Nucleophosmin Mediates Intracellular LPS Signaling in Lung Epithelial Cells

by

Mirna D. Ghazarian

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Master of Science
Institute of Medical Science
University of Toronto
2014

Abstract

**Introduction:** Lipopolysaccharide (LPS) internalization, mediated by intercellular adhesion molecule-1 (ICAM-1), has been demonstrated in lung epithelial cells. We hypothesized that nucleophosmin (NPM) facilitates downstream signaling by interaction with intracellular ICAM-1.

**Methods:** Monodansylcadaverine was used to inhibit LPS internalization and the subsequent IL-8 production in human lung epithelial cells. Co-immunoprecipitation and direct protein-protein binding assays were performed to assess whether NPM interacts with ICAM-1. NPM siRNA was transfected into lung epithelial cells to specifically examine NPM-mediated intracellular LPS signaling.

**Results:** LPS stimulation in lung epithelial cells was associated with IL-8 production that was completely attenuated by inhibition of LPS internalization. ICAM-1 interaction with NPM was associated with intracellular LPS-induced IL-8 production and cytotoxicity. This LPS-induced inflammatory response was facilitated by NPM-mediated regulation of c-Fos expression.
**Conclusion:** Intracellular LPS that stimulates the downstream NPM signaling may contribute to ongoing inflammatory responses in Gram-negative sepsis.

Total Number of Words: 142
Acknowledgments

This thesis represents not only the culmination of my work over the past couple of years, but is a milestone in seven years of education at the University of Toronto. Since I first started as an undergraduate student at the university in 2007, various opportunities and experiences both academically and personally have contributed to the work detailed in these pages—without which, this thesis would not have been possible. This thesis is the result of work by numerous individuals, whom I wish to thank. This thesis is also the result of numerous experiences I have encountered from many remarkable individuals who I also wish to acknowledge.

I would like to express my utmost gratitude to my supervisor, Dr. Haibo Zhang, who saw my potential and gave me the opportunity to work and learn under his supervision. His scientific and clinical expertise, guidance, patience, and understanding added considerably to my graduate experience. He taught me how to be a better critical thinker and problem solver, which is something I will take with me into all of my future endeavors. He always listened to my ideas and encouraged constructive discussions. I don’t remember a single time he was too busy to speak with me; his continual availability and openness to discuss enhanced my graduate experience. It was always nice to know that my supervisor was easy to talk to and interested in my ideas, just as I was interested in his.

I wish to thank the members of my thesis committee, Dr. Mingyao Liu, Dr. Wolfgang Kuebler, and Dr. Sergio Grinstein, for the time that they invested, their enthusiasm, and their intellectual contributions towards my work. At first I was severely intimidated that these great experts would be advising me, but their continual support, kindness, and friendliness towards me throughout my journey broke those walls and we enjoyed numerous fruitful discussions pertaining to my work. Their comments and suggestions were significant to the completion of this thesis and I am most appreciative.

Numerous individuals at the Li Ka Shing Knowledge Institute at St. Michael’s Hospital helped, encouraged, and taught me immensely. A very special thanks goes to Dr. Bing Han,
who never hesitated to help me — from being with me at every single visit to the confocal microscope, to teaching me the majority of experimental techniques I needed to complete my thesis, to reaching for boxes that I could not reach; I could not have asked for a better research associate to work with. He always took time out of his busy work schedule to cater to me and for that, I will always be grateful. To Julie Khang, a very large thanks for her administrative help, technical help, and most of all, her friendship towards me over the last few years. She was nothing but kind to me in 2010 when she first introduced me to the place I would be doing my undergraduate research project in — and little did we know that one and a half years later, I would continue there for a Master’s project. She has been a continual source of moral support, with a collection of some of the funniest stories I have ever heard. She has been just a completely wonderful colleague and friend to me throughout my journey and I will never forget her. To Alice Luo, where to start! We started undergraduate research projects together, and went on to pursue our Master’s degrees together. She was always willing to help me when I needed it the most, and took the time out of her project to help me synthesize ideas for my own. She was with me throughout all of the difficult moments when experiments failed and helped me to come up with potential solutions. She was with me throughout all of the happier moments when experiments were successful too, and cheered on with me. She and I had some of the funniest moments together in the lab and I do not know what my graduate experience would have been like without her. I am very thankful for all of her support both in and out of the lab, and am grateful for the opportunity to develop a meaningful relationship with her. My sincerest gratitude to Diana Islam, Dr. Florence Morriello, Dr. Martin Kneyber, Dr. Yasumasa Morita, Dr. Yuexin Sham, Dr. Raphael Molinaro and Caterina Di Ciano-Oliveira for their knowledge, expertise, and friendship during my graduate duration. They each had special parts to play in the completion of this thesis.

My sincerest and deepest thanks to my special group of friends who have always been there for me and remembered me in their prayers. I do not know what I would have done without their advice, encouragement, and support during some of my most difficult times. They have seen my growth and development the most — not just academically, but personally and spiritually too. Their love, patience, and understanding towards me humble
me and I am so blessed to have a group of friends such as these to stand by my side during the most challenging few years of my life.

I wish to thank my family, who has displayed their love for me in countless ways. I have been blessed with a **Mom** who prays for me incessantly and encourages me when I need it and makes sure I eat when I forget due to the workload throughout my years at the University of Toronto. She dropped me off and picked me up from the subway at the most inconvenient times without complaint, just to make it easier for me. I have been blessed with a **Dad** who continually sacrificed his comfort for me and I will always be grateful for the provision he has provided for me. Even a ride home from him at the end of a long day to avoid the subway rush-hour frenzy was sometimes all I needed. Sometimes, he brought me home stuffed animals to let me know he was thinking of me, and it would brighten up my day, despite how anxious I was about my experiments. I am also blessed with a brilliant brother, **Magar Ghazarian**, who was always willing to sit down with me and go over my presentation slides or data. He would take the time to understand my work and brainstorm ideas with me. He always commended me for my work and gave me a sense of accomplishment that motivated me to keep going. I will always be thankful and hope I can do the same for him while he pursues his future endeavors.

Last but not least, I would like to thank my Heavenly Father, who has been my source of blessing, encouragement, friendship, love, and hope. He has orchestrated everything according to His will and the past seven years at the University of Toronto contain a mesh of interwoven experiences that have come together to form the fabric of who I am in Him today. This thesis represents the culmination of all that He has done for me in bringing me to this point. It has been a privilege to study His creation and I wish to thank and glorify Him for His goodness. “And we know that all things work together for the good of those who love God, to those who are called according to His purpose.” Romans 8:28
Contributions

The work presented in this thesis was principally performed by myself, or otherwise stated.

The work presented in Figure 10 was completed with the help of Dr. Raphael Molinaro.

All confocal photomicrographs presented in Figure 3, Figure 4, Figure 5, and Figure 6, and Figure 8 were acquired with the help of Dr. Bing Han in order for images to be acquired in a blinded fashion.

Julie Khang provided technical assistance for ELISAs and RT-PCR.

Dr. Aye Aye Khine provided guidance for the establishment of the LPS binding assay protocol.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A549</td>
<td>Adenocarcinomic human alveolar basal epithelial cell line</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Arf</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>ATII</td>
<td>Alveolar type II</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
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<tr>
<td>ATF2</td>
<td>Activating transcription factor 2</td>
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<td>BAX</td>
<td>BCL2-associated X protein</td>
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<td>BEAS-2B</td>
<td>Normal human bronchial epithelial cell line</td>
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<tr>
<td>BODIPY</td>
<td>Boron-dipyrromethene</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CD14</td>
<td>Cluster of differentiation 14</td>
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<td>CD54</td>
<td>Cluster of differentiation 54</td>
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<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
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<td>Colo205</td>
<td>Colon epithelial cell line</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
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<td>CXCR2</td>
<td>CXC receptor 2</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>-------------------------------------------------</td>
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<tr>
<td>DNA</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>HBD</td>
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<td>HMGB1</td>
<td>High mobility group box 1</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<td>IFN-β</td>
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<td>Interleukin</td>
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<td>IRF3</td>
<td>Interferon response factor 3</td>
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<td>c-Jun N-terminal kinase</td>
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<td>LBP</td>
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<td>Lactate dehydrogenase</td>
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<td>Lymphocyte function-associated antigen-1</td>
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<tr>
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<td>Monoclonal antibody</td>
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<tr>
<td>Mac-1</td>
<td>Macrophage-1 antigen</td>
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<td>Mal</td>
<td>MyD88 adaptor-like</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
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<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>MD-2</td>
<td>Myeloid differentiation factor-2</td>
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<tr>
<td>MDC</td>
<td>Monodansylcadaverine</td>
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<td>MEF2</td>
<td>Myocyte enhancer factor 2</td>
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<tr>
<td>MeSH</td>
<td>Methanethiol</td>
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<td>miRNA</td>
<td>Micro RNA</td>
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<td>Myeloid differentiation protein 88</td>
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<td>Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Sodium orthovanadate</td>
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<td>Abbreviation</td>
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<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NETS</td>
<td>Neutrophil extracellular traps</td>
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<td>NFκB</td>
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<tr>
<td>PBS-T</td>
<td>Phosphate-buffered saline Tween</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerithrin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>Mouse macrophage cell line</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor interacting protein 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile alpha and HEAT-Armadillo motifs</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline Tween</td>
</tr>
<tr>
<td>TCFs</td>
<td>Ternary complex factors</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAF3</td>
<td>TNF receptor-associated factor 3</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing IFN-β</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Sepsis

Sepsis is a pathological condition in which invasion of microorganisms induces release of inflammatory and cytotoxic mediators in the host [1]. Overwhelming and persistent systemic inflammatory responses can lead to organ injury and eventually death. Sepsis is one of the most challenging syndromes encountered in patients at the intensive care units, mainly due to its rapid onset that can obstruct early diagnosis and the physiological complexities that contribute to the development and propagation of this ailing condition.

This thesis will begin with an overview of sepsis, walking through the history of sepsis definitions and diagnosis criteria, the epidemiology and the general nature of the host’s maladaptive physiological responses. It subsequently funnels into more specific aspects that are relevant to the studies presented herein, including specific molecules and various signaling pathways that trigger and propagate a systemic inflammatory response.

Furthermore, this thesis will focus on the role of the lung epithelium in the pathogenesis of sepsis since lower respiratory tract infections constitute an awful worldwide public health threat, affecting hundreds of millions of people per year [2]. Moreover, although the airway epithelia are traditionally considered as passive airflow ducts and gas exchange barriers, recent evidence indicates that they possess an intrinsic inflammatory capacity that contributes to the pathogenesis of sepsis-associated lung injury [3, 4].

The work contained herein supports an essential role for the lung epithelium in the internalization of LPS released from Gram-negative bacteria, and in the intracellular signaling that is independent of the classical cell surface-TLR4 pathway seen in immune cells. These findings not only challenge the conventional role of the lung epithelium in LPS
signaling, but also offer potential therapeutic targets to protect the lung from ongoing inflammatory responses.

1.2 Sepsis Definition

Sepsis is a systemic inflammatory response syndrome caused by infection [1]. The word “sepsis” was coined by Hippocrates (460-370 BC) and originates from the Greek word sipsi, meaning “decomposition of animal or vegetable organic matter” [5]. Until the 19th century, sepsis was associated with blood putrefaction (bacteremia) and viewed as a dangerous process of malodorous biological decay causing death [5]. Later work by Ignaz Semmelweis (1818-1865) [6], Louis Pasteur (1822-1895) [7], Joseph Lister (1827-1912) [8], and Robert Koch (1843-1910) [9] launched breakthroughs that eventually led to the currently held modern concept of sepsis. They established that infection was transmissible amongst humans and that microorganisms were their cause. Furthermore, their work demonstrated the prophylactic nature of hygiene, sterilization, and heating in the transmission of infection, thus reducing infection-related mortality amongst patients.

For years onward, sepsis was the term used for the clinical display of infection. As knowledge of this syndrome advanced, definitions of sepsis were developed and refined. In 1991, a consensus definition of sepsis was developed [1]. Key definitions included 1) sepsis — the clinical manifestations of infection and systemic inflammatory response syndrome (SIRS); 2) severe sepsis — the clinical manifestations of infection and SIRS, in addition to organ dysfunction; and 3) septic shock — severe sepsis, complicated with persistent arterial hypotension causing acute circulatory failure [1].

Due to its non-specificity for sepsis, the criteria of sepsis was updated at the 2001 International Sepsis Definitions Conference Consensus Conference [10]. The updated criteria of sepsis includes the presence of infection and at least one of the multiple criteria involving a patient’s general parameters (e.g. presence of fever), inflammatory parameters (e.g. leukocytosis), hemodynamic parameters (e.g. hypotension), organ dysfunction parameters (e.g. arterial hypoxemia), and tissue perfusion parameters (e.g. hyperlactatemia). Typically, the diagnosis of sepsis comes alongside a positive culture.
However, patients can encounter a sepsis diagnosis with negative cultures if there is a suspected infection [11]. Septic patients with negative cultures exhibit fewer comorbidities, shorter length of stay, milder illness, and reduced mortality compared to culture-positive septic patients [12].

1.3 Epidemiology

Worldwide, more than 18 million people are affected by sepsis each year [13], and it claims the lives of more than 1.5 million people in North America and Europe alone [14]. Sepsis is the leading cause of death in the ICU [15] with mortality ranging from 30% to 50%, depending on its severity [16]. Specifically in Canada, the mortality of severe sepsis is 38%, according to a prospective observational study incorporating 12 Canadian teaching and community hospitals [17]. Despite advancements in medicine and critical care in the developed world, several reports indicate an increase in the incidence of sepsis in various countries, including the United States [16, 18], United Kingdom [19], and Australia [20] — likely owing to the ageing population, who suffer impaired immunity due to immunosenescence [21]. Although the incidence of sepsis is not as well-defined for the developing world, the high frequencies of infectious diseases (e.g. malaria, tuberculosis) present in these regions arguably make sepsis a greater concern. In fact, sepsis is the underlying cause of death for the majority of children under 5 years old in developing parts of the world today [22, 23].

Despite its high morbidity rate, the case-fatality of sepsis has declined in the past decade [24], chiefly due to advancements in supportive care for critically ill patients. These include the application of bundled care practices (e.g. Surviving Sepsis Campaign) [25, 26] and low tidal volume (protective) ventilation in acute respiratory distress syndrome (ARDS) patients [27, 28].
1.3.1 Sepsis-Associated ARDS

Acute respiratory distress syndrome (ARDS) is a severe medical condition characterized by widespread inflammation in the lungs and impaired gas exchange. ARDS can develop from pulmonary causes (e.g. pneumonia); however, sepsis is a leading risk factor for patient progression to ARDS from non-pulmonary causes [29, 30]. Sepsis-associated ARDS presents a higher mortality than non-sepsis-associated ARDS [31] – sepsis-related ARDS contributes to the highest number of deaths out of all ARDS patients [32].

There is a high level of interplay between the host response in sepsis and the lung, and their relationship is bidirectional. Indeed, respiratory tract infections cause up to one third of sepsis cases [33] and the lung is the most common site of infection [34, 35].

Due to the high level of interplay between the host response in sepsis and condition of the lung, it is incumbent to understand pathophysiological mechanisms that lead one to the other, and vice versa.

1.3.2 Financial Burden

Sepsis also presents a burden on healthcare resources and finances. Septic patients require longer hospital stays [15] and the total cost of care for sepsis patients around the world ranges from $22,000 USD to $50,000 USD [13, 15, 36]. The annual cost of sepsis in the United States is reported to be almost $17 billion [15]. In fact, it is up to 10 times more expensive to treat a patient with sepsis compared to treat a patient without sepsis in the ICU [37].

1.3.3 Long-Term Effects

Patients who have survived sepsis still encounter relatively high morbidity, with poorer health-related quality of life and increased physical dysfunction [38, 39]. Furthermore, 32% of hospitalized sepsis patients are discharged to other care centres, such as rehabilitation facilities, rather than to their homes [16]. As such, sepsis is not only a
devastating disease to battle in the ICU, but it also leaves its remnants outside of the hospital environment in daily living.

1.4 Pathogenesis

The inflammatory response in sepsis is highly associated with infection. The initial event in sepsis involves the invasion of pathogens into normally sterile tissue and their recognition by host receptors, leading to a pro-inflammatory state in the host. With a fewer number of pathogens, the progression to an anti-inflammatory state in the host is sufficient to clear the pathogenic response and resolve inflammation. However, with a high virulence or number of pathogens systemically or locally, the anti-inflammatory state is not able to inhibit the pro-inflammatory reaction that was initiated. Indeed, this anti-inflammatory state also favors secondary infections [40].

Infectious pathogens causing sepsis include mainly bacteria, fungi, and viruses [34]. Bacterial infections are the most common causes of sepsis in humans [34]. Bacterial infections are divided into two groups: Gram-negative and Gram-positive. Gram-negative bacteria are characterized as such by their thinner peptidoglycan cell wall layer, which does not hold the crystal violet dye used in the Gram staining protocol. The most common Gram-negative infections in sepsis include those of the *Pseudomonas* species (20%) and *Escherichia coli* (16%) [35].

Despite the recent increase of Gram-positive infections, an international registry of over 12,000 septic patients from 37 countries showed that 57% had gram-negative bacterial infections, 44% had gram-positive bacterial infections, and 11% had fungal infections (the total is more than 100% because some patients had mixed infections) [34].

A large number of bacteria, viruses, and fungi produce and release conserved toxins that have the ability to disrupt normal cell function and cause injury. These extrinsic toxins may contain pathogen-associated molecular patterns (PAMPs) that interact with pathogen-recognition receptors (PRRs) on antigen presenting cells, which signal to regulate the transcriptome. Typically, the host also releases endogenous mediators that contribute to
the pathophysiology of sepsis. These intrinsic mediators are termed “damage-associated molecular patterns” (DAMPs), which are also able to signal through the same PRRs as PAMPs.

The classical mechanisms through which PAMPs and DAMPs are recognized are by a family of PPRs that include Toll-like receptors (TLRs) — transmembrane receptors that surround extracellular and endosomal compartments — Nod-like receptors (NLRs), and retinoic-acid-inducible gene I (RIG-1)-like helicases (RLHs), which scan cytoplasmic compartments [41]. These PPRs are activated by distinct PAMPs and/or DAMPs, and they induce or propagate cellular activation, as discussed below.

1.4.1 Gram-Negative Bacteria

The chief pathogenic component of Gram-negative bacteria is attributed to their highly negatively charged outer membrane composed of lipopolysaccharide (LPS), or endotoxin, which will be the focus of this thesis. LPS contains three distinct portions: an outer hydrophilic polysaccharide O-side chain, a center core oligosaccharide, and an inner hydrophobic lipid A region [42]. Specifically, the lipid A component, which fixes LPS to the bacterial cell wall, is responsible for its toxic biological activity [43]. Indeed, the administration of purified LPS can induce the same pathophysiological effects as those seen in patients with Gram-negative sepsis [44].

LPS is typically released from the cell wall as a consequence of cell death and lysis, as well as growth by cellular division [45]. When LPS is released, it appears as free LPS and has a significantly increased biological activity in comparison to its bacterial cell-bound counterpart [46]. While antibiotic treatment is necessary to combat infection, antibiotics precipitate the release of LPS by disrupting the outer membrane of bacteria [47]. LPS can activate endothelial cells [48], epithelial cells [49], macrophages [50], and other immune cells [51, 52] to produce pro-inflammatory mediators, including cytokines and chemokines.

Toll-like receptor (TLR) 4 (TLR4) has been implicated as the LPS receptor because mice that carry a null mutation for TLR4 (C57BL/10ScCr) and mice that carry a mutation in the
cytoplasmic signaling domain (C3H/HeJ) are hyporesponsive to LPS [53]. LPS-mediated TLR4 signaling results in the activation of the transcription factors NFκB [54], activating protein-1 (AP-1) [55] and interferon response factor 3 (IRF3) [56], which lead to the production of inflammatory cytokines and chemokines (more discussed below).

Other Gram-negative toxins include Exotoxin A, which is produced by *P. aeruginosea* and enters the cell through receptor-mediated endocytosis [57]. Exotoxin A catalyzes the removal of the ADP-ribosyl moiety from nicotinamide-adenine dinucleotide and transfers it to elongation factor-2 (EF-2)—thus inactivating EF-2 and arresting protein synthesis [58].

### 1.4.2 Gram-Positive Bacteria

Gram-positive bacteria in the staphylococci and streptococci species produce toxic components that can be released without bacterial growth or lysis [59]; they contain peptidoglycan (PepG) and lipoteichoic acid (LTA) in their cell walls [60]. Comparable to LPS, PepG and LTA can act synergistically through TLR2 to activate cells and induce the production of pro-inflammatory mediators through NFκB activation [61].

In addition, Gram-positive bacteria produce relatively small, potent toxins called superantigens (SAg) that directly disrupt normal cell function [63]. *S. pyogenes* produce streptococcal mitogenic exotoxin Z (SMEZ), and streptococcal pyrogenic exotoxin A and C (SPEA, SPEC) [63, 64]. SAgS generated by *S. aureus* include staphylococcal enteroxins A-E (SEA-E) and toxic shock syndrome toxin-1 (TSST-1) [65]. These toxins bind to MHC-II molecules, thus evading the processing by antigen presenting cells, yet still stimulating T cells [66]. Furthermore, SAgS bind to CD28, a costimulatory molecule on T cells that leads to further activity and increased production of cytokines, which mediates lethal toxic shock [67]. Whereas usual antigens activate less than 0.01% of T cells, SAgS can activate more than 20% of T cells due to their direct binding of MHC-II and CD28 [68]. Other examples of SAgS include enterotoxin (toxin A) and cytotoxin (toxin B) produced by *Clostridium difficile*, which are glucosyltransferases that inactivate the Rho family of GTPases [69]. The Gram-positive related products and their signal pathways are not further discussed, as it is out of the scope in the present study.
1.4.3 Summary of Endotoxin-Associated Sepsis Clinical Trials

Since the identification of LPS, clinical trials began using anti-endotoxin antibodies to neutralize LPS in the circulation. Despite many of these trials reaching phase III, these treatments provided no overall benefit to patients with sepsis [70].

Upon the discovery of TLR4 as a receptor for LPS, clinical trials began to look at anti-TLR4 antibodies [71] and antagonists [72, 73] as treatment options for sepsis. However, they too have failed to reach their primary outcomes and have failed to save patients with sepsis. The most recent drug, Eritoran (a synthetic lipodisaccharide that antagonizes the effects of LPS on the cell surface) [74], failed in Phase III as there was no difference in 28-day mortality and IL-6 levels in patients who took the drug versus placebo [72, 73] (Figure 1).
Figure 1. Neutralization of circulating LPS and inhibition of the cell-surface receptor of LPS, TLR4, failed in clinical trials of patients with sepsis. LBP = LPS binding protein; MD-2 = myeloid differentiation factor 2; TLR4 = Toll-like receptor 4.
1.5 Pathophysiology

1.5.1 Macrophages and Neutrophils

If a microorganism crosses the epithelial barrier and begins to multiply in the host tissue, the residential mononuclear phagocytes, or macrophages, of the tissues recognize it. Circulating monocytes continuously mature into macrophages that leave the circulation to migrate into tissues.

Upon receptor engagement on the cell surface by LPS, monocytes and macrophages are activated to release pro-inflammatory cytokines, which include IL-1β, TNFα, and IL-6, as well as anti-inflammatory cytokine, IL-10, to modulate the inflammatory response [4]. These responses are typically mediated through TLR4 [75].

These pro-inflammatory cellular signals recruit neutrophils and other immune cells to the site of infection. Neutrophils, or polymorphonuclear leukocytes (PMNs), are the most abundant phagocytes in the circulation, with a half-life of approximately 6-8 hours [76]. In response to increasing levels of cytokines, endothelial cells rapidly up-regulate their surface expression of adhesion molecules, P- and E-selectin for neutrophil adhesion [77, 78]. Later release of IL-8 from endothelial and epithelial cells further recruit neutrophils and increase the expression of β2-integrins LFA-1 and Mac-1 on neutrophils [79]. These integrins interact with intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial and epithelial cells to further increase adherence and facilitate trans-endothelial and trans-epithelial migration of neutrophils [80, 81].

Both macrophages and neutrophils have the ability to recognize, ingest, and kill many microorganisms. Bacteria are recognized either by their cellular components (such as LPS or PepG), or through opsonization by the host (such as being coated with the complement protein, C5a) [82]. Engagement of the various cell-surface receptors that recognize bacteria leads to phagocytic activity, followed by bacterial killing.

Phagocytosis of bacteria is a dynamic process. The pathogen is first encompassed by the phagocyte membrane, and then internalized in a membrane-bound vesicle called the
phagosome. In addition, neutrophils are granulocytes that contain antimicrobial peptides. The phagosome fuses with one or more granules to generate a phagolysosome—a structure in which the granule contents are released [83]. This creates an adverse local environment with decreased pH and numerous proteases that focus on killing the pathogen [83]. In addition to their microbicidal functions, these anti-microbial peptides also possess pro-inflammatory properties when released extracellularly or into the circulation; for instance, alpha-defensins—the most prevalent antimicrobial peptide in neutrophils—have the ability to stimulate the release of IL-8 from epithelial cells [84].

Neutrophils can also mediate oxygen-dependent killing mechanisms with the production of reactive oxygen species, including hydrogen peroxide, the superoxide anion, and nitric oxide, which are immediately toxic to bacteria [85]. This process, known as “respiratory burst”, is generated by NADPH oxidases in their granules [85].

1.5.1.1 Endotoxin Tolerance

Endotoxin tolerance refers to the state wherein professional immune cells or other cell types are resistant to subsequent endotoxin challenges. Endotoxin tolerance is defined by the reduced capacity of these cells to release pro-inflammatory cytokines in response to LPS or other microbial toxins [86]. Both circulating monocytes harvested from sepsis patients that were stimulated with LPS or normal human monocytes stimulated with LPS at two time points showed a reduced ability to release pro-inflammatory cytokines, such as TNFα, but had unchanged or enhanced production of anti-inflammatory mediators, such as IL-10 (comprehensive review in [86])[87]. Endotoxin tolerance is well-described in monocytes and macrophages, but not well-understood in epithelial cells. Clinically, the persistence of this refractory state is associated with increased mortality and the occurrence of nosocomial infections [88].

The mechanisms involved in endotoxin tolerance are not yet clear. An increase in the release of immunosuppressive mediators, such as IL-10, has been implicated to play a role [86, 89]. Furthermore, a persistent reduction in monocyte human leukocyte antigen-DR expression—a MHC class II surface receptor—has been implicated in endotoxin tolerance
in human monocytes and shown to predict mortality in septic shock [89, 90]. However, microRNAs and epigenetic modifications are also currently being investigated for their roles [86, 91].

Our studies contained in this thesis incorporated a single time point for LPS challenge to cells in all experimental conditions. Our interests pertain to the contribution of epithelial cells to the pro-inflammatory state in the host that arises in sepsis, rather than the possible refractory state of epithelial cells that may occur as the sepsis condition progresses.

1.5.2 Airway Epithelium

Epithelial cells are held together by tight junctions, which are protective against the external environment and are a physical barrier to infection. Epithelial cells are found in the skin, gastrointestinal, respiratory, and urinary tracts, among others. Pathogens can establish an infection only if they can colonize the epithelial barrier, or traverse through it.

Bacteria can bind to specific receptors on mucosal epithelial surfaces, which allow them to enter, or damage the cells for invasion. Mucosal epithelia, such as those of the respiratory tract, have cilia and secrete mucus [92]. Glycoproteins in the mucus can coat bacteria to prevent their adhesion to the epithelium, and ciliary movement expels the foreign invaders [93]. In fact, individuals with defective ciliary movement or mucus secretion have the tendency to develop more bacterial lung infections [94].

Pulmonary epithelial cells are traditionally considered as “innocent bystanders”, wherein immune cell-mediated inflammatory cascades target the epithelium, leading to its destruction [95]. However, the pulmonary epithelium is increasingly being shown to play a part in the inflammatory cascade itself [95]. Particularly, lung epithelial cells participate in the immune response to LPS in various ways. Epithelial cells produce and secrete various antimicrobial peptides [96]. For example, human beta-defensins (HBD1-4) are small cationic peptides expressed by respiratory epithelial cells that are secreted to kill bacteria by damaging their cell membrane [96, 97]. Furthermore, antimicrobial peptides released from the lung epithelia appear to possess immunomodulatory properties separate from
their antimicrobial function. These roles include facilitating posttranslational processing of interleukin (IL)-1β [98] and being chemoattractants for neutrophils [99].

Moreover, lung epithelial cells can contribute to inflammation by producing cytokines, chemokines, and reactive oxygen species in response to LPS stimulation [3, 4]. Of particular attention is the large amount of chemokines produced by primary alveolar type II cells (ATII) isolated from humans, which include monocyte chemoattractant protein (MCP)-1, and IL-8 [4]. Furthermore, media collected from LPS-stimulated ATII cells stimulated migration of leukocytes in an in vitro transwell system, which was significantly blocked by antibodies against IL-8 and MCP-1 [4]. These findings indicate the role of the lung epithelium as an important source of chemokines that prompts leukocyte migration to the lung.

1.5.3 Host Inflammatory Response

Once the cell encounters bacteria, or LPS, receptors are activated to induce cytokine production. The term “cytokines” was proposed by Stanley Cohen in 1974 to classify the soluble family of proteins that are secreted from cells to communicate stimulatory or inhibitory signals to other cells and alter their function [100]. Cytokines typically act through cell-surface receptors that lead to intracellular signaling pathways that modulate the transcriptional activity of a cell [101]. Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are among the earliest secreted cytokines upon LPS stimulation in various cell types [4]. In fact, TNFα is secreted as little as 30 minutes after LPS stimulation to stimulate alveolar macrophages and epithelial cells to release chemokines [4]. Other cytokines include interleukin-6 (IL-6) and interferon γ (IFNγ) [102]. Chemokines, such as interleukin-8 (IL-8) and MCP-1, are a subset of cytokines that function as chemotactic signals to recruit immune cells to the site of infection.

In an ideal situation, this infection-induced host immune response is tightly regulated and localized by various mechanisms including: 1) production of anti-inflammatory cytokines (IL-10) [103]; 2) secretion of antagonists that neutralize extracellular pro-inflammatory cytokines (IL-1 receptor antagonist) [104]; and 3) epigenetic modifications that silence
pro-inflammatory genes [105]. With these regulatory mechanisms in place, homeostasis should be reached within the host’s system. However, sepsis patients exhibit an imbalanced pro-inflammatory response that is unable to be modulated by the host’s regulatory mechanisms, leading to a persistent, hyper-inflammatory state [106].

Indeed the host’s maladaptive and persistent inflammatory response to the foreign pathogen wreaks systemic bedlam and approximately 30% of sepsis deaths occur within the first 3 days of the syndrome [107]. PAMPs, particularly LPS, induce inflammatory cells, as well as other cell types, to produce inflammatory cytokines including TNFα, IL-1β, IL-6, IL-8, and IL-12 [108, 109]. These inflammatory mediators further recruit and activate immune, endothelial, and epithelial cells, which becomes lethal without a sufficient counter-inflammatory response.

TNFα and IL-1β are the typical pro-inflammatory cytokines that mediate endotoxin shock and cause tissue injury [110, 111]. These cytokines are released within the first couple of hours of LPS exposure, and induce activation of a secondary inflammatory cascade [112]. TNFα induces the expression of various pro-inflammatory cytokines including IL-8 [113]. Distinct from cytokine production, TNFα also induces the production of reactive oxygen species and augments the expression of cell adhesion molecules, which lead to increased permeability and immune cell migration into tissues [114, 115].

The high levels of pro-inflammatory cytokines continue to recruit more immune cells in the early phase of sepsis, leading to the further production of pro-inflammatory cytokines and cell injury [116]. Moreover, endothelial and epithelial cells produce pro-inflammatory cytokines as well, which contribute to the hyperinflammatory environment [116]. Furthermore, defensins released by neutrophils and epithelial cells further stimulate cytokine production and recruit immune cells [84, 99]. This overstimulation of immune, endothelial, and epithelial cells, and the positive-feedback loop of pro-inflammatory cytokines causes an uncontrollable, damaging “cytokine storm” that eventually results in cell injury, tissue ischemia, and multi-organ failure if left unresolved [116].
1.5.4 Coagulation

In an effort to prevent spread of the infection, clotting pathways are activated within the host [117]. Sepsis is associated with a pro-coagulative state that promotes fibrin deposition through three known pathways: Tissue factor (TF)-mediated generation of thrombin, deregulated anticoagulation pathways, and decreased fibrinolysis (Reviewed in [118]).

Activation of coagulation is mainly driven by TF. When monocytes or macrophages are stimulated by bacteria, TF is expressed on their surface in the circulation and binds and activates factor VII. The TF/factor VIIa is thus generated, which activates factor X, producing factor Xa, resulting in thrombin generation [117-119].

The most prominent anticoagulation pathways are antithrombin, TF pathway inhibitor, and activated protein C [118]). Antithrombin inhibits thrombin and factor Xa, thus preventing thrombin generation [118]. However, antithrombin activity is decreased in sepsis patients [120], thus promoting coagulation. TF pathway inhibitor is localized to the endothelial cell surface and facilitates inhibition of the TF-initiated coagulation [121]; however, sepsis is associated with decreased TF pathway inhibitor, which further promoting coagulation [122]. Lastly, activated protein C cleaves the coagulation factor Va [123], yet is also downregulated in sepsis, thus promoting coagulation. Furthermore, activated protein C bears protective effects on endothelial barrier function [124]. Severe sepsis patients markedly vary in their ability to produce activated protein C [125].

Fibrin has a known function in stabilizing the formation of clots, as well as a role in immobilizing microbes on the leukocyte surface [117]. Sepsis is also associated with a depression in fibrinolytic processes [126]. Therefore, there is compromised fibrin removal and consequential formation of fibrin deposits in the microcirculation.

In a non-sepsis environment, there is a delicate balance between procoagulant and anticoagulant processes; however, there is a decrease in the anticoagulant pathways during sepsis that results in excessive thrombin generation and fibrin formation [127], as described above. Excessive thrombin signaling increases endothelial cell permeability and leukocyte adhesion, which are mediated largely by induced E-selectin, P-selectin, ICAM-1
and VCAM-1 expression [128-131]. The formation of fibrin clots causes excessive blood clotting, which is detrimental to sepsis patients as blood flow to vital organs becomes impaired. Furthermore, thrombin and fibrin signaling can stimulate the production of IL-8 in endothelial cells, contributing to the pro-inflammatory and pro-coagulative state in the host [132].

Neutrophils are the first line of defense against infection. Alongside their phagocytic activity, activation releases web-like structures of DNA from neutrophils – termed neutrophil extracellular traps (NETs)[133]. NETs contain proteolytic activity that ensnare bacteria and kill them [133]. Despite trapping and killing bacteria, NETs can also induce pro-coagulation effects by acting as stimuli for thrombus formation [134].

1.5.5 Endothelial Dysfunction

Upon pathogen invasion, endothelial cells are locally activated to release pro-inflammatory mediators, recruit leukocytes, and promote clotting in an effort to prevent spread [135]. Indeed, some endothelial cells are lost due to apoptosis or necrosis in these processes, but this is considered an adaptive response that is under host control [135]. However, due to sustained and excessive endothelial activation during sepsis, the host response becomes maladaptive rather than adaptive. The hallmark of endothelial dysfunction is low nitric oxide (NO) availability, likely due to impaired NO synthase activity and/or the increased production of reactive oxygen species, which inactivate NO [136]. Endothelial dysfunction results in reduced vascular relaxation, leading to hypertension [136]. Furthermore, LPS has been shown to also cause physical disruption of endothelial cells [137], which can lead to tissue edema and the presence of inflammatory mediators and cells leaking into the interstitial space [136].

1.5.6 Multi-Organ Dysfunction

If the host response to infection spreads systemically rather than remaining local, the persistent widespread activation and dysfunction of endothelial, epithelial, and immune cells, together with the deregulation of coagulation pathways, lead to lowered tissue
perfusion. Sepsis has been associated with significant heterogeneous distribution of oxygen supply in alveoli and the microvasculature, and increased intercapillary distance, which lead to abnormal oxygen extraction [138-140]. The aforementioned pathophysiological effects discussed herein interact with one another to perpetuate inflammation, coagulation, apoptosis, and other relevant cellular interactions, which can eventually lead to microvascular occlusion, tissue edema and ischemia, and ultimately, organ failure [136].

1.6 LPS and Toll-Like Receptor 4 (TLR4)

The Toll protein was first discovered in Drosophila as an essential regulator of dorsal-ventral patterning during embryogenesis [141]. Sequencing Toll revealed that its carboxyl terminus was appreciably similar to that of the vertebrate IL-1 receptor (IL-1R) [142]. Knowledge of the role of IL-1R in infection provided grounds to investigate the now-established roll of Toll in immune responses to fungi and bacteria [143].

Human Toll-like receptors (TLRs) belong to a family of molecules called the IL-1R/TLR superfamily, which are characterized by their Toll/IL-1R (TIR) signaling domain in the cytoplasmic domain [144]. TLRs are type I transmembrane receptors, with an amino extracellular and carboxyl intracellular domain. There are currently 10 identified human TLRs, expressed in a wide variety of cell types, including immune, epithelial, and endothelial cells [144]. The extracellular domain of TLRs typically contains over 600 residues and is highly polymorphic compared to its intracellular TIR domain, which is highly conserved [145]. The TIR domain contains 150 residues and regulates protein-protein interactions between TLRs and key proteins involved in the signal transduction pathway [145].

TLR4 was identified as an LPS receptor when two strains of mice, C3H/HeJ (Tlr4-signaling inactive) and C57BL/10ScCr (Tlr4-null) displayed defective LPS signaling [53]. C3H/HeJ mice have a single mutation in the TIR signaling domain of TLR4, which renders it dysfunctional. C57BL/10ScCr mice (also called C57BL/10ScNJ) have a mutation in the TLR4 gene, resulting in no TLR4 to be made, as confirmed by mRNA analysis [53, 146].
Despite being implicated as the key pattern-recognition receptor for LPS receptor, direct binding between TLR4 and LPS has not been established.

Rather, free LPS interact with several carrier, adaptor and receptor proteins to transmit its cellular signal in immune cells [147, 148]. LPS binding protein (LBP), a carrier protein, forms a complex with LPS, which then associates with the glycosylphosphatidylinositol-anchored membrane protein, CD14 on cells [147, 148]. CD14 transfers LPS to the adaptor protein MD-2, which binds to TLR4 [149, 150]. As such, MD-2 mediates complete TLR4 activation upon LPS recognition [149]. Once LPS is recognized, a cellular signal is transduced, which activates the transcriptome and leads to cytokine production [144]. Soluble CD14 may also bind to the LBP-LPS complex and is required to activate cells that do not express membrane CD14, such as endothelial and some epithelial cells [144].

1.6.1 TLR4 Signaling Pathways

TLR4 is the chief signaling receptor for LPS in human immune cells. Upon LPS recognition, TLR4 undergoes ligation, which activates the TIR domains to recruit downstream adaptor proteins [145]. A single point mutation in the TIR domain of TLR4 completely abolishes its response to LPS, thus making the TIR domain crucial for signaling [53].

In response to LPS, TLR4 signaling involves 5 TIR-containing adaptor proteins: 1) myeloid differentiation protein 88 (MyD88); 2) TIR domain-containing adaptor protein (TIRAP), also known as MyD88-adapter-like (Mal); 3) TIR domain-containing adaptor inducing IFN-β (TRIF); 4) TRIF-related adaptor molecule (TRAM); 5) and sterile alpha and HEAT-Armadillo motifs (SARM)-containing protein [145].

TLR4 signaling is conventionally divided into two arms. Each arm recruits downstream protein kinases, which activate transcription.

The MyD88-dependent signaling pathway comprises Mal recruitment to the plasma membrane, which subsequently acts as a bridge to recruit MyD88 and TIRAP [145]. Activation of these adaptor proteins induces inflammatory cytokine expression through activation of JNK/MAPK pathways, AP-1, and NFκB, mediated by tumor-necrosis factor-
receptor-associated factor 6 (TRAF6) [151]. Studies using MyD88-deficient macrophages demonstrated that the MyD88-dependent pathway is involved in the production of pro-inflammatory cytokines TNFα, IL-1β, and IL-6 [152].

TLR4 is then internalized into the endosome network where the MyD88-independent (TRIF-dependent) pathway is triggered [153, 154]. Here, TRAM is recruited and subsequently recruits TRIF, which activates TRAF6, TRAF3, and receptor interacting protein 1 (RIP1). TRIF signaling culminates in the late-phase activation of NFκB, and the activation of interferon regulatory factor 3 (IRF3), which is involved in the regulation and production of Type I interferons [151].

NFκB transcription factors are critical for the regulation of cell responses, immunity, apoptosis, and cellular differentiation [155, 156]. There are five transcription factors that comprise the NFκB family: RelA (p65), RelB, c-Rel, p50, and p52 [155]. NFκB transcription factors associate as dimers to κB sites on DNA regulatory sequences to either induce or suppress gene transcription.

NFκB activity is induced by various stimuli, including LPS, cytokines, and oxidative stress [157-159]. In resting conditions, NFκB dimers are bound to IκB proteins, which inhibit NFκB by sequestering the dimers in the cytoplasm [155]. NFκB activation involves the IκB kinase (IKK), which contains two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ [155]. Upon stimulation, IKK phosphorylates IκB—targeting it for ubiquitination and degradation, and releasing NFκB dimers to translocate to the nucleus where they initiate transcription [155]. NFκB regulates gene transcription for a variety of proteins, including cytokines and adhesion molecules [156].

Activator protein-1 (AP-1) is a family of accessory transcription factors that directly interact with DNA regulatory sequences, known as 12-O-tetradecanoylphorbol-13-acetate response elements, or AP-1 sites [160]. AP-1 is composed of either homodimeric or heterodimeric complexes, which include nuclear phosphoproteins in the Fos, Jun, activating transcription factors (ATF), and c-Jun dimersization protein (JDP) families; however, the most common and best understood are c-Fos and c-Jun from the Fos and Jun
families, respectively [161, 162]. AP-1 can be composed of c-Jun/c-Jun homodimers or c-Jun/c-Fos heterodimers. c-Fos cannot form homodimers due to its inability to bind to DNA.

Following cell stimulation, the majority of genes that encode AP-1 proteins are immediate-early response genes, meaning that they are rapidly induced [162]. AP-1 is induced by external stimuli that increase mitogen-activated protein kinase (MAPK) activity [163]. MAPK signaling, including ERK and JNK signaling, activate several transcription factors, including ternary-complex factors (TCFs), activating transcription factor 2 (ATF2) and myocyte-enhancer factor 2 (MEF2), which induce Fos and Jun transcription [163]. Furthermore, c-Fos expression can subsequently induce c-Jun expression [163]. Once induced, phosphorylated c-Fos and c-Jun translocate into the nucleus for target gene activation. A variety of different cytokines and cell-adhesion molecules are regulated by AP-1 transcription, including IL-8 and ICAM-1 [164, 165].

LPS was shown to activate AP-1-mediated MAPK phosphatase-1 transcription—a key phosphatase in innate immunity and MAPK signaling [166]. Moreover, LPS intraperitoneal injection significantly increased AP-1 activity in lungs of mice [167].

1.6.1.1 Measurement of AP-1 Activity

AP-1 activity can be measured by electrophoretic mobility shift assays; however, AP-1 DNA binding activity does not necessarily infer total AP-1 transcriptional activity for a couple of reasons. Firstly, certain phosphorylated AP-1 components, such as phosphorylated c-Fos and phosphorylated c-Jun, have high trans-activating potentials that can promote AP-1 activity without altering DNA binding [161]. Secondly, various proteins in the AP-1 family have the ability to form complexes on AP-1 binding sites, yet differ in their ability to activate target gene transcription [162]. The activation of AP-1 family members (such as c-Jun or c-Fos) can be assessed through protein expression of their phosphorylated states; however, this does not infer AP-1 transcriptional activation of a target gene or complete AP-1 activity. Thus, AP-1 activity is more accurately represented by measuring the transcriptional activity of an AP-1-dependent reporter gene [162].
1.6.2 TLR4 and the Lung

Various findings suggest that LPS may be recognized through a surface-TLR4-independent mechanism in the lung. Reports differ on the expression of surface TLR4 in lung epithelial cell lines (BEAS-2B and A549) [168, 169]; however, there seems to be consensus that primary lung epithelial cells do not express surface TLR4, as confirmed in primary alveolar type II cells and primary human bronchial epithelial cells [102, 168]. Yet, lung epithelial cells express TLR4 intracellularly [102, 168].

Despite the lack of surface TLR4 expression on lung epithelial cells, pulmonary epithelial cells still respond to LPS challenge with the production of pro-inflammatory mediators [168]. Additionally, mice that lack bone-marrow derived TLR4 still respond to LPS in the lung by elevated MPO levels, suggesting increased neutrophil infiltration [170, 171]. Indeed, since lung parenchymal cells express TLR4 intracellularly, LPS is likely recognized through another surface receptor. Unpublished work from our lab demonstrates that both C3H/HeJ (Tlr4 signaling inactive) and C57BL/10ScCr (Tlr4-null) mice display a significant increase in TNFα, MCP-1, and RANTES in lung tissue after intratracheal LPS administration, although the inflammatory response was lower than their wild-type littermate controls. As such, LPS is able to trigger inflammatory responses in the lung, independently of surface TLR4. TLR4 may, however, still play a role in LPS-induced intracellular signaling pathways in the lung. Thus, alternative mechanisms of LPS recognition and signaling may exist in the lung to prevent hyperactivation, as it is a privileged site that comes into continual contact with basal levels of LPS through inhalation.

1.7 LPS Internalization

LPS is internalized in various cell types and mediates inflammatory responses, although the mechanisms remain unclear. Through a series of pulse-chase studies using various endocytosis inhibitors, Lichtman et al. [172] provided evidence that endocytosis is required for LPS to stimulate the production of TNFα in Kupffer cells. Cowan et al. [173] treated cardiomyocytes with the nonspecific inhibitor cytochalasin D, which disrupts the actin microfilament system, prior to LPS stimulation to inhibit LPS internalization. They
showed a cytochalasin D-dose-dependent attenuation of ERK and NFκB activation and reduced TNFα production relative to cells stimulated with LPS alone. In the murine macrophage cell line, RAW 264.7, inhibition of LPS internalization using both monodansylcadaverin (stabilizes clathrin-coated vesicles) and Dynosore (dynamin inhibitor) inhibited TNFα and IL-6 production, and down-regulated JNK, ERK, and NFκB activity [174]. Pre-treatment with monodansylcadaverine in intestinal epithelial cells before LPS stimulation also attenuated NFκB activation [175]. Thus, LPS internalization appears to be associated with the MAPK signaling pathway, leading to the activation of NFκB, and the subsequent transcription and production of pro-inflammatory cytokines.

Human lung epithelial cells have been shown to internalize LPS, and intracellular LPS is localized to the Golgi complex and maintained for at least 24 hours [176]. Yet, the role of intracellular LPS in mediating inflammation and the mechanisms of action in the lung is unknown.

1.7.1 Methods Used to Study LPS Internalization

1.7.1.1 Chemical Approaches

To study the effects of LPS internalization on cytokine production, various endocytosis inhibitors are commonly used, including cytochalasin D and monodansylcadaverine. [173, 174, 177]. Studies have demonstrated that these chemical inhibitors are non-specific due to their confounding downstream effects (For review, see [178]). For instance, cytochalasin D permeates the cell to inhibit actin filament polymerization, yet it also disrupts intracellular trafficking and various other endocytosis pathways [178]. Monodansylcadaverine (MDC) is a more suitable alternative as it does not enter the cell, but stabilizes clathrin-coated vesicles at the cell surface; however, its inhibition of transglutaminase 2, a multifunctional enzyme, may cause other currently unknown effects [179]. Thus, caution must be taken in interpreting data using such methods.
1.7.1.2 Genetic Approaches

In an attempt to circumvent the issues that arise with non-specific endocytosis inhibitors, genetic manipulations have been used to inhibit endocytosis. These approaches include both the depletion of critical proteins involved in endocytosis by siRNA, or the over-expression of mutant forms of these proteins that render them inactive [178]. Nevertheless, over-expressing mutant forms of proteins or knocking down critical endocytosis players may lead to various indirect effects [178]. Furthermore, the time it takes to deplete an endocytosis-associated protein by siRNA (3-7 days) has raised concerns that the cell may adapt or alter gene expression; thus, there is no guarantee that only the protein of interest is affected [178].

1.7.1.3 Transfection/Electroporation Approaches

Recently, three independent groups studied the role of intracellular LPS in macrophages by direct LPS transfection or electroporation into cells [180-182]. Although these methods minimize off-target effects, they also minimize the role of receptor-recognition of LPS or receptor internalization, which may contribute to cellular effects. Thus, transfection and electroporation approaches are artificial means of LPS internalization that lack physiological relevance, yet may be helpful for proof-of-concept studies.

1.7.2 TLR4-Independent LPS Internalization

Various studies have shown that LPS internalization occurs independently of TLR4 [177, 180, 181]. Dunzendorfer et al. [177] isolated peritoneal monocytes and endothelial cells from TLR4 wild type and knock-out mice and showed that LPS uptake was the same in both strains of mice. Furthermore, Rescigno et al. [183] isolated macrophages and dendritic cells from C57BL/10ScCr (TLR4-null) mice and showed that bacterial phagocytosis was similar to cells from the wild type mouse strain. Moreover, A549 lung epithelial cells that do not express surface TLR4 [168] are able to internalize LPS. Unpublished work in our lab demonstrated that TLR4 is not required for LPS internalization in the lung as
C57BL/10ScSnJ (Wild-type) and C57BL/10ScNJ (TLR4-null) mice administered LPS intratracheally displayed internalized LPS in lung tissue.

On the contrary, Latz et al. [184] showed that TLR4-transfected HEK293 cells had some minimal LPS uptake; however, their study seemed to lack non-transfected cell controls. Nevertheless, another consideration is that HEK293 cells may lack several co-factors required for LPS internalization. As such, these results are not conclusive.

Suzuki et al. [185] correlated LPS internalization with increased TLR4 expression in Colo205 intestinal epithelial cells; however, they showed that TLR4 was solely expressed intracellularly, and not on the cell surface. Together, these studies suggest that surface-TLR4 is not involved in LPS internalization; however, the role of intracellular LPS and the potential involvement of intracellular TLR4 in signaling is not yet clear.

Recently, two independent groups showed that intracellular LPS could activate TLR4-independent caspase-11-dependent IL-1β secretion and pyroptosis [180, 181]. Hagar et al. [181] transfected boiled bacterial lysates derived from Gram-negative bacteria (which contain LPS) into macrophages and found activation of caspase-11, compared to no caspase-11 activation from cell lysates derived from Gram-positive bacteria (which do not contain LPS). Because boiled bacterial lysates were administered to cells via transfection, cell-surface TLR4 was not involved in their intracellular effects. Additionally, upon priming Tlr4-/- mice with a TLR3 agonist (to activate the non-canonical inflammasome signaling pathway), Kayagaki et al. [180] showed that Tlr4-/- mice were as susceptible to LPS-induced sepsis as wild-type mice. Nevertheless, it is noteworthy that macrophages are professional phagocytes, which have the inherent ability to take up LPS; thus, transfection of LPS into the cells is an artificial model that lacks physiological relevance.

Despite the presence of these recent findings, very little is known about how LPS is internalized and intracellular LPS signaling in lung epithelial cells.
1.7.3 Intercellular Adhesion Molecule-1 (ICAM-1)-Mediated LPS Internalization

After exposure to bacteria, the interaction between cells is partially mediated by different families of cell-adhesion molecules. Intercellular adhesion molecules (ICAMs) are a family of glycoproteins expressed on a wide variety of cell types, which include myeloid and non-myeloid cells [186]. The ICAM family consists of five different ICAM molecules (ICAM-1 to 5), each containing two or more common Ig-like extracellular domains [186]. Typically, ICAMs are type I transmembrane proteins with a short cytoplasmic domain [186].

ICAM-1, also called CD54, is constitutively expressed in lung epithelial cells [187], and its expression is elevated in response to cytokine or LPS stimulation [81]. Elevated ICAM-1 expression is also observed in the lungs of mice subjected to cecal-ligation and puncture, a common sepsis model in mice [188].

The ICAM-1 polypeptide is heavily glycosylated and the mature glycoprotein is ~80-115 kDa, depending on various cell types [189]. Typically, ICAM-1 is observed as a 95-kDa protein and contains five Ig-like extracellular domains, which interact with β2 integrin molecules present on leukocytes [186]. The first domain (D1) is responsible for its interaction with leukocyte function-associated antigen-1 (LFA-1, or CD11a/CD18) and the third domain (D3) is responsible for its interaction with macrophage adhesion molecule-1 (Mac-1, or CD11b/CD18) on leukocytes [190]. Thus, ICAM-1 mediates the transmigration between epithelial cells and leukocytes [81, 191]. Furthermore, ICAM-1 serves as the receptor for human rhinovirus as its D1 domain binds into the hydrophobic pocket of human rhinovirus and brings it into the epithelial cell [192, 193].

Pilot studies in our lab have shown that ICAM-1 binds to the hydrophobic lipid A portion of LPS in human lung epithelial cells. Furthermore, ICAM-1 associates with Syk and mediates LPS internalization. LPS internalization is correlated with elevated IL-8 levels, which is abrogated with ICAM-1 siRNA transfection in pulmonary epithelial cells. Lastly, ICAM-1 is co-localized with LPS intracellularly in human pulmonary epithelial cells. Since lung epithelial cells do not express surface-TLR4, ICAM-1 may mediate LPS internalization in a surface-TLR4-independent manner; however, ICAM-1 and TLR4 are co-localized
intracellularly, suggesting a role of intracellular TLR4 in intracellular LPS signaling processes. In fact, ICAM-1−/− mice, C3H/HeJ mice, and C57BL/10ScNj mice independently exhibited elevated cytokine levels in the lung upon LPS challenge compared to animals administered phosphate-buffered saline as control (albeit, less than wild-type animals challenged with LPS). However, ICAM-1−/−/TLR4−/− double knockout mice displayed complete reduction of cytokine production. These studies suggest that ICAM-1 binds to LPS directly, brings it into the epithelial cell, and mediates intracellular LPS signaling.

1.7.4 ICAM-1 and its Signaling

In addition to its role in facilitating cell-leukocyte interactions, ICAM-1 has also been implicated in contributing to intracellular signal transduction pathways by “outside-in” signaling [186]. That is, ICAM-1 signaling has been shown to occur by receptor multimerization [186]. The short cytoplasmic tail of ICAM-1 lacks any known protein-protein interaction domains or intrinsic kinase activity; however it contains multiple positively charged amino acids and one tyrosine residue (Y512) [194]. Nevertheless, ICAM-1 has been associated with various signaling molecules and adaptor proteins. In particular, ICAM-1 cross-linking is associated with the cytoskeletal component, α-actinin [195]. Moreover, ICAM-1 clustering mediated by Rho-GTPase activation confers its interaction with adaptor proteins ezrin, radixin, and moiesin (ERM), which stabilize ICAM-1 ligation on the plasma membrane to the cytoskeleton [196-198]. These processes were shown to involve the activation of RhoA and c-Fos [199]. Through its cytoskeletal interactions, ICAM-1 facilitates actin polymerization [200].

The role of ICAM-1 as an inflammatory mediator has been studied in various cell types, with varying results. Blocking pulmonary ICAM-1 expression reduces lung injury in a diet-induced pancreatitis model [201], and attenuates lung injury in mice subjected to cecal-ligation and puncture [188], suggesting that ICAM-1 plays a role in signal transduction and regulation of inflammation in the lung [202]. ICAM-1 has also been implicated as an inflammatory mediator as it activates a number of kinases, which culminate in the activation of proinflammatory gene transcription through AP-1 or NFκB [203-205]. For instance, ICAM-1 cross-linking activated src tyrosine kinase by mediating the production of
reactive oxygen species in pulmonary endothelial cells [203]. Moreover, activation of src tyrosine kinase was essential for the activation of ezrin and MAPK signaling [203]. Studies have also shown the involvement of intracellular calcium signaling and protein kinase C activation associated with ICAM-1 ligation [206].

ICAM-1 cross-linking has been associated with ERK and JNK activation, leading to AP-1 activation and IL-1β transcription [207, 208]. ICAM-1 cross-linking in human vascular endothelial cells induced IL-8 and RANTES production [204]. Moreover, ICAM-1 cross-linking in both lung adenocarcinoma cells (A549) and primary human normal bronchial epithelial (NHBE) cells activated ERK and JNK signaling, and induced RANTES production [208]. Interestingly, ICAM-1 cross-linking did not appear to induce IL-8 production or gene expression in lung epithelial cells, suggesting that the effects of ICAM-1 cross-linking are cell-specific.

1.7.5 ICAM-1 and Nucleophosmin

ICAM-1 does not have many known intracellular binding targets. Perez-Hernandez et al [209] recently identified two nuclear phosphoproteins – nucleolin and nucleophosmin – as proteins that interact with the cytoplasmic domain of ICAM-1 through pull-down assay in human lymphoblasts. Nucleolin is expressed throughout the cell, and surface nucleolin was recently implicated in mediating LPS internalization in alveolar macrophages and associated TNF-α and IL-6 production [210]. Nucleophosmin is expressed intracellularly, yet its role upon LPS stimulation has yet to be elucidated. Interestingly, nucleophosmin was not pulled down with the cytoplasmic domain of ICAM-3, suggesting that NPM specifically interacts with ICAM-1 intracellularly. Although mainly regarded as a nuclear protein, NPM was recently shown to regulate K-Ras plasma membrane interactions that modulate MAPK signaling [211, 212].
1.8 Nucleophosmin (NPM)

The nucleophosmin/nucleoplasmin (NPM) family is comprised of three structurally similar functional proteins—NPM1, NPM2, and NPM3—that act as molecular chaperones within a cell to facilitate the formation of nucleoprotein complexes.

NPM1 (referred to as NPM for the remainder of this thesis) is a 36 kDa multifunctional protein principally localized in the nucleus of cells. NPM (also called B23 or numatrin in mammals, and NO38 in Xenopus) forms an eight-stranded β-barrel at the N-terminus, and five monomers come together to form a stable pentamer [213]. NPM has multiple phosphorylation sites, and phosphorylated NPM is the most abundant protein in the nucleolus [214]. However, NPM shuttles between the nucleoli, nucleoplasma, and cytoplasm to regulate the processing and transport of ribosomal RNA [215]. Over the past three decades, NPM has been shown to play a role in centrosome duplication, cell proliferation, apoptosis, chaperone activity, and the regulation of transcription [216-218] (Figure 2).

NPM is predominately a nuclear phosphoprotein that was initially thought to only play a role in ribosomal maturation, ribosomal assembly, and ribonuclease activity [219-221]. However, NPM was recently shown to be present in low amounts in the inner leaflet of the plasma membrane and regulate Ras interactions and modulate MAPK signaling [211, 212].

NPM2 (also called nucleoplasmin) is a conventional molecular chaperone that natively binds histones H2A and H2B in humans [222] and amphibians [223]; however, NPM2 has predominantly been studied in Xenopus to date, participating in the decondensation of sperm chromatin after fertilization [224] and nucleosome assembly in male pronuclei [225].

NPM3 (also called NO37 in Xenopus) is not as well-characterized as NPM1 or NPM2. In humans, NPM3 is ubiquitously expressed and acts as a chaperone in various tissues, presenting highest in the testes and pancreas, and lowest levels in the lung [226]. NPM3 has been shown to form a complex with NPM1 and is implicated in ribosomal biogenesis.
NPM3 may also play a role in regulating paternal chromatin decondensation [228]. Nevertheless, the precise roles of NPM3 are still largely unclear and under investigation.
Figure 2. **Nucleophosmin is a multifunctional phosphoprotein.** Nucleophosmin is implicated in various processes, including ribosomal biogenesis, centrosome duplication, cell-cycle progression, cell survival/apoptosis, and transcriptional regulation, through its interaction with transcription factors NF-κB and AP-2α. The role of NPM in inflammation is a relatively new concept, as NPM can act as an alarmin and may mediate inflammation through the transcriptional regulation of cytokines. We hypothesize that NPM can mediate LPS-induced inflammation through AP-1 components, c-Fos and c-Jun.
1.9 NPM and Cancer

Most notably, NPM is associated with hematopoietic malignancies, including acute myeloid leukemia [229], anaplastic large-cell lymphoma [230], and acute promyelocytic leukemia [231]. NPM expression is elevated in tumor and growing cells compared to resting cells [232], and certain mutations in the nucleolar localization signal of the NPM1 gene result in aberrant NPM expression in the cytoplasm; cytoplasmic NPM is a biomarker for acute myeloid leukemia [215]. NPM has been shown to act as an antiapoptotic protein [233] and during apoptosis, NPM is cleaved by caspase-3 [234]. However, emerging evidence demonstrates that NPM may also possess tumor suppressive properties by its activity in growth-suppressing pathways, mediated by the nuclear protein, Arf [235].

1.10 NPM and Endotoxin

Until recently, NPM was only studied in developmental or cancer investigations. Proteomic analysis of macrophages (RAW 264.7 cells) stimulated by LPS identified a significant increase in NPM expression in the cytoplasm, possibly due to LPS-induced suppression of NPM degradation [236]. Zhang et al. [236] found that full-length NPM and procaspase-3 were only detected in LPS-stimulated cells, whereas cleaved NPM and caspase-3 were detected in untreated cells. Cleaved NPM was associated with monocytes undergoing differentiation to macrophages [237]. In another study, wild-type or NPM+/− mice were injected with LPS (0.5 mg/kg) by tail vein for 30 or 60 minutes [237]. NPM+/− mice contained higher levels of IL-6, KC, and MCP-1 in plasma, compared wild-type controls [237]. This suggests that NPM plays a protective role in endotoxemia; however, it is important to note that as a positive signal, LPS did not seem to induce chemokine secretion in wild-type mice challenged with LPS, possibly due to macrophage abnormalities that occur in NPM+/− mice [238] — NPM+/− mice develop features of myelodysplastic syndrome, in which the bone marrow produces fewer numbers of blood cells or defective blood cells; this can lead to deregulated cytokine production and altered polarization of macrophages, thus rendering in vivo inflammation results unreliable [238].
Nawa et al. also showed an increase in cytoplasmic NPM expression in LPS-stimulated RAW264.7 cells and in alveolar macrophages isolated from rats subjected to CLP after 16 hours [239]. Moreover, NPM was detected in the peritoneal lavage fluid of CLP rats, suggesting that NPM may act as an alarmin. Recombinant NPM activated ERK and JNK pathways and stimulated the release of TNFα, IL-6, and MCP-1 in RAW264.7 cells [239]. Additionally, ICAM-1 expression was induced by recombinant human NPM in human umbilical vein endothelial cells [239]. Thus, NPM may play a role as an alarmin in sepsis.

1.10.1 Alarmins

Necrotic cells can release alarmins, which can act on TLRs and other receptors to increase cytokine production [240]. Alarmins are host factors that maintain regulatory cell functions in normal circumstances, but become pro-inflammatory and damaging when released to the extracellular space during sepsis [240]. For example, high mobility group B1 (HMGB1) is a chromatin-associated protein that is abundantly expressed in the nucleus, cytoplasm and membrane of cells [241]. It facilitates transcription, is involved in DNA stabilization and repair, and regulates the activity of steroid receptors [241]. HMGB1 is a late mediator of endotoxin shock as necrotic immune and endothelial cells passively release it to the extracellular space [242]. HMGB1 acts through TLR2 and TLR4 to produce pro-inflammatory cytokines IL-1, TNFα, IL-6, and IL-8 [243].

1.11 NPM and Transcriptional Regulation

In the last ten years, NPM has been shown to be associated with transcription factors. NPM co-localizes with AP-1 transcriptional component, c-Fos, in the nucleus and cytoplasm of human neuroblastoma cells activated by retinoic acid [244], and NPM binds to the c-Jun transcription factor in resting and UV-activated cells [245], suggesting that NPM may play a role in AP-1 activity. NPM was also shown to bind to AP-2α in retinoic acid-induced cell differentiation and acts as a co-repressor of transcription [246]. Furthermore, NPM is part of the complex for NFκB activity and can be either a co-activator or co-repressor of transcription [237, 246-249].
Chapter 2
Hypothesis and Objectives

2 Hypothesis and Objectives

2.1 Rationale

Although the traditional LPS-signaling paradigm involves signaling from the surface through CD14-MD2-TLR4, there is accruing evidence showing that LPS can enter inside the cell to activate inflammatory signaling pathways independent of TLR4. LPS does not directly bind to TLR4, and in light of the recently failed TLR4-clinical trials, novel avenues of LPS signaling are emerging. Indeed, it is possible that the inflammatory response is far too large in sepsis patients, such that one drug targeting a single receptor is not enough to ameliorate a septic patient's condition. In conjunction, numerous groups have shown that LPS is internalized in various cell types [173, 174, 176], rendering anti-endotoxin and anti-TLR4 remedies ineffective in targeting the intracellular LPS. In light of emerging evidence showing that intracellular LPS contribute to inflammatory responses, the question arises as to what the intracellular mechanisms are.

The lung is an open organ for bacterial exposure, and the lung epithelium can actively participate in inflammatory responses to LPS by releasing high levels of chemokines, particularly IL-8. It has been shown that LPS is internalized in the A549 lung epithelial cell line [176]. Our group has further demonstrated that ICAM-1 mediates LPS internalization and is co-localized with LPS intracellularly. However, whether LPS internalization contributes to IL-8 production and its downstream signaling pathways in the lung epithelium remain to be elucidated.

ICAM-1 does not have many known intracellular binding partners, yet was recently shown to interact with NPM through its cytoplasmic domain [209]. It has also been shown that LPS-stimulated macrophages induced elevated NPM expression [236, 239]. Furthermore,
NPM has the ability to bind to various transcription factors in the cytosol, including NFκB and AP-2α. Whether NPM contributes to AP-1 activity upon LPS stimulation is unknown. The studies herein provide groundwork in assessing whether NPM is a potential downstream target of ICAM-1 or LPS and its involvement in LPS intracellular signaling through the AP-1 factors c-Fos and c-Jun in human lung epithelial cells.

2.2 Hypothesis

NPM mediates intracellular LPS signaling by interaction with ICAM-1 in lung epithelial cells.

2.2.1 Objective 1

Investigate whether LPS internalization contributes to IL-8 production and cytotoxicity.

2.2.2 Objective 2

Investigate NPM expression in lung epithelial cells and the NPM/ICAM-1 interaction.

2.2.3 Objective 3

Assess the specific role of NPM in LPS-induced IL-8 production and cytotoxicity and its downstream signaling.
Chapter 3
Methods

3 Methods

3.1 Materials

3.1.1 Antibodies, Recombinant Proteins, and Primers

Mouse α-human NPM monoclonal antibody, mouse α-human ICAM-1 monoclonal antibody, and mouse α-human TLR4 monoclonal antibody were purchased from Abcam® (Cambridge, England, UK). Rabbit α-human phospho-c-Fos, c-Fos, phospho-c-Jun, and c-Jun monoclonal antibodies were purchased from Cell Signaling Technology Inc.® (Beverly, MA, USA). Mouse α-human CD14 monoclonal antibody was purchased from R&D Systems® (Menneapolis, MN, USA). Mouse α-human β-actin antibody was purchased from Sigma-Aldrich® (St. Louis, MO, USA). Mouse α-human GAPDH monoclonal antibody was purchased from Biolegend® (San Diego, CA, USA). Rabbit α-human Lamin B1 polyclonal antibody was purchased from Thermo Fisher Scientific. Goat α-rabbit IgG-HRP secondary antibody was purchased from Jackson ImmunoResearch Laboratories Inc. (Westgrove, PA, USA). Goat α-mouse IgG-HRP secondary antibody was purchased from Invitrogen-Thermo Fisher Scientific (Waltham, MA, USA). Rhodamine-Phalloidin F-actin stain was purchased from Invitrogen™ (Burlington, ON, CA). DyLight™ 488-conjugated AffiniPure Goat α-mouse IgG and Cy3-conjugated donkey α-mouse IgG were purchased from Jackson ImmunoResearch Laboratories Inc. (Westgrove, PA, USA).

Recombinant human NPM1 was purchased from MyBioSource Inc. (San Diego, CA, USA). Recombinant human ICAM-1 and recombinant human CD14 were purchased from R&D Systems® (Minneapolis, MN, USA).
**NPM1** forward and reverse primers were purchased from ACGT Corporation (Toronto, Canada). **GAPDH** forward and reverse primers were provided with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific).

### 3.1.2 Lipopolysaccharide

Non-labeled LPS was purchased from List Biological Laboratories Inc® (Campbell, CA, USA). LPS arrived in lyophilized form, isolated from *Escherichia coli* O55:B5 by phenol extraction method [250]. LPS was reconstituted in normal saline at a concentration of 20 mg/ml and incubated at 50°C with intermittent vortexing, then stored at 4°C, as per company recommendations. Dilution of LPS to a final concentration of 1.0 mg/ml was used as the stock dilution for experiments. For a concentration of 0.1 μg/ml, LPS was diluted 1000x in normal saline then 10x in cell culture medium, which was added to the cells for stimulation. Analysis of LPS by the company showed 3.0% 2-Keto-3-deoxyoctonate, 3.9% phosphate, 0.9% protein, and 0.2% nucleic acid content. LPS was sonicated each time prior to use for 30 seconds.

Boron dipyrromethene difluoride (BODIPY®)-labeled LPS (BODIPY-LPS) from *Escherichia coli* O55:B5 was purchased from Molecular Probes (Eugene, OR, USA). Lyophilized LPS was reconstituted in saline and heated at 37°C with vortexing, aliquoted, and stored at -80°C in the dark as per company recommendations. A stock concentration of 1 mg/ml BODIPY-LPS was used, which was diluted 1000x then subsequently 10x in culture medium.

### 3.1.3 siRNA Transfection Reagents

DharmaFECT Transfection Reagent I, ON-TARGETplus Human NPM1 siRNA SMARTpool and ON-TARGETplus Non-targeting Control siRNA were purchased from Dharmacon-Thermo Fisher Scientific (Waltham, MA, USA). NPM1 siRNA arrived as a mixture containing four designed siRNA target sequences for the **NPM1** gene (**Table 1**). siRNAs were reconstituted to a concentration of 5 μM, aliquoted, and stored in -20°C until use.
Table 1. *NPM1* siRNA SMARTpool target sequences.

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<th>ON-TARGETplus Human NPM1 siRNA SMARTpool</th>
<th>Target Sequence</th>
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</thead>
<tbody>
<tr>
<td>ON-TARGETplus SMARTpool siRNA J-015737-05, NPM1</td>
<td>GUAGAAGACAUUAAAGCAA</td>
</tr>
<tr>
<td>ON-TARGETplus SMARTpool siRNA J-015737-06, NPM1</td>
<td>AAUGCAAGCAAGUAUAGAA</td>
</tr>
<tr>
<td>ON-TARGETplus SMARTpool siRNA J-015737-07, NPM1</td>
<td>ACAAGAAUCCUUCACAGAAA</td>
</tr>
<tr>
<td>ON-TARGETplus SMARTpool siRNA J-015737-08, NPM1</td>
<td>UAAAGGCCGACAAGAUUA</td>
</tr>
</tbody>
</table>

3.2 Cell Culture

3.2.1 Human Bronchial Epithelial Cells (BEAS-2B)

The transformed human bronchial epithelial cell line (BEAS-2B) were grown in 10 cm² dishes and kept at a density of 0.1-1x10⁶ cells/ml in Dulbecco’s Minimal Essential Medium (DMEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. Cells were washed with phosphate-buffered saline (PBS), trypsinized with 0.25% Trypsin-EDTA, and neutralized with DMEM. Cells were centrifuged at 150 × g for 5 minutes. Supernatant was aspirated and the cell pellet was resuspended in 1.0 to 5.0 ml medium and counted using Trypan Blue protocol. Cells were seeded in 6- or 24-well plates and incubated at 37°C with 5% CO₂ overnight prior to use for experiments to allow for suitable attachment and stabilization.

3.2.2 Human Primary Small Airway Epithelial Cells (SAEC)

Normal primary human small airway epithelial cells (SAEC) isolated from the distal portion of the lung in the 1 mm bronchiole area were purchased from Lonza Group (Basel, Switzerland) and cultured in Small Airway Epithelial Cell Growth Medium, supplemented
with 0.4% bovine pituitary extract, 0.1% hydrocortisone, 0.1% human epidermal growth factor, 0.1% epinephrine, 0.1% transferrin, 0.1% insulin, 0.1% retinoic acid, 0.1% triiodothyronine, 0.1% Gentamycin, and 1% bovine serum albumin (BSA) (Lonza Group). Cells within the first 15 doublings of growth were used for experiments, as per the company’s recommendation. 5x10^5 cells were thawed and grown in T75 flasks until reaching ~70% confluence, after which they were washed with Hepes-buffered BSS (HBSS), trypsinized with 0.25% Trypsin-EDTA, and neutralized with Trypsin Neutralizing Solution. Cells were centrifuged at 200 × g for 8 minutes. Supernatant was aspirated and the cell pellet was resuspended in 1.0 ml medium and counted using Trypan Blue protocol. After counting, cells were seeded in 6- or 24-well plates according to specific experimental protocols. Cells were incubated at 37°C with 5% CO₂ for 48 hours prior to use for experiments to allow for suitable attachment and stabilization.

3.2.3 Cell Counting

After harvesting cells by Trypsin-EDTA and centrifuging in a Falcon 15-ml conical centrifuge tube (Thermo Fisher Scientific), supernatants were aspirated and fresh warm medium was added to re-suspend the cell pellet. The haemocytometer was cleaned with 70% ethanol prior to use.

10 µl of cells were placed in a microcentrifuge tube, after which 10 µl of trypan blue was added. The cells were gently mixed with the trypan blue by slow pipetting up and down to avoid cell lysis. 10 µl of the cell suspension containing trypan blue was carefully pipetted into the haemocytometer. Using a counter, the number of cells in the 4 sets of 16-squares in the corners were counted. The haemocytometer is designed such that one set of 16-squares in the corner is equivalent to the number of cells in × 10^4 per ml. Thus, to obtain the final cell count, the following calculations were made: Divide the count by 4 and multiply by 2 to adjust for the 1:2 dilution in trypan blue. The final number provides the cell density as × 10^4 cells/ml.
3.3 Immunofluorescence Staining

3.3.1 Cells in Chamber Slides (TLR4 Staining)

4-well chamber slides (Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ System), with a growth area of 1.7 cm² in each well were used.

Primary SAEC were counted and plated in 4-well chamber slides at a density of 2x10⁵ cells/chamber and allowed to attach for 48 hours. After attachment, cells were washed three times for 5 minutes with warm HBSS and subsequently fixed with 300 μl of 4% paraformaldehyde for 10 minutes. Chambers were then washed three times for 5 minutes with PBS and cells were permeabilized with 300 μl of 0.1% Triton X-100 in PBS for 3 minutes. After washing 3 times for 5 minutes with PBS, cells were blocked with 500 μl of 2% BSA in PBS-T (1x PBS with 0.1% Tween-20) for 30 minutes. After washing 1 time for 5 minutes with PBS, TLR4 mAb (1:100, total volume = 200 μl per chamber) or equivalent concentration of IgG control were diluted in 1% BSA in PBS-T was added to chambers and incubated for 1 hour. After primary antibody incubation, chambers were washed 3 times for 5 minutes with PBS and subsequently incubated in the dark with DyLight™ 488-conjugated secondary antibody (1:4000, total volume = 200 μl per chamber, visualized as green fluorescence) diluted in 1% BSA in PBS-T. After washing 3 times for 5 minutes with PBS, F-actin was stained with rhodamine phalloidin (1:2000, total volume = 300 μl per chamber, visualized as red fluorescence) diluted in 1% BSA in PBS-T for 45 minutes in the dark. After washing 3 times for 10 minutes, Hoechst stain (1:10,000, total volume = 300 μl per chamber, visualized as blue fluorescence) was added for 10 minutes for nuclear staining. Finally, chambers were washed 2 more times for 10 minutes with PBS and a coverslip was mounted onto the glass chamber slide using Dako fluorescent mounting medium (Dako Canada Inc., Burlington, ON, CA). Slides were stored in the dark overnight for mounting medium to dry and cells were visualized by confocal microscope (Zeiss LSM 510 inverted confocal microscope) the next day.
3.3.2 Cells on Coverslip (ICAM-1 Staining)

Primary SAEC were counted and plated on 12 mm round glass coverslips (Fisherbrand™) (5x10^4 cells/well) in duplicate and allowed to attach for 48 hours. After attachment, cells were stimulated with 0.1 μg/ml non-labeled LPS or equivalent amount of normal saline vehicle control for 4 hours. After LPS stimulation, cells were washed three times for 5 minutes with warm HBSS. Cells were then fixed with 200 μl of 4% paraformaldehyde for 10 minutes and washed three times for 5 minutes with PBS. Cells were permeabilized with 200 μl of 0.1% Triton X-100 in PBS for 3 minutes, washed 3 times for 5 minutes with PBS and subsequently blocked with 300 μl of 2% BSA in PBS-T (1X PBS with 0.1% Tween-20) for 30 minutes. After washing 2 times for 5 minutes with PBS, ICAM-1 mAb (1:100, total volume = 150 μl) diluted in 1% BSA in PBS-T or equivalent concentration of IgG control were added to the wells and incubated for 1 hour. After incubation with the primary antibody, wells containing the coverslips were washed 3 times for 5 minutes with PBS, and Cy3-conjugated secondary antibody (1:1000, total volume = 150 μl per well, visualized as red fluorescence) diluted in 1% BSA in PBS-T was added to the wells for 1 hour in the dark. After washing 3 times for 10 minutes with PBS, coverslips were mounted to glass slides using a drop of Dako fluorescent mounting medium (Dako Canada Inc., Burlington, ON, CA) and left in the dark overnight for complete mounting. The next day, cells were viewed by confocal microscope (Zeiss LSM 510 inverted confocal microscope).

3.4 Inhibition of LPS Internalization

3.4.1 Immunofluorescence Experiments

3.4.1.1 Cell Preparation

BEAS-2B cells were counted and plated on 12 mm round glass coverslips (Fisherbrand™) (3x10^4 cells/well) in triplicate and allowed to attach overnight.

Primary SAEC were counted and plated on 12 mm round glass coverslips (Fisherbrand™) (5x10^4 cells/well) in duplicate and allowed to attach for 48 hours. More cells were required
to be plated on each coverslip for this cell type due to their tendency not to attach to the glass coverslip when there was a reduced cell density.

3.4.1.2 MDC Pre-Treatment and LPS Stimulation

After proper attachment to the glass coverslips, BEAS-2B cells were washed three times with warm PBS and treated with 100 μM monodansylcadaverine ([MDC], Sigma-Aldrich, St. Louis, MO, USA) or vehicle (DMSO) for 30 minutes. MDC produces irreversible effects. As such, wells were washed two times with warm PBS and subsequently stimulated with 0.1 μg/ml BODIPY-LPS in the dark for 4 hours at 37°C. Sterile normal saline was used as the vehicle control.

After proper attachment to the glass coverslips, primary SAEC were washed three times with warm HBSS and treated with 100 μM monodansylcadaverine ([MDC], Sigma-Aldrich, St. Louis, MO, USA) or vehicle (DMSO) for 30 minutes. Wells were then washed two times with warm HBSS and cells were stimulated with 0.1 μg/ml BODIPY-LPS in the dark for 4 hours at 37°C.

After LPS stimulation, culture medium was aspirated and cells underwent immunofluorescence staining, as outlined in Section 3.4.1.3.

3.4.1.3 Immunofluorescence Staining Protocol

Cells seeded on 12 mm round glass coverslips (Fisherbrand™) were washed three times for 5 minutes with 1x PBS. Cells were then fixed with 250 μl of 4% paraformaldehyde for 10 minutes and washed three times for 5 minutes with 1x PBS. Cells were permeabilized with 200 μl of 0.1% Triton X-100 in PBS for 3 minutes, washed 3 times for 5 minutes with 1x PBS and subsequently blocked with 300 μl of 2% BSA in PBS-T (1X PBS with 0.1% Tween-20) for 30 minutes. F-actin was stained with rhodamine-phalloidin (1:2000, total volume = 150 μl per well). Cells on coverslips were mounted on glass slides with a drop of Dako fluorescent mounting medium (Dako Canada Inc., Burlington, ON, CA) and cells were
visualized by confocal microscope (Zeiss LSM 510 inverted confocal microscope). The plate was placed on a shaking platform during all washes and incubations at room temperature.

3.4.2 Experiments for IL-8 and Cytotoxicity Measurements

3.4.2.1 Cell Preparation

BEAS-2B cells were counted and plated in a 24-well plate (1x10^5 cells/well) in duplicate and allowed to attach overnight. Culture medium from like-treated duplicate wells were pooled for IL-8 ELISA and LDH measurement.

SAEC were counted and plated in a 24-well plate (1.5x10^5) in triplicate and allowed to attach for 48 hours. Culture medium from like-treated triplicate wells were pooled for IL-8 ELISA and LSH measurement.

3.4.2.2 MDC Pre-Treatment and LPS Stimulation

After proper attachment to the wells, BEAS-2B cells were washed three times with warm PBS and treated with 100 μM monodansylcadaverine ([MDC], Sigma-Aldrich, St. Louis, MO, USA) or vehicle (DMSO) for 30 minutes. Wells were washed two times with warm PBS and subsequently stimulated with 0.1 μg/ml non-labeled LPS for 4 hours at 37°C. Sterile normal saline was used as the vehicle control.

After proper attachment to wells, primary SAEC were washed three times with warm HBSS and treated with 100 μM monodansylcadaverine ([MDC], Sigma-Aldrich, St. Louis, MO, USA) or vehicle (DMSO) for 30 minutes. Wells were then washed two times with warm HBSS and cells were stimulated with 0.1 μg/ml non-labeled LPS for 4 hours at 37°C.

After LPS stimulation, culture medium was collected for IL-8 ELISA and LDH Cytotoxicity Assay measurements, as described in Section 3.10.
3.5 NPM Expression

3.5.1 Total NPM Protein Expression in SAEC

SAEC were seeded in 6-well plates (2.0x10^5 cells/well) and allowed to attach for 48 hours. Wells were then washed two times with HBSS and cells were starved with basal medium for 4 hours. Cells were then stimulated with LPS (0.1 μg/ml) for up to 8 hours at 37°C. Total cell lysates were collected and 3 μg total protein was used for western blotting (as described in Section 3.6).

3.5.2 Cytoplasmic and Nuclear NPM Expression in SAEC

SAEC (4.0x10^5 cells) were harvested by Trypsin-EDTA; cytoplasmic and nuclear fractions were isolated using a modified protocol of the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA).

The modified protocol was as follows: After trypsinization, cells were neutralized with Trypsin Neutralizing Solution and transferred to a Falcon 15-ml conical centrifuge tube (Thermo Fisher Scientific). Cells were centrifuged at 300 × g for 8 minutes. The supernatant was discarded, and the cells were washed by suspending the cell pellet with HBSS. The cells were transferred to a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 300 × g for 3 minutes at 4°C. The supernatant was carefully discarded and 100 μl of CER I (NE-PER® Nuclear and Cytoplasmic Extraction Reagents) was added to the cells. Tubes were mixed with a vortex mixer for 15 seconds and incubated on ice for 10 minutes. 11 μl of CER II (NE-PER® Nuclear and Cytoplasmic Extraction Reagents) was then added to each tube. Samples were mixed with a vortex mixer for 5 seconds and incubated on ice for 1 minute. After another mix with the vortex mixer for 10 seconds, tubes were centrifuged at 17,000 × g for 5 minutes at 4°C. Supernatants, which contained the cytoplasmic extract, were immediately transferred to new microcentrifuge tubes and stored at -80°C until use. The remaining pellet was suspended in 100 μl NER (NE-PER® Nuclear and Cytoplasmic Extraction Reagents) and mixed using a vortex mixer for 15 seconds every 10 minutes, for total time of 50 minutes. Tubes were then centrifuged at
17,000 × g for 10 minutes at 4°C. The supernatant (nuclear extract) was immediately transferred to a new microcentrifuge tube and stored in -80°C until use.

3.5.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.5.3.1 Stimulation

SAEC were seeded in 6-well plates (2.5×10^5 cells/well) and allowed to attach for 48 hours. Wells were then washed twice with HBSS and starved with basal medium for 4 hours. Cells were then stimulated with LPS (0.1 μg/ml) for 15 minutes, 30 minutes, or 1 hour at 37°C.

3.5.3.2 RNA Extraction

After stimulation, cells were washed one time with HBSS and total RNA was isolated by TRIzol® Reagent (Life Technologies Corporation, Carlsbad, CA, USA). 1 ml of TRIzol® Reagent was used per well. Cells were then transferred to a microcentrifuge tube. 200 μl of chloroform was added to the samples for phase separation. After centrifugation (12,000 × g for 15 minutes at 4°C), the sample was separated into three distinct phases: a red (organic) phenol-chloroform phase at the bottom containing proteins and lipids, an interphase in the middle containing DNA, and a colourless aqueous phase at the top containing RNA. The aqueous phase was removed and added into a new microcentrifuge tube. 200 μl of isopropanol was added to the aqueous phase and incubated in -20°C for 30 minutes. Samples were then centrifuged at 12,000 × g for 10 minutes at 4°C. RNA pellet was washed with 75% ethanol in RNase and DNase-free water, and centrifuged at 7500 × g for 5 minutes at 4°C. RNA was resuspended in 25 μl of nuclease-free water and stored in -80°C until use.

3.5.3.3 RNA Quantification

RNA concentration and purity was assessed by NanoDrop® ND-1000 Spectrophotometer. 1 μl of RNA was directly placed onto the lower measurement platform of the NanDrop1000,
after which the upper arm was brought down to make contact with the lower pedestal. Spectral measurements were made after selecting RNA-40 in the operating software on the computer and initiating measurement. A 260/280 of approximately 2.0 was accepted as “pure” for RNA purity, as per company recommendations. When the measurement was completed, the upper arm was lifted, and both of the optical surfaces were cleaned using a Kimwipe to prevent sample carryover onto the next measurement, as per company instructions. RNA measurements were between 200 – 500 ng/μl.

3.5.3.4 cDNA Synthesis

Synthesis of first strand cDNA from RNA templates was performed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). 1.0 μg of total RNA was used with random hexamer primer. After the reaction components were added (Table 2), samples were incubated at room temperature for 5 minutes, followed by 42°C for 1 hour. The reaction was terminated by heating at 70°C for 5 minutes and samples were stored in -80°C until use for RT-PCR.
Table 2. cDNA Synthesis Protocol.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>1 µg</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>to 11 µl</td>
</tr>
<tr>
<td><strong>Kit Components</strong></td>
<td></td>
</tr>
<tr>
<td>5x Reaction Buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>RiboLock RNase Inhibitor</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 mM dNTP Mix</td>
<td>2 µl</td>
</tr>
<tr>
<td>RevertAid H Minus M-Mulv Reverse Transcriptase</td>
<td>1 µl</td>
</tr>
<tr>
<td>Random Hexamer Primer</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME</strong></td>
<td>9 µl per sample</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME WITH RNA</strong></td>
<td>20 µl per sample</td>
</tr>
</tbody>
</table>
3.5.3.5 Target Gene PCR

*NPM1* and *GAPDH* PCR were performed using REDExtract-N-Amp™ PCR ReadyMix™, which contains buffer, salts, dNTPs, and Taq polymerase (Sigma-Aldrich®, St. Louis, MO, USA). Final primer concentrations for *NPM1* and *GAPDH* were 0.4 μM each. Primer forward and reverse sequences are listed under Table 3. 10 μl of REDExtract-N-Amp PCR Reaction Mix was added to 4 μl of sample cDNA (Table 4). The specific thermal cycler programs for *NPM1* and *GAPDH* are listed in Table 5 and Table 6, respectively. All thermocycling was conducted using the Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA).

### Table 3. RT-PCR Primer Information

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NPM1</em> Forward Primer</td>
<td>5′–3′ GTCCTTTCCCTGGTGATT</td>
</tr>
<tr>
<td><em>NPM1</em> Reverse Primer</td>
<td>5′–3′ GTTTACATCCTCCTCTTTCC</td>
</tr>
<tr>
<td><em>GAPDH</em> Forward Primer</td>
<td>5′–3′ CAAGGTCATCCATGACAACCTTTG</td>
</tr>
<tr>
<td><em>GAPDH</em> Reverse Primer</td>
<td>5′–3′ GCCACACCCTGGTGCTGTAG</td>
</tr>
</tbody>
</table>

### Table 4. RT-PCR Protocol.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA from RT reaction</td>
<td>4 μl</td>
</tr>
<tr>
<td>REDExtract-N-Amp PCR Reaction Mix</td>
<td>10 μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Water</td>
<td>5 μl</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME</strong></td>
<td><strong>20 μl per sample</strong></td>
</tr>
</tbody>
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Table 5. *NPM1* PCR Program.

<table>
<thead>
<tr>
<th>PCR Program (<em>NPM1</em>)</th>
<th>Temperature °C</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivation</td>
<td>94</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>49</td>
<td>45 sec</td>
<td>32</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. *GAPDH* PCR Program.

<table>
<thead>
<tr>
<th>PCR Program (<em>GAPDH</em>)</th>
<th>Temperature °C</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivation</td>
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<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>30 sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
<td></td>
</tr>
</tbody>
</table>
3.5.3.6  Agarose Gel Electrophoresis

1% agarose gel was prepared with agarose (electrophoresis grade, BioShop Canada Inc.) and 1x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1mM EDTA). After adding the buffer to agarose in a flask, contents were heated in a microwave for approximately 1.5 minutes until all agarose was solubilized. 7 μl RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, Korea) was added, and the solution was poured into a gel mould with a 15-well comb inserted within. The agarose gel solidified after approximately 1 hour. 10 μl of sample PCR product was pipetted into the agarose gel and electrophoresed at 100 V for 1 hour before detection with an ultraviolet transilluminator.

3.6  Western Blot

3.6.1  Sample Preparation from Cell Culture

3.6.1.1  Collection of Total Cell Lysate

After cells were stimulated according to their respective experimental protocols, culture medium was collected after centrifugation at 5000 rpm for 5 minutes (Microcentrifuge 5415 D, Eppendorf, Mississauga, ON, CA), aliquoted, and stored at -80°C until further use. Plates containing the cells were on ice, washed with PBS two times, and 50-200 μl of cold lysis buffer was added to each well. Lysis buffer contained 50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1% proteinase inhibitor cocktail (Thermo Scientific), and 1% phosphatase inhibitor Na₃VO₄, pH 7.54. This Tris-Triton lysis buffer was chosen because it preserves protein-protein interactions/minimizes denaturation, which is particularly useful for our co-immunoprecipitation studies. Since this lysis buffer does not contain detergents, cells must be scraped for lysis to occur. Cells were scraped, collected in microcentrifuge tubes and incubated on ice for 10 minutes with intermittent vortexing. Cells were then centrifuged at 11,000 × g for 15 minutes at 4°C, after which supernatants containing the proteins were transferred into fresh microcentrifuge tubes and stored at -80°C until further use.
3.6.1.2 Determination of Protein Concentration

Protein Assay Reagent (Pierce, Rockford, IL, USA) was used to measure protein concentrations from total cell lysates in a 96-well plate. A protein standard was prepared by diluting BSA (Sigma-Aldrich, St. Louis, MO, USA) in lysis buffer in concentrations of 0 μg/ml, 0.2 μg/ml, 0.4 μg/ml, 0.6 μg/ml, 0.8 μg/ml, and 1.0 μg/ml. 10 μl of standard or protein sample was added to 150 μl protein assay reagent and incubated for 10 minutes at room temperature for the reaction to take place and colour to develop. Absorbance measurements were taken at 660 nm by spectrophotometry (SpectraMax 340, Molecular Devices) and protein concentration was calculated with the constructed standard curve.

3.6.1.3 Sample Preparation for Loading Gel

3- or 25-μg of total protein was diluted in 6X loading buffer to yield a final concentration of 1X loading buffer in the sample. Loading buffer contained 375 mM Tris-HCl, 6% SDS, 48% glycerol, 9% MeSH, 0.03% β-mercaptoethanol, pH 6.8. For denaturation, samples were boiled at 99°C for 5 minutes, then placed on ice. Samples were given a quick-spin (Microcentrifuge 5415 D, Eppendorf, Mississauga, ON, CA) to bring all contents in the tube down, then loaded into the gel.

3.6.2 Preparation of SDS/PAGE Gel

10% handcast gels were prepared for SDS/PAGE electrophoresis. Before gel preparation, the short and tall glass plates, casting frame, and casting stand (BioRad, San Diego, CA, USA) were washed and thoroughly dried prior to use. The short and tall glass plates were placed in the casting frame together and secured in place, after which the casting frame was placed onto the casting stand.

Table 7 lists the recipe used for preparation of the resolving gel. Acrylamide, 1.5M Tris, 10% SDS, and water were added first, followed by ammonium persulphate, and finally the TEMED. Immediately after TEMED was added, approximately 6-7 ml of the gel was poured between the short and tall glass plates on the casting stand apparatus. A layer of water was added on top of the gel to remove any bubbles that may have formed while pouring the gel.
After 1 hour, the resolving gel was polymerized. The excess layer of water was absorbed by filter paper for removal, after which the stacking gel was cast using the recipe listed in Table 8. Similarly to the resolving gel, TEMED was added last in the list of reagents. After the addition of TEMED, approximately 2 ml of stacking gel was added on top of the polymerized resolving gel. Then, a 10-well or 15-well comb was inserted in between the glass plates, into the stacking gel and left for 30 minutes for the stacking gel to solidify.

If the SDS/PAGE gel was not to be used on the same day, the glass plates (with the comb still inserted) were removed from the casting apparatus and wrapped in paper towels moistened with 1x Running Buffer (25 mM Tris-HCl, 0.1% SDS, 192 mM glycine) and stored at 4°C for up to 3-4 days.

3.6.3 Electrophoresis and Transfer

One-dimensional electrophoresis using a gel electrophoresis system (BioRad, San Diego, CA, USA) was used for protein separation. Samples and a molecular weight marker (5 μl) were loaded onto 10% SDS-polyacrylamide gels, and gels were submerged into 1X running buffer (25 mM Tris-HCl, 0.1% SDS, 192 mM glycine). A constant voltage of 100 V was applied for 1.5 hours for proteins to migrate down the gel.

Following electrophoresis, proteins were transferred onto a 0.4-μm pore nitrocellulose membrane using a wet transfer system (BioRad, San Diego, CA, USA). A “transfer sandwich” was prepared in 1x transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) that consisted of filter paper – gel – membrane – filter paper. The contents of the sandwich were held together by a support grid. The support grid was placed vertically inside a transfer apparatus that contained electrodes, and the transfer apparatus was filled to the top with 1x transfer buffer. The transfer apparatus containing the support grids were immersed in ice throughout the complete transfer to mitigate any heat that was produced. A constant current of 400 mA was applied and proteins were transferred for 1.5 hours.
3.6.4 Immunoblotting

Nitrocellulose membranes were blocked with 5% BSA in TBS-T (0.5 M Tris, 1.5 M NaCl, pH 7.4, 0.1% Tween-20) for 1 hour at room temperature on a shaking platform. Membranes were then rinsed three times with TBS-T, after which appropriate primary antibodies were incubated for 1 hour at room temperature, or overnight at 4°C, on a shaking platform.

Primary antibodies were prepared in 5% BSA in TBS-T in the following dilutions: NPM monoclonal antibody (1:3000), ICAM-1 monoclonal antibody (1:1000), phospho-c-Fos monoclonal antibody (1:1000), c-Fos monoclonal antibody (1:1000), phospho-c-Jun monoclonal antibody (1:1000), c-Jun monoclonal antibody (1:1000), β-actin monoclonal antibody (1:1000), Lamin polyclonal antibody (1:1000), and GAPDH monoclonal antibody (1:1000). Membranes were washed for 5 minutes three times with TBS-T. For phospho-c-Fos and phospho-c-Jun blots, membranes were washed for 10 minutes three times with TBS-T. Respective secondary antibodies (1:4000) were added for 1 hour at room temperature. After three 10-minute washes with TBS-T, enhanced chemiluminescence solution was added to the membranes for up to 10 minutes for band development. Phospho-c-Fos and phospho-c-Jun blots were washed for 10 minutes 6 times to minimize non-specific binding.

If stripping the membrane was required for the probing of another protein of a similar size (such as that for phospho-c-Fos and c-Fos or phospho-c-Jun and c-Jun), stripping buffer was used. Harsh stripping buffer was used for our experiments, which contained 20 ml 10% SDS, 12.5 ml of 0.5 M Tris HCl pH 6.8, 67.5 ml water, and 0.8 ml β-mercaptoethanol. 6 to 10 ml of stripping buffer was added to each membrane for 20 minutes, after which membranes were washed 3 times with TBS-T for 5 minutes, blocked with 5% BSA in TBS-T for 30 minutes, and re-probed with the primary antibody of interest.
**Table 7. Resolving Gel Recipe.**

<table>
<thead>
<tr>
<th>Resolving Gel (10%)</th>
<th>10 ml (1 Gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

**Table 8. Stacking Gel Recipe.**

<table>
<thead>
<tr>
<th>Separating Gel</th>
<th>5 ml (2 Gels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>2.98 ml</td>
</tr>
<tr>
<td>0.5 M Tris, pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006 ml</td>
</tr>
</tbody>
</table>
3.7 Co-immunoprecipitation

BEAS-2B cells and SAEC were seeded in 6-well plates (2x10^5 cells/well) and allowed to attach overnight or 48 hours, respectively. Wells were washed two times with PBS or HBSS and cells were starved with basal medium for 4 hours. Cells were then stimulated with LPS (0.1 μg/ml) for up to 6 hours at 37°C. Total cell lysates were collected. 300 μg (BEAS-2B) or 150 μg (SAEC) of total protein was incubated with NPM mAb (1 μg) or IgG (1 μg) for 18 hours at 4°C on a shaker. Bead slurry (30 μl) was added to the lysate and incubated for 2 hours at room temperature, while shaking. A/G beads (Protein AG UltraLink Resin, Thermo Scientific) were precipitated at 8,000 rpm (Microcentrifuge 5415 D, Eppendorf, Mississauga, ON, CA) for 1 minute at room temperature and washed two times in PBS, one time in high-salt HNTG buffer (20 mM Hepes, 500 mM NaCl, pH 7.5, 10% glycerol and 0.1% Triton X-100), and once more in PBS. Proteins were eluted with 40 μl 1x loading buffer and boiling at 99°C for 5 minutes. Proteins were resolved by SDS/10% polyacrylamide gel and transferred onto nitrocellulose membranes, as previously discussed in Section 3.6.2 and Section 3.6.3, and Section 3.6.4.

3.8 Direct Binding Assays

3.8.1 NPM/ICAM-1 Protein-Protein Binding Assay

Three concentrations of recombinant human ICAM-1 (0 μM, 0.5 μM, or 1.0 μM) were coated in a 96-well high affinity Immulon plate overnight at 4°C. The next day, wells were washed 3 times with PBS-T (PBS in 0.05% Tween®20). Washing steps consisted of 300 μl PBS-T added to wells for 30 seconds, then removed with a multichannel pipette, for a total of 3 times. After the last wash, the plate was inverted and tapped dry on absorbent tissue to remove any excess wash buffer. After washing, wells were blocked with 0.2% BSA in PBS-T for 1 hour at room temperature. After 3 washes with PBS-T as previously described, recombinant human NPM or BSA (0 μM, 0.5 μM, or 1.0 μM) were added to respective wells and incubated for 2 hours at 37°C. After 3 washes with PBS-T, NPM monoclonal antibody (2.5 μg/ml) was added to all wells for 1 hour at room temperature, after which the wells
were washed 3 times with PBS-T. Goat α-mouse IgG1-HRP secondary antibody was added for 1 hour at room temperature and washed out with PBS-T, 3 times. TMB substrate was used and the plate was read by spectrophotometer at 620-nm absorbance every 10 minutes until the highest OD\textsubscript{620nm} reached 0.8 or no further increases could be detected. The reaction was stopped with sulfuric acid and the plate was read at 450-nm absorbance for the final values.

### 3.8.2 ELISA-Based LPS Binding Assay

LPS (0.1 or 1.0 µg/ml) was immobilized in a 96-well high-affinity Immulon plate overnight at 4°C. The next day, wells were aspirated and washed 3 times with PBS-T (PBS containing 0.05% Tween\textsuperscript{®}20). To wash, 300 µl PBS-T was added to each well, allowed to sit for 30 seconds, and then removed with a multichannel pipette. After the third wash, the plate was inverted and tapped onto absorbent tissue to remove excess buffer. Wells were blocked with 0.2% BSA in PBS-T for 1 hour at room temperature. After rinsing the wells two times with PBS-T, 100 µl of target recombinant human proteins NPM and CD14, or BSA, were added to the wells and incubated in concentrations of 0 µM, 0.5 µM, and 1.0 µM for 2 hours at 37°C. After the incubation, wells were aspirated and washed 3 times as previously described. After washing, 100 µl of corresponding primary antibodies, NPM monoclonal antibody and CD14 monoclonal antibody (2.5 µg/ml) were incubated for 1 hour at room temperature. After the incubation, wells were washed 3 times with PBS-T as previously described and 100 µl of goat anti-mouse IgG1 antibody conjugated with HRP (1:2500) was added for 1 hour at room temperature. After washing 3 times, 100 µl tetramethylbenzidine ([TMB], Sigma-Aldrich\textsuperscript{®}, St. Louis, MO, USA) substrate was added. The plate was read by spectrophotometer at 620-nm absorbance every 5 minutes until the highest OD\textsubscript{620nm} value reached 0.8 or no further increases were detected. The reaction was stopped through addition of 100 µl sulfuric acid and the plate was read at 450-nm absorbance for the final values.
3.9  siRNA Transfection

3.9.1  Transfection Optimization

3.9.1.1  BEAS-2B

BEAS-2B cells (2.0x10^5) in 6-well plates were treated with varying amounts of DharmaFECT Transfection Reagent I (0 μl/well, 2 μl/well, 3 μl/well, 4 μl/well, or 5 μl/well) to assess cytotoxicity and acquire the optimal concentration for siRNA transfection. Cytotoxicity was assessed by LDH assay. For NPM1 siRNA optimization, 2.5 μl DharmaFECT Transfection Reagent I was used in conjunction with 25 nM, 50 nM or 100 nM NPM1 siRNA. 100 nM scrambled siRNA was used as control. After 24 hours of transfection, the medium was replaced with fresh medium and NPM expression was assessed by Western Blot 24 hours later as described in Section 3.6. 50 nM of NPM1 siRNA and scrambled siRNA were used for all BEAS-2B transfection experiments.

3.9.1.2  SAEC

SAEC (2.0x10^5) in 6-well plates were treated with 4 concentrations of DharmaFECT Transfection Reagent I (0 μl/well, 1 μl/well, 3 μl/well, or 5 μl/well) to assess cytotoxicity and acquire optimal transfection reagent concentration for efficient siRNA transfection. SAEC transfection experiments used 1.5 μl/well of DharmaFECT Transfection Reagent I based on the dose-response curve. Based on results obtained from BEAS-2B cells, 50 nM of NPM1 siRNA and scrambled siRNA were used for all SAEC transfection experiments.

3.9.2  Transfection Protocol

3.9.2.1  BEAS-2B

In one tube, 2.5 μl of DharmaFECT Transfection Reagent I was added to 197.5 μl of serum- and antibiotic-free medium. In a separate tube, 50 nM of NPM1 or scrambled siRNA was
prepared in a total volume of 200 μl in serum- and antibiotic-free medium. Contents in both tubes were incubated at room temperature for 5 minutes, after which, the siRNA was added to the transfection reagent and incubated at room temperature for 20 minutes. After the incubation, 1.6 ml of serum-containing medium (antibiotic-free) was mixed into each tube, and subsequently added to the respective cells (2 ml total volume per well).

3.9.2.2 SAEC

In one tube, 1.5 μl of DharmaFECT Transfection Reagent I was added to 198.5 μl of basal medium. In a separate tube, 50 nM of NPM1 or scrambled siRNA was prepared in a total volume of 200 μl in basal medium. Contents in both tubes were incubated at room temperature for 5 minutes, after which, the siRNA was added to the transfection reagent and incubated at room temperature for 20 minutes. After the incubation, 1.6 ml of serum-containing medium (antibiotic- and retinoic acid-free) was mixed into each tube, and subsequently added to the respective cells (2 ml total volume per well).

3.9.3 Stimulation

3.9.3.1 BEAS-2B

BEAS-2B cells (2.5x10^5) in 6-well plates were transfected with NPM1 or scrambled siRNA (2.5 μl DharmaFECT Transfection Reagent I, 50 nM siRNA) for 24 hours. Wells were then washed 2 times with PBS and replaced with basal medium. 4 hours later, cells were stimulated with LPS (0.1 μg/ml) or saline control in complete medium for 2 or 6 hours, after which culture medium and total cell lysates were collected for analyses.

3.9.3.2 SAEC

SAEC (2.0x10^5) in 6-well plates were transfected with NPM1 or scrambled siRNA (1.5 μl DharmaFECT Transfection Reagent I, 50 nM siRNA) for 24 hours. Wells were then washed 2 times with HBSS and replaced with basal medium. 4 hours later, cells were stimulated
with LPS (0.1 μg/ml) or saline control in retinoic acid-free medium for 2 or 6 hours, after which culture medium and total cell lysates were collected for analyses. Retinoic acid can induce changes in NPM expression and activity [244]; thus, retinoic acid was removed from culture medium in SAEC experiments.

3.10 Endpoint Measurements

Culture medium was collected from stimulated BEAS-2B cells and SAEC and centrifuged at 500 × g for 5 minutes at 4°C. Supernatants were aliquoted and stored at -80°C until use for cytotoxicity and IL-8 measurements.

3.10.1 Cytotoxicity

Cytotoxicity measurements were performed by Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Penzberg, Germany)—a colorimetric assay that quantifies cell death and cell lysis based on the amount of LDH activity released from the cytosol of damaged cells into the culture medium. In this assay, a two-step reaction occurs, resulting in the ability to quantify cell toxicity. In the first step, LDH released from cells gets oxidized to pyruvate. In the second enzymatic reaction, the two hydrogens lost from LDH during oxidation get transferred to the tetrazolium salt by a catalyst, converting it to formazan salt, which can be detected at 490-nm absorbance.

100 μl of sample was incubated with 100 μl Working Solution in a 96-well plate for 30 minutes at room temperature. The Working Solution was prepared with 11.25 ml of Dye solution (containing tetrazolium salt) and 250 μl Catalyst (for 100 tests). The plate was read by spectrophotometer at 490-nm absorbance.

In each experiment, a “low control” (LC) group was included, which contained cells with culture medium, without any treatment. Likewise, each experiment included a “high control” (HC) group that also did not receive treatment, but in which 0.1% Triton X-100 was used to lyse all of the cells at the end of the experimental time points. The LC OD_{490nm} value served as the baseline cytotoxicity and the HC OD_{490nm} value represented 100%
cytotoxicity for calculation purposes. The formulation used to assess percent cytotoxicity was as follows, as per the company’s protocol:

\[
\% \text{ Cytotoxicity} = \frac{\text{Sample OD value} - \text{LC OD value}}{\text{HC OD value} - \text{LC OD value}} \times 100\%
\]

### 3.10.2 IL-8 Concentration

IL-8 measurements were performed by IL-8 Human ELISA Kit (Novex®, Thermo Fisher Scientific). Cell culture supernatant samples were thawed at 4°C and mixed with a vortex mixer prior to use. The Wash Buffer Concentrate was allowed to reach room temperature prior to use to ensure the dissolution of any precipitated salts. The Wash Buffer Concentrate was diluted 1 part Wash Buffer Concentrate to 24 parts deionized water. All other kit reagents were allowed to reach room temperature before use.

Wells pre-coated with monoclonal human IL-8 capture antibody were provided. The human IL-8 Standard was reconstituted to 10.0 ng/ml, and subsequently diluted 10x to a concentration of 1000 pg/ml. Serial dilution of the human IL-8 standard were prepared according to the protocol included in the kit. The standards consisted of 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 15.6 pg/ml and 0 pg/ml.

50 μl of standards and samples were added into individual wells, after which polyclonal biotinylated IL-8 detector antibody was added. After a 90-minute incubation on a shaking platform at room temperature, wells were washed four times with Wash Buffer Solution. Washes consisted of removing the liquid content using a multi-channel pipette, adding 300 μl of Wash Buffer to each well, allowing it to sit for 10 seconds, removing the Wash Buffer solution from each well, and inverting the plate and tapping dry on absorbent tissue. After washing, wells were incubated with streptavidin-conjugated HRP for 30 minutes at room temperature. After washing 4 times again, chromagen was added and reactions were stopped with stop solution 30 minutes later. IL-8 OD values were read by spectrophotometer at 450 nm absorbance.
IL-8 concentration was calculated from the standard curve by plotting optical density against IL-8 standard concentration. If the samples were diluted, the resulting concentration was multiplied by the appropriate dilution factor.

3.11 Statistical Analyses

The data are presented as mean ± standard error. Statistical significance was determined by non-parametric Mann-Whitney test; or two-way ANOVA, followed by a Bonferroni post-hoc test. A P-value less than 0.05 was deemed statistically significant. Analyses were conducted using GraphPad Prism v.5.00c (GraphPad Software Inc., San Diego, CA, USA).
Chapter 4
Results

4 Results

4.1 Objective 1 – Investigate Whether LPS Internalization Contributes to IL-8 Production and Cytotoxicity

4.1.1 TLR4 is Absent on the Surface, but Expressed Intracellularly in Primary Human Lung Epithelial Cells

Although other groups have noted the lack of surface TLR4 expression in primary lung epithelial cells, this has not been confirmed in primary SAEC. Primary SAEC in chamber slides were stained with TLR4 mAb or IgG control. DyLight™ 488-conjugated secondary antibody (green) was used for fluorescent visualization of TLR4. F-actin was stained with rhodamine phalloidin (red) and Hoechst stain (blue) was used for nuclear visualization. Representative serial 1.0-μm Z-plane optical sections for TLR4 and IgG staining are depicted in Figure 3. All cells exhibited diffuse intracellular staining of TLR4 and no staining was visualized in cells incubated with IgG control antibody. Thus, SAEC were used a model wherein TLR4 is not expressed at the surface.

4.1.2 MDC Inhibits LPS Internalization

LPS internalization was completely inhibited in both BEAS-2B cells and SAEC by pre-treatment with 100 μM primary alkyl amine, monodansylcadaverine (MDC), a compound that has been used to inhibit clathrin-mediated endocytosis in various cell types [251, 252]. Typical results of experiments by confocal visualization in both BEAS-2B cells (Figure 5) and human primary SAEC (Figure 6) are depicted. The leftmost column of the photomicrographs shows BODIPY-LPS staining in the respective treatment conditions. The centre column of the photomicrographs depicts F-actin staining by F-actin rhodamine
phalloidin. The rightmost column of the photomicrographs reveals the merged images from the corresponding centre and leftmost columns. Despite variability in the degree of internalization, all lung epithelial cells contained intracellular LPS after 4 hours of LPS stimulation. LPS internalization was abolished by MDC, as no green fluorescence was observed in sections with MDC pre-treatment prior to BODIPY-LPS stimulation. All images in each respective cell type were acquired and captured with identical settings on the confocal microscope. LPS staining (shown in green) amidst F-actin (shown in red) suggests that LPS is internalized within the cell, and staining patterns demonstrate perinuclear localization. This assertion was confirmed by examination of serial 1.0-μm Z-plane optical sections of BEAS-2B cells pre-treated with MDC or not, and stimulated with LPS for 4 hours (Figure 4). These representative images demonstrate the perinuclear distribution of LPS within the inner sections of the cell, signifying that LPS is, indeed, within the cell; pre-treatment with MDC inhibited LPS internalization in human lung epithelial cells.

4.1.3 LPS Internalization is Associated with IL-8 Production and Cytotoxicity

100-μM MDC significantly attenuated LPS-induced IL-8 production and cytotoxicity in BEAS-2B cells (Figure 5) and SAEC (Figure 6). Cells treated with Vehicle+Saline did not exhibit significant differences in IL-8 levels compared to cells treated with MDC+Saline (14.6 ± 6.9 pg/ml vs. 10.2 ± 4.1 pg/ml, respectively in BEAS-2B, P = 0.25; and 75.5 ± 11.3 pg/ml vs. 109.4 ± 16.7 pg/ml, respectively in SAEC, P = 0.13), suggesting that MDC did not alter basal IL-8 concentrations. Upon LPS stimulation, cells without MDC pre-treatment had elevated IL-8 production, which was attenuated by MDC (36.7 ± 2.8 pg/ml vs. 14.3 ± 2.5 pg/ml, respectively in BEAS-2B, P = 0.01; 293.9 ± 64.4 pg/ml vs. 65.4 ± 8.9 pg/ml, respectively in SAEC, P = 0.008). SAEC displayed higher IL-8 levels overall than BEAS-2B, likely due to the higher cell density used for SAEC experiments and the increased sensitivity of primary cells to LPS stimulation. MDC also significantly attenuated LPS-induced cytotoxicity (6.3 ± 1.6% vs. 1.4 ± 0.6% in BEAS-2B, P = 0.025; 10.4 ± 0.7% vs. 3.4 ± 1.7% in SAEC, P = 0.02).
Figure 3. Z-stack of SAEC expressing intracellular, but not surface TLR4. Human primary small airway epithelial cells were stained with TLR4 antibody or isotype control antibody. Fluorescence was detected by DyLight-488-conjugated secondary antibody (green). F-actin was stained with rhodamine phalloidin (red). Nuclei were stained with Hoechst (blue). Z-stack images were captured in 1.0-μm sections at 60x magnification. Images are representative of 3 experiments.
**Vehicle + LPS**

**MDC + LPS**

**Figure 4. Z-stack of LPS internalization with and without MDC.** BEAS-2B cells were seeded on coverslips and pre-treated with **Upper Panel.** Vehicle solution (DMSO) or **Lower Panel.** MDC for 30 min prior to incubation with BODIPY-LPS (green) at 0.1 μg/ml for 4 hours at 37°C. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 2% BSA-PBST, and F-actin was stained with rhodamine-phalloidin (red). Cells were visualized by confocal microscopy. Z-stack images demonstrate progressive 1.0 μm-sections at 60x magnification. Experiments were repeated 3 times.
Figure 5. MDC blocks LPS internalization and attenuates IL-8 production and cytotoxicity in BEAS-2B cells. Cells were seeded on coverslips and pre-treated with vehicle solution (DMSO) or MDC for 30 min prior to incubation with BODIPY-LPS (green) at 0.1 μg/ml for 4 hours. F-actin was stained with rhodamine-phalloidin (red). Cells were visualized by confocal microscopy at 60x magnification. Concentrations of IL-8 were measured in culture medium by ELISA. Cytotoxicity was determined in culture medium by LDH assay. Experiments were performed 3 times. * Denotes statistical significance (P < 0.05) vs. Saline; § denotes statistical significance vs. Vehicle+LPS.
Figure 6. MDC blocks LPS internalization and attenuates IL-8 production and cytotoxicity in human primary SAEC. Cells were seeded on coverslips and pre-treated with vehicle solution (DMSO) or MDC for 30 min prior to incubation with BODIPY-LPS (green) at 0.1 μg/ml for 4 hours. F-actin was stained with rhodamine-phalloidin (red). Cells were visualized by confocal microscopy at 60x magnification. Concentrations of IL-8 were measured in culture medium by ELISA. Cytotoxicity was determined in culture medium by LDH assay. Experiments were performed 4 times. * Denotes statistical significance (P < 0.05) vs. Saline; § denotes statistical significance vs. Vehicle+LPS.
4.2 Objective 2 – Investigate NPM Expression in Lung Epithelial Cells and the Interaction Between NPM/ICAM-1 and NPM/LPS

4.2.1 Total NPM Expression in SAEC upon LPS Stimulation

Before examining whether NPM specifically played a role in LPS-mediated cellular effects, NPM expression was determined. In SAEC, NPM protein content was evaluated by Western Blot in whole cell lysates for up to 8 hours of LPS stimulation (Figure 7). NPM protein content appeared to moderately increase after 2 hours of LPS stimulation (P = 0.25), and decreased to slightly below basal levels between 2 to 8 hours of LPS stimulation.

4.2.2 NPM mRNA Expression upon LPS Stimulation

Previous results showed that LPS induced a slight increase in NPM protein content in SAEC after 2 hours of stimulation. To elucidate whether the increased protein content was due to elevated mRNA expression, RT-PCR was used to analyze NPM mRNA (Figure 7). After LPS stimulation of 15 minutes, 30 minutes, or 1 hour, no significant change was detected in the mRNA expression of NPM. Thus, LPS did not alter NPM mRNA expression within the tested time intervals.

4.2.3 Subcellular NPM Expression in the Presence and Absence of LPS

In order to assess whether NPM is a potential downstream target of ICAM-1, we examined the subcellular expression of NPM in SAEC with or without LPS stimulation by Western Blot (Figure 7). Cytoplasmic and nuclear isolates were prepared from SAEC that were stimulated with Saline (vehicle control) or LPS for 4 hours. The level of NPM expression in the nuclear fraction was higher than the level of NPM expression in the cytoplasmic fraction (implied by the exposure times used for band detection and the absolute band intensity values that were used for measurement). NPM expression in the cytoplasmic fraction appeared to decrease upon LPS stimulation, approaching significance (P = 0.15),
whereas the nuclear fraction displayed unchanged NPM expression upon LPS stimulation (P = 0.75).

4.2.4 Subcellular ICAM-1 Expression in the Presence and Absence of LPS

ICAM-1 expression was assessed by immunofluorescence in SAEC (Figure 8). ICAM-1 expression in cells treated with Saline (vehicle control) displayed strong membrane staining with moderate intracellular staining. Different cells displayed varying staining intensities, and roughly 10% of cells did not appear to express ICAM-1. Upon 4 hours of LPS stimulation, ICAM-1 staining intensity increased. Membrane staining persisted, yet the presence of intracellular ICAM-1 was increased compared to saline control. Cells stained with IgG antibody and Cy3-conjugated secondary antibody displayed no red fluorescence, suggesting that the ICAM-1 staining was specific.
**Figure 7. NPM expression in the presence and absence of LPS stimulation.**

**Upper Left Panel:** SAEC were stimulated with LPS (0.1 μg/ml) for up to 8 hours. Total NPM expression (36 kDa) was detected by Western Blot in total cell lysates. β-actin (42 kDa) was used for the loading control. Experiments were repeated 3 times.

**Upper Right Panel:** SAEC were stimulated with LPS (0.1 μg/ml) for 15 minutes, 30 minutes, or 60 minutes, after which total NPM1 mRNA expression was assessed by RT-PCR. GAPDH mRNA expression was used as the housekeeping control. Two independent experiments were performed, as depicted.

**Lower Panels:** Primary SAEC were stimulated with Saline (vehicle control) or LPS (0.1 μg/ml) for 4 hours, after which cytoplasmic and nuclear fractions were isolated. NPM protein expression was assessed in the subcellular fractions. β-actin was used for the loading control in the cytoplasmic fraction and Lamin was used for the loading control in the nuclear fraction. Three independent experiments were performed, as depicted.
Figure 8. ICAM-1 subcellular localization in the presence and absence of LPS stimulation. Primary human SAEC were stimulated with Saline or LPS (0.1 μg/ml) for 4 hours. Cells were stained with ICAM-1 or IgG antibodies. Cy3-conjugated secondary antibody was used for detection (red). Nuclei were stained with Hoechst (blue). Z-stack images were captured in 1.0-μm sections at 60x magnification. The maximum intensity projection of the IgG Z-stack is depicted. Images are representative of 3 experiments.
4.2.5 NPM Binds to ICAM-1

Co-immunoprecipitation experiments were performed to determine the validity of the hypothetical interaction between NPM and ICAM-1. Precipitated NPM co-precipitated ICAM-1 repeatedly in BEAS-2B cells and SAEC (Figure 9). ICAM-1 co-immunoprecipitation occurred in resting conditions (no LPS stimulation), and was maintained upon LPS stimulation up to 6 hours. Co-immunoprecipitation is a technique that only asserts whether an association takes place (directly or in complex), and conclusions regarding direct binding cannot be made. As such, a protein-protein binding assay was performed between ICAM-1 and NPM. NPM directly bound to ICAM-1 in a dose-dependent fashion (Figure 10). NPM-ICAM-1 direct binding experiments were performed one time, in triplicate.

4.2.6 NPM Binds to LPS

As previous studies in our lab have demonstrated that ICAM-1 and LPS co-localize intracellularly in lung epithelial cells, an ELISA-based LPS binding assay was also performed to assess whether NPM has the ability to bind LPS. NPM bound to LPS in a dose-dependent fashion (Figure 11). CD14, a protein known to bind to LPS, was used as a positive control. Statistical analyses were not performed because LPS binding experiments were performed two times.
Figure 9. Co-immunoprecipitation of ICAM-1 with NPM. BEAS-2B cells or SAEC (as indicated) were stimulated with Saline (vehicle control) or LPS (0.1 μg/ml) for 4 hours, or in a timecourse of 2 to 4 hours. NPM mAb or IgG control was used as the bait. Coimmunoprecipitation of ICAM-1 and NPM were detected by Western Blot in 300 μg or 150 μg total cell lysates. Recombinant human ICAM-1 (rhICAM-1) was used for positive control. Experiments were performed 3 times in BEAS-2B and one time in SAEC for the single timepoint. Timecourse experiments were repeated 4 times in SAEC.
Figure 10. NPM directly binds to ICAM-1 in solution. rhICAM-1 was coated in a high-affinity plate at depicted concentrations. BSA (negative control) or recombinant human NPM (rhNPM) was incubated in the wells (0-1.0 µM) and binding was determined by TMB substrate. Absorbance was read at 450 nm. Experiment was performed one time in triplicate. Error bars represent standard deviation.
Figure 11. NPM directly binds to LPS in solution. LPS (0.1 or 1.0 µg/ml) was coated in a high affinity plate. BSA (negative control), rhNPM, or rhCD14 (positive control) (0-1.0 µM) were incubated in respective wells and binding was determined by TMB substrate. Absorbance was read at 450 nm. Experiment was repeated two times.
4.3 Objective 3 – Assess the Specific Role of NPM in LPS-Induced IL-8 Production and its Downstream Signaling

4.3.1 NPM is Significantly Knocked Down by NPM siRNA

To assess the specific role of NPM on LPS-stimulated cells, NPM was knocked down through siRNA transfection. For efficient transfection, DharmaFECT Transfection Reagent I was optimized to minimize its cytotoxic effects (Figure 12). DharmaFECT Transfection Reagent I was tested at concentrations of 1, 3, 4, and 5 μl per 2 ml well-volume for BEAS-2B cells and 1, 3, and 5 μl per 2 ml well-volume for SAEC, as indicated. Less than 10% cytotoxicity was deemed viable according to siRNA company guidelines. 2.5 μl DharmaFECT Transfection Reagent I was used for BEAS-2B siRNA transfection and 1.5 μl DharmaFECT Transfection Reagent I was used for SAEC siRNA transfection as primary cells were more sensitive to the transfection reagent.

NPM knockdown was optimized in total cell lysates of BEAS-2B cells (Figure 12). For optimization, three concentrations of NPM siRNA were used: 25 nM, 50 nM, and 100 nM. DharmaFECT Transfection Reagent I-only control and scrambled siRNA control (100 nM) were included. Sufficient NPM knock down was observed by all three concentrations. Future NPM siRNA experiments in both BEAS-2B cells and SAEC used 50 nM NPM siRNA and correspondingly, 50 nM scrambled control. NPM knockdown was verified again by Western Blot for all independent experiments (Figure 13).
Figure 12. Optimization of NPM siRNA transfection. Upper Panel: BEAS-2B cells or primary human SAEC (as indicated) were treated with depicted doses of DharmaFECT Transfection Reagent I. Culture medium was collected 24 hours later and cytotoxicity was assessed by LDH assay. Experiments were repeated 2 times in BEAS-2B cells and 2 times in SAEC. Lower Panel: BEAS-2B cells were transfected with DharmaFECT Transfection Reagent I only (2.5 μl), or in combination with depicted concentrations of NPM siRNA or Scrambled siRNA. NPM expression was assessed by Western Blot in 3-μg of total protein lysate. β-actin was used as a loading control. Experiment was performed one time.
4.3.2 NPM Mediates LPS-Induced IL-8 Production and Cytotoxicity

To confirm NPM knockdown, NPM expression was assessed in total cell lysates of BEAS-2B cells and SAEC through Western Blot analysis (Figure 13). In BEAS-2B cells, there were no significant differences observed in NPM expression between cells transfected with DharmaFECT Transfection Reagent 1-only or scrambled siRNA (which contains DharmaFECT Transfection Reagent I) (P = 0.52 with Saline and P = 0.2 with LPS stimulation). Thus, the DharmaFECT Transfection Reagent 1-only group was omitted from experiments in SAEC.

Transfection of cells with scrambled siRNA (50 nM) or NPM siRNA (50 nM) did not produce a difference in basal IL-8 concentration (67.1 ± 27.0 pg/ml vs. 66.4 ± 29.4 pg/ml, respectively in BEAS-2B, P = 1.00; and 58.6 ± 18.5 pg/ml vs. 74.7 ± 11.0 pg/ml, respectively in SAEC, P = 0.5). Upon LPS (0.1 μg/ml) stimulation for 6 hours in SAEC, cells transfected with scrambled siRNA had significantly increased IL-8 production, which was significantly attenuated by transfection with NPM siRNA (151.8 ± 13.3 pg/ml vs. 105.5 ± 4.3 pg/ml, respectively, P = 0.02). BEAS-2B experiments exhibited the same trend, and values approached significance (186.1 ± 31.1 pg/ml vs. 72.0 ± 22.3 pg/ml, respectively; P = 0.055) (Figure 13).

NPM knockdown did not significantly alter basal cytotoxicity levels (2.0 ± 0.8% Scrambled siRNA vs. 2.4 ± 0.9% NPM siRNA in BEAS-2B, P = 0.39; and 1.4 ± 0.09% Scrambled siRNA vs. 2.4 ± 0.5% NPM siRNA in SAEC, P = 0.16). However, NPM siRNA significantly attenuated LPS-induced cytotoxicity in SAEC (7.7 ± 0.2% vs. 5.1 ± 0.7%, P = 0.023). Transfection with NPM siRNA in BEAS-2B cells moderately reduced LPS-induced cytotoxicity, but this was not significant (P = 0.36) (Figure 13).
Figure 13. The use of NPM siRNA attenuates LPS-induced IL-8 production and cytotoxicity in lung epithelial cells. Cells were transfected with NPM siRNA or scrambled control (50 nM) for 24 hours, starved for 4 hours, then stimulated with LPS for 6 hours. NPM expression was assessed by Western Blot in total cell lysates of BEAS-2B cells and SAEC, as indicated. IL-8 concentration and cytotoxicity was measured in the culture media. * Denotes statistical significance (p < 0.05) compared to respective Saline groups; § denotes significance vs. Scrambled siRNA+LPS. Experiments were performed 3 times in BEAS-2B cells and 4 times in SAEC. DF = DharmaFECT Transfection Reagent I only; NPM = NPM siRNA; Sc = Scrambled siRNA.
4.3.3 Transfection with NPM siRNA Reduces c-Fos and c-Jun Expression

The IL-8 promoter sequence contains AP-1 binding sites [164], and NPM has been shown to co-localize with c-Fos during neuroblastoma differentiation and also has the ability to bind to c-Jun [244, 245]. Furthermore, NPM binds to AP-2α in differentiating cells [246]. As such, NPM was assessed for its role in the expression and activity of c-Fos and c-Jun, two components of the AP-1 transcription factor. After siRNA transfection and LPS stimulation for 2 hours, phosphorylated c-Fos (at serine 32), total c-Fos, phosphorylated-c-Jun (at serine 33), and total c-Jun were assessed (Figure 14) by Western Blot. Initial studies in BEAS-2B cells included three groups: transfection with 2.5 μl DharmaFECT Transfection Reagent I-only, transfection with 50 nM NPM siRNA (including transfection reagent), and transfection with 50 nM scrambled siRNA (including transfection reagent). There was no significant statistical difference between cells transfected only with DharmaFECT Transfection Reagent I-only and cells transfected also with Scrambled siRNA pertaining to phospho-c-Fos or c-Fos levels with LPS stimulation (P = 0.57 and P = 0.31, respectively) or without LPS stimulation (P = 0.14 and P = 0.39, respectively). Thus, the DharmaFECT Transfection Reagent I-only was omitted from future experiments.

BEAS-2B cells transfected with scrambled siRNA displayed significantly elevated expression of phospho-c-Fos upon LPS stimulation (P = 0.036). Cells transfected with NPM siRNA displayed significantly reduced phospho-c-Fos expression upon LPS stimulation (P = 0.038). Of note, however, is that cells transfected with NPM siRNA also exhibited reduced total c-Fos expression upon LPS stimulation (P = 0.44), which likely contributes to the associated diminished levels of phosphorylated c-Fos. Nevertheless, c-Fos activation, as displayed by the ratio of phospho-c-Fos/c-Fos, was reduced upon knockdown with NPM siRNA compared to cells transfected with scrambled siRNA (P = 0.025). Cells transfected with NPM siRNA did not express significantly altered phospho-c-Jun expression or total c-Jun expression upon LPS stimulation (P = 0.62 and P = 0.097, respectively); however, a downward trend was observed similar to that of c-Fos (Figure 14).

In primary SAEC, cells transfected with NPM siRNA displayed a reduction in phospho-c-Fos, c-Fos, phospho-c-Jun, and c-Jun expression compared to cells transfected with scrambled
siRNA upon 2 hours of LPS stimulation, consistent with previous results in BEAS-2B cells (Figure 14). However, findings did not reach statistical significance in SAEC. Notably, upon LPS stimulation, the attenuation of total c-Jun expression in cells transfected with NPM siRNA approached significance (P = 0.06). Indeed, these findings suggest that NPM partially regulates c-Fos and c-Jun expression, rather than mediating their activation.
Figure 14. Silencing of the NPM gene by using specific siRNA reduces c-Fos and c-Jun expression upon LPS stimulation. BEAS-2B cells or SAEC (as indicated) were transfected with DharmaFECT transfection reagent I only, NPM siRNA or scrambled siRNA for 24 hours, followed by starving for 4 hours and LPS stimulation for 2 hours. Phospho-c-Fos (62 kDa), c-Fos (62 kDa), β-actin (42 kDa), phospho-c-Jun (42 kDa), c-Jun (42 kDa), and GAPDH (37 kDa) were detected by Western Blot in total cell lysates. * Denotes statistical significance (P < 0.05) vs. respective Saline group; § denotes statistical significance vs. scrambled siRNA+LPS. Experiments were repeated 3 times in BEAS-2B and 5 times in SAEC.
Chapter 5
Discussion and Future Directions

5 Discussion and Future Directions

5.1 Human Lung Epithelial Cell In Vitro Model Considerations

LPS forms the outer membrane of Gram-negative bacteria and contains the Lipid A component that is responsible for its biological activity [43]. Using LPS as a model of sepsis is based on the notion that symptoms of sepsis are caused by the host's maladaptive response to bacterial toxins, rather than the intact bacteria itself. Indeed, LPS is typically released from the cell wall of bacteria and circulates as free LPS [46]. Administration of purified LPS in animals induce the same pathophysiological effects as those seen in patients with Gram-negative sepsis [44], and LPS stimulation has been shown to activate human lung epithelial cells in vitro [49].

As a barrier between the inside of our bodies and the outside world, lung epithelial cells must not only rid the body of infection, but also prevent exaggerated inflammatory responses to the constant microbial stimulation. Such a balance is accomplished in part through the intracellular compartmentalization of TLR4 [253]. In lung epithelial cells, TLR4 is predominantly localized to intracellular compartments [102, 168, 175, 254]. Thus, LPS must be transported to these respective intracellular compartments in order to initiate a signaling response [255]. In this way, inflammatory responses are triggered by bacterial invasion, and are avoided by noninvasive bacteria [253].

Although sepsis can occur from non-pulmonary sources that may eventually injure the lung by the continual activation of immune and endothelial cells, respiratory tract infections (mainly pneumonia) are responsible for up to one third of sepsis cases [33]. Pulmonary epithelial cells, which were traditionally branded as “innocent bystanders”, are increasingly being shown to partake in the inflammatory cascade itself, particularly through their active
release of pro-inflammatory chemokines. In our *in vitro* model, LPS was applied to the apical surface of human lung epithelial cells, which mimics infection occurring from a pulmonary source. Indeed, our model does not directly address the spread of LPS from the circulation, which would eventually reach the lung epithelium from the basolateral cell membrane. As the lung epithelium is an active source of pro-inflammatory cytokines, it is incumbent to understand the pathophysiological mechanisms of the lung epithelium in inducing pro-inflammatory responses to LPS.

5.2 The Role of LPS Internalization in Inflammatory Responses

It is indeed a challenge to study the effects of intracellular LPS in a physiologically relevant and specific manner: Endocytosis inhibitors are non-specific, genetic approaches are costly, time-consuming, and may not provide specific results, and transfection/electroporation are physiologically irrelevant as they eliminate the role of the LPS receptor when transfecting LPS directly into the cell. Nevertheless, scientists have utilized these methods in an effort to further understand the role of intracellular LPS as best as possible in various cell types.

Lichtman *et al* [172] used various endocytosis inhibitors to show that LPS internalization is required for LPS to stimulate TNFα production in Kupffer cells. In the RAW264.7 cell line, Wang *et al* [174] used the non-specific endocytosis inhibitors MDC or Dynosore prior to LPS stimulation and observed a reduction in JNK, ERK, and NFκB activation, with associated reductions in TNFα and IL-6 production. Hornef *et al* [175] pre-treated intestinal epithelial cells with MDC prior to LPS stimulation and observed reduced NFκB activation. Cowan *et al* [173] pre-treated cardiomyocytes with cytochalasin D prior to LPS stimulation and showed the attenuation of ERK and NFκB activation, and a corresponding reduction in TNFα production. To assess whether their observed effects were due to the inhibition of LPS internalization and not the non-specific effects of cytochalasin D, cells were pre-treated with cytochalasin D and stimulated with hydrogen peroxide. Findings showed that ERK was still activated by hydrogen peroxide in the presence of cytochalasin D, suggesting that the attenuation of ERK signaling and TNFα production upon LPS
stimulation in the presence of cytochalasin D was due to the inhibition of LPS internalization itself [173].

Interestingly, intracellular LPS has recently been shown not only to activate the transcriptome, but to distinctly activate the inflammasome as well. Inflammasomes are intracellular multiprotein complexes that are characterized by caspase-1 activation in the presence of stimuli, mainly leading to the subsequent maturation and secretion of pro-inflammatory cytokines, such as IL-1β [256].

Hagar et al [181] recently showed that transfection of boiled bacterial lysates from Gram-negative bacteria into murine macrophages activated caspase-11 and subsequent IL-1β secretion. Kayagaki et al [180] also showed that transfection or electroporation of LPS stimulated caspase-11-dependent IL-1β production in murine macrophages. As humans do not express caspase-11, Jianjin et al [182] studied whether there was an intracellular LPS sensing mechanism in humans. In their studies, LPS was electroporated into various human cell lines, including U937 (monocytes), HeLa (epithelial cells), and HaCaT (keratinocytes). Intracellular LPS was recognized by caspase-4 and induced caspase-4 oligomerization and activation [182]. Furthermore, caspases-4 and -5 (human homologues for caspase 11) directly bound to LPS, further supporting their involvement [182]. However, these studies utilized artificial models by transfection of LPS into cells and were not directly proven in in vivo models.

In summary, these studies indicate that intracellular LPS is not merely a bystander, but that LPS internalization contributes to intracellular cellular signaling and the subsequent production of pro-inflammatory cytokines and cytotoxicity though various pathways.

5.2.1 The Role of Intracellular LPS in Lung Epithelial Cells

Inhibition of LPS internalization by use of various non-specific endocytosis blockers has demonstrated the association of intracellular LPS on the production of inflammatory mediators in cardiomyocytes, monocytes, and macrophages [173, 174, 177], as previously discussed. Genetic approaches to silence proteins required for endocytosis would have
been costly with no added benefit, as they also do not provide specificity [178]. Transfection or electroporation of LPS was a feasible method to use to assess the role of intracellular LPS in the lung epithelium; yet it is an artificial model of internalization. We desired to maintain the natural LPS internalization process with ICAM-1, which would have been eliminated if LPS was directly transfected into cells. Indeed, ICAM-1-mediated LPS internalization may promote specific intracellular localizations and functions that we did not want to compromise. After all considerations, we decided to use MDC to inhibit clathrin-mediated endocytosis; however, considerations for data interpretation must be made:

MDC inhibits clathrin-coated pit assembly as a competitive inhibitor of tissue transglutaminase 2 [257] — an enzyme that regulates actin assembly and dynamics through its activation of Rho GTPases [179]. Transglutaminase 2 activity is required for the self-association of the F-actin motor, myosin II [258]. Therefore, inhibition of transglutaminase by MDC may result in global changes in the structure and dynamics of the actin cytoskeleton. However, such significant side effects of MDC are mainly noted in developing cells or in cells undergoing differentiation [258]. Furthermore, no differences were observed in the F-actin staining of cells used for experiments herein, suggesting that significant cytoskeletal changes were not induced by the dose of MDC used. Moreover, it is documented that other members of the transglutaminase family compensate for transglutaminase 2 in its absence [259].

Nevertheless, results herein provide evidence that intracellular LPS actively contribute to inflammatory processes in the lung epithelium. Interestingly, MDC inhibited LPS-induced IL-8 production and cytotoxicity down to basal levels, suggesting that LPS must be internalized to initiate inflammatory signaling in lung epithelial cells. Consideration of the lung as a privileged site supports these findings: the lung epithelium comes into constant contact with foreign material, such that to prevent hyper-activation at the cell surface, bacteria must be brought into intracellular compartments to initiate a signaling response [255]. Indeed, this is partly accomplished by the intracellular compartmentalization of the implicated LPS receptor, TLR4, in lung epithelial cells [168, 253].
5.2.2 Surface TLR4 is Independent of LPS Internalization and its Signaling

Various groups have begun to identify TLR4-independent signaling pathways associated with LPS stimulation [177, 180, 181, 260]. For instance, alveolar macrophages isolated from C3H/HeJ mice are sensitive to LPS stimulation [261]. Additionally, unpublished work from our lab indicates that C3H/HeJ and C57BL/10ScNJ mice locally respond to intratracheal LPS challenge in the lung. Regarding intracellular LPS, various studies have shown that LPS uptake itself is independent of TLR4 in several cell types [177, 180, 181], and that the production of TNFα and other cytokines are elevated in association with LPS uptake [177].

Furthermore, recent studies demonstrated a completely novel TLR4-independent role of intracellular LPS in monocytes, as previously mentioned. In these studies, LPS directly transfected or electroporated into the cytoplasm activated the caspase-11-dependent inflammasome pathway in murine macrophages, leading to pyroptosis [180, 181]. Another group recently also showed the involvement of caspase-4 and caspase-5 (human homologues for mouse caspase-11) in intracellular LPS-induced pyroptosis [182]. TLR4 was not implicated in these intracellular effects since LPS was electroporated or transfected into the cell, which bypassed cell-surface TLR4 activation by LPS. The receptor responsible for LPS uptake and its intracellular targets are still under investigation; although, Pilla et al. demonstrated the involvement of guanylate binding proteins in cytoplasmic LPS-related pyroptosis [262]. These studies of intracellular LPS support our finding that LPS internalization contributes to intracellular inflammatory signaling.

BEAS-2B cells have been shown not to possess surface-TLR4 expression [168]. In our studies, human primary SAEC were shown not to express surface-TLR4 as well by immunofluorescence. Thus, the model we used for our studies did not involve LPS recognition by TLR4 and surface-TLR4 downstream signaling, as is the conventional LPS signaling paradigm. LPS internalization in the lung is independent of surface-TLR4. Inhibition of LPS internalization by MDC was associated with attenuated IL-8 production and cytotoxicity in human lung epithelial cells. Thus, LPS internalization is associated with IL-8 production and cytotoxicity through a mechanism that is independent of surface-TLR4.
Moreover, unpublished work in our lab has shown that LPS internalization is mediated by ICAM-1 in lung epithelial cells, suggesting that ICAM-1-mediated LPS internalization is independent of TLR4.

5.2.3 Significance of LPS Internalization

In light of the failed clinical trials neutralizing LPS in the circulation or blocking its surface receptor, TLR4, there is a growing need to understand alternate LPS signaling pathways in the cell. Epithelial cells are one of the first cells to encounter LPS in Gram-negative bacterial infections. Since respiratory infections are the largest contributors to sepsis [33, 263], and also since sepsis is the largest risk factor for the development of ARDS [29, 264, 265], studying the role of LPS in lung epithelial cells is of critical importance.

Although it has been demonstrated by various groups, the mechanisms of LPS internalization and the signaling pathways induced by intracellular LPS have been largely overlooked until recently. With accruing evidence supporting that intracellular LPS contributes to inflammatory responses, it is important to investigate the mechanisms of internalization and intracellular signaling for potentially new therapeutic targets.

5.3 The Role of NPM in Mediating Inflammation

5.3.1 Currently Known General Functions of NPM

NPM is principally expressed in the nuclei of cells and plays a key role in ribosomal biogenesis through its inherent ribonuclease activity [221]. NPM is classically defined as a histone chaperone — proteins that are required for DNA-dependent activities including DNA repair and transcription (Reviewed in [215]). Various groups have reported on the role of NPM in regulating transcription through its interaction with transcription factors. NPM can co-activate transcription factors, as with NFκB [247], or suppress transcription factors, as with AP-2α [246].
More recently, NPM was shown to shuttle between the nucleus and cytoplasm, and cytoplasmic NPM has been associated with ribosomal assembly and centrosome duplication [216]. Furthermore, depending on different cell types or stimuli, NPM present in the cytoplasm is reported to possess an anti-apoptotic or pro-apoptotic role (Reviewed in [215]).

5.3.2 The Role of NPM in Macrophages

Despite all that is currently known about NPM, the role of this important phosphoprotein in the immune response — particularly in the presence of LPS is currently unclear and limited. Zhang et al [236] performed a proteomic analysis of macrophages (RAW264.7 cells) stimulated by LPS and identified a 16-fold increase in cytoplasmic NPM expression, which they attributed to the suppression of NPM degradation. However, their studies characterized NPM without addressing any specific roles of NPM in the presence of LPS stimuli.

Similar findings by Nawa et al [239] reported that LPS induced detectable cytoplasmic expression of NPM in macrophages (RAW264.7 cells). However, this group was interested in the potential role of NPM as an alarmin and thus tested the effects of recombinant NPM on cytokine release and activation of MAPK signaling in macrophages. In their studies, recombinant NPM induced TNFα, IL-6, and MCP-1 production and activated ERK and JNK signaling pathways, comparable to the levels induced by LPS. Furthermore, recombinant NPM induced ICAM-1 expression in HUVECs [239].

Guery et al recently showed a protective role of NPM in mice injected with a low dose of LPS (0.5 mg/kg) by tail vein. In their study, NPM+/− mice had a significantly higher IL-6 concentration in plasma after 60 minutes of LPS tail vein injection compared to wild-type mice [237]. Of note, however, is that NPM+/− mice develop features of myelodysplastic syndrome [238], which can lead to deregulated cytokine production and altered polarization of macrophages, thus rendering in vivo inflammation results unreliable.
Furthermore, NPM siRNA transfection in monocyte-derived macrophages increased MCP1 and MIP-1β gene expression after 3 hours of LPS (1 mg/ml) stimulation, further supporting the protective role of NPM that they reported [237]. However, it is important to note that a very high concentration of LPS was used for stimulations, which may be physiologically irrelevant and induce off-target cellular responses. Furthermore, it is peculiar that their data illustrated a near-complete lack of full-length NPM expression in monocyte-derived macrophages that were not stimulated with LPS, which contradicts previously published work illustrating high basal levels of NPM — especially as NPM is characterized as the most abundant phosphoprotein in nucleoli [214, 239]. Thus, these findings are inconclusive.

5.3.3 The Role of NPM in Lung Epithelial Cells

5.3.3.1 NPM Expression

We observed NPM expression in the cytoplasm of resting lung epithelial cells that may be required for interaction with intracellular LPS and/or ICAM-1. Total NPM expression appeared to moderately increase after two hours of LPS stimulation, and then diminish to basal levels by 8 hours of LPS stimulation. Interestingly, however, NPM mRNA expression was not altered between 15 minutes and 1 hour of LPS stimulation.

Contrary to studies in macrophages wherein cytoplasmic NPM was only detected upon LPS stimulation [236, 239], primary SAEC exhibited basal levels of NPM in the cytoplasm, which were reduced upon LPS stimulation. Thus, it appears as though lung epithelial cells display different NPM subcellular localization as compared to macrophages. Reasons for the reduction of cytoplasmic NPM expression upon LPS stimulation are currently unknown. One may speculate that if NPM can act as an alarmin [239], NPM may be released into the extracellular space, thus decreasing in abundance in the cytoplasm. However, studies measuring the concentration of NPM in the cell culture supernatants by ELISA must be performed to address this question.
5.3.3.2 NPM Mediates Inflammation and Cytotoxicity

The role of NPM in mediating inflammation is a novel concept that has not yet been profoundly studied. Recombinant NPM was shown to induce ERK and JNK signaling and cytokine production in a macrophage cell line, yet NPM⁺/- mice exhibited decreased cytokine production in the plasma upon low-dose LPS tail vein injection [237, 239]. As previously mentioned, however, NPM⁺/- mouse models are not ideal for studies involving inflammation parameters. As such, not much is known about the specific role of NPM in inflammation — especially in the lung epithelium. Our studies are the first to report that NPM mediates IL-8 production and cytotoxicity in human lung epithelial cells. Indeed, NPM may mediate IL-8 production and cytotoxicity from the nucleus through its interactions with various transcription factors; however, NPM interaction with ICAM-1 propels us to further consider the role and contribution of cytoplasmic NPM as well, as ICAM-1 is not expressed in the nucleus. Interestingly, in response to ischemic stroke in vivo, cytoplasmic NPM binds to BCL2-associated X protein (BAX)—a key protein in the apoptotic pathway that induces cytochrome c release [266]. Furthermore, knockdown of NPM expression in neuroblastoma cells subjected to staurosporine, a protein kinase inhibitor that induces apoptosis, reduced cytochrome c release [266].

BAX expression is increased in LPS-stimulated endothelial cells and macrophages [267, 268]. It is proposed that p53 translocation to the mitochondria induces the translocation of BAX from the cytoplasm to mitochondria, subsequently reducing mitochondrial membrane potential and causing the cytosolic release of cytochrome c to propagate apoptosis [267]. However, whether BAX is involved in the NPM-mediated cytotoxic response to LPS stimulation in the cytoplasm is currently not known.

5.3.3.3 Significance of NPM/ICAM-1 Interaction

Our results suggest that NPM may be a downstream target of ICAM-1 as ICAM-1 was pulled down with NPM in whole cell lysates. A direct binding experiment was performed to confirm the interaction that was observed by co-immunoprecipitation.
Co-immunoprecipitation provides one of the most convincing evidence that two (or more) proteins physically interact [269]; however, it is recommended to confirm protein-protein interactions by two or more distinct methods [269]. Indeed, co-immunoprecipitation does not infer direct interaction, as proteins within a complex can also be pulled down [269]. However, NPM/ICAM-1 direct binding outside of a cell-based system demonstrated the ability for these two proteins to physically bind to one another.

ICAM-1 was co-immunoprecipitated with NPM in non-stimulated cells, and a large change in NPM and ICAM-1 co-immunoprecipitation was not observed during the course of LPS stimulation for up to 6 hours, which was associated with IL-8 production and cytotoxicity. Until recently, NPM was regarded mainly as a nuclear phosphoprotein and ICAM-1 is not present in the nucleus. However, we detected NPM in the cytoplasm of resting lung epithelial cells. Although ICAM-1 was mainly expressed in the membrane in resting cells, ICAM-1 expression was also detected intracellularly in the cytoplasm. These findings suggest that NPM and ICAM-1 may interact in the cytoplasm. Furthermore, the Tris-Triton buffer used for cell lysis did not contain detergents. Since detergents are required to release membrane-bound proteins, membrane-bound ICAM-1 was discarded after centrifugation of the cell lysate. This information suggests that the ICAM-1 pulled down with NPM in the co-immunoprecipitation was likely cytoplasmic ICAM-1.

Our findings are supported by a recent study that identified NPM/ICAM-1 interaction in human lymphoblasts by mass spectrometry [209]. In fact, NPM was pulled down by the cytoplasmic domain of ICAM-1, suggesting that these proteins interact intracellularly [209] — supporting the notion that NPM may be an intracellular ICAM-1 target.

However, NPM interaction with ICAM-1 is not just limited to cytoplasmic compartments. Interestingly, Inder et al recently detected NPM in the inner leaflet of the plasma membrane in baby hamster kidney (BHK) and HEK293T cells [212]. Furthermore, they identified NPM and nucleolin as novel K-Ras regulators that modulate plasma membrane interactions and activate MAPK signaling [212].

In their studies, proteomic analysis was performed to identify proteins that interacted with K-Ras. Ras proteins transmit signals from the surface to inside the cell. Co-
immunoprecipitation experiments confirmed NPM, nucleolin, and K-Ras interaction [212]. Using electron microscopy and immunofluorescence, NPM was shown to be localized to the inner leaflet of the plasma membrane with K-Ras, although plasma membrane NPM expression was reported to be considerably lower in abundance compared to nuclear expression [212].

Based on their findings and biochemical considerations, Inder et al concluded that nucleolin likely recruits K-Ras to the plasma membrane, where NPM is already present [211]. However, it is currently unknown how NPM is recruited to or is present on the plasma membrane. NPM can then specifically interact with K-Ras, possibly in complex with nucleolin, to increase the amount of K-Ras-GDP and K-Ras-GTP in nanoclusters, leading to increased MAPK activation [211].

Interestingly, nucleolin has already been implicated in mediating LPS internalization and its associated inflammation in alveolar macrophages [210]; however, the involvement of NPM in these processes has not yet been studied. One may speculate that NPM is present at the plasma membrane through its interaction with the cytoplasmic domain of ICAM-1. Furthermore, once LPS is bound and internalized with ICAM-1, NPM is activated, which perhaps through Ras-mediated signaling, can activate MAPK signaling, leading to IL-8 production.

The cytoplasmic domain of ICAM-1 lacks known protein-protein interaction motifs and most of its interactions with cytoskeletal components are through adaptor proteins [186, 270]. Thus, it is possible that NPM and ICAM-1 form a complex that includes various unknown adaptor proteins. However, the direct protein-protein binding between NPM and ICAM-1 in a dose-dependent manner implies that they have the ability to directly bind to one another. In fact, NPM is known to contain an acidic intrinsically disordered region that possesses a negative charge [271], which may potentially bind the multiple positively charged amino acid residues in the cytoplasmic domain of ICAM-1 [194]. Indeed, further proteomic and molecular studies must be undertaken to further characterize the interaction between NPM and ICAM-1, as discussed below.
5.3.3.4 Significance of the NPM/LPS Interaction

We demonstrate that NPM can potentially bind to LPS through a plate-based method. Thus, once ICAM-1 is internalized with LPS, it is possible that LPS is detected by NPM intracellularly, leading to NPM activation and the subsequent production of IL-8. Indeed further studies must be performed to further understand the physical and physiological relationship between NPM and intracellular LPS.

If NPM can be released from cells and act as an alarmin, our findings also allow speculation for the ability of circulating LPS to bind to NPM outside of the cell. The implications of their interaction outside of the cell are currently unknown.

The preliminary goal of this thesis was to investigate the potential relationship between NPM and ICAM-1. Previous studies in our lab showed that LPS and ICAM-1 are co-localized in lung epithelial cells; however, it is currently unknown whether LPS and ICAM-1 are separated at any point during the course of LPS stimulation inside of the cell, or whether NPM is a downstream target of intracellular LPS. As such, investigating the NPM interaction with LPS was a secondary objective to shed light on whether NPM and LPS are able to directly bind in cells to further propagate intracellular LPS signaling.

5.3.3.5 NPM Downstream Signaling is Facilitated by c-Fos and c-Jun

LPS stimulation of epithelial cells induces gene and protein expression, particularly the activation of several pro-inflammatory genes leading to the secretion of pro-inflammatory mediators [272]. The NFκB and AP-1 transcriptional networks largely govern pro-inflammatory gene transcription [273]. Twenty-five years ago, NPM was shown to bind nucleic acids [220], and NPM has since been shown to interact with AP-2α and NFκB as a transcriptional co-repressor or co-activator [237, 246]. For instance, NPM was found to localize to the MCP1 promoter as part of the NFκB regulatory complex to downregulate MCP1 transcription [237]. However, stimulation of macrophages with recombinant human NPM activated ERK/JNK signaling and induced the production of TNFα, IL-6, and MCP-1 [239]. NPM was also shown to bind to c-Jun in activated cells, and co-localized with c-Fos during retinoic acid-induced neuroblastoma differentiation [244, 245]; moreover, NPM
expression regulates cellular sensitivity to the natural AP-1 inhibitor, curcumin, in cancer cells, proposing an interconnectivity between NPM and AP-1 [274].

We were interested to study the role of NPM in c-Fos and c-Jun activity in mediating IL-8 production. In the studies contained herein, NPM appeared to partially mediate the total expression of c-Fos and c-Jun in BEAS-2B cells and SAEC; however, other proteins are likely involved (P = 0.06 for the role of NPM in modulating c-Jun expression in SAEC upon LPS stimulation). NPM was found to mediate phosphorylated c-Fos levels in BEAS-2B cells, which influences c-Fos activity. However, this effect may have been due to the underlying reduced c-Fos expression.

Notably, LPS did not induce significant phospho-c-Jun expression in these experiments, as is expected to be seen with LPS stimulation [275]. It is possible that the peak time for c-Jun activity was missed in these studies. Different groups report differing peak times for c-Jun activity, depending on cell-type. Whereas some report c-Jun peak activity between 30 minutes to 1 hour as an immediate-early response protein [276], others report a peak activity at 4 hours [275, 277]. It is possible that c-Jun activity peaks at an earlier or later time point in lung epithelial cells than that studied here [275, 277], thus explaining the lack of significant difference observed in c-Jun activity between non- and LPS-stimulated cells.

Although detection of phosphorylated c-Fos and c-Jun by Western Blot is a viable method to assess activity of these proteins, it does not infer AP-1 activity. The AP-1 transcription factor can be comprised of various proteins in the Fos and Jun families, alongside various other proteins belonging to the ATF and JDP families [162]. Different proteins within these families contain distinctive activating potentials; thus, phosphorylation at one site of a given protein does not necessarily infer that AP-1 target genes are being transcribed [162]. Although c-Fos and c-Jun were chosen for study due to being the most abundant of the AP-1 proteins, along with being the most studied [161], the Western Blot method of study neglects to assess AP-1 activity completely. Complete AP-1 activity is more accurately represented by measuring the transcriptional activity of an AP-1-dependent reporter gene [162], which is a method that can be adopted in future studies.
Since the *IL-8* promoter region contains AP-1 sites [278], NPM may mediate IL-8 production through its regulation of c-Fos and c-Jun expression upon LPS stimulation. These findings are significant as NPM knockdown altered c-Fos and c-Jun total expression (not merely their phosphorylation) within 2 hours of LPS stimulation, inferring its novel function as an immediate-early response protein in the context of LPS stimulation. Results herein indicate for the first time the regulatory role of NPM in mediating c-Fos expression and activity in BEAS-2B cells, contributing to IL-8 production upon LPS stimulation, and also the potential role of NPM in mediating c-Jun expression in SAEC in a manner that is independent of surface-TLR4 signaling by LPS.

5.4 Conclusion

We tested the hypothesis that NPM mediates intracellular LPS signaling by interaction with LPS and/or ICAM-1 in lung epithelial cells. We demonstrate that primary human lung epithelial cells internalize LPS independent of surface TLR4, resulting in NPM-mediated intracellular signaling, which may involve interaction between NPM and ICAM-1 or LPS.

We demonstrated that NPM directly interacts with LPS and ICAM-1 and their interaction was associated with NPM-mediated IL-8 production and cytotoxicity in lung epithelial cells through modulation of c-Fos and c-Jun expression. Although we showed an association between NPM/ICAM-1 interaction and the production of IL-8 upon LPS stimulation, we did not directly address whether the NPM/ICAM-1 interaction contributes to inflammatory responses. Thus, we conclude that NPM may mediate the intracellular LPS-induced IL-8 production through its interaction with ICAM-1 or LPS intracellularly.

Our findings demonstrate a novel role for NPM in the context of LPS-induced inflammation (*Figure 15*). We speculate that the NPM interaction with ICAM-1 and/or LPS facilitates its activation and downstream signaling, which will be tested in future studies, as discussed below.
Our study suggests that adjunctive therapy may be a future direction to manage patients with sepsis, which include the classical LPS neutralization in the circulation, LPS surface receptor blockade, and inhibition of the on-going intracellular LPS signaling.

**Figure 15. Proposed LPS signaling paradigm in the lung epithelium.** Preliminary findings in our lab demonstrate that LPS binds to and is internalized by ICAM-1 on the cell surface of lung epithelial cells, and that LPS and co-localizes with ICAM-1 intracellularly. ICAM-1 interacts with NPM, which mediates phospho-c-Fos expression, and potentially c-Fos activity, leading to cytokine production.
5.5 Future Directions

5.5.1 In Vitro Studies to Further Study the NPM/ICAM-1 and NPM/LPS Interactions

5.5.1.1 Co-Immunoprecipitation Experiments

We showed that NPM and ICAM-1 have the ability to directly interact, and that their interaction is associated with IL-8 production in lung epithelial cells. In our studies, ICAM-1 was co-immunoprecipitated with NPM in total cell lysate, which includes membrane proteins, cytoplasmic proteins, and nuclear proteins.

To further solidify our findings, we can perform reverse co-immunoprecipitation experiments wherein NPM will be co-immunoprecipitated with ICAM-1. If NPM pull-down is observed, it confirms that NPM and ICAM-1 are associated within the cell.

To further characterize the interaction between NPM and ICAM-1, co-immunoprecipitation of ICAM-1 with NPM can be performed in the cytoplasmic fraction and the membrane fraction of cells. The cytoplasmic fraction can be obtained as the previously described in the Methods chapter. The cytoplasmic supernatant can be further fractionated for membrane protein isolation by ultracentrifugation at 100,000 × g for 1 hour at 4 °C. These co-immunoprecipitation studies will provide further detail as to where NPM and ICAM-1 interact within the cell.

5.5.1.2 Domain-Binding Experiments

We observed direct binding between full-length recombinant human NPM and full-length recombinant human ICAM-1. To understand the molecular basis for their interaction, specific domains of the NPM phosphoprotein can be tested for binding with the cytoplasmic domain of ICAM-1 in a plate-based method. Three domains of NPM can be tested for their binding affinity to the cytoplasmic domain of ICAM-1. These regions are 1) the N-terminal
region of NPM (amino acids 1-119); 2) the acidic domain of NPM (amino acids 120-188); and 3) the C-terminal region of NPM (189-294). By understanding how NPM and ICAM-1 interact, potential therapeutic agents can be designed to target those regions specifically.

We also observed direct binding between full-length recombinant human NPM and LPS. To further understand their interaction, specific domains of NPM can be tested for binding with LPS, as previously mentioned for NPM/ICAM-1. Furthermore, Polymyxin B, which binds and inhibits the lipid A portion of LPS, can be used to assess whether NPM interacts with LPS through its lipid A component.

5.5.1.3 Immunofluorescence Experiments

In addition, to binding experiments, immunofluorescence experiments can be performed to identify NPM/ICAM-1 or NPM/LPS co-localization in lung epithelial cells. Although co-localization studies do not definitely mean that two proteins are bound to one another, the observation of NPM/ICAM-1 or NPM/LPS co-localization inside the cell will further confirm our findings from co-immunoprecipitation and direct protein-protein binding studies.

5.5.2 In Vitro Studies to Further Study the Role of the NPM/ICAM-1 Interaction in the Inflammatory Response to LPS

A limitation of our current studies herein is whether involvement of the NPM/ICAM-1 interaction contributes to inflammatory responses. We merely show an association between NPM/ICAM-1 interaction and IL-8 production. To conclusively support the hypothesis that the NPM/ICAM-1 interaction is involved in the intracellular LPS signaling, leading to IL-8 production, an in vitro model using both ICAM-1 and NPM siRNAs can provide insight into whether they work synergistically to propagate signaling.

Of consideration, however, is that previous unpublished work in our lab has demonstrated that ICAM-1 siRNA transfection severely inhibits LPS internalization in lung epithelial cells. Therefore, ICAM-1 siRNA models will not induce LPS internalization. In this case, LPS can be electroporated into the cells after ICAM-1 and NPM siRNA transfection (and respective
scrambled siRNA control) to achieve the presence of intracellular LPS. ICAM-1 and NPM involvement in inflammatory processes can then be assessed. IL-8 measurement by ELISA is an endpoint that can be used.

5.5.3 Confirmation of Intracellular TLR4 Expression in Lung Epithelial Cells

To confirm our immunofluorescence data that displayed intracellular — not surface — localization of TLR4 in lung epithelial cells, flow cytometry can be used. In these studies, lung epithelial cells that are permeabilized with Triton X-100, or not, can be stained with a PE-TLR4 antibody (and the corresponding IgG control). We expect to not see a shift in the peak for TLR4 staining compared to the IgG control for cells that are not permeabilized, describing the surface expression of TLR4 in lung epithelial cells. However, a shift is expected to be seen for TLR4 staining in permeabilized cells, depicting the presence of intracellular TLR4 in lung epithelial cells.

5.5.4 Studies to Further Study the Role of TLR4 in NPM-Mediated IL-8 Production

5.5.4.1 In Vitro Studies

Our studies in human lung epithelial cells revealed NPM association with ICAM-1 and mediating inflammation in the presence of LPS, in a manner independent of surface TLR4 expression. However, TLR4 is still expressed intracellularly in these cells, and its involvement in propagating NPM-mediated signaling is unknown.

Unpublished work from our lab has demonstrated the intracellular co-localization and co-immunoprecipitation of TLR4 with ICAM-1. To establish whether NPM also interacts with TLR4 intracellularly in the presence of LPS, the following experiments can be performed: 1) co-localization of NPM/TLR4 through immunofluorescence; 2) co-immunoprecipitation of TLR4 with NPM in total cell lysate or the cytoplasmic fraction; 3) direct protein-protein binding experiments in a plate-based system. These studies will first establish whether
NPM and TLR4 interact within the cell, which will allow us to further speculate and formulate hypotheses regarding the role of TLR4 in NPM-mediated signaling.

Once more is known about the interaction between NPM and TLR4, we can study the role of TLR4 in NPM-mediated inflammation in the presence of LPS. TLR4 siRNA can be transfected into lung epithelial cells and NPM/ICAM-1 interaction can first be assessed to see if they are maintained. Further double knockdown experiments using both TLR4 siRNA and NPM siRNA will allow us to conclude whether these two proteins work synergistically to mediate the release of IL-8 upon LPS stimulation. Another method is to generate a stable TLR4 knockdown cell line model, and use NPM siRNA in conjunction for the double knockdown model.

5.5.4.2  In Vivo Studies

To reproduce our in vitro findings in a translational model, C57BL/10ScNJ (TLR4-null) and C57BL/10ScSnJ (control background) mice can be concurrently challenged with LPS (intratracheal instillation), and the specific NPM inhibitor, NSC348884 (intraperitoneal injection). NSC348884 is a commercially available product that directly inhibits NPM multimerization, thus inactivating NPM [279]. After 4-24 hours of LPS challenge, mouse plasma and right lung tissue samples can be collected and analyzed for cytokine measurement. Left lung tissue can be inflated with 4% formalin and processed for histological staining to assess lung injury. Another option is to collect bronchiolavage fluid from the mice and measure cytokine concentrations or albumin concentration as a measure of lung permeability. If the use of the NPM inhibitor attenuates inflammation and lung injury, it can begin to be considered as a potential therapeutic drug for sepsis patients in the years to come. Furthermore, regarding TLR4 involvement in NPM-mediated signaling, C57BL/10ScSnJ mice will only express TLR4 intracellularly in lung epithelial cells, whereas C57BL/10ScNJ mice will not express TLR4 at all. This discrepancy can help to further confirm whether NPM requires intracellular TLR4 for its signaling.
5.5.5 NPM and LPS Internalization

Since ICAM-1-mediated LPS internalization in lung epithelial cells has been established in our lab, it would be interesting to further study whether NPM mediates LPS internalization in lung epithelial cells through its interaction with ICAM-1. Since the NPM family member, nucleolin, was recently shown to mediate LPS internalization [210], and since NPM was recently detected on the inner leaflet of the plasma membrane [212], it is possible that NPM mediates LPS internalization as well. To study this question, human lung epithelial cells can be transfected with NPM or scrambled siRNA prior to LPS stimulation, and LPS internalization can be assessed through confocal microscopy.

In further in vivo studies, C57BL/6 mice can be administered the NPM specific inhibitor (intraperitoneal injection) and purified LPS (20 mg/kg, intratracheal instillation) concurrently and LPS internalization can be assessed in lung tissue harvested 4- or 24-hours later. In this case, the mouse lung would be fixed with 4% formalin and LPS would be visualized by immunofluorescence. However, heat induced epitope retrieval in citrate buffer (pH 6.0) must be performed to break protein crosslinks that are formed by formalin fixation.

5.5.6 In Vivo Experiments Assessing NPM-Mediated Inflammation in Other Organs

Indeed, the role of NPM in intracellular LPS signaling can be assessed in other organs besides the lung in future studies. By understanding the effects of intracellular LPS and its signaling through NPM, more will be known about how NPM signals, and whether it mediates inflammation in a similar or different fashion in various organs. Long-term plans can involve animal models wherein combined therapies are tested. For instance, neutralization of LPS in the circulation, TLR4 blockage at the cell surface, and intracellular NPM inhibition may together be used to reduce inflammation and organ injury, and perhaps be a novel treatment option for sepsis patients in the future.
References


Copyright Acknowledgements

None.