The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

Podocalyxin promotes vascular barrier function

__________________________________________
submitted by Jessica Cait
in partial fulfillment of the requirements for the degree of Master of Science in The faculty of graduate and postdoctoral studies (Experimental Medicine)

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Abstract

The CD34-family sialomucin, podocalyxin (Podxl), is broadly expressed on the luminal face of blood vessels in adult mammals; however, its biological function on vascular endothelial cells (vEC) is not well-defined. Here, we reveal specific functions for podocalyxin in maintaining endothelial barriers using HUVEC monolayers as a model in vitro. Detailed analysis of barrier HUVEC characteristics using electrical cell-substrate impedance sensing (ECIS) and live cell imaging revealed essential roles for podocalyxin in maintaining cell-cell and cell-matrix interactions. Thus, podocalyxin-deficient HUVEC fail to form a functional barrier when plated on several extracellular matrix (ECM) substrates. Regardless of ECM substrate, these monolayers lack adherens junctions and focal adhesions; and display a disorganized cortical actin cytoskeleton. To explore an in vivo function of podocalyxin, we conditionally deleted Podxl in vEC using the Tie2Cre strain (PodxlΔTie2Cre). Although we did not detect altered permeability in naïve mice at steady state, systemic priming with lipopolysaccharides (LPS) disrupted the blood-brain barrier (BBB) in PodxlΔTie2Cre but not WT mice. To study the potential consequence of this BBB breach, we used a selective agonist of PAR-1, a thrombin receptor expressed by neurons and glial cells. As a polar peptide, the PAR-1 agonist (TFLLRN), is normally excluded from CNS parenchyma by the BBB. In response to systemic administration of TFLLRN, LPS-primed PodxlΔTie2Cre mice experienced a dramatic behavioral change marked by a severely dampened neurological electrical activity. We conclude that podocalyxin expression by CNS vECs is required to maintain BBB integrity under inflammatory conditions.
Lay Summary

Blood vessels transport blood and nutrients around the entire body. One important function of blood vessels, in addition to providing a conduit for blood and oxygen transport, is to provide a barrier between the circulation and the surrounding tissue. This “vascular barrier function” is provided by vascular endothelial cells that lie at the interface between blood and tissues and, essentially, comprise the tubing that carries the blood. During inflammation (for example, during a blood infection), autoimmune disease, or stroke, this tubing becomes leaky or permeable and can expose healthy tissue to unhealthy signals and infections in the blood. The goal of this thesis is to better understand the function of one protein, called podocalyxin, in maintaining the integrity of blood vessels. In studying this protein, we hope to develop new drugs and treatment strategies for preventing leakiness of blood vessels during disease.
Preface

I was principally responsible for data analysis and interpretation of results. I have performed all experiments and generated all data and figures presented in this thesis with the exception of:

- Experimental planning and data interpretation done with the help of Dr. Michael R Hughes and Dr. Kelly M McNagny
- Experimental planning for *in vitro* staining assays, which was performed in consultation with Dr. Cal Roskelley
- Sample collection performed with the help of Sabrina Osterhof, Diana Canals, and Dr. Alissa Cait
- EEG imaging and analysis performed by Dr. Allen Chan in the Dr. Tim Murphy lab
- ECIS evaluation performed under the supervision of Dr. Matthew Zeglinski in the Dr. David Grandville lab. (Rb and α measurements were performed using ECIS ZΘ software as operated by Dr. Matthew Zeglinski)
- Electron microscopic analyses which were performed by Dr. Wayne Vogel
- HUVEC were isolated and provided by Dr. Pascal Bernatchez under human ethics certificate number: H10-00643

This work was approved by the Animal Care Committee under Certificate Number: A16-0073

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<td>actin binding proteins</td>
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<tr>
<td>AJ</td>
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<tr>
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<td>Allophycocyanin</td>
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<td>Avertin</td>
<td>2,2,2-tribromoethanol</td>
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<td>BBB</td>
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<td>Cdc42</td>
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<td>Electric cell-substrate impedance sensing</td>
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<td>tumor necrosis factor alpha</td>
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<td>wild-type</td>
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Finally, thank you to my wannabe parabiosis twin, Dr. Alissa Cait. You really are my other half and I am so fortunate to say you are my sister, supervisor, lab mate, teammate, swimmer and best friend.
Dedication

To my family.

And to the mice, for giving their lives in the name of science.
Chapter 1: Introduction

1.1 Blood vessels: structure and function

The mammalian cardiovascular system is a closed circuit made up of a highway of blood vessels responsible for delivering oxygen and nutrients to all cells in the body. Arteries carry blood away from the heart, branch into smaller arterioles that branch into even smaller capillaries. It is in the tiny capillaries where nutrients and cellular waste exchange takes place. From there, the blood starts its journey back to the heart from small venules to the increasingly larger veins. Blood vessels maintain a semi-permeable barrier between circulating blood and surrounding tissue while selectively allowing the exchange of nutrients and passage of immune cells. The vessel lumen is the conduit in which blood flows. Surrounding the lumen, the inner most part of the blood vessel, is the tunica intima. Capillaries possess only a tunica intima, and no other layers (1). The tunica intima consists of a layer of vascular endothelial cells (vECs), the cells that are in direct contact with circulating blood. vECs attach to a basement membrane made up of extracellular matrix components. The mid-section of a larger blood vessel, the tunica media, consists of a smooth muscle layer and an elastin-rich extracellular matrix. The smooth muscle layer is responsible for controlling blood vessel tone by dilating and constricting, a critical step to regulating blood pressure. The tunica media varies in thickness between types of blood vessels: It is thickest where blood pressure is highest (arteries) and thinnest where blood pressure is lowest (veins). Arteries also possess an internal elastic lamina that allows them to be elastic under high-pressure conditions. The outermost layer of the blood vessel is the tunica externa (or tunica adventitia), an external sheath composed largely of collagenous connective tissue. The responsibility of the tunica externa is to help the vessel hold its relative position in the tissue to prevent changes in position.
that could block or alter blood flow. Structure and function of blood vessels is reviewed in Tennant & McGeachie (2).

Figure 1.1. Structure of a blood vessel.

1.2 The vascular endothelium

The endothelium is the inner layer of cells that line blood and lymphatic vessels. The vascular endothelium consists of the layer of cells that line every blood vessel. These cells are continuously in contact with circulating blood components and are therefore the major regulators of the passage of materials in and out of the blood stream. vECs are thin, flat squamous cells of mesodermal origin (3). Endothelial cells have distinct apical and basal polarity; a key characteristic that allows them to form sheets of cells lining the vascular tree and maintaining barrier function (4). Originally
vECs were seen simply as a vessel covering, with the limited and specific function of maintaining selective permeability of small molecules. However, they provide a much wider range of functions including regulating blood vessel tone, hemostasis, leukocyte trafficking, cell proliferation and migration of blood vessels during angiogenesis (5).

1.2.1 Formation of blood vessels – proliferation and migration of endothelial cells

The endothelium is a key mediator of blood vessel formation. Blood vessel formation can be divided into two different processes: Vasculogenesis, the formation of a blood vessel de novo from undifferentiated precursors; and angiogenesis, development of blood vessels branching from pre-existing blood vessels (6). **Vascular endothelial growth factor (VEGF)**, a vEC-specific growth factor, is the main signaling protein that drives vessel formation (7). Vasculogenesis generally refers to the formation of blood vessels during development from endothelial precursor cells (angioblasts) (8). During vasculogenesis, angioblasts aggregate and elongate into cord-like structures, which then differentiate into endothelial cells and create a lumen in which blood flows (8).

Angiogenesis, in contrast, is the main mechanism of vascularization of adult and embryonic tissues and involves the sprouting of new vessels from existing ones. During angiogenesis, vECs are activated and extracellular matrix is degraded. Endothelial tip cells proliferate and migrate through a provisional **extracellular matrix (ECM)** to guide the developing capillary sprout towards a gradient of VEGF (9).
1.2.2 The role of endothelia in blood pressure

The endothelial layer can regulate vascular tone by responding to a variety of regulatory substances. By responding to physical stimuli, hormones and substances released from circulating platelets, vECs release vasoactive substances that communicate with the underlying smooth muscle layer to alter vessel tone (10). Nitric oxide (NO), prostacyclin I_2 (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) are examples of endothelial-derived vasodilators whereas endothelin-1 (ET-1) and thromboxane A_2 (TXA_2) are examples of endothelial-derived vasoconstrictors (11). Vasoactive substances are released by the endothelia in response to circulating physical (e.g. sheer stress) and chemical (e.g. hormones) stimuli. Therefore, the endothelium is critical for healthy regulation of vascular tone. Endothelial dysfunction manifests from a decrease in the bioavailability of the vasodilator NO and an increase in the vasoconstrictor ET-1 (12). The imbalance of vasoactive substances contributes to changes in vasoregulation observed in cardiovascular disease. Endothelial dysfunction has been implicated both as a consequence and a preceding factor of hypertension and hypertension-related cardiovascular morbidity and mortality (10).

1.2.3 Endothelium and leukocyte trafficking

The endothelium regulates the recruitment and extravasation of pro-inflammatory leukocytes in response to tissue damage and infection through expression of cell adhesion molecules and cytokines. Innate immune cells communicate with the endothelium upon recognition of a pathogen by releasing inflammatory cytokines such as interleukin-1 (IL-1) and tissue necrosis factor alpha (TNFα) (13). As vECs are exposed to cytokines, they respond by releasing cytokines of
their own as well as upregulating the expression of the adhesion molecules P-selectin, E-selectin, intracellular-adhesion molecule 1 (ICAM-1) and vascular endothelial adhesion molecule 1 (VCAM-1) (14). Circulating leukocytes in the blood bind these adhesion molecules to initiate rolling, adhesion and extravasation into the surrounding tissues. Opening and resealing of the junctional barrier between vECs must occur to have paracellular transmigration of leukocytes (15). However, dysregulation of vEC junctions can have pathological consequences of chronic inflammation and edema. Therefore, the vascular endothelium and vEC junctions play a central role in regulating leucocyte transmigration during acute inflammation.

1.2.4 Hemostasis

Hemostasis (the process of stopping a bleed) is a complex system regulated by the endothelium. Under normal conditions, the role of the endothelium is to allow blood to flow freely, without systemic bleeding or clotting by tightly regulating the balance between pro- and anti-coagulation (16). Upon vascular injury, cellular and protein materials congregate at the site of injury to create a blood clot and to prevent excessive blood loss. Under inflammatory conditions (e.g. sepsis) the hemostatic system is activated. Ongoing activation can cause widespread microvascular thrombosis (the formation of clots within microvessels) as well as depletion of coagulation factors and platelets leading to extensive hemorrhaging (17). Disseminated intravascular coagulation (DIC) is the condition of systemic activation of coagulation and is characterized by deposition of fibrin in the circulation (18). DIC occurs in 35% of septic patients, however, 50-70% of septic patients present with hemostatic changes (19).
The serum protease thrombin is the central enzyme of hemostasis. Thrombin signaling is mediated in part by G-protein coupled **protease-activated receptors (PARs)**. Each PAR has a tethered ligand that is proteolytically cleaved during PAR activation to expose the activating ligand. PARs are expressed on vECs, platelets and throughout the brain in glial cells and neurons (20). It should be noted that although human platelets express PAR-1, mouse platelets do not. They do however express PAR-3 and PAR-4 (21). In both mouse and human, PARs mediate neuroprotection and neurodegeneration under pathological conditions of neurodegenerative disorders (20). How PARs regulate neuronal survival and death is dependent on the magnitude and length of receptor activation. Thrombin induces apoptosis via PAR-1 on hippocampal neurons and astrocytes *in vitro* (22). Furthermore, *in vivo*, PAR-1 activation increases infarct volume in murine models of ischemic stroke (23). In contrast, low levels of thrombin can be neuroprotective in the brain (20). Low level thrombin can *inhibit* apoptosis in hippocampal neurons treated with cytotoxic levels of glutamate (24). Taken together, these data suggest that, in a diseased state, the presence of thrombin within the brain is a critical mediator of the consequences of neurodegenerative disorders.
1.3 Barrier function

The essential purpose of endothelial cells is to maintain a barrier between blood and tissue. Barrier function encompasses both the passage of molecules through the cell (transcellular) and the passage of molecules between cells (paracellular) (25). Through cell polarization, vECs organize and stabilize adhesive structures to generate a paracellular barrier. Stabilization and localization occurs through an interconnected network of actin filaments that link integral membrane proteins. Two important interactions, cell-matrix and cell-cell binding, allow endothelial cells to act as a selective barrier by sealing interstitial spaces to passage of large molecules. Located at the basolateral domain of the cell surface, integrins are localized to focal adhesions; focal points where the endothelial cells attach to ECM (figure 1.3). Within focal adhesions, integrins associate with actin filaments, which in turn are connected to focal and adherens junction proteins located at cell-cell contacts (26). Through actin networks, indirect communication occurs between cell-cell
junctions, integrins and the endothelial glycocalyx (the carbohydrate-rich layer of transmembrane proteins expressed on the luminal face of endothelial cells). Cross-talk between all aforementioned complexes is required to complete the picture of how endothelial cells generate and maintain dynamic barrier function.

Barrier leakiness is a major contributor to morbidity and mortality of inflammatory disease. Under inflammatory conditions the activated endothelium (see section 1.6) is unable to maintain a healthy physiological barrier. Understanding how endothelial cells maintain a healthy barrier and, conversely, how barrier function is disrupted during disease, is critical to the generation of new drugs and therapies for treating inflammation induced hyperpermeability.
Figure 1.3. A schematic of structures contributing to endothelial barrier function.

Adhesive molecules from vECs as they pertain to barrier function. Green and yellow structures are α and β subunits of integrins, adhering to the basement membrane. Within cells, integrins are found in focal adhesion complexes associated with focal adhesion proteins FAK, vinculin, paxillin, talin and F-actin. Adherens junctions (shown in blue) are associated between two cells, bound to intracellular catenins and bound to F-actin. Tight junction protein representative of claudin-5 is shown in purple. The apical hair-like structure is representative of the glycocalyx.
1.3.1 The actin cytoskeleton in barrier function

The actin cytoskeleton, made up of microfilaments, is a dynamic regulator of cell shape (26). At steady state, the actin cytoskeleton maintains a dynamic equilibrium between polymerization of globular-actin (G-actin) and de-polymerization of filamentous-actin (F-actin) (27). When endothelial cells are activated, intracellular signaling molecules can promote actin polymerization via G-actin ADP/ATP exchange (28). Rho family GTPases regulate actin polarization dynamics to form lamellipodia, filopodia, cortical lattices, and stress fibers and to regulate cell motility (29). A cortical actin web controls cell shape and stability, via the endothelial glycocalyx (30), and can also promote the assembly of cell–cell and cell–matrix adhesions. Therefore, the cytoskeleton acts as the internal scaffold that tethers together integral membrane proteins with intracellular organelles (31). This association is critical to maintaining localization and functioning of both cell-cell and cell-matrix adhesion complexes and therefore is a key component in proper functioning of endothelial barrier function. Thus, there is a critical role for the actin cytoskeleton in barrier function.

1.3.2 Junctional proteins in the endothelium

Junctions are adhesive structures that link adjacent endothelial cells (cell-cell contacts). Their role is to maintain vascular integrity by regulating the paracellular passage of molecules. Endothelial junctional proteins can be broken up into three categories: gap junctions (GJ), adherens junctions (AJ) and tight junctions (TJ). GJs are responsible for creating a conduit for adjacent cells to pass chemical messages. Although critical for cell signaling, GJs do not traditionally contribute to barrier formation (32). AJs and TJs are the key mediators of vascular integrity.
Furthermore, transmembrane junctional proteins interact with intracellular binding partners to communicate and regulate apoptosis, growth and hemostasis (33). Junctional complexes are disrupted under pathological conditions (such as sepsis and ischemia/reperfusion injury) and contribute to disease morbidity and mortality.

1.3.2.1 Adherens junctions

Adherens junctions are arguably the most important junctional proteins responsible for maintaining endothelial cell-cell contacts (34). AJs are made up of several associated protein complexes to make junctions that are dynamic and responsive to environmental signals. AJs localize to the cell border and permit adhesion of adjacent cells. Cadherins are the main transmembrane adhesion proteins in AJs. Endothelial cells uniquely and consistently express vascular endothelial cadherin (VE-cadherin (*Cdh5*)) (35). The C-terminal domain of VE-cadherin binds β-catenin, γ-catenin (plakoglobin) and p120, three proteins responsible for stabilizing junction architecture and mediating downstream signaling (36). In mature endothelial monolayers, β-catenin is sequestered at AJ complexes. During endothelial cell activation, β-catenin translocates to the nucleus where it acts as a transcription factor regulating cell survival and angiogenesis (37).

Beyond direct association of VE-cadherin and intracellular binding partners, AJ complexes are also associated with the actin cytoskeleton through actin binding proteins (ABP). γ and β-catenin bind the ABP α-catenin, vinculin and α-actinin (among others), which link VE-cadherin to the actin cytoskeleton (32). The cadherin-catenin complex can transduce information inside the cell to
mediate actin rearrangement. VE-cadherin controls AJ stability and cytoskeletal rearrangement by regulating activation of Rho GTPases through intracellular binding with catenins (36). Members of the Rho GTPase family are regulators of vascular permeability. Ras homolog gene family, member A (RhoA), is involved in stress fiber organization and weakens barrier function (38). Conversely, Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42) are controllers of the formation of filopodia and lamellipodia and improve barrier function (38).

The most important and best-studied function of AJs is regulation of vascular permeability. Under inflammatory conditions, vasoactive substances such as thrombin and histamine can regulate AJ stability, localization and expression (14). In part, this occurs through Rho GTPases and post-translational regulation of AJs proteins through phosphorylation. Phosphorylation of cadherin-catenin complexes by VEGF stimulation causes increases in permeability and decreased junctional strength (32). Although disassembly and reassembly of AJs is critical for leukocyte transmigration, vascular remodeling and angiogenesis, the chronic or exacerbated disruption of AJs is detrimental during vascular barrier diseases. Chronic disruptions of AJs cause edema and inappropriate passage of blood components into surrounding tissue leading to poor tissue perfusion and increased cell death and inflammation (39).
1.3.2.2  Tight junctions

The role of TJs is to maintain an intercellular barrier to prevent the passage of molecules and ions between adjacent cells. TJs are primarily known for their role in epithelial barrier formation (40). In contrast to their organization in epithelial cells, in non-brain endothelia, TJs are much less abundant, less organized and intermingled with AJs along the intercellular cleft (41).

Endothelial TJs are most commonly studied in the blood-brain barrier (BBB). For a detailed discussion on barrier function in the BBB see section 1.4. Central nervous system (CNS) diseases are associated with BBB dysfunction and TJ disassembly. Unlike endothelial cell in other tissues, brain endothelia highly express TJ proteins that are well organized and situated apically to AJ proteins (32). Claudins and occludins are the transmembrane proteins of TJs (42). Claudin-5 is the main TJ protein of the endothelia, however, deletion of claudin-5 in the BBB only shows minor alterations to BBB permeability suggesting a redundant role for other claudin proteins (43). Occludins are specifically expressed in the BBB and the blood-retinal barrier (44). Under ischemic and inflammatory conditions, dephosphorylation of occludins leads to disruptions in TJ assembly and increases in vascular permeability (45). Claudins and occludins link to intracellular partners including zonula occludins (ZO) important for tight junction assembly (46). TJs interact with AJs in the BBB. VE-cadherin establishment at AJs induces claudin-5 transcription (47). After ischemic stroke, internalization of VE-cadherin causes downregulation of claudin-5 transcript leading to loss of claudin-5 at TJs (39). Therefore, TJ/AJ communication is a critical component of maintaining BBB stability.
1.3.3 The endothelial glycocalyx

The endothelial glycocalyx is a carbohydrate-rich layer made up of proteoglycans and glycoproteins that lines the apical (luminal) face of endothelial cells (48). Beyond protecting the endothelial cell layer from direct blood flow, it plays a major role in regulating leukocyte adherence and hemostasis (49). The negative charge of the glycocalyx regulates hemostasis by inhibiting platelet and red cell adhesion to endothelial cells (48). Furthermore, heparin sulfate molecules of the glycocalyx bind and display anti-thrombin III to inhibit coagulation (50). By disrupting the glycocalyx during endothelial injury, the environment shifts to a pro-inflammatory and pro-coagulant state (51). Structural stabilization of the glycocalyx occurs through a cytoskeletal scaffold network (52). Currently, it is recognized that the glycocalyx plays a role in actin reorganization (53). Under inflammatory conditions, injury to the glycocalyx causes increased paracellular permeability and leakage of albumin and other plasma components into the interstitial space (54). Therefore, the endothelial glycocalyx plays a role in maintaining vascular barrier function. Disruption of the endothelial glycocalyx is observed in sepsis and ischemia/reperfusion injury and has been linked to poor clinical outcome (49).

1.3.4 Integrins

Integrins are basolateral transmembrane proteins that link cells to the substratum. Integrins are heterodimers comprised of an α and β subunit. They possess a short cytoplasmic domain that interacts with binding proteins localized to focal adhesion (FA) complexes. Integrins do not function only as adhesion molecules; they are bi-directional signaling molecules that allow cells to recognize and respond to properties of their environment. Integrin cytoplasmic domains have
several binding partners. In FAs, integrins bind downstream signaling molecules, such as focal adhesion kinase (FAK), to regulate cellular processes such as cell cycle, attachment and migration. Integrins bind ABPs such as vinculin, talin and α-actinin to associate with the actin cytoskeleton, which is required for correct localization and stabilization of FAs. This association allows integrins to influence cytoskeletal rearrangement. Association with the actin cytoskeleton also promotes stabilization of FAs and contributes to barrier formation. The extracellular domain of integrins bind ECM of the basement membrane. The basement membrane is a thin layer of ECM proteins onto which endothelial cells attach. Generally, the basement membrane is made up of laminin, type IV collagen, fibronectin, nidogen, and heparan-sulfate proteoglycan 2 (perlecan) (55) but composition of the basement membrane can vary depending on vessel type, tissue and function. For example, in the cerebrovasculature, each vessel type is composed of different laminin isoforms (56). During angiogenesis, endothelial cells must remodel provisional ECM made up of collagens and fibronectins to generate a laminin rich ECM that leads to endothelial stabilization (57). ECM composition can be altered during disease via degradation by matrix metalloproteinases (MMP) (58). Integrins are activated when intracellular signals induced by interactions at the cytoplasmic domain, cause a conformational change to the extracellular portion of the integrin, increasing affinity of the integrin receptor to the extracellular ligand (59).
1.4 The blood-brain barrier

The BBB is a unique permeability barrier found in brain capillaries required for separation of brain and systemic blood circulation. This barrier is essential for normal brain function. Neuron communication transmitted between synaptic clefts is extremely sensitive to the presence of plasma solutes (60). To protect neurons in the brain and to allow them to signal normally, the BBB is required to form and maintain the most selective endothelial barrier in the body (61). BBB breakdown is implicated as a contributing factor to disease progression and morbidity in a consortium of CNS diseases including multiple sclerosis, stroke, and epilepsy (62). Although the BBB is required for protecting the brain tissue from the presence of harmful substances, it is also a major obstacle for administering treatment for CNS diseases such as neurodegenerative disorders and brain tumors (63). Modulating the BBB, by either enhancing function during disease progression, or decreasing function to deliver lifesaving drugs, is a crucial step in improving outcomes for patients with CNS diseases.

The BBB consists of multiple cell types that contribute to increased barrier tightness, collectively called the neurovascular unit (NVU). The NVU is made up of vECs, pericytes, astrocytes, neurons and microglia (64). The brain endothelium is distinct from other endothelial barriers in the body. Brain vECs have low rates of transcytosis, no fenestrations and distinct organization and expression of TJ proteins (discussed in section 1.3.2.2) (65). Furthermore, brain vECs have unique polarization of luminal and abluminal membranes with distinct localization of transporters, ion channels and receptors for the passage of nutrients and waste products in and out of the brain (66). For example, in non-brain endothelia, VEGFR2 is expressed luminally, whereas in brain
endothelia, VEGFR2 is abluminal (67). Activation of apically expressed VEGFR1 on brain endothelia facilitates cytoprotection whereas activation of abluminal VEGFR2 promotes vascular permeability (68). Pericytes are perivascular support cells found throughout the vascular tree, including the brain. They wrap around endothelial cells and communicate directly with endothelial cells through GJs to mediate vessel maintenance and permeability (69). Astrocytes are situated between neurons and endothelial cells allowing them to respond to synaptic activity. Astrocytic endfeet wrap around vECs to control cerebral blood flow (70) and regulate TJ formation (71). Neurons generally contribute to the BBB via signaling to astrocytes. Neurons can control vEC constriction and dilation by release of vasoactive substances in response to increased metabolic demand (65). Microglia, the immune cells of the brain, can be found perivascularly where they can mediate BBB permeability through pro-inflammatory and anti-inflammatory responses (72).

1.5 Sepsis

Sepsis is a life-threatening condition that arises from an infection and results in an overwhelming systemic immune response. In Canada, 1 in 18 deaths involves sepsis (73). Sepsis is responsible for 53% of all infectious disease deaths (73). The mortality rate for sepsis is estimated to be 20-25% and in many cases sepsis can progress to septic shock (73). The mortality rate of septic shock can be as high as 80% and is the leading cause of death of hospitalized patients in the US and Canada (74). Sepsis, severe sepsis and septic shock represent a continuum of conditions of acute inflammation and organ failure (75). A description of these conditions and their defining criteria can be found in table 1 (76). Currently, sepsis is treated using antibiotics, intravenous fluids and respiratory support but with the lack of preventative treatment the incidence of sepsis is still
increasing (75). The pathophysiology of sepsis is mediated by the release of cytokines, through initial activation of innate immune cells (i.e. neutrophils) by Toll-like receptors (TLRs). Activation of TLRs induces the release of cytokines TNFα, IL-1 and interleukin–6 (IL-6) (77). The full pathophysiology of sepsis is complicated and involves the activation of many different cell types including vECs. Endothelial activation is a key contributor to sepsis related morbidity and mortality (78). Sepsis is also characterized by early acute encephalopathy which contributes to increased morbidity and mortality. Brain dysfunction during sepsis is a result of decreased perfusion of brain vasculature, as well as disruptions to the BBB (79). Recently, the endothelium has been highlighted as a potential therapeutic target during sepsis because dysregulation of vascular barrier function is central to the pathophysiology of this syndrome (reviewed in ref(80)).

1.6 Endothelial activation and its role in sepsis

Sepsis causes endothelial activation and affects almost all aspects of vEC function. Endothelial activation is described a shift of the endothelium towards a proinflammatory and prothrombotic state and is associated with many disease states and most forms of cardiovascular disease (81). Under inflammatory conditions, the endothelium plays a key role in disease progression through mediating inflammatory response, coagulation, immune cell trafficking and blood pressure (82). Unfortunately, under pathological conditions, an activated proinflammatory endothelium can be severely impaired and unable to perform normal endothelial function. In the presence of reactive oxygen species (ROS), and inflammatory cytokines such as TNFα, the glycocalyx is shed, which leads to disruptions in barrier function (78). In sepsis, the bacterial cell wall component LPS activates endothelial cells through soluble CD14 (83) and directly through TLR4/lymphocyte
antigen 96 (MD2) (84) or indirectly via inflammatory cytokines released by monocytes and granulocytes. Inflammatory cytokines IL-1, TNFα and IL-6 (85) can trigger a signaling cascade resulting in morphological changes to endothelial cells and rearrangement of the actin cytoskeleton (86). Cytoskeletal rearrangement causes internalization and cleavage of junctional proteins leading to disruptions in vascular barrier function. During sepsis, disruptions to the vEC barrier causes hypotension, inability to perfuse organs, and leads to organ failure. Furthermore, the presence of blood components, like serum proteases, within the parenchyma can cause damage to vulnerable tissues. In the CNS consequences include glial activation and neuronal death (87).

1.7 Studying endothelial genes in vivo
To study endothelial barrier function in vivo, there are several disease models and techniques available. Using the Cre-lox recombination system (88), genes of interest can be deleted specifically in endothelial cells. Two common transgenic mouse lines in which expression of Cre-recombinase is under the regulatory control of an endothelial-specific promoter are: Cdh5-Cre (VE-cadherin) and Tie2-Cre. Cdh5-Cre mouse strain reporter lines have demonstrated successful and specific Cre-expression in most adult vascular beds (89). However, contrary to conclusions made in the original literature using Cre-reporter alleles, the brain microvasculature of this mouse strain does not consistently result in gene deletion for all floxed alleles. However, the Tie2-Cre mouse strain reporter lines show more robust target gene deletion in brain microvasculature. Under the Tie2 promoter, Cre is expressed in all endothelial cells (including brain microvasculature) as well as in hematopoietic lineages (90).
1.8 Podocalyxin

Podocalyxin is a CD34-related transmembrane sialomucin. The CD34 family consists of 3 morphologically similar, yet distinct molecules: CD34, podocalyxin (Podxl) and endoglycan. Podocalyxin is a single pass transmembrane protein, localized to the apical side of polarized cells. It has a heavily glycosylated extracellular domain, a short cytoplasmic tail which contains a membrane-proximal ezrin-binding domain and a C-terminal PDZ-binding domain (91).

Through its PDZ-binding domain, podocalyxin interacts with several intracellular binding partners including the sodium-hydrogen antiporter 3 regulators 1 and 2 (NHERF-1 and NHERF-2), which possess two tandem PDZ domains linked to an ezrin-, radixin-, moesin- (ERM) binding motif. Thus, podocalyxin has the capacity to bind to the actin cytoskeleton through direct ezrin association, or indirectly through ezrin binding to NHERFs (92).
Figure 1.4. A schematic of podocalyxin and its binding partners.

Structure of podocalyxin adapted from (93). Light blue region is representative of the extracellular mucin domain with N-linked and O-linked carbohydrates. Arrows indicate possible sialic acid residues. Yellow boxes indicate cysteine residues located in the globular domain. The green box is the stalk region, the orange box indicates the transmembrane domain and the cytoplasmic domain is grey. At the C-terminus, a DTHL sequence binds the central PDZ2 domain of NHERF-1/2. Intracellular binding partner ezrin binds to podocalyxin via the ezrin binding site (and/or indirectly through NHERF1/2). Podocalyxin links apical membrane domain with F-actin through ezrin and NHERF-1/2.
1.8.1 Podocalyxin expression

Podocalyxin was originally identified as the predominant sialoglycoprotein of kidney podocytes, where, by virtue of its strong negatively charged mucin domain, it was postulated to play a role in the dissolution of TJs and AJs between immature podocytes as they undergo morphogenesis to form foot processes (94, 95). This notion is further supported by Podxl gene deletion studies where podocalyxin-deficient podocytes fail to form foot-processes and instead retain AJ/TJ interactions between podocytes that leads to renal failure due to anuria (91).

Since its discovery on kidney podocytes, podocalyxin has been found on several other cell types. It is known to be on a small subset of hematopoietic progenitor cells, it has been found on subsets of specialized epithelial cells where upregulation has been shown to be a prognostic marker for several subsets of cancers (96). Podocalyxin is also expressed on high endothelial venules (HEVs) where it has a sialo-Lewis-X modification allowing it to bind L-selectin (97). Podocalyxin has also been published to be expressed on stress erythrocytes in mice and rats (98, 99) and on rat platelets (99). However, expression of podocalyxin on mouse and human platelets has yet to be proven in our hands. Podocalyxin has also been published to be expressed on a subset of neurons, where its function is unknown (100).

Podocalyxin is highly expressed in all vECs in the mammalian tissue. Using electron microscopy, in 1989 it was observed that podocalyxin expression on vECs is patchy, and occurs in clusters on endothelial cells of the lung, kidney and pancreas. Although distribution of podocalyxin was uneven along the luminal surface, sometimes it appeared associated with coated pits (101).
Through proteomic analyses, podocalyxin was also shown to be one of only a few transmembrane proteins upregulated on the BBB endothelia compared to other vascular beds (102), suggesting an important function for podocalyxin in the brain.

1.8.2 Adhesive and antiadhesive functions

Podocalyxin is most commonly described as an anti-adhesive molecule. Mechanistically, we and others have shown that podocalyxin has the capacity to regulate integrin function altering its localization in polarized epithelial cells (103-105). Podocalyxin is potently targeted to the apical domains of epithelial cells and, in its absence, integrin sorting to basolateral domains is attenuated. Thus, although this apical sialomucin does not function as an adhesion molecule per se, its expression, or lack thereof, can dramatically alter the adhesive properties of cells.

1.8.3 The role of podocalyxin in the endothelia

During development, the first blood vessels arise through vasculogenesis. The first and largest blood vessel to form in all mammals is the aorta. During aorta formation vECs assemble into multicellular cords. Before lumen development, junctional proteins exist between cords and the presence of paracellular openings create an initially permeable vessel. As the aorta continues to develop, CD34 family siaolmucins podocalyxin and CD34 migrate towards the apical side of the vEC to form what will become the vessel lumen and coincides with the “unzippering” of adhesion complexes to enlarge the lumen (106). In this way, during vascular lumen formation, apical and basal polarity is established. However, deletion of podocalyxin and CD34 in the developing aorta
shows only a modest delay in lumen formation and they are therefore not required for lumen formation but help promote lumen development (106).

Podocalyxin’s role in the adult endothelium has remained elusive. Although podocalyxin and its close relative CD34 are highly expressed by all adult vECs, little is known of their function in these cells. Previously, using a Cdh5Cre vascular deletion approach (Podxl\textsuperscript{ACdh5Cre}), we observed a steady state hyperpermeability to evan’s blue dye (EBD) in the lung. Furthermore, we saw an additional increase in permeability in Podxl\textsuperscript{ACdh5Cre} mice after intranasal administration of LPS. Although these results were statistically significant, increases in permeability were subtle and not seen in other organs. Podxl-null vECs isolated from mouse lungs had an impaired ability to spread when plated on laminin and an altered gene expression pattern of integrins and matrix proteins (104). Using a Tie2Cre vascular deletion approach (Podxl\textsuperscript{ATie2Cre}), others have reported vasculitis and organ failure in aged mice after Podxl deletion. They have also reported high-dose LPS-induced decreased survival in Podxl\textsuperscript{ATie2Cre} mice and mild disruptions in junctional VE-cadherin after stimulation with thrombin (107). For our studies, we have generated the same Podxl\textsuperscript{ATie2Cre} mice and have not observed the same differences.

1.9 Rationale

Currently, there are only two studies that focus on the function of podocalyxin in adult vasculature (104) (107). \textit{In vitro} studies show subtle defects in cell spreading and adhesion hinting that podocalyxin might play a role in integrin-mediated cell adhesion. \textit{In vivo} work suggested a role for podocalyxin in barrier function however both studies focused solely on lung endothelia. In our
study, we wanted to focus on podocalyxin as a mediator of vascular barrier function. We were interested in generating quantitative measurements of barrier function \textit{in vitro} and describing podocalyxin’s role in endothelial cell-cell and cell-matrix interactions. We concentrated on the BBB since it is the most selective vascular barrier in the body and its breakdown is detrimental in neurological disease. Furthermore, podocalyxin has been shown to be upregulated on the BBB compared to other vascular beds (102), but it’s function in the BBB has not yet been described.

1.10 Hypothesis

We hypothesize that expression of podocalyxin in vascular endothelial cells promotes adhesion to matrix and formation of cell-cell junctions. We hypothesize that podocalyxin has a role in maintaining the vascular endothelial barrier in the CNS under steady-state conditions and during systemic inflammation.

1.10.1 Objectives

\textbf{Aim 1.} Assess the role of podocalyxin in maintaining vascular endothelial cell monolayer interactions and barrier formation \textit{in vitro}.

\textbf{Aim 2.} Assess the role of podocalyxin in maintaining BBB integrity \textit{in vivo}. 
Chapter 2: Materials and methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords supplied by donors with informed consent (Human Ethics # H10-00643). HUVEC were cultured in endothelial basal medium-2 (EBM-2) (Lonza, Basel, Switzerland) supplemented with hydrocortisone, human epidermal growth factor (hEGF), fetal bovine serum (FBS), VEGF, human fibroblast growth factor B (hFGF-B), heparin, ascorbic acid, antibiotics (GA-1000, gentamicin, amphotericin-B) and human recombinant insulin-like growth factor 1 (R3-IGF-1). HUVEC were expanded on 1% gelatin coated plates. HUVEC were maintained at 37°C, high humidity and 5% CO₂ and used between passages 2-8.

2.1.1 Cell line passaging

HUVEC were passaged or harvested for experiments at 70-80% confluence. HUVEC were washed once with Mg²⁺/Ca²⁺-free Hank’s buffered salt solution (HBSS) (Gibco, Burlington, ON). Cells were lifted into suspension using 0.25% Trypsin/EDTA (Invitrogen, Carlsbad, CA) at 37°C. Trypsin was quenched using growth medium with 10% FBS and cells were pelleted for 3 minutes at 453 x g to remove residual enzyme. Cells were resuspended in culture media and plated on coated plates at desired confluence.
2.2 siRNA silencing of PODXL in HUVEC

Suppression of PODXL expression was achieved by transfecting HUVEC overnight with PODXL-targeting or scrambled control siRNA using oligofectamine transfection reagent (Life Technologies, Carlsbad, CA). Briefly, siRNA (25 pmol) and oligofectamine transfection reagent (5µl) were incubated in optiMEM I minimal media (Thermo fisher, Waltham, MA) for 30 minutes at room temperature. Solution was then added to adherent HUVEC at 70% confluence overnight. The next day, media was added, and cells were incubated in a normal cell incubator for another 24 hours. On day 3, podocalyxin protein expression knockdown was confirmed by flow cytometry. Control and PODXL knockdown (PODXL\textsuperscript{KD}) HUVEC were subsequently used for experiments for up to 4 days post-transfection.

2.2.1 Flow cytometry

HUVEC were washed once with Mg\textsuperscript{2+}/Ca\textsuperscript{2+} free HBSS. Next, cells were lifted into suspension using 0.25% Trypsin/EDTA at 37°C. Trypsin was quenched using growth medium with 10% FBS. Cells were pelleted for 3 minutes at 453 x g to remove residual enzyme. After, HUVEC were resuspended in blocking buffer (FACS buffer (PBS, 2mM EDTA, 5% FBS) with 2% normal serum (NS)) for 20 minutes at 4°C. Cells were incubated with 2µg/mL primary rabbit anti-podocalyxin antibody (in-house antibody see (96)) diluted in blocking buffer for 30 minutes at 4°C. A rabbit-IgG antibody was used as a primary antibody isotype control. After three washes with FACS buffer, HUVEC were incubated with 2µg/mL Allophycocyanin (APC)-conjugated donkey anti-rabbit antibody (Life Technologies, Carlsbad, CA) diluted in blocking buffer in the
dark for 20 minutes at room temperature. HUVEC samples were run on a BD LSR II flow cytometer and podocalyxin staining analyzed using FlowJo_V10 software (FlowJo, LLC).

2.3 Barrier function assay

**Electric cell-substrate impedance sensing (ECIS)** electrodes in 8-well plates (8W10E+ PET; Applied BioPhysics, Troy, NY) were stabilized for 30 minutes at **room temperature (RT)** with 500µL cysteine buffer (Applied BioPhysics) and then coated with fibronectin (10 µg/mL; Sigma, St. Luis, MO), laminin (5 µg/mL; R&D systems, Minneapolis, MN), collagen (1% gelatin; Sigma) diluted in culture media for 1h at 37°C or left uncoated. Excess matrix was subsequently aspirated from each culture well. To assess barrier function, adhesion and spreading, scrambled control and *PODXL*<sup>KD</sup> HUVEC were plated in triplicate at 1.5x10<sup>5</sup> HUVEC/well and placed in a cell culture incubator (37°C, high humidity and 5% CO<sub>2</sub>). Impedance was measured at all alternating current (AC) frequencies (<i>f</i>) over 24 hours using the ECIS ZΦ instrument (applied BioPhysics). Frequencies used for further analyses (4 & 64 Hz) were selected based on previous reports (108, 109). Rb (cell-cell) and α (cell-matrix) parameters were calculated using Applied Biophysics mathematical modeling (110).

2.4 Video microscopy

A tissue culture-treated flat bottom 48-well plate (Costar, Washington, D.C.) was coated with fibronectin (10 µg/mL; Sigma), laminin (5 µg/mL; R&D systems), collagen (1% gelatin; Sigma) diluted in culture media for 1 hours at 37°C or left uncoated. Scrambled control and *PODXL*<sup>KD</sup> 3x10<sup>5</sup> HUVEC were plated per well of the 48-well plate coated with ECM components. Each
condition was plated in triplicate. Immediately after plating, cells were placed in a cell culture incubator (37°C, high humidity and 5% CO₂). Phase-contrast images of cultures were taken every 3 hours for 4 days using Incucyte Zoom instrumentation and software (Essen Bioscience, Ann Arbor, MI). For analysis, total confluent area was quantified per well and mean confluence value and standard deviation (SD) was plotted over time.

2.5 Monolayer assessment

Scrambled control and PODXL<sup>KD</sup> HUVEC were trypsinized, counted using a hemocytometer, and plated at identical cell densities on glass cover slips (inserted into 24-well cell culture plate) coated with poly-d-lysine (NeuVitro, Vancouver, WA), fibronectin (NeuVitro), laminin (NeuVitro), or collagen (1% gelatin; Sigma). At the experimental endpoint, cells were washed once with Mg<sup>2+</sup> and Ca<sup>2+</sup> free HBSS (Gibco). Next the cells were fixed onto coverslips using 4% paraformaldehyde (PFA) (Electron microscopy sciences, Hatfield, PA) diluted in PBS for 10 minutes on ice. Coverslips were washed 3x 5 minutes in PBST (PBS, 0.3% triton-X) to wash off residual fixative. Cells were blocked and permeabilized with blocking buffer (PBST, 3% BSA, 10% NS) for 20 minutes on ice. Block was aspirated and primary antibodies diluted in blocking buffer were added onto the coverslips overnight at 4°C. Primary antibodies were anti-vinculin (5µg/mL; Sigma, V9264), anti-β-catenin (1:400; Cell Signaling Technologies, D10A8) and anti-VE-cadherin (2µg/mL; Cell Signaling Technologies, 2500S). The next day, primary antibodies were aspirated and coverslips were washed 1x 30 seconds, 3x 10 minutes with PBST. Secondary antibodies were diluted in blocking buffer and added to wells in the dark for 2 hours at room temperature. Secondary antibodies were goat anti-mouse 594 (2µg/mL), donkey anti-rabbit 647
(2µg/mL) and/or donkey anti-rat 594 (2µg/mL). Secondary antibodies were aspirated, and cells were stained with AlexaFluor 488-phalloidin (5U/mL, Life Technologies, A12379). Next, coverslips were washed 1x 30 seconds, 3x 10 minutes with PBST, removed from 24-well plate and mounted using Prolong Gold Antifade containing DAPI (Life Technologies). Coverslip edges were sealed using clear nail polish and left in the dark to dry. Imaging was performed using a Nikon epifluorescence microscope (Nikon eclipse Ni-U, Tokyo, Japan). Image analysis was performed using ImageJ software (National Institute of Health, Bethesda, MD).

2.6 Mice

Tie2-Cre mice (B6.Cg-Tg(Tek-Cre)12Flv/J mice) were from The Jackson Laboratory (JAX#004128; Bar Harbor, ME). B6-congenic (Cg) conditional podocalyxin knockout mice (PodxlFL/FL) (104) were crossed with Tie2-Cre to delete podocalyxin specifically in vascular endothelia tissue strain (PodxlΔTie2Cre). PodxlΔTie2Cre (B6-Cg) were previously described (104). All mice were maintained under specific pathogen-free conditions at the Biomedical Research Centre, UBC. Experiments were performed humanely based on recommendations of the Canadian Committee on Animal Care with approval of UBC Animal Care Committee (A14-0269 (KMM) and A18-0036 (TM)).
2.7 Animal model of sepsis

Wild-type (WT) and Podxl^{ATie2Cre} mice were treated with 5 mg/kg lipopolysaccharides (LPS-EK, Invivogen, San Diego, CA) intraperitoneal (i.p.) prepared in sterile PBS (1 mg/mL). Mice were left either overnight (16 hours) or 6 hours before mice were either given further treatment (see section 2.7.1 below) or sacrificed for analysis (see section 2.7.2)

2.7.1 Animal model of BBB permeability

After treatment with LPS (see section 2.7), mice were treated with 5 mg/kg PAR-1 agonist (TLFFR-NH$_2$) (Sigma) prepared in PBS or treated with vehicle control (PBS alone) (i.v., tail vein). Mice treated with the PAR-1 agonist were immediately observed for changes in behaviour and video recorded for subsequent analysis. Mice treated with PBS did not demonstrate any change in activity. Activity was scored by reviewing videos based on total motion observed at 10 s intervals (with 1=normal, 0.5=reduced activity and 0=no activity) and time for full recovery (sustained return to score = 1).

2.7.2 In vivo immunofluorescence

Control mice and mice treated with LPS and/or the PAR-1 agonist (as described above) were anesthetized with 2,2,2-tribromoethanol (avertin). Next, mice were injected via the retro-orbital route of administration with Texas Red (TR) dextran (70 kDa) (TR-DEX$_{70kD}$) (125 µg/mouse; life technologies) mixed with FITC-labeled Lycopersicon esculentum (Tomato) lectin (FITC-LEL) (50 µg /mouse; Vector Labs, Burlingame, CA) in a 100 µL total volume. The TR-DEX$_{70kD}$ and FITC-LEL cocktail was allowed to circulate for 10 minutes.
2.7.3 Mouse necropsy and tissue preparation

For mouse sacrifice, mice were humanely euthanized using averitin as a terminal anesthetic. After mice lost pedal reflex and were determined to be in a surgical plane of anesthesia, the chest cavity was opened and the mouse’s right atria was punctured. 10 mL of cold PBS-EDTA (2mM) was perfused through the left ventricle until internal organs were pale, indicating that circulating blood had been successfully removed. Immediately following, 10 mL of 10% buffered formalin (Sigma) was perfused through the same ventricle. After fixation, the skulls were excised and placed in 10 mL of 4% PFA (Electron Microscopy Sciences, Hatfield, PA) overnight at 4°C. The next day, brains were removed from skulls and subsequently placed into 70% ethanol or sucrose for further processing.
2.7.4  Frozen sectioning for \textit{in vivo} immunofluorescence imaging

Post fixed brains were transferred to a 20% sucrose solution overnight at 4°C in the dark. When tissues had sunk, they were embedded in \textit{optimal cutting temperature (OCT)} (Sakura Finetek, Torrance, CA) and snap frozen. Brain sections (30 µm) were cut using a cryostat and floated into a 6-well plate (corning) containing PBS. Floating sections were subsequently placed onto glass slides, mounted using fluoromount (Sigma) and coverslips were sealed using clear nail polish. Imaging was preformed using a Nikon epifluorescence microscope (Nikon eclipse Ni-U). Image analysis was preformed using ImageJ software (National Institute of Health).

2.8  Electron microscopy

For mouse sacrifice, mice were humanely euthanized using avertin as a terminal anesthetic. After mice lost pedal reflex and were determined to be in a surgical plane of anesthesia, the chest cavity was opened, and the mouse’s right atria was punctured. Immediately following, 10 mL of EM fix (0.15 M sodium cacodylate trihydrate (Sigma) in water (pH 7.3) containing 4% formaldehyde and 2% glutaraldehyde (Electron Microscopy Sciences)) was perfused through the left ventricle. After fixation, the skulls were excised and 1 mm thick cerebral cortex sagittal sections were cut using a brain matrix (Zivic Instruments, Pittsburgh, PA) and placed in 10 mL of EM fix. The brains were then prepared for imaging as described in (111).
2.9 EEG

All EEG analysis was performed by Dr. Allen chan in the Brain Research Centre at the Tim Murphy lab under animal protocol number: A18-0036. Methods as described previously in (112).

2.10 Statistical analysis

All data were expressed as means ± SD. Statistical analysis was performed using Prism 7.04 (GraphPad Software, CA). Differences between treatment groups were compared using unpaired Student's t-test, unless otherwise specified. P-value < 0.05 was considered statistically significant. P < 0.05*, P < 0.01**, P < 0.001***
Chapter 3: Results

3.1 Podocalyxin is required for development of endothelial barrier functions.

We assessed the role of podocalyxin in the establishment and maintenance of endothelial barrier function using a real-time electric cell-substrate impedance sensing (ECIS) system comparing primary human umbilical vein endothelial cells (HUVEC) transiently transfected with a $PODXL$-targeting siRNA ($PODXL^{KD}$) or non-targeting control siRNA (CTRL). We consistently achieved a 90% knock down of surface podocalyxin expression based on flow cytometric analysis (Figure 3.1).

![Figure 3.1 siRNA knockdown of podocalyxin in HUVEC.](image)

Knockdown assessed by flow cytometry. $PODXL^{KD}$ cells showed a 90% knockdown of podocalyxin surface expression.
HUVEC were then plated on ECIS electrodes coated with select matrix components (fibronectin, laminin or collagen). To assess cell coverage and barrier function we measured capacitance at an AC frequency ($f$) of 64 kHz or 4 kHz over 24 hours. $PODXL^{KD}$ were able to adhere and spread on fibronectin and collagen matrix but displayed significantly less electrode coverage than control cells on laminin as shown by increased capacitance over the entire 24 hour period ($f$=64 kHz) (Figure 3.2A). In addition, $PODXL^{KD}$ cells consistently display reduced transendothelial electrical resistance ($f$=4 kHz) indicative of an inability to form a mature barrier on all matrix components (Figure 3.2A). This difference was most striking when cells were plated on laminin (Figure 3.2A). Using ECIS mathematical modeling, we separated the contribution of barrier function attributable to cell-cell interactions (Rb) and cell-matrix interactions ($\alpha$) (Figure 3.2B) (110). On all three matrices, $PODXL^{KD}$ cells exhibit decreased Rb, indicative of fewer cell-cell interactions. In addition, although, there was no significant difference when plated on fibronectin, when plated on laminin and collagen, the $\alpha$ scores of control- and $PODXL^{KD}$- cells were significantly increased, indicating reduced matrix binding. We conclude that expression of podocalyxin by HUVEC is required for the formation of a functional endothelial barrier between adjacent cells and between cells and the basal matrix.
Figure 3.2 Podocalyxin promotes endothelial cell adhesion and barrier function.

(A) Electric cell-substrate impedance sensing (ECIS $Z_\Theta$) assay of barrier function of control (scrambled siRNA) and $PODXL$ knockdown ($PODXL^{KD}$) HUVEC. Cells were seeded on matrix-coated 8W10E+ PET wells at a density of $1\times10^5$ cells/well. Electrode coverage and barrier parameters were measured at multiple frequencies (f). (i) Barrier formation measured as resistance ($\Omega$) at 4 kHz. (ii) Electrode coverage measured as capacitance (nF) at 64 kHz. (iii) (B) Modeling of cell-cell interactions (Rb) (iv) Modeling of cell-matrix interactions ($\alpha$)***Significantly different than control with $p < 0.0001$ (n=4 per condition).
3.2 Podocalyxin is required for the maintenance of a functional endothelial architecture

It is possible that $PODXL$-deficient HUVEC fail to develop appropriate barriers simply due to impaired growth and spreading on matrix. To test this, we seeded control- and $Podxl^{KD}$- cells at a super-confluent density (concentration $3 \times 10^5$ cells/well on a 48-well plate) and monitored growth in real-time using video microscopy (IncuCyte®). Contrary to sensitive ECIS measurements, this method only demonstrates gross morphological changes and overall monolayer confluency. Based on observed morphology using bright-field phase contrast microscopy, $PODXL^{KD}$ cells can adhere and form a barrier by 24 hours; however, over the next three days, the consistency of the monolayer becomes progressively disrupted as the cells lose their ability to maintain appropriate spacing and cell-cell contacts (Figure 3.3). At 72 hours, only the control but not $PODXL^{KD}$ cells maintain a monolayer absent of large holes and major morphological changes. Even after plating at high density, clusters of $PODXL^{KD}$ HUVEC fail to maintain normal cell-cell attachments and instead exhibit gaps between neighbouring cells. These gaps were present regardless of the choice of matrix (fibronectin, collagen or laminin).
Figure 3.3 Podocalyxin promotes endothelial cell monolayer maintenance.

Normalized confluence of CTRL and $PODXL^{KD}$ HUVEC monolayers measured by IncuCyte ZOOM™ software over 96 hours. Error bars represent SD (n=3). **Significantly different than WT with $p<0.01$.

To better assess the interactions between cells after 72 hours in culture, we stained fixed monolayers with a fluorochrome labeled anti-VE cadherin antibody. While VE-cadherin was uniformly distributed at the cell-cell contacts in control cells plated on fibronectin, collagen or laminin, VE-cadherin on $PODXL$-deficient HUVEC cultures was sparsely distributed at rare cell-cell contact sites when present and was largely present in a cytoplasmic compartment, particularly when cells were plated on collagen (Figure 3.4). Correspondingly, fluorescence microscopy of DAPI- and phalloidin- stained monolayers revealed similar cell densities at 48 hours between of control and $PODXL^{KD}$ cells but a striking difference in the arrangement of the actin cytoskeleton (Figure 3.5). Although control cells exhibited a normal radial arrangement of polymerized actin
and stress fibers, $PODXL^{KD}$ cells exhibited a decrease in stress fiber formation and non-uniform hair-like projections extending from the cells indicative of retraction fibers. Some cells appear to have a wavy actin appearance compared to the more linear actin fibers observed in the control cells (Figure 3.6A). Furthermore, there was a decreased intensity of phalloidin staining in the $PODXL^{KD}$ cells, indicative of less total filamentous actin (F-actin) (Figure 3.5 & 3.6B). Staining of control HUVEC for β-catenin revealed prominent localization of β-catenin to cell-cell contacts. In contrast, in $PODXL^{KD}$ HUVEC, β-catenin was primarily located throughout the cell— a result consistent with the lack of cell-cell contacts, aberrant AJ formation and poor barrier function in the absence of podocalyxin (Figure 3.5). Finally, we examined the ability of $PODXL^{KD}$ HUVEC to form FA contacts to matrix. Vinculin regulates and marks protein complexes that form FA (113). We observed clear, punctate vinculin staining at the peripheral cell membranes of control cells whereas, in $PODXL^{KD}$ cells, vinculin accumulates in perinuclear structures (Figure 3.5). This disparate vinculin-staining pattern was observed for all matrices. This suggests that podocalyxin expression promotes FA complex formation in HUVEC bound to common ECM matrix components. Intriguingly, assessment of overall vinculin protein expression (by measuring staining intensity) suggests that vinculin is expressed at higher levels in $PODXL^{KD}$ compared to control cells (Figure 3.6D). Thus, vinculin localization, rather than protein expression or stability, is aberrant in $PODXL$-deficient HUVEC.
Figure 3.4 Podocalyxin expression in HUVEC is required to form monolayers with functional junctions between adjacent cells.

VE-cadherin staining (red) of WT and $PODXL^{KD}$ HUVEC 48 hours after seeding cells on the indicated matrix. Blue color is DAPI. Scale bar = 100 µM.
Figure 3.5 Podocalyxin expression in HUVEC is required for F-actin stabilization, association of β-catenin with cell junctions and formation of focal adhesion complexes.

F-actin (phallodin – green), β-catenin (red), and vinculin (aqua) staining CTRL and PODXL<sup>KD</sup> (KD) HUVEC 72 hours after seeding on the indicated matrix. Nuclei are blue (DAPI). Scale bar = 10 μM.
Figure 3.6 Podocalyxin expression in HUVEC is required for F-actin organization and focal adhesion localization.

(A) F-actin (phallodin – green), DAPI (blue) staining in CTRL and PodxlKD HUVEC 72 hour after seeding on cell culture treated plastic. (B) ImageJ quantification of F-actin staining intensity n=10 cells. (C) ImageJ quantification of cell size n=10 cells. (D) ImageJ quantification of vinculin staining intensity n=10 cells. Scale bar = 10 µM.
3.3 Podocalyxin is required for maintenance of tight junctions and BBB integrity in response to systemic inflammation.

To further assess the role of podocalyxin in maintaining endothelial barrier function in vivo, we deleted Podxl in vEC using two separate Cre-deleter strains (Cdh5-Cre and Tie2-Cre)(89, 90). Previously, we showed that Cdh5-Cre mediated deletion of Podxl (Podxl$^{\Delta\text{Cdh5Cre}}$)(104) led to a modest leakage of plasma from circulation into the lung parenchyma and that this effect was exacerbated in response to lung inflammation induced by direct intranasal exposure to LPS suggesting an underlying defect in lung microvascular integrity in the absence of Podxl on vECs (104). In this previous report however, we were not able to detect a leakage defect in any other organs in naïve or LPS-induced Podxl$^{\Delta\text{Cdh5Cre}}$ mice whether delivered intranasally or systemically.

Although the Podxl$^{\Delta\text{Cdh5Cre}}$ mice delete Podxl expression efficiently in lung vECs, they fail to delete Podxl in brain microvessels (among other vascular beds) (89). Because podocalyxin is highly expressed by BBB endothelia (102) and because TJ and AJ play a critical role in the integrity of this barrier to protect against immune-mediated neural inflammation we hypothesized that podocalyxin would play a more crucial role in regulating BBB function. We and others have previously shown that the Tie2-Cre strain is an efficient deleter of floxed genes in brain endothelia and we therefore generated Podxl$^{\Delta\text{Tie2Cre}}$ mice to study podocalyxin function in the BBB (Fig 3.7). To visualize the integrity of the brain vasculature we injected mice with FITC-LEL and TR-DEX$^{70kD}$ to mark the lumen of vascular endothelium and assess leakage of high molecular weight plasma components, respectively. We did not observe TR-DEX$^{70kD}$ in the brain parenchyma in either wild type (WT) or Podxl$^{\Delta\text{Tie2Cre}}$ mice at steady state (naïve - no LPS treatment) suggesting
appropriate BBB integrity (Figure 3.8A). However, six hours after systemic administration of LPS we observed a robust extracellular accumulation of the TR-DEX$_{70kD}$ in $\text{Podxl}^{\text{Tie2Cre}}$ but not WT mice brain tissue (Figure 3.8A). Although the dye accumulated outside of the vessel luminal space, it remained closely associated with microvasculature.

To further evaluate this disruption at the ultrastructural level, vEC in the cerebral cortex were evaluated by transmission electron microscopy (TEM). Intriguingly, there was no major observable differences in junctional complexes or in basement membrane thickness in WT and $\text{Podxl}^{\text{Tie2Cre}}$ vessels (Figure 3.9).

We conclude from these data that, although WT and $\text{Podxl}^{\text{Tie2Cre}}$ mice appear to maintain BBB permeability under steady state conditions, in response to systemic inflammation, podocalyxin is required for maintenance of the BBB.

![Figure 3.7 Deletion of Podxl in brain microvasculature using a Tie2-Cre mouse strain. Scale bars = 100 µM.](image)
Figure 3.8 Podxl expression in vascular endothelia promotes barrier function during LPS-induced inflammation.

Fluorescent micrographs of the cerebral cortex region of brains harvested from naïve and LPS-treated (5 mg/kg i.p. for 6 hours) mice. Two minutes before sacrifice FITC-LEL and TR-DEX\textsubscript{70kD} was circulated via retro-orbital route to label vessel lumens and assess vascular barrier function. Scale bars = 100 µM.
Figure 3.9 Electron micrographs of Podxl<sup>F/F</sup> and Podxl<sup>Tie2ΔEC</sup> brains

(A) Transmission electron microscopy (TEM) images showing cross sections of microvessels from cerebral cortex region of mouse brain after treatment with LPS. (B) Quantification of junctional complex length. (C) Quantification of basement membrane thickness.
3.4 A selective PAR1 peptide-agonist induces cortical spreading depression and transient suppression of electrical activity in brain of PodxlΔTie2Cre mice.

Leakage of plasma constituents into the brain parenchyma propagates neuroinflammation and neurodegeneration. At least part of this pathology is mediated by inappropriate exposure of the CNS parenchyma to normally plasma-restricted, proteases (e.g. thrombin) and proteases activated or expressed in response to inflammation (e.g. matrix metalloproteinase 9 (MMP9))(114). Thrombin is a potent inducer of acute vascular permeability and also promotes platelet activation and aggregation (115). However, in mice, PAR-1 is not expressed on platelets (116) and is instead, with respect to the neurovascular unit, is restricted to endothelial cells (117), glial cells (118) (119) and neurons (120). To more thoroughly assess the impact of disruption of the BBB on neurological function and behaviour, we treated mice with a PAR-1 selective agonist (TLFFR-NH₂) to activate PAR-1 on vascular endothelial and to determine if the peptide agonist entered the CNS parenchyma. We first primed mice with LPS for 6 hours and then administered the PAR-1 agonist (i.v., tail vein) to further enhance permeability and assess CNS response. Although we did not observe a behavioural change in PodxlFF mice, PAR-1 agonist administration to LPS-primed PodxlΔTie2Cre mice led to an immediate loss of voluntary motor control (Figure 3.10A & Appendix A (video)). These mice became completely immobile for a period lasting, on average for five minutes (Figure 3.10B). This was then followed by a rapid return to full activity and normal behaviour. During the period of immobility, PodxlΔTie2Cre mice retain ocular and pedal reflexes; normal respiration and heart rate; and, normal tail vein blood pressure (data not shown). The LPS-priming and PAR-1 agonist did not significantly increase BBB permeability above that obtained through LPS treatment alone (Figure 3.10C). Subsequent to their full recovery, PodxlΔTie2Cre mice
remained refractory to a second PAR-1 agonist treatment for at least 1 hour (not shown, summarized in Table 3.1). Finally, the PAR-1 agonist alone did not promote BBB leakage in the absence of LPS-priming in either WT or in PodxlΔTie2Cre mice (not shown).

Figure 3.10 A selective PAR-1 agonist peptide highlights the consequences of BBB dysfunction during neuroinflammation in LPS-primed PodxlΔTie2ΔEC mice.

(A) Relative activity levels of LPS-primed WT (PodxlF/F; red) and PodxlΔTie2ΔEC (blue) mice following administration of a selective PAR-1 agonist (5 mg/kg i.v.). Activity was scored by video review as follows: 1 = normal activity; 0.5 = subdued; 0 = no activity. (B) Time (s) to recovery of full activity (score = 1). Videos of sample behavior can be viewed in Appendix A. (C) Fluorescent micrographs of the cerebral cortex region of brain harvested from LPS-primed (5mg/kg i.p. 16h), PAR-1 agonist treated mice (5 mg/kg i.v. for 2min). Two minutes before sacrifice FITC-LEL and TR-DEX70kD was circulated via retro-orbital route to label vessel lumens and assess vascular barrier function. Scale bars = 100 μM.
In depth analysis and characterization of this unique phenotype was achieved through assessment of multiple mouse strains and treatment variations (summarized in Table 3.1). Briefly, the only time this phenotype was observed was with \textit{Podxl}^{ΔTie2Cre} mice. No behavioural changes were observed in \textit{Podxl}^{ΔCdh5Cre}, which still express \textit{Podxl} in the brain microvasculature or \textit{Podxl}^{ΔVavCre} mice, which do not express \textit{Podxl} in hematopoietic cells. Deletion of NHERF-1 (an intracellular \textit{Podxl} binding partner) in mice also did not show changes in behaviour after treatment with LPS and a PAR-1 agonist. Furthermore, no behavioral changes were observed in core 2 (\(\beta_1,6\)-glucosaminyl transferase (\textit{Gcnt1})) KO mice, which do not possess the ability to add O-linked glycosylations to proteins, including \textit{Podxl} and other glycocalyx mucins. Treatment of \textit{Podxl}^{ΔTie2Cre} mice with another vasoactive substance, a sphingosine-1-phosphate antagonist (\textit{W146}), caused no change in behavior, supporting the conclusion that the behavioral changes are PAR-1 agonist specific. To assess whether behavioral changes were due to increased coagulation, mice were pre-treated with acetylsalicylic acid (asprin) and clopidogrel (Plavix) to inhibit clotting and aggregration. Treatment with Aspirin and Plavix had no effect on behaviours observed.
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* β1,6-glucosaminyltransferase knockout mouse

Table 3.1 Summary of PAR-1 agonist neurological phenotypes.
To further characterize the effects of the PAR-1 agonist on \textit{Podxl\textsuperscript{ΔTie2Cre}} mice, we recorded brain electrical activity (EEG) in LPS-primed mice. We found that injection of the PAR-1 agonist induced a transient suppression of electrical activity that lasted approximately 5 minutes (Figure 3.11).

To confirm that the BBB leakage and neurological response to PAR-1 was associated with deletion of Podxl from BBB vasculature, we performed similar experiments using \textit{Podxl\textsuperscript{ΔCdh5Cre}} mice. These mice delete Podxl from most vascular beds with the notable exception of BBB endothelia and thus served as an ideal control strain (104). Histologic analyses of podocalyxin expression in the brains of \textit{Podxl\textsuperscript{ΔCdh5Cre}} and \textit{Podxl\textsuperscript{ΔTie2Cre}} mice verified our previous observation that the prominent difference between these two deleter strains is the failure to delete podocalyxin expression from the brain endothelia of ΔCdh5Cre strain (89, 104, 121) (Figure 3.7). Strikingly, BBB integrity in LPS-primed mice \textit{Podxl\textsuperscript{ΔCdh5Cre}} was identical to WT mice and these mice failed to exhibit the same inertia response to PAR-1 agonist administration. Consistent with our \textit{in vitro} results, these data suggest that podocalyxin has a functional role in maintaining BBB integrity during systemic inflammation.
Figure 3.11 A selective PAR-1 agonist peptide highlights the consequences of BBB dysfunction during neuroinflammation in LPS-primed Podxl^{Tie2EC} mice.

(A) Example EEG spectrograms of spontaneous activity from a surface electrode placed near primary somatosensory cortex indicate a transient suppression of activity following administration PAR-1 agonist (5 mg/kg i.v.) to LPS-primed (i) Podxl^{Tie2EC} but not (B) WT mouse. The time of PAR-1 agonist administration (t=0) is indicated with a dashed green line. (C) Power spectral density (mV^2/Hz) of EEG measured for 3 minute periods. Periods of EEG activity (f = 0-5 Hz) were analyzed at t<0 (baseline; black line), t=3.5 minutes (nadir; red line) and t=15 minutes (recovery (>90% of baseline); blue line). Inset, power spectral density (mV^2/Hz) over the full EEG frequency range (log_{10} scale). (D) Integration of EEG power at baseline, t=3.5 and t=15 minutes after PAR-1 agonist administration. EEG power is 0.0155 mV^2 at t=3.5 minutes compared to 0.0786 mV^2 at baseline (approx. 80% loss of activity) over 0-5 Hz frequency range.
Chapter 4: Discussion

4.1 Summary of Key Findings

In this thesis, we have extended previous podocalyxin studies to examine the functional role of podocalyxin in maintenance of vascular endothelia integrity at steady state and during inflammation. For the first time we have shown that loss of podocalyxin from endothelia in vitro leads to a striking reduction in cell spreading on matrix, poor localization of adhesive and cytoskeletal elements to the appropriate cellular microdomains and a reduced ability to form electrically resistant monolayers. Thus, our data would argue that, consistent with previous observations in transformed epithelial cell lines, in primary vascular endothelia, podocalyxin plays a critical role in proper segregation of apical and basolateral membrane structures and targeting of adhesion complexes.

Intriguingly, although we have demonstrated that podocalyxin loss cripples the ability of endothelial cells to form appropriate intact monolayers in vitro, we had previously failed to observe severe vascular defects due to podocalyxin in vivo: These mice only exhibit a modest delay in the opening of vascular lumens during embryogenesis and a modest increase in permeability in adults (104, 106, 107). Thus, in vivo there appear to be sufficiently redundant mechanisms to ensure appropriate formation of patent vessels and allow survival at steady state. For example, the multiple matrix components that make up the ECM in vivo permit redundant functional adhesion and cell-cell interactions in CNS tissue whereas the individual matrix components (laminin, collagen, fibronectin) we use in vitro limit the potential adhesion pathways and highlight defects. We therefore reasoned that in vivo adhesion mechanisms might be compromised in situations of
stress and here we have examined the vascular function of podocalyxin in maintenance of the BBB during systemic inflammation (102). Strikingly, we find that podocalyxin loss compromises the ability of the BBB to maintain integrity in response to LPS-induced inflammation. Disruptions in BBB integrity may permit the entry of disruptive plasma components in Podxl-deficient mice, as we demonstrated with the administration of the selective PAR-1 agonist.

We hypothesized that podocalyxin has a role in maintaining vascular endothelial barrier formation under steady-state conditions and in a model of sepsis. From these studies, we can support this original hypothesis and concluded that podocalyxin is required for maintaining normal endothelial barrier function.

4.2 Podocalyxin as a mediator of barrier function in vitro

Podocalyxin is a mediator of vascular barrier function in vitro and in vivo. In aim 1, we set out to assess the role of podocalyxin maintaining a normal cell monolayer in vitro. Using the ECIS ZΩ system, we were able to perform robust and accurate measurements of monolayer integrity. Although many impedance measurements can be made using this system, a select few measurements were used to assess barrier function of this monolayer. The main readout for barrier resistance is resistance at 4kHz. At this frequency, AC will flow around and between vECs and not directly through the cell membrane, making it an accurate measurement of the resistance under and between cells and not the intracellular resistance (122). By electrical resistance we observed that both CTRL and PODXL KD cells generated some adherence to the ECM. Over time, both CTRL and PODXL KD cells formed monolayers (at around 6h) and matured to their maximal resistance.
(observed when resistance stabilizes over time). This maturation is indicative of complete AJ assembly and adhesion to the basement membrane through FAs. We found that $PODXL^{KD}$ monolayers were never able to reach the same level of resistance as the CTRL counterparts. This indicates that AJ and/or FA assembly never generates a complete, fully functional barrier.

In contrast to measurements at 4kHz, at a high frequency, the AC runs intracellularly, making it a measurement of coverage, rather than barrier function (122). We found that $PODXL^{KD}$ monolayers cover the electrodes to the same extent as the CTRL monolayers on fibronectin and collagen, however, adhesion and spreading was severely impaired on laminin. We have seen similar results previously in Debruin et al. (104) where isolated lung endothelia from Podxl$^{4Cdh5Cre}$ mice had severely impaired spreading on laminin. We hypothesize that the difference we see in electrode coverage is due to the adherence of the cells to the electrode via FA complexes. This is further supported by differences in $\alpha$, which calculates integrity of cell-matrix interaction.

To determine the contribution of cell-cell (junctions) and cell-matrix (FAs) interactions to disruptions in barrier function, we used ECIS mathematical modeling to separate these measurements (110). Rb was used to assess cell-cell interactions and $\alpha$ was used to assess cell-matrix. On all three matrices, $PODXL^{KD}$ cell-cell interactions were less adhesive than CTRL. This indicates disruptions in the function of AJ complexes and their ability to maintain an effective semipermeable vascular barrier. Cell-matrix interactions were also affected by the loss of podocalyxin, on both laminin and collagen. However, in fibronectin, cell-matrix interactions were not different between $PODXL^{KD}$ and CTRL monolayers. This observation is consistent with our
previous observation that *Podxl*-deficient mouse lung endothelial cells can adhere and spread effectively on fibronectin, less effectively on collagen and are strikingly impaired on laminin (104).

Finally, the gross assessment of monolayer maintenance was observed through light microscopy imaging over 96h using the Incucyte Zoom. Through measurement of monolayer confluence, we observed that, over time, monolayer disruptions caused by loss of podocalyxin become increasingly severe. This suggests that podocalyxin does not only have a role in initial barrier formation but in barrier maintenance as well.

### 4.3 Podocalyxin and the actin cytoskeleton

We hypothesize that podocalyxin’s interaction with actin allows it to affect cell adhesion molecules and barrier function. Podocalyxin sequesters NHERF-1 and phosphorylated ezrin to the apical domain through a RhoA-ROCKI-Ezrin feedback loop (105). Furthermore, ERM binding proteins are known to regulate endothelial vascular permeability induced by thrombin by phosphorylation of a conserved threonine residue (123). We postulate that loss of podocalyxin causes disruptions in localization of adhesion proteins: both cell-cell (AJs and TJs) and cell-matrix (integrins). Overexpression of podocalyxin in an epithelial cell line induces localization of β1-integrins to the basolateral domain (111). In kidney podocytes, expression of podocalyxin induces TJ migration between foot processes (111). Finally, expression of podocalyxin in the developing aorta causes the migration of AJs and TJs away from the developing lumen towards cell-cell contact sites (106). Now, we have shown a similar phenotype in adult vascular endothelial cells in
vitro and in vivo. Loss of podocalyxin induces changes to the morphology of the actin cytoskeleton. Not only did we observe a decrease in overall F-actin levels, we also saw major changes to F-actin skeletal rearrangement. In some cases, F-actin filaments appeared to have a wavy appearance. Often, we observed the F-actin rim was more intensely stained, yet there were fewer stress fibers seen throughout the entire cell. We also observed retracted cells with hair-like projections. By measuring total cell area, we observe that cells have retracted and are less spread out than their CTRL counterparts. We suggest that with podocalyxin, Ezrin is localized to the apical membrane where it can stabilize F-actin. When podocalyxin is lost, Ezrin is unable to localize to the apical membrane and tether F-actin.

By sequestering ezrin to the apical domain, podocalyxin regulates FAs and cell-cell junctions. By destabilizing cytoskeletal rearrangement, integrins are not stabilized in FAs at the basolateral cell domain where they can bind ECM proteins to regulate cell survival and barrier maintenance. In parallel with this hypothesis, we observe changes in localization of FAs when podocalyxin is lost. Furthermore, we see an increase in vinculin staining throughout the cell, as opposed to localization to FA complexes. We hypothesize this is due to a compensatory mechanism where vinculin expression is upregulated when it is mislocalized.

In summary, we find that podocalyxin promotes the formation of AJ between cells, the organization of the cortical actin cytoskeleton, and the generation of FA between cells and the ECM matrix. Podocalyxin thus has a critical role in the regulation of endothelial cell architecture and matrix-adhesion required to form a functional barrier.
In vECs of the BBB, it has been observed that during stroke, hyperpermeability is partially due to shedding and degradation of β1-integrins (39). The loss of β1-integrins reduces the association of catenins with VE-cadherin at the AJ complex. This dissociation induces the internalization of VE-cadherin. We see something similar with loss of podocalyxin expression in HUVEC. In HUVEC, loss of podocalyxin leads to loss of AJs. At 48h after cell seeding, we observed the loss of VE-cadherin at junctional complexes and more uniform distribution throughout the cell. At 72h, we see complete loss of AJ protein β-catenin. Therefore, our work highlights a potential link between podocalyxin and BBB in stroke and we hypothesize that podocalyxin could play a role in stroke mediated vascular barrier disruption.

4.4 *In vitro vs in vivo barrier function*

Although ubiquitous deletion of podocalyxin is neonatal lethal, two different Cre-lox deletions of *PODXL* (*Cdh5*-*cre* and *Tie2*-*cre*) yield viable mice with no gross morphological defects. This poses the question, “If deletion of *PODXL* in HUVEC demonstrates such a striking defect in vascular barrier function, how can vascular deletion of *PODXL* in mice yield viable animals?” There are likely several factors that could explain this discrepancy. Generally, unlike an *in vivo* environment, *in vitro* studies were performed on individual matrices which we hypothesized would amplify adhesion defects. Furthermore, *in vitro* studies use vECs alone to focus in on endothelial function, however, in a blood vessel, other cells can stabilize and contribute to barrier function, for example in the BBB where cells of the neurovascular unit can help maintain barrier function.

Next, *in vitro*, we observe that defects in barrier function are exacerbated over time. *In vivo*, the adult vasculature is non-proliferative under steady state. Therefore, inflammatory stimuli may be
required to observe podocalyxin induced defects in vascular permeability. For example, when challenged with VEGF, TNFα or thrombin, vinculin and F-actin bundles are observed at sites of distinct remodeling VE-cadherin junctional complexes. Vinculin- catenin complexes can provide mechanical stability of these VE-cadherin complexes under inflammatory conditions (124). To prevent the opening of these junctional complexes, F-actin bundles are required. Therefore, in the absence of podocalyxin, challenging vECs with vasoactive substances would show increases in vascular permeability that would normally be prevented via F-actin mechanical forces.

4.5 Podocalyxin mediates vascular permeability in the blood-brain barrier

In aim 2 we wanted to assess the role of podocalyxin in maintaining BBB in vivo. Our in vitro data, as well as previous studies in the lung (104), suggest that podocalyxin has a role in maintaining vascular barrier function in the mouse. The BBB is one of the most impenetrable vascular barriers in the body and is critical for healthy functioning of brain tissue. Furthermore, podocalyxin is expressed on all vascular endothelial cells, but is most highly expressed on vEC of the BBB (102). This suggests that podocalyxin could be more critically important for brain vEC function. Although in vitro studies used HUVEC and not brain endothelial cells and therefore have a less organized expression of TJ proteins, we recognized the interplay of AJ and TJ proteins and postulated that podocalyxin would still be critical for brain vEC junction formation. As discussed in (39), AJ assembly in the BBB is required for the maintenance of TJs. For example, under inflammatory conditions, internalization of VE-cadherin at the BBB induces downregulation of the TJ protein claudin-5.
At steady-state, $Podxl^{ΔTie2Cre}$ mice appear to maintain normal barrier function. This was assessed using a 70kDa dextran. We postulate that a smaller probe may reveal minor disruptions to the BBB, however we can conclude that there is no observable permeability to a 70kDa dextran. As we hypothesized, systemic treatment of mice with LPS induced leakage of a 70kDa dextran in the brains of $Podxl^{ΔTie2Cre}$ mice but not in WT mice. In models of severe BBB breakdown (i.e. experimental autoimmune encephalomyelitis), large clouds of dextran would be observed throughout the parenchyma (125). In our model, the leakage is much subtler. Dextran leakage was observed in $Podxl^{ΔTie2Cre}$ tissue situated tightly around the blood vessel and not distal to the endothelial barrier. To assess the formation of junctional complexes in the BBB, we performed TEM imaging of cross sections of microvessels of $Podxl^{ΔTie2Cre}$ and WT mice treated with LPS. TEMs revealed no observable changes to ultrastructural junction formation. Although dextran experiments do reveal a disruption of the BBB, $Podxl^{ΔTie2Cre}$ mice maintain a BBB under steady state conditions, and so perturbations in junction stability would most likely be subtle. Furthermore, complete deletion of claudin-5, the main tight junction protein of brain endothelia, shows only minor changes in tight junction complexes overserved by electron microscopy (43), suggesting that even drastic junctional complex alterations do not show major ultrastructural changes.

4.6 PAR-1 in sepsis and vascular barrier function

To test the integrity of the BBB in LPS-primed mice, we administered a selective PAR-1 agonist. The thrombin receptor PAR-1, has been shown to be highly expressed on neuronal and glial cells within mouse and human brain (126). Activation of PAR-1 in neural tissue has been shown to
promote survival of neurons but also to regulate neurodegeneration and neuroprotection in experimental models of stroke and brain injury (20) and is upregulated in experimental models of brain ischemia (127). In our study, we decided to treat mice with a PAR-1 agonist, for two reasons; firstly, to mimic, in part, the systemic coagulation that occurs during sepsis and secondly to use the agonist as a small molecular weight probe to assess the functional role of the BBB under inflammatory conditions.

Treating PodxlΔTie2Cre mice with the PAR-1 agonist induced a 5-minute period of unresponsiveness, followed by recovery to full activity. In depth analysis and characterization of this unique phenotype was achieved through assessment of multiple mouse strains and treatment variations (summarized in Table 3.1). The only mouse strain that displayed this phenotype was PodxlΔTie2Cre mice. No behavioral changes were observed in PodxlΔCdh5Cre. This transgenic strain also deletes Podxl in the vasculature with the main difference being that it still expresses Podxl in the brain microvasculature. The other difference between Cdh5-cre and Tie2-cre mice is the deletion in the hematopoietic lineage. Although Podxl is not known to be expressed in adult hematopoietic cells, to confirm the PAR-1 induced neurological phenotype was not due to a hematopoietic deletion, a PodxlΔVavCre mouse strain was treated with LPS and PAR-1 agonist and no changes in behavior were seen. Deletion of NHERF-1 (an intracellular podocalyxin binding partner) in mice also did not show changes in behavior after treatment with LPS and a PAR-1 agonist. Furthermore, no behavioral changes were observed in core 2 KO (β1,6-glucosaminyl transferase 1 KO) mice, which do not possess the ability to add O-linked glycosylations to proteins, including podocalyxin and others of the glycocalyx. This suggests that vascular barrier
function in these mice is not due to the negative charge of the extracellular domain like what has been the proposed mechanism of aorta lumen formation and podocyte foot process formation.

Treatment of $Podxl^{ΔTie2Cre}$ mice with another vasoactive substance, a sphingosine-1-phosphate antagonist (W146) caused no change in behavior, supporting the conclusion that the behavioral changes are PAR-1 agonist specific. To assess whether behavioral changes were due to changes in coagulation, mice were pre-treated with Aspirin and Plavix to prevent clotting. Treatment with Aspirin and Plavix had no effect on behaviours observed. Other measurements were taken to characterize potential other non-neurological causes of the PAR-1 induced phenotype. There were no observable differences in blood pressure, glucose levels, heart rate, or body temperature.

Finally, we assessed their neurological response. Treatment with a PAR-1 agonist led to transient suppression of EEG and loss of motor function for a 5 minute period followed by a full recovery. From this we conclude that active neuroinflammation in $Podxl^{ΔTie2Cre}$ mice permits entry of a selective PAR-1 agonist peptide into the brain parenchyma and causes a transient suppression of cerebral cortex electrical activity. The fact that this pathology is only observed in mice lacking Podxl on the brain endothelia (rather than mice lacking podocalyxin on other vascular beds) would argue that this phenotype reflects a selective role for podocalyxin in BBB function. We propose that expression of podocalyxin has a critical neuro-protective role by maintaining BBB integrity during acute neuroinflammation.
4.7 Future directions

Overall, we conclude that podocalyxin has a role in maintaining vascular barrier function \textit{in vitro} and \textit{in vivo} in the BBB. To confirm hypotheses regarding ezrin localization, we should visualize ezrin within a cell. High magnification confocal microscopy of vEC cross sections +/- podocalyxin could show disruptions in ezrin localization. Next, \textit{in vitro} studies could be completed with brain endothelial cells to characterize disruptions in localization and expression of TJ proteins. For \textit{in vivo} studies, inducing BBB breakdown in WT mice and then treating them with a PAR-1 agonist could confirm the BBB specificity of the behavioral phenotype. The next step would be development of podocalyxin targeting drugs that could be administered under inflammatory conditions to protect vascular barrier disruption during disease.

4.8 Conclusion

This thesis provides a novel explanation of podocalyxin and its role in maintaining vascular endothelial barrier function. Although podocalyxin is highly expressed in vECs, previous literature offers minimal explanation of its role or function. Here, we are the first to directly measure barrier function via ECIS and the first to implicate podocalyxin as a mediator of BBB permeability. By elucidating a role for podocalyxin in regulation of BBB vascular integrity, we can acknowledge a number of interesting therapeutic implications. BBB function is critical to the healthy function of the CNS. Hyperpermeability of this barrier is known to contribute to the pathology of a number of acute inflammatory diseases including traumatic brain injury and stroke. Similarly, in chronic neurodegenerative disease including multiple sclerosis, ALS, Parkinson’s disease, meningitis and Alzheimer’s disease, accumulating evidence suggest declining BBB integrity is a harbinger of
poor outcome or, indeed, an initiating insult. Thus, enhancing podocalyxin-dependent integrity may prove therapeutic in both acute and chronic disease. Alternatively, a major block to therapeutic drug delivery to the CNS is the inability of most agents to cross the BBB. Thus, a transient down modulation of podocalyxin-dependent BBB integrity could offer an opportunity for transient drug delivery. Ultimately, we hope to use the knowledge gained in this study to improve clinical outcomes for patients with inflammatory-mediated vascular barrier diseases.
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Appendix A

Video observation of behavioral changes observed after administration of PAR-1 agonist

Description:
Video analysis of mice described in methods section 2.7.1. Video analysis started immediately after i.v. injection with PAR-1 agonist. Two subsequent mice were observed in the video; one WT, one $Podx^{Tie2Cre}$ respectively. Activity was scored by reviewing videos based on total motion observed at 10 s intervals (with 1=normal, 0.5=reduced activity and 0=no activity) and time for full recovery (sustained return to score = 1).

Filename:

ube_2018_september_cait_jessica_video.mp4