Cues Guiding Leukocyte Transendothelial Migration across the Blood-Brain Barrier in Neuroinflammation: Endothelial Heterogeneity, Chemokines, and Extracellular Vesicles

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Leukocyte infiltration into the central nervous system (CNS) underlies the pathology in a wide spectrum of neuroinflammatory and neurodegenerative diseases like multiple sclerosis (MS), stroke, meningitis, and neuroAIDS. While the steps that mediate the initial adhesion of activated leukocytes to the endothelial wall has been well-characterized, not much is known about their subsequent transendothelial migration (TEM) across the blood-brain barrier (BBB), a highly restrictive paracellular barrier established by the specialized CNS endothelial cells, thus severely limiting the treatment options.

In Multiple Sclerosis (MS) ‘focal’ leukocyte infiltration into the CNS parenchyma early in disease is thought to be critical for the inflammatory response, and eventual neurodegeneration. Therefore, to explore the cues that regulate leukocyte TEM across the BBB in a neuroinflammatory milieu, in this dissertation, we evaluated the role of three factors- endothelial heterogeneity, chemokine CCL2 from CNS sources, and extracellular vesicles (EVs) from endothelial cells containing a major tight-junction (TJ) protein, Claudin-5 (CLN-5), in CNS leukocyte infiltration, in an animal model of MS called Experimental Autoimmune Encephalomyelitis (EAE).
Using a novel high-resolution three-dimensional confocal image analysis approach, existence of a functional heterogeneity in microvascular response was found during neuroinflammation in EAE. Specifically BBB damage and leukocyte extravasation in EAE was restricted to venules only. Furthermore, Chemokine CCL2, which only surges in a neuroinflammatory milieu to detectable levels and released predominantly from endothelium and astrocytes in the CNS, was shown to uniquely guide leukocytes across the 'respective' (endothelial and astrocyte) basement membranes. Interestingly, leukocytes invading the CNS early in EAE were found to be coated with TJ protein CLN-5. Brain microvascular endothelial cells (BMECs) were seen to release extracellular vesicles (EVs) in neuroinflammation that contained CLN-5, and could bind to the adherent leukocytes possibly for conveying the TJ protein cargo. These CLN-5+ leukocytes were found to transmigrate more efficiently across cultured BMECs. The obtained results from these studies have shed significant light on previously uncharacterized cues and mechanisms that guide circulating leukocytes across the BBB in disease, and holds the key for novel therapeutic strategies to treat a myriad of neurologic disorders and vasculopathies that display immune infiltration in the CNS.
Cues Guiding Leukocyte Transendothelial Migration across the Blood-Brain Barrier in Neuroinflammation: Endothelial Heterogeneity, Chemokines, and Extracellular Vesicles

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A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut

[2015]
Cues Guiding Leukocyte Transendothelial Migration across the Blood-Brain Barrier in Neuroinflammation: Endothelial Heterogeneity, Chemokines, and Extracellular Vesicles

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[2015]
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Years ago, I dreamt of being a scientist one day. Today, I feel honored to have accomplished an important feat in that direction. I’m extremely grateful to Dr. Joel Pachter, my advisor, for providing me the opportunity to pursue my dream in his laboratory. I thank him for his relentless encouragement, support, riveting discussions and guidance. He always garnered my expertise in asking good scientific questions, designing experiments, and most importantly, communicating complex topics in a lucid manner using examples from day-to-day life.

My sincere thanks to all members of the Pachter lab. Most importantly, thanks to Dr. Shujun Ge for wholeheartedly helping me through the thesis work and providing guidance with experiments. Thanks to Dr. Bandana Shrestha, who is not only a colleague but also a great friend, for being immensely helpful and cheering me up when experiments refused to work. I want to thank Dr. Nivetha Murugesan, for her support during the initial years. Special thanks to Yen Lemire for help with the CCL2 study, and rotation students Timothy Kiprono and Cory Willis, for helpful discussions on the extracellular vesicle project.

Heartfelt thanks to the members of my advisory committee, Dr. Ann Cowan, Dr. Donald Kreutzer and Dr. Robert Cone. I am grateful for your constructive suggestions throughout my dissertation research that immensely contributed to shaping the projects.

None of this would have been possible without the constant support and encouragement from my wonderful family- my mom, dad and sister. I also feel blessed to have an amazing circle of close friends, who made grad life a fun experience for me.
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Publications and Contributions to the Thesis Projects

1. **Chapter II**: In this study, using a novel high-resolution 3D confocal imaging and analysis approach on thick spinal cord cryosections, I highlighted the existence of a functional heterogeneity in microvascular response, i.e., venule restricted leukocyte extravasation in an animal model of multiple sclerosis, called experimental autoimmune encephalomyelitis (EAE), compared to apparent refractory behavior of the contiguous capillaries. I performed all the experiments and analysis described in this study. Dr. S. Ge induced EAE. Dr. A. Cowan provided guidance on 3D image analysis. Dr. J. Pachter supervised the project. I and Dr. J. Pachter wrote the manuscript. This work was published in *Paul, D, Cowan, AE, Ge, S, Pachter, JS. Novel 3D analysis of Claudin-5 reveals significant endothelial heterogeneity among CNS microvessels. Microvascular Res. 2013;86:1-10.*

2. **Chapter III**: In this study, using two conditional knockout mouse, lacking CCL2 in endothelium and astrocytes (two major CNS sources of CCL2) respectively, I showed CCL2 from these sources, uniquely guides leukocytes across the ‘respective’ (endothelial and astrocyte) basement membranes. I performed all the 3D confocal image acquisition and analysis described in this study. Dr. D. Serwanski obtained the EM images. Dr. N. Ruddle provided guidance with EAE induction. Dr. S. Ge performed EAE induction and clinical scoring. Y. Lemire and Dr. E. Jellison contributed to cell proliferation assays and FACS analysis. Dr. J. Pachter supervised the project. I and Dr. J. Pachter wrote the manuscript. This work was published in *Paul, D, Ge, S, Lemire, Y, Jellison, ER, Serwanski, DR, Ruddle, NH, Pachter, JS. Cell-selective knockout and 3D confocal image analysis reveals separate roles for astrocyte-and endothelial-derived CCL2 in neuroinflammation. J Neuroinflammation. 2014;11:10.*

3. **Chapter IV**: For passage across the BBB in neuroinflammation the leukocytes are thought to negotiate the tight junctions (TJs). Here, I showed leukocytes invading the CNS early EAE, are
coated with TJ protein claudin-5 (CLN-5), a BBB determinant. I further demonstrated endothelial extracellular vesicles (EVs) can transfer CLN-5 protein to the circulating leukocytes both \textit{in vitro} and \textit{in vivo} in neuroinflammatory milieu, possibly for transendothelial migration. CLN-5\textsuperscript{+} leukocytes migrated more efficiently across brain microvascular endothelial cells (BMECs) compared to ones lacking them. I performed all the 3D analysis of confocal/ serial EM images, EV isolations and labeling, Western Blotting, and EAE induction described in this work. V. Baena acquired serial EM images under Dr. M. Terasaki’s guidance. Dr. E. Jellison performed FACS analysis. S. Ge undertook leukocyte transmigration assays across BMECs. Dr. D. Agalliu provided the eGFP-Tie2-CLN-5 mice. Dr. J. Pachter supervised the project. A manuscript is under preparation for this study. The abstract of this work was published in Paul, D, Ge, S, Jellison, E, Agalliu, D, Pachter, JS. Extracellular vesicles as possible conveyors of tight junction protein to leukocytes in neuroinflammation. Journal of Extracellular Vesicles. \textbf{2015};4: 27783.
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<tbody>
<tr>
<td>γgt</td>
<td>gamma glutamyl transferase</td>
</tr>
<tr>
<td>2-ME</td>
<td>2- Mercaptoethanol</td>
</tr>
<tr>
<td>ABC transporter</td>
<td>ATP-binding cassette transporters</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin I converting enzyme</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens Junctions</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Astro KO</td>
<td>Astrocyte-specific CCL2 knockout mice</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>BCSFB</td>
<td>Blood-cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMEC</td>
<td>Brain Microvascular Endothelial Cell</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CLN-5</td>
<td>Claudin 5</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>EBA</td>
<td>Endothelial barrier antigen</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent protein</td>
</tr>
<tr>
<td>Endo KO</td>
<td>Endothelial-specific CCL2 knockout mice</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular Vesicles</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>FE-SEM</td>
<td>Field emission scanning EM</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GSST</td>
<td>Glutathione S transferase</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus type I</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional Adhesion molecule</td>
</tr>
<tr>
<td>LAM 1</td>
<td>Laminin 1</td>
</tr>
<tr>
<td>LBRC</td>
<td>Lateral Border Recycling compartment</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser Capture Microdissection</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LNC</td>
<td>Lymph node cell</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser Scanning Confocal microscope</td>
</tr>
<tr>
<td>MAOB</td>
<td>Monoamine oxidase B</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance-associated proteins</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle Tracking Analysis</td>
</tr>
<tr>
<td>NVU</td>
<td>Neurovascular Unit</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial Electrical Resistance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transendothelial migration</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial-cadherin</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very Late Antigen-4</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
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CHAPTER I

INTRODUCTION

1.1 The CNS: A castle protected by specialized barriers

The Central Nervous System (CNS) is the regulatory center of the body- consisting of two main parts, namely, the Brain, and the Spinal Cord. It controls our vital bodily functions, including awareness, movements, sensations, thoughts, speech, memory, and also influences our behavior; both conscious and unconscious. Though, contributing to only 2% of the total body mass, the brain demands a constant supply of nutrients and consumes 20% of the body's energy. As the name suggests, CNS is the 'control tower' where sensory information from the peripheral nervous system is fed, carefully integrated and interpreted, yielding intricate cognitive processing, regulation of motor functions, or emotional responsiveness (Purves et al., 2001). Precisely controlled gradients for ions such as Na⁺, K⁺, Cl⁻, and Ca²⁺ is indispensable in the CNS for eliciting action potentials. Therefore, a tight regulation of CNS homeostasis is required to avoid disturbances in function. Although, CNS is considered an immune-privileged environment, innate and adaptive immune defense mechanisms are operational at this site, as the immune cells can resolve certain viral, bacterial, fungal, and parasitic infections that affect the brain. However, these responses are not always beneficial. Owing to the presence of the rigid bone casing of the brain, i.e., the skull, classic features of an inflammatory response, such as swelling and expansion, can have detrimental consequences. Other pathologies, like the
ones underlying meningitis and encephalitis, might reduce neuronal function or induce their apoptosis (Wilson et al., 2010). Inflammation in the brain can thus contribute to the pathogenesis of a multitude of neurodegenerative diseases, including Multiple Sclerosis (MS), Parkinson disease and Alzheimer’s disease. Therefore, specialized structural and functional barriers, mainly, the blood-brain barrier, have evolved to protect it, and to maintain a precisely regulated milieu for reliable CNS activity while keeping the immune cells at bay.

1.2 The Blood-Brain Barrier (BBB)

The CNS vasculature is equipped to actively supply the brain with essential nutrients and oxygen through specific transport mechanisms. Interestingly, these endothelial cells that line the lumen of the CNS vessels also establish an intricately regulated and specialized barrier to protect the central nervous system (CNS) from unwanted molecules, cytotoxic compounds and immune cells in the systemic circulation, called the Blood-Brain Barrier (BBB). In effect, the BBB is not a rigid barrier, but rather a dynamic structure that receives continuous input from the resident CNS cells it protects, e.g., endothelial cells, astrocytes, pericytes, and neurons. This intricate communication allows for a thorough response to the local demands for oxygen, nutrients, and buffering, which is crucial for the maintenance of CNS homeostasis that favors optimal neuronal function (Mizee et al., 2014). All areas of the brain possess a BBB excluding the ones in direct contact with the ventricular system, termed circumventricular organs, which are protected via the blood-cerebrospinal fluid barrier (BCSFB) instead.
1.3 The History of BBB

The concept of BBB has been recognized for its physiological existence for over a century. The earliest evidence of “blood-brain barrier” dates back to the work of Paul Ehrlich (1885, 1906) and his student, Edwin Goldman (1909, 1913), who found that water soluble dyes injected into the peripheral circulation were excluded from the brain and cerebrospinal fluid (CSF); however the choroid plexus showed heavy staining. This concept of a BBB was further bolstered through subsequent experiments, whereby, injection of dyes into the brain through subarachnoid space showed staining in CSF but not in the peripheral tissues. In 1950, Tschirgi showed through a series of experiments that these dyes can bind to albumin, a plasma protein widely employed as an indicator of BBB permeability in in vitro models.

The existence of BBB in cerebral blood vessels was confirmed by Biedl and Kraus (1898). They observed intravenously injected sodium ferrocyanide or cholic acids had no effects on the central nervous system (CNS) pharmacologically. However, intraventricular administration of the same agent produced neurological symptoms post injection. It was not until 1900 that Lewandowsky first introduced the term “blood-brain barrier”. Later, a series of additional experiments carried out by Goldman in 1909 and 1913 with the Trypan blue injection in animals further proved the existence of BBB.

Later on, Friedemann (1942) found that lipid soluble dye could permeate across the CNS microvessels and stain the brain. In 1941, Broman introduced his findings of two barrier systems in the brain- one at the choroid plexus, called BCSFB, and the other at the cerebral
microvasculature, called BBB. BBB constitutes the largest barrier in the brain, featuring a continuous layer of specialized endothelial cells connected by tight junctions, such as the lining of capillaries (Figure 1). BCSFB exists at the choroid plexus epithelium and at the avascular arachnoid epithelium under the dura mater that completely encases the brain. The barrier function of BCSFB is provided by the junctions between epithelial cells, which are slightly more permeable than those of the brain endothelial cells that form the BBB. These barriers within the CNS serve multiple protective functions for the brain.

Broman (1941) argued that the capillary endothelial cells and not the astrocytic endfeet confers the blood-brain barrier function. Through the development of high resolution electron microscopy, as well as sensitive tracer methods; the BBB was structurally shown to exist at the astrocytic and endothelial level in 1967. It was revealed by Reese and Karnovsky ultrastructurally using electron-dense tracer horseradish peroxidase (HRP), a 39.8 KDa glycoprotein, that endothelial cells in mouse cerebral capillaries form a structural barrier to HRP. They found that HRP could travel through the first luminal inter-endothelial tight junctions. The tight junctions between the adjacent endothelial cells at the BBB appeared to be continuous; pinocytotic vesicles could hardly be found and were not engaged in the transport. Other experiments with smaller protein tracers such as microperoxidase and ionic lanthanum were tested through intravenous injection by Feder in 1971, and Brightman and Reese in 1969, respectively. All these experiments demonstrated the contribution of tight junctions in BBB’s ability to actively keep out specific substances from entering the brain interstitial fluid.
1.4 The building blocks

It has been shown that the microvascular endothelium, astrocytes, pericytes, neurons, and extracellular matrix exist as an intricately regulated structural and functional network, to constitute the “neurovascular unit” (NVU). Therefore, a knowledge of the structural and functional regulation of the NVU is critical for understanding the development and physiology of the BBB (Cohen et al., 1996; Neuwelt, 2004; Wang et al., 2004). The concept of the NVU establishes a framework for an integrative approach to understanding how the CNS responds in vasculopathies or inflammation (Lo et al., 2004), while providing a basis to understand the intricate pathways by which might alter the microvascular permeability in disease.

1.4.1 Component Cells- The Neurovascular Unit

The BBB is an integral component of the neurovascular unit (NVU). The NVU is comprised of CNS microvessels in close proximity to the astrocytic end feet, pericytes, and neuronal processes (Figure 1). Complex intercellular signaling among the NVU components regulates proper neuronal activity. The endothelium separates blood and the brain parenchyma and selectively allows vital nutrients to reach the brain while restricting the passage of neurotoxic substances or circulating immune cells. The endothelial cells that line the CNS microvessels and house the BBB establish a thin basement membrane (i.e. basal lamina) supporting the abluminal surface of the endothelium. This basement membrane surrounds the endothelial cells and the juxtaposed pericytes; the region between which is known as the perivascular space. Astrocytes are closely apposed to the endothelium, with astrocytic end feet comprising
a second basement membrane, termed the \textit{glia limitans}. The vascular contractility is partially contributed by the pericytes, that contain contractile proteins as well as a number of vasoactive mediators (Hamilton et al., 2010).
Figure 1. The neurovascular unit (NVU). Schematic cross-sectional representation of a CNS capillary. The capillary lumen is surrounded by a single endothelial cell (EC). TJs exist at the interface of the two apposing endothelial membranes. Pericytes are juxtaposed on the abluminal surface of the endothelial cell, and these two cell types are ensheathed by a common endothelial basement membrane. The endothelial basement membrane is closely juxtaposed by the astrocyte basement membrane or glia limitans at the astrocyte end-feet and the interneurons. The BBB is a highly restrictive yet dynamic barrier between the blood and the CNS tissue, intricately regulated both structurally and functionally by these components of the NVU.
Astrocytes

It has long been thought that astrocytes are critical in the development of BBB characteristics (Davson and Oldendorf, 1967) and regulate endothelial cell proliferation, survival, migration, differentiation, and vascular branching (Hellstrom et al., 2001). The contribution of astrocytes in the establishment of the BBB was demonstrated through the injection of purified neonatal astrocytes into the anterior chamber of the eye. These injected astrocytes formed aggregates that were rapidly vascularized. Interestingly, intravenous injection of Evans blue showed that these newly formed microvessels excluded the dye from the neighboring astrocytes; similar to that observed in the brain (Janzer and Raff, 1987). Similarly, coculture of brain endothelial cells (BMECs) with astrocytes (Tao-Cheng et al., 1987; Neuhaus et al., 1991) or with the conditioned media from astrocytes (Maxwell et al., 1987) has been shown to strengthen the BBB properties in vitro. It is believed that astrocytes can act as intermediaries acting in concert with neurons to regulate CNS microvascular permeability (Ballabh et al., 2004), particularly through a dynamic Ca$^{2+}$ signaling between astrocytes and the BMECs via gap junctions and purinergic transmission (Braet et al., 2001; Zonta et al., 2003).

Pericytes

Pericytes are flat, undifferentiated, contractile connective tissue cells, which develop closely apposed to the capillary wall. They maintain a close physical association with the endothelium and are ensheathed by a common basement membrane or the basal lamina. Pericytes are thought to influence the brain endothelium, through their specialized junctions, e.g., gap
junctions, tight junctions, and adherens junctions. Pericytes extend cellular processes, which penetrate the basal lamina and cover approximately 20-30% of the microvascular circumference (Frank et al., 1987). Although the molecular mechanism by which pericytes regulate vascular integrity isn’t well understood, perivascular pericytes have been reported to release growth factors and angiogenic molecules might regulate microvascular permeability and angiogenesis. Lack of pericytes can lead to endothelial hyperplasia and irregular vascular morphology (Hellstrom et al., 2001). It is believed that pericytes of the BBB might derive from microglia, since they can phagocytose exogenous proteins in the CNS (Coomber and Stewart, 1985). Additional evidence shows that pericytes are able to mimic astrocyte ability to enhance "tightness" of the BBB (Minakawa et al., 1991). The blood flow in CNS capillaries has been shown to be partly regulated by pericytes via rhythmic contractions and relaxtions (Yemisci et al., 2009).

**Neurons**

The high metabolic demand of neurons and the dynamic pattern of their activity require a tight regulation of the CNS microcirculation to supply essential nutrients and drain out the waste. The coupling of brain activity and CNS blood flow is therefore indispensable for normal neuronal function (Mizee and de Vries, 2013). An intricate crosstalk between the components of the NVU is thus required for the regulation of CNS blood flow by neurons; the vascular contractility however, is partly contributed by the pericytes (Hamilton et al., 2010). In addition, neurons can also directly innervate brain endothelium or juxtaposed astrocytes for mediating
the neuronal-endothelial coupling. Therefore, neuronal damage is often found to be associated with the disruption of BBB integrity underlying pathological changes in CNS blood flow. In support, noradrenergic, serotonergic, cholinergic, and GABA-ergic neurons have been found to directly contact the microvascular endothelium. Although, the exact mechanism is unknown, neurons innervating the NVU are believed to contribute to BBB permeability. For instance, the loss of cholinergic innervation of the CNS microvasculature causes impaired cerebrovascular functioning in Alzheimer’s disease. In summary, neurons in the NVU not only regulate the CNS blood flow, but also directly influence BBB permeability, by innervating the microvascular endothelium.

1.4.2 The Anchor- The Extracellular Matrix (ECM)

Besides the astrocytes, pericytes, and neurons, the ECM of the basal lamina also interacts with the CNS microvascular endothelium for establishment of the BBB (Hawkins and Davis, 2005). Pathological ECM damage correlates with increased BBB permeability (Rosenberg et al., 1993; Rascher et al., 2002). The ECM serves as an anchor or scaffold for the specialized CNS endothelial cells via interaction of laminin and other matrix proteins with the endothelial integrin receptors (Hynes, 1992). Such cell-matrix interactions trigger many intracellular signaling pathways (Tilling et al., 2002), and regulate the expression and localization of endothelial TJ proteins (Tilling et al., 1998; Savettieri et al., 2000). This suggests, endothelial-ECM interactions are required for maintenance of the TJs that pose an impediment to paracellular diffusion of soluble substances and immune cells into the CNS.
1.4.3 The Glue- Junctional proteins

The interendothelial regions of cerebral microvasculature houses junctional complexes that includes adherens junctions (AJ) (Schulze and Firth, 1993), tight junctions (TJ) (Kniesel and Wolburg, 2000; Wolburg and Lippoldt, 2002; Vorbrodt and Dobrogowska, 2003), and perhaps gap junctions as well (Tao-Cheng et al., 1987; Braet et al., 2001; Kojima et al., 2003; Simard et al., 2003). The AJ and TJ serve to restrict the paracellular passage of soluble molecules and immune cells entering the brain parenchyma (Bazzoni and Dejana, 2004).

Tight Junctions (Claudin, Occludin and ZO proteins)

TJs are comprised of three integral membrane protein families (claudins, occludin, and junction adhesion molecules) and several accessory or adaptor proteins including zona occludens (ZO-1, ZO-2, ZO-3), cingulin, paracingulin, and others (Kniesel et al., 2000; Gumbiner et al., 1991; Haskins et al., 1998). Claudins are 22-kDa phosphoproteins that are major components of the tight junctions (Furuse et al., 1999). Over 20 members of the claudin family have been identified so far (Morita et al., 1999). Claudins mostly interact with other claudins on adjacent endothelial cells homotypically and establish the primary seal of the tight junction (Furuse et al., 1999). The carboxy terminus of the claudins interacts with cytoplasmic adapter proteins, like ZO-1, ZO-2, and ZO-3 (Furuse et al., 1999). The ZO proteins together with cingulin and many others are cytoplasmic proteins involved in tight junction formation (Citi et al., 1988; Stevenson et al., 1989). ZO-1 and ZO-2 bridge the integral TJ proteins to the actin cytoskeleton at their carboxy terminus (Furuse et al., 1999). This critical link serves for structural stability of the BBB.
and is an important means of regulating paracellular permeability (Hawkins et al., 2005). Occludin is a 65-kDa phosphoprotein with four transmembrane domains, a long carboxy-terminal cytoplasmic domain, and a short amino-terminal cytoplasmic domain (Ueno et al., 2007; Furuse et al., 1993; Ando-Akatsuka et al., 1996). Two extracellular loops of occludin and claudin from neighboring cells can also establish a paracellular barrier at the tight junctions (Ueno et al., 2007). Occludin is also linked to the ZO proteins and regulates permeability through their association with the actin cytoskeleton (Mitic et al., 2000). Junctional adhesion molecules (JAMs) are 40-kDa membrane proteins that also bind ZO-1 (Ebnet et al., 2000). Of the three JAM molecules identified, only JAM-1 and JAM-3, but not JAM-2, are expressed in brain endothelium (Aurrand-Lions et al., 2001). JAM-1 localizes with actin and is involved in cell-to-cell adhesion (Aurrand-Lions et al., 2001).

**Adherens junctions (VE-cadherin, JAMs)**

AJ establish adhesive contacts between cells and consist of the membrane protein cadherin that connects to the actin cytoskeleton via intermediary proteins, called catenins (Ueno et al., 2007; Watabe et al., 1994). AJJs form homophilic interactions between the exposed extracellular domains of cadherins on the surface of connected cells (Lampugnani et al., 1995). The cytoplasmic domains of cadherins bind to β- or γ-catenin, which are in turn linked to the cytoskeleton via α-catenin (Lampugnani et al., 1995). AJJs interact with TJs via ZO-1 and catenins to influence TJ assembly (Matter and Balda, 2003).
1.4.4 The delivery guys - Transporters

Although the BBB freely allows passage to oxygen, carbon dioxide, and small lipophilic substances, it actively regulates the entry of hydrophilic molecules such as glucose, amino acids, and other nutrients essential to life (Zlokovic, 2008). Thus, a major physiological function of the BBB is the stringent regulation of transport of nutrients and other molecules into and out of the brain tissue. In addition, BBB transporters are also involved in inactivation and reuptake of neurotransmitters (Zlokovic, 2008; Ueno et al., 2007). The TJs at the BBB maintain high electrical-resistance and only allow small lipid-soluble molecules (<400 Da) to enter the brain (Pardridge, 2007). All other substances requires specific transporters on either the apical or basolateral endothelial membrane to cross the BBB (Zlokovic, 2008; Ueno et al., 2007). Specialized carrier-mediated transport systems facilitate transport of nutrients such as glucose and galactose, amino acids, nucleosides, purines, amines, and vitamins down their concentration gradient from the blood to the brain (Zlokovic, 2008; Ueno et al., 2007). Transport of these nutrients is generally regulated by metabolic demand of the brain and the concentration of these substrates in the plasma. Receptor-mediated transport systems are present for aiding the transport of proteins and peptides, e.g., neuroactive peptides, chemokines, and cytokines, into the brain (Rennels et al., 1975; Ueno et al., 2007). Specific receptor-mediated transport systems are also present to allow larger proteins like transferrin, low-density lipoprotein (LDL), leptin, insulin, and insulin-like growth factor into the brain (Jones and Shusta, 2007). Active efflux transporters located on both apical and basolateral endothelial
membranes serve to flush neurotoxic substances from the brain back into the circulation. Many active efflux transporters have been identified, most belonging to the ATP-binding cassette (ABC) transporter superfamily (Zlokovic, 2008; Ueno et al., 2007). ABC transporters use ATP-bound energy for the transport of molecules across the cell membrane and include the multidrug resistance (MDR) transporter P-glycoprotein (P-gp) that mediates removal of toxic lipophilic metabolites and cationic drugs, multidrug resistance-associated proteins (MRP), the breast cancer resistance protein (BCRP), and others (Zlokovic, 2008; Ueno et al., 2007).

1.5 Crossing the line- Leukocyte transendothelial migration across the BBB in neuroinflammation

Leukocyte trafficking is critical for immune surveillance in the central nervous system (CNS). However, in several diseases the entry of leukocytes into the CNS is heightened, which can disrupt the blood-brain barrier (BBB) and trigger neuroinflammation. These pathologic processes result in BBB permeability, gliosis, and neuronal damage and/or demyelination, which contribute to neuroinflammation. The resulting neuronal damage and death are characteristic of many neuroinflammatory conditions including Alzheimer disease, multiple sclerosis, HIV-1 encephalopathy, sepsis, ischemia and reperfusion, and CNS tumors.

1.5.1 Routes of transendothelial migration

Leukocyte migration across the endothelium in an inflammatory milieu is an active process for the migrating leukocytes as well as the endothelial cells, and this entry of leukocytes into the
target tissues is tightly regulated. With increasing literature about the mechanisms controlling the endothelial cell-cell adhesion and barrier properties, this transmigration is predominantly believed to take place at the endothelial junctions, with the leukocyte squeezing through the adjacent endothelial cells—termed the ‘paracellular route’. This passage reportedly occurs in a zipper-like fashion, whereby the traversing leukocyte transiently replaces the homophilic interactions of transmembrane proteins localized at the endothelial junctions. However, this paradigm of leukocyte transmigration across the endothelium is challenged by a number of elaborate in vivo studies that provide evidence for an alternate exit pathway whereby leukocytes migrate through the endothelial cells—the ‘transcellular route’ (Engelhardt and Wolburg, 2004). It has been shown that transendothelial leukocyte migration (TEM) can occur through specialized membrane compartments either attached to the cell surface or proximal to the cell borders called lateral border recycling compartment (LBRC). For instance, TEM of monocytes and neutrophils across human endothelial cells involves trafficking of the LBRC to the site of transcellular diapedesis. In addition to PECAM, the LBRC is known to contain CD99 and JAM-A, but not vascular endothelial cell–specific cadherin (VE-cadherin; cadherin 5, CD144) (Mamdouh et al., 2009). Notably, based on the LBRC hypothesis, since, both paracellular and transcellular TEM happens close to the endothelial cell-cell junctions, it difficult to ascertain the exact route of exit undertaken by the pathogenic leukocytes in vivo. Moreover, the extent of inflammation, type of microvessels involved, and the immune cell subtype might further dictate one route preferentially over the other.
1.6 Exploring the unknown- Cues and routes for leukocyte transmigration across BBB

While the cascade of events that initiate leukocyte adhesion and diapedesis across the peripheral vascular beds has been well characterized (Rossi et al., 2011; Sallusto et al., 2012), we lack an appreciable understanding of the cues required for transendothelial migration (TEM) of leukocytes across the BBB in CNS. A knowledge of cues governing the exit of immune cells across the BBB in neuroinflammation, holds key to novel therapeutic strategies for treating a wide spectrum of diseases like Parkinson disease, Alzheimer disease and Multiple Sclerosis (Mae et al., 2001; Pander et al., 2002; Wiendl, 2002; Weber et al., 2012).

1.6.1 Knowledge of permissible sites- Endothelial Heterogeneity

Although the special attributes of endothelial cells that confer the BBB phenotype, has been nearly exclusively described in the capillaries, the terms “capillaries” and “microvessels” are often used interchangeably. Such a practice is inappropriate and dismissive of the fact that the microvasculature is constituted of morphologically and functionally unique vascular segments. The CNS microvasculature is comprised of arterioles (10–100 µm diameter), capillaries (4–10 µm diameter), and venules (10–100 µm diameter), each branch with its own distinguishing features (Simionescu and Simionescu, 1977). Though the unique properties of these distinct segments arise in part from the contributions of adventitial cells (e.g., pericytes and smooth muscle cells) associated with the microvascular wall, functional differences also originate from the heterogeneous nature of the endothelial cells themselves. Such endothelial heterogeneity raises a possibility that the BBB, as well as other aspects of vascular function in the CNS, is
restricted to specific microvessel subtypes. Considering the role of BBB in maintaining neuronal homeostasis, loss or permeability of this barrier can cause cerebral microvascular dysfunction as reflected in a multitude of neuroinfectious, neuroinflammatory, and neurodegenerative diseases (Floyd, 1999; Farkas and Luiten, 2001; Dietrich, 2002). Therefore, understanding the diversity along the CNS microvessels is required for effectively treating a myriad of neurologic disorders that involve vasculopathies. Thus, while the microvascular endothelium remains the undisputed source of the BBB, it is imperative to ask- Which specific branch or branches harbor the actual BBB properties and how does such heterogeneity affect microvascular response in physiology and pathophysiology? If endothelial heterogeneity reflects functional diversity, this will underline a division of labor along the brain microvascular endothelium, which might warrant differential response among the CNS microvasculature tributaries in disease. Thus, we believe that BBB properties might be endowed within specific microvascular segments. Alternatively, it is possible that is that select aspects of the BBB are differentially expressed by specific microvascular segments; with no one segment representing the BBB in its entirety (Ge et al., 2005).

1.6.2 The negotiation- Leukocyte-Endothelial Crosstalk

Leukocytes devise several tacks to breach the endothelium to enter the perivascular space, and subsequently find a way through the glia limitans, into the brain parenchyma (Sixt et al., 2001; Wu et al., 2009). The classic steps of capture-rolling-tethering, activation, arrest-crawling and transmigration/diapedesis have been well established in the peripheral vasculature and
extensively studied in the CNS microvessels as well. These steps require leukocyte-endothelial interactions via expression of cell adhesion molecules (CAMs) by the endothelial cells, such as intercellular CAM-1 (ICAM-1) and vascular CAM-1 (VCAM-1), as well as their cognate ligands on the leukocytes, e.g., leukocyte functional antigen-1 (LFA-1), and very Late Antigen-4 (VLA-4). Of further importance, is the contribution of extracellular matrix (ECM) components in leukocyte TEM across the BBB, as the laminin composition of the vascular and parenchymal BMs might determine accessibility to the CNS (Wu et al., 2009). Since, the integrins on the surface of resting leukocytes are not constitutively active, they have a limited ability to breach the BBB. However, freshly activated T cells can migrate into the CNS irrespective of their antigen specificity (Ludowyk et al., 1992; Hickey et al., 2001). The TEM of initial leukocytes through the BBB can cause barrier damage, favoring further leukocyte infiltration. Thus, the initial entry of pro-inflammatory leukocytes into the CNS in neuroinflammation might trigger other events, e.g., expression of inflammatory cytokines, reactive oxygen species (ROS) and matrix-metallo proteinases (MMPs), by these leukocytes, inducing further BBB disruption, allowing continued infiltration of pathogenic leukocytes (Larochelle et al., 2011).

1.6.3 Inflammatory chemokines at the BBB

An inflammatory response in the peripheral and central nervous system often begins when the injured cells release inflammatory mediators called cytokines and chemokines, into the immediate microenvironment that act to alter the function of neighboring cells or to attract immune cells into the affected region for repair (Chavarria and Alcocer-Varela, 2004; Ramesh
et al., 2013). Unlike in peripheral tissues, the expression and regulation of lymphoid and inflammatory chemokines within the brain microvasculature has not been explored extensively. Several chemokines are known to be expressed by the CNS microvasculature, including CCL2, CCL4, CCL5, CCL19, CCL21, and CXCL12. These molecules bind chemokine receptors expressed by activated mononuclear cells (Holman et al., 2010).

The chemokine CCL2 (formerly termed Monocyte Chemoattractant Protein-1, MCP-1) has long been established as a crucial mediator of inflammation within and outside the central nervous system (CNS). It can mediate the extravasation of mononuclear leukocytes into CNS and peripheral tissues (Leonard et al., 1991; Mantovani et al., 1993; Bennett et al., 2003; Toft-Hansen et al., 2006; Yadav et al., 2010). Elevated expression of CCL2 in the CNS has been a consistent observation in MS, and its animal correlate experimental autoimmune encephalomyelitis (EAE) (Izikson et al., 2002; Mahad and Ransohoff, 2003; Conductier et al., 2010). This chemokine’s singular importance in EAE progression was demonstrated by global CCL2 knockout (KO) mice (CCL2−/−), which showed diminished severity and delay in onset of disease. Using, adoptive transfer EAE experiments, this study further revealed, effector T cells from CCL2−/− mice could cause EAE in wild-type (WT) recipients but not vice versa (Huang et al., 2001). In another set of elegant bone marrow chimera studies, EAE induction was shown to be markedly diminished when bone marrow from WT mice was engrafted into lethally irradiated CCL2 mice, but not when bone marrow from CCL2−/− mice was delivered to the WT recipients (Dogan et al., 2008). Collectively, these findings underscore the role of CCL2 from
CNS sources in mediating EAE, perhaps for driving the circulating myelin-specific encephalitogenic leukocytes into the CNS tissue. However, the specific CCL2 sources in the CNS that significantly contribute to neuroinflammation, and the mechanism of their pathogenic action, still remains unclear, hindering its therapeutic targeting.

1.6.4 Reading the message in a package- Endothelial extracellular vesicles (EVs)

Recently, a heterogeneous group of nano-sized extracellular vesicles (EVs) shed by multiple cell types, including endothelial cells, into the plasma and other bodily fluids, have emerged as a means of complex intercellular communication that conveys a broad spectrum of bioactive molecules (including protein, mRNA, miRNA, and DNA) over long and short distances (Turturici et al., 2014; Raposo and Stoorvogel, 2013; Ludwig and Giebel, 2012; Camussi et al., 2010; Simons and Raposo, 2009). These EVs can be broadly categorized into exosomes (30nm to < 100nm in diameter) and microvesicles (100nm to 1μm in diameter). The cargo carried by EVs uniquely reflects the identity of the parent cell. Intercellular communication can be accomplished by bioactive molecules on the EV surface (e.g., an adhesion protein or receptor) or ones contained in the intravesicular lumen (e.g., mRNA, miRNA, DNA). Importantly, inflammation in and outside the CNS is known to trigger the release of EVs from the activated endothelial cells (Horstman et al., 2007; Meziani et al., 2008; Chironi et al., 2009; Leroyer et al., 2010; Dignat-George and Boulanger, 2011; Yuana et al., 2013). EVs are also recognized for their immunomodulatory capacity (Chaput et al., 2004; Thery et al., 2009; Chaput and Thery, 2011; Robbins et al., 2014). They can modulate leukocyte activation (Muturi et al., 2013),
and/or adhesion (Liu et al., 2012), and exhibit strong association with several autoimmune conditions including MS. This has led to EVs being characterized as a critical component in the nexus between inflammation and immunity (Morel et al., 2011). As such, EVs are considered critical biomarkers of inflammatory disease in general, and perhaps neuroinflammatory disease in particular (Liu et al., 2012; Colombo et al., 2012; Barteneva et al., 2013; Gupta and Pulliam, 2014; Saenz-Cuesta et al., 2014). EVs can also play a causative role in inflammatory processes. For example, Rautou et al. (2011) reported EVs isolated from atherosclerotic plaques promote monocyte adhesion and TEM across cultured human umbilical vein endothelial cells. Furthermore underscoring the correlation of EVs and CNS leukocyte extravasation in neuroinflammation is the reported elevation in plasma endothelial EVs in MS (Mandel et al., 2012). These EVs can bind and activate monocytes (Jy et al., 2004), and when isolated from plasma of MS patients in exacerbation – but not in remission – forge monocyte-EV complexes with enhanced capacity for TEM across cultured BMEC (Jimenez et al., 2005) Shedding of junction protein+ -EVs from cultured human BMEC (Haqqani et al., 2013), lung microvascular endothelial cells and aortic endothelial cells (Takahashi et al., 2013) further focuses attention on endothelial cells as promising key players in the EV:TJ protein+-leukocyte relationship. Conceivably, EVs from the endothelium may transfer TJ protein and/or mRNA to leukocytes, possible aiding their TEM at the permissive microvascular locales in the CNS.
Novel 3D analysis of Claudin-5 reveals significant endothelial heterogeneity among CNS microvessels

1. Abstract

Tight junctions (TJs) feature critically in maintaining the integrity of the blood-brain barrier (BBB), and undergo significant disruption during neuroinflammatory diseases. Accordingly, the expression and distribution of Claudin-5 (CLN-5), a prominent TJ protein in central nervous system (CNS) microvessels and BBB determinant, has been shown to parallel physiological and pathophysiological changes in microvascular function. Therefore, it is critical to quantify the expression and/or distribution of predominant TJ proteins, like, CLN-5 along the various CNS microvessel subtypes under physiological and pathological conditions, for understanding which microvessels serve as an actual seat for the BBB, and explore whether the BBB damage in neuroinflammatory diseases is restricted to specific subsets of CNS microvessels only. However, efforts to quantify CLN-5 within the CNS microvasculature in situ, by using conventional two-dimensional immunohistochemical analysis of thin sections, are encumbered by the tortuosity of capillaries and distorted diameters of inflamed venules. Herein, we describe a novel contour-based 3D image visualization and quantification method, employing high-resolution confocal z-stacks from thick immunofluorescently-stained thoraco-lumbar

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spinal cord cryosections, to analyze CLN-5 along the junctional regions of different-sized CNS microvascular segments. Analysis was performed on spinal cords of both healthy mice, and mice experiencing experimental autoimmune encephalomyelitis (EAE), an animal model of the neuroinflammatory disease multiple sclerosis. Results indicated that, under normal conditions, the density of CLN-5 staining (CLN-5 intensity/ endothelial surface area) was greatest in the capillaries and smaller venules, and least in the larger venules. This heterogeneity in junctional CLN-5 staining was exacerbated during EAE, as spinal venules revealed a significant loss of junctional CLN-5 staining that was associated with focal leukocyte extravasation, while adjacent capillaries exhibited neither CLN-5 loss nor infiltrating leukocytes. However, despite only venules displaying these behaviors, both capillaries and venules evidenced leakage of IgG during disease, further underscoring the heterogeneity of the inflammatory response in CNS microvessels. This method should be readily adaptable to analyzing other junctional proteins of the CNS and peripheral microvasculature, and serve to highlight their role(s) in health and disease.

2. Introduction

Significant restriction of paracellular movement of soluble substances between the central nervous system (CNS) parenchyma and systemic circulation is one of the properties conferred by the blood-brain barrier (BBB). Such limitation is generally considered to derive from an intricate circumferential network of specialized membrane contacts, tight-junctions (TJs), which exist between CNS microvascular endothelial cells (Furuse, 2010; Blasig and Haseloff, 2011;
Coisne and Engelhardt, 2011).

The integrity of the BBB has been reported to be compromised during neuroinflammatory and neurodegenerative diseases, with disruption of TJs widely thought to contribute significantly to pathology (Petty and Lo, 2002; Hawkins and Davis, 2005; Carvey et al., 2009; Coisne and Engelhardt, 2011; Grammas et al., 2011). Some reports have noted that dysregulated expression, dephosphorylation and/or redistribution of TJ proteins at the BBB precede signs of clinical disease (Morgan et al., 2007; Argaw et al., 2009; Bennett et al., 2010). Such findings have been interpreted that alterations in TJs play a causative role in the inflammatory process. In this regard, disruption of TJs might facilitate leukocyte diapedesis through weakened inter-endothelial contact points (Garrido-Urbani et al., 2008), and/or support extravasation of serum proteins to which leukocytes must attach to invade the CNS parenchyma (Pober and Sessa, 2007). Additional reports have pointed to TJ disruption and associated BBB damage as being a consequence of the leukocyte diapedesis process itself — particularly through the actions of leukocyte-derived matrix metalloproteinases and reactive oxygen species (Gidday et al., 2005; Pun et al., 2009; Moxon-Emre and Schlichter, 2011). And still others have found intermediate ground by linking opening of TJs to initial intimate contact between activated/infected leukocytes and the brain microvascular endothelium (Haorah et al., 2005; Suidan et al., 2008; Ivey et al., 2009). These interpretations are not mutually exclusive, and one mechanism may foster the others leading to protractive BBB dysfunction, TJ disruption, and a degenerative sequence of neurologic sequelae (Carvey et al., 2009).
TJs in the CNS are mainly comprised of three distinct families of integral membrane proteins, namely, occludin, junctional adhesion molecules A, B and C, and claudins (CLNs) – of which there are now more than 20 recognized isoforms in various endothelial and epithelial beds (Liebner et al., 2011; Paolinelli et al., 2011). In turn, these integral proteins are linked to the actin cytoskeleton through several scaffolding proteins, including zonula occludens (ZO) proteins 1, 2 and 3 (Hawkins and Davis, 2005; Abbott et al., 2006), which assist in regulating TJ performance and BBB phenotype through a variety of signal transduction cascades (Ishizaki et al., 2003; Fischer et al., 2005; Haorah et al., 2005; Zhong et al., 2008; Jalali et al., 2010; Morin-Brureau et al., 2011; Ma et al., 2012).

CLN-5 has been localized to endothelial cell junctions of CNS microvessels in situ (Morita et al., 1999; Wolburg et al., 2003; Dobrogowska and Vorbrodt, 2004; Sheikov et al., 2008) and ex vivo (Bake et al., 2009), as well in culture (Song and Pachter, 2003; Calabria et al., 2006; Nakagawa et al., 2009; Gesuete et al., 2011; Luissint et al., 2012). A critical role for CLN-5 in BBB function has further been established. Specifically, overexpression of CLN-5 in cultured brain microvascular endothelial cells was shown to heighten barrier properties (Ohtsuki et al., 2007), while its deficiency imparted size-selective loosening of the BBB in vivo (Nitta et al., 2003). In order to correlate altered status of TJs with BBB dysfunction and disease processes, it is thus imperative to be able to accurately assess expression and distribution of TJs proteins such as CLN-5 at the BBB in situ.
While two-dimensional (2D) assessment of immunofluorescent confocal images obtained from thin sections of CNS tissue has revealed apparent changes in the amount and distribution of TJ proteins with neuroinflammatory disease (Persidsky et al., 2006; Alvarez and Teale, 2007; Argaw et al., 2009), this approach is limited in scope as it is restricted to visualization of only a minor fraction of any given vessel segment. Because of the severe tortuosity of CNS microvessels, 2D analysis of thin sections is largely confined to vessels of larger diameter, e.g., venules or arterioles, cut in cross-sectional profile (Janacek et al., 2011). Longitudinal profiles are not acquired to any significant extent by this tack and, therefore, much of the intercellular TJ network embedded within the long axis of the vascular wall is excluded from quantitative morphometric assessment. The information thus acquired may fail to capture highly focal changes in TJ expression/distribution. Moreover, cross-sections of the smaller diameter, but far more numerous, capillaries cannot accurately be evaluated for TJ expression as their circumference contains but only one to two cells. As endothelial heterogeneity may dictate that arterioles, capillaries, post-capillary venules and venules, differentially contribute to the BBB and inflammatory processes (Vorbrodt et al., 1986; Ge et al., 2005; Bechmann et al., 2007; Macdonald et al., 2010; Saubamea et al., 2012), it is possible that TJ responses and their physiological consequences are highly segment-dependent. Thus, it is important to be able to assay TJ expression qualitatively and quantitatively within all segment types along the CNS microvascular tree. It would further be advantageous to sample thicker tissue sections and access as much of the vascular surface as possible so as not to miss events that might be spatially restricted or polarized, and correlate TJ effects with those occurring in the perivascular
spaces during inflammation such as step-wise penetration of leukocytes from lumen into the CNS parenchyma (Sixt et al., 2001; van Horssen et al., 2005).

With these considerations in mind, and using CLN-5 as an example, herein we describe a protocol aimed at providing more accurate focal information, greater resolution and enhanced spatial perspective regarding junctional TJ proteins during CNS inflammation. Specifically, expression of CLN-5 was analyzed by subjecting microvessels in thick sections to 3D rendering, yielding both qualitative and quantitative information about the status of BBB integrity, and its relationship to inflammatory disease. Using this protocol to analyze CNS tissue from mice inflicted with experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (Mix et al., 2010; Batoulis et al., 2011), examples are presented to show alterations in the amount and distribution of CLN-5 at inter-endothelial junctions of spinal cord microvessels, and how these changes correlate with other vascular manifestations of inflammation.

3. Materials and methods

3.1 EAE induction

Female C57BL/6J mice (Charles River Laboratories), age 8–10 weeks, were used throughout. All animal protocols were in compliance with Animal Care and Use Guidelines of the University of Connecticut Health Center (Animal Welfare Assurance #A3471-01). Active EAE was induced as recently described (Murugesan et al., 2012), following a modification of the procedure of Suen et al. (1997). Subcutaneous flank injection of 300μg MOG35-55 peptide
(MEVGWYRSPFSRVVHLRNGK; synthesized by the Keck Biotechnology Resource Center at Yale University) in complete Freund’s adjuvant (Difco) containing 300 μg M. tuberculosis was performed on day 0 (d0), and supplemented by intraperitoneal injections of 500 ng pertussis toxin (List Biological) on d0 and d2. The typical disease that results from this protocol is monophasic, with acute symptoms beginning ~ d10–d12, and peak clinical disease appearing by ~ d15–d20, associated with ascending paralysis. Chronic disease then continues with disability achieving a plateau or diminishing somewhat by d25. The mice were scored on a scale of 0 to 5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, hind and fore limb paralysis; and 5, moribund.

3.2 Tissue preparation

At designated times post-EAE induction, mice were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, ip) in phosphate buffered saline, pH 7.4 (PBS). Following exposure of the heart by left anterolateral thoracotomy, the mouse was transcardially perfused (via the left ventricle) first with Heparin-PBS (10 usp/ml), to flush out the blood, and then with fixation buffer (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4), using an “in-house” constructed gravity perfusion apparatus.

Laminectomy was performed for harvesting the spinal cord. Briefly, the entire spinal column containing the spinal cord was removed and, after clearing the overlying ligaments and muscle, was incubated in fixation buffer for 2 hours at room temperature. The lamina was then ektomized by opening the spinal canal from the C1 to L5 vertebra, breaking one at a time using
a pair of fine laminectomy forceps. The dissected spinal cords were post-fixed again in fixation buffer for 30 min, and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer, pH 7.4, overnight at 4 °C prior to freeze-embedding in cryomatrix. Subsequently, 12 × 60 μm cryosections were obtained from the thoraco-lumbar region, approximately between the T10 and L3 vertebrae (Figure 2c), using a Thermo Fisher Scientific microtome (maintained at −25 °C), and adhered to poly-l-lysine coated slides.

3.3 Immunostaining

Sections were permeabilized with 1% Triton X-100 in PBS for 30 min, and non-specific binding blocked by incubation with Powerblock® in UltraPure™ (GIBCO) distilled water for 10 min. The microvascular endothelium was stained by rat anti-mouse CD31 (BD Pharmingen; 1:100 dilution) followed by incubation with goat anti-rat Alexa® 555 (Life Technologies; 1:200). The basement membranes were labeled with rabbit anti-mouse Laminin 1 (Cedarlane; 1:100) and goat anti-rabbit Alexa® 555 (Life Technologies; 1:200). Anti-mouse Claudin5-Alexa® 488 (Life Technologies; 1:150) was employed to highlight the TJs at the interendothelial borders. Anti-mouse IgG-Alexa® 488 or IgG-Alexa® 555 Fab’ fragment (Life Technologies; 1:200) was utilized to detect the leaked endogenous serum IgG from inflamed CNS microvessels. Anti-mouse CD4-Alexa® 488 antibody (generously provided by Dr. H. E. de Vries, VU medical center, Netherlands) was used to immunolabel the perivascular leukocytes associated with inflamed microvessels. Additionally, nuclear stain DRAQ5 (Biostatus Ltd., Leicestershire, UK) was utilized to reveal the perivascular cellularity surrounding inflamed CNS microvessels due
to leukocyte extravasation. Sections were mounted in Mowiol® 4-88 (Sigma-Aldrich, Missouri, USA).

3.4 Image acquisition

Spinal cord microvessels (capillaries and venules) from the dorsolateral white matter (between T10 and L3) were imaged and categorized into appropriate segments based on their average diameter (Figs. 2.1a–c). This region, just underneath the meninges, was intentionally selected for analysis as it sustains the earliest inflammation in the spinal cord parenchyma in the MOG35-55 EAE model (Brown and Sawchenko, 2007). Confocal z-stacks were acquired (at 1 μm increments between z-slices) following a multitrack scan, using a Zeiss LSM 510 Meta confocal microscope equipped with a 40 × Zeiss Fluar (NA 1.3, determining voxels of 0.62 × 0.62 × 1 μm3) and a 63 × Plan-neofluar (NA 1.25, determining voxels of 0.39 × 0.39 × 0.51 μm3) oil immersion objective lens. Stitching of z-stacks from overlapping regions (in x, y plane) of the same vessel was performed by XuV stitch v1.8 software (Free Software Foundation Inc., Boston, USA).

3.5 3D quantification of microvascular CLN-5 density

To quantify relative CLN-5 protein expression within select microvessels, confocal z-stacks were imported into Imaris® suite version 7.1 × 64 software (Bitplane Inc., South Windsor, CT). Fluorescence intensities above a background value were assigned for each color channel in the volume rendered image (i.e., 3D reconstruction) and kept constant for all the acquired
z-stacks. For quantification in 3D, manual contour tracing was first performed by cursoring out the vessel of interest in each confocal z-slice and the individual contours merged into a 3D contour surface. Surface area of the generated 3D contour was used as an estimate of the “microvascular surface area” defined by the endothelial layer. This 3D contour surface was subsequently used as a mask to isolate only the microvessel of interest from rest of the dataset by setting all voxel intensities “outside” the 3D contour surface to zero. The CLN-5 channel was isosurface rendered using surface creation wizard and the density of CLN-5 calculated as follows:

\[
\text{Total CLN-5 intensity} = \text{Mean CLN-5 intensity} \times \# \text{ of voxels}
\]
\[
\text{CLN-5 intensity per unit area (i.e., CLN-5 density)} = \frac{\text{Total CLN-5 intensity}}{\text{Microvascular surface area}}
\]

In effect, this method allowed the surface area of the microvascular endothelium to be spread out in 3D space (x, y, z axes) for quantification of CLN-5 density per unit area (Figs. 2.1d–e), while excluding the luminal volume (Figure S1a–b).

3.6 Re-slicing isosurface-rendered images

The Clipping Plane module on Imaris® was employed to optically “re-slice” 3D isosurface rendered microvessels along a desired oblique plane, in a manner perpendicular to axis of the microvessel, so as to uncover hidden views and resolve the interior versus exterior or luminal versus abluminal vascular compartments. This tool further allowed resolution of the microvascular basement membrane into its endothelial and parenchymal counterparts, which split apart during neuroinflammation to accommodate the CNS invading leukocytes.
(Engelhardt and Sorokin, 2009).

To introduce the clipping plane tool into the 3D dataset, its icon in the object list was selected and the clipping plane first placed along a desired orthogonal plane (xy, yz or xz) with the manipulator (Figure S1). To further orient the clipping plane in an oblique plane, and align it perpendicular to axis of the microvessel for a cross-sectional view, the manipulator was selected and rotated as required. Finally, to alter the location of the clipping plane along the same axis, and thereby obtain different depths within the vessel, the manipulator was re-positioned accordingly.

3.7 2D quantification of CLN-5 immunostaining

Background subtracted volume rendered confocal z-stacks of venules from thoraco-lumbar spinal cord were exported from Imaris® as tiff images for ImageJ (NIH, Maryland, USA) based 2D quantification of mean CLN-5 pixel intensities along the interendothelial junctions. Relative intensity values corresponding to the level of CLN-5 immunostaining were measured over 30–50 ROIs, each defining 10 × 10 pixels, traced in a non-overlapping manner along the intercellular borders in each venule, as previously described (Song and Pachter, 2004). For inflamed venules from EAE mice, ROIs were sampled from regions with visible reduction in CLN-5 immunoreactivity, and displaying dense perivascular cellularity. For venules from naïve mice, ROIs were randomly selected along the microvessel profile. Mean pixel values were then obtained by averaging the intensity values of all ROIs.
3.8 Statistical analysis

All statistical analyses were performed employing GraphPad Prism 5 software (La Jolla, CA, USA). Microvascular CLN-5 density values obtained from Imaris® 3D image analysis were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was employed to assess statistically significant differences in junctional CLN-5 density between microvascular segments from naïve and d24 EAE mice, followed by Bonferroni's multiple comparison post-hoc analysis. For assessing the relationship between CLN-5 density and microvessel diameter, a Pearson product–moment correlation coefficient was determined. Comparison of CLN-5 mean pixel intensity values from 2D images of venules from naïve versus d24 EAE mice was performed by one-tailed unpaired Student's t-test. Results were considered significant at a p ≤ 0.05.

4. Results

4.1 Segmental heterogeneity of CLN-5 density in naïve spinal cord microvessels

To initially determine if normal spinal cord microvessels demonstrate segmental heterogeneity with respect to CLN-5 density at endothelial junctions, spinal cord sections from naive mice were immunostained for CLN-5. Isosurface rendering of high-resolution confocal z-stacks obtained from the different-sized microvascular segments were subjected to 3D quantification of endothelial CLN-5 density. Microvascular segments were classified as larger venules (> 20 μm in diameter); smaller, possibly ‘postcapillary’ venules (10–20 μm in diameter); and
capillaries (< 10 μm in diameter). This classification was based primarily on a consensus of established size criteria (Fawcett, 1994, Leeson et al., 1988, Ross and Pawlina, 2006 and Simionescu and Simionescu, 1977), as there is no widely recognized marker that distinguishes postcapillary venules (Owens et al., 2008). The smaller venules were often seen connecting capillaries to larger venules, lending support to the smaller venules being postcapillary in nature. To further verify venule identity, 3D isosurface rendered datasets were re-sliced along a desired plane using the clipping plane module in Imaris® to analyze microvessels in cross section (Figure S1c). The presence of a smooth, non-puckered lumen lent to microvessels (> 20 μm in diameter) being classified as venules rather than arterioles (Macdonald et al., 2010).

Isosurface rendered z-stacks of venules and capillaries revealed appreciable heterogeneity in endothelial CLN-5 density (CLN-5 staining intensity/μm2 microvascular surface) in naïve mice (Figs. 2.2a–f). Specifically, 3D quantification of CLN-5 density in larger venules yielded a significantly lower mean value than that found in smaller, venules (19.95 ± 1.65 vs. 48.89 ± 2.97, respectively). CLN-5 density in larger venules was also significantly lower (2-fold) than that in naïve capillaries (19.95 ± 1.65 vs. 39.77 ± 2.54, respectively). However, junctional density of CLN-5 was not statistically different between capillaries and the smaller diameter venules. These results suggest that, under normal conditions, the density of CLN-5 at endothelial junctions within CNS microvessels tends to vary inversely with microvessel diameter, being greatest in the capillaries and smaller venules, and least in the larger venules (Figure S3). Specifically, a significant negative linear correlation coefficient was
established with a Pearson's correlation coefficient \( r = -0.73 \).

### 4.2 Reduction of CLN-5 density in spinal cord venules during MOG-induced EAE

As MOG-induced EAE has been reported to result in diffuse TJ breakdown and relocalization of the TJ scaffolding protein ZO-1 in CNS microvessels (Bennett et al., 2010), we evaluated if EAE affects junctional CLN-5 density equally in various microvascular segments (capillaries vs. venules) at the peak of disease.

Isosurface rendering of spinal cord venules at d24 EAE revealed significant disruption of CLN-5 distribution at sites of dense perivascular cellularity (Figure 4a and b). These sites largely coincided with CD4\(^+\) leukocyte infiltrates (Figure S2) and thus corroborated the vessels as inflamed. Moreover, there was considerable heterogeneity in junctional CLN-5 loss among venules — possibly reflecting a range in inflammatory status and/or vulnerability within this vessel population. The venules analyzed varied in diameter from \( \sim 20 \) to \( 50 \) μm — a range shown to become inflamed in this and similar EAE paradigms (Bergman, 2012 and Pfeiffer et al., 2011) — and thus could have included initially smaller, postcapillary venules that had distended in size due to disease. In keeping with the prior description that relocalization of the TJ scaffolding protein ZO-1 correlates with sites of inflammatory cell accumulation (Bennett et al., 2010), venules qualitatively showing near complete breakdown of CLN-5 staining pattern and displaying a CLN-5 density of \(< 10\) were considered to be severely inflamed. By contrast, those venules showing small punctate regions of CLN-5 loss and having a CLN-5 density of \( \geq 10\) were considered moderately inflamed. Capillaries in regions with even severely inflamed
venules appeared refractory to CLN-5 loss (Figure 4c). Specifically, quantification of junctional CLN-5 density revealed a 5-fold difference in severely inflamed venules compared to capillaries (4.79 ± 0.78 vs. 24.0 ± 5.85, respectively), while moderately inflamed venules and capillaries did not significantly differ in this measure (12.95 ± 0.49 vs. 24.0 ± 5.85, respectively). As the moderately inflamed venules displayed only small punctate regions of CLN-5 loss, it was unclear if they had yet to suffer diffuse extensive disruption, were less susceptible to disruption, or in stages of repair. As all size vessels analyzed in naïve mice clearly showed continuous CLN-5 staining along interendothelial borders in a 60μm section (Figure 3), it is unlikely that the significant reduction in venular CLN-5 junctional density in EAE mice was due to obstacles to antibody penetration. Instead, it probably reflected a differential response in this vessel population.

4.3 Comparison of junctional CLN-5 density in inflamed venules from EAE mice and normal venules from naïve mice

Having first established the heterogeneity of CLN-5 density at endothelial junctions within the normal spinal microvasculature, and then within the diseased microvasculature during EAE, we next directly compared capillaries and venules from naïve and EAE mice. Results reveal that d24 EAE venules had significantly lower (~ 3.9 fold) CLN-5 density compared to both naïve venules (8.87 ± 1.31 vs. 34.42 ± 6.65, respectively) and naïve capillaries (8.87 ± 1.31 vs. 34.74 ± 3.70, respectively) (Figure 5i). Additionally, there was no statistically significant alteration in CLN-5 density between the naïve and d24 EAE capillaries, or evidence of perivascular
cellularity associated with capillaries during disease. These findings highlight that venules are the primary sites of neuroinflammation-associated junctional CLN-5 staining loss and possibly TJ breakdown, while capillaries appear to maintain their pattern of junctional CLN-5 immunoreactivity during EAE.

To confirm that our 3D analysis of CLN-5 density reflected diminished expression of the TJ protein at interendothelial junctions in venules during EAE, and not just redistribution of CLN-5 over a dilated, wider surface area, conventional 2D analysis of mean pixel intensity was performed along the intercellular junctional regions. Figure S4 shows that CLN-5 staining in inflamed venules from d24 EAE mice had significantly lower mean pixel intensity than that in venules of naive subjects (~4.2-fold). The higher variability of CLN-5 staining during EAE, as indicated by both increased standard deviation and standard error of mean pixel intensity measure, were consistent with the more fragmented, irregular appearance of TJ staining associated with neuroinflammatory disease.

4.4 Heterogeneity of CLN-5 density in contiguous spinal cord microvessels during EAE

Though diverse inflammatory responses of closely located microvessels have provided strong evidence of segmental endothelial heterogeneity (Thurston et al., 2000 and Xu et al., 2005), it is not clear if venules and capillaries directly attached to each other show such disparity within the inflamed CNS. Therefore, a contiguous venule/capillary pair from a d24 EAE spinal cord section was subjected to 3D reconstruction and isosurface rendering for CLN-5 (Figs. 2.5a–b), to visualize segmental changes in CLN-5 density at the endothelial junctions. In what amounts
to yet another clear display of segmental endothelial heterogeneity, a capillary emanating from a severely inflamed venule had intact junctional CLN-5 staining similar to that seen in capillaries of naïve mice. However, the immediately adjacent venule demonstrated near obliteration of CLN-5 junctional organization, along with separation of endothelial and parenchymal basement membranes and increased perivascular cellularity (Figure 6a, insets).

This finding reinforces the view that loss of junctional CLN-5 protein during neuroinflammation reflects an inherent susceptibility of CNS venules — a property not shared even by the most closely juxtaposed capillaries (Figure 6c).

4.5 Heightened microvascular permeability to endogenous serum IgG occurs in both capillaries and venules during EAE

Given the disparate CLN-5 response between venules and capillaries during EAE, we next sought to determine if both microvessel types evidenced inflammation-associated leakage of serum IgGs. Thick spinal cord cryosections from both d6 (early stage) and d24 (late stage) EAE animals were stained for endogenous IgG and the basement membrane protein laminin-1 (Lam-1), followed by 3D reconstruction and isosurface rendering of the z-stacked confocal dataset (Figs. 2.6a–d). IgG leakage was detected as focal deposits around both venules and capillaries at d6. Despite evidence of IgG extravasation at this early time, no loss of junctional CLN-5 was apparent (Figure S5). By d24, extravasation of IgG was so pronounced and diffuse that it obscured boundaries between the microvessel segments, though increased perivascular cellularity was associated with inflamed venules only (Figure 7).
5. Discussion

Given the importance of TJs in neuroinflammatory disease, and increasing awareness of endothelial heterogeneity, this report described a novel microvascular contour-based 3D quantification method of acquiring and analyzing expression of the TJ protein CLN-5, a critical BBB determinant, along different type microvessel segments of the spinal cord during EAE. Venules were shown to display significant loss of CLN-5 at intercellular junctions during EAE, which was accompanied by severe extravasation of leukocytes and disruption of basement membrane integrity. In stark contrast, capillaries showed none of these responses.

This 3D approach also allowed for capture and analysis of small diameter capillaries lying in close proximity to venular structures. Due to their small caliber and tortuosity, such capillaries are typically precluded from conventional 2D analysis of TJs in thin-sectioned material. However, in the current protocol capillaries and venules directly attached to each other could be readily contrasted, allowing for a clearer picture of their diverse phenotypes in physiology and pathophysiology to emerge. In this regard, the density of CLN-5 expression under normal conditions was observed to vary inversely with microvessel diameter, being greatest in the capillaries and smaller venules, and least in the larger venules. That the correlation coefficient was −0.73, and not closer to −1.0, might reflect that CLN-5 density reaches asymptotes at the extreme diameters of the microvascular tree, and/or the 3D quantification approaches its limits of accuracy at these extremes. Nevertheless, this inverse relationship of CLN-5 density with diameter size was significant. This discrepancy in CLN-5 density between capillaries and
venules was further exaggerated during EAE, as loss of CLN-5 expression appeared restricted to venular structures. Loss of junctional CLN-5 might render the venular endothelium more amenable to leukocyte extravasation via the paracellular pathway (Garrido-Urbani et al., 2008). Alternatively, it could be the result of a sustained transendothelial leukocyte migration specifically at venular domains (Xu et al., 2005).

Despite the most significant CLN-5 loss being reserved for venules, IgG leakage was nevertheless detected around all size microvessels during early and late EAE. As capillaries did not sustain significant CLN-5 loss even as late as d24 EAE, this might reflect that inflammation-associated IgG leakage at these sites occurred primarily through transcytosis (Claudio et al., 1989 and Proulx et al., 2012) — a process that, presumably, would not have required TJ breakdown (Kreuter, 2013). Venules, on the other hand, might have similarly employed IgG transcytosis early during EAE, but also engaged in paracellular leakage later following the extensive CLN-5 loss.

Xu et al. (2005) described similar breakdown of TJs and loss of CLN-1/3 and occludin at inter-endothelial contacts within retinal venules during a related condition, experimental autoimmune uveoretinitis. And in a further parallel with our results, they similarly reported retinal capillaries were apparently spared disruption of these TJ proteins — again highlighting endothelial heterogeneity and the differential endothelial response to inflammation. Using confocal microscopy of retinal whole mounts, this group has most recently elaborated a means to portray relative fluorescent intensity values of microvessel-associated CLN-1/3 in a 3D heat map
(Xu and Liversidge, 2011). Our work extends these studies by employing 3D isosurface renderings of individual microvessels, thereby allowing a holistic perspective of TJ protein distribution within the microvascular network, in addition to enabling relative quantification. Furthermore, the acquisition of high-resolution confocal z-stacks from 60μm thick sections supported analysis of local effects, which in thinner sections or 2D analysis might well have been missed.

The d24 time-point of EAE was selected for analysis as it is soon after the apex of clinical disease in this particular paradigm (Suen et al., 1997), and into the chronic phase when inflammatory histopathology is at or near maximum (Pachner, 2011). Thereafter, clinical presentation either plateaus or abates somewhat. The spinal cord region between T10 and L3 vertebrae was the area of focus as disease commences at the lumbo-sacral level and progresses in the caudal-to-rostral direction (Gruppe et al., 2012). We thus reasoned that by the d24 time-point, maximal CLN-5 disorganization would be achieved at the spinal level analyzed. It is significant that IgG leakage was apparent from both capillaries and venules during early (d6) and late (d24) EAE, though no reduction in CLN-5 density was apparent in capillaries at either of these time-points. This scenario underscores a differential responsiveness between CNS capillaries and venules vis-à-vis neuroinflammation-associated changes in CLN-5 density at endothelial junctions. That C57BL/6 mice display a similar overall CNS microvascular architecture and BBB transcriptome from mouse-to-mouse (Macdonald et al., 2010 and Ward et
al., 1990) perhaps contributed to the low variance in CLN-5 density within each group of microvessels analyzed, and aided in highlighting this heterogeneity.

Owing to the fact that microvascular density is greatly reduced in white matter compared to gray matter (Cavaglia et al., 2001), and venules constitute only a small percentage of the microvascular surface area (Berne and Levy, 1988), we were limited in the venule population to sample from the dorsolateral region. A further constraint was trying to capture venules with juxtaposed capillaries, so as to compare both basal and reactive CLN-5 expression by the two types of microvessel segments within the same or similar microenvironment. Nonetheless, sampling of 12 × 60μm sections between T10 and L13 vertebrae enabled sufficient acquisition of venules/capillaries for statistical comparisons, while minimizing differences in endothelial phenotype due exclusively to regional heterogeneity within the CNS (Ge et al., 2005).

In contrasting TJ protein during health and disease, and between different microvessel subtypes, we chose to express the density of CLN-5 expression in relation to microvessel surface area rather than microvessel volume, as volume (πr²h) increases with the square of the radius of a cylinder. As the lumen — which is ‘dead space’ — disproportionally contributes more to the vascular volume of bigger segments, reporting TJ protein density per unit volume would yield artifactually lower values in larger diameter vessels even if the number of TJ proteins per unit area of endothelial membrane were unaltered from vessel to vessel. This is important not only for comparing different vessel subtypes in healthy subjects, but also when evaluating changes in any one vessel subtype during disease, as vessel caliber may swell along with the
separation of basement membranes. We recognize, however, individual endothelial cells of larger diameter vessels may also be bigger than in the smallest capillaries, and that this could also lend toward a skewing of TJ protein density data being highest in the smaller vessels. It is nevertheless significant that Nagy et al. (1984) reported that the 'complexity' of brain TJ protein particles; i.e., the degree to which they comprise long, uninterrupted strands when viewed in freeze-fracture faces, is highest at the capillary end of the vascular tree and much less so at the venular end. Thus, our method of analysis yielded results consistent with the freeze-fracture technique, which displayed the en face vista of the interior of the cell membrane and focused exclusively at the intercellular junctions. That the reduced CLN-5 density in venules during EAE reflects diminished protein expression and not just dilation-associated distortion in endothelial cell size, is reinforced by our observations of reduced mean pixel intensity of CLN-5 immunostaining along the venular endothelial junctions during EAE, and the recent report describing loss of CLN-5 protein in EAE brain (Errede et al., 2012), as evidenced by Western blotting and quantitative optical densitometry. The current method should thus have broad applications in efforts to link changes in the expression and/or distribution of TJ proteins with focal microvascular incidents in the CNS and peripheral tissues.
Figure 2. 3D Contour-based quantification of junctional CLN-5 in spinal cord microvessels. (a) CNS venule from a naïve mouse detailing CLN-5 (Green) staining at intercellular junctions. The image shows microvascular tributaries (e.g. capillaries, post-capillary venules) emerging from a venule, whose lumen has been “optically” cut open to reveal the inner vessel wall. Endothelial cells are highlighted with CD31 (Red). (b,c) To gauge endothelial heterogeneity with respect to CLN-5, spinal cord microvessels (capillaries and venules) obtained in confocal z-stacks from the dorsolateral white matter (between T10 and L3 vertebrae) were imaged and categorized into appropriate segments based on their average diameter. (d) Schematic indicating this method effectively allows the surface area of the microvascular endothelium to be spread out in 3D space (x, y, z axes) for quantification of CLN-5 density per unit area, while excluding the luminal volume. (e) To quantify CLN-5 staining associated with a microvessel in 3D, an individual contour for each confocal z-slice was created by cursoring out the vessel of interest, and the individual z-slice contours then merged into a 3D contour surface. This contour surface was utilized to isolate the microvessel of interest from the rest of the dataset (masking), and its area was used as an estimate of the “microvascular surface area” defined by the endothelial layer. An isosurface for the CLN-5 channel was then created from the selected vessel for statistical analysis. Scale bar = 50µm.
Figure 3. Heterogeneity in CLN-5 density distribution among different-sized microvessels in naïve spinal cord. Isosurface rendering of the CLN-5 channel was performed in confocal z-stacks of different-sized spinal cord microvessels in tissue sections from naïve mice: (a,b) larger venules; (c,d) smaller venules; (e,f) capillaries. Top row, shows CLN-5 (Green) and nuclei/DRAQ5 (Blue). Bottom row, shows CLN-5 only, to emphasize the disparity in junctional CLN-5 immunostaining between the smaller and larger microvessels. (g,h) 3D contour-based quantification of CLN-5 density (intensity per unit surface area of the endothelium) within naïve spinal cord microvessels. Junctional CLN-5 density was greatest in the capillaries and smaller venules, and least in the larger venules. A total of 5 microvessels were analyzed in each group sampled from 3 mice. *p < 0.001. Scale bar = 15µm.
Figure 4. CLN-5 density in spinal cord microvessels during EAE. Isosurface rendering of the CLN-5 channel was performed in confocal z-stacks of spinal cord microvessels at d24 EAE. Top row, shows CLN-5 (Green) and nuclei/DRAQ5 (Blue) to highlight the close association of altered CLN-5 with dense perivascular cellularity. Bottom row, shows staining of only CLN-5 to emphasize significant TJ protein disruption. Inflamed venules demonstrated heterogeneity in CLN-5 loss: (a,b) severely inflamed venules displayed diffuse and extensive disruption of CLN-5; (c,d) moderately inflamed venules showed small punctate regions of CLN-5 loss; and (e,f) capillaries adjacent to severely inflamed venules appeared refractory to CLN-5 loss. 3D quantification of intercellular CLN-5 staining showed a significant reduction in intensity of CLN-5 staining per unit area of the endothelium in the severely inflamed venules compared to the capillaries (g,h). The boundaries of inflamed venules are marked with dashed white lines. A total of 6 microvessels were analyzed in each group sampled from 3 mice. *p < 0.0001. Scale bar = 20µm.
Figure 5. CLN-5 density in spinal cord microvessels from naïve vs. EAE mice. Isosurface rendering of z-stacked images of spinal cord sections from naïve mice and mice at d24 EAE. Top row, shows staining of CLN-5 (Green) and DRAQ5 (Blue) to highlight the close association of altered CLN-5 with dense perivascular cellularity (reflective of leukocyte infiltrates) during EAE. Bottom row, shows staining of only CLN-5 to emphasize significant TJ protein disruption that accompanies disease. (a,b) Venules from naïve mice; (c,d) Venules from d24 EAE mice; (e,f) Capillaries from naïve mice; and (g,h) Capillaries from d24 EAE mice. (i,j) 3D quantification of CLN-5 microvascular staining showed a significant reduction in CLN-5 density in inflamed venules compared to the naïve venules and naïve capillaries. The boundary of inflamed venule is marked with dashed white line. A total of 6 microvessels were analyzed in each group sampled from 3 mice. *p < 0.0001. Scale bar = 15µm.
Figure 6. Heterogeneity in CLN-5 density in a contiguous venule/capillary pair during EAE. (a) Isosurface rendered 3D reconstruction of a contiguous venule and capillary in spinal cord section from d24 EAE mouse, highlighting basement membrane/Lam1 (Red), CLN-5 (Green), and nuclei/DRAQ-5 (Blue). (b) Isosurface rendered CLN-5 channel only, with boundary of the inflamed venule marked with dashed white line. The venule shows severe loss and fragmentation of junctional CLN-5, while the attached capillary displays intact junctional CLN-5 staining. The insets reveal cross sections through the inflamed venule, optically cut using clipping plane module in Imaris®, demonstrating association of venular CLN-5 loss with seminal signs of inflammation: (top) separation of endothelial and astrocyte basement membranes (BM); and (bottom) increased perivascular cellularity. (c) Schematic representation showing, qualitatively, heterogeneity in CLN-5 density distribution in a contiguous venule/capillary pair at d24 EAE.
Figure 7. Endogenous serum IgG leakage from spinal cord microvessels during EAE. (a,c,e) Shows volume rendered images of confocal z-stacks from microvascular segments obtained from naïve mice, and mice at d6 and d24 EAE. (b,d,f) Shows the corresponding isosurface rendered images for purpose of enhanced spatial perspective. Staining of IgG (Green) and basement membrane/LAM 1 (Red) highlights vascular permeability around venules and capillaries. (a,b) Microvessels from naïve mice reveal no visible IgG immunostaining associated with venules or capillaries. (c,d) Microvessels at d6 EAE – prior to evidence of clinical disease – display focal IgG immunoreactivity around both venules and capillaries. (e,f) Microvessels at d24 EAE show pronounced and diffuse IgG immunoreactivity – reflecting endogenous serum protein extravasation – which obscured boundaries between the microvessel segments. Increased perivascular cellularity, indicative of leukocyte infiltration (inset), is highlighted by DRAQ5 staining (Blue). Scale bar = 20µm.
CHAPTER III

Cell-selective knockout and 3D confocal image analysis reveals separate roles for astrocyte- and endothelial-derived CCL2 in neuroinflammation

1. Abstract

Although, the tight junctions (TJs) at the blood-brain barrier (BBB) pose an impediment to entry of the circulating leukocytes into the central nervous system (CNS) under normal conditions, upregulated expression of pathogenic chemokines, like CCL2, by the resident CNS cells in neuroinflammation can mediate the transendothelial migration of pathogenic leukocytes across the BBB. Expression of chemokine CCL2 in the normal central nervous system (CNS) is nearly undetectable, but is significantly upregulated and drives neuroinflammation during experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and considered a contributing factor in the human disease. As astrocytes and brain microvascular endothelial cells (BMEC) forming the blood-brain barrier (BBB) are sources of CCL2 in EAE and other neuroinflammatory conditions, it is unclear if one or both CCL2 pools are critical to disease and by what mechanism(s). Mice with selective CCL2 gene knockout (KO) in astrocytes (Astro KO) or endothelial cells (Endo KO) were used to evaluate the respective contributions of these sources to neuroinflammation, i.e., clinical disease progression, BBB damage and parenchymal...

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2 This work was published in Paul, D, Ge, S, Lemire, Y, Jellison, ER, Serwanski, DR, Ruddle, NH, Pachter, JS. Cell-selective knockout and 3D confocal image analysis reveals separate roles for astrocyte-and endothelial-derived CCL2 in neuroinflammation. J Neuroinflammation. 2014;11:10.
leukocyte invasion in myelin oligodendrocyte glycoprotein peptide (MOG\textsubscript{35-55})-induced EAE model. High-resolution 3-dimensional (3D) immunofluorescence confocal microscopy and colloidal gold immuno-electron microscopy were employed to confirm sites of CCL2 expression, and 3D immunofluorescence confocal microscopy utilized to assess inflammatory responses along the CNS microvasculature. Cell-selective loss of CCL2 immunoreactivity was demonstrated in the respective KO mice. Compared to wild-type (WT) mice, Astro KO mice showed reduced EAE severity but similar onset, while Endo KO mice displayed near normal severity but significantly delayed onset. Neither KOs showed deficits in T cell proliferation, or IL-17 and IFN-g production, following MOG\textsubscript{35-55} exposure \textit{in vitro}, or altered MOG MHC Class II tetramer binding. 3D confocal imaging further revealed distinct actions of the two CCL2 pools in the CNS. Astro KOs lacked the CNS leukocyte penetration and disrupted immunostaining of tight junction protein claudin-5 at the BBB seen during early EAE in WT mice, while Endo KOs uniquely displayed leukocytes stalled in the microvascular lumen. These results point to astrocyte and endothelial pools of CCL2 each regulating different stages of neuroinflammation in EAE, and carry implications for drug delivery in neuroinflammatory disease.

2. Introduction

The chemokine CCL2 (formerly called Monocyte Chemoattractant Protein-1, MCP-1) has long been established as a critical mediator of inflammation within and outside the central nervous system (CNS), and stimulates extravasation of mononuclear leukocytes into CNS and peripheral tissue beds (Leonard et al., 1991; Mantovani et al., 1993; Bennett et al., 2003; Toft-Hansen et al.,
Our laboratory (Song et al., 2004) and others (Stamatovic et al., 2003; Yao et al., 2011) have also revealed a role of CCL2 in the disruption, redistribution, or reduced expression of tight junction (TJ) proteins in cultured BMEC, which might contribute in part to allow the passage of leukocytes across the BBB *in vivo*. Elevated CNS expression of CCL2 has been a consistent observation among the different paradigms of experimental autoimmune encephalomyelitis (EAE) (Izikson et al., 2002; Mahad et al., 2003; Conductier et al., 2010), a CNS demyelinating inflammatory disease that serves as a model for multiple sclerosis. This chemokine’s singular importance in driving EAE was demonstrated by global CCL2 knockout (KO) mice (CCL2−/−), which showed diminished severity and delay in onset of disease in C57BL/6 mice actively immunized with myelin oligodendrocyte glycoprotein35-55 (MOG35-55) (Huang et al., 2001). Adoptive transfer EAE experiments also revealed effector T cells from MOG-immunized CCL2−/− mice could transfer EAE to naïve wild-type (WT) recipients, while encephalitogenic T cells from WT donors were unable to induce EAE in CCL2−/− mice (Huang et al., 2001). Bone marrow chimera studies further showed active immunization EAE was markedly reduced when bone marrow from WT mice was engrafted into lethally irradiated CCL2−/− mice, but not when bone marrow from CCL2−/− mice was transferred into WT recipients (Dogan et al., 2008). Collectively, these findings of induced CNS expression of CCL2 during EAE, together with the adoptive transfer and bone marrow chimera studies, are consistent with a prominent role for CNS CCL2 in mediating EAE and diminish or negate the pathogenic impact of CCL2 from the peripheral leukocyte compartment.
What remains unclear, however, is which specific sources of CCL2 significantly contribute to disease, whether any reside locally in the CNS, their pathogenic mechanisms, and how they might be targeted therapeutically. Astrocytes are a major CNS source of CCL2 in both EAE and multiple sclerosis (MS) (Mahad et al., 2003; Conductier et al., 2010; Giraud et al., 2010). By projecting their endfeet toward the abluminal surface of brain microvascular endothelial cells (BMEC) that form the blood–brain barrier (BBB), astrocytes are ideally situated to intimately modulate BBB function and CNS leukocyte extravasation (Hermann et al., 2012). Our laboratory (Song et al., 2004) and others (Stamatovic et al., 2003; Yao et al., 2011) have demonstrated that CCL2 can disrupt integrity of cultured BMEC along with causing redistribution and reduction in expression of tight junction (TJ) proteins. A priori, CCL2 released from astrocyte endfeet may be partly responsible for the loss of BBB properties that accompanies both EAE (Bennett Jet al., 2010) and MS (Meinl et al., 2008), assisting development of a chemotactic gradient across the microvascular wall to drive the migration of adhered leukocytes past the endothelium, and/or further guiding extravasated leukocytes into the CNS parenchyma (Carrillo-de Sauvage et al., 2012). In addition to astrocytes, BMECs have also been shown to express CCL2 during EAE (Berman et al., 1996) and MS (Subileau et al., 2009). The observations that anti-CCL2 antibody prohibits firm attachment of leukocytes in vivo to CNS pial venules of mice immunized for EAE (dos Santos et al., 2005), and inhibits monocyte transendothelial migration (TEM) across cultured BMEC (Seguin et al., 2003), support the concept that CCL2 presented on the luminal endothelial surface aids in arresting leukocytes prior to their extravasation. The recent description that TEM of lymphocytes is mediated, in part, by intraendothelial vesicle stores of
CCL2 (Shulman et al., 2012), further accents a novel role for the endothelium as a critical source of this chemokine.

To resolve the respective contributions of astrocyte and endothelial cell CCL2 to neuroinflammation, we developed cell-conditional chemokine KO mice, in which the CCL2 gene was selectively eliminated in each of these cell types (Ge et al., 2009). Here we report for the first time that targeted CCL2 gene deletion from either astrocytes or endothelial cells abates EAE pathogenesis, while differentially affecting separate aspects of CNS leukocyte extravasation and clinical disease course.

3. Materials and Methods

3.1 Animals

Astrocyte- and endothelial cell-specific CCL2 KO mice were generated by intercrossing mice containing a floxed CCL2 allele with transgenic mice of GFAP-Cre or Tie2-background, respectively, and previously characterized in detail (Ge et al., 2009). Astrocyte-specific knockout mice are referred to as Astro KO, and endothelial specific knockout mice as Endo KO mice throughout this study. KO mice and their wild-type (WT) littermate controls were housed in specific pathogen-free conditions. All procedures involving animals were performed in accordance with the Animal Care and Use Guidelines of the University of Connecticut Health Center.
3.2 EAE induction

EAE was induced by active immunization with MOG_{35-55} peptide (MEVGWYRSPFSRVSRLYNK; W. M. Keck Biotechnology Resource Center, Yale University), as described (Murugesan et al., 2012). Briefly, on day 0 (d0), female mice 8 - 10 weeks of age were injected subcutaneously into the right and left flanks with a total of 300 mg of MOG peptide in complete Freund’s adjuvant containing 300 mg Mycobacterium tuberculosis (DIFCO). Mice were also injected intraperitoneally with 500 ng pertussis toxin (List Laboratories) in phosphate buffered saline (PBS, Gibco/BRL) on d0 and d2 post-immunization (p.i.).

3.3 Clinical assessment of EAE

Mice were scored daily for clinical disease severity according to the following scale: 0 = normal; 1 = tail limpness; 2 = limp tail and weakness of hind legs; 3 = limp tail and complete paralysis of hind legs; 4 = limp tail, complete hind leg and partial front leg paralysis; and 5 = death. Several disease parameters were calculated as described (Suen et al., 1997). The Mean Day of Onset was calculated by averaging the time when clinical symptoms; i.e., a clinical score of > 1, were first observed for two consecutive days in each mouse. The Mean Maximum Clinical Score was calculated by averaging the highest score for each mouse. The Disease Index was calculated by adding the daily average clinical scores in each group, dividing by the mean day of onset, and multiplying by 100. If an animal showed no disease, the day of onset was arbitrarily counted as one day after the last day of the experiment. Disease Incidence was the fraction of mice experiencing EAE.
3.4 Cell culture and Cytokine assay

MOG MOG$_{35-55}$-immunized mice were sacrificed and draining lymph nodes were dissected on d12. Mashed lymph nodes were pressed through a 70 μm mesh into cold RPMI. Cells were pelleted at 450$g$ at 4°C for 5 min and resuspended in red blood cell lysing buffer (Sigma) on ice for 5 min. After three washes with cold PBS, cells were stained with 0.4% Trypan Blue (Sigma Aldrich) and counted with the Countess® Automated Cell Counter (Invitrogen) to permit discrimination of dead cells.

Single cell suspensions of lymph node cells (LNCs) were prepared and cultured in 24-well plates (Corning) at 1 $\times$ 10$^6$ viable cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1.25% HEPES buffer, 1% sodium pyruvate, 1% penicillin-streptomycin, 1% glutamine, 1% non-essential amino acids, 0.01% 0.05M- 2 mercaptoethanol (2-ME) (Sigma Aldrich). LNCs were restimulated with a combination of 10 mg/ml MOG$_{35-55}$ and 0.5 ng/mL interleukin-12 (IL-12) (R&D Systems). Cytokines present in the cell culture supernatants of LNC were quantified using the multiplex ELISA kit (R&D Systems).

3.5 Proliferation assay

LNCs were prepared as for the cytokine assay. Cells were pulse-labeled with 2μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) in RPMI for 5 min at room temperature (RT). After extensive washing with PBS, the CFSE-labeled cells were suspended and cultured in complete medium as above in 24-well plates (2 x 10$^6$/well) for 72h. Cell viability
was assessed by trypan blue exclusion. The LNC samples were washed in FACS buffer (1% FCS and 0.1% sodium azide in PBS). After blocking with Fc Block (BD Biosciences) at 4°C for 20 min in dark, the cells were washed and stained with fluorochrome-labeled antibodies against murine CD3, CD4, and CD11a (BD Biosciences) at 4°C for 30 min. After the cells were washed, the fluorescence intensities were measured by a FACS LSRII flow cytometer (BD Biosciences) and the data analyzed using FlowJo software (Treestar).

### 3.6 MOG38-49 MHC Class II tetramer binding assay

The procedure was based on that of Cravens et al. (2011). LNCs from MOG35-55-immunized mice were prepared and cultured as for cytokine and proliferation assays for 72 h. Thereafter, LNCs were washed with FACS buffer, and blocked with Fc Block in FACS buffer at 4°C for 20 min in dark. LNCs were washed again, and MOG38-49 MHC class II tetramer-PE or MHC class II control tetramer-PE hCLIP103-117-PE (obtained from the NIH Tetramer Core Facility) were added and incubated at 37°C for 1 hour in the dark. After the incubation, the cells were directly stained with fluorochrome-labeled antibodies against murine CD4, CD11a and CD44 at 4°C for 30 min. LNCs were washed and resuspended in FACS buffer. Samples were acquired on a FACS LSRII flow cytometer (BD Biosciences) and the data analyzed using FlowJo software (Treestar). Cells were gated as single live CD4+ T lymphocytes and examined for CD11a, CD44, and tetramer reactivity. Cells that were MOG tetramer-positive and CD11a+ were considered MOG-specific and previously activated.
3.7 Immunofluorescence and 3D analysis of confocal z-stack images

Tissue was prepared as described by Paul et al. (2013). In brief, following transcardiac perfusion/fixation of mice, spinal cords were removed by laminectomy and freeze-embedded in cryomatrix. Subsequently, 12×60μm cryosections from the thoraco-lumbar region, approximately between the T10 and L3 vertebrae were adhered to poly-L-lysine coated slides. Following staining, sections were mounted in Mowiol® prior to microscopic analysis.

For immunodetection of CCL2, affinity-purified rabbit anti-mouse CCL2 (Peprotech) was utilized with a corresponding anti-rabbit Alexa® 555-conjugated antibody (Life Technologies). Sections were subsequently double-immunostained with either rat anti-mouse CD31 (BD Pharmingen) followed by secondary incubation with anti-rat Alexa® 488 (Life Technologies) to highlight the endothelium, or Alexa® 488-conjugated anti-mouse GFAP (Life Technologies) to identify astrocytes. To specifically enhance detection of CCL2 – which shows dispersed punctate immunoreactivity – confocal z-stacks were first deconvolved using AutoQuant X3 (Media Cybernetics) software, to correct for z-axis distortion. This significantly improved z-resolution and the resulting high-resolution images were exported into Imaris® (Bitplane Inc.). Representative z-slices, showing the co-localization of CCL2 with endothelial CD31 or astrocyte marker GFAP were then obtained.

For 3D quantification of microvascular CLN-5 density, the protocol recently detailed by Paul et al. (2013) was used. The microvascular basement membrane (BM), a fusion of the respective endothelial and parenchymal BMs (Owens et al., 2008), was labeled with rabbit anti-mouse
Laminin 1 (Cedarlane) and anti-rabbit Alexa® 555 (Life Technologies). Anti-mouse Claudin5-Alexa® 488 (Biolegend) was employed to highlight TJs. Additionally, nuclear stain DRAQ5 (Biostatus Ltd.) was utilized to reveal the perivascular cellularity surrounding inflamed CNS microvessels, identified as venules (Paul et al., 2013), due to leukocyte extravasation.

Spinal cord venules from comparable regions of the dorsolateral white matter were imaged. Confocal z-stacks were acquired, at 1µm increments between z-slices, following a multitrack scan, using a Zeiss LSM 510 Meta confocal microscope equipped with a 40X Fluar (NA 1.3) and a 63X Plan-neofluar (NA 1.25) oil immersion lens. Confocal z-stacks were imported into Imaris® (version 7.6) software (Bitplane Inc.) and the venule of interest was segmented out from rest of the 3D dataset by manually tracing the vessel contour in each confocal z-slice, followed by merging the z-slice contours into a 3D contour surface. Surface area of the generated 3D contour was used as an estimate of the microvascular “surface area” defined by the endothelial layer. The CLN-5 channel was isosurface rendered (within the 3D contour surface) and the density of CLN-5 staining calculated as \( \frac{\text{Total CLN-5 intensity}}{\text{Microvascular surface area}} \). CLN-5 density values were expressed as mean ± standard error of the mean (SEM).

To optically isolate (3D segmentation) and resolve the DRAQ5⁺ cellularity associated with leukocyte accumulation (Paul et al., 2013) in the luminal or perivascular compartments of an inflamed venule, confocal z-stacks of venules revealing a cross-sectional view were acquired and imported into Imaris®. The venule of interest was then isolated from rest of the 3D dataset by creating a 3D contour surface, defining the parenchymal BM. This effectively eliminated the
parenchymal cellularity (i.e., extravascular infiltrates). Additionally, the lumen was segmented out in a similar manner, by creating another 3D surface, approximating the contour of the endothelial BM. Spatial location of the observed cellularity between the BMs was considered perivascular.

To graphically resolve the distribution of DRAQ5+ luminal and perivascular cells along microvascular x, y and z axes with respect to the endothelial and parenchymal BMs, a 3D volume was first constructed from the acquired confocal z-stack, followed by 3D segmentation of the luminal and perivascular compartments as described above. Imaris® spot creation wizard was then employed to represent each of the DRAQ+ nuclei as a “spot object” in 3D space. Only DRAQ5+ nuclei >3 µm in diameter were considered. The 3D profiles of the created spot objects, representing the position of luminal and perivascular leukocytes, were then plotted on a 3D Imaris® Vantage plot (Figure 14) for revealing its spatial location.

3.8 Immuno-electron microscopy (Immuno-EM)

Colloidal gold detection of CNS microvascular CCL2 immunoreactivity by immuno-EM was performed as described previously (Li et al., 2010). WT mice at d16 EAE were anesthetized as described above, and subjected to transcardiac perfusion/fixation with Ringer’s solution, pH 6.9, followed by 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Vibratome sections of spinal cord (300-500 µm thick) were cryoprotected with 2M sucrose in PB and plunge-frozen in liquid propane cooled by liquid nitrogen (-186°C). Sections were stained en bloc with 1.5% uranyl acetate in anhydrous methanol at -90°C for 30 hours and infiltrated with Lowicryl HM20 resin (Polysciences), followed by polymerization with UV light for
72 hours in a freeze-substitution instrument (Leica AFS) in a temperature gradient (-45°C to 0°C). Sections (70-80 nm thick) were cut and collected onto 400-mesh gold-gilded nickel grids coated with a Coat-Quick “G” pen (Daido). Tissue sections were incubated with anti-mouse CCL2 antibody (Peprotech), followed by incubation with goat anti-mouse IgG labeled with colloidal gold particles of 12 nm diameter (Jackson ImmunoResearch). After immunoreaction, tissue sections were counterstained with 2% uranyl acetate and then with 2% lead citrate. Primary antibody was omitted in immunoreaction as a control, and yielded no detectable gold labeling.

3.9 Statistical analysis

For analysis of cell-specific KO on clinical EAE parameters, a chi-square (χ2) test was used for comparisons of disease incidence; a Mann-Whitney U-test was used for comparisons of disease severity; and ANOVA, followed by Bonferroni’s multiple comparison post-hoc analysis, was used for comparison of disease onset (Ge et al., 2012). ANOVA/Bonferroni post-hoc test was also employed to assess differences in CLN-5 density values (Paul et al., 2013). To contrast the rate of rise of clinical EAE progression among the different mouse groups, linear regression was performed on data points beginning at the onset of disease through attainment of the plateau or highest score. Statistical analyses were performed employing Prism 5 software (GraphPad), and results were considered significant at a $p \leq 0.05$. 
4. Results

4.1 Astro KO and Endo KO mice show cell-selective loss of CCL2

Immunofluorescent staining of CCL2 in spinal cord during EAE in Astro KO, Endo KO, and WT mice is shown in high-resolution z-stack confocal images in Figure 8. Using identical image acquisition parameters, no CCL2 staining was detected either in naïve mice, or EAE mice in the absence of primary antibody (Figure S6), thus highlighting specific immunoreactivity to inflamed CNS tissue. In WT mice at d16 post-EAE induction, CCL2 staining was vessel-associated as well as within the perivascular space (Figure 8a). Notably, CCL2 staining appeared aligned with inter-endothelial junctions (CD31), and showed a punctate distribution rather than a diffuse appearance throughout the cytoplasm. This could reflect containment of CCL2 within vesicles, as recently described by Shulman et al. (2012) for cultured human umbilical vein endothelial cells (HUVECs). A representative z-slice further revealed intense double staining (CCL2 and CD31) of the endothelial layer. Parenchymal CCL2 staining (Figure 8b) was largely observed in association with GFAP+ astrocytes. Localization within astrocytes was confirmed in a representative z-slice. Some large deposits of CCL2 could also be seen just outside the astrocytes, which may indicate secreted chemokine. Both endothelial and astrocyte immunoreactivity in WT mice with EAE were confirmed by immuno-electron microscopy (Fig 8.1c–f). Notably, CCL2 immunoreactivity was detected within the inter-endothelial junctions (Figure 8c), and could also be seen in association with endothelial vesicular-like structures (Figure 8d). There was also abundant immunoreactivity within cellular processes at the
abluminal side of the endothelium, possibly representing CCL2 destined for or contained within astrocyte endfeet (Figure 8e–f).

The CCL2 staining patterns with both KO mice were markedly different (Figure 9). Astro KO mice at d16 (Figure 9a) showed vessel-associated CCL2 staining but greatly diminished staining in the perivascular space. Conversely, Endo KO mice (Figure 9b) exhibited a near absence of vessel-associated CCL2 staining, while robust CCL2+ astrocytes were still clearly evident. These staining patterns are consistent with previous results from this laboratory showing CCL2 RNA expression by both microvessel and parenchymal fractions of brain and spinal cord from WT mice with EAE (Ge et al., 2012), and argue that CCL2 protein is produced by astrocytes and BMECs, and not merely taken up at these sites. That some vessels in Astro KO mice and some astrocytes in Endo KO mice appeared devoid of CCL2 staining possibly reflects that not all of the respective endothelial cell and astrocyte populations became similarly inflamed, a prospect supported by only a subset of vessels/vessel segments being associated with leukocyte infiltrates (data not shown). It may further be that CCL2 from endothelial cells or astrocytes exerts some positive control over CCL2 expression by the other cell type (Ge et al., 2008; 2009).

4.2 Astro KO and Endo KO mice are both resistant to EAE, but show different clinical phenotypes

Figure 10a,b shows that targeted CCL2 gene eliminations in astrocytes and endothelial cells, respectively, prominently affected development of EAE, consistent with earlier reports of the
effects of global CCL2 knockout (Huang et al., 2001; Dogan et al., 2008). While the incidence of disease was largely unaffected in both KO mice compared to WT, there was clear evidence of altered disease progression in the two cell-selective CCL2 KO groups. Astro KO mice exhibited significantly reduced disease severity (clinical score) compared with WT littermates throughout the time frame observed, along with just a slight, insignificant delay in disease onset. In sharp contrast and nearly mirror image effect, Endo KO mice showed only mild reduction in disease severity, but a significantly protracted delay in the onset of disease. It was further observed that the rate of rise of clinical disease, as reflected by the slope of the linear, ascending region of the disease score graphs, was different between Astro KO and Endo KO mice. Astro KO mice showed a lesser rate of rise of clinical disease than both WT and Endo KO mice, while the latter two mouse groups showed similar rates of disease rise. Thus, it appears that while disease was significantly delayed in Endo KO mice, once disease commenced it proceeded on a normal time course (for the period evaluated). Comparisons of Disease Incidence, Mean Day of Onset, Mean Maximum Clinical Score, and Disease Index in Astro KO, Endo KO, and WT mice are tabulated in Figure 10c.

**4.3 Astro KO and Endo KO mice do not show different MOG-specific T cell responses**

Endo KO mice have the CCL2 gene eliminated from all endothelial cells, central and peripheral. This could mean that observed alterations in EAE disease progression in Endo KO mice might stem from a defect in the afferent immune arm, such as attenuated T cell priming. Indeed, this possibility is prompted by findings that CCL2 is expressed and presented by high endothelial
venules in lymph nodes (Palframan et al., 2001), instrumental in dendritic cell maturation (Jimenez et al., 2010), and released by dendritic cells to attract antigen-specific T cells (Secco et al., 2009). Hence, several T cell proliferation parameters – % Divided, Division Index, Proliferation Index, Expansion Index, and Replication Index – were assayed in CSFE pulse-labeled LNC cultures from MOG-immunized Astro KO, Endo KO, and WT mice (Figure 11a). Following restimulation with MOG, no significant differences were detected among cells from the three types of mice for any of these parameters. This is in agreement with previous findings that indicated no difference in T cell proliferation between LNC cultures from WT and global CCL2⁻/⁻ mice (Huang et al., 2001).

We also assayed the ability of cultured LNCs from each of these mice to produce IFN-γ or IL-17, which are cytokines considered instrumental in autoimmunity and EAE (Damsker et al., 2010), in response to restimulation with MOG. Figure 11b shows that MOG-stimulated expression of neither cytokine was significantly altered in Astro KO or Endo KO mice compared to their WT cohorts. Earlier, Huang et al. (2001) had described diminished production of IFN-γ in LNCs from CCL2⁻/⁻ mice, which might reflect the effects of having depleted a peripheral CCL2 pool distinct from that of endothelial cells. Figure 11c further reveals that, following MOG immunization, the percentage of MOG₃₈₋₄₉ MHC class II tetramer positive CD4 T cells was in line with another report (Cravens et al., 2011) and similar in LNCs from Astro KO, Endo KO, or WT mice, indicating that the frequency of MOG-specific T cells was comparable among these groups. Hence, our results argue against either astrocyte- or endothelial cell-targeted CCL2
gene deletion having significantly impaired peripheral T cell behavior and immune responsiveness to MOG peptide. These findings are further in accord with reports that MOG-specific, encephalitogenic T cells can be generated in mice with global knockout of CCL2 (Huang et al., 2001).

4.4 Astro KO and Endo KO mice display altered inflammatory responses along the CNS microvasculature during EAE

Given the different clinical EAE phenotypes of Astro KO and Endo KO mice with apparent absence of overt impact on T cell priming, we next sought to determine effects of cell selective CCL2 loss on inflammatory events along the CNS microvasculature. 3D perspective projection views of confocal reconstructions were generated using Imaris® to provide a more realistic 3D representation of the z-stack dataset. Notable differences in staining of CLN-5, a prominent TJ protein in CNS microvessels and BBB determinant (Nitta et al., 2003), were found among WT, Astro KO, and Endo KO mice during EAE (Fig 3.5), while naïve mice of all groups showed no evidence of disparities (data not shown). At d9, prior to evidence of clinical disease, WT mice showed focal fragmentation of the CLN-5 staining pattern. This discontinuous appearance of CLN-5 staining in regions of increased perivascular cellularity was not a result of CLN-5 staining being distributed in z-planes not captured during acquisition, as the confocal reconstructions shown represent 3D images generated from 60 × 1-μm thick z-slices. Rather, the areas lacking immunostaining reflected actual sites of CLN-5 disruption associated with extravasating leukocytes. This picture differed significantly from that seen with naïve mice,
where CLN-5 immunostaining appeared continuous, without obvious interruptions, and perivascular cellularity was absent (Figure S7). Hence, for the purpose of highlighting the pathologic role(s) of CCL2 released from astrocyte and endothelial sources in neuroinflammation, statistical comparisons for CLN-5 density were only made between WT, Astro KO, and Endo KO mice during evolving EAE. The significantly altered CLN-5 pattern observed in WT mice during EAE corresponded with a sharp decrease of approximately 60% in the density of CLN-5 staining. In stark comparison, Astro KO mice demonstrated little if any change in CLN-5 staining, while Endo KO mice displayed a somewhat intermediate response at this time-point. By d16- following disease onset- WT mice showed further disruption and loss of CLN-5 staining, down approximately 80% from that demonstrated by naïve mice. Astro KO mice at this later time showed a precipitous loss of CLN-5 staining, declining by approximately 60% (compared to naïve and d9 mice), while Endo KO mice at d16 showed only a moderate, non-significant CLN-5 decrease of approximately 19% (compared to d9).

Differences in leukocyte infiltration patterns were also obvious among the various strains of mice when vessels were viewed in longitudinal- and cross-section (Figure 13). During neuroinflammation, the BM splits into its respective endothelial and parenchymal components (Owens et al., 2008), highlighting two spaces: the subendothelial space (between the endothelium and endothelial BM) and the perivascular space (between the endothelial BM and the parenchymal BM). Both these spaces swell with leukocytes that have recently extravasated across the BMECs, but as the BM becomes extensively fragmented during this process,
demarcation between the spaces is blurred in longitudinal sections. Thus, in referring to leukocyte distribution, the term “perivascular” is used herein to describe all extravasated leukocytes that are vessel-associated. At d16, dense perivascular infiltrates were seen to be associated with venules in WT mice, with cells apparently penetrating the parenchymal BM to enter the parenchyma (Figure 13a,d). Astro KO mice showed similar type clusters of perivascular cells, but no clear evidence of leukocytes in the act of rupturing the parenchymal BM (Figure 13b,e). Venules of both WT (Figure 13d) and Astro KO (Figure 13e) further exhibited lumens within which no cells were detectable. By contrast, Endo KO mice alone displayed aggregated cells apparently stalled in the lumen, as well as a seemingly lesser extent of perivascular cells (Figure 13c,f−h). To further resolve the aggregated cells throughout the microvascular lumen of an entire 60μm section from Endo KO mice, and graphically distinguish this pattern from that in WT and Astro KO mice, the distribution profiles of cells associated with the lumen and perivascular space, respectively, were mapped in 3D (Figure 14). Intra-luminal cells could only be detected in Endo KO mice (Figure 14c). As the actual number of spinal vessels showing any evidence of inflammation was extremely low in Endo KO mice at this time, the few examples detected showed this common appearance of hindered leukocyte migration. Moreover, since tissue was perfusion-fixed, these luminal cells are not likely to have resulted from blood stasis but, instead, suggest a possible deficit in extravasation from CNS microvessels in Endo KO mice.
5. Discussion

While the critical role of CCL2 in EAE has been revealed in global CCL2 knockout studies (Huang et al., 2001), and the chemokine sources mediating this effect are suggested to reside in the CNS (Dogan et al., 2008), the identities of the sources responsible for specific neuroinflammatory events, e.g., effects at the BBB and leukocyte penetration into the CNS, have not been resolved. In the present study, we used mice with targeted CCL2 gene deletion in astrocytes or endothelial cells, along with 3D confocal imaging, to establish - for the first time – that CCL2 from each of these sources regulates different aspects of neuroinflammation and EAE course. Astro KO mice exhibited a similar onset but reduced severity of disease compared to WT, while Endo KO mice displayed nearly the opposite clinical pattern. However, neither of these KO mice showed any significant changes in MOG-specific T cell responses in LNC cultures, consistent with the observed effects of CCL2 gene deletion being limited to the CNS. Further reflecting CNS action, Astro KO mice failed to show the parenchymal leukocyte infiltration and clear disruption of CLN-5 that accompanied WT EAE, while Endo KO mice revealed leukocytes apparently stalled in the lumen of spinal cord microvessels. Significantly, the combined effect of separate astrocyte and endothelial CCL2 elimination on clinical disease closely mirrors the phenotype reported when CCL2 gene ablation was confined to the “central compartment” by adoptive transfer of WT T cells (Huang et al., 2001) or transplantation of WT bone marrow (Dogan et al., 2008) into global CCL2 KO mice; i.e., reduced disease severity along with delayed disease onset. This reinforces the notion that CCL2 from astrocytes and
endothelial cells each contribute to EAE disease in a major, yet different way. The differences in EAE noted between Astro KO and Endo KO mice may reflect direct consequences of cell-specific CCL2 release on the CNS microvascular endothelium, leukocyte migration, or both. Possible actions of CCL2 released by CNS endothelial cells or astrocytes are schematized in Figure 14.

5.1 Astrocyte-derived CCL2 regulates BBB integrity and leukocyte penetration into the CNS parenchyma, while endothelial-derived CCL2 impacts leukocyte transendothelial migration

The failure of only Astro KO mice to show clear disruption of CLN-5 staining at d9 EAE supports the interpretation that CCL2 released from astrocytes figures prominently in destabilizing endothelial TJs at the abluminal microvascular surface early during the neuroinflammatory process (Song et al., 2004). Later loss of CLN-5 in these mice by d16 may, instead, reflect disruption of TJs by extravasating leukocytes. Astrocyte-derived CCL2 may additionally serve to recruit extravasated leukocytes into the parenchyma, as Astro KO mice revealed leukocytes congregated in the perivascular space. This latter role is supported by Carrillo-de Sauvage et al. (2012), who described contact of CNS infiltrating T cells with CCL2-expressing perivascular astrocytes during neuroinflammatory disease.

The disruption of CLN-5-containing TJs during EAE and its prominent control by astrocyte-derived CCL2, is consistent with numerous reports on the action of CCL2 on TJs and BBB properties in cultured BMECs (Song et al., 2004; Stamatovic et al., 2003; Yao et al., 2011;
Dhillon et al., 2008), CNS microvessels \textit{in vitro} (Song et al., 2004), and CNS microvessels \textit{in vivo} in other neurological settings (Stamatovic et al., 2005; Dimitrijevic et al., 2006; Strecker et al., 2013). Notably, however, a recent report describing the effect of pertussis toxin injection in mice constitutively overexpressing CCL2 selectively in oligodendrocytes under direction of the myelin basic protein (MBP) promoter, found no evidence of a disrupted CLN-5 pattern accompanying leukocyte extravasation into brain (Schellenberg et al., 2012). This apparent contradiction may be due, in part, to the high level of chronic over-expression of CCL2 (<100,000 times normal values) having caused down-modulation of CCR2, the cognate receptor for CCL2, on BMECs (Dzenko et al., 2001), as well as inappropriate or inadequate access of oligodendrocyte-derived CCL2 – normally not found in health or disease – to the CNS microvasculature. The present study thus underscores the unique relationship between endogenous, astrocyte-derived CCL2, TJ$s$, and BBB permeability in neuroinflammatory disease (Yao and Tsirka, 2014).

Endothelial-derived CCL2, on the other hand, may facilitate a ‘post-adhesion’ stage of leukocyte extravasation, as the absence of this chemokine pool was uniquely associated with the appearance of leukocytes stalled within the microvascular lumen. In support of this hypothesis, Shulman et al. (2012) recently showed that an intraendothelial vesicle pool of CCL2 within cultured HUVECs is a critical regulator of TEM of adherent effector T cells. Our data, showing both apparently stalled leukocytes in the microvascular lumen of Endo KO mice and punctate CCL2 immunostaining in BMECs of WT mice, may thus represent an extension of
the results of Shulman et al. (2012) to an in vivo scenario and advance a critical role for a CNS endothelial pool of CCL2, possibly vesicle bound, in mediating leukocyte TEM during neuroinflammatory disease. While this differs from the finding that CCL2 facilitates adhesion of leukocytes to pial microvessels (dos Santos et al., 2005.), this distinction may represent the considerable endothelial heterogeneity along the CNS microvasculature (Ge et al., 2005).

The smaller but significant loss of CLN-5 staining noted in Endo KO mice from d9 to d16 may chiefly represent the action of astrocyte-derived CCL2, as lesser extravasation was observed in these mice during this period. Conceivably, the astrocyte CCL2 pool could also have guided the lesser amount of extravasated cells into the parenchyma, resulting in the delayed disease noted.

5.2 Cell-selective CCL2 knockout highlights CNS actions of CCL2 in neuroinflammatory disease

Our collective findings reinforce critical and non-redundant roles of CNS CCL2 in mediating EAE, as previously implicated in adoptive transfer and bone marrow chimera EAE studies with global CCL2 knockout mice (Huang et al., 2001; Dogan et al., 2008). Of further importance, the use of Astro KO and Endo KO mice together with high-resolution 3D confocal imaging in this study was able to resolve apparently unique contributions of astrocyte and endothelial CCL2 pools to EAE pathogenesis. That both types of mice might share some effects of conditional CCL2 deletion is in accord with reports that BMECs can deposit CCL2 abluminally (Chui et al., 2010), and CCL2 can be transcytosed from the abluminal to luminal BMEC surface (Ge et al.,
Because all endothelial cells in the Endo KO mice are deficient in CCL2 expression, at this time it cannot be concluded that CCL2 from CNS endothelial cells, as opposed to peripheral endothelial cells, affected the disease process. However, given the failure of LNCs from these mice to show any deficits in MOG-stimulated proliferation, IFN-γ or IL-17 production, or MOG MHC class II tetramer staining, it is doubtful that the absence of CCL2 from peripheral endothelial cells was a major factor in the aberrant EAE patterns noted. Diminished CLN-5 disruption and heightened presence of luminal leukocytes in Endo KO mice further point to the CNS endothelial pool of CCL2 as featuring critically in EAE.

Though only EAE was analyzed in this study, astrocytes and BMECs have been suspected as critical sources of CCL2 during other neuroinflammatory conditions investigating three different CNS inflammatory scenarios (human glioma, striatal injection of LPS in mice, and adenovirally injected monkeys) reporting that extravasation of lymphocytes is mediated by CCL2-expressing astrocytes independent of the inflammatory situation and species (Carrillo-de Sauvage et al., 2012). Further, Tei et al. (2013) most recently argued that CCL2 expression by both astrocytes and BMECs may contribute to the invasion and parenchymal migration of brain Iba1+/NG2+ cells, descendants of subpopulations of circulating monocytes, following cerebral ischemia. Thus, expression of CCL2 by astrocytes and/or BMECs may be considered a widespread phenomenon associated with neuroinflammation. The seminal importance attributed to these particular sources of CCL2 does not preclude contributions by other CCL2-expressing cell types, e.g., microglia (Dogan et al., 2008; Starossom et al., 2012), which
may further modulate neuroinflammatory disease in their unique ways.

It has nevertheless been firmly established through elegant adoptive T cell transfer (Huang et al, 2001) and bone marrow chimera (Dogan et al., 2008) studies, that CCL2 derived from the peripheral leukocyte compartment is not critical to the development of EAE. Hence, even though Tie-2-driven Cre expression has been reported in cells of the hematopoietic lineage (Tang et al., 2010), any potential loss of CCL2 from this population would not detract from our interpretation that CCL2 elimination from BMEC, rather than leukocytes, predominantly altered EAE disease. The delay of disease phenotype in Endo KO mice, in fact, is consistent with the lack of CCL2 immunostaining in BMEC and apparent stalling of adherent leukocytes observed in these animals.

5.3 Therapeutic targeting of CNS CCL2

Lastly, as the BBB has generally been recognized as the major impediment to drug delivery to the CNS (Pardridge et al., 2005), these results have significant implications for targeting CCL2 in the treatment of neuroinflammatory disease. It is thus notable to point out that injection of a CCL2-neutralizing antibody directly into the brain was effective at suppressing lymphocyte infiltration following striatal lipopolysaccharide injection (Carrillo-de Sauvage et al., 2012), a situation in which astrocytes were observed to be the major CCL2-expressing cell type. Arguably, therapeutic inhibition of endothelial CCL2 would not require circumventing or penetrating the BBB, in contrast to suppressing astrocyte production of this chemokine. However, as our and the recent results of Shulman et al. (2012) point out, merely targeting
CCL2 with antibodies or receptor antagonists may not be effective against vesicle-bound endothelial CCL2 depots. The recent demonstration that the anti-inflammatory compound bindarit, a synthetic indazolic derivative (MWr 324 Daltons) that preferentially inhibits transcription of the monocyte chemoattractant subfamily of CC chemokines (MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7), delayed and suppressed EAE in concert with diminishing CNS microvascular CCL2 expression (Ge et al., 2012), suggests that interfering with intra-endothelial CCL2 might be of high therapeutic value. However, as BBB disruption often accompanies neuroinflammatory disease (Paul et al., 2013; Ge et al., 2005), drugs that inhibit CCL2 synthesis could potentially have opportunity to strike at both endothelial cells and astrocytes, even if only with limited efficiency at the latter, and thus offer better therapeutic prospects than antibodies or antagonists. Recent descriptions of CCL2 involvement in post-ischemic disruption of the BBB (Strecker et al., 2013), beta-amyloid neurotoxicity (Severini et al., 2014), and traumatic brain injury (Ho et al., 2012), further underscore that modulating CNS CCL2 synthesis at the vascular and/or parenchymal level may offer a novel therapeutic option for a wide range of neuropathologies.

6. Conclusions

In light of our results, it is determined that CCL2 from either astrocytes or BMECs separately impacts clinical EAE and associated neuroinflammatory processes in distinct ways and through different mechanisms depending on the source cell type. CCL2 from astrocytes regulates severity of clinical EAE disease, while controlling penetration of leukocytes into the CNS.
parenchyma and disrupting CLN-5 staining pattern along the CNS microvasculature. In contrast, CCL2 from BMECs appears to more so determine disease onset, and effect post-adhesion leukocyte transendothelial migration (Figure 15). Therapeutic targeting of CCL2 expression or action at the parenchymal or vascular levels may thus offer promise in treating neuroinflammatory disease.
Figure 8. CCL2 expression in spinal cord of WT mice during EAE. (a–b) z-stack confocal images from spinal cord cryosections of WT mice at d16 EAE are shown, revealing staining of CCL2 (red), and CD31 or GFAP (green) to delineate the endothelial cells and astrocytes, respectively. CCL2 staining was isosurface rendered for enhanced spatial perspective. (a) WT mice express CCL2 both along the CD31+ microvascular endothelium, where staining appears aligned along the endothelial junctions, and within the perivascular space (left). (b) CCL2 staining is also associated with GFAP+ astrocytes (right). Insets show co-localization of CCL2 with CD31 or GFAP (yellow) in a single z-slice from the respective regions marked by the hatched white boxes, or CCL2 (red) channel alone. (c–f) Colloidal gold immuno-EM localization of CCL2 localization along microvessels in sections of spinal cord from mice at d16 EAE. (c) CCL2 immunoreactivity is localized within the inter-endothelial junction (arrow) and scattered throughout the endothelial cytoplasm. (d) A cluster of CCL2 immunoreactivity (arrow) is shown in close apposition to an endothelial vesicular structure that is near the plasma membrane. (e) Low magnification showing cross-section of a microvessel (possibly a postcapillary venule or small venule) and punctate distribution of CCL2 immunoreactivity in the perivascular space (arrows). (f) Higher magnification, revealing a high density of CCL2 immunoreactivity in and around what may represent astrocyte endfeet (arrows). Results are representative of 5–7 sections sampled from three mice in each group and two independent experiments. Scale bars are noted on the respective images.
Figure 9. CCL2 expression in spinal cord of Astro KO and Endo KO during EAE. Representative z-stack confocal images from spinal cord cryosections of KO mice at d16 EAE are depicted. Cell-specific CCL2-KO mice display loss of CCL2 staining in respective targeted cell types. (a) Astro KO mice show venule-associated CCL2 staining, but lack staining in the parenchymal astrocytes (left). (b) In contrast, Endo KO mice are deficient in vessel-associated CCL2 staining, but maintain astrocyte staining (right). The endothelial boundary is marked with yellow lines. Insets show co-localization of CCL2 with CD31 or GFAP (yellow) in a single z-slice from the respective regions marked by the hatched white boxes, or CCL2 (red) channel alone. Results are representative of 5–7 sections sampled from three mice in each group and two independent experiments.
Figure 10. Astro KO and Endo KO mice show different patterns of clinical EAE. EAE was induced in WT, Astro KO, and Endo KO mice by MOG<sub>35-55</sub> immunization; all mice were observed daily and scored for clinical disease for 30 days. Each group consisted of 5–6 mice, and analysis was performed in triplicate. Graphs represent mean data points from all three analyses ± standard error.

(a) Mean clinical EAE scores. Astro KO mice do not attain as severe disease as WT during the evaluation period, while Endo KO mice approach WT disease severity but do so only after significantly delayed onset. The rate of rise of clinical disease, as reflected by the slope of each regression line (hatched lines) through the respective ascending disease scores for the different mice, is similar in both WT and Endo KO mice, but notably less in Astro KO mice. (b) Disease incidence. All mice show a similar incidence of disease but, compared to WT mice, Astro KO mice show only a mild delay while Endo KO mice show a prolonged delay in disease onset. (c) Summary of various clinical disease parameters among the three mouse groups.
Figure 11. LNCs from MOG

restimulation in vitro. LNCs were prepared from MOG

immunized mice on d12, and restimulated with MOG

for 72 h in culture, after which time different responses were measured. (a) T cell proliferation. LNC were pulse-labeled with 2 μM CFSE for 5 min at the beginning of culture and analyzed after 72 h by FACS, gating on CD3, CD4, CD11a. (b) Cytokine production. The concentrations of IL-17 and IFN-γ were determined in supernatants of LNC after 72 h in culture. (c) Binding of MOG

MHC class II tetramer-PE. Binding was determined after 72 h in culture, and hCLIP103-117 tetramer-PE served as a control for non-specific binding. Plots were gated on CD4 T lymphocytes. The frequency of MOG

I-Ab tetramer+ CD4 T cells is similar among WT, Astro KO, and Endo KO groups, while hCLIP103-117 I-Ab tetramer does not bind cultured T cells. The data shown are representative of at least two independent experiments; data in (a) and (b) reflect mean value ± standard error.
Figure 12. Astro KO and Endo KO mice show differential loss of CLN-5 staining in spinal venules during EAE. (a) Isosurface-rendered images were generated from confocal z-stacks of 60 μm thick thoraco-lumbar spinal cord cryosections at d9 and d16 EAE, as described in Materials and Methods. Staining of CLN-5 (green isosurface) and nuclei/DRAQ5 (blue) is shown. Larger images displaying 3D perspective projection views of confocal reconstructions show CLN-5 channel only, to emphasize the fragmented pattern of TJ protein staining. Insets depict both CLN-5 and nuclei, highlighting the close association of altered CLN-5 staining with dense perivascular cellularity representing infiltrating leukocytes. Arrows demark overt gaps in CLN-5 staining, where the TJ pattern is clearly disrupted. Notably, CLN-5 staining pattern during EAE appears most intact in Astro KO mice, least so in WT mice, and intermediate in Endo KO mice. (b) Quantification of CLN-5 staining as intensity per unit surface area of the endothelium. CLN-5 density in naïve WT mice is included as a reference for the normal state, wherein the pattern of CLN-5 junctional staining is continuous [30]. Statistical comparisons are between groups and within days. (c) Summary of CLN-5 changes. Statistical comparisons are within groups and between days. A total of 12 venules were analyzed in each group sampled from three mice. Data reflect mean value ± standard error. Scale = 20 μm.
Figure 13. Astro KO and Endo KO mice display differences in perivascular cellularity associated with spinal venules during EAE. Isosurface-rendered images were generated from confocal z-stacks of 60 μm cryosections at d16 EAE. Staining of BM Lam 1 (red), CLN-5 (green), and nuclei/DRAQ5 (blue) is shown. (a, b, c) Longitudinal sections reveal the extent of vessel-associated leukocytes. CLN-5 staining is presented to highlight the endothelial boundary. Insets represent enlarged view of areas highlighted in white hatched boxes, while double-headed arrows denote the space between the endothelial and parenchymal BMs. All extravasated leukocytes within this space are considered “perivascular”. In WT mice, a dense accumulation of DRAQ5+ perivascular cells (representing leukocytes) is seen, a few apparently penetrating the fragmented parenchymal BM (arrowhead). In Astro KO mice, a similar dense perivascular cellularity is observed, with visibly intact parenchymal BM and lack of parenchymal leukocyte migration. In Endo KO mice, the BM is also apparently intact, with minimal perivascular cellularity. Scale = 20 μm. (d, e, f) Cross-sections highlight the spatial distribution of vessel-associated leukocytes. In WT mice, the vessel lumen (demarked by white dashes) appears empty and cells are seen in the perivascular space. A few cells are visibly penetrating the parenchymal BM (arrowheads), along with dense parenchymal cellularity (brackets). In Astro KO mice, the lumen again appears empty; congregated cells are evident in the perivascular space, with a few parenchymal clusters. In Endo KO mice, cells are clearly present in the lumen, with apparently fewer cells in the perivascular space as compared to WT and Astro KO mice. Parenchymal clustering is seemingly absent. The diffuse DRAQ5+ cells are likely parenchymal neural cells. (g-h) The red arrow designates the same Endo KO image subject to contour-based 3D segmentation (see Materials and Methods) to further resolve luminal (blue) from perivascular (turquoise) cells. Results are representative of 5–6 microvessels sampled from three mice in each group and two independent experiments. Scale = 10 μm.
Figure 14. Astro KO and Endo KO mice display differences in 3D distribution profiles of luminal and perivascular cells.

Isosurface-rendered images were generated from confocal z-stacks of 60-μm thick cryosections from WT, Astro KO, and Endo KO mice at d16 EAE. The BM is highlighted by staining of Lam1 (red). (Top row) DRAQ5+ nuclei in luminal and perivascular compartments were optically isolated using 3D contour based segmentation (as described in Materials and Methods), and pseudo-colored blue (luminal) and turquoise (perivascular), respectively. Using Imaris® spot creation module, each of these nuclei is shown in the 3D dataset (volume) as a “spot object,” designating its luminal or perivascular location. Scale = 10 μm. (Bottom row) Imaris® vantage plots showing the 3D distributions of luminal and perivascular cells along microvascular x, y, and z-axes in the corresponding vessels from the top row. Scale = 20 μm. (a) Representative WT vessel showing an empty lumen (*). (b) The lumen in the Astro KO vessel also appears empty (*) but partially collapsed, possibly owing to accumulation of perivascular cells that are missing guidance cues from deleted astrocyte-derived CCL2. (c) In contrast, Endo KO vessel shows evidence of congregation of cells in the lumen (blue), possibly reflecting stalled leukocyte transmigration in absence of endothelial-derived CCL2. Box-and-whisker plots are shown indicating the maximum and minimum spread from the median, in μm, of luminal or perivascular nuclei along the x, y, and z-axes.
Figure 15. Differential actions of astrocyte-derived and endothelial cell-derived CCL2 at CNS venules. Based on observations with Astro KO and Endo KO mice during EAE, the schematic depicts endothelial-derived CCL2 facilitating migration across the endothelium (1), a step post-adhesion. Astrocyte-derived CCL2 is shown promoting both breakdown of endothelial tight junctions (2), and penetration of leukocytes across the parenchymal BM into the CNS parenchyma (3).
Endothelial extracellular vesicles transfer tight junction protein Claudin-5 to circulating leukocytes in neuroinflammation

1. Abstract

Leukocyte transendothelial migration (TEM) across the blood-brain barrier (BBB) is a hallmark of neuroinflammatory disease like multiple sclerosis (MS), and its animal model, experimental autoimmune encephalomyelitis (EAE). The neurodegeneration underlying MS stems from focal infiltration of myelin-reactive leukocytes into the central nervous system (CNS) parenchyma, which in turn correlates to BBB dysfunction. While the mechanism(s) of TEM remains unclear, the presence of tight junctions (TJs) at the BBB is generally considered an impediment to leukocyte extravasation in the central nervous system (CNS). Paracellular TEM across the intact BBB must therefore require leukocytes to negotiate the endothelial TJs. To address this, high-resolution 3D confocal imaging was used to highlight disposition of the TJ protein claudin-5 (CLN-5), a major BBB determinant, during experimental autoimmune encephalomyelitis (EAE) – a model of multiple sclerosis (MS). Strikingly, a sub-population of leukocytes immunoreactive for CLN-5 on their surface was seen to infiltrate the CNS of mice early during EAE, in close apposition to inflamed vessels. Confocal imaging and Western blotting confirmed the presence of CLN-5⁺ leukocytes in plasma from EAE mice. As circulating CLN-5⁺ leukocytes have been

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3 The abstract of this work was published in Paul, D, Ge, S, Jellison, ER, Agalliu, D, Pachter, JS. Extracellular vesicles as possible conveyors of tight junction protein to leukocytes in neuroinflammation. Journal of Extracellular Vesicles 2015;4: 27783.
described in MS patients, and extracellular vesicles (EVs) from cultured brain microvascular endothelial cells (BMEC) of the BBB reported to express a variety of adhesion molecules and stimulate leukocyte TEM in vitro, we investigated if BMEC-derived EVs have CLN-5 cargo and bind to leukocytes. Western blotting showed BMEC-derived EVs, corresponding in size to both exosomes and microvesicles, expressed CLN-5. Confocal imaging and flow cytometry further showed EVs from TNFα-stimulated BMECs can bind leukocytes. Additional imaging of inflamed vessels from endothelial targeted, Tie-2-eGFP-CLN-5 transgenic mice showed labeled leukocytes in the CNS during early EAE, consistent with the transfer of CLN-5 from endothelial cells to leukocytes in vivo. eGFP-EVs were also detected by flow cytometry in culture supernatants from both BMEC and plasma of Tie-2-eGFP-CLN-5 mice, and observed by confocal imaging to bind leukocytes. Lastly, serial scanning electron microscopy and 3D contour-based surface rendering revealed a close association of membrane-bound structures resembling EVs between the marginating leukocytes and BMECs in vivo during EAE. Results indicate CLN-5+ EVs released from BMEC can bind to leukocytes and thereby transfer CLN-5 protein. This action may be a prelude to facilitate TEM through the formation of TJ protein bridges between these two cell types.

2. Introduction

Cells require constant communication with neighbors for their normal physiological function or during a pathological insult. Burgeoning evidence further implicates a role of membrane-enclosed sacs shed from the surface of cells, collectively termed extracellular
vesicles (EVs), as vehicles for such communication (Ratajczak et al., 2006b; Mause and Weber 2010). EVs of sizes ranging from 30 to 1000 nm can be of endosomal or plasma membrane origin, and termed exosomes or microvesicles, respectively (Shifrin et al., 2013). Both EV subtypes relay cell-specific repertoires of proteins, lipids, and genetic material to their targets. Owing to their small size and membrane-bound nature, EVs can be rapidly mobilized throughout the circulation, while protecting their cargo from degradation in bloodstream.

In the central nervous system (CNS), neurons, microglia, astrocytes, oligodendrocytes and endothelial cells have been reported to secrete microvesicles and/or exosomes into the extracellular environment. Though their molecular cargo awaits rigorous evaluation, EVs from CNS-resident cells are thought critical for CNS function in health and disease (Verderio et al., 2012; van der Vos et al., 2011). For instance, EVs derived from oligodendrocytes can regulate myelination (Bakhti et al., 2011; Frühbeis et al., 2012), while those released by neurons may aid clearance of pathogenic agents or degenerative proteins like beta-amyloid (Yuyama et al., 2015). These findings imply EVs perform functions necessary for CNS homeostasis.

However, EVs can also act as pathogenic effectors in the CNS, as they have been implicated in the dispersal of toxic proteins in a number of neurodegenerative diseases, e.g., Multiple Sclerosis (MS), transmissible spongiform encephalopathies, Alzheimer’s disease, Parkinson’s disease, tauopathies, and amyotrophic lateral sclerosis (Horstman et al., 2007; Jimenez et al., 2005; Vingtdeux et al., 2007; Vella et al., 2008; Guest et al., 2011; Frühbeis et al., 2013). Microglia-derived EVs contain inflammatory cytokines, such as interleukin (IL) 1β, and
can influence neuronal activity (Turola et al., 2012). MVs of myeloid origin have also been detected in the cerebrospinal fluid (CSF) of humans and rodents, and reported to increase in concentration upon brain inflammation, possibly implicating their role as pathogenic effectors and/or biomarkers of disease activity (Verderio et al., 2012). It is of further high significance that EVs can potentially travel long distances for horizontal transfer of the contained bioactive molecules to these distal sites.

Apart from contributing to the dispersal of toxic bioactive molecules, EVs have also been reported to contribute to immune pathologies in the periphery by modulating multiple facets of leukocyte biology, e.g., their activation, migration, or adhesion (Zech et al., 2012; Jimenez et al., 2005; Lee et al., 2010). For instance, exosomes released by cancer cells can create an immune-privileged environment by inducing apoptosis of activated tumor-specific T cells (Kim et al., 2005). A study on rheumatoid arthritis patients further demonstrated TNFα–containing exosomes from synovial fluid fibroblasts can potentiate the disease by delaying the activation-induced death of infiltrating T cells (Zhang et al., 2006). Additionally, exosomes from bronchial epithelial cells of asthmatic patients have been reported to induce the proliferation and chemotaxis of monocytes (Kulshreshtha et al., 2013).

In accord with their immunomodulatory role in the periphery, EVs have also been recently called into question as possible players in leukocyte entry into the CNS in neuroinflammatory disease like MS (Jy et al., 2004; Jimenez et al., 2005; Horstman et al, 2007) and in stroke (Simak et al., 2006; Jung et al., 2009). Leukocyte infiltration into CNS tissue is often
reported to be associated with a transient focal loss in the endothelial TJs, suggesting that invading leukocytes somehow negotiate the physical obstacle of BBB (Paul et al., 2013; Cosetti et al., 2012). For instance, Xu et al. (2005) used intravital scanning laser ophthalmoscopy to demonstrate activated T cells in mice inflicted with experimental autoimmune uveoretinitis interact with the endothelium, upregulate ICAM-1, and induce transient breakdown of the blood-retinal barrier. In a subsequent study (Xu et al., 2005b), these authors also reported that sites of leukocyte extravasation in retinal venules correlated with areas of TJ protein loss in vivo. Accompanying this TJ protein loss, was the redistribution or loss of ensheathing astrocyte processes at venular sites, but not at adjacent capillaries or arterioles. Plumb et al., (2002) used enhanced MRI to reveal in vivo breakdown of BBB and subtle elevation of microvascular permeability in expanding inflammatory lesions in relapsing-remitting and chronic progressive MS. Analysis of microvascular TJ abnormalities using fluorescence confocal imaging further highlighted beading, discontinuity, absence or diffuse cytoplasmic localization of TJs, suggestive of BBB damage, in forty percent of vessels inside the lesions (Plumb et al., 2002). These findings were also mimicked in EAE, an animal model of MS, where brain and spinal cord microvessels showed a selective loss of claudin-3 immunostaining in venules surrounded by inflammatory cuffs (Wolburg et al, 2003). Such loss of endothelial TJs can also be seen in other human disease like glioblastoma multiforme, where selective loss of claudin-3 immunostaining was reported in altered cerebral microvessels (Wolburg et al, 2003). Using an in vitro model and human brain tissues with HIV-1 encephalitis, Persidsky et al. (2006), showed that monocyte migration across brain microvascular endothelial cells requires dislocation of membrane
recruited TJ protein occludin from the cell junctions.

Interestingly, these TJ abnormalities or increased microvascular permeability in the CNS, suggestive of BBB damage in neuroinflammation, is often associated with a concomitant increase in circulating endothelial EVs (Marcos-Ramiro et al., 2014; Cosetti et al., 2012). A recent study has thus, offered EVs as a possible link between leukocytes and TJ damage. Mandel et al. (2012), reported expression of TJ protein Claudin-1 and Claudin-5 in peripheral blood leukocytes (PBLs), predominantly in B and T lymphocytes and monocytes in multiple sclerosis (MS) patients in relapse. Strikingly, RNA levels for these TJ proteins decreased in these cells following glucocorticoid treatment of patients, which suppress immune activation and inflammation. The study concluded increased TJ proteins on leukocytes were associated with immune activation and overall MS disease activity, implicating leukocyte TJ proteins as significant elements of autoimmunity. However, it is unclear if the leukocytes endogenously express these TJ proteins or acquire it through TJ protein+ EVs released into the circulation by endothelial cells. It is also unclear how leukocyte-associated TJ proteins contribute to TEM across the BBB, a critical feature of a multitude of neuroinflammatory diseases. It is possible that TJ protein+ leukocytes define a small subset of the total leukocyte population, that contribute to the initial opening of the BBB in neuroinflammatory disease, paving a route for the trailing pathogenic leukocytes to extravasate along. Therefore, addressing the role, source, and mode of acquisition of these TJ proteins on the circulating leukocytes, might be critical for understanding how they breach the BBB during the onset and/or progression of neuroinflammation.
3. Materials and Methods

3.1 High resolution 3D imaging of leukocyte transendothelial migration in vivo

Processing of thoraco-lumbar spinal cord tissue, immunofluorescent staining of 60µm thick cryosections and analysis of confocal z-stack images were performed as described previously (Paul et al., 2013; 2014).

3.2 Isolation of EVs from brain endothelial cell line (bEND3) and primary brain microvascular endothelial cells (BMEC)

Brain endothelial cells (bEND3) (ATCC) were cultured in DMEM media supplemented with 10% Debyon, FBS till 90% confluence. The cells were switched to DMEM supplemented with 2% exosome free FBS, and grown for additional 12 hours with 10ng/ml TNFα in the media. The bEND3 supernatant was spun at 300×g for 10 min at 4°C, 2000×g for 10min at 4°C, and finally 8000×g for 30 minutes at 4°C to remove the debris and cell fragments. The clear supernatant was spun first at 20,000×g and 60,000×g for 30 mins for isolating the larger and smaller MVs-sized vesicles respectively, and then at 100,000g for 60 mins to isolate the exosomes-sized vesicles.

Brain microvascular endothelial cells were isolated from mice in a fashion described earlier (Ge et al., 2007). Briefly, after removal of the brain from 4-6 week old mice, the meninges and big vessels were discarded and the cortex homogenized with a 7 mL Dounce tissue grinder (Kimble/Kontes) in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL) supplemented with
antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The homogenates were suspended in 18% dextran (v/v, molecular weight 60,000–90,000; USB Corporation) and centrifuged at 10,000 × g for 15 min. The pellet was resuspended in Ca\(^{2+}\) and Mg\(^{2+}\)-free Hank’s balanced salt solution (HBSS) (Gibco BRL), and filtered through a 40 μm cell strainer (Becton Dickinson Labware). The microvessels trapped on the filter were digested with 0.1% collagenase-dispase (Roche) in HBSS containing 20 U/mL deoxyribonuclease-I, and 0.147 μg/mL of the protease inhibitor tosyllysine chloromethyl ketone at 37°C for 40–60 min. Digestion was continued with occasional agitation, until endothelial cells were seen popping out from most of the microvessel fragments. These microvessels were pelleted at 1000×g for 5 min, and washed once with phosphate-buffered saline (PBS) containing 1% bovine serum albumin. The pelleted vessels purified with anti-murine PECAM-1-coated Dynabeads M-450 (Dynal) at 4°C for 60 min with gentle mixing, and then subjected to immunomagnetic purification (Song et al., 2003, 2004). The bead-purified vessel fragments resuspended in complete culture medium (DMEM containing 10% fetal bovine serum, 10% horse serum, 100 μg/mL penicillin and 100 μg/mL streptomycin, 100 μg/mL heparin, and 100 μg/mL endothelial cell growth factor supplement), and plated onto tissue culture dishes coated with murine collagen IV (BD Biosciences). After 48 h, cultures the cells were resuspended in fresh media after washing off the free magnetic beads. Cultures were allowed to reach about 90 percent confluence prior to their subculture. At that time, purity was gauged to be ≥ 98% BMEC, according to dil-acetylated LDL uptake (Song and Pachter 2003).
BMECs were isolated and cultured from eGFP-CLN-5 transgenic mice in the same way. EVs were isolated from TNFα-treated wild-type and eGFP-CLN-5 transgenic BMECs as described for the bEND3 cells.

3.3 Isolation of leukocytes and EVs from blood

Naïve and EAE mice (n=10) were anesthetized with ketamine (80 mg/kg, ip) and xylazine (10 mg/kg, ip) in PBS, pH 7.4. A 3ml syringe with 25 gauge needle was briefly flushed with 0.109M 3.2% Sodium Citrate (BD) and blood was slowly acquired using transcardiac puncture. 3ml of fresh anticoagulant treated blood was diluted 1:1 with HBSS at 25°C by gently inverting the tube and slowly layered on 3ml of Ficoll-Paque PLUS in a 15ml Falcon tube. Samples were spun at 400×g for 40 min at 25°C in a swing-bucket centrifuge (Eppendorf 5804R) without acceleration or brakes.

The “buffy” lymphocyte layer from the Ficoll-Paque PLUS gradient was carefully aspirated using a 1ml pipette, washed in 3 volumes of HBSS and spun at 100×g for 10 min. After, discarding the supernatant, 1 ml of RBC lysis buffer was added in each tube to remove left over RBC contamination and incubated for 5 min at 25°C. The sample was neutralized with three volumes of HBSS (Gibco) and spun at 100×g for 10 min. The pellet was resuspended in HBSS and the yield was calculated by Trypan blue exclusion technique with a hemocytometer.

The upper layer containing plasma was slowly drawn using a sterile Pasteur pipette and used for microvesicle and exosome isolation. The plasma was spun at 300g for 10min at 25°C,
2000g for 10min at 25°C, and finally 8000g for 30 minutes at 4°C to remove the debris and cell fragments. The clear supernatant was then spun first at 20,000×g and 60,000×g for 30 mins for isolating the larger and smaller MVs and then at 100,000g for 60 mins to isolate the exosomes.

Enrichment of microvesicle and exosome – sized vesicles in these fractions was validated by nanoparticle tracking analysis (NTA) (Figure S9).

3.4 EAE induction and scoring in wild-type and eGFP-CLN-5 mice

EAE was induced, and the disease score analysis was done as published before (Paul et al., 2013, 2014)

3.5 Western blotting

Protein level was assayed by the Micro BCA protein assay kit (Pierce). Leukocytes were analyzed for CLN-5 protein using mouse monoclonal anti-CLN-5 (Life Technologies) as previously detailed (Paul et al, 2013, 2014). Blots were developed using the chemiluminescent HRP substrate kit (SuperSignal West FemtoMaximum Sensitivity; Pierce) and signal detected using a G:Box XX6 digital gel imager (Syngene). Images were acquired by GeneSys software (Syngene) and automated densitometric analysis performed using GeneTools software (Syngene).

3.6 Binding of total EVs to leukocytes

Exosome and microvesicle- sized EVs were labeled with the fluorescent membrane dye PKH67
(green), while isolated PBMCs were labeled with another fluorescent membrane dye, PKH26 (red). PKH67-labeled EVs were incubated with PKH26-labeled leukocytes in Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS supplemented with 2% exosome-free serum (System Biosciences) at 4°C overnight on poly-L-lysine-coated 8-well chamber slides (Nunc). Following washing in HBSS, the leukocyte-EV complex was spun down at 200×g and fixed in 4% PFA. High-resolution images were acquired using a Zeiss LSM 510 Meta confocal microscope equipped with a 100X plan-apochromatic oil immersion lens, 1.3NA, as described above for 3D imaging of CLN-5\(^+\) leukocytes. While leukocytes might need to be activated to bind EVs, circulating leukocytes from EAE mice might already be saturated with EVs bound \textit{in vivo} prior to our isolation. Also, EVs released into the blood during neuroinflammation might differ in binding capacity from those present in the normal state. Thus, for our \textit{in situ} experiments EVs purified from EAE mouse plasma were incubated with leukocytes from naïve mice.

### 3.7 Isolation of CLN-5 leukocytes from eGFP-CLN-5 mice

Transgenic C57BL/6 mice expressing reporter eGFP fused to CLN-5 protein, under the \textit{endothelial Tie-2/Tek-1} promoter/enhancer, in all \textit{endothelial cells}, underwent EAE induction and CLN-5 expression in the CNS microvessels evaluated by high-resolution 3D fluorescence imaging as described earlier (Paul et al., 2013, 2014), and the eGFP-CLN-5 leukocytes studied \textit{in situ} in mice inflicted with EAE in a similar fashion. eGFP-CLN-5 leukocytes were isolated from blood and CNS tissue as described in previous section, and further purified via FACS. Co-localization with anti-CLN-5 staining (Red) and the transgenic eGFP (Green) was performed
to validate epitope specificity of the immunostaining (data not shown).

3.8 Serial scanning electron microscopy (serial EM) and 3D contour surface creation using Imaris®

Serial EM was performed as described before (Terasaki et al., 2013). Mice were anesthetized with Ketamine/Xylazine and transcardially perfused first with PBS to wash off the blood, and then with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M cacodylate buffer through the left ventricle of the heart. The spinal cords were isolated by laminectomy and fixed for an additional 3-4 hours in the same fixative, then rinsed and stored in 0.1 M cacodylate buffer at 4°C. The lumbar section of the spinal cord was cut with a razorblade into ~1mm thick slices and rinsed in 0.1M cacodylate buffer several times. The samples were processed using the ROTO protocol (Tapia et al., 2012), they were dehydrated in grade ethanol solutions and embedded in epoxy resin (Polybed, Polysciences; Warrington, PA).

After the samples were polymerized, the face of each block was shaped to a ~2 x 3 mm rectangle using a diamond trimming knife. Thin sections, 60nm thick, were cut using a microtome (Leica EM UC7, Buffalo Grove, IL) and a diamond knife. Serial sections were collected on kapton tape (glow-discharged to minimize wrinkling of sections) using the ATUM tape collector (Hayworth et al., 2006), the tape with sections was cut into strips and mounted on 4-inch silicon wafers (University Wafers, South Boston, MA), and then carbon coated for electron grounding (Denton 502B, Moorestown, NJ).
The sections were imaged using a field emission scanning EM (Zeiss Sigma FE-SEM; Peabody, MA) in backscatter mode (10 keV electrons, ~5 nA beam current). A high-precision map of the sections on the wafer (± 4 μm) was generated, then the Atlas Large Area Imaging software (Fibics Inc.; Ottawa, Ontario, Canada) was used to automatically image a ~65 μm × 65 μm field of the serial sections at 5-7 nm/pixel resolution (12,288 × 12,288 pixels). The images were aligned using the Linear Alignment with SIFT algorithm (FIJI, Image J), and reconstructed using Imaris®.

To visualize the endothelium and EVs at the site of leukocyte adhesion in 3D, the serial EM slices were first imported into Imaris for volume rendering. Manual contour tracing was then performed by cursoring out the endothelium, EVs and the ‘adherent’ leukocyte of interest in each serial EM z-slice and the individual contours merged into a 3D contour surface, as detailed previously (Paul et al., 2013; Bohórquez et al, 2014).

3.9 Transendothelial migration (TEM) of leukocytes across BMEC

TEM of leukocytes across a mouse BBB model was performed as previously described (Ge et al., 2008). bEND3, brain microvascular endothelial cells (BMEC) (ATCC), were cultured on Transwell inserts (24-well format, 8.0 um pore) till confluent. Leukocytes were isolated from the blood of Tie-2-eGFP-CLN-5 transgenic mice at day 9 post EAE induction and separated based on presence or absence of eGFP-CLN-5 using a flow cytometer (BD FACSArria™ II). 1×10^5 leukocytes were placed on the upper chamber, and chemokine CCL2 (10 nM; Biolegend) added to the lower chamber. After different times (2h, 6hr, and 18hr) at 37°C, transmigrated leukocytes in
the lower chamber were removed and counted by flow cytometry (MACSQuant® Analyzer 10, Miltenyi Biotech).

4. Results

4.1 TEM of CLN-5+ leukocytes during preclinical EAE

To determine the status of blood-brain barrier integrity, with respect to endothelial CLN-5 immunostaining, during the earliest leukocyte TEM events, spinal cord sections from mice at D9 post EAE induction (preclinical phase) were immunostained for TJ protein CLN-5, endothelial marker CD31, and nuclear stain DRAQ5. 3D reconstruction and Isosurface rendering of microvessels in high-resolution confocal z-stacks were performed as detailed previously (Paul et al., 2014). Loss of TJ protein CLN-5 has been previously reported to be restricted to venules and post-capillary venules in EAE (Paul et al., 2013). Venular CLN-5 staining seemed to largely localize with the CD31 staining at this time-point. However, a population of leukocytes transmigrating across inflamed thoraco-lumbar spinal cord venules (meningeal and parenchymal) at this time point was immunostained for CLN-5+ (Figure 16a; Figure S8). Such, leukocytes were mostly visible along the meningeal microvessels or the infiltrating parenchymal vessels at this early time point (Figure S8). Notably, the TEM of CLN-5+ leukocytes appeared to happen in regions proximal to focal discontinuities in CLN-5 immunoreactivity, indicative of BBB damage. The CLN-5 staining on these abluminal leukocytes was apparently distributed all around the leukocyte surface in a discreetly punctate pattern (Figure 16b inset). This could suggest that these leukocytes acquire endothelial CLN-5 either prior to or during the process of TEM by a yet
uncharacterized mechanism, which might be critical for these leukocytes to negotiate their passage across the BBB. These CLN-5+ leukocytes might in effect be the first ones to focally open the BBB, for the others to follow. Using identical staining and image acquisition parameters no evidence of CLN-5+ leukocytes was detected in spinal cord venules from naïve mice (data not shown).

4.2 Expression of CLN-5 on leukocytes in EAE

High resolution 3D reconstruction of confocal images of leukocytes isolated from plasma of mice inflicted with EAE (d7 post induction) revealed punctate CLN-5 immunostaining distributed around the leukocyte surface, similar to the CLN-5+ extravasating leukocytes observed in vivo. The CLN-5 staining could be localized on leukocyte surface under both permeabilizing and non-permeant conditions, suggesting surface binding of the CLN-5 antibody (Figure 17a). FACS analysis of leukocytes from d7 EAE mice immunostained with the same antibody further validated the CLN-5 staining under both permeabilizing and non-permeant conditions, although, permeabilization yielded marginally higher staining intensity compared to the non-permeabilized group, suggesting better accessibility of the antibody epitope when permeabilized (Figure 17b). Specificity of the CLN-5 antibody was further confirmed by using the same antibody clone in immunoblot analysis of lysates obtained from plasma leukocytes at D7 p.i. Immunoblot analysis yielded a band of 23KDa, consistent with the reported molecular weight of CLN-5 protein (Figure 17c). These results could reflect the presence of CLN-5 on extravasating leukocytes prior to their TEM across CNS microvessels.
4.3 Endothelial origin of CLN-5 on leukocytes

Given the reported loss of endothelial CLN-5 in the CNS microvessels during neuroinflammation in EAE (Paul et al., 2013, 2014), to explore if the CLN-5 on these CNS infiltrating leukocytes in EAE is endothelial in origin, transgenic mice expressing eGFP-CLN-5 in endothelial cells, Tie-2-eGFP-CLN, were employed (Knowland et al., 2014). Figure 18a shows CLN-5 staining along inter-endothelial locales in various microvessel subtypes—venules, post-capillary venules and capillaries in spinal cord from naïve eGFP-CLN-5 mice. This picture differed in eGFP-CLN-5 mice inflicted with EAE at D9 p.i., as an emergence of CLN-5+ extravasating leukocytes were clearly seen in inflamed venules at this time (Figure 18b), suggesting a predominantly endothelial origin of the CLN-5 on leukocytes undergoing TEM.

4.4 Expression of CLN-5 on EVs isolated in vitro and in vivo

Since, two main categories of extracellular vesicles (EVs), namely exosomes (50nm -100nm in diameter) and microvesicles (100nm -2µm in diameter), have been reported to bind leukocytes (Lee at al., 2010), mediate their immune responses (Muturi et al., 2013; Zech et al., 2012; Raposo and Stoorvogel, 2013) and adhesion to endothelium (Lee at al., 2010), we investigated if EVs released from brain microvascular endothelial cells (BMECs) both in vitro and in vivo contain CLN-5 protein.

Figure 19a, shows the expected 23 KDa band for CLN-5 in total EV lysates obtained following a 100,000×g spin from BMEC cultures treated with proinflammatory cytokine TNFα. To
further dissect the specific EV subtypes released from TNFα-treated BMECs that might contain CLN-5, three EV fractions were isolated using differential ultracentrifugation at 20,000×g, 60,000×g, and 100,000×g. All the obtained EV fractions displayed the expected 23KDa band, suggesting the presence of CLN-5 in EVs from BMECs in vitro (Figure 19b).

We next assayed the presence of CLN-5 in EVs released during EAE from BMECs in vivo using mice expressing eGFP-CLN-5 in endothelial cells (Tie-2-eGFP-CLN-5) (Figure 19c). FACS analysis of plasma EVs isolated from eGFP-CLN-5 mice at D9 p.i. and co-stained with PKH-26 dye showed evidence of the transgenic eGFP labeled endothelial CLN-5 on EVs at this time point, although, eGFP-CLN-5 EVs comprised only 0.4% of the total PKH-26 labeled EV population (Figure 19d).

4.5 Binding of BMEC-derived EVs to naïve leukocytes

Having shown the presence of endothelial-specific CLN-5 on circulating leukocytes in EAE, and that BMECs-released EVs, we next addressed if BMEC-derived EVs can bind naïve leukocytes in vitro and in vivo. For, in vitro analysis EVs were isolated from TNFα-stimulated bEND3 cultures and separated by differential ultracentrifugation into sizes corresponding to exosomes and microvesicles followed by validation with nanoparticle tracking analysis (NTA) (Figure S9). Figure 20a shows, these exosome and microvesicle –sized vesicles from bEND3 cells labeled with PKH-67 dye, could bind to naïve PKH-26 stained leukocytes. This binding was further confirmed as PKH-67+ PKH-26+ (double-positive) events in FACS analysis (Figure 20b), which revealed 7.1% co-localized events. However, this could have included the possibility of multiple EVs binding to
one leukocyte. Notably, the binding of PKH-67+ EVs to PKH-26− naïve leukocytes was saturable, and could be competed out with a thousand-fold excess of unlabeled EVs, under identical binding conditions.

To explore if CLN-5+ EVs released from ‘inflamed’ BMECs in vitro and in vivo can also bind naïve leukocytes, Tie-2-eGFP-CLN-5 mice was used for obtaining the transgenic reporter (eGFP) labeled CLN-5+ EVs. CLN-5+ EVs isolated from TNFα-treated primary BMECs (data not shown) or the serum of Tie-2-eGFP-CLN-5 mice at d9 post-EAE induction, showed apparent binding to the naïve CD45+ leukocytes (Figure 20d).

4.6 EVs are present in vivo between the endothelium and site of leukocyte attachment

Till date, it is not known if EVs can be released locally by endothelial cells in vivo to aid leukocyte attachment or TEM, an event post attachment. Capturing such an event would require electron microscopic (EM) resolution; although, the likelihood of imaging an adherent or transmigrating leukocyte in its entirety along the microvascular endothelium in vivo at this resolution is very thin. Therefore, 3D serial scanning EM (serial EM) acquiring 300-350 slices each being 60 nm thick, i.e., equivalent of a 20 µm thick section, and sampling of several spinal cord tissue blocks encompassing multiple thoraco-lumbar regions obtained from the spinal cord of a mouse at d13 EAE, were undertaken to visualize the leukocytes adhered to inflamed microvascular endothelium (Figure 21a). Furthermore, using a novel Imaris® based 3D contour tracing approach, which allowed us to trace contours of the endothelium, adherent leukocytes, and vesicular structures in these EM stacks, we found EV-like structures at the leukocyte-endothelial
These EV-like multilamellar structures appeared to be partially embedded in the endothelium proximal to site of leukocyte-endothelial attachment (Figure 21c-e), suggesting endothelial EVs are released in vivo during EAE close to the leukocyte attachment site, perhaps for aiding TEM of the adherent leukocytes.

4.7 CLN-5⁺ leukocytes are more efficient in TEM in vitro

To gauge the role of endothelial CLN-5 on leukocytes in EAE, CLN-5⁺ leukocytes were FACS sorted from the blood of endothelial-specific Tie-2-EGFP-CLN-5 mice at d9 EAE (Figure 22a) and their TEM efficiency across a bEND3 endothelial cell monolayer was compared to CLN-5⁻ leukocytes isolated from the same animals. TEM efficiency of CLN-5⁺ leukocytes seemed to be 1.9, 2, and 2.68 fold higher at 2 hours, 6 hours and 18 hours respectively, compared to the CLN-5⁻ controls (Figure 22b).

5. Discussion

The TEM of leukocytes across the blood-brain barrier (BBB) has been known to underlie the histopathology in a multitude of neurodegenerative conditions, like Multiple Sclerosis (Al-Omaishi et al., 1999; Larochelle et al., 2011). While the mechanism(s) of leukocyte-endothelial adhesion and the molecules involved in the extravasation of circulating leukocytes across the endothelial wall in peripheral microvessels is well understood (Muller, 2011), the TEM of leukocytes across the highly restrictive blood-brain barrier (BBB) remains largely unclear. Several intravital imaging studies have shed some light on the differences in TEM across the CNS microvessels compared
to that in the periphery, e.g., leukocytes have been reported to crawl preferentially against blood flow before their diapedesis across the vascular wall in the CNS (Engelhardt, 2010; Greenwood et al., 2011) under the influence of adhesion molecules (Engelhardt, 2006), and chemokines like CCL2 (Paul et al., 2014; Huang et al., 2001; Dogan et al., 2008). Since, the presence of tight junctions (TJs) at the BBB is generally considered an impediment to leukocyte extravasation in the CNS (Larochelle et al., 2011); the initial leukocytes crossing the almost intact BBB are believed to negotiate these TJ to access the CNS tissue by a yet uncharacterized mechanism.

In this study, we present the first report of a sub-population of leukocytes infiltrating the CNS of mice early in EAE (d9), an animal model of MS, being immunoreactive for TJ protein CLN-5, a BBB determinant, in close apposition to the inflamed microvessels. These CLN-5⁺ cells appeared in microvascular regions displaying dense perivascular cellularity, suggestive of an inflamed status (Paul et al., 2013; 2014). Such, leukocytes were visible only along the meningeal microvessels or the infiltrating parenchymal vessels at this early time point which are known to display higher expression of adhesion molecules (Kivisäkk et al., 2009; Owens et al., 2008) and get inflamed earlier in MS (Choi et al., 2012) and its animal correlate, EAE (Christy et al., 2013).

Using high-resolution 3D confocal imaging, the CLN-5 immunostaining appeared to localize on the surface of these transmigrating leukocytes in a discreetly punctate pattern, and close to inflamed microvessels displaying focal beading, discontinuity, or diffuse pattern of CLN-5 staining, suggestive of BBB damage (Plumb et al., 2002). Leukocyte entry into the CNS tissue has been reported to be associated with a transient focal loss of TJ proteins in both animal models of
neuroinflammation (Xu et al., 2005; Wolburg et al., 2003) as well as in human CNS diseases, like, MS (Plumb et al., 2002), glioblastoma multiforme (Wolburg et al., 2003), and HIV-1 encephalitis (Persidsky et al., 2006).

Interestingly, a recent report described the expression of TJ protein Claudin-1 and Claudin-5 in peripheral blood leukocytes (PBLs), predominantly in B cells, T cells and monocytes in MS patients in relapse (Mandel et al., 2012). Furthermore, following glucocorticoid treatment in these patients, which confers both anti-inflammatory and immunosuppressive properties, RNA levels of these TJ proteins in PBLs decreased. The study reinforced the notion that increased number and/or expression CLN5\(^+\) leukocytes is correlated with immune activation, and overall MS disease activity, suggesting a potential role of leukocyte TJ proteins in physiological states, and autoimmunity. However, the emergence of leukocytes having TJ proteins across the inflamed microvessels, their TEM efficiency, and possible role in BBB damage has never been reported before. These TJ positive leukocytes could define a small subset of the total leukocyte population, that contribute to the initial opening of the BBB in disease, paving a route for the trailing pathogenic leukocytes to extravasate along.

3D confocal z-stack images of isolated leukocytes from mice with EAE at d7 post-induction revealed a similar distinct CLN-5 immunostaining all around the leukocyte surface as seen \textit{in vivo}. It was noteworthy that the intensity of CLN-5 immunostaining on the isolated leukocytes was weak, either suggesting they are not bonafide CLN-5 expressing cells, or they acquire it from other sources. Western Blot analysis on leukocyte lysates obtained d7 EAE mice using the
same antibody clone used on immunohistochemistry yielded the expected 23KDa molecular weight band, consistent with the molecular weight of CLN-5.

Since, CLN-5 on CNS endothelial cells has been shown to be a BBB determinant (Nitta et al., 2003), and leukocytes breaching the BBB were likely to acquire in from the endothelium, high-resolution confocal imaging was used to capture the transmigrating leukocytes across the inflamed microvessels in EAE using a transgenic mice expressing eGFP-CLN-5 predominantly in endothelial cells (Tie-2-eGFP-CLN-5). Leukocytes undergoing TEM across the inflamed microvessels at d9 EAE displayed a similar pattern of CLN-5 labeled with eGFP as seen in the WT mice with EAE, which was absent in the naïve Tie-2-eGFP-CLN-5 mice, indicative of this TEM of CLN-5+ leukocytes being related to the onset and/or progression of the disease.

Since Mandel et al., (2012) reported the presence of CLN-5+ leukocytes in the blood of MS patients, and accumulating evidence indicates that EVs released by resident CNS cells, like, neurons, microglia, astrocytes, oligodendrocytes and endothelial cells can aid in dispersal of pathogenic bioactive effectors (proteins, DNA, or RNA) into the blood in neurodegenerative diseases, e.g., transmissible spongiform encephalopathies, Alzheimer’s disease, Parkinson’s disease, tauopathies, and amyotrophic lateral sclerosis (Vingtdeux et al., 2007; Vella et al., 2008; Guest et al., 2011; Frühbeis et al., 2013) which in turn can modulate the activation, adhesion and migration of the circulating immune cells (Lee et al., 2010), we looked for the presence of CLN-5 in EVs derived from BMECs in vitro and in vivo. Owing to the fact that 80% of the EVs present in blood have been reported to be derived from platelets (Győrgy et al., 2011), we first looked into
presence of CLN-5 in EVs obtained from TNFα-stimulated bEND3 cells. Surprisingly, despite the EVs being known to originate from different subcellular locales (i.e., MVs from cell membrane, and the exosomes from endosomal compartment), the EV fractions isolated by differential centrifugation and characterized according to size using nanoparticle tracking analysis, contained TJ protein CLN-5. Endothelial-specific EVs isolated from the plasma of mice expressing eGFP-CLN-5 (Tie-2-eGFP-CLN-5) at D9 p.i., showed only about 0.4% EVs positive for CLN-5 among the total PKH-26 labeled EV population, as expected considering a major contribution of plasma EVs from the platelets. As, FACS couldn't resolve vesicles lower than 300nm -400nm in diameter, based on calibration with florescent nano-beads (data not shown), exosomes and smaller MVs could have been excluded from our FACS based EV analysis.

PKH-67 labeled EVs from bEND3 cells following differential centrifugation and categorized into exosomes and MVs based on size, clearly appeared to bind PKH-26 stained naïve leukocytes. Total EV population isolated from plasma of WT mice or endothelial-EVs isolated from Tie-2-eGFP-CLN-5 mice with EAE could also bind naïve leukocytes in a similar fashion. Since, incubation of naïve PKH-26+ leukocytes with a 1000 fold excess of unlabeled EVs could almost compete out the binding of PKH-67+ EVs, the binding sites for EVs on the leukocytes could possibly be discreet.

However, in vitro binding analysis of EVs to leukocytes can't accurately model the events happening in vivo in a neuroinflammatory milieu. In additional, the nano-sized EVs can't be visualized at high-resolution in vivo by using conventional epifluorescence or confocal
microscopy. Therefore, using a 3D serial scanning electron microscopy (serial EM) followed by a novel Imaris based 3D contour tracing approach, we provide the first evidence of EV-like structures being released in vivo during EAE proximal to site of leukocyte-endothelial attachment site, perhaps aiding TEM of the adherent leukocytes. These EV-like structures seemed to display multilamellar appearance. Since, the likelihood of capturing an adherent or transmigrating leukocyte in its entirety along the microvascular endothelium in vivo at EM resolution is very thin, serial EM of 300-350 slices each being 60 nm thick, and sampling of several spinal cord tissue blocks enabled us to capture the docked leukocytes.

Finally, using the endothelial specific Tie-2-EGFP-CLN-5 mice, the TEM efficiency of CLN-5\(^+\) leukocytes migrating across a bEND3 monolayer was consistently found 2-3 fold higher with time (at 2h, 6h and 18h) compared to the CLN-5\(^-\) controls isolated from the same mice. Notably, based on the scatter property analysis, the population transmigrated cells lacked monocytes.

In summary, based on the scattered literature on presence of TJ proteins on leukocytes and a role of EVs in immunomodulation, while unifying the two observations, we report for the first time that CLN-5 transmigrating leukocytes can be seen on the abluminal side of inflamed microvessels in EAE and endothelial EVs can transfer CLN-5 to the circulating leukocytes for their efficient TEM in neuroinflammation. An understanding of the contribution of these TJ proteins on leukocytes in TEM across the BBB, a step critical for pathogenesis underlying many inflammatory diseases like MS, might be critical for therapeutic targeting of these pathogenic leukocytes for the treatment of central and/or peripheral inflammation.
**Figure 16. TEM of CLN-5* leukocytes in early EAE.** z-stack confocal images acquired from thoraco-lumbar spinal cord cryosections of WT mice at d9 EAE are shown, revealing staining of TJ protein CLN-5 (green), and CD31 (red) to delineate the endothelial cells, respectively. DRAQ5 staining reveals the cellularity associated with TEM of CNS infiltrating leukocytes. **a, b** and **c** demonstrate a population of leukocytes undergoing TEM being immunostained for CLN-5 in a punctate fashion, around the leukocyte surface. Arrows (yellow) point toward discontinuity in microvascular CLN-5 immunostaining, suggestive of BBB damage.
Figure 17. Expression of CLN-5 on circulating leukocytes in EAE. (a) Representative z-stack confocal images of leukocytes isolated from blood of WT mice at day 7 (d7) post EAE induction and immunostained with CLN-5 in presence or absence of Triton-X 100 detergent, underscoring the presence of CLN-5 binding epitope on the leukocyte surface. (b) FACS analysis of leukocytes from d7 EAE mice immunostained with the same antibody shows CLN-5 staining under both permeabilizing and non-permeant conditions, although, permeabilization with Triton X-100 yields marginally higher staining intensity (along the x-axis) compared to the non-permeabilized group, suggesting better accessibility of the antibody epitope when permeabilized. Leukocytes stained with an isotype antibody were used as control. (c) Western blot analysis of lysates from the same batch of leukocytes and same antibody clone used in (a) and (b) showing a 23 KDa molecular weight band, consistent with the molecular weight of CLN-5.
Figure 18. Transmigrating leukocytes have CLN-5 of endothelial origin. z-stack confocal images acquired from thoraco-lumbar spinal cord cryosections from Tie-2-eGFP-CLN-5 mice showing (a) distribution of eGFP-CLN-5 in naïve microvessels, (b) TEM of eGFP-CLN-5+ leukocytes at day 9 (d9) EAE, suggesting the predominantly endothelial origin of the observed eGFP-CLN-5 on the extravasating leukocytes. Inset shows DRAQ staining for nuclei in these leukocytes.
Figure 19. CLN-5 expression in BMEC derived EVs. Western blot analysis of CLN-5 in EVs from supernatants of TNFα-treated bEND3 cultures. (a) Total EV population pelleted following a 100,000×g and (b) EV pellets obtained following differential ultracentrifugation at 20,000×g, 60,000×g and a 100,000×g spin, showing the expected 23 KDa molecular weight band. (c) Schematic representation of endothelial specific EV isolation strategy from endothelial-specific Tie-2-eGFP-CLN-5 mice. (d) FACS analysis of EVs isolated from Tie-2-eGFP-CLN-5 mice, showing eGFP EVs alone (left), or eGFP EVs co-stained with PKH-67 dye. Ultrapure distilled water (below), and nano-fluorescent size standard beads (now shown) were used for setting the gates.
Figure 20. EVs from ‘inflamed’ BMECs bind naïve leukocytes in vitro. (a) z-stack confocal images showing PKH-26 labeled EVs, enriched for exosome- and microvesicle-sized particles by differential ultracentrifugation, isolated from TNFα-treated bEND3 cells can bind PKH-26 labeled naive leukocytes. Insets further highlight the co-localization on single leukocytes. (b) FACS analysis of PKH-67 labeled Total EVs, isolated following a 100,000×g spin, binding to naïve PKH-26 labeled leukocytes, analyzed as double-positive events. (c) Shows the binding of PKH-67 EVs to naïve leukocytes is ‘saturable’ and can be competed out by adding a 1000-fold excess of unlabeled EVs. (d) Endothelial-specific Tie-2-eGFP-CLN-5± EVs FACS purified from plasma of mice at d9 post EAE induction, as described in Figure 18, can bind naïve leukocytes in culture.
Endothelial EVs are released *in vivo* at sites proximal to leukocyte adhesion. Serial scanning EM images from thoraco-lumbar spinal cord sections of perfusion-fixed d13 EAE mice. (a) 3D reconstruction from 130 serial slices showing cross-section on an inflamed venule highlighting adherent leukocytes, some apparently undergoing TEM. Insets highlight EV like membrane-bound structures at the leukocyte-endothelial interface in single serial slices. (b) Representative 3D reconstruction of a single adherent-leukocyte. (c) Contour surface reconstruction from the ‘traced’ leukocyte, EVs and endothelium in all serial slices, providing 3D view of released endothelial EVs proximal to the site of leukocyte docking. Inset shows the site of leukocyte attachment on the endothelium and the site of EV release. (c, d) Serial EM images containing the 3D surface reconstruction of the same leukocyte in (c) and representative single serial slices highlighting various patterns of released endothelial EVs, close to the sites of leukocyte binding.
Figure 22. CLN-5⁺ leukocytes undergo efficient TEM. (a) FACS sorting of CLN-5⁺ leukocytes isolated from blood of Tie-2-eGFP-CLN-5 mice at d9 post-EAE induction (right). Control leukocytes from naive mice were used to set up the gate (left). (b) FACS analysis of TEM efficiency, represented as fold increase in the number of transmigrated CLN-5⁺ leukocytes over time (in hours) compared to control leukocytes isolated from the same mice, across bEND3 brain endothelial cell monolayer plated on transwell chambers. Each group consisted of leukocytes obtained from 10 mice, and analysis was performed in triplicate. Graphs represent mean ± standard error. p values depicted were obtained from two-way ANOVA analysis followed by Bonferroni post hoc test. *p≤0.01 and ** p≤0.001.
Concluding remarks & future directions

Leukocyte extravasation into the central nervous system (CNS) features critically in the pathogenesis of neuroinflammatory and neurodegenerative disease (Zlokovic, 2008; Glass et al., 2010; Qian et al., 2010; Holman et al., 2011; de Vries et al., 2012; van Noort et al., 2012). Hence, an understanding of the cues governing the exit of circulating immune cells, and thereby, breaching the BBB, holds key to novel therapeutic strategies for treating a wide spectrum of diseases like Parkinson disease, Alzheimer disease and Multiple Sclerosis (Mae et al., 2001; Pander et al., 2002; Wiendl, 2002; Weber et al., 2012). While the cascade of events that initiate leukocyte adhesion in peripheral microvessels has been well characterized (Rossi et al., 2011; Sallusto et al., 2012), we lack an understanding of the cues required for transendothelial migration (TEM) of leukocytes across the BBB in CNS. The projects described in this dissertation have broadened our understanding on how endothelial heterogeneity, inflammatory chemokines, and bioactive factors transferred by endothelial EVs guide leukocyte TEM at the ‘permissible sites’ along the CNS microvessels in neuroinflammation and might be applicable to peripheral inflammation as well.

Venules are the portals of leukocyte entry into the CNS in neuroinflammation

The highly-specialized CNS microvascular endothelial cells serve as the seat for Blood-Brain Barrier; a barricade restricting paracellular flux of solutes, and entry of circulating immune cells
into the tissue parenchyma (Furuse, 2010; Blasig and Haseloff, 2011; Coisne and Engelhardt, 2011). The CNS microvascular network is comprised of arterioles, capillaries, post-capillary venules and venules, yet the respective contribution of each of these to the BBB is not known. In this regard, the brain microvascular endothelial cells (BMEC) from these different tributaries could exhibit structural and functional heterogeneity, which in turn might confer unique physiological and pathophysiological roles. Previously, using gene expression analysis of BMECs retrieved in situ from naïve capillaries or venules followed by immuno-laser capture microdissection coupled with quantitative real-time PCR analysis of 87 BBB structure and function related genes, we demonstrated that although the BBB properties reside in both segments, the capillaries are enriched in mRNAs related to solute transport, while venules demonstrate an increased expression of mRNAs involved in inflammation-related tasks (Macdonald et al., 2010).

However, the integrity of the BBB has been reported to be compromised in neuroinflammatory and neurodegenerative diseases like MS, and this observed disruption of TJs (Carvey et al., 2009; Coisne and Engelhardt, 2011; Grammas et al., 2011; Hawkins and Davis, 2005; Petty and Lo, 2002) might contribute significantly to the pathology, by facilitating the passage of leukocyte (Garrido-Urbani et al., 2008), or by supporting leakage of serum proteins (Pober and Sessa, 2007) along certain microvessel subtypes or segments only. Therefore, knowledge of which microvascular segments are affected and consequently allow leukocytes to breach the BBB is critical for understanding the disease progression and the emergence of
treatment options.

In this study, using a novel microvascular contour-based 3D quantification of TJ protein CLN-5, a critical BBB determinant, we demonstrated a significant heterogeneity microvascular response along the various microvessel segments of the spinal cord during EAE, a model for MS. Specifically, venules displayed a significant loss of CLN-5 at intercellular junctions during EAE, which was accompanied by extravasation of leukocytes, loss of CLN-5 at the inter-endothelial locales, and compromised basement membrane integrity. In stark contrast, even the directly connected adjoining capillaries were completely refractory to such responses. However, the leakage of endogenous serum leakage, an indicator of BBB permeability was detected around all size microvessels during early and late EAE. Since, the capillaries in EAE fail to show CLN-5 loss even in the chroninc EAE phase (D24 p.i.), this observed inflammation-associated IgG leakage along the CNS capillaries could stem from increased transcytosis (Claudio et al., 1989; Proulx et al., 2012).

Mice with EAE typically display an ascending paralysis, progressing from the lumbar spinal cord towards the brain. However, if the neuroinflammatory events also propagate directionally isn't clear. Therefore, it is imperative to explore the spatio-temporal sequence of events involved in BBB damage, e.g., barrier permeability, TJ loss and leukocyte extravasation, along the length of spinal cord to completely understand the progression of neuroinflammation in this model, and eventually in MS.
Chemokine CCL2 has been long known as a critical mediator of inflammation in the periphery and the CNS, by aiding the extravasation of circulating mononuclear leukocytes into the tissue (Leonard et al., 1991; Mantovani et al., 1993; Bennett et al., 2003; Toft-Hansen et al., 2006; Yadav et al., 2010). Although undetectable in the normal CNS, its expression surges orders of magnitude in neuroinflammatory diseases. Pioneering work by Huang et al., (2001), and Dogan et al., (2008), established the role of CCL2 released exclusively from CNS sources in EAE. Mice globally lacking CCL2 (CCL2−/−) display diminished severity and delay in onset of neuroinflammation in C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein35-55 (MOG35-55). However, to date the contribution of individual CNS sources of CCL2 in the aiding the passage of leukocytes across the BBB in this model, mimicking MS pathology, hasn’t been explored. Since, endothelial cells lining the CNS microvessels and the juxtaposed astrocytes are two major CCL2 sources in the CNS, this study aimed at resolving their individual contributions in guiding leukocytes across the BBB. Getting drugs across the BBB has been a persistent challenge over the years (Pardridge, 2005). Therefore, resolution of the CNS CCL2 targets and their role in disease pathology is critical in determining if drugs would require circumventing or penetrating through the BBB.

Here, using two cell-conditional CCL2 knockout (KO) mice previously generated in our lab (Ge et al., 2009), lacking CCL2 gene in astrocytes (Astro KO) or endothelial cells (Endo KO) respectively, we were able to dissect the individual roles of CCL2 from these two major CNS
sources in disease progression and guiding luminal leukocytes across the BBB for the first time.

The dual effect on EAE progression in global CCL2 KO (CCL2−/−) mice; i.e., diminished severity and delay in EAE onset seen by Huang et al. (2001), could be further resolved as effects dependent on CCL2 released either from astrocytes and endothelial cells. Specifically, reduced EAE severity was found in Astro KO mice and a delay in onset in Endo KO mice, reinforcing that CCL2 from astrocytes and endothelial cells are both critical for EAE progression, in a unique manner. Moreover, the lack of CCL2 in the central compartment did not affect the peripheral priming or activity of myelin-specific T cells, as neither of the KO mice showed deficits in T cell proliferation, or IL-17 and IFN-γ production, following MOG35-55 exposure in vitro, or altered MOG-major histocompatibility complex class II tetramer binding.

To substantiate these altered disease progression with the status of BBB damage and leukocyte extravasation in these KO mice, high-resolution 3D confocal image acquisition and microvessel contour based CLN-5 density analysis was performed as reported previously by our lab (Paul et al., 2013). We present the first evidence that endothelial-derived CCL2 may facilitate a ‘post-adhesion’ stages of leukocyte extravasation, as in the absence of this chemokine pool (in Endo KO) an apparent aggregation of leukocytes in the microvascular lumen, and a better preservation of junctional CLN-5 immunostaining or basement membranes compared to the inflamed microvessels in Astro KO was observed, corroborating the delayed disease onset seen in these mice. On the contrary, astrocyte-derived CCL2 may instead facilitate the passage of leukocytes into the parenchyma, as Astro KO mice revealed an apparent congregation of
leukocytes in the perivascular space.

In light of our results, CCL2 from either astrocytes or BMECs uniquely mediates different aspects of clinical EAE and the associated neuroinflammatory processes depending on the source. While, CCL2 from astrocytes affects severity of clinical EAE via guiding leukocytes into the CNS parenchyma and disrupts CLN-5 at the BBB in the process, CCL2 from BMECs affects disease onset, through regulating the leukocyte transendothelial migration post-adhesion. These results hold significant implications for the requirement for designing BBB permeable vs. impermeant drugs for the treatment of neuroinflammatory diseases. Along these lines, we have recently shown that the anti-inflammatory compound bindarit, a synthetic indazolic derivative that selectively inhibits the transcription of the monocyte chemoattractant subfamily of CC chemokines (MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7), can afford protections against EAE while inhibiting the CNS microvascular CCL2 expression (Ge et al., 2012), underscoring the therapeutic benefits of targeting endothelial CCL2.

Transfer of endothelial CLN-5 to the leukocytes through EVs helps their TEM

The presence of tight junctions (TJs) at the BBB is generally considered an impediment to leukocyte extravasation in the CNS. Therefore, paracellular TEM of the initial leukocytes across the intact BBB must therefore require the leukocytes to negotiate the endothelial TJs.

In this study, we provide the first report of the emergence of a sub-population of transmigrating leukocytes immunoreactive for TJ protein CLN-5, on their surface, in the CNS of
mice early during EAE, in close apposition to inflamed thoraco-lumbar spinal cord venules. Using high-resolution 3D confocal imaging and analysis as detailed previously (Paul et. al, 2014), the CLN-5* leukocytes appeared in regions proximal to focal discontinuities in CLN-5 immunoreactivity along the inflamed microvessels, indicative of BBB damage. The CLN-5 staining on these abluminal leukocytes was apparently distributed all around the leukocyte surface in a discreetly punctate pattern. This raised several questions, e.g., if leukocytes acquire TJ proteins while in circulation or during the TEM process, what is the source of this CLN-5 on the leukocytes, and finally how is CLN-5 acquired by the leukocytes.

Confocal imaging and Western blotting analysis further revealed expression of CLN-5 in leukocytes isolated from the blood of mice with EAE, consistent with a recent report (Mandel et al., 2012) showing the expression of TJ protein Claudin-1 and Claudin-5 in peripheral blood leukocytes (PBLs), predominantly in B and T lymphocytes and monocytes from MS patients.

Since, EVs released from cultured brain microvascular endothelial cells (BMEC) of the BBB have been reported to express a variety of adhesion molecules, e.g. ICAM-1 (Intercellular Adhesion Molecule 1), and stimulate leukocyte TEM in vitro by aiding their endothelial attachment, we investigated if BMEC-derived EVs have CLN-5 cargo and bind to leukocytes, suggestive of the delivery of CLN-5 proteins to the recipient cells.

Using 3D high-resolution confocal imaging and Western blot analysis, we demonstrate for the first time that EVs corresponding in the size to both exosomes and microvesicles obtained from cultured TNFα-stimulated BMEC, express CLN-5, and can bind to leukocytes. To
investigate if the source of CLN-5 on the transmigrating leukocytes in EAE is endothelial, we imaged the inflamed venules in thick thoraco-lumbar spinal cord cryosections from endothelial targeted, Tie-2-eGFP-CLN-5 transgenic mice. We observed labeled leukocytes in the CNS of these mice during early EAE, consistent with CLN-5 having been transferred from endothelial cells to the CNS infiltrating leukocytes. Subsequently, FACS sorted purified eGFP-CLN-5 EVs isolated from the plasma of Tie-2-eGFP-CLN-5 mice were shown to bind naïve leukocytes.

However, to date there is no direct evidence if endothelial EVs are released at the sites of leukocyte TEM in vivo in neuroinflammation. In this study, using serial scanning electron microscopy and 3D contour-based surface rendering we report for the first time that membrane-bound structures resembling EVs are present at the interface of endothelium and the marginating adherent leukocytes in vivo in inflamed microvessels from mice with EAE.

Having shown the transfer of CLN-5 via endothelial EVs to leukocytes, we next looked into the transmigration efficiency of leukocytes containing or lacking endothelial CLN-5 across an in vitro model of BBB, by adding eGFP-CLN-5+ or control leukocytes purified from blood of the Tie-2-eGFP-CLN-5 transgenic mice on BMEC monolayers. eGFP-CLN-5+ leukocytes consistently showed a two fold higher TEM after 2 hours and 6 hours, and a three fold increase in TEM after 18 hours compared to the control leukocytes.

Our results indicate CLN-5+ EVs released from BMEC can bind to leukocytes and thereby transfer CLN-5 protein, for facilitating TEM possibly through the formation of TJ protein bridges between these two cell types (Figure 23). However, further studies are required to validate if
shRNA knockdown of CLN-5 in eGFP-CLN-5⁺ leukocytes can reduce their transmigration and if TEM efficiency of leukocytes lacking endothelial CLN-5 can be rescued following incubation with eGFP-CLN-5 EVs isolated from the plasma of Tie-2-eGFP-CLN-5 mice with EAE.
Figure 23. Interactions of endothelial CLN5+-EVs with leukocytes. EVs shed from endothelial cells could potentially transfer CLN-5 protein to leukocytes and foster TEM by several conceivable scenarios: (1) Binding of shed CLN-5+ EVs to undefined sites on the leukocyte surface; (2) Binding of nascent CLN-5+ EVs still associated with the endothelium to endogenous CLN-5 on the leukocyte surface; and (3) Binding of shed CLN-5+ EVs to endogenous CLN-5 on the leukocyte surface, resulting in temporary crosslinking of leukocyte to the endothelium. Binding of CLN-5+ EVs to endogenous CLN-5 on the leukocyte surface could potentially amplify leukocyte:endothelial interactions by increasing avidity of CLN-5 binding partners. Not shown are possibilities EVs might inject endothelial-derived CLN-5 protein and/or mRNA into the leukocyte for surface expression. Concentrated release of EVs near the junctional region could act in a paracrine manner to guide leukocytes to this site for TEM.
Heterogeneity of the CNS Microvascular endothelium

I. Introduction: To be or not be—cellophane

The endothelium is recognized as a specialized epithelium lining the vasculature, the lymphatic vessels and the heart (Dyer and Patterson 2010). German anthropologist and pathologist Rudolph Virchow is credited with the earliest known description of this tissue in the mid-19th century, and the term endothelium (from the Greek end meaning within, and thele meaning nipple) was coined by the Swiss anatomist Wilhelm His soon after (to distinguish it from epithelium; i.e. outside the nipple). Despite its century and half-long medical history, the complexity of the endothelium has only relatively recently come to light. Indeed, Virchow’s banal characterization of the capillary lining as “a membrane as simple as any that is ever met with in the body” (Laubicher et al. 2007), and Lord Florey’s no less flattering account – nearly a century later – of the endothelium as “a sheet of nucleated cellophane” (Florey 1966), initially branded the endothelium as phenotypically invariant and serving only a passive barrier function. This interpretation has since changed considerably.

Two technical advances largely contributed to the sharp trajectory in our understanding about the endothelium. One, was the development of electron microscopy (EM), which provided

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the earliest ability to peer into the endothelial ultrastructure — in particular, the membrane microdomains and junctional interfaces of individual endothelial cells (ECs) forming the endothelium (Bruns and Palade 1968, Simionescu et al. 1975), a tissue that is at once an organ within an organ (Aird 2004, Molema 2010). Two, was he initial descriptions in the 1970s of methods to culture ECs from umbilical vein (Jaffe et al. 1973, Gimbrone et al. 1974), protocols that were adapted before the decade’s end to procure and grow these cells from a multitude of organs and organ systems (reviewed by Balconi and Dejana 1986) – including the CNS (Panula et al. 1978, DeBault et al. 1979, Phillips et al. 1979).

These advances, along with the application of emerging immunohistological and molecular biological techniques, have culminated in awareness that, far from displaying a monolithic and inert phenotype, the endothelium exhibits substantial heterogeneity (a.k.a. endothelial diversity) in form, molecular composition and function (extensively reviewed by (Gerlach et al. 1985, Gerritsen 1987, Garlanda and Dejana 1997, Ghitescu and Robert 2002, Aird 2003, 2007a, b, 2010, Molema 2010). Such heterogeneity among normal vessels can be broadly classified into two major categories: segmental and regional. Segmental heterogeneity refers to differences in endothelial phenotype among the hierarchical branches of the blood vascular tree: arteries and veins (which comprise the macrovasculature; >100μm in interior diameter), and arterioles, capillaries and venules (which form the microvasculature; <100μm in interior diameter). The variety of branch sizes within the CNS microvasculature is depicted in Figure 24. By analogy, the lymphatic vasculature also harbors differences between lymphatic capillaries and high
endothelial venules (Lee et al. 2010). Regional heterogeneity alludes to the EC distinctions that exist in any given type of vessel segment between different organs or anatomical domains within a single organ/organ system (in the CNS, for example: brain vs. spinal cord; gray vs. white matter; or pial vs. parenchymal vessels). Superimposed on this bicameral classification is a microheterogeneity due to endothelial activation state, wherein adjacent ECs can manifest different phenotypes with regard to expression of a single or limited number of genes (Huang 2007). Striking examples of the breadth of both segmental and regional heterogeneity were revealed in the magnum opus work of Chi et al. (2003), who used DNA microarrays to determine the expression profile of 53 cultured ECs. These authors found that ECs from different blood vessels (segmental) and microvascular ECs from different tissues (regional) have distinct and characteristic gene expression profiles.

While endothelial heterogeneity has been long and widely recognized in the peripheral vascular beds (Vanhoutte and Miller 1985, Barbera-Guillem and Vidal-Vanaclocha 1988, Koop et al. 2003, Knosalla et al. 2009, Lee, Choi and Hong 2010, Stevens 2011, Molema and Aird 2012), until recently this issue has received comparatively little attention with regard to the central nervous system (CNS) (Owman and Hardebo 1988). Inasmuch as the cerebral microvascular endothelium is the recognized seat of the blood-brain barrier (BBB) (reviewed by Ge et al. 2005), endothelial heterogeneity has profound implications for understanding both homeostatic and pathogenic mechanisms involving the CNS vasculature. Moreover, the fact that the BBB is represented by biochemical, anatomical and metabolic facets (Ge et al. 2005, Macdonald et al.
heterogeneity can be evident at one or more of these levels. The objective of this review is thus to specifically highlight examples of endothelial cell heterogeneity within the CNS microvasculature, and focus on the significance of these findings to vascular physiology, pathophysiology, and therapeutics.

II. Manifestations of CNS endothelial heterogeneity at the BBB: Hiding in plain site

Using immune-guided laser capture microdissection (LCM) coupled to qRT-PCR (immuno-LCM/qRT-PCR), this laboratory profiled expression of 87 genes considered to play a role in BBB function and/or be enriched in isolated brain microvessels; i.e., reflecting the “BBB transcriptome” (Macdonald et al. 2010). This approach enabled separate global profiling of brain capillaries or venules, with results implying that BBB properties reside in both types of microvascular tributaries, though to varying degrees. Capillaries preferentially expressed some genes related to solute transport, while venules tended toward higher expression of a variety of genes involved in inflammatory-related tasks. These findings and others from a multitude of studies are described below, which highlight the vast accumulation of evidence of endothelial heterogeneity at the respective biochemical, anatomical, and metabolic levels of the BBB.

- **Biochemical:** The biochemical BBB refers to the many plasma membrane transporters and associated enzymatic activities that regulate transcellular solute flux into and out of the CNS. Glycosylation patterns in the luminal and abluminal endothelial surfaces, as revealed by specific lectin-binding domains, may also contribute to this BBB category. Anecdotally, some of the earliest examples of CNS segmental endothelial heterogeneity came from EM studies evaluating...
membrane transporters/enzyme activities and lectin binding sites. The enzymes 5'-nucleotidase, Mg\textsuperscript{2+}-ATPase and N\textsuperscript{+}-K\textsuperscript{+}-ATPase were found to exhibit more intense immunostaining in arteriolar endothelial cells compared to both the capillary and venular endothelium (Vorbrodt et al. 1982, Vorbrodt 1988).

As regards lectins and endothelial heterogeneity, RCA binding sites were described as greater in intensity and regularity on the luminal face of brain capillary as compared to arteriolar endothelium, and being generally less robust in venules than capillaries (Vorbrodt 1988). On the other hand, concanavalin A binding was found to be more intense at both luminal and abluminal surfaces of arterioles than capillaries or venules. These findings offered a primordial hint that the BBB is not homogeneously distributed along the respective segments of the CNS microvascular tree.

More recently, expression of P-glycoprotein (P-gp), a member of the superfamily of ATP-binding cassette transporters and efflux pump for a wide range of hydrophobic amphipathic compounds (Sharom 2011), was reported to show significant segmental heterogeneity in brain. Using confocal microscopy, Virgintino et al. (2002) first called attention to the restricted expression of P-gp to the luminal endothelial surface of only the small microvessels of the human cortex, and Vogelgesang et al. (2004) followed with reporting lower expression in cerebral arterioles in the aged brain. Ge et al. (2005) then provided additional support for microvascular segregation of P-gp, by showing that its expression was concentrated in the smallest diameter microvessel fraction isolated from a bulk population of mouse brain microvessels by differential
sieving. In addition, Saubamea et al. (2012), employing thick-section (100 µm) confocal microscopy, exquisitely resolved P-gp expression still further, highlighting strikingly nonhomogeneous immunofluorescent staining along the brain microvascular tree within the rat cortex and hippocampus. Specifically, P-gp displayed a gradient of expression, being near undetectable in large penetrating arterioles while being uniformly high in capillaries and venules.

Transferrin receptor and Glut-1 transporter, two of the most conspicuous BBB transporters (Orte et al. 1999), have also displayed evidence of segmental heterogeneity. Like P-gp, both these proteins were also observed to segregate with smaller diameter brain microvessels trapped on successive filters of smaller pore size (Ge et al. 2005). Cornford et al. (1996) further demonstrated endothelial heterogeneity of Glut-1 within the capillary compartment of human brain.

The endothelial barrier antigen (EBA), an endothelial-specific protein strongly associated with BBB properties in the rat (Sternberger and Sternberger 1987) but whose precise function remains elusive, has likewise been shown to demonstrate stark heterogeneity in situ (Saubamea et al. 2012). While immunofluorescent staining of this protein was also excluded from cerebral arterioles, and confined to capillaries and venules like that of P-gp, it uniquely demonstrated a mosaic pattern – at the single cell level – in the latter vessel segments. A similar heterogeneous expression of EBA was described in pial microvessels (Allt and Lawrenson 1997). Thus, at least with regard to EBA, it appears endothelial heterogeneity may be the most extreme, with no two brain microvascular endothelial cells displaying the same phenotype.
Expression of the sodium-dependent, glutamine transporter Snat3, a member of the solute carrier (SLC) family of transporters at the BBB, was recently described to be restricted to larger cortical microvessels, whereas a related amino acid transporter, SnaT1, was found distributed on smaller capillaries as well (Ruderisch et al. 2011).

Other examples of biochemical heterogeneity, though not immediately related to solute transport or BBB function, also exist along the CNS microvascular endothelium. For example, Saubamea et al. (2012) found that immunofluorescent staining with RECA-1, a pan endothelial-specific monoclonal antibody (Duijvestijn et al. 1992), yielded the opposite pattern of that seen with P-gp; i.e., being more intense in arterioles than venules. This at once both validated the P-gp findings as not due to immunostaining artifact, and provided yet another example of segmental endothelial heterogeneity.

In further evidence of such heterogeneity, this laboratory described a dramatic variance in expression of the blood clotting glycoprotein, von Willebrand Factor (VWF), among the different type brain microvascular tributaries. Specifically, immunofluorescent analysis revealed that VWF, which is stored within Weibel-Palade bodies (WPBs) of endothelial cells, was highly expressed in venules of normal brain in situ, while being nearly undetectable in contiguous capillaries (Macdonald et al. 2010). Microvessels isolated from brain also showed this discrepancy (Figure 25), with venules displaying the greatest VWF immunostaining, capillaries the least, and arterioles an intermediate amount. This reinforces that the heterogeneous VWF staining previously observed in brain tissue sections was not influenced by antibody penetration, but
rather reflected variable VWF expression by different brain microvascular tributaries. Interestingly, the presence of VWF has been reported to influence BBB permeability, with VWF knockout (VWFKO) mice showing a significant increase compared to wild-type controls, during encephalitis following Bordetella pertussis toxin-induced histamine hypersensitivity (Noubade et al. 2008). VWFKO mice further exhibited earlier onset and greater severity of disease, with heightened inflammation occurring in brain but not spinal cord. These observations, along with both the discovery that certain chemokines can be sequestered in WPBs (Rondaij et al. 2006), and the conventional view that venules are the preferred exit of leukocyte extravasation site in most tissues - including the CNS (Thurston et al. 2000, Wojciechowski and Sarelius 2005, Owens et al. 2008, Nourshargh et al. 2010) - could suggest concentrating VWF expression within these particular tributaries is a means to highly focus and tightly regulate inflammation.

Most recently, Wang et al. (2013), reported ABO blood antigen expression was heterogeneous among human brain capillary endothelial cells, prompting these authors to speculate, “the possibility that blood-brain barrier permeability and cerebral autoregulation may differ over short differences.”

- **Anatomical:** Perhaps the most distinguishing anatomical feature of the cerebral microvasculature is the high density of high resistance tight junctions (TJs) between endothelial cells. Since Reese and Karnovsky (1967) and Brightman and Reese (1969) identified the barrier to CNS penetration of exogenous horseradish peroxidase to lie at the TJs, these structures have been inextricably linked to the BBB phenotype. The later demonstration by Crone and Olesen
(1982) that frog capillaries had a transendothelial electrical resistance (TEER) of ~2000 ohms cm² – nearly that found in frog skin and urinary bladder – Ussing and Windhager (1964) cemented TJs as the cardinal feature of the anatomical BBB.

The protein make-up of TJs is complex and covers a vast original literature. Accordingly, this topic will not be dealt with in-depth here, and the reader is encouraged to consult recent excellent reviews on the subject in this volume (Chapters 1 and 2) and elsewhere (Wolburg et al. 2009, Coisne and Engelhardt 2011, Luissint et al. 2012). TJs are comprised of three main classes of transmembrane proteins: occludin, which is extensively modified at posttranscriptional and posttranslational levels (Cummins 2012), junctional adhesion molecules (JAMs 1, 2 and 3) (Bazzoni 2011), and claudins (CLNs, of which there are now > 20 recognized isoforms in various endothelia and epithelia in and outside the CNS (Elkouby-Naor and Ben-Yosef 2010). There is general consensus that the predominant CLNs expressed at TJs in endothelial cells forming the BBB are CLN-3 and CLN-5 (Morita et al. 1999, Nitta et al. 2003, Wolburg et al. 2003, Ohtsuki et al. 2007, Schrade et al. 2012). Occludin, JAMs and claudins are linked to the actin-based cytoskeleton through numerous scaffolding/adaptor proteins, including zonula occludin proteins (ZO-1, ZO-2 and ZO-3) (Itoh et al. 1999, Wittchen et al. 1999, Bruewer et al. 2004), which assist in regulating TJ performance and BBB phenotype through a variety of signal transduction cascades (Ishizaki et al. 2003, Fischer et al. 2005, Haorah et al. 2005), (Zhong et al. 2008, Jalali et al. 2010, Morin-Brureau et al. 2011, Ma et al. 2012).

Early evidence that TJs were not homogeneously distributed along the CNS
microvasculature was gathered from painstakingly detailed freeze-fracture analysis along the cerebrovascular bed by Nagy et al. (1984). These authors described greater ‘complexity’ of TJ protein particles; i.e., the degree to which these particles comprised long, uninterrupted strands in freeze-fracture ‘faces,’ in brain capillaries compared to post-capillary venules. This difference was even more exaggerated at the level of collecting veins, which possessed TJ stands that were discontinuous, widely separated and free-ending. Consistent with these findings, this laboratory recently observed disparate expression of CLN-5 along the microvascular tree of the mouse spinal cord (Paul et al. 2012). Using a novel contour-based 3D image visualization and quantification method, employing high-resolution confocal z-stacks from thick immunofluorescently stained cryosections, it was determined that the density of claudin-5 staining was greatest in the capillaries and smaller venules, and least in the larger venules. Specifically, a significant negative linear correlation was established indicating that the density of CLN-5 at inter-endothelial junctions varied inversely with microvessel diameter. Allt and Lawrenson (1997) further described a disparity in TJ morphology within pial microvessels, noting two distinct junctional populations: one in which adjacent endothelial membranes appeared fused, and another bearing a discernible gap between apposing membranes. Interestingly, Crone and Olesen (1982) noted a broad distribution of TEER values among frog pial microvessels, which may reflect physiological correlates of heterogeneous TJ protein expression and/or function.

What dictates this heterogeneity in TJs and TEER values along the CNS microvasculature is unclear, but there is increasing evidence of the role of astrocytes in neurovascular coupling and
the induction of the BBB phenotype (Nagy and Martinez 1991, Abbott 2002, Haseloff et al. 2005, Wolburg, Noell, Mack, Wolburg-Buchholz and Fallier-Becker 2009, Willis 2011, Ronaldson and Davis 2012). *A priori*, the degree of astrocyte investment onto the abluminal surface of brain microvessels might contribute to segmental endothelial heterogeneity in the CNS. This hypothesis is lent support by recent observations of (McCaslin et al. 2011), who, using *in vivo* two photon microscopy to study astrocyte-vascular interactions in the somatosensory cortex, noted the highest density of astrocyte processes contacting microvessels was highest for capillaries (on average, 0.96 processes/100μ² surface area), less in venules (0.41 processes/100μ² surface area), and least in arterioles (0.36 processes/100μ² surface area). The fact that astrocytes themselves are recognized to be highly functionally diverse (Bachoo et al. 2004, Hewett 2009, Chaboub and Deneen 2012) might further impart another layer of complexity to this issue, particularly as regards regional endothelial heterogeneity.

Lastly, beside astrocytes and endothelial cells, additional cellular elements including pericytes, microglia and neurons also help forge the neurovascular unit (NVU), which serves as the functional unit of the BBB (Bonkowski et al. 2011, Mae et al. 2011, Sa-Pereira et al. 2012). The perivascular distribution of these other cells, each with their own diverse phenotypes (Sims 2000, Choi and Kim 2008, Olah et al. 2011), could thus potentially further contribute to endothelial heterogeneity.

- **Metabolic**: The metabolic capacity of the BBB refers to enzymes within the CNS microvascular endothelium that modify the biological activities of substrates on route between
the circulation and brain (Minn et al. 1991, Pottiez et al. 2009). Alkaline phosphatase (AP) activity, long considered a BBB marker (Karnushina et al. 1980), was shown to be highest in the pre-capillary arterioles and capillaries in brain, and depreciate in a graded manner toward the venules (Vorbrodt 1988). The stark segregation of AP activity in contiguous brain microvascular segments was later confirmed by this laboratory (Ge et al. 2005). And immuno-LCM/qRT-PCR analysis indicated preferential mRNA expression of gamma glutamyl transferase (γgt) and neutral endopeptidase (NEP) by brain capillaries, while revealing a bias of glutathione S transferase (GSST1), angiotensin I converting enzyme (ACE) and monoamine oxidase B (MAOB) mRNA by venules (Macdonald et al. 2010).

III. Functional correlates of endothelial cell heterogeneity

The general consensus that leukocyte extravasation in the CNS occurs preferentially at venules is an indictment – in functional terms – of segmental heterogeneity along the CNS microvascular endothelium. It further stands to reason that such endothelial heterogeneity should manifest itself in disparate responses of CNS microvascular tributaries in the course of neuroinflammatory conditions. Indeed, this has been found to be the case.

Correlating with venules being the preferred exit site for leukocytes, Xu et al. (2005) reported that, during the ocular inflammatory condition experimental autoimmune uveoretinitis, breakdown of TJs and loss of CLN-1/3 and occludin at inter-endothelial contacts was focused within mouse retinal venules, near completely sparring capillaries. And in complete parallel to these findings, Paul et al. (2012) recently described the disruption of CLN-5 in mouse spinal cord as being
restricted to venules during the related neuroinflammatory condition experimental autoimmune encephalomyelitis – a recognized model for multiple sclerosis. Thus, at least with regard to the mouse CNS microvasculature, endothelial cells of venules not only display a lesser density of TJ protein than do capillaries in the normal state, but also appear more vulnerable to neuroinflammation-associated disruption of their TJ network. This assessment at least partially resembles the remote situation regarding histamine-induced leakage in the periphery, which is a phenomenon restricted almost exclusively to venules (Majno et al. 1961). Whether other endothelial determinants of neuroinflammation – such as cytokines, chemokines, or other vasoactive substances and their cognate receptors, as well as adhesion molecules – demonstrate preferential expression by endothelial cells of CNS venules awaits detailed and systematic analysis. Heterogeneity in endothelial responsiveness during inflammation in peripheral vascular beds has recently been reviewed by (Molema 2010).

IV: Conclusion: consequences of endothelial heterogeneity for BBB research. Since the mid-1970s, cultures of brain microvascular endothelial cells have provided a highly convenient format to peer into the operations of the BBB, and highlight its involvement in physiological and pathophysiological processes. This opportunity has been aided and abetted by extending the natural lifespan of these cells, typically by transforming them with viral, proto-oncogene products. To be sure, such culture systems have provided a vast amount of physiologically and clinically relevant information. But despite their tremendous advantages, no model has emerged that recapitulates the BBB with complete fidelity. While this caveat has long been rationalized as due
primarily to phenotypic drift, stemming from removal of these endothelial cells from their native environment, a closer look may reveal elements of endothelial heterogeneity at play. Moreover, failure to recognize this can potentially lead to erroneous conclusions about BBB function.

Typically, brain microvessels isolated for endothelial culture and use as BBB models are heterogeneous in size, and comprised of capillary, venule and arteriole fragments (Ge et al. 2005). In fact, resulting cultures are most often – and rightly – referred to as brain microvascular endothelial cells (BMEC), in recognition of these cells’ precise derivation being unknown. But as capillaries are by far the most numerous tributaries of the microvascular network (Berne and Levy 1988) – they initially predominate in freshly-established microvessel cultures. This has lead, at times, to the assumption that BMEC cultures are strictly or mostly capillary in nature.

Were it the case that endothelial cells of all microvascular tributaries possess the same growth potential, then this assumption would necessarily hold true. However, observations by DeBault et al. (1979) and Spatz et al. (1997) tell a different story. Specifically, these groups noted the viability and growth of endothelial cells from brain microvascular tributaries in culture was vessel class- and size-dependent, with the cellular outgrowth from larger diameter vessel fragments being far quicker and more extensive than that from small capillaries. These discrepancies are consistent with the contention that during angiogenesis – the formation of new blood vessels from pre-existing ones in vivo – endothelial sprouts arise from venules (Thurston et al. 2000, Baluk et al. 2004, Adams and Alitalo 2007). The basis for the attenuated growth of endothelial cells from capillary fragments in culture is not clear but may, in part, reflect a muted
capacity of capillary endothelial cells to produce their own growth factors. Advancing this hypothesis, we observed that when purified brain capillary segments were cultured in a dual-chamber Transwell format – with capillaries in the top chamber, separated by a porous filter from a mixed population of microvessels in the bottom – endothelial growth from capillaries could be stimulated, though still not achieve that observed in the microvessel population (Figure 26).

In this case, perhaps venules in the microvessel population supplied the missing requisite growth stimulant(s). The outcome of plating brain microvessels may well be that the BMEC cultures that result are largely venular derivatives – and thus, barring any special additives or modifications outside normal endothelial growth conditions, behave more like venules than capillaries. This interpretation is consistent with the relatively low TEER values (compared with the BBB in vivo) obtained from static BMEC cultures containing no adventitial cells of the NVU, and that such cultures typically highly express VWF in a homogeneous manner (like brain venules). Simple BMEC cultures of this type might serve as suitable models to study neuroinflammation, but not so for accurately assessing BBB properties governing solute transport into and/or out of the CNS.

Conversely, BMEC cultures of capillary derivation or somehow made to behave like capillaries, might more faithfully recapitulate transport of soluble ligands between the circulation and CNS, yet be inadequate to study inflammatory-related phenomena – especially those concerning leukocyte extravasation. The burgeoning examples of CNS endothelial heterogeneity would seem to dictate there is no ‘one-size fits all’ model.

What to do? Sophisticated endothelial culture paradigms that utilize human BMEC in
cylindrical format approximating vascular morphologies, and incorporate flow and cellular elements of the NVU (Cucullo et al. 2008, Cucullo et al. 2011) – all of which are likely to be determinants of endothelial heterogeneity – offer the best prospect of contriving models that display CNS vessel-specific characteristics. With mounting efforts to define endothelial ‘markers’ that distinguish the respective branches of the entire vascular tree (Othman-Hassan et al. 2001, Harvey and Oliver 2004, dela Paz and D’Amore 2009, Rivera et al. 2009, Richardson et al. 2010), endothelial heterogeneity of the CNS microvasculature will ultimately cease to be a problem, and instead provide the basis for more precise evaluation of vascular activity within the brain and spinal cord during health and disease.
Figure 24. Segmental heterogeneity along the CNS microvasculature. (a) A CNS venule from a naïve mouse detailing CLN-5 staining (green) at intercellular junctions. The image shows microvascular tributaries (e.g. capillaries, post-capillary venules) emerging from a venule. The nuclei are highlighted with DRAQ5 (blue). (b) Lumbar spinal cord microvessels from a mouse inflicted with EAE, revealing diffuse and fragmented CLN-5 staining (yellow arrows on the zoomed-in inset) in an “inflamed” venule associated with increased perivascular cellularity (blue). Notably, capillaries contiguous with this venule appear to be spared this inflammatory response and present intact CLN-5 staining. Scale bar = 20µm.
Figure 25. Heterogeneity in VWF expression in isolated brain microvessels. Brightfield image shows a crude population of microvessel segments (Ge et al., 2005), containing representatives of all size diameter microvessel tributaries (arterioles, venules and capillaries). Microvessels were double immunofluorescently stained with antibodies to α-smooth muscle actin (green) and VWF (red). α-Smooth muscle actin distinguishes the muscular tunica media of arterioles (arrow). The largest diameter structure (arrowhead) possesses much less α-smooth muscle actin staining, and most likely represents a venule (containing α-smooth muscle actin+ pericytes). The venule stains most intensely for VWF, the arteriole intermediately so, and the small capillary segments apparently not at all.
A crude population of mouse brain microvessel segments of varying size diameter was prepared by immunomagnetic bead sorting, and then separated by sequential passage through filters of the following porosities, as described (Ge et al., 2005): 297 μm, 70 μm, 40 μm and 20 μm. Material retained on the 70 μm and 20 μm filters contained mixed microvessel segments (venules, arterioles and capillaries) or purified capillaries, respectively. The separated capillary and microvessel preparations were then each plated in the top chamber of a collagen IV-coated, dual-chamber Transwell filter (1.0 μm pore). Mixed microvessels were also placed in the bottom chamber of the capillary sample. This arrangement allowed microvessel-conditioned media to bath the capillaries. Top row, shows capillary and microvessel populations immediately after plating. Bottom row, shows BMEC growth from these populations after 24 hr in culture. Growth of BMEC is more extensive on the filters containing microvessels.

Figure 26. Differential BMEC growth from small- and large-sized brain microvessel segments.
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Three-dimensional quantification of Claudin-5 within microvessels of the normal and inflamed CNS: highlights of endothelial heterogeneity

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Figure S1. Microvascular segmentation through 3D contour surface

Figure S2. CD4 staining associated with perivascular cellularity

Figure S3. Correlation analysis of CLN-5 density with microvascular diameter

Figure S4. 2D quantification of mean pixel intensity of CLN-5 immunostaining

Figure S5. Endogenous serum IgG leakage from spinal cord microvessels at d6EAE
Figure S1. Microvascular segmentation through 3D contour surface. A 3D isosurface rendered dataset of a venule was cut using the Clipping Plane module on Imaris®. (a) Longitudinal section (L.S.) of a CNS venule from a naive mouse, detailing CLN-5 (Green) staining at intercellular junctions. The anterior vessel wall has been optically cut away, and a clipping plane can be seen piercing through the lumen and into the back/posterior wall. (b) Oblique view from the side, depicting cross section (C.S.) of the same venule, to demonstrate the generated contour surface defines the endothelial surface boundary and excludes the hollow lumen from TJ quantification. (c) Clipped C.S. of a venule showing its characteristic smooth, non-puckered lumen.
Figure S2. **CD4 staining associated with perivascular cellularity.** 3D volume rendered images of z-stacks obtained from serial-sections of naïve and d24 EAE lumbar spinal cords used in Figure 4, stained for leukocyte marker CD4, CD31, and DRAQ5. (a) Parenchymal venule from naïve mouse showing intact CD31 staining along the intercellular junctions but no CD4 staining or perivascular cellularity (DRAQ5). (b) Parenchymal venule from d24 EAE mouse revealing CD4 staining largely coincident with perivascular cellularity. Severely diminished CD31 staining is also evident; confirming dense perivascular DRAQ5 staining is associated with inflamed vessel status. Scale bar = 15 μm.
Figure S3. Correlation analysis of CLN-5 density with microvascular diameter. For calculation of Pearson's correlation coefficient, 3 representative microvessels were analyzed from each size microvessel group (capillaries, smaller venules, and larger venules) sampled from spinal cords of 3 naïve mice. The density of CLN-5 at endothelial junctions displays a negative linear relationship with microvessel diameter. Pearson's correlation coefficient \( r = -0.73; p < 0.05 \).
Figure S4. 2D quantification of mean pixel intensity of CLN-5 immunostaining. Representative confocal images are shown depicting the placement of ROIs along the intercellular junctional regions of the venular endothelium, so as to determine relative intensity values associated with CLN-5 immunostaining. (a) Venule from a naïve mouse; (b) Venule from a d24 EAE mouse. Insets highlight the dense perivasculaary cellularity associated with inflamed venules. (c,d) 2D quantification of CLN-5 immunostaining, showing significant reduction of mean pixel intensity in 3 representative venules from d24 EAE mice compared to those from naïve controls. The chart of mean pixel intensities reveals not only the diminished mean value associated with d24 EAE (53.13) compared to naïve (11.75), but also the higher variability of this measure in the diseased condition, as indicated by higher standard deviation (Std. Dev) and standard error (SEM). *p < 0.0005. Scale bar = 20 μm.
Figure S5. Endogenous serum IgG leakage from spinal cord microvessels at d6EAE. (a-c) Volume rendered z-stack of microvessels, showing IgG (Red), CLN-5 (Green), and nuclei/DRAQ5 (Blue). (d-f) Corresponding isosurface rendered images, providing enhanced spatial perspective. Leakage of IgG can be seen at this early time during disease, though this is not associated with any apparent disruption of CLN-5 in capillaries or venules. Scale bar = 10 μm.
Cell-selective knockout and 3D confocal image analysis reveals separate roles for astrocyte-and endothelial-derived CCL2 in neuroinflammation

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Content

Figure S6. Specificity of CCL2 immunostaining.

Figure S7. Lack of focal CLN-5 immunostaining loss and perivascular cellularity in naïve spinal microvessels.
Figure S6. Specificity of CCL2 immunostaining. Volume rendered images of z-stacks obtained from serial sections of d9 EAE spinal cords used in Figure 6 (left) and naïve (right) mice demonstrating specific immunoreactivity of the CCL2 antibody. No detectable CCL2 staining (green) was observed in naïve mice upon incubation with CCL2 antibody or in EAE mice in the absence of primary antibody. The endothelium is highlighted with CD31 (red), while DRAQ5 staining reveals the nuclei (blue). Scale = 20 μm.
Figure S7. Lack of focal CLN-5 immunostaining loss and perivascular cellularity in naïve spinal microvessels. Isosurface-rendered images generated from confocal z-stacks of 60-μm thick cryosections from naïve mice showing continuity of CLN-5 staining (green) in naïve spinal microvessels. The lack of perivascular cellularity associated with typical inflamed microvessels is further highlighted with DRAQ5 staining for nuclei (blue). Scale = 20 μm.
Supplemental Information - CHAPTER IV

Endothelial extracellular vesicles transfer tight junction protein Claudin-5 to circulating leukocytes in neuroinflammation

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Content

**Figure S8.** CLN-5' leukocytes along the meningeal and parenchymal thoraco-lumbar spinal cord venules in EAE.

**Figure S9.** Nanoparticle Tracking Analysis of purified EVs from TNFα-treated BMECs.
Figure S8. CLN-5+ leukocytes along the meningeal and parenchymal thoraco-lumbar spinal cord venules in EAE. Z-stack confocal images acquired from a thoraco-lumbar spinal cord cryosection of WT mice at 9 EAE are shown, revealing staining for TJ protein CLN-5 (green), endothelial CD31 (red) and nuclear DRAQ5 staining. Regions A and B are shown to highlight the emergence of CLN-5+ leukocytes along the meningeal microvessels, whereas, C underscores the TEM of CNS infiltrating CLN-5+ leukocytes in parenchymal microvessels during EAE progression.
Figure S9. Nanoparticle Tracking Analysis (NTA) of EVs. High-resolution particle size profiling and concentration measurement were performed on exosomes (left) and microvesicles (right) isolated from supernatant of TNFα-stimulated cultured BMECs.
Appendix

Cell trafficking across the CNS barriers (co-authored publications)

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Alterations in Tight Junction Protein and IgG Permeability Accompany Leukocyte Extravasation Across the Choroid Plexus During Neuroinflammation

Bandana Shrestha, BS, Debayon Paul, MS, and Joel S. Pachter, PhD

Abstract

The choroid plexus (CP) is considered to be a point of leukocyte entry into the CNS during normal immune surveillance and in neuroinflammatory diseases. The structural and functional alterations within the CP that support this migration are not understood. We used quantitative, high-resolution, 3-dimensional (3-D) fluorescence imaging to analyze CP alterations associated with inflammatory responses in C57/B6 mice after the induction of experimental autoimmune encephalomyelitis by immunization with myelin oligodendrocyte glycoprotein (MOG) and complete Freund adjuvant/pertussis toxin (MOG-CFA/PTX) or adjuvants alone (CFA-PTX). The MOG-CFA/PTX and CFA-PTX produced similar effects, although those caused by the former were consistently more marked. Both treatments resulted in the accumulation of serum immunoglobulin G and leukocytes in the CP stroma, consistent with elevated stromal capillary permeability. They also provoked distortions and diminished immunostaining patterns of the tight junction adaptor protein ZO-1 in the choroidal epithelium but no obvious change in the patterns of the tight junction associated protein claudin-2. Only MOG-CFA/PTX triggered visible extravasation of immunoglobulin G and leukocytes across the choroidal epithelium. Our results suggest that CFA/PTX primes the CP for neuroinflammation by inducing structural changes that are exacerbated when there is an immune response to MOG and reinforce the CP as a gateway for leukocytes to enter the CNS by accessing the CSF and leptomeninges.


INTRODUCTION

The choroid plexus (CP) is a specialized out-pocketing of the roofs of the third, fourth, and lateral ventricles and has long been recognized as the site of production of cerebrospinal fluid (CSF), within which it is suspended (1,2). The CP is a highly compartmentalized structure that contains at its core a tortuous capillary plexus displaying a fenestrated endothelium, which is more permeable than that of capillaries forming the blood–brain barrier (BBB) in the CNS parenchyma (3). This vascular core is surrounded by a layer of choroidal epithelium with a relatively high density of tight junctions (TJs) and limited permeability, providing the CP its classification as part of the blood–CSF barrier (4,5). There is a confined stromal space located between the capillaries and choroidal epithelium.

Since the pioneering work of Helen Cserr (6), the CP has been commonly recognized for its role in generating CSF, as most recently reviewed (7). However, a growing number of reports have turned attention to the CP as a site through which T cells might enter an initially uninfamed CNS in the initial stages of neuroinflammatory diseases such as multiple sclerosis and the animal model of multiple sclerosis experimental autoimmune encephalomyelitis (EAE) (8–12). It has been suggested that once encephalitogenic T cells cross the CP and enter the CSF, they can migrate to the subarachnoid space surrounding the brain and spinal cord and there forge immune synapses with resident dendritic cells. Cytokine bursts stemming from such synapses, in turn, are thought to activate the endothelial cell surfaces of nearby microvessels within the subarachnoid space, thereby enabling adhesion and extravasation of leukocytes circulating in the bloodstream (13–15). Interactions between these leukocytes and other dendritic cells in the subarachnoid space would then follow, ultimately propagating an inflammatory wave along the surface-penetrating microvessels that enter the CNS parenchyma.

This scenario begs the question: “Are there changes within the CP anatomy and integrity during evolving neuroinflammation that would allow leukocytes to navigate across the TJs of the choroidal epithelium and enter into the CSF?” Currently, direct evidence of leukocyte extravasation across the CP into the CNS ventricles, or structural arrangements of the choroidal epithelium that might support this process, is lacking. Therefore, we used high-resolution 3-D fluorescence imaging to highlight aspects of CP morphologic and functional changes that have not been previously resolved during the progression of EAE.

Specifically, immunization with myelin oligodendrocyte glycoprotein (MOG) peptide\textsuperscript{35,55}, along with complete Freund adjuvant (CFA) and pertussis toxin (PTX), was used to induce EAE, and qualitative and quantitative assessments were made at different time points for 2 TJ proteins expressed by the CP, that is, claudin-2 (CLN-2) and zonula occludens...
1 (ZO-1) (16–18). Claudin-2 belongs to a large family of claudins (>20 members), which are integral membrane proteins that perform cell-cell bridging. Zonula occludens 1 is a peripheral membrane scaffolding/adaptor protein that directly or indirectly links claudins (and other integral membrane TJ proteins) to the actin cytoskeleton (19–21). Tight junction protein changes were further correlated with leakage of serum immunoglobulin G (IgG), a marker of barrier permeability status (22), and leukocyte extravasation across the successive CP compartments. Because administration of the adjuvants CFA and PTX alone can significantly alter gene expression patterns in the CP (23), MOG<sub>35-55</sub>–immunized mice were compared with those receiving these adjuvants alone or no treatment.

Our results indicate that ZO-1 and CLN-2 immunostaining patterns within the CP choroidal epithelium displayed significant, yet different, changes during the evolution of EAE, that is, the former showed a severely altered morphology and the latter a gradual decrease in staining intensity. Immunoglobulin G was detectable at a low level in the CP stroma even in naïve animals; it was appreciably increased during disease, and later, in acute disease, it was observed to extravasate across the CP choroidal epithelium. A few leukocytes were also detected in the CP stroma of naïve mice. These cells showed an acute transient accumulation early in EAE and then seemed to be reduced to near predisease level. Correlating with this decline, leukocytes could be seen apparently extravasating across the CP choroidal epithelium and into the ventricles, presenting direct evidence supporting the theory that the CP is the entry site into the CNS for leukocytes in the ontogenesis of neuroinflammation (8).

**MATERIALS AND METHODS**

**Animals**

Female C57BL/6 mice, aged 8 to 10 weeks, were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and used in all experiments. Three animals per group were used for each treatment and time point assessed. All animal experimental procedures were performed following Animal Care and Use Guidelines of the University of Connecticut Health Center (Animal Welfare Assurance A3471-01) and approved under protocol 100346-1214.

**EAE Induction**

Experimental autoimmune encephalomyelitis was induced in mice by active immunization with MOG<sub>35-55</sub> peptide (MEGVYRSPFSRVVHLRYNGK), of murine origin (W. M. Keck Biotechnology Resource Center, Yale University, New Haven, CT), as described (24). Briefly, on Day 0 (D0), 1 group of mice was injected subcutaneously with 300 μg of MOG peptide in CFA (DIFCO, Becton-Dickinson, Franklin Lakes, NJ) into the right and left flanks, 100 μL per site. These mice were also injected i.p. with 500 ng pertussis toxin (PTX) List Laboratories, Campbell, CA), in PBS, pH 7.4, on D0 and D2 after the first immunization (referred to as the MOG-CFA/PTX group). Another group of age-matched mice received only CFA and PTX (500 ng) injections on D0 and a second injection of 500 ng PTX on D2 (referred to as the CFA/PTX “control” group). A third group of age-matched mice was left untreated (referred to as “naïve” mice) and represented “normal” tissue. Only the MOG-CFA/PTX group developed EAE. Mice typically showed development of acute clinical signs at approximately D12, followed by ascending paralysis and chronic disease (25). Animals were killed at D0 (naïve), D6, D9, and D15 after injection. They were monitored for clinical disease severity, and mean clinical scores were calculated as follows: 0 = normal; 1 = tail limpness; 2 = limp tail and hind leg weakness; 3 = limp tail and complete hind leg paralysis; 4 = limp tail, complete hind leg and partial front leg paralysis; and 5 = death. The time points selected for analysis, D6, D9, and D15, represent preclinical (score 0), early (scores 0–0.5), and acute stages of EAE (scores 2–5), respectively.

**Tissue Preparation and Sectioning for 3-D Analysis**

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) in PBS. Animals were then transcardially perfused (via the left ventricle) first with heparin-PBS (10 usp/mL) to eliminate vascular blood content and then with fixation buffer (2% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4), as previously described (24). Brains were embedded in OCT cryomatrix compound (Thermo Fisher Scientific, Waltham, MA) before sectioning. Twenty-micrometer-thick cryosections were obtained using a Microm HM 505M cryostat (Mikron Instruments, Oakland, NJ), maintained at −25°C, and deposited onto poly-l-Lysine–coated slides.

**Immunostaining for 3-D Analysis**

Sections were permeabilized with 0.6% Triton X-100 in PBS for 30 minutes, and nonspecific binding was blocked by incubation with Power block for 10 minutes in Ultrapure (GIBCO) distilled water (22). The CP capillary endothelium was stained with rat anti-mouse CD31 antibody (BD Pharmingen) at 1:160 dilution, followed by incubation with goat anti-rat Alexa Fluor 555 antibody (Life Technologies, Foster City, CA) at 1:250 dilution. Choroidal epithelial cells were stained using monoclonal pan-cytokeratin-FITC antibody (Sigma, St. Louis, MO) at 1:160 dilution. Rabbit polyclonal antibodies to CLN-2 (Invitrogen, Billerica, MA) at 1:160 dilution, CLN-3 (Abcam, Cambridge, MA) at 1:100 dilution, ZO-2 (Invitrogen) at 1:100, and ZO-1 (Invitrogen) at 1:160 dilution, followed by Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies) and 1:250 dilution, were used to stain epithelial TJs. Alexa Fluor 488 goat anti-mouse IgG F<sub>ab</sub> fragment (Life Technologies) at 1:160 dilution was used to detect endogenous IgG within the CP. DRAQ5 (Biostatus Ltd., Leicestershire, UK) was used at 1:1000 dilution to stain nuclei. Alexa Fluor 647 anti-mouse CD45 (BioLegend, San Diego, CA) at 1:160 dilution was used to stain for leukocytes. Sections were mounted in Mowiol (Sigma-Aldrich).

**Image Acquisition and Quantitative Analysis of TJ Proteins, IgG, and Leukocytes in the CP**

Confocal z-stacks (multitrack scan) were acquired using a Zeiss LSM 510 Meta equipped with Zeiss Fluar 40X/1.30, 63X Plan-neofluar/1.25, and 100X Plan-apochromatic/1.4 oil immersion objective lenses. Thereafter, z-stacks were imported
into Bitplane Imaris suite version 7.1 x64 software (Bitplane Inc., South Windsor, CT), as previously described (22).

The CP tissues located in the lateral ventricles were evaluated in all experiments because complexity and developmental changes of TJ proteins have been reported to show differences among the CP locales in rats (18). Images of these TJ proteins were first isosurface rendered to obtain a measure of total surface area; the epithelial nuclei were represented as spots to estimate the number of epithelial cells per image. Total areas of TJ protein staining per epithelial cell were calculated as follows:

\[
\text{Total TJ area/epithelial cell} = \frac{\text{Total surface area of green iso surface}}{\text{Number of epithelial nuclei}}
\]

A slightly different method was used for relative 3-D quantification of endogenous IgG that had extravasated from the circulation into the CP. Specifically, a volumetric approach was taken as IgG became diffusely distributed throughout the CP by D15 of EAE. Images of extravasated IgG were isosurface rendered, and mean voxel intensities were determined. Immunoglobulin G staining along the endothelial lining of the capillaries was specifically masked to quantify only the IgG that leaked out of the vasculature. This was done by creating an isosurface of the capillary staining (CD31) and assigning all the included green (IgG) voxel intensities to zero. A mean red voxel intensity value of the capillary isosurface was then obtained. The volume of extravasated IgG per volume of capillaries was calculated as follows:

\[
\text{Volume of IgG/Volume of capillaries} = \frac{\text{Total green intensity}}{\text{Total red intensity}}
\]

\[
\text{Total green intensity} = \text{Mean green intensity} \times \text{no. of green voxels}
\]

\[
\text{Total red intensity} = \text{Mean red intensity} \times \text{no. of red voxels}
\]

Leukocyte quantification was performed using the measurement points module of the Imaris software suite, first representing the CD45-positive cells as “spots” and then counting the total number of spots present in the field of view. The surface area of the capillaries was determined by creating an isosurface of the capillary staining (CD31), and the number of leukocytes per capillary surface area of the CP was calculated as follows:

\[
\text{Total number of leukocytes/capillary area} = \frac{\text{Total no. of spots} \times 10^2}{\text{Total surface area of the red isosurface}}
\]

**Laser Capture Microdissection and Quantitative Reverse Transcription Polymerase Chain Reaction**

Immunohistochemistry-guided laser capture microdissection (LCM) was performed as recently described (23). In brief, naive animals were killed by gradual CO2 inhalation. Brains were immediately removed, snap-frozen in dry ice-cooled 2-methylbutane (Acros, Geel, Belgium), and embedded in cryomatrix compound (Thermo Fisher Scientific, Waltham, MA) for cryosectioning. Coronal sections (7 μm) were cut on a Microm HM 505 M cryostat (Micron Instruments, Oakland, NJ) and affixed to uncoated precleaned glass slides (Fisher Scientific, Pittsburgh, PA). The CP stromal capillaries were stained using a substrate combination of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (Vector Labs, Burlingame, CA) to detect the endogenous alkaline phosphatase activity in endothelial cells. The choroidal epithelial cells were immunostained with monoclonal pan-cytokeratin–FITC antibody (Sigma). A PnXCell IIe laser capture microscope (Life Technologies, Foster City, CA) was used to retrieve CP choroidal epithelial tissue from the lateral ventricles. Laser capture microdissection samples were solubilized in Cell Lysate Buffer (Signosis, Sunnyvale, CA) for direct reverse transcription, and relative cDNA levels were quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using an ABI 7900HT Fast Real-Time PCR System (Life Technologies Corp) and normalized to housekeeping gene RPL-19, as described previously (26). Samples were probed for the TJ proteins CLN-1, CLN-2, CLN-3, CLN-11, occludin, and ZO-1, and the purity of the captured LCM material was assessed by epithelial marker (cytokeratin-8) and endothelial cell marker (CD31) levels.

**Statistical Analysis**

Two CP sections from each animal and a total of 3 animals were used for each group: naive, control (CFA/PTX), and EAE (MOG-CFA/PTX). Data from all experiments were initially assessed for normality using a Shapiro-Wilk test. Accordingly, data from ZO-1, IgG, and leukocyte determinations were subsequently analyzed by a 1-way nonparametric Kruskal-Wallis test followed by Dunn posttest analysis; data from CLN-2 determinations were analyzed by a 1-way analysis of variance followed by Bonferroni posttest. All statistical analyses were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA). Results were considered significant at p ≤ 0.05.

**RESULTS**

**Relative Expression of Genes Encoding TJ Proteins in the CP**

The normal CP choroidal epithelium was first analyzed by LCM to establish the relative expression of TJ components in this tissue layer of naive mice (Fig. 1). Previous descriptions by this laboratory have highlighted the ability of LCM to resolve with high purity the choroidal epithelium from the vascularized core (23). Analysis of LCM-derived choroidal epithelial tissue by qRT-PCR revealed expression of cytokeratin 8 (K8), as well as mRNAs encoding the TJ proteins ZO-1, CLN-1, CLN-2, CLN-3, and CLN-11, which have also been previously identified in the CP in situ by immunocytochemistry as well as in cultured choroidal epithelial cells (16–18,27). Notably, expression of CD31, which is found in all endothelial cells but not choroidal epithelial cells, was barely detectable, reinforcing the high precision of the LCM process. Because of their relatively prominent expression, immunofluorescence studies of ZO-1 and CLN-2 were further pursued to determine whether cytologic changes of these CP TJ components were
correlated with functional aspects of neuroinflammation during evolving EAE.

**Distribution of TJ Proteins, IgG, and Leukocytes Within the CP of Naive Mice**

Initial studies sought to demonstrate the status of the normal CP in naive mice (Fig. 2). With respect to ZO-1 and CLN-2 distribution (Fig. 2A and 2B, respectively), strong immunostaining for both these TJ proteins revealed a smooth contour and continuous pattern, delimiting the junctions of all choroidal epithelial cells. A scarce amount of IgG could be detected within the CP stroma of naive mice (Fig. 2C), along with a few leukocytes (Fig. 2D). The approaches used to perform relative quantification of ZO-1, CLN-2, IgG, and leukocytes in the CP are illustrated in Figure 3.

**Distribution of ZO-1 in the CP After Immunization**

Subsequent studies evaluated immunostaining of TJ proteins and IgG at different time points in both control mice (receiving CFA/PTX) and mice after EAE induction (receiving MOG-CFA/PTX) (Fig. 4A). The staining pattern of ZO-1 was altered in both groups, but the changes varied in extent. In CFA/PTX-treated control mice, immunostaining of ZO-1 was a bit distorted at D6, showing ridgelike irregularities in the contour at some locales that persisted through D15. Treatment with MOG-CFA/PTX to induce EAE resulted in more drastic alterations in ZO-1 staining patterns. Similar ridgelike irregularities were initially obvious along some choroidal epithelial cells at D6, but these became more pronounced during the disease course. By D15, the ZO-1 staining pattern was severely crenulated throughout the choroidal epithelium, giving a palisade appearance. To quantify these changes in TJ staining patterns, total area of ZO-1 staining within the CP epithelium of each image was related to the number of CP epithelial nuclei (Fig. 4B), as shown in Figure 3. Control mice evidenced no changes in this parameter over the time points evaluated, whereas mice with EAE showed a stepwise elevation, with significant changes at D9 and D15 (compared with naive). As this suggested an increase in individual CP epithelial cell surface area during evolving EAE, internuclei distances were also determined as a surrogate for corresponding volume/cell size changes.
At D15 EAE, mean internuclei distance in CP epithelial cells was nearly 3-fold higher than that found in naive mice, suggesting a significant increase in CP epithelial cell volume during neuroinflammatory disease. Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A657 shows an increase in CP epithelial internuclei distance. These observations were further supported by a significant increase in the perimeter of ZO-1 staining in CP epithelial cells in D15 EAE mice compared with naive mice. Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A658 shows an increase in the perimeter of ZO-1 staining within the CP epithelium.
FIGURE 3. Three-dimensional (3-D) quantification of choroid plexus (CP) tight junction protein, IgG, and leukocytes during evolving experimental autoimmune encephalomyelitis (EAE). (A–I) Representative sections of the CP of mice at D15 EAE are depicted to demonstrate the approaches used for quantification of disease-associated changes in CP tight junction protein and IgG leakage. (A, D, G) Composite 3-D data sets of confocal z-stacks. The 3-D frames are presented to display the holistic acquisition of the data. (B, C, E, F, H–I) 2-D depictions of the images in (A), (D), and (G), respectively. The 2-D projections of 3-D images served as the sources from which quantitative data were obtained. Isosurface rendering was performed to highlight the tortuous nature and 3-D disposition of the respective CP compartments. Capillaries were immunostained for CD31 (red) and isosurface rendered throughout. Zonula occludens 1 (ZO-1), an example of tight junction protein within the choroidal epithelium, was immunostained (green) in (A–C); isosurface is rendered in (C). IgG was also shown immunostained (green) in (D–F) to demonstrate serum protein leakage; isosurface is rendered in (F). Choroidal epithelial nuclei are stained with DRAQ5 (blue) in (A–F); leukocytes are stained for CD45 (blue) in (G–I). DRAQ5- and CD45-positive structures were reconstructed as “spheres” in (C) and (I), respectively. Insets in (C) show the total ZO-1 staining (top) and nuclei staining (bottom) used to calculate relative amount of tight junction protein/choroidal epithelial cell. Insets in (F) show total IgG staining (top) and capillary staining (bottom) used to calculate relative amount of leaked IgG/capillary mass. Insets in (I) show the total number of leukocytes (top) and total capillary staining (bottom) used to calculate relative number of leukocytes/capillary surface area.
The response of CLN-2 was different both qualitatively and quantitatively from that of ZO-1 (Fig. 5A). The immunostaining pattern of CLN-2 did not appear to change in control mice receiving CFA-PTX alone, remaining relatively linear at intercellular borders. There was, however, a slight change in the staining intensity by D15. Likewise, the overall CLN-2 staining pattern remained unaltered after EAE induction, although a steeper reduction in staining intensity was observed.

**Distribution of CLN-2 in the CP After Immunization**

The response of CLN-2 was different both qualitatively and quantitatively from that of ZO-1 (Fig. 5A). The immunostaining pattern of CLN-2 did not appear to change in control mice receiving CFA-PTX alone, remaining relatively linear at intercellular borders. There was, however, a slight change in the staining intensity by D15. Likewise, the overall CLN-2 staining pattern remained unaltered after EAE induction, although a steeper reduction in staining intensity was observed.

**Figure 4.** Zonula occludens 1 (ZO-1) distribution in the choroid plexus (CP) during evolving experimental autoimmune encephalomyelitis (EAE). (A) Representative sections of the CP were collected at the indicated time points from control complete Freund adjuvant/pertussis (CFA/PTX) mice and from mice after EAE induction with myelin oligodendrocyte glycoprotein (MOG-CFA/PTX). Capillaries were immunostained for CD31 (red) and isosurface rendered in all images to highlight the swelling of these microvessels during disease. Choroidal epithelial cells were stained with DRAQ5 (blue) to identify their nuclei and immunostained for ZO-1 (green). Inset highlights area of extensive crenulation of ZO-1 immunostaining in mice at D15 EAE. (B) 3-D quantification and analysis of the changes in total area of ZO-1 per epithelial cell nuclei for the CFA/PTX and MOG-CFA/PTX groups. There was a significant increase in immunostained area at D9 and D15 in the mice with EAE versus naive mice. * p < 0.05; *** p < 0.001.
more noticeable in this group, which by D15 revealed sites of sharp discontinuities (arrows). The ratio of total area of CLN-2 immunostaining to number of CP epithelial cell nuclei was calculated as for ZO-1 (Fig. 5B). This parameter did not significantly change in control mice until D15 but was significantly reduced earlier in mice with EAE, that is, showing a decrease at D9 and an even greater decline at D15 of after immunization as discontinuities became more evident.

FIGURE 5. Claudin-2 (CLN-2) distribution in the choroid plexus (CP) during evolving experimental autoimmune encephalomyelitis (EAE). (A) Representative sections of the CP were collected at the indicated time points from control complete Freund adjuvant/pertussis (CFA/PTX) mice and from mice after EAE induction with myelin oligodendrocyte glycoprotein (MOG-CFA/PTX). Capillaries were immunostained for CD31 (red) and isosurface rendered in all images to highlight the swelling of these microvessels during disease. Choroidal epithelial cells were stained with DRAQ5 (blue) to highlight their nuclei and immunostained for CLN-2 (green). White arrows show focal disruptions in CLN-2 staining by D15 EAE. (B) 3-D quantification and analysis of the changes in total area of CLN-2 per epithelial cell nuclei for CFA/PTX and MOG-CFA/PTX groups. There was a significant decrease in immunostaining area corresponding to the loss in focal staining in D9 and D15 EAE versus naive mice. ** p < 0.01; *** p < 0.001. The CFA/PTX group also showed a significant decrease in area by D15. * p < 0.05.
Given the strongly disparate responses between ZO-1 and CLN-2, additional studies were carried out to determine whether the different patterns of TJ protein redistribution might be related to these proteins being of peripheral and integral membrane classes, respectively (Figure, Supplemental Digital Content 4, http://links.lww.com/NEN/A659 shows ZO-2 and CLN-3 staining in the CP from naive and MOG-CFA/PTX-treated mice). Zonula occludens 2, another peripheral membrane TJ protein, demonstrated a response much like that of ZO-1, showing a similarly crenulated pattern while apparently maintaining constant intensity after MOG-CFA/PTX immunization. In contrast, the response of CLN-3, another integral membrane TJ protein, paralleled that of CLN-2, exhibiting a less intense and discontinuous pattern.

**Distribution of IgG in the CP After Immunization**

Increased IgG staining was seen in both control mice and in mice after EAE induction, although to different extents (Fig. 6A). It increased in CFA/PTX-treated control mice by D6 and displayed greater elevation through D15. The IgG appeared to be restricted to the CP stroma, as none could be readily seen to infiltrate the choroidal epithelium. These changes in IgG were even more dramatic in mice with EAE. A visible increase in IgG immunostaining was first noted by D6, becoming more extreme by D15. At this later time, IgG was clearly seen permeating across the choroidal epithelium. Because IgG staining became extremely diffuse by later stages of EAE, relative IgG leakage within the CP was quantified by relating its total volumetric staining to total volumetric staining of capillaries within each image (Fig. 3). An increase in IgG leakage was established in both control mice and those with EAE, with a greater level (~3-fold) seen in the latter by D15 (Fig. 6B).

**Distribution of Leukocytes in the CP After Immunization**

Injection with CFA/PTX alone resulted in increased appearance of leukocytes in the CP compared with that seen in naive mice (Fig. 7A). However, these leukocytes appeared to be in close association with the CP capillaries and/or confined to the stroma. This moderate leukocyte increase was reversed to a normal level by D9, where it remained through D15. Immunization with MOG-CFA/PTX also resulted in an apparent influx of leukocytes in the CP at D6 and D9, which reverted by D15. Notably, at D15, leukocytes could be seen closely associated with the choroidal epithelium, a few apparently caught in the act of extravasating past the epithelial barrier. That these leukocytes were, in fact, outside of the CP stroma was revealed by rotation of 3-D images, wherein some extravasated leukocytes in the CSF could be seen still tethered to the luminal surface of choroidal epithelium. The Video (Supplemental Digital Content 5, http://links.lww.com/NEN/A660) shows leukocytes attached to the CP epithelium. It is important to emphasize here that any leukocytes fully extravasated into the CSF are unlikely to be represented in these images because the cells would not be retained in the ventricles after tissue sectioning and immunostaining. Quantification showed that the number of leukocytes significantly increased in the CP at D6 after CFA/PTX treatment and also did so at D6 and D9 after MOG-CFA/PTX before returning to normal levels (Fig. 7B).

**DISCUSSION**

Although the appearance and developmental regulation of TJs in the CP have been rigorously investigated for nearly 5 decades (16,18,28–39), much less is understood of the alterations of these junctions and their protein constituents during neuroinflammatory disease and their relation to blood–CSF barrier permeability and leukocyte extravasation (40). Hence, high-resolution 3-D imaging of the CP was performed in this study to track and quantify such alterations and their functional correlates (i.e. extravasation of IgG and leukocytes during evolving EAE) and to distinguish MOG immunization-associated changes from those caused by adjuvants alone.

Our use of LCM/qRT-PCR to analyze the CP choroidal epithelium selectively confirmed the relative gene expression of major TJ proteins, particularly in MOG-sensitive C57BL/6 mice; a similar pattern was previously described in the Sprague-Dawley rat by standard qRT-PCR of total RNA from whole CP tissues (18). These results reinforced emerging LCM technology as a critical tool to probe gene changes in discrete compartments of the CP (23) and focused our attention on TJ proteins ZO-1 and CLN-2 for detailed 3-D image analysis.

Strong and smooth immunostaining of both ZO-1 and CLN-2 was associated with all intercellular junctions of CP epithelial cells in naive mice, as has been previously reported in these cells from various species (16,17,27,41). However, ZO-1 and CLN-2 showed markedly different responses to MOG immunization or injection of adjuvants. The pattern of ZO-1 immunostaining became moderately distorted after CFA/PTX alone and more drastically crenulated after MOG immunization. To the best of our knowledge, this is the first description of such alterations in TJ staining in vivo. Recent in vitro reports have given similar accounts of “tuffed” or “wavy” ZO-1 patterns associated with loosening of epithelial TJs of cultured Caco-2 and MDCK cells after application of nanoparticles or mechanical stretch (42,43). Together, these observations seemingly highlight a common manifestation of ZO-1 redistribution that accompanies epithelial barrier disruption. Although the basis and physiologic significance of the peculiar alteration of ZO-1 appearance noted here remain unknown, Samak et al (43) noted the comparable disruption in Caco-2 cells to be associated with JNK-2, e-Src, and MLCK-dependent mechanisms. Interestingly, radical ultrastructural changes in CP epithelial cells, for example, widening of intercellular clefts, have been reported after experimental traumatic brain injury (44), and the 2 findings may be related to observations in the current study. The approximately 3-fold increase in both the mean internuclei distance and ZO-1 perimeter of CP epithelial cells during EAE is further consistent with a significant increase in cell volume and points toward even more alterations associated with EAE than have been previously imagined. Interestingly, while describing distributions of TJ proteins at the CP more than a decade ago, Wolburg et al (16) did not note any changes in ZO-1 distribution in EAE-affected mice and specifically
commented on the difficulties of obtaining quantitative results by immunohistochemistry when using conventional confocal microscopy. The advantages afforded by state-of-the-art, high-resolution, 3-D imaging may thus have uniquely enabled us to uncover and quantify the remarkable ZO-1 responses in the CP choroidal epithelium.

FIGURE 6. Immunoglobulin G distribution in the choroid plexus (CP) during evolving experimental autoimmune encephalomyelitis (EAE). (A) Representative sections of the CP were collected at the indicated time points from control (CFA/PTX) mice and from mice after EAE induction (MOG-CFA/PTX). Capillaries are immunostained for CD31 (red) and isosurface rendered in all images to highlight the swelling of these microvessels during disease; choroidal epithelial cells are stained with DRAQ5 (blue) to highlight their nuclei; IgG was immunostained (green). IgG can be seen extravasating across the choroidal epithelium in mice to increasing extents across time in CFA/PTX mice and with increased severity in MOG-CFA/PTX mice (white arrows, inset). (B) 3-D quantification and analysis of the changes in total IgG per total capillaries for CFA/PTX and MOG-CFA/PTX groups. There was a significant increase in extravasating IgG in D15 CFA/PTX and D9 and D15 EAE mice versus IgG staining in naive mice. * p < 0.05; ** p < 0.01; *** p < 0.001.
Leukocyte extravasation in the choroid plexus (CP) during evolving experimental autoimmune encephalomyelitis (EAE).

Representative sections of the CP were collected at the indicated time points from control mice (CFA/PTX) and from mice after EAE induction (MOG-CFA/PTX). Capillaries are immunostained for CD31 (red); choroidal epithelial cells are immunostained for cytokeratin (green); leukocytes are immunostained for CD45 (blue); isosurface was rendered in all images to highlight the 3-D appearance of these cells and their spatial relation to the different compartments of the CP. In control CP, the number of leukocytes appears to increase at D6 but returns to near naive level by D9 and D15. The leukocytes in these mice appear to remain associated with the capillaries or stroma. In the CP of mice with EAE, there is an increase in leukocytes at D6 and D9, which reverses to naive level by D15. Inset highlights leukocytes that are associated with the apical surface of the choroidal epithelium, likely extravasating into the CSF at D15 EAE. (B) 3-D quantification and analysis of the changes in total number of leukocytes per total surface area of capillaries for CFA/PTX and MOG-CFA/PTX groups. There was a significant increase in accumulation of CD45 leukocyte immunostaining in D6 control mice and in D6 and D9 EAE versus naive mice. * p < 0.05; ** p < 0.01; *** p < 0.001.

FIGURE 7. Leukocyte extravasation in the choroid plexus (CP) during evolving experimental autoimmune encephalomyelitis (EAE). Representative sections of the CP were collected at the indicated time points from control mice (CFA/PTX) and from mice after EAE induction (MOG-CFA/PTX). Capillaries are immunostained for CD31 (red); choroidal epithelial cells are immunostained for cytokeratin (green); leukocytes are immunostained for CD45 (blue); isosurface was rendered in all images to highlight the 3-D appearance of these cells and their spatial relation to the different compartments of the CP. In control CP, the number of leukocytes appears to increase at D6 but returns to near naive level by D9 and D15. The leukocytes in these mice appear to remain associated with the capillaries or stroma. In the CP of mice with EAE, there is an increase in leukocytes at D6 and D9, which reverses to naive level by D15. Inset highlights leukocytes that are associated with the apical surface of the choroidal epithelium, likely extravasating into the CSF at D15 EAE. (B) 3-D quantification and analysis of the changes in total number of leukocytes per total surface area of capillaries for CFA/PTX and MOG-CFA/PTX groups. There was a significant increase in accumulation of CD45 leukocyte immunostaining in D6 control mice and in D6 and D9 EAE versus naive mice. * p < 0.05; ** p < 0.01; *** p < 0.001.
By contrast to the observed effects on ZO-1, changes in CP epithelial CLN-2 after immunization were much more subtle. Claudin-2 showed no obvious alterations in pattern after CFA/PTX treatment and a minimal decrease in the amount of immunoreactivity. A similar trend was observed after MOG immunization but with a sharper decrease in immunoreactivity of CLN-2 that ultimately presented a fragmented appearance. Wolburg et al (16) similarly observed the immunostaining of CLN-2 to be “interrupted” in the CP choroidal epithelium after EAE immunization but did not resolve whether this was caused by MOG or adjuvant effects. Kooij et al (27) also recently described a comparatively tempered loss of CLN-3 immunostaining in this tissue in mice immunized for EAE, possibly suggesting that this type of response is common among the different CLN proteins of the CP during neuroinflammation, as opposed to the more peculiar change in ZO-1 pattern.

That the responses of the 2 classes of TJ proteins, ZO-1/ZO-2 and CLN-2/CLN-3, were vastly different from each other could reflect their respective positions and roles within the TJ complex. Peripheral membrane ZO proteins, lying internal to CLNs and directly connected to the actin cytoskeleton (19–21), might transduce the disruptive inflammatory signal to their integral membrane TJ protein partners. Inasmuch as the epithelial actin cytoskeleton can become severely disorganized during both PTX treatment and inflammation (45,46), this could potentially lead to retraction of ZO proteins from their membrane locale and disengagement from CLNs. No longer bound to ZO proteins, CLNs might be susceptible to rearrangement and/or degradation (22,24). Whatever their cause, the different effects on these 2 proteins underscore the complexity of actions taking place at the level of the TJ in the CP choroidal epithelium, and that the response of individual TJ proteins cannot be assumed to be similar. Disparities in responsiveness among various TJ proteins have also been reported in cultured CP choroidal epithelial cells (47). In this case, CLN-2 showed reduced protein level by Western blotting after treatment with protein kinase C activator phorbol 12-myristate 13-acetate, whereas levels of CLN-1, ZO-1, and occludin were spared.

In line with the characterization of the fenestrated CP capillaries as normally “leaky” (48–50), there was some evidence (albeit scarce) of IgG immunostaining and a few leukocytes in the CP stroma of naive mice. In contrast, while using the same technology as used here, virtually no IgG or leukocytes were detected around parenchymal CNS microvessels in the naive situation (22,24). It would thus appear that although CP capillaries in the healthy state are somewhat more permeable than the BBB, their perception as being “highly permeable” (51), and “allowing the free passage of molecules and cells” (52) may merit some reconsideration. This would concur with the description of fenestrated endothelia in airway exchange vessels having permeabilities to plasma proteins that are about the same as found in continuous endothelia (53).

Choroid plexus capillaries nevertheless seemed to become significantly leakier in response to CFA/PTX or MOG-CFA/PTX, allowing for extravasation of IgG and leukocytes into the stromal compartment. It is possible that some of the IgG appearing in the stroma might have been derived from intrathecal synthesis by B cells. However, the fact that IgG levels continually increased while leukocytes decreased in the CP stroma at later time points argues against B cells being the exclusive or major source of IgG observed and suggests that a significant amount of this protein was serum derived. The basis for this effect is unclear at this time because the presence of TJs or other less-restrictive junctional specializations in CP capillaries has not been confirmed (51). Nevertheless, CLN-5, occludin, and ZO-1 have been reported in this vascular bed (27,35) and could possibly be consequences of CFA/PTX or MOG-CFA/PTX immunization. We previously found that the chemokine CCL2 is elevated within the CP capillary plexus after CFA/PTX or MOG-CFA/PTX treatment (23); this may relate to the enhanced IgG leakiness noted here because this chemokine has been linked to downregulation and/or redistribution of endothelial CLN-5, occludin, and ZO-1 in association with increased permeability (24,54–58). Appearance of IgG leakage across the CP choroidal epithelium was only observed after MOG-CFA/PTX immunization and may be caused by the more severe alterations in TJ proteins that accompany neuroinflammation.

The observation that few leukocytes were present in the CP stromal space in naive mice is consistent with other recent descriptions (27,59) and may reflect a limited permeability of normal CP capillaries to blood cellular elements. Specifically, scant CD3-positive and CD4-positive T cells and CD68-positive cells of myeloid lineage (dendritic cells and macrophages) have been shown to reside in this CP compartment under normal conditions (8,60,61). Both CFA/PTX and MOG-CFA/PTX immunization produced a transient increase in the CP leukocyte population. The pathway(s) by which leukocytes accumulate in the CP stroma remains uncertain, as previous reports using immunohistochemistry at the light and ultrastructural levels noted expression of adhesion molecules, ICAM-1, VCAM-1, or MadCAM-1, on the CP choroidal epithelium but not along the CP capillaries in SJL mice with EAE induced by immunization with CFA/PTX and myelin proteolipid protein (62,63). We recently used LCM to detect upregulated gene expression of VCAM-1, E-selectin, P-selectin, and Smad-3 in the CP capillary bed of C57BL/6 mice after CFA/PTX or MOG-CFA/PTX, with Smad-3 exclusively among those showing superinduction during MOG-CFA/PTX-induced EAE (23). Reinforcing the relevance of these gene findings, E-selectin and P-selectin immunoreactivity has also been described in the CP vasculature of patients with non-neurologic disorders (8). Previously noted examples of upregulated chemokine expression in these microvessels, for example, CCL2, CCL5, and CCL19 (23), may thus act cooperatively with elevated adhesion molecule expression to drive leukocyte extravasation across the CP capillaries into the stroma. At present, we are unaware of any descriptions of the CP bearing specialized postcapillary venules, the preferred sites of exit of leukocytes from the circulation (64), but are mindful of the possibility that leukocyte extravasation from the circulation could occur at the transitional regions between the CP capillary plexus and draining venules (8).

Only MOG-CFA/PTX immunization resulted in clear evidence of leukocyte extravasation across the CP choroidal epithelium, which, for CCR6-bearing Th17 cells, is thought to be mediated in significant part by epithelial expression of...
the chemokine CCL20 (12). That the CD45-positive cells observed clinging to the apical side of the CP choroidal epithelium actually represent extravasating leukocytes is strongly supported by the sequence of images after MOG-CFA/PTX immunization, which showed these cells first appearing in the CP stroma, then in association with the basolateral epithelial surface, and finally attached to the apical epithelial surface facing the CSF. To the best of our knowledge, the scenario shown is the first histologic evidence of such directed transit through the entire CP. This finding is also in accord with the inability of CFA/PTX treatment alone to cause clinical EAE in these mice (23) and directly supports the concept that the rudimentary processes underlying neuroinflammatory diseases such as multiple sclerosis and EAE originate via the trafficking of leukocytes through CP into the CSF (8–12). The previous observation by Schmitt et al (60) that CFA/PTX alone resulted in a subtle but definite increase in the number of myeloid cells in the extraventricular CSF spaces could suggest that this adjuvant combination promotes modest leukocyte extravasation across the CP choroidal epithelium but at a level too low to be readily detected even by high-resolution 3-D confocal microscopy. In this regard, histologically capturing leukocytes in the act of extravasating across the CP choroidal epithelium is particularly challenging because they are in effect washed away after entering the CSF.

As with the observed IgG leakage at this site, the more profound perturbations in TJ proteins caused specifically by MOG-CFA/PTX immunization may enable leukocytes accumulating within the CP stroma to migrate more effectively between the choroidal epithelial cells and into the CSF, as depicted schematically in Figure 8. Alternatively, other factors induced by MOG-CFA/PTX immunization, for example, chemokines and/or adhesion molecules, may facilitate leukocyte extravasation at this level, which, in turn, alters the integrity of the CP choroidal epithelium, leading to the TJ protein manifestations and IgG leakage observed. The fact that the CP stroma showed apparent reduction in the leukocyte population from D9 to D15 may further represent time needed for leukocyte extravasation across the CP choroidal epithelium.

Although no absolute cause-and-effect relationship between the observed TJ alterations and IgG/leukocyte extravasation can be established at this time, it is significant to point out that neither IgG nor leukocytes were seen permeating the CP choroidal epithelium until D15 after MOG-CFA/PTX immunization, whereas changes in both ZO-1 and CLN-2 were observed earlier (D9). This time frame is thus consistent with the noted structural changes in CP choroidal epithelial TJ proteins having functional consequences that encourage extravasation of IgG and leukocytes.

Collectively, our present results reinforce the notion that CFA/PTX instigates a series of molecular and structural changes associated with neuroinflammation that is augmented when this adjuvant combination accompanies MOG immunization. At present, it is unclear whether only one or both adjuvants were responsible for the observed effects, as our objective was to specifically highlight changes in TJ protein

![Figure 8](link-to-image)

**Figure 8.** Schematic diagram of the choroid plexus. “Leaky” fenestrated capillaries [1] are surrounded by a “tight” layer of epithelial cells [2], which have tight junctions [3], forming the blood–CSF barrier (BCSFB). The stroma [4] lies in between the 2 layers. Loss of BCSFB integrity during neuroinflammation is associated with alteration of tight junctions [3], leakage of serum protein IgG [5], and extravasation of leukocytes [6] across the BCSFB into the CSF. Leukocyte extravasation is depicted as occurring paracellularly, that is, between choroidal epithelial cells, as it accompanies junctional disruption, although the precise mode has not been established.
distribution and IgG/leukocyte extravasation stemming from MOG immunization versus collective adjuvant action. Complete Freund adjuvant and PTX have each been shown separately to disrupt TJs and increase permeability at the BBB in other settings (65,66). However, PTX was not found to disrupt barrier function in epithelial Plexus choroidaeus-derived monolayer cultures (67), suggesting that CFA might be responsible for the effects noted here or that the 2 adjuvants cooperate to alter TJs and structural integrity of the CP capillaries and/or choroidal epithelium in vivo in support of IgG and leukocyte extravasation.

We previously reported that CFA/PTX alone elicited significant changes in the expression of numerous immune response associated genes in both CP capillary and choroidal epithelium compartments (23), whereas MOG-CFA/PTX immunization resulted in superinduction of some of these genes as well as stimulated a novel cadre of genes in this same functional category. In the current report, we extend these findings to show that CFA/PTX further instigates structural changes in the CP that may facilitate the autoimmune response to MOG immunization. Thus, although it has been reported that PTX, in particular, can drive CNS autoimmunity via regulation of TLR4 signaling and opening up the BBB (68,69), CFA/PTX appears to act more broadly to “raise the floor” of a collective of inflammatory processes in the CP necessary for a neuroinflammatory disease such as EAE to develop.

Additional implications are worth noting. Attempts to attribute gene or histologic changes seen in EAE as being a specific autoimmune response to MOG immunization might overlook significant contributions by CFA and/or PTX. Also, the fact that CFA/PTX exerts prominent effects in the CP (considered a possible gateway for leukocytes to invade the uninnflamed CNS (70–72)) calls attention to the prospect that other environmental toxins with activities like CFA or PTX might target this brain structure to help trigger CNS autoimmune inflammation (73).

Further clarification of the modes of leukocyte trafficking through the CP should enable more efficient drug targeting to this organ and significantly broaden the prospects for treatment of neuroinflammatory diseases (74,75).

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Human ESC-Derived MSCs Outperform Bone Marrow MSCs in the Treatment of an EAE Model of Multiple Sclerosis

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SUMMARY

Current therapies for multiple sclerosis (MS) are largely palliative, not curative. Mesenchymal stem cells (MSCs) harbor regenerative and immunosuppressive functions, indicating a potential therapy for MS, yet the variability and low potency of MSCs from adult sources hinder their therapeutic potential. MSCs derived from human embryonic stem cells (hES-MSCs) may be better suited for clinical treatment of MS because of their unlimited and stable supply. Here, we show that hES-MSCs significantly reduce clinical symptoms and prevent neuronal demyelination in a mouse experimental autoimmune encephalitis (EAE) model of MS, and that the EAE disease-modifying effect of hES-MSCs is significantly greater than that of human bone-marrow-derived MSCs (BM-MSCs). Our evidence also suggests that increased IL-6 expression by BM-MSCs contributes to the reduced anti-EAE therapeutic activity of these cells. A distinct ability to extravasate and migrate into inflamed CNS tissues may also be associated with the robust therapeutic effects of hES-MSCs on EAE.

INTRODUCTION

Multiple sclerosis (MS) is a chronic neuroinflammatory disease characterized by infiltration of peripheral immune cells into the CNS through an impaired blood-brain barrier (BBB) or blood-spinal cord barrier (BSCB), and loss of myelin with accompanying scarring of axons (McFarland and Martin, 2007). However, most current treatments for MS only offer palliative relief without providing a cure, and many are also associated with adverse effects that limit their long-term utility (Weber et al., 2012).

Transplantation of mesenchymal stem/stromal cells (MSCs) for the treatment of MS has emerged as an attractive therapy due to the immunomodulatory and neuroregenerative properties of these cells (Auletta et al., 2012; Pittenger et al., 1999) and their potential ability to repair the BBB (Chao et al., 2009) with fewer side effects (Lalu et al., 2012). MSCs can home to injured tissues and exert therapeutic effects through the secretion of immunomodulatory and trophic factors as well as through direct cell-cell contact (Uccelli and Prockop, 2010). Importantly, allogeneic MSCs generally do not provoke a host immune response due to lack of expression of immune costimulatory receptors and low expression of major histocompatibility complex (MHC) class II antigens (Uccelli and Prockop, 2010), raising the possibility that cells derived from a single donor may be used to treat a large number of patients.

Human adult-tissue-derived MSCs have shown therapeutic utility in experimental autoimmune encephalitis (EAE) models of MS (Bai et al., 2009; Gordon et al., 2008, 2010; Peron et al., 2012; Zhang et al., 2005) and in clinical trials for MS patients (Connick et al., 2012; Karussis et al., 2010; Mohyeddin Bonab et al., 2007; Yamout et al., 2010); however, the large variability in the efficacy of MSCs hinders their development as a standard MS therapy. Extensive in vitro expansion of MSCs may diminish the efficacy of these cells (Kyriakou et al., 2008), and MSCs derived from younger cell sources (e.g., embryonic, fetal, and umbilical cells) have higher in vitro proliferation potential and can more readily differentiate (Barlow et al., 2008; Giuliani et al., 2011). Thus, deriving MSCs from a young and renewable (i.e., pluripotent) cell source, such as human embryonic stem cells (hESCs), could (1) alleviate the quantity and quality issues involved in the use of adult-tissue-derived MSCs, (2) obviate the need for constant donor recruitment, and (3) reduce the risk of pathogenicity from the use of multiple donors.

Different groups have derived MSCs from hESCs (hES-MSCs) with a morphology and immunophenotype similar to those of BM-MSCs. Previously described derivation methods involve coculturing with mouse OP9 cells and
Figure 1. hES-MSCs Attenuate the Disease Score of MOG35-55/CFA-Immunized Mice

(A) Disease scores of MOG35-55/CFA-immunized mice treated with $10^6$ hES-MSCs (CT2, H9, and MA09) or $10^6$ parental hESCs at day 6 postimmunization. n = 5, ***p < 0.001 by Mann-Whitney. Error bar, SEM.

(B) Immunohistochemical detection of MBP (red) and CD3 for T cells (green) (a and b) and IBA1 for microglia (green) (c and d) on lumbar spinal cord cross-sections from MOG35-55/CFA-immunized mice treated with either hES-MSC (a and c) or PBS (b and d). Scale bars, 250 μm (a and b) and 120 μm (c and d).

(C) Relative fluorescent intensity measurements of MBP expression in digitally captured spinal cord hemi-sections. n = 4–6, **p < 0.02. Error bar, SD. The regions shown are from the lumbar level of the spinal cord. Error bars indicate SD and the p value was determined using two-tailed unpaired Student’s t test of the total fluorescence signal of each section.

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sorting, scraping, or handpicking of cells (Barberi et al., 2005; Brown et al., 2009; Gruenloh et al., 2011; Hwang et al., 2008; Olivier et al., 2006; Vodyanik et al., 2010), which limits the efficiency and purity of the hES-MSCs, as well as the ability to scale up their production. hES-MSCs have been used in some disease models, such as inflammatory bowel disease, lupus, and uveitis (Kimble et al., 2014; Sánchez et al., 2011); however, no one has shown whether hES-MSCs can be used to treat an EAE model of MS or compared the immunosuppressive functions of hES-MSCs and BM-MSCs. Here, using an improved hemangioblast-enriching method (Lu et al., 2007), we generated hES-MSCs from the MA09 ESC line (Kimble et al., 2014) and multiple other hESC lines. We demonstrate that these hES-MSC lines can effectively treat an EAE model of MS and outperform multiple lines of BM-MSCs in therapeutic activities.

RESULTS

hES-MSCs Attenuate EAE Disease in MOG35-55/CFA-Immunized Mice when Administered either Prophylactically or Therapeutically

In this study, we derived MSCs through a hemangioblast-enriched, intermediate stage as described previously (Kimble et al., 2014). We tested the reproducibility of this method by generating independent MSC lines from four different hESC lines: H9 (Thomson et al., 1998), CT2 (derived at UConn; Wang et al., 2009), MA09 (Klimanskaya et al., 2006), and E03-Env (Envy, a GFP+ line derived at ES International; Costa et al., 2005). These hES-MSC lines expressed cell surface markers consistent with those of adult human MSCs (Figure S1A available online) and were capable of differentiating into osteocytes, adipocytes, and chondrocytes (Figure S1B). We also confirmed that the hES-MSCs were karyotypically normal for at least 12 passages (Figure S1C) and did not express telomerase (Figure S1D).

We employed a standard EAE model of MS in which C57BL/6 mice were immunized with an emulsion of MOG35-55 peptide and complete Freund’s adjuvant (CFA) to test the therapeutic utility of our hES-MSC lines. Six days after immunization but prior to disease onset, mice were injected with $1 \times 10^{6}$ hES-MSCs or PBS intraperitoneally (i.p.). hES-MSCs derived from three hESC lines (CT2, MA09, and H9) all significantly attenuated the daily (Figure 1A), cumulative, and maximal disease scores (Figure S1E). H9 hES-MSCs also delayed the disease onset, and MA09 hES-MSCs appeared to lower the disease incidence (Figure S1E). However, mice injected with parental hESCs (CT2) manifested high disease scores similar to those of PBS controls (Figure 1A). To confirm the results, we histologically analyzed microglial inflammatory activity within the spinal cord. Immunostaining for ionized calcium-binding adaptor molecule 1 (IBA1) revealed inhibited development of microgliosis in MOG35-55/CFA-immunized mice treated with hES-MSCs compared with those treated with PBS (Figure 1B). Infiltration of CD3+ T cells into the spinal cord was decreased and the number of interleukin-17 (IL-17) - and interferon γ (IFNγ) -expressing CD4+ T cells in the CNS was also decreased by hES-MSC treatment (Figures 1B, S1E, and S1G). Stronger immunostaining for myelin-binding protein (MBP) suggests that demyelination was prevented in mice treated with hES-MSCs (Figures 1B and 1C).

We also tested the effect of hES-MSC treatment on mice that had already developed EAE (postonset). hES-MSCs were injected on day 18 postimmunization, when all mice had disease scores of 3. We observed a gradual decline in disease scores from 3 down to an average score of 1.7 by day 30 in hES-MSC-treated mice, whereas the PBS-treated mice showed an average score of 2.8 by day 30 (Figure 1D). Collectively, the data presented in Figure 1 show that hES-MSCs can reproducibly decrease disease severity both prophylactically and therapeutically in the mouse EAE model.

Mitotically Arrested hES-MSCs Retain the EAE-Inhibitory Effect

MSCs transplanted into animals may undergo malignant transformation or support tumor growth formed by host cells (Djouad et al., 2003; Wong, 2011). However, since short-term cytokine secretion and cell-cell contact may be sufficient to exert MSC functions (Uccelli and Prockop, 2010), we hypothesized that mitotically arrested MSCs may still execute a disease-modifying effect. To test this, we irradiated hES-MSCs at 80 Gy immediately before injecting cells into MOG35-55/CFA-immunized mice at day 6 postimmunization. This irradiation regimen did not significantly reduce the viability of hES-MSCs that were replated and cultured for 48 hr in vitro (90% of the cells were trypan-blue negative), but was sufficient to completely attenuate cell proliferation as assessed by

(D) Disease scores of MOG35-55/CFA-immunized mice treated with $10^6$ hES-MSCs 18 days postimmunization. n = 6, ***p < 0.001 by Mann-Whitney. Error bar, SEM. At day 17 postimmunization, all mice with a disease score of 3 were pooled into a single group and then randomly assigned to either the PBS-treatment group or the hES-MSC-treatment group. Mice that were immunized but did not show a disease score of 3 on day 17 were removed from the study. See also Figure S1.
bromodeoxyuridine (BrdU) incorporation during this same time period (Figure 2A). Mice that received $2 \times 10^6$, but not $1 \times 10^6$, irradiated cells showed a similar reduction in EAE disease severity as mice who received $1 \times 10^6$ nonirradiated hES-MSC (Figures 2B and S2A). To determine the lifespan of irradiated hES-MSC in vivo, we established a CT2 hESC clone with constitutive expression of luciferase in the hESCs and subsequent hES-MSCs (Figure S2B) by

![Figure 2. Mitotically Arrested hES-MSCs Retain the EAE-Inhibitory Effect](image-url)

(A) Nonirradiated or irradiated hES-MSCs ($1 \times 10^5$) were cultured in vitro for 48 hr in the absence (gray line) or presence (black line) of BrdU. The percentage of proliferating cells, as determined by flow-cytometry staining for BrdU$^+$ cells, is indicated.

(B) Disease scores of MOG$_{35-55}$/CFA-immunized mice treated at day 6 with PBS, $10^6$ nonirradiated or $2 \times 10^6$ irradiated hES-MSCs (MA09); n = 4, ***p < 0.001 by Mann-Whitney for comparison with the PBS control. Error bar, SEM. The table below shows a comparison of cumulative disease score (Cum. D.S.), maximum disease score (Max. D.S.), disease incidence, and disease onset day.

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<tr>
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<th>PBS</th>
<th>hES-MSC</th>
<th>Irr-hES-MSC</th>
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<tr>
<td>Cum. D.S.</td>
<td>51.63±10.85</td>
<td>13±3.36**</td>
<td>10.88±10.88*</td>
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<tr>
<td>Max. D.S.</td>
<td>3.5±0.29</td>
<td>0.6±0.24***</td>
<td>0.75±0.75*</td>
</tr>
<tr>
<td>Incidence %</td>
<td>100</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Onset Day</td>
<td>9.25±0.95</td>
<td>12.6±1.20</td>
<td>11</td>
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(C) Nonirradiated (left) and irradiated (right) luciferase-expressing hES-MSCs (CT2) were tracked in MOG$_{35-55}$/CFA-immunized mice by in vivo bioluminescence imaging using the Xenogen IVIS 100 system. See also Figure S2.
transducing the cells with a lentiviral vector (Pomper et al., 2009). Using these luciferase-expressing hES-MSCs, we found that both irradiated and nonirradiated hES-MSCs had roughly the same lifespan of at least 7 days in wild-type mice as determined by whole-body bioluminescence imaging (Figure 2C).

Teratoma formation is another concern for any cells differentiated from pluripotent cells. To assess this risk, we injected hES-MSCs into immunodeficient SCID-beige mice at 1 x 10^6 cells/mouse, and found no tumor formation at the injection sites within 2 months, whereas teratomas formed in mice injected with the same dose of parental hESCs (data not shown).

**hES-MSCs Have Stronger EAE-Inhibitory Effects than BM-MSCs**

Next, we compared hES-MSCs and BM-MSCs in the prophylactic EAE model. We derived MSCs from six different BM donors (four from frozen monocytes [MNCs] and two from fresh BM) and obtained two BM-MSC lines at passage 1 from the Texas A&M MSC repository. We tested BM-MSC lines anywhere from passage 2 to 4 and found that none of them could consistently attenuate EAE disease scores of MOG35-55/CFA-immunized mice, as shown in Figures 3A (BM-MSC#1–3), 3B (BM-MSC#4), 6 (GFP-expressing BM-MSC#5), and S3F (BM-MSC#7-8). Of note, one BM-MSC line (BM-MSC#6, from Texas A&M) showed moderate but significant disease-modifying effects (Figure 3C) at passage 2 relative to control PBS-treated mice (p < 0.001), but these effects were gone at passage 4 (see Figure 5F). This is in marked contrast to the four independent hES-MSC lines that all showed a strong disease-inhibitory effect when tested up to passage 5, as shown in Figures 1A (H9, CT2, and MA09) and 6 (GFP-expressing Envys).

EAE/MS is accompanied by infiltration of reactive T cells into the CNS (Mcfarland and Martin, 2007). We found that hES-MSC-injected mice had significantly fewer CD4^+ and CD8^+ T cell infiltrates in the CNS, including Th1 and Th17 CD4^+ subsets, than PBS-treated MOG35-55/CFA-immunized mice (Figures 3D, S1F, and S1G), whereas parental hESC line CT2-treated mice had levels comparable to those in controls (Figure S1F). In contrast, BM-MSC-treated mice actually displayed significantly more CD4^+ and slightly more CD8^+ T cell infiltrates than PBS-treated MOG35-55/CFA-immunized mice (Figure 3D, first two panels). This included similar or greater Th1 numbers and consistently greater Th17 numbers compared with the controls (Figure 3D, last two panels). Reduced FluoroMyelin staining of MBP in the spinal cord of both PBS- and BM-MSC-treated mice suggests severe demyelination, whereas MBP levels were preserved in the spinal cord of hES-MSC-treated mice (Figure 3E). The damaged regions in BM-MSC-treated mice also show a high number of DAPI-positive cells (Figure 3E), suggesting more inflammatory cell infiltration. To determine whether the reduced FluoroMyelin staining in the BM-MSC-treated mice was due to reduced levels of myelin, loss of entire axons, or inflammatory infiltrate blocking myelin staining, we performed costaining for MBP and neurofilament (NF) on day 32 lumbar spinal cord cross-sections. Quantification of MBP-positive areas again revealed significantly lower levels of myelination in BM-MSC-treated mice as compared with hES-MSC-treated ones, whereas NF staining revealed similar numbers of axons in both groups (Figures S3A–S3E).

Considering the important role of regulatory T cells (Treg cells detected as CD4^+, Foxp3^+, and CD25^+) in suppressing inflammation, we examined the ratio of Treg cells among infiltrated CD4^+ T cells in the CNS, and found no difference in hES-MSC-treated versus control MOG35-55/CFA-immunized mice (Figure S3G). This is similar to a previous report (Zappia et al., 2005). In vitro, both hES-MSCs and BM-MSCs increased Treg cell proliferation in the presence of IL-2, but there was no remarkable difference between the two groups (Figure S3H). This suggests that enhanced Treg cell proliferation is a common response to both hES-MSCs and BM-MSCs, but is not necessarily a contributor to their divergent effects in the in vivo EAE model.

**Analyzing MSC Effects on T Cell Functions: hES-MSCs Show Stronger Inhibition of Th1 Differentiation than BM-MSCs**

We next compared hES-MSCs and BM-MSCs for their ability to inhibit T cell proliferation in vitro, using mixed leukocyte reaction (MLR) assays. We incubated carboxyfluorescein succinimidyl ester (CFSE)-labeled mouse naive T cells isolated from lymph nodes with increasing amounts of BM-MSCs or hES-MSCs. Both types of MSCs inhibited CD4^+ and CD8^+ T cell proliferation in response to a constant amount of anti CD3/anti-CD28 stimulation to similar degrees (Figure 4A). Likewise, BM-MSCs and hES-MSCs similarly inhibited human peripheral blood mononucleated cell (PBMC) proliferation induced in response to phytohemagglutinin (PHA) over a range of PBMC/MSC ratios (Figure 4B). Together, these data suggest that BM-MSCs and hES-MSCs display similar inhibitory effects on T cell proliferation in vitro.

Since MOG35-55/CFA-immunized mice treated with BM-MSCs had more Th1 and Th17 cell infiltration in the CNS than mice treated with hES-MSC (Figure 3D), we examined these T cell subtypes in vitro in the presence or absence of hES-MSCs and BM-MSCs. Under the Th1 condition, differentiation of naive CD4^+ T cells into Th1 (CD4^+/IFNγ^+) cells was reduced by hES-MSCs, but was unaffected or even enhanced by different BM-MSC lines (Figure 4C, upper panels). Interestingly, even the BM-MSC line (BM-MSC#6) that gave a modest but significant therapeutic
Figure 3. hES-MSCs Have a Stronger EAE-Inhibitory Effect In Vivo than BM-MSCs

(A–C) Disease scores of MOG35-55/CFA-immunized mice treated with PBS, 10^6 BM-MSCs, or 10^6 hES-MSCs (MA09) at day 6. n = 4–5 per group, ***p < 0.001 by Mann-Whitney for comparison with the PBS control. Error bar, SEM. The table below shows a comparison of cumulative disease score (Cum. D.S.), maximum disease score (Max. D.S.), disease incidence, and disease onset day.

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<tr>
<td>PBS</td>
<td>40.50±8.56</td>
<td>2.75±0.25</td>
<td>100</td>
<td>11.75±0.25</td>
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<tr>
<td>BM-MSC#1</td>
<td>43.75±9.80</td>
<td>2.63±0.55</td>
<td>100</td>
<td>11.75±0.25</td>
</tr>
<tr>
<td>BM-MSC#2</td>
<td>38.75±5.99</td>
<td>2.75±0.25</td>
<td>100</td>
<td>13.25±0.25</td>
</tr>
<tr>
<td>BM-MSC#3</td>
<td>51.25±5.20</td>
<td>3.25±0.13</td>
<td>100</td>
<td>13.00±0.57</td>
</tr>
<tr>
<td>hES-MSC</td>
<td>5.00±2.68</td>
<td>0.70±0.20</td>
<td>80</td>
<td>15.00±0.71*</td>
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(D) Total numbers of CD4^+ and CD8^+ cells in the CNS of MOG35-55/CFA-immunized mice treated with PBS, BM-MSCs, or hES-MSCs on day 32 postimmunization. Lymphocytes purified from the CNS were analyzed via flow cytometry for numbers of CD4^+ and CD8^+ cells (left two panels) or IL-17^+ and IFN-γ^+ cells (intracellular stained) poststimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin (right two panels). n = 4, *p < 0.05, **p < 0.01. Error bar, SD; p values were determined using two-tailed unpaired Student’s t test.

E

Fluoromyelin / DAPI

Figure 3 continued...
response in the prophylactic EAE model could not effectively reduce Th1 differentiation in this in vitro assay (Figure 4C, upper last panel). Under the Th17 differentiation condition, both hES-MSCs and BM-MSCs reduced the differentiation of Th17 (CD4+/IL17+) cells (Figure 4C, lower panels). However, under the same Th17-inducing conditions, BM-MSCs, but not hES-MSCs, significantly increased the percentage of IFNα+/IL17− (i.e., Th1) cells (Figure 4C, lower panels). Collectively, these results show that hES-MSCs effectively dampen differentiation of both Th1 and Th17 in vitro, and, surprisingly, BM-MSCs promote Th1 differentiation under a Th17-inducing environment.

BM-MSCs Express Higher Levels of IL-6 than hES-MSC, and IL-6 Blockage Enhances the Disease-Modifying Effects of BM-MSCs in the EAE Model

Many factors have been reported to mediate the immunomodulatory and/or neuroprotective effects of MSCs (Uccelli and Prockop, 2010). We conducted a microarray analysis to identify differences in the expression of these factors between BM-MSCs and hES-MSCs. The overall expression profiles of the hES- and BM-MSC samples were similar (data not shown); however, a small set of genes was expressed differentially. Among these, IL-6 appeared to be much more highly expressed in BM-MSCs than in hES-MSCs. Multiple methods, including quantitative RT-PCR (qRT-PCR; Figure 5A), intracellular flow cytometry (Figure 5B), and cytokine antibody arrays (Figure 5C), confirmed this finding.

Upon IFNγ stimulation, the percentage of IL-6-expressing hES-MSCs did not change; however, the percentage of IL-6-expressing BM-MSC nearly doubled (Figure 5D). We also tested MSC production of IL-6 following coculture with stimulated PBMC/T cells, as the latter produce high levels of IFNγ and TNF-α upon stimulation. IL-6 mRNA expression levels increased for both BM-MSCs and hES-MSCs after coculture, yet the levels in BM-MSC were still ≥10 times higher than in hES-MSCs (Figure 5A). This large difference in IL-6 secretion was a rather unique observation because the expression levels of other secreted cytokines did not differ dramatically between the two cell types (data not shown).

Since IL-6 has been found to enhance T cell differentiation (Dienz and Rincon, 2009), we sought to determine the effects of MSC-secreted human IL-6 on mouse T cell differentiation. First, we confirmed that human IL-6 works just as well as mouse IL-6 at an equivalent dose for directing mouse Th17 differentiation (Figure 5B). Next, under Th0 conditions (i.e., without any mouse cytokines), we found that an anti-human-IL-6 neutralizing antibody with no mouse cross-reactivity (clone MQ2-13A5) reduced the Th1-promoting effects of BM-MSCs on mouse T cells by ~23%–50% (Figure 5E). Lastly, we observed that MOG35-55/CFA-immunized mice treated with human BM-MSCs plus the same anti-hIL-6 antibody showed a significant reduction in EAE disease severity relative to the control group (Figure 5F). This effect was specific for IL-6 produced by the BM-MSCs, as IL-6 antibody alone (no MSCs) or BM-MSCs plus isotype control antibody failed to reduce the EAE disease score significantly. These data suggest that high IL-6 expression by human BM-MSCs contributes to the inability of these cells to modulate EAE disease severity.

Both hES-MSCs and BM-MSCs Home to the Spinal Cord, but Only hES-MSCs Successfully Extravasate into Inflamed Tissue

To determine whether hES- and BM-MSCs home to the injured CNS, we used the constitutively GFP-expressing hESC line “Envy” and GFP-labeled human BM-MSCs (Hofstetter et al., 2002) in MOG35-55/CFA-immunized mice. Cells were injected on day 6 after immunization and spinal cords were analyzed 8 days later (day 14 postimmunization), a time point when disease scores for the GFP+ BM-MSC-injected mice and PBS controls were ~1.5–2.0, and scores for the GFP+ hES-MSC were effectively 0 (Figure 6A). Both BM-MSCs and hES-MSCs homed to the spinal cord in mice subjected to EAE (Figure 6B), yet the vascular association patterns of the two types of MSC were vastly different. At day 14, GFP+ hES-MSCs were immunolocalized in the parenchyma adjacent to spinal cord venules, indicating the ability of these cells to penetrate and move beyond the vasculature to enter the parenchyma (Figure 6B, top row). In marked contrast, GFP+ BM-MSCs appeared to remain closely associated with the parenchymal vessels, seemingly trapped inside the microvascular lumen and/or confined to the perivascular space, and incapable of breaching the bipartite complex of endothelial and parenchymal basement membranes (Owens et al., 2008; Paul et al., 2014) to enter the CNS tissue (Figure 6B, second row). This apparent retarded migration of BM-MSCs persisted even at day 18, when the disease is more severe and both tight junctions and the basement membrane complex of the BBB have been shown to be grossly disrupted (Figure 6B, third row) (Paul et al., 2013, 2014). No GFP

(E) Qualitative analysis of myelin content in spinal cord cross-sections of MOG35-55/CFA-immunized mice treated with PBS, BM-MSCs, or hES-MSCs using FluoroMyelin staining (green) and counterstained with DAPI (blue) for infiltration of nucleated cells. Scale bar, 350 μm. The regions shown are from the lumbar level of the spinal cord. See also Figure S3.
Figure 4. Effects of hES-MSCs and BM-MSCs on T Cell Functions In Vitro

(A) hES-MSCs (MA09) or hBM-MSC#7 were cocultured with $1 \times 10^5$ CFSE-labeled mouse lymphocytes stimulated with anti-CD3/CD28 at various MSC/lymphocyte ratios. After 3 days, the proportion of proliferating CD4$^+$ (left panel) or CD8$^+$ (right panel) T cells was measured by CFSE dilution using flow cytometry. The percent inhibition of T cell proliferation is relative to T lymphocytes stimulated in the absence of MSCs. Lymphocytes from three individual mice were tested and results are average ± SD.

(B) Mitotically inactivated hES-MSCs (MA09) or BM-MSCs were incubated with CFSE-labeled human PBMC at the indicated ratios and stimulated with 2.5 ng/ml PHA. Bars represent the average of three different BM-MSC lines and two independent hES-MSC (MA09) clones ± SD.

(C) hES-MSCs (MA09) or BM-MSCs were incubated with mouse naive CD4$^+$ T cells at a ratio of 1:10, followed by Th1 or Th17 differentiation for 5 days. IFN$\gamma^+$ and IL-17$^+$ CD4$^+$ T cells were detected via intracellular flow cytometry staining after TPA/ionomycin stimulation. Data represent four independent experiments.
signals could be detected in control mice receiving PBS injection alone (Figure 6B, bottom row). Movies S1 and S2, which were made from confocal z-stack reconstructions of the data sets in Figure 6B, provide a magnified 3D perspective of hES-MSC and BM-MSC distributions, respectively. Figure 6C shows a schematic detailing the regions of spinal cord selected for analysis in Figure 6B.

DISCUSSION

In this study, we have shown that multiple hES-MSC lines significantly attenuated disease scores in a mouse EAE model of MS. In stark contrast, only one out of eight independently derived human BM-MSC lines displayed a marginal effect in the prophylactic-treatment EAE model. Our analysis of IL-6 expression and migration of the MSCs suggests that the superior disease-altering effects of hES-MSCs may be related in part to the lower expression of IL-6 and the greater ability of hES-MSCs to extravasate the BBB/BSCB and migrate into inflamed CNS tissue relative to BM-MSCs.

In examining the effects of hES-MSCs on EAE induction, we observed that preonset treatment of MOG35-55/CFA-immunized mice was more effective in attenuating disease scores than postonset treatment. This is not surprising, since preonset treatment begins before the development of severe demyelination, axonal damage, or inflammatory cell infiltration. Costaining for MBP and NF in day 32 lumbar spine sections of treated animals showed that hES-MSC treatment protected against demyelination without affecting the number of surviving axons. These results do not rule out the possibility that hES-MSCs may also contribute to remyelination of axons that have already lost their myelin. This remains to be elucidated by further investigations examining whether and how hES-MSCs may contribute to neural regeneration. Future studies in the postonset model will also be needed to address whether larger doses and/or repeated injections of cells could enhance the therapeutic effects. Of note, irradiated hES-MSCs were also effective in reducing the EAE disease score and had the same lifespan in vivo as their nonirradiated counterparts. Thus, irradiation of cells may provide an additional enhancement to the therapeutic effects. Of note, irradiated hES-MSCs may contribute to functional differences in the treatment of EAE. This idea is supported by our data showing that blocking human IL-6 with a neutralizing antibody experiment (Figure 5F). On the other hand, hES-MSCs consistently resulted in large reductions in the disease score of MOG35-55/CFA-immunized mice when used from passage 2 to 5. This indicates that the therapeutic capacity of BM-MSCs may be more vulnerable to extended in vitro culture than that of hES-MSCs.

It has been reported that BM-MSCs derived from frozen MNCs have less immunosuppressive effects than those derived from fresh MNCs (Samuelsson et al., 2009). We derived different BM-MSC lines from both frozen MNCs and fresh (never frozen) whole BM. Both types had similarly negligible effects in the EAE model when administered prophylactically (Figures 3A, 3B, and 3F). One BM-MSC line (#8), derived from fresh BM, did show a modest efficacy in the EAE model when administered postonset (Figure S3I), but this effect was not as great as that of hES-MSCs (Figure 1D). These data suggest that variability among human BM-MSC lines derived from different donors could influence the efficacy of these cells, whereas hES-MSC lines consistently exhibit a greater EAE-inhibitory effect than human BM-MSC lines.

In this study, we found that IL-6 was much more highly expressed in BM-MSCs than in hES-MSCs in both the basal and IFNγ-stimulated states. Elevated IL-6 levels have been found in blood and brain tissue from MS patients (Patanella et al., 2010), and site-specific production of IL-6 in the CNS can enhance inflammation in EAE (Quintana et al., 2009). Mice lacking IL-6 receptor α are resistant to EAE (Leech et al., 2013), and an IL-6-neutralizing antibody can reduce symptoms in EAE mice (Gijbels et al., 1995). Thus, higher levels of human IL-6 secretion by BM-MSCs relative to hES-MSCs may contribute to functional differences in the treatment of EAE. This idea is supported by our data showing that blocking human IL-6 with a neutralizing antibody partially rescues the disease-modifying effects of BM-MSC in the EAE model (Figure 5F). It is important to note that the anti-hIL-6 antibody in this study does not inhibit the endogenous mouse IL-6. This suggests that human IL-6 produced by the BM-MSCs may act in an autocrine or paracrine manner on the BM-MSCs themselves and/or on surrounding cells, with the net effect of limiting BM-MSC therapeutic activity in EAE.

Recent reports have noted that MSCs can actually promote the differentiation of proinflammatory T cells in certain permissive environments (Carrión et al., 2011; Darlington et al., 2010). Consistently, in vivo and in comparison with PBS controls, we observed reduced CNS infiltration of Th1 and Th17 cells with hES-MSC treatment, but increased CNS infiltration of Th1 and Th17 cells with BM-MSC treatment. In vitro and in contrast to hES-MSCs, we found that BM-MSCs skewed cell differentiation to a Th1 phenotype under both Th0 (nonpolarizing) and Th17 conditions. It is possible that certain factors that are
Therapeutic Efficacy of hES-MSC in an EAE Model

A. IL-6 (qRT-PCR) relative normalized gene expression for hES-MSC and BM-MSC.

B. Cell number for BM-MSC#1, BM-MSC#2, and BM-MSC#6:
- BM-MSC#1: 49.5
- BM-MSC#2: 34.2
- BM-MSC#6: 39.4

C. Control, IL-6, and IL-8 expression in hES-MSC and BM-MSC.

D. Cell number for NC and +IFNγ 10ng/ml in hES-MSC and BM-MSC:
- NC: 2.0
- +IFNγ 10ng/ml: 2.1

E. CD4 expression under Th0 condition with IgGk and anti-IL-6:
- Control: 3.2
- hES-MSC: 3.6
- BM-MSC#2: 39.4
- BM-MSC#3: 41.6
- BM-MSC#6: 36.8

F. Disease score over time for PBS, PBS+anti-IL6, BM-MSC#6+IgGk, and BM-MSC#6+anti-IL6 treatments.

Legend:
- Th0 Condition: + IgGk
- Th0 Condition: + anti-IL-6

(Please refer to the legend on the next page for further details.)
highly produced by human BM-MSCs, but not hES-MSCs, can trigger Th1 differentiation, thus overriding Th17 differentiation under defined in vitro conditions (Lazarevic et al., 2011). An anti-human IL-6 antibody was able to partially reverse the effect under Th0 conditions (Figure 5E), but not Th17 conditions (data not shown), presumably because the presence of abundant exogenous mouse IL-6, which was added for Th17 induction, could not be neutralized by the anti-human IL-6 antibody. Together, these results suggest that high IL-6 secretion by BM-MSCs may impact the local cytokine milieu and augment the overall inflammatory response, resulting in a striking difference in Th1/Th17 CNS infiltration between hES-MSC- and BM-MSC-treated mice.

Lastly, we observed that both GFP-labeled hES-MSCs and BM-MSCs homed to the CNS microvasculature, but only hES-MSCs that showed a high therapeutic potential had the capacity to effectively extravasate and migrate into the parenchyma. This raises the possibility that therapeutic efficacy and MSC extravasation are somehow mechanistically linked. Such a therapeutic requirement for MSCs to extravasate during EAE is consistent with evidence that these cells can downregulate proinflammatory effector functions of parenchymal microglia (Figure 1B; Lee et al., 2012; Sheikh et al., 2011). The lack of penetration into the CNS tissue by BM-MSCs was further remarkable in that it persisted through late disease, a time when severe BBB disruption has been shown to occur (Paul et al., 2013, 2014). Since such a compromised BBB might be expected to facilitate cellular entry during EAE (Lanz et al., 2013), this suggests that hES-MSCs might uniquely express specific transendothelial migratory signals or properties.

BM-MSCs, in turn, might lack one or more of the minimal requirements for effective migration across even a structurally attenuated CNS microvascular endothelium and/or surrounding basement membrane complex. It is significant that in vitro culture and expansion of MSCs have both been implicated as factors that impair homing and transendothelial migration (De Becker et al., 2007; Rombouts and Ploemacher, 2003). A priori, BM-MSCs could be more sensitive to these factors than hES-MSCs, which could at least partially explain the differences in therapeutic efficacy observed between the two MSC types. Determining the dynamic changes and differences in expression of adhesion molecules, chemokines/chemokine receptors, and matrix metalloproteinase between hES-MSCs and BM-MSCs in situ will be critical for delineating the molecular requirements for MSC extravasation and efficacy (De Becker et al., 2007; Teo et al., 2012). In situ gene-expression profiling of both MSC types is currently under investigation and should shed further light on the mechanism(s) responsible for the unique therapeutic efficacy of hES-MSCs in EAE.

**EXPERIMENTAL PROCEDURES**

**Culture of hESCs and Generation of hES-MSCs**

hESC lines were cultured either on Matrigel in TeSR1 medium or on mouse embryonic fibroblasts in Dulbecco’s modified Eagle’s medium/F12 + 20% knockout serum replacement + 10 ng/ml basic fibroblast growth factor. hES-MSCs were generated as described previously (Kimble et al., 2014). Only hES-MSCs at ≤5 passages were used throughout the study. The use of hESCs in this study was approved by the Stem Cell Research Oversight Committee of the University of Connecticut (#2012-005).

**Figure 5. BM-MSCs Express Higher Levels of IL-6 than hES-MSCs, and a Neutralizing IL-6 Antibody Reduces the Influence of BM-MSCs on T Cells and Disease Scores**

(A) qRT-PCR for IL-6 in BM-MSCs versus hES-MSCs. Bars represent the average of three independent experiments ± SD. *p < 0.05 (the p value was determined using two-tailed unpaired Student’s t test).

(B) Intracellular flow cytometry showing the percentage of IL-6-expressing cells (black line) and isotype control (gray line) among BM-MSC from three different donors and hES-MSCs from three hESC lines (MA09, CT2, and H9).

(C) Cytokine antibody arrays showing the level of IL-6 and IL-8 proteins in conditioned medium from hES-MSC or BM-MSC cultures. Cytokine antibodies and controls are spotted in duplicate and labeled next to the corresponding spots. Images are representative of at least five independent experiments.

(D) Intracellular flow cytometry showing the percentage of IL-6-expressing cells (black line) and isotype control (gray line) among hES-MSCs or BM-MSCs treated ± 10 ng/ml IFNγ for 12 hr. NC, negative control.

(E) Intracellular flow cytometry measuring the percentage of CD4+ and IFNγ+ mouse T cells arising from naïve T cells after coculture with hES-MSC (MA09) or BM-MSCs from three different donors under Th0 conditions. The MSC/T cell ratio was 1:10. IgGk isotype control (upper panels) or anti-human IL-6 neutralizing antibody (clone MQ2-13A5) was added to determine the effect of blocking IL-6 on BM-MSC-induced production of IFNγ from T cells (i.e., Th1 differentiation) (lower panels).

(F) Mean disease scores of mice immunized with MOG35-55 and treated with PBS or 1 × 10⁶ human BM-MSC on day 6. Anti-human IL-6 neutralizing antibody (clone MQ2-13A5, 12.5 mg/kg/day), IgGk isotype control antibody (12.5 mg/kg/day) or PBS was administered i.v. on days 6 and 7. **p < 0.01 by Mann-Whitney for BM-MSC+anti-IL-6 versus PBS control (n = 5 per group). Error bar, SEM. The table on the right shows a comparison of cumulative disease score (Cum. D.S.), maximum disease score (Max. D.S.), disease incidence, and disease onset day. See also Figure 5A.
Therapeutic Efficacy of hES-MSC in an EAE Model

A

<table>
<thead>
<tr>
<th></th>
<th>Disease Score (Day 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>BM-MSC#5 (GFP+)</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>hES-MSC (Envy-GFP+)</td>
<td><strong>2.5 ± 0.2</strong></td>
</tr>
</tbody>
</table>

B

- **hES-MSC (Envy-GFP+)** Day 14
  - GFP CD31 DRAQ5
  - ROI Isosurface
  - z-slice

- **BM-MSC#5 (GFP+)** Day 14
  - GFP CD31 DRAQ5
  - ROI Isosurface
  - z-slice

- **BM-MSC#5 (GFP+)** Day 18
  - GFP CD31 DRAQ5
  - ROI Isosurface
  - z-slice

- **PBS Control**
  - GFP CD31 DRAQ5
  - ROI Isosurface
  - z-slice

(legend on next page)
Animal Model of MS
The mouse EAE model was induced as previously described (Stromnes and Goverman, 2006). In brief, C57BL/6 mice were subcutaneously injected with an emulsion of MOG35-55 Peptide, CFA, and pertussis toxin contained in the EAE induction kit from Hooke Laboratories (Cat. No. EK-0114). BM-MSCs or hES-MSCs at 1 × 10^6 cells/mouse or PBS (a vehicle control) were i.p. injected on day 6 (for preonset) or 18 (for postonset) after the immunization. Disease score was monitored every day for up to 31 or more days as follows: 0, no sign of disease; 1, loss of tone in the tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, front limb paralysis; and 5, moribund (Stromnes and Goverman, 2006). For some experiments, cumulative and maximal disease scores were also calculated, and disease incidence and disease onset day were recorded. Injection and scoring were performed double-blinded in Figure S3f. All animal studies were approved by and performed in accordance with policies of the Institutional Animal Care and Use Committee of the University of Connecticut Health Center.

Tracking of GFP* MSCs in Perivascular Regions in the CNS
Spinal cord tissue was prepared as described previously (Paul et al., 2013). In brief, after perfusion/fxation, spinal cords were harvested by laminectomy and cryosectioned for immunostaining. Anti-GFP-Alexa 488, anti-CD31 (BD Bioscience), and Alexa 555 secondary antibody (Life Technologies) were used to detect GFP* and endothelial cells, and DRAQ5 (Biostatus) was used to visualize the nuclei. Sections were then mounted in Mowiol and confocal z-stacks were acquired at 1 μm increments between z slices, following a multitrack scan, using a Zeiss LSM 510 Meta confocal microscope. Images were analyzed with Imaris suite version 7.1 software (Bitplane). The GFP channel was isosurface rendered to provide better spatial perspective for visualizing GFP* cells.

Statistical Analysis
The EAE clinical disease scores for each group were graphed as the mean ± SEM for each day of the study. Differences in EAE disease scores between groups were analyzed using nonparametric Mann-Whitney unpaired U test, and individual time-point differences were analyzed using two-way ANOVA with Bonferroni’s posttest. Percentage data for CNS-infiltrated T cells were arcsine transformed prior to analysis. EXCEL and Prisim 6.0 (GraphPad) software was used for statistical analysis, and p < 0.05 was considered to be statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.04.020.

AUTHOR CONTRIBUTIONS

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P-glycoprotein regulates trafficking of CD8⁺ T cells to the brain parenchyma

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Abstract The trafficking of cytotoxic CD8⁺ T lymphocytes across the lining of the cerebral vasculature is key to the onset of the chronic neuro-inflammatory disorder multiple sclerosis. However, the mechanisms controlling their final transmigration across the brain endothelium remain unknown. Here, we describe that CD8⁺ T lymphocyte trafficking into the brain is dependent on the activity of the brain endothelial adenosine triphosphate-binding cassette transporter P-glycoprotein. Silencing P-glycoprotein activity selectively reduced the trafficking of CD8⁺ T cells across the brain endothelium in vitro as well as in vivo. In response to formation of the T cell–endothelial synapse, P-glycoprotein was found to regulate secretion of endothelial (C–C motif) ligand 2 (CCL2), a chemokine that mediates CD8⁺ T cell migration in vitro. Notably, CCL2 levels were significantly enhanced in microvessels isolated from human multiple sclerosis lesions in comparison with non-neurological controls. Endothelial cell-specific elimination of CCL2 in mice subjected to experimental autoimmune encephalomyelitis also significantly diminished the accumulation of CD8⁺ T cells compared to wild-type animals. Collectively, these results highlight a novel (patho)physiological role for P-glycoprotein in CD8⁺ T cell trafficking into the central nervous system during neuro-inflammation and illustrate CCL2 secretion as a potential link in this mechanism.

Keywords P-glycoprotein · Multiple sclerosis · Transendothelial migration · CD8⁺ T cells · Neuroimmunology · CCL2

Abbreviations
ATP Adenosine triphosphate
CCL2 Chemokine (C–C motif) ligand 2
EAE Experimental autoimmune encephalomyelitis
TNF-α Tumor necrosis factor-α

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Introduction

Leukocyte migration across the endothelium of the brain vasculature plays a key role in the onset of the chronic inflammatory disorder multiple sclerosis. Upon the formation of perivascular infiltrates, clinical symptoms arise leading to severe neurological deficits. Autoreactive T lymphocytes play a critical role in the disease process [12], both in multiple sclerosis as well as its animal model experimental autoimmune encephalomyelitis (EAE) [15]. CD4+ T cells are thought to be essential for the initiation and progression of the disease, whereas CD8+ T cells, which clonally expand within the inflamed CNS, are believed to play a key role in the progression of multiple sclerosis [42].

While the molecular interactions that underlie the migration of CD4+ T cells across the brain endothelium are well-defined, the mechanisms regulating CD8+ T cells entry into the central nervous system remain poorly described. In general, the sequential steps in the diapedesis of lymphocytes include tethering, rolling, capture, firm adhesion and finally migration across the endothelial layer [38]. The continuous cross-talk between immune cells and brain endothelium is an essential element in mediating immune cell migration into the central nervous system parenchyma [14]. Endothelial cells actively participate in this process by expressing adhesion molecules and producing chemokines [2, 10]. Upon the adhesion of lymphocytes, endothelial cells form ring-like membrane structures, so-called migratory cups, which stabilize the leukocytes onto the endothelial cell surface [3] and facilitate their migration. Although great progress has been made in the identification of the molecular interactions that mediate leukocyte transmigration [25, 31], it is becoming increasingly clear that in order to finalize this process, T lymphocytes require additional activation through chemokines presented on or present within the endothelium [35]. However, the precise mechanism that underlies this signaling of the endothelium to the adhered T cell remains to be elucidated.

We here provide evidence that the brain endothelial ATP-binding cassette transporter P-glycoprotein is critical for the release of brain endothelial chemokines, thereby providing a means to control the migration of CD8+ T cells in particular. ATP-binding cassette transporters are highly expressed at the cerebral endothelium, where they actively remove toxic compounds from the brain at the cost of ATP hydrolysis [26]. Although P-glycoprotein was originally described as a prototypic transporter that mediates multidrug resistance of tumor cells [17], most recent work suggests that P-glycoprotein may additionally mediate immune responses possibly through its capacity to mediate secretion of cytokines, chemokines and bioactive lipids [18–21, 39].

Here, we show that silencing of P-glycoprotein activity in brain endothelial cells selectively impairs the adhesion of CD8+ T cells to intercellular adhesion molecule-1 as well as their transendothelial migration through the secretion of endothelial (C–C motif) ligand 2 (CCL2), a critical agent for CD8+ T cell migration. Notably, characterization of human brain microvessels freshly isolated from post-mortem tissue of multiple sclerosis patients revealed high levels of CCL2 transcripts, reinforcing a role for endothelial-derived CCL2 in human neuro-inflammatory disease. Finally, we were able to show that endothelial targeted CCL2 conditional knockout mice that were subjected to EAE contained significantly reduced levels of infiltrated CD8+ T cells, highlighting the importance of secreted CCL2 in regulating CD8+ T lymphocytes entry into the brain under pathological conditions.

Materials and methods

Cell isolation and culture

The immortalized human brain endothelial cell line hCMEC/D3, which establishes the key features of brain endothelium, was cultured as described [43]. Human CD4+ and CD8+ T cells were purified from peripheral blood mononuclear cells using MACS® magnetic cell sorting kit (Miltenyi Biotec) according to manufacturer’s instructions. CD4+ and CD8+ T cell purity was >96 % as assessed by flow cytometry performed (FACSCalibur™) using CELL-Quest™ software (BD Biosciences). Isolated T cells were cultured for 48 h before experiments. Effector T cells were generated by adding IL-2 (10 ng/ml) and phytohemagglutinin (1 µg/ml) during 48 h.

Docking structure imaging

hCMEC/D3 cells were cultured in µSlide VI coated with collagen (Ibidi GmbH) to confluence. CD4+ or CD8+ T cells were labeled with a fluorescent membrane dye PKH26 (Sigma-Aldrich) according to manufacturer’s instructions and added to the endothelial slides for 30 min. Non-adherent cells were removed and slides were fixed with 4 % paraformaldehyde in phosphate buffered saline for 15 min and blocked with 5 % normal donkey serum in phosphate buffered saline with 0.3 % Triton X-100 for 15 min. Cells were incubated with anti ICAM-1 (Santa Cruz) and anti-P-glycoprotein (15D3, BD Biosciences) antibodies (Electronic Supplementary Material 1) and relevant secondary antibodies were used. After each step, the slides were washed three times with phosphate buffered saline. Coverslips were mounted with Vectashield (Vector Labs) or Mowiol 488 reagent (Calbiochem) on microscope slides. Imaging was performed using a confocal laser scanning microscope (Leica TCS SP2).
Silencing P-glycoprotein and qRT-PCR

Plasmids encoding specific P-glycoprotein shRNA or non-targeting control shRNA (NTC-shRNA) were purchased from Sigma. Messenger RNA was isolated from hCMEC/D3 cells using an mRNA capture kit (Roche). cDNA was synthesized with the Reverse Transcription System kit (Promega) and real-time PCR was performed as described previously [19]. All primer sequences are listed in Electronic Supplementary Material 2 and expression levels of transcripts obtained with qRT-PCR were normalized to GAPDH expression levels.

Flow adhesion and migration assays

NTC-shRNA or P-glycoprotein shRNA-transduced hCMEC/D3 cells were cultured to confluence in collagen-coated μSlide VI flow chambers (Ibidi GmbH). Cells were treated with 5 ng/ml TNF-α (Preptech) for 24 h. Cells were mounted onto the microscope stage using a POC-mini chamber system (LaCon) and connected to a perfusion pump. Using physiological flow conditions (flow speed was 500 μl/min, correlating to 0.8 dyn/cm²), 5 × 10⁵ T cells/ml were perfused over the endothelial cells, followed by 20 min of fluid-flow. Transendothelial migration was characterized by the change in appearance of T cells from bright to dim. Migrated cells were quantified by counting per field-of-view. From one experiment, five fields were analyzed. Movies were generated using VirtualDub.

ICAM-1 binding assay

ICAM-1 binding assays were performed as described previously [4] and fluorescence was quantified using a cytometer (Bio-Rad). Results are expressed as the mean percentage of adhesion of triplicate wells.

Electron microscopy

hCMEC/D3 cells were grown to confluence on fibronectin-coated Thermofax slides (Nunc) and fixed with 1 % paraformaldehyde overnight, then embedded in gelatine, infiltrated with sucrose and frozen in liquid nitrogen as described [36]. Ultra-thin sections (60–70 nm) were prepared using a cryotome (UC6; Leica) and labeled with an anti-P-glycoprotein antibody (C219, Alexis) in 1 % bovine serum albumin (Aurion) in phosphate buffered saline for 1 h. Visualization was performed with Protein A gold 10 nm (UMC). Sections were post-stained with uranyl acetate and uranyl oxalate. The sections were examined at 80 keV using a Technai 12 EM (FEI Co), equipped with a side-mounted Megaview II camera (SIS). The specificity of the electron microscopy was controlled by using an isotype control as well as leaving out the primary antibody, which in both cases did not show any gold grains.

Isolation of microvessels from control or multiple sclerosis brains

Brain tissue (eight non-neurological controls and eight multiple sclerosis patients) was obtained from The Netherlands Brain Bank from donors with written consent. Brain microvessels were isolated from non-neurological patient tissue, normal-appearing white matter (NAWM) and multiple sclerosis lesions from post-mortem multiple sclerosis patients and validated by qRT-PCR as described before [34].

EAE induction in mice

Female mice with CCL2-deficient endothelial cells were developed as described previously [13] and together with their littermate controls (C57BL/6 background) were used at the age of 8–10 weeks. All mice were kept under specific pathogen-free conditions and used in accordance with local animal experimentation guidelines. EAE was induced in C57BL/6 mice as described previously [22].

Histology and immunohistochemistry

Brains of the killed animals were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C. For immunohistochemistry, cryosections were fixed in ice-cold acetone for 10 min and blocked with normal mouse serum prior to antibody staining. Immunofluorescence staining was performed in phosphate buffered saline, supplemented with 0.1 % bovine serum albumin as previously described [18], and antibodies are described in Electronic Supplementary Material 1. Immunofluorescent sections were enclosed in Vinol (Air Products) supplemented with DAPI (Invitrogen) and analyzed on a Leica DM6000 fluorescence microscope (Leica Microsystems), equipped with LAS AF (Leica) software.

3-D rendering of extravasating CD8⁺ T cells during EAE

Tissue processing was performed as described recently [32]. In brief, at day 16 of EAE, spinal cords were harvested and cryoprotected in 30 % sucrose overnight prior to cryomatrix embedding. Serial cryosections of 60 μm thickness were obtained from the thoraco-lumbar spinal cord, and incubated with rabbit anti-mouse Laminin 1 (CLPR245679; Cedarlane) antibody to highlight the microvascular basement membrane and with CD8β-FITC antibody for CD8⁺ T cells. DRAQ5 (DR51000; Biostatus Ltd.) was utilized for nuclear staining. For visualization of
the CD8+ T cells in inflamed venules, confocal z-stacks were acquired as described [32] and imported into Bitplane IMARIS suite version 7.1x 64 software (Bitplane Inc.) and a volume-rendered image was obtained in maximum-intensity projection mode. Individual color channels in the volume-rendered image were subjected to iso-surface rendering to enhance visualization of spatial architecture around venules and relative localization of the CD8+ T cells.

Relative quantification of immunohistochemistry and 3-D rendering

Volume-rendered images were fed into ImageJ software (NIH) and a microvascular contour was created manually by cursoring out the inflamed venules of interest. Respectively areas representing the microvessel segment (# of pixels occupied by the microvessel) and associated CD8+ T cell population (# of green pixels) were determined and the percentage vessel area occupied by CD8+ T cell aggregates calculated as follows: (# of green pixels)/(# of pixels occupied by the microvessel) × 100. For immunohistochemistry, a similar approach (using ImageJ) was applied on multiple EAE lesions from 4 WT and 4 mdr1a/1b−/− animals by separating the green (CD4+ or CD8+ T cells) from the blue (CD45+ cells) channel. These images were also subjected to intensity-threshold adjustment and the percentage of CD45+CD4+ or CD45+CD8+ cells was determined.

Statistical analysis

All data were analyzed statistically by means of analysis of variance (ANOVA) and Student’s t test. Statistical significance was defined as *p < 0.05, **p < 0.01, ***p < 0.001.

Results

P-glycoprotein regulates CD8+ T cell migration across an in vitro model of the blood–brain barrier

To determine the potential role for P-glycoprotein in the transendothelial migration of CD4+ and CD8+ T cells under flow conditions, we generated human brain endothelial cells that lack P-glycoprotein activity using a lentiviral approach, resulting in over 80% reduction of P-glycoprotein expression (Electronic Supplementary Material 3a) and function (Electronic Supplementary Material 3b) compared to non-targeting control (NTC) shRNA-transfected cells. Silencing P-glycoprotein activity significantly reduced transendothelial migration (Fig. 1a) and adhesion (Fig. 1b) of both T cell subsets across inflamed (TNF-α-stimulated) brain endothelium (Fig. 1a–c). The percentage of CD8+ T cell that adhere to the endothelial cells and subsequently migrate was significantly impaired up to 50% under P-glycoprotein-silenced conditions (Fig. 1c), whereas the migration capacity of CD4+ T cells after firm adhesion was independent of P-glycoprotein function (Fig. 1c). Upon adhesion, CD8+ T cells appeared immobilized on the endothelial cells (Electronic Supplementary Material 4), whereas CD4+ T cells still displayed crawling behavior followed by transmigration (Electronic Supplementary Material 5). Importantly, silencing P-glycoprotein did not affect the mRNA and protein expression levels of intercellular adhesion molecule-1 (Electronic Supplementary Material 3c, d) and vascular cell adhesion molecule-1 (Electronic Supplementary Material 3d, e). Collectively, these results suggest that P-glycoprotein activity is particularly required to direct CD8+ T cell trafficking upon their firm adhesion to brain endothelial cells.

Fig. 1 P-glycoprotein mediates CD4+ and CD8+ T cell adhesion and migration under flow conditions Non-targeting control (NTC; black bars) or P-glycoprotein shRNA (grey bars) transfected cells were grown in IBIDI slides and were incubated after 2 days with TNF-α for 24 h. Next, freshly isolated primary human CD4+ and CD8+ T cells were added to visualize and quantify their migration (a) and adhesion (b) capacity. Moreover, the number of CD4+ and CD8+ T cells that started to migrate after their initial arrest on the endothelial cells was quantified (c). Experiments were performed in triplicate using three different human T cell donors and were presented as the percentage of migration/adhesion compared to NTC-transfected cells ± SEM. **p < 0.01, ***p < 0.001 as determined by Student’s t test.
As reported earlier, mdr1a/b−/− mice lack CD8+ T cell infiltrates during EAE.

To study if mediators effluxed by P-glycoprotein induce the migration of CD8+ T cells, we harvested supernatants from control or P-glycoprotein-silenced endothelial cells under inflammatory or control conditions, and determined their ability to affect CD4+ and CD8+ T cell adhesion to intercellular adhesion molecule-1, a key player in transendothelial migration. As shown in Fig. 5a, b, conditioned media from TNF-α-stimulated endothelial cells significantly induced the adhesion of both CD4+ and CD8+ T lymphocytes to ICAM-1 compared to conditioned media from untreated endothelial cells. Induced lymphocyte adhesion to intercellular adhesion molecule-1 was inhibited in the presence of pertussis toxin (data not shown), indicating an important role for G-protein-coupled receptors, such as chemokines receptors, herein. Conditioned medium from TNF-α-treated endothelial cells lacking P-glycoprotein activity failed to induce CD8+ T cell adhesion to intercellular adhesion molecule-1, whereas the induction of CD4+ T cell adhesion to intercellular adhesion molecule-1 was largely unaffected (Fig. 5a, b). These results indicate that P-glycoprotein is involved in the secretion of soluble factors from inflamed endothelium that selectively affect CD8+ T cell adhesion to intercellular adhesion molecule-1.

P-glycoprotein localizes in endothelial migratory cups upon T lymphocyte attachment

Binding of both effector CD4+ (Fig. 3a) and CD8+ (Fig. 3b) T lymphocytes to inflamed brain endothelial cells resulted in the recruitment of P-glycoprotein into the migratory cups, as indicated by the partly overlapping expression with an established marker intercellular adhesion molecule-1. However, silencing P-glycoprotein on the endothelium revealed no differences in docking structure formation upon T cell attachment (Electronic Supplementary Material 6), thereby excluding a potential role for P-glycoprotein in migratory cup formation.

Further analysis using electron microscopy revealed that P-glycoprotein localized at the plasma membrane of the endothelial cells at the contact sites of CD8+ T lymphocytes (Fig. 4b; quantification Fig. 4c) and CD4+ T lymphocytes (data not shown), whereas in control endothelium its plasma membrane localization is less predominant (Fig. 4a). Notably, P-glycoprotein expression is also observed in T cells (Fig. 4b), although P-glycoprotein activity is low in either CD4+ or CD8+ T lymphocytes compared to brain endothelial cells (Electronic Supplementary Material 7). Together, these results indicate that P-glycoprotein becomes enriched in endothelial migratory cups upon T cell attachment, where it may locally exert its regulatory role in CD8+ T cell transendothelial migration.

P-glycoprotein regulates CD8+ T cell migration via CCL2 secretion

To study if mediators effluxed by P-glycoprotein induce the migration of CD8+ T cells, we harvested supernatants from control or P-glycoprotein-silenced endothelial cells under inflammatory or control conditions, and determined their ability to affect CD4+ and CD8+ T cell adhesion to intercellular adhesion molecule-1, a key player in transendothelial migration. As shown in Fig. 5a, b, conditioned media from TNF-α-stimulated endothelial cells significantly induced the adhesion of both CD4+ and CD8+ T lymphocytes to ICAM-1 compared to conditioned media from untreated endothelial cells. Induced lymphocyte adhesion to intercellular adhesion molecule-1 was inhibited in the presence of pertussis toxin (data not shown), indicating an important role for G-protein-coupled receptors, such as chemokines receptors, herein. Conditioned medium from TNF-α-treated endothelial cells lacking P-glycoprotein activity failed to induce CD8+ T cell adhesion to intercellular adhesion molecule-1, whereas the induction of CD4+ T cell adhesion to intercellular adhesion molecule-1 was largely unaffected (Fig. 5a, b). These results indicate that P-glycoprotein is involved in the secretion of soluble factors from inflamed endothelium that selectively affect CD8+ T cell adhesion to intercellular adhesion molecule-1.
Fig. 2 Lack of CD8+ T cell infiltrates in EAE lesions of mdr1a/1b−/− mice. Brains were isolated from EAE mice and the cerebellum white matter was analyzed for the infiltration of CD4+ T cells (green; a, b) or CD8+ T cells (green; c, d). Slides were counterstained for the presence of CD45+ infiltrating cells (blue) and laminin positive (red) basement membranes around vessels in WT (a, c) or mdr1a/1b−/− (b, d) mice. Images represent representative tissues from four mice per group. Magnification ×200. e Quantification of immunohistochemistry using ImageJ software on various EAE lesions of 4 mice per group. Data are presented as the percentage of CD4+ and CD8+ T cells of the total number of CD45+ infiltrates per EAE lesion and indicate a lack of CD8+ T cells in mdr1a/1b−/− animals compared to WT during EAE. f Peripheral blood mononuclear cell analysis by fluorescence-activated cell sorting revealed no differences in the number of circulating CD45+ CD4+ or g CD45+ CD8+ T cells in wild type (WT; black bars) or mdr1a/1b−/− (grey bars) mice under control (CFA complete Freund’s adjuvant) or EAE conditions. Experiments were performed in triplicate using four mice per group in two independent experiments and were presented as the mean ± SEM. **p < 0.01, ***p < 0.001 as determined by Student’s t test.
media was significantly inhibited upon brain endothelial P-glycoprotein silencing (Fig. 5e). However, the addition of CCL2 to the conditioned media restored the migratory capacity of CD8\(^+\) T cells, whereas CD4\(^+\) T cell migration was unresponsive to CCL2 (Fig. 5e). These data collectively show that expression of P-glycoprotein by TNF-\(\alpha\)–activated brain endothelial cells is required for release of chemotactic factors that stimulate migration of both CD4\(^+\) and CD8\(^+\) T cells. Moreover, the release of CCL2 appears responsible for the stimulated migration specifically of CD8\(^+\) T cells.

Increased expression levels of CCL2 in brain microvessels from multiple sclerosis patients

To determine endothelial CCL2 expression ex vivo, we isolated post-mortem brain microvessels from active demyelinating multiple sclerosis lesions, NAWM and non-neurological control brain tissue. Real-time PCR analysis revealed high endothelial purity as the expression levels of GAFP (astrocytes) and PDGFR\(\beta\) (pericytes) were just above detection limit, whereas (brain) endothelial markers like ZO-1, claudin-5 and P-glycoprotein were highly expressed (Fig. 5f). CCL2 mRNA gene transcripts were significantly higher in isolated microvessels both in NAWM and active multiple sclerosis lesions compared to transcripts in brain microvessels isolated from non-neurological controls (Fig. 5g), whereas the expression levels of P-gp remained unaffected (Electronic Supplementary Material 9). These results indicate that in NAWM, inflammatory alterations in the brain endothelium are already present.

Endothelial CCL2 knockout mice display reduced CD8\(^+\) T cell infiltrates during EAE

To obtain proof of principle that during neuro-inflammation brain endothelial CCL2 drives CD8\(^+\) T cell trafficking into the CNS, we induced EAE in endothelial CCL2\(^{-/-}\) mice [13] as well as their littermate controls. We evaluated CD8\(^+\) T cell influx using 3D rendering of spinal cord venules on day 16 of EAE (peak of the disease). As shown in Fig. 6a, b, high numbers of CD8\(^+\) T cells are associated with the vasculature of EAE animals. Vessel-associated CD8\(^+\) T cells in WT mice demonstrate extensive clumping with little-to-no obvious space between cell aggregates. By contrast,
infiltrating CD8⁺ T cells in endothelial CCL2−/− mice form smaller aggregates that are clearly resolvable from each other. It thus appears that the density of vessel-associated CD8⁺ T cells is reduced in endothelial CCL2−/− mice with EAE, compared to WT mice at the same time-point during disease. As shown in Fig. 6c, we observed a significant (p < 0.016) reduction in vessel-associated CD8⁺ T cell density in endothelial CCL2−/− mice compared to WT mice. These results reinforce the notion that endothelial CCL2 is a crucial regulator of CD8⁺ T cell trafficking into the central nervous system under pathological conditions.

**Discussion**

In the pathogenesis of multiple sclerosis, CD8⁺ T cells are present within brains of patients and in animals with EAE, where they may clonally expand and subsequently induce tissue damage and, in particular, axonal damage. However, the mechanism by which CD8⁺ T cells gain access to the brain remains largely unknown. Here, we describe a novel regulatory role for multi-drug resistance transporter P-glycoprotein as well as the CCL2/CCR2 axis in this process. Our studies suggest that P-glycoprotein regulates the release of CCL2 by inflamed brain endothelium. In that way, the endothelial cells instruct adhered leukocytes, in particular CD8⁺ T lymphocytes, to migrate.

The prototypic ATP-binding cassette transporters at the brain vasculature are regarded as protectors of the central nervous system by virtue of their active efflux capacity for neurotoxic compounds. Our data extends the role for these efflux pumps, providing first-hand evidence that, under inflammatory conditions, a key member of this family (P-glycoprotein) also regulates leukocyte trafficking into
the brain by releasing CCL2. Under neuro-inflammatory conditions, P-glycoprotein translocates from the cytosol to the plasma membrane and intra-endothelial vesicles at the contact site of the adhered T cell. TNF-α treatment of brain endothelial cells resulted in a similar P-glycoprotein distribution pattern (unpublished data), indicating that the observed effects may be regulated by downstream TNF-α receptor signaling events as also described for brain capillaries [29]. Electron microscopy analysis further confirmed our observations of a membrane and vesicle-like
expression pattern of P-glycoprotein upon T cell attachment. Recent findings show that, besides T cells being activated by chemokines presented at the endothelial surface of peripheral vascular beds, effector T lymphocytes also cross inflamed endothelial barriers by sensing intra-endothelial chemokines stored within vesicles localized at the contact site [35]. Based on our data, it may be postulated that brain endothelial P-glycoprotein plays a role in this process. Subsequent focal release or presentation of endothelial CCL2 may then be mediated by P-glycoprotein, providing a local cue for the adhered lymphocyte to initiate migration while maintaining low levels of CCL2 in the circulation. That adherent CD8$^+$ T cells appeared most inhibited from migrating is consistent with P-glycoprotein/CCL2 regulating a post-adhesion event. A controversial issue remains whether ATP-binding cassette transporters themselves are capable of secreting chemokines directly, or are involved in the secretion of other more lipophilic inflammatory substrates, e.g., like bioactive lipids, which in turn may affect CCL2 secretion as a secondary effect [20]. Further research is warranted to define the nature of the P-glycoprotein-mediated secretome and its potential autocrine effect on endothelial cells (chemokine release) or its paracrine effect on the adhering leukocyte (e.g., leukocyte activation).
CCL2 is a pro-inflammatory chemokine induced during a variety of neuro-inflammatory and neurodegenerative diseases [37]. The cognate receptor for CCL2 is chemokine (C–C motif) receptor 2 (CCR2), and the CCL2/CCR2 axis attracts immune cells like monocytes [28], and activated and memory T cells [6] to the site of inflammation. Several reports have indicated the relevance for CCR2 during neuro-inflammation, as mice that lack CCR2 are resistant to the induction of EAE [11, 16]. Moreover, CCR2-deficient leukocytes show impairments in their adhesion and migration capacity across microvascular endothelium in vitro [9, 23, 44]. Our data indicate P-glycoprotein-mediated release of CCL2 preferentially affects CD8+ T cell migration across TNF-α-activated brain microvascular endothelial cells in culture. Although further research is warranted to confirm these findings in primary human endothelial cells, our data suggest that P-glycoprotein-mediated release of CCL2 may also be responsible for controlling trafficking of this leukocyte subset into the central nervous system parenchyma under neuro-inflammatory conditions. This may be because CCR2 is differentially expressed among effector T cell subsets. Indeed, CCR2 appears to be specifically induced on activated CD8+ T cells, whereas CD4+ T cells express a wider range of chemokine receptors [5]. In addition, CCL2/CCR2 interaction can also rescue CD8+ T cells from antigen/growth factor deprivation-apoptosis [7], enabling them to migrate to sites where antigen is available. Moreover, the generation of effector and central memory CD8+ T cells is abrogated in the absence of CCL2, as is the migration of central memory CD8+ T cells to inflammatory sites [41], indicating CCL2 may influence CD8+ T cells at various levels. In multiple sclerosis, CD8+ T cells may clonally expand and may exert their lytic functions [1], such as axonal damage [30].

Brain microvessels isolated from multiple sclerosis patients were shown to contain high levels of CCL2. Our results with endothelial CCL2−/− mice underscore the significance of these clinical findings, highlighting that vascular-derived CCL2 is a critical determinant in the multi-step process of leukocyte extravasation into the central nervous system. The finding of reduced density of vessel-associated CD8+ T cells in endothelial CCL2−/− mice with EAE could imply that the actual extent of these extravasating cells is diminished in the absence of an endothelial pool of CCL2. This interpretation is consistent with the proposed role of CCL2 as an ‘arrest’ chemokine, functioning at the luminal endothelial surface to increase the affinity and avidity of leukocyte integrins and, thus, to promote to stronger leukocyte–endothelial interaction, thereby selectively inducing transendothelial migration attachment [24]. In this regard, it was reported that addition of anti-CCL2 antibody just prior to intravital microscopy, blocked leukocyte adhesion, but not rolling, along brain pial microvessels during EAE [8]. Endothelial CCL2 released at the abluminal surface can alternatively regulate leukocyte transendothelial migration as an ‘inflammatory’ chemokine to stimulate leukocyte chemotaxis [33], and/or serve to concentrate and orient leukocytes within the remodeled subendothelial and/or perivascular space [40], while they await additional cues to advance further into the brain parenchyma. And a more recently proposed role for endothelial CCL2 is as a facilitator of diapedesis to modulate migration at a ‘post-adhesion’ step, when contained in endothelial vesicles inaccessible to the circulation [35].

In conclusion, herein we show that the ATP-binding cassette transporter P-glycoprotein plays a significant role in the selective recruitment of CD8+ T cells into the central nervous system during neuro-inflammation, and that this process may be mediated by CCL2 derived from brain microvascular endothelial cells. Since CD8+ T cells are capable of clonally expanding within multiple sclerosis lesions and subsequently inducing axonal damage and consequent neurodegeneration, our results may provide novel tools to specifically hamper CD8+ T cell trafficking into the brain, thereby preventing severe tissue damage.

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Conflict of interest The authors declare that they have no conflict of interest.

References


The CCL2 synthesis inhibitor *bindarit* targets cells of the neurovascular unit, and suppresses experimental autoimmune encephalomyelitis

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**Abstract**

**Background:** Production of the chemokine CCL2 by cells of the neurovascular unit (NVU) drives critical aspects of neuroinflammation. Suppression of CCL2 therefore holds promise in treating neuroinflammatory disease. Accordingly, we sought to determine if the compound *bindarit*, which inhibits CCL2 synthesis, could repress the three NVU sources of CCL2 most commonly reported in neuroinflammation – astrocytes, microglia and brain microvascular endothelial cells (BMEC) – as well as modify the clinical course of neuroinflammatory disease.

**Methods:** The effect of *bindarit* on CCL2 expression by cultured murine astrocytes, microglia and BMEC was examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). *Bindarit* action on mouse brain and spinal cord *in vivo* was similarly investigated by qRT-PCR following LPS injection in mice. And to further gauge the potential remedial effects of *bindarit* on neuroinflammatory disease, its impact on the clinical course of experimental autoimmune encephalomyelitis (EAE) in mice was also explored.

**Results:** *Bindarit* repressed CCL2 expression by all three cultured cells, and antagonized upregulated expression of CCL2 in both brain and spinal cord *in vivo* following LPS administration. *Bindarit* also significantly modified the course and severity of clinical EAE, diminished the incidence and onset of disease, and evidenced signs of disease reversal.

**Conclusion:** *Bindarit* was effective in suppressing CCL2 expression by cultured NVU cells as well as brain and spinal cord tissue *in vivo*. It further modulated the course of clinical EAE in both preventative and therapeutic ways. Collectively, these results suggest that *bindarit* might prove an effective treatment for neuroinflammatory disease.

**Keywords:** CCL2, Neuroinflammation, Blood–brain barrier, Neurovascular unit, Brain microvascular endothelial cells, Astrocytes, Microglia

**Background**

The chemokine CCL2 (formerly called MCP-1) is a critical mediator of neuroinflammation in a myriad of diseases states, including multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) [1], HIV-1 encephalitis [2], Guillain-Barré Syndrome [3], Alzheimer’s disease [4], ischemia [5], neurotrauma [6], epilepsy [7], neurogenic hypertension [8] and alcoholism [9]. While its precise mechanisms of action remain to be elaborated, among CCL2’s widely recognized effects are disruption of the blood–brain barrier (BBB) [10-12] and stimulated migration of mononuclear leukocytes into the central nervous system (CNS) [13-17].

These actions and pathogenic role, along with the fact that constitutive expression of CCL2 in the healthy central nervous system is severely limited [18], render CCL2 an ideal target for therapeutic intervention in neuroinflammatory disease [17,19,20]. Indeed, there is already strong suggestion that pharmacological suppression of CCL2 expression [21,22], oligomerization [23,24] or binding to its cognate receptor, CCR2 [25,26], can...
mitigate aspects of EAE. Pharmacologic blockade of CCL2 binding to glycosaminoglycans (GAGs) has also been reported to antagonize an autoimmune inflammatory condition of the neural retina, experimental autoimmune uveitis [27].

While highly effective in moderating neuroinflammation experimentally, many pharmacological agents that abrogate CCL2 expression and/or activity have nevertheless failed clinically. This disappointing performance in clinical trials might stem, in part, from overly broad suppression of microglia and astrocytes, a potential caveat that could curtail beneficial action of these cells in resolving neuroinflammation [28,29], as well as redundancy of chemokine binding sites and targets [30,31]. An alternative approach that more selectively targets CCL2 synthesis might therefore hold therapeutic promise in the treatment of human neuroinflammatory disease.

An attractive candidate in this regard is the well-characterized compound 2-((1-benzyl-indazol-3-yl) methoxy)-2-methyl propionic acid (bindarit) [32]. A small, synthetic indazole derivative that preferentially inhibits transcription of the monocyte chemoattractant subfamily of CC chemokines (MCP-1/CCL2, MCP-2/CCL8 and MCP-3/CCL7) [33], bindarit has shown clinical efficacy in a broad array of experimental inflammatory, autoimmune and vascular disorders involving peripheral organ beds [34-38], as well as success in recent clinical trials for diabetic nephropathy [39] and lupus nephritis [40]. Such efficacy has been associated with bindarit’s ability to interfere with monocyte recruitment, which is also a critical feature in neuroinflammatory disease [13-17].

Given this clinical history of bindarit suppressing various examples of peripheral inflammation, we investigated its effect on expression of CCL2 in culture by the three cell types that represent the most frequently reported CNS sources of this chemokine during neuroinflammation: astrocytes, microglia and brain microvascular endothelial cells (BMEC). These cells serve as integral components of the neurovascular unit (NVU) [41] and, via their expression of CCL2, can impact the BBB and course of neuroinflammatory disease [42,43]. As a complement to these culture studies, the ability of bindarit to suppress LPS induction of CNS CCL2 expression in vivo was well determined. And to gauge bindarit’s potential clinical efficacy, its effect on EAE, a prototypical neuroinflammatory disease [44,45], was also examined. Results indicate bindarit significantly suppressed CCL2 gene expression in culture, as well as blunted lipopolysaccharide (LPS)-induced expression of CCL2 in the CNS. It also inhibited various facets of clinical EAE, and showed signs of promoting disease recovery. Collectively, these data suggest that bindarit might offer promise, either alone or in conjunction with other therapies, in the treatment of human neuroinflammatory disease.

Methods

Reagents

All reagents and antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specified otherwise. Bindarit was synthesized by and obtained from Angelini (Angelini Research Center-ACRAF, Italy). MOG peptide [35-55] was synthesized by the WM Keck Biotechnology Resource Center at Yale University, New Haven, CT, USA.

Preparation of bindarit

For experiments with cultured cells, a stock solution of 100 mM bindarit was prepared in dimethyl sulfoxide (DMSO), and dilutions (50, 100, 300 and 500 μM) of the DMSO stock were made in culture medium. For in vivo experiments, bindarit was prepared as a suspension in 0.5% methylcellulose (MTC) at a concentration of 20 mg/ml as previously described [37].

Mice

C57BL/6 mice were obtained from the Charles River Laboratories, Inc. (Wilmington, MA, USA). All animal studies were performed, and CO2-mediated euthanasia carried out, according to the Animal Care and Guidelines of the University of Connecticut Health Center (Animal Welfare Assurance #A3471-01).

Isolation and culture of mouse astrocytes and microglia

Brain tissue obtained from mice at postnatal days 2 to 3 was used as the source of astrocytes and microglia. After decapitation, brains were removed immediately and separate astrocyte and microglial cultures prepared following a modified version of the protocol described by Ge and Pachter [46]. Cerebral cortices were first cut into small pieces (approximately 1 mm), and the minced tissue incubated in dissecting medium (Hank’s Balanced Salt Solution, from Gibco/BRL, Rockville, MD, USA), containing 0.5% glucose, 0.7% sucrose, 20 mM: hydroxyethyl piperazineethanesulfonic acid (Hepes) (pH 7.4) with 0.03% trypsin at 37 °C for 20 to 30 minutes. The tissue extract was then centrifuged at 1000 × g for 5 minutes and the resulting pellet washed and resuspended in astrocyte culture medium (Earl’s Modified Eagle Medium, from Gibco/BRL) containing 10% fetal bovine serum, 10% horse serum, 2 mM glutamine, 20 mM D-glucose, 4 mM sodium bicarbonate, 100 μg/ml penicillin and 100 μg/ml streptomycin. The tissue was mildly triturated to produce a single cell suspension, and the dissociated cells plated onto tissue culture flasks (T-75 cm²) coated with polylysine (BD Biosciences, Bedford, MA, USA). Cultures were maintained up to 1 week in plating medium in a humidified atmosphere (5% CO2) at 37 °C. After this time, cultures were shaken at 200 rpm for 2 hr at 4 °C, and supernatants containing dislodged microglia collected.
Supernatant material was then centrifuged at 1000 × g for 5 minutes to pellet microglia. Microglia were then resuspended in microglia culture medium (Dulbecco's modified Eagle Medium, from Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin) and cultured in a 24-well plate. Following removal of microglia from the initial mixed glial cultures, the latter were shaken for an additional 18 hr at 37 °C to remove residual neurons. The enriched astrocyte population that remained was further depopulated of remaining microglia by treatment with L-leucine methyl ester (LME) [47]. LME was dissolved in astrocyte culture medium, and the solution adjusted to pH 7.4 and filtered prior to adding to cultures to achieve a final concentration of 50 mM. After 90 minutes of LME treatment, astrocyte-enriched cultures were washed thoroughly and re-incubated with fresh astrocyte culture medium. Cell purity was determined by immunocytochemistry using a monoclonal anti-human glial fibrillary acid protein (GFAP) antibody, and cultures assessed to be ≥ 98% astrocytes (GFAP+).

Isolation and culture of mouse brain microvascular endothelial cells

BMEC were isolated as previously detailed by this laboratory [10,48]. Primary cultures were typically grown for approximately five days prior to sub-culturing for experiments. At that time, purity was gauged to be ≥ 98% BMEC, according to dil-acetylated LDL uptake [48]. BMEC also exhibited common endothelial characteristics, e.g. CD31 and vWF immunostaining, plus displayed expression of the tight junction-associated proteins ZO-1 and occluding, found enriched at the BBB.

Treatment of cultured cells

To examine effects of bindarit on basal CCL2 expression, cultured microglia and BMEC were incubated with different concentrations of bindarit for 4 hr or exposed to 300 μM bindarit for different time. To gauge effects of bindarit on LPS-stimulated CCL2 expression, astrocytes and microglia were pretreated with 300 μM bindarit for 1 hr; then cells were incubated with ± 100 ng/ml LPS (Escherichia coli Serotype 026:B6) for 4 or 20 hr in the continued presence of bindarit. After treatments, cells were extracted for RNA purification.

Separation of brain microvessels and parenchyma

Distinct brain microvessel and parenchymal fractions were obtained using a modification of the method to prepare BMEC [10,48]. In brief, after removal of the brain from the cranium, the meninges and big vessels were discarded, and the whole brain diced into approximately 1 mm pieces. Brain tissue was then homogenized with a 7 mL Dounce tissue grinder (Kimble/Kontes, Vineland, NJ, USA) in PBS. The homogenate was then transferred to a 15 ml conical tube and centrifuged at 400 × g for 15 minutes in an Eppendorf Model 5804R centrifuge/A-4-44 rotor. The resulting pellet was resuspended in 18% dextran for approximately five days prior to sub-culturing for experiments. At that time, purity was gauged to be ≥ 98% BMEC, according to dil-acetylated LDL uptake [48]. BMEC also exhibited common endothelial characteristics, e.g. CD31 and vWF immunostaining, plus displayed expression of the tight junction-associated proteins ZO-1 and occluding, found enriched at the BBB.

Treatment of animals

To determine effects of bindarit on LPS-stimulated CCL2 expression in brain and spinal cord, C57BL/6 female mice were given intraperitoneal (i.p.) injection of bindarit (200 mg/kg) or methylcellulose vehicle, once a day, for 4 consecutive days. At 30 minutes following the last bindarit injection, mice were given i.p. injection of LPS (5 mg/kg; Escherichia coli Serotype 026:B6). Then, 4 hr after LPS injection, mice were euthanized and brain and spinal cord dissected for CCL2 mRNA and protein analysis.

For active induction of EAE, C57BL/6 female mice were immunized with MOG35-55 peptide (MEVGWYRSPFSRVMHYLGNGK) of rat origin, by a modification of the method previously described [49]. Briefly, on day 0 female mice 7 to 9 weeks of age were injected subcutaneously with 150 μg of MOG peptide and 300 μg of Mycobacterium tuberculosis (DIFCO, Detroit, MI, USA) in complete Freund's adjuvant (CFA) (DIFCO) into the right and left flank, 100 μl per site. Mice were also injected i.p. with 200 ng pertussis toxin (List Laboratories, Campbell CA, USA) in PBS on days 0 and 2 following the first immunization. Animals were monitored and scored daily for clinical disease severity according to the following scale: 0 = normal; 1 = tail limpness; 2 = limp tail and weakness of hind legs; 3 = limp tail and complete paralysis of hind legs; 4 = limp tail, complete hind leg and partial front leg paralysis; and 5 = death. Several disease parameters were calculated as described [49]. The mean day of onset was calculated by averaging the time when clinical symptoms, that is, a clinical score ≥ 1, were first observed for two consecutive days in each mouse. The mean maximum clinical score was calculated by averaging the highest score for each mouse. The disease index was calculated by
adding the daily average clinical scores in each group, dividing by the mean day of onset, and multiplying by 100. In the case that an animal showed no disease, the day of onset was arbitrarily counted as one day after the last day of the experiment (for example, day 22). And the disease incidence was the fraction of mice experiencing EAE.

To investigate the effects of bindarit on both the clinical course of EAE and CCL2 level during disease, mice were given daily i.p. injection of bindarit (or vehicle MTC) at 200 mg/kg for three consecutive days, beginning the day before MOG immunization (day -1), then injections every other day till day 20. This schedule was designed to mitigate, as much as possible, trauma associated with daily injections at times of peak neurologic disease and physical compromise.

RNA purification from cell cultures
Total RNA was extracted from cell cultures using the RNeasy kit according to the manufacturer's instructions. RNA was treated with Turbo DNase (Ambion, Austin, TX, USA) according to the protocol provided by the manufacturer. RNA yield and purity were determined by spectrophotometry absorption at 260 and 280 nm.

RNA purification from CNS tissue
RNA and protein were differentially extracted from the same mouse brain and spinal cord samples using the AllPrep RNA/Protein kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. RNA was treated with Turbo DNase (Ambion, Austin, TX, USA) according to the protocol provided by the manufacturer. RNA yield and purity were determined by spectrophotometry absorption at 260 and 280 nm. Protein level was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin as a standard.

Reverse transcription
cDNA was synthesized from the total RNA using a SuperScript III (Invitrogen, Carlsbad, CA, USA) First-strand synthesis system for RT-PCR with a standard protocol. The resulting cDNA was stored at -80 °C until used for further analysis.

CCL2 RNA determination by quantitative RT-PCR
Measurements of cDNA levels were performed by quantitative (q) RT-PCR using an ABI PRISM 7500 Sequence Detection System Version 1.3, and SYBR green (AB Applied Biosystems, Foster city, CA, USA) fluorescence was used to quantify relative amplicon amount. Separate controls included a no template-control and no reverse transcriptase-control, and standard curves were constructed for all primers used. Cycle time (Ct) values for all samples were normalized to RPL-19, the housekeeping gene encoding the 60 S ribosomal protein L19. Specifically, relative amplicon quantification was performed using the formula: (1 + Etarger/Ct(target))/((1 + Etarger)/Ct(ref)) × 100%, with ref: RPL19; target: CCL2; E: primer pair efficiency; and Ct: threshold cycle.

For all cell culture studies and in vivo LPS studies, relative CCL2 gene expression values (after normalization to RPL19) were expressed as percentage of control. For EAE studies, relative CCL2 gene expression values were designated as percentage of RPL-19 expression, as control CCL2 level (time-point 0) was undetectable. The primer sequences used in this study were as following: for mouse CCL2, 5′-GGC TCA GCC AGA TGC AGT TAA-3′ (forward) and 5′-CCA GCC TAC TCA TTG GGA TCA -3′ (reverse); for RPL-19, 5′-CGC TGC GGG AAA AAG AAG-3′ (forward) and 5′-CTG ATC TGC TGA CGG GAG TTG -3′ (reverse).

CCL2 protein determination
The level of CCL2 was measured with mouse JE/CCL2 commercial enzyme-linked immunoassay kit (BioSource International Inc., Camarillo, CA) according to the manufacturer's instructions.

Statistical analysis
Statistical significance of differences between mean values of bindarit-treated cultures and control cultures was analyzed using a paired two-tailed t-test, while comparisons of bindarit treatment on LPS-treated mice were performed using a two-tailed t-test for independent samples. For analysis of bindarit effects on clinical EAE, a chi-squared (χ²) test was used for comparisons of disease incidence; a Mann–Whitney U-test was used for comparisons of disease index; and a two-tailed t-test for independent samples was used for comparison of disease onset. A P-value < 0.05 was considered significant in all cases.

Results
Bindarit differentially suppresses CCL2 expression by cultured CNS cells
The effects of bindarit on cultured glial and BMEC were investigated first (Figures 1, 2, 3). Figure 1 shows that cultured microglia demonstrated both a dose and time dependency of bindarit effect on CCL2 mRNA level. Suppression of basal CCL2 mRNA was seen beginning with the lowest dose of 50 μM for 4 hr, amounting to nearly 75% reduction. Increasing the dose to 300 and 500 μM resulted in still further diminution of CCL2 mRNA to approximately 10% and 5%, respectively, of control level. Treatment with bindarit at 300 μM for as little as 2 hr resulted in near 60% reduction in CCL2 mRNA level, and treatment for longer times at this
concentration resulted in suppression of CCL2 mRNA to ≥90% of control level.

BMEC demonstrated a similar qualitative response in basal CCL2 mRNA to increasing bindarit concentration, but suppression was not as severe as seen with microglia (Figure 2). Significant reduction was not observed until 100 μM, and the maximal suppression achieved was about 20% of control. The time course of bindarit action on BMEC also differed. Maximal suppression by 300 μM bindarit was achieved at the earliest time-point of 2 hr, reaching a level of approximately 20% of that of the control. Longer time-points, however, appeared to result in a lesser effect. It is important to reemphasize that, in the normal CNS, CCL2 expression is barely detectable. This would suggest that both cultured microglia and BMEC, possibly removed from a normally suppressive microenvironment, are in a somewhat activated state.

This situation appears different for astrocytes. In this case, bindarit’s effects on constitutive CCL2 gene expression could not be accurately assessed, as level of this chemokine’s mRNA in murine culture of these cells is very low. Astrocyte cultures were thus stimulated with LPS for different lengths of time to greatly induce CCL2 mRNA, and the effect of bindarit on this CCL2 induction was assayed (Figure 3A). Stimulation with 100 ng/ml LPS for both 4 hr and 20 hr
produced similar elevations in CCL2 gene expression, and bindarit treatment at 300 μM similarly suppressed, by 40 to 60%, the induction of CCL2 mRNA at both time-points.

In light of bindarit’s success at mitigating induction of CCL2 in astrocytes, we next assayed whether it was similarly effective in preventing induction in LPS-stimulated microglia. Figure 3B shows that this was in fact the case, bindarit completely suppressing the induction and reducing CCL2 mRNA level to 15% of the control (basal) value.

Bindarit blocks LPS-induced CCL2 expression in brain and spinal cord

It was next investigated whether bindarit could suppress LPS-induced CCL2 expression in the CNS in vivo (Figure 4). In the normal, resting state, CNS CCL2 mRNA level is barely detectable in C57BL/6 mice [50], but is elevated 50- to 100-fold shortly after peripheral LPS injection [51]. Pretreatment with bindarit was nevertheless able to effectively block this induction both in the brain and spinal cord, by 92% and 86%, respectively. In addition to abrogating LPS-induction of CCL2 mRNA in the CNS, bindarit was also effective at reducing CCL2 protein level in both brain and spinal cord, though not to the same extent as mRNA.

![Figure 3 Bindarit effects on LPS-stimulated CCL2 mRNA in cultured astrocytes and microglia.](image-url)

![Figure 4 Bindarit's effects on CCL2 mRNA and protein in brain and spinal cord following LPS.](image-url)
Bindarit therapeutically modifies clinical EAE

The ability of bindarit to modify clinical EAE was examined next. Figure 5 shows that, with a bindarit schedule of daily injections for the first three days, and beginning at day \(-1\), then injections every other day till day 20, bindarit yielded several therapeutic effects. By the criterion that disease is manifest when a clinical score of at least 1 is demonstrated for at least two consecutive days [49], bindarit delayed onset of EAE. Specifically, control mice developed acute disease beginning at day 8, while bindarit-treated mice did not show evidence of clinical disease until days 14 to 15. A second therapeutic effect observed was reduced disease progression and severity. Control mice showed rapid progression of EAE, proceeding towards a maximum mean clinical score of approximately 2.1 to 2.2 by day 9. Bindarit-treated mice evidenced slower progression, and only reached a maximum mean clinical score of 1.5. A third therapeutic effect was apparent reversal of disease course. After experiencing rapid onset, control mice showed a plateau in disease score typical of this monophasic MOG-induced EAE [52,53]. However, in marked distinction, bindarit-treated mice demonstrated a consistent downward trend in disease score following their delayed and attenuated peak in clinical presentation. A summary of the effects of bindarit treatment on clinical EAE is presented in Table 1.

We next sought to examine how bindarit modifies CCL2 expression in the brain during EAE. First, the temporal expression of CCL2 was determined only in MOG-immunized mice not receiving any bindarit, to gauge the window of opportunity during which bindarit might act. As seen in Figure 6, CCL2 RNA is barely detectable at the time of immunization. Its expression then accelerates beginning around day 9, is significantly

![Figure 5](image-url)

**Figure 5** Bindarit effects on clinical experimental autoimmune encephalomyelitis (EAE). Mice were subject to EAE by active immunization with MOG peptide, beginning on day 0, as detailed in Methods. Bindarit (or vehicle) was injected i.p. at 200 mg/kg according to the schedule indicated, beginning at day \(-1\) (one day before MOG immunization). Mean clinical score (A) and % incidence of EAE (B) were determined. EAE was diagnosed when animals demonstrated a clinical score ≥ 1 for two consecutive days.
elevated by day 14, rapidly declines at day 17, and reaches near basal level by day 21. Because bindarit has been shown to most effectively suppress stimulated, rather than basal, CCL2 expression [33,54], bindarit effects on CCL2 were analyzed selectively during this peak interval. Additionally, brain tissue was resolved into microvascular and parenchymal fractions to further identify targeted cell types. This resolution was deemed important, as both microvessels (BMEC) and parenchymal neural cells (astrocytes and microglia) have been reported as sources of CCL2 during EAE [55-58], though microvessels only contribute < 1% to brain volume [59]. It was thus reasoned that parenchymal effects could overshadow possible bindarit-induced changes in microvascular CCL2 expression if only whole-brain levels were evaluated. Figure 7 reveals that bindarit significantly reduced peak CCL2 expression during EAE in both microvascular and parenchymal fractions, in agreement with what was found in our culture studies. Also, bindarit did not affect CCL2 expression outside the peak window, reinforcing that its action appears restricted to activated cells within and outside the CNS [33,54].

Discussion

Given the success of the CCL2 synthesis inhibitor bindarit in ameliorating several animal disease models and human clinical conditions associated with peripheral inflammatory disease, initial studies were conducted to examine its effects on elements critical to neuroinflammatory disease. Focusing on the three main cell types responsible for CCL2 expression during neuroinflammation, experiments revealed bindarit significantly suppressed CCL2 in cultured BMEC, microglia and astrocytes. Bindarit was further shown to be effective in vivo in two neuroinflammatory paradigms: 1) it blocked LPS induction of CCL2 in both brain and spinal cord; and 2) it therapeutically modified the course of EAE while suppressing CCL2 expression in both brain microvascular and parenchymal compartments.

As to the effects on the seminal sources of CCL2, it was critical to determine whether each was susceptible to bindarit, as the specific cellular pool(s) responsible for CCL2’s pathogenic actions during neuroinflammatory disease remain unclear [51]. While all three cell types responded with significant reduction in CCL2 mRNA, microglia were the most sensitive - experiencing > 90% diminution in this chemokine’s expression. This high sensitivity to bindarit holds particular significance, as microglia are widely considered the primary immune effector cells in the CNS [60-63], and their expression of CCL2 has been linked to monocyte recruitment into the CNS [64,65]. As CCL2 can also direct recruitment and proliferation of microglia [66-68], as well as activation of these cells [68], microglial expression of CCL2 can potentially support a self-sustaining cycle of neuroinflammation. Bindarit action, however, might effectively abrogate such a scenario.

That bindarit also suppressed CCL2 mRNA in BMEC is noteworthy. As these cells form the first line of defense in the BBB [69], their expression of CCL2 might strongly influence incipient steps of neuroinflammation [70]. Indeed, elevated CCL2 expression by BMEC has been reported in MS [71] and EAE [55,56], as well as in autoimmune inflammation of the peripheral nervous system [3]. Furthermore, intravenously administered anti-CCL2 antibody blocked heightened leukocyte adhesion to pial venular endothelium in vivo in mice suffering acute

Table 1 Summary of bindarit effects on clinical experimental autoimmune encephalomyelitis (EAE) parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Sick/total, number</th>
<th>Mean day of onset*</th>
<th>Mean max clinical score</th>
<th>Mean disease indexb</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTC</td>
<td>14/14</td>
<td>11.42 ± 1.76</td>
<td>2.43 ±0.47</td>
<td>163.7 ± 20.53</td>
</tr>
<tr>
<td>BND</td>
<td>8/14</td>
<td>16.85 ± 2.17</td>
<td>1.52 ± 0.88</td>
<td>993 ± 10.21</td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Mean presented ± SD.
*aDay of onset established when clinical score ≥ for two consecutive days.
*bDisease index calculated at day 21.

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Figure 6 CCL2 expression profile during experimental autoimmune encephalomyelitis (EAE). Mice were immunized with MOG peptide to induce EAE. At the indicated days post-immunization, mice were sacrificed and CCL2 mRNA levels determined in the whole brain. CCL2 expression is seen to rapidly rise and fall between days 9 to 21, showing the highest level at day 14.
EAE [72], as well as prevented recurring clinical episodes in a chronic relapsing EAE model [73], possibly by antagonizing CCL2 at the luminal endothelial surface. Supporting this possibility, CCL2 harbors in its C-terminal α-helix a binding site for GAGs typically found on the luminal endothelial surface [74], and has been shown to bind to the luminal surface of cultured endothelial cells and then trigger firm adhesion followed by transmigration of mononuclear leukocytes [75,76]. Binding of CCL2 released from BMEC in culture has most recently been shown to switch from the luminal to the abluminal surface following cytokine-induced activation [77], possibly reflecting the changing roles of this chemokine pool from first promoting leukocyte adhesion to later directing extravasation into the parenchyma. Thus, by targeting the BMEC reservoir of CCL2 during disease, bindarit might be able to blunt neuroinflammation at different stages.

Bindarit action on CCL2 expression by cultured astrocytes had to be studied in the context of LPS stimulation, as these cells exhibit barely detectable CCL2 mRNA in culture or in situ in the naive state [50,51]. Yet despite significant induction, astrocyte CCL2 mRNA was reduced by half or more following bindarit exposure. As astrocytes constitute the most abundant glial cell population in the CNS [78], suppression of their CCL2 production by bindarit in vivo might well exert profound influence on pathologic events.

That bindarit could indeed act in vivo to effectively suppress neuroinflammation was evident in both the LPS and EAE paradigms. Injection of bindarit dramatically reduced LPS-stimulated expression of CCL2 in both brain and spinal cord, dropping mRNA levels to near 10% of vehicle-injected control values, while cutting protein levels approximately by half. In this case, the efficacy of bindarit in suppressing brain CCL2 may have...
been aided by the fact that LPS can severely disrupt the BBB [79,80], and thereby possibly facilitate bindarit entry into the CNS parenchyma. As CCL2 can also disrupt tight junctions leading to elevated BBB permeability [10-12], CCL2 generated early after LPS injection may have contributed to its subsequent suppression by further enabling bindarit CNS access.

The effects of bindarit on clinical EAE suggest that bindarit exerted both preventative and therapeutic actions. Preventative action is indicated by the considerable delay in disease onset in the bindarit-treated group, as well as the reduced incidence and severity of disease displayed by these mice. Possible therapeutic action is conveyed by the steady decline in disease severity following diminished peak clinical score. Such decline was in marked contrast to the typical plateau in clinical score exhibited by EAE mice given vehicle. These results are qualitatively similar to those recently reported by Laborde et al. [81] who, employing a regimen of twice daily oral dosage of a novel heteroaroylphenylurea antagonist of CCL2 function, also described delayed disease onset and resolution of EAE symptoms. In the case of bindarit, however, clinical symptoms seemed to steadily remit following attenuated peak disease, and a reduced incidence was also noted. Both studies nevertheless highlight the prospect that selective targeting of CCL2 activity might prevent EAE, as well as reverse its course.

The effect of bindarit on clinical EAE was accompanied by significant reduction of CCL2 mRNA in both brain microvascular and parenchymal fractions, consistent with bindarit’s mechanism of action being inhibition of CCL2 transcription [33]. Reinforcing this point, global knockout of the CCL2 gene has been shown to similarly delay EAE onset, and reduce both disease incidence and severity, effects that have been attributed to absence of CCL2 expression within the CNS compartment [81,82]. This, along with demonstration that CCL2-deficient mice also exhibit reduced neuroinflammatory responses to peripheral LPS injection [83,84], underscores CCL2’s non-redundant role in neuroinflammatory disease and accentuates its value as a therapeutic target. Results with bindarit and EAE may further suggest that both microvascular and parenchymal sources of CCL2 contribute to pathogenesis. If this is so, it could further imply bindarit would not have to penetrate the BBB in order to reach at least one of its targets, BMEC. In contrast to the acute situation with LPS, which acts directly on the endothelium, it is reasoned that the BBB was more intact in EAE mice receiving bindarit, as mean disease score only reached approximately 1.5. Thus, a likely scenario is that bindarit also sufficiently crossed the BBB to suppress the astrocyte and/or microglial response as well. This lends promise that bindarit can access the CNS parenchyma during the early stages of neuroinflammatory disease, when BBB breakdown is not yet manifested.

Conclusions

In summary, the CCL2 synthesis inhibitor bindarit, previously shown to be highly effective in myriad experimental disease models as well as human conditions having inflammatory involvement [34-40], was observed to significantly reduce steady state and LPS-induced CCL2 expression in cultured microglia, BMEC and astrocytes, as well as LPS-stimulated CCL2 mRNA and protein levels in CNS tissue in situ. Bindarit was further effective in delaying, preventing and attenuating clinical EAE, and evidenced signs of possibly reversing disease course while also suppressing elevation of CCL2 in brain microvascular and parenchymal compartments. Collectively, these data are consistent with the widely proposed critical role for CCL2 in neuroinflammation [18-20], and suggest bindarit, by targeting cells of the NVU [41], might have therapeutic success in the treatment of MS and/or other human neuroinflammatory diseases.

Abbreviations

BBB: Blood–brain barrier; BMEC: Brain microvascular endothelial cells; CFA: Complete Freund’s adjuvant; CNS: Central nervous system; Ct: Cycle time; DMSO: Dimethyl sulfoxide; EAE: Experimental autoimmune encephalomyelitis; GAG: Glycosaminoglycan; GFAP: Glial fibrillary acidic protein; Heps: Hydroxethyl piperazineethanesulfonic acid; i.p.: Intraperitoneal; LME: L-leucine methyl ester; LPS: Lipopolysaccharide; MTC: Methylcellulose; MS: Multiple sclerosis; NVU: Neurovascular unit; PBS: Phosphate buffered saline; PCR: Polymerase chain reaction; RT: Reverse transcription.

Competing interests

The author(s) declare that they have no competing interests.

Acknowledgements

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Authors’ contribution

SG, BS, DP and CK performed all the experiments. SG assisted with design of the experiments and data analysis; prepared the figures, and participated in drafting the manuscript. AG and RC assisted with the data analysis. JP designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Active induction of experimental autoimmune encephalomyelitis by MOG\textsubscript{35-55} peptide immunization is associated with differential responses in separate compartments of the choroid plexus

Nivetha Murugesan, Debayon Paul, Yen Lemire, Bandana Shrestha, Shujun Ge and Joel S Pachter*  

Abstract

**Background:** There is increasing awareness that, aside from producing cerebrospinal fluid, the choroid plexus (CP) might be a key regulator of immune activity in the central nervous system (CNS) during neuroinflammation. Specifically, the CP has recently been posited to control entry of sentinel T cells into the uninflamed CNS during the early stages of neuroinflammatory diseases, like multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). As the CP is compartmentalized into a stromal core containing fenestrated capillaries devoid of typical blood–brain barrier properties, surrounded by a tight junction-expressing choroidal epithelium, each of these compartments might mount unique responses that instigate the neuroinflammatory process.

**Methods:** To discern responses of the respective CP stromal capillary and choroidal epithelial tissues during evolving neuroinflammation, we investigated morphology and **in situ** expression of 93 immune-related genes during early stages of EAE induced by immunization with myelin oligodendrocyte glycoprotein peptide (MOG\textsubscript{35-55}). Specifically, 3-D immunofluorescent imaging was employed to gauge morphological changes, and laser capture microdissection was coupled to an **Immune Panel** TaqMan Low Density Array to detail alterations in gene expression patterns at these separate CP sites on days 9 and 15 post-immunization (p.i.). To resolve CP effects due to autoimmunity against MOG peptide, from those due to complete Freund’s adjuvant (CFA) and pertussis toxin (PTX) included in the immunization, analysis was performed on MOG-CFA/PTX-treated, CFA/PTX-treated, and naïve cohorts.

**Results:** The CP became swollen and displayed significant molecular changes in response to MOG-CFA/PTX immunization. Both stromal capillary and choroidal epithelial tissues mounted vigorous, yet different, changes in expression of numerous genes over the time course analyzed - including those encoding adhesion molecules, cytokines, chemokines, statins, interleukins, T cell activation markers, costimulatory molecules, cyclooxygenase, pro-inflammatory transcription factors and pro-apoptotic markers. Moreover, CFA/PTX-treatment, alone, resulted in extensive, though less robust, alterations in both CP compartments.

(Continued on next page)
Introduction

Though the choroid plexus (CP) is commonly recognized as the production site of cerebrospinal fluid (CSF) [1-3], it has relatively recently gained attention as a critical player in central nervous system (CNS) inflammation [4-6]. Specifically, the CP has been suggested as the site of entry into the uninflamed CNS of pioneer T cells searching for their cognate antigens during immunosurveillance and in the early stages of neuroinflammatory diseases such as multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) [7,8]. Current theory holds that, after crossing the CP into the CSF, pioneer T cells travel to the subarachnoid space (SAS), where antigen-presenting cells reactivate them. In turn, reactivation is thought to set off a burst of cytokines and other mediators that inflames meningeal and parenchymal immune/inflammatory activities between the periphery and CNS. But the extremely close apposition of the different CP layers has posed a significant challenge to studying the depth of their respective contributions to inflammatory processes. In fact, no immune function has yet been ascribed to the CP capillary endothelium, leaving completely unresolved the factors that drive T cell emigration into the stroma. And gene regulatory events surrounding transmigration of T cells across the choroidal epithelium further remain unsettled.

To elaborate the sequence of events in the CP that set the stage for CNS inflammation during EAE induced by active immunization with MOG\textsubscript{35-55} peptide, we used laser capture microdissection (LCM) coupled to qrt-PCR-based microarray [23] to establish the time course of expression of a panorama of immune mediators in the separate stromal (including capillaries) and choroid epithelial layers. Morphological changes in the CP associated with MOG immunization were also examined by quantitative 3-D image analysis following confocal microscopy. Results reveal substantial changes in CP gene expression and morphology occurred in response to specific aspects of the MOG immunization process. These results could hold relevance for how combinations of environmental factors trigger neuroinflammatory disease.

Materials and methods

Animals

Female C57BL/6 mice, age 8–10 weeks and obtained from Charles River Laboratories, Inc. (Wilmington, MA), were used to minimize microvascular heterogeneity due to genetic variability, sex, and age [24]. Animals were euthanized by CO\textsubscript{2} inhalation, following Animal Care and Use Guidelines of the University of Connecticut Health Center (Animal Welfare Assurance # A3471-01). A total of n = 3 animals/group were used for each treatment and time-point assessed.

Induction of experimental autoimmune encephalomyelitis (EAE)

EAE was induced in mice by active immunization with MOG\textsubscript{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK), of murine origin (W. M. Keck Biotechnology Resource
Center, Yale University), as described [25]; following Animal Care and Use Guidelines of the University of Connecticut Health Center (Animal Welfare Assurance # A3471-01). Briefly, on day 0, one group of female mice 7–9 weeks of age was injected subcutaneously with 300 μg of MOG peptide in complete Freund’s adjuvant (CFA, DIFCO) into the right and left flank, 100 μl per site. These mice were also injected i.p. with 500 ng pertussis toxin (PTX, List Laboratories, Campbell CA) in PBS on days 0 and 2 following the first immunization (referred to as the MOG-CFA/PTX group). The second group of age-matched mice received CFA alone and PTX (500 ng) injections on day 0 and a second injection of 500 ng PTX alone on day 2 (referred to as the CFA/PTX group). The third group of naïve age-matched female mice was left untreated. Animals were monitored and scored daily for clinical disease severity according to the following scale: 0 = normal; 1 = tail limpness; 2 = limp tail and weakness of hind legs; 3 = limp tail and complete paralysis hind legs; 4 = limp tail, complete hind leg and partial front leg paralysis; and 5 = death. LCM tissue was acquired at day 9 (score 0) and day 15 (score ~2.0) post-immunizations.

Tissue preparation for Immuno-LCM
Brains were snap-frozen in dry ice-cooled 2-methylbutane (Acros; Geel, Belgium), and stored at −80°C. Frozen brain was embedded in cryomatrix compound (Thermo Fisher Scientific, Waltham, MA) prior to sectioning. Coronal sections (7 μm) were cut on a Microm HM 505 M cryostat (Mikron Instruments; Oakland, NJ) and affixed to uncoated, pre-cleaned glass slides (Fisher Scientific, Pittsburgh, PA) and stored in a slide box at −80°C. Tissue was processed for LCM within a week of sectioning.

Immunostaining for Immuno-LCM
Immunostaining was performed as detailed [24,26,27], with minor modifications. Briefly, sections were fixed in 75% ethanol, on ice, for 3 min prior to staining. The CP stromal capillaries were stained using alkaline phosphatase substrate NBT (nitro-blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt), (Vector Labs, Burlingame, CA) for 3–5 minutes in 100 Mm Tris–HCl (pH 9.5) to detect endogenous alkaline phosphatase activity in the endothelial cells. In this case, endothelial cells were intentionally not immunostained by anti-CD31/ABC alkaline phosphatase [24,27], as it resulted in extensive deposition of chromogenic precipitate, which made the stromal capillaries difficult to resolve from the choroidal epithelial layer. The choroidal epithelial cells were immunostained with monoclonal pan-cytokeratin-FITC antibody (Sigma) for 10 minutes (diluted 1:10 in 1X PBS + 0.5% Tween-20). RNAsin® RNAse inhibitor (Promega, Madison, WI) was added to all staining reagents. Immediately after immunostaining, sections were dehydrated through graded alcohol and xylenes as described [24].

Laser capture microdissection (LCM)
A PixCell Ile laser capture microscope (ABI, Foster City, CA) was used to separately procure CP stromal capillary and CP choroidal epithelial tissues, as previously described for brain parenchymal vessels [24,27,28]. We refer specifically to CP stromal capillary tissue, instead of pure capillary endothelium, as it was not possible to completely resolve vascular from matrix elements (including extravasating leukocytes) within the dense CP stroma. Likewise, the choroidal epithelial tissue may contain some epiplexus cells, and so is not described as pure epithelium. Only choroid plexus material from within the fourth ventricle and lateral recess of the fourth ventricle was retrieved.

Tissue extraction
LCM-retrieved tissue was solubilized in Cell Lysate Buffer® (Signosis; Sunnyvale, CA) for direct reverse transcription. Cell Lysate Buffer®, pre-heated to 75°C, was added and the resulting lysate heated at 75°C for an additional 15 min. Samples were immediately frozen at −80°C.

DNase treatment and cDNA synthesis
Cell Lysate Buffer® extracts were treated with Turbo DNase (Ambion; Austin, TX) according to the manufacturer’s instructions. Specifically, Turbo DNase buffer and DNase were added and samples incubated at 37°C for 30 min. Next, DNase inactivation reagent was added for 2 min at room temperature. Samples were then reverse transcribed using the SuperScript III (Invitrogen) standard protocol with random hexamers (Roche; Indianapolis, IN), and employing an extension temperature of 42°C – optimal for random hexamers – for 60 min. Resulting cDNA was stored at −20°C until used for analysis.

cDNA Pre-Amplification
Pre-amplification was carried for array analysis out using TaqMan® PreAmp Master Mix and a PreAmp Pool containing all the primers for detection by the Mouse Immune Panel TaqMan® Low density Array (TLDA; Life Technologies Corp., Foster City, CA) [23]. This panel contains 93 immune-related genes plus three housekeeping control genes (see Additional file 1: Mouse Immune Panel TLDA). Pre-amplification was carried out with an initial hold at 95°C for 10 min, followed by 14 cycles at 95°C for 15 sec and 60°C for 4 min.
qR-PCR
Relative cDNA levels were quantified by qR-PCR using an ABI PRISM 7500 Sequence Detection System Version 2.3, and reported compared to housekeeping gene GAPDH. Relative quantitation to GAPDH was performed using the standard $2^{-ΔΔCt}$ method of Pfaffl [29], where $ΔΔCt = Ct$ target threshold cycle – Ct reference (GAPDH) threshold cycle. Expression of genes relative to GAPDH was then represented as percent expression of GAPDH. To assure consistency in relating gene expression patterns to a housekeeping gene, GAPDH and two other housekeeping genes, β-actin and 18 S ribosomal RNA, were evaluated for constant expression across treatments. Additionally, gene expression values for a handful of randomly selected immune-related genes were also determined relative to β-actin and 18 S ribosomal RNA (see Additional file 2: Housekeeping control genes). Custom TaqMan® primers/probes were used for the Mouse Immune Panel TLDA. TLDA analysis was conducted as per the manufacturer’s protocol, with 100 μl sample volumes containing a 1/32 dilution of pre-amplified cDNA added to each port of the microfluidic card [23]. For qR-PCR analysis of CD31 and Cytokeratin 8, ‘singleplex’ assays were used as neither of these genes are represented in the mouse Immune Panel TLDA.

Immunostaining for confocal microscopy
Frozen cryosections (60 μm) were fixed with 4% paraformaldehyde, permeabalized with 1% Triton X-100 (in PBS) and incubated with Powerblock® for 10 min. Puriﬁed rat anti-mouse CD31 antibody (BD Pharmingen; 1:150 dilution in 10% NBS in 1X PBS+0.5% TW-20) was used to stain the CP capillary network followed by incubation with goat anti-rat Alexa-555 secondary antibody (1:200). Pan-cytokeratin-FITC 1: 150 dilution, (Sigma) was used to stain the CP epithelium. Next, Alexa-647 anti-mouse CD45 antibody (1:160 dilution) was used to stain leukocytes.

Confocal microscopy
Images were acquired on a Zeiss LSM 510 Meta laser scanning confocal microscope, and optical slices (at 2-μm intervals) obtained using a 40x objective. Acquired z-stacks were background-subtracted, and 3-D isosurface rendering performed using Bitplane IMARIS suite version 7.1 x 64 software (Bitplane Inc. Saint Paul, MN). Each z-stack was thresholded and the “ﬁlament tracker” module used to generate a 3-D traced outline of immunostained vessels in order to determine the diameter range of the CD31-immunostained capillary network within the choroid plexus across different treatments.

Statistical analysis
Relative gene expression values are given as mean ± SEM. Student’s two-tailed test (Microsoft Excel 2003, Redmond, WA) was employed to assess statistical significance in gene expression values between MOG-CFA/PTX and CFA/PTX samples from the CP capillary stroma and CP epithelium groups, separately for the two different time points assessed. Results were considered significant at a $p \leq 0.05$. Additionally, two-way ANOVA followed by post-hoc Bonferroni analysis was performed using GraphPad Prism 5 (GraphPad, La Jolla, CA) to determine interactive effects between immunization treatment and time of analysis post-immunization, and assessed for each CP compartment.

Results
Anatomy of the CP is altered in response to MOG-immunization
First, the anatomy of the CP was investigated using confocal microscopy followed by 3-D isosurface rendering. The close apposition of stromal capillary and choroidal epithelial layers in the CP is depicted in Figure 1. The 3-D analysis highlights the tortuosity of the capillary plexus. At day 15 post immunization (p.i.) with PTX and MOG, peptide in CFA to induce EAE (MOG-CFA/PTX group), or with PTX and CFA alone (CFA/PTX group), which does not produce disease in this paradigm, the capillary plexus can be seen to locally ‘swell’ in certain regions (Figure 1). Specifically, the range in diameter of capillaries in the MOG-CFA/PTX and CFA/PTX groups was 1.24 to 11.39 μm and 1.86 to 10.84 μm respectively, as compared to that found in naïve (1.24 to 6.22 μm) mice. In contrast to that seen within CP capillaries, the morphology of the choroidal epithelial layer remained relatively constant following immunization.

LCM enables resolution of CP stromal capillaries from the choroidal epithelium
Studies were next carried out to confirm the ability of LCM to resolve the stromal capillary and choroidal epithelial layers. Figure 2A shows an example of the highly selective retrieval of both tissues from naïve and EAE brain specimens. Microscopic analysis indicates no appearance of ﬂuorescently-stained choroidal epithelial tissue in the LCM-captured capillaries and, conversely, no alkaline phosphatase-stained capillary tissue in the retrieved epithelial samples. Figure 2B further highlights the purity in qR-PCR detection of LCM tissue from the respective CP compartments. Using equivalent amounts of input LCM tissue (1000 laser ‘shots’) from both CP compartments, the endothelial marker CD31 was signiﬁcantly enriched in the CP capillary tissue, while the epithelial marker cytokeratin 8 was observed in CP epithelial tissue alone. The extremely low level of CD31
mRNA detected in CP epithelial tissue may reflect the few monocytes and/or dendritic cells circulating through this area in the steady-state mouse brain [29,30]. There is thus high confidence that LCM effectively separates CP stromal capillary from choroidal epithelial layers with high purity.

Expression of immune-related genes by stromal CP capillary tissue following immunization

The next series of experiments coupled LCM to TLDA qrt-PCR arrays to further characterize expression patterns of a panorama of 93 immune-related genes in the separate CP compartments at different stages of the neuroinflammatory response to immunization. Expression of these genes relative to housekeeping gene GAPDH (GAPDH was unaffected across treatments; Additional file 2: Housekeeping control genes), was determined in three groups of mice: MOG-CFA/PTX, CFA/PTX and naïve at two time points: day 9 and 15 p.i. Contrasting these three treatment groups enabled effects of the adjuvants CFA and PTX to be distinguished from the autoimmune response to MOG. Furthermore,
examining effects at day 9 p.i. (prior to any evidence of clinical disease) and day 15 p.i. (after disease onset), highlighted the progression of gene changes that may be linked with developing pathology.

At day 9 p.i. (EAE clinical score 0), numerous gene changes were already evident in the CP stromal capillary tissue, despite the lack of onset of any clinical disease signs. Specifically, both MOG-CFA/PTX- and CFA/PTX-immunized mice showed up-regulated expression in 49 of the genes in the panel compared to naïve animals, (Table 1), with there being no statistically significant differences between the two immunized groups. Some prominent inflammatory genes that were equivalently elevated at this time point included: CCL2, CCL5, CXCL10, Sele (E-selectin), Selp (P-selectin), IL1b, Stat1 and Fasl all of which were modulated more than 10 fold higher than naïve levels. It would thus appear that, at this early stage before clinical EAE symptoms are present, the gene responses in the CP stromal capillary tissue following MOG immunization may stem largely from adjuvants CFA and/or PTX.

By day 15 p.i. (EAE clinical score 1.5-2.0), however, the MOG-CFA/PTX-immunized group surpassed the CFA/PTX group in up-regulation of several genes, highlighting what might specifically be the autoimmune response of the CP vascular stroma. These genes included B2m, C3, CCL19, CCL5, CD4, Gzmb, Ptgs2, Ptprc (CD45), Smad3, Stat4, and CD40l – which were selectively augmented in the CP stromal capillary tissue of the MOG-CFA/PTX group (Figure 3). The fold changes in these genes following immunization (Figure 3, bottom) indicate their super-stimulation by MOG-CFA/PTX

Figure 2 Immuno-LCM allows retrieval of tissue from specific CP compartments. A) Evidence of histological purity. Immunofluorescence was performed using FITC-conjugated pan-cytokeratin antibody to highlight the CP epithelium (green), while immunohistochemistry using alkaline phosphatase detection with NBT/BCIP as substrate was carried-out to label the endothelium of CP stromal capillaries (dark brown). LCM was performed on a Pixcell Ile LCM unit. Images both BEFORE and AFTER LCM, as well as LCM retrieved tissue deposited on the cap, are shown to highlight selective retrieval of CP stromal capillary (top row) and CP choroidal epithelial tissues (bottom row). B) Evidence of purity by qrt-PCR. Levels of CD31, an endothelial marker, and Cytokeratin-8, an epithelial marker, were probed to determine the purity of the CP capillary and CP epithelial tissues, respectively, retrieved by LCM.
Table 1 Genes similarly up-regulated in CP stromal capillary tissue from both MOG-CFA/PTX- and CFA-PTX-immunized mice at day 9 p.i.

<table>
<thead>
<tr>
<th>Gene name</th>
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<tr>
<td>B2m*</td>
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</tr>
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</tr>
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<tr>
<td>Gzmbf</td>
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</tr>
<tr>
<td>Hmox1</td>
<td>Fasl**</td>
</tr>
<tr>
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<tr>
<td>Icos</td>
<td>Vegfa</td>
</tr>
<tr>
<td>Il11g**</td>
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</table>

genes with * ≥ 5 and ** ≥ 10 fold increase in expression compared to naïve animals.

Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP stromal capillary tissue from immunized and naïve mice at day 9 p.i. At this early time-point, all 49 immunization-induced genes were similarly stimulated in both MOG-CFA/PTX- and CFA-PTX-immunized mice compared to naïve animals, and only these are listed. t denotes genes that show specific upregulation later on with disease progression on day 15.

Notably, the CFA/PTX group indicated some dampening of immune regulation by this time, as certain genes, e.g., Smad3 and Gzmb, dropped back to levels matching those of naïve mice, after initially displaying an elevation at day 9. Genes that trended towards elevated expression following MOG-CFA/PTX treatment (but with p values slightly > 0.05) are displayed in Additional file 3: Genes that trended towards elevated expression in MOG-CFA/PTX-immunized CP stromal capillary tissue compared to CFA-PTX-immunized mice, at day 15 p.i., while those that were similarly up-regulated in CP stromal capillary tissue of MOG-CFA/PTX- and CFA-PTX-immunized mice compared to naïve mice at day 15 are listed in Additional file 4: Genes similarly up-regulated in CP stromal capillary tissue from both MOG-CFA/PTX- and CFA-PTX-immunized mice at day 15 p.i. Genes that were undetected in the CP capillary tissue in all treated and naïve mice at both time-points were the following: CCR4, CD19, CD3e, CSF3, Ctl4a, Cyp1a2, Cyp7a1, H2-Ea, IL12b, IL13, IL3, IL4, IL5, IL6, IL9, and Lta.

Expression of immune-related genes by CP choroidal epithelium following immunization

Immunization also produced a change in expression of numerous immune-related genes within the CP choroidal epithelium. Moreover, these changes differed from those observed in the capillary stroma, emphasizing the differential immune sensitivities of the two tissues.

At day 9 p.i., the CP choroidal epithelium of only MOG-CFA/PTX-immunized mice displayed increased expression of any immune-related genes compared to that of naïve cohorts. Specifically, the following eight immune-related genes were up-regulated: B2m, CCL19, CCL2, CCR2, CD8a, CXCL10, Sele, and Selp (Figure 4A). B2m (beta 2 microglobulin), a biomarker for certain peripheral inflammatory conditions [37] was increased 2.2-fold in MOG-treated versus naïve mice. The chemokine CCL2 has been demonstrated to play a critical, non-redundant role in directing mononuclear leukocyte extravasation into the CNS during EAE [32,38,39], and was stimulated > 24-fold higher in the CP choroidal epithelial tissue of MOG-CFA/PTX-treated mice compared to that in naïve cohorts. CXCL10 and CCL19 were 28- and 14-fold higher than naïve values, respectively. Sele (E-selectin) and Selp (P-selectin), CCL19 and CD8a further showed pronounced

...
stimulation specifically following MOG immunization, being undetectable in the epithelium of both CFA/PTX and naïve cohorts. What appears to reflect the differential sensitivities of the two CP tissues, CFA/PTX immunization clearly ‘activated’ the stromal CP capillary tissue on day 9 p.i. at both the anatomical and molecular levels (Figure 1 and Table 1), but produced no detectable changes in the CP epithelium at this time. By day 15 p.i., genes B2m and CXCL10 displayed further increases in expression in the CP choroidal epithelium of MOG-CFA/PTX mice compared to that seen in this cohort at day 9 (Figure 4B), showing >19-fold and >800-fold higher levels, respectively, compared to naïve mice. Expression levels of yet additional genes in MOG-treated mice also became elevated at this time; these included Bax, Bcl2L1, C3, CD68, Gusb, H2-Eb1 and Ski (Figure 4B). Moreover, genes CCL19, CCL2, CCR2, CD8a, Sele and Selp, which had previously shown up-regulation only in the MOG-CFA/PTX group at day 9, became similarly induced in the CFA/PTX group at this later time-point. Genes that trended towards elevated expression following MOG-CFA/PTX treatment for both time points (but with $p$ values slightly $>0.05$) are displayed in Additional file 5 and Additional file 6: Genes that trended towards elevated expression in MOG-CFA/PTX-immunized mice at day 15 p.i. (compared to naïve mice) are graphed. The bar graphs depict those specific genes showing statistically significant differences in relative expression values ($p$ values indicated by asterisks) between MOG-CFA/PTX and CFA-PTX experimental groups. RNA values are presented as mean percent expression relative to GAPDH ($\pm$ SEM) in log scale. * and ** represent comparisons made between MOG-CFA/PTX and CFA-PTX-treatment groups; * $p < 0.05$, ** $p < 0.005$, Student’s t-test, $n = 3$ animals/group. Fold changes in gene expression normalized to naïve animals are tabulated below the graph. Fold changes of those genes that were up-regulated after immunizations but undetectable in naïve are denoted as ‘∞’.

Figure 3 Genes super-induced in CP stromal capillary tissue from MOG-CFA/PTX-immunized mice at day 15 p.i. Relative mRNA expression values were determined by immuno-LCM/TDLA in CP stromal capillary tissue from MOG-CFA/PTX-immunized, CFA-PTX-immunized, and naïve mice at day 15 p.i. Those genes that were more stimulated; i.e., ‘super-induced’, in MOG-CFA/PTX versus CFA-PTX-immunized mice at day 15 p.i. (compared to naïve mice) are graphed. The bar graphs depict those specific genes showing statistically significant differences in relative expression values ($p$ values indicated by asterisks) between MOG-CFA/PTX and CFA-PTX experimental groups. RNA values are presented as mean percent expression relative to GAPDH ($\pm$ SEM) in log scale. * and ** represent comparisons made between MOG-CFA/PTX and CFA-PTX-treatment groups; * $p < 0.05$, ** $p < 0.005$, Student’s t-test, $n = 3$ animals/group. Fold changes in gene expression normalized to naïve animals are tabulated below the graph. Fold changes of those genes that were up-regulated after immunizations but undetectable in naïve are denoted as ‘∞’.

Interaction between immunization treatment and time

In order to gain further appreciation of the extent to which time impacted the effect of specific type immunization on the expression patterns of immune-related genes, two-way ANOVA was performed to
deduce interactive effects between immunization treatment (e.g., MOG-CFA/PTX, CFA/PTX or naïve) and time post-immunization. For example, the expression of CCL19 in CP stromal capillary tissue following MOG-CFA/PTX immunization was time-dependent ($p < 0.05$ for positive interaction). Two-way ANOVA was done on all genes that displayed statistically significant modulation after immunization in at least one of the CP

Figure 4 (See legend on next page.)
compartments (as shown in Figures 3 and 4A, B) in either of the time-points analyzed (twenty-three genes in total). Interactive effects differed depending on the CP compartment, further highlighting the unique responses of the two CP tissues analyzed. Specifically, two-way ANOVA of CP stromal capillary tissue revealed the following twelve genes displayed positive interaction between immunization treatment and time post-immunization: B2m, C3, CCL19, CCL5, CD4, Gzmb, Ptgs2, Ptprc, Stat4, CCR2, CD68, Gusb (Figure 5). CP choroidal epithelial tissue, on the other hand, demonstrated positive interaction for another collective of genes: B2m, Bax, C3, CXCL10 (Figure 5).

Figure 6 qualitatively summarizes the differential responses of the CP capillary and CP choroidal epithelial tissues, respectively, to MOG-CFA/PTX immunization versus CFA/PTX immunization, contrasting adjuvant versus autoimmune effects on immune-related gene regulation over the two time-points analyzed.

Discussion

Due to increasing awareness of the CP as fundamental to the development of CNS inflammation [4-6], immuno-LCM coupled to qrt-PCR array was used to separately acquire CP stromal capillary and choroidal epithelial tissues and assess their respective patterns of expression in situ of a wide panorama of immune-related genes. Gene patterns were evaluated during pre-clinical and early clinical stages of EAE to appreciate the switches in gene expression that accompany evolving disease.

It is clear that the CP responds vigorously to MOG immunization at both the anatomical and molecular levels. Interestingly, immunization with CFA and PTX alone produced striking effects. Swelling of the capillary plexus occurred to nearly the same extent with injection of just these agents, as with PTX and MOG in CFA. PTX is an ancillary adjuvant commonly employed to elicit EAE, as well as several other experimental autoimmune diseases [40-44]. And while its mechanism of action in this regard has generally been attributed to increasing vascular permeability [45-47] – most notably that of the BBB [48-51] – additional hypotheses have been put forth [52-55]. However, to the best of our knowledge, this is the first report to turn attention to the CP as a possible target of PTX. It is of further interest to point out that the distension of CP capillaries noted here study bears similarity to that seen following systemic neutralization of VEGF and TGFβ [56]. In the latter case, CP capillary swelling was accompanied by loss of fenestrae from endothelial cells and appearance of multiple caveolae, transport vesicles that transcytose a variety of cargo [57] – including chemokines [58] – and are often associated with heightened vascular permeability and inflammation [59,60]. Engelhardt et al. [4] had also described ultrastructural changes of the CP during EAE (along with CFA and PTX as adjuvants), but noted these were mostly restricted to the CP choroidal epithelium. Moreover, as comparison in this latter study was just between healthy mice and those afflicted with EAE, it is unclear whether the observed epithelial response was autoimmune in nature and/or due to adjuvant action.

Our results suggest that injection of PTX and/or CFA, alone, might trigger an immune response in the CP capillaries that helps “set the stage” for CNS inflammation [61]. The CP capillaries might be uniquely responsive in this regard, as CFA/PTX treatment evoked an early response (day 9 p.i.) in the CP stromal capillary tissue, while the choroidal epithelium experienced neither overt morphological nor gene expression changes at this time. If, as speculated during MS/EAE, Th17 cells first transit through the CP, and then travel in the CSF to reach their cognate antigens in the SAS, then the CP capillaries must somehow initially be rendered capable of supporting T cell extravasation. In the EAE paradigm used here, PTX and/or CFA might provide the stimulus to evoke such capability. In this regard, we noted >10-fold increase in chemokines CCL2, CCL5 and CXCL10 in the CP stromal capillaries of both MOG-CFA/PTX- and CFA/PTX-treated mice at day 9 p.i. Constitutive CCL2 expression within the CP stromal tissue has been reported using in situ hybridization analysis, and shown to be induced following peripheral tissue inflammation [62]. The ability of CFA/PTX treatment to stimulate expression of these chemokines could potentially reflect the actions of one or both of these adjuvants to ‘prime’ the neuroinflammatory process by activating the
endothelium to elicit initial auto reactive T cell extravasation from the circulation into the stromal compartment. This hypothesis is consistent with the recent observation that administration of PTX to transgenic mice over-expressing CCL2 in the CNS causes disruption of the BBB and promotes leukocyte migration into the brain parenchyma [63].

Notwithstanding the effects of CFA/PTX treatment on CP capillary morphology and gene expression, immunization with MOG-CFA/PTX further induced the expression of additional genes – some or all of which might specifically reflect the autoimmune response and associated development of EAE. While perhaps necessary for disease to develop, the CP conditions set in place by PTX and CFA are insufficient for inducing EAE in wild-type C57BL/6 mice. For disease to occur, supernumerary induction of some genes, and de novo induction of others must take place. The findings by Goverman et al. and Brabb et al. [61,64], that injection of PTX alone can “trigger” EAE in TCR-transgenic mice specific for myelin basic protein, by fostering T cell access to the CNS, comports with our results and the view that PTX enables mice to reach the disease threshold. And helping pull this trigger may be additional effects of PTX on T cell behavior. Our observation of increased mRNA for genes CD8a, CD80, CD86, Gzmb (granzyme) and Ptprc (CD45) in the CP capillary stromal tissue of both MOG-CFA/PTX and CFA/PTX cohorts may reflect capture of PTX-activated CD8 T cells in transit across the CP and into the CSF. This interpretation is consistent with the recent finding by Murphey et al. [65], that PTX stimulation of cultured spleen cells results in CD 8 T cell activation via CD80/86 co-stimulation.

As to signals responsible for the extravasation of T cells from the circulation into the CP stroma during MOG-induced EAE, a combination of chemokines may fill this role, as these immune mediators do in guiding

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**Figure 5** Interaction between immunization treatment and time of analysis post-immunization on expression of immune-related genes within CP stromal capillary and choroidal epithelial compartments. Interactive effects of treatment (MOG-CFA/PTX, CFA-PTX and naïve) and time post-immunization were determined by two-way ANOVA on the 23 immune-related genes that were super-induced following MOG-CFA/PTX immunization in either of the two CP compartments (those genes graphed in Figures 3, 4A and B). Of these 23 genes, 13 genes in the CP stromal capillary and 4 genes in the CP epithelial tissue exhibited significant positive interaction between treatment and time post-immunization, and are denoted with their corresponding p values. The remaining genes that showed no significant interaction are labeled by (x). Time-dependent changes in expression of CCL19 in stromal capillary tissue and CXCL10 in choroidal epithelial tissue are graphed as representative examples * p<0.05, ** p<0.005.
parenchymal leukocyte infiltration. In particular, CCL5 level was increased significantly in CP stromal capillary tissue at day 15 p.i., which coincides with high CCL5 protein level reported in whole brain extract of EAE mice at a similar time-point, and argued to mediate leukocyte adherence to the CNS microvasculature [32]. And CCL19 – a CCR7 ligand known to play a crucial role in EAE development through IL-23 producing Th17 cells [66] – was likewise up-regulated dramatically in the CP stromal capillaries of MOG immunized mice at day 15 p.i.

With specific regard to those mechanism(s) further driving T cell migration from the CP stroma into the CSF, recent evidence points toward expression of another chemokine - CCL20 - by the CP choroidal epithelium as directing CCR6⁺ T cells across this layer and into ventricular fluid during MS/EAE [8]. However, as this chemokine:cytokine receptor pair was not represented on the commercial TLDA card used in these experiments, confirmation of this pathway was not performed. Aside from CCL20 providing a driving force for T cell migration into CP epithelium, CCL2 might also serve in this capacity, as Chodobska et al. [67] noted the latter chemokine was rapidly stimulated in this tissue in vivo and then released into the CSF, following traumatic brain injury. Indeed, the significant increase in CCL2 we observed in the MOG-CFA/PTX CP choroidal epithelium at day 9 p.i. might just reflect such a role for this chemokine in EAE. The cognate receptor for CCL2, CCR2 was also seen to be elevated in the choroidal epithelium at this early time-point. Presently, it is unclear if the high levels of CCR2 mRNA indicate activation of the epithelium, which, in the periphery, has been shown to express CCR2 [68,69], or the accumulation of infiltrating CCR2⁺ T cells.

In what might suggest multi-level control of leukocyte extravasation into the CSF, still other chemokines were also significantly up-regulated by the CP during EAE – namely CXCL10 and CCL19. As CXCL10 has been reported to be up-regulated in the sub-ventricular zone (SVZ) during EAE, and postulated to stimulate migration of activated T cells into the SVZ [70], its spike in expression by CP choroidal epithelial tissue at day 9 p.i. and more robust elevation by day 15 p.i., might imply this chemokine is obligate for T cell entry into the ventricles. In analogous manner, CCL19 was also elevated at this site at day 9 p.i. In fact, the timing of the CP epithelial spikes in this chemokine during early stages of EAE noted here, coincides well with that reported by Reboldi et al. [8] for initial T cell entry into the uninflamed CNS through the CP. Recently, Marques et al. [22,71] used hybridization-based microarray to assess the global transcriptome of the whole CP following chronic peripheral LPS stimulation. When compared to our study, there were some common and unique findings. Among the common findings, complement protein C3, and chemokines CCL2 and CCL5 were elevated following either acute or chronic peripheral LPS stimulation, as well as during MOG-induced EAE (complement C3 in both CP capillary tissue and epithelium; CCL2 in CP epithelium; and CCL5 in CP capillary tissue). And Selectin (Sele and Selp) expression was also elevated both following acute peripheral LPS stimulation [22], and in the CP epithelium after MOG-induced EAE. These common gene modulations may thus reflect more generic CP inflammatory response genes. As for unique findings, these too involved chemokines. Marques et al. [71] reported stimulation of CCL7 and CXCL1 in the CP following chronic LPS stimulation, while we detected stimulation of CCL19 and CXCL10 in the CP epithelium and CCL19 in the CP endothelium during
MOG-induced EAE. A priori, up-regulation of these latter two chemokine genes may more distinguish an EAE signature for the respective CP tissue compartments.

Of further note was our observation of a MOG-sensitive increase in expression of B2m at day 15 p.i. in CP stromal capillary tissue, and at both time-points in the choroidal epithelial tissue. Aside from perhaps reinforcing a more ubiquitous role for B2m in inflammation [37], this result complements previous reports of increased B2m mRNA and protein levels in both neuronal and non neuronal cell types during EAE [72,73], a response thought to be due to induction of synaptic plasticity by infiltrating autoreactive immune cells. The sharp induction of complement C3, also noted in both CP compartments of MOG-CFA/PTX mice at the later time point (day 15 p.i.), further supports previous studies highlighting C3 deficiency inhibits development of EAE [74].

As to the specific approach used here, i.e., LCM coupled to TLDA, it offered extraordinary opportunity to probe, in extensive detail, the focused immune response within the distinct CP compartments. Earlier reports, using immuno-histochemistry and in situ hybridization, had shown that the respective CP stromal capillary endothelial cells and the CP choroidal epithelial cells displayed different expression patterns of a small nucleus of adhesion molecules during EAE [4,20]. Specifically, VCAM-1 and ICAM-1 were found to be expressed constitutively by CP choroidal epithelial cells of healthy SJL/N mice, and then further induced following active immunization with spinal cord homogenate. MAdCAM-1 was only seen in these cells after induction of EAE. However, none of these adhesion molecules, nor E- or P-selectin, was detected in CP stromal capillary endothelial cells [75].

We too noted constitutive VCAM-1 expression in the CP choroidal epithelial tissue of healthy naïve mice. Likewise, VCAM-1 trended toward elevation within this CP compartment of MOG-CFA/PTX cohorts at both days 9 and 15 p.i., though it showed no up-regulation in CFA-PTX-immunized mice at either time-point. In further agreement with previous observations [75], our analysis demonstrated induction of E-selectin and P-selectin in the CP choroidal epithelial tissue from MOG-CFA/PTX-immunized mice at day 9. Our results nevertheless displayed some stark differences with earlier reports. Specifically, we also noted a trend of increased VCAM-1 expression by day 15 in CP stromal capillary tissue with MOG immunization, paralleling what has been described in MS brain tissue [6]. And both E- and P-selectin mRNA were also observed to be induced in CP stromal capillary tissue of both MOG-CFA/PTX- and CFA/PTX-immunized mice compared to that of naïve mice at day 9 p.i. E-selectin increased expression in the two immunized groups by >100-fold, while P-selectin was stimulated >10-fold.

A priori, differences in results between these EAE studies could result from several factors, among them being 1) the EAE model employed (e.g., immunization of SJL/6 mice with spinal cord homogenate versus immunization of C57BL/6 mice with MOG35-55 peptide), the time of analysis post-immunization (e.g., before or after disease onset), and 3) the sensitivity of the analytic techniques (e.g., in situ hybridization versus qrt-PCR). As neither MAdCAM-1 nor ICAM-1 were represented on the TLDA card used in these experiments, confirmation of expression or lack thereof was not possible for these genes.

Most recently, Liddelow et al. [76] employed LCM to collect mouse lateral ventricular CP tissue CP for transcriptome analysis of transporter gene expression during normal development. Here, we extended this application, utilizing LCM to resolve – for the first time – the CP capillary stromal tissue from the CP choroidal tissue, and then separately analyzing each for their unique immune responses to MOG immunization.

Conclusions

Induction of EAE in C57BL/6 mice by active immunization with MOG35-55 peptide results in the respective CP stromal capillary and choroidal epithelial compartments each mounting vigorous, yet distinct, immune responses, underscoring the active role of the CP in investigates CNS inflammatory disease. Furthermore, our results make clear that a significant component of the total CP response is due to effects elicited by adjuvants PTX and/or CFA used in the immunization protocol – which might serve to prime the CP to support autoimmune activity necessary for developing MS/EAE. These results are summarized schematically in Figure 6.

Additional files

Additional file 1: Mouse Immune Panel TLDA. The card map for the 96 genes (93 immune-related genes and 3 control genes) on the commercially available mouse Immune panel TLDA is shown, with gene names and corresponding accession numbers.

Additional file 2: Housekeeping control genes. Ct (Threshold cycle) values for the three housekeeping genes – GAPDH, β-Actin and 18 S represented on the mouse Immune-panel TLDA are shown. The housekeeping genes were almost unchanged across treatments (shown in A and B) with < 1 cycle difference between samples. C, Six genes were normalized to each of the three housekeeping gene and expression patterns plotted, indicating identical patterns of expression across housekeeping control gene used.

Additional file 3: Genes that trended towards elevated expression in MOG-CFA/PTX-immunized CP stromal capillary tissue compared to CFA-PTX-immunized mice, at day 15 p.i. Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP stromal capillary tissue from immunized and naïve mice at day 15 p.i. A total of 14 genes trended towards greater induction in the MOG-CFA/PTX group compared to the CFA-PTX group; these genes are listed with their corresponding p values. Analysis was by Student’s two-tailed t-test.
Additional file 4: Genes similarly up-regulated in CP stromal capillary tissue from both MOG-CFA/PTX- and CFA-PTX-immunized mice at day 15 p.i. Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP stromal capillary tissue from immunized and naïve mice at day 15 p.i. At this later time-point, 25 immunization-induced genes were similarly stimulated in both MOG-CFA/PTX- and CFA-PTX-immunized mice compared to naïve animals, and only these are listed.

Additional file 5: Genes that trended towards elevated expression in MOG-CFA/PTX-immunized CP epithelium tissue compared to CFA-PTX-immunized mice, at day 9 p.i. Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP epithelium from immunized and naïve mice at day 9 p.i. A total of 15 genes trended towards greater induction in the MOG-CFA/PTX group compared to the CFA-PTX group; these genes are listed with their corresponding p values. Analysis was by Student’s two-tailed t-test.

Additional file 6: Genes that trended towards elevated expression in MOG-CFA/PTX-immunized CP epithelium tissue compared to CFA-PTX-immunized mice, at day 15 p.i. Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP epithelium from immunized and naïve mice at day 15 p.i. A total of 19 genes trended towards greater induction in the MOG-CFA/PTX group compared to the CFA-PTX group; these genes are listed with their corresponding p values. Analysis was by Student’s two-tailed t-test.

Additional file 7: Genes similarly up-regulated in CP epithelium from both MOG-CFA/PTX- and CFA-PTX-immunized mice at day 9 p.i. Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP epithelium from immunized and naïve mice at day 9 p.i. At this early time-point, 10 immunization-induced genes were similarly stimulated in both MOG-CFA/PTX- and CFA-PTX-immunized mice compared to naïve animals, and only these are listed.

Additional file 8: Genes similarly up-regulated in CP epithelium from both MOG-CFA/PTX- and CFA-PTX-immunized mice at day 15 p.i. Relative mRNA expression values of 93 immune-related genes were determined by immuno-LCM/TLDA in CP epithelium from immunized and naïve mice at day 15 p.i. At this later time-point, 8 immunization-induced genes were similarly stimulated in both MOG-CFA/PTX- and CFA-PTX-immunized mice compared to naïve animals, and only these are listed.

Competing interests
The authors have no competing interests.

Authors’ contributions
N. Murugesan assisted in the design of the experiments, developed the immuno-LCM/TLDA protocol for evaluating the different CP tissues, performed the immuno-LCM/TLDA analyses of CP tissues and microscopic evaluation of CP structure in response to immunization, and contributed to the writing and editing of the manuscript. D. Paul assisted with the 3-D image analysis of CP structure. B. Shrestha assisted with the immuno-LCM/TLDA analyses. Y. Lemire and S. Ge assisted with the immunizations. J. Pachter designed the experiments, wrote the manuscript and provided oversight for all studies. All authors have read and approved the final version of the manuscript.

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Bandana Shrestha, Debayon Paul, and Joel Pachter

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