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The Metalloproteinase-Dependent Role of TIMPs in Regulation of Pulmonary Microvascular Endothelial Cell Barrier Function During Sepsis

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Abstract

Sepsis causes injury and dysfunction of pulmonary microvascular endothelial cells (PMVEC), leading to pulmonary edema. Metalloproteinases, are associated with inflammation and tissue damage, and their regulation by tissue inhibitors of metalloproteinases (TIMPs) may protect against septic PMVEC dysfunction. Thus, I hypothesize that murine septic PMVEC barrier dysfunction is due to disruption of the balance between metalloproteinases and TIMPs leading to increased metalloproteinase activity.

PMVEC were isolated from wild type (WT) and *Timp3*$^{-/-}$ mice. *Timp* and metalloproteinase mRNA expression was altered under septic conditions and this was associated with increased metalloproteinase activity. Global metalloproteinase inhibitors BB-94 and TAPI-2 reduced albumin and dextran flux across septic PMVEC. Further, *Timp3*$^{-/-}$ PMVEC had less cell surface intercellular adhesion molecule (ICAM) 1 vs. WT PMVEC, which was associated with significantly impaired neutrophil-PMVEC adhesion.

Thus, my data suggest TIMPs may protect against septic PMVEC barrier dysfunction, at least partly, through direct inhibition of metalloproteinase activity.

Keywords

Sepsis, Acute Respiratory Distress Syndrome, pulmonary microvascular endothelial cells, tissue inhibitor of metalloproteinase, metalloproteinases, intercellular adhesion molecule, microvascular barrier function, polymorphonuclear leukocyte adhesion

Co-Authorship Statement

All studies and data included in this thesis report were generated by Marcello G. Masciantonio, except for data presented in Chapter 2 (Figure 2-2), which was generated and kindly provided by Cynthia Pape.

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List of Abbreviations

ADAM	A Disintegrin And Metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ARDS	Acute Respiratory Distress Syndrome
ANG	Angiotensin
AUC	Area Under the Curve
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CaM	Calmodulin
CLP	Cecal Ligation and Perforation
CO_2	Carbon Dioxide
C_t	Cycle Threshold
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EB	Evans Blue

EC	Endothelial Cells
ECM	Extracellular Matrix
EGF	Epithelial Growth Factor
eNOS	Endothelial Nitric Oxide Synthase
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycan
HBSS	Hank's Balanced Salt Solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
ICU	Intensive Care Units
IFN	Interferon
IL	Interleukin
IV	Intravenous Fluids
JAM	Junctional Adhesion Molecule

LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MT-MMP	Membrane Type-Matrix Metalloproteinase
MVEC	Microvascular Endothelial Cells
NET	Neutrophil Extracellular Trap
NFκB	Nuclear Factor κB
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PAMP	Pathogen-Associated Molecular Pattern
PAR	Protease-Activated Receptor
PBS	Phosphate Buffered Saline
PECAM	Platelet Endothelial Cell Adhesion Molecule
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear Leukocyte

PMVEC	Pulmonary Microvascular Endothelial Cells
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
PKC	Protein Kinase C
RBC	Red Blood Cells
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RQ	Relative Quantity
rTIMP3	Recombinant Tissue Inhibitor of Metalloproteinase-3
SELE	E-selectin
siRNA	Small Interfering Ribonucleic Acid
TEER	Transendothelial Electrical Resistance
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumour Necrosis Factor
VCAM	Vascular Cell Adhesion Molecule
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor

VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild Type
Zn^{2+}	Zinc
ZO	Zona Occludens

Chapter 1

1 Introduction

1.1 Sepsis

Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. It is a common and important clinical problem associated with significant morbidity and mortality, and in North America, approximately one million cases of sepsis occur annually, leading to severe sepsis 40% of the time and 300, 000 deaths [2]. Additionally, sepsis remains the leading cause of death in intensive care units (ICU). Sepsis also presents a significant economic burden as it consumes up to 45% of total ICU costs in the United States, or $24 billion annually [3,4]. Morbidity and mortality in sepsis are largely due to multiple organ dysfunction and failure, most commonly in the pulmonary, renal, and cardiac systems [3,5,6]. Despite intensive basic and clinical research, treatment of sepsis and related organ dysfunction consists largely of supportive care (i.e. antibiotics and intravenous fluids [IV]), with no specific therapy available for the treatment of systemic inflammation or resulting cellular and organ injury [7].

Part of this dysregulated response is persistent systemic inflammation, causing major pathophysiological changes, including increased microvascular permeability leading to organ edema, damage, and dysfunction distant to the site of infection. Septic organ dysfunction is characterized by the activation and dysfunction of microvascular endothelial cells (MVEC), leading to a loss of microvascular barrier function [8]. In addition, septic inflammation is characterized by the activation of both circulating

(polymorphonuclear leukocyte [PMN]) and tissue-resident inflammatory cells (macrophages), as well as the enhanced activity of soluble inflammatory mediators, including lipopolysaccharide (LPS) and various cytokines (e.g. tumour necrosis factor [TNF] α, interleukin [IL] 1β, interferon [IFN] γ) [8].

1.2 Acute Respiratory Distress Syndrome

Acute respiratory distress syndrome (ARDS) is a critical pulmonary illness characterized by increased pulmonary vascular permeability, which ultimately leads to severe pulmonary edema, increased lung weight, and loss of aerated lung tissue [9]. ARDS is triggered by direct pulmonary insults, such as gastric aspiration, smoke inhalation, and pneumonia, or by indirect insults, such as sepsis, pancreatitis, and trauma [10]. ARDS is also characterized by the activation of alveolar macrophages as well as the influx of circulating neutrophils, resulting in the enhanced production and release of inflammatory mediators including pro-inflammatory cytokines, oxidants, and proteases, ultimately leading to hypoxemia and potentially, death [11]. Persistent inflammation results in injury and dysfunction of the pulmonary microvasculature, which is characterized by a loss of structural integrity and increase in barrier permeability [11,12]. The disruption of junctions between adjacent pulmonary MVEC (PMVEC), specifically adherens junctions comprising vascular endothelial (VE)-cadherin, contributes to this pathology [10]. 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.

Despite advances in understanding ARDS, effective pharmacologic interventions shown to improve patient outcomes remain lacking [10]. While mortality rates associated

with ARDS have decreased recently due to improvements in mechanical ventilation and fluid management, mortality rates remain at approximately 30% [10]. Further, surviving patients deal with long-term pulmonary complications like persistent inflammation and fibrosis leading to an impaired quality of life [10].

1.3 Microvascular Endothelial Cells

The inner lining of all blood vessels consists of a monolayer of endothelial cells (EC), which serves as a selectively permeable barrier between the circulation and surrounding tissue [13–16]. In addition to the endothelial monolayer, blood vessels are comprised of multiple layers of smooth muscle cells, connective tissue, and pericytes [17]. However, capillaries, which are the smallest of all blood vessels, consist only of the single endothelial monolayer [17]. The microvasculature, includes the arterioles, capillaries, and postcapillary venules [17].

The EC is a multifunctional cell. These functions include regulation of angiogenesis, the maintenance of a nonthrombogenic barrier, and the modulation of blood flow and vascular resistance. Further, EC mediate host immune responses via release of pro- and anti-inflammatory mediators, such as cytokines and chemokines, as well as regulation of leukocyte adhesion to the endothelial surface via changes in expression of surface adhesion molecules [15,17,18]. However, the primary role of EC is to act as a selectively permeable barrier between the contents of the vasculature and the surrounding tissue. In order to control the substances passing through the semi-permeable layer, EC have selective transport systems to target specific substrates. For example, larger substrates are thought to pass through the endothelial barrier membrane via a transcellular route mediated by caveolae [19].

1.3.1 MVEC Barrier Function

Inter-MVEC adherens and tight intercellular junctions are required for the establishment and maintenance of MVEC barrier function [13,14,16,20–23]. However, dynamic control of endothelial permeability, which occurs through both paracellular and transcellular pathways, does allow for the regulated exchange of plasma proteins, solutes, and liquid between the vasculature and surrounding tissues [13,14,16,20–23]. The paracellular pathway consists of transport through the intercellular space between adjacent EC, whereas the transcellular pathway, also known as transcytosis, consists of active receptor-mediated transport through the cell itself [13,14,16,17]. Under basal conditions, only small molecules (e.g. ions) cross the endothelial barrier through the paracellular pathway [13,17]. Larger macromolecules are actively transported through membrane receptor/vesicle-mediated transcytosis [13,16,17,24].

1.3.2 Regulation of MVEC Barrier Function

Interaction of adjacent EC through adherens and tight intercellular junctions is the key mechanism regulating microvascular endothelial permeability [13,14,16,20–23]. These transmembrane junctional structures are comprised of multiple cell surface proteins, including VE-cadherin within adherens junctions, and the claudins and occludins within tight junctions [13,14,16,20,21,23].

In addition to inter-MVEC interaction, microvascular permeability is also thought to be regulated by a mesh-like matrix, known as the glycocalyx, found on the luminal surface of the endothelial monolayer [13,25]. The glycocalyx is made up of glycoproteins and proteoglycans, including syndecans and glypicans, in addition to glycosaminoglycans (GAGs) [13,17,25]. Interestingly, the ability of the glycocalyx to facilitate maintenance

of a semi-permeable barrier to fluid and proteins may not occur in all microvascular beds (i.e. pulmonary microvasculature) as degradation of the glycocalyx was not associated with increased albumin flux or edema within the lung [26]. The glycocalyx is also involved in the regulation of circulating leukocyte-MVEC interactions such as trans-MVEC migration [13,25].

The extracellular matrix (ECM) is also known to be a structural component required for the maintenance of endothelial barrier integrity. EC bound to the ECM are generally quiescent, as activation of integrins on the basolateral surface of EC following binding to the ECM generates signals that stabilize adjacent cell-cell and cell-ECM adhesions, which are vital to the formation and preservation of an endothelial barrier [27]. Thus, both the glycocalyx and the ECM are critical to establishing and maintaining endothelial integrity and barrier function.

1.4 MVEC Dysfunction

Dysfunction of the microvasculature under conditions of inflammation or infection is central to the resulting organ injury, dysfunction, and failure. This microvascular dysfunction is primarily characterized by activation and dysfunction of MVEC, leading to a loss of microvascular barrier function as well as upregulation of cell surface leukocyte receptors and upregulation of pro-thrombotic pathways [13,14,20,21,28,29]. Importantly, this dysfunction results in increased tissue inflammation and edema due to enhanced adhesion and diapedesis of leukocytes as well as increased microvascular permeability, both of which can lead to organ dysfunction and failure [13,14,20,21,28,29].

EC dysfunction occurs under many different conditions, including but not limited to local tissue injury, infection, and sepsis, but is also a hallmark of vascular diseases such as atherosclerosis. Within the setting of injury and/or infection, MVEC dysfunction is initiated by the interaction of MVEC with circulating blood components, including PMNs and soluble inflammatory mediators (e.g. bacterial products like LPS, or cytokines like TNFα). In fact, MVEC dysfunction following injury or infection is highly PMN-dependent [8,30–33]. There are several mechanisms of PMN-dependent cell/tissue injury. These include physical interaction (e.g. PMN-MVEC adhesion), release of reactive oxygen species (ROS), proteases/peptides (e.g. metalloproteinases), pro-inflammatory cytokines/chemokines, and cytotoxic neutrophil extracellular traps (NETs) [33,34].

1.4.1 Features of MVEC Dysfunction

The primary features of MVEC dysfunction are the loss of MVEC barrier function and the increased PMN-MVEC interaction. The loss of MVEC barrier function is mediated through several mechanisms, including but not limited to disassembly of inter-MVEC adherens and tight junctions due to cleavage or modification of membrane junctional proteins; gap formation due to actin cytoskeleton rearrangement and microtubule-dependent MVEC contraction; activation of trans-MVEC transport systems; and MVEC death [21,22,35]. Enhanced PMN-MVEC interaction is associated with increased PMN recruitment out of the vasculature into tissues, and is thought to be dependent on a number of factors, including but not limited to increased release of pro-inflammatory cytokines and chemokines, upregulation of MVEC cell surface leukocyte receptors, and shedding of the glycocalyx [36,37].

1.4.2 Mechanisms of MVEC Barrier Dysfunction

MVEC activation by inflammatory cytokines and leukocytes leads to increased MVEC permeability, and this increased permeability is primarily due to loss of inter-MVEC junctions and MVEC retraction [13,14,20,21,28,29]. As VE-cadherin is critical to the formation of MVEC adherens junctions, modifications of its structure along with its associated intracellular catenins, including p120, β- and α-catenin, significantly attenuates barrier integrity [13,14,16,20,21,23,38]. For example, enhanced barrier permeability is correlated with tyrosine phosphorylation of the VE-cadherin/catenin complex [13,14,16,39].

Activation of MVEC by pro-inflammatory cytokines (e.g. TNFα) has also been shown to induce loss of barrier function by promoting cleavage of VE-cadherin and the generation of a soluble extracellular fragment [13,16,40,41]. This VE-cadherin cleavage appears to be at least partially dependent on tyrosine phosphorylation of the VE-cadherin/catenin complex by several kinases (i.e. Protein Kinase C [PKC] and p38 mitogen-activated protein kinase) [13,16,40,41]. Finally, thrombin has been found to bind protease-activated receptor (PAR) 1 on the MVEC surface, and promote barrier dysfunction by increasing cytosolic Ca^{2+} concentrations leading to PKCα-dependent VE-cadherin phosphorylation and subsequent internalization [13,16,42]. Collectively, these studies illustrate the complex intercellular signaling pathways that are involved in the control of MVEC barrier function, and that are often disrupted under pathological conditions, such as tissue injury or infection.

1.5 Metalloproteinases

Metalloproteinases are a diverse group of endopeptidases comprised of many distinct families [43]. Matrix metalloproteinases (MMPs) and the closely related a disintegrin and metalloproteinases (ADAMs) share several common structural domains, including a signal peptide, the pro-peptide domain, and the catalytic domain [44]. The pro-peptide domain contains a cysteine residue that ligates the active site zinc (Zn^{2+}) or Ca^{2+} and maintains the enzyme in an inactive conformation until this interaction is disrupted. MMPs are generally categorized by their domain structure [45,46]. In addition to the domains common to all MMPs and other metalloproteinases (i.e. signal peptide, pro-domain, and catalytic domain), specific subsets of MMPs contain unique domains. Many MMPs contain a hemopexin-like C-terminal region, which is connected to the catalytic region by a short flexible hinge and can mediate protein-protein interactions (i.e. binding to tissue inhibitor of metalloproteinases [TIMPs]). Additionally, the membrane type (MT)-MMPs contain a furin-recognition domain allowing for activation by the pro-protein convertase furin, as well as a C-terminal transmembrane domain [46–48]. ADAMs also contain a furin cleavage site as well as a disintegrin-binding domain, which can interact with integrins and mediate cell-ECM interactions [44].

Metalloproteinases are regulated in four ways – gene expression, compartmentalization, pro-enzyme activation, and enzyme inactivation [46]. Metalloproteinase expression can be mediated by many factors, such as growth factors (e.g. transforming growth factor [TGF] β and vascular endothelial growth factor [VEGF]), cytokines (e.g. IFNγ, IL1β, and IL6), cell-ECM signaling through integrins, and pathogen-associated molecular patterns (PAMPs) [44,49,50].

Studies have shown that metalloproteinases demonstrate considerable overlap in their substrate selectivity *in vitro*. Thus, regulation of metalloproteinase activity is critical and likely occurs at multiple levels. Compartmentalization (e.g. the pericellular accumulation of metalloproteinases in specific microenvironments through cell-enzyme interactions) is thought to be important in regulating extracellular substrate degradation [44,46,50]. Additionally, while many metalloproteinases are secreted in their active form, some (i.e. specific MMPs) are secreted as zymogens, or inactive precursors, and remain in this inactive conformation due to the interaction between the pro-peptide and catalytic domains [47]. Disruption of this interaction, which occurs through multiple mechanisms including proteolytic removal or oxidation of the pro-domain, allows for MMP activity [46,47,49,51]. Finally, metalloproteinase activity is directly inhibited by specific inhibitors, the TIMPs [49,52].

Multiple MMPs are expressed by MVEC, some of which are localized to vesicles likely for rapid secretion following activation. While data on metalloproteinase regulated MVEC dysfunction is limited, changes in metalloproteinase expression in response to proinflammatory cytokines has been characterized in other types of EC. For example, angiotensin (ANG) II treatment of human umbilical vein endothelial cells (HUVEC) stimulates TNFα production that leads to enhanced MMP2 production and release, and co-treatment with anti-TNFα antibodies blocks this increased MMP2 production [53]. Interestingly, stimulation of brain EC with TNFα and IL1β, selectively upregulates MMP9 production, but does not alter MMP2 expression [54]. This suggests vascular bed specificity in MMP expression in response to cytokines. ADAM expression is also mediated by cytokines. Specifically, ADAM17 expression by murine brain EC is strongly

upregulated by many pro-inflammatory cytokines (i.e. TNFα, IL-1β, IFNγ) and growth factors (epithelial growth factor [EGF], VEGF) [55].

EC, including MVEC, also express and release higher levels of metalloproteinases following infection. For example, LPS, a component of the outer membrane of gram negative bacteria, elicits strong increases in the expression and activity of ADAM10 and ADAM17 in human PMVEC [56]. Further, Japanese encephalitis virus, a single stranded ribonucleic acid (RNA) virus, increases expression of MMP9 in human brain MVEC [57]. Additionally, infection of HUVEC with *Chlamydophila pneumoniae*, a bacteria associated with lung infections such as pneumonia, leads to increased MMP9 production while infection of HUVEC with Dengue virus leads to overproduction of MMP2 and to a lesser extent of MMP9 [57–59]. Finally, while it has yet to be linked to EC-specific expression, humans with severe sepsis have significantly elevated plasma MMP2, -3, -7, -8, and -9 [60]. It is important to note, however, that at least part of the EC metalloproteinase response to infection may be mediated through autocrine signaling by cytokines expressed by EC in response to the infection.

1.5.1 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 [42,56,61–63]. For instance, MMP7, ADAM10, and -12 are all capable of cleaving VE-cadherin, the integral cell surface transmembrane protein of endothelial adherens junctions [42,62]. Further, increased MMP2 and -9 expression following HUVEC infection by Dengue virus is correlated with a loss of expression of VE-cadherin

cell-cell adhesion suggesting that MMP2 and -9 may also be capable of cleaving VE-cadherin [59].

MMP2 and -9 along with ADAM17 are also capable of cleaving tight junction associated proteins such as occludin, zona occludens (ZO) 1, and junctional adhesion molecules (JAMs) [56,61,63]. 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 [41,64]. Moreover, knockdown of ADAM10 following small interfering RNA (siRNA) treatment of either EC or activated leukocytes stabilizes the microvascular barrier as well as reduces leukocyte transendothelial migration [42]. Collectively, these studies suggest increased metalloproteinase expression and activity promotes MVEC dysfunction through the cleavage and/or disruption of critical inter-MVEC junctional proteins.

MMPs and ADAMs with thrombospondin motifs (ADAMTSs) have also been found to cleave the ECM surrounding the vasculature, which can modify EC function through disrupted endothelial-ECM interactions, ultimately leading to increased vascular permeability [65–70]. For example, proteolytic degradation of ECM constituents, including fibronectin, laminin, and type IV and V collagens, by MMPs, including MMP2 and -9, results in increased permeability across EC monolayers *in vitro* as well as increased pulmonary edema *in vivo* [69,70]. The cleavage and degradation of other ECM protein constituents, including hyaluronan and aggrecan, have also been shown to augment endothelial permeability [70,71]. Additionally, MMP9 has been shown to be responsible for the cleavage and release of VEGF bound to the ECM, which results in

increased microvascular permeability through internalization of VE-cadherin [72]. Thus, these studies suggest increased metalloproteinase activity drives MVEC dysfunction through the proteolytic processing of integral MVEC structural determinants (i.e. the ECM) resulting in MVEC barrier dysfunction and enhanced microvascular leak.

Supportive evidence for the role of metalloproteinases in microvascular and MVEC dysfunction includes data from several studies using synthetic metalloproteinase inhibitors, such as GM6001. For example, stimulating HUVEC with TNFα increases metalloproteinase expression and activity, and treatment of these HUVEC with GM6001 reduces the TNFα-induced increase in MVEC permeability through inhibition of VE-cadherin cleavage [41,42]. Additionally, in models of brain edema due to dysfunction of the blood-brain barrier, treatment of murine cerebrovascular EC with GM6001 leads to decreased microvascular leak due to the stabilization of tight junction adhesive proteins and decreased EC F-actin stress fiber formation through the inhibition of MMP9 [73,74]. Collectively, these studies highlight the potential role of metalloproteinase inhibition in the control of MVEC barrier dysfunction.

In addition to controlling MVEC barrier dysfunction, metalloproteinases are also involved in mediating PMN-MVEC interaction through multiple potential mechanisms. Specifically, metalloproteinases, including both MMPs and ADAMs, can mediate PMN-MVEC interaction through shedding of leukocyte receptors, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), from the MVEC surface. For example, MMP14, as well as ADAM10 and -17 have been found to cleave ICAM1 [75–77]. Further, the interaction between ICAM1 and MMP14 appears to

be mediated by interaction between the cytoplasmic domains [77]. Additionally, ADAM17 has been shown to cleave VCAM1 from the surface of heart and aorta EC following stimulation with phorbol 12-myristate 13-acetate (PMA) [78]. Cleavage of ICAM1 and VCAM1 by MMPs and ADAMs reduces MVEC surface expression of ICAM1 and VCAM1 leading to decreased PMN-MVEC interaction [78]. These studies suggest that MMPs and ADAMs have the ability to downregulate PMN-MVEC interactions, reduce inflammation, and could also be involved in the resolution of inflammation.

Metalloproteinases have also been proposed to have a role in regulation of PMN-MVEC interaction through cleavage of the glycocalyx. While degradation of the glycocalyx is thought to be primarily mediated by heparanase, glycocalyx degradation still occurs in mice lacking heparanase, and this degradation is associated with increased MMP activity [16,37]. Collectively, this potential degradation of the glycocalyx could lead to increased PMN-MVEC interaction; however, this remains to be confirmed. Thus, the role of metalloproteinases, including both MMPs and ADAMs, in MVEC dysfunction is complex, including promoting the loss of MVEC barrier function and increased leukocyte recruitment through degradation of the glycocalyx, while also decreasing direct leukocyte-MVEC interaction. Further, the specific roles likely depend on the metalloproteinase in question, the specific vascular bed, and the type of inflammation or injury.

1.6 Tissue Inhibitor of Metalloproteinases (TIMPs)

The TIMP family is comprised of four members, TIMP1-4, in humans and other mammals (e.g. mice, rats) [52,79]. TIMPs inhibit active metalloproteinases in a 1:1

stoichiometric inhibitor-to-enzyme ratio [52,79]. Specifically, the N-terminal domain of TIMP molecules interacts with the active site of metalloproteinases in a fashion similar to metalloproteinase substrates, resulting in inhibition of catalytic activity [52,79].

TIMP expression varies in the different vascular beds throughout the body, including both micro- and macrovascular beds; however, all TIMP family members are expressed within at least one vascular bed, supporting the importance of TIMPs to EC function. For example, TIMP1, -3, and -4 are expressed by brain MVEC, and TIMP1, -2, and -3 are expressed by EC isolated from the aorta, iliac artery, and coronary artery [80–82]. Additionally, TIMP3 is expressed by pulmonary MVEC, whereas TIMP2 and -4 do not appear to be [83,84].

Besides EC, TIMPs are also often expressed by multiple other cells associated with the vasculature. For example, TIMP3 is expressed by pericytes, and TIMP2 and -4 are expressed by vascular smooth muscle cells [85–87]. Importantly, TIMPs expressed by these adjacent cell populations are localized within the microenvironment surrounding the MVEC, and in many instances, have been found to be involved in regulation of MVEC function [85–88]. Additionally, while TIMP1, -2, and -4 are secreted and thus function in a soluble manner, TIMP3 is known to be bound to sulfated GAGs located in the ECM [52,79,89].

Within the setting of tissue injury and infection, the expression of the various TIMPs appears to be differentially regulated (i.e. expression of different TIMPs does not change in the same direction) [53,80,81,83,87,88,90,91]. For example, treatment of brain MVEC with pro-inflammatory cytokines, specifically IL1β and TNFα, led to an increase

in *Timp1* and a decrease in *Timp3* messenger ribonucleic acid (mRNA) expression [80]. Additionally, stimulation of brain MVEC with homocysteine, which is associated with endothelial dysfunction, increased TIMP1 expression but decreased TIMP4 expression [81].

Interestingly, the TIMP expression in response to various stimuli also appears to be cell-dependent as stimulation of a transformed brain EC line (hCMEC/D3) with TNFα resulted in increased TIMP3 expression [92]. There is, however, some evidence suggesting that similar expression patterns for TIMPs may also be observed across different vascular beds. For example, while it has yet to be linked to MVEC-specific expression, TIMP1, -2, and -4 levels in plasma are significantly elevated in patients with severe sepsis [60,93,94]. Further, brain MVEC treated with homocysteine have increased TIMP1 protein expression [81]. Conversely, similar to the reduction in *Timp3* expression in brain MVEC under inflammatory conditions, TIMP3 expression (mRNA and protein) was significantly decreased in pulmonary MVEC following treatment with IFNγ, IL1β, and TNFα [83].

1.6.1 Metalloproteinase-Dependent TIMP Functions

In general, all four TIMPs have been shown to collectively inhibit all MMPs *in vitro* [95,96]. There are, however, some differences between TIMPs. For example, TIMP1 does not appear to be able to regulate MT-MMPs [47,50]. In addition, ADAMs and ADAMTSs appear to be primarily inhibited by TIMP3 [47]. Some TIMPs have also been found to form a complex between an active MMP and latent pro-MMP leading to activation of the latent MMP. This has been demonstrated extensively with TIMP2, which binds active MMP14 and latent MMP2, and facilitates MMP2 activation [96].

The majority of our understanding of metalloproteinase and TIMP interactions arises from *in vitro* studies utilizing purified proteins [95]. Hence, much of this data reveals the potential for metalloproteinase-TIMP interaction but does not provide information as to physiological relevance. There have been a number of *in vivo* studies using mice lacking specific TIMPs that have sought to identify physiological interactions. For example, TIMP1 has been found to interact with and inhibit MMP7 (also referred to as matrilysin) as MMP7 activity was increased in the airway epithelium of mice lacking TIMP1 following napthalene exposure [97]. Further, co-immunoprecipitation demonstrated that TIMP1 directly binds to MMP7 [97].

Use of different models of injury and inflammation with mice lacking TIMP3 have found that TIMP3 is the physiological inhibitor of ADAM17 [95]. Specifically, inflammation is generally increased in mice genetically lacking TIMP3 following multiple models of injury (i.e. partial hepatectomy, bleomycin-induced lung injury), and in most cases, this increased inflammation is associated with increased TNFα shedding resulting from augmented ADAM17 activity [98]. TIMP3 has also been associated with multiple other metalloproteinases *in vivo*, primarily through the observation of increased metalloproteinase activity (e.g. MMP9) or increased substrate degradation (e.g. aggrecan cleavage potentially by ADAMTS5) in mice lacking TIMP3 [95,99].

Rescue of the basal and pathological phenotypes in mice lacking specific TIMPs with small molecule metalloproteinase inhibitors also provides further support for the metalloproteinase dependent roles for TIMPs *in vivo* [83,98–101]. Thus, through regulation of metalloproteinase activity, TIMPs are able to finely tune processing of a wide variety of extracellular substrates ranging from degradation of the ECM to cleavage

of cytokines and cytokine receptors from the cell surface, which suggests TIMPs are critical for the regulation of many cellular functions from cell-cell interaction to influx of leukocytes into injured tissues.

1.6.2 Role of TIMPs in Endothelial Function

A number of studies have characterized TIMP expression within multiple EC types under various conditions; however, the direct evidence of a role for EC-derived TIMPs in endothelial function is more limited. Indirect evidence from mice globally deficient for individual TIMPs as well as from studies of recombinant TIMPs suggests that TIMPs play an integral role in the setting of inflammation, as well as potentially in the setting of endothelial dysfunction following tissue injury. For example, TIMP1-3 all appear to support the blood brain barrier (BBB) following injury, although the mechanism may differ between TIMPs. Mice lacking TIMP1 have increased ischemic injury and blood-brain barrier disruption compared to wild type (WT) mice following focal cerebral ischemia and this is associated with increased MMP9 secretion and activity [94]. Further, increased expression of TIMP1 in brain EC treated with ANG (1-7) following hypoxia-induced injury was responsible for restoration of tight junctions and reduced EC permeability [102].

TIMP3 appears to regulate EC barrier function through metalloproteinase-dependent mechanisms. *Timp3*$^{-/-}$ mice have augmented leak of Evans blue (EB)-labeled albumin 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 [68]. *Timp3*$^{-/-}$ mice also have increased pulmonary EB-albumin leak at baseline, which is likely due to loss of PMVEC-derived

TIMP3 as PMVEC from *Timp3*$^{-/-}$ mice also exhibit impaired barrier function *in vitro* compared to PMVEC from WT mice [83]. Further, treatment of *Timp3*$^{-/-}$ PMVEC with global metalloproteinase inhibitor GM6001 appears to restore basal PMVEC barrier function [83]. Together, these studies suggest that TIMP3 may be an important homeostatic regulator of EC function in healthy tissue, such as establishing a selectively permeable barrier, and that this role for TIMP3 is at least partly dependent on the ability of TIMP3 to inhibit metalloproteinases [70,83].

While the classic function for TIMPs is inhibition of metalloproteinase activity, there are multiple lines of evidence suggesting that TIMPs may regulate EC function through metalloproteinase-independent mechanisms. Specifically, mice lacking TIMP2 exhibit blood-brain barrier disruption compared to WT mice following focal cerebral ischemia; however, no changes were observed in MMP activity [94]. Additionally, injection of recombinant TIMP3 (rTIMP3) led to decreased vascular leak across the blood-brain barrier in a model of traumatic brain injury, but this effect was not mimicked by use of a synthetic metalloproteinase inhibitor [103].

TIMP2 and -3 have also been found to inhibit VEGF signaling through metalloproteinase-independent mechanisms [90,91,104]. For TIMP3, this inhibition occurs through TIMP3 binding to the VEGF receptor (VEGFR) and blocking VEGF-VEGFR2 interaction, ultimately inhibiting angiogenesis [90,91]. During angiogenesis, VEGF acts as a potent mediator of increased endothelial permeability through phosphorylation and internalization of VE-cadherin and subsequent disassembly of cell-cell adhesive contacts [13,90,91,105]. Importantly, both TIMP2 and -3 have been found to inhibit angiogenesis, including inhibition of both EC proliferation and migration,

through inhibition of VEGF signaling [90,91,106]. Thus, these studies together suggest that TIMP2 and -3 may promote brain EC barrier function through metalloproteinase-independent mechanisms.

In addition to regulation of MVEC barrier function, TIMPs have also been associated with regulation of PMN-MVEC interaction. Specifically, TIMP3 has been found to inhibit shedding of VCAM1 from the surface of aortic EC following treatment with IL1β, TNFα, or the phorbol ester PMA [106]. Specifically, aortic EC from *Timp3*$^{-/-}$ mice had increased VCAM1 shedding compared to aortic EC from WT mice, and this inhibition of VCAM1 shedding by TIMP3 was likely through inhibition of ADAM17 as knockdown of ADAM17 protected against increased VCAM1 shedding [106]. Thus, TIMPs appear to be critical regulators of MVEC dysfunction following tissue injury/infection, including protection against loss of MVEC barrier function through metalloproteinase-dependent and -independent mechanisms, as well as the regulation of leukocyte recruitment by stabilization of leukocyte receptors on the MVEC surface.

1.7 Rationale

Pathophysiological changes in the microvasculature are important mechanisms of sepsis-induced organ dysfunction and mortality. Specifically, increased pulmonary microvascular permeability during sepsis-induced ARDS, which is due to PMVEC injury, dysfunction and death, is the primary cause of the protein-rich edema fluid that accumulates within the damaged lung tissue [13,14,20–22,29,104]. While many mechanisms contributing to septic PMVEC injury/dysfunction have been described [13,14,20–22,28,29,104,107], no treatment for the septic tissue damage and organ dysfunction has been identified. Moreover, endogenous mechanisms protecting against

sepsis-induced PMVEC dysfunction are poorly characterized. Metalloproteinases are capable of processing many extracellular proteins, including inter-MVEC junctional proteins (i.e. VE-cadherin, JAM-A), and thereby disrupting MVEC barrier function and increasing microvascular permeability **(Figure 1-1).** TIMPs inhibit metalloproteinase activity, and thereby are critical to maintaining MVEC barrier function and restricting PMN-MVEC interaction. While recent studies support a role for metalloproteinases and TIMPs in mediating MVEC barrier dysfunction, many of these studies used *in vitro* models with HUVEC. Importantly, EC from the micro- and macrovasculature have different biological properties and thus, examining the role of metalloproteinases and TIMPs in the microvasculature, specifically in PMVEC, is critical [108,109]. Further, the potential role of the balance between metalloproteinases and TIMPs in regulating PMVEC barrier dysfunction as well as other aspects of PMVEC activation (i.e. leukocyte interactions) have not been examined under septic conditions.

1.8 Objectives

I propose to:

1. Characterize PMVEC expression of metalloproteinases and TIMPs under control and septic conditions *in vitro* and *ex vivo*.
2. Examine the role of the metalloproteinase/TIMP balance in PMVEC activation and dysfunction during sepsis *in vitro*.

For this, I will examine PMVEC metalloproteinase and TIMP expression in WT PMVEC under homeostatic and septic conditions, as well as under conditions in which the metalloproteinase/TIMP balance has been disrupted (i.e. *Timp3*$^{-/-}$ PMVEC). To examine the role of the metalloproteinase/TIMP balance in PMVEC activation and

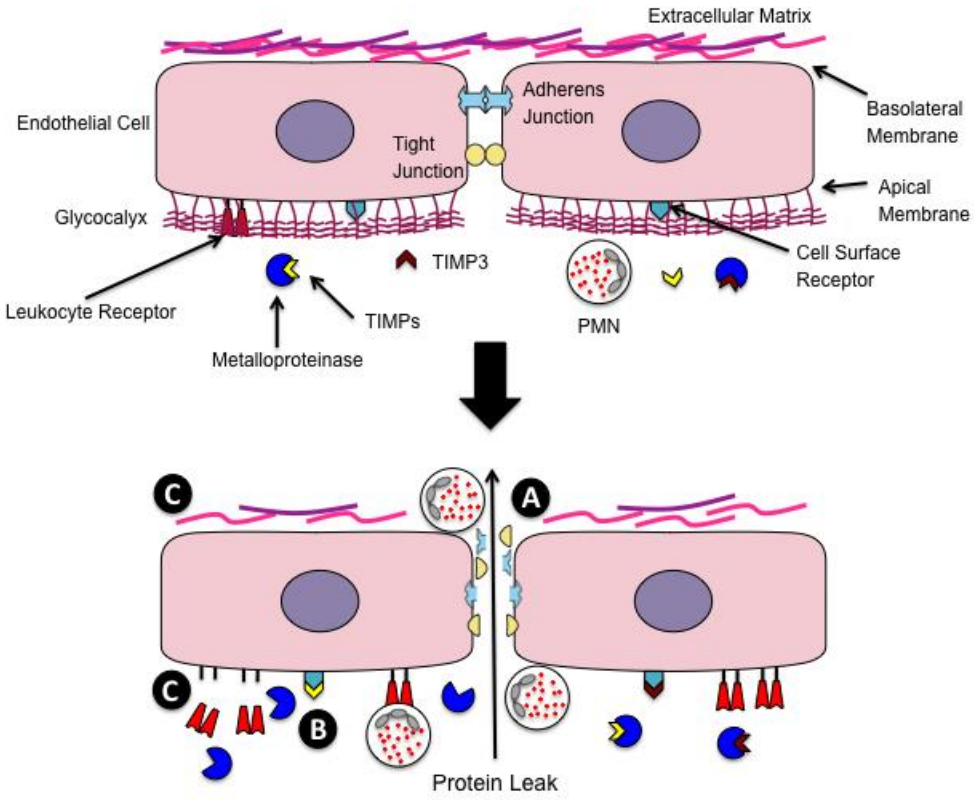

Figure 1-1: Disruption in the balance between metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs) leading to increased metalloproteinase activity promotes microvascular endothelial cell (MVEC) activation and dysfunction following tissue injury or infection. (A) Cleavage of inter-MVEC junctional proteins (i.e. vascular endothelial [VE]-cadherin, junctional adhesion molecule [JAM]-A reduces MVEC barrier function and increases permeability for larger macromolecules. (B) Shedding of leukocyte receptors reduces polymorphonuclear leukocyte (PMN) adhesion to endothelial cells in an attempt to reduce increased PMN migration into the surrounding tissue. (C) Cleavage of the extracellular matrix/glycocalyx increases MVEC permeability and promotes PMN-MVEC interaction. Importantly, TIMPs inhibit metalloproteinase

activity, and thereby are critical to maintaining MVEC barrier function and restricting PMN-MVEC interaction.

dysfunction, I will utilize two complementary approaches: 1) PMVEC treated with synthetic metalloproteinase inhibitors, and 2) PMVEC from *Timp3*$^{-/-}$ mice.

1.9 Hypothesis

I hypothesize that septic murine PMVEC barrier dysfunction contributes to disruption of the balance between metalloproteinases and TIMPs leading to increased metalloproteinase activity.

Chapter 2

2 Methodology

2.1 Pulmonary Microvascular Endothelial Cell (PMVEC) Isolation for *in vitro* Analysis

To study the function of the metalloproteinase/TIMP balance in maintaining pulmonary microvascular barrier function following septic lung injury, murine PMVEC were isolated from the lungs of healthy male WT and *Timp3*$^{-/-}$ mice and cultured for use in all *in vitro* experiments, as previously described [110,111]. In brief, following lung isolation, lung tissue was finely minced and digested using 0.3% collagenase in Hank's Balanced Salt Solution (HBSS, #14170-112, Invitrogen). 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-platelet endothelial cell adhesion molecule (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% [4-2[2-hydroxyetyl]-1-piperazineethanesulfonic acid] [HEPES] buffer [1M], #15630-080, Invitrogen), then seeded into a 1% gelatin-coated cell culture flask and incubated at 37°C with 5% carbon dioxide (CO_2). 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, as well as stained with fluorescently-labelled antibodies against endothelial cell markers (i.e. CD31, CD34, CD146 and CD202) and assessed by 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% CO_2 until the cell monolayer reached confluence. PMVEC at passages 5-12 were used for all experiments

2.2 PMVEC Isolation from Healthy and Septic Mice for *ex vivo* Analysis

To further examine the role of the metalloproteinase/TIMP balance in sepsis, PMVEC were isolated from healthy (naïve and sham surgery) and septic (cecal ligation and perforation [CLP] model of sepsis) mice. For these studies, male WT and *Timp3*$^{-/-}$ mice (8-12 weeks) were randomized to control (naïve and sham surgery) or volume-resuscitated CLP-sepsis for 1, 2, or 4h, as previously described [8,83]. Importantly, naïve mice exhibited no difference in gene expression compared to 4h sham mice, and thus were used in subsequent studies (**Figure 2-1**). PMVEC were isolated using the Mouse Lung Dissociation Kit (#130-095-927, Miltenyi) as per the manufacturer instructions. Briefly, the pulmonary vasculature was flushed with 10 mL saline and the lungs isolated. Lungs were then physically disrupted using the gentleMACS^{TM} Dissociator (Miltenyi) followed by chemical digestion via Collagenase D and DNAse A (#130-095-927, Miltenyi) for 30 min at 37°C. The whole lung disgest was strained through 100 μm and 70 μm cell strainers (#10199-658, VWR; #130-098-462, Miltenyi; respectively) and

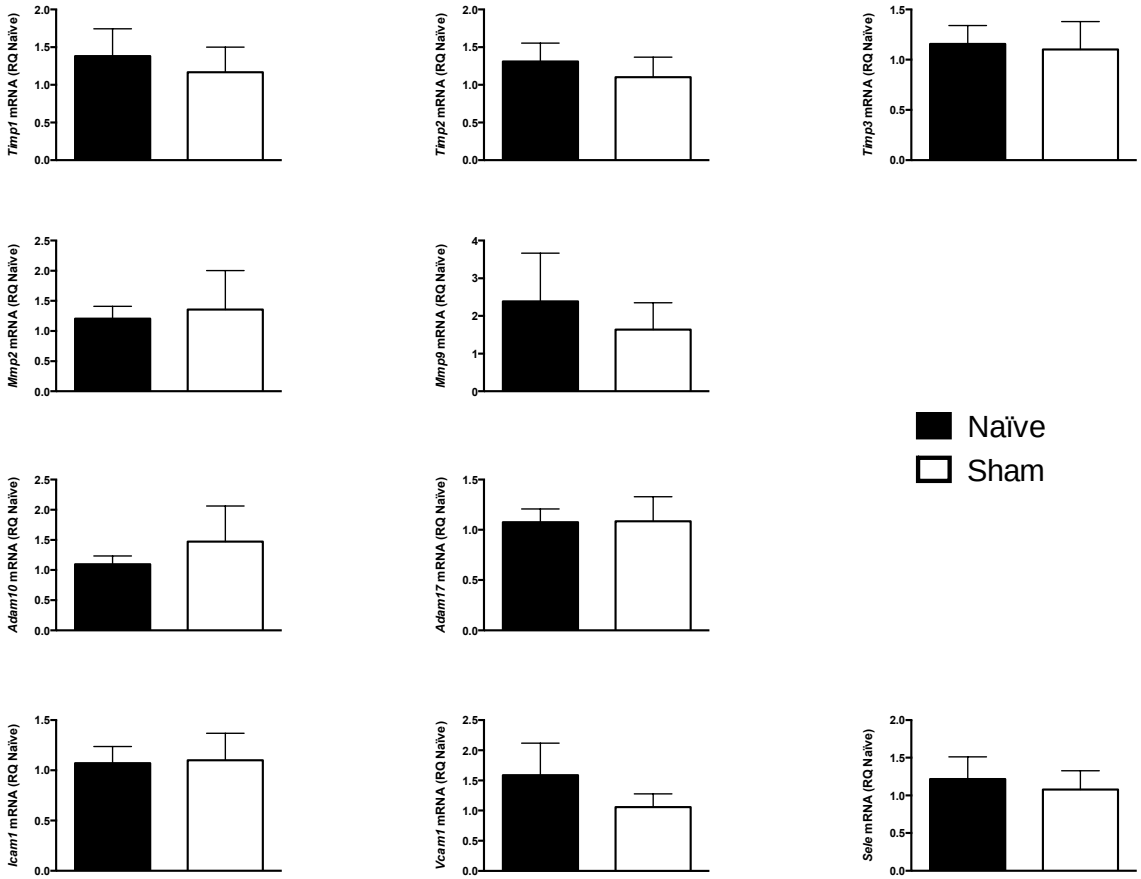

Figure 2-1: Pulmonary MVEC (PMVEC) from sham mice exhibit no significant differences in gene expression compared to PMVEC from naïve mice. *Timp* and metalloproteinase mRNA expression are nearly identical, justifying the decision to use naïve mice instead of 4h sham mice for subsequent PMVEC endothelial cell isolation (n=4-8).

resuspended in DMEM supplemented with 0.5% bovine serum albumin (BSA) and 0.5M EDTA (pH 8.0). Once isolated, the whole lung cell suspension was incubated with CD45 mouse MicroBeads (#130-097-153, Miltenyi) for 15 min at 4°C and then $CD45^+$ cells were magnetically separated using LS columns (#130-042-401, Miltenyi). The $CD45^-$ cells were then incubated with CD31 mouse MicroBeads (#130-097-418, Miltenyi) for 15 min at 4°C and the $CD45^-/CD31^+$ cells magnetically isolated using new LS columns. These cells were spun at 300 g for 5 min at 4°C, resuspended in phosphate buffered saline (PBS) with RNAse inhibitor (4U/ul, #129916, Qiagen), and placed at -80°C for storage. As above, purity of the isolated $CD45^-/CD31^+$ cells was confirmed by flow cytometry using fluorescently-labelled antibodies against endothelial cell markers (i.e. CD31, CD34, CD146 and CD202; **Figure 2-2)**.

2.3 Assessment of PMVEC Expression of *Timp1-4*, Metalloproteinases and Cell Adhesion Molecule Messenger Ribonucleic Acid (mRNA)

PMVEC *Timp1*, *-2*, *-3*, *-4*, *Mmp2*, *-7*, *-9*, *Adam10*, *-17*, *Icam1*, *Vcam1*, and E-selectin (*Sele*) mRNA expression levels were examined in male WT and *Timp3*$^{-/-}$ PMVEC *in vitro*, and in male WT and *Timp3*$^{-/-}$ PMVEC *ex vivo* by quantitative real-time polymerase chain reaction (qRT-PCR). For *in vitro* analysis, cells were cultured in 6-well cell culture plates coated with 1% gelatin. Once confluent, PMVEC were treated for 1, 2, and 4h with PBS (vehicle control), cytomix (an equimolar solution of TNFα, IL1β, and IFNγ, 30 ng/mL, PeproTech, Rocky Hill, NJ), LPS (10 μg/mL, 0111:B4 serotype, #L2630, Sigma) or a combination of both cytomix and LPS (30 ng/mL, 10 μg/mL, respectively), used to mimic septic conditions. Following stimulation, cells were lysed and RNA isolated using the RNeasy Mini Kit following the manufactures directions

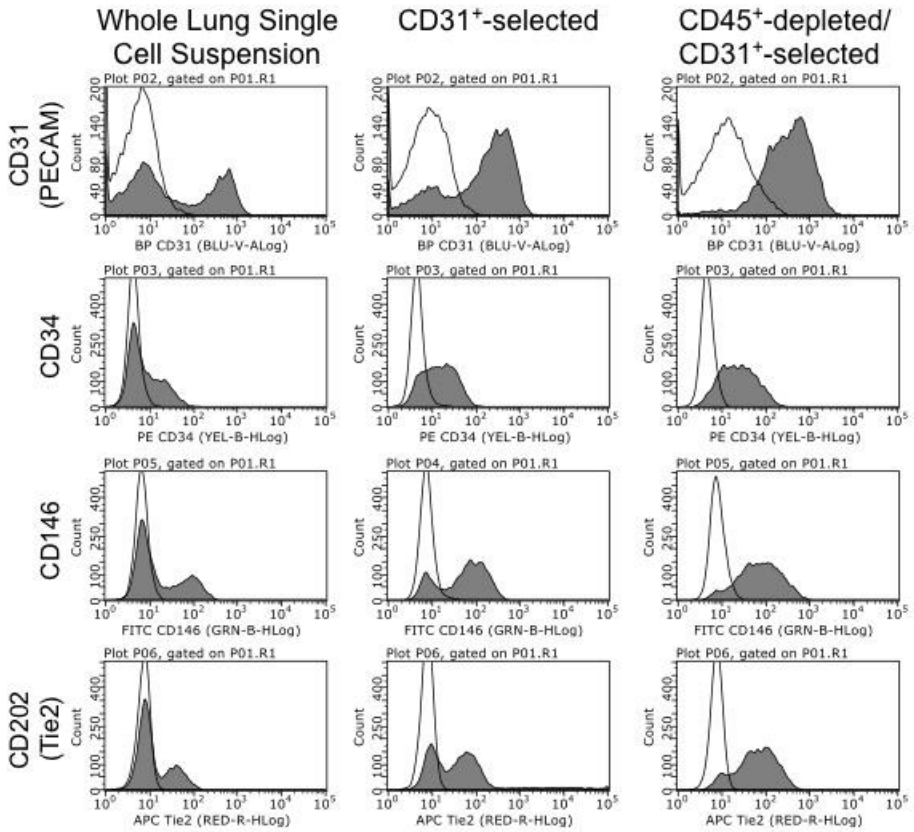

Figure 2-2: CD45 and CD31 MicroBeads assist in selecting for a pure endothelial cell population. Compared to whole lung single cell suspension, $CD31^+$-selected endothelial cells represent a purer population of endothelial cells, illustrated by the shift in fluorescence of CD31, CD34, CD146 and CD202 staining. $CD45^+$-depleted/$CD31^+$-selected endothelial cells represent an even purer population, compared to both whole lung single cell suspension and $CD31^+$-selected endothelial cells.

(#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 14 000 x g for 2 min. 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.

For *ex vivo* analysis, RNA was isolated using the RNeasy Micro Kit (#74004, Qiagen, Hilden, Germany) from PMVEC isolated from naïve, sham, and septic mice as described in section 2.2. Briefly, cells were spun at 14 000 x g for 2 minutes and subsequently run through a genomic column for 1 minute at 10 000 x g.

Flow through was collected and washed, and the RNA eluted from the columns. For all isolated RNA, purity (260/230 and 260/280 ratios) and concentration 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 assessed using TaqMan Gene Expression Assays from Applied Biosystems (Timp1: Mm00441818_m1; Timp2: Mm00441825_m1; Timp3: Mm00441826_m1; Timp4: Mm01184417_m1; Mmp2: Mm00439498_m1; Mmp7: Mm00487724_m1; Mmp9: Mm00442991_m1; Adam10: Mm00545742_m1; Adam17: Mm00456428_m1; Icam1: Mm00516023_m1; Vcam1: Mm01320970_m1; Sele: Mm01310197_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 (C_t) value for each gene and this value was then used to determine gene expression relative to control PMVEC (PBS-treated or isolated from naïve/sham mice). ΔC_t was the normalization of the gene of interest (i.e. *Timp3*) to the housekeeping gene (*Hprt*) within a specific sample (i.e. 2h cytomix-treated PMVEC). ΔΔCt was the normalization of a specific sample (i.e. 2h cytomix-treated PMVEC) to the control sample (PBS-treated PMVEC). Finally, the relative quantity (RQ) was the fold change in expression of a specific sample (i.e. 2h cytomix-treated PMVEC) relative to the control sample (PBS-treated PMVEC). RQ was determined by the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

2.4 Assessment of PMVEC Metalloproteinase Activity

Male WT and *Timp3*$^{-/-}$ PMVEC were grown to confluence in 6-well cell culture plates coated with 1% gelatin, and treated for 4h with PBS (vehicle control), cytomix (30 ng/mL), LPS (10 μg/mL), or a combination of both cytomix and LPS (30 ng/mL and 10 μg/mL, respectively). Following stimulation, the conditioned media was collected and placed at -80°C for storage. Cells were rinsed with PBS and then lysed by directly adding lysis buffer (1X CellLytic extraction buffer [#C2978-50ML, Sigma-Aldrich] into each of the wells. Cells were then placed on ice for 20 min, after which, the cell lysate was collected using cell scrapers and placed at -80°C storage.

2.4.1 Assessment of Global Metalloproteinase Activity

Global metalloproteinase activity in the conditioned media and cell lysate was analyzed using the P126 OmniMMP fluorogenic substrate as per the manufacturer instructions (BML-P126-0001, Enzo Life Sciences). Briefly, 85 μL of conditioned media or 25 μL of cell lysate from each sample was added to each well of a clear bottom 96-well black microplate (#82050-754, VWR). Fluorescently-labelled P126 substrate and assay buffer were then added to each well, including positive controls. Wells containing the reference peptide, P127 (BML-P127-0001, Enzo Life Sciences), were used as positive controls and wells containing sample and assay buffer but no P126 substrate were used as negative controls for background fluorescence. Additionally, to confirm that any observed increase in fluorescence was due to metalloproteinase activity, GM6001 and BB94 were added to a subset of wells. Fluorescence was measured (Excitation peak wavelength: 328 nm [320-340 nm]; Emission peak wavelength: 393 nm [393-405 nm]) using a Victor3 multilabel fluorescence microplate reader (Wallac) at 0, 3, 5, 7, 10, 20, and 30 min, and 1, 2, 3, 4, 5, 21 and 24h. The plate was incubated at 37°C with 5% CO_2 between readings.

2.4.2 Assessment of A Disintegrin and Metalloproteinase (ADAM) 17 Specific Activity

ADAM17 activity was assessed in the cell lysate using the Sensolyte 520 Adam17 Activity Assay Kit as per the manufacturer instructions (#AS-72085, AnaSpec). Briefly, 25 μL of cell lysate from each sample was added to each well of a clear bottom 96-well black microplate (VWR). Fluorescently-labelled substrate and assay buffer were added to each sample, along with positive and negative controls. Wells containing purified

ADAM17 peptide were used as positive controls and wells containing the substrate and assay buffer but no sample were used as negative controls for background fluorescence. The fluorescence was measured (Excitation peak wavelength: 490 nm; Emission peak wavelength: 520 nm) using a Victor3 multilabel fluorescence microplate reader (Wallac) every 5 min for 2.5h. The plate was incubated at 37°C with 5% CO_2 between readings.

2.5 Assessment of PMVEC Barrier Integrity

Endothelial monolayer integrity was assessed in male murine WT PMVEC using an *in vitro* model of PMVEC barrier function. WT PMVEC were seeded at a cell density of $2.5x10^4$ cells/insert on 1% gelatin-coated transwell cell-culture inserts (3.0 μM pore, # CA62406-169, VWR Scientific Inc., Radnor, PA). PMVEC were grown to confluence in supplemented DMEM growth medium (as described above) in 24-well cell-culture plates. Baseline permeability of the PMVEC monolayer was 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 formed.

Once a stable monolayer formed, baseline PMVEC barrier permeability was comprehensively assessed using three complementary techniques: (i) TEER; (ii) fluorescein isothiocyanate (FITC)-labelled dextran flux, which reflects small molecule paracellular permeability; and (iii) EB-labelled albumin flux, which is a clinicially relevant marker of both paracellular and transcellular permeability to large molecules. In general, assessment of PMVEC permeability by all three complementary techniques was conducted at five days after initial cell culture.

2.5.1 Assessment of PMVEC Transendothelial Electrical Resistance (TEER)

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 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). TEER across empty individual inserts not containing cultured PMVEC was used as a control to account for background resistance from the cell-culture inserts and this value was subtracted from the TEER measurements obtained from cell-culture inserts containing PMVEC.

2.5.2 Assessment of trans-PMVEC Macromolecular Flux

To further characterize PMVEC barrier function, movement of both small (dextran; a small, 3 kDa sugar derivative) and large (albumin; a large, 67 kDa protein found within blood) macromolecules from the upper chamber into the lower of the two-chamber transwell system was assessed. Both FITC-labelled dextran and EB-labelled albumin were added directly to the upper chamber of the transwell insert containing the PMVEC monolayer, while the same concentration of unlabelled albumin was added to the lower chamber to remove osmotic pressure. Following 1h, the transwell inserts were removed, and the conditioned media of the lower chamber was collected. In order to measure FITC-labelled dextran flux, the fluorescence of the conditioned medium was measured (Excitation peak wavelength: 488 nm; Emission peak wavelength: 525 nm)

using a Victor3 multilabel fluorescence microplate reader (Wallac). Background fluorescence was measured in dextran-free DMEM. To measure EB-labelled albumin flux, absorbance of the conditioned medium was measured (A_{620}) by an iMark™ Microplate Reader (BioRad Laboratories Inc., Hercules, CA). Background absorbance was measured in EB-albumin-free media.

2.6 Assessment of PMVEC Barrier Function following PMVEC Stimulation

Previous work in my lab reported the effects of cytomix stimulation (30 ng/mL) on TEER and trans-PMVEC macromolecular leak in PMVEC monolayers cultured on transwell cell-culture inserts [83,112]. These studies demonstrated that maximal trans-PMVEC EB-labelled albumin leak occurred 4h following stimulation with cytomix [83]. To assess the effects of global metalloproteinase inhibitors on PMVEC barrier dysfunction under septic conditions, male WT PMVEC were grown to confluence on cell-culture inserts as described above and permeability assessed by all three complementary techniques following a 4h stimulation with either PBS (vehicle control) or cytomix (30 ng/mL) in the presence of vehicle control (dimethyl sulfoxide [DMSO] or distilled water [dH_2O]) or synthetic metalloproteinase inhibitor (GM6001 [10 – 100 μM, #CC1010, EMD Millipore, St. Charles, MO], BB-94 [1 – 100 μM, #196440-5MG, EMD Millipore], or TAPI-2 [10 – 100 μM, #579052-1MG, EMD Millipore]).

2.7 Assessment of PMVEC Cell Surface Adhesion Molecules

Levels of PMVEC surface ICAM1, VCAM1 and E-selectin were examined in WT and *Timp3*$^{-/-}$ PMVEC by flow cytometry using the Guava easyCyte HT Flow

Cytometer (EMD Millipore). PMVEC were grown to confluence in 6-well cell culture plates coated with 1% gelatin, and treated with PBS (vehicle control), cytomix (30 ng/mL), LPS (10 μg/mL) or a combination of both cytomix and LPS (30 ng/mL, 10 μg/mL, respectively) for 4h. Following stimulation, PMVEC were trypsinized with 0.05% Gibco trypsin-EDTA (1x) (Thermo Fisher Scientific)**,** for 10 min, placed into Falcon tubes and spun at 200 g x 5 min, washed with 0.02% BSA, and then resuspended in 100 μL of 0.02% BSA. Fluorescently-labelled antibodies against ICAM1 (0.5 mg/mL; #116111, BioLegend), VCAM1 (0.5 mg/mL; #105711, BioLegend), and E-selectin (0.2 mg/mL; #553751, BD Biosciences) were then added to each tube and the PMVEC placed on a rotator for 40 min to ensure adequate staining. Unstained and stained PMVEC were washed with PBS, resuspended in 250 μL of 0.02% BSA, transferred to a 96 well round-bottomed plate and assessed by flow cytometry.

The effect of metalloproteinase inhibition on cytomix-induced PMVEC surface protein expression levels was also assessed. For these studies, PMVEC were treated with GM6001, BB-94, or TAPI-2 (100 μM) as above, or appropriate vehicle control (DMSO or dH_2O) for 16 hours prior to cytomix stimulation.

2.8 Assessment of Polymorphonuclear Leukocyte (PMN)-PMVEC Interaction

To examine PMN-PMVEC adhesion, WT and *Timp3*$^{-/-}$ PMVEC were grown to confluence on 24-well cell culture plates coated with 1% gelatin and bone marrow PMN were isolated from WT mice. Briefly, bone marrow was collected from the bones of WT mice into eppendorf tubes and spun at 3000 x g for 1 min. PMN were then isolated by density gradient centrifugation using 62% Percoll (#P4937, Sigma-Aldrich). Red blood

cells (RBC) were subsequently lysed with RBC lysis buffer solution (#00-4333-57, eBioscience), and the isolated PMN stained with calcein-AM (#17783, Sigma-Aldrich) for 30 min at 37°C. PMVEC and PMN were then treated separately with either PBS (vehicle control) or cytomix (10 ng/mL) for 4h. Following stimulation, PMN were added to PMVEC at a ratio of 10:1. After 30 min, cells were washed 3x with PBS to remove non-adherent PMN, and the adherent PMN and PMVEC monolayers lysed using 0.05% trypsin-EDTA. Lysate was then transferred to a clear bottom 96-well black microplate (#82050-754, VWR) and fluorescence measured using a Victor3 multilabel fluorescence microplate reader (Excitation: 488 nm; Emission: 525 nm; Wallac). Percentage of total neutrophil adhesion was determined by using a standard curve of labelled PMN. Background fluorescence was determined using unlabeled PMVEC.

2.9 Statistical Analysis

Differences between groups were assessed by t-test or a One-Way ANOVA with a Bonferroni post-hoc test for one independent variable, or by a Two-Way ANOVA with a Bonferroni post-hoc test for two independent variables using GraphPad Prism 6.2. Significance threshold was set at $\alpha = 0.05$. Outliers were assessed using the Grubbs' test.

Chapter 3

3 Results

3.1 *Timp* and Metalloproteinase mRNA Expression in Wild Type (WT) PMVEC

Previous studies demonstrated that septic PMVEC barrier dysfunction appeared to peak after 4h cytomix-stimulation *in vitr*o and *in vivo* [8,83,112]. Further, TIMP3 appeared to be required for normal PMVEC barrier function, as mice lacking TIMP3 had increased basal PMVEC permeability [83]. Thus, PMVEC expression of all *Timps* and key metalloproteinases was examined in PMVEC under basal conditions and 1, 2, and 4h following *in vitro* and *in vivo* models of sepsis by qRT-PCR. Analysis of *Timp1-4* expression found that *Timp2* had the highest basal expression and *Timp4* had the lowest expression (**Table 3-1**; NOTE: C_t values of 35+ were considered as very low expression and C_t values of 40+ were considered to be absent). *Mmp2*, *Adam10* and *Adam17* appeared to be expressed at high levels whereas basal expression of *Mmp9* was fairly low, and *Mmp7* was not detected (**Table 3-1**). Basal expression levels of *Timp1-4* and metalloproteinases in PMVEC isolated from naïve WT mice *ex vivo* were similar to WT PMVEC *in vitro* (**Table 3-2)**.

3.1.1 *Timp* mRNA Expression is Altered in WT PMVEC under Septic Conditions

Septic models had varying effects on *Timp* mRNA expression, with *Timp4* mRNA expression remaining undetectable under all septic conditions (**Figure 3-1**). PMVEC stimulated with cytomix had significantly increased *Timp1* mRNA expression by 4h post-stimulation, but had no changes in *Timp2* and -*3* mRNA expression (**Figure 3-1**).

Table 3-1: Average Basal (Threshold Cycle) C_t values for TIMP/metalloproteinase messenger ribonucleic acid (mRNA) expression in isolated murine wild type (WT) pulmonary MVEC (PMVEC) *in vitro* (n=22).

Timp1	***Timp2***	***Timp3***	***Timp4***	***Hprt1***
28.4	24.7	25.2	36.7	25.6
Mmp2	***Mmp7***	***Mmp9***	***Adam10***	***Adam17***
28.7	40+	36.1	25.6	27.1

Table 3-2: Average Basal C_t values for *Timp*/metalloproteinase mRNA expression in isolated murine WT PMVEC isolated *ex vivo* (n=8).

Timp1	***Timp2***	***Timp3***	***Timp4***	***Hprt1***
38.2	31.7	29.7	38.2	31.4
Mmp2	***Mmp7***	***Mmp9***	***Adam10***	***Adam17***
34.3	40+	36.5	31.6	31.7

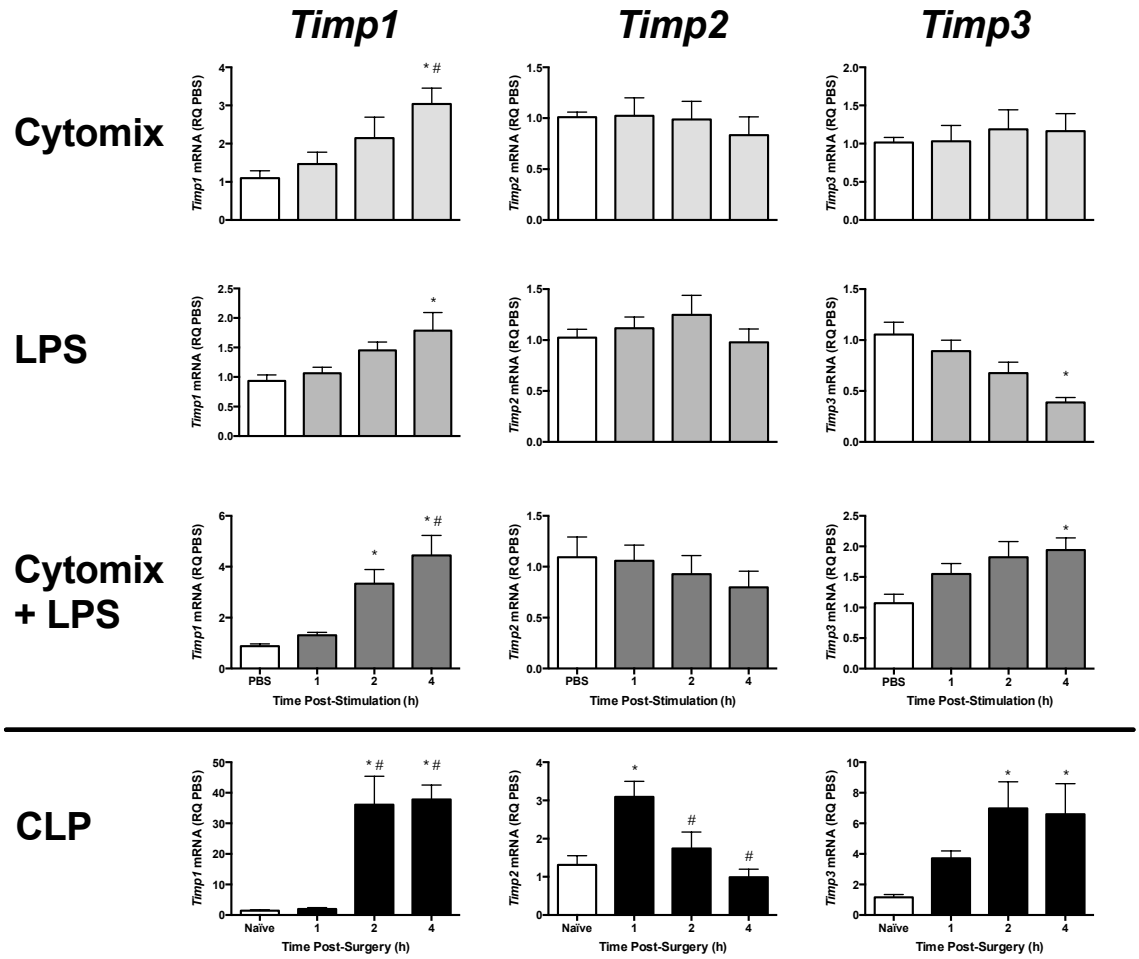

Figure 3-1: *Timp* messenger ribonucleic acid (mRNA) expression is altered in wild type (WT) PMVEC *in vitro* and *ex vivo* under septic conditions. Cytomix-stimulated WT PMVEC had an increase in *Timp1* expression over 4h vs. PBS control (data expressed as relative quantitiy [RQ] of WT PBS). Lipopolysaccharide (LPS)-stimulated WT PMVEC had an increase in *Timp1* expression and a decrease in *Timp3* expression over 4h vs. PBS control (data expressed as RQ of WT PBS). Cytomix + LPS-stimulated WT PMVEC had an increase in *Timp1* and *Timp3* expression over 4h vs. PBS control (data expressed as RQ of WT PBS control). PMVEC from cecal ligation and perforation (CLP)-septic WT mice had an increase in *Timp1*, *Timp2*, and *Timp3* expression vs. PMVEC from naïve WT mice (data expressed as RQ of WT naïve). *Timp4* was not

detected at sufficient levels in all conditions. * $p<0.05$ vs. PBS, # $p<0.05$ vs. 1 h, and + $p<0.05$ vs. 2 h, One-Way ANOVA followed by a Bonferroni post-hoc test, n=5-8.

LPS-stimulation of PMVEC significantly increased *Timp1* mRNA expression by 4h post-stimulation, had no effect on *Timp2* mRNA expression, but significantly reduced *Timp3* mRNA expression by 4h post-stimulation. The combination of cytomix and LPS resulted in significant increases in *Timp1* and *-3* mRNA expression as early as 2h post-stimulation and this increase continued at 4h; however, no effect was observed on *Timp2* mRNA expression (**Figure 3-1**).

PMVEC isolated from CLP-septic mice had significantly increased *Timp1* and *-3* mRNA expression as early as 2h post-CLP vs. PMVEC isolated from naïve mice, and this increase remained significant at 4h post-CLP. *Timp2* mRNA expression was significantly increased by 1h post-CLP, but recovered to basal levels by 2h post-CLP (**Figure 3-1**). Interestingly, *in vitro* the individual stimulations (i.e. cytomix or LPS alone) caused significant changes to a single TIMP; however, the combined stimulation of cytomix and LPS *in vitro* appeared to be more representative of what was observed *in vivo* (**Figure 3-1**).

3.1.2 Metalloproteinase mRNA Expression is Altered in WT PMVEC under Septic Conditions

Because *Timp* mRNA expression levels were significantly altered under septic conditions, I next examined metalloproteinase mRNA expression. Cytomix stimulation of PMVEC *in vitro* significantly increased *Mmp9* and *Adam17* mRNA expression by 4h post-stimulation. *Mmp2* and *Adam10* mRNA expression did not change (**Figure 3-2**). LPS-stimulated PMVEC had significantly increased *Mmp9* and *Adam17* mRNA expression by 4h post-stimulation and no effect on *Mmp2* and *Adam10* mRNA expression (**Figure 3-2**). The combination of cytomix and LPS significantly increased *Mmp9* and

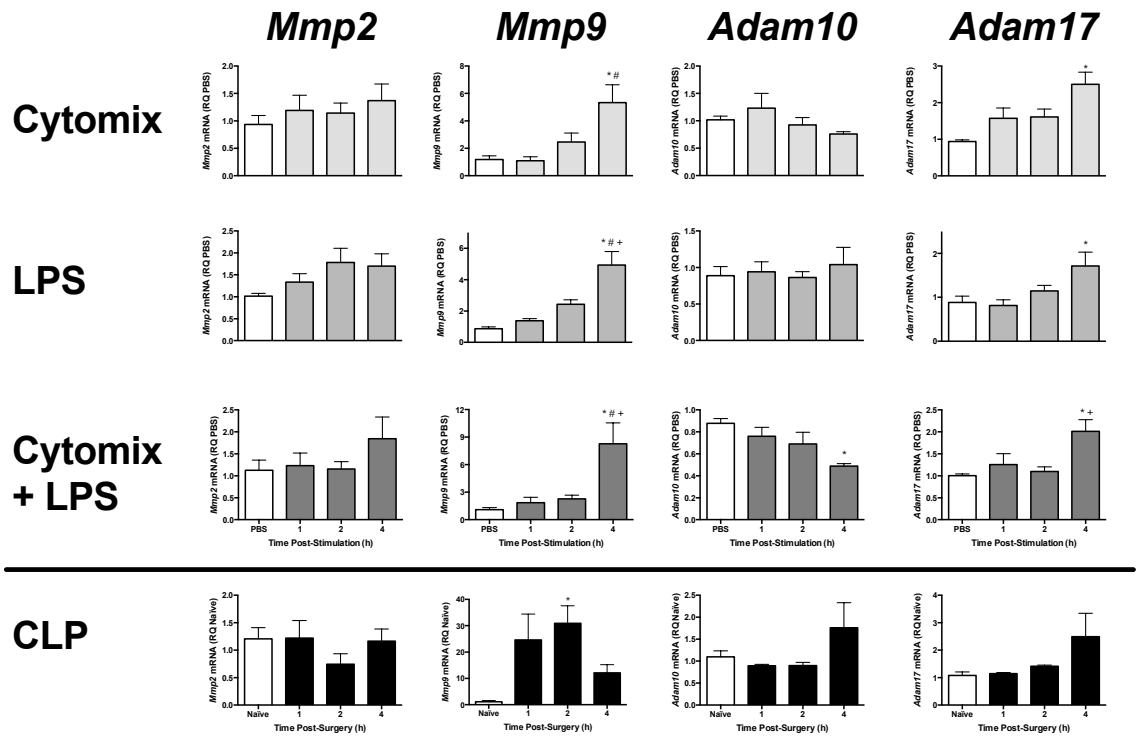

Figure 3-2: Metalloproteinase mRNA expression is altered in WT PMVEC *in vitro* and *ex vivo* under septic conditions. Cytomix-stimulated WT PMVEC had an increase in *Mmp9* and *Adam17* expression over 4h vs. PBS control (data expressed as RQ of WT PBS). LPS-stimulated WT PMVEC had an increase in *Mmp9* and *Adam17* expression over 4h vs. PBS control (data expressed as RQ of WT PBS). Cytomix + LPS-stimulated WT PMVEC had an increase in *Mmp9* and *Adam17* expression and a decrease in *Adam10* over 4h vs. PBS control (data expressed as RQ of WT PBS). PMVEC from CLP-septic WT mice displayed an increase in *Mmp9* expression vs. PMVEC from naïve WT mice (data expressed as RQ of WT naïve). * $p<0.05$ vs. PBS, # $p<0.05$ vs. 1 h, and + $p<0.05$ vs. 2 h, One-Way ANOVA followed by a Bonferroni post-hoc test, n=5-8.

Adam17 mRNA expression by 4h post-stimulation, significantly decreased *Adam10* mRNA expression by 4h post-stimulation, and had no effect on *Mmp2* mRNA expression (**Figure 3-2**).

PMVEC isolated from CLP-septic mice had significantly increased *Mmp9* mRNA expression by 2h post-CLP. Once again, *Mmp2* and *Adam10* mRNA expression did not change; however, *Adam17* mRNA expression had a trend towards increased expression. Thus, while *in vitro* septic models led to significant changes in PMVEC-derived metalloproteinase expression, only *Mmp9* was significantly altered in the *in vivo* model of sepsis (**Figure 3-2**).

3.2 Metalloproteinase Protein Activity in WT PMVEC under Septic Conditions

My expression data suggests that the balance between PMVEC-derived TIMPs and metalloproteinases is disrupted under septic conditions; however, these changes in expression may not be indicative of overall changes in metalloproteinase activity, and thus, altered degradation or cleavage of proteins critical to PMVEC barrier function and PMN-PMVEC interaction. Either or both septic stimulations and expression independently or collectively could affect metalloproteinase activity. To examine the impact of septic stimulation and the resulting altered TIMP/metalloproteinase balance on metalloproteinase activity, I assessed global metalloproteinase activity within PMVEC lysates and in PMVEC-conditioned media, as well as the activity of a specific metalloproteinase, ADAM17, within the cell lysate.

3.2.1 Global Metalloproteinase Activity in the Conditioned Media is not Altered under Septic Conditions

Multiple metalloproteinases expressed by PMVEC, including MMP2 and -9, are considered soluble MMPs and as such, may be released from the cell into the surrounding microenvironment. To examine the activity of these soluble metalloproteinases, I assessed total metalloproteinase within the conditioned media from WT PMVEC under basal conditions and after 4h of septic (cytomix, LPS, or a combination of both) stimulation. Compared to PBS control, soluble metalloproteinase activity was not significantly altered under septic conditions. However following stimulation with cytomix or the combination of cytomix and LPS, there appeared to be a trend towards decreased metalloproteinase activity (Cytomix: 7%; Cytomix + LPS: 10%; **Figure 3-3**).

3.2.2 Global Metalloproteinase Activity in PMVEC Lysate is not Altered under Septic Conditions

While soluble metalloproteinases are detected in the conditioned media, many metalloproteinases, including ADAMs, are found on the cell membrane. Further, soluble metalloproteinases expressed by PMVEC but not yet secreted, or bound to the cell surface through interaction with cell surface receptors such as integrins, are also associated with the cellular compartment. Thus, I next investigated global metalloproteinase activity within the PMVEC lysate. Surprisingly, compared to PBS control, total metalloproteinase activity was not significantly altered under septic conditions; however, there was a trend towards increased activity following cytomix stimulation (9%; **Figure 3-4).**

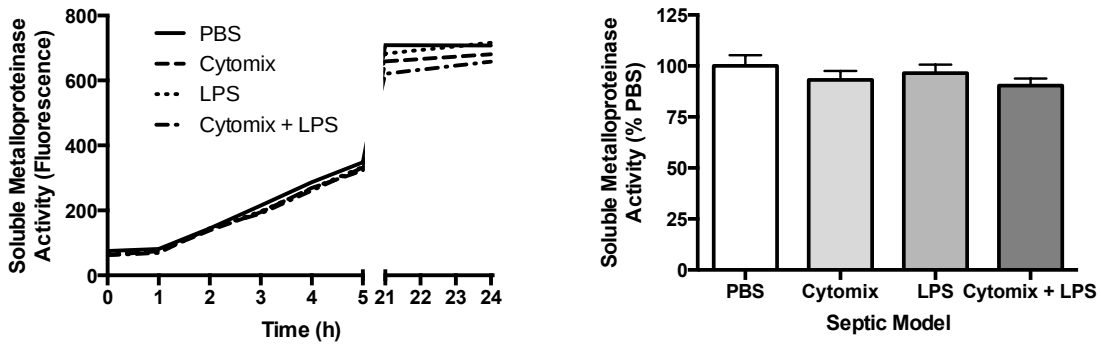

Figure 3-3: PMVEC-derived soluble metalloproteinase activity is not altered under 4h septic conditions. Left panel: Representative graph of metalloproteinase activity (indicated by increase in relative fluorescence over time) in the conditioned media of WT PMVEC under basal and septic conditions. Right panel: Area under the curve (AUC) analysis revealed that compared to PBS control, PMVEC stimulated under septic conditions for 4h had no significant change in soluble metalloproteinase activity. However, PMVEC stimulated with cytomix and the combination of cytomix and LPS appeared to have a trend towards decreased metalloproteinase activity (n=6).

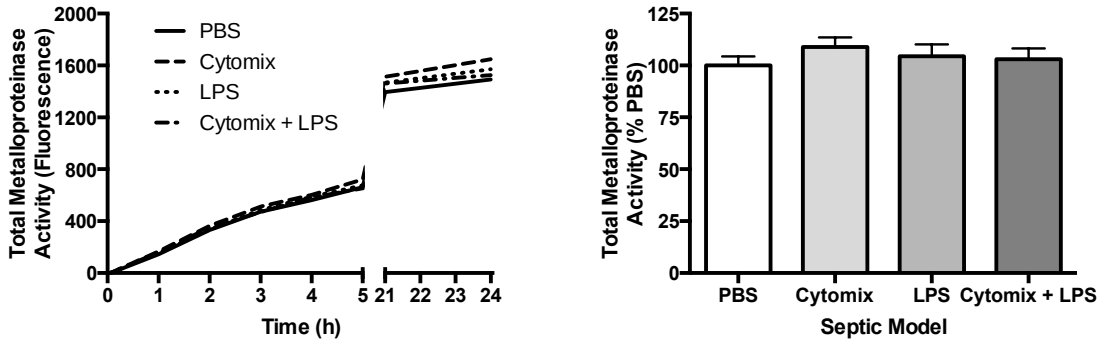

Figure 3-4: Total metalloproteinase activity in WT PMVEC lysate is not altered under 4h septic conditions. Left panel: Representative graph of metalloproteinase activity (indicated by increase in relative fluorescence over time) in the lysate of WT PMVEC under basal and septic conditions. Right panel: AUC analysis revealed that compared to PBS control, the lysate from PMVEC stimulated under septic conditions for 4 hours had no change in global metalloproteinase activity. However, PMVEC stimulated with cytomix appeared to have a trend towards increased metalloproteinase activity (n=4).

3.2.3 ADAM17 Activity is Increased in WT PMVEC under Septic Conditions

As showed in 3.1.2, PMVEC expression of *Adam17*, a metalloproteinase known to be associated with the cell membrane, was significantly increased under septic conditions, which suggests that the observed trend towards an increase in total metalloproteinase activity in PMVEC lysate could be due to increased ADAM17 activity. Analysis of PMVEC lysate ADAM17 activity revealed that ADAM17 activity was significantly increased following the combined stimulation of cytomix and LPS compared to PBS control (**Figure 3-5**). Further, there also appeared to be a trend towards increased ADAM17 activity following both cytomix and LPS stimulation (Cytomix: 8%; LPS: 6%; **Figure 3-5**).

3.3 Inhibition of Metalloproteinase Activity Reduces WT PMVEC Barrier Dysfunction under Septic Conditions

Septic stimulation, including cytomix and LPS, is well known to promote PMVEC barrier dysfunction [8,83,108,110,113,114]. Further, this PMVEC barrier dysfunction appears to be associated with a shift in the expression of metalloproteinases and TIMPs from the basal expression patters, and is also associated with increased metalloproteinase activity, specifically, ADAM17. However, whether this increased metalloproteinase activity is responsible for the septic PMVEC barrier dysfunction is unknown. To address this, permeability across WT PMVEC was assessed *in vitro* under septic conditions in the presence or absence of three global metalloproteinase inhibitors, GM6001, BB-94 or TAPI-2. Specifically, WT PMVEC were stimulated with PBS (vehicle control) or cytomix (30 ng/mL) for 4h in the presence of increasing

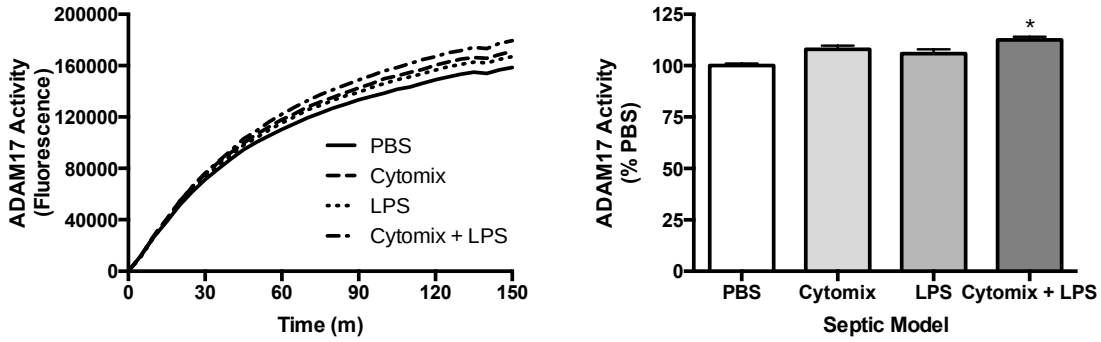

Figure 3-5: A disintegrin and metalloproteinase (ADAM) 17 activity appears to be increased in WT PMVEC lysate under 4h septic conditions. Left panel: Representative graph of ADAM17 activity (indicated by increase in relative fluorescence over time) in the lysate of WT PMVEC under basal and septic conditions. Right panel: AUC analysis revealed that compared to PBS control, PMVEC stimulated with cytomix and LPS together for 4h had significantly increased ADAM17 activity. PMVEC stimulated with cytomix and LPS separately appeared to have a trend towards increased ADAM17 activity. * $p<0.05$ vs. PBS, One-Way ANOVA followed by a Bonferroni post-hoc test, n=4.

concentrations of GM6001, BB-94 or TAPI-2 (1-100 uM) or their vehicle controls (DMSO or dH_2O) and TEER, as well as FITC-labelled dextran and EB-labelled albumin flux across the PMVEC monolayers was measured. WT PMVEC stimulated with cytomix for 4h was associated with a significant increase in PMVEC permeability as measured by decreased TEER and increased dextran and albumin flux (**Figure 3-6 and 3-7)**. Interestingly, compared to vehicle control, increasing concentrations of GM6001, BB-94, and TAPI-2 had no effect on TEER (**Figure 3-6**). Further, compared to DMSO control, increasing concentrations of GM6001 also had no effect on albumin and dextran flux across WT PMVEC under septic conditions (**Figure 3-7**). However, BB-94 significantly reduced albumin flux vs. DMSO control at a concentration of 10 μM, and this reduction persisted at higher concentrations (**Figure 3-7**). Further, treatment with BB-94 also appeared to reduce dextran flux with a significant reduction observed at 25 μM (**Figure 3-7**). Compared to dH_2O, increasing concentrations of TAPI-2 also led to significantly reduced albumin and dextran flux at 25 μM (**Figure 3-7**).

3.4 Basal differences between WT vs. *Timp3*$^{-/-}$ PMVEC

Basal studies have demonstrated that basal *Timp3*$^{-/-}$ PMVEC permeability, as measured by TEER and macromolecular flux, is significantly greater than WT PMVEC [83]. Further, treatment of *Timp3*$^{-/-}$ PMVEC with a synthetic metalloproteinase inhibitor, GM6001, partially restored this increased permeability suggesting that increase was at least partly dependent on metalloproteinase activity [83]. Thus, I next began to further characterize the impaired barrier function in *Timp3*$^{-/-}$ PMVEC assessing *Timp* and metalloproteinase mRNA expression as well as metalloproteinase activity *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC under basal conditions. Analysis of *Timp* expression

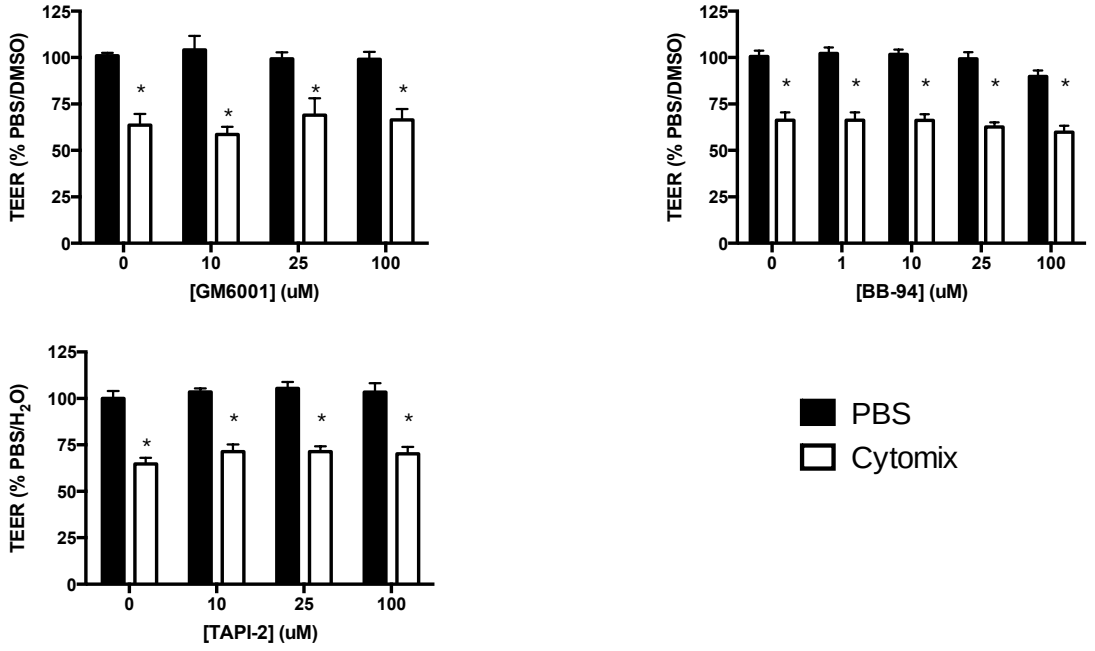

Figure 3-6: Transendothelial electrical resistance (TEER) is not altered following treatment with global metalloproteinase inhibitors. Compared to vehicle control (DMSO or dH_2O), treatment of WT PMVEC with increasing concentrations of GM6001, BB-94, and TAPI-2 had no effect on TEER under basal or septic (cytomix, 30 ng/mL) conditions. * $p<0.05$ vs. PBS, Two-Way ANOVA followed by a Bonferroni post-hoc test, n=4-10.

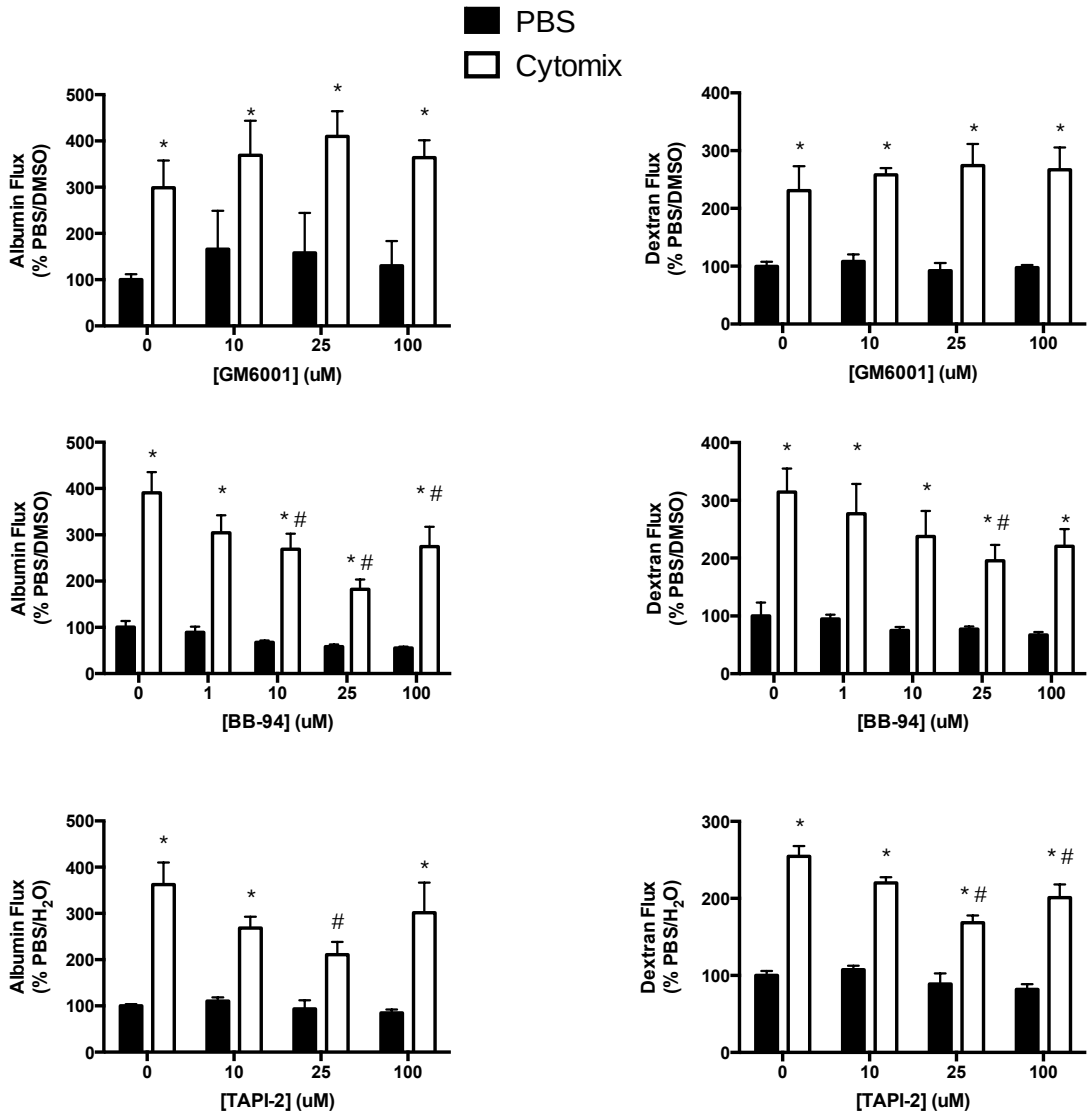

Figure 3-7: Septic macromolecular flux is reduced following treatment with specific global metalloproteinase inhibitors. Compared to vehicle control (DMSO), increasing concentrations of GM6001 has no effect on albumin or dextran flux across WT PMVEC under septic (cytomix, 30 ng/mL) conditions. However, increasing concentrations of BB-94 and TAPI-2 significantly reduced both albumin and dextran flux across WT PMVEC under septic conditions vs. vehicle control (DMSO/dH_2O). * $p<0.05$ vs. PBS, and # $p<0.05$ vs. 0 μM, Two-Way ANOVA followed by a Bonferroni post-hoc test, n=3-5.

found that *Timp2* had the highest basal expression and *Timp4* had the lowest expression (**Table 3-3**). *Mmp2, Adam10* and *Adam17* appeared to be expressed at high levels whereas basal expression of *Mmp9* was fairly low, and *Mmp7* was not detected (**Table 3-3**). *Timp* and metalloproteinase basal expression were similar in PMVEC isolated from naïve *Timp3*$^{-/-}$ mice *ex vivo* (**Table 3-4**).

3.4.1 Metalloproteinase and *Timp* mRNA Expression in *Timp3*$^{-/-}$ PMVEC is Similar to WT PMVEC under Basal Conditions

Analysis of metalloproteinase and *Timp* mRNA expression in WT and *Timp3*$^{-/-}$ PMVEC *in vitro* under basal conditions revealed no significant differences between genotypes, except for *Timp3,* which is not present in *Timp3*$^{-/-}$ PMVEC (**Figure 3-8**). Similarly, in PMVEC isolated from naïve mice, compared to WT, *Timp3*$^{-/-}$ PMVEC had no significance differences in *Timp* or metalloproteinase expression, except for *Timp3* (**Figure 3-9**).

3.4.2 Metalloproteinase Activity Appears Decreased in *Timp3*$^{-/-}$ vs. WT PMVEC under Basal Conditions

Analysis of global metalloproteinase activity in conditioned media from WT and *Timp3*$^{-/-}$ PMVEC under basal conditions demonstrated significantly reduced metalloproteinase activity in the conditioned media from *Timp3*$^{-/-}$ PMVEC compared to WT PMVEC **(**15%; **Figure 3-10A**)**.** Interestingly, no significant differences in global metalloproteinase activity or in ADAM17 activity were noted between genotypes in the cell lysate under basal conditions (**Figure 3-10B and C**).

Table 3-3: Average Basal C_t values for *Timp*/metalloproteinase mRNA expression in isolated murine *Timp3*$^{-/-}$ PMVEC *in vitro* (n=22).

Timp1	***Timp2***	***Timp3***	***Timp4***	***Hprt1***
28.4	25.0	-/-	36.4	25.5
Mmp2	***Mmp7***	***Mmp9***	***Adam10***	***Adam17***
28.5	40+	36.0	26.0	27.1

Table 3-4: Average Basal C_t values for *Timp*/metalloproteinase mRNA expression in isolated murine *Timp3*$^{-/-}$ PMVEC *ex vivo* (n=5).

Timp1	***Timp2***	***Timp3***	***Timp4***	***Hprt1***
37.2	30.2	-/-	38.2	31.4
Mmp2	***Mmp7***	***Mmp9***	***Adam10***	***Adam17***
33.0	40+	35.4	30.0	30.5

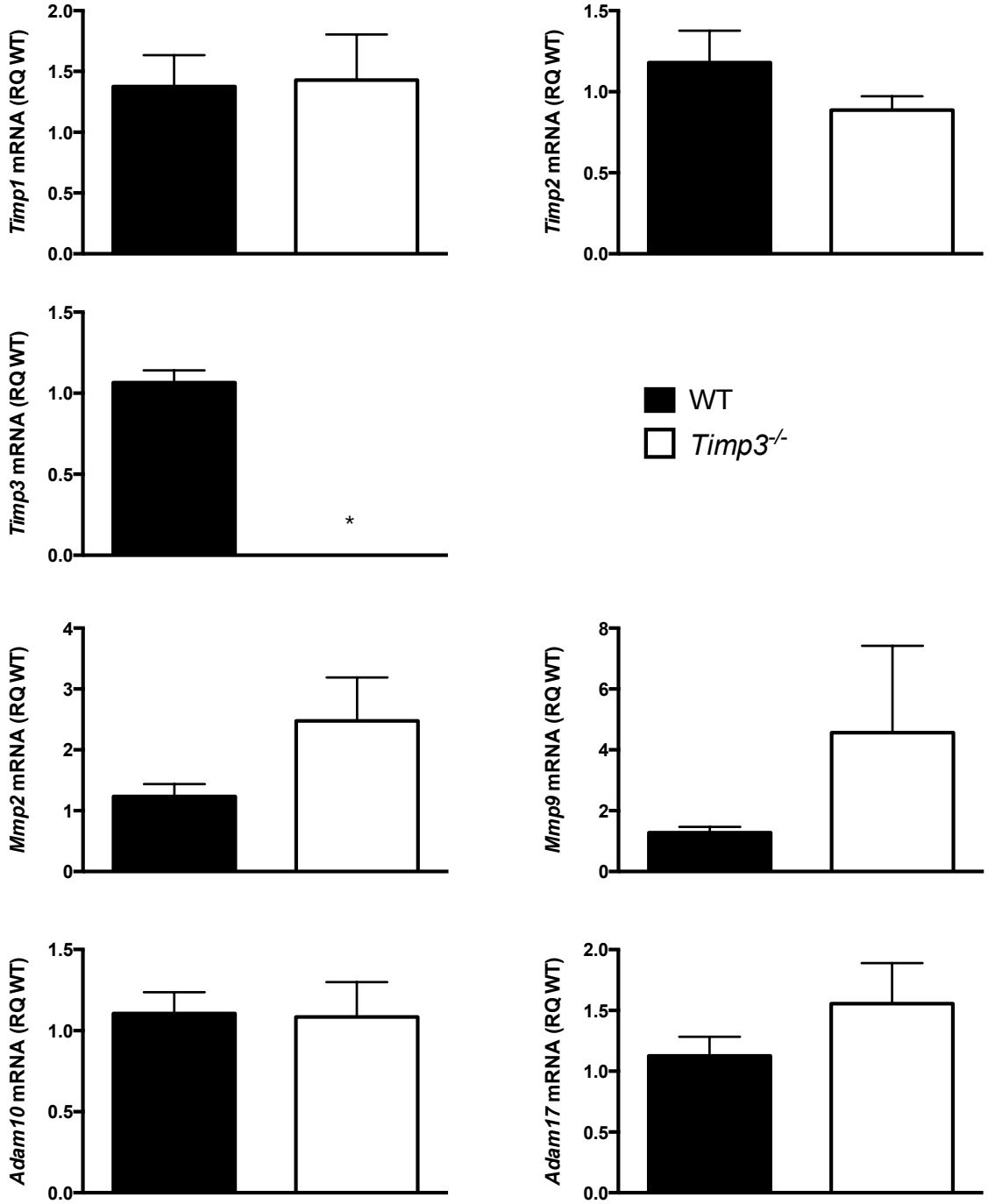

Figure 3-8: *Timp* and metalloproteinase mRNA expression is not altered in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC at basal levels *in vitro*. No significant differences were observed in *Timp* or metalloproteinase expression, except for *Timp3*, in *Timp3*$^{-/-}$ PMVEC

vs. WT PMVEC. Data expressed as the RQ of WT control, * $p<0.05$ vs. WT, paired t-test, n=22.

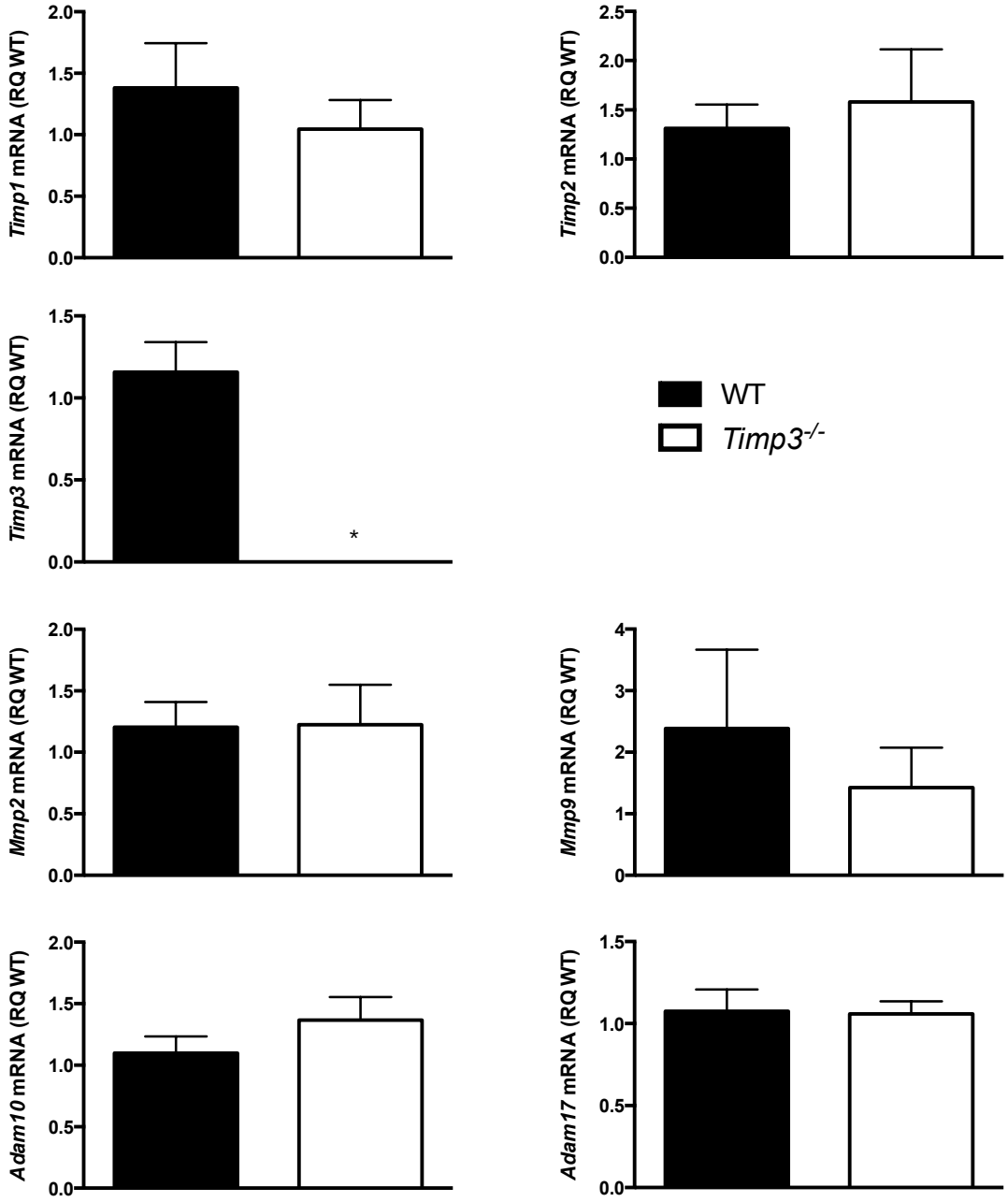

Figure 3-9: *Timp* and metalloproteinase mRNA expression is not altered in *Timp3*$^{-/-}$ PMVEC isolated from naïve mice. No significant differences in *Timp* or metalloproteinase expression, except *Timp3*, were found in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC isolated from naïve mice. Data expressed as the RQ of WT control, * $p<0.05$ vs. WT, paired t-test, n=5-8.

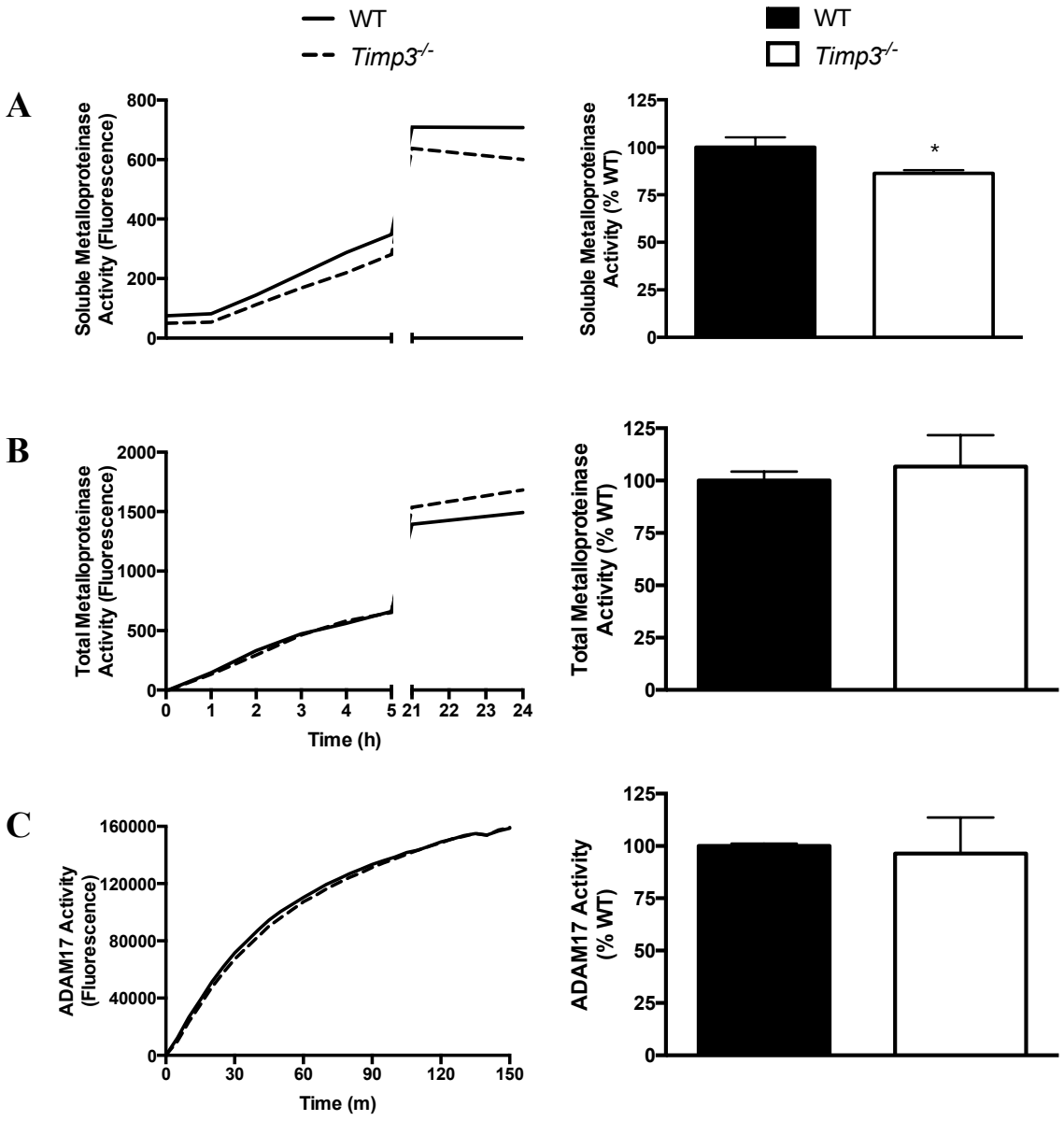

Figure 3-10: Metalloproteinase activity appears reduced in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC under basal conditions *in vitro*. Left panel: Representative graph of metalloproteinase activity (indicated by increase in relative fluorescence over time) in the conditioned media **(A)** and lysate **(B, C)** of WT and *Timp3*$^{-/-}$ PMVEC under basal conditions. Right panel: AUC analysis revealed that compared to WT, *Timp3*$^{-/-}$ PMVEC had **(A)** significantly reduced soluble metalloproteinase activity, **(B)** no change in global

metalloproteinase activity, **(C)** no change in ADAM17 activity. * $p<0.05$ vs. WT, paired t-test, n=4-6.

3.5 *Timp* and Metalloproteinase mRNA Expression in *Timp3*$^{-/-}$ PMVEC

While no differences were observed in metalloproteinase and *Timp* expression between WT and *Timp3*$^{-/-}$ PMVEC under basal conditions, TIMP3 has been previously found to mediate pro-inflammatory signalling and as such, it is possible that the response of *Timp3*$^{-/-}$ PMVEC to septic stimuli could be altered [49,98,100,101,115]. To assess this, I next examined *Timp* and metalloproteinase expression data in *Timp3*$^{-/-}$ PMVEC under septic conditions.

3.5.1 *Timp* mRNA Expression is Altered in *Timp3*$^{-/-}$ PMVEC under Septic Conditions

PMVEC-derived expression of all *Timps* and key metalloproteinases was examined in *Timp3*$^{-/-}$ PMVEC at 1, 2, and 4h following *in vitro* (cytomix, LPS, and both cytomix and LPS) and *in vivo* (CLP) models of sepsis by qRT-PCR. Similar to WT PMVEC, in *Timp3*$^{-/-}$ PMVEC, *Timp1* mRNA expression was significantly increased at 4h post-cytomix, as well as at 2 and 4h post-stimulation with both cytomix and LPS, while *Timp2* mRNA expression was not altered under septic conditions *in vitro* (**Figure 3-11**). Further, *Timp1* mRNA expression was significantly increased at 4h post-CLP in PMVEC from *Timp3*$^{-/-}$ mice (**Figure 3-11**). Additionally, in PMVEC from *Timp3*$^{-/-}$ mice, *Timp2* mRNA expression appeared to trend towards an increase at 1 and 2h post-CLP, but was significantly reduced at 4h post-CLP (**Figure 3-11**).

3.5.2 Metalloproteinase mRNA Expression is Altered in *Timp3*$^{-/-}$ PMVEC under Septic Conditions

I next examined metalloproteinase mRNA expression in *Timp3*$^{-/-}$ PMVEC under septic conditions *in vitro* and *in vivo*. Cytomix stimulation of PMVEC significantly

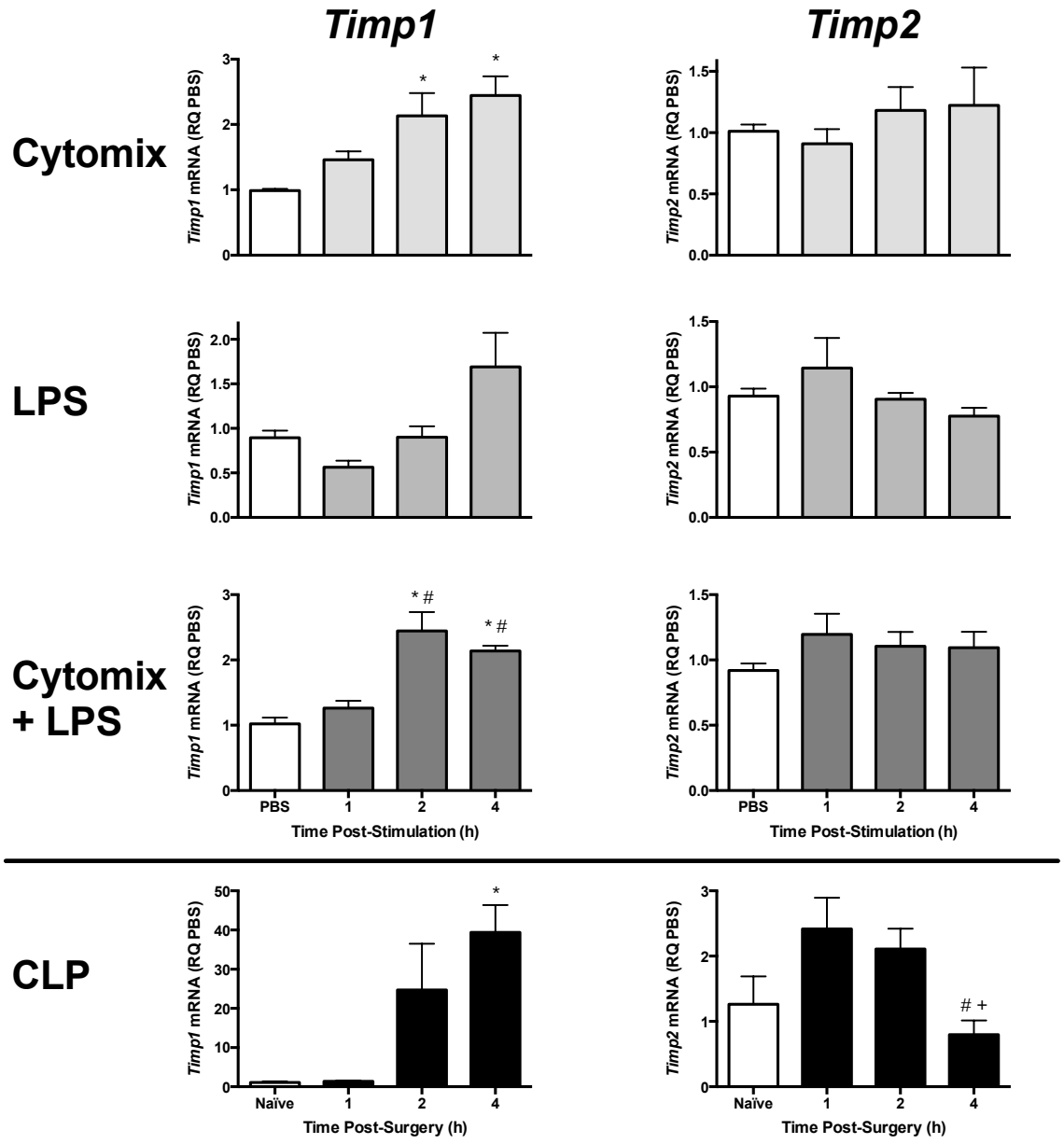

Figure 3-11: *Timp* mRNA expression is altered in *Timp3*$^{-/-}$ PMVEC *in vitro* and *ex vivo* under septic conditions. Cytomix-stimulated *Timp3*$^{-/-}$ PMVEC had an increase in *Timp1* expression over 4h vs. PBS control (data expressed as RQ of PBS). LPS-stimulated *Timp3*$^{-/-}$ PMVEC had no significant changes in *Timp* expression over 4h vs. PBS control (data expressed as RQ of PBS). Cytomix + LPS-stimulated *Timp3*$^{-/-}$ PMVEC had an increase in *Timp1* expression over 4h vs. PBS control (data expressed as RQ of

PBS). PMVEC isolated from CLP-septic *Timp3*$^{-/-}$ mice had an increase in *Timp1* expression over 4h and significantly decreased *Timp2* expression at 4h vs. PMVEC from naïve *Timp3*$^{-/-}$ mice (data expressed as RQ of naïve). *Timp3* and *-4* were not detected at sufficient levels under any condition. * $p<0.05$ vs. PBS, # $p<0.05$ vs. 1 h, and + $p<0.05$ vs. 2 h, One-Way ANOVA followed by a Bonferroni post-hoc test, n=3-8.

increased *Mmp9* and *Adam17* mRNA expression by 4h post-stimulation. *Mmp2* and *Adam10* mRNA expression did not change (**Figure 3-12**). LPS-stimulated PMVEC had significantly increased *Mmp9* by 2h post-stimulation, and this increase remained significant at 4h post-stimulation. Interestingly, *Adam10* mRNA expression significantly reduced by 1h post-stimulation, and this decrease remained significant at 2 and 4h post-stimulation. *Mmp2* and *Adam17* mRNA expression did not change; however, *Adam17* expression appeared to trend towards increased expression (**Figure 3-12**). Further, the combination of cytomix and LPS significantly increased *Mmp2, -9,* and *Adam17* mRNA expression at 4h post-stimulation. *Adam10* mRNA expression, however, remained constant at all time points (**Figure 3-12**). Finally, PMVEC isolated from CLP-septic *Timp3*$^{-/-}$ mice had significantly increased *Mmp9* and *Adam17* mRNA expression at 1 and 2h post-CLP; however, expression of both returned to basal levels at 4h post-CLP. No significant differences in *Mmp2* and *Adam10* mRNA expression were observed in PMVEC isolated from CLP-septic *Timp3*$^{-/-}$ mice (**Figure 3-12**).

3.6 Metalloproteinase Activity in *Timp3*$^{-/-}$ PMVEC under Septic Conditions

Once again, my expression data suggests that the balance between PMVEC-derived TIMPs and metalloproteinases is disrupted under septic conditions; however, these changes in expression may not be indicative of overall changes in metalloproteinase activity. To examine the impact of the altered TIMP/metalloproteinase balance on metalloproteinase activity, I assessed global metalloproteinase activity within the conditioned media and within the cell lysate, as well as the activity of a specific metalloproteinase, ADAM17, within the cell lysate under septic conditions.

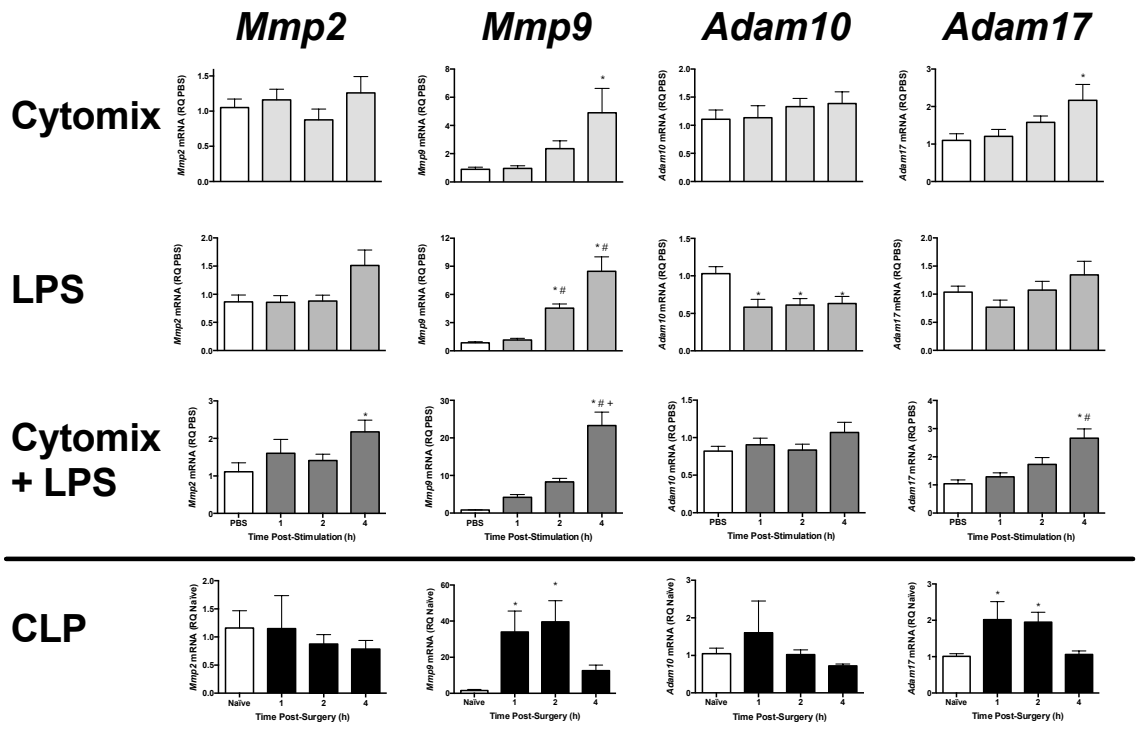

Figure 3-12: Metalloproteinase mRNA expression is altered in *Timp3*$^{-/-}$ PMVEC *in vitro* and *ex vivo* under septic conditions. Cytomix-stimulated *Timp3*$^{-/-}$ PMVEC had an increase in *Mmp9* and *Adam17* expression over 4h vs. PBS control (data expressed as RQ of PBS). LPS-stimulated *Timp3*$^{-/-}$ PMVEC had an increase in *Mmp9* expression and a decrease in *Adam10* expression over 4h vs. PBS control (data expressed as RQ of PBS). Cytomix + LPS-stimulated *Timp3*$^{-/-}$ PMVEC had an increase in *Mmp2*, *Mmp9* and *Adam17* expression over 4h vs. PBS control (data expressed as RQ of PBS). PMVEC from CLP-septic *Timp3*$^{-/-}$ mice had an increase in *Mmp9* and *Adam17* expression over 4h vs. PMVEC from naïve *Timp3*$^{-/-}$ mice (data expressed as RQ of naïve). * $p<0.05$ vs. PBS, # $p<0.05$ vs. 1 h, and + $p<0.05$ vs. 2 h, One-Way ANOVA followed by a Bonferroni post-hoc test, n=3-8.

3.6.1 Global Metalloproteinase Activity in the Conditioned Media is not Altered under Septic Conditions in *Timp3*$^{-/-}$ PMVEC

To examine the activity of soluble metalloproteinases, I assessed total metalloproteinase within the conditioned media from *Timp3*$^{-/-}$ PMVEC under basal conditions and after 4h of septic (cytomix, LPS, or a combination of both) stimulation. Compared to PBS control, soluble metalloproteinase activity was not significantly altered under septic conditions. However following stimulation, there appeared to be a trend towards decreased metalloproteinase activity (Cytomix: 6%; LPS: 11%; Cytomix + LPS: 12%; **Figure 3-13**).

3.6.2 Global Metalloproteinase Activity in *Timp3*$^{-/-}$ PMVEC Lysate is not Altered under Septic Conditions

I investigated global metalloproteinase activity within the cell lysate, which contains many membrane bound metalloproteinases, as well as soluble metalloproteinases, which have not been secreted, or bound to the cell surface through interaction with cell surface receptors. Compared to PBS control, total metalloproteinase activity was not significantly altered under septic conditions. However, following cytomix stimulation and the combined stimulation of cytomix and LPS, there appeared to be a trend towards decreased and increased metalloproteinase activity, respecitvely (Cytomix: 10%; Cytomix + LPS: 23%; **Figure 3-14**).

3.6.3 ADAM17 Activity is not Altered in *Timp3*$^{-/-}$ PMVEC under Septic Conditions

Expression of *Adam17*, a metalloproteinase known to be associated with the cell membrane, was significantly increased under septic conditions, which suggests that the observed trend towards an increase in total metalloproteinase activity in the cell lysate

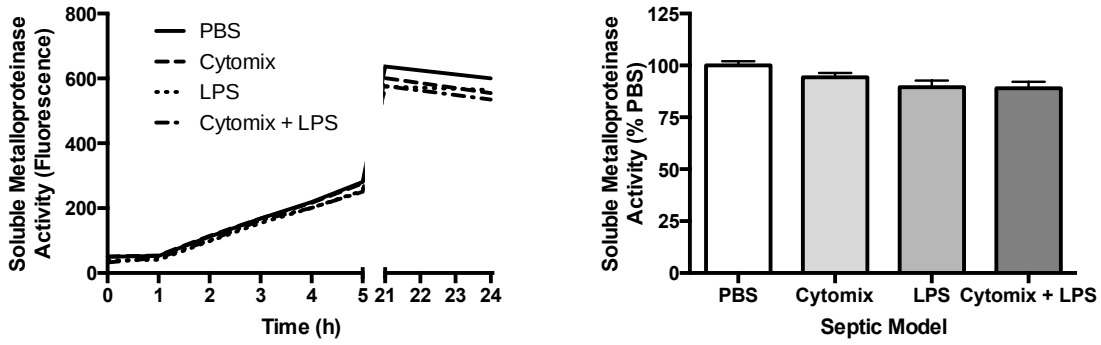

Figure 3-13: *Timp3*$^{-/-}$ PMVEC-derived soluble metalloproteinase activity is not altered under 4h septic conditions. Left panel: Representative graph of metalloproteinase activity (indicated by increase in relative fluorescence over time) in the conditioned media of *Timp3*$^{-/-}$ PMVEC under basal and septic conditions. Right panel: AUC analysis revealed that compared to PBS control, PMVEC stimulated under septic conditions for 4h had no significant change in soluble metalloproteinase activity. However, PMVEC under septic conditions appeared to have a trend towards decreased metalloproteinase activity (n=6).

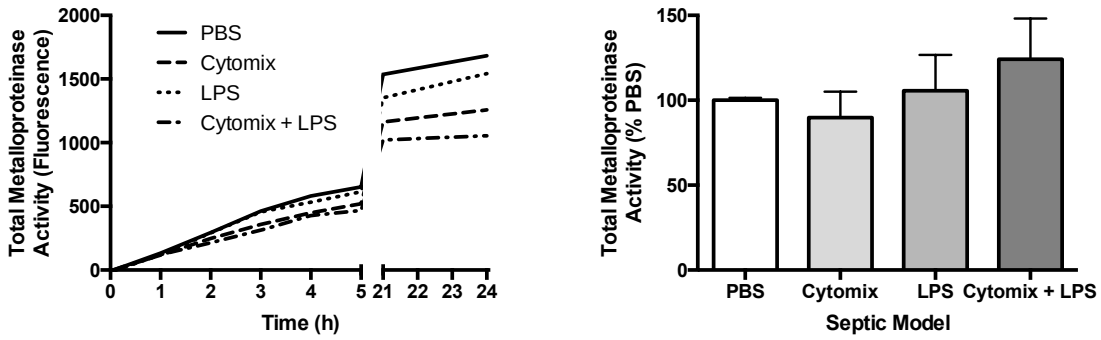

Figure 3-14: Total metalloproteinase activity in *Timp3*$^{-/-}$ PMVEC lysate is not altered under 4h septic conditions. Left panel: Representative graph of metalloproteinase activity (indicated by increase in relative fluorescence over time) in the lysate of *Timp3*$^{-/-}$ PMVEC under basal and septic conditions. Right panel: AUC analysis revealed that compared to PBS control, the lysate from PMVEC stimulated under septic conditions for 4h had no change in global metalloproteinase activity. However, PMVEC under cytomix and cytomix + LPS stimulation appeared to have a trend towards decreased and increased metalloproteinase activity, respectively (n=4).

could be due to increased ADAM17 activity. PMVEC stimulated under septic conditions after 4h had no significant change in ADAM17 activity. However, PMVEC stimulated with LPS and the combination of cytomix and LPS appeared to have a trend towards increased activity (LPS: 23%; Cytomix + LPS: 52%; **Figure 3-15**).

3.7 Endothelial Barrier Dysfunction is Associated with Increased Cell Surface Expression of PMVEC Cell Adhesion Molecules

3.7.1 PMVEC Cell Adhesion Molecule mRNA Expression is Increased in WT and *Timp3*$^{-/-}$ PMVEC under Septic Conditions

Expression of *Icam1*, *Vcam1*, and *Sele* mRNA levels was examined in WT and *Timp3*$^{-/-}$ PMVEC *in vitro* following stimulation with cytomix, LPS, or both for between 1-4h. Cytomix stimulation of WT and *Timp3*$^{-/-}$ PMVEC significantly increased *Icam1*, *Vcam1*, and *Sele* (**Figure 3-16**). LPS stimulation of WT PMVEC significantly increased *Sele* mRNA expression, as well as *Icam1*, *Vcam1*, and *Sele* mRNA expression in *Timp3*$^{-/-}$ PMVEC (**Figure 3-16**). *Icam1* and *Vcam1* mRNA expression trended towards increased expression in LPS-stimulated WT PMVEC (**Figure 3-16**). Under each septic condition, *Sele* mRNA expression decreased at 2 and 4h in both WT and *Timp3*$^{-/-}$ PMVEC (**Figure 3-16**). Cytomix and LPS stimulation of WT PMVEC led to a significant increase vs. PBS control in *Icam1* and *Vcam1* mRNA expression by 1h post-stimulation, which persisted at 2h and continued to increase at 4h post-stimulation. *Sele* mRNA expression significantly increased by 1h post-stimulation, and remained significantly increased at 2 and 4h vs. PBS control; however, *Sele* expression was decreased at 2 and 4h post-stimulation vs. 1h post-stimulation (**Figure 3-16**). *Timp3*$^{-/-}$ PMVEC stimulated with both cytomix and LPS

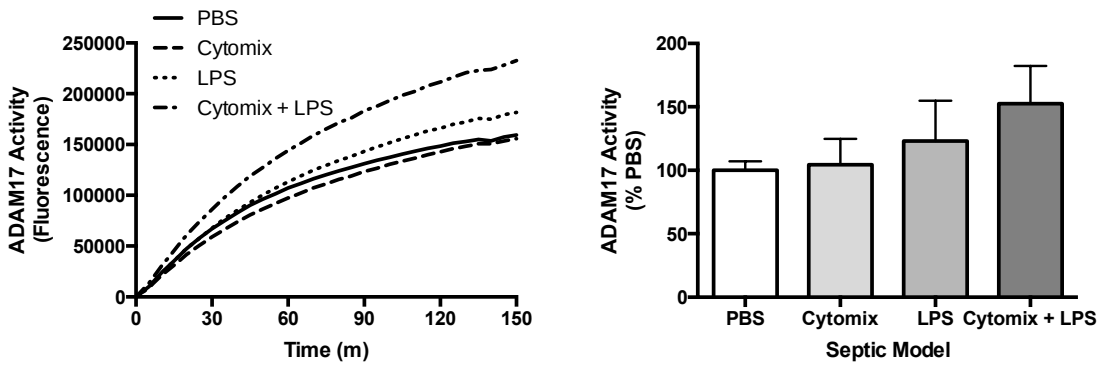

Figure 3-15: ADAM17 activity is not altered in *Timp3*$^{-/-}$ PMVEC lysate under 4h septic conditions. Left panel: Representative graph of ADAM17 activity (indicated by increase in relative fluorescence over time) in the lysate of *Timp3*$^{-/-}$ PMVEC under basal and septic conditions. Right panel: AUC analysis revealed that compared to PBS control, PMVEC stimulated under septic conditions for 4h had no significant change in ADAM17 activity. PMVEC stimulated with LPS and a combination of cytomix and LPS appeared to have a trend towards increased ADAM17 activity (n=4).

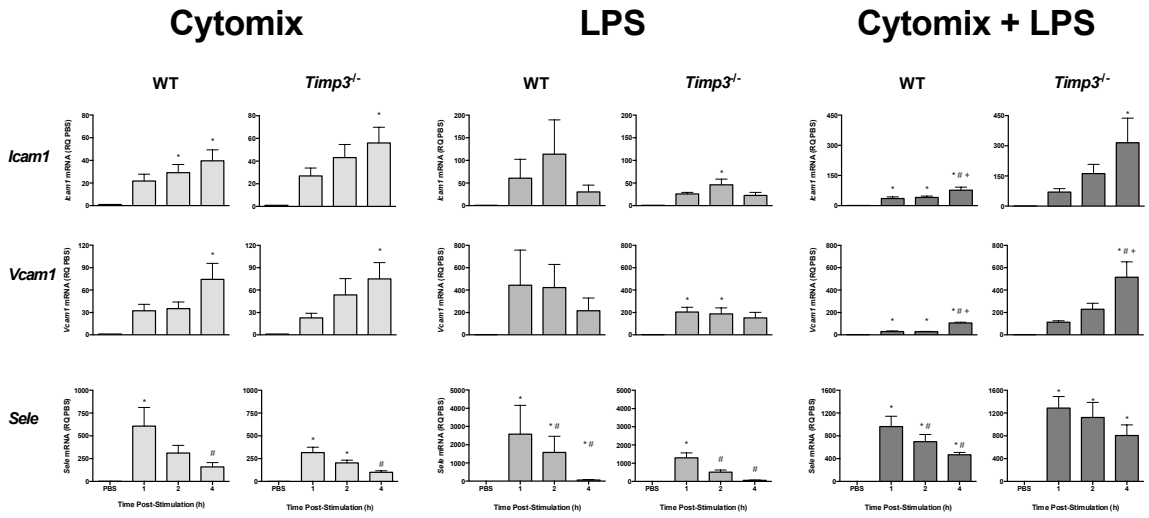

Figure 3-16: Septic models increase expression of PMVEC cell adhesion molecules *in vitro*. *Icam1, Vcam1,* and *Sele* mRNA expression is significantly increased following stimulation with cytomix, LPS, and cytomix + LPS over 4h time course in both WT and *Timp3*$^{-/-}$ PMVEC. When focused on cytomix + LPS stimulated PMVEC, the increased expression, however, appears to be of a greater magnitude in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC. * $p<0.05$ vs. PBS, # $p<0.05$ vs. 1 h, and + $p<0.05$ vs. 2 h, One-Way ANOVA followed by a Bonferroni post-hoc test, n=6-8.

revealed similar trends in *Icam1, Vcam1,* and *Sele* mRNA expression over the entire time course; however, compared to WT PMVEC, *Timp3*$^{-/-}$ PMVEC expression of cell adhesion molecules appeared to be 1.5-4 fold increased under septic conditions (**Figure 3-16**).

3.7.2 Cell Surface Expression of PMVEC Cell Adhesion Molecules is Increased in WT and *Timp3*$^{-/-}$ PMVEC under Septic Conditions

Flow cytometry was used to examine the cell surface levels of ICAM1, VCAM1, and E-selectin on WT and *Timp3*$^{-/-}$ PMVEC under basal and septic conditions. For these studies, PMVEC were treated *in vitro* with PBS, cytomix, LPS, or a combination of both cytomix and LPS for 4h. Compared to PBS control, ICAM1, VCAM1, and E-selectin levels were increased under septic conditions in both WT and *Timp3*$^{-/-}$ PMVEC (**Figure 3-17**). Analysis of the geometric mean fluorescence intensity (MFI) demonstrated that, in general, ICAM1, VCAM1, and E-selectin cell surface abundance increased significantly under septic conditions in both WT and *Timp3*$^{-/-}$ PMVEC (**Figure 3-18**). Specifically, ICAM1, VCAM1, and E-selectin MFI significantly increased in WT and *Timp3*$^{-/-}$ PMVEC following stimulation with cytomix and the combination of cytomix and LPS. ICAM1 and VCAM1 also significantly increased following LPS stimulation in WT PMVEC with a trend towards increased expression in *Timp3*$^{-/-}$ PMVEC. Interestingly, compared to WT PMVEC, ICAM1 cell surface levels were significantly reduced in *Timp3*$^{-/-}$ PMVEC under basal as well as each septic condition – this decreased abundance in the *Timp3*$^{-/-}$ vs. WT PMVEC was not seen for VCAM1 or E-selectin (**Figure 3-18**).

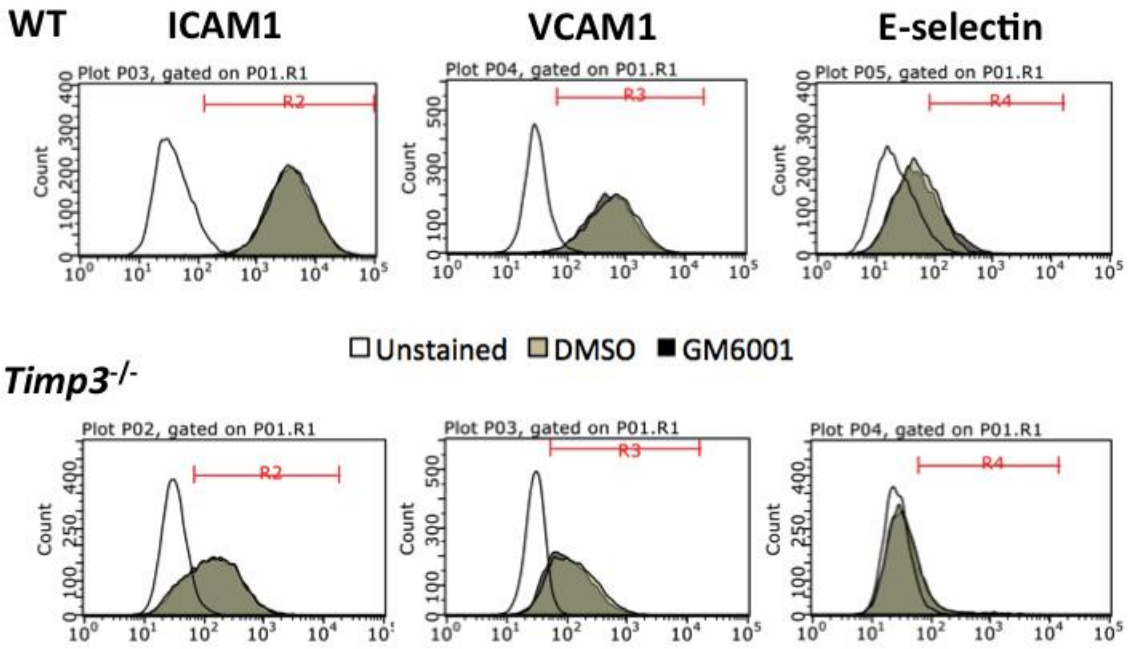

Figure 3-17: Cell adhesion molecule abundance appears increased on the cell surface of PMVEC under septic conditions *in vitro*. Representative flow cytometry histograms from WT and *Timp3*$^{-/-}$ PMVEC demonstrated that PMVEC stimulated with cytomix and LPS for 4h (dark grey histogram) had increased ICAM1, VCAM1 and E-selectin vs. PBS control (tan histogram). Further, under basal and under septic conditions, ICAM appeared to be more abundant on WT vs. *Timp3*$^{-/-}$ PMVEC. Little background fluorescence was observed in unstained cells (white histogram; n=4-5).

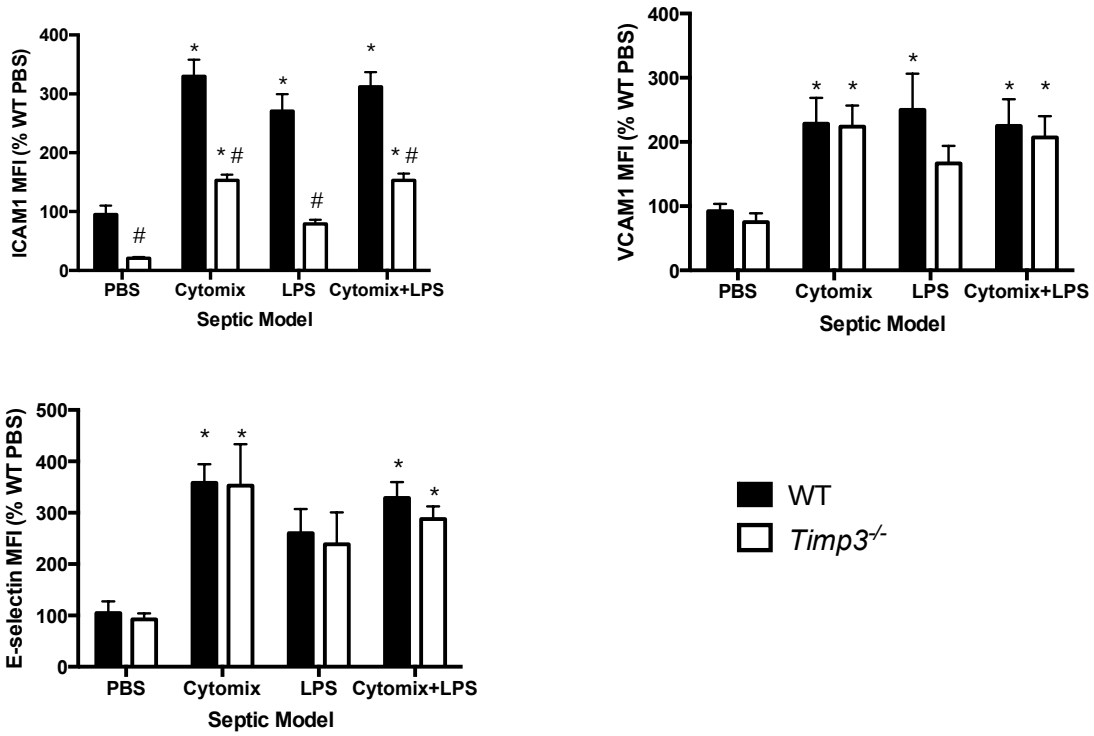

Figure 3-18: Cell adhesion molecule abundance is significantly increased on the cell surface of PMVEC under septic conditions *in vitro.* Analysis of the mean fluorescence intensity (MFI) revealed that ICAM1, VCAM1 and E-selectin cell surface abundance is significantly increased in WT and *Timp3*$^{-/-}$ PMVEC under septic conditions. Further, under basal and septic conditions, ICAM cell surface abundance is significantly increased in WT vs. *Timp3*$^{-/-}$ PMVEC. # $p<0.05$ vs. PBS, # $p<0.05$ vs. WT, Two-Way ANOVA followed by a Bonferroni post-hoc test, n=4-5.

3.8 Cell Surface Intercellular Adhesion Molecule (ICAM) 1 Deficiency in *Timp3*$^{-/-}$ PMVEC under Basal Conditions Appears Metalloproteinase Independent

To begin to determine the mechanism through which TIMP3 regulates PMVEC surface ICAM1 expression under basal conditions, WT and *Timp3*$^{-/-}$ PMVEC were treated with global metalloproteinase inhibitors. Surprisingly, treatment with GM6001, BB94 or TAPI-2 had no effect on ICAM1 cell surface abundance on either WT or *Timp3*$^{-/-}$ PMVEC compared to DMSO or dH_2O control (**Figure 3-19**). Moreover, cell surface ICAM1 remained significantly decreased on *Timp3*$^{-/-}$ vs. WT PMVEC in all treatment groups (**Figure 3-19** and **3-20**). Additionally, no changes were observed in VCAM1 or E-selectin cell surface abundance in any of the treatment groups (**Figure 3-20**).

3.9 *Timp*$^{-/-}$ PMVEC have Decreased PMN Adhesion under Basal and Septic Conditions

The decreased ICAM1 abundance on the surface of *Timp3*$^{-/-}$ PMVEC may interfere with PMN adhesion as ICAM1 is a critical receptor for CD11b on the leukocyte cell surface [116]. Thus, I next investigated the potential role of TIMP3 in regulating PMN-PMVEC adhesion. For these studies, WT and *Timp3*$^{-/-}$ PMVEC as well as WT PMN were stimulated separately with cytomix for 4h before being combined for 30 min to measure static adhesion. Under basal conditions, PMN adhesion to WT PMVEC was approximately 15% of PMN whereas adhesion to *Timp3*$^{-/-}$ PMVEC was approximately 10% (**Figure 3-21**). Cytomix stimulation significantly increased PMN adhesion to both WT and *Timp3*$^{-/-}$ PMVEC (31% and 24%, respectively (**Figure 3-21**). However, under both basal and septic conditions, significantly fewer PMN adhered to *Timp3*$^{-/-}$ vs. WT PMVEC (**Figure 3-21**).

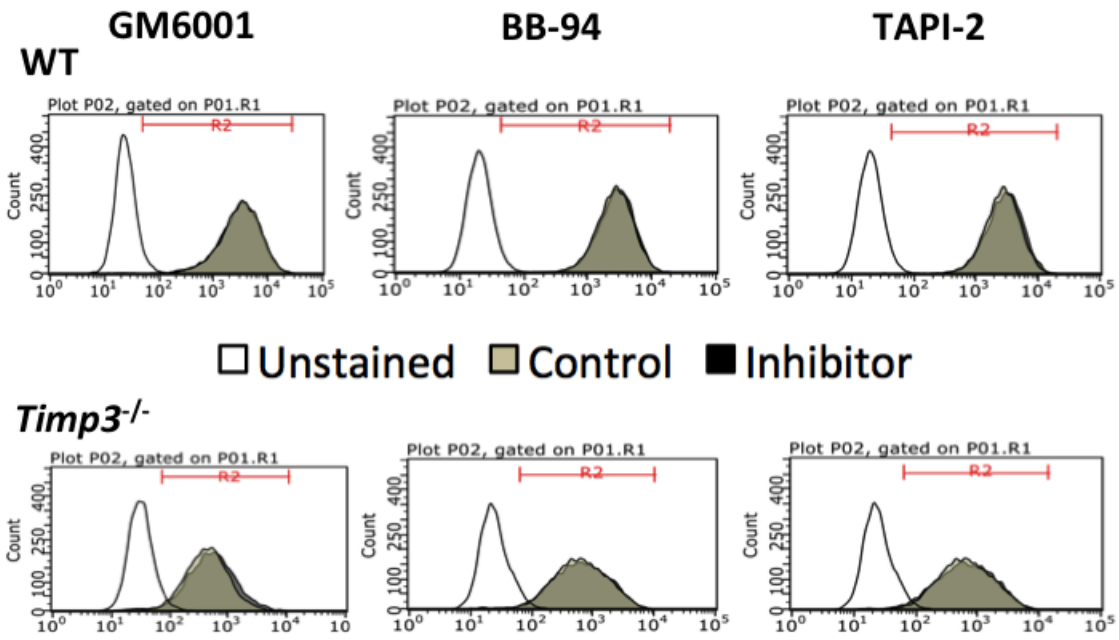

Figure 3-19: Global metalloproteinase inhibitors have no effect on PMVEC intercellular adhesion molecule (ICAM) 1 cell surface abundance *in vitro*.

Representative flow cytometry histograms from WT and *Timp3*$^{-/-}$ PMVEC demonstrated that compared to vehicle control (DMSO and dH_2O; tan histogram), treatment with GM6001, BB-94, and TAPI-2 (black histogram) had no effect on ICAM1 cell surface abundance on WT and *Timp3*$^{-/-}$ PMVEC under basal conditions (n=3).

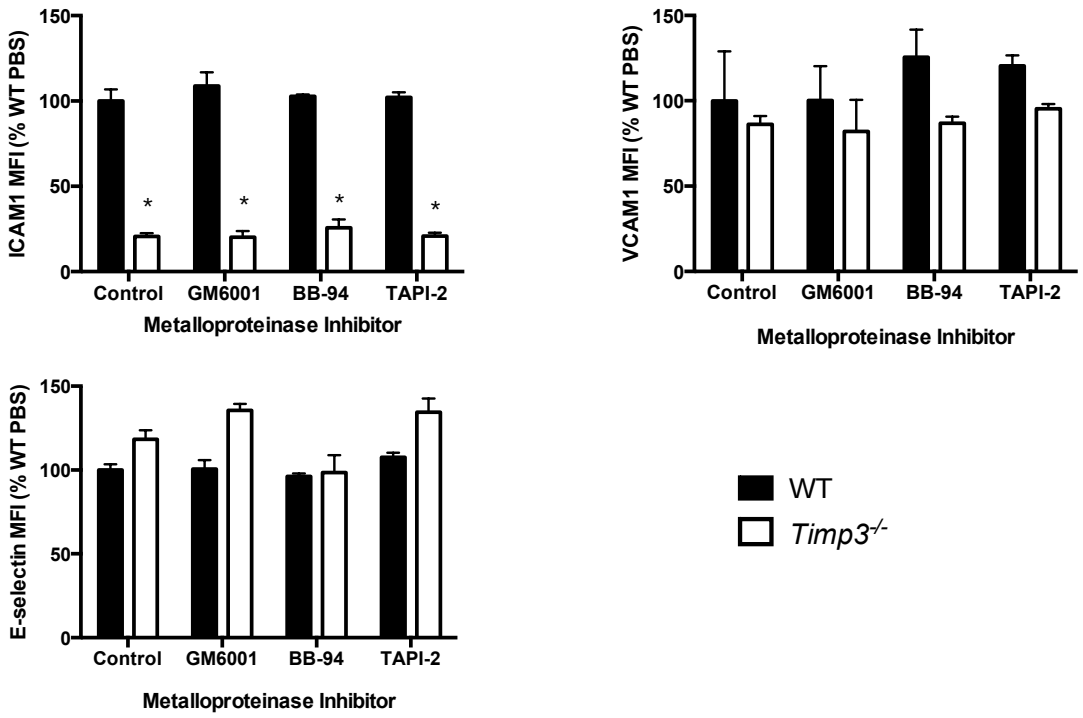

Figure 3-20: Global metalloproteinase inhibitors have no effect on the cell surface abundance of cell adhesion molecules on PMVEC *in vitro*. Analysis of MFI revealed that compared to vehicle control (DMSO and dH_2O), GM6001, BB-94, and TAPI-2 had no effect on ICAM1, VCAM1, or E-selectin cell surface abundance on WT and *Timp3*$^{-/-}$ PMVEC under basal conditions. Further, *Timp3*$^{-/-}$ PMVEC had significantly decreased cell surface ICAM1 abundance vs. WT PMVEC under all conditions. * $p<0.05$ vs. WT, Two-Way ANOVA followed by a Bonferroni post-hoc test, n=3.

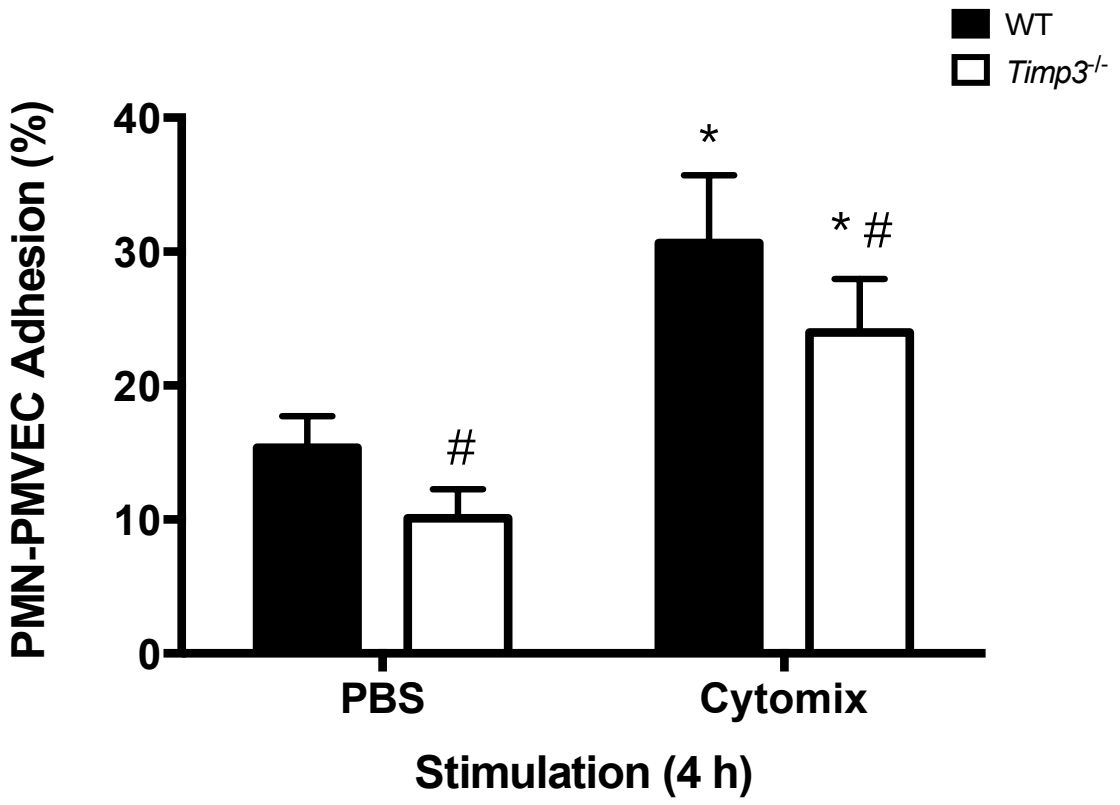

Figure 3-21: *Timp3*$^{-/-}$ PMVEC have reduced polymorphonuclear leukocyte (PMN) adhesion. Compared to PBS, WT and *Timp3*$^{-/-}$ PMVEC have significantly increased PMN adhesion; however, *Timp3*$^{-/-}$ PMVEC have significantly reduced PMN adhesion under both basal and under septic (cytomix, 30 ng/mL) conditions compared to WT PMVEC. * $p<0.05$ vs. PBS and # $p<0.05$ vs. WT, Two-Way ANOVA followed by a Bonferroni post-hoc test, n=7.

Chapter 4

4 Discussion

4.1 Summary of Findings

Sepsis is a common and serious condition characterized by life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Excessive inflammation causes pathophysiological changes, including increased microvascular permeability, leading to organ damage and dysfunction distant to the site of infection. In particular, the accumulation of alveolar protein-rich edema observed in sepsis-induced ARDS is thought to be caused by injury and dysfunction of PMVEC and the alveolar epithelium due to excessive inflammation, characterized by a loss of structural integrity and resulting barrier dysfunction [11,12]. PMVEC are critical in maintaining homeostatic microvascular function by monitoring vascular tone, and controlling both the migration of circulating cells and the movement of macromolecules across the intact microvascular barrier into the lung [14,20–23]. Many mechanisms of septic PMVEC injury and dysfunction have been described. The endogenous mechanisms protecting against sepsis-induced PMVEC dysfunction, however, are not well defined.

Thus, I sought to investigate the metalloproteinase-dependent role of PMVEC-derived TIMPs in the regulation of normal and septic murine microvascular barrier function. My data suggest that the balance between TIMPs and metalloproteinases is disrupted under septic conditions, leading to increased metalloproteinase activity and PMVEC barrier dysfunction. Furthermore, disruption of this balance (loss of TIMP3) is associated with impaired PMN recruitment. Therefore, this study demonstrates that

TIMPs may mediate a novel, endogenous mechanism that promotes PMVEC barrier function under healthy conditions, and that this homeostatic mechanism may be disrupted under septic conditions.

4.2 Contributions of Research to Current State of Knowledge

4.2.1 TIMP/Metalloproteinase Levels in Septic PMVEC

Previous studies have demonstrated that stimulation of murine WT PMVEC monolayers *in vitro* with cytomix at a concentration of 30 ng/mL led to significant barrier dysfunction, as measured by decreased TEER and enhanced trans-PMVEC EB-labelled albumin and FITC-labelled dextran flux, by 4h post-stimulation [83]. Thus, *Timp* and metalloproteinase mRNA expression levels were examined in WT and *Timp3*$^{-/-}$ PMVEC, over the same timeline in order to assess the role of TIMPs in sepsis-induced PMVEC dysfunction. The assessment of *Timp* mRNA expression following cytomix, LPS (10 μg/mL), or a combination of both revealed septic WT PMVEC express significantly higher *Timp1* mRNA levels than PBS-treated WT PMVEC. Significant alterations in *Timp3* mRNA expression levels, which depended on the septic stimuli, were also observed compared to PBS-treated WT PMVEC. Further, *Timp2* mRNA expression did not change under various septic conditions, and *Timp4* mRNA expression was not detected in WT PMVEC.

Overall, my data demonstrates the importance of TIMPs in pulmonary microvasculature, with TIMP1, -2, and -3 all being expressed under basal conditions. TIMP expression varies in the different vascular beds throughout the body, including both micro- and macrovascular beds; however, all TIMP family members are expressed

within at least one vascular bed supporting the broad importance of TIMPs in EC function. For example, TIMP1, -3, and -4 are expressed by murine brain MVEC, and TIMP1, -2, and -3 are expressed by human EC isolated from the aorta, iliac artery, and coronary artery [80–82]. Additionally, TIMP3 is expressed by murine PMVEC; however, TIMP2 and -4 do not appear to be expressed by human PMVEC [83,84]. Interestingly, while my data supports the observed lack of TIMP4 expression by PMVEC, it clearly demonstrates constitutive *Timp2* mRNA expression by murine PMVEC. One potential reason for these disparate findings may be due to species differences as well as differences in mRNA vs. protein expression as Shen and colleagues examined TIMP2 protein expression in human PMVEC [84].

TIMP expression appears to be differentially regulated under conditions of infection and organ injury [53,67,80,81,83,88,90,103]. My observed increase in *Timp1* mRNA expression in septic WT PMVEC is consistent with previous studies in other vascular beds. Specifically, treatment of murine brain MVEC with IL1β and TNFα, or with homocysteine, have been found to increase *Timp1* mRNA expression [80,81]. Moreover, TIMP1 levels in serum have been found to be increased in severe sepsis in humans [92,93]. Thus, these previous studies combined with the strong increase in *Timp1* expression seen in my dual stimulation *in vitro*, as well as CLP-sepsis *in vivo,* strongly suggest a potential role for PMVEC-derived TIMP1 in tissue injury and sepsis.

The decrease in *Timp3* mRNA expression in LPS-stimulated WT PMVEC is consistent with previous studies, in which TIMP3 expression (mRNA and protein) was significantly decreased in murine brain MVEC and PMVEC following treatment with IFNγ, IL1β, and TNFα [80,83]. Conversely, my finding that *Timp3* mRNA expression

increased in PMVEC stimulated with both cytomix and LPS, similar to the observed increase in expression in CLP-septic PMVEC, suggests that more intense septic stimulation is required to induce *Timp3* expression. IL1β and TNFα act synergistically, and the combination of both have led to significantly greater inflammation in human gingival fibroblasts compared to LPS stimulation or either cytokine alone [117]. In human sepsis, as well as murine CLP-sepsis *in vivo*, both cytokines and LPS are present, collectively contributing to excessive inflammation and tissue/organ injury [118,119]. Therefore, an *in vitro* model using both cytokines and LPS may be more reflective of the *in vivo* conditions during sepsis.

The combination of LPS and IFNγ, IL1β, and TNFα was also found previously to maximally upregulate nitric oxide (NO) synthase (NOS) expression in hepatocytes [120]. In addition, the combination of LPS and cytokines (TNFα, IL1β and IL6) incubated together induced a greater decrease in sarcomere shortening in isolated cardiomyocytes compared to stimulation with LPS or individual cytokines alone demonstrating a compounding effect [121]. Collectively, my data shows a greater increase in *Timp1* mRNA expression in PMVEC stimulated with a combination of cytokines and LPS than PMVEC stimulated with either individually. *Timp3* mRNA expression is also induced at higher levels in PMVEC stimulated with both cytomix and LPS, which is additionally seen in CLP-sepsis *in vivo*. Overall, combined septic stimulation *in vitro* more closely reflected *Timp1* and *-3* mRNA expression in PMVEC from *in vivo* CLP-septic mice, suggesting a more effective model than cytomix or LPS individually.

My examination of metalloproteinase mRNA expression in each septic model revealed septic WT PMVEC express significantly greater *Mmp9* and *Adam17* mRNA

levels, whereas *Mmp2* and *Adam10* mRNA expression levels remain stable. Generally, my data are supported by previous studies. For example, stimulation of brain EC with TNFα and IL1β selectively upregulates MMP9 production, but does not alter MMP2 expression [54]. Further, MMP9 expression is also increased in HUVEC following bacterial infection and in brain MVEC following viral infection, as well as in patients with septic shock [43,57,58]. Additionally, ADAM17 expression has been found to be upregulated in murine brain EC following stimulation with multiple pro-inflammatory cytokines (i.e. TNFα, IL1β, IFNγ), and in human PMVEC following stimulation with LPS [55,56]. Thus, MMP9 and ADAM17 expression by PMVEC appears to be highly responsive to inflammatory stimuli suggesting a critical role for these metalloproteinases in septic PMVEC dysfunction.

4.2.2 Metalloproteinase-Dependent Loss of Endothelial Barrier Function

My lab has previously reported that PMVEC barrier function is impaired both *in vitro* and *in vivo* under septic conditions [8,108,110,114,122,123]. PMVEC activation by inflammatory cytokines leads to increased paracellular permeability, predominantly through the disruption of inter-PMVEC junctions and actin cytoskeleton-driven PMVEC retraction [14,16,20,21,23,31,104,124–127]. There are many mechanisms involved in PMVEC barrier dysfunction following sepsis, including VE-cadherin internalization and cleavage, as well as actin cytoskeleton polymerization and reorganization [14,20,21,23,38,104,128–131]; however, endogenous mechanisms protecting against sepsis-induced PMVEC barrier dysfunction are not well understood.

Metalloproteinases, which have the ability to cleave multiple extracellular proteins associated with inflammation, as well as proteins involved in cell-cell and cell-ECM interactions, have been found to be expressed by EC and this expression is altered under the setting of infection and inflammation, which is supported by my data. However, metalloproteinases are regulated at multiple levels, including secretion as zymogens and subsequent extracellular activation following secretion [44,47,95]. Thus, changes in expression may not be consistently reflected in changes in metalloproteinase activity. *Mmp9*, a soluble metalloproteinase, was consistently upregulated with regards to mRNA expression in every septic model, yet total soluble metalloproteinase activity in the PMVEC-conditioned media was not altered under septic conditions *in vitro*. In addition, the PMVEC lysate, which includes both membrane bound and intracellular soluble metalloproteinases, did not exhibit enhanced metalloproteinase activity under septic conditions, even though *Mmp9* and *Adam17* mRNA expression were both upregulated. This disconnect between metalloproteinase expression and activity has been recognized, and suggests metalloproteinase activity may be significantly regulated post-translationally as metalloproteinases are predominantly secreted as inactive zymogens [47].

My examination of metalloproteinase activity under septic conditions found that while global metalloproteinase activity associated with the conditioned media or with the cell lysate remained unchanged, combined stimulation of WT PMVEC led to significantly increased ADAM17 activity. Importantly, this data demonstrates that the observed changes on ADAM17 mRNA expression are reflective of functional changes in ADAM17 levels (i.e. increased activity). It is interesting, however, that despite increased

ADAM17 activity the total metalloproteinase activity was not significantly increased, suggesting that the activity of other specific metalloproteinases may be decreased under septic conditions. Further, because the cell lysate was used, it is important to note that ADAM17 activity included both the membrane bound protein as well as intracellular fraction, although it is unclear which was more active. Biologically, ADAM17 shedding events can increase in occurrence without increasing the quantity of membrane bound protein via an increase in cellular ADAM17 activity [132].

Although it is unclear why total metalloproteinase activity does not change under septic conditions, my studies clearly demonstrate that inhibition of metalloproteinases with synthetic metalloproteinase inhibitors in WT PMVEC under septic conditions attenuates septic microvascular barrier dysfunction. BB-94, a potent broad-spectrum inhibitor for MMP1, -2, -3, -7, -9, -14 and ADAM17, reduced albumin and dextran flux by 50% in cytomix-stimulated WT PMVEC [133]. This is consistent with the literature as previous studies found that BB-94 significantly reduced alveolar-capillary leakage in rats in pancreatitis-associated lung injury, as well as BBB leakage in rats following either acute pancreatitis or methamphetamine treatment [134–136]. My finding that TAPI-2, a potent hydroxamate-based inhibitor of MMPs and ADAM17, reduces albumin and dextran flux by 40% in cytomix-stimulated WT PMVEC is also supported by previous studies [137]. Administration of TAPI-2 was found to significantly attenuate bronchoalveolar fluid protein and TNFα levels in mice following hypercapnia acidosis [138,139].

Interestingly, I also found that GM6001, another broad-spectrum metalloproteinase inhibitor, had no effect on microvascular leak in cytomix-stimulated

WT PMVEC. This is in contrast to previous studies, which have demonstrated the ability of GM6001 to inhibit vascular permeability. Specifically, treatment of HUVEC with GM6001 following recombinant TNFα-induced upregulation of metalloproteinase activity rescued the increase in permeability as measured by macromolecular flux [41,42]. Similarly, treatment of murine cerebrovascular EC with GM6001 following both homocysteine injection *in vitro* and acute liver failure with azoxymethane *in vivo* resulted in decreased microvascular leak due to the reversal of degradation of tight junction adhesive protein and F-actin formation through the inhibition of MMP9 [73,74]. Finally, previous work from my lab demonstrated that the enhanced basal leak across *Timp3*$^{-/-}$ PMVEC was attenuated by treatment with GM6001. The reason for the apparent difference between my current study and previous work on the effects of GM6001 on EC barrier dysfunction is unclear. However, one potential mechanism could be due to the metalloproteinases expressed and active in the different vascular beds or in different models of injury/inflammation. EC from the micro- and macrovasculature have different biological properties. For example, EB-albumin leak was significantly higher across HUVEC than human PMVEC under basal and septic (LPS or cytomix) conditions [108]. HUVEC treated with GM6001 had reduced dextran permeability compared to untreated HUVEC [42]. It is unclear which metalloproteinases are responsible for this permeability; however, my data suggests they are not inhibited well by GM6001. This current data suggest that ADAM17 may be a critical mediator of barrier dysfunction in the lung microvasculature during sepsis. This supports a previous study, which demonstrated that ADAM17 is involved in the regulation of vascular permeability during LPS-induced acute lung injury in murine MVEC *in vivo*, and its inhibition with GW280264X, an

ADAM17-specific metalloproteinase inhibitor, or its absence in *Adam17*$^{-/-}$ mice, resulted in significantly reduced edema formation and vascular permeability [56].

One mechanism through which metalloproteinases can mediate loss of PMVEC barrier function is through cleaving proteins associated with endothelial adherens and tight junctions, such as VE-cadherin, occludin, ZO1, and JAMs as well as other crucial adhesive junctional proteins [41,42,56,61–64]. MMP7, ADAM10, and -12 actively cleave VE-cadherin [42,62], while MMP2 and -9 are capable of cleaving occludin and ZO1 resulting in microvascular dysfunction via increased paracellular permeability [61,63]. MMPs also target the components of the surrounding ECM, including fibronectin, laminin, and type IV and V collagens, leading to disrupted endothelial–ECM interactions and increased vascular permeability [65–70]. Thus, these studies suggest increased metalloproteinase activity drives MVEC dysfunction through the proteolytic processing of integral MVEC structural determinants (i.e. the ECM), resulting in MVEC barrier dysfunction and enhanced microvascular leak.

In addition to changes in metalloproteinase expression, metalloproteinase activity is also regulated by TIMPs. Surprisingly, my data demonstrated that while the overall expression of *Timps* was increased in PMVEC under septic conditions, sepsis was also associated with an increase in specific metalloproteinase activity. The four TIMPs have overlapping abilities to inhibit metalloproteinases, but also exhibit varying degrees of specificity. TIMP1 is secreted as a soluble inhibitor, and predominantly targets MMPs, which are mostly soluble [50,95]. My data showed that metalloproteinase activity in the conditioned media may be decreasing, which could be indicative of TIMP1 expression as TIMP1 is generally found in body fluids (i.e. serum), especially under septic conditions

[93,140,141]. Further, TIMP3 is known to inhibit ADAM17, which actively processes and cleaves membrane bound pro-TNFα to yield the soluble inflammatory cytokine TNFα [142,143]. While the increased ADAM17 activity in the presence of increased *Timp3* expression is surprising, it is possible that TIMP3 upregulation in septic PMVEC *in vitro* and *in vivo* is a compensatory mechanism to limit or restrict ADAM17 activity and thereby promote maintenance of stable PMVEC barrier function.

In addition, the observed differences in *Timp3* mRNA expression may reflect different functions for TIMP3 within the septic microenvironment and during the time course of sepsis. For example, under basal conditions, TIMP3 appears to have a critical role in promoting PMVEC barrier function, at least in part through regulation of VE-cadherin localization [83]. TIMP3, however, may also have a role in mediating the initial inflammatory response to an infection. Specifically, TIMP3 has been found to inhibit shedding of VCAM1 from the surface of aortic EC following treatment with IL1β, TNFα, or the phorbol ester PMA [106]. Aortic EC from *Timp3*$^{-/-}$ mice had increased VCAM1 shedding compared to aortic EC from WT mice, and this inhibition of VCAM1 shedding by TIMP3 was likely through inhibition of ADAM17, as the TIMP3 dependent effect was lost under conditions of ADAM17 knockdown [106]. Thus, TIMP3 appears to be a critical regulator of MVEC dysfunction following tissue injury/infection, including protection against loss of MVEC barrier function and regulation of PMN-PMVEC interactions.

4.2.3 TIMP3 Regulation of PMN-PMVEC Interaction

My studies demonstrate that endothelial cell adhesion molecules, such as ICAM1, VCAM1 and E-selectin, are significantly upregulated in septic WT and *Timp3*$^{-/-}$ PMVEC.

This supports previous studies that found that both ICAM1 and VCAM1 expression on the endothelial cell surface was induced by the release of pro-inflammatory cytokines from macrophages at the site of infection, including TNFα and IL1β [144–147]. Additionally, LPS was found to stimulate translocation of the transcription factor nuclear factor κB (NFκB) to the nucleus to initiate transcription of pro-inflammatory cytokines and increase endothelial expression of ICAM1, both of which are associated with increased endothelial permeability [13,16].

Interestingly, my data shows that *Timp3*$^{-/-}$ PMVEC express significantly less ICAM1 on their cell surface under both basal and septic conditions compared to WT PMVEC, and this was associated with significantly weaker adhesion to PMN, again, under both basal and septic conditions. Previously, ICAM1 deficient mice were found to exhibit impaired peritoneal neutrophil migration in response to chemical peritonitis [148]. Additionally, mice with a deficiency in both ICAM1 and P-selectin showed a significant reduction in neutrophil migration in response to myocardial ischemia and reperfusion [149]. Other studies, however, suggest that ICAM1 is not necessary for PMN adhesion to EC and extravasation into tissues following infection. Specifically, neutrophil migration into the alveolar spaces during acute *Streptococcus pneumoniae*-induced pneumonia was not significantly altered in P-selectin/ICAM1 double deficient mice vs. WT mice [150]. Further, lung ICAM1 was required for spontaneous intravascular effector lymphocyte entrapment, but not for neutrophil entrapment or emigration following instillation of endotoxin [151]. Collectively, my data complements these previous studies and provides further evidence to support a role for ICAM1 in regulation of PMN-PMVEC interaction.

My studies also provide insight into a potential mechanism regulating PMVEC surface ICAM1 expression as the amount of ICAM was significantly less on *Timp3*$^{-/-}$ PMVEC compared to septic WT PMVEC. ICAM1 is constitutively expressed on the cell surface of many cell types, including EC, and it is upregulated primarily through increased transcription [152]. A number of inflammatory mediators including proinflammatory cytokines (IFNγ, IL1β, and TNFα), retinoic acid, ROS and virus infection activate ICAM1 transcription under inflammatory and injurious conditions [152]. My data demonstrated significant PMVEC upregulation of ICAM1 mRNA and protein under septic conditions *in vitro*, which supports these previous studies. However, this data also suggests that *Icam1* mRNA expression may not always correlate with cell surface ICAM1 levels. Specifically, *Timp3*$^{-/-}$ PMVEC stimulated with cytomix had 1.4-fold more expression than WT PMVEC; LPS had 1.3-fold less expression than WT PMVEC; cytomix and LPS had 4-fold more expression than WT PMVEC. However, cell surface ICAM1 levels were similar in PMVEC under each condition, with a significant decrease observed in *Timp3*$^{-/-}$ vs. WT PMVEC. In addition, *Icam1* mRNA expression was upregulated the most in PMVEC stimulated with both cytomix and LPS, and the least in PMVEC stimulated with LPS. However, cell surface quantity of ICAM1 was upregulated approximately the same under each septic condition.

One mechanism regulating cell surface ICAM1 may be proteolytic cleavage [153,154]. For example, MMPs have previously been found to shed ICAM1 from the cell surface under inflammatory conditions [153]. Additionally, human leukocyte elastase, which has been found to cause tissue destruction in a number of inflammatory diseases, also sheds ICAM1 from the cell surface [154]. However, treatment with the broad-

spectrum metalloproteinase inhibitors GM6001, BB-94, and TAPI-2 had no effect on *Timp3*$^{-/-}$ cell surface ICAM1, which suggests that the decreased ICAM1 on *Timp3*$^{-/-}$ PMVEC was not due to increased metalloproteinase activity. TIMPs have also been found to have metalloproteinase-independent functions. For example, TIMP3 inhibits cell proliferation, binding directly to VEGFR2 on EC and blocking VEGF-VEGFR2 interaction, thereby inhibiting phosphorylation of VEGFR2 and downstream signalling leading to decreased EC proliferation [95]. Further, VEGFR2 activation in response to VEGF was also reduced by overexpression of a mutant TIMP3 that lacked the ability to inhibit MMPs suggesting that TIMP3 inhibition of VEGF signaling is a metalloproteinase-independent function [95]. Similarly, TIMP3 also binds the ANG II type 2 receptor, and the presence of both inhibits HUVEC proliferation and angiogenesis through inhibition of PKC and endothelial NOS (eNOS) [155].

Although there are no known mechanisms to explain the reduced ICAM1 on the cell surface of *Timp3*$^{-/-}$ PMVEC, one proposed mechanism could involve ADAM17. Previous studies have targeted ADAM17 to shed VCAM1 and ICAM1 from the cell surface of EC. PMA stimulation of heart and aortic EC induced VCAM1 shedding via ADAM17 [78]. Further investigation determined that PMA stimulated aortic EC from *Timp3*$^{-/-}$ mice had increased VCAM1 shedding compared to stimulated WT mice, suggesting TIMP3-dependent inhibition of ADAM17 [106]. However, my data does not support this due to the lack of observed differences in ADAM17 activity between WT and *Timp3*$^{-/-}$ PMVEC lysate, as well as the lack of observed differences in cell surface ICAM1 on *Timp3*$^{-/-}$ PMVEC treated with GM6001, BB-94, or TAPI-2, suggesting a metalloproteinase-independent mechanism.

4.2.4 Role of TIMP3 in TIMP/Metalloproteinase Balance

In general, the primary function of TIMPs is thought to be inhibition of metalloproteinase activity [47,95,96]. Further, shifting the balance between TIMPs and metalloproteinases in favour of TIMPs is thought to regulate and reduce inflammation and injury, while a shift in favour of metalloproteinases is thought to cause organ damage and dysfunction [46,90,93,98,100,101]. For example, *Timp3*$^{-/-}$ mice have been found to have decreased bronchiole branching and enhanced activity of MMPs throughout lung development, leading to erratic focal ECM proteolysis [100,101]. In addition, lung inflammation from bleomycin-induced injury persisted nearly four-times as long in *Timp3*$^{-/-}$ mice, and was associated with upregulated MMP activity and greater neutrophil chemotactic activity compared to WT mice [98]. In contrast, mice with a specific MMP deficiency tend to have reduced morbidity and mortality in models of infection and injury. *Mmp9*$^{-/-}$ mice were resistant to endotoxin-induced shock following endotoxemia [156]. Bacterial endotoxin-induced systemic inflammation was considerably repressed in mice lacking MMP3 [157]. MMP8 and -13 deficient mice were highly resistant to lethal effect of LPS and CLP, with the latter being associated to reduced TNF release [43].

Interestingly, metalloproteinase activity data in *Timp3*$^{-/-}$ PMVEC in the current report shows a shift in favour of TIMPs. Soluble metalloproteinase activity decreases in *Timp3*$^{-/-}$ PMVEC basally, suggesting that TIMP1, a strong inhibitor of soluble MMPs, is basally expressed at a sufficient quantity to inhibit these MMPs. However, I only assessed the expression of a handful of metalloproteinases, and it is possible that there could be differences in expression of other metalloproteinases (i.e. decreased expression), which would account for the observed decrease in activity under basal conditions. Total

metalloproteinase activity in *Timp3*$^{-/-}$ PMVEC lysate did not differ vs. WT lysate, and one possibility is that other TIMPs may compensate for the lack of metalloproteinase inhibition by TIMP3 in the *Timp3*$^{-/-}$ PMVEC. My studies, however, found no evidence of compensation in *Timp1* and *-2* mRNA expression in *Timp3*$^{-/-}$ PMVEC basally, which suggests that compensation is not involved.

Further, there is the potential for redundancy in the inhibition profiles between TIMPs. However, while *in vitro* studies have demonstrated overlapping metalloproteinase inhibition profiles (i.e. both TIMP1 and TIMP3 have been implicated in the regulation of MMP7 activity and thus, in the absence of TIMP3, TIMP1 may be able to inhibit MMP7 activity even without evidence of increased expression), there are differences that exist between TIMPs in their ability to inhibit specific metalloproteinases, especially ADAMs [79,95,97]. For example, TIMP1 does not appear to be an effective inhibitor of the membrane type MMPs (MT-MMPs; MMP14, -15, -16, -17, -24, and -26) [50]. Additionally, TIMP3 is unique from other TIMPs as it is a very strong inhibitor of ADAMs and, currently, is the only known inhibitor of ADAMTSs [50]. As such, it is surprising that ADAM17 activity was not significantly increased in *Timp3*$^{-/-}$ PMVEC basally. Further, this lack of change in ADAM17 activity in *Timp3*$^{-/-}$ PMVEC makes it unclear whether ADAM17 is responsible for the previously reported increase in basal microvascular albumin flux in *Timp3*$^{-/-}$ PMVEC as a decrease in ADAM17, as well as ADAM10, have been previously demonstrated to reduce vascular permeability [42,56,83]. However, one caveat is that my analysis of ADAM17 expression was in total cell lysate and not solely cell membrane associated. As such, it is unclear if

ADAM17 activity was seen intracellularly in WT PMVEC from ADAM17 being processed or transported to the cell membrane.

4.3 Limitations and Future Directions

4.3.1 Study Limitations

Many of my experiments were done entirely *in vitro*, and thus, under static conditions. However, in an *in vivo* setting, several external forces, such as hemodynamic stimuli including shear stress and changes in vessel size can affect PMVEC physiology and responses to inflammatory stimuli (i.e. sepsis). Modulation of blood flow is achieved by the release of vasoconstrictors (i.e. angiotensin-converting enzyme, endothelin) and vasodilators (i.e. NO, prostacyclin) in order to regulate vascular resistance and systemic blood pressure [15,17]. 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 [21]. Due to technological limitations, I was not able to replicate a flow model that PMVEC would encounter in their native conditions. However, my use of multiple *in vitro* models of septic stimulation created various environments for the endothelium to respond to, one of which appears to be a strong representation of a septic environment *in vivo* based on my *ex vivo* expression analysis. Overall, I believe that the use of multiple models strengthened my *in vitro* analysis, and was also supported by direct *ex vivo* study of PMVEC isolated from septic mice.

Although TNFα, IL1β, and IFNγ are seen in human sepsis, they are considered to be pro-inflammatory cytokines, and not sepsis-specific. Previous studies have identified significant increases in several other cytokines, including monocyte chemotactic protein

1, IL6 and -8, granulocyte colony-stimulating factor, hepatocyte growth factor, and macrophage inflammatory protein 1β, in plasma from patients with severe sepsis [158]. Using a mixture of these sepsis-specific cytokines, in conjunction with the current cytomix, may create a more accurate model of sepsis *in vitro*.

Additionally, PMVEC were cultured on 1% gelatin coated transwell inserts and wells in order to mimic an extracellular matrix for the cells to adhere to. The ECM, however, is a complex network, composed of many different components *in vivo* including collagens, gelatin, fibronectin, laminin, and elastin. These many components mediate PMVEC-ECM interaction and downstream signalling through integrins, releasing cryptic ECM fragments, which activate cell signalling and releasing sequestered growth factors [49,101]. In order to mimic physiological conditions present *in vivo,* a variety of ECM components, including collagens, elastin, proteoglycans, and fibronectin, can be used in cell culture. Furthermore, the glycocalyx is known to be an important regulator of endothelial function, forming a significantly thicker pulmonary endothelial surface layer compared to other regions in the systemic circulation [25]. This endothelial surface layer is critical to inflammation, barrier function and mechanotransduction. Specifically, it regulates the exposure of EC surface adhesion molecules, acting as a barrier to neutrophil adhesion and extravasation [25]. Metalloproteinases have been shown to have the ability to cleave the glycocalyx, which is predominantly cleaved by heparanase; however, the glycocalyx is only observed *in vivo* and appears to be absent *in vitro* [25,159].

Further, while my studies assessed the PMVEC expression of each TIMP under basal and septic conditions, it lacked a comprehensive analysis of metalloproteinase

expression. Specifically, my study focused on three MMPs and two ADAMs; however, metalloproteinases are an incredibly diverse group of enzymes. Finally, while I assessed metalloproteinase and *Timp* mRNA expression, I did not examine metalloproteinase and TIMP protein levels specifically due to a lack of available suitable antibodies. This analysis of metalloproteinase activity was used as a surrogate for examining metalloproteinase and TIMP protein levels as the balance between these components is critical to metalloproteinase activity; however, further analysis of the activity of specific metalloproteinases and the use of specific inhibitors is required.

4.3.2 Future Directions

In future studies, barrier dysfunction in septic WT PMVEC must be further characterized *in vitro* to define the TIMP-dependent mechanisms promoting normal PMVEC barrier function and protecting against septic PMVEC barrier dysfunction. Thus, comprehensive expression and protein analysis of other members of the MMP and ADAM families must be investigated combined with the use of specific activity assays and inhibitors. Further, the use of PMVEC with genetically modified expression of metalloproteinases (i.e. PMVEC isolated from mice genetically deficient, use of siRNA) and more specific MMP/ADAM inhibitors will be required to comprehensively assess their functional role. It will also be critical to utilize mice lacking specific metalloproteinases and TIMPs to confirm my *in vitro* studies using *in vivo* models of sepsis.

The reduced quantity of cell surface ICAM1 in *Timp3*$^{-/-}$ PMVEC should also be further addressed by determining total (cytosolic combined with cell surface) ICAM1 protein expression. If total ICAM1 protein is significantly reduced compared to WT

PMVEC, it suggests there is a disruption in the translational process, whereas if total ICAM1 protein in *Timp3*$^{-/-}$ PMVEC is similar to WT PMVEC, it suggests that the protein is effectively being synthesized, but that localization to the cell surface under basal conditions is impaired. Interestingly, while the mechanisms mediating the decreased ICAM1 on the cell surface of *Timp3*$^{-/-}$ PMVEC is unknown, it is important to note that the increase in ICAM1 on the cell surface of *Timp3*$^{-/-}$ PMVEC under septic conditions does suggest that *Timp3*$^{-/-}$ PMVEC have the ability to produce and localize ICAM1 protein to the cell surface.

In addition, while these studies utilized synthetic global metalloproteinase inhibitors, the use of rTIMP3 is required to confirm the specific role of TIMP3 in both microvascular leak as well as PMN-PMVEC interaction. This is important as injection of rTIMP3 has been reported to decrease vascular leak across the blood-brain barrier in a model of traumatic brain injury [80].

This investigation also focused solely on the metalloproteinase-dependent role of TIMPs in PMVEC barrier dysfunction. There is, however, evidence for several metalloproteinase-independent mechanisms. TIMPs have been found to interact directly with cell surface receptors to activate the receptor and initiate cell signalling (i.e., TIMP1 binds CD63 and β1 integrin to promote epithelial cell proliferation and inhibit apoptosis) [160]. Further, TIMP3 inhibits angiogenesis by binding to the VEGF receptor and blocking VEGF-VEGFR2 interactions [88,90]. Similarly, TIMP3 also binds the ANG II type 2 receptor, inhibiting HUVEC proliferation and angiogenesis through inhibition of PKC and eNOS [155]. Thus, for example, future characterization of the VEGF signalling pathway in WT and *Timp3*$^{-/-}$ PMVEC under basal and septic conditions would allow for

direct examination of the role of TIMP3-dependent VEGFR2 phosphorylation in basal EC permeability and septic EC barrier dysfunction.

Solely male murine PMVEC were used for the current study; however, there is much evidence of sex specific differences in human sepsis (i.e. septic females are less susceptible to organ dysfunction and death). Men are more likely to have a 372 T/C genetic polymorphism on the TIMP1 gene, which is associated with a higher risk of severe sepsis and mortality [161]. Further, plasma levels of MMP3 were significantly higher in male patients with severe bacterial sepsis compared to female patients [162]. Finally, men with severe bacterial sepsis represented a greater percentage of carriers of MMP3 single nucleotide polymorphism allele 6A [162]. Thus, it would be interesting to examine whether there are differences in the roles of metalloproteinases and TIMPs. Preliminary mRNA expression data suggests that overall basal expression of *Timps*, metalloproteinases, and cell surface proteins are similar in both sexes; however, there are some trends towards sex-linked differences (**Figure 4-1**).

Lastly, all experiments were conducted using murine cells, and as such, human cells must be employed in future studies to investigate this endogenous TIMP-dependent mechanism promoting basal PMVEC barrier function *in vitro*, as well as potential involvement in septic PMVEC barrier dysfunction. These studies would provide an opportunity to assess in human PMVEC the molecular mechanisms regulating normal and septic microvascular permeability that I have identified in murine PMVEC, which will give further insight regarding the physiological relevance of these findings, as well as provide strong clinical relevance.

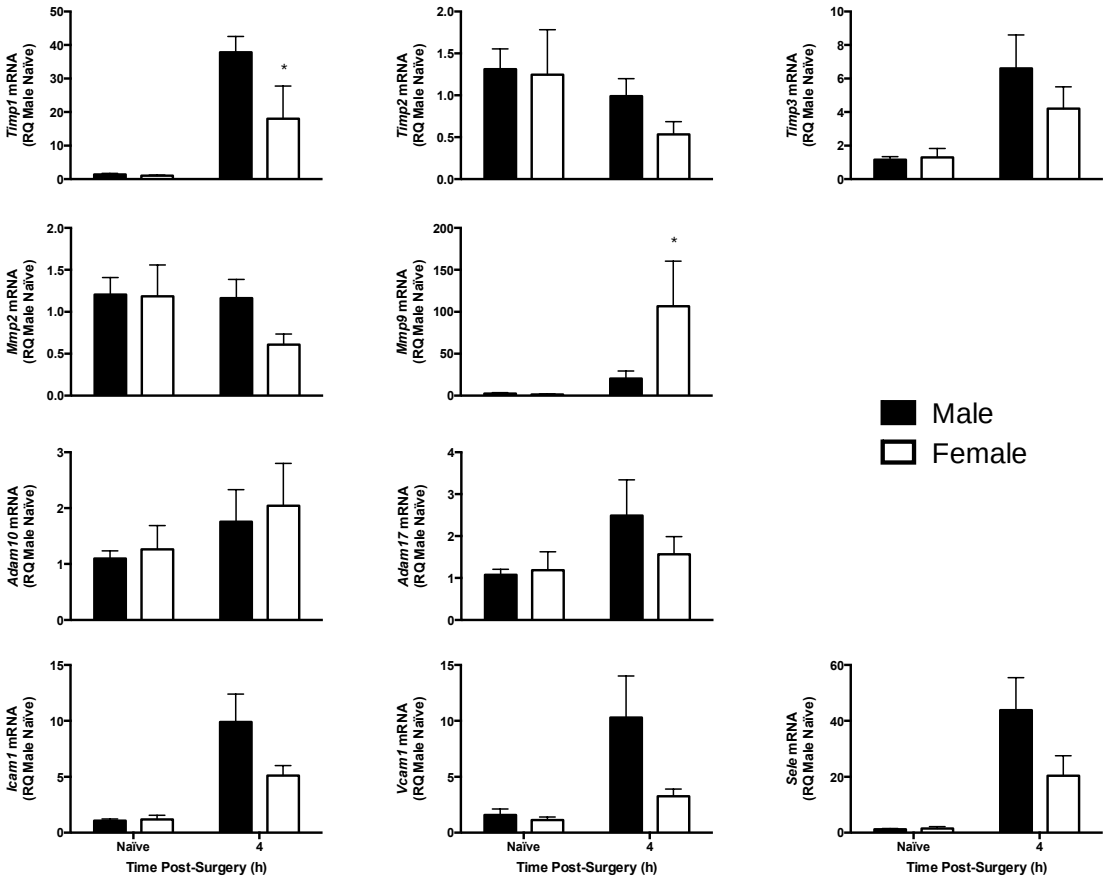

Figure 4-1: Septic changes in TIMP and metalloproteinase expression are different in female vs. male PMVEC. Compared to naive male mice, *Timp* and metalloproteinase mRNA expression is relatively similar in naïve female mice. Compared to 4h CLP male mice, *Timp* and metalloproteinase mRNA expression is significantly altered or appears to be altered in 4h CLP female mice; specifically, *Timp1*, *-2*, *-3*, *Mmp2*, *-9*, *Adam17*, *Icam*, *Vcam*, *Sele*. * $p<0.05$ vs. male, Two-Way ANOVA followed by a Bonferroni post-hoc test, n=4-8.

4.4 Summary and Conclusions

In conclusion, I have identified a disruption in the TIMP/metalloproteinase balance, leading to increases in specific TIMP and metalloproteinase expression and metalloproteinase activity associated with septic PMVEC barrier dysfunction. This septic PMVEC barrier dysfunction is based on a metalloproteinase-dependent mechanism, potentially mediated through ADAM17, resulting in increased microvascular permeability. In addition, *Timp3*$^{-/-}$ PMVEC have reduced ICAM1 on their cell surface, which may be associated with impaired PMN recruitment. Based on my results, I believe PMVEC-derived TIMPs support normal pulmonary microvascular endothelial barrier function via metalloproteinase-dependent mechanisms, through the direct inhibition of metalloproteinase activity, but also through metalloproteinase-independent mechanisms, via regulation of PMN-PMVEC interactions (**Figure 4-2**). A better understanding of the TIMP-mediated endogenous protective mechanism against septic PMVEC barrier dysfunction would support new therapeutic interventions in human sepsis and related septic organ dysfunction, as well as help eliminate PMVEC dysfunction in other vascular diseases.

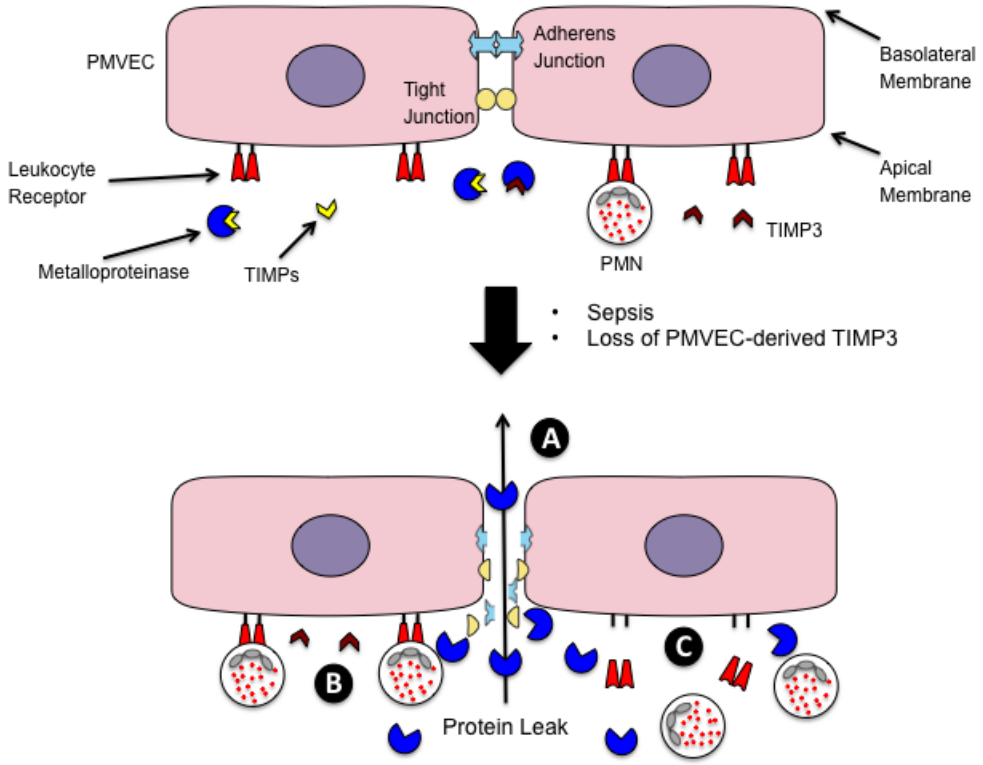

Figure 4-2: Disruption in the balance between metalloproteinases and tissue inhibitors of metalloproteinases leads to PMVEC activation and dysfunction. (A) Septic PMVEC barrier dysfunction is based on a metalloproteinase-dependent mechanism, resulting in increased microvascular permeability. **(B)** PMN-PMVEC adhesion increased under septic conditions due to activation of cell adhesion molecules. **(C)** In the absence of TIMP3 (*Timp3*$^{-/-}$) cell surface ICAM1 is reduced on the cell surface under basal and septic conditions, which may be associated with impaired PMN recruitment; however, it appears to be through a metalloproteinase-independent mechanism.

References

1. Singer M, Deutschman C, Seymour C, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA. 2016;315: 801–810.

2. Ferrer R, Artigas A, Suarez D, Palencia E, Levy MM, Arenzana A, et al. Effectiveness of treatments for severe sepsis. Am J Respir Cell Mol Biol. 2009;180: 861–866.

3. Husak L, Marcuzzi A, Herring J, Wen E, Yin L, Capan DD, et al. National analysis of sepsis hospitalizations and factors contributing to sepsis in-hospital mortality in Canada. Healthc Q. 2010;13: 35–41.

4. Torio C, Moore B. National inpatient hospital costs: the most expensive conditions by payer, 2013. In: Statistical Brief #204 [Internet]. 2016 [cited 25 Jun 2017]. Available: https://www.hcup-us.ahrq.gov/reports/statbriefs/sb204-Most-Expensive-Hospital-Conditions.jsp?utm_source=AHRQ&utm_medium=AHRQSTAT&utm_content=Content&utm_term=HCUP&utm_campaign=AHRQ_SB_204_2016

5. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 2003;348: 138–150.

6. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med. 2001;29: 1303–1310.

7. Glauser MP. Pathophysiologic basis of sepsis: considerations for future strategies of intervenstion. Crit Care Med. 2000;28: S4–S8.

8. Gill SE, Taneja R, Rohan M, Wang L, Mehta S. Pulmonary microvascular albumin leak is associated with endothelial cell death in murine sepsis-induced lung injury in vivo. PLoS One. 2014;9: e88501.

9. Ranieri M, Rubenfeld G, Thompson T, Ferguson N, Caldwell E, Fan E, et al. Acute respiratory distress syndrome: the Berlin definition. JAMA. 2012;307: 2526–2533.

10. Gill SE, Yamashita CM, Veldhuizen RAW. Lung remodeling associated with recovery from acute lung injury. Cell Tissue Res. Cell and Tissue Research; 2017;367: 495–509.

11. Wheeler AP, Bernard GR. Acute lung injury and the acute respiratory distress syndrome : a clinical review. Lancet. 2007;369: 1553–1565.

12. Johnson E, Matthay MA. Acute lung injury: epidemiology, pathogenesis, and treatment. J Aerosol Med Pulm Drug Deliv. 2010;23: 243–252.

13. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. Physiol Rev. 2006;86: 279–367.

14. Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. J Cell Sci. 2008;121: 2115–2122.

15. Sumpio BE, Timothy Riley J, Dardik A. Cells in focus: endothelial cell. Int J Biochem Cell Biol. 2002;34: 1508–1512.

16. Vandenbroucke E, Mehta D, Minshall R, Malik AB. Regulation of endothelial junctional permeability. Ann NY Acad Sci. 2008;1123: 134–145.

17. Ross MH, Pawlina W. Histology: A Text and Atlas : with Correlated Cell and Molecular Biology. Wolters Kluwer/Lippincott Williams & Wilkins Health; 2011.

18. Aird WC. The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood. 2003;101: 3765–3777.

19. Galley HF, Webster NR. Physiology of the endothelium. Br J Anaesth. 2004;93: 105–113.

20. Dejana E, Lampugnani MG, Martinez-Estrada O, Bazzoni G. The molecular

organization of endothelial junctions and their functional role in vascular morphogenesis and permeability. Int J Dev Biol. 2000;44: 743–748.

21. Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Dev Cell. Elsevier Inc.; 2009;16: 209–221.

22. Lee WL, Slutsky AS. Sepsis and endothelial permeability. N Engl J Med. 2010;363: 689–91.

23. Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, et al. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. Proc Natl Acad Sci U S A. 1999;96: 9815–9820.

24. Minshall RD, Sessa WC, Stan R V, Anderson RGW, Malik AB. Caveolin regulation of endothelial function. Am J Physiol Lung Cell Mol Physiol. 2003;285: L1179-83.

25. Yang Y, Schmidt EP. The endothelial glycocalyx. Tissue Barriers. 2013;1: e23494.

26. Dull R, Cluff M, Kingston J, Hill D, Chen H, Heohne S, et al. Lung heparan sulfates modulate Kfc during increased vascular pressure: evidence for glycocalyx-mediated mechanotransduction. Am J Physiol Lung Cell Mol Physiol. 2012;302: L816–L828.

27. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer. 2003;3: 422–433.

28. Bernard G, Vincent J, Laterre P, LaRosa S, Dhainut J, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001;344: 699–709.

29. De Backer D, Creteur J, Preiser JC, Dubois MJ, Vincent JL. Microvascular blood flow is altered in patients with sepsis. Am J Respir Crit Care Med. 2002;166: 98–

104.

30. Mehta S. The effects of nitric oxide in acute lung injury. Vascul Pharmacol. 2005;43: 390–403.

31. Wang L, Taneja R, Razavi HM, Law C, Gillis C, Mehta S. Specific role of neutrophil inducible nitric oxide synthase in murine sepsis-induced lung injury in vivo. Shock. 2012;37: 539–547.

32. Bone R. The sepsis syndrome: definition and general approach to management. Clin Chest Med. 1996;17: 175–181.

33. Skibsted S, Jones A, Puskarich M, Arnold R, Sherwin R, Trzeciak S, et al. Biomarkers of endothelial cell activation in early sepsis. Shock. 2013;39: 427–432.

34. DiStasi MR, Ley K. Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability. Trends Immunol. 2009;30: 547–556.

35. Schouten M, Wiersinga WJ, Levi M, Poll T Van Der. Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol. 2008;83: 536–545.

36. Scholz D, Devaux B, Hirche A, Pötzsch B, Kropp B, Schaper W, et al. Expression of adhesion molecules is specific and time-dependent in cytokine-stimulated endothelial cells in culture. Cell Tissue Res. 1996;284: 415–423.

37. Lipowsky H. Protease activity and the role of the endothelial glycocalyx in inflammation. Drug Discov Today Dis Model. 2011;8: 57–62.

38. Lum H, Malik AB. Regulation of vascular endothelial barrier function. Am Physiol Soc. 1994;267: L223–L241.

39. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci. 1998;111: 1853–1865.

40. Angelini DJ, Hyun S-W, Grigoryev DN, Garg P, Gong P, Singh IS, et al. TNF-α

increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia. Am J Physiol Lung Cell Mol Physiol. 2006;291: L1232–L1245.

41. Sidibé A, Mannic T, Arboleas M, Subileau M, Gulino-Debrac D, Bouillet L, et al. Soluble VE-cadherin in rheumatoid arthritis patients correlates with disease activity: evidence for tumor necrosis factor α-induced VE-cadherin cleavage. Arthritis Rheum. 2012;64: 77–87.

42. Schulz B, Pruessmeyer J, Maretzky T, Ludwig A, Blobel CP, Saftig P, et al. ADAM10 regulates endothelial permeability and T-cell transmigration by proteolysis of vascular endothelial cadherin. Circ Res. 2008;102: 1192–1201.

43. Vandenbroucke RE, Libert C. Is there new hope for therapeutic matrix metalloproteinase inhibition? Nat Rev Drug Discov. Nature Publishing Group; 2014;13: 904–927.

44. Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. Mol Aspects Med. Elsevier Ltd; 2008;29: 258–289.

45. Overall CM, López-Otín C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer. 2002;2: 657–672.

46. Parks WC. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 2004;4: 617–629.

47. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003;92: 827–839.

48. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res. 2006;69: 562–573.

49. Khokha R, Murthy A, Weiss A. Metalloproteinases and their natural inhibitors in inflammation and immunity. Nat Rev Immunol. Nature Publishing Group;

2013;13: 649–665.

50. Murphy G. Tissue inhibitors of metalloproteinases. Genome Biol. 2011;12: 233.

51. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol. 2007;8: 221–233.

52. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 1477. 2000;1477: 267–283.

53. Arenas IA, Xu Y, Lopez-Jaramillo P, Davidge ST. Angiotensin II-induced MMP-2 release from endothelial cells is mediated by TNF-α. Am J Physiol Cell Physiol. 2004;286: C779–C784.

54. Harkness K, Adamson P, Sussman J, Davies-Jones G, Greenwood J, Woodroofe M. Dexamethasone regulation of matrix metalloproteinase expression in CNS vascular endothelium. Brain. 2000;123: 698–709.

55. Bzowska M, Jura N, Lassak A, Black RA, Bereta J. Tumour necrosis factor-α stimulates expression of TNF-α converting enzyme in endothelial cells. Eur J Biochem. 2004;271: 2808–2820.

56. Dreymueller D, Martin C, Kogel T, Pruessmeyer J, Hess FM, Horiuchi K, et al. Lung endothelial ADAM17 regulates the acute inflammatory response to lipopolysaccharide. EMBO Mol Med. 2012;4: 412–423.

57. Paolillo R, Iovene MR, Romano Carratelli C, Rizzo A. Induction of VEGF and MMP-9 expression by toll-like receptor 2/4 in human endothelial cells infected with Chlamydia pneumoniae. Int J Immunopathol Pharmacol. 2012;25: 377–386.

58. Shwetank, Date OS, Kim KS, Manjunath R. Infection of human endothelial cells by japanese encephalitis virus: increased expression and release of soluble HLA-E. PLoS One. 2013;8: e79197.

59. Luplertlop N, Missé D. MMP cellular responses to dengue virus infection-induced vascular leakage. Jpn J Infect Dis. 2008;61: 298–301.

60. Yazdan-Ashoori P, Liaw P, Toltl L, Webb B, Kilmer G, Carter DE, et al. Elevated plasma matrix metalloproteinases and their tissue inhibitors in patients with severe sepsis. J Crit Care. Elsevier Inc.; 2011;26: 556–565.

61. Bauer AT, Bürgers HF, Rabie T, Marti HH. Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain via tight junction rearrangement. J Cereb Blood Flow Metab. 2010;30: 837–848.

62. Ichikawa Y, Ishikawa T, Momiyama N, Kamiyama M, Sakurada H, Matsuyama R, et al. Matrilysin (MMP-7) degrades VE-cadherin and accelerates accumulation of beta-catenin in the nucleus of human umbilical vein endothelial cells. Oncol Rep. 2006;15: 311–315.

63. Liu J, Jin X, Liu K, Liu W. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood brain barrier damage in early ischemic stroke stage. J Neurosci. 2012;32: 3044–3057.

64. Alexander JS, Elrod JW. Extracellular matrix, junctional integrity and matrix metalloproteinase interactions in endothelial permeability regulation. J Anat. 2002;200: 561–574.

65. Sounni NE, Paye A, Host L, Noël A. MT-MMPS as regulators of vessel stability associated with angiogenesis. Front Pharmacol. 2011;2: 1–11.

66. Lemarchant S, Pruvost M, Montaner J, Emery E, Vivien D, Kanninen K, et al. ADAMTS proteoglycanases in the physiological and pathological central nervous system. J Neuroinflammation. 2013;10: 133.

67. Jönsson-Rylander AC, Nilsson T, Fritsche-Danielson R, Hammarström A, Behrendt M, Andersson JO, et al. Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican.

Arterioscler Thromb Vasc Biol. 2005;25: 180–185.

68. Schrimpf C, Xin C, Campanholle G, Gill SE, Stallcup W, Lin S-L, et al. Pericyte TIMP3 and ADAMTS1 modulate vascular stability after kidney injury. J Am Soc Nephrol. 2012;23: 868–883.

69. Partridge CA, Jeffrey JJ, Malik AB. A 96-kDa gelatinase induced by TNF-α contributes to increased microvascular endothelial permeability. Am J Physiol. 1993;Vol 265: L438–L447.

70. Passi A, Negrini D, Albertini R, De Luca G, Miserocchi G. Involvement of lung interstitial proteoglycans in development of hydraulic- and elastase-induced ededma. Am Physiol Soc. 1998;275: L631–L635.

71. Qiao RL, Wang HS, Yan W, Odekon LE, Del Vecchio PJ, Smith TJ, et al. Extracellular matrix hyaluronan is a determinant of the endothelial barrier. Am J Physiol. 1995;269: C103–C109.

72. McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol. 2001;13: 534–540.

73. Chen F, Ohashi N, Li W, Eckman C, Nguyen JH. Disruptions of occludin and claudin-5 in brain endothelial cells in vitro and in brains of mice with acute liver failure. Hepatology. 2009;50: 1914–1923.

74. Lominadze D, Roberts AM, Tyagi N, Moshal KS, Tyagi SC. Homocysteine causes cerebrovascular leakage in mice. Am J Physiol Hear Circ Physiol. 2006;290: H1206–H1213.

75. Ponnuchamy B, Khalil RA. Role of ADAMs in endothelial cell permeability: cadherin shedding and leukocyte rolling. Circ Res. 2008;102: 1139–1142.

76. Rahman A, Fazal F. Hug tightly and say goodbye: role of endothelial ICAM-1 in leukocyte transmigration. Antioxid Redox Signal. 2009;11: 823–839.

77. Sithu SD, English WR, Olson P, Krubasik D, Baker AH, Murphy G, et al. Membrane-type 1-matrix metalloproteinase regulates intracellular adhesion molecule-1 (ICAM-1)-mediated monocyte transmigration. J Biol Chem. 2007;282: 25010–25019.

78. Garton KJ, Gough PJ, Philalay J, Wille PT, Blobel CP, Whitehead RH, et al. Stimulated shedding of vascular cell adhesion molecule 1 (VCAM-1) is mediated by tumor necrosis factor-α-converting enzyme (ADAM 17). J Biol Chem. 2003;278: 37459–37464.

79. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci. 2002;115: 3719–3727.

80. Bugno M, Witek B, Bereta J, Bereta M, Edwards DR, Kordula T. Reprogramming of TIMP-1 and TIMP-3 expression profiles in brain microvascular endothelial cells and astrocytes in response to proinflammatory cytokines. FEBS Lett. 1999;448: 9–14.

81. Shastry S, Tyagi SC. Homocysteine induces metalloproteinase and shedding of β-1 integrin in microvessel endothelial cells. J Cell Biochem. 2004;93: 207–213.

82. Siemianowicz K, Likus W, Francuz T, Garczorz W. Effect of elastin-derived peptides on the production of tissue inhibitor of metalloproteinase-1, -2, and -3 and the ratios in various endothelial cell lines. Exp Ther Med. 2015;9: 2245–2250.

83. Arpino V, Mehta S, Wang L, Bird R, Rohan M, Pape C, et al. Tissue inhibitor of metalloproteinases 3-dependent microvascular endothelial cell barrier function is disrupted under septic conditions. Am J Physiol Hear Circ Physiol. 2016;310: H1455–H1467.

84. Shen Q, Lee ES, Pitts RL, Wu MH, Yuan SY. Tissue inhibitor of metalloproteinase-2 regulates matrix metalloproteinase-2-mediated endothelial barrier dysfunction and breast cancer cell transmigration through lung microvascular endothelial cells. Mol Cancer Res. 2010;8: 939–951.

85. Mascall KS, Small GR, Gibson G, Nixon GF. Sphingosine-1-phosphate-induced release of TIMP-2 from vascular smooth muscle cells inhibits angiogenesis. J Cell Sci. 2012;125: 2267–2275.

86. Dollery CM, McEwan JR, Wang M, Sang QA, Liu YE, Shi YE. TIMP-4 is regulated by vascular injury in rats. Ann N Y Acad Sci. 1999;84: 498–504.

87. Saunders WB, Bohnsack BL, Faske JB, Anthis NJ, Bayless KJ, Hirschi KK, et al. Coregulation of vascular tube stabilization by endothelial cell TIMP-2 and pericyte TIMP-3. J Cell Biol. 2006;175: 179–191.

88. Ebrahem Q, Qi JH, Sugimoto M, Ali M, Sears JE, Cutler A, et al. Increased neovascularization in mice lacking tissue inhibitor of metalloproteinases-3. Investig Ophthalmol Vis Sci. 2011;52: 6117–6123.

89. Yu WH, Yu SSC, Meng Q, Brew K, Woessner JF. TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. J Biol Chem. 2000;275: 31226–31232.

90. Qi JH, Ebrahem Q, Moore N, Murphy G, Claesson-Welsh L, Bond M, et al. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nat Med. 2003;9: 407–415.

91. Hurst LA, Bunning RAD, Couraud PO, Romero IA, Weksler BB, Sharrack B, et al. Expression of ADAM-17, TIMP-3 and fractalkine in the human adult brain endothelial cell line, hCMEC/D3, following pro-inflammatory cytokine treatment. J Neuroimmunol. Elsevier B.V.; 2009;210: 108–112.

92. Lorente L, Martín MM, Solé-Violín J, Blanquer J, Labarta L, Díaz C, et al. Association of sepsis-related mortality with early increase of TIMP-1/MMP-9 ratio. PLoS One. 2014;9: e94318.

93. Lorente L, Martín MM, Labarta L, Díaz C, Solé-Violán J, Blanquer J, et al. Matrix metalloproteinase-9, -10, and tissue inhibitor of matrix metalloproteinases-1 blood

levels as biomarkers of severity and mortality in sepsis. Crit Care. 2009;13: R158.

94. Fujimoto M, Takagi Y, Aoki T, Hayase M, Marumo T, Gomi M, et al. Tissue inhibitor of metalloproteinases protect blood–brain barrier disruption in focal cerebral ischemia. J Cereb Blood Flow Metab. 2008;28: 1674–1685.

95. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim Biophys Acta. 2010;1803: 55–71.

96. English JL, Kassiri Z, Koskivirta I, Atkinson SJ, Di Grappa M, Soloway PD, et al. Individual Timp deficiencies differentially impact pro-MMP-2 activation. J Biol Chem. 2006;281: 10337–10346.

97. Chen P, McGuire JK, Hackman RC, Kim K-H, Black RA, Poindexter K, et al. Tissue inhibitor of metalloproteinase-1 moderates airway re-epithelialization by regulating matrilysin activity. Am J Pathol Pathol. American Society for Investigative Pathology; 2008;172: 1256–70.

98. Gill SE, Huizar I, Bench EM, Sussman SW, Wang Y, Khokha R, et al. Tissue inhibitor of metalloproteinases 3 regulates resolution of inflammation following acute lung injury. Am J Pathol. American Society for Investigative Pathology; 2010;176: 64–73.

99. Sahebjam S, Khokha R, Mort JS. Increased collagen and aggrecan degradation with age in the joints of Timp3-/- mice. Arthritis Rheum. 2007;56: 905–909.

100. Gill SE, Pape MC, Khokha R, Watson AJ, Leco KJ. A null mutation for tissue inhibitor of metalloproteinases-3 (Timp-3) impairs murine bronchiole branching morphogenesis. Dev Biol. 2003;261: 313–323.

101. Gill SE, Pape MC, Leco KJ. Tissue inhibitor of metalloproteinases 3 regulates extracellular matrix-cell signaling during bronchiole branching morphogenesis. Dev Biol. 2006;298: 540–554.

102. Wu J, Zhao D, Wu S, Wang D. Ang-(1-7) exerts protective role in blood-brain barrier damage by the balance of TIMP-1/MMP-9. Eur J Pharmacol. Elsevier; 2015;748: 30–36.

103. Menge T, Zhao Y, Zhao J, Wataha K, Geber M, Zhang J, et al. Mesenchymal stem cells regulate blood brain barrier integrity through TIMP release after traumatic brain injury. Sci Transl Med Novemb. 2012;4: 161ra150.

104. Darwish I, Liles WC. Emerging therapeutic strategies to prevent infection-related microvascular endothelial activation and dysfunction. Virulence. 2013;4: 572–582.

105. Seo D-W, Li H, Guedez L, Wingfield PT, Diaz T, Salloum R, et al. TIMP-2 mediated inhibition of angiogenesis. Cell. 2003;114: 171–180.

106. Singh RJR, Mason JC, Lidington EA, Edwards DR, Nuttall RK, Khokha R, et al. Cytokine stimulated vascular cell adhesion molecule-1 (VCAM-1) ectodomain release is regulated by TIMP-3. Cardiovasc Res. 2005;67: 39–49.

107. Tyml K. Critical role for oxidative stress , platelets , and coagulation in capillary blood flow impairment in sepsis. Microcirculation. 2011;18: 152–162.

108. Shelton JL, Wang L, Cepinskas G, Sandig M, Inculet R, Mccormack DG, et al. Albumin leak across human pulmonary microvascular vs . umbilical vein endothelial cells under septic conditions. Microvasc Res. 2006;71: 40–47.

109. Zetter B. The endothelial cells of large and small blood vessels. Diabetes. 1981;30: 24–28.

110. Farley KS, Wang L, Mehta S. Septic pulmonary microvascular endothelial cell injury: role of alveolar macrophage NADPH oxidase. Am J Physiol Lung Cell Mol Physiol. 2009;296: L480–L488.

111. Razavi HM, Wang LF, Weicker S, Rohan M, Law C, Mccormack DG, et al. Pulmonary neutrophil infiltration in murine sepsis role of inducible nitric oxide synthase. Am J Respir Crit Care Med. 2004;170: 227–233.

112. Wang L, Mehta S, Brock M, Gill SE. Inhibition of murine pulmonary microvascular endothelial cell apoptosis promotes recovery of barrier function under septic conditions. Mediators Inflamm. Hindawi Publishing Corporation; 2017; 3415380.

113. Sun C, Beard RS, McLean DL, Rigor RR, Konia T, Wu MH, et al. ADAM15 deficiency attenuates pulmonary hyperpermeability and acute lung injury in lipopolysaccharide-treated mice. Am J Physiol Lung Cell Mol Physiol. 2013;304: L135–L142.

114. Wang L, Taneja R, Wang W, Yao L, Veldhuizen RAW, Gill SE, et al. Human alveolar epithelial cells attenuate pulmonary microvascular endothelial cell permeability under septic conditions. PLoS One. 2013;8: e55311.

115. Adissu HA, McKerlie C, Di Grappa M, Waterhouse P, Xu Q, Fang H, et al. Timp3 loss accelerates tumour invasion and increases prostate inflammation in a mouse model of prostate cancer. Prostate. 2015;75: 1831–1843.

116. Diamond MS, Staunton DE, De Fougerolles AR, Stacker SA, Garcia-Aguilar J, Hibbs ML, et al. ICAM-1 (CD54): A counter-receptor for Mac-1 (CD11b/CD18). J Cell Biol. 1990;111: 3129–3139.

117. Kent LW, Rahemtulla F, Hockett RD, Gilleland RC, Michalek SM. Effect of lipopolysaccharide and inflammatory cytokines on interleukin-6 production by healthy human gingival fibroblasts. Infect Immun. 1998;66: 608–614.

118. Dejager L, Pinheiro I, Dejonckheere E, Libert C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? Trends Microbiol. Elsevier Ltd; 2011;19: 198–208.

119. Gill SE, Rohan M, Mehta S. Role of pulmonary microvascular endothelial cell apoptosis in murine sepsis-induced lung injury in vivo. Respir Res. Respiratory Research; 2015;16: 109.

120. Geller DA, Nussler AK, Silvio M Di, Lowenstein CJ, Shapiro RA, Wang SC, et al.

Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci USA. 1993;90: 522–526.

121. Hobai IA, Morse JC, Siwik DA, Colucci WS. Lipopolysaccharide and cytokines inhibit rat cardiomyocyte contractility in vitro. J Surg Res. Elsevier Inc; 2015;193: 888–901.

122. Razavi HM, Werhun R, Scott JA, Weicker S, Wang LF, Mccormack DG, et al. Effects of inhaled nitric oxide in a mouse model of sepsis-induced acute lung injury. Crit Care Med. 2002;30: 868–873.

123. Shelton JL, Wang L, Cepinskas G, Inculet R, Mehta S. Human neutrophil – pulmonary microvascular endothelial cell interactions in vitro: differential effects of nitric oxide vs. peroxynitrite. Microvasc Res. 2008;76: 80–88.

124. Groeneveld ABJ. Vascular pharmacology of acute lung injury and acute respiratory distress syndrome. Vascul Pharmacol. 2003;39: 247–256.

125. Farley KS, Wang LF, Razavi HM, Law C, Rohan M, Mccormack DG, et al. Effects of macrophage inducible nitric oxide synthase in murine septic lung injury. Am J Physiol Lung Cell Mol Physiol. 2006;290: L1164–L1172.

126. Wang LF, Patel M, Razavi HM, Weicker S, Joseph MG, Mccormack DG, et al. Role of inducible nitric oxide synthase in pulmonary microvascular protein leak in murine sepsis. Am J Respir Crit Care Med. 2002;165: 1634–1639.

127. Handa O, Stephen J, Cepinskas G. Role of endothelial nitric oxide synthase-derived nitric oxide in activation and dysfunction of cerebrovascular endothelial cells during early onsets of sepsis. Am J Physiol Hear. 2008;295: H1712–H1719.

128. Petrache I, Birukova A, Ramirez SI, Garcia JGN, Verin AD. The role of the microtubules in tumor necrosis factor-α-induced endothelial cell permeability. Am J Respir Cell Mol Biol. 2003;28: 574–581.

129. Petrache I, Verin AD, Crow MT, Birukova A, Liu F, Garcia JG. Differential effect

of MLC kinase in TNF-α-induced endothelial cell apoptosis and barrier dysfunction. Am J Physiol Lung Cell Mol Physiol. 2001;280: L1168–L1178.

130. Shen Q, Rigor RR, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase in microvascular endothelial barrier function. Cardiovasc Res. 2010;87: 272–280.

131. Wójciak-Stothard B, Potempa S, Eichholtz T, Ridley AJ. Rho and Rac but not Cdc42 regulate endothelial cell permeability. J Cell Sci. 2001;114: 1343–1355.

132. Doedens JR, Mahimkar RM, Black RA. TACE/ADAM-17 enzymatic activity is increased in response to cellular stimulation. Biochem Biophys Res Commun. 2003;308: 331–338.

133. Chirivi RGS, Garofalo A, Crimmin MJ, Bawden LJ, Stoppacciar A, Brown PD, et al. Inhibition of the metastatic spread and growth of B16-BL6 murinen melanoma by a synthetic matrix metalloproteinase inhibitor. Int J Cancer. 1994;58: 460–464.

134. Keck T, Iv JHB, Fernández-Del Castillo C, Antoniu BA, Warshaw AL. Matrix Metalloproteinase-9 promotes neutrophil migration and alveolar capillary leakage in pancreatitis-associated lung injury in the rat. Gastroenterology. 2002;122: 188–201.

135. Muhs BE, Patel S, Yee H, Marcus S, Shamamian P. Inhibition of matrix metalloproteinases reduces local and distant organ injury following experimental acute pancreatitis. J Surg Res. 2003;109: 110–117.

136. Urrutia A, Rubio-Araiz A, Gutierrez-Lopez MD, El Ali A, Hermann DM, Shea EO, et al. A study on the effect of JNK inhibitor, SP600125, on the disruption of blood–brain barrier induced by methamphetamine. Neurobiol Dis. Elsevier Inc.; 2013;50: 49–58.

137. Raissi AJ, Scangarello FA, Hulce KR, Pontrello JK, Paradis S. Enhanced potentcy of the metalloproteinase inhibitor TAPI-2 by multivalent display. Bioorg Med Chem Lett. 2014;24: 2002–2007.

138. Finigan JH, Faress JA, Wilkinson E, Mishra RS, Nethery DE, Wyler D, et al. Neuregulin-1-human epidermal receptor-2 signaling is a central regulator of pulmonary epithelial permeability and acute lung injury. J Biol Chem. 2011;286: 10660–10670.

139. Otulakowski G, Engelberts D, Gusarova GA, Bhattacharya J, Post M, Kavanagh BP. Hypercapnia attenuates ventilator-induced lung injury via a disintegrin and metalloprotease-17. J Physiol. 2014;592: 4507–4521.

140. Hoffmann U, Hoffmann U, Bertsch T, Hoffmann U, Bertsch T, Dvortsak E, et al. Matrix-metalloproteinases and their inhibitors are elevated in severe sepsis: prognostic value of TIMP-1 in severe sepsis. Scand J Infect Dis. 2006;38: 867–872.

141. Lauhio A, Hästbacka J, Pettilä V, Tervahartiala T, Karlsson S, Varpula T, et al. Serum MMP-8 , -9 and TIMP-1 in sepsis: high serum levels of MMP-8 and TIMP-1 are associated with fatal outcome in a multicentre, prospective cohort study. Hypothetical impact of tetracyclines. Pharmacol Res. Elsevier Ltd; 2011;64: 590–594.

142. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, et al. TNF-α converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett. 1998;435: 39–44.

143. Amour A, Knight CG, Webster A, Slocombe PM, Stephens PE, Kna V, et al. The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. FEBS Lett. 2000;473: 275–279.

144. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF. Neutrophils in development of multiple organ failure in sepsis. Lancet. 2006;368: 157–169.

145. Cohen J. The immunopathogenesis of sepsis. Nature. 2002;420: 885–891.

146. Hack CE, Zeerleder S. The endothelium in sepsis: source of and a target for

inflammation. Crit Care Med. 2001;29: S21–S27.

147. King EG, Bauzá GJ, Mella JR, Remick DG. Pathophysiologic mechanisms in septic shock. Lab Investig. 2014;94: 4–12.

148. Sligh JE, Ballantynet CM, Richi SS, Hawkins HALK, Smith CW, Bradley A, et al. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc Natl Acad Sci USA. 1993;90: 8529–8533.

149. Briaud SA, Ding Z, Michael LH, Entman ML, Daniel S, Ballantyne CM, et al. Leukocyte trafficking and myocardial reperfusion injury in ICAM-1/P-selectin-knockout mice. Am J Physiol Hear Circ Physiol. 2001;280: H60–H67.

150. Bullard DC, Qin L, Lorenzo I, Quinlin WM, Doyle NA, Bosse R, et al. P-Selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. J Clin invest. 1995;95: 1782–1788.

151. Petrovich E, Feigelson SW, Stoler-barak L, Hatzav M, Solomon A, Bar-shai A, et al. Lung ICAM-1 and ICAM-2 support spontaneous intravascular effector lymphocyte entrapment but are not required for neutrophil entrapment or emigration inside endotoxin-inflamed lungs. FASEB J. 2016;30: 1767–1778.

152. Roebuck KA, Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J Leu. 1999;66: 876–888.

153. Kotteas EA, Boulas P, Gkiozos I, Tsagkouli S, Tsoukalas G, Syrigos KN. The intercellular cell adhesion molecule-1 (ICAM-1) in lung cancer: implications for disease progression and prognosis. Anticancer Res. 2014;34: 4665–4672.

154. Champagne B, Tremblay P, Cantin A, Pierre S, Champagne B, Tremblay P, et al. Proteolytic cleavage of ICAM-1 by human neutrophil elastase. J Immunol. 1998;161: 6398–6405.

155. Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling:

metalloproteinase-independent biological activities. Sci Signal. 2008;1: re6.

156. Dubois B, Starckx S, Pagenstecher A, Oord J Van Den, Arnold B, Opdenakker G. Gelatinase B deficiency protects against endotoxin shock. Eur J Immunol. 2002;32: 2163–2171.

157. Van Hove I, Lefevere E, Groef L De, Sergeys J. MMP-3 Deficiency Alleviates Endotoxin-Induced Acute Inflammation in the Posterior Eye Segment. Int J Mol Sci. 2016;17: 1825.

158. Blom C, Deller BL, Fraser DD, Patterson EK, Martin CM, Young B, et al. Human severe sepsis cytokine mixture increases β 2-integrin-dependent polymorphonuclear leukocyte adhesion to cerebral microvascular endothelial cells in vitro. Crit Care; 2015;19: 149.

159. Potter DR, Damiano ER. The hydrodynamically relevant endothelial cell glycocalyx observed in vivo is absent in vitro. Circ Res. 2008;102: 770–776.

160. Jung K-K, Liu X-W, Chirco R, Fridman R, Kim H-RC. Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. EMBO J. 2006;25: 3934–3942.

161. Lorente L, Martín M, Plasencia F, Solé-violán J, Blanquer J, Labarta L, et al. The 372 T/C genetic polymorphism of TIMP-1 is associated with serum levels of TIMP-1 and survival in patients with severe sepsis. Crit Care. 2013;17: R94.

162. Collazos J, Asensi V, Martin G, Montes AH, Su T. The effect of gender and genetic polymorphisms on matrix metalloprotease (MMP) and tissue inhibitor (TIMP) plasma levels in different infectious and non-infectious conditions. Clin Exp Immunol. 2015;182: 213–219.

Appendices

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Publications:

Masciantonio MG, Gill SE (2017). Tissue Inhibitor of Metalloproteinases. Encyclopedia of Signaling Molecules, 2nd ed [In print]

Masciantonio MG, Surmanski AA (2017). Medical smartphone applications: a new and innovative way to manage health conditions from the palm of your hand. University of Western Ontario Medical Journal [In print]

Masciantonio MG, Lee CKS, Arpino V, Mehta S, Gill SE (2017). The Balance Between Metalloproteinases and TIMPs: Critical Regulator of Microvascular Endothelial Cell Function in Health and Disease. Progress in Molecular Biology and Translational Science. 147: 100-130

Abstracts:

Masciantonio MG, Mehta S, Wang L, Rohan M, Pape C, Gill SE. The metalloproteinase-dependent role of TIMPs in regulation of pulmonary microvascular endothelial cell barrier function during sepsis. Acta Pharmacol Sin 2017;38: 1085-1086

Masciantonio M, Mehta S, Wang L, Rohan M, Pape C, Gill SE. The metalloproteinase-dependent role of TIMPs in regulation of pulmonary microvascular endothelial cell barrier function during sepsis. Am J Respir Crit Care Med 2017;195: A7433