Use of Tat-Hsp70 Fusion Protein to Attenuate Sepsis-Induced Lung Injury in Rodents With Acute Respiratory Distress Syndrome Caused by Cecal Ligation and Double Puncture (2clp)

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Use of Tat-Hsp70 Fusion Protein to Attenuate Sepsis-Induced Lung Injury in Rodents With Acute Respiratory Distress Syndrome Caused by Cecal Ligation and Double Puncture (2clp)

Abstract
Background: Sepsis, a fatal syndrome of dysregulated inflammation, is the leading cause of death in the critically ill. The lung is the organ most often injured, developing into Acute Respiratory Distress Syndrome (ARDS). However, both sepsis and ARDS predispose patients to immobility and debilitating neuromuscular weakness. Interventions that limit the extent of lung injury would provide both direct and indirect benefit. The cecal ligation and double puncture (2CLP) rodent model of sepsis mimics the ARDS and immobility features of the human syndrome. An endogenous mediator that protects cells from injury is Heat Shock Protein 70 (HSP70). 2CLP eliminates HSP70 abundance in the lung and is associated with ARDS. We hypothesized that delivering an HIV-1 Tat-HSP70 fusion protein to enhance HSP70 abundance in lung cells would protect against lung injury and as an indirect benefit improve locomotion. Methods: Tat-HSP70 was injected into the trachea of Sprague-Dawley rats made septic by 2CLP (2CLP-Tat-HSP70). Controls included unoperated, sham operated, and 2CLP-phosphate buffered saline (PBS) treated rats. Immunoassays were used to examine the abundance of HSP70, Myeloperoxidase (MPO), Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1), Macrophage Inflammatory Protein-2 (MIP-2) and Interleukin-6 (IL-6) in lung tissue. Rodent locomotor assessment was measured with telemetry. We used descriptive analysis to describe histologic lung findings. Results: (1) Relative to 2CLP-PBS rats, HSP70 treatment significantly increased HSP70 protein abundance in 2CLP-Tat-HSP70 rats and in histologically normal and abnormal lung regions, at 24 and 48 hr, (2) Relative to 2CLP-PBS rats, we observed decreased histologic lung injury in 2CLP-Tat-HSP70 rats at 24 and 48 hr, (3) Relative to 2CLP-PBS rats at similar time-points, abundance of MIP-2 at 24 hr and MPO at 48 hr were significantly decreased in 2CLP-Tat-HSP70 rats, (4) We were unable to detect a difference in abundance of CINC-1, IL-6 and in locomotion in 2CLP-Tat-HSP70 rats, (5) Relative to 2CLP-PBS rats, Tat-HSP70 improved survival following 2CLP at 48 hr. Conclusion: Intratracheal Tat-HSP70 increased lung HSP70 abundance, reduced lung injury as indicated by lung histology, MIP-2 and MPO abundance. Future studies designed to optimize the use of Tat-HSP70 as a treatment for lung injury secondary to sepsis will be explored.

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Mary Melanie Lyons

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Dedication

For God and the journey, and for my father George Lyons.
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ABSTRACT

USE OF TAT-HSP70 FUSION PROTEIN TO ATTENUATE SEPSIS-INDUCED LUNG INJURY IN RODENTS WITH ACUTE RESPIRATORY DISTRESS SYNDROME CAUSED BY CECAL LIGATION AND DOUBLE PUNCTURE (2CLP)

Mary Melanie Lyons

Dr. Nancy Tkacs

Dr. Clifford S. Deutschman

Background: Sepsis, a fatal syndrome of dysregulated inflammation, is the leading cause of death in the critically ill. The lung is the organ most often injured, developing into Acute Respiratory Distress Syndrome (ARDS). However, both sepsis and ARDS predispose patients to immobility and debilitating neuromuscular weakness. Interventions that limit the extent of lung injury would provide both direct and indirect benefit. The cecal ligation and double puncture (2CLP) rodent model of sepsis mimics the ARDS and immobility features of the human syndrome. An endogenous mediator that protects cells from injury is Heat Shock Protein 70 (HSP70). 2CLP eliminates HSP70 abundance in the lung and is associated with ARDS. We hypothesized that delivering an HIV-1 Tat-HSP70 fusion protein to enhance HSP70 abundance in lung cells would protect against lung injury and as an indirect benefit improve locomotion. Methods: Tat-HSP70 was injected into the trachea of Sprague-Dawley rats made septic by 2CLP (2CLP-Tat-HSP70). Controls included unoperated, sham operated, and 2CLP-phosphate buffered saline (PBS) treated rats. Immunoassays were used to examine the abundance of HSP70,
Myeloperoxidase (MPO), Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1), Macrophage Inflammatory Protein-2 (MIP-2) and Interleukin-6 (IL-6) in lung tissue. Rodent locomotor assessment was measured with telemetry. We used descriptive analysis to describe histologic lung findings. Results: (1) Relative to 2CLP-PBS rats, HSP70 treatment significantly increased HSP70 protein abundance in 2CLP-Tat-HSP70 rats and in histologically normal and abnormal lung regions, at 24 and 48 hr, (2) Relative to 2CLP-PBS rats, we observed decreased histologic lung injury in 2CLP-Tat-HSP70 rats at 24 and 48 hr, (3) Relative to 2CLP-PBS rats at similar time-points, abundance of MIP-2 at 24 hr and MPO at 48 hr were significantly decreased in 2CLP-Tat-HSP70 rats, (4) We were unable to detect a difference in abundance of CINC-1, IL-6 and in locomotion in 2CLP-Tat-HSP70 rats, (5) Relative to 2CLP-PBS rats, Tat-HSP70 improved survival following 2CLP at 48 hr. Conclusion: Intratracheal Tat-HSP70 increased lung HSP70 abundance, reduced lung injury as indicated by lung histology, MIP-2 and MPO abundance. Future studies designed to optimize the use of Tat-HSP70 as a treatment for lung injury secondary to sepsis will be explored.
TABLE OF CONTENTS

Dedication ................................................................................................. iii
Acknowledgements .................................................................................. iv
Abstract ................................................................................................ vi
Table of Contents ................................................................................... viii
List of Figures ......................................................................................... xi
List of Illustration ................................................................................... xii
CHAPTER 1: INTRODUCTION ................................................................. 1
Goals of Dissertation, Specific Aims and Hypothesis ............................. 3
Background and Significance................................................................. 6
What is sepsis? ..................................................................................... 6
What is Acute Respiratory Distress Syndrome (ARDS)? ..................... 9
The Use of Cecal Ligation and Double Puncture (2CLP) .................... 12
Heat Shock Proteins (HSPs) ............................................................... 15
Heat Shock Protein 70 (HSP70) .......................................................... 16
HSP70, Sepsis and Lung Injury ............................................................. 18
Background and Rationale for Study Outcome Measures: ................ 22
1. Assessing Lung Injury in the 2CLP model-Histologic Lung Changes in ARDS-Hematoxylin/Eosin Stains
2. Detection of Neutrophil-Derived Myeloperoxidase (MPO) ............ 26
3. Detection of Cytokine Induced Neutrophil Chemoattractant .......... 28
   Protein-1 (CINC-1), Macrophage Inflammatory Protein-2 (MIP-2) and Interleukin-6 (IL-6)
4. Neuromuscular and Functional Consequences of Critical Illness...... 32
Study Significance and Summary ......................................................... 35
CHAPTER 2: TAT-HSP70 ATTENUATES LUNG INJURY IN A 2CLP .......... 38
MODEL OF ARDS
Abstract .................................................................................................. 39
The Use of Tat-HSP70 fusion protein in attenuating 2CLP Lung Injury

Conclusion

CHAPTER 5: SUMMARY AND CONCLUSION

Introduction

Summary of Findings

Limitation and Observations

Gaps This Study Has Filled

Recommendations of Future Research

APPENDIX A: Table 1. HSP70, MPO abundance and H&E histology data

APPENDIX B: Table 2. Hematoxylin and Eosin stain-itemized comparison between all groups

APPENDIX C: Table 3. Animal survival data-Chapter 2

APPENDIX D: Table 4. CINC-1, MIP-2, IL-6, Locomotion data

APPENDIX E: Table 5. Animal survival data- Chapter 3

REFERENCES
LIST OF FIGURES

Figure 1a: Immunohistochemical stain of Tat-HSP70 abundance in lungs following 2CLP .......... 49
Figure 1b: Graphic representation, Tat-HSP70 increases HSP70 protein abundance in lungs following 2CLP .......... 50
Figure 2: Graphic representation, Tat-HSP70 increases HSP70 protein abundance in abnormal and normal lung sections following 2CLP .......... 52
Figure 3: Histologic representation, Tat-HSP70 attenuates lung injury following 2CLP .......... 54
Figure 4a: Immunohistochemical stain depicting decreased MPO abundance in Tat-HSP70 administered lungs at 48 hr following 2CLP .......... 56
Figure 4b: Graphic representation, Tat-HSP70 decreased MPO abundance in lungs at 48 hr following 2CLP .......... 57
Figure 5: Graphic representation, Tat-HSP70 did not reduce survival (Chapter 2 study) .......... 59
Figure 6: Graphic representation, Tat-HSP70 did not reduce 2CLP mediated abundance of CINC-1 in the lung .......... 79
Figure 7: Graphic representation, Tat-HSP70 reduced abundance of MIP-2 in the lung at 24 hr following 2CLP .......... 81
Figure 8: Graphic representation, Tat-HSP70 increased abundance of IL-6 in the lung following 2CLP .......... 83
Figure 9: Graphic representation, Tat-HSP70 did not have an effect on rat locomotion following 2CLP .......... 85
Figure 10: Graphic representation, Tat-HSP70 did not increase core rat temperature following 2CLP .......... 87
Figure 11: Graphic representation, Tat-HSP70 increased survival at 48 hr following 2CLP (Chapter 3 study) .......... 89
LIST OF ILLUSTRATION

Illustration 1: Roadmap-Dissertation overview............................................................. 37
CHAPTER 1

INTRODUCTION

*That which drugs fail to cure, the scalpel can cure. That which the scalpel fails to cure, heat can cure. That which heat cannot cure, must be determined to be incurable.*

—Hippocrates.


Sepsis, a fatal syndrome of dysregulated inflammation, is a leading cause of death of critically ill patients in the United States (Xu, Kochanek, Murphy, & Tejada-Vera, 2010). There is no cure. Treatment is purely supportive. Left unchecked, sepsis quickly leads to multiple organ-dysfunction syndrome (MODS) and death. The lungs are the first and most common organ system affected in MODS. The most severe sepsis-induced pulmonary abnormality is Acute Respiratory Distress Syndrome (ARDS). The mortality of patients from sepsis-induced ARDS has declined in the past decade yet still exceeds 20% (Spragg et al., 2010). The pathological course of sepsis and ARDS contributes to immobility, muscular deconditioning, and critical illness polyneuropathy that develop almost universally in these patients. As treatment options for sepsis and for ARDS are limited, there is a compelling need for innovative therapy and novel approaches to management. Interventions that limit the extent of lung injury in sepsis would provide both primary and secondary benefit.

One highly conserved endogenous mechanism that protects cells from injury is the Heat Shock Response (HSR) expressing Heat Shock Proteins (HSPs). HSPs are a ubiquitous family of phylogenetically-conserved proteins that confer cytoprotection against a variety of stressful and noxious cellular circumstances. One group in the family
of HSPs have molecular weights around 70 kDa and are referred to collectively as HSP70. Studies in patients of ARDS and severe sepsis found HSP70 abundance in peripheral monocytes to be diminished. This finding suggests that the normal HSR, including that in the lungs, may be decreased (Delogu et al., 1997; Durand et al., 2000; Schroeder et al., 1999; D. S. Wheeler et al., 2005).

*In vivo* studies to evaluate HSP70 abundance in the lungs of patients with sepsis-induced ARDS have been unfeasible. However, the cecal ligation and double puncture (2CLP) rodent model of sepsis closely mimics many of the disease features of the human syndrome including ARDS, and decreased mobility. Prior studies have shown that expression of HSP70 in the lungs of the 2CLP sepsis rodent model is severely diminished (Villar et al., 1993; Weiss et al., 2000). Studies conducted from our laboratory have shown that intratracheal delivery of an adenoviral vector to increase lung HSP70 expression (AdHSP70) attenuated lung injury and reduced mortality at 48 hr in the 2CLP model (Weiss, Maloyan, Tazelaar, Raj, & Deutschman, 2002). However, the use of adenoviral vectors in humans may cause complications, including life-threatening host-immune responses such as excessive inflammation in transduced cells, dysregulated triggering of nuclear factor kappa B (NF-κB) signal transcription of inflammatory pathways, the development of excessive cytolytic responses, and death (Muruve, 2004). Additionally, development of viral traps and sequestration of adenovirus vectors in the liver may limit the effectiveness of the virus (Anderson, 1998; Khare, Chen, Weaver & Barry, 2011). Thus, alternate strategies to increase HSP70 abundance in pulmonary
epithelial cells of the sepsis-induced ARDS model represent an underexplored therapeutic avenue.

The HIV-1 Transactivator of Transcription (Tat) protein-transduction domain (PTD) has been successfully used to transduce proteins of various sizes into human and rodent cells. However, only our laboratory and that of our collaborator, Laurie Kilpatrick, Ph.D., have successfully used the Tat protein to deliver therapeutic proteins into the lungs in the setting of experimental 2CLP sepsis-induced ARDS. This dissertation extends previous work in the Deutschman lab showing improved outcomes in 2CLP rats treated with AdHSP70. The present body of work demonstrates that intratracheal delivery of exogenous HSP70 protein using the Tat fusion mechanism (Tat-HSP70) successfully increases HSP70 abundance in lung tissue. Additionally, we studied the efficacy of Tat-HSP70 in attenuating lung injury measured by histologic and inflammatory markers, increasing survival at 48 hr, and as a secondary outcome, increasing locomotor activity in a 2CLP animal model of ARDS.

Goals of Dissertation, Specific Aims and Hypothesis

The ability of nursing science to promote health begins with a deep understanding of the pathophysiology of disease. With this background, it will be possible to develop and test conceptual models and interventions to improve recovery and promote quality of life (Donaldson & Crowley, 1978).

This dissertation details an innovative preclinical study, testing a novel therapeutic approach to increase HSP70 protein abundance in pulmonary epithelial cells of a 2CLP sepsis-induced ARDS rodent model. The overall hypothesis guiding this work
is that direct delivery of exogenous Tat-HSP70 fusion protein via the trachea into the lung of a 2CLP sepsis-induced ARDS rodent model, an in-vivo methodology that has not been demonstrated before in this model, will successfully increase HSP70 protein abundance in the lung and improve pathological and clinical outcomes at 24 and 48 hr. The specific hypotheses will be tested with the following two specific aims.

Specific Aims

1. To examine the effects of Tat-HSP70 fusion protein delivered into the trachea of a 2CLP rodent model on lung injury.
   
   **Hypothesis A**: Intratracheal delivered Tat-HSP70 increases HSP70 protein abundance in 2CLP sepsis induced lung injury.

   **Hypothesis B**: Intratracheal Tat-HSP70 attenuates 2CLP sepsis-induced lung injury and decreases abundance of Myeloperoxidase (MPO), Cytokine Induced Neutrophil Chemoattractant-1 (CINC-1), Macrophage Inflammatory Protein-2 (MIP-2) and Interleukin-6 (IL-6) in the lung.

   **Hypothesis C**: Intratracheal Tat-HSP70 will confer protection from lung injury for a limited time after 2CLP.

2. To examine the effect of Tat-HSP70 on 2CLP induced changes in locomotion.

   **Hypothesis**: Intratracheal Tat-HSP70 improves locomotion following 2CLP.

This introductory chapter provides a systematic review discussing the relationship between sepsis, ARDS, and the 2CLP rodent model of sepsis and ARDS. Then, an overview is given of HSPs and the evidence for the role of HSP70 in improving sepsis
outcomes. The section concludes with a description and rationale for the study-outcome measures including histologic indicators of ARDS in the lungs; measures of exaggerated neutrophil activation, key chemokine and cytokine inflammatory markers, MPO, CINC-1, MIP-2, and IL-6, and the assessment of neuromuscular weakness/critical illness polyneuropathy by locomotor activity. The abundance of dysregulated inflammatory cytokines and chemokines vary by time-points during sepsis pathology; therefore, evaluation of these markers were conducted at two pre-selected time-points, 24- and 48-hrs (Kellum et al., 2007; Remick, Bolgos, Copeland, & Siddiqui, 2005; Remick, Bolgos, Siddiqui, Shin, & Nemzek, 2002).

Chapters 2 through 4 are comprised of three journal articles, in preparation, that form the core of the dissertation research. The first article describes the significant increase in HSP70 protein abundance in lung epithelial cells following intratracheal delivery of exogenous Tat-HSP70, at different time points, in lung tissue of the 2CLP model. Importantly, considering that ARDS is a heterogeneous lung disease, we distinguished that HSP70 abundance was significantly present in normal and abnormal (or sick areas) of the lung. Article 1 then describes successful attenuation of lung injury via histologic examination, and the significant reduction of MPO abundance in the lungs of Tat-HSP70-treated animals compared to the phosphate buffered saline (PBS)-treated group specifically at the 48-hr time point. The second article describes the effect of Tat-HSP70 treatment in the 2CLP model on key inflammatory markers, CINC-1, MIP-2 and IL-6. This article also describes the effect of Tat-HSP70 treatment on a behavioral outcome of critical illness syndromes such as sepsis and ARDS, effects on spontaneous locomotor activity. The last article describes the novelty and use of Tat as a vehicle to
deliver a protein and peptide therapeutic, Tat-HSP70 and Tat-Protein Kinase C-delta inhibitor (Tat-PKC-δ inhibitor), using two different methods of coupling, into lung epithelial cells in a 2CLP model. Last, in Chapter 5, findings are summarized and inferences are drawn from this body of research. We discuss relevant rationales, pitfalls, limitations, and alternative approaches, and explore additional questions from this study. We conclude by addressing future directions in research on the use of the Tat-HSP70 fusion protein in the 2CLP model of ARDS.

**Background and Significance**

**What is sepsis?**

Sepsis, a leading cause of death in critically ill patients, is a complicated and poorly understood syndrome of disordered inflammation (Levy, Fink, Marshall, Abraham, Angus, et al., 2003; Xu, et al., 2010). In a 1992 consensus conference, the clinical syndrome was defined by the presence of abnormalities in two of the following: temperature, heart rate, respiratory rate, and white blood count in the presence of a known or suspected infection (Bone, Balk, Cerra, Dellinger, Fein, Knaus et al., 1992). This was modified in 2003 by extending the list of clinical signs and symptoms to include general variables, inflammatory variables, hemodynamic variables, organ-dysfunction variables, and tissue-perfusion variables (Levy et al., 2003). Importantly, sepsis can rapidly progress to severe sepsis (sepsis with acute organ failure in the presence of infection or suspicion of it), septic shock (sepsis with organ failure and hypotension unresponsive to fluid resuscitation), multisystem organ failure, and death if treatment is not initiated in a timely manner (Dellinger et al., 2013). Promptness in recognizing
symptoms and systematic implementation of supportive treatment is of grave importance. Of patients with lung infections, 47% will develop sepsis (G.S. Martin et al., 2003).

In 2013, an estimated 900,000 people will be affected by sepsis syndromes annually, increasing at a projected prevalence of 13% per year (Gaieski, Edwards, Kallan, & Carr, 2013). The rate of hospitalizations for patients with a principal diagnosis of sepsis doubled in the past decade, from 11.6 to 24 per 10,000 population, whereas the rate for patients hospitalized with sepsis as a principal or secondary diagnosis (including patients who developed sepsis while in the hospital) increased by 70% (Hall, Williams, DeFrances, & Golosinskiy, 2011). In the United States, the healthcare system is billed approximately $24 billion annually for the treatment of severe sepsis and the adjusted costs for sepsis are projected to increase by 11.9% (Hall et al., 2011; Lagu et al., 2012).

Sepsis is the first (Medicare), third (Medicaid), and eighth (private insurance) most expensive diagnosis in hospital treatment and management (Wier, & Andrews, 2011). Early recognition of the sepsis syndromes by clinicians, and advanced supportive management strategies have helped reduce mortality for sepsis to approximately 25% in the past decade, but this rate remains variable by region (H. E. Wang, Devereaux, Yealy, Safford, & Howard, 2010). Mortality of patients with severe sepsis and septic shock have also decreased from approximately 20 to 50% in the past decade to 14 to 29% (Gaieski et al., 2013).

Vulnerable populations, such as the elderly and the immunocompromised, have greater prevalence, morbidity, and mortality due to sepsis (G. S. Martin, Mannino, Eaton, & Moss, 2003). This presents a public health hazard, with the proportion of the aging population greater than or equal to 65 years expected to double from 35 million to 71
million people between 2010 and 2030 (Centers for Disease Control and Prevention, 2003). Additionally, the number of people living to an age of 80 years or more is projected to increase by 10.2 million during that time. It is estimated that 74% of patients of age 65 years or more who survived severe sepsis for 3 or more years developed functional disability with moderate to severe cognitive disorder (Iwashyna, Cooke, Wunsch, & Kahn, 2012).

The pathophysiology of sepsis is poorly understood. It is not dependent on a singular inciting event or agent, as similar host responses occur when sepsis is induced by infectious agents (gram negative, gram positive or anaerobic bacteria, viruses, fungi) or noninfectious causes such as ischemia-reperfusion injury or hemorrhage (Baumann & Gauldie, 1994; Bone et al., 1992; A. Kumar et al., 2006; Sands et al., 1997). The hallmark of septic pathophysiology lies in dysregulated inflammation, and loss of systemic vasoregulation. Septic pathophysiology may begin with the recognition of danger to the host by the innate immune system that uses either danger - or pathogen-recognition receptors (DRRs, PRRs) sensing the presence of danger associated and/or pathogen-associated molecular patterns (DAMPS, PAMPS). Recognition very quickly activates vascular, solid organ, blood and lymphatic cells to release mediators, including cytokines, complement and coagulation factors. Release of some of these mediators appear to be impaired while others are over-enhanced, some of the mediators themselves are inactive while others are over-active, yielding a dysregulated immune response and haphazard signaling pathways between most organs (Pinsky, 2012; Russell, 2006; Skrupky, Kerby, & Hotchkiss, 2011). For example, there may be equal elevations in the abundance of the anti-inflammatory cytokine interleukin-10 and the pro-inflammatory
cytokine TNF-α in patients dying of sepsis (van Dissel, van Langevelde, Westendorp, Kwappenberg, & Frolich, 1998). At the same time that dysregulation of the immune system occurs there may also be alterations in neural and endocrine integrative systems. Left unchecked, the net result is tissue damage, increased vascular permeability, loss of vasoregulation, and end organ injury that may result in death (Skrupky et al., 2011; Russell, 2006).

**What is Acute Respiratory Distress Syndrome?**

The organ that is most affected by sepsis is the lung. Dysfunction takes the form of lung injury, developing rapidly to the most severe and heterogeneous form, ARDS (Ware & Matthay, 2000). In 2005, 190,000 patients were diagnosed with ARDS leading to 3.6 million hospital days and increasing the age-adjusted incidence to 86 per 10^5 persons (Rubenfeld, 2005). Lung injury can be caused by direct (pneumonia, chest trauma, or inhalation injury) or indirect causes (sepsis, severe systemic trauma, hemorrhage, or cardiopulmonary bypass) and infectious (pneumonia or sepsis) or noninfectious injuries (aspiration or hyperoxia) (G. S. Martin et al., 2009). The use of a low tidal-volume ventilatory strategy is the only proven treatment to decrease mortality and hospital length of stay (ARDSNET, 2000). Mortality rates of patients with ARDS have declined in the past decade to 22%, due to protective ventilation strategies (Spragg et al., 2010). ARDS pathophysiology reflects the effects of dysregulated inflammation contributing to destruction of lung tissue. The predisposition of the lungs to infection suggests that some part of the immune system is not working properly.
ARDS was first described by Ashbaugh and colleagues in 1967 (Ashbaugh, Bigelow, Petty, & Levine, 1967). In 1994, the American–European Consensus Conference redefined acute lung injury (ALI) and ARDS (Bernard et al., 1994). Physiologic differentiation included stratification by the ratio of arterial oxygen to fraction of inspired oxygen (PaO2 to FiO2) and by radiologic and clinical changes, to better recognize and standardize treatment. In 2012, new definitions of ARDS were developed, the Berlin definition of ARDS, and the term ALI was eliminated (ARDS Definition Task Force, 2012). The severity of ARDS has been reclassified to three groups, distinguished by PaO2/FiO2 ratios: (a) mild (incorporating definitions from the previously termed ALI- 200 to ≤ 300 mmHg), (b) moderate (100 to ≤ 200 mmHg), or (c) severe (≤ 100 mmHg). The clinical criteria for ARDS were redefined and key morphologic characteristics were identified. Histologic and morphologic characteristics of ARDS include diffuse alveolar damage attributed to interstitial edema from increased proteinaceous exudate, the development of hyaline membranes, inflammation (from increased neutrophil activation, alveolar macrophage activation, and secretion of dysregulated inflammatory mediators), or hemorrhage. These events led to diffuse disruption of alveolar epithelial Type I and Type II cells, ventilation/perfusion mismatching, reduced gas exchange, and lung dysfunction (Ashbaugh, et al., 1967; Petty & Ashbaugh, 1971).

It has been proposed that the development of alveolar epithelial injury results primarily from the accumulation and dysregulated activation of neutrophils and macrophages (Abraham, 2003; Grommes & Soehnlein, 2011). Injuries to pulmonary epithelial Type I and Type II cells are particularly destructive to adequate pulmonary
function (Bachofen & Weibel, 1977, 1982). Type 1 simple squamous epithelial cells are flat cells that facilitate gas exchange by making up 90% of the alveolar surface and presenting a minimal barrier to gas diffusion. However, Type 1 cells are easily injured. Protein released as Type 1 cells die, producing the characteristic hyaline membrane seen on biopsy and postmortem samples.

Type II epithelial cells are cuboidal, metabolically active, produce and synthesize surfactant, facilitate ion transport, influence activation of the immune system, and, most importantly, proliferate, spreading over denuded basement membrane and differentiating to replace destroyed Type 1 alveolar cells (Bachofen & Weibel, 1977, 1982). However, development of hyaline membranes disrupts contact between basement membranes and Type II cells. The result is excessive proliferation of Type II cells, which then fail to differentiate. The overgrowth of Type II cells initiated during the acute phase leads to lung fibrosis in the subsequent 2 weeks, consequently increasing dead space and decreasing lung compliance. Timely treatment can prevent this progression and prevent development of the advanced fibroproliferative stages. Importantly, lung injury leading to ARDS is the only component of MODS that can be directly attributed to excessive dysregulated inflammation and neutrophil-mediated injury. Interventions that limit neutrophil activation and accumulation, prevent the loss of Type I cells, and attenuate the overproliferation of Type II cells are likely to be extremely useful in the treatment of sepsis-associated and sepsis-independent ARDS (Weiss et al., 2007; Weiss, Raj, Goloubinoff, & Deutschman, 2008).

For those who survive, ARDS is associated with significant postmorbid dysfunction (Herridge et al., 2011). Paramount among these are residual abnormalities of
basic lung functions such as ventilation and gas exchange. However, the prolonged time course of sepsis and ARDS also lead to systemic abnormalities beyond those that result from damage to the lungs. Of particular importance are deficits in cognitive function and neuromuscular dysfunction such as intensive-care-unit (ICU)-acquired weakness and critical-illness polynuropathy that is protracted and can result in severe postsyndrome debilitation 5 years later (Garnacho-Montero, Amaya-Villar, García-Garmendia, Madrazo-Osuna, & Ortiz-Leyba, 2005; Herridge et al., 2011). Early recovery of lung function and early mobility of patients with ARDS improved functional ability considerably at a 1-year follow up (Herridge et al., 2003; Morris et al., 2008).

To summarize, sepsis is a condition of dysregulated inflammation with a dysregulated immune-system response with high morbidity and mortality. Many cases of sepsis are accompanied by ARDS, a lung disorder with equivalent morbidity and mortality that has severe long-term sequelae. Treatment for sepsis and for sepsis-induced ARDS are few and are mainly supportive. The past several decades of sepsis and ARDS research have produced frustratingly few evidence-based effective therapies. The current body of work builds on research underlying pathophysiology at the cellular and molecular level of this syndrome. We assessed the efficacy of a novel in vivo cellular treatment for ARDS in a rodent sepsis model.

**The Use of Cecal Ligation and Double Puncture (2CLP) in Rodents as a Model of Sepsis-Induced Acute Respiratory Distress Syndrome (ARDS)**

2CLP in rodents has been a well-established rodent model of severe sepsis and ARDS (Rittirsch, Huber-Lang, Flierl, & Ward, 2009; Wichterman, Baue, & Chaudry,
1980). It has been in use for over 30 years and is recognized as the most widely used model of the complex sepsis syndrome. 2CLP is a surgical procedure performed under anesthesia. Briefly, following full surgical preparation, the rodent abdomen is opened, the cecum is exposed, the distal stalk of the cecum is ligated with suture to promote necrosis, the ligated portion of the cecum is punctured twice with an 18 gauge needle, stool is extruded, the cecum is placed back in the abdomen and the incision is closed with suture. 2CLP results in septic peritonitis with gram negative and polymicrobial infection. The lungs are rapidly subjected to the surge of inflammation.

The 2CLP animal model of sepsis has parallels to human sepsis, exhibiting similar physical and histologic changes, including the development of ARDS. Within 24 hr, there is diffuse alveolar neutrophilic damage, intra-alveolar proteinacious exudate, alveolar collapse, hyaline-membrane formation, and the formation of microthrombi (Matute-Bello et al., 2011; Matute-Bello et al., 2008). Prior studies have established that survival at 3, 6, and 16 hrs following 2CLP is 100%. Survival at 24 hr is 50%. Survival at 48 hr is 25%. Previous studies using Sprague-Dawley male rats demonstrated that they reliably develop ARDS after 2CLP (Weiss et al., 2000, 2002, 2001). Rats were chosen over mice because their organs are large enough for our proposed experiments, in addition, 2CLP results in a significant, early, and progressive decrease in locomotor activity, a behavioral manifestation of infectious illness that can be attributed to critical illness polyneuropathy and or sickness behavior (Cankayali et al., 2007; Dantzer & Kelley, 2007; Nayci et al., 2005; Petronilho, et al., 2012; Remick et al., 2005; Tuon et al., 2008; Wichterman et al., 1980).
Alternate immune-stimulation models such as endotoxemia (induced by injection of bacterial lipopolysaccharide (LPS)), live-bacteria models, or cell culture models lack either the infectious focus, kinetics, or magnitude in producing mediators during the immuno-inflammatory reaction of human sepsis. Indeed, studies show that 2CLP reproduces cytokine profiling that is closer to a human response compared to an LPS challenge (Remick, Newcomb, Bolgos, & Call, 2000; Reutershan & Ley, 2004). Choice of animal model for such studies allows the investigator to vary rodent species, age of the animal, sex, lack of comorbid disease conditions in animals, and impact of anesthetic on the immune system. Further variation is possible in methods of drug delivery and drug absorption, such as drug delivery by intraperitoneal, intravenous, intratracheal, pellet implantation, or alternate forms. Finally, investigators can focus on particular types of specimens obtained from animals to extrapolate assays and data, including tissue homogenate, bronchoalveolar (BAL) lavage, plasma, serum, fresh-frozen tissue, and or paraffin-embedded samples (Domínguez-Punaro, Segura, Radzioch, Rivest, & Gottschalk, 2008; Riedemann, Guo, & Ward, 2003; Wagner, Radermacher, & Stahl, 2010; Zanotti-Cavazzoni & Goldfarb, 2009). Male rodents are used, as female reproductive hormones are known to provide protection from mediators of shock. Knowledge of rodent type and their history of cross breeding ensures the development of genetic consistency when producing knockout models.

Large animal models (dogs, sheep, or nonhuman primates) may mimic pathophysiologic changes that are in line with the human response. However, the use of large numbers of these animals to extrapolate data that is not guaranteed to completely replicate human data makes it ethically and financially improbable. Recent studies have
questioned the validity and generalizability of findings from CLP studies in mice to the human inflammatory syndrome in humans, but as presented above there are many factors that must be considered prior to extrapolating disease-sensitive data (Iskander et al., 2013; Seok et al., 2013). The CLP-Methicillin Resistant Staphylococcus Aureus or CLP-Pseudomonas pneumonia two-hit model of inflammation may be a close consideration for correlation to human syndromes but each method presents advantages and disadvantages (Jung et al., 2011). To date, 2CLP in rodents has been an acceptable model for sepsis, ARDS, and sepsis-induced lung histological and behavioral alterations in mobility that can be monitored in response to putative interventions; but as research suggests, new approaches should be continually explored to determine the most promising correlation to the human syndrome.

**Heat Shock Proteins (HSPs)**

HSPs are highly conserved cell-protective molecules that were serendipitously discovered in 1962. Ritossa and coworkers found that exposing *Drosophila* larvae to noxious thermal stress or elevated temperatures produced characteristic patterns of altered gene expression (Ritossa, 1962). Ten years after Ritossa’s discovery, Tissières, Mitchell, and Tracy (1974) demonstrated that these patterns reflected upregulation of genes encoding HSPs. HSPs are ubiquitously present in prokaryotes, eukaryotes, yeast, and bacteria. Their primary role is as cellular molecular chaperones, maintaining proper protein folding and unfolding, preventing protein denaturation and aggregation, promoting protein transport across membranes, and directing defective polypeptides to specific areas in cells for degradation (Lindquist & Craig, 1988). Some members of the
HSP family are inducible whereas others are constitutively present. HSPs may also be found in the extracellular compartment, although function outside the cell differs considerably from activity in cells. HSPs are induced by noxious cell stimuli that include hyperthermia, exposure to heavy metals, and oxidants, ischemia, anoxia, radiation, hypoxia, oxidants, microbial infections, and ethanol. Additionally, HSP expression is induced by exposure to nicotine, salicylates (Aspirin), steroids/steroid hormones (Dexamethasone/estrogen), cocaine, alpha adrenergic agonists, and numerous other drugs (Allan & Harmon, 1986; Y. P. Chen, Voegeli, Liu, Noble, & Currie, 2007; J. E. Dyson et al., 1986; Lindquist & Craig, 1988; Ohyama, Yamada, Ohkawa, & Watanabe, 1985).

HSPs are classified by molecular weight and function. Constitutively present HSPs (HSCs) are also referred to as cognate proteins and are found primarily in the endoplasmic reticulum, mitochondria, and lysosomes (Goldfarb et al., 2006). Inducible HSPs are predominantly cytoplasmic or present in the nucleus. HSP chaperone function typically is Adenosine-5'-triphosphate dependent. Importantly, studies have suggested that inducible or exogenous HSPs delivered into cells have potential pharmacotherapeutic benefits by limiting cell injury (Hoshino et al., 2011; Lazar et al., 2007; Srivastava, 1994; Srivastava & Amato, 2001; Tamura, Peng, Liu, Daou, & Srivastava, 1997; X. Y. Wang, Kazim, Repasky, & Subjeck, 2003; Weiss et al., 2002; Wirk, 2011). When HSPs are induced, cells are also known to develop thermotolerance and to be protected from cell injury.

**Heat Shock Protein 70 (HSP70)**
HSP70 is a subfamily of highly inducible intracellular members of the HSP family. Increased cytoplasmic concentrations of HSP70 provide cytoprotection in critical illness (Lindquist & Craig, 1988). Members of the HSP70 group consist of many isoforms that include both cognate/constitutive and inducible proteins ranging in size from 66 to 78 kDa (Tavaria et al., 1996). Constitutive HSP73 is moderately inducible to provide cell protection and also maintain cell-machinery functions (Manzerra, Rush, & Brown, 1997). GRP78/Bip, an isoform found in the mitochondria and endoplasmic reticulum, is expressed during cell stress or cell starvation. However, the highly inducible HSP70, found localized in the cytoplasm, also known as isoform HSP72, specifically responds to cell stress and protein unfolding, and inhibits protein denaturation (Flynn, Pohl, Flocco, & Rothman, 1991; Gething & Sambrook, 1992; Parsell & Lindquist, 1993; Schein, 1989; Taura, Kusukawa, Yura, & Ito, 1989).

Early studies showed that one mechanism of HSP70 cytoprotection during inflammation occurred through direct repression of NF-κB, a critical regulatory transcription factor (Amorim & Moseley, 2010). NF-κB is responsible for activation of a number of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12; activation of genes involved in cell proliferation; regulation of mechanisms in the pro- and antiapoptotic pathways; and altering expression in adhesion molecules, growth factors, and immunoreceptors (Brazier, 2006). In an indirect manner, HSP70 is purported to repress mitogen-activated protein kinases (involved in cell proliferation, cell differentiation, cell movement, and cell death) activation, inhibit c-Jun N-terminal kinases and mitogen-activated protein kinases signal pathways, and suppress release and
translocation of high-mobility group box-1 protein (a proinflammatory mediator) (Amorim & Moseley, 2010).

**HSP70, sepsis and lung injury**

*Early studies on the therapeutic effects of HSP70.* Early studies on the inducible effect of HSP expression in sepsis and other disease models subjected septic rodents to whole-body heat stress. Ryan, Flanagan, Moseley, and Gisolfi (1992) found that raising rat whole-body temperature to 42 °C for 5 hr prior to injecting endotoxin 24 hr later, provided thermotolerance and reduced bacterial LPS-associated mortality by 71%. Villar and coworkers (1993) demonstrated that subjecting rats to whole-body heat pretreatment (42 °C) 18 hr prior to intratracheal administration of Phospholipase A2 to induce lung injury was protective. This study demonstrated both an increase in HSP70 mRNA and protein abundance in pulmonary tissue, and a 27% reduction in mortality. They found that HSP70 mRNA was detected in the lungs within 1 hr of thermal pretreatment and levels peaked at 12 hr. Villar and colleagues (1994), also found that heat stress prior to 2CLP attenuated both lung injury (less atelectasis, less hyaline membrane formation, and less inflammation) and liver injury, and decreased mortality by 48%. Last, a study by Singleton and Wischmeyer (2006), showed that HSP70.1/3 knockout mice showed greater vulnerability to CLP sepsis-induced ARDS, exhibiting increased lung injury, increased activation and prolonged binding/activation of NF-κB in lung tissue, increased expression of TNF−α and IL-6, and increased mortality.

*In vitro* studies also support a protective role of HSP70 against cellular injury. Wong, Ryan, and Wispé (1997) showed that exposing cultured human bronchial-
epithelial cells to 1.5 hr of thermal stress at 43 °C was protective against oxidative injury from nitric oxide. Importantly, increasing the amount of HSP70 using an intracellular plasmid-gene-expression vector system protected cells of a rodent epithelial cell line against S-nitroso-N-acetyl penicillamine, strongly suggesting that the cytoprotective effects of heat shock were mediated by HSP70. Additionally, Wong, Ryan, Menendez, Denenberg, and Wispé (1997) showed that induction of the HSP70 response by thermal stress (43 °C) for 1 hr in a human pulmonary-epithelium cell-culture line was associated with inhibition of IκBα dissociation from NF-κB, preventing degradation of I-κBα and subsequent translocation and activation of NF-κB in the nucleus. Thus, data from in vivo and in vitro studies support the cytoprotective thermotolerant role of the inducible HSR, as well as the specific protective role of HSP70.

**The inducible and protective effects of HSP70 in the 2CLP model.** In vivo studies by Weiss and coworkers (2000) in our laboratory, showed that 2CLP caused extensive lung injury and failed to increase lung HSP70. With intratracheal administration of HSP70 using an adenoviral vector (AdHSP70), Weiss and coworkers (2002), demonstrated that restoration of HSP70 abundance in the pulmonary epithelium significantly decreased lung injury and reduced mortality by 31% at 48 hr. AdHSP-mediated abundance of lung HSP70 was highest 48 hr following 2CLP, but declined thereafter and was at basal levels within a week.

Weiss and coworkers (2007) found that AdHSP70 administration in 2CLP rats had a direct or indirect affect on proinflammatory signal-transduction pathways such as NF-κB and IkappaB kinase (IκB /IKK). AdHSP70 treatment stabilized smaller, less
active oligomers of NFκB/IκB/IKK and limited NF-κB activation in intracellular-mediated cell-injury pathways. In addition, AdHSP70 preserved pulmonary epithelial alveolar Type I cells and attenuated proliferation of Type II cells, a process that is believed to lead to pulmonary fibrosis and scarring by stabilizing the retinoblastoma/E2F complex, preventing dissociation of a critical cell-division transcription factor (Bromberg, Raj, Goloubinoff, Deutschman, & Weiss, 2008). Last, AdHsp70 abundance impaired apoptotic cellular pathways by interfering with complex activation of the apoptosome and caspase-8, and directly bound to caspase-9 and caspase-3 to limit pulmonary alveolar cell apoptosis (Aschkenasy, Bromberg, Raj, Deutschman, & Weiss, 2011). Despite these demonstrations of efficacy of AdHSP70 administration in the 2CLP model, adenovirus-based gene therapy has been associated with complications of severe adverse immune response from the host, including death. Thus, the focus of the present work was to assess an alternative delivery system to increase HSP70 protein abundance in the lungs in the 2CLP rodent model.

**The use of Tat to deliver exogenous HSP70 to the lungs.** Large proteins such as HSP70 are generally unable to cross the cell plasma membrane bilayer by simple or facilitated diffusion, making it difficult to use them as therapeutic agents. One promising strategy for intracellular protein delivery is by coupling the protein to sequence 47–57 (YGRKKRRQRRR), of the Tat peptide, an 11 amino acid PTD derived from the Type 1 HIV virus (Derossi et al., 1996; Green & Loewenstein, 1998; Gump & Dowdy, 2007; Nagahara et al., 1998). Tat is not toxic or biologically infective and is incapable of replicating itself. There have been many theories hypothesizing mechanisms of how Tat
delivers a cargo protein into a cell. One of the more prevalent recent theories hypothesized that protein transduction occurs through an endocytosis mediated pathway referred to as macropinocytosis (Gump & Dowdy, 2007; Kaplan, Wadia, & Dowdy, 2005). Transduction is quick, is concentrated and energy dependent, and is receptor independent.

Tat has been used to introduce more than 50 proteins, some with potential to treat disorders, ranging in size from 15 to 120 kD, into cells, *in vitro* and *in vivo* (Asoh, Ohsawa, Mori, Katsura, et al., 2002; Begley, 2004; Cao et al., 2002; Hotchkiss et al., 2006; Kilpatrick et al., 2011; Klein et al., 2005; Schwarze, Ho, Vocero-Akbani, & Dowdy, 1999; Simon, Kang, Gao, Banta, & Morrison, 2010; Zhou, Du, Koretsky, Bagby, & Pang, 2008). In the few human studies, Tat conjugated to a PKC-δ inhibitor peptide has been approved for a Phase I/II clinical trial in the treatment of myocardial ischemia (DELTA MI Investigators, 2008).

*In vitro* studies by D. S. Wheeler, Dunsmore, and Wong (2003) used the Tat protein to build HSP70 abundance in a murine pulmonary embryonic fibroblast cell line that lacked the HSP70 gene. This transduction conferred protection against lethal thermal stress and hyperoxia. In a groundbreaking study, working in collaboration with our laboratory, Kilpatrick and colleagues (2011) successfully used the Tat protein to introduce a signaling protein PKC-δ inhibitor into the lungs of rats subjected to 2CLP. The Tat- PKC-δ inhibitor was delivered via intratracheal injection, similar to work done by Weiss using AdHSP70 (Weiss et al., 2002). The Tat- PKC-δ inhibitor successfully diminished lung PKC-δ phosphorylation, improved lung injury, and decreased abundance of proinflammatory chemokine levels in BAL specimens at 24 hr.
Our current study uses the Tat mechanism to increase HSP70 protein abundance in lung epithelial cells and evaluates the effectiveness of the Tat-HSP70 treatment on similar outcomes in the 2CLP model.

To summarize, in the preceding introduction we present two linked clinical syndromes, sepsis and ARDS that together produce severe morbidity and result in very high mortality rates. We propose use of the protein delivery mechanism, Tat, to increase the abundance of HSP70, a cytoprotective protein, in the lungs of a 2CLP sepsis induced ARDS model. We hypothesize that direct delivery of Tat-HSP70 into lungs will reduce lung injury after 2CLP as evidenced by histologic exam, decreased abundance of MPO (a marker of neutrophil activation), and decreased abundance of pro-inflammatory proteins CINC-1, MIP-2 and IL-6. As a secondary outcome measure, we hypothesize that amelioration of lung injury with Tat-HSP70 will improve rodent locomotion. The following section will present the background of the general methods in evaluating the specific aims and the rationale for those methods. The results of analyzing these methods will be presented in chapters two and three.

Background and Rationale for Study Outcome Measures

1. Assessing Lung Injury in the 2CLP model—Lung Histologic Pathologic Changes in ARDS are Detected by Hematoxylin/Eosin Stains

Pathologic changes in human ARDS can be observed as diffuse alveolar damage over time in three phases: exudative, proliferative, and fibrotic progression. Necropsy histopathologic studies conducted on patients identified with ARDS in 1967 showed hyperemia, dilated engorged capillaries, areas of alveolar atelectasis, interstitial and
intraalveolar hemorrhage, along with edema, presence of alveolar macrophages, neutrophils, hyaline membranes, scattered diffuse interstitial inflammation and fibrosis (Ashbaugh et al., 1967; Petty & Ashbaugh, 1971). To date, these remain key histologic markers depicting the progression of ARDS from mild to moderate and severe (Thille et al., 2013). The present study focused on changes evaluated during the exudative and proliferative phases that develop within 24 to 48 hr in the 2CLP model.

**The exudative phase.** In humans, within 7 days of a noxious insult to the lung causing injury, patchy areas of inflamed alveoli, interstitial and intraalveolar proteinaceous edema, focal hemorrhage, leukoagglutination, necrosis of endothelial cells, and platelet fibrin thrombi develop (Tomashefski, 2003). Hyaline membranes form as proteinaceous fluid and fibrin accumulate and leak through the alveolocapillary membrane. Type I pneumocytes are destroyed, exposing basement membranes, and Type II cell hyperplasia develops. Neutrophil and monocyte extravasation from alveolar capillaries leads to endothelial cell damage. With the loss of surfactant and increase in alveolar surface tension, there is reduced alveolar volume and segmental atelectasis. Histologic specimens reveal punctate airspaces of dilated alveolar ducts or collapse, alveolar edema, with distinctive presence of hyaline membranes along the alveolar septa. A computed tomographic scan reveals increased involvement of dependent lung regions. Similarly, in the rodent 2CLP model of ARDS, exudative changes evaluated at 24 hr confirm septal thickening, interstitial proteinacious fibrin/edema, alveolar collapse, hyaline membranes, accumulation of lymphocytes, plasma cells, and neutrophils, and inflammatory areas (encompassing proliferation of alveolar epithelial cells, hyperplasia, and fibrosis).
**The proliferative stage.** Within 7 to 10 days, inflammatory mediators and endogenous fibroblasts accumulate causing increased fibrin deposits and Type II pneumocytes to proliferate along the septa (Bachofen & Weibel, 1977, 1982). Epithelial cells proliferate and regenerate as Type II cells are stimulated to cover the denuded basement membrane. Water accumulates in lung connective tissue while activated neutrophils fill these spaces, injuring and causing alveolar collapse (Hällgren, Samuelsson, Laurent, & Modig, 1989). As ARDS progresses, the lungs become noncompliant, weighted down with edema and unable to turn off the enhanced fibroproliferative response. There are regions of newly formed connective tissue, and regions of dilated airspaces with increased fibrous tissue, leading to abnormal gas exchange (Tomashefski, 2003). In humans, this stage is followed by a fibrotic stage; however, this stage is not observed in the rodent 2CLP model due to the high mortality that generally precludes survival to a fibrotic stage. Nevertheless, the 2CLP model captures aspects of both the exudative and proliferative stage; as mentioned above, it would be impossible for any animal model to mimic all aspects of the human syndrome.

**The central role of neutrophils in sepsis-induced lung injury.** As first responders of the innate immune system, neutrophils are an essential component of host defenses and rapidly accumulate in areas of injury and inflammation to help contain and clear pathogens. Nevertheless, toxic mediators secreted in excess by activated neutrophils contribute to the pathogenesis of lung injury. In sepsis, neutrophils aggregate, are activated, degranulate, and release cytotoxic proteases, leukotrienes, platelet-activating factor, and reactive oxygen-derived free radicals that contribute to the production of additional pro- and anti-inflammatory mediators, chemokines, and cytokines (Grommes
Once activated, neutrophils can form neutrophil extracellular traps that regulate severity of infection, and activate epithelial, endothelial, and mast cells, as well as macrophages and T cells, the latter inducing the adaptive immune system (V. Kumar & Sharma, 2010). This results in pulmonary alveolar-epithelial cell and capillary-endothelial cell injury, resulting in loss of alveolar membrane integrity. Early in the course of sepsis-induced lung injury, neutrophils accumulate in the lungs by transmigration, traversing endothelium, interstitium, and alveolar epithelium into alveolar spaces. Fibrin deposits in the latter exudative stage of ARDS continue to draw large numbers of neutrophils into the alveolar airspace.

In a rodent model of CLP, increase in circulating levels of lymphocytes, monocytes, and neutrophils were observed up to day 21, however resection of the abscessed cecum reduced circulating neutrophil counts (Xiao, Siddiqui, & Remick, 2006). In mice made neutropenic, lung injury from endotoxemia-induced sepsis was severely reduced compared to controls (Abraham, Carmody, Shenkar, & Arcaroli, 2000). Further, activation of proinflammatory factors—NF-κB, IL-1β, TNF-α, and MIP-2 (an IL-8 homologue measured in our study)—were also decreased in neutropenic mice.

Clinically, increased neutrophil production found in BAL of patients within 2 weeks of a sepsis-induced lung injury was inversely correlated to patient survival (Steinberg et al., 1994). BAL-measured levels of neutrophil elastase, the primary protease product of neutrophil degranulation, are positively correlated with increased severity of lung injury in ARDS patients (Pittet, Mackersie, Martin, & Matthay, 1997). Excessive activation of
neutrophils has also been shown to be an important mediator of lung injury in several
rodent ARDS models (Abraham et al., 2000).

However, patients can be neutropenic and still develop ARDS (Ognibene et al.,
1986). Morphologic studies of BAL from patients who are at risk for ARDS or who have
established ARDS show a low proportion of neutrophil apoptosis. In addition, the
relatively low rate of neutrophil apoptosis in this patient population was related to the
protective presence of granulocyte-colony stimulating factor and granulocyte-
macrophage colony-stimulating factor (Matute-Bello et al., 1997). Indeed, while
decreased neutrophil apoptosis may prolong neutrophil-induced lung injury during
ARDS, alveolar-epithelial-cell apoptosis exacerbates lung injury (T. R. Martin,
Hagimoto, Nakamura, & Matute-Bello, 2005). This was confirmed by in an animal model
by Aschkenasy and colleagues (2011) who showed that 2CLP caused pulmonary
apoptosis in alveolar epithelial Type I and II cells but not in neutrophils.

Based on the above findings, outcome measures in the current study aimed, in
part, to detect altered levels of neutrophils and neutrophil-related markers in 2CLP rats
treated with TAT-HSP70.

2. Detection of Neutrophil-Derived Myeloperoxidase (MPO)

MPO is an enzyme contained in neutrophil granules that generates microbicidal
oxygen radical-containing metabolites. It is used as a marker of appropriate or
inappropriate regulation of neutrophil activation. MPO catalyzes reactions using
hydrogen peroxide to produce toxic hypochlorous acid as a bactericidal agent during
neutrophil respiratory burst (Kutter, Devaquet, Vanderstocken, et al., 2000). In addition,
MPO oxidizes tyrosine-producing toxic tyrosyl radical during the neutrophil respiratory
burst (Heinecke, Li, Francis, & Goldstein, 1993). Neutrophils are phagocytes responsible for engulfing and destroying pathogens. Following bacterial phagocytosis, neutrophil granules fuse with the phagosome, forming a phagolysosome. Production of reactive oxygen species such as hypochlorous radical and release of elastases into the phagolysosome kills the bacteria. However, these toxic molecules can also spill over to extracellular spaces, damaging host cells. Reactive oxygen species are damaging to cell structures causing plasma-membrane breakdown and denaturation of nucleic acids and proteins, promoting apoptosis, and stimulating proinflammatory effects. In the few human studies, MPO was elevated in the BAL of patients with lung injury and ARDS (Pittet et al., 1997; Weiland et al., 1986).

In a rodent model of acute inflammation, induced by LPS, MPO was generated by stimulated phagocytes in the endothelium and subendothelium and resulted in the toxic release of nitric oxide (Eiserich et al., 2002). Relative to rodents in control groups, LPS-induced lung injury increased MPO activity significantly within 6 hr in lung microvasculature (Buesing et al., 2011). In vivo studies in which MPO and other peroxidases were infused through the trachea showed lung injury that was reduced by catalase and superoxide dismutase, suggesting that production of toxic oxygen-derived free radicals from MPO granules are capable of direct lung injury (Johnson, Fantone, Kaplan, & Ward, 1981). MPO deficient mice (/-) with CLP polymicrobial sepsis were more likely to die from inflammation relative to wildtype mice, supporting a role for MPO in the response to severe bacterial insult (Gaut et al., 2001). Last, MPO abundance in lung homogenate of animals following 2CLP were significantly abundant relative to
sham-operated control animals at 24 and 48 hr (Weiss et al., 2001). Thus, MPO is likely to be mechanistically involved in lung injury during ARDS, and is used as a marker of neutrophil accumulation and generation in the present study.

3. Detection of Cytokine Induced Neutrophil Chemoattractant Protein-1 (CINC-1), and Macrophage Inflammatory Protein-2 (MIP-2)

Cytokine-induced neutrophil chemoattractant protein-1 (CINC-1), is a potent proinflammatory chemokine expressed in rodent models of sepsis. CINC-1 is homologous to human proinflammatory IL-8, both belonging to the CXC chemokine family of chemotactic proteins for neutrophils (Kunkel, Standiford, & Kasahara, 1991; Standiford et al., 1990; Watanabe, Konishi, Fujioka, Kinoshita, & Nakagawa, 1989). Macrophage Inflammatory Protein-2 (MIP-2), known as Chemokine C-X-C motif ligand 2 (CXCL2), also belongs to the CXC chemokine family (Driscoll, 1994). MIP-2 genes have been implicated as the rodent homolog of IL-8 (Huang, Paulauskis, Godleski, & Kobzik, 1992). In rodents with lung injury, studies show that CINC-1 and MIP-2 are secreted by alveolar macrophages and monocytes. The CXC chemokine family mediates lung inflammation through the recruitment and movement of neutrophils into the lung during sepsis (Guo et al., 2006; Haelens et al., 1996). Studies using the 2CLP model of sepsis and ARDS show increased levels of the CXC chemokines, CINC-1 and MIP-2, from either plasma, serum, or tissue homogenate (Guo et al., 2006; Remick et al., 2000; Strieter, Keane, Burdick, Sakkour, Murray, & Belperio, 2005).

The role of these mediators during lung injury is supported by evidence that blocking the CXC chemokine receptor CXCR2 pathway correlates with increased
mortality in animals with pneumonia (Mehrad et al., 1999). Neutrophil counts were reduced when the CXCR2 ligand pathway was neutralized, and mortality was higher when compared to the control group where neutrophil presence provided some resistance against bacterial influx. In the study by our collaborators, Kilpatrick and colleagues (2011) found that relative to 2CLP control rats with ARDS morphology, intratracheal delivered Tat-PKC δ inhibitor attenuated lung injury and significantly reduced BAL levels of both CINC-1 and MIP-2 at 24 hr.

In humans, IL-8 is typically secreted by neutrophils, alveolar macrophages, epithelial and endothelial cells, fibroblasts, and hepatocytes. Following stimulation by pathogens or inflammatory mediators, IL-8 is secreted into the extracellular space subsequently attracting neutrophils to the site of injury. IL-8 levels are elevated in sepsis conditions and in multiorgan system dysfunction inclusive of pulmonary dysfunction (Friedland et al., 1992; Marty et al., 1994). Of significance, elevated plasma levels of IL-8 in patients with sepsis correlate with mortality (Hack et al., 1992; Marty et al., 1994). IL-8 has been implicated in the exudative stage of ARDS as a key promoter of neutrophil rolling, a preliminary step in neutrophil migration into tissues. Studies related to ARDS show that elevated levels of IL-8 in BAL specimens of patients with ARDS correlate with elevated levels of neutrophils and death (Chollet-Martin et al., 1993; Miller et al., 1992).

In summary, we included the study of CINC-1 and MIP-2 to evaluate levels of these rodent homologs of the proinflammatory chemokine IL-8 in the 2CLP model of sepsis induced ARDS.

**Detection of Interleukin-6 (IL-6)**
IL-6 is produced by T cells, macrophages, and endothelial cells and has a role in diverse physiological and pathophysiological processes including acute inflammation, chronic inflammatory disorders, hematopoiesis, and oncogenesis (Kishimoto, 2010). IL-6 has direct and indirect effects that may lead to both proinflammatory and anti-inflammatory roles (Opal & DePalo, 2000). Elevated plasma levels of IL-6 have been associated with or predictive of mortality at early time points in patients diagnosed with inflammatory or sepsis syndromes and rodents made septic by CLP (Hack et al., 1989; Yende et al., 2008). In a female mouse model of CLP, elevated plasma IL-6 levels significantly correlated with mortality at 6 hr (Remick et al., 2002). In the later phase following 2CLP, at 72 hr, decreased serum levels of IL-6 were also associated with a 90% mortality (Deutschman, Cereda, Ochroch, & Raj, 2006). However, mortality in IL-6−/− knockout mice was reported at 100% at 12 hr following 2CLP (Deutschman et al., 2006). These studies point to the complexity of the role of IL-6 following 2CLP and sepsis morbidity and mortality. Timing of measurement of this biomarker, in the context of other measures of ARDS and sepsis progression, is critical (Deutschman, 2001).

The measurement of circulating (plasma/serum) versus tissue homogenate levels of cytokine or chemokine mediators can produce variable results that, again, must be put into context with the time of measurement and clinical state. In a study evaluating the role of IL-6 as a hepatic biomarker during the acute phase response following 2CLP, Andrejko, and coworkers (1998) found that relative to sham-operated animals and the less severe single puncture (CLP) model of sepsis, serum IL-6 levels in the 2CLP model were elevated at 6 and 16 hr but significantly decreased to baseline at 24 hr and below the levels of the sham-operated and CLP models by 48 hr. 2CLP animals also had
significantly lower increases of intrahepatic transcription factor STAT-3 activity, and reduced expression of the acute phase protein α2-macroglobulin relative to CLP animals. Higher mortality was also seen in 2CLP animals. The authors concluded that, although intrahepatic IL-6 abundance and serum IL-6 levels correlate with mortality in sepsis, intrahepatic IL-6 abundance and serum IL-6 levels did not correlate with intrahepatic activity. These findings demonstrate a potential disconnection between systemic cytokine activity and IL-6-mediated intracellular-signaling pathways.

Studies in patients who presented to the emergency department and/or were admitted to an ICU with sepsis, observed that elevated plasma levels of IL-6 (and IL-8) was useful as a marker of poor prognosis and as a marker (IL-6 levels only) for progression to the development of severe sepsis (Oberhoffer, Vogelsang, Russwurm, Hartung, & Reinhart, 1999; Terregino, Lopez, Karras, Killian, & Arnold, 2000). Early studies by Schütte and coworkers (1996) found that serum and BAL levels of IL-6 were increased in patients diagnosed with ARDS or severe pneumonia, but not in patients diagnosed with pulmonary compromise from cardiogenic pulmonary edema, supporting the usefulness of IL-6 as a marker of lung infection and inflammation. A decade later, elevated levels of plasma IL-6, at early time-points (up to 72 hr), are still prognostic of adverse clinical outcomes for patients with ARDS (Parsons et al., 2005). As a precaution, elevation in plasma IL-6 values, and all biomarkers, should be evaluated in the context of comorbid diseases (acute renal failure), as they could falsely alter its predictive value even in the setting of a critical illness such as sepsis (Simmons et al., 2004). Simmons and coworkers (2004) found that plasma IL-6 and IL-8 levels of patients with acute renal
failure, monitored over a 2-week period, were significantly elevated in non-survivors relative to survivors regardless of presence or absence of sepsis.

In summary, IL-6 expression is a complex but necessary as part of the acute-phase immune response in sepsis and ARDS. We present data to support the hypothesis that elevated IL-6 levels may be associated with poor prognosis during the early, acute time of inflammation; however, decreased levels of IL-6 past 24 hr may also be associated with worse outcomes. Carefully designed studies to evaluate defined mediators at different times to study the correlation with assessments of syndrome severity are still needed.

4. Neuromuscular and Functional Consequences of Critical Illness

Both sepsis and ARDS can produce debilitating acute and long-term respiratory and neuromuscular dysfunction, making recovery of function and return of quality of life protracted, and costly. Early recovery of lung function is paramount to better functional recovery.

Patients who survived ARDS and were followed up at 3 months post hospital discharge experienced persistent lung dysfunction with evidence of mild restrictive disease and reduced diffusion capacity (Herridge et al., 2003). A follow-up study by the same study group evaluating 1-year outcomes in survivors of ARDS reported pronounced muscle weakness, muscle wasting, and fatigue that led to persistent functional limitations. Five years following recovery, patients who survived ARDS reported experiencing persistent limitations in pulmonary function and exercise endurance, limitations in ambulation, and limitations in cognitive and psychosocial behavior culminating in
anxiety and depression. All of these sequelae contributed to an inability to transition back into the work force (Herridge et al., 2011). Following hospitalization and discharge for ARDS, the burden of costs are overwhelming, and are attributed to loss of wages, hospital readmissions, medications, home-care therapies, and stress to caregivers. However, findings in the 1-year follow-up study of these patients demonstrated that early recovery from lung injury and multiorgan dysfunction during the acute phase predicted better long-term functional recovery (Herridge et al., 2003; Marini &Gattinoni, 2004). In addition, patients diagnosed with ARDS who were able to ambulate and had a mobility protocol initiated within 48 hr of ARDS diagnosis had fewer days in bed, in the ICU, and a shorter hospital stay (Morris et al., 2008).

Although the studies mentioned in this section focus on long-term patient outcomes that are more functionally relevant, the global causes that lead to the culmination of ICU acquired weakness are many and will be discussed briefly next.

**Critical illness polyneuropathy/myopathy and other causes of weakness.** There is a significant body of work that focus on the development and diagnoses of critical illness polyneuropathy (CIP) and or critical illness myopathy (CIM) as a significant root cause of weakness in the critically ill. More than 50% of patients diagnosed with sepsis, MODS, and ARDS will develop either CIP or CIM, contributing to ICU acquired weakness (Bolton, Gilbert, Hahn, & Sibbald, 1984; Hermans, De Jonghe, Bruyninckx, & Van den Berghe, 2008; Latronico, 2005; Stevens et al., 2007). Approximately 25% of patients receiving supportive mechanical ventilation therapy for greater than 7 days, will experience diaphragmatic atrophy, and significant musculoskeletal weakness with
myopathic changes seen on muscle biopsy. Additionally, muscle alterations can be abnormal from 6 months to 2 years post discharge from intensive care (Angel, Bril, Shannon, & Herridge, 2007; Fan, Zanni, Dennison, Lepre, & Needham, 2009; Wilcox & Herridge, 2010).

CIP is characterized by peripheral axonal disintegration, affecting sensory and motor fibers, whereas CIM, which develops as early as 48 hr into the course of the syndrome, includes the development of thick filaments, myosin loss, and decreased myosin to actin ratio (Zink, Kollmar, & Schwab, 2009). Pathogenesis of CIP and CIM are thought to be attributed to inactivation of sodium channels, cytokine and chemokine-mediated activation of myofiber-related ubiquitin/proteasome degradative pathways and overproduction of nitric oxide, causing mitochondrial dysfunction (Brealey et al., 2002; Bolton, 2005; Di Giovanni et al., 2004; Fenzi, Latronico, Refatti, & Rizzuto, 2003; Novak et al., 2009). Differentiating between CIP and CIM is difficult and requires electromyogram studies, nerve conduction studies, and nerve or muscle biopsy. Neuromuscular dysfunction, whether due to CIP, CIM, or both, can progress to severe skeletal-muscle weakness affecting respiratory muscles that results in difficulties in ventilator weaning (Garnacho-Montero et al., 2005; Jubran, 2006).

Alternate proposed causes of musculoskeletal weakness during sepsis and ARDS are diverse and include cytokine-mediated changes, increase in oxidative stress, impaired microcirculation, unbalanced protein synthesis and breakdown, altered calcium homeostasis causing changes in excitation-contraction coupling, hyperglycemia, exposure to steroids, use of neuromuscular blockade, heterotopic ossification, and
peroneal and ulnar palsies (Bolton, 2005; Di Giovanni, Mirabella, D’Amico, Tonali, & Servidei, 2000; Di Giovanni et al., 2004; Hermans et al., 2007; Klaude et al., 2007; Rossignol et al., 2008; van den Berghe, Schoonheydt, Becx, Bruyninckx, & Wouters, 2005).

*Animal studies of locomotor activity alterations during sepsis/ARDS.* Numerous studies using the CLP model have illustrated decreased locomotion associated with sepsis and ARDS. Studies that assessed neural conduction or muscle biopsies implicating CIP and CIM found that electrophysiological alterations occurred as early as 4 to 24 hr from the onset of CLP sepsis and continued up to Day 7 (Cankayali et al., 2007; Latronico et al., 1996; Nayci et al., 2005; Rossignol, Gueret, Pennec, Morel, Rannou, Giroux-Metges, et al, 2008). Some studies have attributed critical illness weakness to decreased locomotion, attributed to neurocytokines and immune system mediators during sepsis (Dantzer & Kelley, 2007; Remick et al., 2005; Wichterman et al., 1980). Studies portraying CLP-induced immunomodulatory sickness behavior are few, but there is evidence to support exploring the relation between CLP and sickness behavior further (Petronilho et al., 2012; Tuon et al., 2008). We propose that delivery of Tat-HSP70 would attenuate lung injury, facilitating recovery of a key organ function and thus promote early locomotion, preempting further debilitation in neuromuscular function.

**Study significance and Summary**

The research presented here describes the use of the HIV-1 Tat PTD as a carrier to augment HSP70 abundance in lungs and limit pathological changes in rats with septic peritonitis. Our research may contribute to the current therapeutic approaches in the
critical care management of patients with sepsis induced ARDS. As a secondary outcome, our proposed experiments investigated the effects of our therapy on locomotion. Sepsis, ARDS and immobility have profound effects on patients’ recovery, their care-givers and costs to the health care delivery system. As such, our body of work may have direct implications for prescribing practitioners, bedside nursing clinicians and other members of the multidisciplinary healthcare team. Illustration 1.
Illustration 1

Roadmap - Dissertation overview

Insult

Triggers: Pathogen Associated Molecular Patterns/Danger Associated Molecular Patterns

Dysregulated inflammation
(Chemokines and cytokine marker that damage lung)
- CINC-1
- MIP-2
- IL-6

Generation of excessive activated Neutrophils
- Myeloperoxidase

Failed expression of inducible HSP70 in a 2CLP model

Intervention
Intratracheal Tat-HSP70

ORGAN FAILURE
- SEPSIS → ARDS

ORGAN RECOVERY
CHAPTER 2

TAT-HSP70 ATTENUATES LUNG INJURY IN A 2CLP MODEL OF ARDS
Abstract

Sepsis, a poorly understood syndrome of uncontrolled inflammation is the leading cause of death in critically ill patients. The lung is the organ most affected, quickly developing into Acute Respiratory Distress Syndrome (ARDS). The Heat Shock Response, during which Heat Shock Proteins (HSPs) are expressed, is an endogenous mechanism to protect cells from injury. We have found that abundance of Heat Shock Protein 70 (HSP70), a member of the family of HSPs, is insufficient to provide pulmonary cytoprotection in a cecal ligation and double puncture (2CLP) rodent model of sepsis induced ARDS. Here, we use the HIV-1 protein transduction domain, Tat to increase HSP70 protein levels in the lung. We found that Tat-HSP70, introduced via the trachea, significantly increased HSP70 abundance in the lung over time following 2CLP (2CLP-Tat-HSP70). Relative to phosphate buffered saline (PBS) treated controls (2CLP-PBS), treatment of septic rats with Tat-HSP70 significantly increased HSP70 abundance in histologically abnormal lung regions. In addition, there was a significant decrease in Myeloperoxidase abundance in lung tissue of 2CLP-Tat-HSP70 treated animals at 48 hr. Finally, staining of lung tissue showed less pronounced inflammation in 2CLP-Tat-HSP70 rats over time than in 2CLP-PBS rats. These findings suggest that the fusion of Tat-HSP70 system is a viable and novel delivery mechanism to increase HSP70 protein in the lung following 2CLP sepsis and can reduce markers of lung injury.
Introduction

Sepsis, an often fatal syndrome of disordered inflammation that may lead to multiple organ dysfunction syndrome (MODS), is the leading cause of death in Intensive Care Units (ICUs) (Xu, et al., 2010). The most recent data, accrued in 2013, indicate prevalence for sepsis associated syndromes was in excess of 900,000 each year in the United States (Gaieski, et al., 2013). Treatment of this complex disorder is expensive - sepsis cost the United States healthcare system 24 billion per year, rising from 17 billion in the past decade, over 40% of total ICU costs (Gaieski, et al 2013). Mortality from sepsis syndromes remain high, at 29% (Gaieski, et al., 2013). Finally, because the pathobiology of sepsis is so poorly understood, therapeutic approaches to sepsis have not been effective and management of the syndrome is almost entirely supportive (Ranieri, et al., 2012).

The lung is the organ that is most often affected by sepsis. Indeed, 47% of sepsis related infections are from a pulmonary source (Martin et al, 2003). Pulmonary dysfunction in sepsis, called the Acute Respiratory Distress Syndrome (ARDS), may progress rapidly from mild to moderate to severe injury (ARDS Definitions Task Force, 2012; Ashbaugh, et al., 1967). With early identification and protective ventilation strategies, mortality of patients with ARDS has declined from 40 % to 22% in the past decade (ARDSNET, 2000; Spragg, et al., 2010). Nevertheless, in survivors of the sepsis and ARDS syndromes, functional, cognitive and psychosocial disabilities makes recovery prolonged and costly (Herridge, et al., 2011; Iwashyna, et al., 2012).

Pathologic findings in ARDS include diffuse alveolar damage, interstitial and
alveolar edema, altered blood flow and capillary disruption, and disruption of Type I and II alveolar epithelial cells (Ashbaugh, et al., 1967; Bachofen & Weible, 1977, 1982; Petty & Ashbaugh, 1971). These pathologic changes lead to ventilation/perfusion mismatching that reduces gas exchange and produces hypoxemia and, in more profound cases, hypercarbia. It is believed that these changes result from dysregulated inflammation with disordered activation of neutrophils and macrophages injuring and often killing alveolar epithelial cells (Abraham, et al., 2003; Aldridge, 2002; Lee & Downey, 2000). Type I epithelial cells, which make up 90% of the alveolar cellular compartment and constitute the surface through which gas exchange occurs, are easily injured and may become necrotic or apoptotic. Protein released from injured or dead Type I cells accumulates in alveoli and form the hyaline membranes that are characteristic of the syndrome. Type II epithelial cells produce and synthesize surfactant and other protein complexes, facilitate ion transport, and influence activation of the immune system. When Type I cells are damaged and shed from the alveolar surface, Type II cells proliferate, spread over denuded basement membrane and differentiate into Type I cells to replace those that have been destroyed (Bachofen & Weible, 1977, 1982). Thus, interventions that limit and regulate neutrophil accumulation and activation, prevent the loss of Type I cells and limit the over-proliferation of Type II cells are required (Weiss, et al., 2007, 2008, 2002). One such potential treatment is the 70kDa heat shock protein, HSP70.

Expression of inducible HSP70 in the lungs of a rat model of sepsis-induced ARDS by cecal ligation and double puncture (2CLP) is severely diminished (Villar, et al., 1993; Weiss, et al., 2000). Using an adenoviral vector delivery system to introduce HSP70
mRNA (AdHSP70) to the lung, Weiss and co-workers successfully increased HSP70 abundance in the pulmonary epithelium and showed significantly decreased lung injury and reduced mortality at 48 hr in the 2CLP rodent model of sepsis induced ARDS. Additionally, AdHSP70 limited 2CLP-mediated activation of NF-κB, blocked caspase induced cell death, suppressed stress kinases involved with mitochondrial damage in the apoptotic pathway, attenuated excessive cell division and proliferation of Type II pulmonary cells and apoptosis of Type I cells (Aschkenasy, et al., 2012; Bromberg, et al., 2008; Weiss et al., 2002, 2007). The use of adenoviral vectors may present problems in animal and human hosts. Immune responses to adenoviral proteins are unpredictable and may induce severe adverse reactions in the host. These include lymphocytic inflammatory infiltrate, development of viral traps and sequestration of the adenovirus vector in the liver diminishing the effectiveness of the virus, and severe immune responses leading to multi-system organ failure and death (Alba, Bosch & Chillon, 2005; Anderson, 1998). Thus, development of alternative delivery strategies that enhance HSP70 abundance in pulmonary epithelial cells are warranted.

One potential protein delivery system is the Trans- Activator of Transcription, Tat, an eleven amino acid protein transduction domain derived from the Type 1 HIV virus (Green & Loewenstein, 1998; Gump & Dowdy, 2007; Nagahara, et al., 1998). Tat is not toxic or biologically infective and is incapable of replication. Recent studies have shown that protein transduction occurs quickly, is energy-dependent and may occur through fluid phase macropinocytosis (Kaplan, et al., 2005). Tat has been used to introduce more than 50 proteins, ranging in size from 15 to 120 kD, in vivo and in vitro,
into human and rodent cells (Begley, 2004; Ezhevsky, et al., 1997; Hotchkiss, et al., 2006; Nagahara, et al., 1998; Renigunta, et al., 2006). In the study detailed here we use the Tat delivery system to enhance HSP70 abundance in pulmonary epithelial cells. To accomplish this goal we injected Tat-HSP70, a fusion protein that combines HSP70 with the HIV 1-Tat protein transduction domain, into the trachea of septic and non-septic rats.

Materials and Methods

Production of Tat-HSP70

A replication vector containing the Tat-HSP70 fusion protein, kindly provided by Hector Wong MD, Cincinnati Children’s Hospital, was expressed in *Escherichia Coli* as previously described (Wheeler et al, 2003). Recombinant Tat-HSP70 fusion protein was reproduced by Impact Biologicals, Inc., Swarthmore, Pennsylvania, as follows. His-Tat-HSP70 was expressed in *E. coli* BL21(DE3)pLysS cells. For induction, cells were exposed to 1 mM IPTG for 3.5 hr at 37°C (Celsius). After induction, cells were pelleted, then frozen at -20°C. For purification, the cell pellets were thawed and suspended in Tris-NaCl buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5). The resulting lysate was treated with benzonase and Mg2+ to reduce viscosity. Insoluble material was then removed by centrifugation. The resulting clarified lysate was applied to a His-Select (nickel) column. After washing with Tris-NaCl, the His-Tat-HSP70 was eluted from the column with Tris-NaCl that had been supplemented with 100 mM imidazole. The eluted protein was dialyzed against 10 mM sodium phosphate, 140 mM NaCl at 4°C. After dialysis, the material was centrifuged briefly to remove the small amount of insoluble
material that had formed. Glycerol was added to the supernatant, to a final concentration of 10%. To remove LPS, the preparation was passed through a polymyxin column. The purity of the resulting protein is ~90%. The recovered protein was sterilized by passing through a 0.2 micron filter, then stored at -80°C Celsius (C).

**Animal protocol and induction of sepsis**

All animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and were conducted in an approved facility overseen by the University Laboratory Animal Resources Center at the University of Pennsylvania. Studies were performed on male eight to twelve week old Sprague Dawley (SD) rats, weighing 250 to 300 grams, (Charles River, Boston, Massachusetts, USA). Rats were housed in a climate controlled, 12h light/12h dark cycle facility and allowed free access to food and water. Previous studies have used these specific SD rats and they are known to reliably develop ARDS after 2CLP (Weiss, et al., 2000).

2CLP was performed using 2% Isoflorane anesthesia as previously described (Weiss et al., 2000). Intratracheal instillation of Tat-HSP70 or phosphate buffered saline (PBS) was conducted via the method of Weiss et al (2002). 200 µl of PBS containing 100 µg of Tat-HSP70, a dose chosen after pilot studies demonstrated that a 50 µg dose failed to increased HSP70 abundance, was injected into a tracheal cannula inserted through a small incision. A like volume of PBS vehicle alone was administered to control animals. Injection was followed by two ml of air to assure equal distribution of agent into lungs (Weiss, et al., 2002). The wound was closed and the animals were allowed to recover.
Immediately following surgery and every 24 hr thereafter, animals were fluid resuscitated with 50 ml/kg of 0.9% saline subcutaneously.

A total of 22 rats were separated into five groups with analysis at two time-points: unoperated, untreated controls (T0), septic, treated animals, subjected to 2CLP who received Tat-HSP70-containing PBS (2CLP-Tat-HSP70) or septic, untreated animals, subjected to 2CLP but administered PBS alone (2CLP-PBS). Table 1 (page 137).

Animals were pre-designated for sacrifice at 24 or 48 hr post 2CLP. Data from at least three surviving animals in each group at each time point were collected for analysis. Prior studies have established that survival is approximately 50% at 24 hr and approximately 25% at 48 hr and that statistical significance could be achieved with three surviving animals at each time point. Designated rats were euthanized and lung lobes were gently infused with Tissue-Tek OCT Compound fixative (Electron Microscopy Sciences, Hatfield, PA). Lung lobes were flash frozen in liquid nitrogen, sectioned for staining or sectioned and mounted on slides and stored at -80° C until ready for use.

**Immunohistochemistry and quantification of HSP70 and MPO abundance in lung parenchyma**

We performed immunohistochemical stains on frozen lung sections to determine HSP70 and Myeloperoxidase (MPO) protein abundance (Weiss, et al., 2002). Five micron sagitally sliced sections of thawed lung tissue were adhered to glass slides and incubated with the Thermo Scientific Starting Block blocking buffer (Thermo Fisher Scientific Inc., Rockford, IL) for 10 minutes. To detect HSP70 protein abundance, fixed
sections were incubated first with a HSP70 goat polyclonal antibody -SC 1060 (Santa Cruz Biotechnology, Inc., California, USA), diluted 1:100 with PBS, for two hr at room temperature. This was followed by a 1-hr incubation with a secondary fluorescence-tagged antibody, donkey anti-goat coupled to Alexa Fluor 555 (Molecular Probes, Invitrogen, California, USA) diluted 1:800 in PBS. DAPI or 4',6-diamidino-2-phenylindole was used to allow visual identification of cell nuclei (Molecular Probes, Eugene, OR). To detect MPO protein abundance, a similar procedure was used substituting the rabbit polyclonal antibody to MPO, SC 33596, as the primary antibody (Santa Cruz Biotechnology, Inc., California, USA). All slides were washed with 1X PBS buffer solution. Fluorescent mounting medium from KPL (Gaithersburg, MD) was added prior to microscopic examination. Tissue and cells were visualized with a Nikon Eclipse E600 microscope equipped with fluorescence optics. Quantification of cytoplasmic HSP70 and MPO activity was done using the iVision for Macintosh Scientific Image Processing program, iVision 4.0.14 (Biovision Technologies, Chester Springs, PA). Nucleated cells positive for HSP70 or MPO staining were counted as were total DAPI-positive cells in per each high-powered field. The mean fluorescence intensity per cell was calculated. Cell counts on 10 high-powered fields per lung lobe section per rat were assessed, counted and averaged.

**Lung histology and morphology**

Five micron lung sections were stained with hematoxylin and eosin (H&E). A veterinary pathologist blinded to procedure and intervention performed histologic evaluation of the lung tissue. Sections were evaluated for minimal, mild, or moderate
changes consistent with ARDS using the following criteria: location of lesion, septal thickening, amount of interstitial proteinacious material (fibrin/edema), alveolar collapse, hyaline membrane, presence of lymphocytes and plasma cells and neutrophils. In addition, lung sections were evaluated for percentage of inflamed areas present per lobe (Bachofen & Weibel, 1977; Petty & Ashbaugh, 1971). See Table 2 (page 138) in Appendix for the itemized grading system and results. The Aperio Image Scope program, v11.0.2.725 for PC (Pathology Core Laboratory, Children's Hospital of Philadelphia Research Institute, Philadelphia, PA) was used to visualize and calculate percentage of inflamed areas.

**Statistical analysis**

Statistical significance comparing mean HSP70 and MPO protein abundance was determined using a two-tailed ANOVA with a Bonferroni adjustment for multiple comparisons. For survival studies Kaplan Meier and Cox proportional hazard regression analyses were performed. Significance level was set at p<0.05. All statistical analysis were conducted on SAS software, version 9.3 (SAS corporation). A descriptive analysis was provided for histologic examination of lung tissues.

**Results**

**Intratracheal administration of Tat-HSP70 increased HSP70 protein abundance in lungs following 2CLP**

2CLP decreases inducible HSP70 protein abundance in the lung. Prior work shows that failure of endogenous HSF to induce HSP70 expression and abundance in the
lung fails to protect against lung injury. To determine the effects of Tat-HSP70 on increasing HSP70 abundance in the lungs, we performed immunohistochemical staining on frozen lung samples of T0, 2CLP-PBS and 2CLP-Tat-HSP70 rats. We present quantitative data comparing changes in mean HSP70 protein abundance in Figures 1a and b. We show presence of baseline endogenous HSP70 abundance in T0 rats. We did not observe a significant difference in HSP70 abundance between T0 and 2CLP-PBS rats at 24 and 48 hr confirming the inability of HSF to induce HSP70 abundance in pulmonary epithelium following 2CLP. Relative to T0 rats, Tat-HSP70 significantly increased HSP70 abundance following 2CLP at 24 (p<0.04) and 48 (p<0.01) hr. Relative to 2CLP-PBS rats, a significant increase in HSP70 protein abundance was detected in 2CLP-Tat-HSP70 rats at both 24 (p<0.01) and 48 (p<0.001) hr.
Figure 1a. Tat-HSP70 increased HSP70 protein abundance in lungs following 2CLP

Note: Immunohistochemical stain of HSP70 protein within the cytoplasm of lung alveolar sections. HSP70 antibody appears as magenta surrounding individual cell nuclei stained with DAPI -that appears as blue. We observe baseline endogenous HSP70 abundance in T0 rats. Relative to 2CLP-PBS rat lung stains at 24 and 48 hr, we observe significantly increased HSP70 abundance in 2CLP-Tat-HSP70 rat lung stains at 24 and 48 hr. We present three control groups as depicted by lung sections of T0 rats without HSP70 antibody staining, and a positive and negative control with and without HSP70 antibody stain in sectioned samples of frozen human lung with malignant lung cancer.
Figure 1b.

Note: Graphic comparison (mean ± Standard Deviation (SD)) of absorbance ratios of cytoplasmic positive HSP70 protein fraction of lung sections per nuclei/high-power field. Counts from 10 high-powered field sections/slide, one slide/rat, three rats/each group of intervention are shown. Relative to T0 animals, HSP70 abundance was significantly increased in 2CLP-Tat-HSP70 animals at 24 (p<0.04) and 48 (p<0.01) hr. Relative to 2CLP-PBS groups, HSP70 abundance was significantly increased at 24 (p<0.01) and 48 hr (p<0.001) in the 2CLP-Tat-HSP70 groups. ∧= statistically different from T0. * = statistically different from 2CLP-PBS at same time point.
Intratracheal administration of Tat-HSP70 increased HSP70 protein abundance in abnormal and normal lung sections following 2CLP

The distribution of lung injury in ARDS is heterogeneous. We know that there are normal as well as injured areas contributing to the complex categories used to characterize severity of the ARDS syndrome. To determine distribution and uptake-effect of Tat-HSP70, we evaluated fixed lung tissue to determine whether the presence or absence of injury affected HSP70 abundance. Data are depicted in Figure 2. Relative to T0 rats, endogenous HSP70 abundance remained unchanged in both injured or normal lung parenchyma in the 2CLP-PBS rats at 24 and 48 hr. However, relative to T0, we observed significant increase of HSP70 abundance in 2CLP-Tat-HSP70 rats in both injured and normal regions of lung sections at 24 (p<0.01) and (p<0.01) and 48 (p<0.02) and (p<0.01) hr, respectively. Relative to 2CLP-PBS rats, we see significant increase in HSP70 abundance in 2CLP-Tat-HSP70 rats, in both injured and normal lung regions at 24 (p <0.01) and (p <0.007) and 48 (p<0.0008) and (p<0.001) hr, respectively. Prior work by our colleagues using AdHSP70 increased HSP70 abundance by turning on mRNA expression. This may produce a homogenous expression of HSP70 abundance in the lung. Distribution of HSP70 abundances in injured or normal areas of the lung has never been shown. In our analysis, we show that direct delivery of Tat-HSP70 distributes evenly to all areas of the lung. Additionally, we aim to show that increased HSP70 abundance in injured as well as normal areas of the lung may contribute to an overall improvement of lung injury as depicted by histologic findings.
Figure 2. Tat-HSP70 increased HSP70 protein abundance in abnormal and normal lung sections following 2CLP

Note: Graphic comparison (mean ± SD) of HSP70 protein abundance in histologically normal and abnormal lung areas per nuclei/high-power field. All counts were performed on 10 randomly selected high-power sections per slide, one slide/rat, three rats/each group of intervention time-point. We were unable to detect a significant difference in HSP70 abundance in normal or injured lung tissue between T0 and 2CLP-PBS rats at 24 or 48 hr. Relative to T0, we observed significant increase in HSP70 abundance in 2CLP-Tat-HSP70 rats in histologically abnormal and normal regions of lung sections at both 24 (p<0.016 and p<0.012) and 48 (p<0.02 and p<0.01) hr, respectively. Relative to 2CLP-PBS, we observed significant increase in HSP70 abundance in injured lung parenchyma of 2CLP-Tat-HSP70 rats at both 24 (p <0.016) and 48 (p<0.0008) hr. Additionally, there were significant increase in HSP70 abundance in 2CLP-Tat-HSP70 rats in histologically normal lung regions at 24 (p <0.007) and 48 (p<0.001) hr compared to the 2CLP-PBS group. ^ = statistically different in abnormal and normal lung regions compared to T0. * = statistically different in abnormal and normal regions compared to 2CLP-PBS groups at same time points.
Intratracheal administration of Tat-HSP70 attenuated lung injury following 2CLP

Figure 3 shows representative H&E histologic stains of lung lobe sections from normal (T0), 2CLP-PBS and 2CLP-Tat-HSP70 rats over time. There was an incremental and heterogeneous distribution of lung injury from 2CLP-PBS rats at 24 and 48 hr. In contrast, no injury was noted in lungs from T0 rats and minimal injury was noted in 2CLP-Tat-HSP70 rats at 24 and 48 hr. See Table 2 in Appendix (page 138) for an itemized depiction of the H&E results. Treatment with Tat-HSP70 following 2CLP limited lung injury consistent with ARDS.
Figure 3. Tat-HSP70 attenuated lung injury following 2CLP

Note: Lung lobes were examined as a whole lobe using the Aperio Image Scope program at magnifications from 2x to 100x, one slide/rat, three rats/each group of intervention time-point. The images shown are magnified by 10x.
Intratracheal administration of Tat-HSP70 decreased MPO abundance at 48 hr following 2CLP

To determine the abundance of activated neutrophils migrating into lungs generating toxic metabolites, we examined frozen lung sections for MPO abundance in T0, 2CLP-PBS and 2CLP-Tat-HSP70 rats. Data is depicted in Figures 4a and b. We show presence of baseline MPO abundance in T0 rats. However, relative to T0 rats, MPO abundance was significantly increased in 2CLP-PBS rats at 24 (p<0.01) hr and profoundly increased at 48 (p<0.0006) hr. Relative to T0 rats, MPO abundance was also significantly increased in 2CLP-Tat-HSP70 rats at 24 hr (p<0.01) but there were no difference in levels observed at 48 hr. 2CLP did not significantly increase MPO abundance between PBS and Tat-HSP70 groups at 24 hr. However, treatment with Tat-HSP70 significantly reduced MPO abundance following 2CLP at 48 (p<0.03) hr. This demonstrates that Tat-HSP70 abundance attenuates MPO abundance with sicker animals at 48 hr following 2CLP.
Figure 4a. Immunohistochemical stain depicting MPO abundance at 48 hr following 2CLP

<table>
<thead>
<tr>
<th></th>
<th>Dapi stain- nucleus/alveolar cells</th>
<th>MPO stain-alveolar cells</th>
<th>Dapi/MPO labeled alveolar cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>2CLP-PBS-48h</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-48h</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Note:** Double labeled immunohistochemical stains for MPO abundance at different time points in pulmonary cells of T0 and 2CLP-PBS and 2CLP-Tat-HSP70 rats at 48 hr. Blue-nuclear marker depicts DAPI while MPO stains appear as bright red. Magenta stain indicates double-labeled DAPI+MPO cells. Magnification at 20X.
**Figure 4b.** Tat-HSP70 decreased MPO abundance at 48 hr following 2CLP

![Graphical representation of MPO abundance](image)

**Note:** Graphic comparison (mean ± SD) of MPO abundance in cytoplasm/nuclei, in lung regions. Counts were performed on 10 randomly selected high-power sections of lung lobe per slide, one slide/rat, three rats/each group of intervention time-point. Relative to T0 rats, we observed significant increase in MPO abundance in 2CLP-PBS rats at 24 (p<0.01) and 48 (p<0.0006) hr. Relative to T0, we observed significant increase in MPO abundance in 2CLP-Tat-HSP70 rats at 24 hr. We were unable to detect a difference in MPO abundance between T0 and 2CLP-Tat-HSP70 rats at 48 hr. Relative to 2CLP-PBS rats at 48 hr, there was significant decrease in MPO abundance in 2CLP-Tat-HSP70 rats at the same time point (p<0.03). * = statistically different from 2CLP-PBS at same time point. ^ = statistically different from T0.
Intratracheal administration of Tat-HSP70 did not increase survival following 2CLP

All rats in the 2CLP groups displayed visible physical and behavioral manifestations of sepsis as previously described (Wichterman et al., 1980). We observed lethargy with decreased movement, diarrhea, crusting exudate expressed from eyes, piloerection and lack of preening. These changes were less pronounced in the 2CLP-Tat-HSP70 groups especially at 48 hr. However, when survival analysis were conducted, we were unable to detect a significant difference in survival between 2CLP-PBS and 2CLP-Tat-HSP70 groups at 24 or 48 hr.
Figure 5: Tat-HSP70 did not increase survival following 2CLP

![Graph showing survival probability over time for different groups.](image)

Note: Kaplan Meier survival analysis of T0, 2CLP-PBS rats at 24 and 48 hr, and 2CLP-Tat-HSP70 rats at 24 and 48 hr. N of 3 rats in T0, 2CLP-PBS, and 2CLP-Tat-HSP70 groups. N of 8 and n of 5 in 2CLP-PBS and 2CLP-Tat-HSP70 rat groups at 48 hr respectively. We were unable to detect a difference in mortality between 2CLP-PBS and 2CLP-Tat-HSP70 rat groups at 24 hr. We observed mortality of 38% in the 2CLP-PBS rat group at 48 hr and mortality of 20% in the 2CLP-Tat-HSP70 rats at the similar time point but it was without statistical significance (p<=0.5).
Discussion

Use of the Tat peptide as a protein delivery agent was associated with an increase in the abundance of HSP70 in pulmonary epithelial cells of rats with lung injury induced by 2CLP. We show that direct intratracheal administration of Tat-HSP70 protein builds HSP70 abundance in pulmonary cells at 24 and 48 hr, in both injured and normal lung regions, reduced MPO abundance at 48 hr and attenuated histological evidence of lung injury.

Tat-HSP70 as a bio-therapeutic protein

Work by Weiss et al. (2007, 2008, 2011) demonstrated that administration of AdHSP70 directly in the lung decreased 2CLP induced activation of NF-κB, proliferation of Type II pulmonary epithelial cells and apoptosis of Type I cells. In each case, HSP70, presumably derived from AdHSP70, formed protein complexes that reduced the activity of the complex enzymes involved in these processes. AdHSP70 presumably stabilized proteins in complexes that were less active than those that form in the absence of HSP70. In the case of NF-κB, HSP70 stabilized smaller, less active oligomers of NF-κB/IKB/IKK. During apoptosis, HSP70 interfered with activation of the apoptosome and caspase-8, and directly bound to caspase-9 and caspase-3 to limit their activity. In the case of cell proliferation, HSP70 stabilized the retinoblastoma/E2Fi complex, preventing dissociation.

Among the mediators activated by degradation of NF-κB are neutrophils. Studies depicting an inflammatory model using lipopolysaccharide show that high levels of
IkBα in the nucleus inhibits NF-κB activity and causes neutrophil inactivity and apoptosis (Castro-Alcaraz, Miskolci, Kalasapudi, Davidson, & Vancurova, 2002). Increased activity of NF-κB in neutrophils propagates inflammation and is associated with lung injury and sepsis (Wright & Christman, 2003; Yang, Arcaroli & Abraham, 2003). A therapeutic goal in sepsis and ARDS is the development of agents to regulate mediators and mechanisms leading to neutrophil activation in the lung. MPO, a product of activated primary neutrophil (azurophil) granules generates toxic free radical oxygen metabolites (Kutter, et al., 1997). MPO has been used as a marker of neutrophil activation, whether appropriately or inappropriately regulated, from BAL specimens of patients with ARDS (Pittet, et al.,1997; Weiland, et al., 1986). We show that both intratracheal Tat-HSP70 and AdHSP70 significantly reduced MPO abundance, an indicator of neutrophil abundance at 48 hr.

In summary, AdHSP70 introduced an mRNA encoding HSP70 into cells to increase HSP70 abundance. Therefore, the process required translation as well as a potentially dangerous viral vector. In contrast, the current study used Tat conjugated to HSP70 protein as an alternate method to directly increase HSP70 protein abundance, an approach that makes enhanced abundance independent of endogenous intracellular pathways. Of interest, the increase in HSP70 abundance occurred preferentially in abnormal and normal regions of 2CLP lung injured cells. Although Tat-HSP70 increased HSP70 protein abundance in injured lung sections and normal lung sections, we do not know if the normal areas were unaffected in the first place, something that is known to
occur in ARDS, or because they were damaged but then recovered following HSP70 abundance. It may be that there was no injury to repair in the normal regions.

Additionally, our study did not explore the role of Tat-HSP70 protein in repair of Type I and/or Type II specific pulmonary cells. Nevertheless, studies by Weiss et al., (2002, 2008, 2011) indicated that uptake of HSP70 following delivery of AdHSP70 was primarily in Type II cells, reducing pulmonary apoptosis that occurred in both Type I and II cells, and reduced Type II cell proliferation. Physiologic ventilation and perfusion testing to determine the extent of improvement in lung injury contributing to return in functional capacity would also be of immense value.

**Delivering Tat-HSP70 across cell membrane**

The difficulty in targeted protein delivery lies in the inability of the hydrophilic peptides or proteins to cross the hydrophobic lipid bilayer of the cell membrane. The use of protein transduction domains (PTDs) has gained favor since their discovery over a decade ago. The most common and well-studied PTDs include the Drosophila melanogaster transcription factor protein antennapedia (Antp), the herpes simplex virus-1 DNA-binding structural protein (VP22), and the HIV-1 Tat protein. It is not known if each of these PTDs traverses the lipid membrane via the same mechanism. PTDs have been used in several different ways. Green and coworker (1988) and Frankel and coworker (1988) independently found that residues one-86 of the Tat protein encode a region that could cross cell membranes and trans-activate viral replication in tissue culture. The specific Tat protein region that allows for trans-activation and transduction is
sequence YGRKKRRQRRR of the basic domain region (amino acids 47 to 57) (Green & Loewenstein, 1988). Tat has been used extensively to deliver peptide or proteins both in \textit{in vitro} and \textit{in vivo} models (Dass & Choong, 2006; Fawell, et al., 1994; Rapoport & Lorberboum-Gaiski, 2009). Tat has been conjugated and successfully delivered via intraperitoneal, intravenous, subcutaneous, and intratracheal routes (Hotchkiss, et al., 2006; Inagaki, et al., 2003; Kilpatrick, et al., 2011; Schwarze, et al., 1999; Simon, Kang, Gao, Banta & Morrison, 2010).

Several mechanisms exist to explain how Tat and its cargo peptide enter cells. A currently popular mechanism hypothesizes that Tat binds to a lipid raft of the plasma membrane and transduction occurs through an energy dependent, receptor independent, macropinocytosis (an endocytosis) mediated pathway (Gump and Dowdy, 2007; Kaplan, et al., 2005). The Tat-cargo peptide is taken up into a cellular compartment of macropinosomes and released through an endosomal escape mechanism into the cytoplasm. Questions remain about possible entrapment of the PTD in endosomal vesicles or macropinosomes and whether this would contribute to delayed or negligible release of the cargo peptide once Tat is able to get through the plasma membrane. Nevertheless, characteristics of Tat that are relevant to the process of transduction include its highly cationic and hydrophilic nature facilitating transduction through electrostatic interaction with the plasma membrane, rich arginine and lysine residues, physiological pH, and ability to transduce despite covalent binding with a cargo protein (Gump & Dowdy, 2007; Del Gaizo Moore & Payne, 2004).
In a conceptually-related study, our collaborator Dr. Laurie Kilpatrick (Kilpatrick et al., 2011) showed that direct intratracheal injection of a PKC-δ inhibitor peptide that was chemically coupled to the YGRKKRRQRRR basic domain region of the Tat PTD via an N-terminal Cys-Cys bond facilitated peptide delivery into pulmonary cells. This approach attenuated lung injury in the 2CLP rat model. In contrast, our Tat-HSP70 fusion protein was created by using a bacterial expression vector capable of producing large amounts of fused protein (Nagahara, et al., 1988; Wheeler, et al., 2003). The open reading frame of the cDNA protein of interest is cloned in frame downstream of an N-terminal leader sequence with a 6-histidine residue purification tag and a Tat-PTD sequence. Indeed, in our in vivo model, Tat-HSP70 does work to reduce lung injury in a similar fashion to the chemically couple peptide.

Implications of Tat-HSP70 in attenuating lung injury.

Optimization of the novel Tat-HSP70 protein requires determining dose-dependent efficacy, uncovering cytotoxic properties and identifying the time-points that HSP70 protein administration is most beneficial to attenuate lung injury in the 2CLP model. Our work focused on one intratracheal delivered dose, 100 µg, following a pilot study that used doses of 50 µg (results not published). Prior investigations using a mammalian embryonic fibroblast cell line demonstrated that a dose of 300 nM of Tat-HSP70 was sufficient to protect against hyperoxic cell injury but 800 nM was toxic (personal communication with Katherine Dunsmore, research assistant in the lab of Dr. H. Wong, Cincinnati, Ohio).
PTDs are known to be cytotoxic to select in vitro cell lines. In a study evaluating toxicity effects from the use of either the Tat, Antp, Rev and VP22 PTDs, Sugita et al. (2008) observed that use of higher concentrations of the Antp PTD to transduce cargo proteins in HeLa and Jurkat cells resulted in loss of plasma membrane integrity, leakage of cargo protein and limited specificity of transduced proteins inside the cell. Further, studies performed in in vitro models observed that choice of PTD and selection of specific in vitro cell lines used contributed to cytotoxic effects (Khaja & Robbins, 2009). Our study did not reveal overt adverse effects or toxicity from the use of Tat-HSP70 when compared to 2CLP-PBS animal groups at equivalent time points. Nevertheless, protein-drug dosage studies evaluating therapeutic dose response curve are necessary prior to extrapolating data for clinical practice.

Work with AdHSP70 showed that HSP70 expression peaked at 48 hr following administration, returning to baseline within one week (Weiss et al, 2002). Additionally, administration of AdHSP70 did not increase HSP70 expression until 16 hr following 2CLP. We believe that the protein will not be effective before 16 hr because disease is not evident prior to that time. Because mortality from 2CLP can reach 75% at 48 hr in a rodent model, the Tat-HSP70 dose response experiments targeting the lung may not prove fruitful past the 48 hr point. Future studies would also evaluate systemic distribution and possible uptake of HSP70 by other organs following delivery of intratracheal Tat-HSP70.

There are several advantages of delivering Tat-HSP70 directly into lungs including targeted delivery, rapid uptake and easy dosage concentration dependence.
Nevertheless, there are many facets of the process that remains to be explored. These include the exact mechanism of lung recovery, adverse effects, potential toxicity, distribution and bioavailability in an *in vivo* model. In conclusion, studies into the feasibility of Tat-HSP70 as a useful treatment for lung injury secondary to sepsis appears promising and should be explored further.
CHAPTER 3

THE USE OF TAT-HSP70 AND EXPRESSION OF INFLAMMATORY BIOMARKERS IN THE LUNG FOLLOWING 2CLP
Abstract

Sepsis, a syndrome of dysregulated inflammation, is the leading cause of death in the intensive care unit. The organ most injured is the lung, with abnormalities taking the form of the Acute Respiratory Distress Syndrome (ARDS). To date, only the use of low tidal volume ventilation has been proven to decrease mortality and hospital length of stay. However, use of exogenous respiratory support compounds immobility and critical illness polyneuropathy (CIP). Interventions that limit the extent of lung injury would provide both direct and indirect benefit. The cecal ligation and double puncture (2CLP) rodent model of sepsis mimics many of the features of the human syndrome including ARDS, CIP, and decreased locomotion. The Heat Shock Response, characterized by expression of Heat Shock Proteins (HSPs) is an endogenous mechanism to protect cells from injury. Our research focuses on one of these proteins, HSP70, which is not induced by 2CLP. We hypothesize that enhancing HSP70 expression would protect against 2CLP-induced lung injury. To accomplish this we delivered an HIV-1 Tat-HSP70 fusion protein into the lung. We found that, relative to septic non-treated animals (2CLP-PBS), rats treated with Tat-HSP70 (2CLP-Tat-HSP70) had significantly increased HSP70 abundance and less pronounced histologic lung injury but we were unable to detect significant effects in reduction of lung Chemokine Induced Neutrophil Chemoattractant-1, Macrophage Inflammatory Protein-2 and Interleukin-6 protein abundance. Further, relative to 2CLP-PBS rats, we were unable to detect a difference in rodent locomotion in the 2CLP-Tat-HSP70 groups. We conclude that further investigation of the use of Tat-HSP70 to treat 2CLP-induced lung injury is required.
Introduction

Sepsis, a fatal disorder of dysregulated inflammation, is the leading cause of death in critical care. The most prevalent study to date compiled in 2013 estimated that sepsis affected 1 million people annually (Gaieski et al., 2013). The financial toll incurred by patients and the healthcare system was estimated at $24 billion annually, 40% of intensive care unit (ICU) costs (Lagu et al., 2011). If unchecked, sepsis progresses to multiple organ dysfunction syndrome and death. To date, the available treatment approaches are merely supportive. The most common organ system abnormality is the Acute Respiratory Distress Syndrome (ARDS) in the lungs most often occurring within 72 hr of the onset of sepsis (Martin et al., 2009). Mortality rates of patients with sepsis and ARDS have been reported to be between 25-30% (Gaieski, et al., 2013; Spragg, et al., 2010; Wang, et al., 2010).

ARDS survivors have significant post-morbid dysfunction. Chief among these are impaired ventilation and gas exchange. However, the prolonged time course of ARDS leads to additional abnormalities. Of particular importance is prolonged neuromuscular dysfunction that can leave patients with severe post-syndrome debilitation (Garnacho-Montero, et al., 2005). Studies evaluating one and five-years outcome of ARDS survivors documented persistent limitations in pulmonary function and exercise endurance that normalized by year five, pronounced muscle weakness, wasting and fatigue that resulted in persistent functional limitations (Herridge, et al., 2003, 2011). The burden of costs attributed to loss of wages, re-hospitalizations, medications, home care therapies and stress to caregivers were overwhelming. Importantly, early recovery of organ function...
was associated with a better functional ability at one-year follow-up (Herridge, et al., 2003). In addition, initiation of a mobility protocol within 48 hr of diagnosis resulted in fewer days in bed, a shorter ICU course and reduced length of hospital stay (Morris, et al., 2008). Therefore, interventions that can prevent or reverse the intensity of sepsis and ARDS and permit early mobilization may reduce functional impairment in survivors.

The Heat Shock Response (HSR), characterized by enhanced expression of Heat Shock Proteins (HSPs) is one highly conserved endogenous mechanism that can protect cells from injury. In particular, the inducible 70 kDa HSP70 family, which specifically responds to protein unfolding and inhibits further denaturation, provides an essential element of cellular protection (Parsell & Lindquist, 1993). In addition, intracellular HSP70 limits the intensity of the inflammatory response to cell stress or injury. However, cecal ligation and double puncture (2CLP) rodent model of sepsis was associated with a failure of HSP70 expression in the lung (Weiss, et al., 2000; Villar, et al., 1994). In previous studies, we found that restoration of HSP70 abundance in the lungs using an adenovirus HSP70 expression system (AdHSP70) significantly decreased 2CLP-induced ARDS pathology, and increased survival by 31% at the 48 hr time point (Weiss, et al., 2002, 2001). AdHSP70 limited 2CLP-mediated activation of NF-κB, a transcription factor that regulates the expression of key pro-and anti-inflammatory mediators (Weiss, et al., 2008), attenuated proliferation of Type II pulmonary epithelial cells, a process that is believed to lead to pulmonary fibrosis and scarring and reduced apoptosis (Bromberg et al., 2012; Weiss et al., 2007). However, adenovirus-based gene therapy in humans is problematic. Associated complications include severe adverse host immune responses as
adenovirus therapy in humans may provoke excessive inflammation in the transduced cells, dysregulated triggering of NF-κB signal transcription of inflammatory pathways and the development of excessive cytolytic responses (Muruve, 2004). Additionally, development of viral traps and liver sequestration of the adenovirus vectors that limit the effectiveness of the virus may occur (Anderson, 1998; Khare, et al., 2011; Marshall, 1999; Alba, Bosch & Chillon, 2005; Weiss et al., 2002). This limits the clinical use of these approaches and suggests that the development of alternative means of protein delivery is desirable.

One potential protein-delivery vehicle is the Trans-Activator of Transcription (Tat), an eleven amino acid protein transduction domain derived from the Type 1 HIV virus (Gump & Dowdy, 2007; Green & Loewenstein, 1998; Nagahara, et al., 1998). Tat is not toxic or biologically infective and is incapable of replicating itself. Recent work shows that protein transduction occurs quickly, in an energy dependent but receptor independent mechanism. Several theories exist regarding Tat’s protein transduction mechanism, but current evidence supports a macropinocytosis-endocytosis pathway (Gump and Dowdy, 2007; Kaplan, et al., 2005). Tat has been used to transduce more than 50 proteins, ranging in size from 15 to 120 kD, into human and rodent cells (Begley, 2004; Ezhevsky, et al., 1997; Hotchkiss, et al., 2006; Nagahara, et al., 1998).

A central mechanism in lung injury from sepsis is the accumulation, adhesion, migration and activation of neutrophils (Grommes, & Soehnlein, 2011). IL-8, a member of the CXC family of chemokines actively participates in the inflammatory response as a leukocyte chemotactic agent in the lungs (Kunkel, et al., 1991; Standiford, et al., 1990).
In rodents, the mediators that are homologous to IL-8 are Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1) protein and Macrophage Inflammatory Protein-2 (MIP-2) (Driscoll, 1994; Watanabe, et al., 1989). Studies show that elevated levels of IL-8 in bronchoalveolar (BAL) specimens of patients with ARDS correlate with elevated levels of neutrophils and death (Miller, et al., 1992; Chollet-Martin, et al, 1993). Studies using the 2CLP model of ARDS show excessive production of CXC chemokines, especially CINC-1 and MIP-2 from either plasma, serum or tissue homogenate (Guo et al., 2006; Strieter, et al., 2005).

Another key mediator in the synthesis of acute phase proteins responding to pathogens and injury is the pro-and anti-inflammatory cytokine IL-6 (Friedland, et al., 1992; Hack, et al., 1989; Remick, et al., 2002). Studies evaluating plasma, serum or BAL levels of IL-6 in patients at varying time-points of the sepsis syndrome and or ARDS have found IL-6 levels prognostic of morbidity or mortality (Hack, et al., 1989; Parsons, et al., 2005; Yende, et al., 2008). Numerous studies using the 2CLP model have correlated measurements of IL-6 levels at varying time-points to disease severity and mortality.

In a previous paper, we used intratracheal delivery of the fusion protein Tat-HSP70 to increase HSP70 protein abundance in the lung 24 and 48 hr following 2CLP. We showed that Tat-HSP70 preferentially increased HSP70 abundance in abnormal regions of the lung, improved lung histology at 24 and 48 hr and significantly decreased neutrophil associated Myeloperoxidase abundance at 48 hr. In this article, we sought to determine the effects of Tat-HSP70 on CINC-1, MIP-2 and IL-6 in lung tissue following
2CLP as a measure of efficacy. Additionally, we hypothesized that decreased lung injury in the 2CLP-Tat-HSP70 treated rats facilitates an indirect outcome of improved locomotor activity.

**Material and Methods**

**Production of Tat-HSP70**

A replication vector containing the Tat-HSP70 fusion protein, kindly provided by Hector Wong MD, Cincinnati Children’s Hospital, was expressed in *Escherichia Coli* as previously described (Wheeler et al, 2003). Recombinant Tat-HSP70 fusion protein was reproduced and purified by Impact Biologicals, Inc., Swarthmore, Pennsylvania.

**Animal protocol and induction of sepsis**

This study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. The rodent experiments were conducted in an approved facility overseen by University Laboratory Animal Resources personnel. Studies were performed on male Sprague Dawley (SD) rats, weighing 250 to 300 grams, purchased from a regulated breeder, Charles River Labs (Boston, Massachusetts, USA). Rats were housed in a climate controlled, 12h light/12h dark cycle facility and allowed free access to food and water. Previous studies have used SD rats and they are known to reliably develop ARDS after 2CLP (Weiss, et al., 2000).

2CLP was performed using Isoflorane anesthesia as previously described (Weiss et al., 2000). Intratracheal instillation of Tat-HSP70 or Phosphate Buffered Saline (PBS)
via a tracheotomy was conducted as previously described (Weiss et al., 2002). A total of 200 µl of either Tat-HSP70 solution (100 µg dose diluted in PBS) or PBS was injected. This was followed with two ml of air to assure equal distribution of agent into lungs (Weiss, et al., 2002). The wound was closed and the animals were allowed to recover. Immediately following surgery and every 24 hr thereafter, animals were fluid resuscitated with 50 ml/kg of 0.9% saline. A dose of 100 µg was chosen after a pilot study demonstrated that a 50 µg dose failed to increased HSP70 abundance.

A total of 54 rats were separated into four groups of animals: (i) unoperated controls (T0), (ii) sham operated (SO) controls (consisting of animals subjected to laparotomy without 2CLP or tracheotomy), (iii) animals subjected to 2CLP and immediately thereafter administered PBS via tracheotomy (2CLP-PBS), and (iv) animals subjected to 2CLP and immediately thereafter administered PBS containing 100 µg of Tat-HSP70 (2CLP-Tat-HSP70). Data presented in Table 4, Appendix D (page 140), details the number of samples/rats used from each rat intervention group, analyzed for the T0, 24 and 48 hr groups. Prior studies have established that survival from 2CLP at 24 hr and 48 hr is approximately 50% and 25% respectively, and that statistical significance can be achieved with three surviving animals at each time point. Designated rats were euthanized and lung lobes were removed en bloc, gently perfused with 0.9% saline and homogenized for whole lung protein extraction. Samples were stored in freezer set at -80°C.

**Surgical insertion of E-mitter transponder**
The E-mitter probe (Respirronics, Inc., 2011) was surgically inserted into the animals approximately seven days prior to SO or 2CLP procedures and assessing locomotion. This is recommended for optimal acclimatization and recovery prior to assessing for data related to locomotion. Following adequate anesthesia using Isoflurane, animals were prepped in a sterile fashion, a midline laparotomy incision was performed and a small pre-sterilized E-mitter transponder (less than one inch in length) was implanted into the peritoneal cavity of the animal. The E-Mitter probe was placed along the vertical plane, dorsal (the back side) to the digestive organs. The incision was closed in two layers. As systemic administration of postoperative opioids alters septic responses and causes respiratory depression, post-op pain was treated with a single subcutaneous injection of Bupivicaine, 0.8 ml/kg of 0.25% solution, between muscle and skin at the end of the procedure (Bottoms & Adams, 1995; Roughan & Flecknell, 2004). Animals were resuscitated with 50 ml/kg of 0.9% saline subcutaneously. Animals were allowed to awaken, placed in individual cages where they had access to water and food. Post operative monitoring for complications were managed as discussed above. Recovery from laparotomy is rapid, locomotion and circadian core body temperature normalized within a day, and body weight, food and water intake returned to normal within two days in a previous study of transmitter implantation (Leon, Walker, DuBose & Stephenson, 2004).

**Whole lung protein extract**

Frozen lung lobes were diced into small particles and thawed in cold Radio-Immuno- Precipitation Assay buffer (RIPA) with Protease/phosphate inhibitors. RIPA
buffer contained 50 mM Tris-HCL, titrated to a pH of 8, 150 mM Sodium Chloride, 5 mM Ethylenediaminetetraacetic acid (EDTA), 10mM Sodium Flordie, 1% Tergitol-type NP-40, 0.5% Sodium Deoxycholate, and 0.1% Sodium Dodecyl Sulfate (SDS). Tissue was homogenized at 4°C. The homogenate was transferred to microcentrifuge tubes and spun at 10,000 xG for 10 minutes at 4°C. The supernatant was removed and re-centrifuged to obtain the final protein extract. Final protein extract was subsequently filtered through an Amicon Ultra-4 50K Centrifugal Filter Device (Merck Millipore, Billerica, MA) to maximize protein sample recovery. Protein concentration for each sample was determined using the Microplate BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Protein samples were aliquoted and stored at -80°C until assays were performed.

**Rodent locomotor activity**

Rodent locomotor activity was examined using the Vital View Series 4000 Mini-Mitter Telemetry monitoring system (Respironics Inc., 2011; Harkin, O’Donnell, & Keley, 2002). This system collects core body temperature and movement. The system consists of: (a) an E-mitter flatbed receiver that is placed under the cage of the animal and uses a radiofrequency field to collect transponder data, and (b) a data port connected to the receiver and managed by a Windows PC based data acquisition system (Harkin, et al., 2002). Animals were acclimatized to the room in which locomotion was assessed for approximately one hr prior to actual locomotion monitoring. Locomotion in each animal was assessed for a period of one hr, at one time point, beginning at 7 pm (the start of increased nocturnal activity). Rat gross motor activity was detected as alterations in
transmitter signal strength with each movement and data were recorded as counts in one second bins (Respironics, Inc., 2011; Harkin, 2002). Locomotion counts were categorized as following: (i) one to 100 counts: sedentary to light movement, (ii) 101 to 500 counts: light movement to moderate movement, and (iii) 501 counts to over 1000 counts: moderate movement to vigorous movement.

**CINC-1, MIP-2 and IL-6 protein activity**

Abundance of CINC-1 (Rat CXCL1/CINC-1, R&D Systems, Minneapolis, MN), MIP-2 (Rat GRO-b, Antigenix America, Huntington Station, NY) and IL-6 (Invitrogen, Camarillo, CA) protein in whole lung homogenate were measured by Enzyme-Linked Immunosorbent Assay (ELISA) kits per the individual manufacturer’s instructions. Results were determined by spectrophotometry using a microplate reader (SpectrMax 190, Molecular Devices, Sunnyvale, CA). The inter-assay coefficient of variation assessing variability was ≤ 10 %.

**Statistics**

Comparisons of mean abundance of CINC-1, MIP-2 and IL-6 protein levels, mean locomotion counts and mean in temperature, between and within multiple groups were performed using a two-tailed ANOVA with a Bonferroni adjustment for multiple comparisons. For survival studies, Kaplan Meier and Cox proportional hazard regression analyses were performed on SAS version 9.3 (SAS corporation). Significance level was set at p<0.05.
Results

Tat-HSP70 did not reduce 2CLP-mediated abundance of CINC-1 but reduced MIP-2 protein abundance at 24 hr in the lung

To investigate the levels of neutrophil chemoattractants in the lung during 2CLP mediated lung injury, we assayed protein levels of CINC-1 and MIP-2 in whole lung homogenate.

We observed minimal abundance of CINC-1 in T0 rats, as was the case in SO rats at both 24 and 48 hr. In contrast, relative to T0, CINC-1 abundance following 2CLP was significantly higher in PBS rats at 24 hr (p<0.01) but levels were not significant at 48 hr. Relative to SO rats, CINC-1 abundance following 2CLP was significantly higher in PBS rats at 24 hr (P<0.007) but levels were not significant at 48 hr. Similarly, relative to T0 and SO rats, CINC-1 abundance was significantly increased in 2CLP-Tat-HSP70 rats at 24 hr (p<0.03) and (p<0.01) respectively, but not at 48 hr. We were unable to detect a significant difference in abundance of CINC-1 between 2CLP-PBS rats and 2CLP-Tat-HSP70 rats at either 24 or 48 hr (Figure 6).
Figure 6: Tat-HSP70 did not reduce abundance of CINC-1 in the lung following 2CLP

Note: Graphic representation of lung homogenate mean protein levels ± SD of CINC-1 abundance. Levels measured in T0, SO, 2CLP-PBS and 2CLP-Tat-HSP70 rats at 24 and 48 hr. N of 2 animals in T0, n of 3 in both SO groups, n of 7 in 2CLP-PBS at 24 and 48 hr and n of 6 and 8 in the 2CLP-Tat-HSP70 group at 24 and 48 hr respectively. Relative to T0, CINC-1 abundance following 2CLP was significantly higher in PBS rats at 24 hr (p<0.01). Relative to SO rats, CINC-1 abundance following 2CLP was significantly higher in PBS rats at 24 hr (P<0.007). Similarly, relative to T0 and SO rats, CINC-1 abundance was significantly increased in 2CLP-Tat-HSP70 rats at 24 hr (p=0.03) and (p<0.01) respectively. ^= statistically different compared to T0. *= statistically different compared to SO at same time point.
We detected significant increase in MIP-2 abundance between T0 and SO 24 hr rats relative to 2CLP-PBS rats at 24 hr (p<0.01) and (p<0.001) respectively. Relative to 2CLP-PBS rats at 24 hr, we detected significant decrease in MIP-2 abundance in 2CLP-Tat-HSP70 rats (p<0.03) at the similar time-point. We were unable to detect significant differences between any other groups (Figure 7).
Figure 7: Tat-HSP70 reduced abundance of MIP-2 in the lung following 2CLP at 24 hr

Note: Graphic representation of lung homogenate mean protein levels ± SD of MIP-2 abundance. Levels measured in T0, SO, 2CLP-PBS and 2CLP-Tat-HSP70 rats at 24 and 48 hr. N of 2 animals in T0, n of 4 rats in SO group at 24 hr, n of 7 rats in 2CLP-PBS group at 24 hr, n of 9 rats in 2CLP-Tat-HSP70 group at 24 hr, n of 4 rats in SO group at 48 hr, n of 8 rats in 2CLP-PBS group at 48 hr and n of 8 rats in 2CLP-Tat-HSP70 group at 48 hr. Relative to TO rats and SO at 24 hr, we detected significant increase in MIP-2 abundance in 2CLP-PBS rats (p<0.01) and p<0.001 respectively. Relative to 2CLP-PBS rats at 24 hr, we detected significant reduction of MIP-2 abundance in 2CLP-Tat-HSP70 rats at 24 hr (p<0.03). We were unable to detect significant differences between combinations of any other group. ^= statistically different compared to T0 and SO 24 hr. *=statistically different to 2CLP-PBS at similar time point.
Tat-HSP70 increased abundance of IL-6 in the lung following 2CLP

To determine the effects of Tat-HSP70 abundance on lung IL-6 abundance, we compared T0 to SO, 2CLP-PBS and 2CLP-Tat-HSP70 rats at 24 and 48 hr (Figure 8). We observe relative to T0, we were unable to detect a significant difference in IL-6 abundance in any other groups. Relative to SO, we were unable to detect a significant difference in levels between any other groups. Relative to 2CLP-PBS rats at 48 hr, we detected a significant increase (p< 0.01) in IL-6 abundance in 2CLP-Tat-HSP70 rats at the same time point. Last, we observed a significant increase in IL-6 abundance in 2CLP-Tat-HSP70 rats at 48 hr (p<0.02) when compared to 2CLP-Tat-HSP70 rats at 24 hr. IL-6 is known to have variable expression levels at different time points of sepsis and 2CLP induced ARDS. Correlation between baseline IL-6 levels to levels following 2CLP-PBS rats at 24 and 48 hr and the significant increase in IL-6 levels in the 2CLP-Tat-HSP70 rats at 48 hr should be explored further.
Figure 8: Tat-HSP70 increased abundance of IL-6 in the lung following 2CLP

Note: Graphic representation of lung homogenate mean protein levels ± SD of IL-6 abundance. Levels measured in T0, SO, 2CLP-PBS and 2CLP-Tat-HSP70 rats at 24 and 48 hr. N of 2 animals in T0, n of 3 in all other groups. Relative T0 or SO groups, we were unable to detect a significant difference in IL-6 abundance in any other groups. Relative to 2CLP-PBS rats at 48 hr, we detected a significant increase (p< 0.01) in IL-6 abundance in 2CLP-Tat-HSP70 rats at the same time point. Last, we observed a significant increase in IL-6 abundance in 2CLP-Tat-HSP70 rats at 48 hr (p<0.02) when compared to 2CLP-Tat-HSP70 rats at 24 hr. *=statistically different to 2CLP-PBS at same time point.
^=statistically different to 2CLP-Tat-HSP70 rats at 24 hr.
Tat-HSP70 did not increase rat locomotion following 2CLP

To evaluate if Tat-HSP70 improved motor activity following 2CLP, we assessed gross exploratory locomotor activity in SO, 2CLP-PBS and 2CLP-Tat-HSP70 rats at 24 and 48 hr (Figure 9). Relative to SO rats at 24 hr, we observed significant decrease in locomotion in 2CLP-PBS rats (p<0.04) and 2CLP-Tat-HSP70 rats at 24 hr (p<0.003), respectively. Relative to SO rats at 48 hr, we observed significant decrease in locomotion in 2CLP-PBS rats (p<0.01) at 48 hr but we did not observe any significance in the 2CLP-Tat-HSP70 group at the same time-point. Relative to 2CLP-Tat-HSP70 rats at 24 hr, we observed significant increase in locomotion in 2CLP-Tat-HSP70 rats at 48 hr (p<0.05). We were unable to detect a significant increase in locomotion in 2CLP-Tat-HSP70 rats compared to 2CLP-PBS rats at either 24 or 48 hr.
Figure 9: Tat-HSP70 does not have an effect on rat locomotion following 2CLP

Note: Graphic representation of mean locomotion counts per second measured over an hr ± SD in rats. Levels measured in SO, 2CLP-PBS and 2CLP-Tat-HSP70 rats at 24 and 48 hr. N of 6 rats in the SO group at 24 hr, n of 7 rats in SO at 48 hr, n of 4 rats in 2CLP-PBS at 24 hr, n of 9 rats in 2CLP-PBS at 48 hr, n of 8 rats and n of 7 rats, respectively, in 2CLP-Tat-HSP70 groups at 24 and 48 hr respectively. Relative to SO at 24 hr, we observed significant decrease in locomotion in 2CLP-PBS rats (p<0.04) and 2CLP-Tat-HSP70 rats at 24 hr (p<0.003), respectively. Relative to SO rats at 48 hr, we observed significant decrease in locomotion in 2CLP-PBS rats (p<0.01) at 48 hr but not in the 2CLP-Tat-HSP70 group at the same time point. Relative to 2CLP-Tat-HSP70 rats at 24 hr, we observed significant increase in locomotion in 2CLP-Tat-HSP70 rats at 48 hr (p<0.05). We were unable to detect a significant increase in locomotion in 2CLP-Tat-HSP70 rats compared to 2CLP-PBS rats at either 24 or 48 hr. *= statistically significant compared to SO at similar time-points. ^=statistically significant compared to similar group at 24 hr.
Tat-HSP70 did not increase core rat temperature following 2CLP

To determine if Tat-HSP70 had an effect on regulating temperature following 2CLP we monitored core rat temperature as measured by the E-mitter transponder placed in the intraperitoneal space. Relative to SO rats at 24 hr, we observed a significant decrease in temperature of 2CLP-PBS rats at 24 hr (p<0.05). However, we were unable to detect difference in temperature between all other groups (Figure 10). Prior studies have established baseline rat temperature for male SD rats taken by intraperitoneal probe to be at 37.4° C (Tkacs, Li & Strack, 1997).
Figure 10. Tat-HSP70 did not increase core rat temperature following 2CLP

Note: Graphic representation of mean temperature (Celsius) in rats ± SD. N of 6 rats in the SO group at 24 hr, n of 7 rats in SO at 48 hr, n of 4 rats in 2CLP-PBS at 24 hr, n of 9 rats in 2CLP-PBS at 48 hr, n of 8 rats and n of 7 rats, respectively, in 2CLP-Tat-HSP70 groups at 24 and 48 hr respectively. Relative to SO rats at 24 hr, we observed a significant decrease in temperature of 2CLP-PBS rats at (p<0.05) 24 hr *=statistically significant compared to SO at 24 hr.
Tat-HSP70 increased survival at 48 hr following 2CLP

To determine if Tat-HSP70 had an effect on survival, we analyzed mortality data of rats at the designated 24 and 48 hr time points (Figure 11). We analyzed combined data on rats depicted in Table 3 and 5, listed in Appendix. Of 14 rats administered Tat-HSP70 following 2CLP, 86% survived to 48 hr. Of 20 rats that only received PBS following 2CLP, 65% survived at 48 hr, p<=0.09, comparing survival plots, indicating a trend toward survival in the 2CLP-Tat-HSP70 group at 48 hr.
Figure 11: Tat-HSP70 increased survival at 48 hr following 2CLP

Note: Kaplan Meier survival analysis of T0, 2CLP-PBS rats at 24 and 48 hr, and 2CLP-Tat-HSP70 rats at 24 and 48 hr. N of 3 rats in T0, n of 8 rats in 2CLP-PBS and n of 9 rats in 2CLP-Tat-HSP70 at 24 hr, respectively, and n of 12 rats in 2CLP-PBS and n of 9 rats in 2CLP-Tat-HSP70 at 48 hr, respectively. We observed mortality of 10% in both 2CLP-PBS and 2CLP-Tat-HSP70 rat groups at 24 hr, mortality of 35% in the 2CLP-PBS rats at 48 hr and mortality of 14% in the 2CLP-Tat-HSP70 rats at the similar time point (p<=0.09, indicating a trend toward survival in the 2CLP-Tat-HSP70 48 hr group.)
Discussion

We previously showed (Chapter 2), that it is possible to increase HSP70 protein abundance in the lung of a 2CLP model by exogenous administration using the HIV-1 Tat-HSP70 fusion protein. Further, we showed that relative to septic non-treated rats, direct intratracheal administration of Tat-HSP70 increased HSP70 protein abundance in both abnormal and normal regions of the lung. Intratracheal administration of Tat-HSP70 ameliorated the histo-pathologic progression of ARDS by limiting changes in septal thickening, the presence of hyaline membrane formation, the development of fibrin/edema, areas with alveolar collapse, presence of lymphocytes and plasma, influx of neutrophils and percentage of area damaged attributed to inflammation. Last, intratracheal treatment with Tat-HSP70 significantly decreased lung Myeloperoxidase abundance at 48 hr (limiting the generation of toxic metabolites in lung injury).

In this paper, we evaluated the effect of intratracheal administration of Tat-HSP70 on abundance of CINC-1, MIP-2 and IL-6, chemokines and a cytokine that are known to mediate inflammation in the pathogenesis of ARDS. Unbalanced activation or heterogeneity of the immune response are common in both the sepsis and ARDS syndromes, therefore these responses were assessed at the 24 and 48 hr time points (Kellum, et al., 2007; Remick et al., 2005; Yende, et al, 2008). To assess a potential indirect benefit of lung recovery, we evaluated a secondary behavioral outcome, the effect of Tat-HSP70 administration on locomotor activity.
**Intratracheal admistration of Tat-HSP70 reduced MIP-2 abundance at 24 hr but did not decrease abundance of CINC-1 at 24 and 48 hr following 2CLP**

Excessive influx of neutrophils is a hallmark of lung injury in sepsis induced ARDS. Neutrophil activation contributes to the early pathogenesis of lung injury by aggregation and degranulation, releasing proteases, leukotrienes, platelet-activating factor, toxic oxygen-derived free radicals, chemokines and cytokines (Fujishima & Aikawa, 1995). Early in the course of sepsis-induced lung injury, neutrophils begin to accumulate in the lungs via transmigration from blood to alveolar spaces.

The CXC family of chemokines regulates movement of neutrophils during inflammation, including sepsis. Both CINC-1 and MIP-2 belong to the CXC chemokine family of neutrophil chemotactic proteins and are homologous to human IL-8 (Kunkel et al., 1991; Standiford, et al., 1990; Watanabe, et al., 1989; Guo, et al., 2006; Haelens, et al., 1996). Studies using the CLP model of sepsis and ARDS show excessive production of CXC chemokines, especially CINC-1 and MIP-2 from BAL, serum and plasma specimens (Kilpatrick, et al., 2011; Zhang, Wu, Qiang, Zhou & Wang, 2010; Guo et al., 2006; Strieter, et al., 2005; Ebong, et al., 1999). Thus, the present study assessed levels of these chemokine proteins in whole lung homogenate as an indicator of their abundance in 2CLP rats treated with Tat-HSP70 versus vehicle controls.

Similar to findings by Kilpatrick and colleagues (2011), our findings show that Tat was successful in delivering HSP70 into the cell and was effective in reducing the abundance of MIP-2 at 24 hr. Although, levels of MIP-2 at 48 hr were slightly reduced in
the treated groups, we did not detect a significant difference. Relative to the septic non-treated groups, levels of CINC-1 abundance in the 2CLP-Tat-HSP70 groups appear slightly reduced at 24 hr and at best equivocal at 48 hr. However, we were unable to detect significant differences between these groups. Of interest, while most prior studies were conducted in BAL, serum or plasma, we conducted our study using lung homogenate. In addition, while lung homogenate samples are reflective of localized protein concentrations, BAL, serum and plasma specimens may be more reflective of systemic abundance. Nevertheless, our findings for lung MIP-2 protein levels at 24 hr are remarkable and supports the need for further explorative studies evaluating the effects of targeted and localized therapy and correlation in reduction of systemic circulatory inflammatory chemokines. Additionally, we have yet to determine the pathways, localized or alternate, that are affected in attenuating lung injury by reduction of these chemokines. In addition, our sample size may have been too small. These factors may or may not have had an influence on our findings.

**Intratracheal administration of Tat-HSP70 increased lung IL-6 protein levels**

IL-6 is a cytokine produced by T-cells, macrophages, and endothelial cells that activates the acute phase response, among other physiological actions (Kishimoto, 2010). IL-6 has direct and indirect effects on cells and possesses both pro-inflammatory and anti-inflammatory roles (Opal et al., 2000). Our study showed that IL-6 protein levels were lowest in 2CLP-PBS, septic rats, compared to all other groups, T0, SO and 2CLP-Tat-HSP70 groups at 24 and 48 hr time points. We were unable to detect significance between any of these groups except between 2CLP-Tat-HSP70 and 2CLP-PBS at 48 hr.
This finding is similar to prior studies that show decreased IL-6 protein levels in the lung and liver of a 2CLP model, where levels drop as sepsis advances (Deutschman, et al., 2006; Andrejko et al., 1998; Barton, Shortall, & Jackson, 1996). In the present study, intratracheal administration of Tat-HSP70 significantly increased IL-6 lung protein levels at 48 hr. One interpretation of this finding is that HSP70 abundance may limit IL-6 degradation at the 48 hr time point when levels would be expected to fall. In the previous chapter, we demonstrated that Tat-HSP70 decreased lung injury on histo-pathologic exam at 48 hr. The effect of Tat-HSP70 on IL-6 in the 2CLP models bears further investigation.

**The complex role of IL-6 in sepsis.** The use of IL-6 as a biomarker in sepsis has led to complex findings. Elevated plasma levels of IL-6 have been found predictive of mortality at early time points in patients with sepsis and in the 2CLP model (Yende, et al., 2008; Hack, et al., 1989). Elevated levels of plasma IL-6 correlated with mortality at six hr in a female murine model of CLP (Remick, et al., 2002).

Mortality in IL-6 −/− mice was 100% within 12 hr of 2CLP but 0% in 2CLP-treated IL-6 +/+ mice before 24 hr (Deutschman, et al., 2006). However, mortality for 2CLP IL-6 +/+ mice at 72 hr was 90%. Exogenous recombinant human IL-6 (rhIL-6) administration to build IL-6 abundance has also been evaluated as a treatment modality in the 2CLP model in IL-6 wild-type and knockout mice. Four 2CLP groups were evaluated: IL-6 +/+ , IL-6 −/−, IL-6 +/+ with rhIL-6 and IL-6 −/− with rhIL-6. Findings revealed that mortality was highest in IL-6 +/+ mice that received excess rhIL-6 at 12 hr. However, despite administration of exogenous IL-6, increased levels of serum IL-6 returned to baseline by
16 hr in all groups (except the IL-6−−−− group). These findings illustrate the complex nature of IL-6 function during sepsis: baseline IL-6 activity is necessary in the early phase of 2CLP to survive but the meaning of excessive IL-6 levels at later time points has not been determined at this time.

In a two hit rodent model, CLP is followed by intratracheal injection of Methicillin Resistant Staphylococcus aureus pneumonia three days later, to mimic the human clinical development of sepsis and ARDS pathology. Jung and co-workers (2011) found, despite high mortality in the two-hit animals, the BAL and systemic IL-6 response were blunted at six, 12 and 24 hr compared to sham/MRSA combined animals.

Despite the complex nature of systemic and tissue IL-6 responses in the response to septic insult, we provide evidence here that direct intratracheal administration of Tat-HSP70 enhances lung IL-6 abundance at the relatively late 48 hr time-point. This is also the time at which we see decreased lung and clinical pathology, suggesting that IL-6 may be exerting protection from 2CLP induced lung injury.

**Tat-HSP70 did not have an effect on gross motor activity/locomotion following 2CLP**

In this study, we were unable to observe any significant group differences in locomotor function in our treated septic rats compared to our non-treated septic rats at either 24 or 48 hr. Nevertheless, we were able to detect a slight improvement in locomotion of our septic and treated rats at 48 hr compared to the non-treated group at the same time point. Further, similar to other studies, we were able to establish that
locomotion following 2CLP is significantly diminished at both 24 and 48 hr when compared to SO rats at similar time-points.

Prior studies have established that more than 50% of patients who are diagnosed with sepsis, MODS and ARDS develop either critical illness polyneuropathy (CIP) or critical illness myopathy (CIM) (Bolton, 1984; Hermans, et al., 2008; Latronico, 2005; Stevens et al., 2007). Further, the exogenous use of mechanical ventilation compounds critical illness as treatment with a ventilator for greater than seven days in greater than 25% of patients show diaphragmatic atrophy, and significant musculoskeletal weakness with myopathic changes seen on muscle biopsy samples six months to two years post discharge from an intensive care (Angel, et al., 2007; Fan, et al., 2009; Wilcox et al.,2010). Long-term effects may be even more profound with studies reporting 1 and 5 year patient outcomes experiencing pronounced muscle weakness, muscle wasting and fatigue that lead to persistent functional limitations in addition to cognitive and psychosocial limitations (Herridge, et al, 2003; Herridge, et al., 2011). Importantly, early recovery of organ function was associated with better functional ability one year after recovery (Herridge, 2003; Marini & Gattinoni, 2004).

Causes of musculoskeletal weakness attributed to CIP, CIM or ICU acquired weakness during sepsis and ARDS are many, some of which include cytokine mediated inflammation, increased oxidative stress, impaired microcirculation, altered proteolytic synthesis and breakdown, altered calcium homeostasis causing changes in excitation-contraction coupling, hyperglycemia, the increased use of steroids, the use of neuromuscular blockade drugs, heterotopic ossification, and peroneal and ulnar palsies.
Numerous animal studies using the CLP model have illustrated decreased gross motor activity associated with sepsis and ARDS and our results confirm these findings (Cankayali et al., 2007; Latronico et al., 1996; Nayci et al., 2005; Rossignol, 2008).

Although the delivery of Tat-HSP70 did not significantly alter 2-CLP-induced patterns of CINC-1, MIP-2, and locomotor activity, promising trends in some of these outcome variables were noted at 48 hr. These findings, in addition to the positive findings in our previous work, warrant continued study of this intervention in larger numbers of animals and with additional outcome variables.

**Tat-HSP70 increased survival at 48 hr following 2CLP**

To assess the cumulative potential and benefit of treatment with Tat-HSP70 following 2CLP on survival, we conducted a survival analysis using the combined number of all animals in experiments spanning both our papers. We found that mortality was higher, 35%, in the septic non-treated group at 48 hr compared to 14% in the septic Tat-HSP70 treated group of animals. Kaplan Meier survival analysis revealed a positive statistical trend, \( p \leq 0.09 \) toward survival in our treated group at 48 hr.

**Conclusion**

The use of Tat to deliver cargo therapeutic proteins in an in vivo setting for the 2CLP model of sepsis and ARDS is in its infancy. Work by our collaborator, Kilpatrick and colleagues using the Tat-PKC-\( \delta \)-inhibitor in this model have been promising and
supports similar findings in our study. Nevertheless, much remains to be explored not limited to dose response curves, transduction of fusion proteins into cells and effects of either Tat and or the cargo protein of interest to other organs, variability in time-points that Tat-HSP70 may be more effective, and the reflection of targeted and localized management of inflammatory mediators to circulating systemic effects. As evidence in the use of HSP70 to attenuate sepsis-induced lung injury continues to accrue, the efficacy and safety of Tat-HSP70 as an intervention in sepsis and ARDS should be explored further.
CHAPTER 4

INTRACELLULAR DELIVERY OF TAT-HSP70 AND TAT-PKC-δ INHIBITOR ATTENUATES LUNG INJURY IN A 2CLP MODEL OF ARDS

(Methods paper- with Dr. Laurie Kilpatrick)
Abstract

Sepsis is a leading cause of death in intensive care units. Sepsis triggers an elaborate and systemic dysregulated release of innate immune and inflammatory mediators that left unchecked can lead to organ failure and death. The lung is the organ most affected developing into Acute Respiratory Distress Syndrome (ARDS). During sepsis and ARDS, key regulatory protein levels are altered with pro- and anti-inflammatory pathways up-regulated and down-regulated during the developing inflammatory response. The purpose of this review is to briefly describe two pathways that are dysregulated and contribute to sepsis-induced lung pathology, to review developments in the field of intracellular delivery of peptide and protein mediators, and to provide an overview of studies in our labs using intratracheal administration of peptide/protein interventions to reduce lung pathology in a sepsis/ARDS model. The rodent model of cecal ligation and double puncture (2CLP) mimics many aspects of ARDS presentation in humans. In the evolution of lung damage/ARDS in the 2CLP model, activation of the pro-inflammatory Protein Kinase C-delta (PKC-δ) is upregulated, and the cytoprotective molecular chaperone Heat Shock Protein 70 (HSP70) is down-regulated. We used the HIV-1 trans-activator of transcription, Tat, protein transduction domain to deliver: (1) A peptide inhibitor of PKC-δ or (2) the HSP70 protein to the lungs, and noted beneficial effects of these interventions on selected outcomes in the 2CLP model. This supports further exploration of the safe and effective use of the Tat protein transduction domain for targeting therapeutic peptides and proteins in treatment of sepsis-induced ARDS.
Introduction

Sepsis, a fatal syndrome of dysregulated inflammation is a leading cause of death in Intensive Care Units (ICUs) in the United States costing the healthcare system approximately 24 billion dollars annually (Lagu, et al., 2012; Hall et al, 2011; Xu, et al., 2010). Sepsis affects approximately 900,000 people annually with a prevalence that is increasing at a rate of 13% (Gaieski, et al., 2013). Timely recognition and supportive treatment has reduced mortality for sepsis to approximately 25% but this rate remains variable by region (Wang, et al, 2010).

Approximately 18% of patients with sepsis will develop Acute Respiratory Distress Syndrome (ARDS) (Critical Care Statistics in the United States, 2012). ARDS is a heterogeneous syndrome of alveolar-capillary compromise as a result of direct or indirect injury to the lung causing severe defects in gas exchange. Hallmark characteristics of ARDS include a dysregulated influx of activated neutrophils, monocytes, alveolar macrophages and the generation of proinflammatory mediators damaging pulmonary endothelial and epithelial cells. This contributes to increased permeability of the alveolar-capillary barrier resulting in a protein-rich pulmonary edema flooding the bronchoalveolar space (Matthay, Ware & Zimmerman, 2012). First described by Ashbaugh and colleagues in 1967, clinical presentation includes acute onset (within seven days of a known insult contributing to lung injury) and increasing hypoxemia that typically requires mechanical ventilation (Ashbaugh et al., 1967).
Sepsis pathophysiology is thought to center on dysregulation of the inflammatory response. Injury or cell stress from sepsis and invading pathogens provokes the innate immune system, alerting a family of pathogen recognition receptors (PRRs) to recognize pathogen associated molecular patterns (PAMPS) and/or danger associated molecular patterns (DAMPs). Common PRRs that are membrane bound include members of the Toll Like Receptor (TLR) family and Receptor Kinases while more complex PRRs exist within the complement and coagulation system, blood and lymphatic systems (macrophage, lymphocytes) and vascular and tissue cells (endothelial and epithelial cells) (Reinhart, Bauer, Riedmann & Hartog, 2012). PAMPS recognized by PRRs include unique pathogen cell wall molecular structures such as lipopolysaccharide and peptidoglycan. DAMPs typically are made up of endogenous proteins such as Heat Shock Proteins (HSPs), high-mobility group box-1, and nucleotides that are released from damaged or dying cells. Binding of PAMPs to PRRs activates several inflammatory pathways and mediators, including pro-inflammatory transcription factors such as NF-κB, the release of acute phase reactants, expression of pro-inflammatory cell surface markers and activation of complement system mediators. Activation of NF-κB, and other proinflammatory transcription factors leads to the synthesis and release of pro-inflammatory cytokines such as TNF-α, IL-6 and chemokines such as IL-8.

A hallmark of lung injury progression is the capture, rolling, migration, and adhesion of activated neutrophils to the pulmonary endothelium and epithelium. Activated neutrophils release proteases (neutrophil elastase), reactive oxygen species, pro- and anti-inflammatory cytokines, prostaglandins and leukotrienes leading to
increased permeability of the alveolar capillary barrier, and accelerating pulmonary edema formation. Damage to pulmonary epithelial Type 1 and Type II cells also contributes to lung dysfunction during this inflammatory progression. As insight into the cellular and molecular mechanisms involved in the pathogenesis of sepsis and ARDS become clearer, researchers have shifted their focus to new avenues targeting repairs in intracellular signaling pathways, recognizing patterns of gene expression, inhibiting pro-inflammatory transcription factors and or intracellular mechanisms in cytokine, chemokine or mediator secretion.

Cecal Ligation and Double Puncture (2-CLP) in Rodents as a Model of Sepsis-Induced ARDS

The 2CLP rodent model of sepsis has been a validated, established rodent model of sepsis and ARDS for the past 30 years and the closest of all models mimicking the physiological and pathological changes seen in the human syndrome. 2CLP results in a functional picture that is consistent with ARDS and animals exhibit physical limitations that are consistent with sickness behavior and critical illness polyneuropathies (Matute-Bello, et al., 2008; Wichterman, et al., 1980). Work by investigators in the pre-clinical field of sepsis-induced ARDS have used the 2CLP model to effectively elucidate cellular and molecular mechanisms such as neutrophil accumulation and dysregulation of inflammatory biomarkers leading to lung damage.

The Role of Protein Kinase C- Delta (PKC- δ) in Sepsis, Lung Injury, and 2CLP-ARDS
PKC-δ is activated by pro-inflammatory mediators involved in the septic response including lipopolysaccharide and cytokines such as TNF-α and IL-1 (Kilpatrick et al., 2002; Page et al, 2003; Puneet et al, 2010; Vancurova, Miskolci, & Davidson, 2001). Moreover, PKC-δ is activated in the lungs of a rodent model of 2CLP induced ARDS (Kilpatrick, et al., 2011). Studies with PKC-δ deficient mice and PKC-δ inhibitors indicate a role for PKC-δ in regulating neutrophil trafficking to the lung in response to pulmonary inflammation triggered by asbestos exposure, stroke/reperfusion injury, or pancreatitis (Chou et al., 2004; Ramnath, Sun, & Bhatia, 2010; Shukla et al., 2007).

**Deficiency of HSP70 in 2CLP-ARDS**

The Heat Shock Response (HSR) expressing HSPs is a highly conserved endogenous mechanism that protects cells from injury. While intracellular HSPs are inducible and cytoprotective, extracellular HSPs can be immunogenic and inflammatory; thus, targeted intracellular delivery is critical (Asea et al., 2000; De Maio, 2011; Lindquist & Craig, 1988; Multhoff et al., 2006, 1999). Sepsis induced ARDS in a rodent model of 2CLP failed to increase the expression of HSP70, an inducible HSP protein, in the lungs (Weiss, et al., 2000). This contributed significantly to increased lung injury consistent with ARDS and subsequently increased mortality. Augmentation of lung HSP70 using an intratracheal delivered adenovirus vector, AdHSP70, significantly decreased ARDS pathology in the 2CLP model and decreased mortality by 31% at 48 hr (Weiss, et al., 2002).
Need for Targeted Bio-therapeutics to the Lung to Prevent and Limit Sepsis-Induced ARDS

In the past decade, there have been many attempts to develop interventions to limit sepsis induced lung injury. Currently, the only mechanism known to reduce 28 day mortality for patients with ARDS is the use of exogenous ventilator support (ARDSNET, 2000). Alternate therapies that have been tried included the use of inhaled or intravenous beta agonist (Albuterol), exogenous surfactant administration, the use of inhaled vasodilators (nitric oxide, prostacyclin), extracorporeal techniques (extracorporeal membrane oxygenation), anti-inflammatory therapies (corticosteroids, prostaglandin E1), antioxidants (glutathione), and dietary oil supplementation. None of these efforts have been successful in reducing mortality (Cepkova & Matthay, 2006; Jain & DalNogare, 2006). As the cellular and molecular mechanisms of sepsis and ARDS pathogenesis continue to be identified, researchers have shifted their focus to new treatment targets. These include interventions altering intracellular signaling pathways, gene expression, pro-inflammatory transcription factors and intracellular mechanisms of cytokine, chemokine or mediator secretion.

Special considerations in delivery of peptide, protein, and other lung interventions

Delivering peptide or protein bio-therapeutics to the lung to target injured pulmonary cells requires careful consideration of delivery methods and efficacy. Evidence exists that small peptides can be absorbed across alveolar epithelial cells by diffusion, active and passive transport mechanisms, paracellular and transcellular
transport, pore formation, and vesicle-mediated endocytosis and transcytosis. However, there may be differences in these processes when alveolar cells are damaged during the evolution of ARDS (Kim & Malik, 2003). Common methods and routes for targeted pulmonary drug delivery include aerosol inhalation, intranasal delivery, intratracheal delivery, the use of perfluorocarbon liquids or compounds, manufactured drug carriers such as nanostructures, microparticles and liposomes, the use of viral vectors for gene delivery, and the use of PTDs or cell penetrating peptides to facilitate targeted cargo delivery into the cell (Andrade, Videira, Ferreira, & Sarmento 2011; Courrier, Butz, & Vandamme, 2002).

Proteins or peptides delivered through intratracheal instillation should pass efficiently via the conducting airways to the lower highly absorbent surface area of the lung. As the pathologic features of ARDS progress, pulmonary edema, inflammation, and release of toxins or insoluble particles can affect the permeability of the alveolar epithelial layer (Audi, Roerig, Ahlf, Lin & Dawson, 1999). In addition, changes in the composition of diseased lung tissue can cause sequestration of pharmacotherapeutic agents. Conversely, epithelial injury or metabolic activity within epithelial cells or most all cells may affect biopermeability of certain drug compounds. Proteolytic activity, such as chymotrypsin activity found in alveolar fluid, could digest peptides and proteins delivered by the airways. Fluids that are secreted by the cells lining the airways and alveoli may pose further barriers to drug absorption. Studies have shown that interactions between the make-up of the surfactant phospholipids and inhaled drugs either enhance
solubility, limit absorption, or increase clearance, and could lead to retention or excess of a drug in the alveoli (McAllister, Alpar, Teitelbaum et al., 1996).

Last, careful consideration should be given to the instillation volume and suspension of a potential drug. For example, in a rat model, an instillation volume of one to two milliliters/kilogram of body weight would correspond to approximately six% to 31% of a rat tidal volume and equate to 31 to 156 milliliters of liquid administered to an average adult human who would have an average tidal volume of 500 ml (Hlastala & Berger, 1996).

**Protein Transduction Domains Can Deliver Peptides and Proteins Into Cells**

One approach that may contribute to development of targeted *in vivo* therapeutics to treat sepsis induced ARDS is the identification of appropriate carriers for peptide or protein cargos that are normally unable to cross the plasma membrane of target cells. Protein transduction domains are unique, basic amino acid rich sequences that have been found to mediate rapid movement of peptides and proteins across cell membranes. This method of peptide and protein intracellular delivery has been referred to as the “Trojan Horse” approach (Derossi, Chassaing, Prochiantz, 1998). The Drosophila Antennapedia’s homeotic transcription factor Antp and the herpex simplex virus-1 DNA binding protein VP22 both contain protein transduction domains (Wadia and Dowdy, 2002). The Human Immunodeficiency Virus-1 (HIV-1), Trans- activator of transcription (Tat) is a 101 amino acid protein required for virus replication. Contained within the Tat protein sequence is a
11 amino acid, highly basic, protein transduction domain (PTD), that allows the whole protein to gain entry to cells (by transduction).

**Production of Tat-Coupled Peptides and Proteins**

Three methods have been described to couple desired peptides and proteins to protein transduction domains. First, PTDs can be linked to a cargo protein using a covalent conjugated chemical coupling (Lewin, et al., 2000). Of the three PTDs, both Tat and Antp have been successfully used in this manner. Conjugation of cargo protein to the PTD using peptide synthesis with a reversible cysteine disulfide bond rather than an irreversible covalent bond, was more effective and resulted in higher intracellular activity of the protein of interest (Inagaki, et al., 2003; Chen, et al., 1999; Stein, et al., 1999). Conjugation of PTDs to cargo proteins could also be successfully done by using a thiazoline ring, thioester, amide or other linkages (Wagstaff, & Jans, 2006).

A second, indirect, way to generate PTD/cargo linkage is by first transfecting an expression vector that has a genetically fused cDNA coding region of the protein/to the carboxy-terminus tail of the PTD (Elliott, & O’Hare, 1997). The PTD-cargo peptide vector is then transduced into cells. By transfecting an expression vector, the protein is reproduced by the cellular machinery within 12 to 24 hr. However, recombinant protein levels may differ and may be dependent on the closeness of the primary transfected cell. This method is primarily used with the VP22 PTD. The last proposed method is by using bacterial expression vectors that are capable of producing large amounts of the Tat-fused protein (Nagahara, et al., 1988). Here, the open reading frame of the cDNA protein of
interest is cloned in frame downstream of an N terminal leader sequence with a six-histidine residue purification tag and a Tat-PTD sequence. The recombinant protein is removed from the bacterial pellet through a protocol of denaturants, purification, and desalting.

**Uptake of Tat-Coupled Proteins Into Cells**

Fawell and coworkers (1994), discovered that the Tat domain could be cross linked to other proteins, carrying the cargo protein of interest into cells. The rate of Tat transduction is rapid, within 10 minutes *in vitro*, in cultured cells, and less than 20 minutes *in vivo*, and is detected in major organs following IP injection in a mice model (Nagahara et al., 1998; Schwarze, et al., 1999). Tat crosses the cell membrane into the cytoplasm and typically targets the nucleus. To ensure cargo delivery to cytoplasm rather than the nucleus requires a disulfide covalent bond between PTD and cargo protein, which is cleaved in the cytoplasm, allowing the cargo protein to remain there (Schwarze, et al., 1999). Intravenous administration of Tat-linked proteins are found in cells of highly vascular organs and tissues such as the heart, liver, spleen, lung, and skeletal muscle. Although early research showed that Tat-linked proteins had poor uptake into cells of the brain and kidney, improved transfection and purification methods resolved early difficulties (Schwarze, et al., 1999). As would be expected, Tat-linked proteins were also found in endothelial cells, Kupffer cells and splenic macrophages.

Nagahara and coworkers (1998), generated a bacterial expression vector, encoding the pTat-HA PTD, capable of fusing Tat to proteins of interest. The pTat-HA
plasmid codes for a protein with an N terminal histidine leader (containing six histidine residue purification tags), an 11 amino acid Tat protein transduction domain, glycine residues on each side, a hemaglutinin (HA) tag, a polylinker, and an Ampicillin resistance gene. ATG, as the start codon, allows for fusion with Tat as the upstream leader. Transformation of plasmid is completed in a high yield bacterial vector. The cDNA open reading frame of the protein of interest is cloned in frame downstream. Transduction across the lipid bilayer membranes of cells was easily facilitated by denaturing the structure of the Tat-fusion protein (using 8 M urea), followed by protein purification (Gottesman, Wickner, & Maurizi, 1997; Nagahara et al., 1988; Schwarze & Dowdy, 2000). It is hypothesized that denaturing the fusion protein helps with, (i) disaggregation of any insoluble proteins making the desired protein more functional (additionally, most Histidine-fused proteins become easily aggregated in the bacterial milieu), and (ii) separates the protein of interest into smaller pieces, allowing a higher energy ($\Delta G$) to transduce the denatured smaller protein structures more easily into a cell (than a lower energy, larger and fully folded functional protein) (Becker-Hapak, McAllister, & Dowdy, 2001; Nagahara, et al., 1998). Once the Tat-fusion protein transduces inside the cell, chaperone proteins help to refold the denatured protein (Schneider, et al., 1996). Tat expression can be detected by SDS-PAGE or immunoblotting (Becker-Hapak, et al., 2001). Tat protein transduction by pTat-HA can be seen at concentrations of 100-200 nM (Becker-Hapak, et al., 2001).

Nagahara and colleagues (1998), illustrated through various Tat-fusion proteins conjugated to fluorescein tags in culture media, that transduction occurred rapidly in
100% of the cells analyzed. Transduction was observed in peripheral blood lymphocytes, stem cells, and carcinoma cell lines, among others. Similarities between PTDs include the presence of the basic amino acids arginine and lysine that are required for transduction. Early hypotheses regarding the mechanism of entry of PTD-cargo protein into cells assumed that the positively charged PTD interacted with the negatively charged lipid layer developing a momentum that propelled the covalently bonded cargo protein into the cytoplasm (Green, et al., 1988; Schwarze, et al., 2000; Nagahara, et al., 1998). Transduction of Tat–cargo protein across the membrane was more successful when the fusion protein was produced by a bacterial expression vector compared to cross linking. Early studies did not show adverse systemic or neurologic effects when up to 1mg/kg of Tat fusion protein was administered per day over 2 weeks in a mouse model. Last, protein transduction occurred in vitro at temperatures as low as 4° C, which was interpreted as implicating a non-receptor, non-energy-requiring mechanism (Becker-Hapak, et al., 2001; Derossi, et al., 1998).

Characteristics of Tat that are relevant to transduction include its highly cationic, arginine and lysine-rich structure facilitating transduction through electrostatic interaction with the negatively charged plasma membrane, physiologic pH, its hydrophilic state and ability to move across the membrane despite the addition of a cargo protein that may greatly increase molecular size (Del Gaizo Moore, & Payne, 2004; Gump & Dowdy, 2007). Altering the sequence by replacing arginine residues with noncharged amino acids decreases Tat transduction. Recent studies show that Tat may bind to cell surface targets such as polar heads of phospholipids, to a plasma membrane lipid raft, or membrane-
associated components such as Heparan Sulfate Proteoglycans or glypicans, facilitating transduction (Fittipaldi, & Giacca, 2005; Gump et al., 2007).

Current evidence supports a macropinocytosis mechanism of transduction (Gump and Dowdy, 2007; Kaplan, et al., 2005). Macropinocytosis, unlike a passive process, is an energy dependent but receptor independent mechanism involving actin-mediated intake of discrete membrane regions to which the PTD+cargo protein molecules have bound into an endocytotic vesicle. Researchers propose that first Tat binds to cell surface lipid rafts through ubiquitous glycan chains. Second, Tat conjugated to the protein of interest is taken up into a cellular compartment of macropinosomes. Last, a process of endosomal escape releases the peptide into the cytoplasm (possibly through acidification in the endosomes). Some investigators recommend mechanisms to rupture the endosome (using a lipid reagent mixed with the transporter peptide) to ensure release or disassociation of the entrapped cargo proteins (Yamaguchi, Inoue, & Goshima, 2011).

Unlike earlier studies, transduction of Tat-cargo protein has not always been demonstrated in temperatures of 4°C. Kaplan and coworkers (2005) found that approximately 30% of the fusion protein disassociated at that temperature. (Sugita, et al., 2008). As successful as Tat mediated protein transduction has been, not all proteins can be expressed adequately in a bacterial vector. Further, proteins that need post-translational modification may need alternate vectors for expression. Protein transduction may not work for all proteins. It is unknown whether all protein transduction will occur in a similar fashion and localize to the cell cytoplasm and nucleus (Schwarze, et al., 2000).
Finally, construction of a fusion protein that permits targeting to diseased cells without affecting other cells would be highly desirable.

The Use of Tat Peptides in Therapeutics (Preclinical and Clinical Studies)

The use of Tat to deliver protein bio-therapeutics has enormous potential. Early in vitro studies by Vocero-Akbani, Heyden, Lissy, Ratner, and Dowdy (1999), transduced an active form of Tat-caspase-3 fusion protein to target and kill HIV infected cells in a cell culture model. Vocero-Akbani and coworkers engineered HIV proteolytic cleavage sites as a substitute for endogenous cleavage sites in the Tat construct that activated after differentiating between normal and abnormal cells. Their construct selectively processed and promoted apoptosis only in HIV-infected cells and left uninfected cells untouched.

In vivo experiments have been performed to investigate the role of CLP sepsis induced apoptosis in lymphocytes. Hotchkiss and coworkers (2006) treated Bcl-x(L) transgenic mice and normal mice with Tat conjugated to anti-apoptotic peptides, Bcl-x(L) and BH4. Tat was conjugated to the Bcl-x(L) protein via bacterial transfection, purified, and extracted while the BH4 peptide was conjugated to Tat by solid phase protein synthesis. Both peptides were loaded and delivered via an implanted pump in the subcutaneous tissue of mice subjected to 2CLP. In a separate experiment Tat-BH4 was also administered intraperitoneally. Both Tat-Bcl-x(L) fusion protein and Tat-BH4 peptides successfully decreased lymphocyte apoptosis and reduced mortality in this sepsis model.
To date, a few human studies have been feasible using Tat conjugated interventions. Following pre-clinical rodent and primate testing, Hill and colleagues (2012) successfully completed a clinical study using a Tat-conjugated inhibitor of the N-methyl-D-aspartate receptor, NR2B9c (NA-1), delivered intravenously, to limit ischemic brain damage following iatrogenic stroke following endovascular aneurysm repair. In another first-of-its-kind clinical study, a PKC-δ inhibitor, KAI-9803, was found safe, well tolerated and showed trends in reducing creatinine kinase MB levels when injected directly into coronary vessels for patients with acute ST elevated myocardial infarction (Delta MI Investigators, 2007). The KAI-9803 study was founded on positive findings conducted first in an animal model of acute myocardial infarction. In the pre-clinical study, animals subjected to receive the PKC-δ inhibitor showed decreased cardiac injury following the ischemia reperfusion insult typically seen following an acute myocardial infarction (Inagaki, et al., 2003).

**The Use of Tat- PKC-δ Peptide Inhibitor to Attenuate 2CLP Lung Injury**

PKC-δ belongs to a family of kinases, Protein Kinase C, that regulates multiple signaling pathways including inflammatory pathways (Anderson, 1994; Duquesnes, Lezoualc'h, & Crozatier, 2011). Prior studies have implicated a role for PKC isoforms in human diseases varying from cancer, diabetes, stroke and Alzheimer’s (Mochly-Rosen, Das & Grimes, 2012). Studies show that activation of PKC-δ contributes to lung injury through mechanisms involving neutrophil adhesion, neutrophil migration, neutrophil anti-apoptosis signaling, regulation of neutrophil related signaling pathways, and activation in
endothelial and epithelial cells, (Brown, Stewart, Liu, Ha, & Yaffe, 2003; Chakrabarti & Patel, 2008; Chou et al., 2004).

Neutrophils have an important role as first responders and are recruited within minutes as a host defense mechanism against pathogens. Nevertheless, recruitment and activation of neutrophils during lung injury contributes to release of tissue-damaging mediators including proteases and reactive oxygen species, further compounding pulmonary endothelial and epithelial injury (Grommes & Soehnlein, 2011; Manicone, 2009; Ware, 2006). In addition, intercellular gaps that form in the pulmonary endothelium from lung injury increase capillary permeability and neutrophil transmigration into the interstitium and alveolar spaces. Studies have shown that PKC-δ alone among PKC isotypes is responsible for catalyzing the phosphorylation and reducing the response of the pro-inflammatory TNF-α (p60TNFR) receptor (Kilpatrick, et al., 2000). Down regulation of PKC-δ contributed to decreased activation of neutrophil associated inflammatory mediators such as NF-κB and neutrophil anti-apoptosis. Shulka and coworkers (2007) found that relative to a normal mice, levels of inflammatory mediators such as IL-1β, IL-4, IL-6 and IL-13, and abundance of polymorphonuclear cells, macrophages and natural killer cells were decreased in PKC-δ knockout mice (PKC−/−) with peribronchiolar cell proliferation caused by asbestos exposure. Studies in an in vitro model of neutrophils demonstrated that intracellular delivery of a Tat peptide conjugated to a PKC isoenzyme inhibitor, V1.1d-PKC–Tat peptide, effectively limited TNF related antiapoptotic signaling (Kilpatrick, Sun, Mackie, 2006). Additional studies show that PKC-δ inhibition has been cardioprotective against cardiac ischemia related
reperfusion injury in a pig model, has attenuated renal cell apoptosis after nephrotoxic chemotherapy in a mouse model, reduced tumor growth and angiogenesis in an *in vivo* xenograft model of multiple myeloma, and attenuated lung injury from pulmonary edema in a hydrogen peroxide perfused guinea pig model (Inagaki et al., 2003; Johnson, Philips, Hocking, Tsan, & Ferro, 1989; Pabla et al., 2011; Podar et al., 2007).

PKC activation is complex and dependent on many factors. PKCs are made up of two regions, the regulatory and catalytic region, hinged together by a conserved domain, C1 and C2 (differs per isoform), that must disassociate to become active. The regulatory domain for each PKC isoform contains a unique, conserved, variable, V, region encompassing the C1 and C2 domains. PKC activation is dependent on its translocation, selectively anchoring to subcellular sites, through these specific V regions, to specific isoenzyme receptors for activated C-kinase (RACK) docking sites (Mochly-Rosen, 1995; Mochly-Rosen et al., 2012). Therefore, selectively designing targeted isoform inhibitors or activators within these regulatory regions would be of value.

Studies by the Mochly-Rosen group have identified the unique V1 region of PKC-δ, δ V1–1, amino acids 8–17 (SFNSYLEGLS), as the region for successful inhibition of RACK anchoring, inhibiting translocation of activated PKC-δ and decreasing activity (L. Chen, Hahn, et al., 2001; Churchill, Qvit, Mochly-Rosen, 2009). This finding led to a molecular target for the development of selective PKC-δ isoenzyme inhibitors. L. Chen, Hahn, and colleagues (2001) demonstrated in an *in vitro* and *in vivo* model of cardiac ischemia, that transducing PKC-δ into cardiac cells by synthesis of the highly selective
δ V1–1 region conjugated to a Tat peptide PTD (amino acids YGRKKRRQRRR) using a cysteine-cysteine (disulfide) bond successfully activated PKC-δ and increased cardiac damage.

In a follow-up study, we hypothesized that targeting pulmonary cells with Tat conjugated to a PKC-δ inhibitor, delivered directly into the trachea of a 2CLP rodent model of sepsis induced ARDS would inhibit and limit PKC-δ phosphorylation and attenuate lung injury (Kilpatrick, et al., 2011). To determine effects of Tat-PKC-δ inhibitor we hypothesized, (i) intratracheal administration of Tat- PKC-δ inhibitor would successfully be delivered into pulmonary cells, (ii) intratracheal administration of Tat- PKC-δ inhibitor would attenuate 2CLP lung levels of CINC-1 and MIP-2, (iii) intratracheal administration of Tat- PKC-δ inhibitor would improve 2CLP ARDS histology and morphometric analysis. Our animal groups consisted of, (ii) animals subjected to SO at 24 hr (control), (ii) animals subjected to 2CLP and intratracheal administered PBS, 2CLP-PBS (control), or (iii) 2CLP-Tat- PKC-δ inhibitor. Outcome measures were assessed at 24 hr.

We determined activation of PKC-δ inhibitor by quantifying phosphorylation of Thr505 (immunoblot analysis using a phosphor-specific PKC-δ antibody) in lung tissue homogenates. Hematoxylin and eosin (H&E) stains were examined for ARDS related lung histology in a blinded fashion by a pathologist. Inflammatory markers of CINC-1 and MIP-2 were analyzed from plasma and bronchoalveolar lavage (BAL) fluid. In addition, lung permeability index, a measurement of pulmonary capillary permeability
where high levels are reflective of an inflammatory process, were measured from total BAL. To create the Tat-PKC-δ inhibitor, the unique region of the PKC-d inhibitor, SFNSYELGSL, was coupled by peptide synthesis, disulfide bond, to the Tat protein transduction region, sequence YGRKKRRQRRR. Due to the cytoplasmic reducing environment toward an irreversible coupling of PKC, the Tat- PKC-δ peptide inhibitor was conjugated through a reversible disulfide bond (Chen et al., 2001). In addition, peptide synthesis using a disulfide bond facilitates cleavage and unrestricted delivery of PKC-δ into cell cytosol and not the cell nucleus. The dose was 200ug/kg delivered in 200 ul vehicle (PBS).

Findings from this study were multifold. First, 2CLP triggers PKC-δ activation in the lung as measured by PKC phosphorylation. Relative to the 2CLP-PBS group, lung tissue homogenate levels of PKC-δ phosphorylation in the 2CLP-Tat- PKC-δ peptide inhibitor group was significantly reduced. Second, both bronchoalveloar and plasma lung levels of CINC-1 and MIP-2 were significantly higher in the 2CLP-PBS groups compared to sham group. Treatment of 2CLP animals with the Tat- PKC-δ peptide inhibitor significantly reduced both CINC-1 and MIP-2 levels. Third, H&E stains evaluating lung histology revealed significant improvement in ARDS pathology in the 2CLP-Tat- PKC-δ peptide inhibitor treated group reflecting decreased septal thickening, cellularity, edema formation, and inflammatory infiltrate. Morphometric analysis revealed less neutrophil infiltration and macrophage presence in the alveolar and interstitial spaces of the 2CLP-Tat- PKC-δ inhibitor treated group. Additionally, relative to 2CLP-PBS group, lung expansion index was significantly increased in the 2CLP-Tat-
PKC-δ group. Lastly, there were less total protein concentration levels in BAL of the 2CLP-Tat- PKC-δ peptide inhibitor treated group indicating reduced lung permeability and injury.

This study was a first of its kind to attenuate lung injury in a rodent 2CLP sepsis induced ARDS model by direct delivery of the PKC-δ peptide inhibitor using the Tat PTD. Treatment with Tat- PKC-δ peptide inhibitor successfully limited lung injury following 2CLP at 24 hr.

**The Use of Tat-HSP70 Fusion Protein to Attenuate 2CLP Lung Injury**

Studies have consistently shown that 2CLP induced ARDS reduces or eliminates HSP70 expression in the lungs (Villar, et al., 1994; Weiss, et al., 2000). Previous investigations in our lab using the 2CLP model demonstrated that exogenous restoration of HSP70 in pulmonary epithelium using an adenoviral vector, AdHSP70, targeting the transcription of mRNA, successfully increased HSP70 protein abundance in the lung. This correlated with significant improvement in histologic evidence of lung injury, reduction in lung neutrophil accumulation, both representative of ARDS pathology, and rodent mortality at 48 hr (Weiss et al., 2002). In our follow up studies, we aimed to use the Tat-HSP70 fusion protein to deliver exogenous HSP70 in the 2CLP model.

For our studies, the Tat-HSP70 plasmid vector was kindly provided to us by Dr. Hector Wong (Cincinnati Children’s Hospital Medical Center and Children’s Hospital Research Foundation, Cincinatti, OH). The human HSP70 cDNA was cloned with the 11 amino acid Tat peptide and subcloned/transformed in an *Escherichia coli* bacterial
expression vector creating the Tat-HSP70 fusion protein (Wheeler et al, 2003). The recombinant Tat-HSP70 fusion protein was produced and purified by Impact Biologicals, Inc., Swarthmore, Pennsylvania.

Intratracheal administration of Tat-HSP70 significantly increased cellular HSP70 protein abundance in lungs of 2CLP rats at 24 and 48 hr. Additionally, we found that HSP70 protein abundance was significantly present in abnormal regions of the lung at both 24 and 48 hr. Histopathology indicated that Tat-HSP70 reduced lung injury at 24 hr, with the greatest improvement at 48 hr. Relative to 2CLP-PBS animals, we detected significantly decreased levels of the chemokine Macrophage Inflammatory Protein-2 in lung homogenate of the 2CLP-Tat-HSP70 group at 24 hr, and significantly decreased levels of neutrophil marker Myeloperoxidase in the 2CLP-Tat-HSP70 group at 48 hr. There were no significant differences in levels of lung homogenate abundance of CINC-1, and IL-6, or significant changes in rodent locomotion between treated and untreated groups. For survival studies, Kaplan Meier and Cox proportional hazard regression analyses indicated a trend (p<0.1) toward survival in the 2CLP-Tat-HSP70 48 hr group. No adverse effects were detected in the Tat-HSP70 treated rats in our study.

Conclusion

In this article, we briefly reviewed the clinical challenges of sepsis and ARDS, and the contribution of PKC-δ and HSP to sepsis and ARDS pathology. We then described the methodology involved in promoting cell penetration of peptide and protein mediators via linking to protein transduction domains. We conclude by surveying the
results from our two pre-clinical studies of interventions to improve outcomes of sepsis and ARDS. Two PTD-coupled agents, Tat- PKC-δ peptide inhibitor and Tat-HSP70 protein, were administered intratracheally for delivery into the lungs of a 2CLP rodent model of sepsis induced ARDS. We presented two methods, fusing and coupling, of our peptide and protein of interest to the Tat PTD for intracellular delivery. Both HSP70 and PKC-δ have variable roles in the intracellular or extracellular environment, thus the need for targeted delivery of Tat-HSP70 and Tat- PKC-δ inhibitor to prevent adverse effects from immunosuppression and in ability to effectively clear pathogens. We reviewed the challenges and considerations in choosing the method and route for delivery of protein therapeutics and proposed mechanisms of how the Tat PTD carries a peptide into the cytoplasm. PKC-δ is a known regulator of NF-κB activation in many types of cells, including neutrophils, epithelial cells and endothelial cells. We propose that one mechanism of action for HSP70 and PKC-δ peptide inhibitor may be at the level of reducing NF-κB; although, other mechanisms such as activation regulation of ROS production does not require activation of NF-κB.

Future studies will explore the effects of sepsis and lung injury on optimal drug uptake in the lung. In addition, uptake by alveolar macrophages, epithelial cells, endothelial cells, and the systemic circulation affecting other organs remains to be explored. Studies with fluorescence or radioactive tagged Tat conjugates could be used to evaluate distribution within the lung or to other organs. These approaches would also permit evaluation of more specific cellular distribution of the transduced peptide/protein.
CHAPTER 5

SUMMARY AND CONCLUSION
Introduction

The preceding chapters reviewed the literature indicating that sepsis induced lung injury presents debilitating effects of grave morbidity and mortality. Further, both sepsis and Acute Respiratory Distress Syndrome (ARDS) predispose patients to debilitating physical, cognitive and psychosocial burdens that last for years. Epidemiological data regarding sepsis and ARDS prevalence, and the increased morbidity and mortality in older adults, indicate that these conditions are public health concerns that are in need of additional evidence-based treatment options. The primary pathology of the sepsis syndrome lies in a dysregulated inflammatory and immune system. Our body of research elaborated on prior work in our lab that showed augmenting HSP70 abundance in the lung, by targeting delivery with an adenovirus (AdHSP70), ameliorates lung injury in a 2CLP model. HSP70 abundance conferred cytoprotection by its role in mediating pro-inflammatory pathways, limiting cell proliferation and reducing apoptosis. The present work extended these findings by using the fusion protein, Tat-HSP70 to increase lung HSP70 abundance in 2CLP rats. This chapter will discuss the summary of our findings and describe observations generating new information from our hypothesis and specific aims. This is followed by a discussion of limitations, gaps and recommendations for future directions from our research.

Summary of Findings

Specific Aim 1: To examine the effects of Tat-HSP70 on lung injury.

Principal findings: (i) Tat-HSP70 significantly increased HSP70 abundance in the
lungs at 24 and 48 hr after 2CLP, (ii) Tat-HSP70 significantly increased HSP70 protein abundance in abnormal and normal lung sections at 24 and 48 hr following 2CLP, (iii) Tat-HSP70 attenuated lung injury at 24 and 48 hr following 2CLP, (iv) Tat-HSP70 significantly decreased MPO abundance at 48 hr following 2CLP in the lung, (v) Tat-HSP70 did not reduce 2CLP-mediated abundance of CINC-1 but significantly reduced levels of MIP-2 at 24 hr following 2CLP in the lung, and (vi) Tat-HSP70 significantly increased abundance of IL-6 following 2CLP at 48 hr in the lung.

In specific aim one, to determine if the Tat-HSP70 fusion method would work, we hypothesized, first, that direct delivery of Tat-HSP70 through the trachea into the lung could increase HSP70 protein abundance in a 2CLP model of lung injury. Our results demonstrated that, compared to 2CLP-PBS rats, there was a significant increase of HSP70 protein abundance in pulmonary cell cytoplasm in the 2CLP-Tat-HSP70 group at both 24 and 48 hr. Ours is the first study to date, to introduce direct delivery of Tat-HSP70 in an *in vivo* model of 2CLP induced ARDS. However, we did not determine if abundance was prevalent within pulmonary type one or type two cells. Additionally, we did not conduct assays to specifically identify the Tat protein within lung tissue. To determine if some of the fused HSP70 proteins were unable to get into the cell or if transduction occurred in cell nuclei, tagging or labeling the Tat protein with Fluorescein Isothiocynate (FITC) would have been helpful. Additionally, we do not know if Tat was absorbed systemically and contributed to abundance of HSP70 and recovery or adverse effects in other organs.
We next determined if HSP70 abundance was concentrated in select areas of the injured lung. This analysis was novel and had not been done in prior studies with administration of AdHSP70. We observed that there was significant HSP70 abundance in both normal and abnormal regions of the lung. This is promising for future work that may need to target bio-therapeutics to specific areas of lung injury. This may also be helpful in identifying appropriate delivery mechanisms of protein bio-therapeutics to capture the heterogeneous nature of injured lung cells in ARDS.

Next, to determine if Tat-HSP70 abundance improved lung injury, we examined histologic findings in the lung sections of our treated groups compared to the non-treated septic groups. We looked for known markers that were consistent with the pathology of ARDS. Since our assessment of Tat-HSP70 abundance was not conducted in a blinded fashion, we sought verification in assessment of histologic slides for ARDS pathology from an independent veterinary pathologist reviewer who was blinded as to treatment group. The results of the analysis confirmed that HSP70 abundance in our treated groups were consistent with amelioration of lung injury at both 24 and 48 hr.

A hallmark in lung injury and ARDS is the disordered activation of neutrophils present in pulmonary vasculature. Next, to determine if Tat-HSP70 abundance attenuated lung injury by reducing recruitment and/or activation of neutrophils, we analyzed the abundance of MPO, a product of activated primary neutrophil granules capable of generating toxic free radical oxygen metabolites. We observed a significant reduction of MPO abundance in our 2CLP-Tat-HSP70 rat group at 48 hr compared to our non-treated septic group at the same time-point. Last, to determine if Tat-HSP70 reduced the
abundance of known chemokines and cytokines involved in neutrophil recruitment and mediation of pulmonary injury, we analyzed the abundance of the CINC-1, MIP-2 and IL-6 protein mediators in lung tissue. We were unable to detect a significant difference for either CINC-1 or IL-6 proteins in the 2CLP-Tat-HSP70 groups at either 24 or 48 hr when compared to the non-treated septic rat groups. Nevertheless, relative to the 2CLP-PBS rat group at 24 hr we detected a reduction in MIP-2 protein abundance in the lung of the 2CLP-Tat-HSP70 group at the similar time point. We confirm that intratracheal delivery of Tat-HSP70 was successful in ameliorating a key chemokine that is synthetized and exacerbate the inflammatory response in the lung similar to work done by our collaborator. A power analysis completed to determine if the number of animals per group were sufficient to possibly detect an effect in differences in protein levels revealed that adding more animal samples per group could potentially yield sufficient power to detect differences in these outcomes. In addition, analysis of samples from a larger pool of animals may reduce the variability of levels noted amongst samples.

**Specific Aim 2: To examine the effect of Tat-HSP70 on 2CLP induced changes in locomotion.**

*Principal findings: (i) Tat-HSP70 did not increase rat locomotion following 2CLP, (ii) Tat-HSP70 did not increase core rat temperature following 2CLP, and (iii) Tat-HSP70 increased survival at 48 hr following 2CLP*

We were unable to detect any significant differences in rodent locomotion in the 2CLP-Tat-HSP70 groups compared to the septic non-treated rat groups. Nevertheless, the power analysis revealed that we may not have had sufficient animals to detect an effect.
Additionally, we were unable to detect any significant differences in core rodent temperature in the 2CLP-Tat-HSP70 groups compared to the septic non-treated rat groups. Of interest, we observed a trend for rodent survival at the 48 hr time-point in the 2CLP-Tat-HSP70 group compared to the non-treated septic rats. This is promising and consistent with findings by our colleagues with the use of AdHSP70.

In summary, increasing HSP70 abundance in the lungs of an in vivo 2CLP model using the Tat-protein system is a viable and novel delivery mechanism. TaT-HSP70 may be a useful treatment for lung injury secondary to 2CLP but additional feasibility studies are needed.

Limitations and Observations

We presented a comprehensive background in the introductory chapter, covering select significance, controversy or considerations that must be taken regarding key aspects in this body of research. The following highlights key limitations and observations during this study.

Is 2CLP the right model of sepsis to mimic a human response? Controversy remains in choosing the right animal model to mimic the human response especially with syndromes as heterogeneous as sepsis and ARDS. Nevertheless, the use of animal models to understand and predict successful therapeutics in human syndromes or diseases (from cancer therapeutics to transplant medicine) has produced critical and valuable data. Caution must be exercised before extrapolating results from animal to human disease. The 2CLP model has been the best available model of human sepsis that leads to lung
injury that closely resembles ARDS. However, there are important caveats. There are significant differences between rats and humans in cardio-dynamics and metabolism. The use of anesthetics in this surgical animal model has been known to attenuate or modulate the inflammatory response (Bedirli, et al., 2012; Li, Zhu, Jiang, Xu, & Sun, 2009). These introduce important biases. In addition, while 2CLP is highly reproducible it still is operator-dependent. Time and training must be maximized to ensure validity of all experimental findings. Last, it is important for investigators to remain current and aware of studies that show limitations of animal models, and who are able to clarify the relevant limitations while continuing to define the benefits of such models. As always in animal research, recognition of the ethics of animal use and welfare is a primary mandate to the investigator.

**Variability of mediator response.** Some studies using the sepsis model have shown a high degree of variability when evaluating the expression of cytokine, chemokine and other mediators across specimen type. For example, levels or abundance of mediators obtained from a BAL specimen may differ from levels or abundance of mediators obtained from plasma, serum, intraperitoneal fluid or tissue homogenate (Guo & Ward, 2007; Kajikawa, Goodman, Johnson, Konishi & Martin, 1996; Matute-Bello, et al., 2011). In addition, repeated freeze/thaw cycles may lead to specimen degradation (Natarajan & Remick, 2012). Failure to adhere to protocol details in a meticulous manner such as in maintaining pH, concentration of buffer solutions or detergents used may alter data validity. In our study, we observed increased variability when the CINC-1 protein elisa was performed using re-thawed samples and when fresh samples versus dated
samples were analyzed. Adherence to best practices in the lab aims to reduce such sources of variability and error.

**Limitations of intratracheal delivery of TAT-HSP70.** Intratracheal delivery of Tat-HSP70 is simple and direct. Studies in SD rats using AdHSP70 indicate that delivery is uniform and successfully reaches alveoli, rather than only reaching more proximal portions of the tracheobronchial tree. Similar uniformity was observed by our colleague, Dr. Laurie Kilpatrick, in her studies with Tat-PKC δ. Nevertheless, potential limitations include localization and uneven distribution of agent within the airway or in one lung. To determine if this is the case, lung sections were examined carefully with immunohistochemistry for HSP70. Alternative approaches that could be tested in future studies include aerosolization of Tat-HSP70.

**Limitations of the use of TAT-HSP70.** The advantages of delivering Tat-HSP70 directly into lungs include targeted delivery, rapid uptake and straightforward determination of dosage dependence. Nevertheless, little is known about adverse effects, potential toxicity and bioavailability. Adverse effects, toxicity and bioavailability studies can be determined by dose response experiments and further studies using a broader range of time points.

**Gaps this study has filled**

Previous studies have suggested that HSP70 had potential as a treatment for ARDS. However, delivery is problematic and direct administration of the protein itself in the extracellular space (intravenously) enhances the extra-cellular, pro-inflammatory
effects of HSP70. Use of viral vectors has been questioned because of inflammatory potential and the appearance of toxic side effects. Therefore, an alternative delivery system is needed. This proposal outlines a previously unexplored delivery system that may make the augmentation of HSP70 in the lungs clinically feasible. This may add an important therapeutic approach to the management of care.

Similarly, others have used the Tat protein to deliver proteins and peptides into specific cells in order to reverse a pathologic defect or augment endogenous responses. However, only our laboratory (in conjunction with our collaborator Dr. Laurie Kilpatrick), has used the Tat protein as a delivery system into the lungs and in ARDS. The combination of the Tat protein and HSP70 as a treatment for ARDS is truly novel.

Finally, studies examining the effects of potential bio-therapeutics and 2CLP on locomotion are limited. Immobility has profound effects on patients’ recovery, their caregivers and the health care delivery system. Our experiments investigated the effects of therapy for ARDS on locomotion. Earlier and increased mobility will have profound implications in therapeutic options to promote recovery of functional status and quality of life of patients with ARDS. Together, in a culture of interdisciplinary and multidisciplinary teamwork and collaboration, from bench to bedside, our body of research aims to globally improve patients’ outcomes.

**Recommendations of Future Research**

We believe that Tat-HSP70 may mediate amelioration of lung injury similar to AdHSP70, by affecting mechanisms involving neutrophil activation, cytokine or
chemokine synthesis, cell apoptosis, pro or anti-inflammatory signaling pathways and or cell proliferation. Administration of AdHSP70 introduced an mRNA encoding HSP70 into cells, meaning the increase in HSP 70 protein will depend on gene activation, transcription, and translation. In contrast, the current studies increased abundance of HSP70 protein abundance directly through the Tat protein transduction domain. The preponderance of our results points to an overall level of efficacy of Tat-HSP70 as a therapeutic agent in sepsis-induced ARDS. Further studies are needed to optimize dose, to increase the number of animals studied, and to extend the outcomes assessment to other variables of interest.
## APPENDIX A

Table 1. Chapter 2.

**HSP70, MPO abundance and H&E histology data**

<table>
<thead>
<tr>
<th>Group</th>
<th>N of rats</th>
<th>H&amp;E (+)</th>
<th>HSP abundance – overall</th>
<th>HSP abundance healthy lung</th>
<th>HSP abundance unhealthy lung</th>
<th>MPO abundance</th>
<th>^ Animal Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>3</td>
<td>None</td>
<td>69 +/- 4%</td>
<td>69 +/- 4%</td>
<td>N/A</td>
<td>14010 +/- 473</td>
<td>None</td>
</tr>
<tr>
<td>2CLP-PBS-24</td>
<td>3</td>
<td>Minimal-Moderate</td>
<td>65 +/- 11%</td>
<td>68 +/- 12%</td>
<td>63 +/- 9%</td>
<td>24101 +/- 967</td>
<td>#</td>
</tr>
<tr>
<td>^ Data depicting significance between T0 and 2CLP treated or 2CLP non-treated groups.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-24</td>
<td>3</td>
<td>None-Minimal</td>
<td>85 +/- 9%</td>
<td>88 +/- 8%</td>
<td>81 +/- 10%</td>
<td>24157 +/- 779</td>
<td>#</td>
</tr>
<tr>
<td>* Data depicting significance between 2CLP treated and 2CLP non-treated groups at equivalent time points. All data shown as mean +/- standard deviation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2CLP-PBS-48</td>
<td>3</td>
<td>Mild-Minimal-Moderate-Marked</td>
<td>60 +/- 7%</td>
<td>65 +/- 5%</td>
<td>56 +/- 4%</td>
<td>30588 +/- 671</td>
<td>##</td>
</tr>
<tr>
<td>^ Data depicting significance between T0 and 2CLP treated or 2CLP non-treated groups.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-48</td>
<td>3</td>
<td>Minimal-Mild</td>
<td>87 +/- 13%</td>
<td>88 +/- 1%</td>
<td>86 +/- 3%</td>
<td>21934 +/- 723</td>
<td>#</td>
</tr>
<tr>
<td>* Data depicting significance between 2CLP treated and 2CLP non-treated groups at equivalent time points. All data shown as mean +/- standard deviation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>^ H&amp;E pathology: Septal thickening, fibrin/edema, alveolar collapse, hyaline membranes, lymphocytes and plasma cells, neutrophils and percentage of area damaged attributed to inflammation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># Animal Observations: Lethargy with decreased movement, diarrhea, crusting exudate expressed from eyes, piloerection and lack of preening.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- ^ Data depicting significance between T0 and 2CLP treated or 2CLP non-treated groups.
- * Data depicting significance between 2CLP treated and 2CLP non-treated groups at equivalent time points. All data shown as mean +/- standard deviation.
- + H&E pathology: Septal thickening, fibrin/edema, alveolar collapse, hyaline membranes, lymphocytes and plasma cells, neutrophils and percentage of area damaged attributed to inflammation.
- # Animal Observations: Lethargy with decreased movement, diarrhea, crusting exudate expressed from eyes, piloerection and lack of preening.
Table 2. Chapter 2.

Hematoxylin and eosin stain-itemized comparison between all groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Slide #</th>
<th>Location of Lesions</th>
<th>Septal Thickening</th>
<th>Interstitial Proteinaceous Material</th>
<th>Alveolar Collapse</th>
<th>Hyaline Membrane</th>
<th>Lymphocytes and Plasma Cells</th>
<th>Neutrophils</th>
<th>% damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>2</td>
<td>Focal</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>No</td>
<td>Minimal</td>
<td>Minimal</td>
<td>0.40%</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0.60%</td>
</tr>
<tr>
<td>2CLP- PBS-24</td>
<td>4</td>
<td>Multifocal Peripheral</td>
<td>Minimal</td>
<td>Minimal</td>
<td>Minimal</td>
<td>No</td>
<td>Minimal</td>
<td>Mild</td>
<td>0.70%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Multifocal (mostly peripheral)</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Yes</td>
<td>Moderate</td>
<td>Minimal</td>
<td>8.50%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Focal Peripheral</td>
<td>Minimal</td>
<td>Minimal</td>
<td>Minimal</td>
<td>None</td>
<td>Minimal</td>
<td>Minimal</td>
<td>0.50%</td>
</tr>
<tr>
<td>2CLP- Tat- HSP70-24</td>
<td>7</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>17A</td>
<td>Multifocal (mostly peripheral)</td>
<td>Minimal</td>
<td>Minimal</td>
<td>Minimal</td>
<td>No</td>
<td>Minimal</td>
<td>Minimal</td>
<td>2.50%</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Focal Peripheral</td>
<td>Mild</td>
<td>Mild</td>
<td>Minimal</td>
<td>No</td>
<td>Minimal</td>
<td>Minimal</td>
<td>0.90%</td>
</tr>
<tr>
<td>2CLP- PBS-48</td>
<td>10</td>
<td>Multifocal</td>
<td>Mild</td>
<td>Mild</td>
<td>Mild</td>
<td>Yes</td>
<td>Minimal</td>
<td>Mild</td>
<td>11.40%</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Multifocal</td>
<td>Mild</td>
<td>Minimal</td>
<td>Minimal</td>
<td>No</td>
<td>Minimal</td>
<td>Minimal</td>
<td>0.90%</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Multifocal Peripheral</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Yes</td>
<td>Marked</td>
<td>Marked</td>
<td>27.10%</td>
</tr>
<tr>
<td>2CLP- Tat- HSP70-48</td>
<td>29</td>
<td>Multifocal Peripheral</td>
<td>Mild</td>
<td>Mild</td>
<td>Minimal</td>
<td>Yes</td>
<td>Minimal</td>
<td>Minimal</td>
<td>2.06%</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Multifocal Peripheral</td>
<td>Mild</td>
<td>Mild</td>
<td>Mild</td>
<td>Yes</td>
<td>Minimal</td>
<td>Minimal</td>
<td>4.48%</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Focal</td>
<td>Minimal</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>Minimal</td>
<td>Minimal</td>
<td>0.70%</td>
</tr>
</tbody>
</table>
APPENDIX C

Table 3. Chapter 2.

*Animal survival data*

<table>
<thead>
<tr>
<th>Group</th>
<th>Total N of rats</th>
<th>N deaths</th>
<th>N alive at designated time-point</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2CLP-PBS-24</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-24</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2CLP-PBS-48</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-48</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>
**APPENDIX D**

Table 4. Chapter 3.

*CINC-1, MIP-2, IL-6, Locomotion data*

<table>
<thead>
<tr>
<th>Group</th>
<th>Total n of rat samples analyzed per group (CINC/ MIP/ IL6/ Locomotion)</th>
<th>CINC-1 (pg/ml)</th>
<th>MIP-2 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>Locomotion (counts/second/1 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>2</td>
<td>9 +/- 1</td>
<td>3.7 +/- 0.02</td>
<td>921 +/- 23</td>
<td>N/A</td>
</tr>
<tr>
<td>SO-24</td>
<td>3/6/3/6</td>
<td>10 +/- 2</td>
<td>4.5 +/- 0.05</td>
<td>814 +/- 95</td>
<td>688 +/- 108</td>
</tr>
<tr>
<td>2CLP-PBS-24</td>
<td>7/8/3/4</td>
<td>344 +/- 260</td>
<td>11.9 +/- 0.3</td>
<td>763 +/- 34</td>
<td>209 +/- 132</td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-24</td>
<td>6/9/3/8</td>
<td>212 +/- 92</td>
<td>7.4 +/- 0.08</td>
<td>822 +/- 141</td>
<td>151 +/- 93</td>
</tr>
<tr>
<td>SO-48</td>
<td>3/4/3/7</td>
<td>27 +/- 17</td>
<td>4.4 +/- 0.02</td>
<td>994 +/- 193</td>
<td>803 +/- 99</td>
</tr>
<tr>
<td>2CLP-PBS-48</td>
<td>7/8/3/9</td>
<td>203 +/- 122</td>
<td>9.1 +/- 0.1</td>
<td>787 +/- 44</td>
<td>374 +/- 88</td>
</tr>
<tr>
<td>2CLP-Tat-HSP70-48</td>
<td>8/8/3/7</td>
<td>223 +/- 154</td>
<td>7.5 +/- 0.1</td>
<td>1203 +/- 1222</td>
<td>520 +/- 99</td>
</tr>
</tbody>
</table>

All data shown as mean +/- standard deviation. CINC-1, MIP-2 and IL-6 listed assessed in picogram/milliliter (pg/ml)
APPENDIX E

Table 5. Chapter 3.

Animal survival data

<table>
<thead>
<tr>
<th>Group</th>
<th>Total N of rats</th>
<th>N deaths</th>
<th>N alive at designated time-point</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>SO-24</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>SO-48</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>2CLP-PBS-24</td>
<td>8</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><strong>2CLP-Tat-HSP-24</strong></td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2CLP-PBS-48</td>
<td>12</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td><strong>2CLP-Tat-HSP-48</strong></td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>
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