Septic Murine Pulmonary Microvascular Endothelial Cell Barrier Dysfunction is Regulated by Tissue Inhibitor of Metalloproteinases 3

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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SEPTIC MURINE PULMONARY MICROVASCULAR ENDOTHELIAL CELL BARRIER DYSFUNCTION IS REGULATED BY TISSUE INHIBITOR OF METALLOPROTEINASES 3

(Thesis format: Monograph)

by

Valerie Arpino

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Sepsis causes dysfunction of pulmonary microvascular endothelial cells (PMVEC) leading to severe pulmonary edema. Metalloproteinases regulate endothelial function through processing of cell surface proteins, which can be associated with increased permeability. Tissue inhibitor of metalloproteinases 3 (TIMP3) regulates metalloproteinase activity in the lung following injury. Thus, we hypothesize TIMP3 promotes PMVEC barrier function through inhibition of metalloproteinase activity.

PMVEC were isolated from WT and Timp3−/− mice. TIMP3 levels (mRNA and protein) were decreased in WT PMVEC under septic conditions. Analysis of leak (transendothelial electrical resistance, dextran, and albumin flux) revealed Timp3−/− PMVEC had significantly higher permeability under resting conditions vs. WT PMVEC. Increased basal Timp3−/− PMVEC permeability was associated with disrupted surface vascular endothelial-cadherin localization, both of which were rescued by treatment with GM6001, a synthetic metalloproteinase inhibitor.

Our data suggest TIMP3 supports normal PMVEC barrier function, and septic downregulation of TIMP3 may be an important contributor to septic PMVEC barrier dysfunction.

Keywords
Sepsis, Acute Respiratory Distress Syndrome, capillary, pulmonary microvascular endothelial cells, metalloproteinases, tissue inhibitor of metalloproteinases 3, intercellular junctions, VE-cadherin, microvascular barrier function
Co-Authorship Statement

All studies and data included in this thesis report were generated by Valerie Arpino, except for data presented in Chapter 2 (Figure 2-2), which was generated and kindly provided by Lefeng Wang.
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<th>Description</th>
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<tr>
<td>ADAM</td>
<td>A Disintegrin And Metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A Disintegrin And Metalloproteinase with Thrombospondin Motifs</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal Ligation and Perforation</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EB</td>
<td>Evans Blue</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-Colony Stimulating Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HMVEC-L</td>
<td>Human Lung Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
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<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>JAMs</td>
<td>Junctional Adhesion Molecules</td>
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<tr>
<td>JMD</td>
<td>Juxtamembrane Domain</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin Light Chain</td>
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<tr>
<td>MLCK</td>
<td>Myosin Light Chain Kinase</td>
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<tr>
<td>MLCP</td>
<td>Myosin Light Chain Phosphatase</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MT-MMP</td>
<td>Membrane Type-Matrix Metalloproteinase</td>
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<tr>
<td>MVEC</td>
<td>Microvascular Endothelial Cells</td>
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<tr>
<td>NFκB</td>
<td>Nuclear Factor κB</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen Activator Inhibitor</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease Activated Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PECAM</td>
<td>Platelet Endothelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Neutrophil</td>
</tr>
<tr>
<td>PMVEC</td>
<td>Pulmonary Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-Selectin Glycoprotein Ligand</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>ROCK</td>
<td>RhoA Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative Quantity</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumor Necrosis Factor-α Converting Enzyme</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin Activatable Fibrinolysis Inhibitor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TEER</td>
<td>Transendothelial Electrical Resistance</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VE</td>
<td>Vascular Endothelial</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona Occludens</td>
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Chapter 1

1 Introduction

1.1 Acute Respiratory Distress Syndrome

The pulmonary system consists of a set of paired lungs in addition to a series of air passages both to and from the lungs, known as respiratory bronchi and distributing bronchioles [1]. The main physiological functions of the pulmonary system include air conduction and filtration as well as gas exchange [1]. Within the lungs, respiratory bronchioles become increasingly smaller until the smallest air spaces, known as the alveoli, are reached [1]. Each alveolus is surrounded by a network of blood capillaries [1]. The intimate relationship between the alveoli and pulmonary capillaries allows for gas exchange to occur [1]. In other words, deoxygenated blood within the pulmonary capillaries that surround the alveoli becomes oxygenated through the diffusion of inhaled oxygen from the alveoli to capillaries, while waste carbon dioxide (CO₂) from deoxygenated blood diffuses in the opposite direction into the alveoli and is exhaled [1].

Acute respiratory distress syndrome (ARDS) is a common critical illness characterized by profound pulmonary inflammation, proteinaceous edema, and impaired gas exchange, ultimately leading to hypoxemia and in some cases, death [2]. ARDS may be caused by various direct and indirect pulmonary insults including, but not limited to, pneumonia, gastric acid aspiration, and severe sepsis [1]. Patients present with diffuse neutrophilic alveolar infiltrates accompanied by the accumulation of protein-rich pulmonary edema as the result of injury and dysfunction of both the pulmonary microvascular capillary endothelium and alveolar epithelium [2,3]. Specifically, ARDS is
clinically defined by the acute onset of symptoms within 7 days of injury or infection, severe hypoxaemia (PaO$_2$/FiO$_2$ ≤ 300mmHg), diffuse bilateral pulmonary infiltrates present in a frontal portal chest radiograph, and pulmonary edema in the absence of left arterial hypertension [2,3].

The alveolar-capillary barrier within the lung is composed of the alveolar epithelium, interstitium, and pulmonary microvascular endothelium [3]. Following the injurious direct or indirect stimulus to the lung, the release of pro-inflammatory cytokines and chemokines promotes the influx of circulating inflammatory cells, such as polymorphonuclear neutrophils (PMNs) and macrophages, via adhesion to and migration through the microvascular endothelium [2,3]. This persistent inflammation results in the dysfunction of the alveolar-capillary barrier. In particular, microvascular endothelial cell (MVEC) dysfunction is characterized by a loss of structural integrity and increase in barrier permeability [2,3]. This results in the accumulation of protein-rich edema in the interstitial space, which ultimately crosses the injured epithelial barrier into the alveoli, impeding proper gas exchange and oxygenation of the pulmonary capillaries (Figure 1-1) [2,3].

While no treatment currently exists for ARDS, mortality rates associated with ARDS have decreased in recent years due to refinements in mechanical ventilation and fluid management [2,3]. However, approximately 40% of ARDS cases remain fatal, and of those patients that do survive, persistent inflammation and fibrosis result in long-term pulmonary complications [1]. Thus, new approaches aimed at better understanding and targeting the underlying pathophysiologic causes of ARDS are critical in developing future novel therapeutic interventions for patients.
Figure 1-1: Simplified schematic representation of the injured alveolus during ARDS. During ARDS, an overwhelming inflammatory process occurs characterized by the activation of both circulating (e.g. neutrophils) and tissue resident (e.g. macrophages) inflammatory cells, which results in the enhanced production and release of a plethora of inflammatory mediators including pro-inflammatory cytokines, oxidants, and proteases. The influx of circulating inflammatory cells occurs via adhesion to and migration through the microvascular endothelium. Persistent inflammation results in injury and dysfunction of pulmonary microvascular endothelial cells, which is characterized by a loss of structural integrity and increase in barrier permeability. This results in the accumulation of protein-rich edema in the interstitial space, which ultimately crosses the injured epithelial barrier into the alveoli, impeding proper gas exchange and oxygenation of the pulmonary capillaries. Adapted from Ware and Matthay, 2000, *N Engl J Med.*
1.2 Sepsis

Sepsis is a common life-threatening condition associated with significant morbidity and a 30-50% mortality rate, [2,4,5]. The disease is characterized by a systemic inflammatory response to infection, often resulting in multiple organ dysfunction and failure, most notably within the pulmonary, cardiac, and renal systems [2,4,5]. In particular, sepsis is known to be the principle indirect cause of ARDS [2]. The condition is referred to as severe sepsis if organ dysfunction does in fact occur, whereas septic shock defines cases of severe sepsis accompanied by acute circulatory failure characterized by persistent systemic arterial hypotension [4]. With a staggering 18 million cases of severe sepsis worldwide each year, increased hospital resources are required along with prolonged stays in intensive care units (ICU) resulting in the consumption of up to 45% of total ICU costs [4,5]. Despite intensive basic and clinical research, treatment of sepsis and related organ dysfunction widely consists of supportive care [2].

1.3 Microvascular Endothelial Cells

Septic organ dysfunction is due to an overwhelming systemic inflammatory process characterized by the activation of circulating and tissue-resident inflammatory cells, as well as the enhanced production and release of various inflammatory mediators including pro-inflammatory cytokines such as tumour necrosis factor (TNF) α, and interleukin (IL) 1β [4–6]. Organ dysfunction is also largely associated with altered vascular function, including changes in blood flow due to disrupted systemic hemodynamics, as well as injury and dysfunction of the microvasculature [6–10].
The inner lining of all blood vessels within the cardiovascular system consists of a monolayer of endothelial cells, also known as the endothelium, which serves as a selectively permeable barrier between the systemic circulation and surrounding tissue [1,8,11–13]. The microvasculature is the smallest system of blood vessels within the cardiovascular system and includes arterioles, postcapillary venules, and capillaries [1]. In addition to the inner endothelial monolayer, blood vessels, including arterioles and postcapillary venules within the microvasculature, consist of multiple layers composed of smooth muscles cells, connective tissue, and pericytes [1]. Capillaries, however, possess the smallest diameter of all the blood vessels, as their structure only consists of the single endothelial monolayer [1]. Due to their thin wall structure and close association with metabolically active cells and tissues, capillaries are very well suited for the exchange of gases, metabolites, and fluids [1]. Particularly, within the pulmonary circulation, blood is delivered by the pulmonary artery to the capillaries surrounding the alveolus, allowing for gas exchange and oxygenation of the blood to occur [1]. Oxygenated blood is then collected by the postcapillary venules and returned to the heart [1].

The endothelial cell is a multifunctional cell associated with several basal and inducible physiological functions. In addition to its role in maintaining a selectively permeable barrier, the endothelium is involved in angiogenesis, the maintenance of a nonthrombogenic barrier, modulation of blood flow and vascular resistance, and the regulation of host immune responses via cellular trafficking and release of pro-inflammatory and anti-inflammatory mediators [1,6,8]. Importantly, endothelial participation in host immune responses is achieved by leukocyte adhesion to the endothelial surface via upregulation of endothelial adhesion molecules, in addition to the
secretion of various cytokines and chemokines [1,8].

1.3.1 MVEC Barrier Function

Dynamic regulation of endothelial permeability occurs in order to control the exchange of plasma proteins, solutes, and liquid between the bloodstream and surrounding tissues via the paracellular and transcellular pathways [1,8,11–13]. The paracellular pathway consists of transport through the intercellular space between adjacent endothelial cells, whereas the transcellular pathway, also known as transcytosis, consists of active receptor-mediated transport through the plasma membrane of the cell itself [1,11–13].

Under basal conditions, the endothelial barrier is known to be restrictive or selectively permeable as only small molecules with a molecular radius of up to 3nm (e.g. ions, urea, glucose) are able to passively cross the barrier paracellularly [1,11]. Macromolecules with molecular radii larger than 3nm must be actively transported transcellularly via membrane receptor/vesicle-mediated transcytosis [1,11,13,14]. The vesicular carriers present within the transcellular pathway are known as caveolae, defined as small invaginations or microdomains within the plasma membrane that are rich in cholesterol and glycosphingolipids [11,13,14]. Caveolin-1 is the principal protein constituent of caveolae and coats the inner surface, or cytoplasmic side, of the plasma membrane [11,14]. The binding of plasma proteins to their appropriate receptor located within the cell surface caveolae initiates association of the membrane-bound receptor with caveolin-1, and subsequent phosphorylation of caveolin-1 and dynamin-2 (responsible for the scission of the newly formed vesicle) by Src kinases [11,14]. Fission of caveolae and internalization of membrane-bound macromolecules then occurs, and the
formed caveolae vesicles migrate through the cytoplasm to the basal membrane, where the contents are then released by exocytosis [11,14]. In particular, caveolae-mediated transcytosis is the mechanism responsible for albumin transport, via binding of albumin to its receptor albumin-binding glycoprotein (gp60) present on the cell membrane [11,14].

1.3.2 MVEC-MVEC Interactions

Interaction of adjacent MVEC through formation of adherens and tight intercellular junctions is one of the key factors regulating microvascular endothelial permeability [7,11–13,15–17]. These complex transmembrane structures contain multiple cell surface proteins belonging to families of ubiquitously expressed cell adhesion molecules, including cadherins, claudins, and occludins [11–13,15–17]. Adherens junctions mainly facilitate the formation of cell-cell contacts and ensure their proper maturation, while tight junctions act to regulate the passage of small ions and solutes via the paracellular route [16]. Gap junctions involved in the direct passage of signalling molecules and ions between adjacent cells are also present in the endothelium, but are not known to play a role in the regulation of endothelial permeability [1,12]. In addition to mediating paracellular interactions, the adhesive molecules present in adherens and tight junctions interact with an intracellular network of cytoskeletal proteins in order to further regulate endothelial cell morphology and integrity [11–13,16,18].

Adherens junctions account for the majority (approximately 80%) of the intercellular junctions present in the endothelium, while the remaining 20% consists of tight and gap junctions [11,13]. Exceptions do occur, however, in endothelia, such as arterial endothelium and brain capillaries, that must maintain a strict control of
permeability to ions and solutes, and therefore are predominately enriched with tight
junctons [15]. Vascular endothelial (VE)-cadherin is the primary transmembrane
structural adhesive protein present in endothelial adherens junctions, as it plays a
principal role in the assembly of adherens junctions and maintenance of a restrictive
endothelial barrier [11–13,15–17]. Previous studies have demonstrated that deletion of
VE-cadherin in mice (VE-cadherin<sup>−/−</sup>) is embryonically lethal due to incomplete vascular
development [11]. VE-cadherin consists of an extracellular fragment composed of five
cadherin-like repeats, that are responsible for multimeric interactions between endothelial
cells as they associate homotypically with the VE-cadherin extracellular domain on the
adjacent cell in a calcium (Ca<sup>2+</sup>)-dependent manner [11,13,19]. Additionally, VE-
cadherin contains a short transmembrane domain, and two cytoplasmic domains. The
first, known as the juxtamembrane domain (JMD), binds p120-catenin, while the other C-
terminal domain binds plakoglobin (also known as γ-catenin) along with β- and α-
catenin, which directly link VE-cadherin to the actin cytoskeleton [11–13,15,16].

Inter-endothelial tight junctions are formed by the homotypic interaction between
occludins, junctional adhesion molecules (JAMs), and claudin-5, which is the only
member of the claudin family that is expressed in endothelial cells [11,13,15,16]. Zona
occludens (ZO) 1-3 encompass the main scaffold proteins present in the endothelial
cytoplasm that tether occludins, JAMs, and claudin-5 to the actin cytoskeleton in order to
further establish cellular integrity and a functional barrier as seen in adherens junctions
[11,13,15,16]. Within the pulmonary microvasculature, ZO1 and JAMA are
predominately expressed [20]. Therefore, both adherens and tight junctions play critical
roles in facilitating adjacent MVEC interactions in order to form an intact and functional
restrictive barrier.

1.3.3 Structural Determinants of the Microvasculature: The Glycocalyx and Extracellular Matrix

Structural determinants that play a role in the maintenance of endothelial barrier function include the endothelial glycocalyx and extracellular matrix. Within the microvasculature, the luminal surface of the endothelial monolayer is lined with a mesh-like framework which forms a substantial surface layer critical to barrier function, known as the glycocalyx [11,21]. The glycocalyx is predominately composed of sialic acid-containing glycoproteins and proteoglycans, including syndecans and glypicans, in addition to glycosaminoglycans (GAGs) [1,11,21]. Interestingly, the pulmonary glycocalyx is considerably thicker than those present within the systemic microvasculature [21]. The glycocalyx resides directly between circulating inflammatory cells and the endothelial surface [11,21]. Thus, it plays a significant role in PMN adhesion and extravasation under inflammatory conditions, as it regulates the exposure of endothelial adhesion molecules in addition to the release of PMN-specific chemokines [11,21]. Furthermore, inflammatory conditions, such as severe sepsis and the resulting ARDS, present with increased plasma concentrations of fragmented GAGs, suggesting an association of glycocalyx degradation with inflammation-induced endothelial hyperpermeability [21,22]. Previous studies have shown that glycocalyx degradation is directly associated with the activation of endothelial heparanase, a glucuronidase responsible for the hydrolysis and resulting degradation of heparan sulfate, one of the principle GAGs found within the glycocalyx [21,23]. Heparanase-mediated glycocalyx degradation allows for the exposure of endothelial surface adhesion molecules, such as
intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), and the subsequent adhesion and extravasation of circulating PMNs [21,23]. Within the pulmonary microvasculature, however, it remains uncertain whether the glycocalyx plays a role in maintaining a semi-permeable barrier to fluid and proteins. Previous studies using isolated mouse and rat lungs perfused with 4% Evans Blue-labelled albumin found that neither fluid nor protein transendothelial flux occurred following glycocalyx degradation [24]. Additionally, in vivo studies have demonstrated that no increase in pulmonary edema is found following intravenous injection of heparanase [21].

The extracellular matrix (ECM) is also known to be a structural component required for the maintenance of endothelial barrier integrity. The ECM is a complex network that provides mechanical and structural support for cells, in addition to providing cues for extracellular signalling from both cryptic or concealed ECM fragments and sequestered growth factors [1]. The ECM is mainly composed of collagens, elastic fibers, proteoglycans including versican and aggrecan, glycoproteins including fibronectin and laminin, and GAGs including keratan sulfate and hyaluronan [1,11]. During vasculogenesis and angiogenesis, ECM assembly is initiated by endothelial cell synthesis and secretion of laminin polymers, anchored to the basolateral surface of the cell by $\beta_1$-integrins, which then associate and bind to collagen IV polymers in order to construct a structural framework onto which other ECM proteins are assembled to produce the basement membrane [25]. Endothelial cells bound to the ECM are generally quiescent, as the association of the ECM with integrins present on the basolateral surface of endothelial cells allows for the generation of signals that inhibit cell proliferation and
migration, while stabilizing adjacent cell and endothelial-ECM adhesion, which are both vital in the formation and preservation of a restrictive endothelial barrier [25]. There is extensive support for this phenomenon, as studies that have employed the use of trypsin infusion on isolated sheep lungs reveal a significant increase in endothelial permeability as shown by increased macromolecular flux, as the result of degradation of ECM-derived fibronectin [11,26]. Additionally, *in vitro* studies with cultured endothelial monolayers have revealed increases in albumin transendothelial flux following fibronectin degradation and release [27,28], with treatment with plasma fibronectin rescuing this increase in endothelial permeability [29]. The cleavage and degradation of other ECM protein constituents, including hyaluran and aggrecan, have also been shown to augment endothelial permeability [30,31]. Particularly, enzymatic degradation of such ECM constituents by matrix metalloproteinases (MMPs), including MMP2 and MMP9, have resulted in increased permeability in cultured endothelial monolayers as well as induced pulmonary edema [32,33]. Thus, these findings suggest a crucial role of the ECM in maintaining endothelial integrity and barrier function.

1.4 Septic Microvascular Endothelial Cell Dysfunction

Dysfunction of the microvasculature, in particular the pulmonary microvasculature in sepsis-induced ARDS, is primarily characterized by enhanced PMN-microvascular interactions, upregulation of pro-thrombotic pathways, and a loss of pulmonary microvascular barrier function [10–12,15,16,34]. The loss of pulmonary microvascular barrier function is accompanied by increased permeability due to pulmonary microvascular endothelial cell (PMVEC) injury, dysfunction, and death following sepsis [10–12,15,16,34].
1.4.1 Activation of Inflammatory and Coagulation Pathways

The pathophysiology of sepsis, including hypotension, coagulopathy, and multiple organ dysfunction, is not related to the invading pathogen causing the initial infection itself, but rather the surplus of host-derived inflammatory mediators that cause systemic inflammation [35]. The inflammatory process that takes place under septic conditions relies on the release of pro-inflammatory cytokines and chemokines in addition to PMN-MVEC interactions. The initial immune response and secretion of pro-inflammatory cytokines, including TNFα, IL1β, and interferon (IFN) γ, by mononuclear cells is stimulated by bacterial cell wall components present on the invading pathogen, such as lipopolysaccharide (LPS) [35–37]. Upon MVEC interaction with these pro-inflammatory cytokines, basal functions are altered as the endothelium becomes activated. Endothelial activation encompasses the expression of inflammatory adhesion molecules, release of inflammatory mediators including cytokines and chemokines, production of vasoactive agents, the switch from an anti-thrombotic to pro-thrombotic surface, and an increase in MVEC barrier permeability [38].

PMNs are an essential part of the innate immune system that eliminate pathogenic bacteria through phagocytosis and subsequent degradation via the production of proteolytic enzymes and reactive oxygen species (ROS) [36]. However, these factors can be released into the infected tissue resulting in local damage and persistent inflammation [36]. In sepsis-induced ARDS, the vast number of invading PMNs is associated with high levels of PMN-derived proteolytic enzymes within the broncoalveolar lavage, resulting in tissue damage and impaired lung function [39]. Under basal conditions, platelet endothelial cell adhesion molecule (PECAM) 1, as well as ICAM1 (whose expression is
additionally upregulated by cytokine stimulation), are constitutively expressed on the endothelium [36–38]. However, following activation by pro-inflammatory cytokines, endothelial cells release granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) in order to stimulate the production, activation, and release of monocytes and granulocytes, including PMN from the bone marrow into the bloodstream [36–38]. Additionally, chemotactic stimuli released from the site of infection, such as IL8, actively recruit PMN to the sites of infection where they adhere to the endothelium in order to migrate into the surrounding tissue [8,36–38].

Adherence of PMNs to the endothelium is a highly controlled process associated with the sequential activity of selectins and integrins. Present on the PMN surface, L-selectin recognizes and binds to specific oligosaccharide motifs associated with endothelial glycoproteins, whereas E-selectin and P-selectin, which are present on the endothelium, bind to specific carbohydrate motifs found on PMN cell surface [36–38]. These interactions allow the PMNs to make further contact with inflammatory mediators produced by the endothelium, such as platelet-activating factor (PAF), while upregulating β1 and β2 integrins, which bind to endothelial ICAM1 and VCAM1, respectively, before subsequently migrating through the endothelium and into the surrounding tissue [8,35–38,40]. Both ICAM1 and VCAM1 expression on the endothelial cell surface is induced by the release of pro-inflammatory cytokines from macrophages at the site of infection, including TNFα and IL1β [36–38,40]. In ARDS specifically, once adhered to the endothelium, PMNs respond to chemotactic stimuli and migrate through the endothelial barrier, pass through the interstitial space, and then continue to migrate across the alveolar epithelial barrier into the alveolar space [39]. Once activated, PMNs release
proteolytic enzymes and ROS into the extracellular space, which can result in tissue damage [36,39].

The inflammatory and procoagulant responses that occur during sepsis are very closely related. In addition to their role in inflammation and the recruitment of activated leukocytes, cytokines, such as TNFα and IL1β, and bacterial components, including LPS, activate coagulation via stimulating the release of tissue factor (TF) from circulating monocytes and the endothelium [6,34,35,37,40,41]. The release of TF results in the production of thrombin and formation of a fibrin clot [34,35,37,40,41]. Pro-inflammatory cytokines and thrombin act to further promote the formation of microthrombi by directly impairing fibrinolytic activity. This is achieved by stimulating endothelial cells and circulating platelets to secrete plasminogen activator inhibitor 1 (PAI1), a protease inhibitor that inhibits the generation of plasmin from plasminogen, and hence impairs fibrinolysis [34,35,37,40,41]. Additionally, thrombin further suppresses fibrinolysis by promoting the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) [34,35,37,40,41]. Thrombin stimulation also initiates the secretion of von Willebrand factor (vWF) from activated endothelial cells [6,8,35]. vWF further amplifies the pro-thrombotic pathway by binding to and inhibiting the degradation of factor VIII (a blood-clotting protein), and promoting platelet aggregation via platelet-platelet and platelet-fibrin adherence [6,8,35].

Furthermore, protein C is a zymogen present in the systemic circulation [34]. Following the interaction of thrombin with thrombomodulin, an endothelial transmembrane glycoprotein present on the endothelial cell surface, protein C binds to this complex and becomes activated [34]. Activated protein C (APC) possesses several
anti-thrombotic roles, as it inactivates factors V<sub>a</sub> and VIII<sub>a</sub>, both necessary for the production of thrombin, in addition to having the ability to directly inhibit PAI-1 activity [34,35,37,40,42,43]. However, under septic conditions, thrombomodulin levels are significantly decreased, resulting in extensive deposition of fibrin and formation of microthrombi, leading to capillary plugging, inadequate tissue perfusion, and organ failure [34,35,37,40,42,43]. Together, the inflammatory and coagulation pathways that take place during sepsis significantly contribute to MVEC dysfunction observed during sepsis-induced ARDS.

1.4.2 Microvascular Endothelial Cell Barrier Dysfunction

A loss of microvascular endothelial barrier stability leading to increased permeability is the result of MVEC injury, dysfunction, and death following sepsis [10–12,15,16,34]. The principal mechanisms that result in the increase in trans-MVEC permeability following MVEC activation by inflammatory cytokines and leukocytes include MVEC retraction as well as loss of intercellular junctions.

Within both adherens and tight junctions, the primary role of scaffold proteins, including adherens junction-associated catenins (α, β, and γ) and tight junction-associated ZO 1-3, is to connect the intercellular junctional membrane proteins to the intracellular actin cytoskeleton [11,13,15,16]. This interaction is required in order to maintain junctional stability and proper barrier function. The reorganization of the endothelial actin cytoskeleton into contractile stress fibres following MVEC activation facilitates cellular retraction, which disrupts adhesion of adjacent intercellular junctional proteins by pulling the MVEC inward, and results in the formation of intercellular gaps [11,13,44–48]. Actin-mediated endothelial retraction is caused by the direct
phosphorylation of the regulatory myosin light chain (MLC) by myosin light chain kinase (MLCK), whose activation is dependent on the binding of calcium (Ca$^{2+}$) to calmodulin (CaM) [11,13,44–49]. The small GTPase RhoA, through its downstream effector RhoA kinase (ROCK), also promotes MLC phosphorylation via the inhibition of MLC phosphatase (MLCP) as well as cofillin, an actin-binding depolymerizing protein [11,13,44–47]. Studies suggest that activation of the RhoA pathway is protein kinase C (PKC)-dependent following increased cellular influx of Ca$^{2+}$ [11,13,44,45]. Additionally, PKC has the ability to directly induce MLC phosphorylation to facilitate endothelial contraction [11,13,44,45,50]. Finally, following the release of pro-inflammatory cytokines, microtubule disassembly has also been implicated in endothelial contraction by initiating the rapid assembly of actin filaments [18,44,45]. During sepsis, TNF$\alpha$ stimulation has been shown to induce cytoskeletal reorganization by increasing MLC phosphorylation, in addition to evoking a decrease in stable tubulin content, resulting in microtubule disassembly and subsequent increases in barrier permeability [18,48].

Although the contractile forces generated by cytoskeletal reorganization following endothelial activation cause the disassembly and cellular internalization of intercellular junctional proteins, several other mechanisms are known to directly target these proteins and disrupt the crucial intercellular tethering complexes. As VE-cadherin is the integral adhesive determinant present in endothelial adherens junctions, modifications of its structure along with its associated catenins by permeabilizing agents during sepsis significantly attenuates barrier integrity [11–13,15–17,50]. Several studies have demonstrated correlations between enhanced barrier permeability and tyrosine phosphorylation of the VE-cadherin/catenin complex. Such permeability-increasing
agents include histamine, TNFα, PAF, and vascular endothelial growth factor (VEGF) [11–13,51]. Additionally, increased tyrosine phosphorylation of the VE-cadherin/catenin complex has been associated with early cell confluency and weak junctional strength, with less phosphorylation occurring at full confluency when stable adhesive junctions have been established [12,52]. p120 catenin, which is bound to the cytoplasmic juxtamembrane domain of VE-cadherin, is a very important determinant of VE-cadherin stabilization. p120 phosphorylation results in cadherin disassembly and internalization, along with increased cytoplasmic p120 content [11–13,16]. This increased cytoplasmic p120 content facilitates a compensatory feedback mechanism which results in a decrease in active barrier destabilizing RhoA, and an increase in Rac1 and Cdc42 [11–13,16].

Rac1 and Cdc42 are members of the Rho family of small GTPases that act to promote reassembly of the adherens junctions and stabilize the endothelial barrier [11–13,16]. Rac1 acts to disrupt the association between β-catenin and Ras GTPase-activating-like protein IQGAP1, allowing for β-catenin to associate with VE-cadherin and establish stable adherens junctions [11,13]. However, Rac1 activity has also been shown to play an injurious role in other cell types, such as cardiomyocytes, under septic conditions [53].

Previous studies have shown LPS-induced activation of Rac1 is responsible for TNFα expression as well as enhanced superoxide (O₂⁻) production in cardiomyocytes, as this response, which results in septic-induced cardiac dysfunction, was inhibited in Rac1 deficient cardiomyocytes [53]. Conversely, Cdc42 is thought to migrate from the cytoplasm to the plasma membrane and promote the re-establishment of the VE-cadherin/catenin complex following disassembly by initiating binding of α-catenin to β-catenin [11,13]. Thus, it is believed that p120 catenin may play a crucial role in
reestablishment of adherens junctions following endothelial activation. In addition to p120 catenin, phosphorylation of alternative catenins, including β- and α-catenin, results in the reduction of their affinity for the VE-cadherin cytoplasmic tail leading to modified VE-cadherin interaction with the actin cytoskeleton [11–13,16].

Several permeability-inducing agents present during sepsis facilitate these described mechanisms. For example, LPS stimulates translocation of the transcription factor NFκB to the nucleus to initiate transcription of pro-inflammatory cytokines and increased endothelial expression of ICAM1, both of which are associated with increased endothelial permeability. ICAM1, in turn, has the ability to increase the expression of endothelial RhoA through a positive feedback mechanism [11,13]. Additionally, activated PMN adherent to the endothelium during sepsis produce ROS, which act to increase barrier permeability. This is achieved by reducing plasma membrane fluidity, resulting in increased membrane permeability to macromolecules and fluid, in addition to activating MLCK and RhoA-dependent actin cytoskeleton contraction [11,13]. Furthermore, under inflammatory conditions, endothelial NO levels are elevated due to increased expression of inducible nitric oxide synthase (iNOS), which increases endothelial permeability through activation of MLCK, in addition to disruption of interendothelial junctions and endothelial apoptosis [11,13,54]. Studies have demonstrated that TNFα induces increased barrier permeability by driving the cleavage VE-cadherin and generating a soluble extracellular fragment [11,13,19,55]. This was found to be dependent on tyrosine phosphorylation of the VE-cadherin/catenin complex by several kinases including Src kinase, protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK) [11,13,19,55]. Thrombin, on the other hand, binds to protease-activated
receptor (PAR)-1 present on the endothelial surface, and promotes barrier dysfunction by
initiating increased cytosolic Ca\(^{2+}\) concentrations leading to PKC\(\alpha\)-dependent VE-
cadherin phosphorylation and subsequent internalization [11,13,56]. Collectively, these
studies illustrate the complex intercellular signalling pathways that are involved in the
control of MVEC barrier function, and that are often disrupted under pathological
conditions, such as sepsis.

1.5 Metalloproteinases

Metalloproteinases are endopeptidases that utilize a zinc (Zn\(^{2+}\)) or Ca\(^{2+}\) ion in
their active site, and function to degrade and process a large range of extracellular
proteins [57,58]. Several different families of metalloproteinases exist. However, the
majority of past research has focused on the function of two distinct metalloproteinase
families: the matrix metalloproteinase (MMP) family and the ‘a disintegrin and
metalloproteinase’ (ADAM) family [58]. MMPs are comprised of a family of 25 zinc-
dependent proteases, whose principle role has classically been defined as degradation of
the ECM through the turnover of connective tissue proteins including collagens and
elastins, as well as basement membrane components [59]. However, MMPs are involved
in the control of several other biological functions including, but not limited to, cleavage
and activation of growth factors such as transforming growth factor \(\beta\) (TGF\(\beta\)), chemokine
processing, regulation of cellular apoptosis, and cleavage of cell surface-associated or
transmembrane proteins [59–61]. ADAMs, on the other hand, function as transmembrane
proteases or “sheddases” that act to cleave the extracellular domain of several cell surface
proteins and receptors, a process referred to as ectodomain shedding [58].

Metalloproteinase activity is regulated at multiple levels including regulation of gene
expression and protein synthesis, compartmentalization, substrate availability and affinity, and inhibition by tissue specific inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) [62].

Although some structural features may vary among individual members, the basic structure of most MMPs and ADAMs is similar and includes a catalytic domain that contains a Zn$^{2+}$ binding site, and a pro-peptide domain, which keeps the enzyme in its inactive form [61,63]. The interaction of Zn$^{2+}$ with its binding site found in the catalytic domain of MMPs and ADAMs is responsible for the functional activation of these endopeptidases [58,61–63]. Additionally, activation is regulated by the pro-domain [61–63]. A conserved cysteine reside within the pro-domain is associated with the catalytic Zn$^{2+}$ ion, which would otherwise be used to invoke catalytic activity [61–63]. When the pro-domain is removed by cleavage, or its interaction with the catalytic Zn$^{2+}$ ion is destabilized through cleavage or other processes, such as oxidation, the catalytic domain becomes available to bind the Zn$^{2+}$ ion, allowing metalloproteinases to be secreted in their active form [61–63]. ADAMs, however, differ from most MMPs by way of a disintegrin-binding domain as well as a transmembrane region [58,63].

### 1.5.1 Role of Metalloproteinases in Sepsis-Induced ARDS

Metalloproteinases are known to regulate several inflammatory and repair processes during ARDS through processing of pro-inflammatory cytokines and chemokines, as well as promoting disruption of the alveolar capillary barrier [57,61]. Bronchoalveolar lavage (BAL) fluid from patients diagnosed with ARDS contain elevated levels of MMP1, 2, 3, 8, 9, and 13 [64–66]. In addition, elevated levels of MMP1 and MMP3 correlate with the severity of lung injury, incidence of multiorgan
failure, and death [64–66]. Previous studies have demonstrated that ADAM28 has the ability to bind to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, which results in enhanced binding to P-selectin present on the endothelial surface [58]. Furthermore, activated PMNs adhered to PMVEC during ARDS express MMP9 and ADAM10 and release a number of oxidants including ROS [56,57,61,62,67]. These oxidants have the ability to enhance MMP gene expression through the activation of MMP-associated transcription factors, as well as directly activate MMPs via oxidation of the MMP pro-domain [56,57,61,67]. Interestingly, in addition to activation, MMPs can be inactivated by ROS via modification of amino acids necessary for catalytic activity [57,61].

MMPs also have the ability to both directly and indirectly regulate chemokine and cytokine activity under inflammatory conditions [57,61]. MMPs can directly cleave specific chemokines leading to enhanced activity or degradation. MMPs can also process substrates that bind or associate with chemotactic molecules, which indirectly results in either augmented or diminished chemotactic activity [57,61]. MMP2, 3, and 9 have the ability to cleave and activate the precursor of pro-inflammatory cytokine IL1β [61]. Furthermore, ADAM17, also known as TNFα-converting enzyme (TACE), actively processes and cleaves membrane-bound pro-TNFα to yield the soluble inflammatory cytokine TNFα [61]. Conversely, two MMPs are known to possess anti-inflammatory characteristics during ARDS. MMP8 and MMP13 attenuate lung inflammation by cleaving and inactivating macrophage inflammatory protein 1α and monocyte chemoattractant protein 1, respectively [64]. Together, these studies suggest that through their catalytic activity, metalloproteinases play a critical role in regulating the inflammatory response present in sepsis and ARDS.
1.5.2 Role of Metalloproteinases in Endothelial Dysfunction

Multiple MMPs and ADAMs have been shown to cleave inter-MVEC junctional proteins, and thereby mediate microvascular dysfunction through increased vascular permeability [20,56,68–70]. For instance, MMP7, ADAM10, and ADAM12 are all capable of cleaving VE-cadherin, the integral cell surface transmembrane protein of endothelial adherens junctions [56,69], while MMP2 and 9 along with ADAM17 are capable of cleaving tight junction associated proteins such as occludin, ZO1, and JAMs [20,68,70]. In addition to cleavage, tyrosine phosphorylation-dependent disassembly of crucial adhesive junctional proteins may also be MMP dependent, as permeability induced by tyrosine phosphatase inhibitors have been shown to be blocked by MMP inhibitors [19,67]. Moreover, knockdown of ADAM10 in endothelial cells as well as activated leukocytes by the utilization of small interfering RNA (siRNA) reduces leukocyte transendothelial migration and stabilizes the vasculature [56].

MMPs and ADAMs with thrombospondin motifs (ADAMTSs) have also been found to cleave the ECM surrounding the vasculature, which can modify endothelial cell function through disrupted endothelial-ECM interactions, ultimately leading to increased vascular permeability [31,32,71–74]. For example, previous studies have shown that the enzymatic degradation of ECM constituents, including fibronectin, laminin, and type IV and V collagens, by MMPs, including MMP2 and MMP9, have resulted in increased permeability in cultured endothelial monolayers as well as induced pulmonary edema in vivo [31,32]. Additionally, MMP9 has been shown to be responsible for the cleavage and release of VEGF bound to the ECM, which is responsible for increased microvascular permeability through internalization of VE-cadherin [60]. Thus, these studies suggest
metalloproteinase activity is associated with MVEC dysfunction through the cleavage of integral MVEC structural determinants, resulting in enhanced microvascular leak.

### 1.6 Tissue Inhibitors of Metalloproteinases

The TIMP family, a family of robust inhibitors of metalloproteinases, is comprised of four members, TIMP1-4 [75,76]. TIMPs are able to inhibit active metalloproteinases in a 1:1 stoichiometric inhibitor-to-enzyme ratio [75,76]. Specifically, the N-terminal domain of TIMP molecules interacts with the active site of metalloproteinases in a similar fashion to that of metalloproteinase substrates, resulting in inhibition of metalloproteinase catalytic activity [75,76]. While TIMP1, 2, and 4 are secreted and thus function in a soluble manner, TIMP3 is known to be bound to sulfated glycosaminoglycans located in the ECM [75–77]. Therefore, although each TIMP has the ability to inhibit most metalloproteinases, differences in inhibition efficiency due to metalloproteinase specificity and localization occur between members of the TIMP family. For example, TIMP2 and 3, unlike TIMP1 are effective inhibitors of the membrane type (MT)-MMPs, while TIMP3 appears to be the primary inhibitor of many of the sheddases such as ADAM17 [75,76].

TIMP3, a robust inhibitor of MMPs, ADAMs, and ADAMTSs, is highly expressed in multiple tissues, including the lung, heart, brain, and kidney [76,78–83]. Through its primary function in the inhibition of multiple metalloproteinases, TIMP3 has been shown to be greatly involved in the regulation of the inflammatory and repair response following injury [58]. Additionally, several biological processes which require TIMP3 have been discovered through the use of a TIMP3 knockout (Timp3−/−) model.
TIMP3 has been shown to act as a major facilitator of ECM sparing, or inhibition of ECM degradation, in several homeostatic processes, including lung and bone development and remodelling as well as mammary gland involution [84]. During fetal development, mice lacking TIMP3 (Timp3\(^{-/-}\) mice) have impaired bronchiole branching morphogenesis (branching of the airways) due to enhanced metalloproteinase activity and ECM degradation [85,86]. Treatment with GM6001, a broad-spectrum synthetic inhibitor of metalloproteinases, rescues the enhanced ECM degradation and impaired bronchiole branching in Timp3\(^{-/-}\) lungs both in whole organ culture or in vivo in pregnant mice [85,86]. Furthermore, Timp3\(^{-/-}\) mice develop spontaneous alveolar air space enlargement that is evident as early as two weeks after birth and progresses with age, until premature death occurs beginning at 13 months of age, which is attributed to heightened MMP activity and ECM degradation [87]. The hind-limb joints of Timp3\(^{-/-}\) mice also possess significantly reduced amounts of articular cartilage exhibited by extensive cleavage of collagen and aggrecan due to increased MMP and ADAM activity, which suggests that decreased expression of TIMP3 may play a pathophysiologic role in the development of osteoarthritis [84,88]. Additionally, studies have shown that mice lacking TIMP3 experience accelerated mammary gland involution characterized by earlier fragmentation of fibronectin due to increased gelatinase activity [84,89]. These events, however, are rescued with the biochemical reconstitution of recombinant TIMP3 [84,89]. Lastly, Timp3\(^{-/-}\) mice display a higher incidence of cardiac rupture following myocardial infarction, which is associated with decreased cardiac myofibroblast cell number and collagen synthesis, as well as increased MMP activity [90]. Therefore, TIMP3 plays a
critical role in proper tissue development and inhibition of ECM degradation through the inhibition of metalloproteinase activity.

1.6.1 Role of TIMP3 in Lung Injury

Previous studies have demonstrated that TIMP3 plays a crucial role in both proper lung development in addition to the resolution of inflammation following lung injury. Following bleomycin as well as LPS-induced lung injury, $\text{Timp}^{3/-}$ mice display impaired resolution of inflammation compared to wild type (WT) mice [91]. This delayed recovery was associated with intensified and persistent inflammation in $\text{Timp}^{3/-}$ lungs as indicated by the prolonged presence of neutrophils in the BAL fluid, likely due to continued neutrophil influx following lung injury [91]. $\text{Timp}^{3/-}$ mice also have increased lung compliance compared to WT mice at baseline, and this increase was further augmented following cecal ligation and perforation (CLP)-induced sepsis [92]. Together, these studies suggest a pivotal role for TIMP3 in the lung, as the absence of TIMP3 results in impaired lung development as well as tissue repair following inflammatory-mediated lung injury.

1.6.2 Role of TIMP3 in Endothelial Dysfunction

TIMP3 is expressed by multiple cells, including endothelial cells, and is known to affect multiple aspects of vascular function, including regulation of angiogenesis as well as the stabilization of the vasculature [74,93–96]. For example, treatment of brain microvascular endothelial cells with IL1β and TNFα leads to a decrease in $\text{Timp}3$ mRNA expression, and injection of recombinant TIMP3 (rTIMP3) leads to decreased vascular leak across the blood-brain barrier in a model of traumatic brain injury [93,94]. Interestingly, $\text{Timp}^{3/-}$ mice, which have increased metalloproteinase activity in a number
of organs including the lungs, have augmented leak of Evans Blue dye into their snout and kidneys at baseline, which is thought to be due to the absence of TIMP3 in pericytes resulting in enhanced microvascular permeability due to increased global metalloproteinase activity [74].

While the more prevalent function for TIMP3 is inhibition of metalloproteinase activity, TIMP3 has also been found to have metalloproteinase-independent functions. One such function is inhibition of VEGF signalling by binding to the VEGF receptor and blocking VEGF-VEGFR2 interaction, ultimately inhibiting angiogenesis [95,96]. During angiogenesis, VEGF acts a potent mediator of increased endothelial permeability [11,95–97]. VEGFR2, located on the endothelial cell surface, is generally maintained in an inactive state. Once VEGF binds to its receptor, VEGFR2, a signalling cascade occurs resulting in the direct phosphorylation of VE-cadherin by Src kinase, which subsequently initiates VE-cadherin internalization via clathrin-coated pits [11,97]. This process promotes disassembly of cell-cell adhesive contacts resulting in increased microvascular permeability and endothelial cell migration [11,97]. Thus, these data suggest that through metalloproteinase-dependent and –independent functions, TIMP3 acts to stabilize the vasculature and promote restrictive endothelial barrier function. However, several aspects pertaining to the role of TIMP3 in stabilization of the vasculature, particularly the pulmonary microvasculature following sepsis-induced ARDS, remain unknown.

1.7 Rationale

Sepsis-induced multiple organ dysfunction and mortality are mainly due, in large part, to perturbations in the microvasculature. In particular, the accumulation of protein-rich edema fluid within the lung tissue in sepsis-induced ARDS is thought to be primarily
caused by a loss of pulmonary microvascular stability and increased permeability due to PMVEC injury, dysfunction, and death following sepsis [7,10–12,15,16,97]. PMVEC are integral to maintaining homeostatic microvascular function by modulating vascular tone, and controlling the passage of circulating cells and macromolecules through an intact permeability barrier [7,11–13,15–17]. Multiple mechanisms contributing to septic PMVEC injury/dysfunction have been reported [7,10–12,15,16,34,41,97]; however, the endogenous homeostatic mechanisms protecting against sepsis-induced PMVEC dysfunction are poorly characterized.

Metalloproteinases are capable of cleaving adhesive transmembrane proteins integral to PMVEC function, including inter-PMVEC junctional proteins as well as PMVEC surface proteins involved in PMN-PMVEC interactions [19,20,56,67–70]. Previous studies have shown that TIMP3, a robust inhibitor of metalloproteinases, which is highly expressed within multiple tissues including the lung, stabilizes the vasculature and inhibits increases in microvascular permeability and leak following injury [56,74–76,79,93–96]. Collectively, previous studies suggest that TIMP3 may have a role in regulating microvascular leak following lung injury. However, the specific role of TIMP3 in pulmonary vascular stability and enhanced septic lung microvascular permeability remain to be determined.

1.8 Objectives

Analysis of PMVEC dysfunction and changes in barrier integrity under homeostatic and septic conditions through the use of inflammatory stimuli will enable us to deduce the mechanisms through which TIMP3 regulates microvascular endothelial activation, injury, and dysfunction following sepsis-induced ARDS. We propose to:
1. Examine the role of TIMP3 in regulating PMVEC barrier function under both homeostatic and septic conditions \textit{in vitro}.

2. Determine the specific mechanisms through which TIMP3 regulates PMVEC barrier function under homeostatic and septic conditions \textit{in vitro}.

1.9 Hypothesis

We hypothesize that TIMP3 supports normal pulmonary microvascular endothelial barrier function and attenuates the increase in pulmonary microvascular permeability following sepsis through the inhibition of metalloproteinase activity.
Chapter 2

2 Methodology

2.1 Pulmonary Microvascular Endothelial Cell (PMVEC) Isolation

To study the regulatory function of TIMP3 in maintaining pulmonary microvascular barrier function following septic lung injury, murine PMVEC were isolated and cultured for use in all in vitro experiments. PMVEC were isolated from the pulmonary tissue of healthy WT and Timp3−/− mice, as previously described [98,99]. In brief, following lung isolation, lung tissue was finely minced and digested using 0.3% collagenase in Hank’s Balanced Salt Solution (HBSS). Following filtration through a 100 µm pore mesh sieve, cells were incubated with magnetic microbeads (Dynabeads M-450 sheep anti-rat IgG, #11035, Dynal Biotech Inc., Lake Success, NY), coupled to anti-PECAM (CD31) antibodies (Rat anti-mouse CD31 monoclonal antibody, #557355, BD Pharmingen, Franklin Lakes, NJ). Microbead-bound PMVEC were magnetically captured (MPC magnet, Dynal Biotech Inc., Lake Success, NY) and subsequently washed and suspended in growth medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% Heat inactivated Fetal Bovine Serum (FBS), #11885-092, Invitrogen, Carlsbad, CA; 1% Penicillin/Streptomycin (10 000 U/mL), #15140122, Invitrogen; 2% HEPES buffer (1M), #15630-080, Invitrogen), then seeded into a 1% gelatin-coated cell culture flask and incubated at 37°C with 5% CO2. Cells were assessed weekly to ensure appropriate morphology and once approximately 90% confluent, cells were stained with fluorescent acetylated-low density lipoprotein (LDL) (Biomedical Technologies, # BT902, Stoughton, MA) and assessed by immunofluorescence and flow
cytometry to ensure purity. Collectively, these processes result in 99% PMVEC culture homogeneity. PMVEC were then grown in supplemented DMEM growth medium and incubated at 37°C with 5% CO₂ until the cell monolayer reached confluence. PMVEC at passages 4-11 were used for all experiments.

2.2 Assessment of PMVEC-derived Timp3 mRNA levels (qRT-PCR)

PMVEC-derived Timp3 mRNA expression levels were examined in male WT PMVEC, in addition to Cdh5 (VE-cadherin) mRNA expression levels in female WT and Timp3⁻/⁻ PMVEC by quantitative real-time polymerase chain reaction (qRT-PCR). Cells were cultured and grown to confluence on 6-well 1% gelatin-coated cell culture plates, and treated with either phosphate buffered saline (PBS, vehicle control) or cytomix (an equimolar solution of TNFα, IL1β, and IFNγ used to mimic a septic response, 30 ng/mL, PeproTech, Rocky Hill, NJ) for 2, 4, and 8h in order to assess Timp3 mRNA expression, or for 6 and 24h in order to assess Cdh5 mRNA expression. Following stimulation, cells were lysed and RNA isolated using the RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany). Briefly, 200 μL RLT buffer containing 2 μL beta-mercaptoethanol was added to each well. Wells were then scraped, and cell lysate collected and placed in Qiashredder tubes (#79656, Qiagen). Samples were spun at 10 000 x g for 30 seconds. Flow through was collected from the Qiashredder tubes, placed into RNeasy spin columns, washed with a series of buffers, and the RNA eluted from the columns by water, as per the manufacturer instructions.

Purity (230/260 and 260/280 ratios) and concentration of isolated RNA was determined by analysis with the NanoDrop 1000 spectrophotometer (Thermo Scientific,
Waltham, MA). Isolated RNA (2 µg) was reverse transcribed using a High capacity cDNA reverse transcription kit (#4368814, Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Gene expression was then analyzed using TaqMan Gene Expression Assays from Applied Biosystems (Timp3: #Mm00441826_m1; Cdh5, #Mm00486938_m1, Invitrogen) and the CFX96 Real Time System (BioRad Laboratories Inc., Hercules, CA). Hypoxanthine-guanine phosphoribosyltransferase (Hprt), a housekeeping gene, was used to normalize expression of genes of interest (# Mm00446968_m1, Invitrogen). qRT-PCR generated the cycle threshold (Ct) value for each gene and this value was then used to determine gene expression relative to PBS-treated PMVECs. ΔCt was the normalization of Timp3 or Cdh5 to Hprt within a specific sample (i.e. 2h cytomix-treated PMVECs), ΔΔCt was the normalization of a specific sample (i.e. 2h cytomix-treated PMVECs) to the control sample (PBS-treated PMVECs), and the relative quantity (RQ) was the fold change in expression of a specific sample (i.e. 2h cytomix-treated PMVECs) relative to the control sample (PBS-treated PMVECs). RQ was determined by the following equation: RQ = 2^-ΔΔCt.

2.3 Assessment of PMVEC Barrier Integrity

Endothelial monolayer integrity was assessed in female murine WT and Timp3^-/- PMVEC using an in vitro model of PMVEC barrier function (NOTE: key experiments were repeated using male murine WT and Timp3^-/- PMVEC to examine any gender-specific effects). WT and Timp3^-/- PMVEC were seeded at a cell density of 2.5x10^4 or 5.0 x10^4 cells/insert and grown to confluence on 1% gelatin-coated transwell cell-culture inserts (3.0 µm pore, # CA62406-169, VWR Scientific Inc., Radnor, PA) in supplemented DMEM growth medium in 24-well cell-culture plates. The baseline
permeability of the PMVEC monolayer was then assessed every second day using transendothelial electrical resistance (TEER), which is the most sensitive measure of paracellular permeability (e.g. to charged ions), to identify the time point at which a stable monolayer is formed.

Once a stable monolayer was formed, baseline barrier permeability of female WT and Timp3<sup>−/−</sup> PMVEC seeded at 2.5x10<sup>4</sup> cells/insert only was comprehensively assessed using three complementary techniques: (i) TEER; (ii) Fluorescein isothiocyanate (FITC)-labelled dextran flux, which reflects small molecule paracellular permeability; and (iii) Evans Blue (EB)-labelled albumin flux, a marker of both paracellular and transcellular permeability to large molecules, which is most clinically relevant. Assessment of PMVEC permeability by all three complementary techniques was conducted at 10 and 14-15 days after initial cell culture.

### 2.3.1 Assessment of TEER across PMVEC monolayers

Intact endothelial barriers exhibit high levels of TEER, and decreased TEER is indicative of physiological changes in PMVEC barrier function leading to increased trans-PMVEC permeability. TEER was measured across murine male and female WT and Timp3<sup>−/−</sup> PMVEC monolayers by placing individual cell-culture inserts into the Endohm chamber (World Precision Instruments, Sarasota, Florida) in order to measure the electrical resistance in Ohms (Ω) using the EVOM2 Endothelial Voltohmmeter (World Precision Instruments, Sarasota, Florida). In order to account for background contribution to TEER measurements from the cell-culture inserts themselves, empty individual inserts not containing cultured PMVEC were used as a control. The TEER of these inserts was measured as described above, and the resulting value was subtracted
from the TEER measurements obtained from the cell-culture inserts containing PMVEC. Basal TEER values that were considered to be indicative of a stable PMVEC monolayer that was acceptable to conduct permeability studies on ranged from 25-30 Ohms (following cell-culture insert background correction), and occurred by 10 days after initial cell culture.

2.3.2 Assessment of trans-PMVEC macromolecular flux

To best characterize the extent of endothelial dysfunction, both large (albumin; a large, 67 kDa blood protein) and small (dextran; a small, 3 kDa sugar derivative) molecular compounds were utilized. The levels of trans-PMVEC macromolecular flux were measured based on movement of the macromolecules from the upper chamber into the lower of the two-chamber transwell configuration (Figure 2-1). Both EB-labelled albumin and FITC-labelled dextran were added directly to the upper chamber of the transwell insert containing the PMVEC monolayer, and following 1h, the transwell inserts were removed, and the conditioned media of the lower chamber was collected. In order to measure EB-labelled albumin flux, absorbance of the conditioned medium was measured (A620) by an iMark™ Microplate Reader (BioRad Laboratories Inc., Hercules, CA). To measure FITC-labelled dextran flux, the fluorescence of the conditioned medium was measured (Excitation peak wavelength: 488 nm and Emission peak wavelength: 525 nm) using a Victor3 multilabel fluorescence microplate reader (Wallac).

2.4 Assessment of PMVEC Barrier Function following PMVEC stimulation

In order to assess PMVEC barrier dysfunction under septic conditions, permeability of confluent female WT and Timp3−/− PMVEC (2.5x10⁴ cells/insert)
PMVEC are cultured on 1% gelatin-coated semi-porous transwell cell-culture inserts in fully supplemented DMEM medium. Confluent PMVEC monolayers are then treated with either PBS (vehicle control) or cytomix (an equimolar solution of TNFα, IL1β, and IFNγ used to mimic a septic response). Both EB-labelled albumin and FITC-labelled dextran are added directly to the upper chamber of the transwell cell-culture insert containing the PMVEC monolayer, and the levels of trans-PMVEC macromolecular flux are subsequently measured based on movement of the macromolecules from the upper chamber into the lower of the two-chamber transwell configuration.

**Figure 2-1: Schematic representation of *in vitro* model of PMVEC barrier function.**
monolayers was measured 7 days after initial cell culture by all three complementary techniques following a 4h stimulation with either PBS (vehicle control) or cytomix (30 ng/mL). We have previously reported the effects of cytomix stimulation on TEER and trans-PMVEC macromolecular leak in PMVEC monolayers cultured on transwell cell culture inserts. In addition to measuring TEER, EB-labelled albumin was utilized to measure trans-PMVEC macromolecular leak following different concentrations of cytomix (0.3 – 100 ng/mL) or over a time course (4-24h). Stimulation of PMVEC with 30 ng/mL cytomix for 4h resulted in maximal trans-PMVEC EB-labelled albumin leak (Figure 2-2).

PMVEC permeability was additionally assessed in the presence or absence of GM6001, a broad-spectrum synthetic metalloproteinase inhibitor. Using the two-chamber transwell configuration, female WT and Timp3\(^{-/-}\) PMVEC (2.5x10\(^4\) cells/insert) were treated with either dimethyl sulfoxide (DMSO, vehicle control) or GM6001 (1 µg/mL, #CC1010, EMD Millipore, St. Charles, MO) every second day, and permeability was subsequently assessed by all three complementary techniques after 10 days in culture.

2.5 Assessment of PMVEC-derived TIMP3 and VE-cadherin protein abundance (Western Blot)

Western blot analysis was performed on PMVEC lysate. Male and female WT and Timp3\(^{-/-}\) PMVEC were cultured and grown to confluence on 6-well 1% gelatin-coated cell culture plates, and treated with either PBS (vehicle control) or cytomix (30 ng/mL) for 6 and 24 hours. Following stimulation, cells were lysed by directly introducing lysis buffer (1x Laemmli buffer with 5% β-mercaptoethanol plus protease inhibitor cocktail [DMSO solution, #P8340, Sigma-Aldrich, St. Louis, MO]) into each of the wells. This
Figure 2-2: Time course and dose-dependent effects of cytomix on PMVEC permeability. PMVEC cultured on cell-culture transwell inserts were stimulated with various concentrations of cytomix (0.3-100 ng/mL). (A and B) Stimulation of PMVEC with cytomix at a concentration of 30 ng/mL resulted in maximal trans-PMVEC permeability vs. PBS vehicle control, as measured by TEER and trans-PMVEC EB-labelled albumin flux. (C and D) Stimulation of PMVEC with cytomix at a concentration of 30 ng/ml over a time course (4, 8, and 24h) resulted in maximal trans-PMVEC permeability vs. PBS vehicle control at 4h post-stimulation, as measured by TEER and trans-PMVEC EB-labelled albumin flux (mean ± SEM, N=8, * indicates P<0.05 and ** indicated P<0.001, (A and B) One-Way ANOVA followed by Dunnett’s post-hoc test, (C and D) Two-Way ANOVA followed by a Bonferroni post-hoc test).
lysis buffer was allowed to lyse the cells slowly on ice over the course of 20 minutes, and wells were subsequently scraped and samples were stored in Eppendorf tubes. Samples were further sonicated and stored at -80°C before use. Protein samples were boiled for 3 minutes and electrophoresed on 4-15% SDS-PAGE gels (Mini-PROTEAN 4-15% TGX Precast Gels, #4561084, BioRad Laboratories Inc.). Protein was transferred to PVDF and individual blots were then blocked with 5% nonfat milk in Tris-buffered saline (TBS). Individual blots were incubated with primary antibodies against TIMP3 (rabbit anti-mouse polyclonal, 1:3000 dilution, #AB6000, EDM Millipore), VE-cadherin (goat anti-mouse polyclonal, 1:500 dilution, #sc-6458, Santa Cruz Biotechnology, Dallas, Texas) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, rabbit anti-mouse polyclonal, 1:5000 dilution, #ab9485, Abcam, Cambridge, England) diluted in TBS-T (TBS and 0.1% Tween 20) plus 1% nonfat milk. Following washes in TBS-T, blots were incubated with the appropriate anti-rabbit or anti-goat horseradish peroxidase-conjugated secondary antibody (1:5000 and 1:1000 dilution for rabbit and goat respectively, #656120 and #811620 respectively, Invitrogen). Following additional washes in TBS-T, bands of interest were then detected by enhanced chemiluminescence (ECL) reagent (Clarity Western ECL Substrate, #170-5061, BioRad Laboratories Inc.), using the MicroChemi camera system (FroggaBio, Toronto, ON, Canada). Subsequent densitometry measurements were calculated using ImageJ software with the relative abundance of TIMP3 and VE-Cadherin normalized to GAPDH.

2.6 Immunohistochemistry

Female WT and Timp3<sup>−/−</sup> PMVEC were cultured and grown to confluence on 1%
gelatin coated glass coverslips and stimulated with either PBS (vehicle control) or cytomix (30 ng/mL) for a duration of 4h. Following stimulation, cells were fixed with 4% PBS-buffered paraformaldehyde (#P6148, Sigma-Aldrich) and permeabilized with 0.1% Triton X-100 detergent (#CA97063-864, VWR). Cells were then blocked using 3% bovine serum albumin (BSA) in TBS, and incubated with primary antibodies against VE-cadherin (goat anti-mouse polyclonal, 1:100 dilution, #sc-6458, Santa Cruz Biotechnology) followed by incubation with red-fluorescent Alexa Fluor® 594 secondary antibody (donkey anti-goat polyclonal, 1:500 dilution in 1% BSA/PBS, #A110055, Invitrogen). A second set of PMVEC were also stained with Texas Red®-X Phalloidin (1:40 dilution in 1% BSA/PBS, #T7471, Invitrogen) in order to visualize the actin cytoskeleton (cortical actin vs. stress fibre formation) under resting and septic conditions and assess PMVEC activation. Following three washes with PBS, cell nuclei were stained with Hoechst 33342 in PBS (1:5000 dilution, #H3570, Invitrogen) to identify individual cells. Coverslips were mounted with fluorescent mounting medium (Dako, #S3023), and examined by fluorescent microscopy (Zeiss Axiovert 200M Inverted Microscope; Carl Zeiss Canada Ltd., Toronto, Canada). Negative controls (i.e. cells stained with secondary antibody alone) were used to identify an exposure time giving minimal background signal, and once set, exposure times were kept constant for all subsequent images.

2.7 Assessment of WT and Timp3<sup>-/-</sup> PMVEC viability and proliferation

Female WT and Timp3<sup>-/-</sup> PMVEC were cultured on 1% gelatin-coated 96-well cell-culture treated plates at a cell density of 5x10<sup>3</sup> or 1x10<sup>4</sup> cells/well. Following 24, 48, and 72h in culture, cellular viability was assessed using an MTT (3-(4,5-dimethylthiazol-
2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (Cell Proliferation Kit I (MTT), #11465007001, Roche, Basel, Switzerland). MTT labeling reagent (10 µL, final concentration 0.5mg/mL) was added to each individual well containing cells. The cell-culture plate was then incubated in a humidified atmosphere (37°C, 5% CO₂) for 4h. Following the incubation period, 100 µl of solubilization solution (10% SDS in 0.01M HCl) was added to each individual well, and the cell-culture plate was further incubated at 37°C with 5% CO₂ overnight. The solubilized product produced by the cleavage of MTT by metabolically active cells was then quantified by measuring spectrophotometrical absorbance (A₅₉₀ (absorbance wavelength) - A₆₄₂ (reference wavelength)) using a Victor3 multilabel microplate reader (Wallac). The amount of product formed, as measured by absorbance, directly correlates with the number of metabolically active or viable cells. Additionally, following 2, 4, and 7 days in culture, cell proliferation was assessed by direct cell counts using a hemocytometer.

2.8 Statistical Analysis

Raw data was examined using GraphPad Prism 5 in order to carry out various statistical analyses depending upon the number of variables associated with the given experiment. Specifically, differences between groups were assessed by t-tests for one measured variable, or by a Two-Way ANOVA with a Bonferroni post-hoc test for two independent variables. Significance threshold was set at α = 0.05.
Chapter 3

3 Results

3.1 *Timp3* mRNA expression is decreased in septic (cytomix-treated) PMVEC

As stated, treatment of PMVEC monolayers with cytomix led to detectable macromolecular leak, as measured by EB-labelled albumin flux, between 4-24h post-stimulation (*Figure 2-1*). Thus, *Timp3* mRNA expression levels were examined in WT PMVEC over the same time course in order to assess the role of TIMP3 in sepsis-induced PMVEC dysfunction. Analysis of *Timp3* mRNA expression by qRT-PCR revealed a significant decrease in *Timp3* mRNA expression as early as 2h post-cytomix stimulation, along with a continued decrease at 4 and 8h. Importantly, no signs of recovery in *Timp3* mRNA expression following cytomix treatment were demonstrated (*Figure 3-1*).

3.2 TIMP3 protein abundance is decreased in septic (cytomix-treated) PMVEC

As septic conditions resulted in the downregulation of *Timp3* mRNA expression, we then sought to investigate whether cytomix stimulation would have a similar effect on TIMP3 protein abundance. Western blot analysis utilizing antibodies against TIMP3 was employed in order to examine TIMP3 protein abundance in cell lysates obtained from female WT PMVEC under septic conditions. Results demonstrated that cytomix-treated (septic) WT PMVEC possess significantly lower TIMP3 protein abundance in cell lysates at 6 and 24h post-cytomix treatment compared to control PBS-treated PMVEC (*Figure 3-2*). Additionally, analysis of TIMP3 protein abundance under septic conditions by
Figure 3-1: *Timp3* mRNA expression is decreased in septic (cytomix-treated) PMVEC. Compared to PBS treatment, cytomix-treated male PMVEC displayed progressively reduced *Timp3* mRNA expression over 8h demonstrated by qRT-PCR. Data is expressed as the relative quantity (RQ) of PBS control (mean ± SEM, N=6-8, * indicates P<0.01 vs. PBS, One-Way ANOVA followed by a Tukey post-hoc test).
Figure 3-2: TIMP3 protein abundance is decreased in septic (cytomix-stimulated) female PMVEC. (A) Septic PMVEC displayed significantly lower TIMP3 protein abundance in cell lysates at 6 and 24h post cytomix vs. PBS-treated PMVEC (western blotting; the normalized ratio of TIMP3 to GAPDH levels are shown as a percentage of PBS-treated PMVEC for each time point. Mean ± SEM, N=4, * indicates P<0.05 vs. PBS, t-test). (B) A representative immunoblot from an independent cell lysate extraction probed with antibodies against TIMP3 and GAPDH (loading control).
western blot was also performed on cell lysates obtained from male WT PMVEC with identical results. Cytomix treatment of WT PMVEC led to significantly lower TIMP3 protein abundance in cell lysates at 6 and 24h post-cytomix treatment compared to control PBS-treated PMVEC (Figure 3-3).

3.3 Endothelial Barrier Dysfunction in TIMP3 deficient (Timp3−/−) PMVEC

3.3.1 Loss of TIMP3 (Timp3−/−) is associated with decreased transendothelial electrical resistance (TEER) and enhanced trans-PMVEC macromolecular leak

In order to examine the if the observed decrease in TIMP3 levels under septic conditions could affect PMVEC barrier function, monolayer integrity was assessed in WT and Timp3−/− PMVEC using an in vitro model of PMVEC barrier function. WT and Timp3−/− PMVEC monolayers were stimulated with PBS (vehicle control) or cytomix (30 ng/mL) for 4h. Following the 4h stimulation, TEER, as well as EB-labelled albumin and FITC-labelled dextran flux across PMVEC monolayers was measured. Treatment of WT PMVEC with cytomix for 4h was associated with a significant increase in endothelial permeability as measured by decreased TEER, and increased FITC labelled-dextran and EB-labelled albumin flux (Figure 3-4). Furthermore, Timp3−/− PMVEC displayed significantly increased leak compared to WT PMVEC demonstrated by decreased TEER and enhanced FITC labelled-dextran and EB-labelled albumin flux, both at baseline (PBS control) and following stimulation with cytomix (Figure 3-4). Timp3−/− PMVEC, however, displayed no increase in leak (via TEER, dextran flux, or albumin flux) following stimulation with cytomix vs. PBS control (Figure 3-4).
Figure 3-3: TIMP3 protein abundance is decreased in septic (cytomix-stimulated) male PMVEC. (A) Septic PMVEC displayed significantly lower TIMP3 protein abundance in cell lysates at 6 and 24h post cytomix vs. PBS-treated PMVEC (western blotting; the normalized ratio of TIMP3 to GAPDH levels are shown as a percentage of PBS-treated PMVEC. Mean ± SEM, N=3, * indicates P<0.05 vs. PBS, t-test). (B) Representative immunoblots from 3 independent cell lysate extractions transferred by western blotting onto a PVDF membrane and probed with antibodies against TIMP3 and GAPDH.
A. TIMP3/GAPDH Protein

(bars) % PBS

Time Post-Stimulation (h)

PBS

Cytomix (30 ng/mL)

B.  

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TIMP3 6h
(24 kDa)

GAPDH 6h
(37 kDa)

TIMP3 24h
(24 kDa)

GAPDH 24h
(37 kDa)
Figure 3-4: TIMP3 deficiency (*Timp3*-/- PMVEC) is associated with PMVEC barrier dysfunction. Under basal (PBS-treated) conditions, *Timp3*-/- PMVEC displayed consistently higher basal permeability vs. WT PMVEC by 3 complementary assays: lower transendothelial electrical resistance (TEER; A), and enhanced trans-PMVEC macromolecular leak including fluorescein isothiocyanate (FITC)-labelled dextran (B), and Evans blue (EB)-labelled albumin (C). This basal *Timp3*-/- PMVEC barrier dysfunction was greater than in septic (cytomix-treated) WT PMVEC. Moreover, the septic PMVEC dysfunction response seen in WT PMVEC was completely attenuated in *Timp3*-/- PMVEC (mean ± SEM, N=3-6, * indicates P<0.05 *Timp3*-/- vs. WT and # indicates P<0.05 cytomix vs. PBS, Two-Way ANOVA followed by a Bonferroni post-hoc test).
A.

TEER (% WT PBS)

PBS  Cytomix

Stimulation (4 h)

B.

Dextran Leak (% WT PBS)

PBS  Cytomix

Stimulation (4 h)

C.

Albumin Leak (% WT PBS)

PBS  Cytomix

Stimulation (4 h)

WT  Timp3⁻/⁻
3.4  \textit{Timp3}^{−/−} PMVEC barrier dysfunction is associated with disrupted VE-cadherin intercellular localization

To identify the mechanism leading to enhanced permeability in \textit{Timp3}^{−/−} vs. WT PMVEC, the pericellular localization of VE-cadherin was examined via immunofluorescence, as VE-cadherin is the integral transmembrane adhesive protein associated with intercellular adherens junctions. WT and \textit{Timp3}^{−/−} PMVEC monolayers were stimulated with PBS (vehicle control) or cytomix (30 ng/mL) for 4h, and subsequently fixed and incubated with antibodies against VE-cadherin. Under septic conditions, WT PMVEC exhibited markedly disrupted inter-PMVEC VE-cadherin localization compared to PBS-treated WT PMVEC. Importantly, \textit{Timp3}^{−/−} PMVEC exhibited similarly disrupted VE-cadherin localization both in the absence of stimulation (PBS treatment) as well as following cytomix stimulation (Figure 3-5A). The disruption in intercellular VE-cadherin demonstrated by immunofluorescence was then quantified by determining the percentage of PMVEC cell surface stained positive for VE-cadherin and expressed as a percentage of PBS-treated WT PMVEC (Figure 3-5B). Quantification revealed a significant decrease in inter-PMVEC VE-cadherin circumferential stain in PBS-treated \textit{Timp3}^{−/−} vs. WT PMVEC VE-cadherin, similar to the significant decrease in septic (cytomix-treated) WT PMVEC vs. PBS-treated WT PMVEC.

3.4.1 \textit{Timp3}^{−/−} PMVEC barrier dysfunction is associated with decreased VE-cadherin protein abundance

VE-cadherin protein abundance in cell lysates collected from female and male WT and \textit{Timp3}^{−/−} PMVEC following either PBS (vehicle control) or cytomix (30 ng/mL) stimulation for 6 or 24h was analyzed by western blot. Interestingly, no significant differences in VE-cadherin protein abundance in female \textit{Timp3}^{−/−} vs. WT PMVEC were
Figure 3-5: *Timp3*<sup>−/−</sup> PMVEC barrier dysfunction is associated with disrupted vascular endothelial (VE)-cadherin intercellular localization. (A) The linear circumferential VE-cadherin staining (red) in PBS-treated WT PMVEC was markedly disrupted in *Timp3*<sup>−/−</sup> PMVEC under basal (PBS-treated) conditions, similar to septic (cytomix-treated) WT PMVEC. Large panels: 63X; inset: 100X. (B) This disrupted intercellular VE-cadherin was quantified by determining the percentage of PMVEC cell surface stained positive for VE-cadherin and expressed as a percentage of PBS-treated WT PMVEC. A significant decrease in inter-PMVEC VE-cadherin circumferential stain in PBS-treated *Timp3*<sup>−/−</sup> vs. WT PMVEC VE-cadherin, in addition to a significant decrease in septic (cytomix-treated) WT PMVEC vs. PBS-treated WT PMVEC and septic (cytomix-treated) *Timp3*<sup>−/−</sup> PMVEC vs. PBS-treated *Timp3*<sup>−/−</sup> PMVEC was demonstrated (mean ± SEM, N=3, * indicates P<0.05 *Timp3*<sup>−/−</sup> vs. WT and # indicates P<0.05 cytomix vs. PBS, Two-Way ANOVA followed by a Bonferroni post-hoc test.
A. WT  

PBS  

Cytomix  

20 μm  

B. PMVEC Circumferential VE-Cadherin Stain (% WT PBS)  

WT  

Timp3/-  

PBS  

Cytomix  

Treatment (4h)
found, although a decrease in VE-cadherin protein abundance was demonstrated in $Timp3^{-/-}$ PMVEC under resting and septic conditions vs. PBS-treated female WT PMVEC (Figure 3-6). At 24h post cytomix stimulation, however, a significant decrease in VE-cadherin protein abundance was observed in septic female WT PMVEC vs. PBS control (Figure 3-6). Additionally, results revealed a significant decrease in VE-cadherin protein abundance in male septic (cytomix-treated) WT PMVEC vs. PBS control at both 6 and 24h post cytomix stimulation (Figure 3-7). VE-cadherin abundance was also significantly decreased in $Timp3^{-/-}$ PMVEC under resting and septic conditions vs. PBS-treated male WT PMVEC (Figure 3-7).

As barrier dysfunction was found to be associated with decreased VE-cadherin protein abundance, both under septic (cytomix-treated) conditions in WT PMVEC and in $Timp3^{-/-}$ PMVEC at baseline, $Cdh5$ (VE-cadherin) mRNA expression levels were examined in male WT and $Timp3^{-/-}$ PMVEC to assess whether mRNA expression levels would demonstrate a similar pattern to that of VE-cadherin protein abundance. Analysis of VE-cadherin mRNA expression by qRT-PCR did not reveal significant differences in $Cdh5$ mRNA expression in WT and $Timp3^{-/-}$ PMVEC following septic (cytomix) stimulation at 6h post stimulation; however, there was a trend towards increased $Cdh5$ expression in both genotypes under septic conditions (Figure 3-8A). Interestingly, a significant increase in $Cdh5$ mRNA expression levels was exhibited by $Timp3^{-/-}$ PMVEC following 24h of cytomix stimulation vs. control (PBS-treated) $Timp3^{-/-}$ PMVEC (Figure 3-8B).
**Figure 3-6**: *Timp3*+/− PMVEC barrier dysfunction is associated with decreased VE-cadherin protein abundance in female PMVEC. (A) VE-cadherin protein content was not significantly decreased between treatment groups or genotypes at 6h post cytomix treatment. (B) Following 24h of cytomix stimulation, however, septic (cytomix-treated) WT PMVEC had significantly decreased VE-cadherin protein content compared to PBS-treated WT PMVEC (western blotting; the normalized ratio of TIMP3 to GAPDH levels are shown as a percentage of PBS treated PMVEC. Mean ± SEM, N=3, # indicates P<0.05 cytomix vs. PBS, Two-Way ANOVA followed by a Bonferroni post-hoc test). (C) Representative immunoblots from an independent cell lysate extraction following 6h and 24h of PBS or cytomix stimulation probed with antibodies against VE-cadherin and GAPDH.
A. VE-Cadherin Abundance (% WT PBS)

- **Treatment (6h)**
  - PBS
  - Cytomix

B. VE-Cadherin Abundance (% WT PBS)

- **Treatment (24h)**
  - PBS
  - Cytomix

C. WT vs. Timp3^-/-

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VE-Cadherin (125 kDa)

GAPDH (37 kDa)
Figure 3-7: Timp3<sup>−/−</sup> PMVEC barrier dysfunction is associated with decreased VE-cadherin protein abundance in male PMVEC. VE-cadherin protein content was significantly decreased in PBS-treated Timp3<sup>−/−</sup> vs. PBS-treated WT PMVEC, similar to the decrease in septic (cytomix-treated) WT PMVEC vs. PBS-treated WT PMVEC at both 6h (A) and 24h (B) post cytomix treatment (western blotting; the normalized ratio of TIMP3 to GAPDH levels are shown as a percentage of PBS-treated PMVEC. Mean ± SEM, N=3, * indicates P<0.05 Timp3<sup>−/−</sup> vs. WT and # indicates P<0.05 cytomix vs. PBS, Two-Way ANOVA followed by a Bonferroni post-hoc test). (C and D) Representative immunoblots from an independent cell lysate extraction following (C) 6h and (D) 24h of PBS or cytomix stimulation probed with antibodies against VE-cadherin and GAPDH.
**A.**

Comparison of VE-Cadherin abundance (% WT PBS) between PBS and Cytomix treatments after 6h of treatment.

**B.**

Comparison of VE-Cadherin abundance (% WT PBS) between PBS and Cytomix treatments after 24h of treatment.

**C.**

Comparison of VE-Cadherin and GAPDH expression levels in WT and Timp3^-/- genotypes under PBS and Cyto treatments.

**D.**

Comparison of VE-Cadherin and GAPDH expression levels in WT and Timp3^-/- genotypes under PBS and Cyto treatments.
Figure 3-8: Septic treatment appears to be associated with an increase in Cdh5 (VE-cadherin) mRNA expression in WT and Timp3−/− PMVEC. No basal differences in Cdh5 mRNA expression were observed between genotypes at baseline (PBS treatment). Compared to PBS treatment, however, qRT-PCR revealed a trend toward increased Cdh5 mRNA expression in cytomix-treated WT and Timp3−/− PMVEC following 6 and 24h post cytomix treatment, with a significant increase in Cdh5 mRNA expression levels in Timp3−/− PMVEC following 24h of cytomix stimulation vs. control (PBS-treated) Timp3−/− PMVEC. Data is expressed as the relative quantity (RQ) of PBS-treated WT PMVEC control (mean ± SEM, N=3, # indicates P<0.05 cytomix vs. PBS, Two-Way ANOVA followed by a Bonferroni post-hoc test).
3.4.2 $\text{Timp3}^{-/-}$ PMVEC barrier dysfunction is associated with increased stress fibre formation

As cytoskeleton-mediated PMVEC retraction is an indication of PMVEC activation and a mechanism of barrier dysfunction, cytoskeletal rearrangement and the formation of stress fibres under septic conditions were assessed by visualization of the PMVEC actin cytoskeleton via immunofluorescence microscopy of phallolidin staining. Following septic (cytomix) treatment, WT PMVEC exhibited increased stress fibre formation compared to WT PMVEC at rest (Figure 3-9). Additionally, $\text{Timp3}^{-/-}$ PMVEC exhibited enhanced stress fibre formation both in the absence of stimulation (PBS-treated), as well as following cytomix stimulation vs. PBS-treated WT PMVEC (Figure 3-9).

3.5 TIMP3 deficiency ($\text{Timp3}^{-/-}$ PMVEC) is associated with delayed PMVEC barrier establishment

Based on the significant differences in basal permeability observed between WT and $\text{Timp3}^{-/-}$ PMVEC at a single time point, baseline barrier permeability in WT and $\text{Timp3}^{-/-}$ PMVEC over a number of days was examined by means of TEER in order to assess whether this difference was persistent over time, and finally, whether $\text{Timp3}^{-/-}$ PMVEC could ever form an intact or impermeable barrier. Following 10 days of culture, WT PMVEC TEER had reached a plateau, indicating a stable monolayer and intact permeability barrier had been established (Figure 3-10A and B, 3-11 A and B). In support of our findings at a single time point, $\text{Timp3}^{-/-}$ PMVEC persistently exhibited significantly lower basal levels of TEER at all time points compared to WT PMVEC as analyzed by differences in area under the curve (AUC) (Figure 3-10C and D, 3-11 C)
Figure 3-9: *Timp3*−/− PMVEC appear to be activated under basal conditions compared to WT PMVEC. Actin cytoskeleton stress fibre formation is enhanced in *Timp3*−/− PMVEC vs. WT PMVEC under basal conditions (PBS-treatment). WT PMVEC had similarly increased stress fibre formation, but only under septic conditions (following treatment with cytomix). Fluorescent microscopy: 63X.
Figure 3-10: Female \( Timp3^{+/−} \) PMVEC exhibit low levels of TEER at baseline. Under basal conditions, \( Timp3^{+/−} \) PMVEC persistently exhibited significantly lower TEER vs. WT PMVEC. TEER time course of WT and \( Timp3^{+/−} \) PMVEC seeded at a cell density of 2.5\( \times 10^4 \) cells/insert (A) and 5.0\( \times 10^4 \) cells/insert (B). Analysis of cumulative TEER over time of WT and \( Timp3^{+/−} \) PMVEC seeded at a cell density of 2.5\( \times 10^4 \) cells/insert (C) and 5.0\( \times 10^4 \) cells/insert (D) revealed that TEER levels were significantly lower in \( Timp3^{+/−} \) vs. WT PMVEC across the time course as measured by Area Under the Curve (AUC) (mean ± SEM, \( N=7-8 \), * indicates \( P<0.05 \) \( Timp3^{+/−} \) vs. WT, t-test).
Figure 3-11: Male Timp3<sup>−/−</sup> PMVEC exhibit low levels of TEER at baseline. Under basal conditions, Timp3<sup>−/−</sup> PMVEC persistently exhibited significantly lower TEER vs. WT PMVEC. TEER time course of WT and Timp3<sup>−/−</sup> PMVEC seeded at a cell density of 2.5x10<sup>4</sup> cells/insert (A) and 5.0x10<sup>4</sup> cells/insert (B). Analysis of cumulative TEER over time of WT and Timp3<sup>−/−</sup> PMVEC seeded at a cell density of 2.5x10<sup>4</sup> cells/insert (C) and 5.0x10<sup>4</sup> cells/insert (D) revealed that TEER levels were significantly lower in Timp3<sup>−/−</sup> vs. WT PMVEC across the time course as measured by Area Under the Curve (AUC) (mean ± SEM, N=8, * indicates P<0.05 Timp3<sup>−/−</sup> vs. WT, t-test).
and D). Interestingly, while the increase in TEER in Timp3−/− PMVEC was impaired vs. WT PMVEC, Timp3−/− PMVEC appeared to establish an intact barrier following 14-15 days of culture, as basal TEER measurements became similar to the stable levels seen in established WT PMVEC monolayers (Figure 3-10A and B, 3-11 A and B). Similar results were found in WT and Timp3−/− PMVEC isolated from male and female mice (Figure 3-10 and 3-11).

As WT PMVEC TEER appeared to reach a plateau following 10 days of culture and Timp3−/− PMVEC appeared, by TEER measurements, to similarly establish an intact barrier following 14-15 days of culture (Figure 3-10, 3-11), permeability studies in WT and Timp3−/− PMVEC analyzing FITC-labelled dextran, and EB-labelled albumin flux were then conducted following 10 as well as 14-15 days of culture (Figure 3-12). Following 10 days of culture in the absence of stimulation, Timp3−/− PMVEC consistently displayed significantly higher basal permeability vs. WT PMVEC by all 3 complementary permeability assays: lower TEER, and higher macromolecular leak measured by both FITC-labelled dextran and EB-labelled albumin flux (Figure 3-12 A-C). However, following 14-15 days of culture, when TEER levels of Timp3−/− PMVEC are nearly equivalent to those of WT PMVEC, differences between genotypes in macromolecular leak, while still significant, appeared to be greatly reduced (Figure 3-12 D-F).

3.6 TIMP3 deficiency (Timp3−/−) does not affect PMVEC viability or proliferation

TIMP3 has previously been found to regulate cell proliferation in hematopoietic cells and pericytes [74,100]. As such, cellular proliferation in WT and Timp3−/− PMVEC
Figure 3-12: TIMP3 deficiency (Timp3−/− PMVEC) is associated with delayed PMVEC barrier establishment. Following 10 days of culture, under basal (PBS-treated) conditions, Timp3−/− PMVEC had consistently higher permeability vs. WT PMVEC by 3 complementary assays: lower TEER (A), and higher macromolecular leak including FITC-labelled dextran (B), and EB-labelled albumin flux (C). However, following 14-15 days of culture, TEER levels in Timp3−/− PMVEC are no longer significantly different from WT PMVEC TEER levels (D). Furthermore, after 14-15 days in culture, differences between WT and Timp3−/− PMVEC in FITC labelled-dextran (E), and EB-labelled albumin flux (F), while still significant, were greatly reduced (mean ± SEM, N=7-8, * indicates P<0.05 Timp3−/− vs. WT, t-test).
was examined to determine if altered proliferation might account for the observed difference in TEER demonstrated over time between WT and Timp3<sup>−/−</sup> PMVEC, and the subsequent delayed formation of a functional barrier in Timp3<sup>−/−</sup> PMVEC. Cellular viability was first assessed by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay. No significant differences in cell viability were observed between WT and Timp3<sup>−/−</sup> PMVEC at 24, 48, and 72h following initial cell culture (Figure 3-13A and B). Cellular proliferation was then further assessed by means of direct cell counts using a hemocytometer, in order to confirm the results shown by the MTT cell viability assay. No significant differences in cell number were noted between genotypes at 2, 4, and 7 days following initial cell culture (Figure 3-13C).

3.7 Barrier dysfunction in Timp3<sup>−/−</sup> PMVEC under basal conditions is partly metalloproteinase dependent

PMVEC permeability was additionally assessed in the presence or absence of GM6001, a broad-spectrum synthetic metalloproteinase inhibitor, in order to investigate the role of metalloproteinase activity in the impaired Timp3<sup>−/−</sup> PMVEC monolayer barrier function. Following 10 days of culture on transwell cell-culture inserts in the presence or absence of GM6001, PMVEC monolayer permeability was comprehensively assessed using all three complementary techniques: TEER, FITC-labelled dextran flux, and EB-labelled albumin flux. Interestingly, the barrier dysfunction exhibited by Timp3<sup>−/−</sup> PMVEC under basal conditions (significantly lower TEER, higher trans-PMVEC dextran and albumin leak) was partially rescued by treatment with GM6001 (1 µg/mL) vs. control (DMSO) (Figure 3-14). Importantly, the significant differences in basal FITC-labelled dextran and EB-labelled albumin flux observed between WT and Timp3<sup>−/−</sup> PMVEC were
Figure 3-13: PMVEC proliferation in cell culture is not significantly different between WT and Timp3−/− PMVEC. WT (blue) and Timp3−/− (red) PMVEC were seeded at a cell density of 5x10^3 or 1x10^4 cells/well, and cell proliferation was examined by two different techniques. (A and B) At 24, 48, and 72 hours of culture, cell viability was assessed by an MTT cell viability assay. No differences in cell viability were noted between genotypes at any time point (mean ± SEM, N=3-4). (C) At 2, 4, and 7 days, cells were harvested and counted. No differences in cell number were noted between genotypes at any time point (mean ± SEM, N=3-4).
Figure 3-14: Barrier dysfunction in *Timp3*−/− murine PMVEC under basal conditions is partly metalloproteinase dependent. Barrier dysfunction in *Timp3*−/− PMVEC under basal conditions (lower TEER; A), higher trans-PMVEC FITC-labelled dextran (B) and EB-labelled albumin flux (C) is partly rescued by treatment with GM6001 (1 µg/mL), a global metalloproteinase inhibitor, vs. control (DMSO) (mean ± SEM, N=6-7, * indicates P<0.05 *Timp3*−/− vs. WT and # indicates P<0.05 GM6001 vs. DMSO, Two-Way ANOVA followed by a Bonferroni post-hoc test).
abolished following treatment with GM6001 (Figure 3-14C and D). Additionally, treatment with GM6001 significantly decreased EB-labelled albumin flux in \( \text{Timp}^{3/-} \) PMVEC vs. DMSO-treated \( \text{Timp}^{3/-} \) PMVEC (Figure 3-14D).

Moreover, the localization of VE-cadherin in WT and \( \text{Timp}^{3/-} \) PMVEC following treatment with the broad-spectrum metalloproteinase inhibitor GM6001 was examined by immunofluorescence. WT and \( \text{Timp}^{3/-} \) PMVEC monolayers were treated with DMSO (vehicle control) or GM6001 (1 µg/mL) for 4h, and subsequently fixed and incubated with antibodies against VE-cadherin. Interestingly, the fragmented pericellular VE-cadherin staining in control (DMSO-treated) \( \text{Timp}^{3/-} \) PMVEC appeared to become more linear and circumferential, similar to WT PMVEC under basal conditions, following treatment with GM6001 (Figure 3-15A). Quantification of the disruption in intercellular VE-cadherin demonstrated by immunofluorescence revealed a significant increase in inter-PMVEC VE-cadherin circumferential stain in GM6001-treated \( \text{Timp}^{3/-} \) vs. DMSO-treated \( \text{Timp}^{3/-} \) (Figure 3-15B).
Figure 3-15: Disrupted VE-cadherin intercellular localization in \textit{Timp3}^{-/-} \textit{PMVEC}

under basal conditions is metalloproteinase-dependent. (A) The fragmented pericellular VE-cadherin staining (red) in control (DMSO-treated) \textit{Timp3}^{-/-} \textit{PMVEC} appeared to be more linear and circumferential (as in WT PMVEC) following treatment with GM6001 (1 µg/mL), a global metalloproteinase inhibitor. Large panels: 63X; inset: 100X. (B) VE-cadherin stain was quantified by determining the percentage of PMVEC cell surface stained positive for VE-cadherin and expressed as a percentage of DMSO-treated WT PMVEC. A significant increase in inter-PMVEC VE-cadherin circumferential stain in GM6001-treated \textit{Timp3}^{-/-} vs. DMSO-treated \textit{Timp3}^{-/-} was demonstrated (mean ± SEM, N=4, * indicates P<0.05 \textit{Timp3}^{-/-} vs. WT and # indicates P<0.05 GM6001 vs. DMSO, Two-Way ANOVA followed by a Bonferroni post-hoc test).
A. WT  \textit{Timp3}^{-/-}

\begin{itemize}
\item DMSO
\item GM6001
\end{itemize}

\begin{itemize}
\item [20 \mu m]
\end{itemize}

B. Treatment (4h)

\begin{itemize}
\item PMVE Circumferential VE-Cadherin Stain (% WT DMSO)
\end{itemize}

\begin{itemize}
\item DMSO
\item GM6001
\end{itemize}

\begin{itemize}
\item WT
\item \textit{Timp3}^{-/-}
\end{itemize}

\begin{itemize}
\item *\textsuperscript{,} \textsuperscript{,}
\item \#\textsuperscript{,} \textsuperscript{,*}
\end{itemize}
Chapter 4

4 Discussion

4.1 Summary of Findings

Sepsis, a systemic inflammatory response to infection, often leads to multiple organ dysfunction mainly due to perturbations in the microvasculature [6–10,101]. In particular, the accumulation of alveolar protein-rich edema observed in sepsis-induced ARDS is thought to be primarily caused by a loss of pulmonary microvascular stability and increased permeability due to PMVEC injury, dysfunction, and death following sepsis [102–105]. PMVEC are integral to maintaining homeostatic microvascular function by modulating vascular tone, and controlling the passage of circulating cells and macromolecules through an intact permeability barrier [7,12,15–17]. However, the homeostatic mechanisms protecting against sepsis-induced PMVEC dysfunction are less defined.

Thus, we sought to investigate the role of PMVEC-derived TIMP3 in the regulation of normal and septic microvascular barrier function. Our data suggests that loss of TIMP3 (under septic conditions or in Timp3−/− PMVEC) leads to increased PMVEC permeability, which is associated with altered localization of VE-cadherin. Furthermore, this increased PMVEC permeability is associated with the loss of metalloproteinase inhibition, as treatment with GM6001 attenuated both the enhanced leak and disrupted VE-cadherin intercellular localization exhibited by Timp3−/− PMVEC. Therefore, our study demonstrates that TIMP3, a potent inhibitor of metalloproteinases, may mediate a novel, endogenous PMVEC protective barrier mechanism under healthy
conditions, and that this homeostatic mechanism may be disrupted under septic conditions (Figure 4-1).

4.2 Contributions of research to current state of knowledge

4.2.1 TIMP3 Levels in Septic (cytomix-treated) PMVEC

Our studies have demonstrated that stimulation of PMVEC monolayers with cytomix at a concentration of 30 ng/mL led to detectable barrier dysfunction, as measured by decreased TEER and enhanced trans-PMVEC EB-labelled albumin flux, over a period of 24 hours post-stimulation. Thus, Timp3 mRNA expression and protein abundance levels were examined in WT PMVEC over a similar timeline in order to assess the role of TIMP3 in sepsis-induced PMVEC dysfunction. The assessment of Timp3 mRNA expression and protein abundance levels following cytomix stimulation revealed cytomix-treated (septic) WT PMVEC consistently possess significantly lower Timp3 mRNA expression levels as well as protein abundance compared to PBS-treated WT PMVEC.

Our data revealing decreased Timp3 mRNA expression in septic PMVEC is consistent with previous studies, in which treatment of brain microvascular endothelial cells as well as astrocytes with IL1β and TNFα resulted in a substantial decrease in Timp3 mRNA expression [93]. Furthermore, our work expands on these studies by examining the levels of TIMP3 following treatment with IL1β, TNFα, and INFγ over a time course, revealing a lack of recovery of Timp3 mRNA expression up to 8h following septic treatment. Importantly, we also demonstrate that decreased mRNA expression is associated with decreased TIMP3 protein abundance. It has been demonstrated that the
Figure 4-1: PMVEC-derived TIMP3 promotes normal PMVEC barrier function through inhibition of metalloproteinase activity. In the absence of TIMP3 (Timp3<sup>−/−</sup>) or septic downregulation of TIMP3, metalloproteinase activity is increased resulting in PMVEC barrier dysfunction, and ultimately increased protein leak into tissues/organs.
expression of several MMPs is enhanced in the presence of IL1β and TNFα in endothelial cells, as well as macrophages and T-cells [106–108]. In particular, MMP14 expression is up-regulated [93]. This cell surface-associated MMP is expressed in vascular endothelial cells [109], and acts as a broad-spectrum proteinase involved in the breakdown of the extracellular matrix, possibly through the activation of other MMPs, including MMP2 and MMP9 [110]. As MMP14 is effectively inhibited by TIMP3 [111], decreased Timp3 mRNA expression and protein abundance levels under septic conditions (cytomix stimulation) may result in increased MMP14 activity leading to degradation of the extracellular matrix and destabilization of the vasculature.

Additionally, TIMP3 is known to inhibit ADAM17, also known as TNFα-converting enzyme (TACE) [112,113], which actively processes and cleaves membrane-bound pro-TNFα to yield the soluble inflammatory cytokine TNFα [112,113]. Diminished TIMP3 abundance may also lead to accelerated pro-TNFα processing by ADAM17, resulting in further PMVEC activation and dysfunction. Thus, we believe TIMP3 may play a critical role in the maintenance of a stable PMVEC barrier, and that following sepsis, this function for TIMP3 is lost due to the observed decrease in TIMP3 mRNA expression and protein abundance.

4.2.2 Endothelial Barrier Function and TIMP3

We have previously reported that PMVEC barrier function is impaired both in vitro and in vivo under septic conditions [98,105,114–117]. It is well known that the inflammatory response under septic conditions results in PMVEC activation by inflammatory cytokines leading to increased paracellular permeability [13,16,102–
Several mechanisms are responsible for the observed increase in permeability in WT PMVEC following cytomix stimulation, including disruption of inter-PMVEC junctions and actin cytoskeleton-driven PMVEC retraction [12,15–17,97]. Inflammatory cytokine-mediated phosphorylation of adherens junction proteins in addition to enzymatic metalloproteinase activity results in VE-cadherin internalization and cleavage, respectively [12,15–17,97]. Furthermore, the actin cytoskeleton undergoes polymerization and reorganization to form stress fibres following myosin light chain (MLC) phosphorylation, resulting in cell retraction [48–50,120]. As these studies suggest, there are many mechanisms involved in PMVEC barrier dysfunction following sepsis; however, the mechanisms protecting against sepsis-induced PMVEC barrier dysfunction are not well understood.

Our data suggests that TIMP3 may be one such endogenous factor protecting against PMVEC barrier dysfunction under homeostatic conditions, and the loss of TIMP3 expression under septic conditions may play a causative role in sepsis-induced PMVEC barrier dysfunction. Furthermore, our data are not only consistent with previous studies, but they also provide important novel information expanding the current knowledge on the role of TIMP3 in PMVEC barrier function. Previous studies in Timp3−/− mice demonstrated an increase in metalloproteinase activity in multiple organs, along with increased leak of Evans Blue dye into the snout and kidneys at baseline and following injury, respectively, which was thought to be due to the absence of TIMP3 in pericytes [74]. Furthermore, previous studies have demonstrated the importance of TIMP3 in other forms of injury such as traumatic brain injury, as the administration of mesenchymal stem cells attenuates enhanced blood-brain barrier (BBB) permeability following injury due to
the beneficial role of TIMP3 produced by these stem cells [94]. The downregulation of TIMP3 expression in mesenchymal stem cells has been shown to abrogate their protective effect on endothelial stability following traumatic injury [94]. Our studies expand on such previous work through the employment of a different model. Particularly, we utilized PMVEC under septic (cytomix) stimulation, in order to investigate the role that PMVEC-derived TIMP3 may potentially play in the protection against sepsis-induced PMVEC barrier dysfunction present during ARDS. Importantly, our data suggests the mechanism of action in which TIMP3 protects against PMVEC barrier dysfunction is through inhibition of metalloproteinase activity, as treatment of PMVEC with GM6001 rescues the enhanced microvascular permeability observed in the absence of TIMP3.

4.2.3 Metalloproteinase-Dependent Loss of Endothelial Barrier Function

Metalloproteinases, including both MMPs and ADAMs, are known to mediate endothelial cell barrier function. For example, several studies have discovered the ability of multiple MMPs and ADAMs to cleave proteins associated with endothelial adherens and tight junctions [56,68–70]. MMP7, ADAM10, and ADAM12 have been shown to actively cleave VE-cadherin [56,69], while MMP2 and 9 are capable of cleaving tight junction associated proteins such as occludin and ZO-1[68,70], resulting in microvascular dysfunction via increased paracellular permeability. A previous study has shown that treatment of human umbilical vein endothelial cells (HUVEC) with MMP7 results in decreased VE-cadherin abundance and β-catenin accumulation in the nucleus, suggesting enhanced VE-cadherin cleavage in the presence of increased metalloproteinase activity
Recently, it has been reported that endothelial cell activation via cytokine stimulation results in increased ADAM10 and 12 expression in addition to subsequent cleavage of VE-cadherin [55]. Moreover, inhibition of ADAM12 via siRNA knockdown reduced VE-cadherin cleavage and shedding [56].

Several studies have also shown that treatment of endothelial cells with GM6001, a synthetic metalloproteinase inhibitor, reverses microvascular dysfunction through the inhibition of metalloproteinase activity. For example, treatment of HUVEC with GM6001 following upregulation of metalloproteinase activity rescues the increase in permeability as measured by macromolecular flux, as well as VE-cadherin cleavage and the subsequent release of a soluble ectodomain [19,56]. Additionally, in models of cranial edema due to dysfunction of the blood-brain barrier, treatment of murine cerebrovascular endothelial cells with GM6001 resulted in decreased microvascular leak due to the reversal of degradation of tight junction adhesive proteins and F-actin formation through the inhibition of MMP9 [121,122]. Collectively, these studies highlight the importance of metalloproteinase inhibition to regulation of PMVEC barrier dysfunction, and provide support for our conclusions that the observed increase in basal permeability, as well as decreased VE-cadherin abundance and altered localization, in \textit{Timp3}^{-/-} \ PMVEC is likely due to increased metalloproteinase activity. Importantly, our data expands on these previous findings by identifying TIMP3 as the key metalloproteinase inhibitor regulating metalloproteinase function and PMVEC barrier dysfunction under normal homeostatic conditions. Moreover, our studies suggest that decreased expression of TIMP3 is a putative contributor to septic PMVEC barrier dysfunction.

In addition to cleavage of cell surface proteins, metalloproteinases have also been
found to cleave many components of the ECM [31,32,57–59]. Importantly, endothelial cells bound to the ECM are generally quiescent [25]. This is due to the fact that cell-matrix interactions allows for the generation of signals that inhibit proliferation and migration while stabilizing adjacent cell-cell and cell-ECM interactions, which are both vital in the formation and preservation of a restrictive endothelial barrier [25]. Studies have shown that the enzymatic degradation of ECM constituents including fibronectin, laminin, and types IV and V collagens by MMPs, including MMP2 and MMP9, have resulted in increased permeability in cultured endothelial monolayers as well as induced pulmonary edema *in vivo* [31,32]. Therefore, it is also possible that *Timp3*−/− PMVEC exhibit low levels of TEER at baseline due to increased metalloproteinase activity leading to increased cleavage and degradation of ECM components and impaired PMVEC-ECM interactions.

4.2.4 Metalloproteinase-Independent Functions of TIMP3 and PMVEC Barrier Function

In addition to cleavage or internalization of VE-cadherin and actin cytoskeleton-driven PMVEC retraction, disruption of PMVEC barrier function is also the result of increased VE-cadherin dissociation via the binding of VEGF to VEGFR2, and initiation of the VEGFR2-Src-VE-cadherin signalling pathway [97]. While the more prevalent function for TIMP3 is inhibition of metalloproteinase activity, TIMP3 has also been found to have metalloproteinase-independent functions [95,96]. In particular, TIMP3 inhibits angiogenesis by binding to the VEGF receptor and inhibiting VEGF-VEGFR2 signalling [95,96]. Studies have revealed that VEGF signalling is increased in *Timp3*−/− mice following kidney injury [74], and decreased vascular leak following addition of
rTIMP3 in a model of traumatic brain injury is mediated through the inhibition of VEGF signalling [94]. These studies suggest that TIMP3 also regulates vascular function through metalloproteinase-independent mechanisms. While we did not specifically examine VEGF signalling, our data that the observed increased in PMVEC permeability and altered VE-cadherin localization in Timp3−/− PMVEC is rescued by treatment with GM6001 suggests that TIMP3 regulation of PMVEC barrier function is dependent on the inhibition of metalloproteinase activity. Our data combined with previous studies, however, provide evidence that TIMP3 may regulate vascular leak through both metalloproteinase-dependent and-independent mechanisms.

4.2.5 Role of TIMP3 in Formation of PMVEC Barrier

We also sought to examine baseline leak in WT and Timp3−/− PMVEC over a number of days in order to assess whether differences in basal permeability observed between WT and Timp3−/− PMVEC at a single time point was persistent over time. Multiple studies have utilized TEER as a method to measure microvascular permeability as TEER offers advantages over measures of macromolecular flux (i.e. changes in paracellular permeability to charged ions are rapidly detected and can be followed over the course of PMVEC barrier formation) [44,48,123–125]. Endothelial cell membranes are composed of lipophilic molecules (e.g. phospholipids, cholesterol) that act as electrical insulators [126]. When the endothelium is damaged, fluid filled pores at cell-cell junctions function as electrical conductance pathways, consequently decreasing electrical resistance [126]. For example, Sedgwick and colleagues demonstrated that treatment of human lung microvascular endothelial cell (HMVEC-L) monolayers with inflammatory cytokines TNFα, IL1β, and INFγ resulted in a dose-dependent decrease in
baseline TEER [123]. Thus, decreased TEER indicates physiological changes in PMVEC barrier function, which result in increased trans-PMVEC permeability. Our study demonstrates that $Timp3^{-/-}$ PMVEC persistently exhibit significantly lower levels of TEER at baseline compared to WT PMVEC. However, while the increase in TEER in $Timp3^{-/-}$ PMVEC is impaired vs. WT PMVEC, $Timp3^{-/-}$ PMVEC appear to establish an intact barrier following 14-15 days of culture, as basal TEER values approximated those of WT PMVEC.

In addition to low levels of TEER, $Timp3^{-/-}$ PMVEC consistently displayed higher basal macromolecular leak vs. WT PMVEC measured by both EB-labelled albumin and FITC-labelled dextran flux. However, as TEER levels of $Timp3^{-/-}$ PMVEC became equivalent to those of WT PMVEC following 14-15 days of culture, differences in macromolecular leak between genotypes persisted, but were greatly diminished. Together, this data suggests that in the absence of TIMP3, PMVEC can begin to establish an impermeable barrier; however, this process is delayed.

One possible mechanism leading to the impaired or delayed barrier formation would be decreased proliferation in $Timp3^{-/-}$ vs. WT PMVEC. TIMP3 has previously been found to regulate cell proliferation in different cell types including hematopoietic stem cells, endothelial cells, pericytes, and cardiomyocytes [74,100,127,128]. Previous studies demonstrate conflicting results pertaining to whether TIMP3 acts to promote or inhibit cell proliferation, which seems to depend on the specific cell type. Within $Timp3^{-/-}$ pericytes and cardiomyocytes, cell proliferation was significantly increased vs. WT control, suggesting TIMP3 inhibits cell proliferation [74,127]. However, TIMP3 has been shown to enhance hematopoietic stem cell proliferation, while having no effect on
HUVEC proliferation in vitro [100,128]. In order to assess any differences in cell proliferation in Timp3<sup>−/−</sup> vs. WT PMVEC, two approaches were used: an MTT assay and quantification of cell number. The lack of any significant difference in cell proliferation between genotypes suggests that in PMVEC, TIMP3 does not appear to regulate cell proliferation and as such, impaired proliferation is not likely the cause of the delayed barrier formation observed in Timp3<sup>−/−</sup> PMVEC.

Previous studies have shown that at early stages of cell confluency, VE-cadherin, in addition to β-catenin and p120, are highly phosphorylated at tyrosine residues [52]. This state of phosphorylation, however, is abolished once cultured endothelial cells become confluent and adherens junction maturation is complete [52]. Additionally, stable VE-cadherin conformation confers contact inhibition during cellular growth and proliferation [15,52,129]. However, truncated VE-cadherin, through cleavage by metalloproteinases for example, no longer has the ability to regulate cellular contact inhibition, nor endothelial barrier stabilization [15,129]. Therefore, in addition to possessing cell adhesive properties, VE-cadherin may also have the ability to transmit intercellular contact inhibition signals, but its binding to β-catenin and/or plakoglobin is required.

Schulz and colleagues have demonstrated that following cleavage and shedding of the VE-cadherin ectodomain (N-terminal domain), the remaining C-terminal domain is subject to proteolysis by γ-secretase [56]. Previous studies have also implicated that following cleavage of epithelial cadherin (E-cadherin), subsequent cytoplasmic domain degradation results in the release of β-catenin from intercellular junctions into the
cytoplasm [130]. Thus, it is believed that the VE-cadherin cytoplasmic domain no longer has the ability to associate with accompanying catenins, such as β-catenin, in addition to the actin cytoskeleton, and therefore, stable adherens junctions cannot be formed [56,130].

Furthermore, elevated cytoplasmic levels of β-catenin have been implicated in the Wnt signalling pathway [15,16,130]. Generally, free β−catenin present in the cytoplasm is marked for proteasomal degradation by glycogen synthase kinase 3 (GSK3) [15,16,130]. However, the binding of Wnt to its receptor inactivates GSK3, and results in the accumulation of free cytoplasmic β-catenin, which is then able to translocate to the nucleus and induce transcription of genes imperative for cell proliferation and survival [15,16,130]. Studies employing the use of mouse embryonic fibroblasts discovered enhanced neuronal cadherin (N-cadherin) and β-catenin levels at the cell membrane in ADAM10 deficient (ADAM10−/−) mice [130]. However, in the presence of ADAM10, enhanced cleavage of N-cadherin, in addition to increased cytoplasmic levels of β−catenin, is present [130]. Collectively, these studies make it tempting to speculate that in \textit{Timp3}\textsuperscript{−/−} PMVEC, increased metalloproteinase activity results in the enhanced cleavage of VE-cadherin, which causes its dissociation from associated catenins and the actin cytoskeleton, therefore rendering VE-cadherin unable to mediate intercellular signalling and regulate cell contact inhibition. Thus, although there are no differences observed in cell proliferation rate between WT vs. \textit{Timp3}\textsuperscript{−/−} PMVEC, \textit{Timp3}\textsuperscript{−/−} PMVEC do not experience contact inhibition and in fact continue to proliferate and grow on top of one another, forming multiple layers of cells, rather than forming a stable monolayer.
4.3 Limitations and Future Directions

4.3.1 Study Limitations

All experiments were done entirely *in vitro*, as our study employed the use of murine PMVEC seeded on gelatin-coated transwell inserts to model PMVEC barrier function. Therefore, the PMVEC are cultured in static conditions. However, in an *in vivo* setting, PMVEC are subject to several external forces, such as hemodynamic stimuli including shear stress and changes in vessel size. The microvasculature is an extremely dynamic structure, however, and modulation of blood flow and vasomotor tone is achieved by the release of vasoconstrictors (i.e. angiotensin-converting enzyme, endothelin) and vasodilators (i.e. NO, PGI₂) in order to regulate vascular resistance and systemic blood pressure [1,8]. Additionally, intercellular junctions along with the actin cytoskeleton continuously reshape in order for the endothelial barrier to adapt to conditions to which it may be exposed [16]. Adhesive junctional proteins, such as VE-cadherin, may also act as flow sensors and transmit intracellular cues that signal the cell to react to changing conditions [16]. Due to technological limitations, we are not able to replicate a flow model that PMVEC would encounter in their native conditions. However, as the endothelium dynamically responds to changing conditions without altering barrier permeability or stability, our model emulates relevant endothelial barrier function conditions, as PMVEC cultured in static conditions are still able to communicate with one another through intercellular junctions, similar to what would occur *in vivo*.

Additionally, PMVEC were cultured on 1% gelatin coated transwell inserts in order to mimic an extracellular matrix for the cells to adhere to. However, the ECM is a complex network, mainly composed of collagens, proteoglycans, and glycoproteins
including fibronectin and laminin \textit{in vivo} [1,11]. Under \textit{in vivo} physiological conditions, where the endothelium is subjected to external forces such as blood flow, the ECM in which the endothelium is adhered to is of greater importance compared to \textit{in vitro} conditions. Nevertheless, in order to best mimic physiological conditions present \textit{in vivo}, future studies should employ a variety of ECM components within cell culture. Moreover, the glycocalyx, which lines the luminal surface of the endothelium, forms a substantial surface layer critical to barrier function \textit{in vivo} [11,21]. The glycocalyx is known to play critical roles in the regulation of barrier function following injury, and has also been shown to be cleaved by metalloproteinases, but however, is only observed \textit{in vivo} and is absent \textit{in vitro} [21,131].

Furthermore, our studies focus solely on the role of PMVEC-derived TIMP3 in the regulation of normal and septic microvascular barrier function. The potential involvement of other members of the TIMP family, however, was not assessed. Possible compensation by other members of the TIMP family, or the activity of one or multiple TIMPs making up for the loss of another, may potentially occur in the absence of TIMP3. However, evidence supporting compensation amongst MMP and TIMP knockout models is lacking [59]. Additionally, while metalloproteinase global activity in \textit{Timp3}^{-/-} PMVEC was assessed through the use of GM6001, changes in expression, as well as activity, of specific metalloproteinases in the absence of TIMP3 were not directly assessed.

\subsection*{4.3.2 Future Directions}

In future studies, endothelial barrier dysfunction in \textit{Timp3}^{-/-} PMVEC must be further characterized \textit{in vitro} in order to define TIMP3-dependent protective mechanisms of normal PMVEC barrier function. Thus, specific metalloproteinases that are inhibited
by TIMP3, or in other words, whose activity is increased in the absence of TIMP3 (Timp3\(^{-/-}\) PMVEC), must be identified. Additionally, expression of other members of the TIMP family must be investigated in order to assess whether any sort of compensation is occurring in Timp3\(^{-/-}\) PMVEC, which may account for the ability of Timp3\(^{-/-}\) PMVEC to establish a stable monolayer following a prolonged timeline in culture. Examination of the expression of other members of the TIMP family may be determined at a transcriptional level by qRT-PCR, as well as at a protein abundance level via western blot. Importantly, future studies should also investigate potential metalloproteinase-independent mechanisms that may play a role in the ability of TIMP3 to regulate PMVEC barrier function under normal and septic conditions. As TIMP3 has the ability to bind to the VEGF receptor present on the PMVEC cell surface and inhibit VEGF-VEGFR2 signalling [95,96], both WT and Timp3\(^{-/-}\) PMVEC could be cultured in the presence and absence of a VEGFR2 antagonist in order to mimic the effects of TIMP3 on VEGFR2 phosphorylation and endothelial permeability.

In addition to VE-cadherin, other endothelial intercellular junctional proteins such as claudin-5, ZO1, and JAMA, which are present in endothelial tight junctions, are also cleaved by metalloproteinases [68,70]. Therefore, intercellular localization of these junctional components in WT and Timp3\(^{-/-}\) PMVEC should be examined by immunofluorescence under basal and septic conditions. Once any junctional proteins with disrupted cell-surface localization in Timp3\(^{-/-}\) vs. WT PMVEC have been identified, it could be determined whether this is due to enhanced cleavage/shedding of the given protein(s), or reduced mRNA/protein expression through the use of qRT-PCR and western blotting. If altered localization is in fact due to increased protein shedding, we
predict increased levels of protein will be present in the conditioned media of *Timp3*−/− vs. WT PMVEC, with decreased levels present in the cell lysate. Additionally, as the intracellular localization and expression of catenins, including β-catenin and α-catenin, are strongly associated with junctional proteins and required for the maintenance of stable endothelial barrier function; catenin expression and localization should be assessed in WT and *Timp3*−/− PMVEC under basal and septic conditions. This will help to allow us to identify any downstream intracellular targets or signalling mechanisms which may occur following the cleavage/shedding of integral endothelial junctional proteins by enhanced metalloproteinase activity.

Moreover, we wish to confirm our results obtained following cytomix treatment of WT and *Timp3*−/− PMVEC through the use of septic plasma acquired from a clinically relevant murine cecal ligation and perforation (CLP)-induced peritonitis model of sepsis *in vivo*. In addition to examining PMVEC barrier permeability in response to septic plasma, we will also expand our studies beyond a model that utilizes only a single cell line to include co-culture studies of WT and *Timp3*−/− PMVEC with WT and *Timp3*−/− PMNs and/or macrophages, in order to assess the role of TIMP3 in the interaction of inflammatory cells with PMVEC.

Lastly, as all experiments were conducted *in vitro*, animal models must be employed in future studies in order to investigate this putative endogenous TIMP3-dependent mechanism and support of basal PMVEC function *in vivo*, as well as potential involvement in septic PMVEC barrier dysfunction. This will allow for *in vivo* demonstration of the molecular mechanisms regulating normal and septic microvascular permeability that we have identified *in vitro*, which will give further insight regarding the
physiological relevance of these findings. Additionally, in vitro studies employing the use of human PMVEC would also provide strong clinical relevance of our findings.

4.4 Conclusions

In conclusion, sepsis-induced ARDS is associated with pulmonary microvascular barrier injury and dysfunction, resulting in increased permeability and the accumulation of alveolar protein-rich edema. Our assessment of WT and Timp3−/− PMVEC barrier integrity under basal and septic conditions has identified a TIMP3-dependent endogenous protective mechanism against PMVEC barrier dysfunction, as well as a possible disruption of this homeostatic mechanism under septic conditions through the septic downregulation of TIMP3. Based on our results, we believe PMVEC-derived TIMP3 supports normal pulmonary microvascular endothelial barrier function, and may attenuate the increase in pulmonary vascular permeability following lung injury through inhibition of metalloproteinase activity. A better understanding of the TIMP3-mediated endogenous protective mechanism against septic PMVEC barrier dysfunction would support new therapeutic approaches for patients suffering from sepsis-induced ARDS, as well as help elucidate PMVEC dysfunction in other vascular diseases.
References


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