August 2013

Role of iNOS in septic pulmonary microvascular endothelial cell activation

Zahra Asad
The University of Western Ontario

Supervisor
Dr Sanjay Mehta
The University of Western Ontario

Graduate Program in Pharmacology and Toxicology

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

© Zahra Asad 2013

Follow this and additional works at: http://ir.lib.uwo.ca/etd

Part of the Cardiovascular Diseases Commons, Immune System Diseases Commons, Pharmacy and Pharmaceutical Sciences Commons, Respiratory Tract Diseases Commons, and the Veterinary Medicine Commons

Recommended Citation
Asad, Zahra, "Role of iNOS in septic pulmonary microvascular endothelial cell activation" (2013). Electronic Thesis and Dissertation Repository. 1412.
http://ir.lib.uwo.ca/etd/1412

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca.
Role of iNOS in septic pulmonary microvascular endothelial cell activation

By
Zahra Asad

Graduate Program in
Pharmacology and Toxicology

A Thesis submitted in partial fulfillment of
the requirement for the Degree of
Master of Science in Pharmacology and Toxicology

School of Graduate and Postdoctoral Studies
Western University
London, Ontario

© by Zahra Asad 2013
ABSTRACT

Background. Neutrophils and nitric oxide (NO) derived from inducible NO synthase (iNOS) contributes importantly to the pathophysiology of acute lung injury (ALI) and pulmonary microvascular endothelial cell (PMVEC) injury. However, the mechanism of neutrophil and neutrophil iNOS dependent PMVEC injury has not been addressed. In our studies, we assessed PMVEC activation under septic conditions, and defined the role of PMVEC vs. bone-marrow polymorphonuclear leukocytes (PMN) iNOS in this septic PMVEC activation.

Methods and Results. We isolated PMVEC from iNOS+/+ and iNOS-/- mice lungs magnetically by microbeads attached to anti-PECAM antibodies, sorted by flow cytometry (FACS) by DiI-acetylated low density lipoprotein (Dil-Ac-LDL) uptake by cells. Bone-marrow PMNs were isolated from femurs and tibia of iNOS+/+ and iNOS-/- mice followed by percoll gradient isolation. Cultured PMVEC monolayers showed that basal E-selectin expression was significantly lower in iNOS-/- vs iNOS+/+ PMVEC (MFI 16±2 vs 59±7, p<0.05). After lipopolysaccharide (LPS) stimulation, E-selectin expression in iNOS+/+ PMVEC increased at 1 hr (MFI 146±15, p<0.01), peaked at 2 hrs (MFI 284±38, p<0.01), and then gradually declined over 12 hrs. iNOS-/- PMVEC had a similar response to iNOS+/+ PMVEC in the timing and magnitude of E-selectin upregulation, with a similar peak expression (MFI 257±29, p=NS vs iNOS+/+). PMVEC responded to cytomix similarly to LPS, with PMVEC E-selectin upregulation in a time dependent and dose dependent manner which was similar in iNOS+/+ and iNOS-/- PMVEC. As compared to E-selectin, basal plasminogen activation inhibitor (PAI)-1 expression was similar in iNOS+/+ and iNOS-/- PMVEC. LPS-induced upregulation of PAI-1 in
iNOS+/+ PMVEC peaked at 4 hrs (MFI 3.4±0.1 vs 1.7±0.1 for control medium, p<0.01), and was sustained until 12 hrs and this pattern of LPS-stimulated PAI-1 expression was similar in iNOS-/- PMVEC. In contrast, cytomix induced PAI-1 in iNOS+/+ PMVEC peaked at 2 hrs and was sustained until 8 hrs, and was similar in iNOS-/- PMVEC. The presence of PMN with PMVEC synergistically enhanced LPS induced PMVEC E-selectin expression in a dose dependent manner. However, this LPS-induced PMN-dependent PMVEC E-selectin induction was independent of PMN iNOS. The presence of either iNOS+/+ or iNOS-/- PMN with PMVEC did not upregulate PMVEC E-selectin post cytomix stimulation. Moreover, iNOS+/+ or iNOS-/- PMN did not affect PMVEC PAI-1 protein expression or mRNA expression after either LPS or cytomix treatment.

Conclusions. PMVEC are activated under different septic conditions (both LPS and cytomix) as reflected by increased E-selectin and PAI-1 expression. However, PMVEC iNOS did not affect LPS or cytomix induced PMVEC activation. PMN enhanced septic PMVEC E-selectin expression following LPS treatment, and this endothelial activation was independent of PMN iNOS. These data suggest that septic PMVEC activation in isolation or in the presence of PMN, is independent of both PMVEC and PMN iNOS.

Keywords: Acute lung injury; sepsis; nitric oxide; iNOS; PMVEC; edema; E-selectin; PAI-1; PMN; LPS; cytomix
GRANT SUPPORT
This research presented in this thesis was funded by a grant from the Heart and Stroke foundation of Canada to Dr Sanjay Mehta (#T6461).
ACKNOWLEDGMENT
First of all, I would like to thank Allah Almighty for giving me the ability to complete this work, for providing me with helping colleagues and for giving me the patience to get through tough times during the course of the thesis. I would like to extend my deepest gratitude to my supervisor, Dr. Sanjay Mehta, who facilitated my research and helped me all the way. This thesis could not have been completed without his continued support and encouragement. I am extremely humbled and thankful for his supervision of my thesis. Dr. Sanjay’s secretary Mary Leckie, deserves thanks for solving logistical issues related to my thesis. I thank Dr. Lina Dagnino, and Jane Rylett, Chair of Graduate studies program, Dept. of Physiology and Pharmacology, for her support during my studies at Western University, for understanding and helping me out. I am thankful to Dr. Berry Tepperman, Ex-Chair of committee meeting for his guidance. My appreciation goes out to the committee members Dr. Martin Sandig, Dr. Tianqing Peng, and Dr. Ian ChinYee for their support.

My colleagues helped tremendously throughout my thesis. Particularly, I would like to acknowledge Dr. Lefeng Wang and Dr. Marta Rohan whose expertise and support helped me in learning experiment techniques and without whose help my work would not be done. I would like to thank Dr. Scott Swarbreck who performed RT-PCR analysis and Dr. Mike Keeney, Dr. Ben Hedley, Dr. Wendy Brown whose expertise helped me with FACS analysis. I appreciate all of them for their help and mentorship during my thesis. It helped me gain confidence in what I was doing and enabled me to carry out experiments in a better manner.

I would also like to thank the Department of Physiology and Pharmacology for its resources that were used during the thesis. I appreciate the Heart and Stoke Foundation of Canada that
supported my work with a grant through my supervisor. Last but not the least; I am thankful to the Graduate School at Western University for their support during the thesis.
DEDICATION
To my father Retd. Colonel Syed Sajjad Haider and mother Dr. Zarafat Haider and my
Supervisor Dr. Sanjay Mehta
TABLE OF CONTENTS

I. ABSTRACT .......................................................................................................................... ii
II. GRANT SUPPORT ............................................................................................................... iv
III. ACKNOWLEDGMENT ....................................................................................................... v
IV. DEDICATION ...................................................................................................................... vii
V. CHAPTER 1: INTRODUCTION ............................................................................................ 1
VI. SECTION A .......................................................................................................................... 1
VII. A. SEPSIS ......................................................................................................................... 1
   A.1 Definition and Clinical features .................................................................................... 1
   A.2 Epidemiology ............................................................................................................... 2
   A.3 Pathophysiology of Sepsis .......................................................................................... 2
      A.3.1 Sources of Sepsis .................................................................................................. 2
      A.3.2 Pathophysiology .................................................................................................. 3
      A.3.3 Role of LPS in sepsis .......................................................................................... 3
      A.3.4 Role of soluble inflammatory mediators in sepsis ................................................. 4
      A.3.5 Neutrophils .......................................................................................................... 5
      Normal Physiology ......................................................................................................... 5
      Neutrophils in Sepsis ...................................................................................................... 6
   A.4 Diagnosis and Treatment of Sepsis, severe Sepsis or Septic shock ............................. 8
      A.4.1 Diagnosis .............................................................................................................. 8
      A.4.2 Treatment ............................................................................................................. 9
VIII. SECTION B ....................................................................................................................... 15
IX. B. ACUTE LUNG INJURY/ACUTE RESPIRATORY DISTRESS SYNDROME ..................... 15
   B.1 Definition .................................................................................................................... 15
   B.2 Epidemiology ............................................................................................................. 16
2.5 RT-PCR analysis ........................................................................................................78
2.6 Effect of presence and type of sub cellular matrix on PMVEC basal E-selectin expression......78
2.7 Sample Size and statistical analysis ........................................................................79

XVI. CHAPTER 3: RESULTS.................................................................................................80
3.1 Analysis of PMVEC activation by FACS ..................................................................80
3.2 Effects of PMVEC iNOS on Basal PMVEC E-selectin expression .........................81
3.3 Effects of PMVEC iNOS on PMVEC E-selectin expression under septic conditions ..........82
3.4 Effect of PMVEC iNOS on PMVEC PAI-1 expression under septic conditions ..........83
3.5 Effect of PMN iNOS on PMVEC E-selectin expression in PMVEC under septic conditions ...85
3.6 Effect of PMN iNOS on PMVEC PAI-1 expression under septic stimulation\ ..................87

XVII. CHAPTER 4: DISCUSSION........................................................................................109
4.1 Role of EC iNOS ........................................................................................................110
4.2 Role of PMN and PMN iNOS ..................................................................................113
4.3 Limitations .............................................................................................................116
4.4 Summary ...............................................................................................................117

XVIII. CHAPTER 5: THESIS SUMMARY.......................................................................118
5.1 Hypothesis 1 –Pulmonary microvascular endothelial cells (PMVEC) iNOS contributes to PMVEC activation under septic conditions in vitro .................................118
5.2 Hypothesis 2 – Bone marrow neutrophil (PMN) iNOS contributes to pulmonary microvascular endothelial cell activation (PMVEC) under septic conditions in vitro. ............................119

XIX. CHAPTER 6: GENERAL DISCUSSION.....................................................................121
6.1 Introduction .............................................................................................................121
6.2 Assessment of EC activation ..................................................................................121
6.3 Endothelial iNOS effect on EC activation ..............................................................122
6.4 Neutrophil iNOS effect on endothelial activation under septic conditions ...............123
6.5 General implications of thesis ...............................................................................124
6.6 Future directions ................................................................. 125

XX. Reference List ..................................................................... 126

XXI. APPENDIX 1: ANIMAL ETHICS APPROVAL .............................. 194

XXII. APPENDIX 2: RESUME OF ZAHRA ASAD ................................. 195
List of Figures

Figure 3.1: Single channel flow cytometric analysis shows induction of E-selectin expression in iNOS +/- PMVEC in response to LPS. ..........................................................88

Figure 3.2: Single channel flow cytometric analysis shows induction of PAI-1 expression in iNOS +/- PMVEC in response to LPS. ..........................................................89

Figure 3.3: The effect of trypsin and EDTA methods of cell harvest on cell-surface E-selectin and PAI-1 expression by FACS in iNOS+/- murine pulmonary microvascular endothelial cell (PMVEC). .................90

Figure 3.4: The effect of the presence of sub-cellular matrix on basal E-selectin expression in iNOS+/- vs. iNOS-/- PMVEC. ...........................................................................91

Figure 3.5: The effect of inhibition of nitric oxide synthase (NOS) on basal E-selectin expression in iNOS+/- PMVEC under non-septic conditions. .................................................................92

Figure 3.6: Time course of LPS (1000 ng/ml) induced E-selectin expression in iNOS+/- vs. iNOS-/- PMVEC. ..................................................................................93

Figure 3.7: Dose-dependent effect of LPS on E-selectin expression in iNOS+/- vs. iNOS-/- PMVEC. ...............................................................94

Figure 3.8: Time course of cytomix (10ng/ml) induced E-selectin expression in iNOS+/- vs. iNOS-/- PMVEC. .............................................................95

Figure 3.9: Dose-dependent effect of cytomix on E-selectin expression in iNOS+/- vs. iNOS-/- PMVEC. ................................................................................................96

Figure 3.10: Time course of LPS (1000ng/ml) induced PAI-1 expression by FACS in iNOS+/- vs. iNOS-/- PMVEC. .................................................................97

Figure 3.11: Dose-dependent effect of LPS on PAI-1 expression in iNOS+/- vs. iNOS-/- PMVEC. ......98

Figure 3.12: Time course of cytomix (10ng/ml) induced PAI-1 expression in iNOS+/- vs. iNOS-/- PMVEC. .................................................................99

Figure 3.13: Dose-dependent effect of cytomix on PAI-1 expression in iNOS+/- vs. iNOS-/- PMVEC. ................................................................................................99

Figure 3.14: The effect of endothelial iNOS on septic stimulation (Cytomix 10 ng/mL vs. LPS 1000 ng/mL) induced PAI-1 mRNA expression. .................................................................100

Figure 3.15: The effect of septic stimulation (cytomix vs. LPS) on the activation of iNOS+/- vs. iNOS-/- bone-marrow neutrophils (PMN). .................................................................102

Figure 3.16: The effect of iNOS+/- vs. iNOS-/- BM PMN on PMVEC E-selectin expression after LPS stimulation. ..................................................................................103
Figure 3.17: The effect of different RBC lysis methods on LPS/PMN induced E-selectin expression in iNOS+/+ PMVEC..........................................................104

Figure 3.18: The effect of iNOS+/+ vs. iNOS-/- BM PMN on E-selectin expression in iNOS+/+ PMVEC after cytomix stimulation..........................................................105

Figure 3.19: The effect of iNOS+/+ vs. iNOS-/- BM PMN on PAI-1 expression in iNOS+/+ PMVEC after LPS stimulation..........................................................106

Figure 3.20: The effect of iNOS+/+ vs. iNOS-/- BM PMN on PAI-1 expression in iNOS+/+ PMVEC after cytomix stimulation..........................................................107

Figure 3.21: The effect of BM PMN iNOS on septic stimulation (Cytomix vs. LPS) induced PAI-1 mRNA expression in iNOS+/+ PMVEC..........................................................108
List of Abbreviations, symbols and Nomenclature

1400W  N- (3- aminomethylbenzyl acetamidine)

A2BAR  Adenosine 2B receptor

ACCP  American College of Chest Physicians

ACTH  adrenocorticotropic hormone

AECC  American European consensus conference

ALI  Acute lung injury

APC  activated protein C

APRV  Airway pressure release ventilation

ARDS  Acute respiratory distress syndrome

BAL  Bronchoalveolar lavage

BALF  Bronchoalveolar lavage fluid

BP  Blood pressure

cAMP  Cyclic adenosine mono phosphate

CLP  Cecal ligation and perforation

COPD  Chronic obstructive pulmonary disease

COX-2  cyclooxygenase-2
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CVP</td>
<td>Central venous pressure</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
</tr>
<tr>
<td>DIC</td>
<td>disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>ECCO$_2$R</td>
<td>Extra-corporeal carbon dioxide removal</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extra corporeal membrane oxygenation</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>E-selectin</td>
<td>endothelial selectin</td>
</tr>
<tr>
<td>ESL-1</td>
<td>E-selectin ligand -1</td>
</tr>
<tr>
<td>EVLW</td>
<td>extravascular lung water</td>
</tr>
<tr>
<td>FGF basic</td>
<td>fibroblast growth factor-basic</td>
</tr>
<tr>
<td>FiO$_2$</td>
<td>fraction of inspired oxygen</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-monocyte colony stimulating factor</td>
</tr>
<tr>
<td>HFOV</td>
<td>High-frequency oscillatory ventilation</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group box 1 protein</td>
</tr>
<tr>
<td>HMG-Co A reductase</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1 Ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>iNO</td>
<td>Inhaled nitric oxide</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN inducible protein-10</td>
</tr>
<tr>
<td>IRV</td>
<td>Inverse ratio ventilation</td>
</tr>
<tr>
<td>ITP</td>
<td>Idiopathic thrombocytopenic purpura</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
</tbody>
</table>

xvii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC</td>
<td>keratinocyte derived chemokine</td>
</tr>
<tr>
<td>LBP</td>
<td>lipopolysacharide binding protein</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LIP</td>
<td>lower inflection point</td>
</tr>
<tr>
<td>LL-37</td>
<td>Cathelicidin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>L-selectin</td>
<td>Leukocyte selectin</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose binding lectin MBL</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MIP-1 α</td>
<td>Macrophage inflammatory protein-1 alpha</td>
</tr>
<tr>
<td>MIP-1 β</td>
<td>Macrophage inflammatory protein-1 beta</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Matrix metalloproteinase-1</td>
</tr>
<tr>
<td></td>
<td>xviii</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MODS</td>
<td>multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistant streptococcus aureus</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kB</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OLV</td>
<td>one lung ventilation</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Partial pressure of arterial CO₂</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activation inhibitor - 1</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of arterial oxygen</td>
</tr>
<tr>
<td>PCWP</td>
<td>Pulmonary capillary wedge pressure</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>platelet endothelial cell adhesion molecule - 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>PFC</td>
<td>Perfluorocarbon</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>P-selectin</td>
<td>platelet selectin</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand -1</td>
</tr>
<tr>
<td>PT</td>
<td>prothrombin time</td>
</tr>
<tr>
<td>PTT</td>
<td>Partial thromboplastin time</td>
</tr>
<tr>
<td>PVR</td>
<td>Pulmonary vascular resistance</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated and normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>SaO₂</td>
<td>Arterial oxygen saturation</td>
</tr>
<tr>
<td>SCCM</td>
<td>Society of Critical Care Medicine</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
</tbody>
</table>

xx
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A</td>
<td>Surfactant protein-A</td>
</tr>
<tr>
<td>SvO₂</td>
<td>Mixed venous oxygen saturation</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>THAM</td>
<td>Tromethamine</td>
</tr>
<tr>
<td>TLR 4</td>
<td>Toll like receptor- 4</td>
</tr>
<tr>
<td>TNF α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TREM-1</td>
<td>Triggering receptor expressed on myeloid cells-1</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>VALI</td>
<td>Ventilator associated lung injury</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VCV</td>
<td>Volume controlled ventilation</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

SECTION A

A. SEPSIS

A.1 Definition and Clinical features

In 1992 the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) defined systemic inflammatory response syndrome (SIRS) as

- Body temperature less than 36°C or greater than 38°C
- Heart rate greater than 90 beats per minute
- Tachypnea with greater than 20 breaths per minute; or, an arterial partial pressure of carbon dioxide less than 4.3 kPa (32 mmHg)
- White blood cell count less than 4000 cells/mm$^3$ (4 x $10^9$ cells/L) or greater than 12,000 cells/mm$^3$ (12 x $10^9$ cells/L); or the presence of greater than 10% immature neutrophils (band forms)
- SIRS can be diagnosed when two or more of these criteria are present

Sepsis is defined as SIRS due to infection (1). Sepsis can progress to severe sepsis, which is defined as sepsis including organ failure and hypotension or hypoperfusion. Severe sepsis can progress to septic shock which is characterized by persistent hypotension [systolic blood pressure (BP) < 90mmHg or Mean arterial pressure (MAP) < 65mmHg], acute circulatory failure and hypotension despite adequate fluid resuscitation (2). Other clinical features also include hypoxemia, hyperglycemia, hyperbilirubinemia, elevated serum lactate levels (hyperlactatemia), oliguria, and uremia, coagulopathy, thrombocytopenia, altered gastrointestinal (GI) motility
(Ileus, intestinal distention) and altered mental status due to uremic or hepatic encephalopathy (3; 4).

A.2 Epidemiology

Sepsis, which is a leading cause of death among intensive care unit (ICU) patients and tenth leading cause of death in USA, affects 750,000 patients and causes 200,000 deaths each year (5). The incidence of sepsis is 50 – 95 cases per 100,000 of population and are increasing by 9% each year in USA (6). According to epidemiological study in 2001, one third of patient with SIRS have sepsis and sepsis may occur in 25% of ICU patients, 50% of septic patients proceed to severe sepsis and 25% of patients with severe sepsis developed septic shock. The mortality rates for sepsis, severe sepsis and septic shock have been reported as 20%, 20-40% and 30-70% respectively (7; 8).

A.3 Pathophysiology of Sepsis

A.3.1 Sources of Sepsis

Sepsis is caused by many factors (9). Sepsis occurs due to gram negative bacteria, gram positive bacteria, fungi and viruses (10; 10-12). For sepsis to occur bacterial inoculum must breach to overwhelm host defence. Sepsis due to gram positive bacteria such as staphylococci originates from skin and enterococci originate from GI tract whereas gram negative bacteria originate from GI and genitourinary tract (13; 14). Other sources of sepsis are respiratory, skin and soft tissue, impaired splenic function, community acquired methicillin resistant staphylococcus aureus (MRSA), clostridial myonecrosis, bones and joints and central venous
catheter through intravenous (IV) infusion (15-20). Therefore, inappropriate use of antibiotics, presence of pulmonary, intra-abdominal infections, neutropenia or multiple organ dysfunction are also associated with increased mortality during sepsis (9).

A.3.2 Pathophysiology

During sepsis inflammatory cells are activated such as macrophages and release tumor necrosis factor alpha (TNFα), interleukin (IL-1β), 2, 6, 11, interferon gamma (IFNγ) that act on hypothalamus for fever, tachycardia, tachypnea (21-23). Moreover, endothelial cells (EC) participate in inflammatory cascade by becoming adhesive to leukocytes, procoagulant and cause barrier dysfunction and release NO and therefore alter vascular tone that results in vasodilation (24). Therefore, microbial pathogens, immune response, inflammatory and coagulation cascade interactions contribute to pathophysiology of sepsis. Gram positive sepsis is initiated when cells interact with bacterial cell wall components peptidoglycans or lipotechoic acids (LTA) activate cells through cluster of differentiation (CD14), Toll like receptor-2 (TLR2) and may be TLR6 or through CD14 and TLR4. Gram negative sepsis is initiated when cell recognizes lipopolysaccharide (LPS) by lipopolysaccharide – lipopolysaccharide binding protein complex (LPS-LBP), CD14 and TLR4. (25).

A.3.3 Role of LPS in sepsis

LPS, a bacterial cell wall component consists of three domains, lipid A, oligosaccharide and O antigen polysaccharide. Lipid A domain is active during human infection whereas oligosaccharide chain determines whether LPS is smooth or rough (26; 27). LPS exists as a free
form or bacterial protein bound form LBP (LPS binding protein). LPS binds to LBP to form LPS–LBP complex which then binds to cell surface receptor CD14 which is present on various inflammatory cells. CD14 also exists as a free soluble state that is detached from membrane bound CD14 (28). This complex then binds to cell surfaces of those cells that lack CD14 receptor like endothelial, some epithelial cells and thus initiates activation by releasing cytokines, NO, ROS and procoagulant factors (29; 30). In humans, intravenous low dose of LPS (2ng/kg iv) is sufficient to induce systemic inflammation and sepsis (31). However, intravenous high dose (1 mg/kg) was sufficient to develop septic shock syndrome in human patient to treat cancer that includes all parameters like increased cardiac output due to hypotension, coagulopathy, thrombocytopenia, hepatic and renal dysfunction and non-cardiogenic pulmonary edema (32).

A.3.4 Role of soluble inflammatory mediators in sepsis

Host response to sepsis can result either in eradication of microbes or it can result in detrimental outcome (33). Therefore, the interaction of invaded pathogens and host response triggers the release of inflammatory mediators such as cytokines, chemokines, and lipid mediators such as prostaglandins, leukotrienes, PAF and oxidized phospholipids. Other proteases and enzymes such as cyclooxygenase-2 (COX-2), 5-lipoxygenase, phospholipase A2, metalloproteinase 9, elastase, inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NADPH), mast cell dipeptidyl peptidase 1, glycogen synthase kinase-3 also contributed to initiation of inflammatory responses. It has been documented that cell marker of stress high mobility group box 1 protein (HMGB1), vasoactive substances such as histamine
and serotonin, plasma factors Ligand of Triggering receptor expressed on myeloid cells-1 (TREM-1), Anaphylatoxin C5A, Mannose binding lectin (MBL), neuromediators such as substance P, neurokinin and norendrenalin plays a major role in sepsis. Furthermore, coagulation factors such as tissue factor (TF), plasminogen activating inhibitor (PAI-1), thrombin, thrombin activable fibrinolysis inhibitor (TAFI), free radicals such as nitric oxide (NO) and superoxide (O$_2^-$) and purine nucleoside (Adenosine via A$_2$A receptor) by inflammatory cells are involved in sepsis. (34-37).

Glycoproteins or cytokines are produced by leukocytes and work in an autocrine, paracrine or endocrine manner (35; 38). During early sepsis, several studies have identified that various pro-inflammatory cytokines [IL-6, IL-8, IL-12, IL-17, INF-γ, TNF, IL-1β, keratinocyte chemoattractant (KC), macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), granulocyte colony stimulating factor (GC-SF)] in animal and human plasma are associated with mortality (6; 39; 40). Moreover, anti-inflammatory serum cytokine (TNF soluble receptors, IL-10, IL-1 receptor antagonist) levels are also higher in human patients who do not survive (6; 39; 41). During onset of sepsis, this “cytokine storm” results in activation and influx of neutrophils, as well as activation of many other cells, including macrophage/monocyte, epithelial cells, EC, and natural killer (NK) cells; all of which results in production of reactive oxygen species and reactive nitrogen species that are detrimental in sepsis (6; 6; 42-45).

A.3.5 Neutrophils

Normal Physiology
Neutrophils are generated in bone marrow through a process of granulopoiesis in the presence of granulocyte colony stimulating factor (G-CSF) (46). Neutrophils are short-lived cells and are removed by apoptosis by resident tissue macrophages in liver, spleen and bone marrow. The half-life of neutrophils is 6-10 hrs post migration from bone marrow (47). During sepsis, neutrophils are transmigrated to the site of infection/injury. Normally, they express cell surface receptors that are useful markers for differentiating different stages during cell development (48). For example CD34 expressed on early precursor cells, CD11/18 β2 integrins are expressed late during development (49). It has 3 isoforms. CD11a/CD18 and CD11c/CD18 are involved in adhesion and migration whereas CD11b/CD18 is involved in cellular adhesion (50; 51). In lung, transendothelial migration of neutrophils can be either CD18 dependent and independent, depending on the inflammatory stimulus (52).

Neutrophils in Sepsis

Activated neutrophils transmigrate into lung tissue across the endothelium during sepsis or initiation of inflammatory cascade (53). Activated neutrophils undergo cytoskeletal derangements and deform to pass through capillary network (54). Transmigration occurs in 3 steps, capturing and rolling on endothelium, adhesion and transmigration through pores due to junctional proteins disruption. Ligation of L-selectin on neutrophil to CD34 on endothelium facilitates rolling. L-selectin is induced for few minutes thus, ligation of P-selectin on endothelium to PSGL-1 on neutrophils accomplishes slowing and rolling of neutrophils on endothelium. Ligation of E-selectin on endothelium to ESL-1 on neutrophil facilitates adhesion. Firm adhesion is accomplished when neutrophils Mac-1 (CD11b/CD18) ligates with ICAM-1 on endothelium and neutrophils β1 integrins very late antigens (VLA-4 and VLA-5) ligates with
VCAM-1 (55-57). Finally migration occurs when PECAM-1 on neutrophils ligate with PECAM-1 on endothelium in some model of sepsis however, some reported JAM-1 contributed in neutrophil trans-endothelial migration during inflammation and sepsis (58; 59). Moreover, ICAM-2, JAM-A, and PECAM-1 also contributes to neutrophil migration through venular walls in vivo (60).

PMN activation, migration and sequestration contribute to the development of sepsis (57). There is release of degradative enzymes (elastase and cathepsin G), ROS and reactive nitrogen species (RNS) produced by neutrophils in tissue injury (61-65).

Moreover, PMN neutrophil extracellular traps (NETs) formation has been important feature of sepsis and infections. However, depletion of PMN NETs by rhDNase can worsen the parameters of sepsis (66). The literature suggests that in neutropenic septic (PMN-/E coli) animals, there is significant histopathologic damage in the adrenal glands and liver and large accumulation of albumin leak in kidneys. However, multiple organ dysfunction was prominent in neutrophil septic animals which was attenuated with PMN depletion (67). Moreover, neutrophil depletion in chest trauma induced ALI has shown to decrease IL-1β, IL-6, bronchoalveolar lavage protein concentration and pulmonary myeloperoxidase activity in mice (68). Microvascular protein leak in sepsis-induced ALI is uniquely dependent on iNOS in inflammatory cells with no obvious contribution of iNOS in pulmonary parenchymal cells (69). Recently, we have shown that pretreatment with anti-CD18 before cecal ligation/perforation significantly reduced pulmonary myeloperoxidase, bronchoalveolar lavage neutrophils, microvascular EB-albumin leak, and 8-isoprostane content (70). Neutrophils are directly involved in causing endothelial activation, barrier dysfunction by increasing permeability and
cytoskeletal derangement during sepsis (45; 71). However, role of mice PMN iNOS in PMVEC activation post LPS or cytomix has not been identified.

A.4 Diagnosis and Treatment of Sepsis, severe Sepsis or Septic shock

A.4.1 Diagnosis

As described above, sepsis is caused due to infection and to diagnose infection various laboratory tests are performed. To diagnose bacteria during SIRS/sepsis, blood culture is done and a complete blood test is also performed to evaluate erythrocytes, white blood cells (WBCs) and platelets (72). Blood test is also performed to evaluate arterial blood gases, lactate level, blood glucose, electrolyte imbalance and C reactive protein (73). Prothrombin time (PT) or partial thromboplastin time (PTT) tests are performed to analyse blood clot formation duration (74).

Urine analysis is done for presence of Escherichia coli or Klebsiella enterococci for urinary tract infection (UTI) or gall bladder infection or bacteriodes fragilis for pelvic or colonic infection (75; 76). If MRSA (methicillin resistant streptococcus Auresus) is diagnosed then there is possibility of occurrence of lower respiratory tract infections or pneumonia (76; 77).

Chest X ray is done to evaluate haemorrhage, emboli, pleural effusion, cancer, pneumonothorax, fluid overload/edema, congestive heart failure and acute myocardial infarction (78). Abdominal ultrasound is important to diagnose any biliary tract obstruction or infection or infection in ovaries (79). Abdominal computerized tomography (CT) scan or magnetic resonance imaging (MRI) is important for diagnosing appendicitis or pancreatitis, nephritis or soft tissue abscesses like in spine (73).
Procalcitonin levels are used as a marker to distinguish sepsis from nonseptic systemic inflammation. Plasma concentration of soluble TREM-1 (triggering receptor expressed on myeloid cells), a member of the immunoglobulin superfamily are increased in septic patients in the presence of bacterial products. The clinical usefulness of Procalcitonin and TREM-1 is still in its preliminary phase. More clinical trials are still required for clinicians to suggest routine use.

A.4.2 Treatment

As described earlier, sepsis is due to infection. Therefore, antibiotic therapy for the underlying infection is critical (80). Empiric antibiotic treatment is given intravenously even if a specific bacterial infection is not diagnosed yet. The empiric treatment should be meropenem, cefoperazone, or cefepime plus additional coverage for staphylococci (81). If MRSA is prevalent in the hospital, then linezolid, vancomycin, or daptomycin can be given. Vancomycin should be avoided if coagulase-negative staphylococci are detected from the blood because this is a low-virulence organism. If intravenous lines or catheter tubes are source of infection then it should be replaced but if cannot be replaced in patient with MRSA or coagulase negative staphylococcal infection due to any medical or clinical reasons then vancomycin can be given. Linezolid, daptomycin and tigecycline are almost universally active against MRSA(82).

Preferred therapy for biliary-tract infections (cholecystitis/cholangitis) due to Escherichia coli, Klebsiella species, and Enterococcus faecalis is with imipenem, meropenem, piperacillin, or cefoperazone. Many physicians prefer penems such as imipenem, meropenem, piperacillin (extended spectrum penicillin)/tazobactam (beta lactamase inhibitor), or ampicillin/sulbactam to treat intra-abdominal and pelvic infections due to gram-negative bacilli and B fragilis,
enterococci. However, many prefer combination therapy such as clindamycin or metronidazole plus aztreonam, levofloxacin, or an aminoglycoside for intra-abdominal and pelvic infections. Surgical drainage is also required.

Urosepsis is mainly caused by gram-negative bacilli, such as coliforms or enterococci. Empiric treatment for urosepsis due to gram-negative bacilli can be monobactam such as aztreonam, floroquinolones such as levofloxacin, third- or fourth-generation cephalosporins, or an aminoglycoside. Urosepsis due to enterococci (E faecalis) can be treated with ampicillin or vancomycin when the patient is penicillin-allergic.

S aureus sepsis is usually associated with infection caused by devices or acute bacterial endocarditis. Empiric antibiotic therapy can be nafcillin (beta lactamase resistant), an anti-staphylococcal, cephalosporin, a carbapenem, linezolid, or clindamycin with or without rifampin. Pneumococcal or meningococcal sepsis may be treated with penicillin G or a beta-lactam. Early appropriate treatment of antibiotics can reduce mortality in sepsis or septic shock patients (83).

A.4.2.1 Supportive (Non-Pharmacological and Pharmacological strategies)

In adults, treatment of sepsis, severe sepsis or septic shock is largely supportive. Supplemental oxygenation or mechanical ventilation may be necessary to facilitate breathing if SaO2 is less than 93% (84). Non-invasive ventilation may be required to support the increased respiration that follows sepsis, for airway protection as encephalopathy and altered mental level of consciousness complicate sepsis (85).
If patient is in severe sepsis or septic shock [mean arterial pressure (MAP) <65 mmHg, central venous pressure (CVP) is less than 8, urine output <0.5ml/kg/hr, central venous oxygen saturation<70%, mixed venous oxygen saturation<65%] careful use of 20mL/Kg of saline solution should be given within 1 hour or diuretics (furosemide, Lasix) to optimize fluid status (86-88). It has been described that fluid resuscitation is important for Lactate clearance or to improve central venous hemoglobin saturation (ScvO$_2$) in septic, severe septic or septic shock patients. In a clinical trial when septic patients had resuscitation for lactate clearance $\geq$10 percent or improve ScvO$_2$ $\geq$70 percent, there was no difference in hospital mortality, length of stay, ventilator-free days, or incidence of multiple organ failure (89). Hypokalemia, hypocalcemia, hypomagnesemia and hypophosphatemia has been manifested due to diuretics during acute renal failure recovery (90).

The appropriate use of crystalloids such as saline solution, ringers lactate and dextrose and colloids such as hydroxyethyl starch and gelofusine is still under debate in septic shock (86). If hematocrit is less than 30%, red blood cells (RBCs) should be transfused until hematocrit is greater than 30 (87). If MAP is still less than 65 mmHg post fluid administration, vasopressors (norepinephrine) and vasopressin should be given to increase blood pressure and provide cardiac support (86).

Serum free cortisol (glucocorticoid) is a method to assess adrenal function during sepsis (91). Before 1990, high dose corticosteroids have shown detrimental effects in improving sepsis or septic shock (92). It has been stated that high dose of hydrocortisone for reversal of sepsis or septic shock can be harmful as excess hydrocortisone can cause hyperglycemia, cushing syndrome and adrenal suppression (93). High dose methylprednisolone has shown to reduce
TNFα induced (HBMVEC) human brain microvascular endothelial cell ICAM-1 and VCAM-1 expressions (94).

During early 2000, low dose corticosteroids have shown benefits in improving conditions of septic shock. The ability to show beneficial effects of cortisol in septic or septic shock patients is still under debate. Low dose hydrocortisone had shown some beneficial effects but in a most recent clinical trial even low dose corticosteroid administration did not reverse sepsis or septic shock in patients (86). In 2005, low dose hydrocortisone has reversed septic shock and reduced IL-1 and IL-6 plasma levels (95). In late 2000s low dose hydrocortisone has shown detrimental effects in improving septic conditions or septic shock (96). In contrast, recently documented that mild dose dexamethasone (0.25 mg/Kg) M/DEX or high dose (2.5 mg/kg) H/DEX significantly attenuated cytokine messenger RNA expression in the lung, liver, and kidney. However, low dose (0.05mg/kg) L/DEX could not significantly reduced plasma level cytokines but reduced cytokine messenger RNA expression in lung, liver, and kidney tissue and reduced the occurrence of bacteremia in mice post CLP (97). Low dose corticosteroids should be administered in septic shock patients where fluid resuscitation or vasopressors does not work. However, more clinical trial may be required for use of appropriate concentration and duration of low dose corticosteroid administration in septic patients (98). As such, many practitioners in US are still reluctant to use steroids therapy for severe septic shock as there is no survival benefit (99).

In case of organ failure in severe sepsis and septic shock, supportive measures are provided such as dialysis for kidney failure. (86). Some studies have shown that analgesics given during mechanical ventilation have prolonged duration of mechanical ventilation but interrupted analgesic treatment has decreased mechanical ventilation duration (86; 100; 101).
Glucose level should be maintained <150 mg/dL and during hyperglycemia in septic patients IV insulin infusion should be monitored every hour initially and then every 4 hours (86). Studies have shown reduction in mortality in Intensive care unit (ICU) patients who received insulin infusion. Moreover, in mice post 2 hit hyperglycemic model i.e femoral fracture followed by CLP, insulin therapy was associated with improvement in survival rates and few neutrophils infiltrates and reduced IL-6 and IL-10 mRNA expression in lung and liver. Therefore, insulin had a direct anti-inflammatory impact that was independent of reducing blood glucose levels (102).

Enteral feeding eicosapentaenoic acid (EPA), gamma-linolenic acid (GLA), and antioxidants with during severe sepsis or septic shock have shown improve oxygenation, more ventilator and ICU stay free days which is correlated with reduced mortality rate (103).

A.4.2.2 Experimental Strategies

Many anti-septicemic, anti-inflammatory and anti-coagulant therapies has been tried to improve survival (104; 105). Endotoxin neutralizing agents, bactericidal permeability increasing protein has shown improvement in preventing morbidity but when it was tried in children with fulminant meningococcemia, it did not improve survival (106; 107). Intravenous Immunoglobulin had been proposed as an adjuvant therapy for septic treatment but it is not recommended by physicians as there is a need of large clinical trials to evaluate the effect of doses and duration of this treatment (108).

Numerous clinical trials have been conducted to block inflammatory responses but still researchers are unable to answer if mortality due to sepsis can be controlled. It has been documented that increased dosage of endotoxin or cytokines has caused mortality (109; 110).
Anti-TNF α therapy has increased or decreased mortality in septic patients or animals (111; 112). In contrast, this does not reflect clinical scenario of sepsis in which people with sepsis had detectable TNF-α (5-10 pg/ml) and IL-1β levels and septic patients treated with TNF-α blocking agents has improved survival in some study and many have not been successful (113). Antagonist to TNF alpha, IL-1 beta, bradykinin, cyclooxygenase and caspases also have variable effect on survival of septic animals and in human (114-116).

Based on a presumed role of activation of coagulation pathways in sepsis, and reduced levels of activated protein C (APC), administration of APC (drotrecogin alpha, Xigris) as an anticoagulant, and possibly as an anti-inflammatory agent, can be beneficial in patients with severe sepsis or septic shock who have high risk of death. APC has been shown to reduce mortality in septic or septic shock patients but some studies have not shown any effect. (105). Some studies reported that it has adverse effect on bleeding so it should be avoided when there is a risk of bleeding (117). However, because of risk and lack of consistent benefit, APC was withdrawn as a treatment for patients in 2012.

Statins [(3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (HMG-Co A reductase inhibitors)] such as simvastatin have been used to reduce mortality in severe septic patients as compared to patients who did not receive it (104; 116; 118). Despite of advancement in the treatment of septic, severe septic or septic shock patients, mortality rate is still high. More research is required into cellular pathways of sepsis, including neutrophils and endothelial cells. There is currently no accepted therapy which targets the inflammatory aspect of sepsis, or the injury at the cellular level.
SECTION B

B. ACUTE LUNG INJURY/ACUTE RESPIRATORY DISTRESS SYNDROME

B.1 Definition

The definition of ALI/ARDS was initially recommended by American European consensus conference (AECC) in 1994 has been used in many clinical trials and research. According to Berlin criteris which has modified the features explained by AECC, diagnostic criteria for ALI include acute onset, the presence of diffuse radiographic pulmonary opacities, severe hypoxemia quantified by the ratio of partial pressure of arterial oxygen ($\text{PaO}_2$) to the fraction of inspired oxygen ($\text{FiO}_2$) less than 300 mmHg (ALI) or less than 200 mmHg (ARDS). The presence of pulmonary edema due to cardiac disease (mitral stenosis, left ventricle failure) is diagnosed by measuring pulmonary capillary wedge pressure (PCWP) clinically, or by measuring vascular permeability in research setting. Pulmonary edema due to non-cardiogenic cause is diagnosed when PCWP is less than 18 mmHg (119).

ALI/ARDS is caused by direct and indirect insult. Direct insult due to direct injury to the lung include pneumonia, acid or gastric aspiration, toxic inhalation, drowning, fat or amniotic fluid embolism, pulmonary contusion, alveolar haemorrhage, pleural effusion drainage, embolectomy, unilateral lung re-implantation and trauma. Indirect insult that is associated with sepsis, shock, blood transfusion, cardiopulmonary bypass, pancreatitis, salicylate or narcotic overdose (119; 120). Sepsis is one of the common indirect cause of ALI and has been focus of interest for clinicians and scientists.
B.2 Epidemiology

In 1967, Ashbaugh and colleagues termed ARDS (acute respiratory distress syndrome) in 12 critical ill patients in ICUs suffering with acute respiratory failure, decreased lung compliance and diffuse opacities that was evident on chest radiograph (121). Since then diagnostic criteria has been modified (122). ALI/ARDS is one of the leading causes of death in ICUs. Some have reported low mortality in patients that were admitted to non-ICU wards in US hospital (123). The incidence of acute lung injury varies between studies: 17-54 cases per 100,000 people in US in 1 study in 2005 which was higher than Europe, Australia and other developed countries (124), but another study suggested a higher incidence of 30 – 75 cases per 100,000 people in United states each year (125). The in hospital mortality rate was 38.5 percent for ALI and 41.1 percent for ARDS. In 2005, approximately 200,000 patients were affected annually in US with mortality rate of 40 % (126; 126). Rubenfeld and colleagues also stated that the incidence of acute lung injury was increased with age as they reported that 16 cases were affected per 100, 000 people with ages between 16- 19 and 306 cases were affected per 100, 000 people with ages between 75- 88 years (126).

ARDS patients, who survived, suffered from cognitive abnormalities, depression, muscle weakness, fatigue and post-traumatic stress (127). Mortality rate of ALI/ARDS patients has been decreased to almost 31% due to advancements in new ventilator strategies like low tidal volume ventilation that suggests that ALI/ARDS due to other non-hypoxemic cause like sepsis induced multiple organ failure also contributes to this deadly syndrome (128). Many ALI/ARDS patients
accompanied by severe sepsis or shock can cause non-pulmonary failures such as cardiovascular failure thus requires vasopressors, renal failure thus requires dialysis, liver failure and haematological disorders leads to anemia, thrombocytopenia (129). Thus, mortality rate of these ALI/ARDS patients due to non-pulmonary failures is still high so etiology and pathophysiology of ALI/ARDS has been subject of great interest by researchers.

B.3 Pathophysiology

ALI is subdivided into three different phases (130). During initial 12-72 hours of onset of ALI, there is increased accumulation of interstitial and alveolar edema that is associated with alveolo-cappillary membrane damage. This exudative phase which lasts for 1-7 days is characterised by increased inflammatory cells sequestration, thrombus formation, decreased blood flow and perfusion leads to hypoxia induced vasoconstriction, altered surfactant function due to changes in phospholipids, fatty acid, neutral lipid, imbalanced surfactant apoprotein composition, plasma protein interaction or any inhibitors in edema exudate, inflammation, epithelial and endothelial injury or incorporation of surfactant phospholipids with fibrin strands due to increased PAI-1 in alveolar compartment (131; 132; 132-135). It has been reported that surfactant therapy has improved gas exchange and normalized surfactant phospholipid and protein content in neonates (136). However, alveolar surface activity was significantly impaired before surfactant treatment and only partially improved after surfactant administration. Moreover, there was twofold increase in neutral lipid content and altered neutral lipid profile in broncho alveolar lavage fluid in patients with ARDS compared with healthy controls or even after large dose of surfactant administration (130; 137; 138).
This exudative phase leads to fibro-proliferative phase which causes collagen matrix production persists for 5-7 days of onset of ARDS. This collagen deposition leads to fibrosis, which correlates with increased mortality in ARDS patients (135). Moreover, recently it has been suggested that epithelial-mesenchymal transition (EMT) leads to pulmonary fibrosis (130; 137; 139).

Damage to pulmonary vasculature leads to activation of endothelial cells. Therefore, increased levels of plasma PAI-1 and broncho-alveolar lavage PAI-1 are increased in ALI (140; 141). Overwhelming inflammation is a prominent feature during exudate phase of ALI due to over production of cytokines (IL-1β, IL-6, IL-6ra) and cellular mediators such as NF-κB and sFasL and activated neutrophils contribute to epithelial damage and apoptosis (142; 142; 143).

B.3.1 Inflammatory cells
B.3.1.1 Neutrophils in ALI

ALI is characterized by increased protein rich edema fluid in the pulmonary interstitium and airspaces, which suggests pulmonary micro vascular endothelium is damaged. There is increased influx of activated neutrophils in the lung during development of ALI. Neutrophils are the first that recruits to the site of inflammation (142; 143).

Neutrophils recruitment in the lung occurs in stages, similar to the systemic circulation. However, rolling is not an important feature of neutrophil-PMVEC interaction in the pulmonary circulation. Neutrophil-PMVEC adhesion can be either β2 integrin-dependent or independent in acute lung injury, depending on the inflammatory stimulus. Adhesion can also be mediated
through L, P and E-selectin, but firm adhesion, especially in sepsis, is usually mediated through β2 integrin interaction with intracellular adhesion molecule (ICAM-1) and β1 (VLA-4 and VLA-5) integrins which interact with vascular cell adhesion molecule (VCAM-1) on endothelium (55-57). In human, deficiency of β2 integrins also called leukocyte adhesion deficiency (LAD), leads to recurrent infections (144). Inhibition of β2 integrins has reduced neutrophil recruitment (145). Integrin dependent firm adhesion on endothelial cells results in respiratory burst in neutrophils that further causes damage to endothelial cells (146).

The next step is neutrophil transmigration across PMVEC in a transcellular or paracellular manner. Migration occurs when PECAM-1 on neutrophils ligate with PECAM-1 on endothelium in some model of septic ALI. However, some have reported JAM-1 contributed to neutrophil trans-endothelial migration during inflammation and septic ALI (58; 59). Moreover, ICAM-2, JAM-A, and PECAM-1 also contributes to neutrophil migration through venular walls in vivo (60). Repeat explained above

Neutrophils are directly involved in endothelial protein leak in septic animals in model of ALI. Various studies have shown that neutrophils are involved in endothelial activation in sepsis induced ALI models (55-57). Prevention of neutrophil activation, neutrophil adhesion and neutropenia has attenuated edema in various animal model of septic ALI. Moreover, neutrophil depletion in chest trauma induced ALI has proved attenuated levels of IL-1 beta, IL-6, bronchoalveolar lavage protein concentration and lung myeloperoxidase activity in mice (68). Microvascular protein leak in sepsis-induced ALI is dependent on iNOS in inflammatory cells with no obvious contribution of iNOS in pulmonary parenchymal cells (69). Recently, we have shown in model of septic ALI that pretreatment with anti-CD18 before cecal ligation/perforation
markedly reduced septic increases in pulmonary myeloperoxidase, bronchoalveolar lavage neutrophils, microvascular EB-albumin leak, and 8-isoprostane content (70). Neutrophils are directly involved in endothelial activation, barrier dysfunction by increasing permeability and cytoskeletal derangement during sepsis (45; 71);(68). This suggests important contribution of neutrophil sequestration in edema formation during sepsis induced lung injury.

Neutrophil granules have played an important role during lung injury. Azurophilic (primary) and specific (secondary) are released when neutrophils enter the tissue, gelatinase (tertiary) are released during neutrophil transmigration and secretory vesicles are during endothelial and neutrophil contact. (147; 148). Neutrophil Protease such as elastase and cathepsin G is found in bronchoalveolar lavage fluid (BALF) and blood of ALI/ ARDS patients (149-151). Neutrophil Cathpsin G, elastase and proteinase-3 has caused endothelial injury and increased permeability in ALI (152; 153). Moreover, Neutrophil elastase and Cathepsin G has reduced only gram negative infections but not gram positive (154). Neutrophils Protease cathepsin G, elastase and proteinase 3 have shown to degrade surfactant A and D, anti-inflammatory proteins and increased lung permeability and coagulation (155; 156). Neutrophil protease elastase inhibitor, sivelestat sodium or neutrophil radical scavenger edaravone has shown attenuation of IL-6 and TNFα in rat lungs in a model of LPS induced lung injury (157). Neutrophils antimicrobial polypeptides lactoferrin levels are correlated with decreased pulmonary edema, inflammatory cells infiltration and pulmonary myeloperoxidase activity in LPS induced ALI in mice whereas, alpha defensins have detrimental effects in ALI as it is elevated in BALF in ALI/ARDS patients and in animals that are chemoattractants for monocytes and macrophages (158; 159).
B.4 Treatment of ALI/ARDS

To date there is no exact treatment for ALI/ARDS, therefore, combination of therapies supportive/non-pharmacological and pharmacological are being used to improve patient survival.

B.4.1 Non pharmacological Strategies

B.4.1.1 Mechanical Ventilation

The goal is to achieve $\text{PaO}_2 > 60 \text{mmHg}$ or arterial oxyhemoglobin saturation ($\text{SaO}_2$) > 90% with $\text{FiO}_2 < 60\%$ through mechanical ventilation either noninvasive (masks) or invasive (endotracheal intubation). During early 48 hours target oxygenation should be achieved otherwise there is a risk of oxygen toxicity which can damage lung airways, lung parenchyma, bronchopulmonary dysplasia in neonates, retinopathy of prematurity or retrolental fibroplasias. The main goal of mechanical ventilation is to provide adequate oxygenation, adequate alveolar ventilation, avoid overdistension and auto-PEEP.

The most commonly used supportive and protective therapy includes techniques of mechanical ventilation using low tidal volumes to limit end inspiratory plateau pressure ($<30 \text{cmH}_2\text{O}$). If the plateau pressure is $>30 \text{ cm of water}$, then tidal volume should be decreased to 5 mL/kg or as low as 4 mL/kg if required. It has been documented that high tidal volume can cause pneumothorax and low tidal volume can cause hypoventilation (160; 161). Moreover, mortality is also reduced in ARDS patients who were treated with using low tidal volume (6-8mL/Kg) as compared to high tidal volume group. Permissive hypercapnia is an important risk associated with low tidal volume in patients with ALI/ARDS. Hypoventilation increases $\text{PaCO}_2$
which results in respiratory acidosis. To treat respiratory acidosis respiratory rate should be increased. Moreover, physicians also recommend sodium bicarbonate infusion, Carbicarb or Tromethamine (THAM) if blood pH < 7.15.

Positive end expiratory pressure (PEEP) should be titrated according to lung compliance, inflation pressure curve, and stress index. PEEP is titrated on mechanical ventilators according to Vt (tidal volume) and frequency. PEEP is applied to reduce the need for supplemental oxygen i.e FiO$_2$≤0.6 (60%). PEEP increases mean airway pressure, improves gas distribution, reduces atelectasis, increases functional residual capacity (FRC), minimizes pulmonary edema, reduces shunt, improves ventilation perfusion mismatch and improves oxygenation and which allows physicians to reduce FiO$_2$. In addition, increased PEEP may cause alveolar overdistension and produce ventilator associated lung injury (VALI). Moreover, PEEP may increase intrathoracic pressure that decreases venous return and cardiac output (CO), and lowers blood pressure or results in hypotension in patients with hypovolemia and ALI. Furthermore, impact of PEEP on hemodynamics is still controversial. It has also been reported that high PEEP is beneficial for ARDS patients not for ALI. Experimental studies revealed that PEEP levels greater than traditional values of 5 to 12 cm H$_2$O can reduce mortality cyclical alveolar collapse and shearing pulmonary injury in patients with edema, alveolar collapse or ARDS.

Sometimes continuous positive airway pressure (CPAP) ventilation alone may be sufficient to improve oxygenation and it has better effect on blood pressure than PEEP due to increase in MAP with CPAP. However, in small number of patients with ARDS/ALI and in neutropenic patients, the use of this technique has allowed some patients to avoid intubation. The overall aim is to maintain acceptable gas exchange and to minimize adverse effects during procedure. It has
been reported that CPAP has been used to reduce FIO₂ below 65%. To find optimal or appropriate level of CPAP in patients with ARDS is under discussion. In patients with ALI, CPAP may reduce shear forces that accelerate ventilator-associated lung injury by keeping the alveoli in an expanded state throughout the respiratory cycle.

Mechanical ventilated patients are also at the risk of deprived sleep, malnutrition, hypotension, oliguria and gastrointestinal bleeding. Antacid, proton pump inhibitors or histamine blockers (H₂) are used to reduce gastrointestinal complications.

B.4.1.2 Alternative or Rescue Ventilator Modes in ARDS

To treat severe hypoxemia or hypercarbia with acidemia despite optimal treatment with low-tidal volume mechanical ventilation in patients with severe ARDS, alternative ventilator strategies are used.

B.4.1.3 Inverse ratio ventilation (IRV) and Airway pressure release ventilation (APRV)

Some physicians use pressure controlled ventilation strategy to manipulate the mean airway pressure by prolonging inspiration, and this method has shown to improve oxygenation without increasing peak or plateau pressures and it also improves gas distribution at the end of inspiration. During hypoxemia in ARDS patients, longer Inspiratory times than expiration (inverse I:E ratio ventilation, ratio 7:1) may be beneficial. However, patient feels discomfort and really needs heavy sedation that can reduce this effect of auto-PEEP and hypotension. Neuromuscular paralysis has improved ventilator-patient synchrony and often improved
oxygenation in patients with ALI. In patients with severe ARDS (PaO$_2$/FiO$_2$ ratio<150), 48 hours of neuromuscular paralysis with cisatracurium (Nimbex) has improved oxygenation and improved mortality.

Newer pressure control modes such as Bilevel/APRV (Airway pressure release ventilation) have been used to reduce discomfort that occurred with IRV in patients with ALI/ARDS. APRV has attenuated airway pressures, minute ventilation and dead space ventilation and increased spontaneous breathing and also reduced use of sedation and increased cardiac output.

High-frequency oscillatory ventilation (HFOV) is a ventilator mode that uses low tidal volumes and high respiratory rates. In largest randomized controlled trial, it has been found that the HFOV group had early improvement in oxygenation that could not persist beyond 24 hours as compared to conventional ventilation. Mortality is also reduced in HFOV group and it may be the most useful for patients with bronchopleural fistulae (160-162). Recently, it has been documented that HFOV is not beneficial in patient’s survival as in hospital mortality was 47% in the presence of 6ml/kg tidal volume and adequate PEEP as compared with control which was 35%.

B.4.1.4 Liquid ventilation

Novel modes of liquid ventilation have been studied, and are occasionally used clinically. These include perfluorocarbon (PFC) for partial liquid ventilation, perfluorohexane for PFC Vaporization and perfluorooctance for aerosolization (163-165). Resolution of gas exchange
impairment with liquid ventilation PFC has been beneficial in preterm neonates as compared to PEEP that can lead to broncho pulmonary dysplasia (166). Partial liquid ventilation has also been tried in ARDS but mortality is still higher (167). Vaporization of Perfluorohexane or aerosolization of perflurooctane has been shown to improve gas exchange in oleic acid-induced lung injury in sheep (168).

B.4.1.5 Prone position

It is used to open or increase surface area of atelactic lung and improve oxygenation but still this strategy is failed in reducing mortality in ALI/ARDS patients. In contrast, in most severe ARDS patients prone positioning has reduced mortality. It is still considered a rescue therapy for refractory hypoxemia (169). The most common complications of prone positioning ventilation include extubation and the development of pressure ulcer.

B.4.1.6 Extracorporeal membrane oxygenation (ECMO)

ECMO is not routinely used as a rescue therapy for ALI/ARDS but still it has been used to treat refractory hypoxemic and severe ARDS patients to improve oxygenation (170). ECMO is associated with complications and increased mortality in ARDS patients such as hemorrhage (171). However, scientific evidences for ECMO in ARDS patients with life threatening hypoxemia and VALI is also lacking. Thus, there is a need for more clinical studies.

B.4.2 Pharmacological Strategies
Fluid Management. The most recent trial in ALI management is to restrict fluid intake specially ARDS due to pneumonia, inhalation injury, aspiration but ARDS due to inflammation and infection or septic shock may require initial fluid therapy to stabilize patient. In ARDS or ALI clinical trial study of a fluid-conservative strategy versus a fluid-liberal strategy in the management of patients with ARDS or acute lung injury (ALI) found no statistically significant difference in 60-day mortality between the 2 groups 72 hours after presentation with ARDS (172). Patients treated with the fluid-conservative strategy had an improved oxygenation index and lung injury score and an increase in ventilator-free days, without an increase in nonpulmonary organ failures. Fluid restriction strategy has reduced duration of mechanical ventilation but has no effect on mortality in large clinical trial studies in patients with ALI who were not in shock (173).

Diuretics are used to minimize the left ventricular filling pressure by fluid restriction. Even if PCWP is less than 18 mmHg, reduced oncotic pressure can cause septic ALI however, combination of Albumin and furosemide therapy has significantly improved oxygenation, net negative fluid balance and hemodynamic stability but not mortality in ALI/ARDS patients (174).

Transfusion of packed RBCs in patients with low hematocrit, however, recently documented that in patients with new-onset ALI, sepsis and shock, there is no correlation between RBC transfusion and mortality or ventilator free days. This suggests RBC transfusion has no effect on ALI patients mortality rate (175).
Antibiotics. Evidences suggested that more than 60% ARDS patients have pulmonary infection either before or after the onset of lung injury. Thus, appropriate antibiotic therapy must be administered as already discussed in sepsis section (83).

Sedatives. To avoid patient ventilator synchrony that causes anxiety, sedatives are given. In patients with severe ARDS receiving mechanical ventilation, administration of neuromuscular blockers such as cisatracurium showed improvement in 90-day survival and increased time off the ventilator (101; 176).

Vasopressors. In order to maintain MAP more than 70 mmHg, vasopressors and vasopressins may be required and norepinephrine and dopamine is mostly preferred (86).

Steroids. Particularly in sepsis induced ALI, early, high-dose steroid administration may decrease pathogen clearance, causes myopathy and reduces wound healing (130). It has been stated that high dose of hydrocortisone for improvement in ARDS can be harmful as it did not reduce mortality (93; 177). Literature suggested a benefit in starting corticosteroids within 72 hours of ARDS onset whereas others supported the use of corticosteroids in ALI prior to 14 days from ALI onset (178). Low dose hydrocortisone had shown some beneficial effects but in a most recent clinical trial even low dose corticosteroid administration did not reverse sepsis or septic shock in patients with ALI (86).

Surfactants. Surfactant and edema proteins interactions lead to surfactant inactivation. As a result there is reduction in surface tension in alveolar compartment that leads to atelectasis. Surfactant administration has improved oxygenation in neonatal RDS, as well as infants, adult patients and in animal model of ALI/ARDS but not mortality (135). Inhaled nitric oxide has shown to
improve oxygenation in patients with ALI/ARDS, but could not improve mortality and has been associated with acute kidney injury (179). It is used only as a rescue therapy for refractory hypoxaemia (169).

Nutrition. Standard supportive care should be given to ALI/ARDS patients that include adequate nutrition omega-3 fatty acid (eicosapentaenoic acid) along with gamma-linolenic acid caused a marked attenuation in risk of mortality in some studies, risk of developing new organ failures, time on mechanical ventilation, and ICU stay (180).

Anti-coagulants. Other therapies include prevention of DVT by using anticoagulants such as LMW heparin or thrombolytics such as urokinase, streptokinase, tissue plaminogen activator (181; 182).

Control of hyperglycemia. Hyperglycemia can be controlled by infusion of insulin, PPAR agonist, ACE-1, metformin, HMG Co-A reductase inhibitors in septic patients with ALI (183).

B.4.3 Experimental Strategies

Human model of ALI, one lung ventilation (OLV), a technique required to facilitate lung resection surgery, has been used to investigate potential biomarkers of VALI. Some studies have shown that use of low tidal volume during OLV was associated with reduced biomarkers of pulmonary and systemic inflammation whereas, high tidal volume and airway pressure resulted in development of ALI (184; 185).

Several lines of evidence suggest that using beta agonists to treat acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) may be beneficial (186). Terbutaline, a short-
acting beta 2 adrenergic receptor agonist, increases the clearance of alveolar edema in lung explants (187). Salbutamol, beta 2 adrenergic receptor agonist has also been administered in animals for edema clearance (186). Intravenous salbutamol decreases alveolar-capillary permeability in patients with ARDS, possibly by simulating alveolar wound repair (188). Albuterol is not beneficial in improving ALI parameters during mechanical ventilation (189). Inhaled salmeterol, a long-acting $\beta_2$ adrenergic receptor agonist, reduces the incidence of high-altitude pulmonary edema (190). cAMP agonist and adenosine dependant signalling pathways (A2BAR) are being studied for lung edema clearance during VALI (191).

Intravenous bone marrow mono nuclear cells administration has reduced inflammation, alveolar collapse, interstitial edema (192). Intratracheal and intravenous administration of mesenchymal stem cells have improved oxygenation and lung function as well as sepsis in mice but in human engraftment of MSC in lung in patients with severe ARDS is still in trail phase (193; 194).

Statins (simvastatin) have shown to reduce pulmonary dysfunction HARP-2 in ALI patients (195).

B.4.4 Future approaches to ALI/ARDS management

Extra-corporeal carbon dioxide removal (ECCO$_2$R) systems can be used to detect novel biomarkers for VALI. In ALI/ARDS patients ECCO$_2$R can be used to detect its effectiveness along with low tidal volume strategies and inflammatory biomarkers or lung injury biomarkers, (procollagen peptide III in lavage) can be detected.
SECTION C

C. ENDOThelial CELLS

C.1 Normal Physiology

The vascular endothelium is composed of 1-6 x10^{13} squamous epithelial cells that forms the inner lining of blood vessels and lymphatic vessels and covers an area of 1-7\text{m}^2 (196). The healthy vascular endothelium regulates many aspects of vascular function, including vasomotor tone, cellular and nutrient trafficking, blood fluidity, and participates in angiogenesis. Endothelial cells release vasoactive molecules that include vasodilators nitric oxide (NO) and prostacyclin and the vasoconstrictors (endothelin-1, ACE, thromboxane A2, and platelet activating factor) that regulate arteriolar tone and maintain blood pressure.

Normal vascular endothelium is responsible for establishing an anti-thrombotic environment. Endothelium maintains hemostasis by regulating the coagulation and fibrinolysis cascade through production of von Willebrand’s Factor (VWF), which is stored in Weibel-Palade bodies (0.1 \text{µm} \text{ wide by 3 } \text{µm} \text{ long membrane-bound structures}), thrombomodulin (TM), and tissue factor plasminogen activator (t-PA). In part, the anti-thrombotic surface consists of the endothelial glycocalyx, which is comprised of proteoglycans and glycoproteins incorporating mediators secreted by EC, such as NO, prostacyclin, heparin and activated protein C, that prevent platelet aggregation and clotting. (197-201).

The pulmonary endothelium participates in the exchange of water and solutes between the blood and the interstitium through passive transport. Water transport occurs through endothelial water transport channels called aquaporins. Under normal conditions, a small amount of fluid is
filtered across the endothelial monolayer and drained through lymph. Tight and adherens junctions between endothelial cells control this fluid filtration. Albumin and other macromolecules are actively transported through cellular vesicles.

The endothelium is extremely important in the regulation of both innate and adaptive immune responses, through interaction with pro- and anti-inflammatory soluble mediators as well as with circulating inflammatory cells. The specialized morphology of endothelium controls trafficking of leukocytes to the site of infection as well as permeability (202).

C.2 Endothelial structural heterogeneity

Although all vascular endothelial cells (ECs) share common functions, there is significant heterogeneity between macrovascular and microvascular endothelial cells, as well as regional variation in different vascular beds. Arteriolar endothelium plays an important role in controlling vasomotor tone whereas post capillary venules are specialized for leukocyte adhesion and transmigration.

Endothelial cells are classified as continuous or discontinuous. Continuous endothelial cells are fenestrated or non-fenestrated. Nonfenestrated continuous endothelium is found in arteries, veins, and capillaries of the lung, brain, skin and heart and is characterized by the presence of tight junctions and adherens junctions. Molecules cross this through active transcytosis by caveolae and vesiculo-vacuolar organelles or through clathrin coated pits for endocytosis. Caveolae (70 nm in diameter) are present in all types of endothelium but are most prevalent in capillaries that contain continuous nonfenestrated endothelium with the exception of blood brain barrier where caveoli are rare (203-205).
Fenestrated continuous endothelium is found in capillaries of exocrine and endocrine glands, gastric and intestinal mucosa, choroid plexus, glomeruli, and a subpopulation of renal tubules for increased filtration or increased transendothelial transport. Fenestrae are transcellular pores (≈70 nm in diameter) that extend through the full thickness of the cell. The majority of fenestrae that are transcellular pores (≈70 nm in diameter) possess a thin 5 - 6-nm nonmembranous diaphragm. Fenestral diaphragms possess integral membrane glycoprotein PV-1 with the exception of the glomerular endothelium (206). Discontinuous fenestrated endothelium is found in certain sinusoidal vascular beds, liver and bone marrow because they lack a well-formed basement membrane. It has large heterogeneous fenestrae (100 to 200 nm in diameter) without diaphragms (205-207). It has few caveolae and contains clathrin-coated pits and vesicles that play an important role in receptor-mediated endocytosis (208).

C.3 Endothelial functional heterogeneity

Endothelial cell function heterogeneity is also critical to understand pathophysiology of various diseases. For example, pulmonary arterial endothelium expresses a large amount of endothelial nitric oxide synthase (eNOS), and releases more nitric oxide (NO), than capillary endothelium. Pulmonary microvascular and macrovascular endothelial cells exhibit certain functional differences that include barrier properties, signal transduction, apoptosis, growth rate, flow alignment and mechanosensing, proliferation, shape, organelle distribution, gene expressions, cytoskeletal arrangements, ion channel expression and function.

Leukocytes transmigrate from blood to infected tissue through a series of cascade that include capturing, adhesion, rolling, firm adhesion and transmigration. Initial capturing and
adhesion of leukocytes involves ligands and endothelial selectins such as P-selectin and E-selectin. E-selectin is highly restricted to activated endothelial cells mainly in postcapillary venules and in bronchial circulation (209; 210). Endotoxemia in mice has increased cell surface P-selectin and E-selectin in all tissues including lung and mesentery, heart, brain, stomach, pancreas, intestine, and muscle, but largest amounts of both selectins were found in lung, small intestine, and heart (211). In another study, lipopolysaccharide administration to mice has increased E-selectin mRNA expression 14.6-fold in the heart, 5.6-fold in the lung, 3.8-fold in the liver, 18-fold in kidney, 5.3-fold in brain, and 0.6-fold in the spleen (212).

The important function of endothelium is to maintain hemostasis and maintains a balance between coagulation and fibrinolysis. On procoagulant side, endothelial cells produce TF, PAI-1 and VWF (213). It has been documented that t-PA expression in mice endothelium was confined to arteries of pulmonary system and central nervous system. However, after maturity t-PA levels in brain becomes less in large arteries but persists in small vessels. PAI-1 expression is highest in lung, followed by heart, brain, spleen, liver, and kidney in mice (207; 212). However, endotoxemia has shown 187-fold increase in PAI-1 in the liver and 3-fold increase in spleen (207).

Endothelial cells have remarkable heterogeneity in function, but still researchers are keen to explore more functions that contribute to pathophysiology of many diseases.

C.4 Endothelial activation in sepsis

During sepsis, microvascular function is altered. Since microvascular EC regulate microvascular function, septic microvascular dysfunction is largely due to endothelial cells being
activated, injured or become apoptotic (196; 211; 214). Many studies have shown that EC can be injured by many mediators in sepsis, such as iNOS, COX-1, COX-2, ACE, ECE, Matrix metalloproteinase, viral or bacterial and other non-bacterial pathogens, cytokines, hypoxia, glucose, ionizing radiation, temperature, mechanical trauma, drugs and poisons (196; 202; 211; 214-224).

There is no standard way to define endothelial activation in sepsis (225). However, many researchers have described it as phenotypic change that occurs during sepsis from anti-adhesive to proadhesive. As a result EC express adhesion molecules on the cell surface, such as P-selectin, E-selectin, ICAM-1, and VCAM-1. These cell surface markers are involved in increased rolling, strong adherence, and transmigration of leukocytes into tissue (197; 199; 226). Activated ECs also recruit increased numbers of platelets to the blood vessel wall (227). Many studies have reflected the importance of these adhesion molecules in sepsis (228).

Many have correlated endothelial activation with physiological phenomenon such as coagulation, inflammation, sepsis, and related diseases. For example, changes in EC expression of PAI-1 have been suggested as an important marker of EC activation. Others have characterized endothelial activation as induction of cell surface proteins, adhesion molecules, cytokines release, cytoskeletal disruption, cell shape, permeability (229-232).

There are many other molecules which have been studied and proposed to indicate EC activation, such as VEGF, endothelin, ACE, and others. For example, under normal conditions, VEGF signaling plays a critical role in homeostasis and is protective against EC barrier dysfunction. VEGF binds to two receptors on endothelial cells, VEGF receptor (VEGFR) 1 and
2. VEGFR1 is also known as Flt-1. In sepsis circulating levels of VEGF are increased which may lead to increased vascular leak, leukocyte adhesion/trafficking, and thrombosis (233-236).

There is evidence that endothelial activation is clearly associated with poor prognosis in various diseases including sepsis.

C.4.1 E-selectin

C.4.1.1 Role of E-selectin

E-selectin or ELAM (endothelial leukocyte adhesion molecule) is a 107-115 kDa endothelial transmembrane glycoprotein (237; 238). It facilitates the binding of leukocytes and certain lymphocytes to activated endothelium (239; 240). Phosphorylation of the cytoplasmic tail of E-selectin may cause endothelial cytoskeletal changes that lead to permeability and facilitate diapedesis of leukocytes (241; 242).

E-selectin shares the same PSGL-1 on leukocytes just like P-selectin (243). It has been shown that soluble E-selectin is deficient in PSGL-1 knockout mice, suggested importance of PSGL-1 in solubilization of E-selectin (244). Moreover, during inflammation E-selectin activates β2 integrins, modulates leukocyte movement, involved in augmentation of reactive oxygen species (245). Adhesion of leukocytes to endothelium also provide a link between E-selectin and cytoskeletal proteins such as α-actinin, vinculin, filamin, and paxillin, focal adhesion kinase (FAK), a tyrosine kinase localized to focal adhesions of adherent cells that facilitate leukocyte transmigration through endothelium (246; 247).
C.4.1.2 Expression of E-selectin

E-selectin is inducible. In non-placental tissues E-selectin expression appeared truly endothelial specific. Recently, cardiac fibroblast has expressed E-selectin in response to cytokines stimulation (248; 249). It is stimulated by LPS, IL-1, IL-13, TNF-α and IFN-γ (249; 250). Combinations of cytokines like TNF-α and IFN-γ increases synthesis of E-selectin (251). E-selectin is transcriptionally regulated by NF-κB (252) and activator protein-1 (AP-1) (253) and can be blocked by inhibitors of transcription, translation or cytokines, including actinomycin D, cyclohexamid or transforming growth factor-β (TGF-β) (254-256). In addition, transcription factors such as ATF-2/Jun plays an important role in the transient induction of the E-selectin mRNA in EC (257). Moreover, endothelium-specific histone modifications and chromatin remodeling at the E-selectin promoter also contributes to the induction of E-selectin gene due to involvement of coactivators p300 and IkB kinase (258; 259). Some other unidentified regulatory factors such as HOXA9, also contribute to induction of the E-selectin gene post TNF alpha stimulation (260).

In HUVEC, strong expression of E-selectin has been detected at 4 and 24 hrs after IL-1β stimulation and returned to baseline after 24 hrs (239; 261; 262). Four important regulatory elements have been identified in E-selectin promotor that contributes to E-selectin expression upregulation during sepsis. Three of these are NF-κB binding sites whereas, one is ATF binding element. NF-κB binding elements are not sufficient for E-selectin expression induction post cytokines stimulation. Therefore, ATF binding sites plays a significant role in E-selectin expression upregulation after cytokine stimulation. TNF alpha has been shown to upregulate E-
selectin expression through activation of three important signaling pathways NF-κB, JNK1, P38 (263).

C.4.1.3 E-selectin in diseases

Polymorphism of the E-selectin gene in humans is associated with early onset, severity of sepsis (264). E-selectin gene polymorphism is caused when serine is replaced by arginine at position 128(265). Studies have shown that polymorphism has altered E-selectin ligand binding by enabling E-selectin to recognize heparin or the non-fucosylated sialyl lactosamine precursor of sLeX abnormally (266). The defect in the GDP-fucose transporter and lack of α1,3 fucosylated carbohydrate ligands that binds to selectins resulted in leukocyte adhesion deficiency II (LAD II) in humans. Patients with LAD II suffer not only from a less severe form of infection but also from severe psychomotor and growth retardation. The endothelium in LADII also retains reduced expression of fucosylated leukocyte L-selectin ligands. Human LADII patients resemble mice that lack L, P and E selectin genes and therefore there is leukocytosis, reduced rolling migration of neutrophils to the site of infection (267).

Appropriate fucosylated selectin ligands, namely Lewis carbohydrate antigens, are critical in leukocyte recognition of selectins and in leukocyte migration to the site of inflammation. Phosphorylation of the cytoplasmic tail of E-selectin may cause endothelial cytoskeletal changes that lead to permeability and facilitate diapedesis of leukocytes (241; 268). Ligation of E-selectin by L-selectin, PSGL-1 and/or CD44 expressed on leukocytes causes activation of αMβ2 (CD11b/CD18, Mac-1) on the leukocyte surface through phosphorylation of
p38 and p42/44 MAPKs, which causes neutrophil recruitment through the endothelium to inflamed tissue (269). However, p38 MAPK specific inhibitor BIRB 796 BS has significantly reduced plasma E-selectin expression after LPS administration in human (270).

During inflammation E-selectin has activated β2 integrins, modulated leukocyte movement and involved in augmentation of reactive oxygen species (269; 271). Adhesion of leukocytes to endothelium also provide a link between E-selectin and cytoskeletal proteins such as a-actinin, vinculin, filamin, and paxillin, focal adhesion kinase (FAK), 2′1 tyrosine kinase localized to focal adhesions of adherent cells that facilitate leukocyte transmigration through endothelium.

C.4.1.4 E-selectin in sepsis

Various studies have used endothelial E-selectin as a marker of sepsis, severe sepsis, septic shock and other sepsis related diseases. In human patients, E-selectin gene expression, soluble E-selectin expression and endothelial cell surface E-selectin are elevated in sepsis and sepsis related disorders like ALI (239; 261-263). In another study, during early sepsis, exposure to hypoxia and arterial hypertension was associated with increased E-selectin expression in human patients (272).

In experimental animals and in cell culture, a variety of endothelial cells have expressed E-selectin in response to septic stimulation (239; 261-263). In mice, E-selectin mRNA levels in both pulmonary and hepatic microvascular endothelial cells are dramatically increased at 3 hours post CLP, then they returned to basal level at 12 hours post CLP (272; 273). In another study,
soluble E-selectin levels are elevated post CLP and in mice 12 and 24 hrs (272; 274). LPS has been shown to activate TLR2 that has affected endothelial activation, coagulation in sepsis (272; 272; 275; 276). TLR2 agonists, such as peptidoglycan-associated lipoprotein, Pam3Cys, and murein lipoprotein have been shown to induce E-selectin expression in HUVEC, human lung microvascular endothelial cells (HLMVEC) (272; 272; 277; 278).

Similarly, mice when intravenously administered with TLR2 agonists, mRNA levels of E-selectin in lung and soluble E-selectin level in plasma were augmented. In mice, cytokines like TNFα have shown to increase E-selectin expression in lung microvascular endothelial cells. Interestingly, TNF receptor 1 (TNFR1) or p 55 signaling contributes to E-selectin expression upregulation in lungs in sepsis (279). TNFR1 is shed by proteolytic cleavage by the metalloproteinase TNF α converting enzyme (TACE or ADAM17) in sepsis from HUVEC and mice LMVECs. Recently, it has been documented that by blocking TNFR1 shedding, inflammation is increased in mice. Similarly, preventing TNFR1 shedding from endothelial cells by inhibiting mitochondrial Ca++ or ROS (H2O2) has also augmented E-selectin expression in lung microvessels under septic conditions. This suggests mitochondrial ROS protects endothelial activation or decrease E-selectin expression in sepsis (280).

In a co-culture model, septic stimulation has increased or decreased E-selectin expression in endothelial cells in the presence of other cells. In a co-culture cell model, neutrophils have enhanced E-selectin expression in endothelial cells (HUVEC) after septic stimulation like TNF alpha (281). In a co culture model, HUVEC in the presence of whole blood cells or mononuclear cells pretreated with biomaterial (polysterene) augmented E-selectin expression in HUVEC after 4 hours of static co-culture. Similarly, the presence of ‘more secretory VEGF’ human aortic
smooth muscle cells with human aortic endothelial cells has increased E-selectin in HAEC after stimulation with biomaterial treated mononuclear cells or fMLP (282). In another cell culture model, E-selectin in medium and on HUVEC cell surface is increased when HUVEC were treated with THP-1 culture medium containing LPS/human plasma (283). There is little direct evidence for neutrophil effects on pulmonary microvascular EC E-selectin expression.

Many other cells have shown to suppress E-selectin expression in endothelial cells in sepsis. Such as, in a bilayer with EC, alveolar epithelial cells A549 reduced expression of E-selectin on the EC in response to LPS, but not after TNF-alpha stimulation (284).

Surface expression of E-selectin leads to either shedding, or slow internalization from the endothelial surface to lysosomes for degradation. Due to smaller size and MW, circulating E-selectin is considered as a microparticle that possesses the ability of leukocytes binding. It has been reported that lower cell surface levels of E-selectin found in sepsis are not necessarily related to shedding of E-selectin. E-selectin is clustered in lipid rafts and clathrin coated pits but is internalized by clathrin coated pits. Localization of E-selectin at both regions facilitate neutrophil rolling under flow free conditions (285).

Circulating levels of soluble E-selectin (sE-selectin) are increased in a variety of diseases, such as sepsis and other inflammatory disorders, hypertension, diabetes, and hyperlipidemia (233; 240; 269; 283; 286). Soluble E-selectin has been detected in supernatants of cytokine-activated endothelial cells and is elevated in serum levels of septic animals, and in patients with sepsis, ALI, hypertension, diabetes, and hyperlipidemia. For example, single intravenous injection of lipopolysaccharide (LPS; 4 ng/kg) has shown to increase sE-selectin in
early septic human patients (287). As well, human patients with severe acute pancreatitis (SAP) had elevated levels of sE-selectin and endothelial permeability in patients with sepsis as compared to people without sepsis or healthy volunteers (288; 289). Low dose prednisolone has shown beneficial effects in the treatment of the ARDS and septic shock. Levels of sE-selectin were prevented by a low dose of (3mg) prednisolone but maximal inhibition occurred with the highest dose of prednisolone 30 mg in a human endotoxemia (289).

In short, the evidence strongly suggests that E-selectin expression is a marker of EC activation under inflammatory conditions, such as sepsis.

C.4.2 PAI-1

C.4.2.1 Role of PAI-1

PAI-1 is a 50-kDa glycoprotein that is a member of the serine protease inhibitor (serpin) family. PAI-1 is involved in vivo in inhibition of both tissue- and urokinase-type plasminogen activators (290). In addition, PAI-1 acts as an acute-phase protein in periods of acute inflammation. PAI-1 has 3 isoforms in the blood circulation: active, inactive, and a latent form. The active form converts transiently into the latent form with a half-life of 1 h. The latent form is more stable and can also be reconverted into the active form when complexed with vitronectin. PAI-1, urokinase and tissue type plasminogen activator inhibitor, is constitutively expressed by endothelial cells and then stored in sub cellular matrix bound to vitronectin (291; 292). The half-life of PAI-1 can be increased when endothelial or platelet derived PAI-1 is complexed with vitronectin. Vitronectin/PAI-1 complex is an inhibitor of activated protein C (APC) and
thrombin in the absence of heparin. The detachment of PAI-1 from extracellular matrix induces F-actin in endothelial cells that causes cytoskeletal rearrangement and cell morphology changes (293)

C.4.2.2 Expression of PAI-1

PAI-1 is synthesized by a number of cells in vitro e.g., hepatocytes, endothelial cells, platelets, smooth muscle cells, adipocytes, cardiac myocytes and tumor cells. It has been reported that PAI-1 is found in the alpha-granules of platelets, where PAI-1 is primarily stored in the latent, free form (∼90%) and is released after vessel trauma. The normal human plasma level of active PAI-1 is 5-10 ng/ml. PAI-1 can be measured either as PAI-1 activity or as PAI-1 antigen. PAI-1 antigen exists as an active, inactive, latent or free form, or in complex with vitronectin, tissue plasminogen activator, or both (291; 294). The secretion of PAI-1 is regulated by a number of agents, such as TNF-α, IL-1β, transforming growth factor–β, endotoxin, very-low-density lipoprotein, glucose, glucocorticoids, and insulin, angiotensin II and estrogen. PAI-1 is removed from the circulation by the liver and also is regulated by endothelial inactivation. PAI-1 plasma level undergoes a circadian pattern, which shows peak levels in the early morning.

C.4.2.3 PAI-1 in diseases

It has been documented that endothelial cell PAI-1 mRNA level, cell surface expression and soluble levels in circulation are increased during sepsis, sepsis induced lung injury and other cardiovascular diseases. Moreover, increased PAI-1 levels play important role in the pathogenesis of acute lung injury, pulmonary edema, and DIC. PAI-1 may also contribute to
vascular permeability during early lung inflammation. However, at later hours PAI-1 contributes to resolution of lung inflammation as disruption of PAI-1 at later stages increased mortality in P. aeruginosa induced pneumonia in mice (295).

C.5 Role of Endothelial cells in sepsis

C.5.1 Increased permeability

Sepsis induces endothelial damage that leads to increased capillary permeability. Sepsis upregulates endothelial adhesion molecules that interact with neutrophils and result in rolling, adhesion and migration of neutrophils through endothelium that results in increased endothelial damage and increased permeability. Moreover, angiopoietins such as angiopoietin-2 is involved in sepsis induced increased permeability and associated with increased mortality in septic patients (296). In contrast, endothelial damage is prevented when angiopoietin-1 binds with Tie-2 receptor (297). In addition, VEGF and its receptor (fms like tyrosine kinase-1) are also associated with increased permeability during sepsis or septic ALI (298).

C.5.2 Proadhesive properties

Activated endothelial cells exhibit proadhesive property during sepsis or septic ALI. As a result leukocytes or platelet adhere to damaged endothelium. Adhesion of leukocytes on endothelial cells triggers ROS production, increases upregulation of P-selectin, E-selectin, ICAM-1, VCAM-1 on cell surface. Adhesions of platelets to endothelial cells result in platelet aggregation and thrombosis (233; 240; 269; 283; 286; 280)
C.5.3 Procoagulant properties

There are many factors that activate endothelial cells such as complement, cytokines, chemokines, serine proteases, fibrin, activated platelets and leukocytes, hyperglycemia, and/or changes in oxygenation or blood flow. Inflammatory mediators activate endothelial cells to induce a net procoagulant phenotype. Many studies have shown that LPS and cytokines can inhibit expression of the anticoagulant factor TM, decrease APC, t-PA, heparin and increase expression of TF and PAI-1 to generate procoagulant microparticles (291; 292; 293). Sepsis associated changes in TM expression varies between organs. In mice, the administration of LPS has resulted in reduction in total tissue TM antigen in the lung and brain, but not in the kidney (299). In sepsis, endothelial cells activate neutrophils, platelets, monocytes that are also capable of initiating coagulation. Moreover, endothelial cells undergoing apoptosis also amplify coagulation process.

In various models of sepsis, and in humans fibrin deposition has been observed in various organs. In a mouse model of endotoxemia, LPS administration has resulted in fibrin deposition in the kidney, adrenal gland, lung (300). Still others have shown that LPS injection in wild-type mice has increased fibrin levels in the kidney, liver, and myocardium, but not the lung (301). In a baboon model of sepsis, the lethal doses of E.coli administration has resulted in increased fibrin deposition in the marginal zone and sinusoids of the spleen, the hepatic sinusoids, the glomeruli, and peritubular vessels of the kidney, but little or no fibrin in the portal vessels of the liver,
cerebral cortex, skin, myocardium, or aorta (302). These studies reflect the association between sepsis and coagulation.

C.6 Role of Endothelial cells in ALI-ARDS

C.6.1 Endothelial activation

During septic ALI, endothelial cells are activated and upregulate adhesion molecules (P-selectin, E-selectin, ICAM-1, VCAM) that are important for leukocyte recruitment, adhesion and transmigration into interstitium and alveolar region (210; 211; 302). In various diseased animal models and clinical models, these adhesion molecules are increased and found in lung and serum. These molecules are upregulated after endotoxemia/ LPS and cytokines (TNF alpha, IL-1 beta) through various signaling pathways (220; 263). Transudation of plasma proteins into airways promotes formation of a fibrin matrix deposition that can lead to pulmonary fibrosis due to increased levels of PAI-1. Whereas, Plasminogen activators (PA), urokinase PA (uPA) and tissue-type PA (tPA), convert plasminogen to plasmin, which in turn lyses fibrin matrices and thereby exerts anti-fibrotic and protective effects in ALI patients (201; 287).

C.6.2 Endothelial Injury / Barrier Dysfunction

Sepsis induces endothelial damage that causes endothelial barrier dysfunction. Lung inflammation is associated with increased pulmonary microvascular (PMV) permeability leading to exudation of plasma proteins into alveolar spaces, interstitial edema, impaired gas exchange,
Increased morbidity and mortality. Numerous efforts have been made to understand how endothelial barrier function is lost during early sepsis, but this knowledge will be beneficial to prevent excess permeability from developing and to improve clinical outcome (223; 303). Cultured pulmonary microvascular endothelial cells (PMVEC) represent an attractive model for the study of barrier function.

Increased alveolar capillary barrier dysfunction is a result of endothelial actin cytoskeletal damage, epithelial cell damage, loss of integrity between cell connections (tight and adherent junctions) which leads to pore formation (223; 303).

Evidences suggested that there is an interaction between iNOS, cytoskeleton and junctional proteins and protein leak. iNOS is induced by LPS and cytokines TNF alpha and IFN gamma. iNOS plays an important role in disrupting junctional proteins. It has been documented that disrupted F actin formation has potentiated LPS induced iNOS expression. Moreover, iNOS induction followed by cytokines has relocated VE cadherin to the site of leak (304). It has been stated that LPS mediates protein leak / endothelial barrier dysfunction through various pathways. In animal model of ALI, LIMK1 which is activated by RhoA/Rho kinase pathway, is involved RhoA-dependent disruption of endothelial barrier function. Inhibition of LIMK1 by using LIMK-/- animals has reduced protein leak and neutrophil influx in lungs (216). In other animal models of sepsis induced ALI, fusadil (specific ROCK inhibitor) reduces protein leak and leukocyte adhesion by reducing iNOS in endothelial cells and increasing eNOS. This suggests that iNOS expression is also altered when Rho is down-regulated due to changes in actin cytoskeleton structure. Clinical evidences suggested that there is elevated soluble cadherin levels found in ARDS patients serum and in animals.
C.6.3 Endothelial apoptosis

Apoptosis is a process that is important for development and homeostasis. Resting endothelial cells exhibit very low apoptotic cells whereas during sepsis and related ALI/ARDS, pathogens are capable of inducing endothelial cell apoptosis. Much evidence suggests that LPS, TNFα, IL-1β, IFNγ, oxygen free radicals, hypoxia can induce apoptosis in endothelial cells. LPS can up-regulate Bcl-2 homologue, A1, and the zinc finger protein, A20, in cultured endothelial cells. It has been stated that LPS or cytokines administration in mice has caused pulmonary endothelial cell apoptosis. Apoptotic endothelial cells contribute to IL-1–dependent paracrine induction of ICAM-1 and VCAM-1, increased production of reactive oxygen species (ROS), increased procoagulant activity, decreased production of prostacyclin, and activation of complement. Endothelial cells undergoing apoptosis has shown increased binding to nonactivated platelets – This suggests this binding of apoptotic endothelial cells may contribute to thrombotic events.

C.7 Treatment of Endothelial Dysfunction

Various non-pharmacological and pharmacological approaches have been shown to improve endothelial dysfunction.

C.7.1 Non-Pharmacological Approaches
Stem cells such as mesenchymal, bone marrow, placental, amniotic fluid, neuronal, adipose tissue derived, hair follicle stem cells have improved endothelial dysfunction.

C.7.2 Pharmacological Approaches

Oxidative stress, the main pathophysiologic mechanism that impairs NO bioavailability and endothelial dysfunction, has become subject of interest of many researchers. The use of high-dose antioxidant vitamins (Vitamin C and E) became effective in restoring normal endothelial function in many studies (305). Other antioxidant compounds, such as the flavonoids contained in red wine and chocolate, have been found to improve endothelial function in animals (306).

Other mechanisms include the upregulation of eNOS expression, the enhanced NO release, their antioxidant activity, and the reduced expression and synthesis of endothelin-1 can improve endothelial function.

Antihypertensive β-blockers and diuretics have little or no effect on endothelium-dependent vasodilation. In contrast, newer β-blockers such as Nebivolol cause vasodilation by a direct effect on NO synthase and by its antioxidant effect and carvedilol was able to reduce ROS generation and improve endothelial dysfunction.

ACE inhibitors and angiotensin receptor blockers have shown antioxidant and anti-inflammatory effects. Accordingly, ACE inhibitors and angiotensin receptor blockers have been shown to improve endothelium-dependent vasodilation in many experimental studies. Calcium-channel blockers have been consistently shown to reverse impaired endothelium-dependent
vasodilation, mainly in the microcirculation. Statins has improved endothelial dysfunction by reducing LDL cholesterol levels and increasing HDL cholesterol.

Glitazones (insulin-sensitizing agents used to treat patients with type 2 diabetes) have been beneficial for endothelial cell function. Both Rosiglitazone and Pioglitazone have improved endothelial function in obese and diabetic patients. In septic patients, Glitazones has reduced levels of asymmetric dimethylarginine, a competitive inhibitor of eNOS, and decreased ROS production and inhibited vascular inflammation.
SECTION D

D. NITRIC OXIDE

Nitric oxide (NO) is a free radical, produced by conversion of L-arginine to L-citrulline in the presence of nitric oxide synthase (NOS). NOS enzyme is made up of 2 identical monomers which have 2 domains, Carboxy-terminal reductase domain and amino or N-terminal oxygenase domain. C-terminal reductase binds to flavin adenine dinucleotide FAD, NADPH, FMN flavin adenine mononucleotide. Whereas, the N-terminal oxygenase binds to co-factors like haem, BH4 and L-arginine. Electrons are transferred from NADPH of reductase domain to haem in oxygenase domain of opposite NOS dimer in the presence of calmodulin binding site and as a result there is conversion of L-arginine to L-citrulline and NO. The N-terminal oxygenase domain catalyses the conversion of arginine into citrulline and NO (307; 308).

Synthesis of NO occurs in two steps. First, NOS hydroxylates L-arginine to Nv-hydroxy-L-arginine which remains attached to the enzyme and in the second step, NOS activates O2 and oxidizes Nv-hydroxy-L-arginine to L-citrulline and NO. However, L-citrulline has been introduced as another NO donor through NOS dependent pathway via conversion of L-citrulline to L-arginine (308). Whereas, NO\textsubscript{2}, NO\textsubscript{3} also contributes to NO synthesis via NOS independent pathway.

D.1 NO Physiology

NO is highly reactive lipophilic free radical produced by variety of mammalian cells. Normal levels of nitric oxide (NO) in the body are important to regulate homeostasis in the cells,
tissues and organs, neurotransmitter of central nervous system, regulation of cell growth, mitochondrial respiration, prevent platelet aggregation and leukocyte binding to endothelial cells.

NO is released for short periods of time (few seconds) following the enzymatic activation of constitutively expressed endothelial NO synthase (eNOS; type 3) or neuronal NO synthase (nNOS; type 1). In contrast, inducible NO synthase (iNOS; type 2) has been suggested to be responsible for the profound and long-lasting production of NO. Nitric oxide diffuses within endothelial cells, to the luminal surface of the endothelium, and into the smooth muscle cells of the vascular wall, where it initiates signaling via guanylate cyclase to produce cGMP and by direct S-nitrosylation of cysteine residues in proteins, to activate transcription factors NF-κB and AP-1 and to inhibit Ca^{2+}-dependent vasoconstriction (309).

D.2 Nitric Oxide Synthesis

D.2.1 eNOS

The main source of endothelial NO is eNOS which is associated with caveole but it is also present in cardiac myocytes, platelets, and certain neurons, in syncytio-trophoblasts of the human placenta and in LLC-PK1 kidney tubular epithelial cells. It is also present in golgi apparatus membranes. It is constitutively and abundantly expressed in the lungs.

Increased intracellular calcium lead to increased levels of calmodulin and the increased binding of calmodulin to eNOS and nNOS that causes increased NO production. eNOS activity depends on Ca^{2+} for generation of NO from eNOS. Its expression increases by hypoxia, shear
stress, VEGF, insulin, bradykinin, estrogen (310-313). After fluid shear stress stimulation, eNOS can also be activated and do not produce sustained release of intracellular Ca\(^{++}\), but still release long lasting NO through phosphorylation of eNOS protein on serine (Ser), threonine (Thr), and tyrosine (Tyr) residues. Ser1177 and Thr495 are important phosphorylation sites in human endothelial NOS. However, Ser1177 is not phosphorylated in resting human endothelial cells.

Endothelial cell-derived NO plays an important role in vascular physiology, regulating blood flow and vessel wall remodeling through direct effects on vascular smooth muscle cells. Many studies reported that by deleting eNOS gene in animals, blood pressure is increased (313; 314). Nitric oxide by eNOS that is released towards the vascular lumen inhibits platelet aggregation and adhesion to endothelium (315). It also inhibits the release of platelet-derived growth factors that stimulate smooth muscle proliferation and its production of matrix molecules.

eNOS derived NO inhibits leucocyte adhesion to endothelium (316) by either interfering with the ability of the leucocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface or by suppressing CD11/CD18 expression on leucocytes.

eNOS-derived NO plays a critical role in post-natal angiogenesis. The lung phenotype of eNOS-deficient mice mimics alveolar capillary dysplasia in humans that leads to vascular dysfunction and respiratory disorders. Endothelium derived NO prevents endothelial cell apoptosis and contributes to anti-inflammatory process.

D.2.2 nNOS
Neuronal NOS (nNOS) is found in neural tissue in brain, in spinal cord, in the sympathetic ganglia and adrenal glands, in peripheral nitrergic nerves (nerves that contain nNOS and generate NO), in epithelial cells, in kidney macula densa cells, in pancreatic islet cells, and in the vascular smooth muscle and in skeletal muscle and in myocardium.

nNOS contains a PDZ domain to interact with the PDZ domains of other proteins to determine enzyme activity. Similar to eNOS, Ca2+-dependent calmodulin activation is important for the regulation of nNOS activity to generate NO. nNOS is also constitutively expressed. However, its expression is changed by hypoxia. nNOS-derived NO acts as a neurotransmitter, plays an important role in synaptic signaling events like nNOS mediates long-term regulation of synaptic transmission, learning, memory, regulation of blood pressure. Inhibition of nNOS leads to hypertension (317; 318). Therefore, nNOS plays an important role in maintaining vascular and microvascular tone.

D.2.3 Mitochondrial NOS (mt NOS)

Mitochondrial NOS (mtNOS) was first recognized as the neuronal isoform (nNOSα) with postranslational modifications in heart and liver. It is also found in skeletal muscle, diaphragm and kidneys and in endothelial cells and breast cancer cells. The localization of NOS in mitochondria is still in debate. Whether it is bound to mitochondria or any other organelles or cytoplasm is still a question. Previous evidences suggested that it can be inhibited by antibodies to iNOS but not to nNOS or eNOS. Further studies regarding its localization will help to understand mechanisms. Mitochondrial NOS contributes to septic shock myocardial depression (319).
D.2.4 iNOS

iNOS has been recognized by almost every cell type and is localized to membranes and cytosol of cells (320). Unlike eNOS and nNOS, iNOS activity is independent of calcium levels because of very tight calcium-calmodulin binding. Thus, iNOS can bind to calmodulin at very low concentrations of calcium but its activity is not increased in response to any fluctuations in calcium level. Consequently, iNOS tends to generate long lasting and much higher concentrations of NO than nNOS and eNOS. It has been reported that iNOS yields NO approximately 100 to 1000-folds for several hours after septic stimulation.

The functional iNOS enzyme is a homodimer. Homodimerization of iNOS depends on the availability of cofactors such as tetrahydrobiopterin (BH4), haem and L-arginine (321). iNOS has much more tendency to uncouple and generates superoxide in case of limited L-arginine or BH4. L-arginine availability can be reduced due to production of NO. Competition between L-arginine and arginase (competitive substrate) occur due to Vmax of arginase is greater than 1000 fold that reduces the bioavailability of L-arginine for NOS, therefore limiting NO production. Consequently, there is increased formation of L-proline and polyamine which contributes in collagen synthesis and cell proliferation that contributes to pathophysiology of lung injury, hypertension and asthma. Super oxide when reacts with surrounding NO, it produces peroxynitrite. Therefore, reduced bioavailability of L-arginine also affects cell proliferation and Tetrahydrobiopterin (BH4) another co-factor, which is also a form of reduced biopterin and can be oxidized to biopterin when reacts with peroxynitrite. Peroxynitrite oxidation of BH4 can be responsible for raised superoxide production instead of NO yield (322).
iNOS activity is also affected by other proteins such as kalirin blocks iNOS activity by inhibiting enzyme dimerization. Another protein named NAP110 (a 110-kDa protein) has been found in mice macrophages can directly interacts with the amino terminus of iNOS and inhibits dimerization and thus affects NOS activity. In addition, iNOS has been reported to interact with calcium/calmodulin-dependent protein kinase II (CaMKII) in rat vascular smooth muscle cells.

iNOS or iNOS activity is responsible for several physiological functions in cells. For instance, high concentrations of NO produced by endothelial cells interferes with DNA and causes DNA fragmentation (323). Furthermore, endothelial cells can lyse tumor cells post cytokine stimulation. Similarly, nitrogen oxide formed by hepatocytes post cytokine stimulation has been useful in killing malarial sporozoites. In addition, iNOS derived NO inhibits enzymes that contain iron such as complexes I and II (mitochondrial electron transport chain), ribonucleotide reductase (involved in DNA replication) and cis-acotinase (involved in kreb cycle). In brief, iNOS activity contributes enough to modulate cellular physiology or associated mechanisms.

D.3 Role of NO in disease

NO is highly reactive gaseous free radical produced by all mammalian cells. NO overproduction has been implicated in pathogenesis of various infections and cardiovascular diseases such as hypertension, atherosclerosis, coronary heart disease and cardiomyopathy, sepsis, ALI/ARDS, asthma, diabetes, renal failure. Moreover, NO, NO metabolites nitrates, nitrites and other RNS such as peroxynitrite (ONOO’) and nitrogen dioxide (NO₂) are thought to
be involved in these diseases. High levels of NO metabolites has been found in plasma and BAL of septic patients and in animal model of sepsis and sepsis induced lung injury (309; 317; 318; 321; 323-328). However, the mechanism of NO involved in pathogenesis of sepsis or sepsis induced lung injury is still controversial.

BH4 performs various functions in regulating NOS activity such as it converts the NOS haem iron to a high-spin state, promotes arginine binding; BH4 bound to NOS acts as a redox-sensitive co-factor; BH4 increases substrate affinity of NOS; BH4 facilitates in electron transfer as it converts to BH3, and it stabilizes NOS dimer formation, which is important for functional NOS activity. When BH4 is limited, NOS dimer is altered that result in uncoupling of NOS dimer which is associated with many diseases (322). As it has been documented that BH4 deficiency converts neuronal NOS into peroxynitrite synthase, which increases neuronal vulnerability/susceptibility to hypoxia-induced mitochondrial damage and necrosis and affects phenylalanine hemostasis and other disorders like phenylketonuria (PKU). Addition of BH4 to eNOS and nNOS results in production of NO whereas, deletion of BH4 results in uncoupling of oxygen reduction. Increased oxidized forms of BH4, especially dihydrobiopterin (BH2), has also been reported in oxidative stress related diseases like arteriosclerosis. Therefore, anti-oxidant like ascorbic acid/vitamin C has played an important role in maintaining BH4 in vascular disorders. BH4 has also played a beneficial role in diseases. BH4 administration has increased iNOS gene level and improved survival in Ischemic /reperfusion animals by increasing NO derived from iNOS. During hypertension, BH4 administration improves endothelial dysfunction.

It has been reported that L-arginine can influence NO production. Under conditions of reduced substrate (like L-arginine) or cofactor availability like BH4, all NOS can also produce
superoxide and peroxynitrite mediated tissue injury. Reduced and increased concentrations of L-arginine contribute to uncoupling of iNOS. Moreover, lack of L-arginine or its availability has shown detrimental effects on cells and tissues post septic stimulation which results in ONOO⁻ production. Various mechanisms of L-arginine deficiency has been documented in sepsis and cardiovascular diseases. Increased activity of arginase-1 and iNOS has contributed to decreased plasma arginine and L-arginine availability for eNOS during sepsis and endotoxemia. However, L-arginine supplementation could not increase intracellular NO production despite increased plasma arginine availability. Contrary to this, L-citrulline supplementation during sepsis and endotoxemia has improved intestinal microvascular perfusion, plasma and tissue concentration of L-arginine, citrulline and intracellular NO production, eNOS phosphorylation and decreased iNOS protein level.

Abnormal nNOS derived NO signaling has been involved in pathophysiology of many diseases like Stroke, Alzheimer, Parkinson, multiple sclerosis. Not only in these diseases, nNOS derived NO has played a detrimental role in sepsis, septic shock and ALI (329). Evidences suggested that NO produced from nNOS has impaired vasodilation in rat skeletal muscles under septic conditions. Nonselective NOS inhibition in sepsis models has improved sepsis-induced hemodynamic disorders. Recently it is stated that selective inhibition of nNOS has reduced ALI by reducing oxidative and nitrosative stress markers (330).

It has been documented that mtNOS derived NO has been involved in pathophysiology of many diseases including sepsis and septic shock (319). It has been found that NO produced by mt NOS is upregulated by LPS and other septic conditions like CLP. However, its expression is downregulated by ANG II, insulin and thyroid hormones and environmental conditions as cold.
Nitric oxide has been involved in several cellular functions including reversible regulation of mitochondrial respiration. NO reacts with superoxide to form peroxynitrite that has been involved in modulating cellular functions and mitochondrial dysfunction and organ failure. Mt derived NO also causes nitration and oxidization of mitochondrial and cell proteins and lipids that plays a critical role in neurogenerative and metabolic disorders.

iNOS expression can be constitutive in some cells/tissues, but in many cells, its expression is commonly and highly induced by a variety of inflammatory mediators like LPS, TNF alpha, IL-1 beta, INF gamma in almost all type of cells. However, recently it has been reported that human blood monocytes could not induce iNOS gene expression after LPS/cytomix treatment (309; 311; 320; 329; 330). In many cells types its gene expression is detectable at 2-4 hrs and peaked at 8 hrs and then declines after stimulation. iNOS is also regulated by phosphorylation. Previous literature reported that protein kinase B (Akt), transcription factors such as nuclear factor (NFκB) (311; 331) and activator protein-1 (AP-1), mitogen-activated protein kinase (MAPK) p42/44 or extracellular signal-regulated kinase (ERK1/2) plays a significant role in regulation of iNOS activity or expression in cardiovascular tissues.

D.3.1 Role of NO in sepsis

NO is highly reactive free radical and is produced by variety of cells during sepsis and ALI. Its co factor L-arginine availability has been contributed to the production of NO. It has been documented that impaired arginine production from citrulline also reduces NO production in septic human patients and in mice patients. L-arginine administration during endotoxemia has
also proved to be beneficial in improving intestinal microvascular perfusion and increased NO production, increased eNOS phosphorylation and decreased iNOS protein (332).

NO donors or exogenous NO supplementation showed variability with respect to the NO synthesis cascade and caused low blood pressure, which limits their use in septic shock models. Recently, early phase of endotoxemia is protected against LPS-induced lung injury by NO administration along with S-nitroso-N-acetylpenicillamine. Tolerance property, especially to organic nitrates, also restricts their therapeutic application. Another new approach of using stable S-nitroso-human--serum-albumin (S-NO-HSA) in the treatment of septic shock in an endotoxemic rat model has been launched.

NO also decreases platelet and leukocytes adhesion to the endothelium. Role of NO is still under debate depending on source of NO production under different septic conditions. NO derived from constitutive endothelial nitric oxide synthase eNOS and nNOS have downregulated P-selectin, E-selectin, ICAM-1 VCAM-1 and as a result there is decreased platelet and leukocyte adhesion during sepsis (333). During early phase of endotoxin administration, NO produced by eNOS plays a cytoprotective role of NO in lungs. eNOS does not contribute to basal E-selectin upregulation and after septic stimulation in endothelial cells. Recently, eNOS is found to be activated by TNF-α through S1P receptors, activated by sphingosine-1-phosphate (Sph1P) produced through neutral-sphingomyelinase-2 (N-SMase2) and sphingosine-kinase-1 (SK1) activation contributes to inhibition of E-selectin in HMVEC and HUVEC. This variability can depend on cell type being studied.
In contrast, iNOS derived NO has shown detrimental effects in sepsis or septic shock animal models and human. Evidence suggests that during LPS administration in septic or septic shock models, iNOS derived NO caused abrupt release of lipid mediators (platelet-activating factor, thromboxanes, leukotrienes) and cytokines (tumor necrosis factor-α, interleukin (IL-2, IL-6, IL-8) (333; 334). In addition, during later phase of LPS administration, iNOS derived NO has decreased blood pressure along with vasoplegia, tissue hypoperfusion, microvascular damage, and multiple organ injury. NO produced from iNOS has increased prostaglandins production in \textit{in vivo} and \textit{in vitro} models of sepsis. It is also involved in reduction of eNOS during sepsis. During sepsis, heat shock protein 90 has been involved in regulation of NO. In various septic models, hsp 90 inhibitors has improved survival, lung injury and decreased formation of hsp90-iNOS complexes and NO metabolites.

Selective iNOS inhibitors and low doses of non-selective NOS inhibitors have been proved beneficial in sepsis whereas high doses of non-selective inhibitors have proved detrimental effects during sepsis (327-330). Selective iNOS inhibition has decreased hypotension in septic shock animals. NO synthesis inhibitors have been shown to improve hemodynamic variables and survival in several animal models of septic shock. In contrast, iNOS selective inhibitor has reduced hypotension in rats but could not reduce mortality following sepsis. Moreover, in sepsis induced acute kidney injury iNOS derived NO and peroxynitrite has played significant role in renal injury. Therefore, selective inhibition of iNOS has improved sepsis induced kidney injury.

In addition to this many animal studies has reported the benefits of selective inhibition as compared to non-selective inhibition of iNOS in improving survival in sepsis induced renal
disease. A recent study using mice with iNOS deleted gene has confirmed that NO can also exert anti-inflammatory effects in the intestinal mucosa (335). In contrast, iNOS knockout mice developed more severe intestinal inflammation and increased perivascular leukocyte recruitment in a model of acetic acid colitis. In addition, some literature reported that iNOS knockout mice have been shown resistance to mortality while some studies have shown decreased defence against bacteria.

iNOS-derived NO contributes to production of cytokines and chemokines under septic conditions (334). In contrast, iNOS deficiency has reduced brain protein levels of the cytokines TNF-a, IL-1beta and IL-6 and brain mRNA levels of 2 major chemokines (MIP-1a and MIP-2) in pneumococcal meningitis.

Coagulopathy is important feature in sepsis which is also associated with endothelial activation. It has been documented that selective inhibition of iNOS by L-NIL has prevented increased plasma nitrate/nitrite and isoprostanes concentrations, protected hypotension and acidosis, inhibited raised plasma vWF and TAT (thrombin anti-thrombin complexes), prevented decreased t-PA activity in porcine model of sepsis. However, selective inhibition of iNOS could not reduce PAI-1 levels and increase platelet count. Thus, iNOS beneficial and detrimental effects are still controversial (336; 336; 337).

During sepsis or septic shock, prostanoids, derived from inducible isoform of cyclooxygenase-2 (COX-2) are increased. It has been stated that iNOS inhibition has played a significant role in reduction of COX-2 and therefore prostanoids under septic conditions. Contrary to this, it has been published that prostanoids like prostacyclin (PGI2) and
prostaglandin E2 has increased iNOS in cultured cells. Interestingly, thromboxane A2 (vasoconstrictor) plays a protective role by suppressing iNOS derived NO in mice when treated with thromboxane A2 receptor agonist, U-46619 under septic conditions.

It is already been documented that neither nNOS nor iNOS is completely responsible for the morbidity and mortality of septic mice. Recently, researchers proposed the idea of inhibiting both iNOS and nNOS to see the effect in septic animals and in ALI models. In sheep model of ALI, the combination of early nNOS and delayed iNOS selective pharmacological inhibition showed reduction in the degree of airway obstruction and improved pulmonary gas exchange, VEGF, 3- nitrotyrosine, poly (ADP ribose) in pulmonary tissue (325; 326). In mice model of sepsis induced lung injury, non-selective NOS inhibition has reduced VEGF expression whereas, combined deletion of nNOS and iNOS genes has reduced VEGF and pulmonary edema. By contrast, non-selective NOS inhibition has increased mortality in septic mice by showing no effect on oxidative and nitrosative stress markers in lung. Whereas, deletion of both nNOS- and iNOS-gene has significantly decreased oxidative and nitrosative stress markers, but could not improve survival. These studies suggested that combined deletion of iNOS and nNOS has improved sepsis and ALI but still could not reduce mortality (325; 326). Interestingly, the idea of simultaneous selective inhibition of nNOS and iNOS showed beneficial results in a sheep model of burn and smoke induced acute respiratory distress syndrome by inhibiting increased lymph and plasma nitrate/nitrite levels, pulmonary shunting, ventilatory pressures, lung lymph flow, and wet/dry weight ratio and significantly improve PaO$_2$/FiO$_2$ ratio. In addition, the idea of selective inhibition of nNOS or iNOS at different time points struck researcher’s mind when they found that nNOS inhibition at 4 h has improved survival and significantly attenuated
contents of lung nitrite/nitrate and liver malondialdehyde and iNOS blockade at 8 hours has decreased liver malondialdehyde (338). In short, simultaneous selective inhibition of nNOS and iNOS or selective inhibition of NOS isoforms at appropriate time points can be beneficial therapeutics for the treatment of sepsis or ALI/ARDS.

D.3.2 Role of NO in ALI/ARDS

Increased NO production is a key feature of ALI. It has been found that increased levels of NO are generated by iNOS during ALI/ARDS in patients and in animal models of ALI. At cellular level various studies reported augmentation of macrophage or neutrophil NO after septic stimulation. Moreover, iNOS derived NO modulate cytokines and chemokines during ALI. This suggests NO can increase or decrease various cytokines. NO also regulate gene expression or protein expression. It regulates or activates NF-kappa B activity. NO has been shown to modulate inflammatory condition through inhibition of iNOS expression induced by cytokines such as IL-1 beta via inhibition of NF-Kappa-B and IKB-alpha in endothelial cells and VSMCs (331; 339; 339). Mechanisms of iNOS derived NO in pathophysiology of ALI are still under debate.

Reactive nitrogen species such as NO, peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂) are vital contributors towards tissue injury. However, NO readily reacts with thiol groups and with molecular oxygen or superoxide to form ONOO⁻, which results in DNA and membrane damage by causing peroxidation of membrane phospholipids and nitration of tyrosine residues.
and forms 3 nitro tyrosine and this disrupts endothelial cytoskeleton and cause protein leak. Increased levels of NO, NO metabolites nitrates, nitrites are found in human and animals BALF and serum in sepsis, severe sepsis or sepsis induced lung injury (318; 329).

NO is elevated not only in lung during ALI but various clinical studies have shown elevated urine NO or NO/Creatinine levels during early ALI days also associated with use of low tidal volume strategy. It has been stated that increased endogenous NO production may be beneficial during ALI regardless of the fact that higher NO levels are the result of less severe endothelial epithelial damage.

Inhaled NO (iNO) has generated keen interest in many clinical trials to improve lung injury. Inhaled NO has reduced transvascular albumin leak in patients with acute respiratory distress syndrome (ARDS). By contrast, some studies have documented that inhaled NO was associated with small improvements in the ratio of partial pressure of oxygen to fraction of inspired oxygen and the oxygenation index but this was short lived and disappeared after 24-48 hours. Therefore, inhaled NO therapy is not recommended as routine treatment for ALI/ARDS, but can reduce refractory hypoxemia on an individual case basis due to reduction in intrapulmonary shunting that is due to lack of inadequate blood oxygenation.

NO has been shown to be involved in various cell-cell interactions and injury models in the lung, and is produced by alveolar epithelial cells, alveolar macrophages, and endothelial cells of the lung. Previous studies have shown that NO can protect type II pneumocytes from stretch injury. NO is most important vasodilator in circulation which allows for increased perfusion of tissues, improved ventilation-to-perfusion matching, and ameliorated oxygenation. Higher NO
levels could help prevent further tissue damage by improving oxygen and nutrition delivery to the tissue

iNOS derived NO has shown detrimental and beneficial effects in sepsis or septic ALI animal models and in human patients. Moreover, iNOS derived NO modulate cytokines and chemokines during ALI. This suggests NO can increase or decrease various cytokines. NO also regulate gene expression or protein expression. It regulates NF-kappa B activity. Our lab has reported reduced protein leak in iNOS knockout mice after septic stimulation (69). In contrast, many has shown increased protein leak after septic stimulation in iNOS deficient mice. We have also assessed the role of iNOS derived NO in neutrophil migration during sepsis induced lung injury model. In our CLP model of acute lung injury, iNOS knockout mice had reduced neutrophil sequestration to endothelium and increased neutrophil migration as compared to wild type mice (341). We have also shown cell source specific effects of iNOS derived NO during ALI. Our lab has shown it is only inflammatory cells (neutrophils and macrophages) iNOS that contributed to increased protein leak during sepsis induced ALI model (342). Recently we have shown by using reciprocal BM neutrophils depleted/reconstituted with neutrophil iNOS+/- chimeric mice that septic ALI is dependent on neutrophil iNOS in vivo (340). Many ongoing and further researches will be helpful in exploring the mechanism of iNOS derived NO in pathophysiology of ALI.

Like sepsis, iNOS derived NO and iNOS gene expression itself has been contributed to variety of diseases like ALI/ARDS. Still the pathophysiology of disease is in debate. iNOS in ALI has shown both beneficial and detrimental effects. Similarly, iNOS-/- animals has significantly increased or decreased protein leak during sepsis induced ALI. Some has reported
that iNOS -/- mice after LPS treatment have increased protein leak while others have shown that selective inhibition of iNOS has prevented leak in rats following sepsis. Similarly, in our lab, iNOS derived NO in mice has increased protein leak, increased neutrophil sequestration and reduced migration after CLP. However, iNOS-/— mice has shown reduction in protein leak, neutrophil sequestration but increased neutrophil migration in CLP model of ALI (341).

iNOS plays an important role in disrupting cellular junctional proteins. As mentioned earlier that disrupted F actin formation has potentiated LPS induced iNOS expression. Moreover, iNOS induction followed by cytokines has relocated VE-cadherin to the site of leak. It has been stated that LPS mediates protein leak / endothelial barrier dysfunction through various pathways. In animal model of ALI, LIMK1 which is activated by RhoA/Rho kinase pathway is involved in RhoA-dependent disruption of endothelial barrier function. Inhibition of LIMK1 by using LIMK-/- animals has reduced protein leak and neutrophil influx in lungs. In other animal models of sepsis induced ALI, fusadil (specific ROCK inhibitor) reduces protein leak and leukocyte adhesion by reducing iNOS in endothelial cells and increasing eNOS. This suggests that iNOS expression is also altered when Rho is down-regulated due to changes in actin cytoskeleton structure. Disruption of cytoskeletal structure is also associated with dislocation but not expression of E-selectin after TNF alpha treatment. Therefore, it modulates neutrophil attachment under septic conditions. Interestingly, Rho (a small GTP binding protein) is involved in clustering of E-selectin for neutrophil adhesion. Recently, another signaling pathway class 1, PI3K contributed to E-selectin dislodgement by cytoskeleton disruption under septic conditions.

iNOS, iNOS derived NO, ROS and RNS has played a significant role in endothelial activation and dysfunction in sepsis induced lung injury. Moreover, iNOS has been played an
important role in upregulation of endothelial E-selectin in CLP induced ALI animal models. In contrast, some has reported no effect of iNOS in endothelial activation following sepsis. In ALI, NADPH oxidase has played a significant role in promoting lung injury or edema formation. In addition to this, NADPH oxidase derived from neutrophils contributed to upregulation of endothelial NFκB dependent ICAM-1 expression under septic conditions. Interestingly, PI3K has shown to be involved in regulation of ICAM-1 and E-selection through NFκB signaling. Therefore, deletion of p110γ blocked TNFα-activated ROS generation, NF-κB activation and reduced MLVEC ICAM-1 and E-selectin expression in mice. Consequently, neutrophils adherence to endothelial cells is failed.

iNOS and iNOS derived NO also contribute to surfactant disruption during ALI. An inverse relationship between exogenous natural surfactants (beractant, calfactant or colfosceril) and surfactant phospholipid (dipalmitoyl phosphatidylcholine, DPPC) and NO, iNOS and proinflammatory cytokines production in AMs has been proposed. This suggests that anti-inflammatory therapies can improve surfactant function in NRDS (neonatal respiratory distress syndrome).

During ALI, Increased NO synthesis is associated with increased plasma nitrogen oxide species (NOS) concentration, which was associated with increased microvascular permeability and pulmonary edema. It has been documented that the combined NOS inhibitor and ONOO•-blocker mercaptoethylguanidine prevented these effects when administered 1 h after the burn and smoke injury in animals. Superselective iNOS synthesis (dimerization) inhibitor BBS-2 was shown to improve pulmonary gas exchange, lung compliance, and airway obstruction and decreased tracheal blood flow, lung lymph flow, and capillary leakage in another animal model.
Some studies have shown that this effect is not due to differences in hydrostatic pressure but due to peroxynitrite inhibition.

To date, iNOS has been expressed by all almost all cell kinds. Therefore, in our lab we assessed the role of cell source specific effects of iNOS and iNOS derived NO in sepsis induced lung injury. As such, we reported that parenchymal (endothelial and epithelial cells) iNOS does not contribute to protein leak under septic conditions but inflammatory cell (neutrophils and macrophages) contributed to enhanced protein leak post septic stimulation (342). Our research did not end here but we also determined the role of inflammatory cell ROS production in protein leak formation under septic conditions. We have reported that parenchymal ROS and RNS (peroxynitrite) could not affect protein leak but instead inflammatory cells ROS from NADPH oxidase and peroxynitrite contributed to protein leak post septic treatment (344).

The role of iNOS in ALI/ARDS is still uncertain with respect to reduction in mortality. Current research focuses on inhibition of all NOS isoforms or combination of NOS isoforms inhibition during ALI models. Many ongoing and future studies on the role all NOS isoforms in ALI/ARDS will be helpful in patient’s survival. My research focused on role of iNOS in endothelial activation in support of previous evidences provided by our lab.

D.4 Cell source specific effects of NO

D.4.1 Endothelial cells
Endothelium derived nitric oxide is a potent vasodilator in the vasculature. Nitric oxide restricts platelet aggregation, leucocyte migration, and cellular adhesion to the endothelium, and reduces vascular smooth muscle cell proliferation and migration. Moreover, nitric oxide can suppress activation and endothelial adhesion molecules, and influence production of superoxide anion. NO derived from constitutive endothelial nitric oxide synthase eNOS and nNOS have downregulated P-selectin, E-selectin, ICAM-1 VCAM-1 and as a result there is decreased platelet and leukocyte adhesion during sepsis (210; 211; 302). During early phase of endotoxin administration, NO produced by eNOS plays a cytoprotective role of NO in lungs. Loss of endothelium derived nitric oxide would promote vasoconstriction in vasculature. High concentrations of NO stimulate apoptosis in endothelial cells and cause cell detachment also called anoikis. Anoikis is initiated by the action of NO. Therefore, NO interferes with focal adhesion kinase tyrosine phosphorylation and restricts endothelial cell adhesion, spreading and formation of focal adhesions. In contrast, low concentrations of NO induce anti-apoptotic effect in endothelial cells through activation of sGC leading to the formation of cGMP.

Nitric oxide (NO) reacts with superoxide anion and forms peroxynitrite which results in DNA and membrane damage by causing peroxidation of membrane phospholipids and nitration of tyrosine residues and forms 3-nitro tyrosine and this disrupts endothelial cytoskeleton and cause protein leak.

Recently, endothelial nNOS pharmacological inhibition (using L-NPA) or siRNA further increased cytokine-mediated up-regulation of VCAM-1 and proinflammatory cytokines, as well as increased leukocyte recruitment.
D.4.2 Neutrophils

Nitric oxide (NO) is a major contributor in neutrophil dysfunction in sepsis. However, NO synthesis inhibition in sepsis resulted in increased neutrophil death despite restoring neutrophil migration. TLR activation in human neutrophils leads to decreased chemotaxis due to chemotactic receptor internalization and increased G protein-coupled receptor kinase 2 expression through involvement of NO-sGC-protein kinase G. Similarly, it has been documented that NO and ONOO- downregulate neutrophil migration by decreasing adhesion and migration. In addition, over production of NO induced by Toll like activation attenuates CXCR2 in circulating neutrophils which correlates impaired neutrophil migration in sepsis.

iNOS derived NO has been involved in pathophysiology of many diseases including ALI/ARDS. It has shown both beneficial as well as detrimental effects. Our lab has reported reduced protein leak in iNOS knockout mice after septic stimulation (69). In contrast, many has shown increased protein leak after septic stimulation in iNOS deficient mice. We have also assessed the role of iNOS derived NO in neutrophil migration during sepsis induced lung injury model. In our model of CLP induced acute lung injury, iNOS knockout mice had reduced neutrophil sequestration to endothelium and increased neutrophil migration as compared to wild type mice (341). We have also shown cell source specific effects of iNOS derived NO during ALI. Our lab has shown it is only inflammatory cells (neutrophils and macrophages) iNOS that contributed to increased protein leak and oxidant stress during sepsis induced ALI model without contribution of parenchymal iNOS (342). Moreover, we have shown neutrophil ONOO\(^{-}\) contributes to trans-endothelial protein leak in septic ALI (343).
D.4.3 Macrophages

Alveolar macrophage iNOS derived NO contributes to production of cytokines and chemokines by alveolar macrophages. However, some studies showed that NO reduces production of cytokines in alveolar macrophages. Moreover, iNOS derived NO produced by alveolar macrophages has increased trans-endothelial protein leak under septic conditions. In addition to this, alveolar macrophage NADPH derived ROS has caused protein oxidation and lipid peroxidation of PMVEC under septic conditions. Scavenging AM iNOS derived NO and AM NADPH derived ROS resulted in reduction of PMVEC injury under septic conditions (344). This suggests alveolar macrophages contribute to extent of septic lung injury.

SECTION E

E. RATIONALE OF STUDIES

The exact role of iNOS derived NO is uncertain in the pathogenesis of sepsis and sepsis-induced lung injury. Studies have shown iNOS-derived NO has beneficial as well as detrimental effects in septic ALI. Indeed, iNOS inhibition or genetic deletion of iNOS can improve the degree of lung injury in many models, but does not necessarily improve mortality in animal models of sepsis and ALI. Our lab has reported iNOS contributes to increased pulmonary protein leak and oxidant stress in septic mice and in isolated PMVEC. Moreover, we have also determined the role of iNOS derived NO in neutrophil migration during sepsis induced lung injury. In our CLP induced septic ALI, iNOS knockout mice had reduced neutrophil
sequestration to endothelium and increased neutrophil migration as compared to wild type mice. Moreover, we have pursued the idea of cell-source specific effects of iNOS-derived NO, using reciprocal BM depleted/reconstituted chimeric mice. We have found that it is specifically neutrophil iNOS that mediates septic ALI in vivo, as well as PMVEC septic injury in vitro, without any apparent contribution of parenchymal (e.g PMVEC) iNOS during septic ALI. However, the mechanism of neutrophil iNOS-dependent pulmonary microvascular endothelial cell (PMVEC) injury is not known. Therefore, we assessed septic PMVEC activation in vitro, and defined the specific role of neutrophils in septic PMVEC activation, and the selective effects of PMVEC vs neutrophil iNOS in septic PMVEC activation.

E.1 Hypotheses

1. Endothelial iNOS mediates PMVEC activation under septic conditions in vitro

2. Neutrophil iNOS mediates PMVEC activation under septic conditions in vitro

E.2 Objectives

1. To assess the role of endothelial iNOS in PMVEC activation under septic conditions.
   
   a. To characterize PMVEC cell activation under septic conditions in vitro

   b. To determine the effect of endothelial iNOS on PMVEC cell activation under septic conditions in vitro

2. To assess the role of neutrophil iNOS in endothelial cell activation under septic conditions.
a. To characterize neutrophil activation under septic conditions in vitro

b. To determine the effect of neutrophils on endothelial activation under septic conditions in vitro

c. To determine the effect of neutrophil iNOS on endothelial cell activation under septic conditions in vitro

CHAPTER 2: MATERIALS & METHODS

2.1 Animal Preparation

Male C57BL6 iNOS+/+ and iNOS-/- mice (6-8 weeks) were purchased (Jackson Laboratories, Bar Harbour, Maine, USA) and used for isolation of PMVEC and bone marrow PMN. All animals’ experimental protocols were approved by the Animal Care and Use Committee of the University of Western Ontario, and were carried out under supervision of a veterinarian.

2.2 PMVEC isolation and culture

Murine PMVEC isolation and culture techniques are currently routinely being performed in our laboratory, as previously described (Razavi et al; 2004). PMVEC were isolated from subpleural lungs by finely chopping lung tissue, digesting with collagenase (0.2 % in PBS or DMEM for 30-45 mins at 37\(^{\circ}\)C, time), and filtered through 70 um mesh. Cell suspension was then incubated with magnetic microbeads bonded to anti-PECAM-antibodies (1.15 - 1.5 dilution
for 1 hr at 4°C). Magnetically-isolated primary PMVEC in culture were grown to 90% confluence in 2% gelatin-coated flasks, during regular purification every 1-2 days by physical removal of non-PMVEC cells, which yields PMVEC homogeneity >99% (Razavi et al; 2004). When cells were grown confluent, they were sorted with FACS by Dil-acetylated LDL uptake.

The cells were then cultured in DMEM (Sigma-Aldrich) supplemented with 1% streptomycin/penicillin (Sigma-Aldrich, city, state), 50 ug/ml endothelial cell growth supplement ECGS, 20mM HEPES buffer (Sigma-Aldrich) and 20% FCS (Gibco, London, ON). PMVEC were maintained in culture conditions (humidified 5% CO2 in incubator at 37°C) and used for experiments between passages 4 and 8.

2.3 FACS Analysis of PMVEC activation

For in vitro PMVEC activation analysis, cultured PMVECs were treated with LPS 1000 ng/ml (Escherichia coli 0111:B4; Sigma) or cytomix 10 ng/ml (equimolar mixture of cytokines TNF-α, IL-1β and INF-γ) for t= 1, 2, 4, 8, 12 hrs for analysis of E-selectin expression and t= 2, 4, 8, 12 hrs for analysis of PAI-1 expression in PMVEC. Following trypsinization, PMVEC were suspended in FACS buffer and were incubated with flourochrome (PE or FITC) conjugated antibodies against E-selectin (BD pharmingen, Mississauga, ON, Canada), PAI-1 (Innovative-research, MI, USA), and PECAM-1 (Biolegend, Burlington, ON, Canada) or with isotype control antibodies at a concentration of 5 ug/mL or 10ug/ml and analysed by FACS. Cultured PMVECs treated with medium only served as control. To ensure no contamination of other cells, PECAM-1 expression on PMVEC surface was detected which was gated based on FACS forward scatter.
(FSC) and all PECAM-1 positive cells were served as control. The mean fluorescence intensity (MFI) ratio was normalized to respective isotype control antibody MFI by dividing MFI of E-selectin or PAI-1 stained cells by Isotype stained cells. 10,000 cells per sample were collected and analyzed with FACS using 488 nm argon laser.

To assess the role of endothelial iNOS in PMVEC activation, PMVEC from iNOS+/+ and iNOS-/- mice were isolated and assessed for E-selectin and PAI-1 upregulation after septic (LPS, cytomix) stimulation. Moreover, in order to assess the effects of PMVEC iNOS on basal E-selectin expression, PMVEC isolated from iNOS+/+ mice were pretreated for 24 hrs with either the non-selective NOS inhibitor L-NAME (100uM) (Cayman chemical, Ann Arbor, Michigan), or the iNOS selective inhibitor N-(3-aminomethylbenzyl acetamide (1400W; 100uM) (Cayman chemical), followed by FACS analysis of basal E-selectin expression.

2.4 PMN isolation and co-culture with PMVEC

Bone marrow (BM) PMN were isolated from iNOS+/+ and iNOS-/- mice femur, tibia and fibula bones as previously reported by our laboratory (Razavi et al; 2004). BM PMNs were isolated by three step percoll gradient density centrifugation. Following centrifugation, the BM suspension was separated in 5 bands. We carefully removed top two layers containing plasma and monocytes and the band containing PMNs was carefully isolated and RBCs were lysed by using d.d H2O or NH4CL based lysis method for 5 mins. PMNs were counted by haemocytometer and almost 6-15 million PMNs were isolated from each iNOS+/+ or iNOS-/-. 
mice bone marrows. PMNs were confirmed with Gr-1 staining. PMNs were then incubated with control medium or septic stimulation.

To assess the potential effects of the presence of PMN and specifically PMN iNOS on PMVEC activation, PMN-PMVEC co-cultures were treated with septic stimulation vs. control medium, prior to FACS assessment of E-selectin or PAI-1 expression in PMVEC. $10^6$ PMVEC were grown to 90% - 100% confluence and $10 \times 10^6$ PMN were applied on the apical surface of PMVEC, which mimics the in vivo condition as the apical PMVEC surface is exposed to blood elements in the lumen of the capillary.

Prior to PMN-PMVEC co-culture, BM PMNs were pre-treated with LPS (100ng/ml) or cytomix (10ng/ml) for 4 hours (37 C, 5% CO2). To assess the potential effects of PMN on PMVEC E-selectin expression, PMN and PMVEC were co-cultured and further stimulated with LPS or cytomix for 2 hrs prior to assessment of PMVEC E-selectin expression. To analyse the potential effects of PMN presence on PMVEC PAI-1 expression, PMN and PMVEC were co-cultured for 4 hrs under LPS stimulation or 2 hrs during cytomix stimulation prior to assessment of PAI-1 expression by FACS.

To analyze the role of PMN iNOS in PMVEC activation, PMN isolated from iNOS+/+ vs. iNOS-/- mice bone marrow (BM) were co-cultured with iNOS+/+ PMVEC under septic (LPS, cytomix) vs. non-septic control conditions. E-selectin and PAI-1 expression were then analyzed by FACS.
2.5 RT-PCR analysis

To analyze the effect of PMVEC iNOS on PAI-1 mRNA in PMVEC, RT-PCR analysis was performed. The potential effect of presence of iNOS+/+ vs. iNOS-/- PMN on PAI-1 mRNA in iNOS+/+ PMVEC was also considered. Trizol (Invitrogen, Burlington, ON) according to the manufacturer's directions was used to extract mRNA from cells. RNA was treated with DNASE I (Sigma-Aldrich) followed by reverse transcription using Superscript II reverse transcriptase (Invitrogen). Quantitative real time PCR (qPCR) was used to determine the mRNA expression levels of PAI-1 (primers: forward 5'-GACAGCACTGTCAGGGTCCATAG-3' and reverse 5'-GCCGTCCTCCTACAAAGCTC-3') in the cells. β-actin (primers: forward 5'-TCGTGGGCGCTCTTAGGCACCA-3' and reverse 5'-GTTGGCCTTAGGGTTCAAGGGG-3') was used as reference gene. The qPCR was carried out using Quantifast SYBR Green master mix (Qiagen, Mississauga, ON) on a Mini-opticon cycler (Bio-Rad, Mississauga, ON). We followed the following cycling protocol: 95°C for 5 minutes followed by 40 cycles of 95°C for 10s, 60°C for 30 s and 80°C for 30s. SYBR green fluorescence was acquired at 80°C in each amplification cycle. A melt curve was generated starting at 60°C taking measurements every 0.2°C until 95°C was reached.

2.6 Effect of presence and type of sub cellular matrix on PMVEC basal E-selectin expression

PMVEC are usually cultured on 1% gelatin. The potential effect of the type of matrix (gelatin vs. matrigel) or the absence of matrix on PMVEC E-selectin expression was assessed. To achieve this, 1ml of matrigel (Beckton Dickinson) at a dilution of 1:8 in ice cold H₂O was used to coat wells in a 6-well plate, and 500ul of liquid was aspirated, and the wells allowed to
air dry for 1 hour at room temperature (Martin et al; 1999). 1 ml of 1% gelatin was used to coat the wells and then aspirated. Wells were then rehydrated with 500 ul of medium, prior to plating of PMVEC, culture, and FACS analysis.

2.7 Sample Size and statistical analysis

One way ANOVA was used to analyze differences across multiple groups or across multiple time points, followed by post hoc two sample Dunnett’s t test and Bonferroni correction for individual comparisons. Statistical significance was accepted when P<0.05.
CHAPTER 3:

CHAPTER 3: RESULTS

3.1 Analysis of PMVEC activation by FACS

We first established the ability to reproducibly assess PMVEC surface expression of E-selectin and plasminogen activator inhibitor-1 (PAI-1) by FACS. Control staining of cells with isotype antibodies for E-selectin and PAI-1 were used. The mean fluorescence intensity (MFI) for E-selectin and PAI-1 for all in vitro experiments were each normalized by dividing by the MFI of the respective isotype-control antibodies. Under basal, unstimulated conditions, wild-type PMVEC expressed low levels of E-selectin, compared to a negligible signal with isotype-control antibody. PMVEC E-selectin expression was markedly increased following LPS treatment (Figure 3.1). Similarly, wild-type PMVEC expressed very low levels of PAI-1 under basal conditions, which was enhanced following LPS stimulation (Figure 3.2). Based on this analysis we can reliably measure E-selectin and PAI-1 expression in PMVEC by FACS.

We next assessed the potential effects of the cultured PMVEC dissociation method on PMVEC cell surface E-selectin or PAI-1 expression, by comparing the effects of EDTA vs. trypsin. Following either trypsinization or non-enzymatic/EDTA harvest, PMVEC had similar levels of basal E-selectin expression and PAI-1 expression (Figure 3.3). Following trypsinization, E-selectin expression septic upregulation in PMVEC was observed. Although septic E-selectin also appeared to be upregulated in PMVEC released by EDTA harvest, but the MFI of isotype antibody varied a lot following EDTA cell dissociation method suggested high
non-specific binding and giving high non-specific fluorescence. In contrast, the septic increase in PAI-1 was only detectable using trypsinization method. As such, tryps inization was used as the PMVEC dissociation method for all subsequent experiments.

3.2 Effects of PMVEC iNOS on Basal PMVEC E-selectin expression

Next, we analyzed the effect of endothelial iNOS on PMVEC E-selectin expression by comparing iNOS+/+ and iNOS-/- PMVEC. Basal, unstimulated E-selectin expression in iNOS+/+ PMVEC was significantly higher as compared to iNOS-/- PMVEC (isotype-normalized MFI 59±7, n=22 vs. 16±2, n=36, p<0.05). The effect of the presence and composition of a subcellular matrix on basal E-selectin in iNOS+/+ and iNOS-/- PMVEC under non-septic conditions was analyzed. Basal E-selectin expression in iNOS+/+ PMVEC was consistently higher than in iNOS-/- PMVEC when cells were grown in the absence of any subcellular matrix, or in the presence of either matrigel or gelatin (Figure 3.4). This suggests that the difference in basal E-selectin expression between iNOS+/+ vs. iNOS-/- PMVEC is not likely due to the effects of the sub-cellular matrix.

In another approach, we explored the difference between iNOS+/+ and iNOS-/- PMVEC basal E-selectin through assessment of the effects of NOS inhibitors on iNOS+/+ PMVEC under non-septic conditions. We assessed the effects of 24-hours treatment of iNOS+/+ PMVEC with L-NAME, a non-selective NOS inhibitor (100µM) and 1400W, an iNOS selective inhibitor (100µM) on E-selectin expression. Complete NOS inhibition (L-NAME) or selective iNOS
inhibition (1400W) had no effect on basal E-selectin in iNOS+/+ PMVEC at 24 hrs (Figure 3.5). This suggests that the greater basal E-selectin expression in iNOS+/+ PMVEC is not directly related to the activity of iNOS or ecNOS.

3.3 Effects of PMVEC iNOS on PMVEC E-selectin expression under septic conditions

PMVEC E-selectin expression rapidly increased, peaking at 2 hrs after septic stimulation (LPS 1000ng/ml) (Figure 3.6). E-selectin expression peaked at a similar level at 2 hrs (MFI 284±38 vs. MFI 257±29, respectively, p=NS) in iNOS+/+ and iNOS-/PMVEC. The basal difference in E-selectin expression between iNOS+/+ and iNOS-/PMVEC persisted at one hour after LPS treatment. Overall, there was rapid and widespread expression of E-selectin in both iNOS+/+ and iNOS-/PMVEC after LPS stimulation: Two hours following LPS treatment, 95.3±1.0% of iNOS+/+ PMVEC expressed E-selectin (p<0.05 vs control medium-treated) and 97.9±0.4% of iNOS-/PMVEC cells expressed E-selectin (p<0.05 vs control medium-treated).

In a dose-response study, the significant difference in E-selectin expression between iNOS+/+ and iNOS-/PMVEC under basal conditions was persistent after LPS stimulation, specifically at low-moderate level doses, but was lost with high dose LPS stimulation (Figure 3.7).

In a cytomix (10ng/ml) stimulation time course study, PMVEC E-selectin expression rapidly increased, peaking at 2 hrs after stimulation in both iNOS+/+ and iNOS-/PMVEC (Figure 3.8). E-selectin expression peaked at a similar level at 2 hrs (MFI 220±22 vs. MFI 256±40, respectively, p=NS) in iNOS+/+ and iNOS-/PMVEC. The basal difference in E-selectin expression between iNOS+/+ and iNOS-/PMVEC persisted at one hour after cytomix treatment. Two hours following cytomix stimulation, 95.4±0.6% of iNOS+/+ PMVEC and
96.6±0.9% of iNOS-/− PMVEC expressed E-selectin (both p<0.05 vs respective control medium-treated cells).

The difference in E-selectin basal expression between iNOS+/+ and iNOS-/− PMVEC was present following low dose cytomix stimulation, but was lost with moderate-high dose cytomix (Figure 3.9). The dose-response for cytomix was otherwise similar between iNOS+/+ and iNOS-/− PMVEC.

These data confirm that PMVEC iNOS presence itself does not contribute to PMVEC E-selectin expression upregulation after septic stimulation with either LPS or cytomix.

3.4 **Effect of PMVEC iNOS on PMVEC PAI-1 expression under septic conditions**

We next assessed the role of endothelial iNOS on PAI-1 expression in iNOS+/+ vs. iNOS-/− PMVEC before and after septic stimulation. There was no difference in basal, unstimulated PAI-1 expression in iNOS+/+ vs. iNOS-/− PMVEC (Figure 3.10). In a LPS treatment time course study, PAI-1 expression in iNOS+/+ and iNOS-/− PMVEC was increased by 4 hrs after LPS stimulation, and sustained at 8-12 hrs (Figure 3.10). 99.7±0.2% of cells expressed PAI-1 in iNOS+/+ PMVEC and 97.8±0.4% of cells expressed PAI-1 in iNOS-/− PMVEC post LPS stimulation at peak of 12 hrs (both p<0.05 vs respective control medium-treated cells). Following LPS treatment, PAI-1 was dose-dependently increased in both iNOS+/+ and iNOS-/− PMVEC to a similar extent (Figure 3.11).

In a cytomix treatment time course study, PAI-1 expression in iNOS+/+ and iNOS-/− PMVEC was increased by 2 hrs after cytomix stimulation and was then sustained at 4-8 hrs
100±0% of cells expressed PAI-1 in iNOS+/+ PMVEC and 100±0% of cells expressed PAI-1 in iNOS-/PMVEC after cytomix stimulation at peak of 2 hrs (p<0.05 vs respective control medium-treated cells).

Similarly, in cytomix dose response studies, PAI-1 was dose-dependently increased in both iNOS+/+ and iNOS-/ PMVEC to a similar degree (Figure 3.13).

These data suggest that endothelial iNOS does not contribute to PAI-1 expression in iNOS+/+ or iNOS-/ PMVEC under basal conditions and post-septic stimulation with either LPS or cytomix.

It has been reported that PAI-1 is transcriptionally induced after septic stimulation but surface expression of PAI-1 is transient followed by cleavage and release of PAI-1 into the circulation. In order to verify induction of PMVEC PAI-1 after septic stimulation, we performed RT-PCR analysis. The effect of endothelial iNOS on PAI-1 mRNA expression/level in iNOS+/+ vs. iNOS-/ PMVEC was analyzed under septic vs. basal/unstimulated conditions. The basal PAI-1 mRNA expression was similar in iNOS+/+ vs. iNOS-/ PMVEC. Following 4-hrs septic stimulation (LPS or cytomix), the increases in PAI-1 mRNA levels were also similar in iNOS+/+ and iNOS-/ PMVEC (p<0.01 by ANOVA for each iNOS+/+ and iNOS-/ PMVEC) (Figure 3.14).

Given the absence of any measurable effect of PMVEC iNOS on PMVEC activation under septic conditions (LPS or cytomix), as reflected by PMVEC surface E-selectin expression or PAI-1 expression by FACS, or any effect on PMVEC PAI-1 mRNA expression, all subsequent studies of PMN-PMVEC co-cultures were carried out using only iNOS+/+ PMVEC.
3.5 Effect of PMN iNOS on PMVEC E-selectin expression in PMVEC under septic conditions

We next defined the roles of PMN presence in PMN-PMVEC co-culture and PMN iNOS on PMVEC activation (E-selectin and PAI-1 expression) in iNOS+/+ PMVEC under basal, unstimulated and septic conditions. In all these PMN-PMVEC co-culture studies, we used PMNs isolated from mouse bone marrow (BM), as they can be obtained in larger numbers than from blood.

Isolated iNOS+/+ and iNOS-/- mouse bone marrow PMN were first assessed to confirm activation after septic stimulation. PMN were assessed for presence of CD11b/CD18 (Mac-1 integrins) post septic stimulation vs. unstimulated PMN. There were no differences in basal, unstimulated CD11b and CD18 expression in iNOS+/+ vs. iNOS-/- PMN (Figure 14A, B). Following treatment of iNOS+/+ and iNOS-/- PMN with LPS (100 ng/ml) for 4hrs, there were marked increases in the expression of both CD11b (MFI 505±64 vs. MFI 537±46, respectively, p<0.001 for each vs. baseline) and CD18 (MFI 157.96±16 vs. MFI 147.63±11, respectively, p<0.01 for each vs. baseline) without any difference between iNOS+/+ and iNOS-/- PMN. In contrast, cytomix (1 or 10 ng/ml) for 4 hrs was not associated with upregulation of either CD11b or CD18 expression in both iNOS+/+ and iNOS-/- PMN (Figure 3.15). PMN activation marker (CD11b/CD18) MFI was normalized by subtracting MFI of respective isotype stained cells.

We next examined the role of PMN presence and PMN iNOS on E-selectin expression in iNOS+/+ PMVEC after septic (LPS or cytomix) stimulation. Co-culture of either iNOS+/+ or iNOS-/- PMN with PMVEC in the absence of septic stimulation, was associated with mildly enhanced E-selectin expression in iNOS+/+ PMVEC as compared to PMVEC alone (Figure
When iNOS+/+ PMN were pre-stimulated with LPS (100ng/ml) for 4 hrs and co-cultured with PMVEC during further LPS (0.3ng/ml) stimulation for 2 hrs, PMVEC E-selectin expression was significantly enhanced as compared to both unstimulated PMN/PMVEC co-cultures and LPS-treated PMVEC alone (Figure 3.16). There was no difference in PMVEC E-selectin upregulation following LPS treatment of PMN-PMVEC co-cultures using either iNOS+/+ or iNOS-/- PMN.

Since we used BM PMNs to access the PMN iNOS effect on endothelial activation, the BM samples required processing to remove other cellular components, e.g. RBC. Thus, isolated BM cells were treated with NH₄Cl buffer to lyse RBCs and obtain purified PMN prior to septic stimulation and FACS analysis of PMN. It has been reported that NH₄Cl can iodinate PMNs (Philips et al; 1983), which could affect PMN or PMVEC activation, and possibly confound interpretation of iNOS+/+ vs iNOS-/− PMN differences. Thus, we compared the effects of two common RBC lysis methods on PMVEC E-selectin upregulation in LPS-treated PMN-PMVEC co-cultures (Figure 3.17). Following distilled water or NH₄Cl-based RBC lysis methods, we observed similar upregulation of PMVEC E-selectin expression in LPS-treated PMN-PMVEC co-cultures with either iNOS+/+ or iNOS-/− PMN (Figure 3.17).

In contrast to LPS-treatment of PMN-PMVEC co-cultures which enhanced PMN-dependent PMVEC E-selectin expression, cytomix treatment of PMN-PMVEC co-cultures was not associated with any upregulation of E-selectin expression in iNOS+/+ PMVEC as compared to cytomix-stimulated iNOS+/+ PMVEC alone (Figure 3.18). Moreover, this lack of cytomix/PMN response was similar with either iNOS+/+ or iNOS-/− PMN in co-culture with PMVEC.
3.6 Effect of PMN iNOS on PMVEC PAI-1 expression under septic stimulation

We next examined the role of PMN presence and PMN iNOS on PMVEC PAI-1 expression in PMN-PMVEC co-cultures under septic (LPS or cytomix) stimulation. Co-culture of either iNOS+/+ or iNOS-/- PMN with PMVEC in the absence of septic stimulation was not associated with any upregulation of PAI-1 expression in iNOS+/+ PMVEC as compared to PMVEC alone (Figure 3.19, 3.20). When iNOS+/+ PMN were pre-stimulated with LPS (100ng/ml) for 4 hrs and co-cultured with PMVEC during further LPS (10 ng/ml) stimulation for 4 hrs, PMVEC PAI-1 expression was not enhanced as compared to both unstimulated PMN-PMVEC co-cultures and LPS-treated PMVEC alone (Figure 3.19). Similarly, cytomix (1 ng/mL) treatment for 4 hrs of PMN-PMVEC co-cultures had no obvious stimulatory effect on PMVEC PAI-1 expression vs. unstimulated PMN-PMVEC co-cultures and cytomix-treated PMVEC alone for both iNOS+/+ and iNOS-/- PMN (Figure 3.20).

The effect of PMN presence and PMN iNOS on PMVEC PAI-1 mRNA expression in PMN-PMVEC co-cultures was also analyzed by RT-PCR. LPS or cytomix treated PMN-PMVEC co-cultures using either iNOS+/+ vs. iNOS-/- PMN did not demonstrate any enhancement of PMVEC PAI-1 mRNA expression (Figure 3.21).
Figure 3.1: Single channel flow cytometric analysis shows induction of E-selectin expression in iNOS +/- PMVEC in response to LPS.

PMVEC were treated with LPS (1000ng/mL; red peak) vs. control medium (black peak) for 4 hrs. PMVEC were stained with monoclonal antibody to E-selectin and PE-conjugated secondary antibody and analyzed by FACS. Isotype-control antibodies were used for all studies, but the signal is not shown here.
Figure 3.2: Single channel flow cytometric analysis shows induction of PAI-1 expression in iNOS +/- PMVEC in response to LPS.

PMVEC were treated with LPS (1000ng/mL; white peak) vs. control medium (black peak) for 4 hrs. PMVEC were stained with polyclonal antibody to PAI-1 or isotype control antibody (grey peak) and PE-conjugated secondary antibody and analyzed by FACS.
Figure 3.3: The effect of trypsin and EDTA methods of cell harvest on cell-surface E-selectin and PAI-1 expression by FACS in iNOS+/+ murine pulmonary microvascular endothelial cell (PMVEC).

iNOS+/+ PMVEC were treated with cytomix (10ng/mL) vs. control Medium for 2 hrs, and then harvested by either trypsin or EDTA treatment, and analyzed for PMVEC surface E-selectin or PAI-1 expression by FACS. For each sample, E-selectin and PAI-1 MFI were Isotype normalized. *, p<0.05 vs. respective Medium, #, p<0.01 vs. cytomix treated PMVEC; N=3 per group. Data are presented as mean ± SEM.
Figure 3.4: The effect of the presence of sub-cellular matrix on basal E-selectin expression in iNOS+/+ vs. iNOS-/- PMVEC.

iNOS+/+ vs. iNOS-/- PMVEC were cultured in the absence of any sub-cellular matrix, or the presence of Matrigel (1:8 dilution) or Gelatin (1%). N=3 per group. Data are presented as mean ± SEM.
Figure 3.5: The effect of inhibition of nitric oxide synthase (NOS) on basal E-selectin expression in iNOS+/+ PMVEC under non-septic conditions.

iNOS+/+ mPMVEC were treated with the NOS inhibitors L-NAME and 1400W (100uM) vs. control Medium/DMSO for 24 hrs. N=4 for each group. Data are presented as mean ± SEM.
Figure 3.6: Time course of LPS (1000 ng/ml) induced E-selectin expression in iNOS+/+ vs. iNOS-/- PMVEC.

**, p<0.01 and ***, p<0.001 for LPS-treated vs. respective control Medium; #, p<0.001 vs. similarly-treated iNOS+/+ PMVEC; N=4-9 for iNOS+/+ and N=9-17 for iNOS-/- PMVEC. Data are presented as mean ± SEM.
Figure 3.7: Dose-dependent effect of LPS on E-selectin expression in iNOS+/+ vs. iNOS-/PMVEC.

iNOS+/+ vs. iNOS-/ mPMVEC were treated with the indicated doses of LPS vs. control medium for 2 hrs. *, p<0.05 and **, p<0.01 vs. respective Medium, #, p<0.001 vs. Medium-treated iNOS+/+ PMVEC; N=4-13 per group. Data are presented as mean ± SEM.
Figure 3.8: Time course of cytomix (10ng/ml) induced E-selectin expression in iNOS+/+ vs. iNOS-/- PMVEC.

*, p<0.05 and **, p<0.01 vs. respective Medium; ##, p<0.001 vs. medium-treated iNOS+/+ PMVEC; N=3 per group. Data are presented as mean ± SEM.
Figure 3.9: Dose-dependent effect of cytomix on E-selectin expression in iNOS+/+ vs. iNOS-/- PMVEC.

iNOS+/+ vs. iNOS-/ mPMVEC were treated with the indicated doses of cytomix vs. control Medium for 2 hrs. *, p<0.05, ***, p<0.001 vs. respective Medium; N=3-5 per group. Data are presented as mean ± SEM.
Figure 3.10: Time course of LPS (1000ng/ml) induced PAI-1 expression by FACS in iNOS+/+ vs. iNOS-/- PMVEC.

*, p<0.05 and ***, p<0.001 vs. respective control Medium; N=4-13 for all groups. Data are presented as mean ± SEM.
Figure 3.11: Dose-dependent effect of LPS on PAI-1 expression in iNOS+/+ vs. iNOS-/-

PMVEC.

iNOS+/+ vs. iNOS-/– mPMVEC were treated with the indicated doses of LPS vs. control Medium for 12 hrs. *, p<0.05, **, p<0.01 and ***, p<0.001 vs. respective control Medium; N=4-13 for each group. Data are presented as mean ± SEM.
Figure 3.12: Time course of cytomix (10ng/ml) induced PAI-1 expression in iNOS+/+ vs. iNOS-/- PMVEC.

*, p<0.05 and **, p<0.01 vs. respective control Medium; N=3 per group. Data are presented as mean ± SEM.
Figure 3.13: Dose-dependent effect of cytomix on PAI-1 expression in iNOS+/+ vs. iNOS-/− PMVEC.

iNOS+/+ vs. iNOS-/− mPMVEC were treated with the indicated doses of cytomix vs. control Medium for 2 hrs. *, p<0.05, **, p<0.01 vs. respective control Medium; N=3-4 per group. Data are presented as mean ± SEM.
Figure 3.14: The effect of endothelial iNOS on septic stimulation (Cytomix 10 ng/mL vs. LPS 1000 ng/mL) induced PAI-1 mRNA expression.

PMVEC PAI-1 mRNA transcript levels were quantified by RT-PCR and normalized to β-actin. *, p<0.05 and **, p<0.01 vs. respective Medium; N=4-9 per group. The data is represented as fold change relative to Medium.
Figure 3.15: The effect of septic stimulation (cytomix vs. LPS) on the activation of iNOS+/+ vs. iNOS-/- bone-marrow neutrophils (PMN).

iNOS+/+ vs. iNOS-/- PMN were pre-treated with LPS (100ng/ml) vs. cytomix (1 and 10ng/ml) vs. control Medium for 4 hrs, and then PMN CD11b and CD18 expression were analyzed by FACS. **, p<0.01 and ***, p<0.001 vs. Medium-treated respective PMN; N=3-4 per group.
Figure 3.16: The effect of iNOS+/+ vs. iNOS-/- BM PMN on PMVEC E-selectin expression after LPS stimulation.

iNOS+/+ vs. iNOS-/- PMN were pre-treated with LPS (100ng/ml) vs. control Medium for 4 hrs and then co-cultured with PMVEC for 2 hrs under LPS (0.3ng/ml) stimulation. *, p<0.05 and **, p<0.01 vs. control medium; §, p<0.05 and §§, p<0.001 vs. respective Medium-treated PMN/PMVEC co-cultures; N=4 per group. Data are presented as mean ± SEM.
Figure 3.17: The effect of different RBC lysis methods on LPS/PMN induced E-selectin expression in iNOS+/+ PMVEC.

Following percoll gradient method to isolate bone marrow PMN, PMN were treated with double distilled water (dd H₂O) or NH₄Cl based RBC lysis buffer. iNOS+/+ vs. iNOS/- PMN were pre-treated with LPS (100ng/ml) for 4 hrs and then co-cultured with PMVEC for 2 hrs under LPS (0.3ng/ml) stimulation. *, p<0.05, **, p<0.01 and ***, p<0.001 vs. Medium; N=3 per group. Data are presented as mean ± SEM.
Figure 3.18: The effect of iNOS+/+ vs. iNOS-/- BM PMN on E-selectin expression in iNOS+/+ PMVEC after cytomix stimulation.

iNOS+/+ vs. iNOS-/- PMN were pre-treated with cytomix (1ng/ml) vs. control Medium for 4 hrs and then co-cultured with PMVEC for 2 hrs under cytomix (0.3ng/ml) stimulation. N=4 per group. Data are presented as mean ± SEM.
Figure 3.19: The effect of iNOS+/+ vs. iNOS-/- BM PMN on PAI-1 expression in iNOS+/+ PMVEC after LPS stimulation.

iNOS+/+ vs. iNOS-/- PMN were pre-treated with LPS (100ng/ml) vs. control Medium for 4 hrs and then co-cultured with PMVEC for 4 hrs under LPS (10ng/mL) stimulation. **, p<0.01 vs. Medium; §, p<0.05 and §§, p<0.01 vs. respective Medium-treated PMN/PMVEC co-cultures; N=3-6. Data are presented as mean ± SEM.
Figure 3.20: The effect of iNOS+/+ vs. iNOS-/- BM PMN on PAI-1 expression in iNOS+/+ PMVEC after cytomix stimulation.

iNOS+/+ vs. iNOS-/- PMN were pre-treated with cytomix (1ng/ml) vs. control Medium for 4 hrs and then co-cultured with PMVEC for 2 hrs under cytomix (0.3&1ng/ml) stimulation. §, p<0.05 vs. respective Medium-treated PMN/PMVEC co-cultures; N=3. Data are presented as mean ± SEM.
Figure 3.21: The effect of BM PMN iNOS on septic stimulation (Cytomix vs. LPS) induced PAI-1 mRNA expression in iNOS+/+ PMVEC.

PMVEC PAI-1 mRNA transcript levels were quantified by RT-PCR and normalized to β-actin. N=2-3 per group. The data is represented as fold change relative to Medium.
CHAPTER 4: DISCUSSION

These studies assessed activation of PMVEC under different septic conditions such as LPS and cytomix, as reflected by expression of E-selectin and PAI-1. However, PMVEC iNOS did not affect LPS or cytomix induced PMVEC activation. PMN enhanced septic PMVEC E-selectin expression following LPS treatment, and this endothelial activation was independent of PMN iNOS. Contrary to this, PMN failed to enhance PMVEC E-selectin expression post cytomix stimulation. In addition, PMN failed to upregulate PMVEC PAI-1 expression post LPS or cytomix stimulation. Our data suggest that septic PMVEC activation in isolation or in the presence of PMN, is independent of both PMVEC and PMN iNOS.

In the pathophysiology of ALI, many cells contribute such as inflammatory cells and parenchymal cells. Mainly, inflammatory cells such as neutrophils contribute to the initiation of inflammatory cascade during septic ALI (345-347). Similarly, EC activation and injury are also important features of sepsis and sepsis-induced lung injury. During activation, EC increase permeability and exhibit adhesive and procoagulant state (348; 349).

In this study, we focused on 2 common markers of EC activation. Endothelial E-selectin mediates leukocyte rolling and adhesion to EC during sepsis, whereas endothelial PAI-1 correlates with increased coagulation and fibrin deposition and endothelial injury during sepsis or septic ALI (350). Indeed, increased endothelial E-selectin and PAI-1 mRNA levels, as well as protein expression on the cell surface and also as a soluble form after cleavage from membrane bound receptors are found in plasma of septic patients and in animals model of sepsis or septic
ALI. Under basal, non-septic conditions, wild-type PMVEC expressed a low-level of E-selectin, which was significantly lower in iNOS-/− PMVEC. The basal E-selectin expression differences were not abrogated when iNOS+/+ and iNOS-/− PMVEC were incubated with either non-selective NOS inhibitor, L-NAME or iNOS selective inhibitor, 1400W. This suggests that difference in iNOS+/+ vs. iNOS-/− PMVEC basal E-selectin expression is not simply due to iNOS or cNOS activity. Moreover, iNOS+/+ and iNOS-/−PMVEC when cultured on different subcellular matrix materials, such as matrigel or gelatin, showed the same difference in basal E-selectin expression. This suggests PMVEC basal E-selectin difference is not related to the subcellular matrix culture condition.

Wild-type PMVEC E-selectin expression was significantly upregulated under septic conditions, and to a similar extent post LPS or cytomix stimulation. LPS stimulation also upregulated PMVEC PAI-1 expression at 4 hrs post stimulation of PMVEC. In contrast, cytomix induced PMVEC PAI-1 expression at 2 hrs post stimulation. This suggests PMVEC behaved differently when exposed to LPS or cytomix in terms of PMVEC PAI-1 upregulation, different from the similar patterns of LPS- and cytomix-stimulated PMVEC E-selectin upregulation. Our results are in agreement with others in terms of EC E-selectin and PAI-1 expression upregulation under septic conditions, although shown to different degrees in different types of EC (HUVEC and human PMVEC) (291; 291; 351-353).

4.1 Role of EC iNOS

During sepsis or septic ALI, NO production and iNOS expression are enhanced, which is believed to increase the degree of vascular and lung injury (354). Our lab has previously shown
in a model of septic ALI that iNOS deficient mice post CLP has reduced neutrophil sequestration, protein leak and increased neutrophil migration (355). iNOS is expressed by a variety of cells including parenchymal and inflammatory cells. Specifically, it has been reported that endothelial cells express iNOS and produce iNOS derived NO under septic conditions like LPS via MAPK dependent pathway (356).

In the present study, iNOS+/+ PMVEC E-selectin expression was upregulated to a similar extent as in iNOS-/- PMVEC post LPS or cytomix treatment regardless of differences in basal E-selectin expression in iNOS+/+ PMVEC vs. iNOS-/- PMVEC. Moreover, iNOS+/+ or iNOS-/- PMVEC E-selectin expression followed a similar time course and dose-response, independent of iNOS, post LPS or cytomix stimulation. This suggests that the very rapid, early maximum upregulation of PMVEC E-selectin may facilitate a high-degree of neutrophil adhesion to PMVEC within the first 2 hrs of septic stimulation.

Similarly, there was no difference in PMVEC PAI-1 expression between iNOS+/+ PMVEC vs. iNOS-/- PMVEC at baseline, or following LPS or cytomix stimulation. It has been recognized that PMVEC PAI-1 contributes to lung vascular permeability during early lung inflammation. However, at later stages, PAI-1 contributes to resolution of lung inflammation as disruption of PAI-1 at later stages increased mortality in P. aeruginosa induced pneumonia in mice (295). In our study, we found sustained elevation of PMVEC PAI-1 expression at 12 hrs, which also supports the hypothesis that enhanced coagulation occurs in more delayed fashion, eg. several hours after septic (LPS or cytomix) stimulation.
In summary, these above findings suggest that PMVEC iNOS does not affect LPS or cytomix induced endothelial E-selectin or PAI-1 expression. The possibility that endothelial iNOS may promote endothelial injury or activation under inflammatory (e.g. septic) conditions is still controversial. Indeed, we found endothelial iNOS does not contribute to endothelial activation under different septic conditions. Similarly to our findings, Hickey in 2001 found no difference in lung endothelial E-selectin expression upregulation in iNOS+/+ or iNOS/- septic mice following LPS treatment(357). In contrast, Lush found increased lung tissue endothelial E-selectin expression in iNOS+/+ vs. iNOS/- septic mice following CLP (358). Moreover, in both of these latter in vivo murine septic studies, there were no differences in basal endothelial E-selectin expression in unstimulated iNOS+/+ or iNOS/- mice.

We also found that iNOS+/+ PMVEC E-selectin expression was higher than iNOS/- PMVEC, and this difference was not related to the use of different subcellular matrix conditions. Moreover, NOS inhibitors (both iNOS-selective 1400W and non-selective L-NAME) did not affect this PMVEC basal E-selectin expression difference between iNOS+/+ and iNOS/- PMVEC. In contrast to our findings, TNF alpha-stimulated PMVEC E-selectin expression was attenuated with L-NAME or when PMVEC were isolated from eNOS-deficient mice (359). We speculate that our observed difference could be due to in vitro culture conditions, which may not reflect in vivo differences, or possibly to other unexpected differences between iNOS+/+ vs iNOS/- PMVEC, as a result of genetic absence of iNOS.

NOS can also modulate endothelial PAI-1 expression during septic ALI, although the effects remain controversial. For example, endogenous NOS activator L-arginine induced PAI-1 antigen in an EC line (EA hy 926) after TNF alpha stimulation, whereas the non-selective NOS
inhibitor L-NAME reduced EC surface PAI-1 antigen (360). In contrast, others have reported that GTPase Rac1 dependent ecNOS-derived NO production in EC reduces PAI-1 upregulation (361). Finally, it was also reported that NO does not contribute to LPS induced PAI-1 expression in HUVEC (362). These data suggest that the role of NO in EC PAI-1 upregulation under inflammatory/septic conditions is still uncertain. Therefore we investigated role of EC iNOS in PMVEC PAI-1 expression and mRNA level upregulation under different septic conditions. Our results are consistent with Perez Ruiz that showed septic PMVEC PAI-1 upregulation was independent of EC iNOS after LPS or cytomix stimulation.

4.2 Role of PMN and PMN iNOS

Neutrophils are involved in endothelial activation, injury and apoptosis during sepsis and septic ALI (357; 363). Furthermore, our lab has shown in mice model of septic ALI, anti-CD18 treatment in mice prior to CLP has significantly reduced pulmonary MPO levels, BAL neutrophils, protein leak and 8-isoprotane levels (Wang L et al;2012). This suggests neutrophils contribute importantly in development of septic ALI. In the present study, the presence of PMN in co-culture with PMVEC synergistically enhanced LPS induced PMVEC E-selectin expression in a dose-dependent manner. However, this PMN-dependent PMVEC E-selectin induction was similar between iNOS+/+ and iNOS-/- PMN, and thus septic (LPS) PMN-dependent PMVEC activation also appears to be independent of PMN iNOS. In contrast, the presence of PMN in co-culture with PMVEC under cytomix stimulation did not enhance PMVEC E-selectin expression, and this was similar between iNOS+/+ PMN and iNOS-/- PMN. These data suggest that there is differential activation of PMN under different septic conditions, e.g. Cytomix vs LPS, and a
resulting difference in PMN-dependent PMVEC activation. Importantly, septic PMN-dependent PMVEC activation, as reflected by E-selectin expression, was independent of both PMVEC and PMN iNOS.

In contrast to LPS-induced PMN-dependent PMVEC E-selectin upregulation, the presence of PMN in co-culture with PMVEC under LPS or cytomix treatment did not upregulate PMVEC PAI-1 expression. Moreover, iNOS+/+ or iNOS-/- PMN with PMVEC could not affect PMVEC PAI-1 expression post LPS or cytomix stimulation. Similarly, iNOS+/+PMN or iNOS-/-PMN did not affect PMVEC PAI-1 mRNA expression after LPS or cytomix treatment.

Among diverse functions of neutrophil during sepsis, many have shown a neutrophil contribution towards cultured EC activation and injury (363). For example, It has been reported that PMN when co-cultured with HUVEC post fMLP treatment has increased procoagulant factors such as TF (364). Similarly, human neutrophils when co-cultured with HUVEC in the presence of TNF α have shown to upregulate HUVEC E-selectin expression at 24 hrs (365).

During neutrophil activation, there are many potential mechanisms of EC activation/injury. These include neutrophil-derived cytokines, NO, ROS, and proteases. For example, neutrophil TNF α has induced endothelial adhesion molecules such as E-selectin, P-selectin, ICAM-1, VCAM-1 whereas, neutrophil IFN-γ gamma has shown to increase ICAM-1 , P-selectin, E-selectin and reduce VCAM-1 under shear stress (366).

We had hypothesized that neutrophil iNOS could contribute to endothelial activation under septic conditions. We have previously shown in septic mice BM-derived neutrophil iNOS dependent pulmonary microvascular trans-EC protein leak, pulmonary oxidant stress and
nitrosative stress in vivo, and reduced trans-endothelial migration in vivo and in vitro (69); (355). Moreover, we found that inflammatory cell (neutrophil) source of iNOS contributes importantly to trans-human PMVEC albumin leak in vitro, without any contribution of PMVEC iNOS (367). Recently we have proved by using reciprocal BM neutrophils depleted/reconstituted with neutrophil iNOS+/+ chimeric mice that septic ALI is dependent on neutrophil iNOS in vivo (70). In the present study, our results showed that PMN iNOS does not contribute to PMVEC E-selectin or PAI-1 upregulation post LPS or cytomix treatment. There are limited studies in similar PMVEC, however, the NO donor, SNAP did enhance PMN-induced HUVEC E-selectin expression (368). This discrepancy may be due to many factors, including interspecies differences, micro vs macrovascular EC heterogeneity, and type of septic stimulus used.

Other neutrophil mediators likely explain PMVEC activation in the current study. Neutrophil peptides and proteases such as antimicrobial proteins, elastase, Cathepsin G, and others have been shown to modulate endothelial integrity and cytoskeleton during septic conditions. For example, it has been reported that recombinant cationic antimicrobial protein of molecular weight 37 kDa (CAP37), a neutrophil-derived protein, upregulated the expression of VCAM-1 and E-selectin in EC (369). In addition, neutrophil proteases contributed to upregulation of endothelial PAI-1 in sepsis induced liver injury. As it is evident from our results that under different septic conditions, activation or non-activation of neutrophils did not affect PAI-1 upregulation. This suggests neutrophils behave differently under different septic conditions to activate endothelial cells in a manner that can potentiate or fine tune inflammatory response.
4.3 Limitations

We appreciate the constraints posed by the use of cytomix to mimic sepsis. The in vitro model (i.e., cytomix) does not exactly reflect human sepsis but only provides exposure to pro-inflammatory mediators of sepsis which are involved in pathogenesis of sepsis or septic ALI. Cytomix has been used extensively to model sepsis in in vitro studies using endothelial and inflammatory cells, but the exact composition of cytomix is extremely variable (345; 346; 346; 367; 370). Our choice of cytomix components and concentrations was based on preliminary in vitro data from our lab on PMN-dependent EC injury, characterized by albumin leak.

We only focused on the role of iNOS specifically in PMVEC and PMN, based on our extensive work in vivo in murine sepsis. Clearly, eNOS could be an important source of NO which may have some modulatory effects in our model. For example, NO derived from eNOS has shown to reduce endothelial activation by attenuating P-selectin, E-selectin, ICAM-1, VCAM-1 levels during sepsis (371). Moreover, NO donors have shown to reduce PMVEC E-selectin expression after TNF\(\alpha\) stimulation (291).

Our assessment of PMN-dependent effects under septic conditions depends upon adequate stimulation of PMN. The lack of PAI-1 response to cytomix suggested that may be cytomix failed to activate PMN adequately. Indeed, our PMN activation results showed that LPS significantly upregulated CD11b/CD18 expressions but cytomix failed to upregulate CD11b/CD18 expressions on PMN. Our results of PMN activation by LPS are consistent with other literature (372).
We assessed surface E-selectin by FACS as a marker of PMVEC activation. The surface E-selectin signal could have been affected by other biologic processes. For example, endothelial E-selectin is also shed as a soluble E-selectin in plasma or it is clustered by lipid rafts or clathrin coated pits or internalized by clathrin coated pits (353; 373). However, we clearly found a significant upregulation of surface E-selectin, suggesting that septic E-selectin shedding does not necessarily result in reduction in endothelial cell surface E-selectin expression.

4.4 Summary

PMVEC E-selectin and PAI-1 are activated under different septic conditions and this upregulation is independent of PMVEC iNOS. Neutrophils behave differently to activate endothelial cells under different septic conditions. PMN can upregulate PMVEC E-selectin expression post LPS in a dose dependent manner but not during cytomix treatment, and PMN-dependent PMVEC activation was independent of PMN iNOS. Furthermore, PMN did not upregulate PMVEC PAI-1 expression post LPS or cytomix stimulation. Moreover, PMN iNOS did not affect PMVEC E-selectin or PAI-1 upregulation post LPS or cytomix stimulation. This study suggests that PMN iNOS dependent PMVEC injury does not appear to be mediated through PMN iNOS dependent PMVEC activation. Therefore, further studies will be required to further explore potential mechanisms of the PMN iNOS-derived NO role in septic PMVEC injury.
CHAPTER 5: THESIS SUMMARY

5.1 Hypothesis 1 –Pulmonary microvascular endothelial cells (PMVEC) iNOS contributes to PMVEC activation under septic conditions in vitro.

In these studies, we investigated the role of iNOS in isolated PMVEC activation under septic conditions. We characterized activation of PMVEC by assessing upregulation of the adhesion molecule E-selectin and the procoagulant factor PAI-1 under different septic conditions, specifically LPS and cytomix. To pursue the role of PMVEC iNOS in PMVEC activation, we isolated, cultured and studied PMVEC from iNOS+/+ and iNOS-/- mice. We assessed PMVEC basal and septic upregulation of E-selectin and PAI-1 expression by flow cytometry.

We found iNOS+/+ PMVEC basal E-selectin expression was higher than iNOS-/- PMVEC, but this difference appeared to not be due to either iNOS or cNOS activity, or to the specific subcellular matrix used in PMVEC culture. However, iNOS+/+ or iNOS-/- PMVEC E-selectin expression is upregulated post LPS or cytomix stimulation. Thus, we found PMVEC iNOS does not contribute to induction of PMVEC E-selectin expression under septic conditions. Moreover, PAI-1 from iNOS+/+ or iNOS-/- PMVEC followed similar pattern of upregulation of expression post LPS or cytomix stimulation vs. control or medium. However, the expression was only detectable in PMVEC. Our result suggested that PMVEC iNOS does not contribute to PMVEC PAI-1 expression upregulation under septic conditions. In addition, we also determined the role
of PMVEC iNOS in PAI-1 mRNA levels post LPS or cytomix stimulation. Our results suggested that PAI-1 gene expression post septic stimulation vs. control is not PMVEC iNOS dependent. We conclude PMVEC iNOS does not contribute to PMVEC E-selectin or PAI-1 expression under basal conditions or following septic stimulation.

5.2 Hypothesis 2 – Bone marrow neutrophil (PMN) iNOS contributes to pulmonary microvascular endothelial cell activation (PMVEC) under septic conditions in vitro.

In these studies, we assessed one potential mechanism of PMN iNOS-dependent septic PMVEC injury, by investigating the role of PMN iNOS in PMVEC activation under septic conditions. We analyzed PMVEC E-selectin and PAI-1 expression by flow cytometry in PMN-PMVEC co-cultures in vitro. PMVEC isolated from WT mice were incubated with LPS or cytomix pretreated PMN isolated from bone marrow of iNOS+/+ and iNOS-/- mice.

We found both iNOS+/+ or iNOS-/ PMN synergistically enhanced PMVEC E-selectin expression post LPS stimulation in a dose dependent manner. However, PMN iNOS did not affect PMVEC expression upregulation post LPS stimulation. In contrast, neither PMN nor PMN iNOS affected PMVEC PAI-1 expression or PAI-1 mRNA level induction post LPS or cytomix treatment. We next assessed PMN activation after LPS or cytomix stimulation. We assessed activation of beta 2 integrins (CD11b/CD18) by flow cytometry. Our results showed a dramatic enhancement of CD11b and CD18 expression post LPS stimulation. In contrast, cytomix could not increase any of CD11b or CD18 expression. This study suggests that PMNs behave differently under different septic conditions to activate endothelial cells. However, PMN iNOS
does not appear to contribute to PMVEC E-selectin or PAI-1 upregulation under septic conditions. Therefore, we conclude that septic PMN iNOS-dependent PMVEC injury is likely not mediated through PMN iNOS-dependent PMVEC activation.
6.1 Introduction

This thesis assessed the presence of PMVEC activation under different septic conditions in vitro, assessed the role of PMVEC iNOS in septic PMVEC activation and the specific involvement of iNOS in both the PMVEC and in co-cultured PMN. PMN behaved differently with regards to PMVEC activation under different septic conditions. PMN significantly enhanced PMVEC E-selectin expression in a dose dependent manner after LPS stimulation, and this PMN-dependent effect was independent of PMN iNOS. PMN had no effect on PMVEC E-selectin expression during cytomix treatment, and did not affect PMVEC PAI-1 upregulation or mRNA level after LPS or cytomix stimulation. Interestingly, LPS showed a dramatic elevation of CD11b and CD18 expression in isolated PMN, but cytomix failed to activate PMN.

ALI is characterized by impaired gaseous exchange, diffuse alveolar injury, increased endothelial injury and permeability and increased neutrophil influx (70; 374; 375). PMVEC are activated under different septic conditions as shown in our results (chapter 6). We hypothesized that PMVEC iNOS and PMN iNOS contributes in PMVEC activation as our lab has previously shown the important contribution PMN iNOS-dependent PMVEC injury under septic conditions in vivo and in vitro, and septic PMVEC injury was independent of PMVEC iNOS (69).

6.2 Assessment of EC activation

Endothelial cell activation and injury are important features in the development of sepsis and septic ALI. During activation of endothelial cells, they become highly permeable, pro-
adhesive, and procoagulant. Expression of the EC adhesion molecule, E-selectin, has been assessed by a number of researchers for its contribution to leukocyte-EC adhesion. E-selectin is endothelial specific and is a common marker of endothelial activation (376). Procoagulant factor PAI-1 presence and expression has also been defined in experiments by many researchers. Although PAI-1 is also expressed by platelets, it can be used as a marker of endothelial activation (377). In this study, we used flow cytometry to assess EC surface protein expression. For our in vitro protocol, we used trypsinization as a cell dissociation method prior to analysis of PMVEC E-selectin or PAI-1 by FACS. Our PMVEC were grown to confluence and then treated with control medium vs. LPS or cytomix stimulation prior to being assessed by FACS.

6.3 Endothelial iNOS effect on EC activation

Endothelial iNOS is upregulated during sepsis and septic ALI. Moreover, endothelial iNOS has been shown to contribute to vascular abnormalities during sepsis and impairment of several lung physiological functions such as regulation of pulmonary vascular tone, immunocompetence, and neuronal signaling (378). Endothelial iNOS contributed to endothelial dysfunction of vascular and conduit arteries during endotoxemia (379). Endothelial iNOS has been shown to contribute to the progression of delayed circulatory failure in septic shock (380).

In previous work from our lab, endothelial iNOS expression did not contribute to septic ALI in septic mice, or EC injury in vitro (356; 367). In the present study, we investigated the role of endothelial iNOS in PMVEC septic activation. We found that PMVEC iNOS does not appear to contribute to LPS or cytomix induced PMVEC E-selectin or PAI-1 expression. Similar to our findings, Hickey in 2001 found no difference in lung EC E-selectin expression upregulation in
iNOS+/+ or iNOS-/- septic mice post LPS stimulation (357). In contrast, Lush in 2001 found increased lung EC E-selectin expression in iNOS+/+ vs. iNOS-/- septic mice post CLP (357; 358). However, the difference in lung EC E-selectin expression in vivo could have been due to the effects of iNOS in other cells and not specifically in the EC themselves.

In the present study, we found higher iNOS+/+ PMVEC basal E-selectin expression as compared to iNOS-/- PMVEC. Interestingly, two previous in vivo studies did not find any difference in basal, unstimulated endothelial E-selectin expression between iNOS+/+ or iNOS-/- mice (357; 358). Moreover, it was previously reported that the iNOS-specific inhibitor 1400W did not modulate PMVEC basal E-selectin expression, however, non-selective NOS inhibition reduced TNFα-induced E-selectin expression in PMVEC (359). The role of endothelial iNOS in endothelial activation is still under debate.

6.4 Neutrophil iNOS effect on endothelial activation under septic conditions

Upregulation of neutrophil iNOS contributes importantly to the pathophysiology of sepsis and especially sepsis-induced ALI. Using bone-marrow transplanted chimeric mice, our lab has previously shown murine neutrophil iNOS-dependent trans-endothelial pulmonary microvascular protein leak, pulmonary oxidant stress and nitrosative stress in sepsis in vivo, as well as neutrophil iNOS-dependent regulation of murine trans-endothelial PMN migration in sepsis (355);(367). Recently we also definitively showed using reciprocal neutrophil-specific iNOS chimeras that septic ALI was almost completely dependent on neutrophil iNOS in vivo (70). Moreover, we found both in murine and human cell culture that neutrophil iNOS contributes...
importantly to trans-EC albumin leak in vitro (367). These data strongly support an essential role of neutrophil iNOS in septic lung injury and specifically PMVEC injury.

In the present studies, we investigated the role of neutrophil iNOS in PMVEC activation under different septic conditions in vitro. We found neutrophils synergistically upregulated PMVEC E-selectin expression post LPS treatment in a dose dependent manner. However, we found that neutrophil iNOS does not contribute to septic PMVEC E-selectin upregulation.

Unfortunately, we could not demonstrate PMN-dependent PMVEC activation under cytomix treatment, nor was there any PMN-dependent effects on septic PMVEC PAI-1 expression. Thus, we also conclude that PMN are differentially activated under different septic conditions.

6.5 General implications of thesis

Pulmonary microvascular endothelial cells are activated during septic ALI, and this could influence neutrophil-endothelial interactions, which could contribute to septic endothelial injury. Activated endothelial cells can also enhance coagulation and microvascular thrombotic events during septic ALI. PMVEC E-selectin was significantly upregulated post LPS or cytomix stimulation, which can mimic systemic septic activation of endothelial cells, and results in enhanced inflammatory cell-EC adhesion. PMVEC showed maximum upregulation of E-selectin expression at early hours post septic stimulation, which is consistent with observations that leukocyte and endothelial interactions occur early during sepsis. In contrast, septic PMVEC PAI-
1 expression was detectable at later timepoints during septic stimulation. This suggests that thrombotic events may occur later during sepsis or septic ALI.

We also demonstrated that septic PMVEC activation, as reflected by E-selectin and PAI-1 upregulation, is independent of both PMVEC and PMN iNOS. Given previous reports of PMN iNOS-dependent septic PMVEC injury/dysfunction, our current findings suggest that this PMN-dependent injury is not mediated through PMVEC activation. As such, the results of the present studies suggest that PMN iNOS-dependent septic PMVEC injury may be mediated through other mechanisms, such as cytoskeleton disruption and/or junctional protein damage.

6.6 Future directions

1. Assessment of the effect of PMN and PMN vs PMVEC iNOS on other markers of EC activation.

2. Assessment of the possible role of PMN iNOS in septic human PMVEC activation as a potential mechanism of documented PMN-dependent septic human PMVEC injury in vitro

3. Assessment of other potential mechanisms of PMN iNOS-dependent septic murine PMVEC injury/dysfunction, specifically PMVEC cytoskeletal arrangement or junctional protein disruption under septic conditions
Reference List


146. Calderwood JW, Williams JM, Morgan MD, Nash GB and Savage CO. ANCA induces beta2 integrin and CXC chemokine-dependent neutrophil-endothelial cell


195. McAuley DF, Laffey JG, O'Kane CM, Cross M, Perkins GD, Murphy L, McNally C, Crealey G and Stevenson M. Hydroxymethylglutaryl-CoA reductase inhibition with


361. **Petry A, Belaiba RS, Weitnauer M and Gorlach A.** Inhibition of endothelial nitric oxide synthase increases capillary formation via Rac1-dependent induction of hypoxia-


APPENDIX 1: ANIMAL ETHICS APPROVAL

Western

07.01.08
This is the 1st Renewal of this protocol
*A Full Protocol submission will be required in 2011

Dear Dr. Mehta

Your Animal Use Protocol form entitled:

Mechanisms of Effects of NO in Septic Lung Injury

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from 07.01.08 to 06.30.09

The protocol number for this project remains as 2007-002

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
4. If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

e.e. Approved Protocol  - S Mehta, T Kirkpatrick
   Approval Letter   - T Kirkpatrick

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, London, Ontario, CANADA N6A 5C1
PH: 519-661-2111 ext. 86770  FL 519-661-2028 www.uwo.ca/animal
APPENDIX 2: RESUME OF ZAHRA ASAD

ZAHRA ASAD

OBJECTIVE
Seeking a career position as a Research Assistant with a well-established organization commensurate with my experience and professional skills.

RESIDENCE STATUS
Canadian Citizen

PROFESSIONAL EXPERIENCE

2007 – 2013
London Health Sciences Centre, Western University, London, Ontario

- Ex-vivo and mice cultured cell surface analysis of endothelial cell surface proteins (cellular adhesion molecules such as E-selectin, PECAM-1, VE-Cadherin, vWF, PAI-1)
- Isolation of endothelial cells from mice lung
- Isolation of PMN from mice tibia and femur
- Tracheostomy for mice broncho alveolar lavage to collect macrophages
- Lung perfusion
- Cecal ligation and perforation of mice to create a model of sepsis
- RT-PCR analysis for analysis of endothelial PAI-1 during sepsis
- Performed co-cultured experiments for analysis of cell surface proteins by flowcytometry (FACS)

Jan 2006 – May 2006
Armed forces Institute of Pathology Research Associate

- Polymerase chain reaction (PCR) analysis for cystic fibrosis in human patients, extraction of DNA from sample, screening test for mutation in gene responsible for cystic fibrosis
- PCR, RT-PCR to investigate Hepatitis C virus genotype in patients
- Analysis of blood blast cells CD34 expression and CD59 in patients with leukemia and lymphoma by FACS

2005
Medical Centre Laboratory Technologist

Through laboratory testing covered the field of hematology, Urinalysis, Immunology and clinical testing blood glucose monitoring, infectious disease diagnosis, histology and report abnormal findings to supervisors and doctors
SKILLS

- Flow cytometric analysis
- PCR, RT-PCR
- Electrophoresis, Agarose gel electrophoresis, SDS-page electrophoresis, elution of DNA band from agarose
- Protein analysis with spectrophotometer
- Western blotting, Northern blotting, Southern blotting
- Excellent knowledge of safety procedures and practices
- Strong communication, excellent facilitation skills, problem solving, self-management, team skills and continuous improvement

TECHNICAL

PRESENTATIONS

"Role of iNOS in septic pulmonary mircovascular endothelial cell activation"

EDUCATION

2013
Master of Science
Pharmacology, Western University

2000
Master of Science
Biochemistry Agriculture University, Faisalabad, Pakistan

1998
Bachelor of Science
Chemistry & Physics University of Punjab, Lahore, Pakistan

LANGUAGE SKILLS

English, Urdu: Fluent-read, write, speak

REFERENCES

Furnished upon request