THE MOLECULAR AND CELLULAR RESPONSE
TO PULMONARY CONTUSION

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS  iv

LIST OF ILLUSTRATIONS  viii

ABSTRACT  xi

Chapter  page

I. INTRODUCTION  1
V. “Mechanism of neutrophil recruitment to the lung after pulmonary contusion” in preparation for publication in Shock  101
VI. “Pulmonary contusion primes systemic innate immunity responses” accepted for publication in J Trauma (Jan. 2009)  126
VII. DISCUSSION  151

SCHOLASTIC VITA  165
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABG</td>
<td>Arterial Blood Gas</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute Lung Injury</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C5a</td>
<td>Complement 5a</td>
</tr>
<tr>
<td>CCI</td>
<td>Cortical Contusion Impactor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CINC-1</td>
<td>Chemokine (C-X-C motif) Ligand 1</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal Ligation and Puncture</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Axial Tomography</td>
</tr>
<tr>
<td>CXCR2</td>
<td>CXC Receptor 2</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated Molecular Patterns</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunoassay</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorter</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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</table>
Gp96    Glycoprotein 96
H&E    Hematoxylin and Eosin
H₂O₂    Hydrogen Peroxide
HMGB-1   High Mobility Group Box 1
HRP    Horseradish Peroxidase
HSP    Heat Shock Protein
HTAB    Hexadecyltrimethylammonium Bromide
HTS    Hypertonic Saline
ICAM-1   Intracellular Adhesion Molecule 1
IL-1β    Interleukin 1 beta
IL1-ra    Interleukin 1 Receptor Antagonist
IL-6    Interleukin 6
IL-8    Interleukin 8
IL-10    Interleukin 10
IL-18    Interleukin 18
IFNα    Interferon alpha
IFNβ    Interferon beta
IRAK    Interleukin 1 Receptor Associated Kinase
IP    Intraperitoneal
IT    Intratracheal
KC    Keratinocyte-derived chemoattractant (CXCL1)
KO    Knockout
LPS    Lipopolysaccharide
LR    Lactated Ringer’s
LTA    Lipoteichoic acid
LTB4    Leukotriene B4
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>Ly6G</td>
<td>Lymphocyte Antigen 6</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1 (CCL2)</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid Differentiation Protein-2</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage Inflammatory Protein-1 (CCL3)</td>
</tr>
<tr>
<td>MIP-2α</td>
<td>Macrophage Inflammatory Protein-2 (CXCL2/3)</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple Organ Dysfunction</td>
</tr>
<tr>
<td>MOF</td>
<td>Multiple Organ Failure</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro Blue Tetrazolium in 70% dimethylformamide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>P/F ratio</td>
<td>PaO₂/FiO₂</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated Molecular Patterns</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet/Endothelial Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Pulmonary Contusion</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Cells (neutrophils)</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted (CCL5)</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>sTNFr</td>
<td>Soluble Tumor Necrosis Factor Receptor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Synthesis</td>
</tr>
<tr>
<td>TBST</td>
<td>10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF Receptor Associated Factor 6</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adapter protein inducing IFN-beta</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Trizma Hydrochloride</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>V/Q</td>
<td>Ventilation/Perfusion</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

## Chapter I

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acute Lung Injury</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>TLR signaling</td>
<td>6</td>
</tr>
</tbody>
</table>

## Chapter II

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A.</td>
<td>The experimental design and animal cohorts used for the experiments in this study are shown.</td>
<td>47</td>
</tr>
<tr>
<td>1B.</td>
<td>The Cortical Contusion Impactor used to deliver direct impact injury of variable and known force to rat lungs.</td>
<td>47</td>
</tr>
<tr>
<td>2.</td>
<td>Morphological changes and pulmonary edema are observed with lung contusion.</td>
<td>48</td>
</tr>
<tr>
<td>3.</td>
<td>PaO₂/FiO₂ ratios show decreased lung function in injured rats.</td>
<td>49</td>
</tr>
<tr>
<td>4.</td>
<td>The contused lung shows profound leukocyte infiltration.</td>
<td>50</td>
</tr>
<tr>
<td>5.</td>
<td>Systemic IL-1RA cytokine levels show a rapid and transient response.</td>
<td>51</td>
</tr>
<tr>
<td>6.</td>
<td>Systemic chemokine expression is consistent with recruitment of inflammatory cells to the injury.</td>
<td>52</td>
</tr>
<tr>
<td>7.</td>
<td>Localized inflammatory response of cytokine and chemokine expression is distinct from the systemic inflammatory response.</td>
<td>53</td>
</tr>
<tr>
<td>8.</td>
<td>Lung contusion and inflammatory mediators recruit activated immune cells to the site of injury.</td>
<td>54</td>
</tr>
</tbody>
</table>

## Chapter III

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Lung histopathology after pulmonary contusion.</td>
<td>71</td>
</tr>
<tr>
<td>2.</td>
<td>Activation of inflammatory responses after pulmonary contusion.</td>
<td>72</td>
</tr>
<tr>
<td>3.</td>
<td>Activation of innate immune responses after pulmonary contusion.</td>
<td>73</td>
</tr>
</tbody>
</table>
Figure 4. Lung histopathology after pulmonary contusion in TLR2 deficient mice.

Figure 5. Decreased inflammatory response in TLR2 deficient mice after pulmonary contusion.

Figure 6. Activation of innate immunity in TLR2 deficient mice after pulmonary contusion.

Chapter IV

Figure 1. Lung dysfunction after pulmonary contusion is dependent on the TLR4 pathway.

Figure 2. Lung pathology after pulmonary contusion is dependent on the TLR4 pathway.

Figure 3. Neutrophil recruitment to the lung after pulmonary contusion is dependent on the TLR4 pathway.

Figure 4. The TLR4 pathway is required for pulmonary inflammatory response to contusion.

Figure 5. The TLR4 pathway is required for systemic innate immune responses to pulmonary contusion.

Chapter V

Figure 1. Neutrophil depletion improves post-injury pulmonary function.

Figure 2. CXCR-2 dependent neutrophil recruitment to the injured lung after lung contusion.

Figure 3. CXC chemokine dependent neutrophil recruitment to the injured lung after lung contusion.

Figure 4. ICAM-1 dependent neutrophil recruitment to the injured lung after lung contusion.

Chapter VI

Figure 1. Lung histology shows that pulmonary contusion primes innate immunity for enhanced neutrophil response.
Figure 2. Pulmonary contusion primes innate immunity for enhanced neutrophil response.

Figure 3. Pulmonary contusion primes the systemic innate immune response.

Figure 4. HTS resuscitation reduces the neutrophil response to a second hit.

Figure 5. HTS resuscitation reduces the systemic innate immune response to a second hit.
ABSTRACT

Multi-system trauma is a significant source of patient morbidity and mortality, accounting for 140,000 deaths annually. Thoracic injuries occur in the majority of blunt trauma patients and are present in up to 75% of all trauma-related deaths. Pulmonary contusion is a common and potentially lethal manifestation of blunt chest trauma, affecting 10-17% of all trauma admissions with estimates of mortality between 10-25%. The mechanisms responsible for both the local and systemic inflammatory processes associated with pulmonary contusion are poorly understood.

There is mounting evidence that the lung injury is also inflammatory in nature. In addition to structural damage to the lung, an inflammatory cell infiltrate composed primarily of neutrophils ensues. Various inflammatory mediators are then produced, and dysfunction occurs in immunocompetent cell populations located both within the lung and remotely. Alterations in apoptosis, defective bacterial clearance, and an increased susceptibility to subsequent septic challenge have been described after lung contusion.

Despite advances in our understanding of the pathogenesis of pulmonary contusion, there has been little meaningful improvement in outcome. Specifically lacking is an understanding of the mechanisms responsible for the inflammatory
response to pulmonary contusion. This is due to several factors, including an inadequate characterization of the pathobiology at the molecular and cellular level, and a deficiency of valid small animal models in which to study this injury. In this study, we establish reproducible rodent models for blunt chest trauma that results in a physiological significant injury with lung dysfunction. We develop a paradigm of innate immune responses to blunt chest trauma that involve activation of inflammatory mediators, like CXC chemokines, that participate in the recruitment of neutrophils to the lung after injury. We found that neutrophil accumulation occurs and correlates with the severity of lung dysfunction. The innate immune response to pulmonary contusion was found, at least in part, to activate TLR dependent responses that we characterize herein.
CHAPTER I

INTRODUCTION

Trauma and pulmonary contusion. Multi-system trauma is a significant source of patient morbidity and mortality, accounting for approximately 170,000 deaths annually (1). Thoracic injuries are present in the majority of blunt trauma patients and in as many as 75% of all trauma-related fatalities (2,3). Notably, pulmonary contusion is a common injury after blunt chest trauma and affects 10-17% of all trauma admissions (3,4). The mortality associated with pulmonary contusion is estimated to be 10-25% (4). The sequelae of pulmonary contusion varies widely, ranging from mild dyspnea to prolonged mechanical ventilation, infection (pneumonia), local organ failure (Acute Respiratory Distress Syndrome, ARDS), and remote organ failure (Multiple Organ Dysfunction/Failure, MODS/MOF) (5,6). In survivors, disabling dyspnea and abnormalities in functional residual capacity are common at 6 month follow-up (7). As such, pulmonary contusion is a relatively common injury following blunt chest trauma and is associated with dramatic morbidity and is potentially lethal.

It is reasonable to speculate that the maladies caused by pulmonary contusion are a result of direct pulmonary damage; however, there is mounting evidence that the lung injury is also inflammatory in nature. In addition to structural damage to the lung, an inflammatory cell infiltrate composed primarily of neutrophils ensues. Various inflammatory mediators are then produced, which lead to the breakdown of pulmonary capillary basement membranes, hypoxia, increased pulmonary capillary resistance,
myocardial dysfunction, production of toxic oxygen metabolites, and alterations in inflammatory cell function (8-11). The magnitude of this localized inflammatory response has been associated with the onset of lung organ failure (ARDS) and infection (7,9,10,12). Interestingly, the inflammatory response in the lung often bears no resemblance to the systemic response in the blood, where clinical markers of inflammation are often measured. Dysfunction occurs in immunocompetent cell populations located both within the lung and remotely (13). Alterations in apoptosis, defective bacterial clearance, and an increased susceptibility to subsequent septic challenge have been described after lung contusion (14).

Despite advances in our understanding of the pathogenesis of pulmonary contusion, there has been little meaningful improvement in outcome. Specifically lacking is an understanding of the mechanisms responsible for the inflammatory response to pulmonary contusion. This is due to several factors, including an inadequate characterization of the pathobiology at the molecular and cellular level, and a deficiency of valid small animal models in which to study this injury.

**Acute lung injury.** Acute lung injury resulting from direct or indirect mechanisms is a common cause of mortality and morbidity in critically injured trauma patients (1,15,16). The hallmark of acute injury is the presence of infiltrating leukocytes (17,18). Extravasation of neutrophils from the circulation to site of injury is a complex process that is thought to be dependent upon NF-κB activation, early response cytokine expression, the production of chemotactic molecules, such as chemokines, complement 5a (C5a), and leukotriene B4 (LTB4), and the upregulation of cell surface-adhesion
molecules (Figure 1, reviewed in 19). The accumulation of activated neutrophils in the interstitium and alveolar space, results in the production of reactive oxygen species and the release of proteolytic enzymes leading to acute inflammation and ultimately pulmonary damage and dysfunction. Neutrophil accumulation in the lung is a key event in the early development of acute lung injury and ARDS in both animal models and in human studies(20-24).

**Figure 1. Acute Lung Injury.**
Chemokines mediate neutrophil chemotaxis and accumulation in the injured lung. Integrin interaction with ICAM-1 and other selectins cause adhesion and extravasation of neutrophils from the vasculature to the pulmonary interstitium, where tissue injuring proteins are released. Neutrophils also transmigrate into the alveolar space and are found in the bronchoalveolar lavage of injured lungs.
There are four major families of chemokines, CXC, CC, C, and CXC₃, that behave as potent chemotactic factors for leukocytes (25). The CXC family is primarily chemotactic for neutrophils and appears to play a role in all phases of acute lung injury (19). CXC chemokines are produced by immune and nonimmune cells in the lung, including alveolar macrophages, endothelial cells, and alveolar epithelial cells (26,27). In mice, keratinocyte-derived chemoattractant (KC, CXCL1) and macrophage inflammatory protein-2α (MIP-2α, CXCL2/3) are members of the CXC family and are orthologues of CXCL8 (IL-8) in man. Both bind to and activate a common receptor, CXCR2, which is also expressed on immune and nonimmune cells in the lung (28,29), but have different receptor affinities, levels of expression and effects on neutrophil function (30-36). The pivotal role of CXC chemokines and their receptor, CXCR2, in neutrophil chemotaxis has been demonstrated in models of ischemia/reperfusion, peritonitis, and lung injury (26,27,32-34,37). Adhesion receptor expression on endothelial and alveolar epithelial cells is essential to neutrophil diapedesis into areas of infection or injury (19,29,38-41). These receptors include the selectins (E- and P-selectin) and members of the immunoglobulin receptor superfamily (ICAM-1) (38,39,42). IL-1β and TNF-α stimulate ICAM-1 expression in endothelial and alveolar epithelial cells (43,44). The interaction between ICAM-1 and the β2-integrin CD11b/CD18 (murine Mac-1), results in “firm attachment” (adhesion) of neutrophils and migration into areas of lung inflammation, although CD18-independent pathways have been described (43,45-48). Factors
that increase neutrophil β2-integrin expression include CXC chemokines and C5a. Studies using antibody neutralization or knockout mice have demonstrated that pulmonary ICAM-1:β2-integrin interactions mediate neutrophil recruitment, accumulation and activation in models of pneumonia, immune complex alveolitis, ischemia/reperfusion, and peritonitis (39,40,49). These data support the theory that neutrophil recruitment is a key step in the pathogenesis of acute lung injury and requires CXC chemokine and adhesion receptor expression. The mediators of neutrophil chemotaxis in acute lung injury after pulmonary contusion are unknown.

Toll-like receptors and acute lung injury. TLRs are a phylogenetically conserved receptor superfamily that act as pattern recognition receptors for highly conserved pathogen associated molecular patterns. To date, 13 distinct mammalian TLRs have been identified (50), that bind bacterial and viral products and initiate the innate immune response to infection (51). In addition to pathogenic ligands, a growing number of endogenous TLR ligands have been identified in the lung such as hyaluronan, heparin sulfate, heat shock proteins (HSP60, HSP70) High Mobility Group Box 1 protein (HMGB-1), and surfactant protein A (52-57).

TLR2 and TLR4 are the primary receptors for peptidoglycan/lipoteichoic acid and lipopolysaccharide, respectively (51). These receptors are found on immune and
nonimmune cells in the lung and contribute to both acute (ARDS or pneumonia) and chronic (chronic obstructive pulmonary disease, COPD or asthma) inflammation in the lung (58,59). A common cytoplasmic adapter protein, MyD88 (50) mediates many innate immune responses (cytokine, chemokine and adhesion receptor expression, Figure 2) through activation of NFκB. MyD88 knockout mice are hyporesponsive to TLR stimulation (eg. 50,60); however, a TLR4-MyD88-independent signaling pathway associated with delayed NF-κB activation and IFNα/β expression has been described (50,51).

![Figure 2. TLR signaling.](image)

Cytokines, chemokines and adhesion molecules are expressed in response to TLR stimulated NFκB activation. TLR2 and TLR4 are known to share some common infectious and endogenous ligands and share the intracellular mediator, MyD88.
Toll-like receptors and noninfectious injury. The inflammatory responses to infection and trauma are virtually indistinguishable and suggest that TLRs may be a mutual participant. Studies evaluating the role of TLR4 in models of hemorrhagic shock and ischemia/reperfusion have found that TLR4 deficiency attenuates neutrophil influx to the lung and results in reduced hepatic injury, cardiac infarction size, and systemic cytokine release (61-64). In a femur fracture model, TLR4 deficiency resulted in reduced IL-6 expression and remote organ (liver) dysfunction (65). In models of colitis, TLR4 deficiency reduced neutrophil infiltration and TNF-α expression, and increased bacterial translocation and frequency of bleeding (66,67). Other studies suggest that TLR4-mediated responses may be enhanced after traumatic injury, a potential explanation for the “2nd hit” phenomenon (17,68,69) that is often described as the onset or exacerbation of ARDS or MODS/MOF following trauma.

Although it is probably phenotypically similar to TLR4, little is known about the role of TLR2 in response to non-infectious injury. In a bleomycin–induced model of lung injury (53), TLR2, TLR4 and MyD88-deficient mice had reduced pulmonary neutrophilia, CXC chemokine expression, and, interestingly, higher mortality when compared to wild-type mice. Low molecular weight hyaluronan was implicated as the mediator of injury, and protection was associated with a TLR mediated antiapoptotic effect on alveolar epithelial cells. Similar findings were found in hyperoxic lung injury models, in which deficiencies in TLR signaling were associated with alterations in alveolar epithelial cell apoptosis and worse outcomes (70,71). Conversely, in a sepsis model (celiac ligation and puncture, 56), expression of HSP70 (a TLR2/4 ligand) in
pulmonary epithelial cells reduced acute lung injury. Thus, TLRs are likely to participate in many physiological responses to injury, either directly or indirectly, and the ultimate effect is dependent on the injury mechanism. These data support the concept that TLR participate in the pathogenesis of infectious and non-infectious acute lung injury. The role of TLR in the response to blunt chest trauma and pulmonary contusion is unknown.

Enhanced TLR reactivity after injury. It has long been recognized in the clinical arena that significant traumatic injury seemingly primes the cells of the immune system for an exaggerated response to subsequent infectious challenge. Studies in both humans and animals have shown that innate immune cells such as neutrophils, macrophages/monocytes, and dendritic cells produce increased levels of inflammatory mediators in response to microbial stimuli after injury. This has been termed the “second hit” phenomenon, and is supported by epidemiologic studies linking the magnitude of the initial systemic inflammatory response to injury with the onset of late post-injury multiple organ failure and death due to invasive infection. Prior studies have linked the second hit response to enhanced Toll-like receptor (TLR) activity in cells of the innate immune system. Specifically, enhanced TLR4-mediated signaling has been shown to be involved in the second hit response in animal models of burn injury and ischemia/reperfusion. However, it remains unknown whether isolated
**blunt chest trauma primes the innate immune system for a second hit response and enhanced TLR4 reactivity.**

*An rodent model of lung contusion.* Historically, most data regarding the pathophysiology of lung contusion came from large animal studies and focused on cardiorespiratory physiology, not acute inflammatory lung injury. Our rationale for developing a rodent model had several objectives: (1) to obtain (relatively) high throughput data on the pathophysiological response to pulmonary contusion; (2) to deliver a reproducible injury, precisely quantified by velocity, energy force and displacement; (3) to study pulmonary contusion as an isolated injury; (4) to better understand the inflammatory response to injury (5) to identify biomarker predictors of severe and life-threatening injury response and (6) to develop targeted therapies that would alleviate and/or abrogate injury outcomes.

Toward this end, a rodent model of pulmonary contusion was developed using a computer-driven cortical contusion impactor (CCI). The CCI allows for precise definition of forces applied to the chest and/or lung. A reproducible contusion in a rodent model that measures clinically relevant parameters of lung function and inflammation after injury will contribute to our understanding of the pathophysiology of lung contusion and may lead to the development of interventions for the care of post-traumatic injuries. Two rodent models have been utilized in the studies presented here: (1) an *open* chest model in the rat where impact was delivered directly to the lung and (2) a *closed* chest model in the mouse where impact was delivered to the right chest. Each of these models has their experimental advantages/disadvantages that are discussed.
Within this proposal, we will describe a small animal model of pulmonary contusion, originally an open chest model in a rat and subsequently a closed chest model in the mouse. Using these models, a clinically significant lung injury will be demonstrated associated with activation of innate inflammatory mechanisms. Our objectives are to determine the role of TLR signaling in the initial inflammatory response to chest injury, the mechanisms surrounding, and importance of, neutrophil recruitment to the injured lung, and lastly to determine if blunt chest trauma systemically primes the animals for an exaggerated response to the TLR4 agonist, LPS.

REFERENCES


CHAPTER II

The following manuscript was published in *J Trauma* 2006;61(1):32-44 and is reprinted with permission. Stylistic variations are due to the requirements of the journal.

**Title:** The Pathogenesis of Pulmonary Contusion: An Open Chest Model in the Rat

**Running title:** A rat model of lung contusion

**Authors:** J. Jason Hoth, MD., Joel D. Stitzel, PhD., F. Scott Gayzik, MS., Noel A. Brownlee, MD. PhD., Preston R. Miller, MD., Barbara K. Yoza, PhD., Charles E. McCall, MD., J. Wayne Meredith, MD., and R. Mark Payne, MD

**ABSTRACT:**

**Background:** Chemokines direct leukocytes to areas of inflammation or injury. In general, CC chemokines (MCP-1, MIP-1α, RANTES) are chemoattractants for mononuclear cells and CXC (CINC-1, MIP-2α) for polymorphonuclear cells (PMNs). Herein we describe an open chest model of pulmonary contusion (PC) in a rodent (rat) and have identified a possible role for CC and CXC chemokines in the pathogenesis of PC.

**Methods:** Sprague-Dawley rats (350g) underwent thoracotomy. The exposed lung was struck with a piston at 5.2 m/s (150 J/M²). Blood, bronchoalveolar lavage (BAL), and lung tissue were collected at 3H and 24H after injury. PaO₂/FiO₂ (P/F) ratio was calculated at 15 minute intervals for 3 hours following contusion. Serum was evaluated for cytokine and chemokine expression using ELISA. Cell count/differential was performed on BAL, and lung tissue was obtained for histologic analysis, protein expression and wet to dry...
weights. Data are reported as pg/ml ± SE. Data were analyzed using Student’s T-test to identify significant differences (p ≤ 0.05 significant) between sham and injured animals.

**Results:** Piston impact caused PC based upon morphologic and histologic criteria. BAL cell count and lung wet to dry weights were increased and P/F ratio was decreased after PC. Systemic levels of IL1-ra, MCP-1 and the CXC chemokines MIP-2α and CINC-1 were significantly elevated at 3 hours when sham and injured animals were compared. All chemokines were found to be significantly elevated at 24 hours, consistent with the early PMN and subsequent mononuclear infiltration observed in the contused lung. Pulmonary expression of TNF-α, IL-1β, CINC-1, MIP-2α, ICAM-1 and elastase were increased and activated systemic neutrophils showed increased CD-11b.

**Conclusion:** A model of PC is described in which innate inflammation is activated locally and systemically. Systemic levels of CC and CXC chemokines are increased after PC. This correlates with elevated PMN CD-11b expression, enhanced pulmonary ICAM-1 expression and mononuclear and PMN infiltration into the lung and alveolar space. Elevated levels of CC and CXC chemokines are seen after PC and may be involved in the lung’s inflammatory response to injury.

**Key Words:** pulmonary contusion, chemokines, inflammation, rodent model, innate immunity, trauma.

**INTRODUCTION:**

Multi-system trauma continues to be a significant source of patient morbidity and mortality in society, accounting for countless hours of lost productivity and roughly 140,000 deaths annually¹. Thoracic injuries are present in the vast majority of blunt
trauma patients and are associated with significant mortality and morbidity\textsuperscript{2}. Pulmonary contusion (PC) is a common injury identified after blunt chest trauma, affecting 10-17\% of all trauma admissions\textsuperscript{3}. The sequela of pulmonary contusion varies widely, ranging from mild dyspnea to prolonged mechanical ventilation, infection (pneumonia), and organ failure (Acute Respiratory Distress Syndrome, ARDS)\textsuperscript{4-6}. In survivors, disabling dyspnea and abnormalities in functional residual capacity are common at 6 month follow-up\textsuperscript{7}. The mortality associated with pulmonary contusion is difficult to predict but is estimated to be 10-25\%\textsuperscript{3-8}. As such, pulmonary contusion is a relatively common injury following blunt trauma with dramatic morbidity and is potentially lethal.

Direct damage to the lung parenchyma resulting in intra-pulmonary shunting is thought to play a major role in the development of respiratory failure after pulmonary contusion as it has been demonstrated that contusion size is related to the onset of ARDS after pulmonary contusion\textsuperscript{6}. However, there is mounting evidence that the lung injury is also inflammatory in nature\textsuperscript{9-11}. Alveolar levels of the pro-inflammatory mediators interleukin (IL)-1\textsubscript{\beta}, IL-8, IL-18, and IL-6 are elevated in patients with blunt chest trauma\textsuperscript{12,13}. Furthermore, alveolar levels of the anti-inflammatory mediators soluble tumor necrosis factor receptor (sTNFr), IL-1 receptor antagonist (IL-1ra), and IL-10 have been shown to rise precipitously following blunt chest injury\textsuperscript{13}. Interestingly, the localized inflammatory response seen in the lung bears no resemblance to that of the systemic response measured in the serum, and it is the magnitude of this localized inflammatory response that has been associated with the onset of local organ failure (ARDS) and infection\textsuperscript{12-14}. Indeed, the lung is an active participant in the inflammatory
response to trauma; however, the role pulmonary contusion has in modulating this response remains ill defined. This may be due to a number of factors including a poor understanding of the associated pathophysiology at the molecular level and a deficiency of valid small animal models in which to study pulmonary contusion. Thus there is a strong need to develop new small animal models of acute lung trauma that take advantage of newer technologies in molecular biology and imaging to evaluate the lung’s response to contusion.

The aim of this study is to develop a novel model of pulmonary contusion wherein a consistent, reproducible unilateral contusion is created in an open chest model in the rat and to characterize the innate immune response to injury. Morphologic and histologic changes within both the ipsilateral and contralateral lung’s cytokine/chemokine expression, neutrophil (PMN) activation and adhesion molecule expression are characterized and clinical severity of the injury is graded using the PaO₂/FiO₂ (P/F) ratio.

**MATERIALS AND METHODS:**

**Animal protocol.** Male Sprague-Dawley rats (Charles River, Boston, MA) weighing approximately 350 grams were used in the study. Anesthesia was induced using 1.5% isoflurane and the animals were orotracheally intubated and mechanical ventilation (FiO₂ of 40%, respiratory rate 50, tidal volume 10cc/kg) was initiated using a Microvent 1 ventilator (Hallowell EMC, Pittsfield, MA). Continuous electrocardiography and pulse oximetry were monitored using Cardell veterinary vital signs monitor model 9403 (Sharn Veterinary Inc. Tampa, FL). Buprenorphine (0.1mg/kg) was administered for analgesia. The animal’s right chest was shaved, cleaned with Clinidine solution
Clinipad Corporation, Guilford, CT) and the right lung was exposed via a right posterolateral thoracotomy. To prevent injury to the underlying lung, the animal was taken off the ventilator for 10 seconds prior to opening the parietal pleura to enter the chest. A retractor was placed and the right lung was exposed. The lung was held in inspiration at a pressure of 16 mm Hg and impacted using the Cortical Contusion Impactor (Custom Design and Fabrication, Richmond, VA) at a velocity of 5.2 m/s to deliver an impact with energy of 150 J/M². After impact, the retractor was removed, the chest and skin closed with 4-0 chromic suture (US Surgical, Norwalk, CT) and residual air was evacuated from the chest using a 16G angiocatheter (BD Biosciences, San Jose, CA). The animal was extubated when spontaneously breathing. Sham animals underwent identical procedures without contusion. The entire procedure on each animal took approximately 20 minutes. A total of 87 rats used in this study were experimentally grouped as shown in Figure 1A. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (#O5-186) at Wake Forest University School of Medicine (Winston-Salem, NC) and are in accordance with State, Federal and National Institutes of Health regulations.

Cortical Contusion Impactor. The (Electric) “Cortical Contusion Impactor” (E-CCI) device was built by Custom Design and Fabrication (Richmond, VA) and is shown in Figure 1B. The CCI was constructed with an aluminum frame to increase rigidity, ensuring impact accuracy. The support base, animal platform, and impactor head are anodized, and the assembly includes the base and support frame, adjustable animal positioner with an aluminum animal platform that was used in conjunction with
stereotactic mounts. The model utilized an enhanced linear motor driven impactor and controller. The impact head houses a photo-optics sensor to determine velocity, impact depth, and dwell and provides reproducible impacts in its native form.

**Measurement of lung wet:dry ratio.** At 3 hours and 24 hours after contusion, sham and contused animals were sacrificed by isoflurane overdose. Whole lung specimens were removed, weighed and placed in an oven at 37°C for 24 hours to dry and then reweighed. Wet:dry ratios were determined and reported as the relative lung weight change for sham and injured animals at 3H or 24H after contusion.

**Arterial blood gas measurements.** As a physiological measure of lung function, P/F ratios were obtained at 15 minute intervals for 3 hours following contusion. Arterial partial pressure of oxygen (PaO₂) was measured in 5 contused animals and 8 sham animals. After the initiation of mechanical ventilation, samples were obtained by cannulating the common carotid artery via a midline neck incision prior to thoracotomy. Samples were obtained (0.3 ml) in heparinized syringes prior to (0) and at 15 minute intervals up to 3 hours after contusion and analyzed with a Nova Stat 9 Analyzer (Waltham, MA). All animals were maintained on 40% FiO₂ prior to and during blood gas measurement.

**Serum cytokine and chemokine levels.** At 3 hours and 24 hours after contusion, sham and injured animals were sacrificed by isoflurane overdose. Blood was collected in heparinized syringes at 0 hours (prior to thoracotomy) via the tail vein and at 3 hours and 24 hours by direct cardiac puncture. Blood was centrifuged at 250 x g, 20°C for 10
minutes and the serum was collected and stored at -70°C. IL-1β, TNF-α, IL-1ra, cytokine-induced neutrophil chemotactic factor (CINC)-1, macrophage inflammatory protein (MIP)-2α, monocyte chemotactic protein (MCP)-1, MIP-1α and regulated upon activation, normal T cell expressed and secreted (RANTES) protein levels in serum were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Histopathology. Lung tissue samples from 3 sham and 3 injured animals were obtained immediately after sacrifice at 3 and 24 hours. The lungs were fixed in 10% buffered formalin at a pressure of 15mm H₂O for 24 hours prior to sectioning. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for examination. All histologic examinations were done by an experienced pathologist (N.B.) blinded to animal status.

Bronchoalveolar lavage and cell counts. Sham and injured animals at 3 or 24 hours underwent bronchoalveolar lavage (BAL) of the right and left lung individually with 10 ml of phosphate buffered saline (PBS, Sigma Biochemical, St. Louis, MO) at 4°C. After sacrifice, BAL was performed by cannulating the trachea via a midline neck incision with a 14 gauge angiocatheter. A sternotomy was performed and the catheter was advanced into the right or left mainstem bronchus and lavage performed. Average return from each lavage was 8.1 ml. BAL fluid was then centrifuged at 300 x g, 4°C for 10 minutes. Supernatant was collected and stored at -70°C. The cell pellet was resuspended in 5 ml of 0.2% saline for 20 seconds and supplemented with 5 ml of 1.8% saline for red cell lysis.
The suspension was centrifuged at 300 x g, 4°C for 10 minutes and the resulting cell pellet was resuspended in 1 ml of PBS and counted by hemocytometer. Cytospin samples were prepared for each specimen. Differential counts for each sample were calculated based on the assessment of at least 200 cells.

**Immunohistochemistry,** Specimens were dried and deparaffinized by rinsing in fresh xylene for 10 minutes, rehydrated, and rinsed in PBS. Endogenous peroxidase activity was blocked by incubating slides with 0.3% hydrogen peroxide in methanol for 30-40 minutes. Non-specific binding was blocked by incubating slides with 10% goat serum in PBS for 45 minutes at room temperature. The primary antibody (IL-1β, Pierce, Rockford, IL; TNF-α, R&D Systems, Minneapolis, MN; elastase, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in 3% serum in PBS and incubated for 1 hour at room temperature in a humidity chamber. Negative controls were incubated with 3% serum in PBS only. Slides were washed with PBS, incubated for 30 minutes with secondary-biotinylated antibody (anti-rabbit IgG, Vector Laboratories, Burlingame, CA) and washed again with PBS. Specimens were then incubated with Streptavidin-RTU (Vector Laboratories, Burlingame, CA) for 30 minutes and washed with PBS. Chromagen (AEC or DAB, Vector Laboratories, Burlingame, CA) was prepared according to the manufacturer’s instructions. Development was observed under the microscope and stopped by dipping the slide in water. Slides were counterstained with Gill’s hematoxylin, washed with running water, and blued with Scott’s Tap water substitute for 10 dips. Results shown are representative of 3 independent experiments.
**Western blot.** Lung tissue samples (3 or 24 hours) were thawed and suspended in homogenization buffer (10mM Tris, 0.5% DDT, 0.1% SDS) and homogenized (TissueMiser, Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 3000 x g, 20°C for 10 minutes and the supernatant centrifuged again at 10,000 x g, 20°C for 10 minutes. The supernatant was saved and the pellet discarded. Protein concentrations were determined using Coomassie Protein Assay Reagent according to the manufacturer’s instructions (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred to an Immuno-Blot® PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Blots were blocked for 1 hour with 5% milk in TBST (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween-20). Anti-CINC-1 (Sigma Biochemical, St. Louis, MO), anti-MIP-2α (US Biological, Swampscott, MA) or anti-ICAM-1 (Serotec, Raleigh, NC) antibodies were diluted (1:1000, 1:500, and 1:500, respectively) in 5% milk/TBST and the membranes were incubated for 1 hour. Antibody-antigen complexes were detected using appropriately matched secondary antibodies conjugated with HRP (ICAM) or alkaline phosphatase (all others). Proteins were visualized using enhanced chemiluminescence (ECL, Pierce) or BCIP/NBT color development substrate solution (Promega, Madison, WI) according to the manufacturer’s instruction. Relative protein levels were quantitated on W. blots using ImageQuant 5.2 (Molecular Dynamics, Amersham BioSciences, Sunnyvale, CA). Results shown are representative of 3 independent experiments.

**Flow cytometry.** At the time of sacrifice, 5 ml of blood was obtained via cardiac puncture in heparinized syringes. Red blood cells were lysed as previously described,
and cell pellet resuspended in 2ml 2% BSA (Pierce, Rockford, IL) in PBS. Cells were counted and cell viability assessed by trypan blue exclusion. 1 x 10⁶ cells were then incubated with 10 µl of anti-CD11b-FITC or isotype control antibodies (BD Pharmingen, Minneapolis, MN) for 30 minutes. Cells were washed twice with PBS and FACS data obtained with a BD Biosciences FACS Calibur flow cytometer. A total of 10,000 events were counted and data were analyzed using CellQuest software (BD Immunocytometry Systems). The PMN population was gated after identification on a standard scattergram. The percent of PMNs expressing CD-11b is reported.

**Statistical analysis.** Data are reported as the mean ± SE of independent observations as indicated in the Figure legends. Statistical analysis to determine significant differences between sham and injured data used the Student’s t-Test (p<0.05 significant) and graphic presentations were performed using Microsoft Excel XP statistical analysis software.

**Results:**

*Contused lungs show pathophysiological changes and pulmonary edema.* All animals survived the injury and no animals died prior to sacrifice. This is consistent with our previous study using this mild to moderately severe injury model, where animals survived to 28 days post-injury¹⁵. In injured animals, morphologic evidence of contusion was apparent by visual inspection at all time points examined after injury. Morphologic changes, including intra-pulmonary hemorrhage and laceration, were observed by 3 hours (data not shown) and increased by 24 hours when compared to sham lungs (Figure 2, compare A and B). Interestingly, although the contusion was unilateral,
morphologic changes were also seen in the contralateral lung by 24 hours (Figure 2B, arrow). As an indication of pulmonary edema, lung wet and dry weights were measured and found to be significantly different when comparing sham animals’ with injured animals’ lungs (Figure 2C). At 3 hours after contusion, there was a significant increase (20%) in lung weight when comparing the wet to dry weight of lungs from sham animals to injured animals. By 24 hours after contusion, significant lung weight increases up to 28% between sham and contused lungs were observed. The increased pulmonary edema suggests that lung dysfunction continues to progress in severity over time.

**Lung function is compromised after contusion.** The ratio of partial pressure of oxygen (PaO\(_2\)) to fractional concentration of oxygen (FiO\(_2\)) (P/F ratio) is a clinical measure of lung oxygenation and is frequently used to describe the extent of lung dysfunction after injury with the degree of hypoxemia as expressed by a lower P/F ratio. As shown in Figure 3, P/F ratios began to decrease significantly at 30 min. after contusion and a significant difference was seen in injured animals for the duration of the experiment (3H). This indicates that contusion resulted in a physiologically significant lung dysfunction almost immediately after impact.

**Infiltration of inflammatory cells into the contused lungs.** Lung tissue was examined for histological changes indicating acute inflammation as a result of contusion. A pronounced interstitial mononuclear and polymorphonuclear (PMN) cellular infiltrate was seen by 24 hours in both the contused (right) and contralateral (left-contralateral to contused) lungs when compared to sham animals (Figure 4, compare Sham (A) and
Contused (B). Initially, a predominantly neutrophilic infiltrate was seen, but by 24 hours, the predominant cells were mononuclear cells. In addition to the inflammatory cell infiltrate, intraalveolar hemorrhage, alveolar disruption with obliteration of airspace, alveolar septal edema, and atelectasis were seen in contused lung specimens. Aside from intra-alveolar hemorrhage that was occasionally seen in sham animals, these changes were distinct to contused lungs. Consistent with the morphological changes described above, minor histopathology was observed at 3 hours that became more pronounced at 24 hours in the non-contused contralateral lung. Injury to the myocardium was not seen morphologically or histologically (data not shown). Using this contusion model, we had previously shown a slow decrease in inflammatory cell infiltrate (7 days, ref.15); however, there remained significant alveolar structural distortion and hemorrhage. By one month after contusion, there was some repair of damage; however, there continued to be significant alveolar wall thickening, and scar formation present on the surface of the lung as well as within the parenchyma of the lung itself as demonstrated by Masson’s Trichrome stains (ref. 15).

Further evidence of inflammatory cell infiltration into the lungs was observed in the BAL fluid from injured animals (Figure 4C). Comparing sham and injured animals, there was a significant increase in BAL cell counts in both the right (ipsilateral) lung and the left (contralateral) lungs of the injured animals at 3 and 24 hours. At 3 hours after contusion the predominant cell type was the neutrophil (62%); however, by 24 hours, the predominant cell type becomes the macrophage (65%). Lymphocyte numbers increased from 3% to 8% at 3 and 24 hours respectively. In addition to the change in the
inflammatory cell populations, there remained a massive cellular influx to the lungs of the injured animals at 24H after contusion.

*Systemic cytokine and chemokine levels are increased after pulmonary contusion and are temporally consistent with mediating the recruitment of inflammatory cells to the contused lung.* IL-1β and TNF-α expression was evaluated as enhanced expression of these pro-inflammatory cytokines has been associated with activation of innate immunity and has been demonstrated in other models of acute lung injury\(^{16-20}\). IL-1ra was likewise chosen for its association with activation of innate immunity and anti-inflammatory properties\(^{21-23}\). Systemic levels of IL-1β and TNF-α were increased after contusion but not significantly different when compared to sham animals (Figure 5, A and B). In contrast, systemic levels of IL-1ra were dramatically and significantly elevated at 3 hours after contusion when compared to sham animals (Figure 5C). By 24 hours after contusion, IL-1ra, IL-1β, and TNF-α levels decreased to control levels.

Given the influx of innate immune cells (mononuclear cells and PMN) observed in BAL and within the contused lung, systemic expression of the CXC chemokines CINC-1 and MIP-2α and CC chemokines MIP-1α, MCP-1 and RANTES were evaluated. These chemokines have been demonstrated to be elevated in other models of acute lung injury\(^{24-26}\). CXC chemokines are generally chemotactic for granulocytes while CC chemokines are typically chemotactic for mononuclear cells\(^{25-26}\). As shown in Figure 6 (right panels), elevated systemic levels of the CINC-1 and MIP-2α were observed at 3 hours after contusion. At 24 hours after contusion the level of the CXC chemokines had declined, but remained significantly different from sham levels (compare 3H and 24H).
Of the CC chemokines (left panels), only MCP-1 levels showed a significant early increase when compared to sham levels; however by 24H, all the CC chemokines examined were found to be significantly elevated. The systemic levels of these chemokines are consistent with histology in the lung tissue and BAL levels of inflammatory cells with a characteristic initial predominance of PMN infiltrate at 3 hours (mediated by the CXC chemokines) and subsequent mixed neutrophil and mononuclear infiltrate at 24 hours mediated by the increases in CC chemokines.

**Localized inflammatory response by cytokine and chemokine expression is distinct from the systemic response to pulmonary contusion.** The importance of IL-1β and TNF-α as primary mediators involved in the inflammatory response to acute lung injury has been demonstrated by others [16-20]. As circulating levels of IL-1β and TNF-α were not markedly increased with pulmonary contusion, the local expression of these cytokines was evaluated in the contused lung by immunohistochemistry. As shown in Figure 7, contused lung tissue showed enhanced local production of IL-1β (Figure 7A) and TNF-α (Figure 7B) at 3 hours after contusion that decreased by 24 hours (data not shown). Immunoblot of protein lysates from the contused lung also showed increased expression of the CXC chemokines CINC-1 and MIP-2α that remained elevated at 24 hours (Figure 7 C and D, respectively). These data indicate that, in the contused lung, the localized inflammatory response can be distinct from the systemic inflammatory response and suggests that organ-specific cytokine production rather than systemic levels may be a more accurate index of inflammation in pulmonary contusion.
Pulmonary contusion induces an innate immune response that recruits activated neutrophils to the site of injury. The localized and systemic innate immune response to injury is characterized by the expression of numerous mediators that recruit inflammatory cells to the site(s) of injury. In our model of PC, we have shown that these cytokines and chemokines are expressed and demonstrate the infiltration of inflammatory cells to the contused lung. To show that these inflammatory cells are activated in response to contusion, we used immunohistochemistry and flow cytometry to assess expression of elastase and CD-11b, respectively, by PMN in our model of lung contusion. After entering areas of inflammation, elastase is released by activated PMN and causes tissue damage. CD-11b is a member of the β2 integrin family of adhesion receptors and mediates neutrophil firm adhesion to the endothelial cell facilitating migration from the circulation into areas of inflammation. We also sought to characterize pulmonary ICAM-1 expression after contusion, as ICAM-1 is the endothelial receptor counterpart of CD-11b.

As, shown in Figure 8, we observed a significant and progressive increase in neutrophil CD-11b expression at 3 and 24 hours after contusion when compared to sham animals (Figure 8A). Increased ICAM-1 expression in the contused lung was also observed (Figure 8B). Finally, we found increased elastase production in the lungs of injured animals when compared to sham animals (Figure 8C). These data are consistent with a model of contusion that activates an innate immune response that includes elaboration of pro-inflammatory cytokines and chemokines. These inflammatory mediators, in turn, activate neutrophils to express CD-11b and, as suggested by
increased lung expression of ICAM-1, recruit inflammatory cells to the site of contusion. Release of elastase and other proteases in the area of injury may contribute to pathophysiologies associated with pulmonary contusion.

**DISCUSSION:**

Pulmonary contusion is a relatively common injury seen following blunt chest trauma and has been demonstrated to be associated with increased morbidity and mortality\(^5^\)\(^-\)\(^8^\). Data is accumulating that suggests pulmonary contusion is inflammatory in nature\(^1^1^\)\(^-\)\(^1^2^\); however, most of this information comes from observational human studies which in patients with multiple injuries are difficult to interpret and, until recently, large animal studies in which a full complement of molecular probes, gene arrays, and antibodies are not available. Thus there is a need to develop viable, small animal models of pulmonary contusion so that a fundamental understanding of the molecular mechanisms involved in modulating the posttraumatic inflammatory response to this injury may be understood and so that newer, molecularly based therapeutic interventions can be developed.

Over the past three decades, several large animal models have been described to study the physiologic and inflammatory sequelae of pulmonary contusion. Early work in models utilizing mongrel dogs demonstrated progressive histologic changes consistent with contusion and impairment in bacterial clearance in the injured lung\(^2^5^\)\(^-\)\(^3^6^\). Studies in pig models demonstrated increased wet to dry weight, increased BAL albumin content, contralateral lung changes after unilateral contusion (secondary injury), and activation of complement following contusion, suggesting systemic
inflammation. Further studies in pig models demonstrated that cross transfusion of blood into non-injured animals resulted in cardiopulmonary changes similar to those found in injured animals. All these changes were ameliorated by the pretreatment of donor animals with indomethacin, suggesting a primary role for prostanoids in the pathogenesis of pulmonary contusion. Consistent with this hypothesis, Davis and colleagues demonstrated elevated systemic levels of prostacyclin and thromboxane after contusion. Pretreatment with indomethacin inhibited prostanoid production and attenuated pulmonary failure, but did not prevent a secondary injury from developing in the contralateral lung, suggesting other mediators must be involved in the inflammatory response to pulmonary contusion.

Recently, two small animal models of pulmonary contusion associated with closed chest injuries have been described. Knoferl et al utilized a blast injury model in mice while Raghavendran et al utilized a blunt chest injury model in rats. Both models have the capability of increasing injury severity, and both have shown that with increasing energy imparted to the chest, there is a more severe injury noted histologically and potential for animal mortality. These models also showed a progressive deterioration in P/F ratios in injured animals consistent with a clinically significant lung injury. Activation of innate immune responses was observed that included systemic elevation of TNF-α and IL-6 at 24 hours after blast injury in the mouse model. Similarly, elevated levels of IL-1β, IL-6 and CXC/CC chemokines were found in the BAL of the injured rats. The importance of neutrophil influx in mediating lung injury was suggested by pretreating rats with vincristine, thus reducing neutrophil influx and
resulting in improved P/F ratios. Further evidence of immune involvement was revealed by impaired cytokine production by splenocytes and splenic macrophages at 24 hours after chest injury. These results were consistent with observed increased susceptibility to sepsis (CLP) after contusion in the mouse model.

In this study, we have characterized pulmonary contusions and innate immune responses that are a result of an open chest contusion. While distinct from the clinical scenario in which most pulmonary contusion occurs, this model has several significant experimental advantages: 1) it minimizes and quantitates the resultant soft tissue injury as sham animals undergo thoracotomy alone, thus this confounding variable is eliminated; 2) it unquestionably avoids injury to adjacent organs (heart, liver, gastrointestinal tract); 3) it eliminates the protective effects of the chest wall that absorb a variable amount of energy directed towards the lungs so that the injury to the lungs themselves are isolated, measured and characterized; and 4) it allows the induction of a unilateral lung contusion so that the effects on the contralateral lung may be studied.

In this open chest contusion model, we describe the histologic and morphologic evidence of contusion dependent upon the amount of energy imparted to the isolated lung. We found progressive deterioration of P/F ratio suggesting a clinically significant lung contusion. Localized production of proinflammatory mediators, like IL-1β, TNF-α and CXC chemokines was found to occur early in the injury response. In an important expansion of previously described contusion models, we have demonstrated elevated systemic levels of CXC and CC chemokines. The early temporal expression of CXC chemokines correlated with the early neutrophil influx to the lung; CC chemokine
expression lags behind as does the mononuclear infiltrate to the lungs. These results suggest a role for these mediators in coordinating the influx of immune cells to the site of injury. Further evidence of the activation of a systemic immune response is our novel finding of enhanced pulmonary ICAM-1 expression and systemic neutrophil CD-11b expression after pulmonary contusion. These observations are consistent with known downstream effects of IL-1β, TNF-α and CXC chemokines that cause upregulation of these receptors by endothelial cells and neutrophils that can facilitate extravasation into the injured parenchyma. Finally, once the activated neutrophil reaches the contused tissue, we found a markedly enhanced elastase expression, that could further contribute to lung dysfunction and progression of injury severity.

Taken together, these data indicate that contusion results in activation of innate immunity by local production of the cytokines, systemic activation of neutrophils and a potential mechanism for chemokine mediated neutrophil adhesion and diapedesis into the contused lung. Once in the pulmonary parenchyma, the activated neutrophil releases granule contents consisting of hydrolases and proteases (elastase) causing tissue injury. Interestingly, we have also observed an association between systemic CC chemokine response and injury force, where increased force showed increased response (data not shown). It is tempting to speculate that these mediators of “late” responses to injury (activation and recruitment of mononuclear cells) may be more dependent on the severity of the contusion and can be tested with further studies in this model. Towards this end, we are developing a biomechanical model in which the predictive metrics (eg. stress and/or strain) associated with this contusion may be correlated with innate
immune responses and progression to more severe complications like ARDS\textsuperscript{15}. In
addition, it would be valuable to identify an “injury threshold” or impact injury force at
which innate immune responses are activated (or not). A detailed characterization of the
activation of innate immune responses, particularly the systemic response, may be
important for recognizing patients that are at increased risk for further complication(s)
associated with the traumatic injury (eg. ARDS). In addition, the correlation of local
and/or systemic immune response with injury force may be important in the
improvement of protective devices and, more importantly, in the development and
design of therapeutic interventions.

We developed this model in the rat for several reasons: 1) The rat is an extensively
used animal with well understood physiology; 2) The rat genome has been completely
sequenced and molecular probes are readily available including DNA arrays and
proteomics; 3) There are multiple rat strains with differing characteristics in which we
can test hypotheses relative to host response (eg. hypertensive rat); and 4) The rat’s
small size makes high-throughput studies possible yet they are large enough for
physiologic measurements (CT scanning, serum analysis, etc.). An open chest model that
is able to isolate the pulmonary injury is likely to be distinct in the inflammatory
response to lung contusion than closed chest models that mimic the blunt chest trauma
often seen in a clinical setting and may involve injury to the lungs as well as other
organs. Our rodent model is further distinct from the clinical scenario in that the animals
were mechanically ventilated for injury induction with the added risk of ventilator-
induced lung injury. The ventilator settings were identical for both the sham and
injured animals to control for and to minimize the effects of ventilator induced injury in our model. Small animal models are better able to facilitate characterization of serial changes in response to injury than larger animal models. However, direct damage and indirect damage caused by a systemic inflammatory reaction possibly affecting initially uninvolved areas of the lung and even the contralateral lung may be different in humans when compared to any animal models. However, it is of note that the physiologic and immunologic response seen in this injury model appears to parallel what is seen in humans who sustain pulmonary contusion. In conclusion, we feel that the data presented within this report describe a viable model of pulmonary contusion and accurately reflects the innate inflammatory response associated with this injury. The open chest model of pulmonary contusion described in this study can further our understanding of the inflammatory responses of the lung and may reveal novel approaches to predicting and intervening in acute lung injury.

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Figure 1. (A) The experimental design and animal cohorts used for the experiments in this study are shown. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (#O5-186) at Wake Forest University School of Medicine (Winston-Salem, NC) and are in accordance with state, federal and National Institutes of Health regulations. One additional sham rat was included at 3H and 24H for histology studies. (B) The Cortical Contusion Impactor used to deliver direct impact injury of variable and known force to rat lungs. The assembly has an adjustable animal positioner with stereotactic mounts and an enhanced linear motor driven impactor and controller. The impact head houses a photo-optics sensor to determine velocity, impact depth, and dwell resulting in reproducible impacts. Velocities of 5.2 m/s deliver an impact with energy of 150 J/M² to the exposed right lung of an intubated rat.
Figure 2. Morphological changes and pulmonary edema are observed with lung contusion. Lungs were sham operated (A) or contused (B). After 24H lungs, were isolated and compared. The sham rat (A, thoracotomy alone) does not show the severe hemorrhage and laceration observed in right lung of the injured rat (B). Morphologic changes were also seen in the contralateral (left) lung of the injured rat by 24 hours (arrow). (C) Wet to dry weight lung weights were measured and compared at 3 and 24H after contusion. At 3H, wet to dry weight of lungs of injured animals (■) was significantly different from sham animals (□). At 24 hours after injury, a significant increase in lung weight (up to 28%) was observed contused lungs.
Figure 3. PaO2/FiO2 ratios show decreased lung function in injured rats. Rats were sham operated or injured with a pulmonary contusion to the right lung. Prior to and every 15 min. for 3H following contusion, arterial samples were obtained for blood gas measurements. Data are reported as the average PaO2/FIO2 (± SEM) for sham (□) or contused (■) at each time. * p<0.05 injured versus sham for samples obtained at 30 min. and for all samples/times thereafter.
**Figure 4.** The contused lung shows profound leukocyte infiltration. Animals were injured (B) on the right lung as described in the methods. After 24H lungs were excised and stained for the presence of inflammatory cells. The non-contused contralateral left lung is shown on the left (L-contralateral to contused), the contused right lung is shown on the right (R-contused). A sham operated/non-contused control right and left lung (A) is shown for comparison. (C) The BAL from contused lungs shows increased differential cell counts. Rats were sham operated ( ■) or injured ( □) with a pulmonary contusion to the right lung. At 3H and 24H, left and right lungs were lavaged and BAL fluid analyzed for differential cell counts. Contused lungs showed significant differences when compared to the sham lungs, with a profound cell infiltration into the contused lung (right lung) at 24H.

![Figure 4](image-url)
Figure 5. Systemic IL-1RA cytokine levels show a rapid and transient response. Blood levels at 3 and 24 hours after PC of IL-1β (A), TNFα (B) and IL-1ra (C) were measured by ELISA and compared in sham (♦) and contused (■) animals. The data shown are the average (pg/ml) ±SE levels for each condition. Systemic levels in control animals (no thoracotomy and injury) are shown for reference (□). Systemic IL-1ra shows a rapid increase (3H) that decreases to sham levels 24H after contusion.
Figure 6. Systemic chemokine expression is consistent with recruitment of inflammatory cells to the injury. Blood levels of CXC chemokines, (shown on the right) MIP-2, and CINC-1 and CC chemokines (left panels), MCP-1, MIP-1α, RANTES were measured in control (□), sham (■) and injured (■) animals by ELISA at 3 and 24 hours after contusion. Average levels (pg/ml ±SE) for each condition are shown. Significant differences between sham and contused animals are indicated (*). MIP-2 and CINC-1 chemokines showed a rapid and significant increase at 3H post-injury, consistent with the recruitment of PMN after contusion. All chemokines showed a significant increase at 24H after contusion, with CC chemokines playing an important role in mononuclear cell immune response.
Figure 7. Localized inflammatory response of cytokine and chemokine expression is distinct from the systemic inflammatory response. Rats were sham operated or injured with a pulmonary contusion to the right lung. At 3H after sham operation or contusion, the right lungs were removed and tissue prepared for immunohistochemistry (A and B) or lysates prepared at 3H and 24H for immunoblot (W. blot, 20µg protein, C and D) as described in the methods. IL-1β (A) or TNF-α (B) expression is increased in tissue from contused lungs (right panels) when compared to tissue from sham (left panels) operated lungs. CINC-1 (C) and MIP-2α (D) expression is also increased in lysates from contused lungs (C) when compared to sham (S) lungs, and the expression is sustained for at least 24 hours. Expression levels are quantitated on W. blots as described in the methods. Data shown are representative of at least 3 independent experiments.
Figure 8. Lung contusion and inflammatory mediators recruit activated immune cells to the site of injury. Sham (■) and contused (■) (3 and 24H post contusion) animal blood neutrophils were analyzed by flow cytometry for activation by CD-11b expression as described in the methods (A). A total of 10,000 events were counted and data presented as average percent (± SE) of cells expressing CD-11b. A representative histogram of results from injured animals at 3H and 24H is shown on the right. (B) Sham and contused lungs were isolated and proteins analyzed by immunoblot for ICAM-1 expression at 3H and 24H after contusion. W.blot (30ug protein) shows increased ICAM-1 expression in the contused lung. (C) Sham (left panel) and contused (right panel) lungs were isolated and proteins analyzed by immunohistochemistry for elastase expression at 24H. Contused lungs show increased elastase expression. The results shown are representative of at least 3 independent experiments.
CHAPTER III

The following manuscript was published in *Shock* 2007;28(4):447-452 and is reprinted with permission. Stylistic variations are due to the requirements of the journal.

Title: Toll-like receptor 2 participates in the response to lung injury in a murine model of pulmonary contusion

Running title: TLR2 and pulmonary contusion

Authors: J. Jason Hoth, William P. Hudson, Noel A. Brownlee, Barbara K. Yoza, Elizabeth M. Hiltbold, J. Wayne Meredith and Charles E. McCall

ABSTRACT:
Blunt chest trauma resulting in pulmonary contusion with an accompanying acute inflammatory response is a common but poorly understood injury. We report that Toll-like receptor 2 participates in the inflammatory response to lung injury. To show this, we use a model of pulmonary contusion in the mouse that is similar to that observed clinically in humans based on histologic, morphologic, and biochemical criteria of acute lung injury. The inflammatory response to pulmonary contusion in our mouse model is characterized by pulmonary edema, neutrophil transepithelial migration, and increased expression of the innate immunity pro-inflammatory cytokines, interleukin-1β and interleukin-6, the adhesion, intracellular adhesion molecule-1, and chemokine (C-X-C motif) ligand-1. Compared with wild-type animals, contused *Tlr2(-/-)* mice have significantly reduced pulmonary edema and neutrophilia. These findings are associated with decreased levels of circulating chemokine (C-X-C motif) ligand-1. In contrast,
systemic interleukin-6 levels remain elevated in the Toll-like receptor 2 deficient phenotype. These results show that Toll-like receptor 2 has a primary role in the neutrophil response to acute lung injury. We suggest that an unidentified non-infectious ligand generated by pulmonary contusion acts via Toll-like receptor 2 to generate inflammatory responses.

Key words: neutrophil, blunt chest trauma, mouse model, innate immunity

INTRODUCTION:

Multi-system trauma is a significant source of patient morbidity and mortality, accounting for 175,000 deaths annually (1). Blunt chest trauma resulting in pulmonary contusion is a common injury, affecting 10-17% of all trauma admissions with estimates of mortality at 10-25% (2;3). Sequelae of pulmonary contusion vary widely and include infection (pneumonia), local organ failure (Acute Respiratory Distress Syndrome, ARDS), and remote organ failure (Multiple Organ Dysfunction/Failure, MODS/MOF)(4). Some lung dysfunction is a direct result of hemorrhagic trauma; however, there is mounting evidence that lung contusion generates an innate immune response with acute inflammation. In both animal and human studies, contusion results in activation of innate immunity with the localized production of a variety of inflammatory cytokines and chemokines, followed by neutrophil infiltration into the areas of injury (5-7). The magnitude of this response has been associated with both ARDS and infection (8). Altered apoptosis and cytokine production in immunocompetent cells, located both within the lung and remotely, has been observed (9). Defective bacterial clearance and an increased susceptibility to sepsis have also been reported (10).
With infection, the activation of the innate immune system is mediated by Toll-like receptors (TLRs). TLRs are a phylogenetically conserved receptor superfamily that confers specificity to innate immune responses based on cell specific expression, intra- and extra-cellular co-factors, and cross talk between family members with shared signaling components (11). The best studied TLR ligands are components of infectious bacteria and viruses (e.g. double stranded RNA) and are potent inflammation and innate immunity activators. For example, TLR4 is the putative receptor for lipopolysaccharide and TLR2 is the receptor for the gram positive and negative bacterial cell wall components lipoteichoic acid (LTA), lipoprotein and peptidoglycan. Additionally, there are a number of noninfectious TLR ligands that have been identified, such as hyaluronan, heparin-sulfate, heat shock protein (HSP) 60, HSP70, High Mobility Group Box-1 (HMGB-1) protein and surfactant protein-A.

Little is known about what initiates inflammation following tissue injury, although there is mounting evidence supporting a role for TLR signaling after noninfectious lung injury. Recently, a protective role for both TLR2 and TLR4 was demonstrated after bleomycin-induced and lethal oxidant lung injury (12-14). Additionally, exogenous expression of HSP70 in pulmonary epithelial cells has been associated with improvement of ARDS and reduced pulmonary neutrophilia (15). However, the role for TLR in mediating innate immune and inflammatory responses to pulmonary contusion, which does not depend on the presence of infection, is unknown.

In this report, we describe a novel model of pulmonary contusion in the mouse resulting in an acute lung injury based on histologic, morphologic, and biochemical
criteria that is analogous to what is described clinically. Post-injury inflammation is associated with pulmonary neutrophilia, edema, adhesion receptor expression, and systemic chemokine (CXC motif) ligand-1 (CXCL1) chemokine expression. To test whether TLR2 mediated the post-injury inflammatory response, we used \( Tlr2^{-/-} \) mice in our model of pulmonary contusion. In TLR2 knockout mice we found reductions in pulmonary neutrophilia and edema that correlated with reduced systemic CXCL1 expression. We conclude that the inflammatory response to pulmonary contusion is dependent, at least in part, on TLR2 activation.

**MATERIALS AND METHODS:**

**Animals.** Male, age-matched wild-type C57/BL6 and \( Tlr2^{-/-} \) mice were utilized in this study. The TLR2 knockout mice were originally provided by S. Akira (Osaka University, Osaka Japan) and were bred and maintained under specific pathogen-free conditions at the animal facility at Wake Forest University School of Medicine. A total of 121 animals, 86 wild type and 35 \( Tlr2^{-/-} \) were used in these studies.

**Blunt chest injury model.** A blunt chest injury was induced using the Cortical Contusion Impactor (CCI) (16), with the following modifications. Mice were anesthetized with 2% isoflurane at a flow rate of 1L/min. The mouse was positioned left lateral decubitus and during inspiration, the right chest was struck with the CCI along the posterior axillary line, 1cm above the costal margin. A known velocity (5.8 m/s) and depth of penetration (6.3 mm) transmits a precisely defined amount of energy (152 J/M\(^2\)) that results in a reproducible pulmonary contusion. Control animals received anesthetic alone. Mice were sacrificed at 3, 24, or 48 hours after injury by cervical dislocation. Serum and tissue
samples were collected and stored at -70°C until use. The mechanical and technical specifications of the CCI have been previously described (16). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (#O5-186) at Wake Forest University School of Medicine and are in accordance with State, Federal and National Institutes of Health regulations.

**Histopathology.** Lung tissue samples were obtained immediately after sacrifice at 3, 24 or 48 hours after injury. Liver tissue samples were obtained at 24 hours after injury from 3 wild type and 3 TLR2 knockout mice. Samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for examination. All tissue samples were evaluated by an experienced pathologist (N.B.) blinded to animal status as previously described (16).

**Bronchoalveolar lavage (BAL).** After cannulation of the trachea, lavage was performed using 4ml of phosphate buffered saline (PBS, Sigma Biochemical, St. Louis, MO) at 4°C. BAL fluid was centrifuged at 300 x g, 4°C for 10 minutes. The supernatant (BAL) was collected and stored at -70°C. The cell pellet was resuspended in PBS, counted and differentiated as previously described (16).

**Lung wet:dry weight.** Whole lung specimens were removed, weighed (wet weight) and placed in an oven at 37°C for 24 hours to dry and reweighed (dry weight). Wet:dry weight ratios were determined are reported as a measure of pulmonary edema.

**Cytokine and chemokine expression.** Interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and CXCL1 (keratinocyte derived chemoattractant, KC) were measured by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.
Immunoblot. Lung tissue extracts were prepared in homogenization buffer (10mM Tris, 0.5% DTT, 0.1% SDS) using the TissueMiser (Fisher Scientific, Pittsburgh, PA). The homogenates were cleared by centrifugation at 3000 x g, 20°C for 10 minutes followed by a high speed centrifugation at 10,000 x g, 20°C for 10 minutes. Protein concentrations were determined by microassay using Coomassie Protein Assay Reagent according to the manufacturer’s instructions (Pierce, Rockford, IL). After separation by SDS-PAGE, proteins were transferred and immunoblotting performed as previously described (16). Anti-Intracellular Adhesion Molecule-1 (ICAM-1, Serotec, Raleigh, NC) antibody-antigen complexes were visualized by enhanced chemiluminescence (ECL, Pierce) according to the manufacturer’s instructions. Relative protein levels were quantitated using ImageQuant 5.2 (Molecular Dynamics, Amersham BioSciences, Sunnyvale, CA).

Statistical analysis. Data were analyzed using GraphPad Prism software (San Diego, CA) and are reported as the mean ± SE. One-way analysis of variance (1way ANOVA, 95% confidence interval) with Bonferroni multiple comparison post-test was used to compare the means between injury groups. P-values <0.05 was considered to be significant.

RESULTS:

A reproducible model for blunt chest trauma and pulmonary contusion in mice. We first established a reproducible model of pulmonary contusion in a closed chest model of acute lung injury in the mouse. This blunt chest injury creates a model of lung contusion that is similar to that observed in human lung injury. A blunt chest injury of precise force reproducibly delivered to mice using the CCI resulted in 8% (6/75 injured mice)
mortality, with all deaths occurring within 5 minutes of impact. Postmortem examination in these animals revealed massive intrathoracic hemorrhage associated with pulmonary parenchymal laceration and/or avulsion of the pulmonary pedicle. Grossly, all animals displayed evidence of pulmonary contusion involving the upper lobes of the right lung. No evidence of cardiac pathology was encountered. Liver histopathology from contused mice showed no evidence of injury (data not shown).

*Activation of inflammatory responses after pulmonary contusion.* Given that a hallmark of acute inflammation is the presence of infiltrating neutrophils, we evaluated pulmonary histology and BAL specimens for the presence of a neutrophilic infiltrate at 3, 24, and 48 hours after injury. Histologic examination of injured lungs showed evidence of intra-alveolar hemorrhage, alveolar septal edema and neutrophilic infiltrate as early as 3 hours, with maximum severity encountered at 24 hours after injury and a decline by 48 hours (Figure 1). We also found significant neutrophilia in the BAL specimens at 24 hours after injury (Figure 2A). Pulmonary edema, an indicator of acute lung injury, as measured by wet:dry lung weight was significantly elevated at all timepoints in all injured animals (Figure 2B). These results demonstrate that pulmonary contusion results in an acute lung injury with a neutrophilic influx to areas of injured pulmonary parenchyma.

*Pulmonary contusion activates innate immune responses.* Extravasation of activated neutrophils from the circulation to the site of injury requires the activation of innate immunity and early cytokine expression. In particular, expression of IL-1β and IL-6 are elevated in patient and animal models with blunt chest trauma and appear? to play a
critical role in the initial innate immune response and recruitment of neutrophils to the injury site (7;17-19). Activation of innate immune responses in our model is supported by significantly increased alveolar expression of IL-1β in the BAL (Figure 3A) and elevated systemic levels of IL-6 in the serum (Figure 3B). Additionally, increased expression of adhesion receptors, specifically ICAM-1, has been demonstrated to play an important role in neutrophil trafficking to areas of injury/inflammation. Consistent with this, injured lung tissue in this model also showed significantly higher levels of ICAM-1 (Figure 3C) in concert with the neutrophil influx observed on lung histology and in the BAL. This result is further supported by markedly increased serum levels of the neutrophil chemoattractant CXCL1 at 3 hours after injury, preceding the localized inflammatory response of migration of neutrophils to the lung (Figure 3D). In summary, we developed a model of pulmonary contusion where we found activation of innate immune responses and localized acute lung inflammation in blunt chest injured mice.

**TLR2 dependent activation of innate immunity and local inflammatory response after pulmonary contusion.** Although much is known about TLR signaling in the inflammatory response to infectious lung injury, little is known about the role of TLR signaling after traumatic lung injury. As both alveolar macrophages and pulmonary epithelial cells express TLRs, we sought to determine whether TLR2 mediated a response to pulmonary contusion. We utilized age- and sex-matched mice deficient in TLR2 and subjected them to blunt chest injury using the CCI. TLR2 knockout mice demonstrated intra-alveolar hemorrhage and alveolar septal edema to a lesser degree than observed in mice that expressed TLR2. There was a profound reduction in interstitial neutrophilia in the TLR2
knockout mice (Figure 4). No liver histopathology was observed at 24hrs after injury in TLR2 deficient mice (data not shown). Furthermore reduced leukocyte counts were observed in TLR2 deficient mice (Figure 5A). Pulmonary edema, as measured by wet to dry lung weight ratio was also reduced in TLR2 knockouts when compared to wild type mice (Figure 5B). These findings support a TLR2 dependent lung injury response. We next determined whether Tlr2(-/-) mice had reduced expression innate immune mediators after pulmonary contusion. Serum IL-6 (Figure 6A) and tissue ICAM-1 (Figure 6B) levels were not significantly different between groups of animals. In contrast, there was a decrease in CXCL1 expression in TLR2 knockout mice when compared to injured wild-type animals (Figure 6C). These data suggest that neutrophil accumulation in the lung after contusion may depend on TLR2 activation of CXCL1 expression.

**DISCUSSION:**

We used a reproducible murine model of lung contusion and its accompanying acute neutrophilic inflammation to examine molecular events that generate the innate immune response and acute inflammation. This model simulates the clinically relevant lung injury that follows blunt chest trauma. We provide the following mechanistic concept for lung contusion and inflammatory injury with lung edema: Lung contusion induces an unknown endogenous ligand(s) that act through TLR2 to induce neutrophilic influx into lung tissue. This influx appears to depend on TLR2 dependent induction of CXCL1, as suggested by our findings that mice not expressing TLR2 have marked reductions in both neutrophil accumulation and expression of this chemokine. Members of the CXC
chemokine subfamily are primarily chemotactic for PMNs and appear to play a role in all phases of the inflammatory response in the lung (20). In contrast, the acute lung injury response that leads to increased IL-6 expression does not depend on TLR2.

Increasing evidence implicates TLR-mediated signaling in the inflammatory response to noninfectious tissue injury. In models of vascular injury, ischemia-reperfusion, colitis, hyperoxic lung injury, hemorrhagic pancreatitis, and femur fracture, a role for TLR signaling has been reported (12-14, 21-25). TLR2 is required for pulmonary neutrophil transepithelial migration in a MyD88-dependent manner after bleomycin-induced lung injury (12). A reduction in CXCL1 and CXCL2 chemokine production was seen in TLR2/4 double knockout and TLR2 deficient animals, respectively. Unlike our results, TLR2 and TLR4 were found to ultimately be protective in bleomycin injury in that TLR deficient mice demonstrated a higher mortality when compared to wild-type mice. Increased pulmonary epithelial cell apoptosis and diminished NF-κB activation occurred in TLR deficient animals. Hyaluronan fragments appeared to mediate this injury. In a model of hemorrhagic pancreatitis, acute lung injury and TNFα expression was dependent upon pulmonary expression of TLR2 and this effect was abrogated by nitric oxide (23). Likewise, in a model of vascular injury, inflammatory gene expression (TNFα, IL-1β, IL-6), reactive oxygen species production, and neointimal formation were all attenuated in TLR2 deficient mice (25). These findings implicate a central role for TLR2 activation in the regulation of the inflammatory response to noninfectious tissue injury. We have confirmed the importance of TLR2 in regulating noninfectious innate
immune and inflammatory responses and extended the role for TLR2 in acute noninfectious lung injury.

In our model of blunt chest trauma, we found evidence for a reduced severity of injury as indicated by a reduction in pulmonary edema (wet wt.:dry wt.) and reduced pulmonary neutrophilia in both the interstitium and alveolar space of TLR2 deficient animals. We found significantly decreased serum levels of CXCL1 suggesting that TLR2 activation may upregulate chemokine expression in response to pulmonary contusion, either in direct response to lung trauma or through some as yet unknown mechanism. No difference in mortality was seen between wild type and TLR2 deficient animals, nor were differences in systemic IL-6 and pulmonary ICAM-1 levels seen. Our results show specific TLR2-dependent responses that are distinct from other pathways that may be activated by pulmonary contusion. One potential signaling pathway may involve TLR4 activation. There is a clear association between TLR4 and TLR2 activation and subsequent CXC chemokine and ICAM-1 expression, primarily established in infectious models of injury (26-30). In a femur fracture model of injury, animals deficient in TLR4 were found to have reduced systemic IL-6 levels however, chemokine expression and neutrophil influx were not evaluated (24). The response to pulmonary contusion in TLR4 knockout mice will be the focus of future studies.

These data also support an important role for neutrophil influx in mediating lung injury after pulmonary contusion. This finding is in agreement with the findings observed by Raghavendran et al in a rat model of lung contusion (19). Pre-injury depletion of neutrophils resulted in a lesser lung injury as indicated by a reduction in
PaO$_2$/FiO$_2$ ratios after injury, indicating a neutrophil-dependent lung injury. However, bleomycin-induced and hyperoxic models of lung injury were associated with reduced pulmonary neutrophilia in the TLR2/4 double knockout and TLR 4 deficient animals, respectively, and were associated with higher mortality (12-14). These discrepancies may be explained, at least in part, by differences in the injury mechanisms in each model. Bleomycin-induced lung injury is more analogous to a chronic lung disease state characterized by lung fibrosis and hyperoxic-induced lung injury relies upon the production of reactive oxygen species to mediate lung injury (31). Neither of these injury mechanisms has been shown to be involved in the initial inflammatory response to pulmonary contusion.

Non-infectious endogenous ligands for TLR2 or other TLRs may be released by physical lung damage and degradation of components the extracellular matrix or the presence of necrotic cells (31-33). These include HSP60, HSP70, HMGB-1, Gp96, and matrix proteins such as hyaluronan (11). Any of these or an as yet unidentified ligand may be generated during a response to pulmonary contusion. Disrupting TLR2-dependent responses may reveal novel approaches to limit blunt chest injury in humans.

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Figure 1. Lung histopathology after pulmonary contusion. Uninjured and injured lungs were formalin fixed, stained and analyzed by an experienced pathologist. Lung sections of the contusion are shown (250X magnification) at 3, 24 and 48 hours after injury. An uninjured lung section is shown for comparison. Intra-alveolar hemorrhage and alveolar septal edema are observed. Neutrophil infiltration appears to peak at 24H after injury. Specimens shown are representative of at least 3 independent experiments.
Figure 2. Activation of inflammatory responses after pulmonary contusion. (A) The BAL from uninjured (open bars) and injured lungs (closed bars) were analyzed for neutrophil (PMN) infiltration at 3, 24 and 48 hours after injury (n=12, 12, 9 and 4, respectively; **p≤.001 relative to uninjured). (B) Whole lungs from uninjured (open bars) and injured lungs (closed bars) were weighed before (wet wt.) and after drying (dry wt.) as described in the methods. Pulmonary edema is expressed as the ratio of wet wt.:dry wt. at 3, 24 and 48 hours after injury (n=7, 5, 4, and 4 respectively; **p≤.001 or *p≤.05 relative to uninjured).
Figure 3. Activation of innate immune responses after pulmonary contusion. IL-1β (A) and IL-6 (B) were measured in the BAL and serum, respectively, by ELISA and ICAM-1 (C) measured by immunoblot as described in the methods. Uninjured (open bars) and injured lungs (closed bars) were analyzed at 3, 24 and 48 hours after injury. (A) n=6, 8, 10, and 6; (B) n=12, 11, 10 and 8; (C) n=2, 3, 3, and 3; *p<.05 relative to uninjured. (D) Serum levels of CXCL1 (KC) were measured by ELISA as described in the methods. Uninjured (open bars) and injured lungs (closed bars) were analyzed at 3, 24 and 48 hours after injury (n=4, 11, 11, and 6, **p<.001 relative to uninjured).
Figure 4. Lung histopathology after pulmonary contusion in TLR2 deficient mice. Injured lungs were formalin fixed, stained and analyzed by an experienced pathologist. Lung sections of the contusion are shown at 3H or 24H (250X magnification) after injury. The injured WT (left) lung sections are shown for comparison with sections from injured TLR2 knockout (right) mice. Intra-alveolar hemorrhage, alveolar septal edema, and neutrophil infiltration in the wild type injured mice are reduced in TLR2 deficient mice. Specimens shown are representative of at least 3 independent experiments.
Figure 5. Decreased inflammatory response in TLR2 deficient mice after pulmonary contusion. (A) The BAL from WT (closed bars) and TLR2 deficient (hatched bars) injured lungs were analyzed for neutrophil (PMN) infiltration at 3 or 24 after injury (n=12, 4, 9 and 7, respectively, as shown on the graph; **p<.001 relative to WT). (B) WT (closed bars) and TLR2 deficient (hatched bars) injured lungs were weighed before (wet wt.) and after drying (dry wt.) as described in the methods. Pulmonary edema is expressed as the ratio of wet wt.:dry wt. at 3 and 24 after injury (n=5, 6, 4, and 6 respectively as shown on the graph; **p<.001 relative to WT).
Figure 6. Activation of innate immunity in TLR2 deficient mice after pulmonary contusion. IL-6 (A) and CXCL1 (C) were measured serum by ELISA and ICAM-1 (B) measured by immunoblot as described in the methods. WT (open bars) and TLR2 deficient (hatched bars) injured lungs were analyzed at 3 and 24 hours after injury. (A) n=11, 9, 10 and 10; (B) n=3, 3, 3 and 3; (C) n=11, 11, 12 **p≤.001 relative to WT).
CHAPTER IV

The following manuscript was published in Shock 2008;31:376-381 and is reprinted with permission. Stylistic variations are due to the requirements of the journal.

Title: Toll-like receptor 4 dependent responses to lung injury in a murine model of pulmonary contusion

Running title: TLR4 and pulmonary contusion

Authors: J. Jason Hoth, Jonathan D. Wells, Noel A. Brownlee, Elizabeth M. Hiltbold, J. Wayne Meredith, Charles E. McCall, and Barbara K. Yoza

ABSTRACT:
Blunt chest trauma resulting in pulmonary contusion with an accompanying acute inflammatory response is a common but poorly understood injury. We previously demonstrated that toll-like receptor 2 participates in the inflammatory response to lung injury. We hypothesized that the toll-like receptor 4, in a MyD88-dependent manner, may also participate in the response to lung injury. To investigate this, we used a model of pulmonary contusion in the mouse that is similar to that observed clinically in humans and evaluated post injury lung function, pulmonary neutrophil recruitment and the systemic innate immune response. Comparisons were made between wild type mice and mice deficient in toll like receptor 4 or MyD88. We found toll-like receptor 4 dependent responses to pulmonary contusion that include hypoxemia, edema, and neutrophil infiltration. Increased expression of interleukin 6 and chemokine (C-X-C motif) ligand-1 in the bronchoalveolar lavage and serum was also dependent on TLR4.
activation. We further demonstrated that these responses to pulmonary contusion were dependent on MyD88, an adapter protein in the signal transduction pathway mediated by toll-like receptors. These results show that toll-like receptors have a primary role in the response to acute lung injury. Lung inflammation and systemic innate immune responses are dependent on toll-like receptor activation by pulmonary contusion.

**Key words:** inflammation, blunt chest trauma, mouse model, innate immunity

**INTRODUCTION:**

Blunt chest trauma resulting in pulmonary contusion is a common injury, affecting 10-17% of all trauma admissions with estimates of mortality at 10-25% (1;2). Sequelae of pulmonary contusion vary widely and include infection (pneumonia), local organ failure (Acute Respiratory Distress Syndrome, ARDS), and remote organ failure (Multiple Organ Dysfunction/Failure, MODS/MOF) (3). In both animal and human studies, lung contusion results in activation of innate immunity with the localized production of a variety of inflammatory cytokines and chemokines, followed by neutrophil infiltration into the areas of injury (4-6). The magnitude of this response has been associated with both ARDS and infection (7;8).

The innate inflammatory response to noninfectious tissue injury is, in many ways, indistinguishable from that initiated by infection. Activation of innate immunity during infection is mediated by activation of the Toll-like receptors (TLR) (9;10). At least 13 TLRs have been identified (11), recognizing conserved components of infectious bacteria (flagella, LPS, PGN) and viruses (eg. double stranded RNA). All but TLR3 utilize the intracellular adapter protein MyD88 in order to induce NFκB activation and subsequent
inflammatory mediator expression (12). An evolving paradigm has suggested that endogenous TLR ligands are liberated after cell injury or necrosis and the resulting “danger signal” serves to activate innate immune mechanisms warning the host of tissue injury (13). Indeed, evidence is accumulating that supports a role for TLR signaling after noninfectious lung injury including identification of a number of endogenous TLR ligands such as hyaluronan, heparin-sulfate, heat shock proteins (HSP) 60, HSP70, High Mobility Group Box-1 (HMGB-1) protein and surfactant protein-A which are potentially liberated after lung trauma (14-19). We have previously demonstrated a role for TLR2 induced activation of innate immunity after lung contusion involving TLR2 dependent CXCL1 expression and neutrophil influx to the injured lung (20). Complementary findings were seen after bleomycin-induced and lethal oxidant lung injury, in which TLR2 and TLR4 deficiency resulted in reduced pulmonary neutrophilia and CXC chemokine expression (14;21;22). Additionally, exogenous expression of the TLR 2 and TLR4 ligand, HSP70 in pulmonary epithelial cells has been associated with improvement of ARDS and reduced pulmonary neutrophilia (23). These data clearly indicate that TLR mediated events play an important part in the initial innate immune response to noninfectious lung injury.

Little is known about the role of TLR4 and MyD88 in the inflammatory response to pulmonary contusion. The purpose of this study was to determine, using clinically relevant criteria, if TLR4 and MyD88 participate in the response to blunt chest injury. We delivered a blunt chest injury to wild type and mice deficient in TLR4 or MyD88 expression and evaluated systemic inflammatory mediator expression, pulmonary
neutrophil recruitment, and post injury lung function. We found a TLR4 dependent inflammatory response to pulmonary contusion that is characterized by edema, neutrophil infiltration, and increased expression of the innate immunity pro-inflammatory mediator interleukin 6 and the chemokine (C-X-C motif) ligand-1 (CXCL1). We further demonstrate that these responses share a common signal transduction pathway that utilizes the TLR adapter protein, MyD88. Our results indicate that lung inflammation and systemic innate immune responses are dependent on TLR activation after pulmonary contusion.

METHODS:

Animals: Male, age matched (8-9 weeks) wild-type (C57/BL6), TLR4-/-, and MyD88-/- mice were utilized in this study. The MyD88-/- mice were provided by S. Akira (Osaka University, Osaka Japan) and backcrossed onto a C57/BL6 background for at least 8 generations. The TLR4-/- were obtained from Jackson Laboratories (Bar Harbor, ME) and the allele introgressed into the C57/BL6 background for at least 5 generations. All animals were bred and maintained under specific pathogen-free conditions at the animal facility at Wake Forest University School of Medicine. The protocol used in this study was approved by the Animal Care and Use Committee (#A06-068).

Blunt Chest Injury Model: Injury was induced using the Cortical Contusion Impactor (CCI) as described previously (20). Briefly, mice are anesthetized with 2% isoflurane at a flow rate of 1L/min. The mouse is positioned left lateral decubitus and during inspiration, the right chest is struck with the CCI along the posterior axillary line, 1cm above the costal margin. Control animals receive anesthetic alone. Mice were followed
for various times after injury as specified in the figure legends. Serum and tissue samples were collected at the time of death by isoflurane overdose and cervical dislocation.

**Arterial Blood Gas:** Arterial blood gas (ABG) samples were obtained from the ileac artery. The animals were induced with isoflurane anesthesia (3%) and then maintained on an admixture of 1.5% isoflurane with 100% oxygen at a flow rate of 1L/min. After 5 minutes, a midline laparotomy incision was created from just above the pubis to just below the sternum. The animal is eviscerated and the ileac artery exposed. A 26 gauge needle was then used to cannulate the iliac artery to obtain the blood. The partial pressure of oxygen (pO2) was measured in each sample using a Stat Profile pHOx Blood gas Analyzer (Nova Biomedical, Waltham, MA) according to the manufacturer’s instructions.

**Histopathology:** Lung specimens harvested at time of death were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin (H&E). Slides were evaluated by an experienced pathologist (NAB) and graded for the presence of interstitial neutrophilic infiltrate, intra-alveolar hemorrhage, and pulmonary septal edema as described previously (20).

**Bronchoalveolar lavage (BAL):** After sacrifice, BAL was performed by cannulation of the trachea and lavage is performed using 4ml of phosphate buffered saline (PBS, Sigma Biochemical, St. Louis, MO) at 4°C. BAL was centrifuged at 300 x g, 4°C for 10 minutes and supernatant collected and stored at -70°C until use. The cell pellet was counted and differentiated as previously described (20).
**Lung wet:dry weights:** Whole lung specimens were removed, weighed and placed in an oven at 37°C for 24 hours to dry and then reweighed. Wet weight:dry weight ratios were calculated.

**Cytokine and Chemokine expression:** BAL and/or serum levels of IL-6 and CXCL1 were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Samples were assayed in duplicate.

**Immunoblot (Western blot) analysis:** Lung tissue samples were thawed and suspended in homogenization buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 0.1% SDS, protease inhibitors) and homogenized (TissueMiser, Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 3000 x g, 4°C for 10 minutes and the supernatant centrifuged again at 10,000 x g, 4°C for 10 minutes. Solubilized protein concentrations were determined using Coomassie Protein Assay Reagent according to the manufacturer’s instructions (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred to an Immuno-Blot® PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Blots were blocked for 1 hour with Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE). Anti-neutrophil elastase (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin (Sigma-Aldrich, St. Louis, MO) antibodies were diluted in Odyssey Blocking Buffer with 0.1% Tween-20 and the membranes were incubated overnight at 4°C. Antibody-antigen complexes were detected using appropriately matched IRDye® secondary antibodies (Li-Cor Biosciences, Lincoln, NE) and visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Relative
protein levels were quantitated using Odyssey v1.2 Software (Li-Cor Biosciences, Lincoln, NE) and reported as integrated intensity.

**Immunohistochemistry:** Lung sections were deparaffinized with three changes of xylenes and hydrated through a series of graded alcohol changes (3 washes of 100% EtOH for 5min; 95% EtOH for 5min; 75% EtOH for 5min). Endogenous peroxidase was quenched with 0.3% H₂O₂ in MeOH for 30min. An antigen retrieval step was performed by heating sections in 10mM citrate buffer pH6.0 for 45min. Sections were incubated with a 1:50 dilution of anti-neutrophil elastase antibody (Santa Cruz Biotechnology, Santa Cruz CA) and visualized using VECTASTAIN ABC and DAB peroxidase substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Sections were counterstained with Mayer’s Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO) and mounted using VectaMount Mounting Medium (Vector Laboratories, Burlingame, CA).

**Statistical analysis:** Data are reported using GraphPad Prism (v 4.03, San Diego, CA) and expressed as the mean ± SE of independent observations as indicated in the Figure legends. One-way analysis of variance (1way ANOVA) with multiple comparison post test (Bonferroni) was used to compare the means between injury groups. A p-value <0.05 was considered to be significant.

**RESULTS:**

*Lung dysfunction after pulmonary contusion is dependent upon the TLR4 pathway.* We use the CCI to induce a pulmonary contusion in our mouse model of blunt chest injury. Blood gas measurements showed that hypoxia induced by contusion was significant at 3 hours
after injury and continued to increase for at least 24 hours (Figure 1A). We found significantly less hypoxia in TLR4 deficient animals suggesting that TLR4 participates in the response to acute lung injury. Consistent with this observation, we further found that MyD88 deficiency also reduced hypoxia in injured animals (compare TLR4 and MyD88 with WT, Fig. 1B). As a further indicator of acute lung injury, pulmonary edema was significantly elevated with blunt chest injury, but significantly less edema was found in MyD88 deficient mice (Figure 1C). These results showed that acute lung injury activated TLR4 and intracellular signaling through MyD88.

*Lung pathology after pulmonary contusion is dependent on the TLR4 pathway.* Acute lung injury is often correlated with the presence of neutrophils in the BAL of patients with pulmonary contusion. In our animal model, we evaluated pulmonary histology and BAL specimens for the presence of a neutrophilic infiltrate after injury. Histologic examination of injured lungs from WT mice showed evidence of intra-alveolar hemorrhage, alveolar septal edema and neutrophilic infiltrate as early as 3 hours, with maximum pathologies observed at 24 hours after injury (Figure 2). Injured lungs from TLR4 and MyD88 deficient mice also showed evidence of hemorrhage, edema and neutrophils. Pathologic evaluation (blinded) of lung injury in knockout animals reported similar intra-alveolar hemorrhage to WT mice, but reduced alveolar septal edema and neutrophil infiltration.

*Neutrophil recruitment to the lung after pulmonary contusion is dependent on the TLR4 pathway.* To quantitate neutrophil recruitment after injury, we used BAL to measure alveolar neutrophil infiltration. As found in our previous studies (4;20), we observed a
significant increase in neutrophils in the BAL from injured mice (Figure 3A). The number of BAL neutrophils was significantly decreased in TLR4 and MyD88 deficient mice, supporting that the local inflammatory response is dependent on TLR activation.

To further assess the role of neutrophils in our model of pulmonary contusion, we used immunoblot to measure neutrophil elastase levels in injured tissue. As shown in Figure 3B, we found low levels elastase in tissue homogenates from uninjured lungs. Consistent with increased neutrophil recruitment and activation by pulmonary contusion, we found increased levels of elastase in injured lung tissue. Elastase levels were decreased in TLR4 and MyD88 deficient (KO) mice. Immunohistochemical staining of lung tissue for neutrophil elastase (Figure 3C) was consistent with these results. Taken together, these results showed that blunt chest injury and pulmonary contusion significantly increases neutrophil infiltration and activation. The recruitment of neutrophils in this model of acute lung injury is dependent on the TLR4 pathway and intracellular signaling through MyD88.

*The TLR4 pathway is required for increased pulmonary and systemic IL-6 and CXCL-1 levels after pulmonary contusion.* Elaboration of cytokines and chemokines are characteristic of inflammatory responses observed in patients with pulmonary contusion and in experimental models of acute lung injury. We measured IL-6, CXCL1, CXCL2/3, and MIP-1α levels in the BAL from mice at various times after injury. Overall, we found the only significant and measurable increases in IL-6 and CXCL1 in the BAL at 3H after injury (Figure 4). We observed significant increases in these mediators in the BAL from WT mice when compared to knockout mice, supporting that local inflammatory
responses are dependent on the TLR4 pathway and MyD88 intracellular signaling. Finally, we measured levels of inflammatory mediators in the blood after pulmonary contusion. In the serum of injured animals we found a rapid, robust and transient increase in both IL-6 and CXCL-1 (Figure 5). These levels were significantly decreased in TLR4 and MyD88 deficient mice. CXCL2/3 and MIP-1α levels were not elevated in the BAL or the serum (data not shown) suggesting that these chemokines are not involved in the inflammatory response to lung contusion. These results show that systemic mediators of inflammation are increased in blunt chest injury with pulmonary contusion. Taken together our results show that local and systemic responses to injury are dependent, at least in part, to TLR4 activation and are mediated by the intracellular adapter, MyD88.

**DISCUSSION:**

Mounting evidence supports a role for TLR-mediated signaling in the initial inflammatory response to noninfectious tissue injury with numerous potential endogenous TLR ligands being identified (14-19). A paradigm is evolving in which these endogenous ligands, when liberated from injured or necrotic tissue, act as “danger signals” warning the host of tissue injury and initiating the ensuing inflammatory response (13). We have used a mouse model of blunt chest trauma to study the initial innate inflammatory response as might be observed in patients with pulmonary contusion. We found that our model approximates the human injury using pathophysiologial and biochemical markers. Furthermore, our previous studies showed that the innate inflammatory response to pulmonary contusion depends on
TLR2 induced signaling (20). We found that TLR2 deficient animals have a less severe lung injury. The reduction in neutrophil chemotaxis to the contused lung correlated with decreased in CXCL1 expression and appeared to depend, at least in part, on TLR2 activation.

Having previously identified a role for TLR2 dependent signaling, in this report we have extended our investigations into the mechanisms regulating the response to traumatic lung injury and pulmonary contusion. As the TLR4 pathway shares many components with TLR2 signaling, we hypothesized that TLR4 may also participate in this injury response. We tested for TLR4 dependent responses in our injury model and found a clinically less severe lung injury, reduced pulmonary neutrophilia, and a diminished innate response in TLR4 deficient mice. In a manner similar to TLR2, TLR4 activation by pulmonary contusion appears to direct neutrophil migration to the lung through the expression of the chemokine, CXCL1 and independent of CXCL2/3. This is consistent with TLR4 mediated signaling that has been implicated in several other models of noninfectious tissue injury and systemic inflammation (17,18,24-29).

Specifically, in bleomycin-induced and hyperoxic models of lung injury, TLR4 deficiency was associated with reduced pulmonary neutrophilia, diminished chemokine expression and increased mortality. However, our study supports a model where TLR4 deficiency is protective in a primary acute lung injury model as demonstrated by better ABG seen following lung contusion with no differences seen in mortality over the study period. Others have reported findings similar to ours in models of ischemia/reperfusion and sterile tissue injury where the absence of TLR4 is associated with a reduced innate
inflammatory response and improved organ function. One explanation for the divergent findings seen in noninfectious lung models involves differing mechanisms of injury with a greater dependence upon neutrophil mediated tissue injury after contusion.

Our results support the concept that neutrophil recruitment to the lung after blunt chest injury is dependent on CXCL1 and independent of CXCL2/3. There are 4 major chemokine families, and in general, neutrophil chemotaxis is highly dependent upon CXC chemokine expression (30). CXCL1 and CXCL2/3 are the murine equivalents of IL-8, the principle chemokine involved in neutrophil chemotaxis after traumatic lung injury in humans (31). Alveolar macrophages and to a lesser extent, alveolar epithelial cells have been demonstrated to produce CXC chemokines after lung injury (32). Both CXCL1 and CXCL2/3 have been demonstrated to be elevated in other models of infectious and noninfectious lung injury, and divergent roles for both have been described (14;22;33-35). Our results suggest that TLR4 and MyD88 induced neutrophil recruitment to the lung after contusion is dependent on CXCL1, raising the possibility that differing pharmacokinetics, ligand specificity, cellular involvement, and/or disruption of normal cell-cell interactions occur after pulmonary contusion when compared to other lung injury models. The involvement of other chemokines such as LTB4 or C5a cannot be excluded based upon our results.

Activation of TLR4 initiates intracellular signaling by recruitment of one or more adaptor proteins. MyD88 was the first adapter protein identified as involved in TLR4 signal transduction and NFκB activation (9;10). TLR4 utilizes both MyD88 dependent and MyD88 independent signaling pathways that result in activation of NFκB and
subsequent inflammatory cytokine expression. MyD88 also participates in TLR2 signaling. In our injury model, MyD88 deficient animals showed improved oxygenation as well as reduced pulmonary neutrophil influx and innate response when compared to animals deficient in TLR4. These findings support that TLR4 utilizes the MyD88-dependent signaling pathway when activated after lung contusion; however, the MyD88 independent pathway utilizing the adapter protein, TRIF, cannot be completely excluded. Our study also supports that the response to lung contusion is not entirely dependent on TLR4 signaling, but other receptors are likely involved in mediating this response. MyD88 is a component of the intracellular signaling pathways for IL-1, IL-18, and all known TLRs with the exception of TLR3. We have previously shown that the innate immune response to pulmonary contusion is also TLR2 dependent and the more protective effect of MyD88 deficiency may reflect the sharing of this intracellular mediator by TLR2 and TLR4.

We are uncertain how TLR signaling is initiated in the lung after contusion and what, if any, coreceptors are utilized. Thus far, HMGB-1 and hyaluronan are potential TLR4 activators in models of soft tissue injury; however, a large number of endogenous TLR4 ligands exist, including surfactant, heat shock, heme, and other matrix proteins, and HMGB-1 (14-19). Many of these mediators are likely present in the lung after contusion, and some of these ligands have specificity for more than one TLR, likely with differing affinities. Thus, it is possible that several different ligands act in concert through different TLRs in order to generate a response. Ligand gradients and localization may also contribute to injury responses. Participation of coreceptors such as
CD14 in the TLR4 response to pulmonary contusion also remains a possibility. For example, surfactant proteins are endogenous TLR4 ligands that require CD14 in order to activate TLR signaling (36;37). In contrast, other TLR4 ligands such as HMGB-1 and hyaluronan are CD14 independent after sterile tissue injury (17;18). Low molecular weight hyaluronan fragments are thought to promote formation of a TLR4 complex with MD-2 and CD44 (17) for signal transduction.

Cell type specificity of the TLR response to noninfectious injury is also not known. Alveolar macrophages, Type II alveolar epithelial cells, pulmonary endothelial cells, and tracheobronchial cells participate in the lung’s innate response to both infectious and noninfectious lung injury (14;32;34;38;39). Germane to this report, Mollen (13) used chimeric mice in a model of hemorrhagic shock and bilateral femur fracture to address the question of cell type specific responses. They found that both bone marrow-derived and parenchymal cells are required in order to generate the ensuing TLR-dependent inflammatory response, suggesting that multiple cell types are involved in the generation of the innate response to noninfectious lung injury. Similar findings were reported by Wu (25) in a model of renal ischemia/reperfusion; however, it was suggested that renal parenchymal cells rather than immune cells, initiated the inflammatory response. Thus, as there are likely multiple endogenous ligands and TLRs involved in the response to pulmonary contusion, there appear to be multiple cell types acting in concert that are responsible for the inflammatory response to noninfectious lung injury.
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Figure 1. Lung dysfunction after pulmonary contusion is dependent on the TLR4 pathway. Arterial blood from uninjured (open) and injured animals (closed) was isolated and blood gas analysis performed as described in the methods. (A) PaO₂:FiO₂ ratios were calculated and are shown for WT animals at 3, 6 and 24 hours after injury (n=5/time). There is a significant decrease in PaO₂:FiO₂ in WT animals after injury (*p<0.01), with the most significant decrease at 24H (**p<0.01 relative to other injury times). (B) Blood gas analysis on injured TLR4 and MyD88 deficient animals (gray) at 24H shows significantly increased PaO₂:FiO₂ ratios when compared to WT injured animals (#p<0.05, n=7/genotype). (C) WT (black) and TLR4 and MyD88 deficient (gray) injured lungs were isolated at 24H after injury and weighed before (wet wt.) and after drying (dry wt.) as described in the methods (n=5/genotype). Pulmonary edema (wet wt/dry wt) is significant (*p<.05) in all animals and is significantly decreased in MyD88 deficient mice (**p<.05 compared to WT).
Figure 2. Lung pathology after pulmonary contusion is dependent on the TLR4 pathway. Injured lungs were formalin fixed, stained and analyzed by an experienced pathologist. Lung sections of the contusion are shown (100X magnification) at 24 hours after injury. Hemorrhage is evident in all injuries (left panels) and masked by red filter (right panels) to highlight neutrophil staining. Septal edema and neutrophil infiltration is reduced in TLR4 and MyD88 deficient mice when compared to WT. Specimens shown are representative of lung injuries in at least 3 animals/genotype.
Figure 3. Neutrophil recruitment to the lung after pulmonary contusion is dependent on the TLR4 pathway. (A) The BAL from WT (closed) and TLR4 and MyD88 deficient (gray, KO) injured lungs were analyzed for neutrophil infiltration. Compared to uninjured (n=5), a significant increase in PMN in the BAL from WT mice is observed (**p<0.001) that is significantly decreased in KO mice (*p<0.001, n=6/genotype). (B) Lung homogenates from uninjured (open), WT (closed) and TLR4 and MyD88 deficient (gray) injured lungs were analyzed by immunoblot for neutrophil elastase (n=4). A significant increase in elastase in WT lung extracts is observed (**p<0.001) that is significantly decreased in KO mice (*p<0.01). (C) Immunostaining for neutrophil elastase in lungs from WT, and TLR4 or MyD88 deficient animals (100X magnification). Specimens are representative of immunohistochemistry for least 3 animals/genotype. Data shown are obtained at 24 hours after injury.
Figure 4. The TLR4 pathway is required for pulmonary inflammatory response to contusion. IL-6 and CXCL-1 in the BAL from uninjured (open) and injured (filled) animals (n=5 for each genotype) were measured by ELISA as described in the methods. Levels in all samples were low and results are reported if the difference between uninjured and injured was statistically significant (**p < 0.001). (A) IL-6 levels in the BAL from injured MyD88 deficient animals (gray) at 3H shows a significant decrease when compared to WT injured animals (*p < 0.01). (B) CXCL-1 levels in the BAL from injured TLR4 and MyD88 deficient animals (gray) at 3H shows a significant decrease when compared to WT injured animals (*p < 0.001). Uninjured (IL-6 and CXCL-1) and CXCL-1 in MyD88 deficient animals’ BAL was below sensitivity (2pg/ml) of the assay.
Figure 5. The TLR4 pathway is required for systemic innate immune responses to pulmonary contusion. Innate immune proteins in the serum of uninjured (open) and after injury (closed) were measured by ELISA as described in the methods. A rapid and significant (**p < 0.001) increase in IL-6 (A) and CXCL-1 (B) levels in the serum from injured WT mice at 3H is observed. IL-6 and CXCL-1 levels in the serum from knockout animals at 3H are significantly less (*p < 0.001). (A) 3H n=9/genotype; 24H n=5/genotype; (B) 3H, n=8/genotype; 24H n=6/genotype.
CHAPTER V

The following manuscript is being prepared for submission to *Shock*. Stylistic variations are due to the requirements of the journal.

**Title:** Mechanism of neutrophil recruitment to the lung after pulmonary contusion

**Running title:** PMN recruitment and pulmonary contusion

**Authors:** J. Jason Hoth, Jonathan D. Wells, Elizabeth M. Hiltbold, J. Wayne Meredith, Charles E. McCall, and Barbara K. Yoza

**ABSTRACT:**
Blunt chest trauma resulting in pulmonary contusion with an accompanying acute inflammatory response is a common but poorly understood injury. We previously demonstrated that the response to lung injury is similar to the inflammatory response that follows innate immune activation. We hypothesized that the innate immune and inflammatory activation of neutrophils may also participate in the response to lung injury. To investigate this, we used a model of pulmonary contusion in the mouse that is similar to that observed clinically in humans and evaluated post injury lung function and pulmonary neutrophil recruitment. Comparisons were made between injured mice with and without neutrophil depletion. We further examined the role of chemokines in neutrophil recruitment to the injured lung. We found that lung injury and resultant physiological dysfunction after contusion was dependent upon the presence of neutrophils in the alveolar space. We also show that CXCL1, CXCL2/3, and CXCR2 are involved in neutrophil recruitment to the lung after injury. Our results also indicate that
ICAM-1 is locally expressed and actively participates in neutrophil recruitment to the lung. These results show that neutrophils have a primary role in lung dysfunction and suggest that the response to noninfectious tissue injury is similar to the response to infection.

Key words: neutrophil, pulmonary contusion, inflammation, chemokine, cytokine, mouse model

INTRODUCTION:

Pulmonary contusion is a relatively common injury seen after blunt trauma that is associated with significant morbidity, organ dysfunction (ALI/ARDS), and is potentially lethal\(^{1,2,3}\). Increasing evidence suggests that the maladies seen after lung contusion are not simply a mechanical phenomenon but are inflammatory in nature\(\textsuperscript{4-7}\). In addition to structural damage to the lung, an inflammatory cell infiltrate composed primarily of neutrophils (PMNs) ensues. Various inflammatory mediators are then produced, which lead to the breakdown of pulmonary capillary basement membranes, hypoxia, increased pulmonary capillary resistance, myocardial dysfunction, production of toxic oxygen metabolites, and alterations in inflammatory cell function\(\textsuperscript{4-7}\). Dysfunction occurs in immunocompetent cell populations located both within the lung and remotely\(\textsuperscript{8}\). Alterations in apoptosis, defective bacterial clearance, and an increased susceptibility to subsequent septic challenge have been described after lung contusion\(\textsuperscript{9}\). These data strongly indicated that lung contusion initiates an inflammatory response associated with neutrophil recruitment to the lung that results in an acute lung injury.
The hallmark of acute lung injury is the presence of infiltrating leukocytes. Among leukocytes, neutrophils figure prominently in the initial inflammatory response. Activation, localization and extravasation of neutrophils from the circulation to site of injury is a complex process that is thought to be dependent upon early response cytokine expression (IL-1β, TNF-α); the production of chemotactic molecules, such as chemokines, complement 5a (C5a), and leukotriene B4 (LTB4); and the upregulation of cell surface-adhesion molecules such as ICAM-1. The accumulation of activated neutrophils in the interstitium and alveolar space, results in the production of reactive oxygen species and the release of proteolytic enzymes leading to acute inflammation and when well orchestrated, this will clear the alveolar space of invading pathogens. However, activated neutrophils may independently contribute to pulmonary damage and dysfunction. In a number of animal models of lung injury and in human studies, neutrophil accumulation in the lung is a key event in the early development of acute lung injury and ARDS.

There are four major families of chemokines, CXC, CC, C, and CXC3, that behave as potent chemotactic factors for leukocytes. The CXC family is primarily chemotactic for neutrophils and appears to play a role in all phases of acute lung injury. CXC chemokines are produced by immune and nonimmune cells in the lung, including alveolar macrophages, endothelial cells, and alveolar epithelial cells. In mice, keratinocyte-derived chemoattractant (KC, CXCL1) and macrophage inflammatory protein-2α (MIP-2α, CXCL2/3) are members of the CXC family and are orthologues of CXCL8 (IL-8) in man. Both bind to and activate a common receptor, CXCR2, which is
also expressed on immune and nonimmune cells in the lung\textsuperscript{20-21}, but have different receptor affinities, levels of expression and effects on neutrophil function\textsuperscript{22-24}. The pivotal role of CXC chemokines and their receptor, CXCR2, in neutrophil chemotaxis has been demonstrated in models of ischemia/reperfusion, peritonitis, and lung injury\textsuperscript{18-20,24-25}.

Adhesion receptor expression on endothelial and alveolar epithelial cells is essential to neutrophil diapedesis into areas of infection or injury\textsuperscript{26-29}. These receptors include the selectins (E- and P-selectin) and members of the immunoglobulin receptor superfamily (ICAM-1)\textsuperscript{26-29,30}. IL-1β and TNF-α stimulate ICAM-1 expression in endothelial and alveolar epithelial cells\textsuperscript{31,32}. The interaction between ICAM-1 and the β2-integrin CD11b/CD18 (murine MAC-1), results in “firm attachment” (adhesion) of neutrophils and migration into areas of lung inflammation, although CD18-independent pathways have been described\textsuperscript{31,33-36}. Factors that increase neutrophil β2-integrin expression include CXC chemokines and C5a\textsuperscript{11}. Studies using antibody neutralization or knockout mice have demonstrated that pulmonary ICAM-1:β2-integrin interactions mediate neutrophil recruitment, accumulation and activation in models of pneumonia, immune complex alveolitis, ischemia/reperfusion, and peritonitis\textsuperscript{26-27,37}.

In a murine model of traumatic lung injury, we previously demonstrated localized expression of early response cytokines, elevated systemic levels of CXC chemokines, upregulated pulmonary ICAM-1 and neutrophil CD11b expression, and a robust pulmonary neutrophilia associated with a clinically significant reduction in the PaO\textsubscript{2}/FiO\textsubscript{2} (P/F) ratio following lung contusion\textsuperscript{6,7,38}. Based upon these findings, we hypothesize that neutrophil recruitment to the lung is in part responsible for the
pulmonary dysfunction seen after lung contusion and is dependent upon CXC chemokine and ICAM-1 expression. Here we demonstrate that neutrophils are primarily responsible for pulmonary dysfunction after lung contusion. We also demonstrate that neutrophil recruitment to the injured lung is dependent upon expression of CXC chemokines, the CXCR2 receptor, and ICAM-1.

**METHODS:**

**Animals:** Male, age-matched wild-type C57/BL6 mice were utilized in this study. Mice were maintained were bred and maintained at the animal facility at Wake Forest University School of Medicine. The protocol used in this study was approved by the Animal Care and Use Committee (#A07-246).

**Blunt Chest Injury Model:** Injury was induced using the Cortical Contusion Impactor (CCI) as described previously. Briefly, mice are anesthetized with 2% isoflurane at a flow rate of 1L/min. The mouse is positioned left lateral decubitus and during inspiration, the right chest is struck with the CCI along the posterior axillary line, 1cm above the costal margin. Control animals receive anesthetic alone. Mice were followed for various times after injury as specified in the Figure Legends. Serum and tissue samples were collected at the time of death by isoflurane overdose and cervical dislocation.

**Bronchoalveolar lavage (BAL):** After sacrifice, BAL was performed by cannulation of the trachea and lavage was performed using 4ml of phosphate buffered saline (PBS, Sigma Biochemical, St. Louis, MO) at 4°C. BAL was centrifuged at 300 x g, 4°C for 10
minutes and supernatant collected and stored at -70°C until use. The cell pellet was counted and differentiated as previously described⁴.

**Arterial Blood Gas:** Arterial blood gas (ABG) samples were obtained from the dorsal tail artery. Animals were induced with isoflurane anesthesia (3%) and then maintained on an admixture of 1.5% isoflurane with 100% oxygen at a flow rate of 1L/min. After 5 minutes, the dorsal tail artery was identified, severed, and blood was collected and used to determine the partial pressure of arterial oxygen (PaO₂). PaO₂ was measured in each sample using a Stat Profile pHOx Blood gas Analyzer (Nova Biomedical, Waltham, MA) according to the manufacturer’s instructions.

**Neutralization/blocking studies:** Neutrophils were depleted using the functional grade RB6-8C5 monoclonal antibody (eBioscience, San Diego, CA) that reacts with mouse Ly6G. Twenty-four hours prior to lung injury mice were given 100ug in PBS of α-Ly6G or an IgG isotype control by intraperitoneal injection. Neutrophil depletion was confirmed by flow cytometry and evaluation of peripheral blood smear with differential staining. Blocking of the CXCR2 receptor was performed using the hexapeptide antileukinate, Ac-RRWWCR-NH₂ (AnaSpec, Inc., Fremont, CA), as previously described²⁰⁻³⁹. Mice were given a 52mg/kg dose of antileukinate or PBS subcutaneously 30 minutes prior to injury. Neutralization of CXCL1 (KC) and CXCL2/3 (MIP-2) chemokine activity was performed using anti-KC and anti-MIP-2 monoclonal antibodies (R&D Systems, Minneapolis, MN). Mice were given 25ug of α-KC and/or 25ug of α-MIP-2 in a single dose intravenously 30 minutes prior to injury. Isotype controls were administered in a similar fashion. Neutralization of ICAM-1 was performed using α-
ICAM-1 monoclonal antibody (R&D Systems, Minneapolis, MN). Mice were given 50 mcg of α-ICAM-1 30 minutes prior to injury. Isotype controls were administered in a similar fashion.

**ICAM-1 Immunohistochemistry:** Paraffin embedded sections were deparaffanized in three changes of xylenes for 5 minutes each and hydrated through three changes for 3 minutes each of 100% EtOH, 95% EtOH, and 80% EtOH. Endogenous peroxidase was quenched with 3% H₂O₂ in PBS for 10 min. An antigen retrieval step was performed by heating sections at 95°C in 10 mM citrate buffer at pH 6.0 for 25 min. Sections were blocked for non-specific binding with 10% FBS in PBS for 60 minutes. A three-step staining procedure was performed using a 1:100 dilution of hamster α-mouse ICAM-1 antibody (BD Biosciences, San Jose, CA), a 1:100 dilution of a biotinylated mouse α-hamster secondary antibody (BD Biosciences, San Jose, CA) and Streptavidin HRP (BD Biosciences, San Jose, CA). The ICAM-1 staining was visualized with DAB substrate for peroxidase (Vector Laboratories, Burlingame CA). Sections were counterstained with Mayer’s Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO) and coverslips were mounted using VectaMount Mounting Medium (Vector Laboratories, Burlingame, CA).

**RESULTS:**

*Neutrophil depletion prior to lung contusion improves post-injury pulmonary function.* We have previously reported an association between reduced pulmonary neutrophilia and improved pulmonary function in injured TLR 2 and 4 deficient animals. However, those studies were not designed to investigate the mechanisms and importance of neutrophil recruitment to the injured lung. Thus, through the use of neutralizing
antibody, we first sought to evaluate the importance of neutrophil recruitment to the lung after lung contusion. Mice were treated with α-Ly6G, a monoclonal antibody with specificity for neutrophils, 24hrs prior to lung injury. As confirmed by peripheral blood smear and flow cytometry, α-Ly6G treatment resulted in depletion of greater than 90% of circulating neutrophils (data not shown). Consistent with neutrophil depletion, BAL PMN counts were significantly diminished at 24hrs after lung injury when treated with α-Ly6G (Figure 1A). In neutropenic animals, pulmonary function improved significantly as evidenced by a marked increase in P/F ratios (Figure 1B). Injured mice treated with IgG isotype control antibody showed PMN infiltrate in the BAL and P/F ratios that were not significantly different from injured mice without pre-treatment. These data correlate PMN in the BAL with lung dysfunction and is similar to what can be observed clinically in patients with pulmonary contusion.

CXC chemokines and the CXC chemokine receptor 2 (CXCR2) receptor mediate neutrophil recruitment to the injured lung after lung contusion. Having demonstrated a correlation between of pulmonary neutrophilia and postinjury pulmonary dysfunction, we next sought to determine the mechanisms that mediate neutrophil recruitment to the injured lung. The CXC chemokines, CXCL1 and CXCL2/3, have been shown to be involved in PMN recruitment in models of acute lung injury6,12,18. We have previously reported increased systemic levels of CXCL1 at 3hrs after lung contusion6,7. Based upon these findings, we hypothesized that CXCL1 and CXCL2/3 were involved with neutrophil recruitment to the lung after lung contusion. As both CXC chemokines act through a common receptor, CXCR2, we used antileukinate, a hexapeptide inhibitor of CXCR2, to
inhibit CXCR2 signaling in our model of lung contusion\textsuperscript{20–29}. Treatment with antileukinate resulted in a significant reduction in BAL neutrophils at 24hrs after lung contusion (Figure 2A). The decrease in PMN in the BAL correlated with an improvement in pulmonary function after injury (Figure 2B).

We next sought to determine the roles of CXCL1 and CXCL2/3 after lung contusion using blocking antibodies. Inhibition of either CXCL1 or CXCL2/3 alone had no significant effect on PMN counts in the BAL. However, when both CXCL1 and CXCL2/3 were inhibited, there was a significant reduction in BAL PMN numbers (Figure 3). Collectively, these data suggest that CXC chemokines mediate neutrophil trafficking to the lung after lung contusion, but other complimentary mechanisms exist outside the CXC family of chemokines.

\textit{ICAM-1 expression is induced by lung injury and mediates neutrophil accumulation.} ICAM-1 interacts with the β2 integrin, CD11b/18, on the surface of the neutrophil and mediates firm adhesion, an essential step for neutrophil diapedesis into areas of inflammation\textsuperscript{31–33}. We have previously demonstrated increased CD11b expression on the surface of circulating neutrophils shortly after lung contusion, leading us to hypothesize that ICAM-1 is involved in this inflammatory process\textsuperscript{7–38}. We initially sought to determine whether lung contusion induces expression of ICAM-1 in the injured lung. As shown in Figure 4A, there is a marked increase in pulmonary ICAM-1 expression at 3hrs after lung injury.

Studies administering blocking antibodies were then performed to determine whether ICAM-1 participated in neutrophil accumulation in the lung. In order to reach
the alveolar space, neutrophils must leave the vascular space and traverse the pulmonary interstitial space. These steps require interaction with endothelial and epithelial cells and ICAM-1 has been shown to be involved with both of these processes\textsuperscript{11, 37}. Therefore, we administered blocking antibody intraperitoneally and intratracheally to assess the endothelial and epithelial contributions to this process, respectively (Figure 4B). Our results indicate that ICAM-1 is an active participant in neutrophil recruitment to the lung after contusion. Interestingly, the route of administration made little difference as no additive or synergistic response was seen.

**DISCUSSION:**

We found that lung injury and resultant dysfunction after contusion was dependent upon the presence of neutrophils in the alveolar space. Acute lung injury caused by direct or indirect mechanisms is associated with the presence of neutrophils in the microvasculature and alveolar space of the lung. Upon activation and arrival to the lung, neutrophils release several cytotoxic substances including reactive oxygen species, eicosanoids, cationic proteins, and proteolytic enzymes\textsuperscript{14}. During infection, these substances play an important role in host defense, but these same mediators potentially can damage pulmonary parenchyma giving rise to the theory that neutrophils are central to the pathogenesis of lung injury\textsuperscript{14}. This has been demonstrated in human and animals studies\textsuperscript{40-44}. In patients with ARDS, the degree of pulmonary neutrophilia is directly proportional to mortality\textsuperscript{45}. In animal models of ischemia/reperfusion, immune complex alveolitis, and endotoxin mediated lung injury, the inhibition of neutrophils
and their function improves outcome, lung function, and prevents lung injury (reviewed\textsuperscript{21}).

The theory that neutrophils mediate acute lung injury remains controversial. For example, acute lung injury has been demonstrated to occur after bleomycin and hyperoxic induced lung injuries in neutropenic animals and in the absence of pulmonary neutrophilia\textsuperscript{46}. Enhanced apoptosis of type 2 alveolar epithelial cells was postulated to be central to the pathogenesis of lung injury. Others have shown that inhibition of Fas/FasL signaling using of siRNA, Fas deficient animals, and Fas neutralizing antibody results in reduced pulmonary inflammation in models of direct and indirect lung injury\textsuperscript{47-49}. At present, we have not evaluated alveolar epithelial apoptosis in our model of lung injury. Although a direct relationship between lung dysfunction and pulmonary neutrophilia is demonstrated, it is possible that neutrophils may influence epithelial cells through cell to cell interactions or through the release of inflammatory mediators that activate intrinsic or extrinsic apoptotic pathways that ultimately result in lung injury. Further studies are needed to determine if neutrophil presence alone or neutrophil function is responsible for posttraumatic lung injury. In either case, it appears that neutrophils play a salient role as an initiator of lung physiological dysfunction.

CXC chemokines are primarily chemotactic for neutrophils and have been demonstrated to be involved in neutrophil recruitment to the lung in several models of lung injury\textsuperscript{45}. CXCL1 and CXCL2/3 are the principle CXC chemokines in mice and are homologues of IL-8 in man. IL-8 is perhaps the most extensively studied chemokine in
man. It has been demonstrated to be elevated in the BAL in patients with ARDS, and has been independently associated with organ dysfunction, organ failure, and death after injury\textsuperscript{50-54}. Although CXCL1 and CXCL2/3 act through a shared receptor, CXCR2, they have different receptor affinities, levels of expression and differentially effect neutrophil migration, apoptosis, respiratory burst, and phagocytosis\textsuperscript{20, 24, 55-56}. For example, only CXCL1 is selectively transported to the blood whereas CXCL2/3 is retained in the pulmonary compartment\textsuperscript{55}. CXCL1 systemically primes circulating neutrophils to migrate to the lung in response to MIP-2\textsuperscript{55}. Endothelial cells, alveolar macrophages and alveolar epithelial cells produce CXC chemokines and express CXCR2 on their surface\textsuperscript{19}.

In our model of lung injury, we have previously reported elevated levels of CXCL1, and suggested that CXCL1, CXCL2/3, and CXCR2 were involved in neutrophil recruitment to the lung after pulmonary contusion\textsuperscript{6, 7}. The studies present here confirm and extend this observation. We also show that inhibition of CXCR2 did not completely abrogate neutrophil accumulation in the lung and that inhibition of CXCL1 or CXCL2/3 alone did not significantly affect pulmonary neutrophilia. These data support that other signaling pathways and mechanisms are involved in neutrophil recruitment to the injured lung. Candidate chemotactic mediators for neutrophils in this model include LTB4, C5a, C3a, CC chemokines (MIP-1a), and other members of the CXC chemokine family (CXCL5 and CXCL15) (reviewed\textsuperscript{57}). Alveolar macrophages and type 2 alveolar epithelial cells express these mediators and may participate in the injury response to pulmonary contusion. This is the subject of future investigation.
PMN recruitment from the blood into the lung occurs at the postcapillary venules. The initial steps of this cascade include E-, P-, and L-selectin mediated rolling of neutrophils along the surface of the endothelial cell. Subsequent to this, firm adhesion occurs as a result of interaction between β2 integrins on the surface of the neutrophil and members of the immunoglobulin superfamily of intracellular adhesion receptors on the endothelial cell. The CD11b/18 integrin complex on neutrophils interacts primarily with ICAM-1 on the surface of the endothelial cell. The importance of this interaction in neutrophil recruitment to areas of inflammation in the lung has been demonstrated in other models of lung injury where deficiency or inhibition of either CD11b/18 or ICAM-1 reduced neutrophil migration to the lung. ICAM-1 is involved in neutrophil movement across the endothelial cell into the interstitium and the epithelial cell into the alveolar space. Crossing the epithelial barrier has been shown to be pivotal for inducing lung injury.

We previously demonstrated increased CD11b expression in circulating neutrophils after lung contusion and suggested that ICAM-1 was likely involved in neutrophil migration to the lung (and our unpublished observations). Here we show that ICAM-1 is locally expressed and actively participates in neutrophil recruitment to the lung after contusion. As either route of administration (IP or IT) was effective at reducing neutrophil infiltration, it appears that ICAM-1 expression on endothelial and epithelial cells participate in this process. Furthermore, similar to CXCL1 and CXCL2/3, it appears that other mediators in addition to ICAM-1 are involved in neutrophil recruitment. Neutrophil migration to the lung has been described to be mediated by CD11b/18...
dependent and independent pathways depending on the stimulus. Pulmonary neutrophilia due to Gram negative bacteria or IL-1 is CD11b/18 dependent while that due to gram positive infection and C5a are CD11b/18 independent. Additionally, other members of the immunoglobulin superfamily of receptors aside from ICAM-1 such as VCAM-1 and PECAM-1 may be involved in this process.

In summary, we sought to determine whether: 1) pulmonary neutrophilia was responsible for reduced pulmonary function after lung contusion; and 2) to determine the mechanisms responsible for neutrophil recruitment to the lung. We used blocking techniques to test the effect of various mediators on neutrophil recruitment. Our results showed that neutrophil recruitment to the injured lung is dependent upon CXC chemokines, the CXCR2 receptor, and localized expression of ICAM-1. We found that pulmonary neutrophilia is correlated with lung dysfunction after lung contusion.

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Figure 1. Neutrophil depletion improves post-injury pulmonary function. Mice were pre-treated with anti-Ly6G (α-Ly6G) or IgG at 24H prior to injury. BAL and arterial blood from uninjured (Control, open bars) and injured animals (filled bars) were isolated at 24H after injury. (A) The cell pellet was counted and differentiated as described in the methods. There is a significant increase in PMN in the BAL in injured mice (*p<0.001). α-Ly6G pretreated, neutropenic injured mice showed a significant decrease in PMN (#p<0.001). There is no significant difference in PMN between injured and IgG treated mice (**p>0.05). n=6,6,6,3 for experimental groups, respectively, as shown. (B) Blood gas analysis showed significantly decreased PaO2:FiO2 in injured animals (*p<0.001). α-Ly6G pretreated, neutropenic injured mice showed significantly better PaO2:FiO2 (#p<0.001). There is no significant difference in PaO2:FiO2 between injured and IgG treated mice (**p>0.05). n=5 for all experimental groups.
**Figure 2. CXCR-2 dependent neutrophil recruitment to the injured lung after lung contusion.** Mice were pre-treated with anti-leukinate (α-Leukinate) or vehicle (PBS) at 30min. prior to injury. BAL and arterial blood from uninjured (Control, open bars) and injured animals (filled bars) were isolated at 24H after injury. (A) The cell pellet was counted and differentiated as described in the methods. There is a significant increase in PMN in the BAL in injured mice (*p<0.001). α-Leukinate treated injured mice showed a significant decrease in PMN (#p<0.001). There is no significant difference in PMN between injured and PBS treated mice (**p>0.05). n=6,6,6,3 for experimental groups, respectively, as shown. (B) Blood gas analysis showed decreased PaO₂:FiO₂ in injured animals (*p<0.001). α-Leukinate treated injured mice showed significantly better PaO₂:FiO₂ (#p<0.05). There is no significant difference in PaO₂:FiO₂ between injured and IgG treated mice (**p>0.05). n=5,5,5,3 for all experimental groups, respectively, as shown.
Figure 3. CXC chemokine dependent neutrophil recruitment to the injured lung after lung contusion. Mice were pre-treated with α-CXCL1, α-CXCL2/3 or both (α-CXCL1+2/3) at 30min. prior to injury. BAL from injured animals was isolated at 24H after injury. The cell pellet was counted and differentiated as described in the methods. There was no significant difference in PMN the BAL in injured mice or injured mice treated with either antibody alone (*p>0.05). In contrast, injured mice treated with both antibodies showed a significant decrease in PMN (#p<0.05) when compared to untreated injured mice. n=6 for all experimental groups.
Figure 4. ICAM-1 dependent neutrophil recruitment to the injured lung after lung contusion. (A) Immunostaining for ICAM-1 in lungs from uninjured (left panel), and injured (right panel) animals at 3H after injury (40X magnification). Specimens are representative of immunohistochemistry for least 3 animals. (B) Mice were treated with α-ICAM-1 antibody either intraperitoneally (IP), intratracheally (IT) or both routes (IP and IT) at 30min. prior to injury. BAL was counted and differentiated as described in the methods. There is a significant decrease in PMN in the BAL between injured mice with and without treatment (*p<0.001). There is no significant difference in decreased PMN level with different routes of administration (p>0.05). n=6 for experimental groups.
CHAPTER VI

The following manuscript was accepted for publication in J Trauma (Jan. 2009). Stylistic variations are due to the requirements of the journal.

Title: Pulmonary contusion primes systemic innate immunity responses

Running title: Mouse model of a “second hit”

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ABSTRACT:

Introduction: Traumatic injury may result in an exaggerated response to subsequent immune stimuli such as nosocomial infection. This “second hit” phenomenon, and molecular mechanism(s) of immune priming by traumatic lung injury, specifically pulmonary contusion, remains unknown. We used an animal model of pulmonary contusion to determine if the injury resulted in priming of the innate immune response and to test the hypothesis that resuscitation fluids could attenuate the primed response to a second hit.

Methods: Male, 8-9 wk, C57/BL6 mice with a pulmonary contusion were challenged by a second hit of intratracheal administration of the Toll like receptor (TLR) 4 agonist, lipopolysaccharide (LPS, 50mcg) 24hrs after injury (injury+LPS). Other experimental groups were injury+vehicle or LPS alone. A separate group were injured and resuscitated by 4cc/kg of hypertonic saline (HTS) or Lactated Ringer’s (LR) resuscitation prior to LPS challenge. Mice were euthanized 4hrs after LPS challenge and blood,
bronchoalveolar lavage (BAL), and tissue were isolated and analyzed. Data were analyzed using one way ANOVA with Bonferroni multiple comparison post-test for significant differences (*, \( p<0.05 \)).

**Results:** Injury+LPS showed immune priming observed by lung injury histology and increased BAL neutrophilia, lung myeloperoxidase, and serum IL-6, CXCL1 and MIP-2 levels when compared to injury+vehicle or LPS alone. After injury, resuscitation with HTS, but not LR was more effective in attenuating the primed response to a second hit.

**Conclusion:** Pulmonary contusion primes innate immunity for an exaggerated response to a second hit with the TLR4 agonist, LPS. We observed synergistic increases in inflammatory mediator expression in the blood and a more severe lung injury in injured animals challenged with LPS. This priming effect was reduced when HTS was used to resuscitate the animal after lung contusion.

**Key Words:** trauma, pulmonary contusion, second hit, immune priming, resuscitation, chemokines, inflammation, rodent model, innate immunity.

**INTRODUCTION:**

It has long been recognized in the clinical arena that significant traumatic injury seemingly primes the cells of the immune system for an exaggerated response to subsequent infectious challenge. This has been termed the “second hit” phenomenon, and is of clinical significance in that it is associated with the onset of multiple organ dysfunction and death\(^1\). Prior studies have linked the second hit response to enhanced Toll-like receptor (TLR) activity in cells of the innate immune system\(^2,3\). TLRs are a phylogenetically conserved family of receptors responsible for the initiation of
inflammatory responses to putative pathogen-associated molecular patterns, such as bacterial lipopolysaccharide (LPS) and peptidoglycan (PGN), as well as several endogenous ligands (danger-associated molecular patterns). Of the 13 known TLRs, TLR4 is essential for cellular responsiveness to LPS, a component of the outer membrane of gram negative organisms. Once triggered, TLR4 signaling activates innate immune mechanisms resulting in NFκB activation and expression of a number of pro-inflammatory mediators including IL-1β, IL-6, and the chemokine, IL-8. Enhanced TLR4-mediated signaling has been shown to be involved in the second hit response in animal models of burn injury and ischemia/reperfusion.

The use of fluids in the resuscitation of critically injured patients has been investigated in clinical trials as well as in several animal models. Resuscitation with hypertonic fluids results in a transient increase in serum osmolarity. The subsequent redistribution of fluid from the extravascular to the intravascular space reduces fluid volume for effective resuscitation. The advantages of such a resuscitation strategy are a reduction in consequences of third space fluid sequestration (cerebral edema and pulmonary edema) and improved cardiac contractility. There is mounting evidence that suggests that resuscitation with 7.5% saline has immunomodulatory properties as well. In vitro and in vivo studies have shown that neutrophil activation, chemotaxis, and degranulation are reduced in the face of hypertonicity. Likewise, hyperosmolar conditions have been shown to prevent macrophage activation in a dose-dependent manner. Moreover, in a second hit model of ischemia/reperfusion followed by LPS challenge, hypertonic resuscitation resulted in abridged neutrophil activation and
oxidative burst and a reduction in alveolar macrophage NFκB translocation\textsuperscript{20}. Other studies have suggested that hyperosmolar conditions may cause phenotypic changes in circulating monocytes and reduce expression of proinflammatory mediators\textsuperscript{18}. Thus, hypertonic saline is a beneficial resuscitation fluid, not only for its immediate effects on hemodynamics and fluid balance, but also due to immunomodulatory properties and potential to inhibit the second hit response after injury.

Blunt chest trauma resulting in pulmonary contusion is a common injury, affecting 10-17\% of all trauma admissions with estimates of mortality at 10-25\%\textsuperscript{21,22}. Sequelae of pulmonary contusion vary widely and include infection (pneumonia), local organ failure (Acute Respiratory Distress Syndrome, ARDS), and remote organ failure (Multiple Organ Dysfunction/Failure, MODS/MOF)\textsuperscript{23}. We have previously demonstrated in an animal model that lung contusion results in activation of innate immunity with the localized production of a variety of inflammatory cytokines and chemokines, followed by neutrophil infiltration into the areas of injury\textsuperscript{5,24,25}. Additionally, we have shown that the initial innate response to lung contusion is at least in part mediated through TLR dependent signaling mechanisms\textsuperscript{5,25}. However, it remains unknown whether isolated blunt chest trauma primes the innate immune system for a second hit response and enhanced TLR4 reactivity. We hypothesized that blunt chest trauma would prime effector cells of innate immunity for a second hit and observed the exaggerated response to the TLR4 ligand, LPS, when administered after injury. We used this second hit model to evaluate the effect of fluid resuscitation and to test the hypothesis that resuscitation would attenuate the second hit response.
METHODS:

**Animals:** Male and age matched (8-9 weeks) C57/BL6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were utilized in this study. All animals were bred and maintained under specific pathogen-free conditions at the animal facility at Wake Forest University School of Medicine. The protocol used in this study was approved by the Animal Care and Use Committee (#A07-246).

**Animal injury model:** Blunt chest injury resulting in pulmonary contusion was induced using the Cortical Contusion Impactor (CCI) as described previously. Briefly, mice were anesthetized with 2% isoflurane at a flow rate of 1L/min. The mouse was positioned left lateral decubitus and during inspiration, the right chest was struck with the CCI along the posterior axillary line, 1cm above the costal margin.

**Second hit model without and with resuscitation:** At 24 hours following blunt chest injury, the animals were re-anesthetized with isoflurane. A midline neck incision exposed the trachea, and 50mcg of LPS in 50mcl of Phosphate Buffered Saline (PBS, Sigma, St. Louis, MO) was injected directly into the trachea of the mouse using a 26G needle and the incision was closed. Control animals were (1) injured and received intratracheal instillation of PBS alone or (2) received intratracheal LPS without prior injury. Resuscitated animals received 4cc/kg of HTS (7.5% saline) or Lactated Ringer’s (LR) via tail vein injection 1hr prior to the second hit with LPS. At 4 hours serum and tissue samples were collected after death by isoflurane overdose and cervical dislocation.
**Histopathology:** Lung specimens were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin (H&E). Slides (100X magnification) were evaluated and graded for the presence of interstitial neutrophilic infiltrate, intra-alveolar hemorrhage, and pulmonary septal edema as described previously525.

**Bronchoalveolar lavage (BAL):** After sacrifice, BAL was performed by cannulation of the trachea and lungs lavaged with 4ml of PBS (Sigma Biochemical, St. Louis, MO) at 4°C. BAL was centrifuged at 300 x g, 4°C for 10 minutes and supernatant collected and stored at -70°C until use. The cell pellet was counted and differentiated as previously described525.

**Cytokine and chemokine expression:** IL-6, CXCL1 and MIP-2 (CXCL2/3) were measured in the serum using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Samples were assayed in duplicate.

**Myeloperoxidase activity:** MPO activity was used as a quantitative measure of neutrophil accumulation in the lung. Lung tissue samples were collected in RPMI 1640 with 5% FCS at 4°C. The lung was cut into small pieces and digested for 60 minutes at 37°C in 10ml of PBS containing 300U/ml collagenase type I (Worthington Biochemical Corp., Lakewood, NJ), and 150µl of a DNase I (10mg/ml, Worthington Biochemical Corp., Lakewood, NJ). The lung digest was passed through a 40µm sieve with two washes of RPMI 1640 with 5% FCS, and cells collected by centrifugation at 300g and 4°C. After lysis of red blood cells with ACK lysis buffer (Lonza, Walkersville, MD), the remaining cells were washed twice with PBS and cell extracts prepared by three freeze-thaw cycles in 1ml HTAB (Sigma-Aldrich, St. Louis, MO) buffer (0.5% HTAB in MPO
buffer; MPO buffer contains 6.8g KH$_2$PO$_4$ + 8.7g K$_2$HPO$_4$ in 1L dH$_2$O). The extracts were cleared by centrifugation at 10,000g for 10 minutes. Seven microliters of extract was assayed for MPO activity with 200ul development reagent (16.7mg O-dianisidine dihydrochloride in 100ml of a solution containing 90 ml dH$_2$O, 10ml MPO buffer and 0.005% H$_2$O$_2$) by measuring the change in absorbance (450nm) over a period of 5 minutes and results reported as $V_{max}$.

**Neutrophil CD11b expression:** Whole blood was collected in a heparinized syringe, transferred to a 1.5ml microcentrifuge tube and mixed thoroughly before placing at 4°C. Fc receptor binding was reduced by adding 0.5ug of Mouse BD Fc Block™ (BD Biosciences, San Jose, CA) to 100ul of whole blood for 5 minutes at 4°C. All subsequent steps were carried out in the dark at 4°C. Staining of CD11b was done by adding 0.5ug of FITC anti-mouse CD11b (eBioscience, Inc., San Diego, CA) to blocked samples for 30 minutes. A FITC labeled isotype control (eBioscience, Inc.) sample was stained in parallel to assess non-specific antibody binding. Next, red blood cells were lysed with an RBC lysis buffer (eBioscience, Inc.) for 5 minutes, cells collected by centrifugation at 300g, washed twice with PBS, and resuspended in fixation buffer (BD Biosciences). After 20 minutes, fixed cells were centrifuged, washed with PBS and resuspended in stain buffer (FBS) (BD Biosciences). Fixed and stained cells were analyzed by flow cytometry using a BD FACSCalibur™ system (BD Biosciences) and BD CellQuest™ Pro software (BD Biosciences).

**Statistical analysis:** Data are reported using GraphPad Prism (v 4.03, San Diego, CA) and expressed as the mean ± SEM of independent observations as indicated in the Figure.
RESULTS:

*Pulmonary contusion primes innate immunity for an exaggerated response to a second hit with the TLR4 agonist, LPS.* To determine if pulmonary contusion primes the innate immune response for a second hit, we compared the response of animals undergoing blunt chest trauma followed by intratracheal instillation of LPS (IT LPS) with animals administered vehicle alone (saline) after injury and animals given IT LPS only with no antecedent trauma. Upon histological examination, second hit animals had a dramatic pulmonary neutrophilic infiltrate and septal edema when compared to animals given IT LPS only and injury+vehicle animals (Figure 1). Hemorrhage and structural distortion were evident in animals receiving blunt chest trauma. To evaluate the involvement of remote organs in this process, we examined hepatic histology. No evidence of acute hepatic inflammation was observed, suggesting that the priming response was localized in the lung (data not shown). As the presence of a neutrophilic infiltrate strongly correlates with acute lung injury we examined bronchoalveolar lavage specimens and measured lung MPO activity to assess neutrophil infiltration in the primed response. As shown in Figure 2, second hit animals had significantly increased BAL neutrophilia (Figure 2A) and lung MPO activity (Figure 2B) when compared to animals given LPS alone or animals with injury+vehicle. A maximal priming effect was observed when the second hit was given at 24hrs after injury (data not shown).
We next evaluated the systemic innate response of second hit animals by measuring serum levels of IL-6 and the chemokines, CXCL1 and MIP-2. We previously demonstrated that both mediators play a central role in the pathogenesis of lung injury after pulmonary contusion. Relevant to our second hit model, these mediators are also produced in an NFkB-dependent fashion after LPS challenge. We now show a synergistic increase in the expression of these mediators with injury+LPS when compared to animals given LPS alone or animals with injury+vehicle (Figure 3). These data support the observation that isolated blunt chest trauma primes for an enhanced innate immune response to TLR4 activation.

*Small volume resuscitation with 7.5% hypertonic saline ameliorates enhanced TLR4 reactivity seen after chest injury.* Given the priming effect observed in our model of blunt chest trauma, we next sought to test two post-injury resuscitation strategies for the ability to attenuate the second hit response. Small volume resuscitation with HTS was compared to an equivalent volume of resuscitation with Lactated Ringer’s (LR). Resuscitation was performed after lung injury but prior to the second hit. As shown in Figure 4, HTS resuscitation prior to the second hit with LPS significantly reduced BAL neutrophilia and lung MPO activity. Resuscitation with LR showed no significant effect in reducing the neutrophil response to a second hit. Serum levels of IL-6 and MIP-2 were significantly reduced with HTS resuscitation in second hit animals (Figure 5). In contrast, serum CXCL1 levels were unaffected by either HTS or LR resuscitation. Finally, to evaluate neutrophil activation, we measured CD11b expression in second hit animals and in animals resuscitated with HTS or LR. As shown in Figure 5, we observed an
anticipated rise in systemic neutrophil CD11b in second hit animals. We further found that CD11b expression was reduced in animals resuscitated with HTS, but not after resuscitation with LR. These data indicate that after isolated chest injury, innate immune responses to a second hit can be attenuated by resuscitation with HTS.

DISCUSSION:

Without question, injury disrupts the normal host immune response and predisposes patients to the development of opportunistic infection. Clinicians have recognized that initial serious injury can result in a priming of the immune system for an exaggerated response to subsequent infectious challenge. Studies in both humans and animals show that innate immune cells such as neutrophils, macrophages/monocytes, and dendritic cells produce increased levels of inflammatory mediators in response to microbial stimuli after injury. This “second hit” phenomenon is supported by epidemiologic studies linking the magnitude of the initial systemic inflammatory response to injury with the onset of late post-injury multiple organ failure and death due to invasive infection.

We sought to determine whether pulmonary contusion would prime innate immune mechanisms in our murine model of isolated blunt chest trauma. As gram negative organisms often cause the nosocomial infections seen after injury, we used the TLR4 ligand, LPS (injury+LPS), to test for post-injury immuno-hyperreactivity in response to this second hit. Synergistic increases in pulmonary neutrophilia and systemic mediator expression (IL-6, CXCL1, and MIP-2) were seen in second hit animals when compared to injury+vehicle or LPS alone. These results indicate that isolated blunt...
chest trauma results in a primed effect on innate immune responses that enhance TLR4 reactivity and augmented lung injury. Our findings of enhanced post-injury TLR reactivity are in agreement with other animal models of injury and enhanced TLR responses to a second hit\textsuperscript{2,3,7}. However, in a blast chest injury model and using cecal ligation and puncture (CLP) as the second hit, Perl et al. found that chest injury had a suppressive effect on macrophage and lymphocytic function and resulted in increased mortality\textsuperscript{31}. These results may, in part, be explained by the use of a blast injury model that imparts significantly more energy to a broad area of the chest than in our model of direct, concussive chest injury. In addition, CLP is a primary, polymicrobial peritoneal infection that is remote from the lung and will likely activate many TLR dependent responses compared to our specific intratraceal instillation of LPS. The diffuse chest injury and variable infectious stimuli of CLP (and potentially other non-TLR stimuli) are likely to activate immune responses that may be significantly different than in our second hit injury model that uses a focused and localized injury and a well characterized TLR4 ligand.

There are multiple cell types present in the lung both before and after injury that express TLR4 and are capable of producing IL-6 and CXC chemokines including alveolar macrophages, endothelial cells, and alveolar epithelial cells\textsuperscript{32,33}. Paterson et al., in a burn injury model, demonstrated that macrophage and dendritic cells show enhanced IL-6 production after an LPS-mediated second hit\textsuperscript{2}. Interestingly, they did not observe a change in TLR4 expression. They later found that injury enhanced TLR4 response was mediated, in part, by enhanced intracellular activity of the mitogen activated protein...
kinase, p38 and suggested a role for CD4+CD25+ T regulatory cells in controlling this innate response. Moreover, in a model of ischemia/reperfusion, Fan et al. demonstrated enhanced CINC-1 expression primarily by alveolar macrophages in animals challenged with LPS intratracheally shortly after reperfusion. They also showed that shock had little effect on TLR4 expression in the lung and that ischemia appeared to prevent LPS-induced reduction in TLR4 mRNA. The mechanisms involved in TLR4 priming after blunt chest injury and which cells within the lung that mediate enhanced TLR4 response(s) are currently being investigated.

HTS resuscitation from hemorrhagic shock has been demonstrated to be an effective resuscitation strategy in the polytrauma patient. HTS has immunomodulatory properties that led us to hypothesize that HTS given after injury could potentially attenuate the clinically important second hit response in our mouse model of isolated chest injury. Distinct from the ischemia/reperfusion injury models, our model does not involve obvious blood loss. We found that HTS resuscitation after chest injury did reduce immune response to TLR4 as demonstrated by a marked reduction in lung PMN influx and reduced systemic levels of IL-6 and MIP2 (CXCL2/3) while resuscitation with LR did not have these effects.

Typically, neutrophil recruitment to the lung after injury involves localized CXC chemokine expression, neutrophil activation and adhesion receptor expression (CD11b/18), neutrophil rolling (selectins), and firm adhesion (β2 Integrins:ICAM-1). Our previous studies showed that CXCL1 is primarily involved in the innate response to traumatic lung injury. CXCL1 and MIP-2 (CXCL2/3) are the murine equivalents of IL-
8, the principle chemokine involved in neutrophil chemotaxis after traumatic lung injury in humans. Alveolar macrophages and to a lesser extent, alveolar epithelial cells have been demonstrated to produce CXC chemokines after lung injury. In our study, we found that both CXCL1 and MIP-2 were increased in second hit animals, and unexpectedly, resuscitation with HTS decreased MIP2, but not CXCL1, expression. Other investigators have observed a reduction in CXC chemokine expression when HTS was used as a resuscitation fluid. In a second hit model of ischemia/reperfusion followed by IT LPS in the rat, Fan et al. reported a reduction in alveolar macrophage expression of the CXC chemokine, CINC-1, but not MIP-2, when HTS was used. They concluded that CINC-1 was primarily involved in the second hit response. Our findings demonstrate a similar differential effect on chemokine expression in second hit animals resuscitated with HTS and suggest that CXCL1 and MIP-2 have divergent roles in a second hit response. Differing pharmacokinetics for these chemokines may also participate in the second hit and may also explain our results. Evaluation of immune response kinetics will help to define the participation of these mediators.

Systemic neutrophil activation is indicated by changes in surface expression of the adhesion receptor, CD11b. CXCL1 and MIP-2 have both been demonstrated to increase CD11b receptor expression in neutrophils. In our study, the decrease in systemic MIP-2 expression with HTS resuscitation correlated with a marked reduction in neutrophil CD11b expression. The reduction in pulmonary neutrophilia and neutrophil CD11b expression in HTS animals may be related to changes in systemic CXCL2/3 chemokine expression, or alternatively, a result of hypertonicity-induced changes in
circulating neutrophils. Hypertonicity might impair neutrophil CD11b expression by impeding neutrophil swelling during transmigration and/or alterations in the actin cytoskeleton preventing translocation of the receptor in response to LPS. We have not measured serum osmolarity; however, this is a likely explanation for our findings as changes in serum osmolarity after equivalent doses of intravenous HTS have been shown to last for up to 6 hours, with the highest levels being seen 1 hour after administration. HTS has also been shown reduce neutrophil L-selectin shedding and reduce pulmonary levels of ICAM-1, effects that could be mediated through hypertonicity or changes in CXC chemokine expression. Thus, in our study, it is likely that decreased neutrophil recruitment and CD11b expression in HTS resuscitated animals involves multiple mechanisms.

In addition to alterations in chemokine expression, resuscitation with HTS showed a dramatic decrease in systemic IL-6 levels. Previous studies implicate the alveolar macrophage as the likely cellular source. We evaluated hepatic histology and observed no apparent indication of involvement in our injury model; however, these findings are neither comprehensive nor conclusive and further study to identify extra-pulmonary organ involvement is indicated. HTS resuscitation has been shown to affect alveolar macrophage function. In vitro studies have shown that hypertonic pretreatment of macrophages reduces proinflammatory mediator production. In animal studies, resuscitation with HTS reduced alveolar macrophage priming resulting in diminished NFκB nuclear translocation in response to LPS. Others have suggested that resuscitation with HTS may also induce a phenotypic change in the monocyte
population towards a less inflammatory profile. The exact mechanisms surrounding these changes in macrophage function remain indistinct. Our data is consistent with the implication that HTS is having an effect on macrophage function in addition to its effects on PMN function.

From a clinical standpoint, the second hit response remains an attractive target for intervention. There have been multiple randomized studies evaluating the efficacy of HTS in the early management of injury patients. All of these studies included patients who had prehospital hypotension and were treated with a single dose of HTS with or without dextran either in the field or early in the emergency department. No consistent survival advantage has been identified. The theoretic advantage of using HTS as a resuscitation fluid may lie in its ability to manipulate the subsequent inflammatory response to later infection. As suggested in our study, HTS significantly attenuated post-injury immune responses; decreased infectious morbidity and mortality may result. It remains unknown whether a single dose of HTS given in the prehospital setting is sufficient to modulate the ensuing inflammatory response or if repeated dosing is required. In this study, HTS was administered 1 hr prior to the second hit, a difficult scenario to mimic clinically; however, it would suggest that repeated dosing may be beneficial. Lastly, clinical lung function was not measured in this study, although others have reported improvement with HTS resuscitation. Continued resuscitation studies and identification of the molecular and cellular mechanisms mediating the second hit are indicated.
In conclusion, our data demonstrate that blunt chest injury resulting in pulmonary contusion primes innate immunity for an exaggerated TLR4 response. Furthermore, resuscitation with HTS after blunt chest injury reduced the second hit response as indicated by decreased pulmonary neutrophilia and the decreased levels of systemic innate immune mediators. These data suggest that HTS may have a significant role in the treatment of patients with blunt chest trauma and pulmonary contusion.

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Figure 1. Lung histology shows that pulmonary contusion primes innate immunity for enhanced neutrophil response. We compared lung histology of animals given LPS with no antecedent trauma (LPS alone), animals undergoing blunt chest trauma followed by intratracheal instillation of LPS (Injury+LPS) and injured animals administered intratracheal saline (Injury+vehicle). Second hit animals (Injury+LPS) show an increased pulmonary neutrophilic infiltrate and alveolar septal edema when compared to animals given LPS alone. Hemorrhage and structural distortion is evident in injured animals. Data shown (100X magnification) is representative of histology for at least 3 animals in each group.
Figure 2. Pulmonary contusion primes innate immunity for enhanced neutrophil response. We compared the neutrophil response of animals given LPS with no antecedent trauma (LPS alone) to animals given a blunt chest trauma followed by IT instillation of LPS (Injury+LPS) or injured animals administered IT saline (Injury+vehicle). Second hit animals (Injury+LPS) have increased BAL neutrophilia (A) and lung MPO activity (B) when compared to animals given LPS alone or injured animals administered IT saline (Injury+vehicle). Data shown is the mean ± SEM. Differences are significant (p<0.01) between all groups (n=5 for each group).
Figure 3. Pulmonary contusion primes the systemic innate immune response. We compared the serum levels of IL-6, CXCL1 and MIP-2 in animals given LPS with no antecedent trauma (LPS alone) to animals given a blunt chest trauma followed by IT instillation of LPS (Injury+LPS) or injured animals administered IT saline (Injury+vehicle). Second hit animals have increased IL-6 (A, n=6), CXCL1 (B, n=5) and MIP-2 (C, n=5) levels when compared to animals given IT LPS or injured animals administered IT saline. Data shown is the mean ± SEM. Differences are significant (p<0.05) between all groups; the exception is MIP-2 levels in Injury+vehicle compared to LPS alone where no significant difference (p>0.05) is observed.
Figure 4. HTS resuscitation reduces the neutrophil response to a second hit. We compared the neutrophil response of injured animals resuscitated with HTS or LR prior to a second hit with IT LPS. Second hit animals resuscitated with HTS show a significant decrease (*p<0.001) in BAL neutrophilia (A) and lung MPO activity (B) when compared to second hit animals without resuscitation (Injury+LPS). There is no significant effect of LR resuscitation (p>0.05) on BAL neutrophilia or MPO activity. Data shown is the mean ± SEM (n=5).
Figure 5. HTS resuscitation reduces the systemic innate immune response to a second hit. We compared the serum levels of IL-6, CXCL1, MIP-2 and CD11b expression in injured animals resuscitated with HTS or LR prior to a second hit with IT LPS. HTS resuscitated animals have a significant decrease (*p < 0.05) in serum IL-6 (A, n=8), MIP-2 (C, n=5) and CD11b expression (D, n=5) compared to second hit animals. There is no significant change (p>0.05) in CXCL1 by either resuscitation strategy (B, n=5, 8, 8 for Injury+LPS, HTS, and LR, respectively). LR resuscitated animals are also not significantly changed (p>0.05). Data shown is the mean ± SEM.
CHAPTER VII

DISCUSSION:

Background. Trauma continues to be a significant social issue, accounting for approximately 170,000 deaths annually and countless hours of lost productivity\(^1\). Blunt injuries as would be encountered after a motor vehicle accident represent the most common mechanism of injury. Specifically, blunt chest trauma complicates approximately 60% of trauma center admissions, and pulmonary contusion is seen in up to 25% of those patients\(^2\)\(^-\)\(^3\). The clinical manifestations of pulmonary contusion vary from simple shortness of breath to profound hypoxia requiring mechanical ventilation and possibly death. Thus, lung contusion is a common injury that has significant morbidity and mortality associated with it. The maladies associated with pulmonary contusion have long been ascribed to direct mechanical damage to the lung with hypoxia being ascribed to V/Q mismatching\(^4\). However, as our work has shown, pulmonary contusion is indeed an inflammatory injury that is dependent upon activation of innate immune mechanisms resulting in the localized production of inflammatory mediators and the activation and recruitment of neutrophils to the alveolar space.

The open chest model of pulmonary contusion in the rat. As most studies of pulmonary contusion were in large animals, we initially sought to develop a model of lung contusion is a rodent model to take advantage of currently available genetic and molecular probes so that the innate response to injury could be evaluated. An open chest
model has several benefits: 1) it minimizes and quantitates the resultant soft tissue injury as sham animals undergo thoracotomy alone, thus this confounding variable is eliminated; 2) it unquestionably avoids injury to adjacent organs (heart, liver, gastrointestinal tract); 3) it eliminates the protective effects of the chest wall that absorb a variable amount of energy directed towards the lungs so that the injury to the lungs themselves are isolated, measured and characterized; and 4) it allows the induction of a unilateral lung contusion so that the effects on the contralateral lung may be studied. From a biomechanical standpoint, this model is superior to a closed chest model in that the energy imparted to the system by the CCI is precisely quantified (velocity, depth, dwell time) and through the use of currently available imaging technology, the size of the contusion in our model could be quantitated very accurately.

RESULTS AND SIGNIFICANCE:

_The application of the open chest lung injury model to advance injury prediction and to develop injury prevention devices_. Through the use of these variables, we have created an equation that correlates principle strain (force) with contusion size and developed a finite element model of the injury. The importance of predictive modeling in this type of injury is that it potentially impacts injury prevention, allowing for the development of new auto safety devices which can reduce the incidence and severity of chest injury after motor vehicle crash and ultimately improve outcome. It is important to note that the only proven method to improve outcome from traumatic injury is injury prevention. Furthermore, we have translated the principles of these studies into the clinical arena where we are attempting to correlate the size of contusion with the magnitude of the
ensuing inflammatory response. Using initial chest CT, segmentation software that our group has developed and validated is being used to quantitate the size of pulmonary contusion, and this information is being correlated with various outcome parameters such as death, pneumonia, ARDS, and remote organ dysfunction. We further hope to correlate size of contusion with the magnitude of the inflammatory response in these patients measuring the levels of various mediators in the BAL and serum. This information will allow clinicians to potentially identify patients who are at risk to have complications after injury and who may benefit from molecular based intervention.

The closed chest model of pulmonary contusion in the mouse. Despite the many potential benefits of our results in the open chest rat model, this injury mechanism is not analogous to what is encountered clinically and limited its utility. Thus, we developed a closed chest model of blunt chest trauma resulting in pulmonary contusion in the mouse using the CCI. A mouse model was chosen as they have a well described immune system, have a wide array of genetic and molecular probes available, and offer gene knockout animals that allow precise mechanistic questions to be addressed. The literature describes two other small animal models, one is a blast model in the mouse, and the other is a blunt chest injury mechanism in the rat. Both result in lung contusion and acute lung injury. However, a blast model, like the open chest model, has very limited clinical applicability to civilian trauma and as previously mentioned, we felt that the rat model limited the potential for mechanistic investigation.

The innate immune response to pulmonary contusion. With infection, the activation of the innate immune system is mediated by Toll like receptors (TLRs). The list of known
TLR ligands consists primarily of conserved components of infectious bacteria and viruses (eg. LPS, LTA, flagellin) that are shared by large groups of pathogens. These “Pathogen Associated Molecular Patterns” (PAMPs) are potent inflammation and innate immunity activators. The initial inflammatory response to injury is very similar to that seen during infection. This observation has resulted in investigation into the signaling mechanisms that regulate the inflammatory response to noninfectious injury, specifically focusing on TLR participation. TLR-mediated signaling has been demonstrated to participate in the inflammatory response in models of ischemia/reperfusion, acute pancreatitis, colitis, femur fracture, and hyperoxic lung injury. Concomitant with these studies, an enlarging number of endogenous TLR ligands have been identified. As a result of these studies, a paradigm has emerged in which injury leads to cellular necrosis and interstitial matrix disruption at the tissue level resulting in widespread release of normally intracellular endogenous ligands. These “danger-associated molecular patterns” (DAMPs) or “danger signals” are detected by TLRs, resulting in activation of innate immune mechanisms.

Most studies have focused on the participation of TLR4 and TLR2 in these responses. Specifically, pulmonary involvement of TLR4 and TLR2 have been demonstrated in a bleomycin-induced and hyperoxic lung injury models in which TLR deficiency resulted in reduced pulmonary neutrophilia, reduced CXC chemokine expression, and interestingly, increased mortality. In both models, enhanced alveolar epithelial cell apoptosis was seen, leading the authors to speculate that lung injury in these models was dependent upon epithelial cell apoptosis.
RESULTS AND SIGNIFICANCE:

Lung dysfunction after pulmonary contusion is dependent on the innate immune response. In our model of noninfectious lung injury, TLR4 and TLR2 deficiency resulted in a markedly diminished inflammatory response and reduced pulmonary neutrophilia, but an improvement in overall lung function was seen, suggesting that neutrophils mediate lung injury in this model. As both TLR4 and TLR2 utilize a MyD88 dependent pathway we also examined the injury response in MyD88 deficient mice where we observed a significantly lessened injury than that observed with either TLR deficiency. This further supported a role for the innate immune response in pulmonary contusion, but did not rule out the participation of non-MyD88 dependent responses. The protective effect of TLR deficiency that we have described has been demonstrated by others in other acute injury models. In a bilateral femur fracture model, TLR4 deficiency improves organ (specifically hepatic) function. Similar findings have been reported in models of ischemia/reperfusion, acute brain injury, and colitis where TLR deficiency seemingly had a beneficial effect. These divergent effects of TLR signaling reported in the literature may be explained by the oxidant versus non-oxidant injuries associated with the different injury models.

EMERGING PARADIGMS FOR TRANSLATIONAL RESEARCH:

Endogenous injury ligands. Identification of participating cells, ligands, TLRs, and co-receptors remain an exciting area for future investigation. Numerous endogenous TLR ligands have been identified that could participate in the inflammatory response to noninfectious lung injury. Potential candidates include S100 proteins, HMGB-1,
hyaluronan, Hsp70, fibronectin, and surfactant protein A15. Hyaluronan, HMGB-1, and Hsp70 in particular have been shown to participate in this response in various models of injury but their contribution after lung contusion is not known16-20,21. Likely, multiple ligands with differing TLR specificity are involved in this response. Adoptive transfer studies used to determine the participating cell types have demonstrated divergent results. Mollen, et al22 in a model of hemorrhage/femur fracture, found that both parenchymal and marrow-derived cells participate in TLR-signaling after injury; however, Wu, et al23 in a model of renal ischemia/reperfusion, showed a major role for parenchymal cells in TLR dependent responses. These results suggest multiple cell types may participate in this response and that there may be organ specificity to this response. In the lung, there are multiple cell types that express TLRs and are capable of initiating an inflammatory response to noninfectious injury including epithelial cells, endothelial cells, and alveolar macrophages. The contribution of each of these cell types in lung contusion is unknown.

**Injury response receptors and signal transduction pathways.** The participation of other TLRs, the intracellular signaling pathways involved and other co-receptors involved in the inflammatory response to injury are areas of active investigation by multiple investigators. While there are some reports of TLR324,25 and TLR926 being involved in the inflammatory response to injury, there is no information regarding the role of any TLRs aside from TLR2 and TLR4 in noninfectious lung injury. TLR4 mediated responses after noninfectious injury has been shown to be largely CD14 independent12, and it has been demonstrated that TLR4 forms a complex with CD44 and MD2 to initiate intracellular
signaling after noninfectious injury. The participation of these TLR co-receptors in the response to lung contusion is not known, although we have preliminary data that indicates that CD14 is involved in this response. Additionally, the intracellular signaling pathways have yet to be determined. The possibility of a MyD88-independent signaling pathway utilizing TRIF has not been fully explored nor have the downstream signaling molecules (eg. IRAK/1/4, TRAF6) involved in this process been delineated. Lastly, regulation of TLR signaling after lung contusion both at the transcriptional and translational levels are potential areas of future investigation.

**Injury outcome prediction and downstream adverse events.** The “second hit” phenomenon seen after lung contusion is an attractive area of future study. The clinical relevance of this mechanism is that there is a window in which interventions such as the quantity and type of resuscitation fluid may be utilized to improve patient outcome. Thus, understanding the cells involved and the mechanisms whereby injury primes innate immune mechanisms for a second hit are important. Alveolar macrophages have been demonstrated by others to largely participate in this response; however, the contribution of parenchymal cells such as alveolar epithelial cells and endothelial cells has not been investigated. Additionally, the mechanisms surrounding downregulation of TLR signaling after injury have not been explored. Towards this end, alterations in CD25+ T regulatory cell and Gr1+ macrophage populations, both immunosuppressive, have been demonstrated to occur after burn injury and are hypothesized to participate in the second hit. The involvement of the SOCS proteins and/or IRAK-M, both of which have been shown to be negative regulators of TLR4 signaling, have not been
investigated in this response. Lastly, the potential involvement of miRNA, specifically miR-146, and other perturbations in transcriptional regulation of TLR signaling are potential areas of investigation34.

The molecular and cellular response to pulmonary contusion. Lung injury in this model is mediated through the recruitment of activated neutrophils to the lung. We have shown that neutrophil recruitment is dependent upon CXC chemokine and ICAM-1 expression after injury. However, our data also indicates that other mediators are involved in this process. Potential mediators include C5a, C3a, LTB4, and members of the CC and CXC chemokine family. In particular, C5a has been shown to be increased systemically after noninfectious lung injury35 and is purported to figure prominently in neutrophil recruitment to the lung36. Further study delineating whether complement activation occurs, the manner in which complement is activated (ie classical, mannose-binding lectin, or alternative pathways), and how TLR signaling influences complement activation after lung contusion are areas of potential investigation. Likewise, our data indicates that ICAM-1 figures prominently in neutrophil recruitment to the lung; however, other receptors may be involved in this process. The role of the selectin family of receptors in neutrophil recruitment to the lung as well as CD11a/18 and CD11c/18 are all potential mechanisms where by neutrophils localize to the site of injury and leave the vascular compartment. The importance of these receptors has been demonstrated in other models of lung injury but their contribution after lung contusion is not known.

In this model, lung injury appears to be dependent upon the presence of neutrophils in the lung, but the exact manner in which neutrophils mediate lung injury is not
known. Neutrophils harbor several cytotoxic substances including eicosanoids, cationic proteins, and proteolytic enzymes, and have the ability to generate multiple reactive oxygen species upon reaching sites of inflammation. All of these have the propensity to cause or propagate tissue damage. Moreover, in sepsis, a dysregulation of leukocyte apoptosis has been demonstrated wherein neutrophils are longer lived\textsuperscript{37}. Thus, lung injury in this model may be the result of neutrophil activation, migration, and degranulation as well as disruption of normal apoptotic signals where neutrophils “live” longer and cause more tissue damage after noninfectious lung injury. Additionally, neutrophils interact with multiple different cell types when migrating to the alveolar space, indicating that cell to cell interactions may play an important role in causing lung injury. As has been previously shown, alterations in alveolar epithelial cell apoptosis has been linked to lung injury in TLR deficient mice after bleomycin-induced\textsuperscript{16} and hyperoxic lung injury\textsuperscript{18} despite a significant reduction in pulmonary neutrophilia. Perhaps, neutrophils interact with the epithelial cells via a cell surface receptor resulting in alterations in apoptosis that are injury/model specific. Studies to further identify the role of the neutrophil in lung injury will focus on neutrophil function (ie oxidant-mediated injury) and/or neutrophil interactions with other cells in the injured lung. More information regarding the control of both neutrophil and alveolar epithelial cell apoptosis after lung contusion may shed further light on the mechanisms of lung injury in this model. The response of the contralateral lung after pulmonary contusion is an area of further study.
SUMMARY AND CONCLUSION:

In conclusion, we have developed novel rodent models of pulmonary contusion that results in an acute lung injury as demonstrated by physiologic, histologic, and biochemical data. Our data indicate TLR 2 and 4 are implicitly involved in the initiation of this response; lung contusion activates innate immune mechanisms; lung injury in this model is dependent upon the presence of neutrophils in the alveolar space; CXC chemokines and ICAM-1 participate in neutrophil recruitment to the contused lung; and lung contusion will prime innate immune mechanisms for a subsequent exaggerated TLR4-dependent response. We conclude that this injury model can be used to further study the participation of other TLRs in this response and identify endogenous injury ligands. This injury model can provide a further understanding of the mechanisms surrounding immune priming and identify therapeutic interventions to reduce the severity of the “second hit”. An understanding of the molecular and cellular response to pulmonary contusion in the rodent will provide insight into potential areas for treatment of patients after trauma.

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