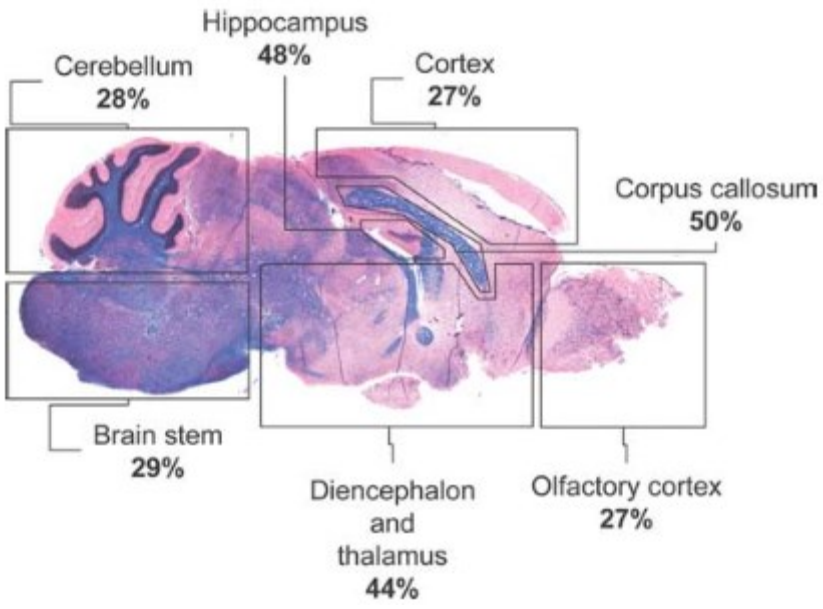

Figure 7: Plasminogen accumulation in various CNS regions of AGTnull animals.

Sagittal sections of the CNS of AGT-/- animals were immunostained for plasminogen and caveolin-1. X-Y planar images were acquired by confocal microscopy and the proportion of vessels around which plasminogen accumulation was detected was compared to the total number of caveolin-1^{+} vessels per field (% $plasminogen^{+}$ vessels). Representative sagittal section of AGT-/- CNS stained with hematoxyllin and eosin and counter stained with Luxol fast blue is shown. N=2 animals, 3 fields per region.

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SECTION 3 MANUSCRIT 2

Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system

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Contributions

Le projet a été élaboré et initié par le Dr Alexandre Prat et moi-même. Les expériences ont été faites en majorité par moi-même. La Dre Karoline Wosik, le Dr Igal Ifergan, Hania Kebir, le Dr Sebastian Krug et la Dre Nathalie Arbour ont participé aux expériences et aux discussions scientifiques dans le laboratoire. Le Dr Robert Moumdjian et le Dr Alain Bouthillier nous approvisionnent en biopsies cérébrales à partir desquelles les CE-BHE sont isolées. Le Dr Arsalan Haqqani et la Dre Danica Stanimirovic ont contribué aux expériences protéomiques. La Dre Jennifer Berard, le Dr Sam David, la Dre Katharina Kreymborg et le Dr Burkhard Becher ont participé aux expériences *in vivo* dans l'EAE. Le manuscrit a été rédigé par le Dr Alexandre Prat, Hania Kebir et moi-même.

Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system

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Abstract

Adhesion molecules of the immunoglobulin superfamily are crucial effectors of leukocyte trafficking into the central nervous system. Using a lipid raft-based proteomic approach, we identified ALCAM as an adhesion molecule involved in leukocyte migration across the blood-brain barrier (BBB). ALCAM expressed on BBB endothelium co-localized with CD6 on leukocytes and with BBB endothelium transmigratory cups. ALCAM expression on BBB cells was up-regulated in active multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) lesions. Moreover, ALCAM blockade restricted transmigration of $CD4^+$ lymphocytes and monocytes across BBB endothelium *in vitro* and *in vivo*, and reduced the severity and time of onset of EAE. Our findings point to an important role for ALCAM in leukocyte recruitment into the brain and identify ALCAM as a potential target to therapeutically dampen neuroinflammation.

INTRODUCTION

Leukocyte transmigration across vascular endothelium is a highly regulated process and plays a central role in inflammation and immune responses. Under physiological conditions, a limited number of immune cells cross the blood-brain barrier (BBB) and enter the central nervous system (CNS) in a process called immune surveillance[1,2]. In multiple sclerosis (MS) and other neuroinflammatory processes, BBB dysfunction is associated with increased leukocyte trafficking into the CNS, resulting in intense perivascular leukocytic infiltration, a hallmark of MS lesions[3,4]. The attachment of blood-borne leukocytes to the vascular bed, followed by diapedesis of immune cells across the endothelial barrier and migration into the brain parenchyma are early events in CNS-directed immune responses[5-9]. These steps are mediated by the interaction of leukocyte adhesion molecules with their cognate ligands on endothelial cells (ECs).

Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are well-described adhesion molecules expressed on inflamed BBBECs and are crucial for the transmigration of lymphocytes and monocytes, respectively[10-13]. Recent observations indicate that during diapedesis, ICAM-1 is recruited to specialized membrane microdomains called transmigratory cups[12] where it mediates cytoskeletal rearrangement through activation of Rho and ROCK GTPases[14-16]. Transmigratory cups are thus involved in the directional apico-basal guidance of the transmigrating leukocyte, through ICAM-1–LFA-1

specific interactions, intra-endothelial cell adhesion molecule-dependent signaling and cytoskeletal rearrangement[12,17].

The immunoglobulin superfamily activated leukocyte cell adhesion molecule (ALCAM, also known as CD166)[18,19], was originally characterized as a ligand for CD6[20], a co-stimulation molecule involved in lymphocyte activation and differentiation[21]. ALCAM-CD6 interactions stabilize the formation of the immune synapse between antigen-presenting cells and lymphocytes[22]. Besides heterotypic interactions (e.g. ALCAM-CD6), weaker homotypic interactions (e.g. ALCAM-ALCAM) have also been described[23]. In addition, ALCAM participates in different cellular migration processes such as neurogenesis, blastocyst implantation, neurite outgrowth, and melanoma invasion[19]. More recent data indicate that ALCAM mediates migration of THP-1 monocytes across rat pulmonary ECs[24], and regulatory T cells within pancreatic tumors[25].

To define additional adhesion molecules of the BBB, we used a proteomic-based approach to analyze specialized membrane microdomain-associated molecules of the human BBB. Here, we identified ALCAM as a putative candidate, and we describe ALCAM expression on human and mouse CNS microvascular endothelium, especially during neuroinflammatory processes. In addition we present data indicating an important role for ALCAM in the migration of leukocytes from the peripheral blood into the CNS.

RESULTS

Proteomic analysis of BBBEC membrane microdomains

To identify effectors of immune cell migration across CNS ECs, we isolated cholesterol-enriched membrane microdomains of human BBBECs grown in primary culture[26,27]. These membrane microdomains act as essential signaling platforms and co-localize with the ICAM-1–enriched transmigratory cup[12]. Lipid rafts were isolated from monolayers of human BBBECs using the detergent Brij-58 and sucrose gradient centrifugation[28]. Immunoblot performed on fractions 1-12 (**Supplementary Fig. 1a**, online) demonstrated that fractions 4 and 5 were highly enriched in cholesterol and phospholipids and contained GM1-ganglioside and CD59 but not transferrin receptors, and were thus identified as lipid rafts. Nanoflow liquid chromatography tandem mass spectrometry (NanoLC-MSpect/MSpect) analysis of raft fractions 4 and 5 revealed the presence of proteins involved in cell-cell adhesion processes (22%), lipid, protein and carbohydrate metabolism (18%), intracellular trafficking (7%), intracellular signaling and cytoskeleton (42%) and proteins of unknown function (11%) (**Supplementary Table 1,** online). Of these molecules, ALCAM was found in 2/3 of experiments performed.

Next we confirmed ALCAM expression on cultures of primary human BBBECs by RT-PCR, immunoblot and flow cytometry (**Fig. 1a-c**). *ALCAM* mRNA was detected in BBBECs using two distinct pairs of primers specific for the 5' and 3' end regions of human *ALCAM* (**Fig. 1a**). We also demonstrated that ALCAM protein expression was stronger in

CNS derived-ECs compared to non-CNS ECs including pulmonary artery ECs (HPAECs), lung microvascular ECs (HLMECs), dermal microvascular ECs (HDMECs) and umbilical vein ECs (HUVECs) (**Fig. 1b**). We further confirmed by flow cytometry the presence of ALCAM on the surface of human BBBECs and demonstrated that 93% of human BBBECs in primary culture express both ICAM-1 and ALCAM, whereas VCAM-1 expression is weaker (**Fig. 1c**). ECs obtained from CNS vessels expressed ALCAM, irrespective of vessel size (**Fig. 1d**), suggesting that ALCAM expression was not restricted to microvessels of the CNS. Immunoblot performed on lipid rafts obtained from primary cultures of resting BBBECs revealed that ALCAM was partially recruited to fractions enriched in GM1-ganglioside and CD59, two prototypical markers of lipid rafts (**Fig. 1e** and **Supplementary Fig. 1a**). Taken together, these results demonstrated that ALCAM is expressed in primary cultures of human CNS ECs and is present at the EC surface in specific membrane microdomains. These observations suggest that ALCAM, similar to ICAM-1 might serve as an adhesion molecule involved in leukocyte trafficking across the CNS endothelium[29].

ALCAM ligands on human leukocytes

To ascertain that human leukocytes have the ability to bind ALCAM on ECs, we measured the expression of the ALCAM ligands, CD6 and ALCAM, on leukocytes isolated from the peripheral blood of healthy donors. Whereas ALCAM was barely detectable on *ex vivo* human $CD4^+$ and $CD8^+$ T lymphocytes, as much as 94% of these cells expressed CD6 (**Supplementary Fig. 2**, online). Conversely, the majority of $CD19^+$ B lymphocytes and $CD14^+$ monocytes (54 and 55%, respectively), expressed ALCAM on their surface but

expression of CD6 was weak on both these populations (**Supplementary Fig. 2**, online). Hence, human peripheral blood leukocytes expressed either CD6 or ALCAM, ligands capable of interacting with endothelial ALCAM.

We next studied the association between ALCAM on BBBECs and CD6 on $CD4^+$ T lymphocytes during the transmigration process. Human $CD4^+$ T lymphocytes from peripheral blood were allowed to migrate for 2 h across human BBBECs; cells were then fixed, stained for ALCAM and CD6 and visualized by confocal microscopy. ALCAM immunoreactivity concentrated around the migrating lymphocytes in a structure reminiscent of the transmigratory cup previously described by Carman *et al*. (**Fig. 2d**)[12]. A 10 μm z stack reconstruction obtained during the transendothelial migration process demonstrated co-localization of CD6 and ALCAM and their accumulation around migrating lymphocytes (**Fig. 2d**), suggesting that ALCAM-CD6 interactions are involved in the migration of lymphocytes across BBBECs.

Inflammation-induced ALCAM expression

It is well-documented that pro-inflammatory cytokines upregulate the expression of ICAM-1 and VCAM-1 on various EC populations[30]. Therefore, effects of the proinflammatory cytokines tumor necrosis factor (TNF) and interferon-□ (IFN-□) on the expression of ICAM-1, VCAM-1 and ALCAM were determined in cultures of primary BBBECs. BBBECs grown under basal culture conditions expressed ALCAM in amounts comparable to those of ICAM-1, and higher than those of VCAM-1 (**Fig. 2a**). Activation with TNF and IFN-γ elicited a robust upregulation of both ALCAM and ICAM-1, but weaker upregulation of VCAM-1 in

BBBECs (**Fig. 2a,b**). After treatment with TNF and IFN-γ, ALCAM expression in BBBEC lipid rafts significantly increased from 39 ± 4% of total ALCAM (in untreated cells) to 61 ± 5% ($P < 0.05$, $n = 3$) (**Fig. 2c**). Taken together, these data strongly suggest that pro-inflammatory cytokines upregulate ALCAM expression on BBBECs and promote its mobilization into cholesterol-enriched membrane microdomains.

Perivascular infiltration of immune cells in the CNS is a hallmark of active MS lesions[3,31]. Therefore, the expression of ALCAM was determined in highly infiltrated plaques in post-mortem brain material of five MS patients and compared to normal-appearing white matter (NAWM) of the same MS patients, as well as to samples from five control brains (non-neurological traumatic deaths). To confirm expression of ALCAM on ECs, brain sections were stained for Caveolin-1 (Cav-1), a marker of CNS EC membranes[32]. Cav-1 expression was not affected by immune cell infiltration or by vessel size (**Fig. 3**). In contrast, ALCAM expression, while weak in small and large vessels of control and NAWM sections, was substantially increased in vessels of active MS lesions. Merged images of ALCAM and Cav-1 staining confirmed the localization of ALCAM on the surface of CNS ECs (**Fig. 3**).

X-y planar images (0.1 μm thickness) were recorded on two distinct channels. Three μm z stack images were then reconstructed and Cav-1 and ALCAM signal intensities were measured in each blood vessel (**Fig. 4**). ALCAM staining was significantly stronger in leukocyte-infiltrated vessels in active MS lesions than in vessels in NAWM or control CNS tissue (**Fig. 4d**). Additional analysis of ALCAM expression on CNS sections from Parkinson's

disease-affected individuals revealed no difference in ALCAM expression between controls, NAWM and Parkinson's CNS material (n = 2, data not shown). These data confirm that ALCAM is strongly expressed on CNS ECs in active MS lesions, and suggest that ALCAM could partake in the recruitment of leukocytes into the CNS.

ALCAM in EAE

To determine whether ALCAM functionally contributes to immune-mediated damage of the CNS in EAE, ALCAM expression was measured in the spinal cord of C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$. ALCAM staining in spinal cord homogenates and sections of MOG-immunized animals was substantially increased compared to naive controls (n = 4) (**Supplementary Fig. 3**, online). These findings are reminiscent of the upregulated expression of ALCAM in human MS lesions.

Next, EAE was induced in C57BL/6 animals by immunization with $MOG_{35–55}$. Animals were then injected intra-peritoneally (i.p.) with either ALCAM-blocking antibody (250 μg, n = 10 animals) or an isotype control antibody (250 μg, n = 10) on days 7, 9, 11 and 13. Animals injected with ALCAM-blocking antibody had significantly lower clinical scores than their isotype control-treated counterparts (day 10 to day 13, **Table 1**). The number of animals with EAE scores of 4 or greater was also significantly lower until day 13 in the ALCAM antibody-treated group (**Table 2**). These results suggest that ALCAM blockade delays the progression and reduces the severity of EAE and demonstrate the important role of ALCAM in immune cell migration into the CNS *in vivo*.

The ALCAM antibody-treated group also displayed a significant reduction in the number of inflammatory demyelinating lesions per section, as well as a marked reduction in the surface area of demyelinated lesions (**Fig. 5a–c**). The decrease in the number of lesions and area of demyelination was more evident in spinal cord samples taken from the cervical and thoracic regions than from the lumbar spine (data not shown). The number of $CD3^+$, $CD4^+$, $CD8^+$ and Mac-1^+ immune cells in EAE lesions was evaluated in animals treated with ALCAM-blocking and isotype control antibodies. In ALCAM antibody-treated animals, inflammatory lesions had significantly lower numbers of $CD3^+$, $CD4^+$ and Mac-1^+ cells, compared to isotype-treated group (**Fig. 5d, e**). ALCAM blockade did not influence the number of $CD8^+$ immune cells present in inflammatory lesions (**Fig. 5d**).

To ensure that the effect the ALCAM antibody on the course of EAE was not a consequence of a blockade of lymphocyte co-stimulation and subsequent activation, we assessed the proliferation and analyzed the cytokine profile of human and mouse lymphocytes following ALCAM blockade. Human peripheral blood $CD4^+$ T lymphocytes were stimulated with autologous $CD14^+$ monocytes and CD3-specific antibody in the presence or absence of ALCAM-blocking antibody. Neither T cell proliferation nor the percentage of cells expressing CD25, IFN-γ or IL-17 were affected by ALCAM blockade (**Supplementary Fig. 4 and supplementary Table 2,** online). Antigen-specific T cell responses to hemmaglutinin A (HA), MOG_{35-55}, myelin-associated oligodendrocytic basic protein $(MOBP)_{28-38}$, and whole myelin basic protein (MBP) were not influenced by ALCAM blockade (**Supplementary Table 2,**

online). To further demonstrate that ALCAM neutralization *in vivo* did not impact leukocyte co-stimulation during priming in EAE, lymph nodes and spleen from anti-ALCAM– and isotype control-treated C57BL/6 animals were collected 9 days after immunization with $MOG_{35–55}$ or keyhole limpet hemocyanin (KLH) (prior to disease onset). IL-17 and IFN-γ production by T cells, assessed by ELISPOT, was similar in animals treated with anti-ALCAM and those treated with the isotype control antibody (**Supplementary Fig. 5**, online) and no differences in proliferation were detected (data not shown). These data confirmed that the role of ALCAM in the development of EAE is related to its ability to mediate immune cell recruitment and migration into the CNS, rather than to its co-stimulatory role in the periphery.

ALCAM blockade restricts migration across BBBECs

The contribution of ICAM-1 and VCAM-1 to the migration of freshly isolated (*ex vivo*) human leukocytes has been studied previously using an *in vitro* model of the human BBB[33,34]. To evaluate the contribution of ALCAM to the recruitment of immune cells into the human CNS, we isolated $CD14^+$, $CD4^+$, $CD19^+$ and $CD8^+$ leukocytes from the peripheral blood of healthy donors and evaluated their migration across human BBBECs in the presence of ALCAM-blocking or isotype control antibodies. ALCAM blockade significantly restricted the migration of $CD14^+$ monocytes, $CD4^+$ T lymphocytes and $CD19^+$ B lymphocytes (**Fig. 6a**). Antibody blockade of CD6 also suppressed the migration of $CD4^+$ T lymphocytes (49,980 ± 5,020 isotype control vs. 36,170 ± 4,028 anti-CD6, $n = 8$, $P < 0.05$, data not shown). In contrast, $CD8^+$ T lymphocyte transmigration was not influenced by blockade of either

ALCAM (**Fig. 6a**) or CD6 (data not shown). These data provided evidence that endothelial ALCAM is functionally involved in the transmigration of human $CD4^+$, $CD19^+$ and $CD14^+$ leukocytes across the BBB.

To further characterize the importance of ALCAM, relative to ICAM-1, in immune cell transmigration, we used anti-ALCAM and anti-ICAM-1 antibodies, alone or in combination. Anti-ALCAM and anti-ICAM-1 antibodies acted synergistically to reduce the migration of $CD4^+$ T lymphocytes across the BBB by 76% (**Fig. 6b**). These results indicated that both ALCAM and ICAM-1 play important and partially redundant roles in the migration of immune cells across human BBBECs.

Discussion

Adhesion molecules are essential for the attachment of leukocytes to the vascular endothelium and play an important role in diapedesis[5,6,8]. Here, ALCAM was identified as an important mediator involved in leukocyte migration into the CNS. ALCAM was found to concentrate in cholesterol-enriched membrane microdomains on the surface of brain ECs, in a structure reminiscent of both the transmigratory cup[12] and the immunological synapse[26]. ALCAM expression was upregulated in both BBBEC after exposure to inflammatory cytokines, and in infiltrated vessels within active MS and EAE lesions. We provided evidence that ALCAM blockade *in vivo* reduces the clinical severity of $MOG_{35–55}$-induced EAE, the mouse model of MS, as well as the number of inflammatory lesions, the area of demyelination and the migration of $CD3^+$, $CD4^+$ and Mac-1^+ leukocytes into the CNS. Finally, ALCAM was shown to participate in the transmigration of human $CD4^+$, $CD19^+$ and $CD14^+$ leukocytes across human BBBECs in an *in vitro* model of the BBB. Taken together, the work presented herein strongly supports an important role for ALCAM in peripheral blood leukocyte recruitment into the CNS, especially in the context of MS and EAE plaque formation.

The pathological hallmark of active MS lesions is the presence of demyelination and mononuclear immune cell infiltrates. These perivascular infiltrates consist of myelin-loaded macrophages, $CD4^+$ and $CD8^+$ T lymphocytes, with a few B lymphocytes[3]. Whereas ICAM-1 is recognized as the adhesion molecule preferentially involved in T lymphocyte migration[35], VCAM-1 is known for its ability to specifically recruit cells of the monocyte-macrophage

lineage[36,37]. One of the key finding in this study is that ALCAM, unlike ICAM-1 and VCAM-1, mediated the transmigration of both lymphoid ($CD4^+$, $CD19^+$) and myeloid ($Mac\text{-}1^+$, $CD14^+$) leukocytes. In addition, although ALCAM and ICAM-1 acted in a partially redundant way, our data also suggested a greater role for ALCAM than ICAM-1 in leukocyte diapedesis across CNS endothelium.

In addition to binding to cognate ligands and providing a "docking" site for leukocytes, cellular adhesion molecules can initiate intracellular signaling cascades that allow ECs to respond to leukocyte adhesion and participate in diapedesis[16]. Despite a short cytoplasmic tail, ALCAM can bind, directly or indirectly, to a number of intracellular proteins in ECs. ALCAM homophilic and heterophilic interactions depend on protein kinase C-□, but not on the GTPases RhoA and Cdc42[38]. Recent studies indirectly linked ALCAM to the actin cytoskeleton remodeling through interactions with α-catenin[39], the ezrin-radixin-moesin family of proteins, the Rac-specific guanine nucleotide exchange factor Tiam1[40] and the actin bundle proteins fascin and filamin[38].

Cholesterol-enriched membrane microdomains, also called lipid rafts, have recently been shown to play a crucial role in immune cell diapedesis[12,26,29]. Lipid rafts are involved in multiple physiological processes related to transmigration including the acquisition and maintenance of morphological and functional asymmetry, the spatial enrichment of proteins and the organization of the initiation and the propagation of intracellular signaling events associated with migration. It is thus believed that during diapedesis, lipid rafts act as crucial signaling platforms required for proper leukocyte transmigration. Adhesion molecules

expressed by ECs, such as E-selectin[41], ICAM-1[12] and VCAM-1[12] also accumulate in lipid rafts during transmigration and mediate intracellular signaling events important for diapedesis. Such signaling events result in activation of small GTPases, tight junction remodeling and actin cytoskeleton rearrangement[17], a phenomenon which coincides with the formation of the transmigratory cup. We report herein that pro-inflammatory cytokines induced the recruitment of ALCAM into lipid rafts; raft-localized ALCAM physically associated with CD6 expressed on leukocytes, and participated in the formation of the transmigratory cup. Whether signaling through ICAM-1 and ALCAM provide redundant intracellular signals and lead to Rho-induced cytoskeletal rearrangements, remains to be established.

Blocking immune cell migration across the BBB has long been regarded as an attractive therapeutic approach for CNS-directed autoimmune diseases[30]. The application of proteomic techniques to human primary CNS cell culture has allowed us to gain a new insight into the immune elements involved in the pathogenesis of CNS-directed immune reactions and to identify potential targets to inhibit neuroinflammatory processes. Although pharmacological agents specifically targeting adhesion molecules significantly reduce the extent of CNS inflammation in MS[42], they also impede on the ability of the immune system to provide protection against chronic (latent) CNS viral infections, such as the polyoma Jonh Cunningham virus (JCV) -induced oligodendrogliopathy, leading to progressive multifocal leukoencephalopathy[43]. Since ALCAM blockade did not affect $CD8^+$ T cell migration *in vitro*, we hypothesize that CNS immune protection against viruses would not be compromised by

ALCAM blockade *in vivo*. ALCAM could thus be considered as an attractive, and possibly safe, therapeutic target for MS.

Materials and Methods

BBBEC isolation and culture

BBBECs were isolated from non-epileptic material (resection path) according to a previously published protocol[10,28,44]. Informed consent and ethic approval were given prior to surgery (CHUM research ethic committee approval number HD04.046). Characterization and purity was performed as previously published[10,28]. HLMECs, HDMECs, HUVECs and HPAECs were purchased from Cambrex Corporation and cultured according to the manufacturer's instructions.

Tissue and immunostaining

Formaldehyde-fixed CNS specimens from five MS-affected individuals were examined, (2 males, both 42 years of age and 3 females, 54, 62 and 66 years of age); evolution of disease was between 2 and 20 years. Five control brains from patients who died of non-neurological traumatic injury (34, 46, 51, 69 years of age and unknown, 3 males and 2 females) were used as controls. Autopsies were performed within 6–12 h from time of death. Active MS lesions were identified by LFB and haematoxilin and eosin (H&E) staining and defined as areas of demyelination associated with intense perivascular immune cell infiltration (58.2 ± 3 nuclei/field centered on a vessel, n = 15 lesions studied). NAWM (from MS cases) and control brains (non-neurological traumatic death) were defined as areas of normal myelin staining in the absence of immune cell infiltration (21.6 ± 4 nuclei/vessel and 23.2 ± 5 nuclei/vessel, respectively, n = 15 per group). For immunohistofluorescence, 5 μm thick sections were

deparaffinized and stained with antibodies specific for ALCAM (polyclonal goat, 1/40, R&D Systems Inc.), Cav-1 (polyclonal rabbit, 1/75, Santa Cruz Biotechnology), or a with donkey goat-specific antibody (1:400, biotin-conjugated, followed by streptavidin-Cy3; Dako and Jackson ImmunoResearch Laboratories, respectively) or a fluoroscein isothiocyanate (FITC)-conjugated goat rabbit-specific antibody (1:400, 30 min; Jackson ImmunoResearch). Nuclei were stained with TOPRO-3 (1:300 in PBS; Molecular Probes). Control staining was performed with an isotype control antibody. For immunocytochemistry human BBBECs were treated, or not, with IFN-γ and TNF (100 U/ml) for 16 h. Isolated peripheral blood $CD4^+$ T lymphocytes migrated for 1 h and were then fixed with 4% paraformaldehyde. ALCAM, ICAM-1 and CD6 were detected with anti-ALCAM (polyclonal rabbit, 1:40, Santa Cruz Biotechnology), anti-CD6 (mouse monoclonal coupled to FITC, 1:20, BD Biosciences) and anti-ICAM-1 (mouse monoclonal, 1:50, R&D Systems Inc.), respectively. All images were acquired using a Leica SP5 confocal microscope and analyzed using the Leica LAS AF software (Leica Wetzlar). 10 μm z stack reconstructions were performed using at least 67 x-y planar images (0.15 μm thickness). Semi-quantitative analysis was performed using 3 μm z stack images of 30 x-y planar images (0.1 μm thickness) recorded sequentially. Signal intensity was collected in duplicate and microscopy data were recovered by two investigators blinded to the disease group.

RT-PCR

RT-PCR was performed using a previously established protocol[28,45]. All reagents used were from Invitrogen. ALCAM and GAPDH primers used are listed in Supplementary table 3, online.

Immune cell isolation and BBB transmigration assay

A modified Boyden chamber assay was used to model the BBB, as previously reported[10,28]. 25×10^3 BBBECs were grown on 3 μm pore size Boyden chambers (Collaborative Biomedical Products) in culture media supplemented with 20% astrocyte media for 72 h. Antibodies specific for ALCAM (30 μg/ml) or ICAM-1 (10 μg/ml) (or the appropriate isotype control 40 μg/ml) were added 1 h prior to immune cell migration. Venous blood samples were obtained from consenting healthy donors, in accordance with institutional guidelines (CHUM research ethic committee approval number SL05.022 and 023), and immune cells were isolated as previously published[10]. Data were collected by two individuals blinded to the treatment group.

Flow cytometry

BBBECs treated with IFN-γ and TNF (100 U/ml) were recovered in cold PBS and EDTA, and incubated with anti-ICAM-1, anti-VCAM-1, anti-ALCAM or anti-CD6 antibodies (all from BD Biosciences, respectively clones HA58, 51-10C9, 3A6 and M-T65). Fluorescence acquisition was performed on a BD LSR II (BD Biosciences) and analyzed using the BD FACS Diva software (BD Biosciences).

Lipid raft isolation and analysis

Lipid raft were isolated from confluent BBBECs treated, or not, with IFN-γ and TNF (100 U/ml, 16 h) using a previously published protocol[28,46]. Briefly, BBBECs were lysed in 1% Brij-58 (Sigma). The lysate was mixed with 1 ml of 85% sucrose (Sigma), overlaid with 35% sucrose and 5% sucrose (5 ml each) and centrifuged for 24 h at 39,000 r.p.m. at 4 ˚C. Twelve 1 ml fractions were collected, top to bottom. The cholesterol, phospholipid and protein concentrations in each fraction were assayed using cholesterol assay kit (Molecular Probes), Phospholipids B colorimetric method kit (Wako) and BCA Protein assay kit (Pierce). Specific protein detection was performed by immunoblot as described below.

Immunoblot

Forty micrograms of BBBEC lysate was analyzed by standard SDS-PAGE using anti-ALCAM (rabbit, 1/200 Santa Cruz Biotechnology). Specific binding was revealed with horseradish peroxydase (HRP)-conjugated anti-rabbit (1/1,000, DakoCytomation) using the ECL system (Amersham Biosciences). □-actin (1/5,000, MP Biomedicals) was used as a loading control. Digital images obtained from the Bio-Rad Gel Doc system were used to quantify the band intensity with the Quantity One software (Bio-Rad).

EAE disease induction, scoring and pathology

Female C57BL/6 mice were immunized subcutaneously with 300 μg MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK; Sheldon Biotechnology Center) in a 100 μl emulsion of incomplete Freund adjuvant supplemented with up to 4 mg/ml *Mycobacterium tuberculosis* (Fisher Scientific). Pertussis toxin (300 ng, Sigma-Aldrich) was injected i.v on days 0 and 2.

Animals were then injected i.p on days 7, 9, 11 and 13 with either anti-ALCAM (250 μg, n = 10 animals) or isotype control (250 μg, n = 10). The scoring system used was a follows: 0, normal; 1, limp tail; 2, mild hind-limb weakness (quick righting-reflex); 3, severe hind-limb weakness (slow righting-reflex); 4, hind-limb paralysis; 5, hind-limb paralysis and partial fore-limb weakness. Mice were scored by an investigator blinded to the treatment group. On day 20, four animals from each group were sacrificed, and CNS organs were recovered and frozen. The spinal cords were stained with LFB and H&E as previously described[28], meningeal and parenchymal inflammatory lesions were counted per spinal cord section as described by Kalivas *et al*. [47]. Demyelination area was assessed as a loss of LFB staining and area was calculated using the Open Lab microscopy software. Counts of $CD3^+$, $CD4^+$, $CD8^+$ and Mac-1^+ immune cells were done on immunofluorescence stained sections (biotin-labeled Ab, 1:50 dilution, all from BD Bioscience). Stainings were visualized with streptavidin-FITC (1:400, Dako) on a SP5 laser scanning confocal microscope (Leica TCS SP5). Number of lesions and area of demyelination were evaluated in cervical, thoracic and lumbar spinal cords of 4 animals (per treatment group). The number of immune cells per lesion was quantified in 15 random lesions in the three levels of the spinal cords (4 animals per treatment group). All counts were performed by two investigators blinded to the treatment group. All procedures were approved by the McGill University Animal Care committee and followed guidelines of the Canadian Council on Animal Care.

Statistical analyses

Statistical analyses were performed using PRISM Graphpad™ software and included ANOVA followed by (paired, when possible) Student *t*-test, Bonferroni post-test, Fisher test or Tukey post-hoc test, depending on the number of comparisons to controls. Only P values < 0.05 were considered statistically significant. Data are expressed as mean ± standard error of the mean from at least $n = 3$ independent experiments, each conducted in duplicate.

Figures

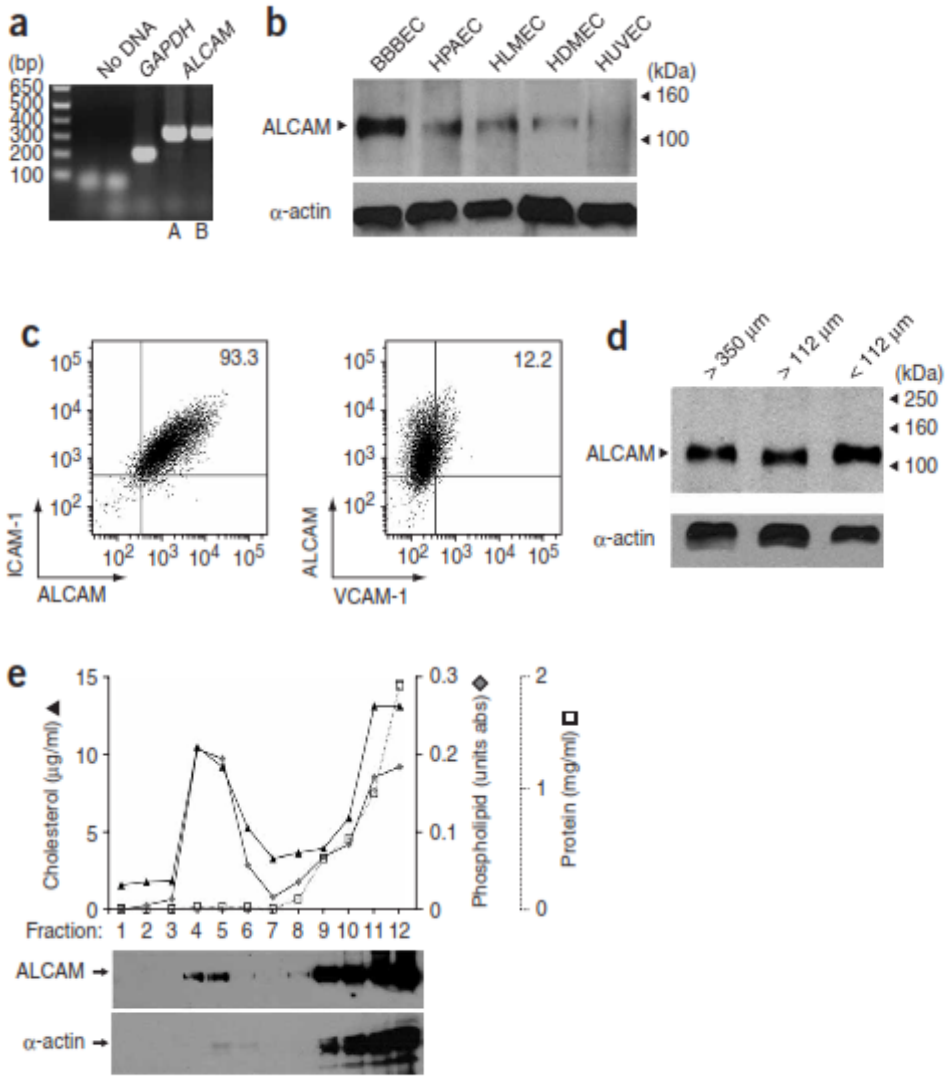

a
No DNA
GAPDH
ALCAM
(bp)
650
500
400
300
200
100
A B
b
BBBEC
HPAEC
HLMEC
HDMEC
HUVEC
(kDa)
160
100
ALCAM
α-actin
c
93.3
12.2
ICAM-1
ALCAM
VCAM-1
d
> 350 μm
> 112 μm
< 112 μm
(kDa)
250
160
100
ALCAM
α-actin
e
Cholesterol (μg/ml)
Phospholipid (units abs)
Protein (mg/ml)
Fraction: 1 2 3 4 5 6 7 8 9 10 11 12
ALCAM
α-actin

Figure 1: ALCAM is expressed by resting human BBBECs and partially localizes to lipid rafts.

(**a**) Expression of *ALCAM* mRNA in BBBECs. *ALCAM* mRNA was amplified using primers located in the 3' and 5' regions (primer pair a and b, respectively). Expected *ALCAM* amplicon 350 bp; *GAPDH* 196 bp. (n = 4) (**b**) Expression of ALCAM protein (105 kDa) in human BBBECs, pulmonary artery ECs (HPAECs), lung microvascular ECs (HLMECs), dermal microvascular ECs (HDMECs) and umbilical vein ECs (HUVECs). (n = 3) (**c**) Surface expression of ICAM-1, VCAM-1 and ALCAM on BBBECs *in vitro*, as assessed by flow cytometry (n = 5). (**d**) ALCAM expression on ECs of CNS vessels of different diameter (microvessels <112 μm, and larger vessel diameters >112 μm or >350 μm, n = 4). (**e**) ALCAM localization to lipid rafts of BBBECs under basal conditions (n = 3). The presence of cholesterol and phospholipids confirms the lipid raft nature of fractions 4 and 5.

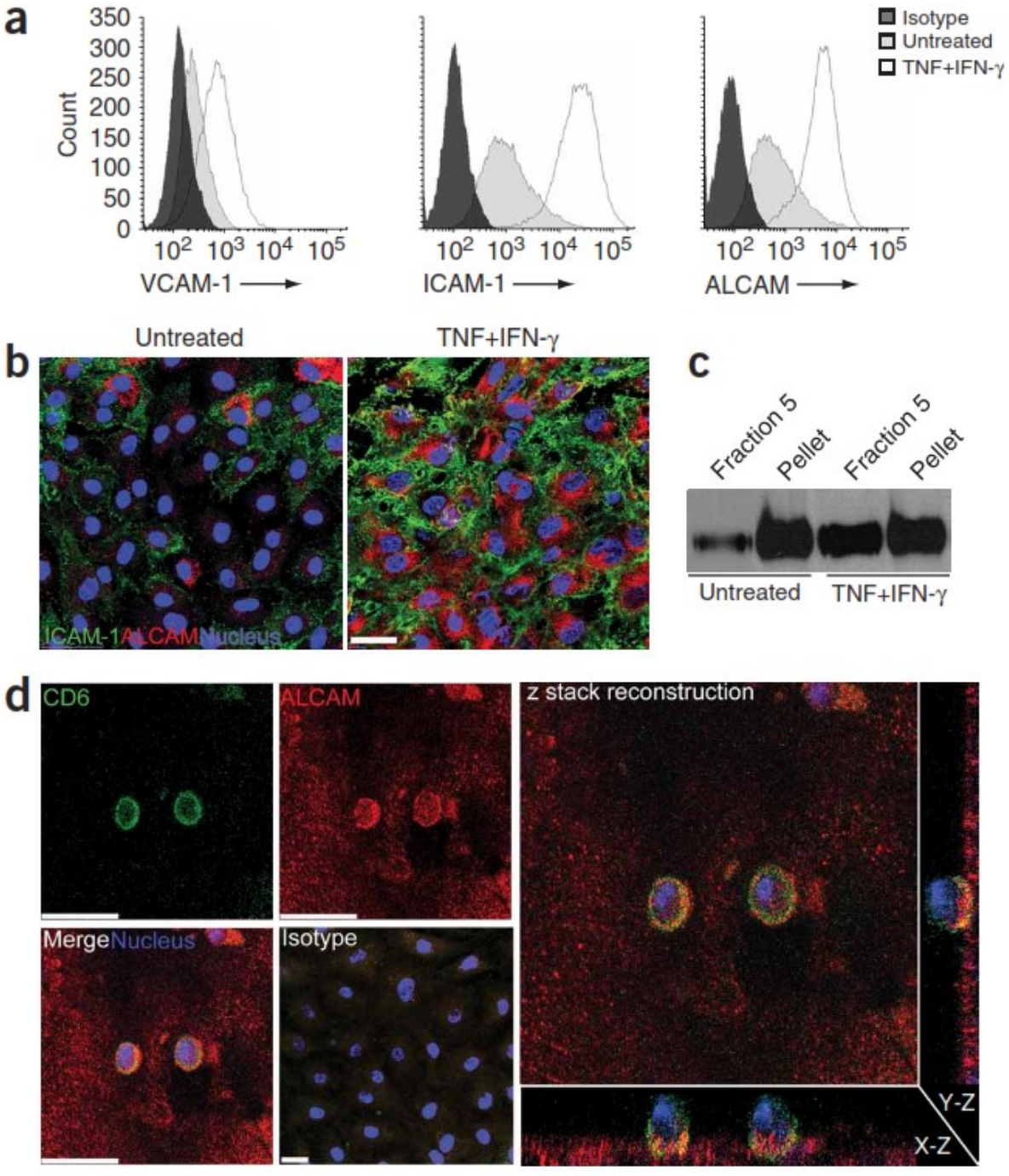

a
Count
350
300
250
200
150
100
50
0
10^2 10^3 10^4 10^5
VCAM-1
ICAM-1
ALCAM
Isotype
Untreated
TNF+IFN-γ
b
Untreated
TNF+IFN-γ
ICAM-1ALCAMNucleus
c
Fraction 5
Pellet
Fraction 5
Pellet
Untreated
TNF+IFN-γ
d
CD6
ALCAM
z stack reconstruction
MergeNucleus
Isotype
Y-Z
X-Z

Figure 2: Pro-inflammatory cytokines upregulate ALCAM on BBBECs and promote ALCAM translocation to lipid rafts.

(**a**) VCAM-1, ICAM-1 and ALCAM expression on primary cultures of human BBBECs, as assessed by flow cytometry. BBBECs were left untreated or incubated with TNF and IFN-γ (100 U/ml each) for 16 h. (*n* = 4, in duplicate). (**b**) ICAM-1 (green) and ALCAM (red) and nuclei (blue) on the surface of BBBECs treated or not with TNF and IFN-γ (100 U/ml each for 16 h) (*n* = 3). Scale bar is 25 μm and magnification is x 40. Nuclei were stained with TOPRO-3. (**c**) Effect of TNF and IFN-γ on the accumulation of ALCAM into BBBEC lipid rafts. Densitometry analysis of ALCAM expression in fraction 5 indicated in **Fig. 1e** in BBBECs left untreated or treated with IFN-□ and TNF (100 U/ml each) for 16 h. Untreated BBBECs, 39 ± 4% of ALCAM in fraction 5; TNF and IFN-γ-treated BBBECs, 61 ± 5% of ALCAM in fraction 5. (*n* = 3) (**d**) Purified human $CD4^+$ T lymphocytes were seeded over a monolayer of confluent BBBECs and stained with antibodies specific for CD6 (green) and ALCAM (red). Nuceli were stained with TOPRO-3 (blue). X-y confocal acquisition (left panels) and 10 μm z stack reconstruction (y-z and x-z, right panel) were used to assess colocalization of ALCAM and CD6 (yellow) in the transmigratory cup surrounding the lymphocytes at points of EC entry. (*n* = 4) Scale bar is 25 μm and magnification is x 63.

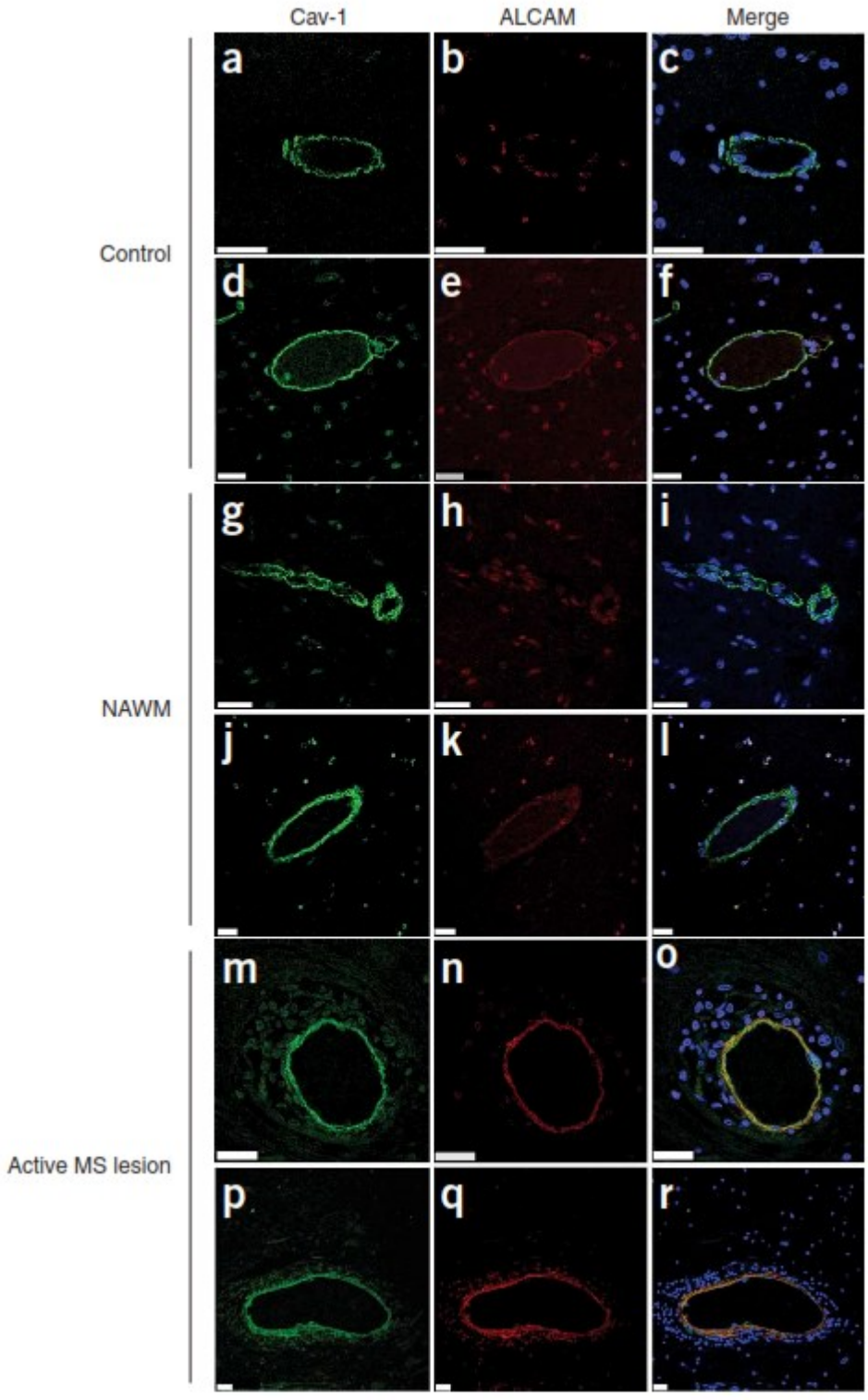
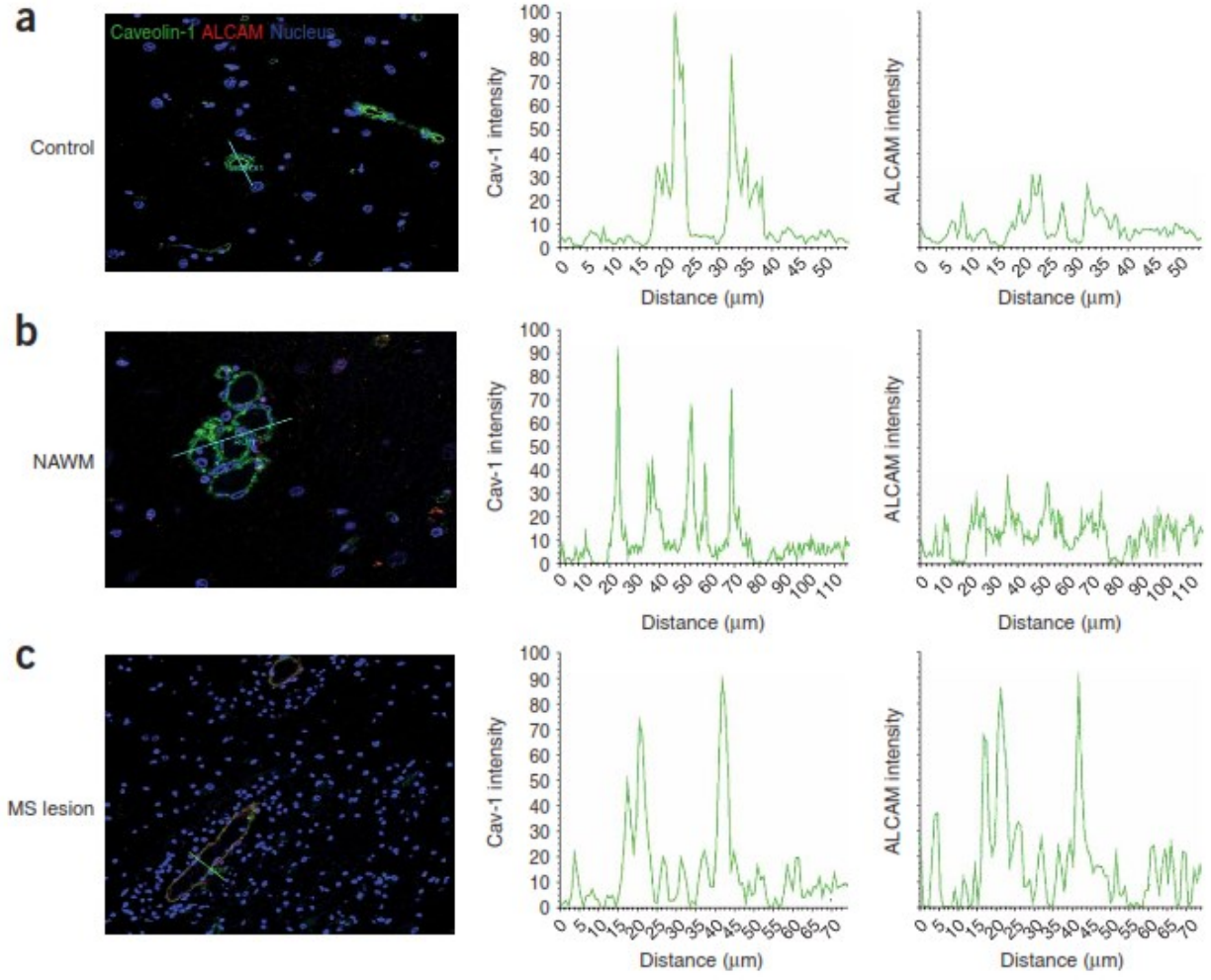

Cav-1
ALCAM
Merge
Control
NAWM
Active MS lesion
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Figure 3: ALCAM is upregulated on CNS vessels in active multiple sclerosis lesions.

Paraffin-embeded material from control human CNS (**a**-**f**), normal-appearing white matter (NAWM) from MS-affected individuals (**g**-**l**) and active MS lesions (**m**-**r**) are shown. Sections were stained with antibodies specific for Cav-1 (green) and ALCAM (red). Nuclei were stained with TOPRO-3 (blue). Images were acquired by confocal microscopy. Data shown are representative of 5 control patients and 15 active MS lesions from 5 distinct 5 MS patients. Scale bar is 25 μm, magnification x 63 (**a**-**c**, **g**-**i**, **m**-**o**) and x 40 (**d**-**f**, **j**-**l**, **p**-**r**).


a
Caveolin-1 ALCAM Nucleus
Control
Cav-1 intensity
ALCAM intensity
Distance (μm)
b
NAWM
c
MS lesion

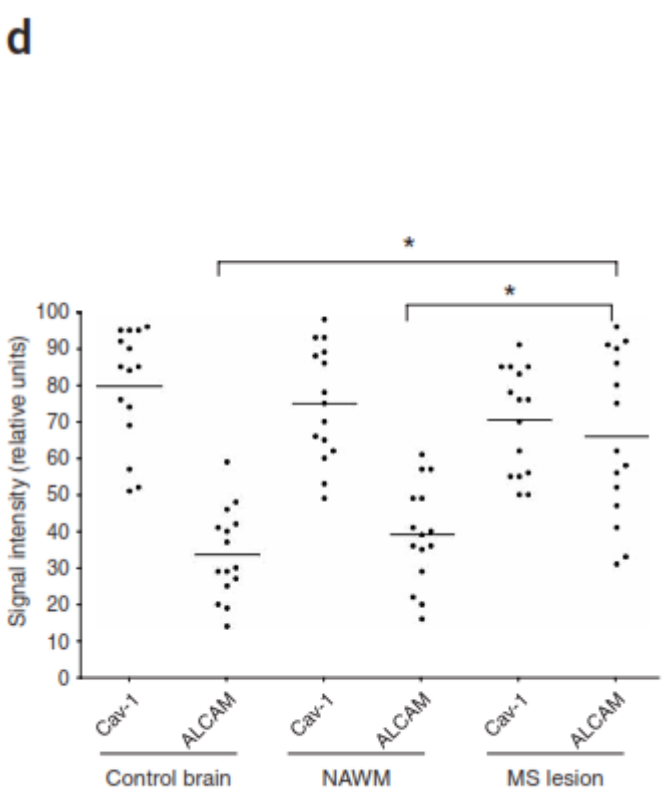
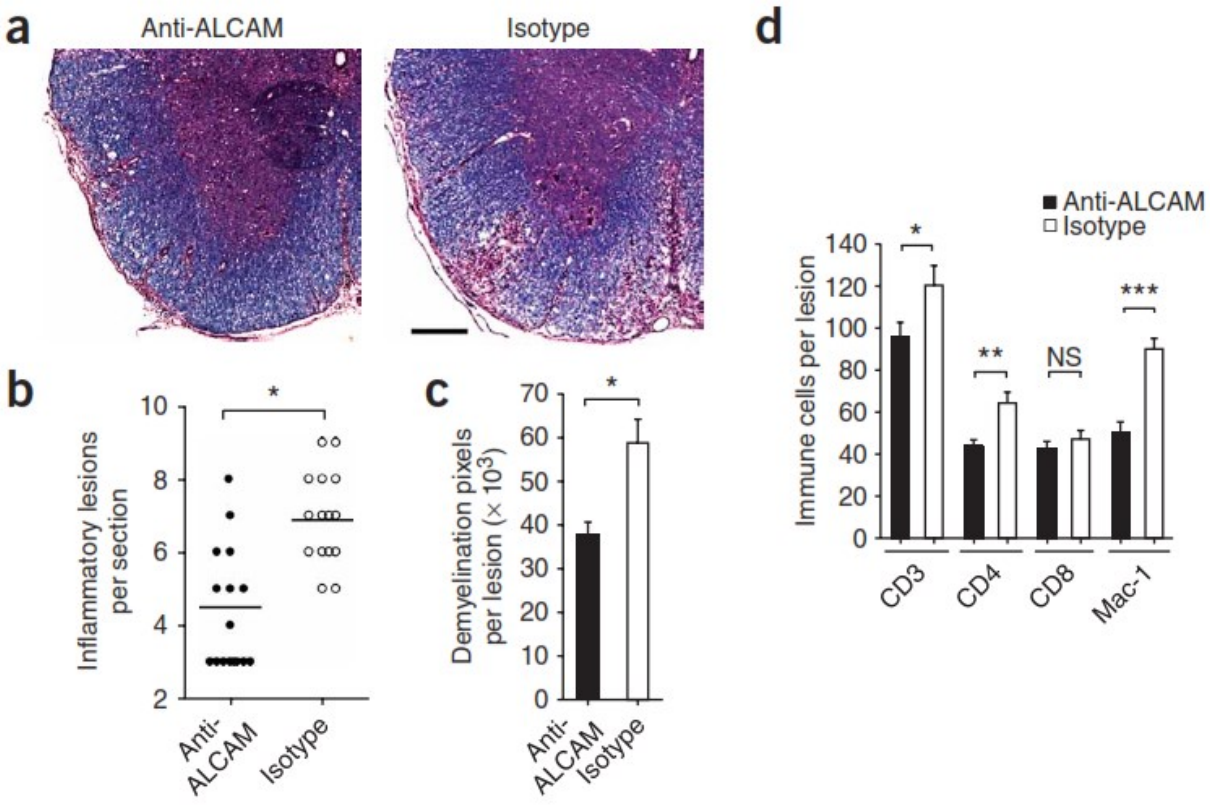


Figure 4: Semi-quantitative analysis of ALCAM expression in MS lesions.

Signal intensity from 3 µm z stack reconstruction along the line marker in three representative merged images of staining for Cav-1 (green) and ALCAM (red). (**a**) Control CNS samples. (**b**) Normal-appearing white matter (NAWM) from MS–affected individuals. (**c**) Active MS lesion with increased cellular infiltration. Representative signal intensities for Cav-1 (**a–c**, center) and ALCAM (**a–c,** right) are shown as histograms. Peak fluorescence was averaged from two distinct measurements on each vessel, and is presented in a graphical view in (**d**). Three representative vessels were analyzed in five distinct CNS specimens, from five donors for

each group (***, $P < 0.001$; $n = 15$ per group). Each point represents one vessel image. Panel (**c**) is shown at lower magnification to demonstrate cell infiltration. Magnification is x 63 for (**a**) and (**b**), x 40 for (**c**).


a
Anti-ALCAM
Isotype
b
Inflammatory lesions per section
Anti-ALCAM
Isotype
c
Demyelination pixels per lesion (× 10³)
Anti-ALCAM
Isotype
d
Anti-ALCAM
Isotype
Immune cells per lesion
CD3
CD4
CD8
Mac-1
NS

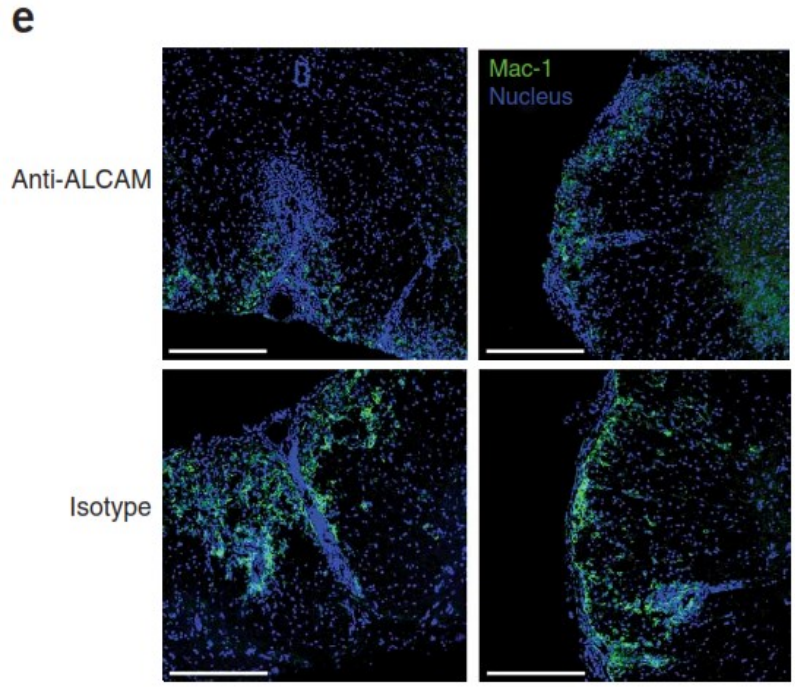


Figure 5: Reduced inflammatory lesions, demyelination and leukocyte transmigration in EAE animals treated with ALCAM-blocking antibody.

EAE was induced by $MOG_{35–55}$ immunization of C57BL/6 mice, and mice treated with i.p injections of ALCAM-specific or isotype control antibodies on days 7, 9, 11 and 13. Mice with similar disease scores were sacrificed at day 20, perfused and CNS material was recovered. (**a**) Representative luxol fast blue and haematoxilin and eosin stainings of spinal cord sections from mice treated as indicated. Scale bar is 250 μm and magnification x 10. (**b**) Number of inflammatory foci per spinal cord section from mice treated as indicated (***, $P < 0.001$; $n = 4$ animals per treatment group). (**c**) Area of demyelination in the spinal cord sections from mice treated as indicated (**, $P < 0.005$; $n = 4$ animals per group, $n \geq 15$ lesions

per animal). (**d**) Number of $CD3^+$, $CD4^+$, $CD8^+$ and Mac-1^+ leukocytes in EAE lesions in mice treated as indicated (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.005$; $n = 4$ animals per group, 15 lesions per animal). (**e**) Representative photomicrographs showing Mac-1^+ immune cells in the spinal cord of mice treated as indicated. Scale bar is 250 μm and magnification x 20.

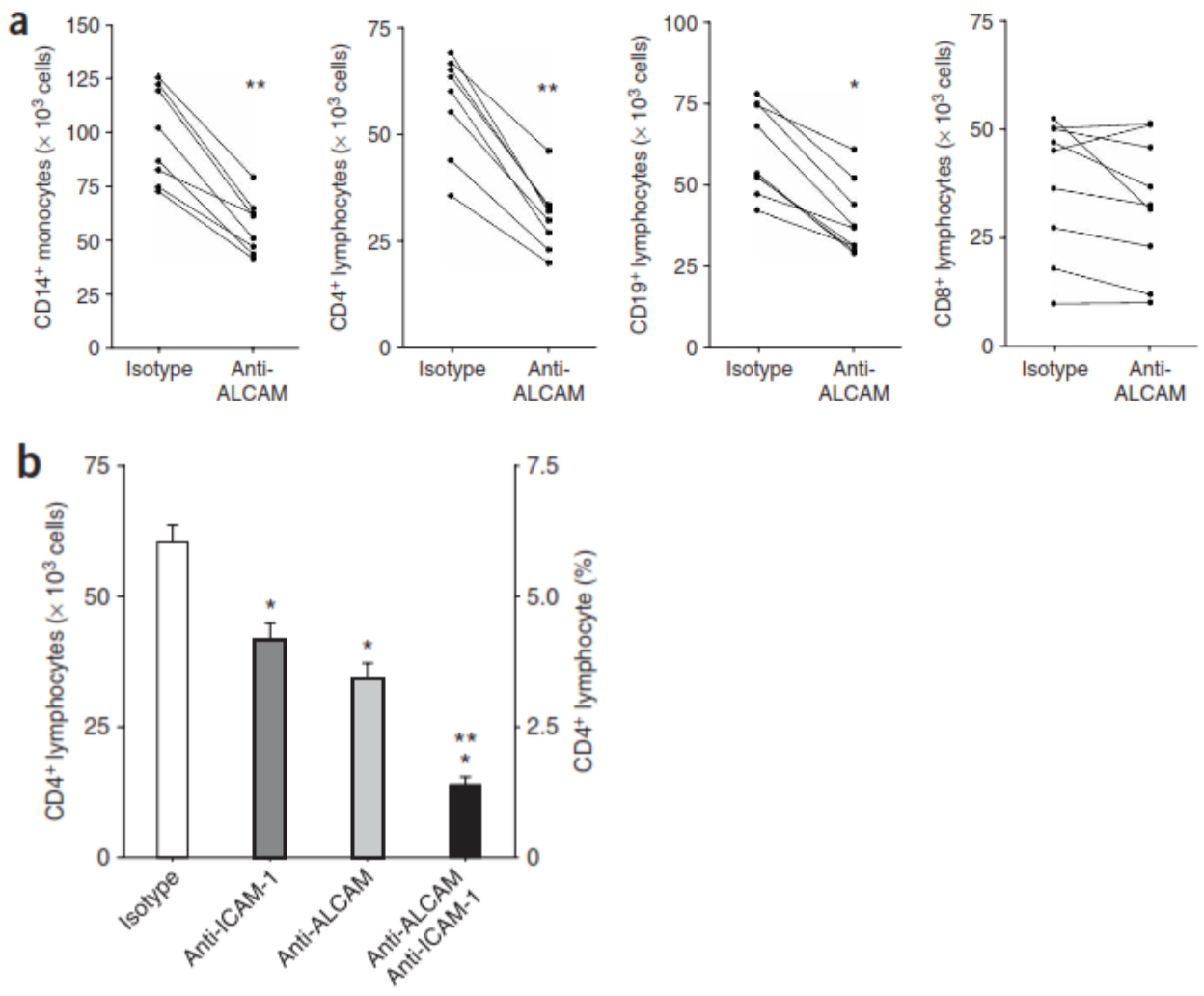


Figure 6: ALCAM contributes to human leukocyte migration in an *in vitro* model of the BBB.

ALCAM-blocking or isotype control antibodies (both at 30 μg/ml) were added to purified leukocytes migrating across human BBBECs in a modified Boyden chamber assay. (**a**) Migration of purified $CD4^+$, $CD8^+$, $CD19^+$ lymphocytes and $CD14^+$ monocytes obtained from healthy donors was assessed in the presence of BBBECs treated as indicated (each point

represents the mean from duplicates, $n = 8$, ***, $P < 0.001$, ** $P < 0.005$). (**b**) Migration of $CD4^+$ T lymphocytes across BBBECs in the presence of antibodies specific for ALCAM (30 μg/ml) or ICAM-1 (10 μg/ml) alone and in combination (***, $P < 0.001$, compared to isotype control; $n = 4$, ‡, $P < 0.0001$).

Table 1 Clinical scores of C57BL/6 mice immunized with MOG(35–55)

		Clinical score						
Treatment group	Incidence	Days 0–7	Day 10	Day 11	Day 12	Day 13	Day 15	Day 18
Isotype	10/10	0 ± 0	0.85 ± 0.3	2.25 ± 0.4	3.35 ± 0.2	3.75 ± 0.3	3.85 ± 0.1	3.95 ± 0.2
ALCAM	10/10	0 ± 0	0.35 ± 0.2*	0.95 ± 0.3**	2.65 ± 0.5***	3.05 ± 0.1****	3.45 ± 0.3	3.75 ± 0.3

Clinical scores of mice injected intraperitoneally with 250 μg of isotype control antibody or anti-ALCAM on days 7, 9, 11 and 13 after immunization with MOG (35–55). *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.01$; ****, $P < 0.01$ (two-way repeated-measures analysis of variance with post-hoc Tukey tests). Data (mean ± s.e.m.) are representative of one experiment with ten mice per group.

Table 2 EAE severity in C57BL/6 mice with EAE scores of over 4

	Time after immunization										
	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17
Isotype	0/10	0/10	0/10	0/10	3/10	4/10	7/10	8/10	7/10	8/10	8/10
ALCAM	0/10	0/10	0/10	0/10	0/10*	0/10*	3/10*	7/10	6/10	6/10	7/10

Analysis of mice injected intraperitoneally with 250 μg of isotopye control antibody or anti-ALCAM on days 7, 9, 11 and 13 after immunization with MOG (35–55); data represent mice with an EAE score of 4 or more/total mice analyzed. *, $P < 0.05$ (unpaired *t*-test) or $P < 0.001$ (Fisher's exact test). Data (mean ± s.e.m.) are representative of one experiment with ten mice per group.

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Author contributions

R.C. performed all the experiments unless stated otherwise; K.W. established BBBEC primary cultures and lipid raft purification; A.D.-D., I.I, S.K., N. A. and H.K. performed some immunostainings and flow cytometry and discussed data; J.L.B. and S.D. assisted with EAE experiments; A.H. and D.S. performed lipid raft proteomic analysis; R.M. and A.B. provided the human CNS material from the operating room; B.B and K.K. performed *in vivo*

restimulation assays; R.C. and A.P. designed the experiments and prepared the manuscript; AP secured the funding.

Competing interest statement

The authors declare that they have no competing financial interests.

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Supplementary Material

Lipid raft isolation and analysis

Lipid raft were isolated from confluent BBBECs treated, or not, with IFN-γ and TNF (100 U/ml, 16 h) using a previously published protocol[1]. Cholesterol, phospholipid and protein concentrations in each fraction were assayed using commercially available kits: Cholesterol assay kit (Molecular Probes), Phospholipids B colorimetric method kit (Wako) and BCA Protein assay kit (Pierce). Specific protein detection was performed by Western blot as described below.

Immunoblot of crude cell lysates and membrane lipid rafts

Forty □g of spinal cord homogenate were analyzed by standard SDS-PAGE using anti-ALCAM antibody (rabbit, 1/200 Santa Cruz Biotechnology). Lipid raft markers were assessed by SDS-PAGE (GM1, horse radish peroxidase (HRP) -cholera toxin B subunit, Molecular Probes; goat anti-CD59, 1/200, R&D Systems; mouse anti-transferrin receptor, 1/1000, BD Biosciences). Specific binding was revealed with HRP-conjugated anti-rabbit antibody using the ECL system and □-actin was used as a loading control.

BBB-ECs lipid raft proteomic analysis

Lipid rafts were dialyzed against denaturing buffer (50 mM Tris-HCl, pH 8.5, 0.1% SDS) and precipitated with 10 volumes of cold acetone. The precipitated proteins were re-suspended in denaturing buffer and reduced and alkylated using dithiothrietol (4 mM, 10 min at 95°C) followed by iodoacetamide (10 mM, 20 min at room temperature in dark). The proteins were

trypsin-digested using 5 μg of trypsin gold (Promega, 12-18 h at 37°C) and the resulting peptides were purified on CE column (POROS® 50 HS, 50-μm particle size, 4.0 mm ×15 mm, Applied Biosystems) as per manufacturer's protocol. The samples were analyzed by tandem mass spectrometry on a hybrid quadrupole time-of-flight Q-TOF™ Ultima instrument (Waters) with an electrospray ionization source and with an online reverse phase nanoflow liquid chromatography column (nano-LC; PepMap C18 capillary column, Dionex/LC-Packings). Peptides were separated on the nanoLC column using a gradient of 5–75% acetonitrile, 0.2% formic acid in 90 min, 350 nL/min supplied by a CapLC HPLC pump (Waters). At least one 40-min blank was ran in-between samples to prevent cross-contaminations. MSpect/MSpect spectra were acquired in the auto- MSpect/MSpect mode (data-dependent scanning) on 2+, 3+, and 4+ ions. These were then submitted to a probability-based search engine, Mascot version 2.1.0 (Matrix Science Ltd., London, U.K.) to search against the NCBI's non-redundant human protein database. Searches were performed with a specified trypsin enzymatic cleavage with two possible missed cleavages and variable modifications for oxidation at methionine (+16 Da) and carbamidomethyl at cysteine (+57 Da). The MSpect/MSpect spectrum of each identified peptide was manually examined and confirmed.

EAE tissue immunofluorescence

For immunofluorescence, 14 □m thick spinal cord frozen sections were used to study ALCAM expression in EAE. Abs used were: ALCAM (polyclonal goat, 1/40, R&D Systems

Inc.), biotin conjugated donkey anti-goat antibody followed by streptavidin-Cy3 (1/400, Dako and Jackson ImmunoResearch Laboratories). Nuclei were stained with TOPRO-3 (1/300 in PBS; Molecular Probes). Control stainings were performed with an isotype control antibody. All images were acquired using a Leica SP5 confocal microscope and analyzed using the Leica LAS AF software (Leica Wetzlar).

Human immune cell isolation and *in vitro* CD4$^+$ T lymphocyte activation assay

Venous blood samples were obtained from consenting healthy donors, in accordance with institutional guidelines (CHUM research ethic committee approval number SL05.022 and 023), and immune cells were isolated as previously published[2]. CD14$^+$ monocytes, CD4$^+$, CD8$^+$ and CD19$^+$ lymphocytes were isolated using the MACS isolation columns, according to the manufacturer's protocol (Miltenyi). Immune cell purity was assessed by flow cytometry using anti-CD14-FITC (fluorescein isothiocyanate), anti-CD8-R-PE (R-Phycoerythrin), anti-CD19-PE-Cy5 and anti CD4-pacific blue (all antibodies from BD PharMingen) and was routinely >95%. CD4$^+$ lymphocytes were labeled with the vital dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and were cultured in the presence of autologous CD14$^+$ monocytes, anti-CD3 (OKT3), in the presence of ALCAM specific antibody (30 μg/ml) or isotype control (30 μg/ml) for 7 days in RPMI 1640 supplemented with 5% human serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma). Similar assay were done in the absence of anti-CD3 and in the presence of purified peptides: influenza hemagglutinin A ($HA_{306-318}$: PKYVKQNTKLAT, 50 μg/ml), human MOG_{35-55}

(MEVGWYRSPFSRVVHLYRNGK, 50 μg/ml), human myelin oligodendrocyte basic protein $(MOBP)_{20-38}$ (HFSIHCCPPFTFLNSKKE, 50 μg/ml) and human whole myelin basic protein (whole MBP, 100μg/ml). MOG_{35-55} was purchased from Sheldon Biotechnology Centre, all the other peptides were generous gifts of Dr. N. Arbour, CHUM Research Center, University of Montreal, Montreal. After 7 days, the proliferation of the $CD4^+$ T lymphocyte, the presence of the activation marker CD25 and the capacity to produce IFN-γ and IL-17 were assessed by flow cytometry. For intracellular cytokine staining, $CD4^+$ lymphocyte and the corresponding APC co-cultures were activated for 18 h with 1 μg/mL ionomycin and 20 ng/mL phorbol 12-myristate 13-acetate (PMA) in the presence of 2 μg/mL brefeldin A (Sigma) for the last 6 h of co-culture. Cells were stained for surface markers and were then fixed and permeabilized in 4% (w/v) paraformaldehyde with 0.1% (w/v) saponin in Hank's Balanced Salt Solution for 10 minutes at room temperature. Intracellular staining was performed by incubating cells with antibodies against IFN-γ and IL-17 (eBioscience, San Diego, CA) (1 mg/mL) for 30 m on ice in PBS buffer containing 0.1% (w/v) saponin, 1% FBS, 0.1% (w/v) NaN_3, followed by two washes and resuspended in staining buffer (1% (v/v) FBS, 0.1% (w/v) NaN_3 in PBS). All cell staining were acquired on a BD LSR II (Becton Dickinson) and analyzed using the BD FACSDiva software (BD Bioscience).

***In vivo* CD4$^+$ T cell activation**

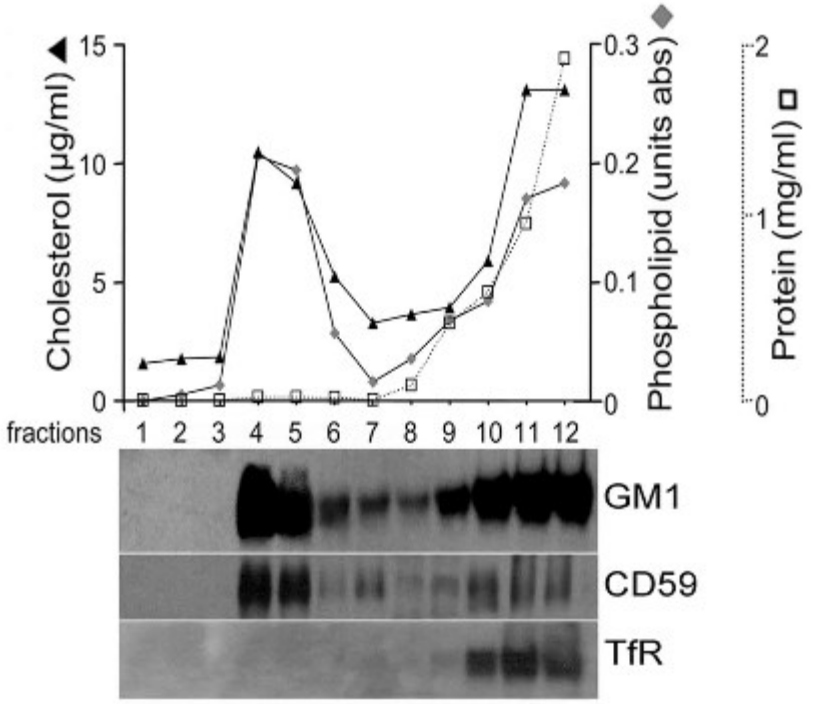
C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and were bred under specific pathogen-free conditions. Mice were immunized subcutaneously with 200

μg of $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) or KLH emulsified in CFA and received 250 μg of anti-ALCAM antibody or isotype control i.p. on days 0 and 4. On day 9, mice were sacrificed using CO_2, spleens and axiliary and inguinal lymph nodes were isolated, treated with 0.5mg/mlDNase and 1mg/ml Liberase (Roche) for 30 min at 37° and homogenized. 2×10^5 cells/well were plated in complete RPMI medium containing either 50 μg/ml of $MOG_{35–55}$, KLH or $OVA_{323–339}$ (ISQAVHAAHAEINEAGR; obtained from Research Genetics, Huntsville, Alabama, USA) or 5 μg/ml of concanavalin A (ConA) in 96-well plates coated with 7.5 μg/ml of anti-IFN-γ (AN18; Mabtech) or 2 μg/ml of anti-IL-17A (TC11-18H10; BD Pharmingen). Plates were incubated at 37 °C in 5% CO_2 for 24h. Cells were discarded and plates washed with PBS and 0.5 μg/ml of biotin-conjugated anti-IFN-γ (R4-6A2; Mabtech) or 1 μg/ml of biotin-conjugated anti-IL-17A (TC11-8H4.1; BD Pharmingen) was added and incubated at 25 °C for 2h. After plates were washed with PBS, streptavidin–alkaline phosphatase (Mabtech) was added, followed by incubation for 1 h at 25 °C. Plates were washed with PBS and 75 μl of the substrate solution BCIP/NBTplus (5-bromo-4-chloro-3-indolylphosphate–nitro blue tetrazolium; Biosource) was added to the wells, which were developed until distinct spots emerged. Plates were analyzed with an enzyme-linked immunospot reader (ImmunoSpot; CTL). Animal experiments were approved by the Swiss Veterinary Office (#69/2003 and #70/2003).

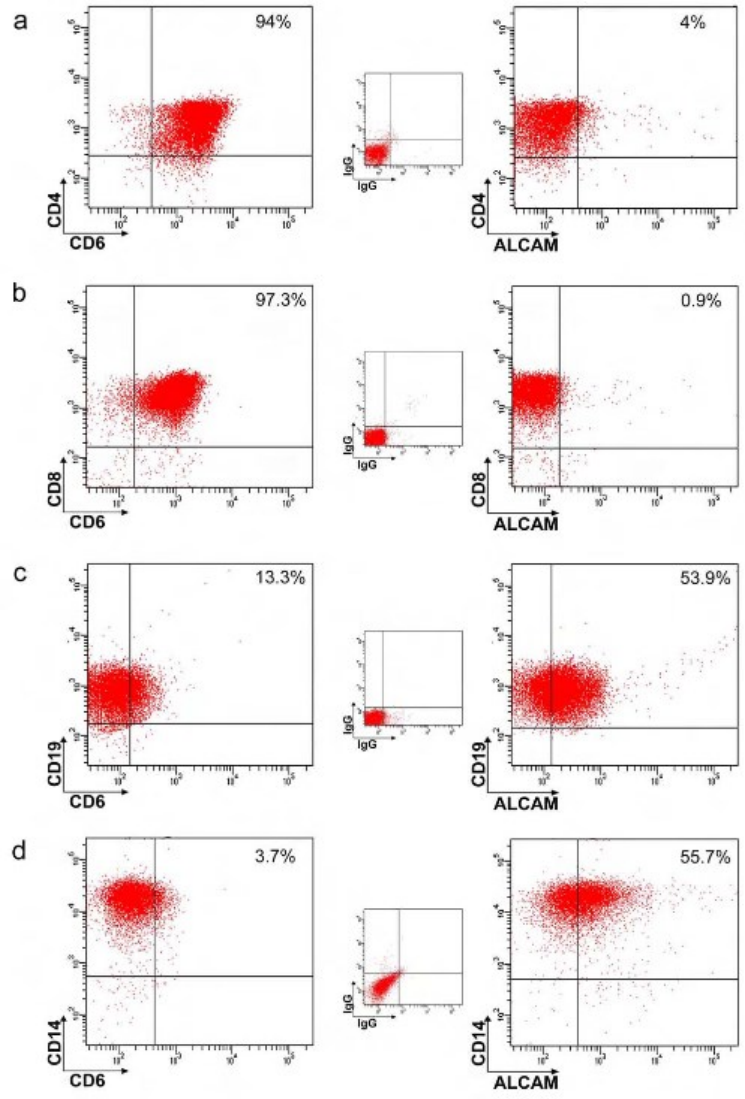
1. Nusrat,A. *et al.* Tight junctions are membrane microdomains. *J Cell Sci.* **113 (Pt 10)**, 1771-1781 (2000).

2. Ifergan,I. *et al.* Statins reduce human blood-brain barrier permeability and restrict leukocyte migration: relevance to multiple sclerosis. *Ann. Neurol.* **60**, 45-55 (2006).

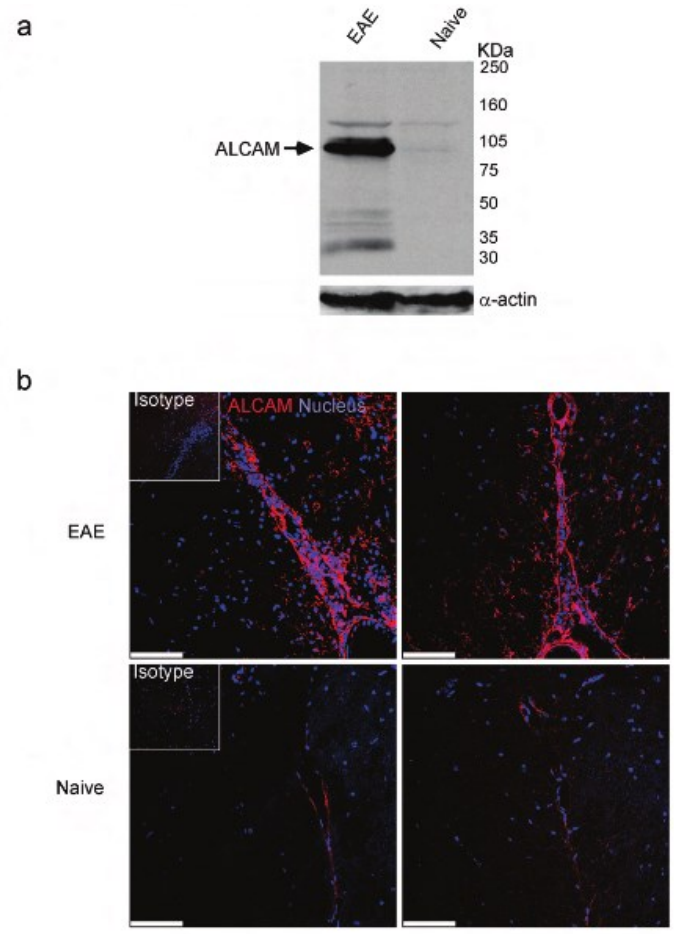
Supplementary figures



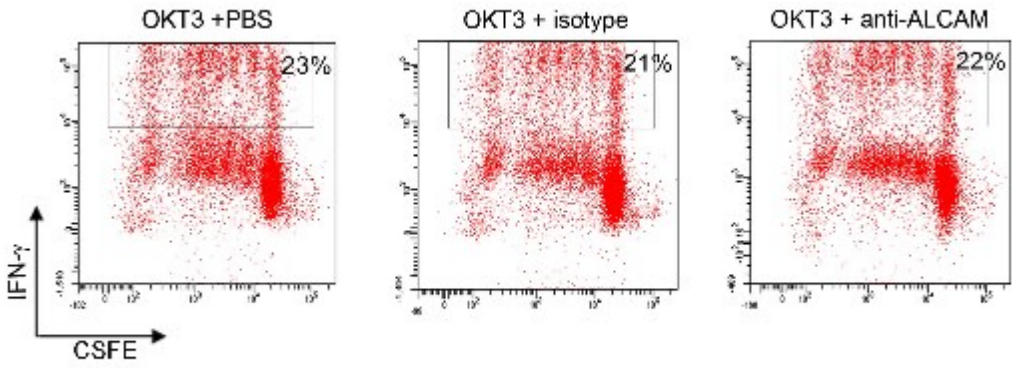
Supplementary Figure 1: BBBEC lipid raft membrane microdomains. Top, characterization of cholesterol, phospholipid and protein content in BBBEC sucrose density fractions. Bottom, immunoblot of lipid raft markers GM1 ganglioside and CD59, and non-raft marker transferrin receptor (TfR), in indicated fractions. Data representative of $n = 5$ independent ECs preparations. Proteomic analysis of lipid raft membrane microdomains is presented in supplementary table 1.



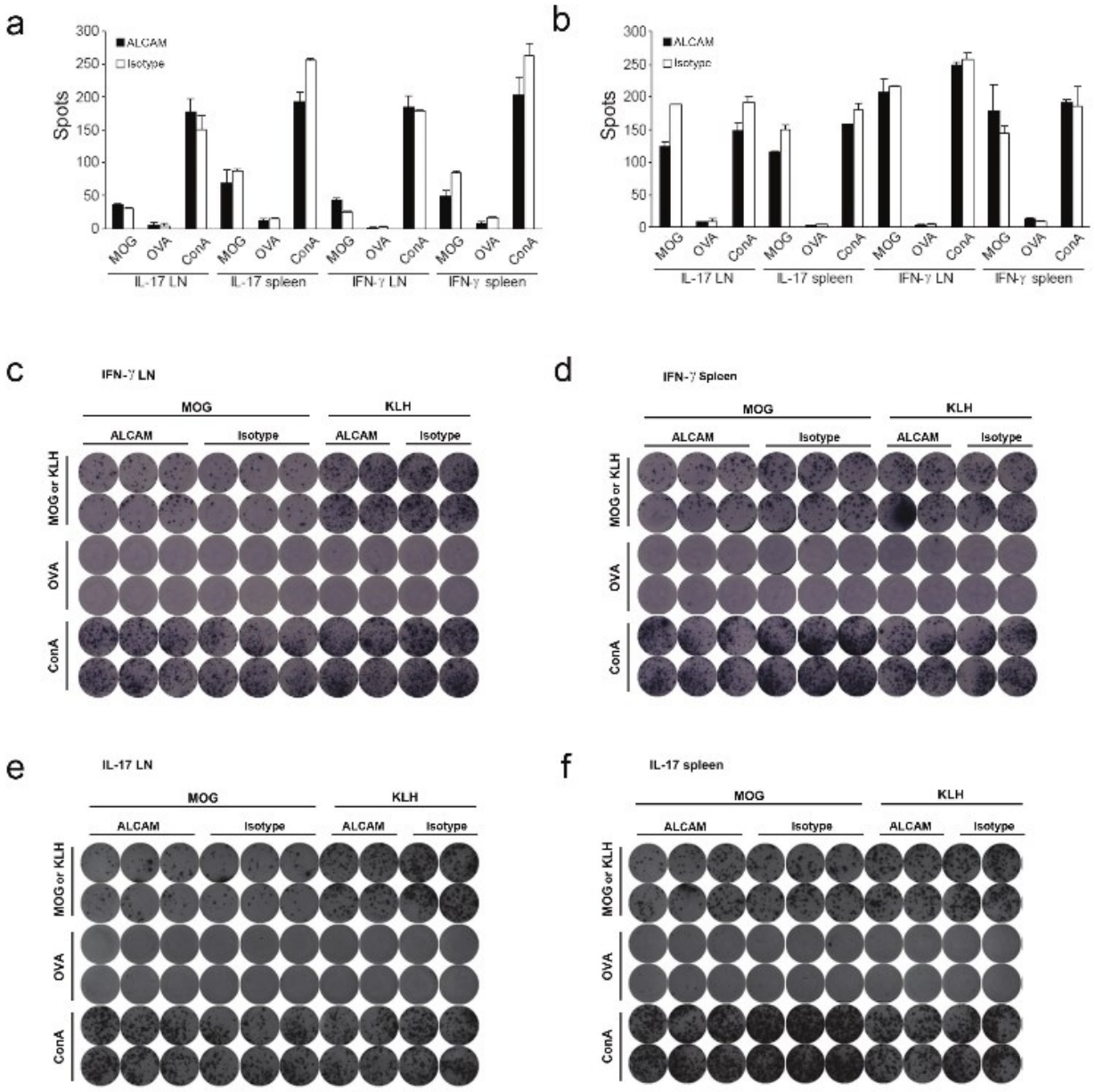
Supplementary Figure 2: ALCAM ligands on human peripheral blood leukocytes. Freshly isolated peripheral blood CD4+ (**a**), CD8+ (**b**), CD19+ (**c**) and CD14+ (**d**) leukocytes were stained with indicated antibodies and analyzed by flow cytometry. Representative isotype control for CD6 and ALCAM staining are shown in the central panel. Data representative of $n = 5$ healthy donors.



Supplementary Figure 3: Increased ALCAM expression in EAE lesions. (**a**) Immunoblot of whole spinal cord homogenates from naïve and EAE (clinical score = 4) mice. Thirty micrograms of protein was loaded into each well and equal loading was demonstrated by probing for a-actin (*n* = 4). (**b**) Immunohistological analysis of ALCAM (red) on CNS endothelium in EAE lesions (EAE), as compared to the spinal cord vasculature of naïve animals (Naïve) (*n* = 4). Blue, nuclei stained with TOPRO-3. Magnification x 20 and scale bar, 75 micrometer.



Supplementary Figure 4: ALCAM blockade does not influence in vitro proliferation and activation of human CD4+ T lymphocytes. Representative flow cytometry plots of CFSE-labeled human CD4+ T lymphocytes cultured for 7 d in the presence of syngeneic CD14+ monocytes, anti-CD3 (OKT3) and either anti-ALCAM or isotype control antibody (30 mg/ml), and stained for intracellular IFN-γ. The numbers in the plots represents the percentage of interferon-g positive cells. *n* = 4 donors.



Supplementary Figure 5 : ALCAM blockade does not influence in vivo cytokine production by primed mouse CD4+ T lymphocytes. C57BL/6 mice were immunized with KLH or MOG peptides and were injected i.p. with 150 micrograms anti-ALCAM or isotype control antibody on days 1 and 4. Mice were sacrificed on day 9 before

disease onset, spleen and LN were collected, and IFN-γ and IL-17A production was assessed by ELISPOT. n = 10 mice per group. (**a**) Spleen and LN cells from mice immunized with MOG peptide were restimulated with MOG or OVA peptide, or with concavilin A (ConA), and cytokine spots were counted. Bars depict mean ± s.d.

(**b**) Spleen and LN cells from mice immunized with KLH peptide were restimulated with KLH or OVA peptide or with ConA, and cytokine spots were counted. Bars depict mean ± s.d. (**c-f**) Representative ELISPOT data. Each column represents one mouse. Cells were restimulated as indicated above.

Supplementary table 1
Proteomic analysis of BBBECs lipid rafts.

Category	Name	Accesion number
Signal transduction and cytoskeleton (42%)		
	Kv channel interacting protein 4	NP_671710
	calcium channel, voltage-dependent, alpha 2/delta subunit 1	NP_000713
	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	NP_003679.1
	guanine nucleotide binding protein (G protein), beta polypeptide 4	NP_067642
	guanine nucleotide binding protein (G protein), alpha 13	NP_006563
	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	NP_002061
	bradykinin receptor B2	NP_000614.1
	RAS-like, family 11, member A	NP_996563
	guanine nucleotide binding protein (G protein), gamma 12	NP_061329
	RAS p21 protein activator 4	NP_008920
	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1	NP_005757
	membrane-spanning 4-domains, subfamily A, member 8B	NP_113645
	ectonucleotide pyrophosphatase/phosphodiesterase 5	NP_067547
	AHNAK nucleoprotein (desmoyokin)	NP_001611
	mitogen-activated protein kinase kinase 1 interacting protein 1	NP_068805
	related RAS viral (r-ras)	NP_006261
	protein kinase, DNA-activated	NP_008835.5
	alpha 1 actin precursor	NP_001091
	vimentin	NP_003371
Cell-cell adhesion (22%)		
	selectin E	NP_000441.1
	vascular cell adhesion molecule-1	NP_001069
	melanoma cell adhesion molecule	NP_006491
	activated leukocyte cell adhesion molecule	NP_001618
	contactin 4	NP_783200.1
	integrin, beta 1	NP_002202
	integrin, beta 4	NP_001005619
	integrin, beta 1	NP_002202
	integrin, alpha 1	NP_852478
	CD44	NP_000601
	Gap junction protein beta 6, connexin 30	NP_006774
	cadherin 1, type 1and type 2	NP_004351
	catenin, beta 1	NP_001895
Protein, lipid and carbohydrate metabolism (18%)		
	gamma-glutamyltransferase-like activity 1	NP_004112
	UDP glucuronosyltransferase 2 family, polypeptide B11	NP_001064
	CD59 antigen p18-20	NP_000602
	solute carrier family 3	NP_001012679
	mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	NP_002399
	amyloid beta precursor protein binding protein 1	NP_003896
	5'-nucleotidase, ecto	NP_002517
Intracellular protein trafficking (7%)		
	caveolin 1	NP_001744
	synaptophysin-like 1	NP_006745
	vesicle transport through interaction with t-SNAREs homolog 1B	NP_006361
	clathrin, light polypeptide	NP_001824
Unknowns (11%)		
	hypothetical protein LOC150159	NP_714922
	hypothetical protein FLJ25082	NP_653261
	THAP domain containing 9	NP_078948
	echinoderm microtubule associated protein like 2	NP_036287
	zinc finger 261	NP_005087
	leucine-rich repeat-containing G protein-coupled receptor 6	NP_001017403

Supplementary table 2

Restimulation of human $CD4^+$ T lymphocytes in the presence of anti-ALCAM antibody and isotype control.

	Proliferating cells (%)	$CD25^+$ (%)	IFN-□$^+$ (%)	IL-17^+ (%)
CD4 alone	1.8 ± 0.4	2.0 ± 0.3	1.1 ± 0.7	0.4 ± 0.3
OKT3 + Isotype	43.2 ± 2.6	28.1 ± 5.8	25.8 ± 6.5	2.2 ± 0.3
OKT3 + ALCAM	40.1 ± 3.6	32.3 ± 9.3	24.2 ± 4.5	2.5 ± 0.7
$HA_{306-318}$ + Isotype	5.5 ± 1.1	29.4 ± 5.9	3.3 ± 1.9	2.2 ± 1.7
$HA_{306-318}$ + ALCAM	4.9 ± 1.3	27.7 ± 7.6	4.5 ± 1.9	2.9 ± 1.5
MOG_{35-55} + Isotype	4.7 ± 1.2	2.7 ± 0.6	2.9 ± 0.5	0.9 ± 0.5
MOG_{35-55} + ALCAM	4.1 ± 0.7	3.2 ± 0.5	3.5 ± 0.6	1.1 ± 0.6
$MOBP_{20-38}$ + Isotype	6.7 ± 1.0	2.5 ± 1.7	2.6 ± 2.0	3.1 ± 1.2
$MOBP_{20-38}$ + ALCAM	7.2 ± 0.7	2.9 ± 1.1	3.3 ± 1.6	2.5 ± 1.0
whole MBP + Isotype	6.1 ± 1.9	5.6 ± 0.9	3.9 ± 2.0	2.6 ± 1.0
whole MBP + ALCAM	6.9 ± 1.6	7.8 ± 2.1	3.3 ± 1.5	3.4 ± 1.8

30 mg/ml of antibody was added at day 0 and proliferation and activation markers were assessed at day 7.

OKT3 activation and antigen-specific recall assay were done on isolated human $CD4^+$ T lymphocytes.

Supplementary table 3

ALCAM and GAPDH primers.

Name	Forward primer sequence (5’ to 3’)	Reverse primer sequence (5’ to 3’)
ALCAM pair a	CGT CTG CTC TTC TGC CTC TT	TAG GTG CCT CAA ACA CGT TG
ALCAM pair b	TCT GCA GGA GGT TGA AGG AC	GCC TCA TCG TGT TCT GGA AT
GAPDH	CAA AGT TGT CAT GGA TGA CC	CCA TGG AGA AGG CTG GGG

SECTION 4 MANUSCRIT 3

Blood-Brain-Barrier Promotes Differentiation of Human Fetal Neural Precursor Cells into Neurons and Oligodendrocytes

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Satyan Chintawar, Romain Cayrol, Jack Antel, Massimo Pandolfo, Alexandre Prat

Contributions

Le projet a été élaboré et initié par le Dr Alexandre Prat, le Dr Satyan Chintawar et moi-même. Les expériences ont été faites par le Dr Satyan Chintawar et moi-même. Le Dr Jack Antel nous a donné accès à des échantillons de SNC fœtaux a partir desquels les CSN sont isolées et a contribué aux discussions scientifiques. Le manuscrit a été rédigé par le Dr Alexandre Prat, le Dr Massimo Pandolfo, le Dr Satyan Chintawar et moi-même.

Blood-Brain-Barrier Promotes Differentiation of Human Fetal Neural Precursor Cells

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Keywords: human fetal neural precursor cells, blood-brain barrier, endothelial cells, trans-endothelial migration, monocyte chemoattractant protein-1, endothelial signalling

Abstract

Neural stem cells are an attractive source for cell transplantation and neural tissue repair after central nervous system injury. After systemic grafting, neural stem cells are confronted with the specialized blood-brain barrier (BBB) endothelial cells before they can enter the brain parenchyma. We investigated the interactions of human fetal neural precursor cells with human brain endothelial cells in an *in vitro* model using primary cultures. We demonstrated that human fetal neural precursor cells efficiently and specifically migrate to sub-endothelial space of human BBB-endothelium, but not pulmonary artery endothelial cells. When migrated across BBB-endothelial cells, fetal neural precursor cells spontaneously differentiate to neurons, astrocytes and oligodendrocytes. Effective migration and subsequent differentiation was found to be dependant on the chemokine CCL2/MCP-1, but not CXCL8/IL-8. Our findings suggest that an intact blood-brain barrier is not an intrinsic obstacle to neural stem cell migration into the brain and that differentiation of neural precursor cells occur in a sub-endothelial niche, under the influence of the chemokine CCL2/MCP-1.

Introduction

Stem-cell therapy is valued for its potential to restore damaged or degenerating tissues, particularly when cells with very limited regeneration capacity as central nervous system (CNS) neurons are involved. When focal loss of neurons in a relatively small CNS region is responsible of most disease manifestations, as is the case in Parkinson disease (PD), transplantation of neural stem cells (NSCs) at the site of injury has provided some functional improvement (1). This type of cell delivery, however, has limitation in neurodegenerative or neuroinflammatory diseases where neurodegeneration is widespread and symptoms derive from the diffuse involvement of multiple CNS regions, like Alzheimer disease (AD), Huntington disease (HD), the degenerative ataxias or multiple sclerosis (MS). Systemic delivery would be an effective way of cell transplantation in those affected individuals, as it should in principle allow widespread distribution. In the case of non-CNS tissues, systemic delivery of stem cells has already proven effective. Examples include intrarterial delivery of mesoangioblast stem cells to skeletal muscles in an animal model of muscular dystrophy (2, 3), and of intraperitoneal implantation of human mesenchymal stem cells which undergo hematopoietic and lymphopoietic tissues implantation and site-specific differentiation (4).

In the CNS, the blood-brain-barrier (BBB) is a natural obstacle to systemic delivery of stem cells. In that regard, when NSCs were injected i.v. in normal naive animals, no such cells could be found in the CNS (5). Conversely, when the BBB is disrupted, as in the animal model of MS experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (5) or in cerebral ischemia (6), systemically injected NSCs reach the site of injury

and appear to provide some functional improvement. Though most neural precursor cells (NPCs) in EAE seem to maintain undifferentiated features and exert unexpected immune-like functions favorably modifying the microenvironment and stimulating endogenous repair processes (7), numerous oligodendrocyte precursors of donor origin were found to remyelinate axons (5). In EAE, systemically grafted NPCs were also found to be attracted into the inflamed white matter by inflammatory cytokines; such cytokines profoundly affect their proliferation and differentiation (8). However, there is no comprehensive study on the interaction of NSCs with an intact BBB, either *in vivo* or in an appropriate *in vitro* model. Lack of migration in normal animals may be due to the lack of any attractive signal from an intact CNS rather than to an inherent inability of NSCs to cross the BBB. Furthermore, there are many indications that endothelial cells interact with NSCs and affect their ability to replicate and differentiate, though the observed effects vary in different experimental conditions (9, 10). It is critical to understand the niche and its components which influence stem cells and their fate. We investigated the interaction of human fetal neural precursor cells (hfNPCs) with the BBB using a previously validated *in vitro* human BBB model (11-13).

Results

Generation of Neurospheres derived from human fetal brain tissues

Human fetal neural precursor cells (hfNPCs) are neural stem/progenitor cells present in human fetal brain that are thought to be committed to generate neural tissue, including neurons, astrocytes and oligodendrocytes. We derived hfNPCs from 18-22 weeks old human fetal tissues and cultured hfNPCs as free-floating neurospheres (Fig 1 A). More than 99% of the cells in a neurosphere were immunoreactive for nestin, Sox2 and GFAP (Fig 1 F, G and H). Neurosphere-derived hfNPCs were tested for their ability to mature into neurons and glial cells using a differentiation assay. After culturing hfNPCs for 8 days on matrigel without growth factors and in the presence of serum, cells from neurospheres had stopped dividing, adhered to matrigel and had taken neuronal, astroglial or oligodendroglial morphology. The presence of immunoreactivity for neuronal (β-tubulin-III, Fig 1J), astrocytic (glial fibrillary acidic protein, GFAP, Fig 1I), or oligodendrocytic (O4, Fig 1K) markers was accompanied by morphological changes, confirming the multipotential characteristics of the hfNPCs used in our assay system. Self-renewal properties were confirmed by clonal assay, in which single cells were derived from neurospheres and cultured until they gave rise to new neurospheres (Fig. 1B to E). Clonally derived neurospheres were also assessed for multipotentiality (supplementary Fig. 3).

Trans-BBB-migration of hfNPCs and phenotypic profile

Primary cultures of BBB-endothelial cells (BBB-ECs) derived from the <112 μm vessel fraction were characterized for their BBB phenotype. These cells express factor VIII related antigen, UAE-1 binding sites, HT-7 antigen, occludin, zonula-occuldens-1 and up-regulate VCAM-1 and ICAM-1, when stimulated with pro-inflammatory cytokines (11-13). Immunoreactivity for α-myosin and GFAP could not be detected on the BBB-ECs, suggesting the absence of contaminating astrocytes and smooth muscle cells. To establish the *in vitro* model of BBB, BBB-ECs were seeded on a 3 μm pore size gelatin coated-Boyden chamber membrane and allowed to form a monolayer in ACM-ECM. hfNPCs were plated in the Boyden chamber onto the BBB-ECs in NSCM, in the absence of ACM. Three days later, hfNPCs-derived cells migrated and remained in the sub-endothelial space, without having migrated to the lower compartment. The membrane was removed for morphological and immunocytochemical analysis by confocal microscopy. Surprisingly, we observed that hfNPCs had undergone differentiation, as shown by the loss of nestin immunoreactivity (Fig. 2A to C), by morphology and the presence of immunocytofluorescent markers of neurons (Fig. 2G to I), astrocytes (Fig. 3G to I), or oligodendrocytes (Fig. 3A to C). They remained in intimate contact with BBB-ECs. By z-stack reconstruction and analysis, we found that most of the cells were below the BBB-ECs monolayer and some cell bodies were located in the same plane as endothelial cells, intermingling with them, while their processes spread between BBB-ECs and the underlying membrane, sometimes penetrating through the pores of the membrane (see fig. 5). When BBB-ECs were allowed to form a monolayer in Boyden chamber in only ECM, in the absence of ACM, similar outcome was found when hfNPCs

were plated in NSCM (data not shown). hfNPCs plated onto BBB-ECs differentiated faster as compared to the direct differentiation assay, as opposed to hfNPCs plated on matrigel coated coverslip for 3 days, where 42% less neurons were generated (see fig. 6A) and there was no presence of oligodendrocyte (see fig. 6B). hfNPCs derived differentiated cells plated on BBB-ECs appeared to be better interconnected, with longer processes and a morphology that is more typical of mature CNS cells. In summary, BBB-ECs did not block the migration of hfNPCs, but rather promoted their differentiation (see Table 1). In control experiments, hfNPCs directly plated on the porous Boyden chamber membrane or on gelatin-coated porous Boyden chamber membrane without BBB-ECs and cultured in the same media failed to differentiate and formed the spherical clusters as they do in proliferating culture (Supplementary Fig. 1).

MCP-1/CCL2 blockade reduces trans-BBB migration and differentiation of hfNPCs

It is well established that various chemokines are involved in the specific recruitment of immune cells to the brain. In the developing brain, chemokines and their receptors are expressed and there is a growing body of evidences that they may play a role in direct cell migration, proliferation and/or differentiation. It has been recently reported that astrocyte differentiation selectively upregulates CCL2/MCP-1 in cultured human brain-derived progenitor cells (16). It has been previously shown that BBB-ECs *in vitro* constitutively secrete the chemokines, CCL2/MCP-1 and CXCL8/IL-8 which regulates the migration of lymphocytes and monocytes across BBB-ECs (11, 17). We investigated further if these chemokines secreted by BBB-ECs in culture could play a role in trans-BBB migration and/or

differentiation of hfNPCs. Adding monoclonal anti-human CCL2/MCP-1 or monoclonal anti-human CXCL8/IL-8 blocking antibodies separately at the time of migration neutralised MCP-1 and IL-8 produced by BBB-ECs. The migration of hfNPCs was reduced by 33% when blocking CCL2/MCP-1 and the number of neurons and oligodendrocytes derived from hfNPCs following trans-BBB migration was reduced by 31% and 81% respectively. Blocking CXCL8/IL-8 had no effect on migration or differentiation of hfNPCs (see Table 1).

hfNPCs did not migrate but differentiated on human pulmonary artery endothelial cells only after addition of recombinant MCP-1

We then tested if the effect of BBB-ECs was specific, or whether non brain-derived endothelial cells could promote the differentiation of hfNPCs. For this purpose, human pulmonary artery endothelial cells (HPAECs) were allowed to form a monolayer on the Boyden chamber membrane, in the same experimental conditions as the BBB-ECs. After 3 days, the ECM was replaced with NSCM and hfNPCs were plated onto HPAECs. Three days later, membranes were removed for immunocytochemistry and the majority of the cells were immunoreactive for nestin (Fig. 2D to F) and GFAP (Fig. 3J to L), although $GFAP^+$ cells did not adopt an astrocytic morphology. Membranes were also immunostained for the presence of neuronal and oligodendrocytic antigens: the cultures were found to be β-tubulin-III^- (Fig. 2J to L) and $O4^-$ (Fig. 3D to F). ELISA assays have shown that BBB-ECs produce 25-times more MCP-1 than HPAECs when cultured in NSCM (see Supplementary Fig. 2C). To determine the role of BBB-ECs produced MCP-1 on the fate of hfNPCs, recombinant MCP-1 was added to

HPAECs monolayers at the same concentration of MCP-1 produced by BBB-ECs. Additional recombinant MCP-1 was added while seeding hfNPCs. Three-days later we found that many hfNPCs lost Nestin (Fig. 4G to I) immunoreactivity and differentiated as shown by the expression of β-tubulin-III (Fig. 4A to C). Culturing HPAECs in ACM did not change the phenotype of hfNPCs as they continue expressing Nestin (Fig. 4J to L) and were β-tubulin-III^{-} (Fig. 4D to F). These studies demonstrate that MCP-1 produced by BBB-ECs is important for hfNPC migration and differentiation and that the lack of HPAECs produced MCP-1 may explain why hfNPCs do not migrate and differentiate in the presence of HPAECs.

Discussion

The interactions between NPCs and the BBB play a key role in neural stem cell biology and have practical implications for cellular therapies. Systemically injected NPCs have been shown to easily cross a ruptured BBB, as found in CNS inflammatory diseases, but do not penetrate the brain parenchyma of intact animals (5). We hypothesized that migration and differentiation of NPCs critically depend on their interaction with the BBB and on attractive signals from the underlying brain.

In this study, we first isolated NPCs from human fetal brain tissue and characterized their self-renewal and their ability to generate mature CNS progeny. All hfNPCs expressed Nestin, Sox2 and GFAP, in agreement with previous studies (18). We then used an experimental setup designed to mimic the human brain microvascular endothelial layer to explore the interaction between hfNPCs and human BBB-ECs. The validity and biological relevance of this *in vitro* setup has been previously shown in studies of inflammatory cell migration through the BBB. With this *in vitro* model, we wished to address two closely related questions: whether hfNPCs can cross an intact BBB endothelium and how their proliferation and differentiation properties are affected by this interaction. As complementary questions, we investigated if any observed effect was specific for BBB endothelium and we tried to identify some of the signaling molecules involved. We expected BBB specificity because, while embryonic stem cells create their own niche during the development, stem/precursor cells already directed to a particular lineage are often surrounded by the heterologous cell types

which are key elements of the niche. For example, it has been previously shown that blood vessel endothelium induces insulin expression in isolated endoderm (19).

We observed that hfNPCs rapidly move across a monolayer of human BBB-ECs. The same cells did not migrate across a monolayer formed by a different type of human ECs, indicating that a specific interaction is important between stem cells and endothelium from the same organ. After hfNPCs moved through the BBB-EC monolayer, they remained in close contact with it and rapidly differentiated, giving rise to all three types of brain cells they can generate. Again, this was specific to BBB-ECs, as it could not be reproduced by ECs from pulmonary arteries, that left hfNPCs in an undifferentiated state where they continued expressing nestin and GFAP. Additionally, hfNPCs did not differentiate by being in contact with the underlying extracellular matrix. Differentiation of hfNPCs in the presence of BBB-ECs was remarkably efficient when compared with the *in vitro* differentiation assay, suggesting a specific and potent differentiation promoting activity of BBB-ECs on NPCs. Our findings therefore partially differ from a previous report showing that ECs promote self-renewal, not differentiation of neural stem cells, though eventually neuron production was enhanced (20). The fact that our model more closely mimics the BBB, as it uses an astrocyte-conditioned microvascular brain EC monolayer directly in physical contact with NPCs rather than simple co-culture system of NPCs with brain ECs, may be responsible for these differences. Furthermore, we used a human, not a rodent cell culture system, which may be more relevant to the understanding of human brain regeneration and the development of cellular therapies.

Our results are in broad agreement with a wealth of data indicating that BBB-ECs are not a simple physical barrier to migration of blood-borne NPCs into the brain parenchyma, but are active players in NPCs biology able to profoundly affect their proliferation and differentiation. During embryonic development, interaction between endothelium and stem cells occurs in the nervous system as well as in non-nervous tissues (21). In the brain, proliferative clusters consisting of neural precursors, committed neuroblast, glia, and endothelial precursors (i.e. angioblast) are centered around blood vessels forming a vascular niche (20, 22-24). *In vivo* neurogenesis requires stem cells-ECs interaction, both during embryogenesis and in the adult animal. As an example, in the adult hippocampus neurons are only generated from NPCs in the subgranular zone (SGZ), located at the border between the granule cell layer and the hilus (or CA4), where they are localized within a vascular niche, while precursor cells in other areas of the hippocampal formation are not found within such a vascular niche and do not generate neurons (22). In recent independent investigations, it was shown that stem cells in the adult brain reside in close proximity to the CNS vasculature. These papers defined a vascular niche for stem cells in the CNS (25-27). We consider that our experimental setup partilly recapitulates the vascular stem cell niche and can be used to investigate its properties.

The hfNPC differentiation-promoting effect of the BBB-EC monolayer was partly mediated by the chemokine MCP-1/CCL2. The involvement of MCP-1/CCL2 hfNPC differentialtion is in agreement with previous finding obtained in mouse hippocampal slices. These data demonstrated that the migration of grafted NPCs toward the inflammatory sites was reduced in slices prepared from MCP-1/CCL2 knock-out mice or when NPCs were

derived from CCR2 knock-out mice (28). Furthermore, Edman LC et. al. (29) have shown that MCP-1/CCL2 did not affect proliferation, rather acted as a pro-differentiation factor to murine NPCs. In our culture system, we quantified the production of MCP-1 chemokine by ELISA and found that BBB-ECs produce 25 times more MCP-1 than HPAECs (see supplementary fig 2). Presence of additional recombinant MCP-1 in HPAECs cultures induced hfNPCs differentiation which further strengthens the role of this chemokine in differentiation of hfNPCs. We confirmed the presence of MCP1 chemokine receptors CCR1 and CCR2 on hfNPCs by RT-PCR (see supplementary fig 4). Concerning rodent NSCs, it has been reported that MCP-1 induces migration (28, 30), differentiation (29) and migration and differentiation (31) of rodent NSCs. Our data shows that these rodent data are relevant to a human model system. In addition to MCP-1/CCL2, other molecules are likely to be involved in the induction of NSC trans-endothelial migration and differentiation. The adhesive interaction between human NSCs and the inflamed vascular endothelium is mediated by a subclass of integrins, alpha2, alpha6, and beta1 (32). These molecules may also be involved in the migration across healthy human BBB. Takeuchi et al reported that intravenously transplanted human NSCs migrate preferentially to the injured rat spinal cord and differentiate into neuronal and glial subpopulations and this homing is not due to BBB disruption but the release of chemokines at the site of injury (33). In disease conditions, brain cells release chemokines that can trigger the migration of NSCs in a chemokine concentration gradient manner.

In conclusion, we showed that an *in vitro* model of the human BBB can be crossed by hNPCs and that this interaction induces their rapid differentiation while still in close contact with endothelial cells. These results are not reproduced when using a non-BBB endothelium and

partly dependent on the cytokine MCP1. It is conceivable that, in a corresponding *in vivo* situation, a similar interaction would allow migration into the brain parenchyma of a differentiated NPCs progeny only in the presence of attractive signals. The latter are lacking in intact animals, but are likely to be present in neurodegenerative disorders. Accordingly, intravascularly administered NPCs can potentially reach degenerating brain areas, but they will remain excluded from normal brain. The fact that some migration of NPCs into the brain was observed after treatments that disrupt BBB integrity does not contradict the above hypothesis, as it could just be due to a passive diffusion phenomenon. More generally, understanding the niche should help us in recruiting endogenous pool of new stem cells in case of disease, intervene in developing new therapeutic strategies for cancer and also to understand the development of the brain.

Materials and Methods

Human fetal NPCs isolation and culture

Human NPCs were obtained from 18 to 22-weeks old fetal brain specimens provided by Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY) following approved guidelines from the Canadian Institutes of Health Research (CIHR). Fetal brain tissues were washed with cold PBS containing 0.6 % glucose. Tissues were digested with 0.1 % papain (Worthington, NJ, USA) for 1 hr at 37°C followed by DNAse (10 μg/ml) treatment for 15 min and then triturated using polished pasteur glass pipette. Cells were cultured as free floating neurospheres in 75-cm^2 flasks with growth medium containing DMEM (high glucose, L-glutamine)/Ham's F12 and supplemented with 15 mM HEPES, 0.6% D-glucose, 9.6 mg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin, 2 μg/ml heparin, 2 mM L-glutamine, penicillin and streptomycin, 20 ng/ml recombinant human EGF (Peprotech, NJ, USA), 10 ng/ml recombinant human bFGF (Peprotech, NJ, USA), 10 ng/ml recombinant human LIF (Chemicon, CA, USA).

Cells were passaged every 10-12 days. Cultured spheres were harvested in 0.025% trypsin/0.053 mM EDTA plus 0.6% glucose dissolved in calcium- and magnesium- free Dulbecco's phosphate buffered saline and incubated for 5 min at 37°C with periodic trituration. Cells were triturated with pipette to ensure separation of individual cells into a single cell suspension. Cell quantification was performed by using tryphan blue exclusion and hemacytometer counting. After passage, neural precursor cells were seeded into 75-cm^2 flasks in 15 ml of fresh medium and incubated at 37°C with 5% CO_2. Half of the volume of the

culture medium was replaced by fresh growth medium every 4-5 days. Cells from passage numbers 3 to 5 were used for the experiments.

Multipotentiality and Clonal analysis

To induce differentiation, neurospheres were dissociated to single cells and seeded on matrigel-coated glass coverslips in 24 well-plates at a density of 60 000 cells/well and cultured in the medium containing only b-FGF (20 ng/ml) as a growth factor. Two days later, the medium was replaced with medium lacking all growth factors and supplemented with 10% FBS. The cells were cultured for a total of 8 days before fixation for immunocytochemistry.

For clonal analysis, NPCs of passage 4 were first dissociated into single cells and counted using a hemacytometer. Then, the cells were diluted in complete medium to a concentration of 1cell/200 μL and seeded into 96-well plates at a dilution of 1 cell per well. After 24 h, wells containing only single cells were scored. On day 21, neurospheres derived from single cell were removed and assessed for clonal multipotentiality by inducing differentiation as described above.

Isolation and culture of primary endothelial cells and astrocytes

For transendothelial migration, BBB-ECs were isolated and cultured as previously described (11-13). In brief, temporal lobe specimens from young adults undergoing surgery for the treatment of epilepsy were obtained from the hospital's operating room, with agreement from the local ethics committee. Tissue was minced and homogenized in phosphate-buffered saline,

filtered through a 350 μm Nitex mesh and then twice through a 112 μm mesh. Filtrate was collected, spun down and digested for 10 min with collagenase type IV (Sigma, Mississauga, On, Canada) at 37°C. Cells were collected by centrifugation and seeded on 0.5% gelatin coated flasks, in M199 cell culture media (GIBCO-BRL, Burlington, On, Canada) supplemented with FCS 10%, 20% human normal serum, insuline-transferrin-selenium and endothelial cell growth supplement (all from Sigma, Oakville, On, Canada). At day 10, visible colonies were collected and expanded in fresh media.

For astrocyte culture, fetal brain specimens of 18–22 weeks old were obtained from the same source as mentioned above. Astrocytes were cultured as previously described (13, 14) in complete DMEM media (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, penicillin and streptomycin. Astrocyte-conditioned media (ACM) was harvested once a week from confluent flasks and added to the BBB-endothelial cell-culture media.

Human pulmonary aortic endothelial cells (HPAECs) were purchased from Cambrex Corporation, USA and cultured in EGM-2MV media according to manufacture's instructions.

Transendothelial migration

All migration assays were conducted in Boyden chambers (3 μm pore size membranes) pre-coated with 0.5% gelatin (Collaborative Biomedical Products, Bedford, MA). BBB-ECs (25,000 cells) were seeded on the membrane 4 days prior to the migration experiment in EC culture media (ECM) containing 20% (ACM). 3 days later, ECM was replaced with neural stem cell media (NSCM). 24 hrs later, 50,000 hfNPCs were seeded to the upper chamber in 700 μl of NSCM. MCP-1 and IL-8 produced by BBB-ECs was blocked by monoclonal anti-

human CCL2/MCP-1 and monoclonal anti-human CXCL8/IL-8 antibodies (R & D Systems) separately. The medium in the upper and lower chamber was replaced with fresh medium every 24 h with fresh antibodies added. 72 h later the membranes of the Boyden chambers were fixed with 4% paraformaldehyde and removed for phenotypic analysis and quantification of transendothelial migration. For quantitative analysis hfNPCs trans-migration, hfNPCs were prelabeled with CFSE dye (carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, Eugene, OR). hfNPCs were resuspended at a concentration of 5 X 10^6 cells/ml in warm RPMI 1640 and incubated for 10 min at 37° C with 5 mM CFSE. Labeling was quenched with human serum. After two washes, cells were resuspended in NSCM and plated on Boyden chamber upper compartment. Three days post trans-BBB migration, Boyden chamber was vigorously and extensively washed with phosphate buffered saline (PBS). The $CFSE^+$ cells were counted in 10 fields at random in control and with blocking MCP-1 and IL-8 chemokine. For control conditions, HPAECs were plated on Boyden chambers at the same density and grown in the same manner as BBB-ECs with and without ACM. In Boyden chamber with HPAECs without ACM, recombinant MCP-1 was added as a control.

Immunocytochemistry and Phenotypic Analysis

The cells on the Boyden chamber membrane and glass-coverslips were fixed with 4% paraformaldehyde (PFA) and washed three times with PBS. Cells were permeabilized (except for O4 staining) with 0.1% Triton X-100/PBS for 15 min at room temperature and then blocked with 10% normal goat serum, 2% horse serum in HBSS (Hanks balanced salt solution) containing 1 mM HEPES and 0.01% sodium azide for 30 min at room temperature.

After blocking, cultures were incubated with following diluted primary antibodies overnight at 4 ^{0}C: rabbit anti-nestin and anti-Sox-2 (1:200; Chemicon, CA, USA) for undifferentiated cells, mouse anti-GFAP (1:400; Dako) for undifferentiated cells and astrocytes, mouse anti-β-tub-III (1:300; Chemicon, CA, USA) for neurons, mouse anti-O4 (1:200; Chemicon, CA, USA) for oligodendcrocytes. Following washings, cultures were incubated for 45 min at room temperature in the dark with either biotinylated goat anti-rabbit (1:400; DakoCytomation) (for nestin) or species-specific fluorophore-conjugated secondary antibodies Cyanine3 or FITC (1:400; Jackson Immunoresearch). Biotinylated staining was then visualized using a streptavidin-FITC conjugate (1:1000; DakoCytomation). All the cultures were counter-stained with Hoechst 33258 pentahydrate (Molecular Probes) for nuclei, and mounted using Fluorsave. For z-stack reconstruction and analysis, BBB-ECs on Boyden chamber membrane were visualized by phalloidin antibody coupled to rhodamine (Invitrogen). Images were taken using Leica DM6000B and a Leica SP5 laser scanning confocal microscope.

Statistical analysis

Quantitative data is presented as mean ± SEM. **Mann-Whitney Rank Sum Test** was used to analyse the data and differences were considered significant with a p value < 0.05.

Figures

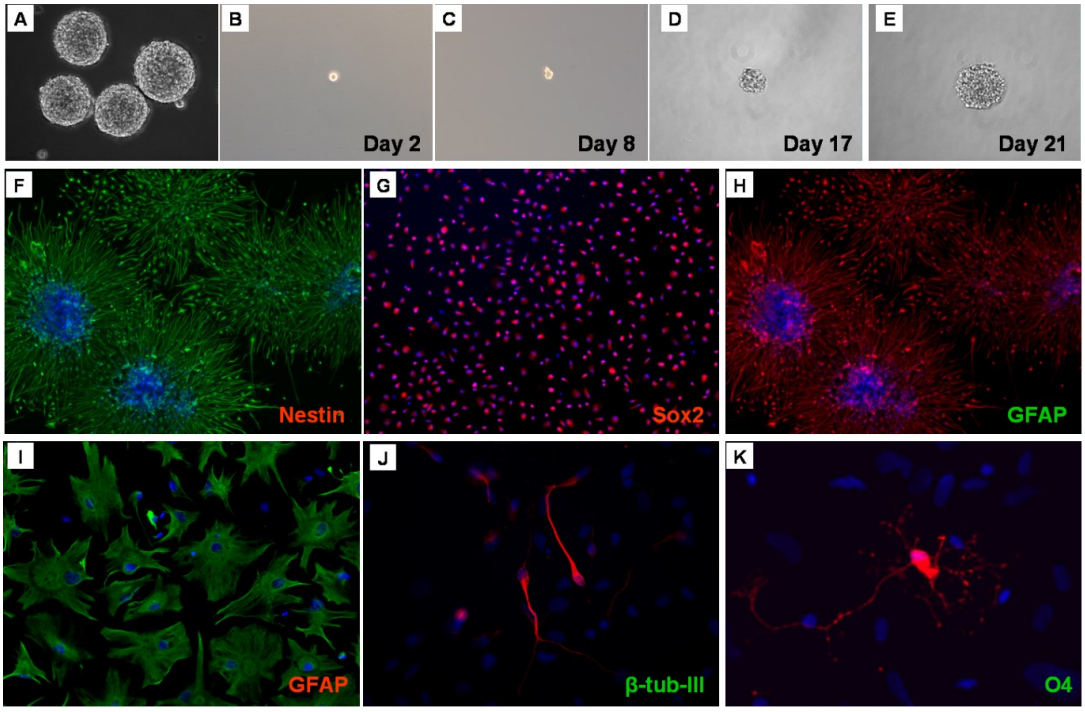

Figure 1: Characterization of hfNPCs.

(A) Neurospheres in proliferation medium at passage 5. (B-E) hfNPCs clonal analysis, single cells were seeded and followed for their capacity to form neurospheres. One out of three cells grew into a neurosphere in 21 days. hfNPCs plated on matrigel for 24 h in complete medium, are immunoreactive for undifferentiated cellular markers of Nestin (F), Sox2 (G), GFAP (H). After 8 days in culture with matrigel hfNPCs differentiate to $GFAP^+$ (I) astrocytes, β-tub-III^+ (J) neurons and $O4^+$ (K) oligodendrocytes. The micrographs were taken at the magnification of 20X (A to E), 10X (F to H) and 40X (I to K).

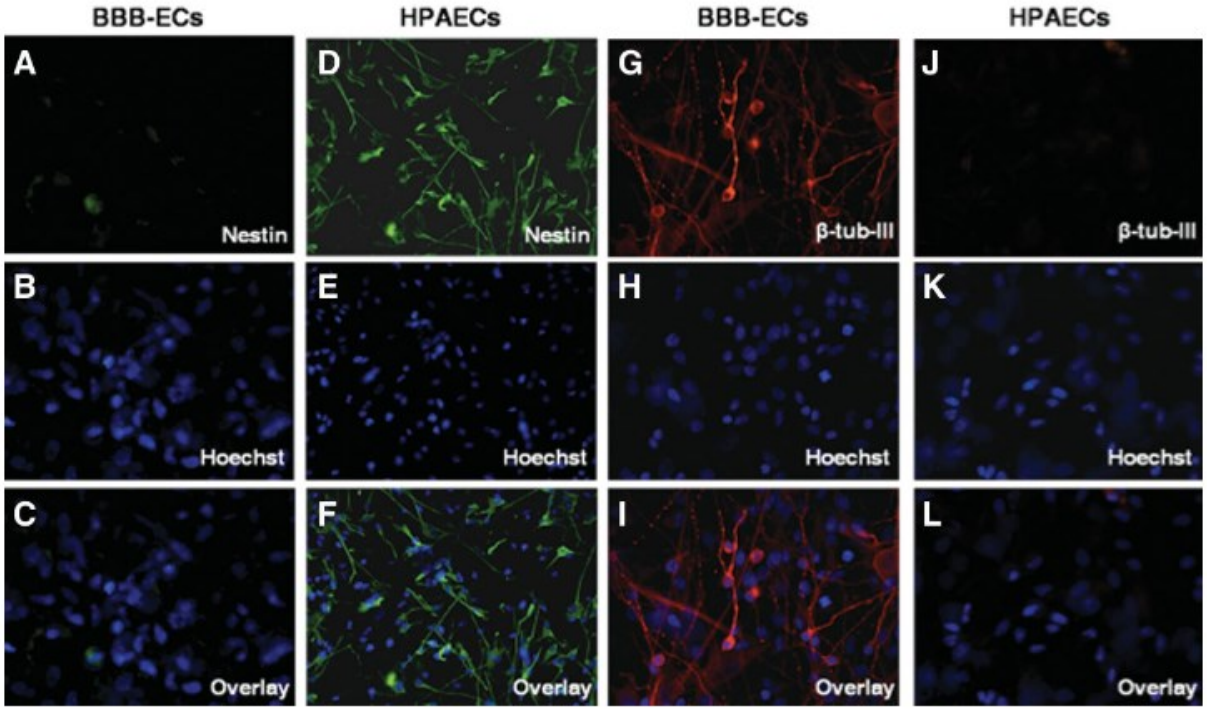

Figure 2: Trans-BBB-migration of hfNPCs.

Three days after being plated on a monolayer of BBB-ECs in a Boyden chamber, hfNPCs had migrated to the sub-endothelial space and nestin expression was lost (A-C) and hfNPCs showed a differenciated morphology and immunoreactivity for the neurolnal marker beta-tubIII (G-I). In control experiments, no such loss of nestin immunoreactivity (D-F) was observed when hfNPCs were plated on a monolayer of HPAECs and culture did not show immunoreactivity for beta-TubIII (J-L). The micrographs were taken at the magnification of 20X.

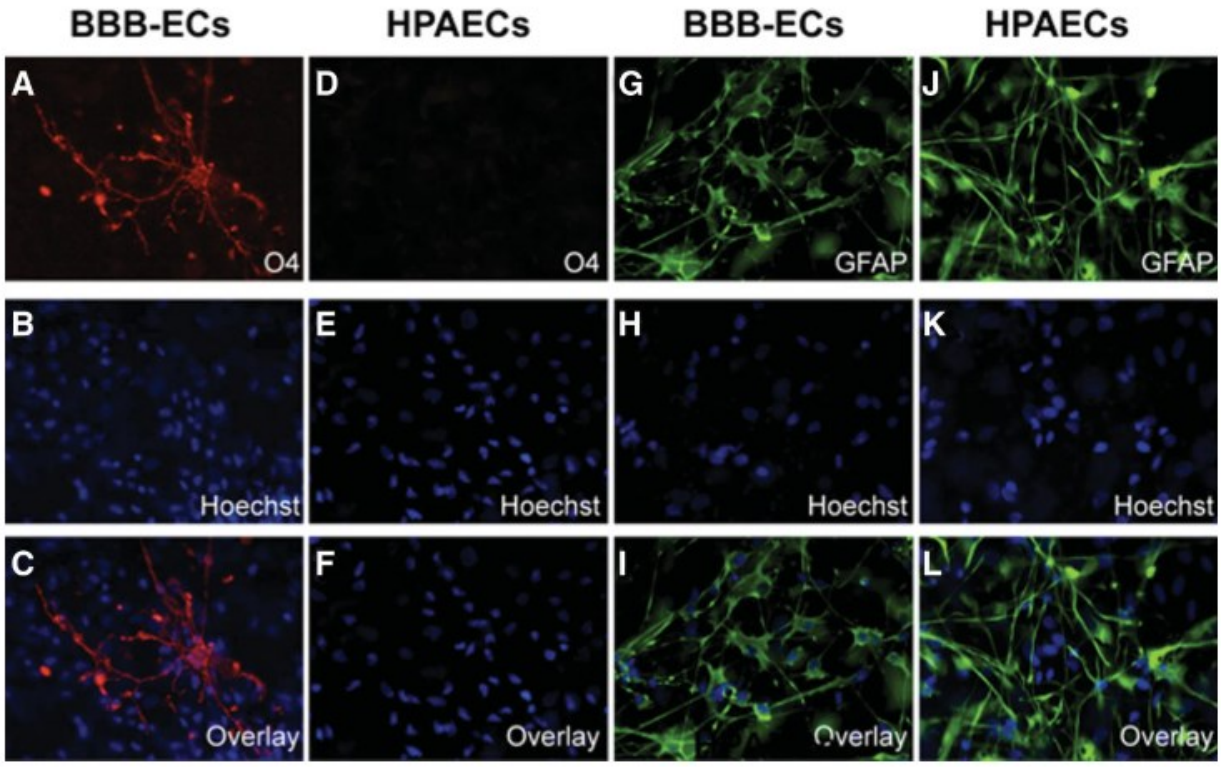

Figure 3: Trans-BBB-migration and differentiation of hfNPCs to neurons.

After migration through BBB-ECs, hfNPCs showed a differentiated morphology and immunoreactivity for the oligodendrocyte marker O4 (A-C) or the astrocytic marker GFAP (G-I). In control experiments we could not detect O4+ cells when hfNPCs were plated on a monolayer of HPAECs (D-F), where they continued expressing GFAP (J-L) but did not adopt an astrocytic morphology. The micrographs were taken at the magnification of 20X.

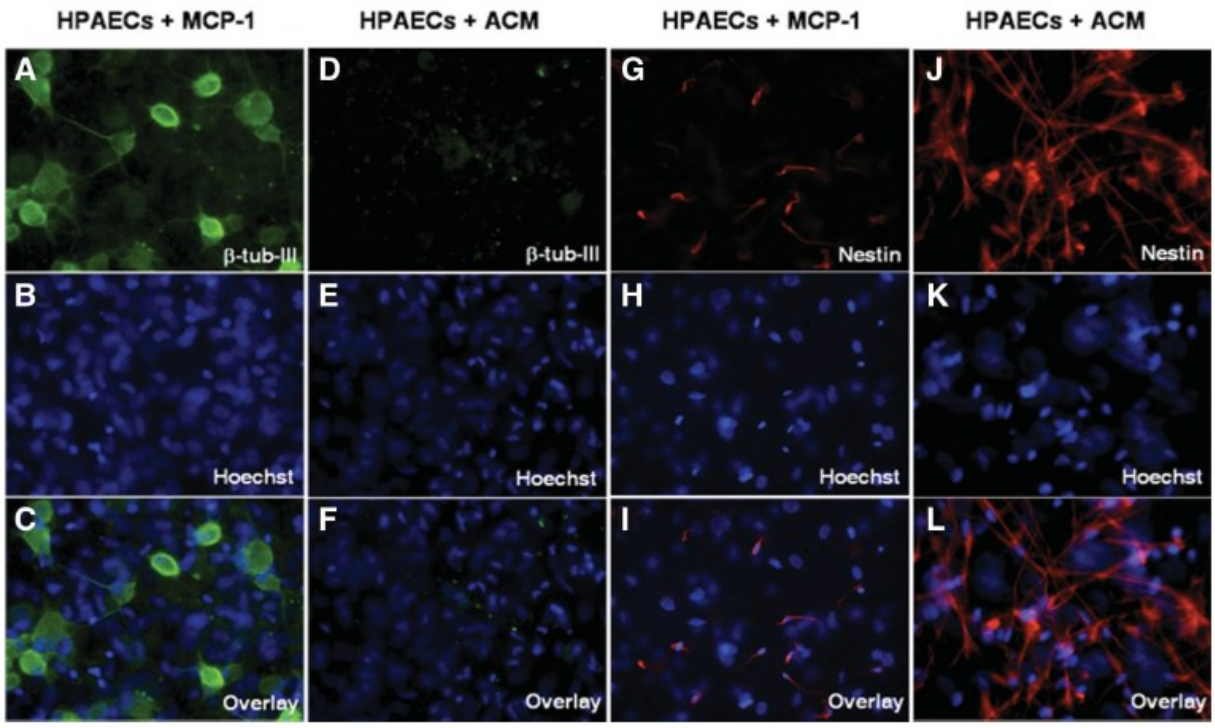

Figure 4: Differentiation of hfNPCs on HPAECs after adding MCP-1.

Addition of recombinant MCP-1 at 100 ng/ml while seeding hfNPCs on a HPAECs monolayer in the upper compartment of the Boyden chamber induces hfNPCs differentiation to neurons, as shown by immunoreactivity for the neuronal marker β-tub-III (A-C) and by loss of Nestin expression (G-I). Allowing HPAECs to form the monolayer in the Boyden chamber in the presence of ACM did not change the phenotype of hfNPCs as they did not express neuronal marker (D-F) and continued expressing Nestin (J-L). The micrographs were taken at the magnification of 20X.

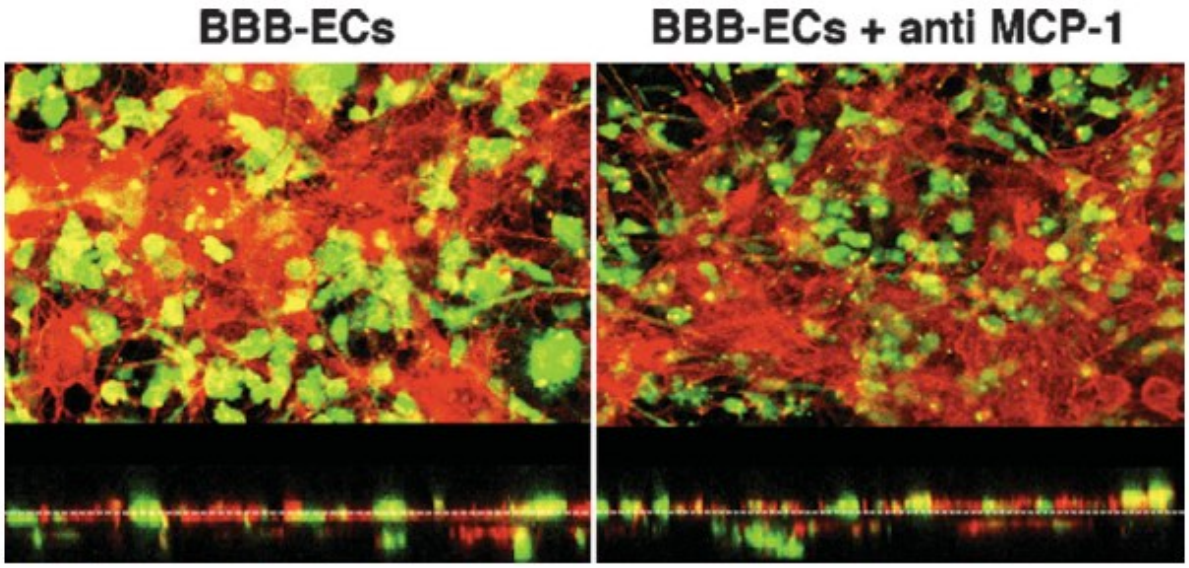

Figure 5: Migration of hfNPCs and derived cells in sub-endothelial space after blocking CCL2/MCP-1

Representative confocal image and z-stack reconstruction analysis shows that more cells migrate to sub-endothelial space as compared to blocking MCP-1. Phalloidin (red) antibody was used to stain BBB-ECs and hfNPCs were labeled with CFSE (green) before trans-BBB migration.

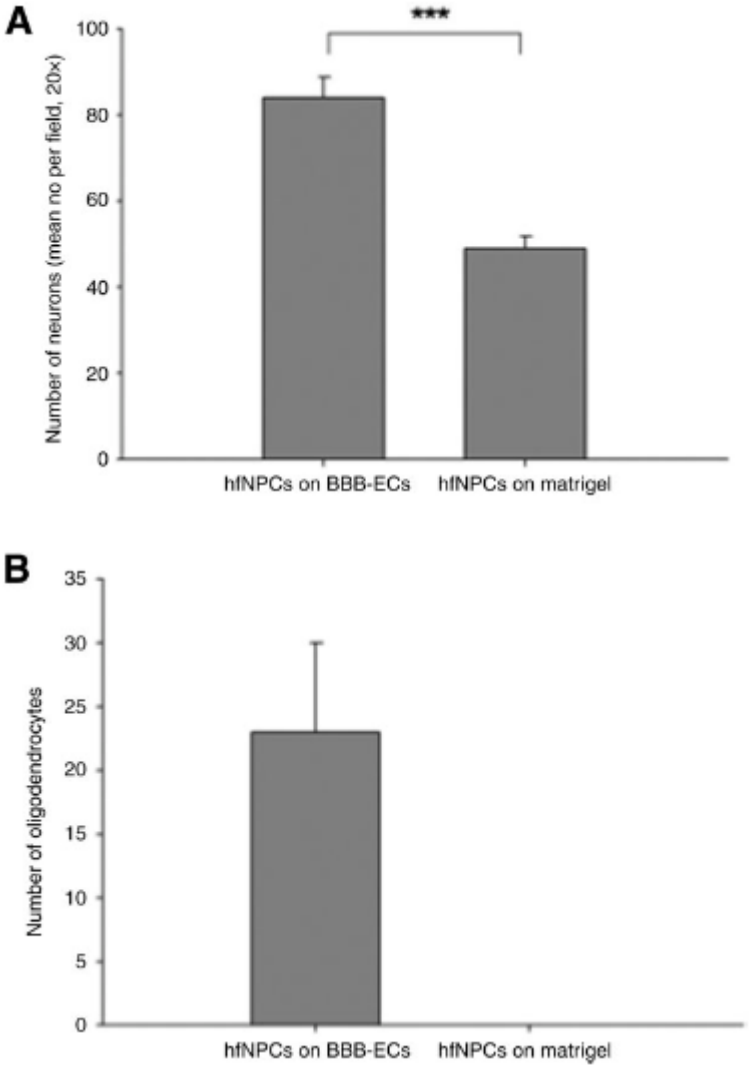

Figure 6: Comparative analysis of differentiation of hfNPCs on BBB-ECs and on matrigel.

hfNPCs were plated on BBB-ECs and on matrigel at the same density. hfNPCs plated on matrigel were grown without growth factors and in the presence of serum and on BBB-ECs in a complete medium (as described earlier). Three days later cultures were fixed and stained for β-tub-III for neurons and O4 for oligodendrocytes. (A) For neuronal counting, mean number

per field (20X, n=30 from 3 independent experiments) was taken into consideration and found that 42% less neurons were generated after plating them on matrigel as compared to BBB-ECs. (B) For oligodendrocytes, total number was counted and the mean of 3 independent experiments is presented and we were unable to detect the presence of oligodendrocyte three days after plating hfNPCs on matrigel.

Chemokine effect on migration and differentiation of hfNPCs on trans-BBB migration

Groups	Migration	Differentiation	
	hfNPCs derived cells Mean number per field (40X) ± SEM, n=30 from 3 independent experiments	Neurons Mean number per field (20X) ± SEM, n=30 from 3 independent experiments	Oligodendrocytes Total number from 3 independent experiments
Control	68.66 ± 3.16	84 ± 4.7	69
anti-MCP-1	47.27 ± 2.62 (%inhibition 31.2)	59.2 ± 3.9 (%inhibition 29.5)	13 (%inhibition 81.2)
anti-IL-8	61.23 ± (%inhibition 10.8)	81 ± 4.8 (%inhibition 3.6)	64 (%inhibition 7.2)

Table 1: Effect of the chemokines MCP-1/CCL2 and IL-8/CXCL8 on the migration and differentiation of hfNPCs after trans-BBB-migration.

Antibody blocking of MCP-1 significantly inhibited migration and differentiation of hfNPCs, decreasing hfNPCs migration by 31%, neuronal production by 30% and oligodendrocyte production by 81%. Blocking IL-8 had no effect. The data shown represents the mean number per field ± SEM of 30 fields selected at random from 3 independent experiments for counting migrated hfNPCs and neurons and total number of oligodendrocytes generated in 3 independent experiments for counting oligodendrocytes.

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Supplemental Data

Materials and Methods

RT-PCR

A previously published protocol was used for RT-PCR (13,15). All reagents were from Invitrogen; primers for CCL2/MCP-1 receptors, CCR1 and CCR2 and synthetic DNA as a positive control were purchased from R&D Systems.

Permeability assay.

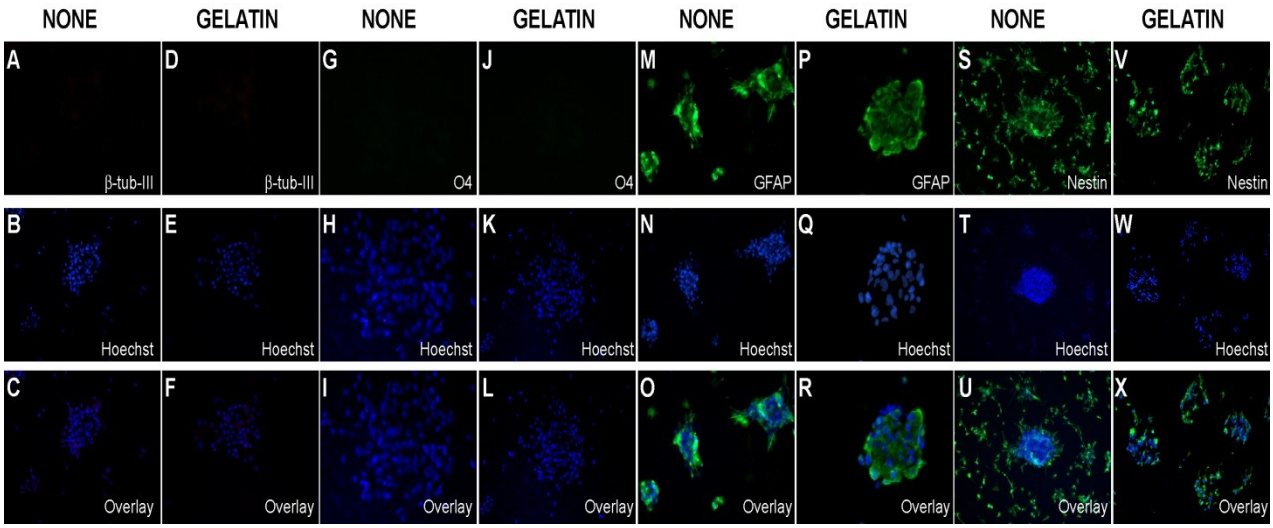
Permebility assay was performed as previously performed (13, 15). Briefly, BBB-ECs were allowed to form a monolayer in astrocyte conditioned-endothelial cell media (ACM-ECM). Three days later, media was replaced with either fresh ACM-ECM or NSCM containing 50 μg/ml of fluorescein isothiocyanate-labeled BSA (Invitrogen) to the upper chamber. Fifty μl samples were taken from the upper and lower chambers at different time points over a 3 d period and the fluorescence intensity was measured using a FL600 microplate fluorescent reader (Biotek). The diffusion rate, a measure of the permeability of BBB-ECs, was expressed as a percentage and calculated as follows: [BSA lower chamber] × 100 / [BSA upper chamber].

ELISA.

Confluent BBB-ECs were cultured either in ACM-ECM or NSCM for 24 h and supernatants were collected and assessed for secretion of CCL2/MCP-1 and CXCL8/IL-8 by ELISA, using a commercially available kit from BD Biosciences. ELISAs were carried out following the manufacturer's instructions as previously published (12). Standards and samples were run in duplicate.

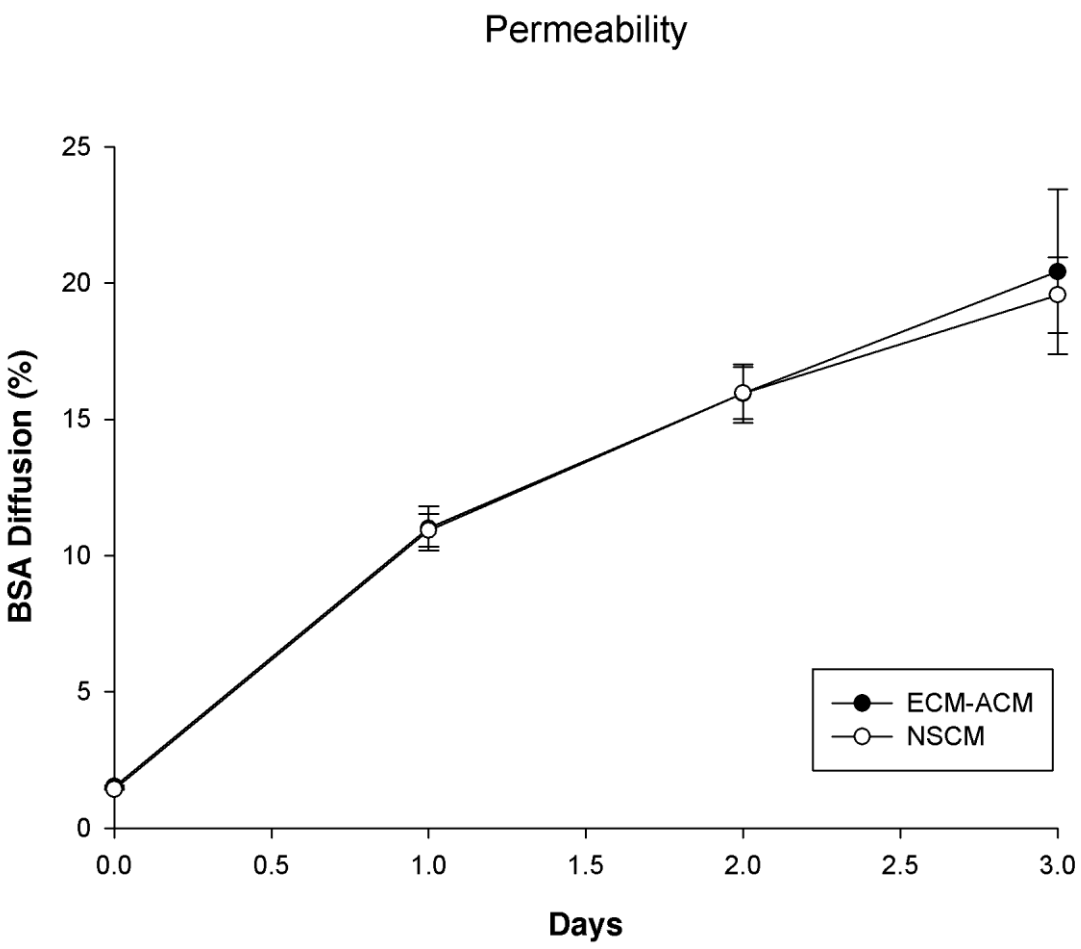
Western blots for tight junction proteins.

Western blots were done as previously published (15). Briefly BBB-ECs were lysed in NP-40 buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl2 and 0.5% NP-40) supplemented with protease inhibitors. Fifty μg of total protein were electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to a polyvinylidene fluoride membrane (Biorad) and blocked for 1 h at room temperature in 5% milk. We incubated the membranes overnight with rabbit antibody specific to human occludin, rabbit antibody specific to human ZO-1 (1/250, Zymed), mouse antibody specific to human JAM-1 (1/500, BD Bioscience) rabbit antibody specific to human claudin-5 (1/250, Zymed) or mouse antibody specific to human actin (1/1,000, 1 h at room temperature, MP Biomedicals) followed by an incubation with horseradish peroxidase (HRP)-conjugated goat antibody specific to rabbit immunoglobulins or rabbit antibody specific to mouse immunoglobulins (1/1,000, 1 h at room temperature, DakoCytomation). Specific binding was visualized using the ECL system (Amersham Biosciences).

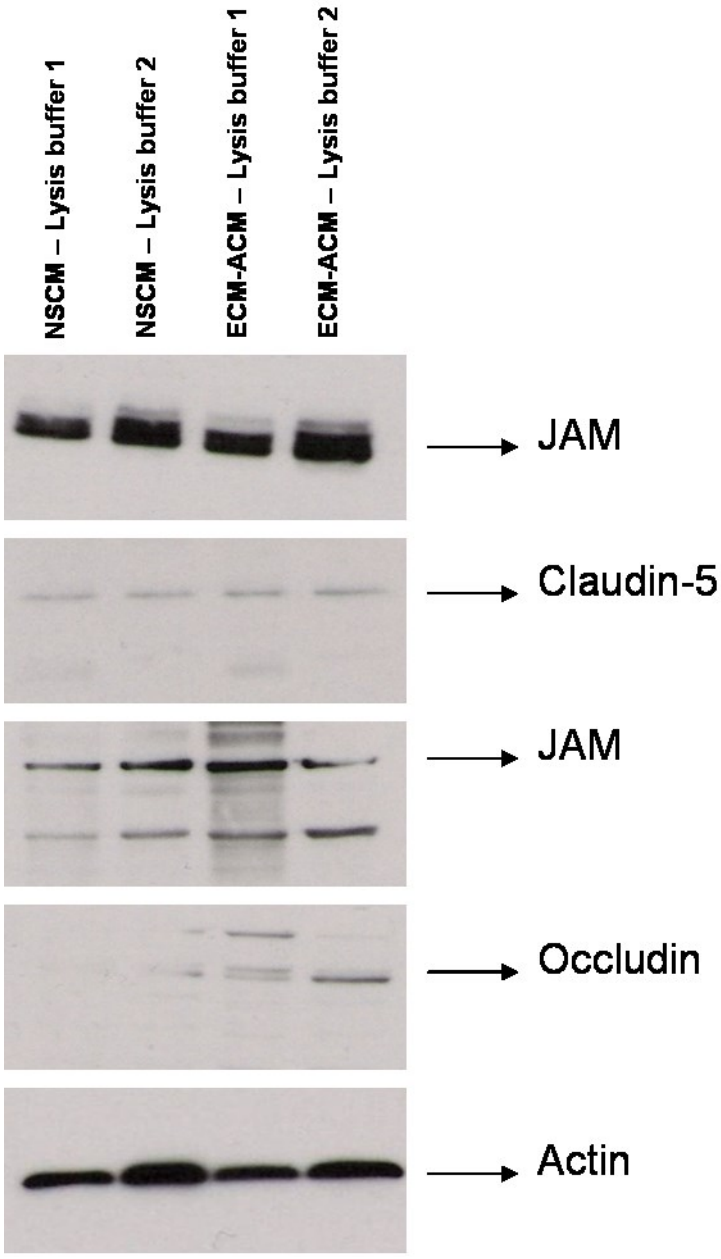


Supplementary Figure 1: hfNPCs on Boyden Chamber membrane with and without gelatine coating in the absence of BBB-ECs.

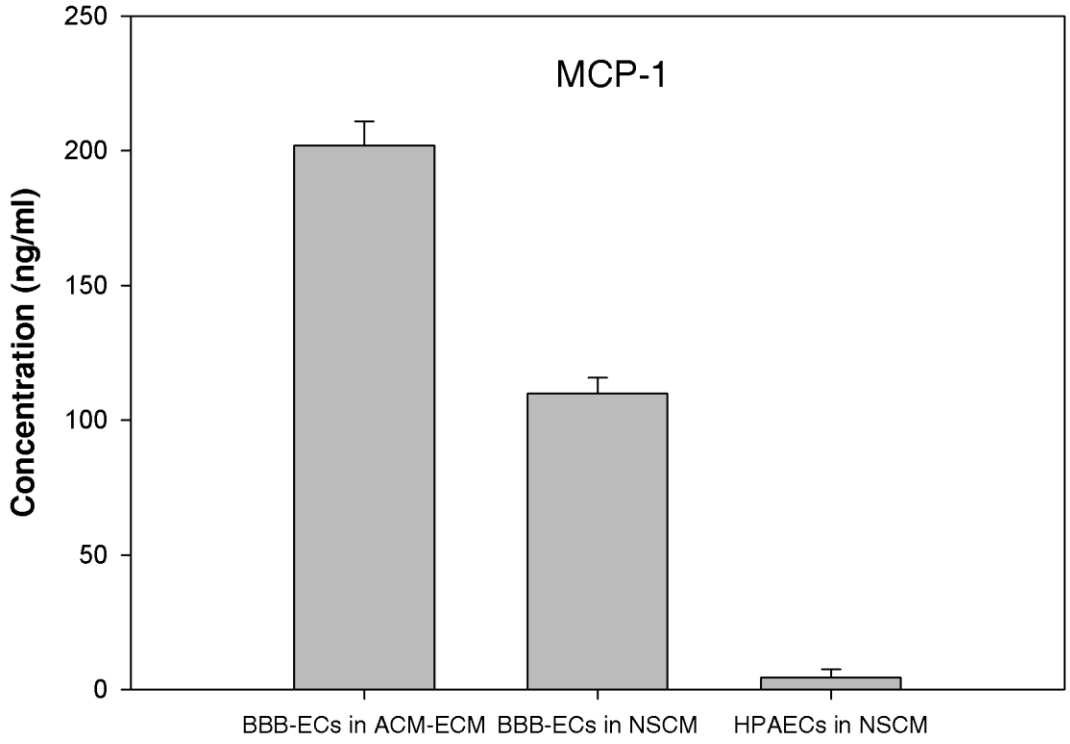
Three days after being plated on porous Boyden chamber membrane or gelatine coated membrane, hfNPCs did not express neuronal marker β-tub-III and the oligodendrocyte marker O4 (A-L). Almost all the cells express GFAP (M-R) and Nestin (S-X).



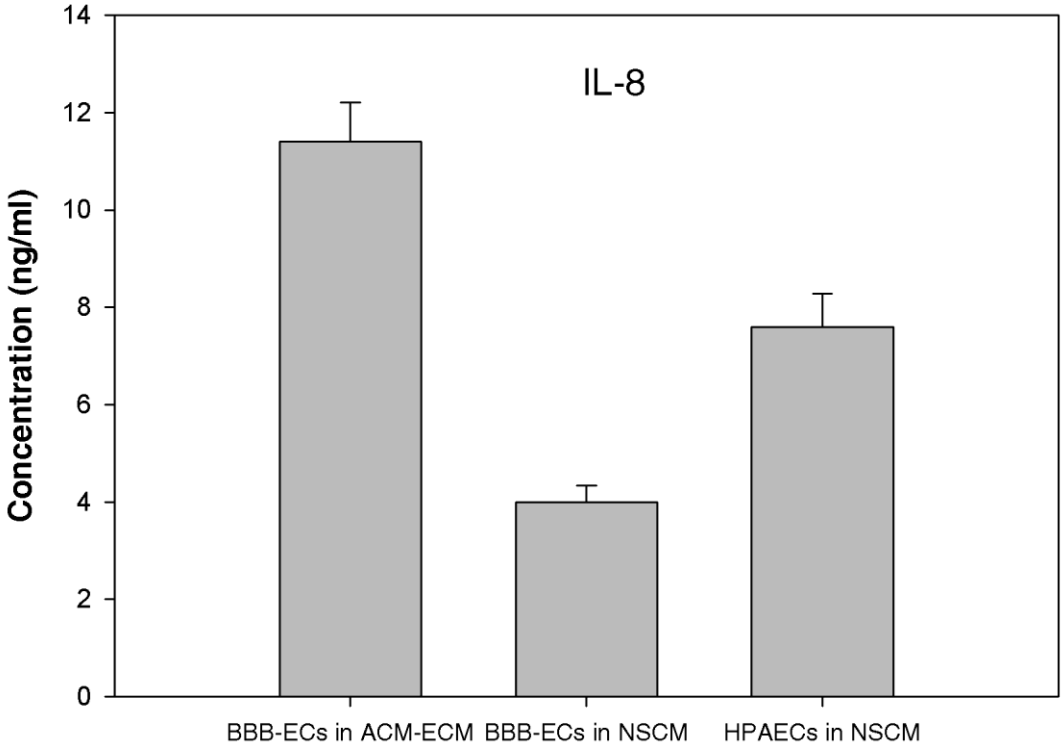
Supplementary Fig. 2 A



Supplementary Fig. 2 B



Supplementary Fig. 2 C

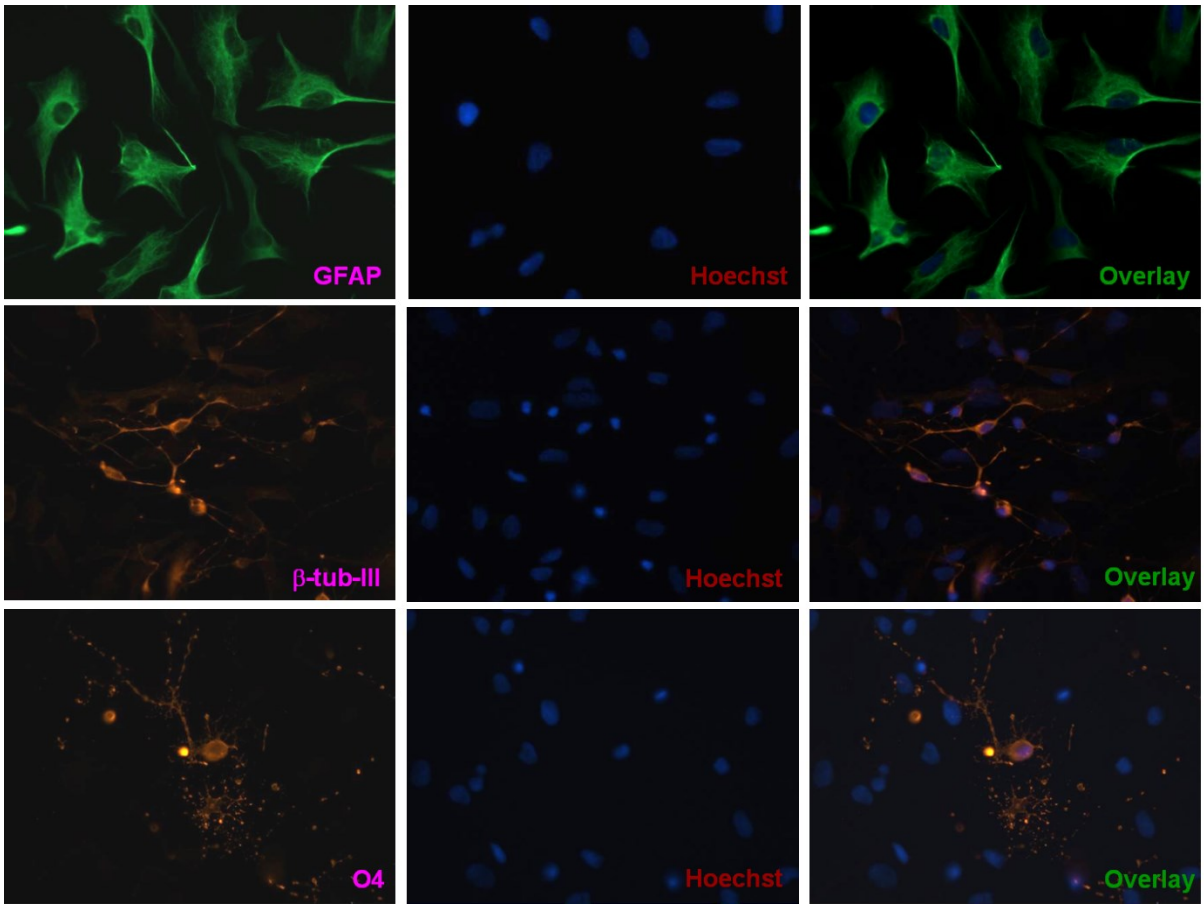


Supplementary Fig. 2 D

Supplementary Figure 2: Characterization of BBB-ECs in neural stem cells media.

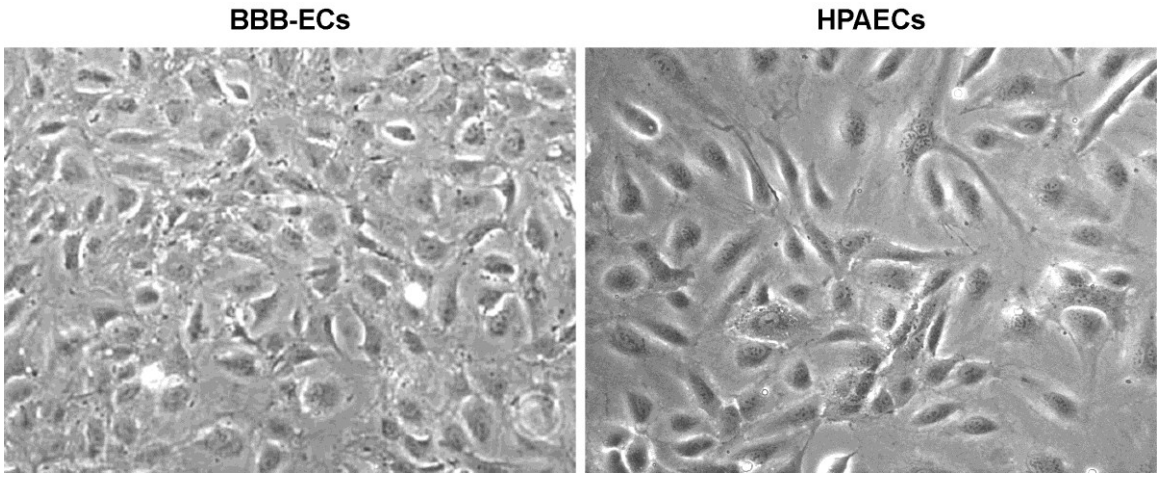
(A) Media in the upper and lower compartment was collected and FITC-BSA diffusion was determined by microplate reader and used as a measure of permeability of BBB-ECs. There was no difference between the permeability of BBB-ECs in ACM-ECM and in NSCM when the BBB-ECs were allowed to form a monolayer in ACM-ECM. Permeability was assessed for 3 days, the same duration as that of transendothelial migration assay. Values represented as average % permeability of triplicates ± SEM. **(B)** BBB-ECs tight junction proteins of Zo-1,

occludin, JAM-1, claudin-5 did not show any change in expression when cultured in ACM-ECM and NSCM for 3 days as determined by western blot analysis. **(C-D)** Confluent BBB-ECs when cultured for 24 h in NSCM and have 50% and 65% reduction in MCP-1 and IL-8 respectively as compared to ACM-ECM. HPAECs produce 25 times less MCP-1 and twice more IL-8 as compared to BBB-ECs when cultured in NSCM.

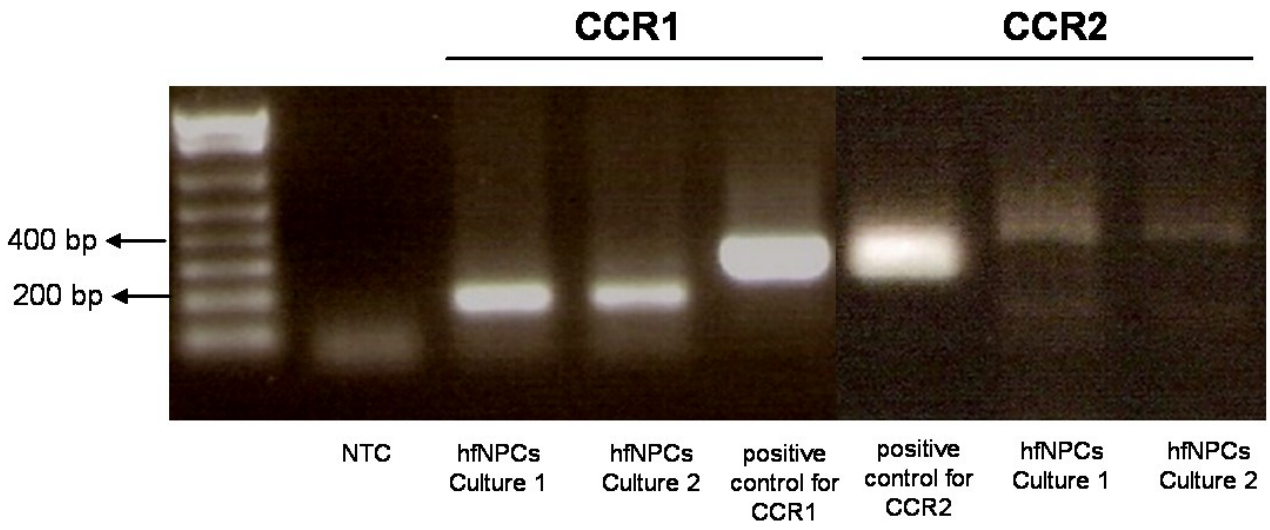


Supplementary Figure 3: Differentiation of clonally derived neurospheres

Clonally derived neurosphere were plated on matrigel coated coverslips. Differentiation was induced by growth factor withdrawal and addition of serum (see Materials and Methods for details). Eight days after plating, coverslips were fixed with paraformaldehyde and stained for differentiated cell markers. Morphology and the expression of neuronal (□-tub-III), astrocytic (GFAP) and oligodendrocytic (O4) markers confirm that clonally derived neurospheres are multipotent in nature.



Supplementary Figure 4: Culture of BBB-ECs and HPAECs at passage 4.



Supplementary Figure 5: Expression of CCL2/MCP-1 receptors CCR1 and CCR2 by RT-PCR

Expression of CCR1 (201 bp) and CCR2 (406 bp) by two different cultures of hfNPCs was confirmed by RT-PCR. Two synthetic DNA of size 320 bp were used as a positive control for CCR1 and CCR2.

SECTION 5 DISCUSSION, PERSPECTIVES ET CONCLUSIONS

La SEP est une maladie chronique débilitante qui affecte une partie importante de la population nord-américaine. Bien que l'étiologie de la maladie reste obscure, on retrouve chez ces patients des plaques caractéristiques de démyélinisation focale, accompagnées d'infiltration leucocytaire causant une réaction neuroinflammatoire pathologique[44]. La vasculature spécialisée du SNC, la BHE, joue un rôle clef dans les interactions entre le SNC et les leucocytes du sang et devient une cible thérapeutique élégante pour limiter les réactions neuro-immunes. Dans ce contexte, ce travail de doctorat vise à étudier les mécanismes moléculaires qui régissent les propriétés de la BHE pour pouvoir ensuite les moduler à des fins thérapeutiques. Cette thèse présente l'étude de trois propriétés de la BHE qui ont le potentiel d'influencer les réactions neuro-immunes et d'amener à de nouvelles stratégies thérapeutiques en SEP.

Le premier volet de ce travail cible les mécanismes qui régissent l'étanchéité de la BHE et identifie le rôle local du SRA dans l'UNV. Une nouvelle boucle signalétique est caractérisée entre les astrocytes et les CE-BHE où l'AGT sécrété par les astrocytes potentialise l'imperméabilité de la BHE à travers les récepteurs AT1 des CE. Ce travail démontre une perturbation de cette boucle signalétique dans les plaques de SEP et identifie une nouvelle avenue thérapeutique potentielle pour moduler la perméabilité de la BHE. L'angiotensine II (AngII) agit à travers le récepteur AT1 exprimé par les CE-BHE sur la phosphorylation et à la relocalisation membranaire de l'occludine pour limiter la perméabilité de la BHE. De plus, cette étude démontre que la relocalisation de l'occludine au sein de domaines membranaires spécialisés, les radeaux lipidiques, est essentielle pour conserver l'imperméabilité de la BHE. Pour mieux comprendre le rôle de ces micro-domaines membranaires dans les propriétés de la BHE, une étude protéomique des radeaux lipidiques des CE-BHE a été faite (ANNEXE I).

La deuxième section de cette thèse présente l'identification de la protéine ALCAM, que l'on retrouve dans les radeaux lipidiques et qui agit comme une molécule d'adhérence au niveau de la BHE. Ce deuxième volet identifie une nouvelle protéine qui participe aux interactions entre les leucocytes et les CE-BHE. L'inhibition des interactions qui dépendent d'ALCAM limite la transmigration de leucocytes à travers la BHE *in vitro* et *in vivo* dans l'EAE. Les interactions à travers ALCAM affectent différemment les différents sous-types de leucocytes. En effet, la transmigration des lymphocytes CD4+ et des monocytes est significativement limitée en présence d'anticorps bloquant contre ALCAM, alors que celle des lymphocytes CD8+ n'est pas affectée. Cette deuxième partie expérimentale identifie ALCAM comme une molécule importante pour la diapédèse des leucocytes à travers la BHE et comme une cible thérapeutique potentielle pour influencer l'infiltration leucocytaire au sein du SNC.

Le troisième volet de ce travail étudie les interactions entre les CSN et la BHE. Les CSN sont une avenue thérapeutique qui gagne de l'intérêt[379-387] dans la SEP, où les traitements actuels n'ont qu'une efficacité clinique limitée pour les formes progressives de la maladie. En effet en phase progressive, l'atteinte neuro-dégénérative prend de l'importance, les stratégies immuno-modulatrices sont peu efficaces et les stratégies qui influencent la neuro-protection et la neuro-régénération sont à privilégier. L'administration exogène de cellules souches est donc une avenue thérapeutique élégante pour ces patients. Ce troisième chapitre démontre la capacité de transmigration des CSN à travers la BHE, *in vitro*. Cette habileté à migrer dans l'espace sous-endothélial prend place préférentiellement avec les CE-BHE plutôt qu'avec des CE d'origine non-cérébrale. Le MCP-1/CCL2 est identifié comme une chimiokine préférentiellement sécrétée par les CE-BHE qui favorise la transmigration et la différenciation des CSN dans l'espace sous-endothélial. Ce travail suggère que l'injection intraveineuse de CSN est une voie d'administration potentielle chez l'humain et identifie le MCP-1 sécrété par les CE-BHE comme un facteur important qui module localement la maturation et la différenciation des CSN.

Les prochains paragraphes résument les résultats des trois sections expérimentales et discutent de leur impact potentiel sur le développement de nouvelles stratégies thérapeutiques pour la SEP.

5.1 Le rôle de l'angiotensine dans la régulation de la BHE

5.1.1 Objectifs, sommaire et critique des résultats

Le SRA qui produit l'octapeptide angiotensine II (AngII) à partir de l'AGT est une cascade enzymatique qui a un rôle essentiel en périphérie dans la régulation du volume sanguin, de la pression artérielle et de la filtration rénale[388]. L'AGT est hydrolysé par la rénine pour former le décapeptide AngI, qui est lui-même métabolisé par ACE-1 pour produire l'AngII. L'AngII a des effets sur de multiples types cellulaires à travers les récepteurs AT1 et AT2. Plusieurs études indiquent que le SRA a un rôle local dans plusieurs organes comme le cœur[389], le pancréas[390], le cerveau[391] et le système immunitaire[392]. Une observation importante faite par Kakinuma Y *et al.* dans les souris déficiente en AGT suggère un rôle local de l'angiotensine dans la perméabilité de la BHE[393]. Ce travail vise à comprendre le rôle local du RAS dans la régulation de la BHE et à caractériser le RAS dans les lésions de SEP.

Une méthode expérimentale utilisant des cultures primaires de CE-BHE humaine et d'astrocytes fœtaux humains a été choisie pour étudier les interactions entre les astrocytes et les CE *in vitro*. Les résultats obtenus ont été corrélés avec des études d'immunofluorescence *in situ* dans les lésions de SEP et dans le SNC de souris déficientes en AGT. L'expression de l'AGT et de l'enzyme ACE-1 a été détectée au niveau des astrocytes humains *in vitro* et *in situ*. Le métabolite AngII se retrouve dans le surnageant d'astrocytes en culture *in vitro*. En contrepartie, les CE-BHE expriment les récepteurs de l'AngII AT1 et AT2. La fonction de l'AngII a été étudiée en utilisant un modèle *in vitro* de la BHE élaboré dans le laboratoire[394-397]. Des CE-BHE sont mises en culture sur une membrane contenant des pores de 3

micromètres. Les traceurs solubles sont ajoutés à la chambre supérieure et la perméabilité est évaluée en fonction du temps par leur accumulation dans la chambre inférieure. Ces expériences démontrent que l'AngII sécrétée par les astrocytes diminue la perméabilité des monocouches de CE aux traceurs solubles comme la BSA-FITC et le sucrose. La BSA traverse la BHE par la route paracellulaire et par transcytose alors que le sucrose utilise la voie paracellulaire. Les effets de l'AngII sur la perméabilité des CE-BHE sont médiés via les récepteurs AT1. Les inhibiteurs spécifiques à l'AT1 limitent les effets de l'AngII alors que les agonistes de l'AT1 reproduisent les effets de l'AngII. Les agonistes et antagonistes de l'AT2 n'ont pas d'effets sur la perméabilité des CE-BHE *in vitro*. L'AngII influence la perméabilité paracellulaire via des modifications post-translationelles de l'occludine. En effet l'AngII modifie la phosphorylation de l'occludine et augmente son association avec des micro-domaines membranaires enrichis en cholestérol, les radeaux lipidiques. L'effet des médiateurs inflammatoires sur cette boucle signalétique a été étudié *in vitro* et les cytokines pro-inflammatoires IFN-gamma et TNF-alpha diminuent la sécrétion de l'AGT et de l'ACE-1 par les astrocytes. De plus, dans les lésions de SEP, on note une diminution du nombre d'astrocytes périvasculaires qui expriment l'AGT, ce qui coïncide avec la diminution de l'expression de l'occludine au niveau des JS de la BHE. Enfin les souris qui n'expriment pas l'AGT ont un endothélium cérébral qui possède des jonctions intercellulaires altérées et qui est plus perméable aux protéines sériques. Ces souris ont une diminution de l'expression de l'occludine au niveau des JS et on note une infiltration diffuse et discrète des protéines sériques albumine et plasminogène dans le parenchyme cérébral. Ces observations démontrent le rôle local de l'AGT sécrété par les astrocytes, qui potentialise l'étanchéité de la BHE en jouant sur la phosphorylation et la localisation de l'occludine dans l'UNV. Ce travail identifie aussi une altération du SRA au sein des plaques de SEP, ce qui peut en partie expliquer les dysfonctions de la BHE que l'on retrouve dans la SEP.

Cette étude identifie un nouveau facteur astrocytaire, l'AGT, qui stabilise et potentialise les propriétés de la BHE. L'AngII est un des facteurs qui se retrouvent dans le milieu conditionné par les astrocyte et qui contribue à l'induction et à la maturation des

propriétés spécifiques à la BHE dans les CE *in vitro*. Les récepteurs de cette boucle signalétique se retrouvent sur les CE-BHE et permettent la déphosphorylation de l'occludine sur les résidus tyrosine et sa phosphorylation sur les résidus thréonine. Ces modifications de l'occludine sont associées à un enrichissement de l'occludine dans les radeaux lipidiques, ce qui favorise l'étanchéité de la BHE. L'AGT s'ajoute ainsi aux facteurs astrocytaires déjà connus qui potentialisent l'imperméabilité de l'endothélium cérébral à l'état physiologique. Par contre, l'AngII n'est pas nécessaire à la BHE, comme le démontrent les expériences effectuées dans les souris déficientes en AGT. À l'état physiologique, l'endothélium de ces souris est subtilement altéré, mais la déficience en AngII ne semble pas avoir d'effet clinique au niveau de la BHE. Il y a très probablement de nombreuses boucles signalétiques redondantes et compensatoires dans l'UNV qui permettent une BHE fonctionnelle dans les animaux déficients en AGT. L'AngII semble donc potentialiser localement les propriétés de la BHE, mais n'est pas nécessaire à l'induction d'une BHE fonctionnelle *in vivo*[393]. Nos observations indiquent que la BHE chez ces animaux est potentiellement fragilisée, ce qui ne risque d'avoir des conséquences que lors de situations pathologiques. Une telle hypothèse concorde avec les observations du Dr Kakinuma[393], où les souris déficientes en AGT ne montrent une BHE anormale que dans des conditions pathologiques, par exemple lors d'une insulte cérébrale par le froid. Notre étude, qui montre que l'AngII agit sur l'occludine, vient également appuyer des résultats publiés précédemment qui démontrent que l'occludine n'est pas nécessaire à l'imperméabilité de la BHE[161], mais qu'elle potentialise cette dernière. Ce travail confirme le rôle des astrocytes dans l'UNV comme un type cellulaire qui stabilise et favorise une BHE imperméable.

Ces expériences confirment le rôle de la phosphorylation de l'occludine sur les résidus thréonine et la déphosphorylation des résidus tyrosine dans une BHE imperméable. Ces résultats sont en accord avec de nombreuses études qui démontrent que la fonction de l'occludine dépend de sa phosphorylation[168, 192, 202, 398-402]. En effet, la phosphorylation des résidus tyrosines inhibe l'association de ZO-1 et de l'occludine et déstabilise les TJ. De plus, notre étude associe un changement dans la phosphorylation de l'occludine avec

l'augmentation de l'expression de celle-ci dans les radeaux lipidiques et avec une régulation de l'étanchéité de la BHE. Les données de McCaffrey *et al.* confirment l'importance de ces micro-domaines membranaires dans l'oligomérisation de l'occludine, ce qui semble être essentiel pour l'imperméabilité de la BHE[162, 165]. Notre étude lie ces observations et suggère que la phosphorylation des résidus thréonine et la déphosphorylation des résidus tyrosine de l'occludine agit sur sa fonction, en augmentant sa localisation dans les radeaux lipidiques et en augmentant ainsi l'imperméabilité de la BHE.

De plus, ce travail est une des premières démonstrations de l'importance des micro-domaines membranaires dans l'étanchéité de la BHE. En effet, les agents qui agissent sur le cholestérol et limitent la formation de ces domaines membranaires diminuent l'imperméabilité de la BHE *in vitro*. Les radeaux lipidiques agissent comme des plateformes membranaires dynamiques qui permettent l'association optimale de protéines membranaires et intracellulaires[403, 404]. Des études récentes concordent avec la nôtre et démontrent un rôle important de ces domaines dans la régulation et les fonctions des jonctions intercellulaires[162, 165, 405]. Les interactions optimales entres toutes les différentes protéines des JS sont importantes pour l'étanchéité de la barrière et il est probable que les radeaux lipidiques favorisent ces complexes macromoléculaires et stabilisent ainsi les jonctions intercellulaires. L'association entre les JS et ces micro-domaines membranaires semble altérée dans de multiples situations où la perméabilité de la BHE est augmentée, comme dans des modèles d'hypoxie cérébrale, de douleur inflammatoire et de réactions neuroinflammatoires.

Les cytokines inflammatoires affectent le SRA localement en diminuant l'expression de l'AGT comme démontré *in vitro* et suggéré *in situ* dans les plaques de SEP. Ces résultats peuvent, en partie, expliquer l'augmentation de la perméabilité de la BHE dans les plaques de SEP où on retrouve des quantités importantes de médiateurs inflammatoires. Ces facteurs inflammatoires peuvent ainsi limiter l'expression de l'AGT par les astrocytes, et donc affecter l'étanchéité de la BHE. Les résultats obtenus dans les souris déficientes en AGT indiquent que le système SRA agit principalement sur la perméabilité vasculaire aux protéines sériques et pas sur l'infiltration leucocytaire. En effet, les souris déficientes en AGT ont une vasculature

cérébrale subtilement déficiente avec des JS altérées et une accumulation périvasculaire diffuse de protéines sériques, mais pas d'infiltration leucocytaire détectable. Il serait d'intérêt d'étudier l'EAE dans les souris déficientes en AGT, ce qui n'a pas été fait lors de cette étude.

Par contre le tableau se complique, en effet certaines publications semblent décrire un rôle contradictoire du SRA dans le SNC. Le groupe du Dr William Banks étudie l'encéphalopathie hypertensive et les dysfonctions de la BHE qui y sont associées[406, 407]. Les travaux de ce laboratoire décrivent l'effet de l'AngII qui augmente la perméabilité des CE-BHE à travers l'AT1 en stimulant l'activation de la protéine kinase C (PKC). Ces différences semblent difficilement conciliables avec nos résultats, mais peuvent probablement s'expliquer par les différences entre les modèles et les protocoles expérimentaux. Le laboratoire du Dr. Alexandre Prat étudie les CE-BHE humaines en culture primaire et le groupe de Banks utilise des CE-BHE murines. Les protocoles expérimentaux de ces deux études diffèrent sensiblement : nos expériences regardent l'effet de l'AngII sur la maturation des propriétés spécifiques de la BHE et les études du laboratoire du Dr Banks étudient les effets de l'AngII sur une BHE mature. Le groupe du Dr Banks utilise des doses 10 fois plus élevées d'AngII que celle utilisée dans notre étude. De plus, le rôle de la PKC, et de ses différents isoformes, dans la régulation de la perméabilité vasculaire est encore controversé[400, 408]. Certaines études associent son activation à une augmentation de la perméabilité[409], tandis que d'autres démontrent son rôle dans la maturation des JS et une étanchéité accrue[410].

Les groupes du Dr Lawrence Steinman et du Dr Ralph Linker ont ensuite décrit des résultats sensiblement discordants avec les nôtres où ils montrent que certains inhibiteurs spécifiques à l'AT1 et à l'ACE1 ont une efficacité clinique pour limiter l'EAE chez la souris[411-413]. Les études du groupe du Dr Steinman démontrent que l'EAE chez les souris SJL est améliorée avec le traitement au linisopril et au cadesartan, un inhibiteur de l'ACE-1 et un antagoniste de l'AT1, respectivement. Ces agents limitent l'activation des sous-types de lymphocytes Th1 et Th17 et favorisent les cellules T régulatrices. De plus, ces agents diminuent la production de TGF-bêta par les cellules gliales dans les lésions d'EAE, ce qui limite localement la réaction neuroinflammatoire. Quant aux travaux du Dr Linker, ces

derniers ont démontré que des inhibiteurs de la rénine, de l'ACE-1 ou de l'AT-1 limitent tous la sévérité de l'EAE. Ces agents diminuent la production de chimiokines par les cellules présentatrices d'antigène, dont celle du MCP-1/CCL2, ce qui limite le recrutement leucocytaire et donc la réaction neuroinflammatoire. Ces expériences démontrent un effet cliniquement significatif à bloquer le SRA dans l'EAE qui, dans ces études, agit surtout au niveau du système immunitaire et des cellules gliales sur la production de TGF-béta. À la lumière de ces résultats, il semble que le rôle local du SRA dans l'UNV est cliniquement moins important dans l'EAE que son rôle au niveau du système immunitaire et des autres cellules du SNC. Ces travaux indiquent que le SRA est exacerbé dans les réactions neuroinflammatoires et contribue en tant que médiateur inflammatoire dans le développement de l'EAE. Le SRA semble donc avoir des rôles différents dans le système immunitaire et localement dans l'UNV.

Seules les études futures pourront statuer sur l'importance clinique du SRA dans les réactions neuro-immunes. Il est néanmoins clair que le SRA a la capacité d'agir à plusieurs niveaux dans le SNC : au niveau de l'UNV, du système immunitaire et des autres cellules du SNC (oligodendrocytes, microglies et neurones). Enfin, il est clair que le SRA joue de multiples rôles dans les réactions inflammatoires du SNC, même si les observations effectuées jusqu'à présent ne sont pas toutes conciliables. L'intérêt d'utiliser ce système pour moduler les réactions neuroinflammatoires reste très important, étant donné l'usage courant et bien toléré des modulateurs pharmacologiques du SRA.

5.1.2 Perspectives et conclusions

Ce travail confirme l'importance des boucles signalétiques locales dans l'UNV pour le contrôle des propriétés de la BHE et identifie une altération du SRA dans l'UNV au sein des plaques de SEP. Étant donné qu'il y a une controverse sur le rôle exact du SRA dans les réactions neuroinflammatoires, il faut maintenant clarifier l'importance clinique des différents rôles du SRA dans le SNC pour élaborer une stratégie thérapeutique cliniquement viable. Les

données les plus récentes dans l'EAE indiquent que le SRA potentialise les réactions neuro-immunes et que l'AngII agit comme un médiateur inflammatoire dans l'EAE.

Nos travaux ont aussi permis d'identifier une nouvelle boucle de régulation entre les astrocyte et la BHE qui module l'imperméabilité de cette dernière. Mais encore cette étude confirme l'importance des radeaux lipidiques comme des structures membranaires qui participent aux propriétés spécifiques à la BHE. Ces micro-domaines membranaires, enrichis en cholestérol, forment des radeaux organisés dans la membrane lipidique des cellules eucaryotes[414]. Ces structures de 10-200 nanomètres sont hétérogènes, dynamiques et associées à un enrichissement de différentes protéines membranaires et intracellulaires. Les radeaux lipidiques peuvent s'associer et former des plateformes membranaires qui ont un rôle critique dans la signalisation intracellulaire. Les domaines membranaires sont associés à de multiples procédés cellulaires[403, 404, 414-423] et récemment aux jonctions intercellulaires[162, 165, 405]. Ces observations suggèrent fortement que les radeaux lipidiques des CE participent à de nombreux procédés cellulaires clés de la BHE, incluant la formation de jonctions intercellulaires étanches.

Pour découvrir des nouveaux mécanismes moléculaires de la BHE, une collaboration a été établie avec un groupe du CNRC (Conseil national de recherches Canada) pour sonder la composition protéique des radeaux lipidiques de la BHE. Le protocole expérimental élaboré a été publié (ANNEXE I) et a permis l'isolation et l'analyse protéomique de radeaux lipidiques de CE-BHE afin d'identifier les protéines qui s'y retrouvent[375, 424]. C'est à partir de ces expériences de protéomique que la molécule ALCAM a été identifiée et que plusieurs autres projets ont vu le jour dans le laboratoire[307, 425, 426].

5.2 Le rôle d'ALCAM dans l'infiltration leucocytaire au SNC lors des réactions neuroinflammatoires

5.2.1 Objectifs, sommaire et critique des résultats

Ce deuxième volet visait à décrire de nouveaux mécanismes de la BHE impliqués dans les interactions avec les leucocytes. À partir d'une étude protéomique des radeaux lipidiques de la BHE, ALCAM a été identifiée comme une protéine exprimée par les CE-BHE. ALCAM est une protéine de la grande famille des immunoglobulines, elle fait partie d'un sous-groupe qui inclue MCAM/CD146 (*melanoma cell adhesion molecule*) et B-CAM (*B-cell adhesion molecule*). ALCAM est une glycoprotéine transmembranaire formée de deux domaines extracellulaires variables et de trois domaines constants. Cette molécule interagit avec elle-même, ou avec la protéine CD6 (exprimée par les lymphocytes T), comme c'est le cas dans la synapse immunitaire. Plusieurs études ont également associé ALCAM à de nombreux procédés de migration cellulaire, comme la neurogénèse, la formation du réseau lymphatique, l'implantation blastocytaire, l'invasion tumorale et, plus récemment, la diapédèse des monocytes[427-434]. Ce travail a pour objectif la description du rôle et de la fonction d'ALCAM au niveau de la BHE, plus particulièrement lors de l'infiltration leucocytaire du SNC.

Le deuxième manuscrit de cette thèse démontre qu'ALCAM est exprimée par les CE-BHE *in vitro* et *in situ*. Comme c'est le cas pour les molécules d'adhérence prototypiques ICAM-1 et VCAM-1, l'expression d'ALCAM est augmentée en présence des cytokines inflammatoires IFN-gamma et TNF. Au niveau des leucocytes du sang périphérique, les monocytes et les cellules B expriment ALCAM, alors que les lymphocytes T expriment CD6. Les études de microscopie confocale démontrent qu'ALCAM est enrichie autour des leucocytes lors de la diapédèse, dans une structure qui ressemble à la coupe transmigratoire (*transmigratory cup*). De plus, dans le modèle *in vitro* de la BHE, un anticorps bloquant contre ALCAM limite la transmigration des lymphocytes T CD4+, des lymphocytes B et des monocytes, mais n'affecte pas la migration des lymphocytes T CD8+. Dans le SNC, on

observe *in situ* une augmentation significative de l'expression d'ALCAM sur l'endothélium cérébral dans les lésions actives de SEP. Ces observations sont corrélées aux résultats *in vivo,* où ALCAM se retrouve surexprimée sur l'endothélium cérébral associé aux lésions d'EAE chez les souris C57BL/6. Finalement, ALCAM montre un potentiel thérapeutique intéressant pour limiter les réactions neuroinflammatoires *in vivo* dans l'EAE, puisque l'injection d'anticorps bloquant contre ALCAM limite les signes cliniques et pathologiques de la maladie. En effet, l'inhibition des interactions d'ALCAM *in vivo* limite l'infiltration des lymphocytes T CD4+ et des CPA dans les lésions d'EAE, et diminue le nombre et la taille des plaques de démyélinisation. Finalement, les études *in vitro* démontrent qu'ALCAM et ICAM-1 ont des rôles synergiques et partiellement redondants dans la diapédèse. Ce travail caractérise donc le rôle d'une nouvelle protéine impliquée dans la diapédèse des leucocytes à travers la BHE et identifie ALCAM comme une cible potentielle pour influencer la diapédèse et l'infiltration leucocytaire au SNC. Des études génétiques de la SEP ont récemment décrit des polymorphismes dans les gènes CD6 et ALCAM qui augmentent le risque de développer la maladie, avec des ratios de côtes de 1.18 et 2.34 respectivement[25, 26]. Ces études appuient nos observations et confirment l'importance de la protéine ALCAM dans la SEP.

Le mécanisme moléculaire par lequel ALCAM agit n'est toujours pas connu. En effet, ALCAM peut tenir le rôle d'un crochet permettant l'adhésion du leucocyte aux CE-BHE. ALCAM interagit et est régulé par certaines protéines membranaires comme la tétraspanine CD9[435]. ALCAM semble avoir des rôles redondants avec la protéine RAGE, une protéine important dans la régulation des réaction inflammatoires[436]. Plusieurs études démontrent qu'ALCAM a aussi des propriétés importantes de signalisation via son domaine intracellulaire. En effet, ALCAM s'associe avec le cytosquelette et avec des protéines intracellulaires telles que l'alpha-caténine[433, 437, 438] et les protéines ERM[439]. Ces protéines sont impliquées dans le contrôle des JA et dans la réorganisation du cytosquelette cellulaire. La liaison et l'agrégation d'ALCAM est également associée à la polymérisation de l'actine[440], à la motilité cellulaire[441], à la régulation de l'activité des métalloprotéases[442, 443], à l'imperméabilité paracellulaire[438] et à la régulation des AJ[441]. Ainsi, ALCAM a le potentiel

d'agir comme un lien adhésif entre les leucocytes et les CE-BHE, mais elle peut aussi contribuer à la signalisation pour coordonner la diapédèse et réguler les jonctions intercellulaires, au même titre que les molécules d'adhérence prototypiques ICAM-1[444] et VCAM-1[253, 257].

ALCAM s'associe aux radeaux lipidiques, qui contribuent à différentes étapes dans la transmigration cellulaire ainsi que dans les changements de morphologie cellulaire, dans l'enrichissement spatial de certaines protéines, dans l'association au cytosquelette et dans la propagation de signaux intracellulaires[403, 404, 414-423]. Les protéines prototypiques de la diapédèse (sélectines, VCAM-1 et ICAM-1) s'associent elles aussi aux radeaux lipidiques, ce qui semble faciliter et optimiser la propagation de signaux intracellulaires essentiels à la diapédèse[253, 270, 445]. Les cytokines pro-inflammatoires IFN-gamma et TNF augmentent l'association d'ALCAM avec ces microdomaines membranaires, ce qui pourrait également favoriser la diapédèse. Ces résultats suggèrent fortement que les radeaux lipidiques de la BHE forment des plateformes signalétiques essentielles à de multiples procédés, comme la diapédèse et la régulation des jonctions intercellulaires. Ces observations de la section I et II, combinées aux expériences de protéomique, nous ont amené à proposer une hypothèse selon laquelle les radeaux lipidiques forment des microdomaines membranaires hétérogènes qui jouent des rôles clés dans plusieurs fonctions de la BHE (ANNEXE II) [446]. L'association dynamique des radeaux lipidiques peut ainsi lier et coordonner de multiples procédés cellulaires, comme c'est probablement le cas dans la coupe transmigratoire lors de la diapédèse (adhésion/signalisation, réarrangement de la morphologie cellulaire et des jonctions intercellulaires). Ces plateformes membranaires favoriseraient la formation transitoire de complexes macromoléculaires qui sont potentiellement des facteurs déterminants dans la diapédèse et dans plusieurs autres situations.

5.2.2 Perspectives et conclusions

Il reste encore beaucoup à faire avant de considérer la modulation d'ALCAM comme une stratégie de traitement viable en SEP. Tout d'abord, ALCAM est exprimée dans de

multiples types cellulaires, dont les CE rétiniennes, les leucocytes, certains épithéliums, les cellules souches, les neurones ainsi que certaines tumeurs. Il est essentiel de comprendre et de clarifier les rôles d'ALCAM *in vivo* dans tous ces différents types cellulaires à l'état physiologique et dans les conditions pathologiques. ALCAM semble déjà remplir plusieurs fonctions différentes, certaines études impliquant ALCAM dans la régulation des AJ[441], dans la régulation locale de la sécrétion de protéase[443], dans l'activation du système immunitaire[447] et dans la régulation de la morphologie cellulaire[440]. Les souris déficientes en ALCAM sont viables et fertiles. Ces souris n'ont pas de phénotype clinique notable. Elles démontrent par contre de subtiles altérations au niveau du trajet des axones de la rétine[448]. Les études actuelles dans le laboratoire tentent de déchiffrer le rôle exact d'ALCAM *in vivo* dans l'EAE, dans l'activation immunitaire et dans la transmigration leucocytaire en utilisant les souris déficientes en ALCAM.

Le rôle exact d'ALCAM dans la diapédèse au niveau des CE-BHE doit aussi être caractérisé, afin de définir si cette molécule agit comme une molécule d'ancrage ou si elle participe à la signalisation intracellulaire nécessaire à la diapédèse, ainsi que la ou les voies signalétiques qu'elle utilise. Il reste aussi à mieux définir le rôle d'ALCAM dans les différentes routes de diapédèse; paracellulaire et transcellulaire. La fonction d'ALCAM dans la transmigration de différents sous-types cellulaires doit, elle aussi, être mieux caractérisée. Nos résultats démontrent que la transmigration des lymphocytes T CD8+, qui expriment le ligand CD6, n'est pas affectée par l'anticorps bloquant contre ALCAM. En effet, ALCAM participe préférentiellement dans la diapédèse de certains sous-types de leucocytes comme cela semble aussi être le cas pour les cellules T régulatrices (Treg) dans le cancer du pancréas[449]. Il est donc nécessaire d'expliquer ces observations pour mieux comprendre leurs implications cliniques et considérer ALCAM comme une cible thérapeutique d'intérêt dans la modulation des reactions neuroinflammatoires.

Ce travail peut aussi avoir un impact important dans toutes les situations où ALCAM est impliquée dans la diapédèse cellulaire. C'est possiblement le cas dans certains cancers, où l'expression d'ALCAM est associée à une augmentation des métastases (mésothéliomes[450],

cancers de l'œsophage[451] et mélanomes[452]). Dans ces situations, il est probable qu'ALCAM participe à la transmigration à travers les parois vasculaires. L'inhibition de ces interactions pourrait être une stratégie thérapeutique pour limiter les métastases tumorales et l'invasion tissulaire. De plus, il sera intéressant d'évaluer le rôle possible d'ALCAM dans de multiples pathologies où les réactions neuroinflammatoires sont pathologiques.

Une étude récente démontre que chez les patients VIH+ il y a une augmentation de la transmigration de monocytes au SNC et cette infiltration est exacerbée par l'utilisation de la cocaïne. La cocaïne augmente l'expression d'ALCAM sur les CE-BHE et potentialise l'infiltration monocytaire au SNC[453]. D'autres études indiquent qu'ALCAM joue un rôle dans les CE rétiniennes et favorise la transmigration de monocytes infectés par *Toxoplasma gondii*[454, 455]. Dans de telles situations la modulation d'ALCAM pourrait être une thérapie potentielle pour limiter les réactions inflammatoires dans l'encéphalite au VIH et pour limiter la propagation de l'infection dans les infections rétiniennes au toxoplasme.

5.3 Interactions entre la BHE et les cellules souches neurales

5.3.1 Objectifs, sommaire et critique des résultats

En SEP, toutes les thérapies actuelles visent le contrôle de la réaction neuroinflammatoire. Cependant, de nombreuses études indiquent qu'il y a une composante neurodégénérative importante, surtout dans la phase progressive de la SEP[385]. Au sein des plaques de SEP, on observe non seulement des dommages inflammatoires chroniques qui conduisent à la démyélinisation, mais aussi des défauts de la réaction de remyélinisation post-insulte[42, 57, 456, 457]. Bien que la capacité de régénération du SNC est très limitée, il existe dans cet organe des niches de cellules souches neurales (CSN) qui persistent même à l'age adulte[458]. Ces CSN permettent une certaine plasticité au SNC, afin de répondre localement aux procédés pathologiques en permettant la régénération limitée d'astrocytes, d'oligodendrocytes

et de neurones[459]. Ces CSN forment une population de cellules hétérogènes, ayant des capacités d'auto-renouvellement et de différentiation, et jouent des rôles clés dans de nombreux procédés comme la remyélinisation post-insulte via les cellules progénitrices d'oligodendrocytes[460]. Ces concepts récents ont révolutionné notre compréhension du SNC, où l'on croyait qu'il n'y avait pas de régénération cellulaire possible[383, 461]. Au sein des plaques de SEP, la remyélinisation est imparfaite, incomplète et amène ultimement à des dommages axonaux et à une neurodégénération focale lorsque la capacité régénératrice locale du SNC est dépassée. Des études en 2003 ont démontré que l'injection exogène de CSN a la capacité de limiter les signes cliniques de l'EAE. Les CSN se différencient dans les lésions de démyélinisation et favorisent la régénération locale du SNC[462]. Depuis, de multiples études ont été faites avec des sources diverses de cellules souches dans différents modèles d'EAE (cellules souches embryonnaires ou adultes, humaines ou murines, soumises à différentes méthodes de culture), afin de comprendre les mécanismes d'action de ces cellules dans les réactions neuroinflammatoires[384, 463-466]. Actuellement, le mécanisme par lequel les CSN agissent n'a pas encore été identifié, bien que ces cellules aient clairement la capacité de migrer dans les plaques d'EAE et de participer à la remyélinisation[464, 465, 467]. Les CSN ont aussi des propriétés trophiques, importantes pour la régénération locale, et des propriétés immuno-modulatrices anti-inflammatoires[382, 383, 461, 468]. Les études actuelles visent à déterminer le mode optimal d'administration et le type de cellules souches à utiliser selon la maladie à traiter. Le troisième volet expérimental de cette thèse s'insère dans ce contexte, puisque la voie d'administration de prédilection des CSN en SEP est la voie intraveineuse. Cette étude a pour objectif la caractérisation *in vitro* des déterminants des interactions entre les CSN et l'endothélium vasculaire cérébral.

Ce travail utilise des CSN humaines foetale (hfCSN) mises en culture *in vitro* sous forme de neurosphères. Les hfCSN ont la capacité d'auto-renouvellement et expriment les marqueurs cellulaires typiques des CSN (Nestin, Sox-2 et GFAP). Les hfCSN peuvent se différencier *in vitro* en astrocytes, en oligodendrocytes et en neurones, comme démontré après 8 jours sur le matri-gel sans facteurs de croissance mais en présence de sérum. Les CSN

adhèrent au matri-gel, adoptent la morphologie des cellules gliales et se différencient en cellules du SNC. Les CSN démontrent la perte de l'expression de nestine et l'acquisition du marqueur GFAP pour les astrocytes, bêta-tubuline pour les neurones et O4 pour les oligodendrocytes. Les études dans le modèle *in vitro* de la BHE démontrent tout d'abord que les hfSNC ont la capacité de migrer à travers une BHE intacte. De plus, les CSN s'accumulent dans l'espace sous-endothélial et s'y différencient rapidement et de façon plus efficace qu'avec le matri-gel seul. Ces observations ne peuvent pas être reproduites en absence de CE-BHE ou en présence de CE d'origine pulmonaire, ce qui indique qu'une propriété spécifique aux CE-BHE est impliquée. Nos résultats démontrent également que la chimiokine MCP-1/CCL2 sécrétée par les CE-BHE est un des facteurs importants impliqués dans les interactions entre l'endothélium cérébral et les hfCSN. En bloquant spécifiquement le MCP-1/CCL2, la transmigration et la différenciation des hfCSN sont significativement diminuées. À l'inverse, l'addition de MCP-1/CCL2 aux CE pulmonaires induit la différenciation des hfCSN sur les CE pulmonaires.

Ces expériences démontrent que les CE-BHE permettent la diapédèse de hfCSN *in vitro*. Comme pour les leucocytes, le MCP-1/CCL2 a un effet chimiotactique important sur les CSN et potentialise leur transmigration[469]. D'autres études ont aussi démontré que, comme les leucocytes, les cellules souches utilisent les sélectines, les chiomiokines et les molécules d'adhérence pour la diapédèse[470-473]. Par contre, contrairement aux leucocytes, les CSN s'accumulent dans l'espace sous-endothélial *in vitro,* où elles se différencient rapidement. Ces résultats suggèrent que les CSN empruntent un mécanisme de transmigration sensiblement similaire à celui des leucocytes, bien que la caractérisation des molécules et des mécanismes moléculaires impliqués dans les interactions entre les CSN et les CE-BHE soit loin d'être complétée[472]. Nos observations *in vitro* corrèlent avec les publications qui décrivent un rôle local du MCP-1/CCL2 dans le recrutement des CSN à travers la BHE *in vivo*[474, 475].

Ce travail décrit aussi un nouveau rôle pour la chimiokine MCP-1/CCL2 dans la différenciation des CSN. Le MCP-1/CCL2 est la première chimiokine humaine à avoir été caractérisée et a des propriétés chimiotactiques importantes[469, 476]. Les chimiokines sont des

cytokines chimiotactiques de petit poids moléculaire (8-14 kDa), qui forment une famille de plus de 50 molécules impliquées dans de nombreux procédés biologiques[238, 477-479]. Le MCP-1/CCL2 attire principalement les leucocytes de la lignée myéloïde (monocyte, macrophage), mais a aussi un effet chimiotactique sur les lymphocytes T, les cellules NK, les basophiles et les astrocytes *in vitro*[469, 480, 481]. Au niveau du SNC, le MCP-1/CCL2 semble avoir des rôles variés et complexes. Cette chimiokine peut être produite par les astrocytes, les microglies et les CE-BHE[482-484]. Lors de réactions neuroinflammatoires, le MCP-1/CCL2 est sécrété par les cellules du SNC et par les leucocytes infiltrants, ce qui peut potentialiser localement l'infiltration leucocytaire. Le MCP-1/CCL2 active les intégrines des leucocytes et favorise la diapédèse. De plus, le MCP-1/CCL2 peut moduler la perméabilité de la BHE en agissant sur les TJ[480, 485, 486]. Certaines études indiquent que le MCP-1/CCL2 a aussi des propriétés trophiques et peut influencer la réparation tissulaire dans le SNC en agissant sur la prolifération des cellules gliales[487] et sur la migration de CSN au site d'une lésion inflammatoire[475]. En SEP, les rôles du MCP-1/CCL2 dans les plaques de démyélinisation ne sont pas clairs. Cette molécule se retrouve dans les lésions actives de SEP, mais les niveaux de MCP-1/CCL2 dans le LCR de patients de SEP sont significativement diminués[488-490]. Les souris déficientes en MCP-1/CCL2 montrent également une réaction immunitaire altérée, présentant un défaut de recrutement de monocytes/macrophages en réponse à un stimulus inflammatoire[491]. Ces souris présentent une réduction de l'infiltration de macrophages dans les plaques d'EAE, accompagnée de signes cliniques moins sévères[492-494]. Nos données suggèrent un nouveau rôle local du MCP-1/CCL2 sécrété par les CE-BHE dans la différenciation efficace des CSN dans l'espace périvasculaire.

Ces résultats renforcent l'importance de la BHE dans la biologie des CSN, puisque celle-ci n'agit pas comme une simple barrière mais semble affecter la prolifération et la différenciation des CSN. En effet, dans le SNC, les niches de CSN se retrouvent dans l'espace périvasculaire[458]. Les CE cérébrales participent activement dans la régulation de l'activité des CSN dans ces niches par la sécrétion de nombreux facteurs comme le PEDF (*Pigment Epithelium Derived Factor*) et le BDNF (*Brain Derived Neurotrophic Factor*)[458]. Cette étude

identifie donc un nouveau joueur sécrété par les CE-BHE, la chimiokine MCP-1/CCL2, qui a le potentiel d'affecter les CSN endogènes dans les niches périvasculaires. Ce phénomène réitère le rôle proactif de la BHE dans l'homéostasie du SNC.

En conclusion, il a été possible d'identifier une propriété de la BHE qui affecte les CSN, et ces résultats apportent une meilleure compréhension de la complexité des interactions entre les CSN et les CE-BHE. La modulation de la sécrétion de MCP-1/CCL2 par les CE-BHE a le potentiel d'influencer la transmigration et la différenciation de CSN exogènes, mais peut-être aussi celle des CSN endogènes des niches périvasculaires. Dans la SEP, le MCP-1/CCL2 se retrouve dans les lésions actives, alors que dans les lésions chroniques on note plutôt une diminution de son expression[488]. À la lumière de nos résultats, les défauts de remyélinisation dans les plaques de SEP sont potentiellement exacerbés par le déficit en MCP-1/CCL2 qui limite le recrutement et la différenciation des CSN localement. Cette observation est d'importance en SEP, car la capacité régénératrice des CSN cible particulièrement les plaques chroniques où l'on retrouve des dommages axonaux importants. La modulation de la production de MCP-1/CCL2 par les CE-BHE dans les plaques chroniques de SEP pourrait, théoriquement, augmenter la transmigration de CSN localement, une étape souvent limitante dans l'administration *intraveineuse* de CSN[379]. En SEP, le MCP-1/CCL2 localement sécrété par les CE-BHE pourrait contribuer à la transmigration et à la différenciation des CSN dans le SNC. Une meilleure compréhension des différents facteurs qui influencent les CSN localement et qui favorisent leur migration et leur différenciation au sein des plaques de SEP permettra d'optimiser les thérapies neuro-régénératrices basées sur l'injection systémique de CSN.

5.3.2 Perspectives et conclusions

En conclusion, il reste encore beaucoup de travail à faire avant de pouvoir utiliser les CSN comme une thérapie pour la SEP. Actuellement, seules les thérapies expérimentales de

transplantations de cellules souches mésenchymateuses sont à l'étude chez l'humain. L'administration de CSN n'est pour l'instant pas étudiée chez l'humain, mais ces cellules restent une avenue thérapeutique d'intérêt. Il est nécessaire de mieux comprendre la biologie des CSN à l'état physiologique et lors de réactions neuroinflammatoires pour pouvoir ensuite les utiliser de façon thérapeutique. De nombreuses questions comme l'administration, le type cellulaire, la provenance des cellules, la sécurité et les questions éthiques doivent être résolues avant de considérer les CSN comme une thérapie cliniquement envisageable pour la SEP. Une meilleure compréhension de la biologie des CSN permettra d'optimiser les stratégies thérapeutiques.

5.4 Conclusions générales

Ce travail de doctorat décrit trois mécanismes moléculaires de la BHE qui ont le potentiel d'influencer les réactions neuroinflammatoires. Nos résultats confirment l'importance de la BHE dans la biologie du SNC et comme cible pour limiter les réactions neuro-immunes. Cette thèse traite de trois propriétés cruciales de la BHE : la perméabilité paracellulaire, la transmigration leucocytaire et la sécrétion de chimiokines.

La première partie expérimentale cible des interactions entre les astrocytes et les CE-BHE. Cette étude implique l'AngII d'origine astrocytaire dans le contrôle de la perméabilité paracellulaire via l'expression de l'occludine. Bien que l'importance clinique de cette boucle signalétique ait été remise en question dans deux études ultérieures utilisant le modèle murin de l'EAE, ce travail identifie un nouveau médiateur astrocytaire qui potentialise l'imperméabilité de la BHE et dont l'expression est altérée dans les réactions neuroinflammatoires. Des travaux ultérieurs dans le laboratoire ont ensuite confirmé l'importance d'une autre boucle signalétique, la voie hedgehog, dans l'UNV et dans les réactions neuroinflammatoires[307].

Le deuxième article traite des mécanismes moléculaires de la transmigration des leucocytes à travers la BHE et caractérise le rôle d'ALCAM, une molécule d'adhérence

exprimée par l'endothélium cérébral. Ces résultats identifient une nouvelle protéine impliquée dans la diapédèse des leucocytes au sein du SNC, et démontrent que l'inhibition des interactions d'ALCAM limite l'infiltration leucocytaire *in vitro* et *in vivo* dans l'EAE. Cette protéine devient donc une cible thérapeutique potentielle pour limiter les réactions neuroinflammatoires. La modulation d'ALCAM pour influencer les réactions neuro-immunes est actuellement à l'étude dans le laboratoire.

De plus, ces deux premières sections expérimentales ont permis d'identifier l'importance des radeaux lipidiques dans les propriétés de la BHE. Les expériences de protéomique des radeaux lipidiques réalisées sur les CE-BHE ont amené à la caractérisation de deux autres molécules d'adhérence de la BHE impliquées dans les réactions neuro-immunes[425, 426, 495].

Le troisième manuscrit étudie les interactions entre les CSN et les CE-BHE. Cette étude confirme le rôle du MCP-1/CCL2, préférentiellement sécrété par les CE-BHE, dans le recrutement de CSN à travers la BHE. De plus, un nouveau rôle du MCP-1/CCL2 est identifié dans la différenciation des CSN. Ces résultats identifient la sécrétion préférentielle de MCP-1/CCL2 comme un déterminant important des interactions entre les CSN et les CE-BHE.

En conclusion, cette thèse identifie la BHE comme un élément dynamique et proactif du SNC qui influence les réactions neuro-immunes par de nombreux mécanismes moléculaires. Nos études contribuent aux publications récentes qui reconnaissent de plus en plus l'importance de la BHE. Une meilleure compréhension de ces mécanismes est nécessaire pour établir de nouvelles stratégies thérapeutiques qui ciblent spécifiquement la BHE pour moduler les réactions neuroinflammatoires.

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ANNEXE

Annexe I: Isolation of human brain endothelial cells and characterization of lipid raft-associated proteins by mass spectroscopy.

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Isolation of Human Brain Endothelial Cells and Characterization of Lipid Raft Associated Proteins by Mass Spectroscopy

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Summary

The blood brain barrier (BBB) limits the movements of molecules, nutrients and cells from the systemic blood circulation into the central nervous system (CNS), and vice versa, thus allowing an optimal microenvironment for CNS development and function. The brain endothelial cells (BECs) form the primary barrier between the blood and the CNS. In addition, pericytes, neurons, and astrocytes that make up the neurovascular unit support the BEC functions and are essential to maintain this restrictive permeability phenotype. To better understand the molecular mechanisms underlying BBB properties we propose a method to study the proteome of detergent resistant microdomain, namely lipid rafts, from human primary cultures of BECs. This chapter describes a robust human BECs isolation protocol, standard tissue culture protocols, ECs purity assessment protocols, lipid raft microdomain isolation method and a mass spectrometry analysis technique to characterize the protein content of membrane microdomains.

Key Words: Lipid raft; Membrane microdomains; Human brain endothelial cell; Blood-brain barrier; Proteomic; Liquid chromatography; Mass spectrometry; Detergent resistant membrane; Human primary culture.

1. Introduction

The central nervous system (CNS) microenvironment is regulated by the blood-brain barrier (BBB) and the other components of the neurovascular unit (NVU) which include cell types such as, pericytes, microglia, astrocytes and neurons ***(1,2)***. The BBB is a property mainly of non-fenestrated brain endothelial cells that protects the brain and regulates the movement of ions, nutrients, molecules and cells from the blood into the CNS and vice versa. Brain endothelial cells (ECs) form the primary barrier between the CNS and the systemic blood circulation and many other cell types such as, pericytes, microglia, astrocytes and neurons interact with the BECs to create a tightly regulated homeostatic environment for the CNS ***(3)***. Complex networks of soluble factors and physical contacts mediate the interactions between the different cell types to regulate the entry of blood-borne molecules and immune cells into the CNS. BECs express multi-protein tight and adherens intercellular junctions to limit paracellular permeability, exhibit low levels of pinocytosis and express large amounts of metabolic enzymes and transporters to regulate CNS homeostasis ***(4,5)***. The restrictive nature of the CNS vasculature has been known for the past 100 years and significant research is still under way to identify BBB attributes, their origin and regulation. During pathological processes the BBB can respond in a number of ways to limit CNS blood leakage while

allowing an optimal microenvironment for repair and healing ***(6)***. The inherent complexity of the NVU has limited our understanding of this system. In the 1970's, the advent of primary *in vitro* tissue culture techniques for CNS cells allowed researchers to isolate different components of the mammalian NVU and study the individual cell types of the NVU under controlled conditions. Presently many laboratories use *in vitro* models of the BBB ***(7)***.

Due to the restrictive nature of the BBB the movement of nutrients, molecules and cells between the blood and the CNS requires interactions with plasma membrane proteins to signal and initiate transport across the BBB ***(1,8)***. Detergent resistant microdomains (DRMs) enriched in cholesterol, namely lipid rafts, are small (10-100 nm) dynamic membrane microdomains that are thought to be crucial for numerous plasma membrane functions. Because of their specific lipid composition these microdomains form micro-platforms, or rafts, of "ordered" plasma membrane in the loosely packed fluidic phase of the plasma membranes. Rafts float in the plasma membrane, act as important functional components of the BBB and can participate in many biological processes such as: plasma membrane signal transduction, membrane protein compartmentalization, cell polarization, cell adhesion and migration and cytoskeleton attachment ***(9-11)***. Even though the relevance of detergent resistant membranes in biological systems has been questioned, the isolation and the study of membrane microdomains have greatly increased our understanding of membrane biology and remain an important tool to study the plasma membrane ***(12)***. Current data generated on lipid rafts strongly suggest that these microdomains are biologically important and form a functional unit in the plasma membranes of cells.

Historically lipid rafts and DRM have been defined as membrane fractions enriched in cholesterol and phospholipids isolated from detergent treated cells after sucrose gradient ultracentrifugation ***(10,13)***. Differential solubility of the microdomains in detergent is a function of the specific biophysical properties of DRM compared to the fluidic membrane. DRM isolation has been done in many cell types and using different detergents and DRM isolation protocols have even been refined to isolate lipid rafts without the use of detergents ***(14,15)***.

Global profiling techniques such as genomics and proteomics are powerful tools to study comprehensive gene and protein expression analysis in defined samples ***(16-18)***. Instead of looking at one, or a few genes or proteins these techniques allow the study of hundreds to thousands of molecules simultaneously. Global profiling protein expression techniques using mass spectrometry allow for the analytical identification of the chemical composition of a sample based on mass to charge ratios of charged particles. Mass spectrometry is a highly sensitive method and can be used for qualitative and quantitative purposes (protein identification, isotopic composition identification, amount quantification, etc.). The association of high sensitivity large-scale proteomic techniques (mass spectrometry) with BEC cultured *in vitro* allows for the global profiling of BBB protein expression (proteome) under various controlled conditions ***(10)***. An important improvement of this proteomic technique is the use of chromatography to separate the sample peptides before they are introduced in the mass spectrometer allowing for a better mass-charge resolution. The identification and the characterization of BBB proteins previously undefined will enhance the understanding of the molecular mechanism underlying BBB functions but might also identify potential therapeutic

targets to modulate BBB phenotype. In recent years quantitative global profiling techniques have been optimised and used to quantify protein expression differences between samples (quantitative genomics and proteomic techniques, isotope-coated affinity tag spectrometry-ICAT) ***(17-19)***. In order to identify "novel" protein effectors of the BBB our laboratory has used tissue culture, DRM isolation and tandem liquid chromatography and mass spectrometry proteomic techniques to probe the proteome of membrane microdomain functional units and identify novel effectors of the BBB (**Fig. 1**). Using this method we have been able to identify lipid raft associated proteins previously undefined at the BBB (*see* **Table I**) and we have described novel molecular mechanisms underlying BBB functions ***(16)***.

This chapter describes the techniques required to isolate and propagate human brain ECs. We propose robust protocols to study BECs properties and functions (immunofluorescence and permeability to small and large tracers). Furthermore this chapter contains a protocol for DRM isolation from human BECs using a discontinuous sucrose gradient and protocols to prepare and analyse DRM samples by mass spectrometry.

2. Materials

2.1. Isolation of Primary Human BECs

1. Sterile phosphate-buffered saline (PBS): 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4 in Milli-Q® water (*see* **Note** 1).
2. Sterilized Nalgene Filtration Apparatus (VWR, Mississauga, Ontario, cat # 28199-440).
3. Sterile 350 and 112 μm nylon or polypropene Nitex filters (BSH Thompson, Montreal, Quebec).

4. Sterile forceps.
5. Sterile scalpel (Paragon medical, Coral Springs, Florida).
6. Six well tissue culture plate, T25, T75 and T150 tissue culture flasks (BD Falcon, San Jose, California).
7. Dounce tissue homogenizer, 30 mL with a tight fitting piston (Wheaton Science Products, Millville, New Jersey).
8. Medium 199 medium (Invitrogen Corp., Burlington, Ontario) filter sterilized with 0.22 µm filters.
9. 0.5% gelatin (BD Difco, San Jose, California) diluted in water, and sterilized with 0.22 µm filters.
10. Sterile heat inactivated human serum (Sigma-Aldrich, Oakville, Ontario).
11. Sterile heat inactivated fetal calf serum (BD Gibco).
12. Tissue culture grade Collagenase type IV (Sigma-Aldrich, 1mg/mL in sterile M199).
13. Endothelial cell growth supplements (ECGS, 1.5 mg/mL in water, 0.22 µm filter sterilized, BD Bioscience).
14. Insulin transferring Selenium (ITS, 1000X concentrate, Sigma-Aldrich, 0.22 µm filter sterilized, Collaborative Medical Products, Bedford, Massachusetts).
15. Mouse melanoma conditioned medium (medium collected at day 8 from a confluent monolayer of clone M3 cells grown in DMEM supplemented with 10% fetal calf serum. The medium is 0.22 µm filter sterilized and kept at -20 ºC.
16. Endothelial cell medium (ECM) #1: 60% M199, 10% human serum, 10% fetal calf serum, 20% M3 conditioned medium, ECGS (6 µg/mL), ITS (1X concentrate).

17. ECM # 2: 65% M199, 5% human serum, 10% fetal calf serum, 20% M3 conditioned medium, ECGS (3 μg/mL), ITS (1X concentrate) (*see* **Note** 1).
18. ECM # 3: 65% M199, 5% human serum, 10% fetal calf serum, 20% M3 conditioned medium, ITS (1X concentrate).

2.2. Maintenance of Human BECs

1. Tissue culture grade trypsin (BD Gibco, 0.5% diluted in PBS).
2. Gelatin 0.5%.
3. Astrocyte conditioned medium (ACM). Human fetal astrocytes are isolated and grown in culture as previously described with 90% DMEM, 10% fetal calf serum ***(13;20)***. The cultures are derived from human embryos obtained from pregnancy terminations done at 17-23 weeks for medical reasons. The latter is in accordance with ethical guidelines of the Canadian Institute of Health Research and the Royal College of Physicians and Surgeons of Canada. After 7 days in culture the supernatant from the fetal astrocyte monolayer is collected, filtered through a 0.22 μm filter and kept frozen at -20 ºC, until use.

2.3. Immunofluorescence Analysis of purity of BEC cultures

1. 4% paraformaldehyde (Sigma-Aldrich) diluted in PBS, pH7.2.
2. Tissue culture plastic coverslips or 8-well chamber slides (Nalgene, Rochester, New York).
3. Anti-Von Willebrand Factor antibody (1/100 dilution, Dako Cytomation, Glostrup, Denmark).

4. Anti-Glucose transporter-1 (Glut-1) antibody (1/200 dilution, Sigma-Aldrich).
5. Ulex europaeus-1 lectin conjugated to fluorescein (1/200, Sigma-Aldrich).
6. Anti-endothelial HT-7 antibody (1/400 dilution, Sigma-Aldrich).
7. Tumor necrosis factor (Biosource-Invitrogen, 100 U/mL, 50 ng/mL).
8. Anti-intercellular adhesion molecule (ICAM)-1 antibody (1/100 dilution, R&D Systems, Minneapolis, Minnesota).
9. Anti-vascular cell adhesion molecule (VCAM)-1 antibody (1/200 dilution, R&D Systems).
10. Anti-zonula occludens (ZO-1) antibody (1/150 dilution, Zymed, San Francisco, California).
11. Anti-Occludin antibody (1/75 dilution, Zymed).
12. Anti-Beta-tubulin antibody (1/400 dilution, Sigma-Aldrich).
13. Anti-Glial fibrillary acidic protein (GFAP) antibody (1/500 dilution, Dako).
14. Anti-Alpha myosin antibody (1/500 dilution, Sigma-Aldrich).
15. Anti-CD68 antibody (1/100 dilution, Dako).
16. Nuclear stain Hoescht or Topro-3 (1/350 dilution, Invitrogen).
17. HHG blocking solution: 1 mM HEPES, 88% Hanks buffered saline solution (Sigma-Aldrich), 2% horse serum (Sigma-Aldrich), 10% goat serum (Sigma-Aldrich) and 0.01% sodium azide. PBS supplemented with 10% of the serum from the same species as the secondary antibody can also used.
18. Triton X-100.

19. Mounting medium, gelvatol: 20% glycerol, 10% poly-vinyl alcohol, 0.1M Tris HCl pH 8.0, incubate while stirring overnight, centrifuge 2000×g for 20 min (some precipitate may appear, use the supernatant of the gelvatol solution).

2.4. Permeability of Human BECs to Small and Large tracers.

1. Boyden chambers and 24 well tissue culture plates (BD Falcon).
2. Bovine Serum Albumin coupled to fluorescein (Molecular probes, 10 µg/mL diluted following the manufacturer's instructions).
3. ^{14}C labelled sucrose (MP Biomedicals, Aurora, Ohio).

2.5. Lipid Raft Isolation and Purification

1. Brij 58 detergent (1% in separating buffer, Sigma-Aldrich).
2. Cell scrapers (Sarstedt, Saint-Leonard, Quebec).
3. Protease inhibitors cocktail (BD Bioscience).
4. Separation buffer: 150 mM NaCl, 25 mM Tris HCl, pH 7.4.
5. Sucrose (Sigma-Aldrich) solutions: 85%, 35%, and 5% diluted in separation buffer.
6. Dounce homogenizer (7 mL with a loose fitting piston, Kontes Glass company, Vineland, New Jersey)
7. 12 mL ultracentrifuge tubes (Beckman, Mississauga, Ontario).
8. Swinging bucket rotor SW41 (Beckman).
9. Ultracentrifuge (Beckman optima ultracentrifuge).
10. Cholesterol assay kit, Amplex red cholesterol assay kit, Molecular Probes.

11. Phospholipid colorimetric method kit (Wako, Richmond, Virginia).

12. BCA protein assay kit, Pierce.

13. Anti-CD59 antibody (R&D Systems).

14. Anti-GM1 Cholera Toxin B (Molecular Probes)

2.6. Liquid Chromatography and Mass Spectrometry

1. Denaturing SDS buffer: 50 mM Tris-HCl, pH 8.5, 0.1% SDS.
2. Dithiothrietol (DTT). Freshly prepare ~250 mM in Milli-Q® water.
3. Iodoacetamide (IAA). Freshly prepare ~500 mM in Milli-Q® water and protect from light.
4. Trypsin Gold, Mass Spectrometry Grade (Promega, Nepean, Ontario, cat. # V5280).
5. Cartridge holder (Applied Biosystems, Foster City, California, cat. # 4326688).
6. Cation Exchange (CE) Cartridge: POROS® 50 HS, 50-μm particle size (4.0 mm × 15 mm) (Applied Biosystems, cat. # 4326695).
7. CE Load buffer: 10 mM KH2PO4, pH 3.0, 25% acetonitrile.
8. CE Elute buffer: CE Load buffer + 350 mM KCl.
9. CE Clean buffer: CE Load buffer + 1 M KCl.
10. CE Storage buffer: CE Load buffer + 0.1% NaN3.
11. MS buffer: 5% acetronitrile, 1% acetic acid.
12. SpeedVac® Concentrator SPD111V (Thermo Scientific, Waltham, Massachusetts).
13. A mass spectrometer (MS) equipped with an electrospray ionization source (ESI) and capable of tandem MS (MS/MS) analysis. For example, a hybrid quadrupole time-of-flight Q-TOF™ Ultima (Waters, Millford, Massachusetts).

14. An online nanoflow liquid chromatography (nanoLC) system. For example, CapLC HPLC pump (Waters).
15. A reverse phase nanoLC column. For example, 75 μm × 150 mm PepMap C18 nanocolumn column (Dionex/LC-Packings, San Francisco, California).
16. Software for identifying peptide sequences from the nanoLC-MS/MS data using database searching. For example, Mascot® version 2.1.0 (Matrix Science Ltd., London), PEAKS (Bioinformatics Solutions Inc., Waterloo, Ontario), or PeptideProphet™ (part of the Trans-proteomic pipeline http://en.wikipedia.org/wiki/Trans-Proteomic_Pipeline).
17. Acetonitrile and formic acid (Sigma-Aldrich).

3. Methods

3.1. Isolation of Primary Human BECs (*See* **Note** 1)

1. Coat 6-well tissue culture plates with 0.5 % gelatin, 2 mL per well and let the gelatin harden on plates for a minimum of 1 h.
2. Wash the human CNS specimens thoroughly using 1-2 L of PBS to remove blood clots. Human temporal lobe resections are obtained from patients undergoing surgery for the treatment of intractable temporal lobe epilepsy at CHUM-Notre-Dame Hospital. Informed consent and ethical approval was given before surgery (ethics protocol HD04.046). BECs were isolated from the non-epileptogenic resection material.
3. Remove meninges and the large pial blood vessels with sterile forceps.
4. Cut tissue into small cubes of 0.1 to 0.5 mm^3 with sterile scalpels.
5. Aspirate pieces with a sterile pipette and transfer pieces to a 50 mL sterile conical tube.

6. Fill tube with PBS, shake, and allow pieces to sediment. Wait 30 min for the pieces to settle and discard the supernatant. At least 45 mL of PBS should be discarded after each wash.
7. Repeat washes until the specimen is free of contaminating blood (3 to 5 washes are necessary, *see* **Note** 2).
8. Put tissue in a dounce tissue homogeniser and homogenise using only 4 to 5 strokes (*see* **Note** 3).
9. Filter homogenate in Nalgene filtration apparatus previously equipped with the 350 µm filter.
10. Remove filter and place it in a 50 mL sterile tube, with the cells and debris facing towards the exterior. This will be the 350 µm fraction.
11. Pass the filtrate through a filtration unit with a 112 µm filter.
12. Remove the filter and place it a 50 mL sterile tube, with the cells and debris facing outwards.
13. Repeat **steps 12** and **13**, with a new 112 µm filter. The two 112 µm filters will be the 112 µm fraction.
14. Recover the filtrate in a 50 mL tube. This will be the < 112 µm fraction.
15. Fill tubes with the filters with PBS and shake tubes vigorously until all visible cells and debris are removed from the filter membrane. Remove filters from the tubes with sterile forceps.
16. Centrifuge all tubes (< 112 µm, the two 112 µm and the 350 µm fractions) at 1000×g for 15 min at room temperature (RT).

17. Remove supernatant and dissociate pellets vigorously. Add 5 mL of collagenase IV (1 mg/mL), use 2.5 mL for both 112 μm fractions and combine them in one tube. A total of three fractions should remain: 350, 112 and < 112 μm fractions.
18. Incubate for 15 min at 37 ºC.
19. Inactivate collagenase with ECM # 1, and fill tubes up to 50 mL.
20. Centrifuge 10 min at 500×g.
21. Remove supernatant and resuspend pellets in 12 mL of ECM # 1 each for 350 and 112 μm fractions. Resuspend < 112 μm pellet in 24 mL of ECM # 1.
22. Remove gelatin from the 6-well plates, by aspiration. Do not rinse the plates with PBS.
23. Plate 350 and 112 fractions in one 6-well plate each, 2 mL per well. Plate < 112 μm fraction in two 6-well plates, 2 mL per well.
24. Incubate for 48 hrs at 37 ºC, with 0.5% CO_2.
25. Remove supernatant cell culture medium from each well.
26. Wash debris off by gently adding 2-3 mL of PBS per well.
27. Remove PBS and add 2 mL of fresh ECM # 1 medium.
28. Incubate cells at 37 ºC, with 0.5% CO_2.
29. Every 7 days, remove 1 mL of medium from each well and add 1 mL of fresh medium. Human brain derived ECs produce autocrine growth factors important for their growth and survival.
30. Every 4-5 days endothelial cell colonies become visible. BECs are adherent and form a monolayer in culture, **Fig. 2.**

31. As the colonies of BECs expand, passage clones that cover over 60% of the surface of the well as a homogenous monolayer. ECM # 1 is used to grow the clones in the 6-well plate. For all other BECs expansion in tissue culture we use ECM #2 and for all experimental protocols we grow the cells in ECM # 3 (*see* **Note** 4).
32. Passage BECs in a T25 sterile flask and grow to confluence with ECM # 2 (passage 2).
33. Passage BECs in a T75 sterile flask and grow to confluence with ECM # 2 (passage 3).

3.2. Maintenance of Human BECs

1. Cells are passaged every 7 days or until the BECs form a confluent monolayer (**Fig. 2**).
2. Coat tissue culture flask with 0.5% gelatin for at least 1 h. Heat gelatin to 37 ºC for 15 min and use 5 mL for a T25 flask and 10 mL for a T75 flask.
3. Remove culture medium from the BECs and gently add PBS to the cells.
4. Remove PBS from the cells and add 0.5% trypsin previously warmed to 37 ºC. Add 5 mL for T25 flasks and 10 mL for T75 flasks.
5. Incubate cells at 37º C for 5 min or until cells detach. Gently tap the flask to allow cells to detach.
6. Add an equivalent volume of ECM #2 to inactivate the trypsin.
7. Collect cells and centrifuge for 10 min at 300×g.
8. Discard supernatant and resuspend pellet in warmed ECM # 2.
9. Remove gelatin from the flask.
10. Plate cells in a flask, grow in ECM # 2 and incubate at 37 ºC with 0.5% CO_2. Cells are usually diluted 1:3 during passage but they can be diluted up to 1:5-dilution (*see* **Note** 5).

3.3. Immunofluorescence Analysis of purity of BEC cultures

1. Grow cells on plastic coverslips (Nunc) or on plastic chamber slides (Nalgene), *see* **Note** 6.
2. Gently remove medium and add PBS.
3. Gently repeat PBS wash.
4. Remove PBS and fix cells with 4% paraformaldehyde, cold methanol or cold ethanol for 10-20 min at RT.
5. Rinse cells with PBS three times.
6. Incubate cells with HHG or another suitable blocking solution supplemented with 10% serum of the same species used for preparation of the secondary antibody. For intracellular antigens, permeabilize cells with 0.3% Triton X-100 in HHG, incubate 30 min at RT.
7. Remove medium, add primary antibodies at appropriate dilutions (diluted in HHG or other appropriate solution with 3% serum of the same species as the secondary used plus 0.1% Triton X-100) and incubate for 1 hr at RT.
8. Remove antibody and wash three times with PBS.
9. Add secondary antibodies at appropriate dilutions (diluted in HHG) and incubate for 1 h at RT.
10. Rinse 3 times with PBS.
11. Add nuclear stain if needed.
12. Rinse with PBS.

13. Mount slide with coverslip using gelvatol mounting medium or commercial aqueous mounting medium.
14. Analyse using a fluorescence microscope (see **Fig. 3**) (*see* **Note** 7).

3.4. Permeability of Human BECs to Small and Large tracers

1. Coat the bottom of the Boyden chamber transwell with 0.5% gelatin and allow it to sit for at least 1 h.
2. Gently rinse BECs monolayer with 37 ^{o}C warmed PBS.
3. Remove PBS and add warmed trypsin for 5 min to detach BECs.
4. Inactivate trypsin with ECM # 2 and collect cells.
5. Centrifuge at 300×g for 10 min.
6. Resuspend pellet in ECM # 2.
7. Count cells with a hemacytometer.
8. Remove gelatin from Boyden chambers.
9. Seed a defined number of BECs in each Boyden chamber and grow in ECM # 3 with or without 40% ACM. We plate 25,000 cells per Boyden chamber and allow them to grow for at least three days until the monolayer is confluent.
10. After 3-5 days in culture the Boyden chambers can be used.
11. Remove treatment medium and replace with new ECM # 3. Add fluorescein conjugated BSA (50 µg/mL) or ^{14}C-sucrose (250 nCi/mL) to the upper chamber.

12. Sample the upper and lower chamber at regular time intervals and place the medium collected in a 96-well plate (1, 3, 6, 12, 24, 48, 72, 96 hrs for BSA-FITC and every 30 min for 6 hrs and then every h for 24 h for sucrose). Collect 10-25 μL per time point.
13. Read fluorescence in a fluorescent plate reader and radioactivity in a gamma counter.
14. Calculate permeability using the following formula: (lower chamber) * 100/ (upper chamber). The diffusion rate is expressed as the diffusion (%) X tracer/time (*See* **Note** 8).

3.5. Lipd Raft Isolation and Purification

1. Let BECs grow as described in **Subheading 3.2**, until they form a confluent monolayer.
2. Treat cells as required by removing the medium and adding fresh ECM # 3 with the experimental treatment of interest (in the laboratory we routinely compare BECs grown under normal culture conditions and BECs treated with the inflammatory cytokines Tumor Necrosis Factor-alpha and Interferon-gamma at 100 units/mL).
3. Incubate for 16 hrs overnight at 37 ºC with 0.5% CO_2. Use a minimum of 4, T75 flasks per conditions and up to 4, T150 flasks per condition.
4. All solutions and materials must be ice-cold and/or stored at 4 ºC before use.
5. Cool flasks on ice for 5 min.
6. Remove medium and wash monolayer by gently adding ice cold PBS.
7. Using a cell scraper to remove adherent cells from the flask bottom and collect cells with ice cold PBS (rinse the flasks several times and make sure that the detached cells are not

forming clumps. Resuspend by moving the pipette up and down a few times to remove aggregates.

8. Centrifuge at 1200×g for 15 min.
9. Combine pellets of the same treatment, wash with ice-cold PBS and centrifuge 15 min at 1200×g.
10. Remove supernatant and carefully resuspend pellet in 1 mL of 1% Brij58 solution supplemented with protease inhibitors. Avoid forming air bubbles. Other detergents can be used as well. In our laboratory, Brij58 was found to be the optimal detergent to fractionate BECs membrane microdomains ***(14)***.
11. Gently rotate at 4 ºC for 30 min.
12. Transfer to a dounce homogenizer and homogenize sample with 10 strokes. Total volume is 1 mL.
13. Mix homogenized sample with 1 mL of 85% sucrose solution. Mix well but avoid bubble formation. Total volume is now 2 mL in a 42.5% sucrose solution.
14. Place the 2 mL of 42.5% sucrose cell membrane mix at the bottom of a 12 mL ultracentrifuge tube. This is the 42.5% sucrose fraction.
15. Carefully overlay 42.5% sucrose fraction with 5 mL of 35% sucrose.
16. Carefully overlay 35% sucrose fraction with 5 mL of 5% sucrose solution (the discontinuous sucrose gradient has a total volume of 12 mL).
17. Centrifuge in an ultracentrifuge having a Beckman SW41 rotor at 250,000×g for at least 16-24 hrs with no rotor deceleration. Avoid using the brakes to stop the rotor after the spin since it will destabilise the sucrose gradient.

18. Carefully remove tubes from rotor being careful to avoid any abrupt movements that can de-stabilize the sucrose gradient.
19. Harvest twelve 1.0 mL fractions from the meniscus of the liquid at the top of the tube until the bottom is reached making sure to keep the pipette tip or needle tip at the meniscus. Cholesterol enriched fractions are at the interface of the 5% and 35% sucrose solutions, usually fractions 4 to 6.
20. Fractions can be stored frozen at -20 ºC.
21. We routinely assess cholesterol, phospholipids and protein content of each fraction using commercially available kits such as the Cholesterol assay kit, Molecular Probes (*see* **Note** 9).

3.6. Liquid Chromatography and Mass Spectrometry

3.6.1. Preparation of Samples for Liquid Chromatography

1. Dialyse frozen detergent resistant microdomain fractions (usually fractions 4 to 6) for 16 hrs against denaturing SDS buffer (*see* **Note** 10).
2. Precipitate proteins by adding 10-volumes of cold acetone and precipitating at -20 °C for 1 h. Precipitation can be continued overnight if the amount of protein is expected to be low. Pellet the protein by centrifugation at 5000×g for 5 min and re-dissolve the pellet in the denaturing SDS buffer.
3. Add freshly prepared DTT to a final concentration of 4 mM to each sample.
4. Vortex to mix, and spin at 10, 000×g for 10 s to bring solution down.
5. Incubate samples at 95 °C for 10 min to reduce the disulfide bonds of cysteine residues in proteins.

6. Vortex to mix, and spin at 10,000×g for 10 s to bring solution down.
7. Cool tubes at RT for 2 min.
8. Add freshly prepared IAA to a final concentration of 10 mM to each sample.
9. Incubate sample at RT for 20 min in the dark to alkylate cysteine residues in proteins. IAA is light sensitive.
10. Vortex to mix, and spin at 10,000×g for 10 s to bring solution down.
11. Add 50 µL of 100 ng/µL trypsin (in Milli-Q® water) to each sample.
12. Tap each sample tube to mix, and spin at 10,000×g for 10 s to bring solution down.
13. Incubate at 37 °C for 12 to 16 h.
14. Vortex to mix, and spin at 10,000×g for 10 s to bring solution down.
15. Samples may be stored at -20 °C for up to 4 weeks.

3.6.2. Cleaning Up Digested Samples Using a CE Cartridge

1. Transfer the trypsin-digested sample to a new tube labelled "PreCE" and add 2 mL CE load buffer.
2. Check the pH. If it is not < 3.3, adjust by adding more CE load buffer.
3. Clean the CE cartridge by injecting 1 mL of CE clean buffer. Divert to waste.
4. Condition the CE cartridge by injecting 2 mL of CE load buffer. Divert to waste.
5. Slowly inject (~1 drop/sec) all the contents of the "PreCE" tube. The flow through may be collected although not required in the subsequent steps.
6. Wash by injecting 1 mL of CE load buffer. Divert to waste.

7. Elute peptides by slowly injecting (~0.5 drop/sec) 0.5 mL of CE elute buffer and collecting the eluted sample in a new tube.
8. If there are additional trypsin-digested samples, repeat **steps 1** to**7**.
9. Wash the cartridge by injecting 1 mL of CE clean buffer and 2 mL of CE storage buffer. Store the cartridge at 2-8 °C.
10. Evaporate each sample to dryness in a SpeedVac®.
11. Dissolve each sample in 300 µL of MS buffer.
12. Samples may be stored at -20 °C for up to 4 weeks.

3.6.3. Liquid Chromatography and Mass Spectrometry

1. Inject 5-10 µL of each sample in MS buffer onto the nanoLC online to the Q-TOF™ Ultima MS.
2. Separate peptides by gradient elution (5–75% acetonitrile, 0.2% formic acid for 90 min, 350 nL/min) and analyze in the automated MS/MS mode.
3. Acquire MS survey on ions between mass/charge (m/z) 400 and 1600 using 30 counts/sec threshold requirements to switch from MS survey to MS/MS acquisition (see **Note** 11).
4. Acquire MS/MS on 2+, 3+ and 4+ ions between m/z 50 and 2000 and return back to MS survey once the total ion current reaches 2500 counts/sec or after 6.6 sec regardless.
5. In-between samples, inject at least one 40-min blank run (MS buffer only) to avoid cross-contamination between samples.
6. Generate peaklist as a single text file (usually a .pkl file from Waters instruments) for each nanoLC-MS/MS run using ProteinLynx™. Each file contains m/z and charge values

of all the precursor ions and their corresponding m/z and intensity values of fragment ions.

7. Submit each file to Mascot® search engine or any other probability-based engine using the following parameters: (i) specify trypsin enzymatic cleavage with one-to-two possible missed cleavages; (ii) allow variable modification for oxidation (+15.99 Da) at the methionine residues; (iii) allow variable modification for carbamidomethyl (iodoacetamide derivative; +57.02 Da) at the cysteine residues if proteins were alkylated; (iv) set parent ion tolerance ≤ 0.5 Da; (v) set fragment ion tolerance ≤ 0.2 Da.
8. The mass spectrometer spectrum of each identified peptide is then manually examined and confirmed.
9. Protein identification is considered specific if a set of peptides can only be assigned to a single protein. Protein identification is considered non-specific if the peptides cannot be assigned to more than one possible protein.
10. Proteins are then be classified into molecular functions and biological processes using the Panther Classification System and online databases (www.pantherdb.org). Proteins can also be classified by sub-cellular localization (Swiss-Prot and GO databases).
11. Several validation approaches should be used to confirm the validity of the MS data. We validate candidate proteins of interest by literature mining, by using alternative detection methods in the same *in vitro* system (RT-PCR, Western blot or immunofluorescence) and by direct *in vivo* validation.

4. Notes

1. All components used for BECs isolation and tissue culture should be sterile before use. All procedures should be done aseptically in a sterile tissue culture hood. This protocol is not optimised for mouse CNS specimens.
2. The volume of tissue can be grossly estimated by looking at the volume of the CNS pieces that settle at the bottom of the tube. At least 3 mL of CNS fragments is required to obtain endothelial cells. A maximum of up to 15 mL of minced CNS tissue (18-20 gms) has been used in our laboratory.
3. A mechanical tissue grinder Dyna-mix set at medium speed, (Fisher Scientific, Ottawa, Ontario) is used in our laboratory.
4. Other techniques have been used to isolate and enrich primary cultures of BECs. These include the use of puromycin as a selection agent for P-glycoprotein expressing BECs ***(21)*** and the use of dextran to isolate brain microvessels from the CNS material ***(22)***. These methods have been used on murine BECs and in our hands these additional steps are not required for primary cultures of adult human BECs.
5. Occasional CD68 positive cells (microglia) are seen (less than 5% at passage 2) and their numbers decreased with passage, due to their strong adherence to plastic and their non-proliferative nature.
6. In our experience BECs do not grow well on glass. They can grow on poly-lysine coated glass surfaces but their morphology is abnormal.
7. In the laboratory BECs are routinely analyzed by flow cytometry to confirm ICAM-1 and VCAM-1 up regulation with inflammatory cytokine treatment. Due to space constraints the detailed cytometry protocol is not described here ***(23)***.

8. Human immune cells are routinely isolated from the peripheral blood of healthy donors. The immune cells are isolated using antibody coated magnetic beads (Miltenyi Biotech, Toronto, Ontario). A known amount of purified immune cells are added to the upper chamber of the Boyden transwell and allowed to migrate for 16 hrs. The immune cells of the bottom chamber are then sampled and counted to assess BECs permeability to immune cells ***(13;16;23)***.

9. Kits that are currently used in the laboratory are the Phospholipid colorimetric method kit (Wako), the Cholesterol assay kit (Molecular Probes) and the BCA protein assay kit (Pierce). We also assess the presence of membrane microdomain markers such as GM-1 and CD59 in each fraction by SDS-PAGE.

10. It is essential that the protein sample be free of potential contaminants for LC-MS such as high levels of sucrose, salt, acid, detergents and/or denaturants since these may interfere with the subsequent steps. High amounts of detergents or denaturants such as SDS can deactivate trypsin activity. High salt, acid or detergents can prevent peptides binding to CE cartridge. These contaminants may be removed by dialysis against the DS buffer and precipitation with acetone.

11. For both the MS survey and MS/MS, we use 1.0 sec for scan duration and 0.1 sec for inter-scan delay. In addition, we usually do not discard MS survey scans that do not contain ions selected for MS/MS analysis.

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Figures

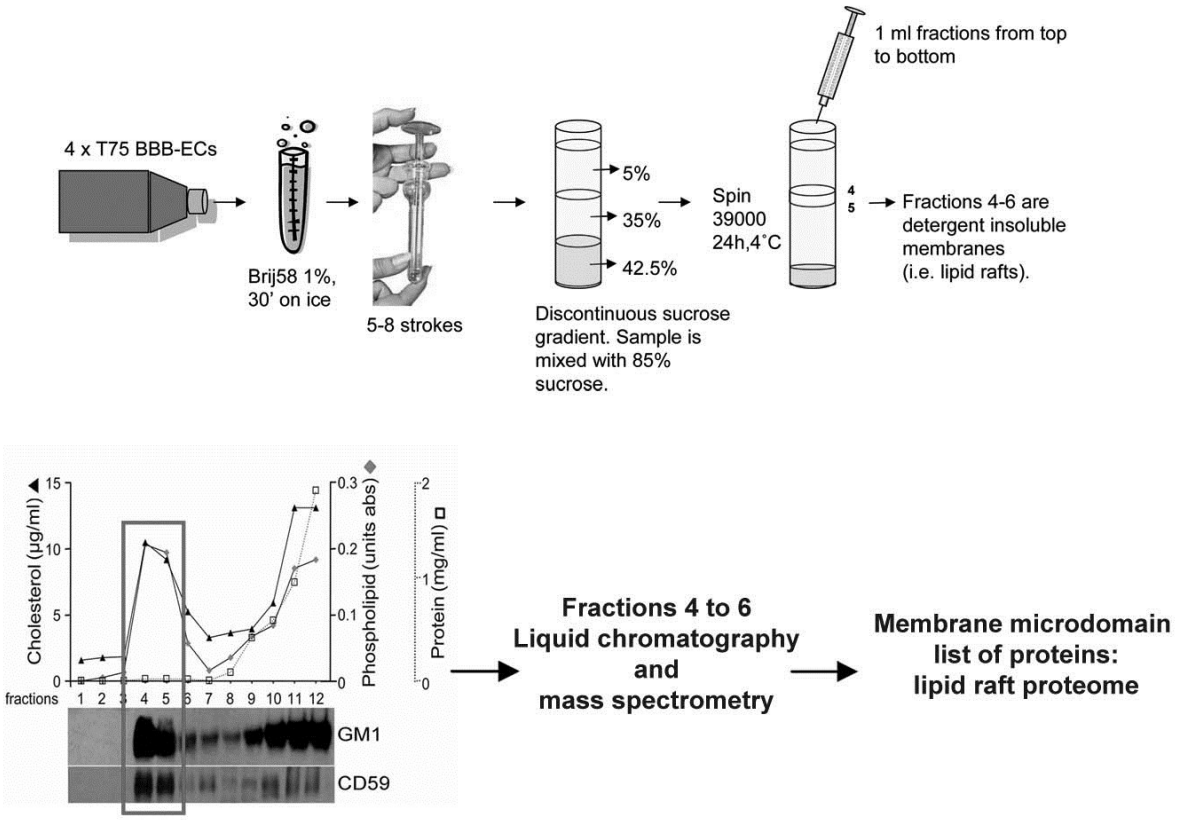

Fig. 1. Detergent resistant membrane isolation and mass spectrometry flow chart.

Lipid raft, or detergent resistant microdomain isolation steps are illustrated in the upper panel. The lower panel describes the major steps that allow for proteome analysis of the lipid rafts.

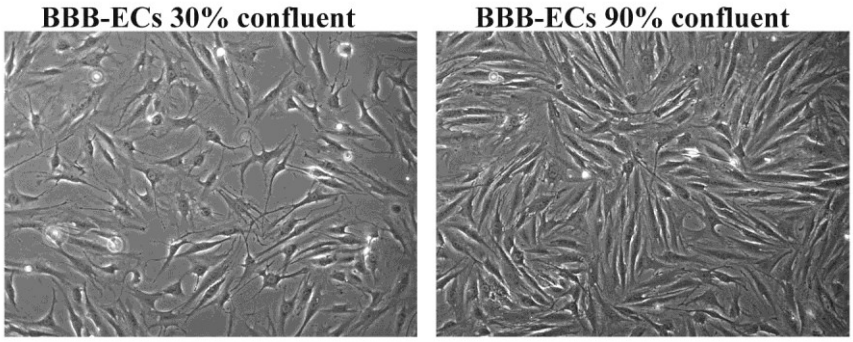

Fig. 2. Phase contrast micrographs of BEC monolayers. The left panel shows a monolayer after one day in culture and the right panel shows the same monolayer after 48 hrs in culture at around 90% confluency.

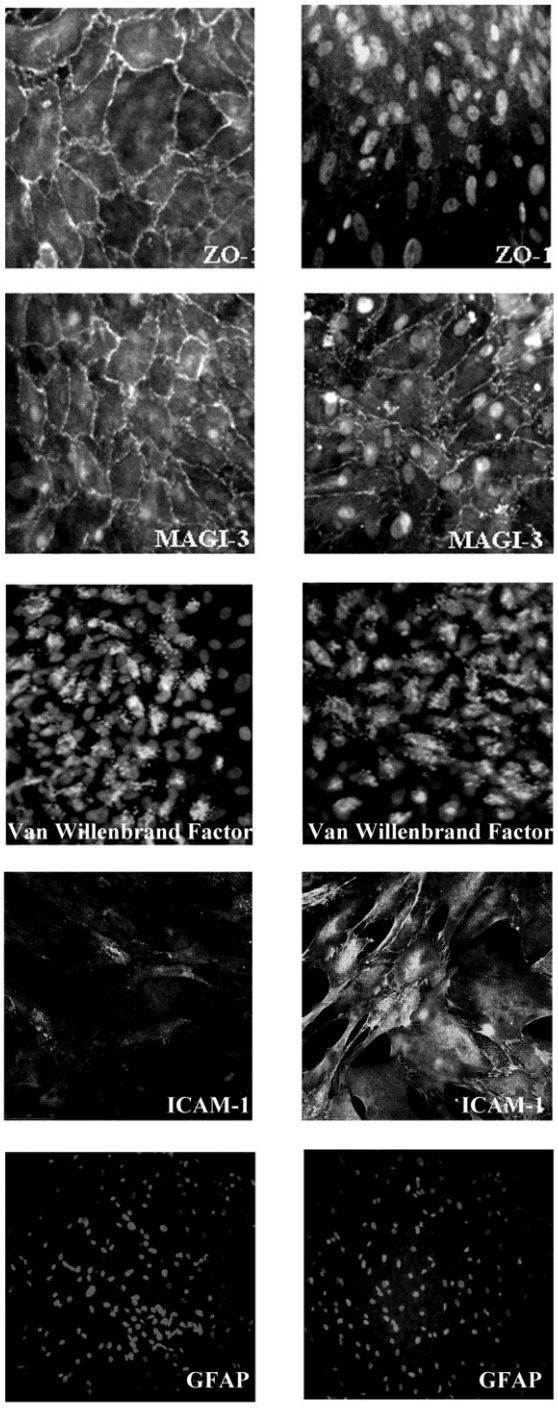
ZO-1
ZO-1
MAGI-3
MAGI-3
Van Willenbrand Factor
Van Willenbrand Factor
ICAM-1
ICAM-1
GFAP
GFAP

Fig. 3. Primary cultures of human BECs showing ZO-1, MAGI-3, Van Willebrand factor, ICAM-1 and GFAP by immunofluorescence. The BECs have been stained with a nuclear stain.

The micrographs on the left show BECs under normal culture conditions and the right panels show BECs treated with the inflammatory cytokines TNF and IFN-gamma for 16 hrs at 100 U/mL. ZO-1 (a) and MAGI-3 (b) staining patterns show an intercellular staining under normal condition and this intercellular staining is decreased with inflammatory cytokine treatment. Panel (c) shows that Van Willebrand factor expression in BECs is not influenced by inflammatory cytokine treatment, while the panel (d) shows that ICAM-1 expression is increased with cytokine treatment. In panel (e) GFAP positive cells are present in the human BECs cultures which show only nuclear staining.

Table I Representative groups of lipid raft-associated proteins derived from BECs are shown.

Category	**Name**
Signal transduction and cytoskeleton (42%)	
	Kv channel interacting protein 4
	calcium channel, voltage-dependent, alpha 2/delta subunit 1
	calcium/calmodulin-dependent serine protein kinase (MAGUK family)
	guanine nucleotide binding protein (G protein), beta polypeptide 4
	guanine nucleotide binding protein (G protein), alpha 13
	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2
	bradykinin receptor B2
	RAS-like, family 11, member A
	guanine nucleotide binding protein (G protein), gamma 12
	RAS p21 protein activator 4
	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1
	membrane-spanning 4-domains, subfamily A, member 8B
	ectonucleotide pyrophosphatase/phosphodiesterase 5
	AHNAK nucleoprotein (desmoyokin)
	mitogen-activated protein kinase kinase 1 interacting protein 1
	related RAS viral (r-ras)
	protein kinase, DNA-activated
	alpha 1 actin precursor
	vimentin
Cell-cell adhesion (22%)	
	selectin E
	vascular cell adhesion molecule-1
	melanoma cell adhesion molecule
	activated leukocyte cell adhesion molecule
	contactin 4
	integrin, beta 1
	integrin, beta 4
	integrin, beta 1

	integrin, alpha 1
	CD44
	Gap junction protein beta 6, connexin 30
	cadherin 1, type 1and type 2
	catenin, beta 1
Protein, lipid and carbohydrate metabolism (18%)	
	gamma-glutamyltransferase-like activity 1
	UDP glucuronosyltransferase 2 family, polypeptide B11
	CD59 antigen p18-20
	solute carrier family 3
	mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase
	amyloid beta precursor protein binding protein 1
	5'-nucleotidase, ecto
Intracellular protein trafficking (7%)	
	caveolin 1
	synaptophysin-like 1
	vesicle transport through interaction with t-SNAREs homolog 1B
	clathrin, light polypeptide
Unknowns (11%)	
	hypothetical protein LOC150159
	hypothetical protein FLJ25082
	THAP domain containing 9
	echinoderm microtubule associated protein like 2
	zinc finger 261
	leucine-rich repeat-containing G protein-coupled receptor 6

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Annexe II: Functions of lipid raft membrane microdomains at the blood-brain barrier.

Functions of lipid raft membrane microdomains at the blood-brain barrier

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Abstract

The blood-brain barrier (BBB) is a highly specialized structural and functional component of the central nervous system (CNS) that separates the circulating blood from the brain and spinal cord parenchyma. Brain endothelial cells (BECs) that primarily constitute the BBB are tightly interconnected by multi-protein complexes, the adherens junctions (AJ) and the tight junctions (TJ), thereby creating a highly restrictive cellular barrier. Lipid-enriched membrane microdomain compartmentalization is an inherent property of BECs and allows for the apico-basal polarity of brain endothelium, temporal and spatial coordination of cell signaling events and actin remodeling. In this manuscript we review the role of membrane microdomains, in particular lipid rafts, in the BBB under physiological conditions and during leukocyte transmigration/diapedesis. Furthermore, we propose a classification of endothelial membrane microdomains based on their function, or at least on the function ascribed to the molecules included in such heterogeneous rafts: 1) rafts associated with inter-endothelial junctions and adhesion of BECs to basal lamina (*scaffolding rafts*); 2) rafts involved in immune cell adhesion and migration across brain endothelium (*adhesion rafts*); 3) rafts associated with transendothelial transport of nutrients and ions (*transporter rafts*).

Key words: blood-brain barrier, central nervous system, diapedesis, lipid raft, DRM, neuroimmunology, multiple sclerosis, leukocyte, transmigration, brain endothelial cell, astrocytes, basal lamina, extracellular matrix, inflammation, myelin, EAE, MS, Rho, Rac, GTPase, actin, neuroinflammation, tight junction, adherens junction, cell adhesion molecule, integrin, permeability, cholesterol, phospholipids

Introduction

The term blood-brain barrier (BBB) refers to the structurally and functionally unique microvasculature of the CNS parenchyma. While the initial description of the BBB defined its' physical barrier characteristics by its ability to restrict diffusion of water soluble acidic dyes from the blood to the CNS parenchyma and vice versa (Goldman, Reese and Karnowsky), contemporary morphological definition(s) of the BBB have refined this concept and allowed for a functional and physical extension of the BBB from the capillary beds to microvascular structures of the post-capillary venules. This morphological definition of the BBB arises from the combination of functional, cellular and molecular studies, which demonstrate that CNS microvessels, whether capillaries or post-capillary venules, are structurally and functionally distinct from microvessels in other organs. These differences include *i)* the presence of vessel-associated pericytes, *ii)* two dense and compact basement membranes, *iii)* astrocytic foot processes en-sheathing CNS vascular endothelium and *iv)* the unique molecular composition and complexity of CNS microvessel tight junctions.

At the functional level, the BBB controls the diffusion of ions and of toxic molecules into the CNS[1], while providing nutrients and oxygen through expression of BBB-specific transporters, such as glucose transporter-1 (GLUT1), sodium/glucose co-transporters and tranferrin receptor (TfR)[1]. Intercellular junctional complexes between BECs, namely tight junctions (TJs) and adherens junctions (AJ) provide endothelial polarity in addition to high transendothelial electrical resistance (TEER) and restrictive permeability[2]. Pericytes and

perivascular astrocytes, through complex communications and signalling events with BECs, induce BBB functions and promote TJ maintenance, specialized transporter expression and immune quiescence of BECs[1, 3-10]. In the current review, we make use of the term BBB to include molecular and functional events, which occur at the level of the highly specialized capillaries and of post-capillary venules of the CNS.

Lipid rafts are dynamic plasma membrane microdomains associated with membrane compartmentalization, actin rearrangements and plasma membrane receptor-mediated signaling events[11-14]. The first experimental line of evidence for biological lipid rafts came from observations made using epithelial cells in which glycosphingolipids trafficked to the apical membrane of polarized epithelial monolayers[15, 16]. Subsequent studies demonstrated that membrane clusters of glycolipids were insoluble in detergents at 4°C, forming detergent resistant membranes (DRMs). These observations, and others, have led to the *lipid raft hypothesis*, which states that lipid (or membrane) rafts, structurally defined as 10-200 nm structures, are heterogeneous and highly dynamic biological membrane microdomains enriched in cholesterol and sphingolipids[11, 13]. A wide variety of proteins are known to be targeted to lipid rafts and raft microdomains have thus been associated with a wide range of biological processes, including endocytosis, signal transduction[17,18], apoptosis, cell polarization[19, 20], adhesion, migration, synaptic transmission and cytoskeleton tethering[12]. Although lipids rafts have been extensively studied in T cell activation and migration[17, 19], junctional proteins, adhesion molecules and transporters can be physically located into lipid raft membrane microdomains of human and mouse BECs, and their expression and localization is regulated by inflammatory and glial-derived factors. In this manuscript we will

review the role of raft microdomains in BBB maintenance and during leukocyte transmigration processes.

Evidence for functionally distinct membrane rafts

The plasma membrane of eukaryotic cells is composed of a complex mixture of membrane proteins and lipid molecules, including glycerophospholipids, sterols and sphingolipids. Glycerophospholipids tend to adopt a mobile loosely-packed fluid phase while sterols and sphingolipids possess distinct biophysical properties and readily group tightly together in a more ordered state. The differential lipid packing abilities lead to phase separation and to the organization of sphingolipid- and cholesterol-rich membrane microdomains, commonly referred to as *rafts*[11, 13, 15, 21].

Experimental studies have demonstrated that membrane clusters of glycosphingolipids and sterols were insoluble in detergents at 4°C and form detergent resistant membranes (DRMs) that can be isolated by ultracentrifugation in a sucrose gradient. These observations have led to the *lipid raft hypothesis* that defines lipid rafts as nanoscale heterogeneous structures enriched in cholesterol and sphingolipids[11, 13]. While most of these lipid raft isolation protocols[22] have been performed using Triton X (TX)-100 at 4°C[22, 23], many other detergents have also been used to prepare DRMs, including Lubrol[24], Brij-58,-96,-98[25], NP40, CHAPS and octylglucoside[22, 26]. Non-detergent fractionation methods have also been used to isolate membrane rafts, and likely reproduce more accurately the *in vivo* protein oligomerizations that occur in microdomains[27, 28]. As a result, the lipid, cholesterol and protein compositions within DRMs can vary between raft preparations, giving rise to biophysically

diverse rafts. However, the heterogeneity of the lipid rafts, in addition to being an outcome of detergent selectivity, may also represent the underlying diversity of lipid and protein compositions. This could in turn reflect the intrinsically different functions attributed to membrane rafts.

A wide variety of proteins are targeted to lipid rafts, notably, but not exclusively, via fatty acylation (myristate and palmitate modifications) and glycosylphosphatidylinositol (GPI)-anchors. Small rafts may be stabilized to form larger platforms through protein-protein or protein-lipid interactions. The raft microdomains have thus been associated with a wide range of biological processes, including endocytosis, signal transduction[18], apoptosis, cell polarization[19], adhesion, migration, synaptic transmission and cytoskeleton tethering[12]. In addition, the existence of distinct lipid constituents within membrane microdomains is important for cellular polarization. Apically, the membrane is enriched in glycosphingolipids and sphingomyelin, favoring the targeting of GPI-anchored proteins. Conversely, proteins important for contacting neighboring cells and the underlying connective tissue are confined to the basolateral membrane, suggesting the presence, within individual cells, of functionally different membrane domains[29]. Segregation of functionally diverse microdomains is an attractive notion, allowing the clustering of molecules that serve similar purposes together in order to optimize efficient intra-cellular signaling. These observations also suggest that membrane rafts have an active role in asymmetric redistribution of membrane proteins during cell migration. Compartmentalized lipid rafts have been described in leukocytes, and most research has focused on membrane raft function during lymphocyte migration[30, 31]. In ECs, lipid membrane rafts also exist but their role and function are still not well defined[32].

A specialized subset of rafts known as caveolae, defined as small cave-like surface invaginations of 50-100 nm in diameter, have been shown to mediate vesicular transport and sophisticated cell signaling. Caveolin-1, the main protein component of these structures, functions as a scaffolding protein and as a possible cholesterol sensor, regulating raft polymerization and lipid trafficking[33, 34]. Unlike lipid rafts, caveolae are not present in all cell types, but are found abundantly in ECs and aid in regulating numerous endothelial functions such as transcytosis, vascular permeability and angiogenesis, and can serve as docking sites for glycolipids and GPI-linked proteins, as well as various receptors and signaling molecules[35]. In addition, due to their similar biophysical properties, caveolae represent a subset of the DRM population and can be further purified using magnetic beads coupled to antibodies against caveolin-1[36].

Elegant studies by Barreiro *et al*. have shown that tetraspanin-enriched membrane microdomains, different from lipid rafts, may be crucial for ECs function by acting as adhesion and signaling structures[37]. Tetraspanin platforms are formed by external homophilic and heterophilic interactions and intracellular associations with signaling proteins and adhesion molecules[38, 39]. The tetraspanins are a group of membrane-spanning molecules and are reported to form an integral part of the leukocyte docking structure, or transmigration cup[37, 40]. CD9 (tetraspanins) is proposed to form an integral web linking cell adhesion molecules (CAMs) such as intercellular CAM-1 (ICAM-1) to vascular CAM-1 (VCAM-1) via its extracellular domain and help organize a plasma membrane signaling platform. Even though tetraspanins have been associated with leukocyte-ECs interactions, the exact role(s) and function(s) of tetraspanins in ECs in health and disease remain to be established.

Raft microdomains and the BBB

The role of membrane microdomains in ECs has only recently been recognized as an important part of many vascular processes. Isolation of lipid rafts from human primary cultures of BECs has been performed using different techniques and detergents[5, 28]. Figure 1 shows different raft isolation preparations using four distinct mild detergents and illustrates variability, especially with regard to the concentration of cholesterol, caveolin-1 and GM1, all markers used to identify rafts. Using these protocols, BEC-derived DRMs are enriched in caveolin-1 and also incorporate TJ proteins, integrins, specific transporters and CAMs. We thus propose a classification of BEC lipid rafts based on the function of molecules found in these membrane compartments: 1) rafts associated with inter-endothelial TJ formation and EC adhesion to the basal lamina (*scaffolding rafts*); 2) rafts involved in immune cell adhesion and recruitment across EC barriers (*adhesion rafts*) and 3) rafts associated with transendothelial transport activity (*transporter rafts*).

1) ***Scaffolding rafts***

TJs consist of at least three known types of transmembrane proteins, including occludin, claudins and the junctional adhesion molecules (JAMs)[1, 2, 41, 42]. Overall, the expression levels of TJ proteins have been found to decrease in many neurological disorders that are characterized by loss of BBB permeability integrity, such as in multiple sclerosis (MS)[5, 43-45]. AJs of BECs are primarily composed of vascular endothelial cadherin (VE-cadherin), which interact with intracellular proteins, including the different catenin isoforms (α-, β-, γ-catenin) and ZO-1 to create the association with the actin cytoskeleton[46-51].

In order to maintain a stable and tight functional barrier, scaffolding rafts serve to regulate integrin-mediated attachment to the basal lamina, as well as TJ- and AJ-mediated cell-cell association[52, 53]. Both AJs and TJs are important for the regulation of paracellular permeability of ions, solutes and nutrients and both junctional complexes localize, at least in part to membrane rafts. Nusrat *et al.* first proposed that TJ molecules of epithelial cells were enriched in lipid raft membrane microdomains. Using the T84 intestinal epithelial cell line and TX-100 raft isolation procedures, occludin and ZO-1 were found to be significantly increased in the insoluble fractions compared to the non-raft soluble fractions[54]. This functional membrane raft idea was adopted by others and recent publications confirmed the close association of membrane rafts with various TJ and AJ proteins in both endothelial and epithelial cells[55, 56]. Figure 2a shows examples of TJ proteins found in membrane rafts of human primary cultures of BECs, isolated using Brij 58 and sucrose gradient centrifugation.

Distribution of TJ proteins to lipid rafts can occur via specific post-translational fatty acid modifications. Inhibition of palmitoylation, required for optimal claudin function, inhibits claudin enrichment into DRM domains and leads to a decrease in TJ barrier function[57]. Using a novel, detergent-free OptiPrep density-gradient method to fractionate rat cerebral microvessels, McCaffrey and co-workers observed TJ proteins in isolated raft fractions and found that the molecular weights of claudin-5, ZO-1 and occludin contained in rafts were twice as large as the predicted weights. This suggests that raft microdomains could be an environment in which TJ oligomerization occurs [27, 28]. Other studies show that alterations in caveolin-1 expression induces alterations of the TJ and AJ complexes[33, 58] and caveolin-1 knockout mice suffer from microvascular hyperpermeability[59]. Song and colleagues observed

that caveolin-1 knock-down in BECs induced a shift in occludin, VE-cadherin and β-catenin subcellular distribution from the DRM to the soluble fraction along with an increase in endothelial permeability[33]. Taken together, these data demonstrate that specialized rafts contribute to barrier permeability through clustering of TJ and AJ proteins.

Several chemicals, described to affect cholesterol synthesis or availability, are known to modulate raft integrity. Modulation of cell membrane cholesterol by treating cells with the cholesterol chelator methyl-β-cyclodextrin (MβCD) induces an initial rise and subsequent fall in TEER measures, associated with actin redistribution and reduced membrane expression of occludin and ZO-1[60, 61]. Moreover, it has been demonstrated that MβCD treatment leads to specific displacement of occludin, claudin-3, -4, -7 and JAM-A out of the Lubrol WX-resistant lipid rafts and coincides with a decrease in TJ integrity[62]. Nusrat *et al.* reported that cholesterol lowering agents, such as HMG-CoA reductase inhibitors (i.e. statins) induced the mobilization of occludin outside of the raft compartment in epithelial cells[54]. In the human *in vitro* system however, statins were shown to decrease BBB permeability without altering subcellular localization of occludin, JAM-A, VE-cadherin, ZO-1 or ZO-2[63]. This suggests that protein localization into rafts is not uniquely regulated by cholesterol concentration, and that additional effects of statins, unrelated to their cholesterol lowering effects might account for these discrepancies. In that sense, we and others have also shown that the barrier promoting effect of statins was dependent on their effect on geranylation, and independent on the cholesterol concentration[63, 64].

TJ enrichment within membrane rafts is positively influenced by astrocyte-secreted angiotensin metabolites and is negatively regulated by proinflammatory cytokines[5]. Similarly,

additional proteins secreted by astrocytes can modulate the BEC-TJ microdomains, such as the chemokine CCL2 acting via its receptor CCR2[65]. MCP-1 was reported to induce changes in caveolin-1 expression and to impact on ZO-1 and occludin localization in membrane microdomains, suggesting that during inflammatory conditions, BBB destabilization can occur through inflammatory cytokine-induced reorganization of TJ proteins on the BEC surface[66].

The endothelial basement membrane and glia limitans formed by BECs and perivascular astrocyte end feet are composed of several extracellular matrix (ECM) components, including fibronectin, different laminin isoforms, collagen type IV and heparan sulfate proteoglycans[8, 52, 67, 68]. Its dense and compact nature provides the ability to function as a substrate for soluble protein immobilization and cell attachment. The ECM is also an important obstacle to blood cell entry into the CNS parenchyma and is thought to be a crucial structure for cellular differentiation and gene expression induction. BECs constitutively express a number of integrins important for adhesion to the endothelial basal lamina, such as $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$. There are several reports associating integrins with caveolae and lipid rafts[69]. Membrane rafts in ECs are known to be enriched in $\alpha_5\beta_1$ integrin, which binds to ECM components and mediates EC attachment to the basal lamina[53]. In that respect, although the significance of lipid raft associated integrins remains to be established, our data presented in Figure 2b suggest that Brij 58-derived lipid rafts from human BECs also contain integrin proteins, such as α_5, α_v and β_1, but not β_3 integrins. This confirms that distinct BEC membrane rafts are involved in the clustering of TJ, AJ and specific integrins, which collectively mediate attachment of ECs to adjacent cells and to the vessel wall.

2) Adhesion rafts

Under physiological conditions, only low number of immune cells continuously cross the BBB and enter the CNS perivascular space in a process called immune surveillance. This process is highly regulated in both leukocytes and ECs and lipid rafts have been associated with all the steps of leukocyte recruitment across the endothelium[70, 71].

Leukocyte rolling is dependent on selectins and their respective carbohydrate ligands or on α4-integrins, at least in non-CNS organs. Selectin association with lipid rafts is important for selectin dependent signaling[72, 73] and thus lipid rafts can influence selectin dependent leukocyte-ECs interactions. Although the exact role of endothelial selectin-dependent outside-in and inside-out intracellular signaling events can influence the migration of some immune cells in peripheral organs, there is still controversy on its role in the CNS[74-78].

Lymphocyte and myeloid cell diapedesis across CNS endothelium involves prototypic BBB CAMs such as ICAM-1 and ICAM-2 (for lymphocytes[79, 80]) and VCAM-1 (for myeloid cells) (3 Ref: Elga, Seguin et Ifergan-brain) which are expressed on the cell surface and/or at intercellular junctions, and can associate with membrane microdomains (Figure 2c) [81-83];. PECAM-1 is also known to act on leukocyte migration, but has rather been documented to halt leukocyte emigration, as PECAM-1 knock out animals have enhanced immune cell infiltration into the CNS[84]. ICAM-1 and PECAM-1 are enriched in lipid rafts and signal through lipid raft platforms provided by membrane rafts[55, 85, 86]. The association of CAMs with cholesterol-enriched membrane microdomains is important for optimal signal transduction events during both transendothelial and transjunctional migration. Accordingly, knockdown of lipid raft-associated protein, caveolin-1, in ECs was shown to inhibit transendothelial leukocyte migration[87]. Recent studies using peripheral derived endothelium and epithelial cultures have

identified novel molecules that coordinate leukocyte transmigration, including JAMs, CD44, CD47, CD73[88], CD90[89], CD137[90], CD81[91], CD99[92], CD99L and CD166/ALCAM[93]. Most of these adhesion molecules are enriched in lipid rafts derived from primary cultures of human BECs[93].

During the final phase of transmigration, complex intracellular signaling events take place in both the leukocyte and the EC to prepare the cells for transmigration/diapedesis. These events are dependent on adhesion molecule signaling and include cytoskeleton remodeling, TJ and AJ disassembly and reassembly and cell body retraction. Recent data from Greenwood and colleagues support the notion that VE-cadherin re-localization in BECs during diapedesis is dependent on ICAM-1 signaling events mediated by possible membrane raft signaling platforms. ICAM-1 signaling lead to VE-cadherin phosphorylation and allowed paracellular leukocyte migration[55]. As recently described, a key feature of transendothelial migration (also called emperiopolesis) is the formation of an EC cup-like structure surrounding the migrating leukocytes[37, 40, 70, 71, 94-97]. According to Carman *et al.*, endothelial membranes positive for caveolin-1 are associated with the transmigrating leukocyte in the transmigration cup, which is dependent on the presence of lipid rafts and their recruitment of CAMs[87]. In this context, lipid rafts are believed to coordinate outside-in signaling in ECs and regulation of actin remodeling by lipid raft associated small GTPases, such as Rho [98-100] and Rac[101, 102], myosin dependent contractions[103] and junctional protein regulation[5, 104]. These transendothelial migration events are highly organized around CAM-mediated signaling within lipid raft structures and support the concept of functionally distinct endothelial *adhesion rafts*.

Recently tetraspanin-enrich membrane microdomains have been suggested to be equally important in acting as adhesion and signaling structures for leukocyte transmigration. Tetraspanins are small proteins (CD9, CD81, and CD151) that can organize as tetraspanin web microdomains (or nanoplatforms) and associate with numerous integral membrane proteins (ICAM-1 and VCAM-1) to provide a scaffold for specific interactions and outside-in signaling induction[39]. The exact differences between lipid rafts and tetraspanin platforms and their contribution during leukocyte migration are under active investigation. Nevertheless the importance of tetraspanins in leukocyte migration to the brain has been shown by CD81 antibody blockade which inhibits monocyte migration and reduces clinical signs in experimental autoimmune encephalomyelitis (EAE), a validated animal model for MS[91]. Taken together, there is ample evidence to support the concept that lipid rafts and/or tetraspanin platforms contribute to the highly regulated process of immune cell transmigration across BECs.

3) TRANSPORTER RAFTS

Transport of nutrients to the CNS is regulated by the expression of specific transporters in BECs. Expression of active transport systems for glucose, amino acids, nucleosides, fatty acids, minerals, vitamins, peptides, proteins and ions define the prototypic nature and function of the BBB. In our liquid chromatography-mass spectrometry analysis of rafts obtained from primary cultures of human BBB endothelium[93], several members of the transporter system were identified. Due to the expected high number of false-negative data generated by mass spectrometry, this list can only be considered partial or incomplete, but includes many

different transporter systems (see Table I). These findings indicate that rafts might also play an active role in trans-BBB trafficking of numerous lipid insoluble molecules.

3. Conclusion

The presence of DRMs or membrane raft microdomains at the level of BECs appears to play a critical role in BBB function under physiological conditions and during complex cellular interactions. We have presented evidence and reviewed the role of the BBB and the function of membrane microdomains in BEC physiology and during immune cell transmigration. We further propose a classification of membrane raft microdomains based on their function in the cells studied. While the proposed classification is not exhaustive, it is useful to identify functionally similar microdomains in heterogeneous populations of lipid rafts (Figure 3). At the level of the BBB, we define *scaffolding rafts* as rafts associated with inter-endothelial junctions and adhesion of BECs to basal lamina, *adhesion rafts* as rafts involved in immune cell adhesion to and migration across brain endothelium and *transporter rafts* as rafts involved in the transendothelial movements of solutes.

Table I: Membrane raft microdomain associated transporters identified by proteomic analysis

Transport System	**Lipid raft associated transporter**
Anion/cation transporters	*VDCA1, VDCA3*
Glutamate receptor	*GRIN2B*
Non voltage-gated sodium channel	*SCNN1A*
Voltage dependant calcium channel	*CACNA2D1*
Calcium transporting ATPase	*ATP2B4*
Potassium/chloride solute carrier family	*SLC12A6, SLC4A3, SLC35E1, SLC41A2, HCN2*
ABC transporters	*ABCA6, ABCB7 and ABCA1*
Carbohydrate transporters	*SLC2A6, SLC45A4, SLC2A14*
Lipid and fatty acid transporters	*ApoB, ABCA6, PITPNM2, ABCA1*
Glycosyl, acetyl, methyl or nucleoside transferases	*GGTLA1, MGAT2, UGT2B11, B4GalNac-T3, THUMPD2, MAT1A*

Human primary cultures of BECs were grown to confluence, lysed in Brij58 detergent and cholesterol enriched membrane microdomains were isolated by sucrose gradient flotation. The raft fraction was subjected to trypsin digestion, separated by liquid chromatography and analyzed by mass spectrometry, as described in Cayrol et al. [93].

Figures

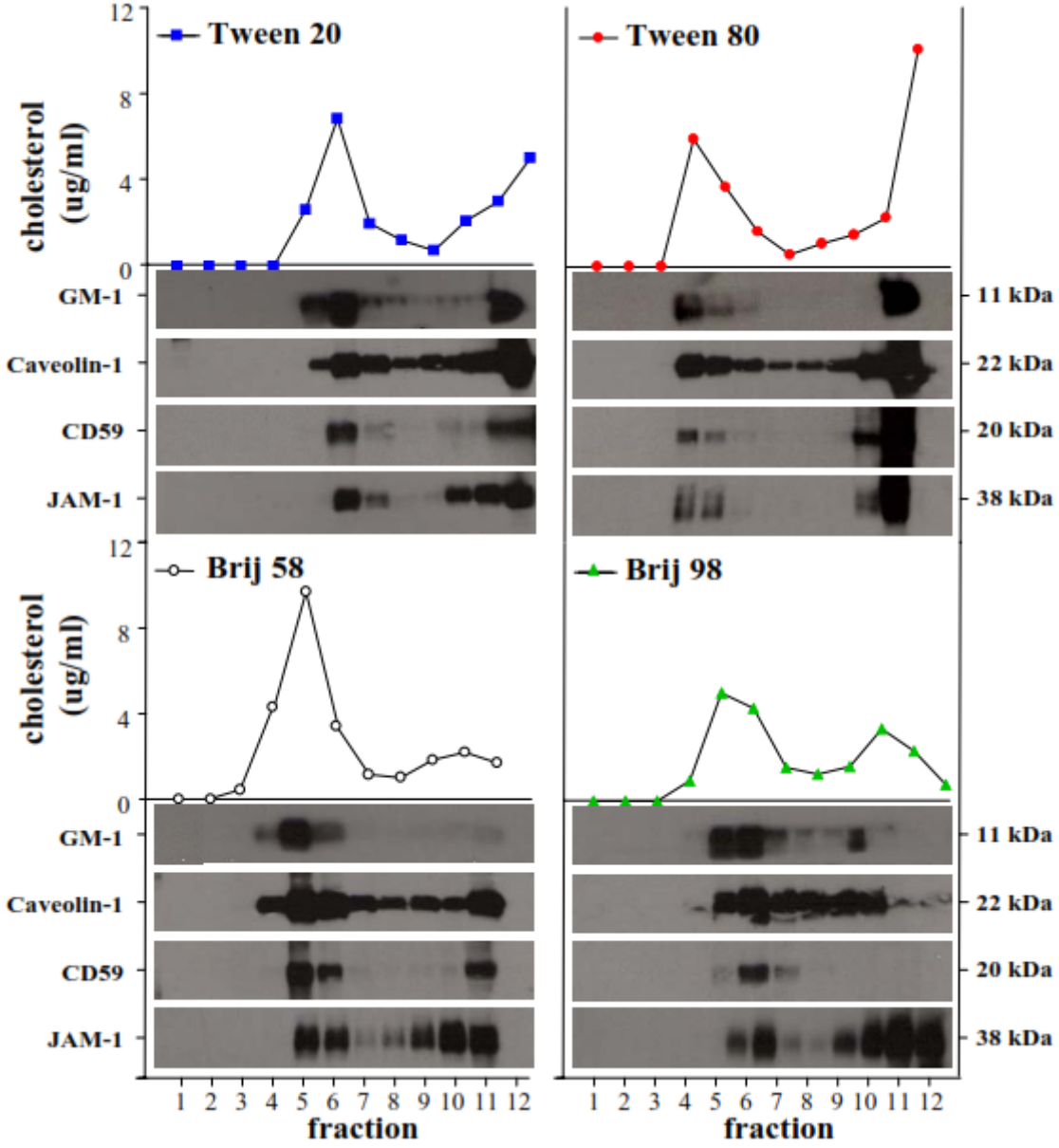

Figure 1: *Use of different detergents to isolate cholesterol-enriched microdomains from human primary cultures of BECs.* Four detergents were used to optimize cholesterol-enriched

DRM isolation from primary cultures of human BECs. Following treatment of the cells with the detergent, membrane microdomains were separated by sucrose-density centrifugation, as described in Wosik et al.[5] and Cayrol et al.[93] Cholesterol and phospholipid concentrations, as well as GM-1, caveolin-1, CD59 were used to identify raft fractions by Western blot. JAM-1 is shown as an example of a tight junction protein expressed in the raft fraction and its modulation according to the detergent used. A detailed protocol of raft preparation is available in the Supplementary material section.

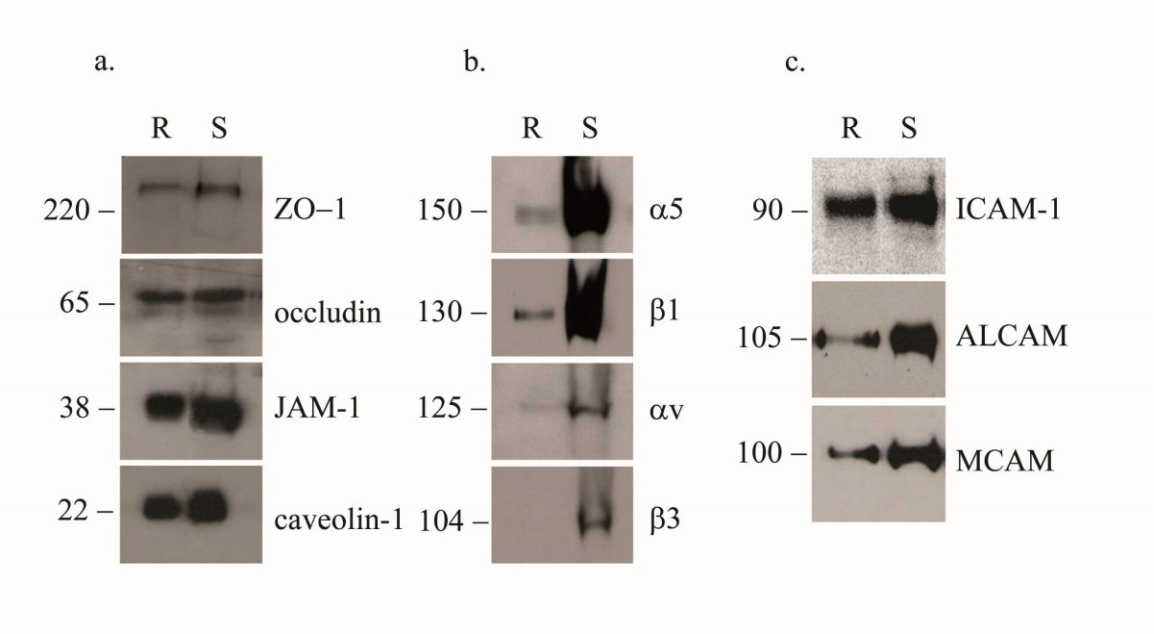

Figure 2: *Representative Western blots of isolated lipid rafts probed for scaffolding, TJ proteins and adhesion molecules.* Lipid raft were generated with treatment of primary cultures of human BECs with Brij 58 detergent followed by sucrose gradient centrifugation, as described in Wosik et al.[5] Representative raft fraction (R) and detergent soluble fraction (S) were used to assess the presence of TJ, integrins and adhesion molecules. The TJ proteins ZO-1, occludin and JAM-1 (A) are present in the lipid raft fraction, in addition to caveolin-1. (B)

Integrins α_5 and β_1 are readily detected in BEC lipid rafts. Integrin α_v is seen at lower levels and integrin β_3 does not seem to be associated with lipid raft under physiological conditions. (C) The cell adhesion molecules ICAM-1, ALCAM and MCAM can be detected in lipid raft fractions of BECs under normal culture conditions.

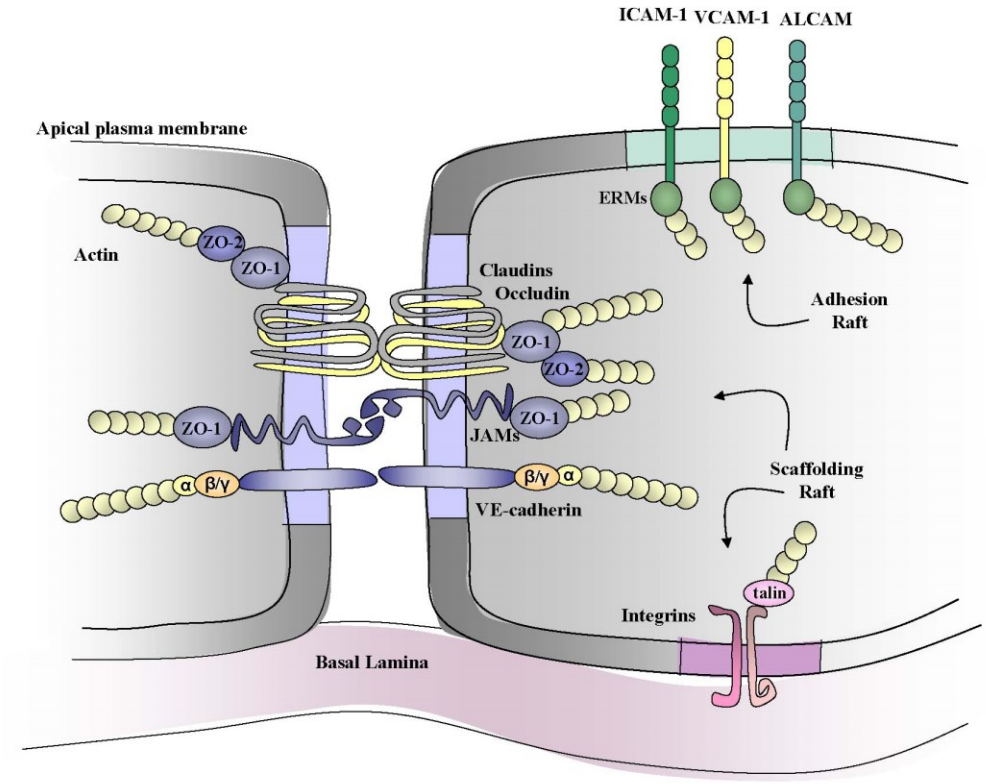

Figure 3: *Hypothetical model representing the functionally and spatially different lipid membrane rafts found in BECs.* Scaffolding rafts secure the BECs to their surrounding and include rafts associated with inter-endothelial cell junction proteins (tight junction and adherens junction) and rafts associated with extracellular matrix attachment proteins. Adhesion rafts include microdomains associated with adhesion molecules found on the BECs

and include rafts that would be enriched in proteins mediating interaction with peripheral leukocytes. Not shown in this model are the transporter rafts.

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Annexe III CURRICULUM VITAE

Études et formation

Juillet 2013	**Résidence en Pathologie,** Université de Montréal, Faculté de Médecine
Juin 2013	**Doctorat en Médecine,** Université de Montréal, Faculté de Médecine Programme MD/PhD médecine-recherche.
Sept. 2005 à Sept. 2013	**Doctorat en Neuroimmunologie**, Université de Montréal, Département de Microbiologie et d'Immunologie, superviseur Dr. A. Prat, recherche fondamentale et clinique sur la sclérose en plaques. **Titre**: Régulation moléculaire de la barrière hémo-encéphalique
2000 à 2003:	**Maitrise de Science**, Université McGill, Département de Biochimie, superviseur Dr. P. Gros, recherche sur le cancer. **Titre**: Études de chimères de la P-glycoprotéine de type I et de type II.
1996 à 00:	**Baccalauréat en Science**, Université McGill, Département de Biochimie. Graduation avec honneurs.
1984 à 96:	**Études secondaires et collégiales,** Collège Stanislas, Montréal, CANADA. Spécialisation en Sciences Biologiques, graduation avec mention assez bien.

Expériences de recherche

Sept. 2005 à Sept. 09	**Doctorat en Neuroimmunologie,** Université de Montréal, laboratoire du Dr. A. Prat.
Sept. 2000 à Sept. 2003:	**Maitrise en Science**, Université McGill, Département de Biochimie. Laboratoire du Dr. P. Gros.
Été 2000:	**Stages de recherche printemps et été dans le laboratoire du Dr. P. Gros**, Université McGill.
Été 1999:	**Stage d'été dans le laboratoire du Dr. P. Gros**, Université McGill. Travaille avec un étudiant gradué à la conception de mutants de la P-glycoprotéine et à la caractérisation des mutants.
Sept. 1997 à 2002:	**Travail mi-temps avec la compagnie Packard\BioSignal,** Montréal, CANADA. Responsable de la recherche et la livraison de références scientifiques pour les scientifiques de la compagnie.
Été 1997:	**Stage d'été en Pharmacologie, Packard\BioSignal,** Montréal, CANADA. Travail de laboratoire (compétition et saturation) sur des membranes enrichies en récepteurs surexprimés.

Prix et distinctions

Sept. 2009 :	Bourse MD-PhD des IRSC
Juin 2009 :	Bourse de voyage pour le congrès FOCIS 2009, San Francisco.
Mars 2009 :	Prix « Cerveaux en tête » de l'Institut de recherche en sante du Canada.
Dec. 2008 :	Bourse de voyage du Cold Spring Harbor.
Mai 2008 :	Prix pour présentation par affiche, Symposium de Neuroinflammation.
Avril 2008 :	Bourse de doctorat du FRSQ (refusée).
Dec. 2007 :	Meilleure présentation par affiche (PhD), congrès des étudiants du CHUM.
Dec. 2007 :	Bourse d'excellence du congrès des étudiants du CHUM 2007-2008.
Juillet 2007 :	Bourse de doctorat de la société canadienne de la sclérose en plaques.
Juin 2007 :	Bourse de voyage de l'association américaine contre l'auto-immunité.
Juin 2007 :	Bourse de voyage du programme de neuroinflammation.
Mars 2006 :	Bourse de voyage du programme de neuroinflammation.
2005-2007 :	Bourse de doctorat du programme de neuroinflammation.
2001-2002 :	Bourse Canderel pour la maitrise du centre du cancer de McGill.
2000-2001:	Prix de la faculté des études graduées et de la recherche, Université McGill.
1999:	Graduation avec honneurs du programme de biochimie, Université McGill.

Langages

Maitrise parlée et écrite du français et de l'anglais. Connaissance de bases de l'espagnol et de l'allemand.

Compétences informatiques

IBM compatible, traitement de texte (MS Word, WordPerfect), DOS, Windows, bases de données (MS Exel). Connaissances de l'internet et application scientifiques de bases (programme d'analyses de données, d'analyses statistiques et de présentation). Connaissance de programmes graphiques (Freehand et Photoshop) et compréhension des programmes d'analyses génétique (Quantitaive Trait Loci analysis).

Expertise de laboratoire

J'ai une expertise de laboratoire en biologie moléculaire, biologie cellulaire et en biochimie. Je suis entrainé pour travailler avec des animaux (souris et rats), avec des produits radioactifs et avec des matières dangereuses. Pour de plus amples détails sur mes compétences de recherche en laboratoire s'il vous plait n'hésitez pas à me contacter.

Publication dans des journaux scientifiques

Melanoma cell adhesion molecule identifies encephalitogenic T lymphocytes and promotes their recruitment to the central nervous system.

Larochelle C**, Cayrol R**, Kebir H, Alvarez JI, Lécuyer MA, Ifergan I, Viel É, Bourbonnière L, Beauseigle D, Terouz S, Hachehouche L, Gendron S, Poirier J, Jobin C, Duquette P, Flanagan K, Yednock T, Arbour N, Prat A. Brain. 2012 Oct;135(Pt 10):2906-24.

Isolation of human brain endothelial cells and characterization of lipid raft-associated proteins by mass spectroscopy.

Cayrol R, Haqqani AS, Ifergan I, Dodelet-Devillers A, Prat A. Methods Mol Biol. 2011;686:275-95.

Disruption of central nervous system barriers in multiple sclerosis.

Alvarez JI, **Cayrol R**, Prat A. Biochim Biophys Acta. 2011 Feb;1812(2):252-64.

Effector functions of antiaquaporin-4 autoantibodies in neuromyelitis optica.
Cayrol R, Saikali P, Vincent T. Ann N Y Acad Sci. 2009 Sep;1173:478-86. Review.

Functions of lipid raft membrane microdomains at the blood-brain barrier.
Dodelet-Devillers A, **Cayrol R**, van Horssen J, Haqqani AS, de Vries HE, Engelhardt B, Greenwood J, Prat A. J Mol Med (Berl). 2009 Aug;87(8):765-74. Review.

Anti-aquaporin-4 auto-antibodies orchestrate the pathogenesis in neuromyelitis optica.
Saikali P, **Cayrol R**, Vincent T. Autoimmun Rev. 2009 Dec;9(2):132-5. Review.

Blood-Brain-Barrier Endothelial Cells Promote Differentiation of Human Fetal Neural Precursor Cells.
Satyan Chintawar, Romain Caryol, Jack Antel, Alexandre Prat et Massimo Pandolfo. Stem Cells, Feb. 2009, 27(4): 838-46.

Functional consequences of neuromyelitis optica-IgG astrocyte interactions on blood-brain barrier permeability and granulocyte recruitment.
Vincent T, Saikali P, **Cayrol R**, Roth AD, Bar-Or A, Prat A, Antel JP. Journal of Immunology, Oct 2008, 181(8): 5730-37.

ALCAM promotes leukocyte trafficking into the central nervous system.
Romain Cayrol, Karolina Wosik, Jennifer L. Berard, Aurore Dodelet-Devillers, Igal Ifergan, Hania Kebir, Arsalan S. Haqqani, Sebastian Krug, Samuel David, Danica Stanimirovic et Alexandre Prat. Nature Immunology, Feb 2008, 9(2) : 137-45.

T_H-17 lymphocytes promote blood-brain barrier disruption and CNS inflammation.

Hania Kebir, Katharina Kreymborg, Igal Ifergan, Aurore Dodelet-Devillers, **Romain Cayrol**, Fabrizio Giuliani, Nathalie Arbour, Burkhard Becher & Alexandre Prat. Nature Medicine 2007 Oct;13(10):1173-5.

The blood-brain barrier induces differentiation of migrating monocytes into Th17-polarizing dendritic cells.

Igal Ifergan, Hania Kébir, Monique Bernard, Karolina Wosik, Aurore Dodelet-Devillers, **Romain Cayrol**, Nathalie Arbour et Alexandre Prat. Brain, 2008 March 131(3) : 785-99.

Angiotensin II controls occludin function and is required for blood-brain barrier maintenance: relevance to Multiple Sclerosis.

Karolina Wosik *, **Romain Cayrol *,** Aurore Dodelet-Devillers , France Berthelet, Monique Bernard , Robert Moumdjian , Alain Bouthillier, Timothy L. Reudelhuber et Alexandre Prat. Co-premier auteur, J of Neuroscience 2007 Aug 22;27(34):9032-42.

NKG2D-mediated cytotoxicity toward oligodendrocytes suggests a mechanism for tissue injury in multiple sclerosis.

Saikali P, Antel JP, Newcombe J, Chen Z, Freedman M, Blain M, **Cayrol R**, Prat A, Hall JA, Arbour N. J of Neuroscience. 2007 Jan 31;27(5):1220-8.

Statins reduce human blood-brain barrier permeability and restrict leukocyte migration: Relevance to multiple sclerosis.

Ifergan I, Wosik K, **Cayrol R**, Kebir H, Auger C, Bernard M, Bouthillier A, Moumdjian R, Duquette P, Prat A. Annals of Neurology 2006 May 25;60(1):45-55

Mutational Analysis of Conserved Carboxylate Residues in the Nucleotide Binding Sites of P-Glycoprotein.

Ina L. Urbatsch, Michel Julien, Isabelle Carrier, Marc-Etienne Rousseau, **Romain Cayrol**, Philippe Gros. Biochemistry; 2000; 39(46); 14138-14149.

Compte rendue de conférence et de recherche:

Immunology now, April 2008 vol. 8(2) : 7-8.

Brain Brief, vol.3 #2, 2006, The First Annual Montreal Neuroinflammation Symposium 2006.

Brevet

MCAM modulation and uses thereof

Inventeurs : Alexandre Prat, **Romain Cayrol**, Nathalie Arbour, Catherine Larochelle

 # Application USPTO: 20110014183 - Classe: 4241301 (USPTO) 20-01-2011

Conférences scientifiques

Résumé et présentation de 10 minutes, FOCIS San Francisco, Juin 2009.

MCAM is expressed by blood brain barrier endothelial cells and Th17 and is involved in lymphocyte migration into the central nervous system.
Romain Cayrol, Hania Kebir, Igal Ifergan, Aurore Dodelet-Devillers, Simone Terouz, Arsalan S Haqqani, Josée Poirier, Danica Stanimirovic, Nathalie Arbour, Pierre Duquette & Alexandre Prat.

Résumé et présentation de 10 minutes, Cold Spring Harbor meeting, Decembre 2008.

MCAM is expressed by blood brain barrier endothelial cells and is potentiallu involved in lymphocyte migration into the central nervous system.
Romain Cayrol, Hania Kebir, Igal Ifergan, Aurore Dodelet-Devillers, Simone Terouz, Arsalan S Haqqani, Josée Poirier, Danica Stanimirovic, Nathalie Arbour, Pierre Duquette & Alexandre Prat.

Résumé et affiche, American Association for Neurology, Chicago 2008.

Functional consequence of NMO-IgG on BBB permeability , astrocyte injury and granulocyte recruitment.

Vincent T, Saikali P, **Cayrol R**, Roth AD, Bar-Or A, Prat A, Antel JP.

Résumé et présentation de 10 minutes, Canadian Society for Immunology, Mt-Tremblant 2008.

MCAM is an adhesion molecule involved in lymphocyte recruitment to the brain

Romain Cayrol, Hania Kebir, Igal Ifergan, Aurore Dodelet-Devillers, Simone Terouz, Arsalan S Haqqani, Josée Poirier, Danica Stanimirovic, Nathalie Arbour, Pierre Duquette & Alexandre Prat.

Résumé et présentation de 10 minutes, ECTRIMS, Montréal 2008.

MCAM is an adhesion molecule of the vasculature, defines a unique T lymphocyte subset and is potentially involved in lymphocyte migration to the central nervous system.

Romain Cayrol**, Hania Kebir, Igal Ifergan, Aurore Dodelet-Devillers, Simone Terouz, Arsalan S Haqqani, Josée Poirier, Danica Stanimirovic, Nathalie Arbour, Pierre Duquette & Alexandre Prat.**

Résumé et présentation de 10 minutes, International society for neuro-immunology, Texas 2008.

MCAM is an adhesion molecule involved in lymphocyte recruitment to the brain.

Romain Cayrol**, Hania Kebir, Igal Ifergan, Aurore Dodelet-Devillers, Simone Terouz, Arsalan S Haqqani, Josée Poirier, Danica Stanimirovic, Nathalie Arbour, Pierre Duquette & Alexandre Prat.**

Résumé et présentation de 25 minutes, BBB 2008 meeting, Amsterdam 2008.

ALCAM is an adhesion molecule of the BBB.

Romain Cayrol **& Alexandre Prat.**

Résumé et présentation de 10 minutes, Federation of Clinical Immunology Meeting, San Diego 2007.

ALCAM promotes leukocyte trafficking into the Central Nervous System.

Romain Cayrol, Karolina Wosik, Jennifer L. Berard, Aurore Dodelet-Devillers, Igal Ifergan, Hania Kebir, Arsalan S. Haqqani, Sebastian Krug, Samuel David, Danica Stanimirovic et Alexandre Prat.

Résumé et présentation de 10 minutes, 2nd Annual retreat of the Center for excellence in Neuromics, Orford 2007.

ALCAM/CD166 is a novel adhesion molecule of the inflamed endothelium et promotes leukocyte trafficking to the central nervous system.

R. Cayrol, K. Wosik, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. Haqqani, D. Stanimirovic et A. Prat.

Résumé et affiche, CHUM symposium étudiant, Montréal 2007.

MCAM is an adhesion molecule potentially involved in leukocyte migration to the brain.

R. Cayrol, K. Wosik, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. Haqqani, D. Stanimirovic et A. Prat

Résumé et affiche, Conference EndMS, Banff December 2007

ALCAM promotes leukocyte trafficking into the Central Nervous System.

Romain Cayrol, Karolina Wosik, Jennifer L. Berard, Aurore Dodelet-Devillers, Igal Ifergan, Hania Kebir, Arsalan S. Haqqani, Sebastian Krug, Samuel David, Danica Stanimirovic et Alexandre Prat.

Résumé et affiche ECTRIMS 2007, Prague 2007.

ALCAM promotes leukocyte migration to the central nervous system : relevance to multiple sclerosis.

R. Cayrol, Dedelet-deviller A, Berard J, David S, Stanimirovic D, Prat A.

Résumé et affiche ECTRIMS 2007, Prague 2007.

NMO-IgG specifically bind primary human astrocytes

T. Vincent, Saikali P, Cayrol R, Colman DR, Bar-or A, Prat A, Antel J.

Résumé et affiche, Annual 7th Cerebral Vascular Biology International Conference, Ottawa 2007.

ALCAM is a novel adhesion molecule of the inflammed endothelium involved in leukocyte trafficking to the central nervous system

R. Cayrol, K. Wosik, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. Haqqani, D. Stanimirovic et A. Prat.

Résumé et affiche, Annual 7th Cerebral Vascular Biology International Conference, Ottawa 2007.

Human Il-23-Dependent Th-17 memory lymphocytes promote blood-brain barrier disruption, CNS inflammation et neuronal killing.

Kebir H, Ifergan I, Kreymborg K, Dodelet-Devillers A, **Cayrol R**, Giuliani F, Arbour N, Becher B, Prat A.

Résumé et affiche, Annual 7th Cerebral Vascular Biology International Conference, Ottawa 2007.

Endothelial cells of the human blood-brain barrier induce the differenciation of monocytes into professional antigen presenting cells.

Ifergan I, Kebir H, Bernard M, **Cayrol R**, Moumdjian R, Bouthilier A, Arbour N, Prat A.

Résumé, federation of Clinical Immunology Meeting, San Diego 2007.

Cytotoxic human Il-23 expressing Th17 lymphocytes promote immune cell migration into the central nervous system.

Kebir H, Ifergan I, Dodelet-devillers A, Giuliani F, Cayrol R, Arbour N, et Prat A.

Résumé et affiche, the Annual American Association of Neurology 2007.

Human Blood-Brain-Barrier Endothelial Cells Promote Differentiation of Human Fetal Neural Precursor Cells.

Satyan Chintawar, Romain Caryol, Jack Antel, Alexandre Prat et Massimo Pandolfo.

Résumé et affiche, 2nd Annual retreat of the Center for excellence in Neuromics, Orford 2007.
Il-23 but not TGF-b, promotes cyotoxic et helper functions of human il-22-secreting Th17 memory lymphcytes.
Kebir H, Ifergan I, Dodelet-devillers A, Giuliani F, Cayrol R, Arbour N, et Prat A.

Résumé et affiche, 2nd Annual retreat of Neuroinflammation training program, Bromont 2007.
ALCAM/CD166 is an adhesion molecule of the inflamed endothelium involved in leukocyte trafficking to the central nervous system.
R. Cayrol, K. Wosik, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. Haqqani, D. Stanimirovic et A. Prat

Résumé et **présentation de 10 minutes,** 74th ACFAS (Association Canadienne Francaise pour l'Avancement de la Science) meeting, Mai 2006.
Identification de nouvelles protéines des jonctions sérrées de la barrière hémato-encéphalique humaine.
Cayrol R, Wosik K, Prat A, Centre de Recherche du CHUM, Hôpital Notre Dame.

Résumé et affiche, Annual American Association for Neurochemistry meeting in Portlet, March 2006.
Tight Junction proteins of the human Blood Brain Barrier localize to the endothelium lipid rafts.
Cayrol, R , Wosik, K, Haqqani, A , Stanimirovic, D , Prat, A

Résumé et affiche, CHUM symposium étudiant, Montréal Décembre 2006.
ALCAM/CD166 is an adhesion molecule of the inflamed endothelium involved in leukocyte migration to the central nervous system.
R. Cayrol, K. Wosik, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. Haqqani, D. Stanimirovic et A. Prat

Résumé et affiche, 22nd ECTRIMS, September 2006.
Statins restrict leukocyte migration and promotes human blood-brain barrier characteristics.
Ifergan I, Wosik K, **Cayrol R**, Kébir H, Duquette P, Prat A.

Intérêts

- La science et la médecine en général, les sciences biomédicales et le traitement des maladies humaines plus particulièrement.

- Le voyage, je suis allé en Europe, en Afrique du Nord, en Amérique centrale, en Asie et en Amérique du Nord.
- Le sport : je suis intéressé par le sport de compétition (arts martiaux, natation, hockey et ballon sur glace) et par les sports de loisir (cyclisme, skie, soccer).
- J'ai un intérêt pour l'audio-visuel et pour le spectacle.
- J'ai été volontaire pour les levées de fonds pour la lutte contre le cancer (défi Canderel) en 2001 et en 2002.
- Je participe aux levées de fonds pour la recherche en sclérose en plaques (marche SEP 2006, 2007 et 2008), ainsi qu'a des journées sclérose en plaques (rencontre avec la clinique de Bois-Brillant 2008).
- J'aime le camping, la lecture, le cinéma, le théâtre, la musique et les spectacles de danses.