The immune response to bacterial pneumonia following traumatic brain injury

https://hdl.handle.net/2144/11057

Boston University
THE IMMUNE RESPONSE TO BACTERIAL PNEUMONIA FOLLOWING TRAUMATIC BRAIN INJURY

by

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Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
2013
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ACKNOWLEDGEMENTS

I would like to acknowledge the people that made getting this far possible.

My family: Andrea, Gary, and Meredith Stepien who have tirelessly supported me through all of these years of school. We've been through the good times and the bad but always have and always will be there for each other no matter what.

Dr. Remick and the entire Remick lab for being my friends and family here in Boston and getting me through the trials and tribulations of science.

Debbie Kiley and Liz Duffy for making the sun rise and set, the wind blow, the rain fall, oceans wave, and all other earthly things. You run the show!
THE IMMUNE RESPONSE TO BACTERIAL PNEUMONIA FOLLOWING TRAUMATIC BRAIN INJURY

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PhD Degree Requirements completed in 2013
Dual M.D.-Ph.D. degrees expected in 2015
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ABSTRACT

Traumatic brain injury (TBI) is an important clinical problem affecting 1.7 million Americans annually. TBI affects peripheral organs beyond the nervous system with particularly profound effects on the lung, predisposing TBI patients to develop respiratory dysfunction due to bacterial pneumonia. Previous clinical and basic science studies have suggested TBI induces an immune depressed state rendering TBI patients more susceptible to pneumonia. As no mechanism has been proven as a cause for TBI-induced immune modulation we created an animal model of TBI and bacterial pneumonia to investigate the effects of TBI on pulmonary immune function. Our model revealed that instead of increasing susceptibility to bacterial pneumonia, TBI results in a more robust neutrophil recruitment in the lung that allows for faster bacterial clearance and increased survival after bacterial challenge. This response is paradoxically accomplished with significant decreases in pro-inflammatory cytokine production. An important neural mediator of pulmonary inflammation is substance P which acts through the neurokinin-1 receptor (NK-1R) to recruit neutrophils to the lung and increase...
pulmonary vascular permeability. Treatment with an NK-1R antagonist abolished the increased bacterial killing and recruitment in TBI mice but treatment of sham injury animals with an NK-1R agonist increased their lung neutrophil recruitment and bacterial killing. These findings point to an important role of substance P after TBI and in the immune response to pneumonia. We complemented the findings in our animal model with patient data from the National Trauma Database comparing the incidence of pneumonia among TBI and non-neurotrauma patients. After matching patients by demographics, vital signs, hospital, and importantly injury severity score, we found TBI patients had a decreased incidence of pneumonia. This finding is contrary to the findings of previously published studies that did not account for the confounding factor of injury severity. Our studies offer a new perspective on immune function after TBI and possibly a new therapeutic approach to pneumonia in TBI and non-TBI patients alike.
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<td>Arterial Blood Gas</td>
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<td>ALI</td>
<td>Acute Lung Injury</td>
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<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
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<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<td>BDL</td>
<td>Below Detection Limit</td>
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<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
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<td>CFU</td>
<td>Colony Forming Unit</td>
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<td>CXC(R)</td>
<td>Cysteine-X-Cysteine Motif (Receptor)</td>
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<td>DAI</td>
<td>Diffuse Axonal Injury</td>
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<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
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<td>EKG</td>
<td>Electrocardiogram</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>GCS</td>
<td>Glasgow Coma Scale</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin Stain</td>
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<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<tr>
<td>HF HRV</td>
<td>High Frequency Heart Rate Variability</td>
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<td>HPA</td>
<td>Hypothalamic Pituitary Adrenal</td>
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<td>ICP</td>
<td>Intracranial Pressure</td>
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<td>ICU</td>
<td>Intensive Care Unit</td>
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<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>KC</td>
<td>Keratinocyte-derived Chemokine</td>
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<tr>
<td>LD</td>
<td>Lethal Dose</td>
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<td>LFP</td>
<td>Lateral Fluid Percussion</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MIP-2</td>
<td>Macrophage Inflammatory Protein-2</td>
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<td>MMCS</td>
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<td>MPO</td>
<td>Myeloperoxidase</td>
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<td>nAChR</td>
<td>Nicotinic Acetylcholine Receptor</td>
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<td>NF-kB</td>
<td>Nuclear factor-kB</td>
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<td>NK-1R</td>
<td>Neurokinin-1 Receptor</td>
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<td>NPE</td>
<td>Neurogenic Pulmonary Edema</td>
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<td>NS</td>
<td>Normal Saline</td>
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<td>NTDB</td>
<td>National Trauma Data Bank</td>
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<td>PA</td>
<td><em>Pseudomonas aeruginosa</em></td>
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<td>PNA</td>
<td>Pneumonia</td>
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<tr>
<td>PNS</td>
<td>Parasympathetic Nervous System</td>
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<td>SNS</td>
<td>Sympathetic Nervous System</td>
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<td>SP</td>
<td>Substance P</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>TBI</td>
<td>Traumatic Brain Injury</td>
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<td>TH</td>
<td>T-helper</td>
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<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>WBP</td>
<td>Whole Body Plethysmography</td>
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<td>WDI</td>
<td>Weight Drop Injury</td>
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Chapter 1: Introduction

**Traumatic Brain Injury**

Traumatic brain injury (TBI) affects 1.7 million people every year resulting in 275,000 hospitalizations and 53,000 deaths making TBI the leading cause of injury death in the United States (25). The wars in Iraq and Afghanistan have resulted in another 320,000 TBI patients suffering TBI (51). Acute care for these injuries has cost an estimated $60 billion each year and chronic care of TBI patients carrying $9.2 billion in lifetime medical costs and $51.2 billion in lost productivity (25).

TBI is defined as a bump, blow, jolt, or penetrating injury to the head that disrupts normal brain function (25). This can vary from a mild concussion with brief change in consciousness to severe injury with coma and permanent neurological deficits (45). TBI severity is assessed using the Glasgow Coma Scale (GCS) that scores the neurological function of a patient in 3 categories: eye opening, verbal response, and motor response (63). The sum of these scores reflects the cognitive function of TBI patient and the severity of their injury. A score of 15-13 represents a mild TBI, 12-9 moderate TBI, 8-3 severe TBI (45). Coupled with imaging studies, GCS scoring is an important tool for predicting patient prognosis and has been shown to correlate with patient outcome (63, 132, 148).
Pathophysiology of TBI

TBI involves two distinct components: primary and secondary injury. Primary injury is the direct result of the mechanism of trauma to the brain (57, 137). Secondary injury encompasses the cellular response to trauma that is initiated by the primary injury but may continue for days (137). Consideration of both arms of TBI is crucial to understanding the pathology of brain injury.

Primary Injury

Falls, motor vehicle accidents, and objects striking the head comprise the leading causes of TBI in the US (25). Each of these can result in multiple different injuries to the brain parenchyma. The patterns of primary injury can determine the course and outcome of traumatic brain injury patients (57).

Hematomas

Hematomas are collections of blood in the cranium that are restricted in their expansion by the bony confines of the skull. These are the result of disrupted blood vessels that, depending on their location, can have different effects on the brain parenchyma. Epidural hematomas involve rupture of the middle meningeal artery as it courses between the skull and the outer covering of the brain, the dura mater. Subdural hematomas are more common and stem from disrupted veins between the dura and arachnoid layers surrounding the brain (57). In both
types of hematoma, the build-up of blood in the skull cannot expand without compressing the surrounding structures. This increases intracranial pressure (ICP) that, if it exceeds the pressure of blood entering the brain, can close off blood vessels and cause global brain ischemia. Expanding hematomas can also directly disrupt and damage brain tissue and herniate brain structures through the structures within the skull corresponding to a very poor outcome (137).

Contusions

Cerebral contusions are bruises of the brain tissue with disruption of small vessels and local extravasation of blood in the brain parenchyma (57). These are usually the result of impact forces and collisions of the brain with the skull. In impact injuries to the skull, typically there will be a contusion to the brain underlying the area of impact and a contusion on the direct opposite side of the brain where the brain bounced off the contralateral skull surface as it accelerated away from the point of impact. These are known as the coup and contracoup injuries respectively. If the brain is accelerated in a side-to-side or front-to-back direction, the undersurface of the brain moves over the sharp irregular surface of the skull floor. This pattern of contusion is known as a gliding contusion or "washerboard" injury. Contusions most commonly occur in the protuberances and undersurfaces of the frontal and temporal lobes. These lesions vary in severity but can result in significant brain swelling that, like expanding
hematomas, can increase ICP and initiate progression into catastrophic brain damage.

**Diffuse Axonal injury**

Acceleration/deceleration injuries can also cause diffuse axonal injury (DAI), a widespread disruption of the white matter tracts of the brain containing axonal projections from the grey matter. DAI is the result of shearing force where one plane of neuronal tissue slides over the other severing or stretching the axons that traverse them. This injury can be mild like a concussion or severe with patients entering a persistent vegetative state and over 90% mortality (57).

**TBI and the Immune System**

Central nervous system injury has deleterious effects on immune function that may last months to years after recovery from the primary insult (21, 30, 78, 140). Initially, TBI causes a release of acute phase proteins from the liver including pro-inflammatory cytokines and chemokines (21, 30). As the acute phase wanes, TBI patients develop widespread apoptosis of lymphocytes and profound decreases in plasma levels of IL-2, IFN-γ, and TNF-α, cytokines crucial to lymphocyte activation and proliferation (21, 51, 89). Monocytes from TBI patients produce significantly lower amounts of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α in response to LPS stimulation and express less major histocompatibility complex II (MHCII) on their surfaces resulting in impaired antigen presentation (51, 139). Circulating neutrophils exhibit decreased reactive
oxygen species formation and phagocytic function (40). Several mechanisms have been proposed to explain these changes that implicate different nervous inputs and their interactions with the cells of the immune system.

The current hypothesis of TBI-induced immunomodulation stems from the brain's ability to detect and respond to inflammation in the periphery. During the normal immune response to infection in the periphery, a small portion of the inflammatory cytokines in the circulation cross the blood-brain-barrier and stimulate several immunomodulatory centers in the brain, namely the hypothalamus and solitary nucleus (21, 72, 140). These centers then signal through their respective efferent pathways to peripheral immune cells to limit the inflammatory response to protect the body from damage caused by an unchecked immune response. When the brain itself is injured, the inflammatory cytokines released from injured brain tissue are also detected by these centers. The abnormally high levels of cytokines within the central nervous system are interpreted as reflecting high concentrations in the peripheral circulation, indicating an over-active peripheral inflammatory response (86, 140). These neural immunomodulatory pathways then go into overdrive with high levels of efferent signaling to quell this perceived excessive peripheral inflammation. While no single pathway has been shown to account for all of the observed immune alterations following TBI, each may play an important role in this multi-faceted response.
The Hypothalamic-Pituitary-Adrenal (HPA) Axis

The hypothalamus is an important sensor and modulator of inflammation. Initial investigations into immune alterations after TBI suggested the stimulated release of the glucocorticoid cortisol from the adrenal cortex was responsible for depressing immune cell function (140). Cortisol has potent anti-inflammatory effects and induces lymphocyte apoptosis and decreased pro-inflammatory cytokine production. Intracerebral injections of the inflammatory cytokine IL-1B to simulate tissue injury within the brain stimulate significant cortisol release (140). While this decreased pro-inflammatory cytokine production in the peripheral blood, blocking cortisol action in this model only mildly decreased the anti-inflammatory response. Thus the HPA axis contributes to, but is not the only mechanism of TBI immunomodulation.

The Sympathetic Nervous System

The sympathetic nervous system (SNS) is termed the “fight or flight” system. It mediates increases in heart rate, blood pressure, and respiration through catecholamine release. The “catecholamine storm” in patients recovering from TBI is a well-documented phenomenon characterized by an abnormally elevated release of catecholamines from both the adrenal medulla and neuron terminals of the SNS (10). With rich innervation of lymph nodes, the spleen, and liver, these catecholamines interact directly with cells of the immune system through β-adrenergic receptors expressed on their surfaces (21, 116, 122).
Christian Woiciechowsky, a neurosurgeon and early pioneer in TBI neuroimmunology investigated the role of catecholamines and the SNS following TBI and their effect on immune cell function. His group found that peripheral blood monocytes from rats that underwent a sustained increase in ICP produced high levels of the anti-inflammatory cytokine IL-10 (138). IL-10 is a potent suppressor of the inflammatory response blocking NF-κB activation, JAK-Stat signaling, and TH1 lymphocyte proliferation. They also demonstrated this effect in patients that had suffered brainstem compression during neurosurgical procedures. Treatment with beta-adrenergic receptor blockers reduced the IL-10 production and increased monocyte production of pro-inflammatory cytokines (39, 138).

While these findings suggested a catecholamine-mediated mechanism for the immune paralysis following TBI, the studies were not conducted specifically in brain injury but only increased ICP and brain stem compression. The pathophysiology of TBI does include changes in ICP but also traumatic tissue injury and intracranial cytokine elaboration that make the neuroimmune response different from isolated rises in ICP (137, 145). Clinical studies have found that, while briefly elevated immediately after injury, SNS signaling and catecholamine surges predominate in the later phases of TBI (>14 days) making it a less likely mechanism for the acute changes in immune function but it may explain the persistence of immune dysfunction long after recovery (13, 73).
Parasympathetic Nervous System and the Vagus Nerve

The parasympathetic nervous system (PNS) is responsible for a "rest and digest" response in contrast to that of the SNS. Increased PNS signaling results in a general slowing of the heart rate in a characteristic pattern detectable on EKG termed high-frequency heart rate variability (HF HRV) (13, 62). In the acute phase after injury, EKG studies of TBI patients demonstrate sustained high levels of HF HRV indicating continuous predominant parasympathetic signaling to the periphery (73). The majority of the PNS signaling to the periphery is mediated by the vagus nerve. Branches of the vagus supply the parasympathetic innervation of the thoracic and the abdominal viscera. Each branch carries both efferent motor fibers and afferent visceral sensory fibers. The efferent and afferent limbs both have distinct immunomodulatory functions that regulate the immune response in target organs (101).

Afferent Sensory Signaling

Vagal sensory neurons project from the viscera to the solitary nucleus in the medulla oblongata. These neurons detect mechanical, chemical, and inflammatory stimuli in the viscera and relay back to the solitary nucleus that, in turn, activates the dorsal nucleus of the vagus to increase efferent signaling out to the viscera. Sensory neurons are also capable of efferent signaling through the release of the neuropeptide substance P (SP) (54).
SP is an 11 amino acid peptide neurotransmitter produced in sensory neurons that is responsible for nociception, the perception of noxious stimuli and pain (54). SP is transported to both the peripheral and central nerve terminals stored in vesicles. Depolarization of the neuron through noxious stimuli like acid, heat, LPS, or pro-inflammatory cytokines causes release of SP into the tissues where it binds to the neurokinin-1 receptor (NK-1R) (77, 98). NK-1R is present on mast cells, vascular endothelium, macrophages, and neutrophils. In the presence of other inflammatory stimuli, SP binding will cause acute vasodilation, histamine release, pro-inflammatory cytokine production, and neutrophil recruitment. Interestingly, in the absence of other inflammatory stimuli (LPS, tissue damage, etc.), SP is not sufficient to recruit neutrophils or activate macrophages (20, 92). It can, however, prime these cells making them more responsive to subsequent chemokine and LPS exposure (129).

SP plays an important role in the inflammatory arm of neurogenic pulmonary edema (NPE) after brain injury. The increased efferent vagal parasympathetic stimulation that follows TBI can induce SP release from sensory nerve terminals (76, 77, 144). This causes vasodilation and inflammatory cell recruitment to the lung tissue already damaged by the high hydrostatic pressures following the catecholamine storm (10, 108). It is unclear whether SP release occurs in conjunction with the catecholamine storm or precedes it and primes the vasculature and inflammatory cells for an exaggerated response later. In an animal model of TBI, prior blockade of SP release prevents the extravasation of
fluid and inflammatory response to NPE indicating a critical role for SP in NPE pathophysiology (76). In stroke patients, treatment with drugs that increase substance P levels were shown to decrease susceptibility to pneumonia (5, 97). The effect of substance P on pneumonia susceptibility in TBI, where pulmonary substance P levels are increased, has not yet been investigated.

**Efferent Cholinergic Signaling**

The efferent arm of the vagus mediates parasympathetic control of the viscera and vasculature. Efferent neurons release the neurotransmitter acetylcholine that binds nicotinic or muscarinic acetylcholine receptors (AChRs) on smooth muscle, endothelium, glands, cardiac muscle, and macrophages. Macrophages express a specific α7 subtype of the nicotinic acetylcholine receptor (α7-nAChR) that, when stimulated, has profound anti-inflammatory effects (134). Kevin Tracey and co-workers discovered the cholinergic anti-inflammatory pathway in 2000 after they observed vagus nerve stimulation significantly reduced the release of TNF-α by macrophages in response to LPS challenge (16). Further investigation revealed that cholinergic stimulation of macrophage α7-nAChR inhibits release of IL-1, IL-6, and TNF-α through c-fos mediated inhibition of NF-κB activity (119, 131, 134). This is not accompanied by an increase in IL-10 production as in catecholamine stimulation and allows for neutrophil recruitment and phagocytosis despite lower levels of pro-inflammatory cytokine production (128). The role of this pathway in
TBI has yet to be investigated in animal models but has been linked to suppressed inflammatory cytokine production in TBI patients (73).

**TBI and the Lung**

While neurological injury is often the primary focus of medical management of TBI, brain injury also has serious detrimental effects on organ systems beyond the central nervous system and the lungs are the most commonly affected (21, 40, 44, 59). Respiratory dysfunction develops in up to 81% of severe TBI patients and is associated with significantly poorer neurological outcomes (59). The main causes of pulmonary dysfunction after TBI are neurogenic pulmonary edema and respiratory infection (59, 148, 149).

**Neurogenic Pulmonary Edema**

Neurogenic pulmonary edema (NPE) is increased extravascular fluid in the lungs developing at any time up to 14 days after neurological injury (124). NPE can occur after TBI, stroke, seizures, or even transient increases in ICP and results in increased vascular permeability with fluid accumulation in the alveoli and interstitium of the lung preventing gas exchange and causing hypoxemia (10, 47, 108). Accompanying the fluid build-up is an inflammatory response that can damage the lung tissue and further impair respiratory function and even progressing to acute respiratory distress (58). Multiple factors contribute to the development of NPE and can be divided into hemodynamic mechanisms and inflammatory mechanisms.
Hemodynamic Mechanism

Immediately after injury, TBI can trigger a sudden release of the catecholamines epinephrine and norepinephrine into the circulation from the adrenal glands (138). These induce vasoconstriction of the pulmonary vasculature but a simultaneous increase in cardiac output and blood pressure. With the heart pushing more blood under higher pressure into a constricted pulmonary vascular bed, fluid is forced into the lung tissue by hydrostatic pressure (10). This surged shift in hemodynamics is called the "blast effect" of TBI (124). While this mechanism may enhance the fluid shift into the airspaces, it does not explain the increased vascular permeability and inflammation that accompanies of NPE.

Inflammatory Mechanism

Inflammation is a key aspect to the initiation and progression of NPE. Pro-inflammatory cytokines like IL-1, IL-6, and TNF-α are increased within the lung and the circulation in NPE and are associated with increased vascular permeability and inflammatory cell infiltrates that damage the lung tissue (47). The exact source of these cytokines is controversial, different studies have implicated the brain itself, circulating and tissue monocytes, and vascular endothelium (50, 108). Direct sympathetic and parasympathetic nervous stimulation of the lung has also been investigated as a possible source of the inflammatory arm of NPE. Stimulation of either input to the lung increases vascular permeability and edema through the release of inflammatory
neuropeptides like substance P, calcitonin gene related peptide (CGRP), and neuropeptide Y from nerve terminals (9, 21, 76, 77, 128).

**Respiratory Infection**

Respiratory infections develop in up to 40% of hospitalized TBI patients. The incidence of infection increases with the severity of injury and results in longer hospitalizations, increased duration of mechanical ventilation, and poorer neurological outcomes (6, 40, 59, 70). These pneumonias are most often hospital-acquired (developing 48 hours or later into hospitalization) and the most commonly isolated organisms are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (149). Development of pneumonia after TBI may be due to several factors: intra-tracheal intubation and mechanical ventilation, aspiration of oropharyngeal bacteria while unconscious, and poor pulmonary toilet (55, 149). With an inoculum in the lung, alveolar macrophages detect and phagocytose the bacteria and release pro-inflammatory cytokines like IL-1, IL-6, and TNF-a as well as the crucial neutrophil recruitment chemokine IL-8 (MIP-2 and KC in mice) (87). Neutrophils then follow the chemotactic trail to the site of infection where they phagocytose bacteria, release degradative enzymes and reactive oxygen species, and extracellular chromatin traps to kill bacteria and clear them from the airspaces (87, 88). Studies in an animal model of ischemic stroke and pneumonia have shown deficiencies in T-lymphocyte function in the lung but did not investigate the innate immune response (34, 110).
The effects of TBI on the pulmonary innate immune response to pneumonia have not been investigated but could yield therapeutic approaches to treatment and prevention of pneumonia in TBI and non-TBI patients alike.
Chapter 2: Assessing Lung Injury in Animal Models of Traumatic Brain Injury and Bacterial Pneumonia

Introduction

After initial resuscitation, medical management of TBI is primarily focused on preventing deficits in neurological function. While this aspect of TBI care is important, respiratory dysfunction is often of lower priority. In severe TBI up to 81% of patients experience respiratory dysfunction during their hospitalization making it the most common extra-neurological complication (58, 59, 148). The most common causes are respiratory infection and neurogenic pulmonary edema (10, 47, 55). Respiratory dysfunction results in significant excess morbidity, and poorer neurological outcome among TBI patients as well as longer hospital stays and increased healthcare costs (51, 55, 58, 59). The majority of studies have been directed at severe brain injury but none have looked at respiratory dysfunction in the much more common moderate TBI (6, 65, 81). Moderate TBI still results in axonal damage, aberrant cholinergic and adrenergic activity, and continuing neurological sequelae that could affect pulmonary function (51).

Studying the consequences of traumatic brain injury requires the use of animal models. Replicating human TBI in an animal model has often-conflicting requirements. While the pattern and severity of injury must be clinically relevant and reproducible, it must also take into account the inherent diversity of the trauma patient population. The most prominent TBI models are lateral fluid...
percussion (LFP) and weight-drop injury (WDI) (99, 145). LFP requires a small skin incision and craniotomy be made for placement of a fluid catheter. This catheter is attached to a fluid reservoir with a diaphragm that is struck with a weighted hammer. This transmits a fluid shock wave into the extradural space and compresses the brain parenchyma inducing injury (43). WDI uses a craniotomy to expose the extradural space and a weighted impactor is positioned to strike the dural surface and injure the cortex beneath it (79). While these models produce excellent specific injury to the brain for neurological studies, the skin and bone incisions required by both models cause significant immune reactions that could confound immunological studies (145). Therefore an injury model is needed that requires no incision or craniotomy.

Acute lung injury (ALI) and the more severe form, acute respiratory distress syndrome (ARDS) can be caused by a variety of pathological processes that affect the lung either directly or indirectly (135). In TBI, a variety of neural signaling pathways are implicated in the development of ALI. Hemodynamic instability due to aberrant sympathetic stimulation and inflammation due to tachykinin release have been shown to contribute to the development of NPE after TBI while anti-inflammatory sympathetic and parasympathetic signaling are thought to predispose TBI patients to nosocomial pneumonia (10, 36, 71, 108, 111, 140). Previous work in a mouse model of stroke demonstrated brain infarction induced both lung injury and the development of spontaneous
pneumonia (110). Our study seeks to investigate the development of ALI following moderate TBI from both of these mechanisms.

As a positive control for lung injury, we needed a mouse model of nosocomial pneumonia. *P. aeruginosa* is a common cause of gram-negative nosocomial pneumonia (136) capable of causing severe or fatal infections (46). Previous experimental studies show that *P. aeruginosa* leads to the development of ALI, characterized by substantial alveolar protein-rich edema (12). The recruitment of airway neutrophils is also a major component of the initial host immune response to *P. aeruginosa* (88). Multiple cytokines that regulate host lung defense and inflammation are increased during this bacterial infection. A variety of histological abnormalities are seen in the lung including fibrinous exudate, polymorphonuclear leukocytes, hemorrhage, and alveolar septal necrosis (142). Impaired oxygenation may significantly affect pulmonary physiology and survival in *P. aeruginosa* infection.

In our study, lung function and histological characteristics of TBI mice and pneumonia mice were obtained to evaluate for the presence of pulmonary injury. Since inflammation may occur without injury, several parameters of pulmonary inflammation were also analyzed. TBI mice were sampled and sacrificed at 48 hours, the point at which respiratory dysfunction most commonly develops in human TBI patients while pneumonia mice were similarly analyzed 18 hours after bacterial challenge (58).
Methods

Animals

Female ICR mice (Harlan-Sprague Dawley, Indianapolis, IN) were used for all experiments. All mice were 8-10 weeks of age, weighed 24-30 grams, and were housed for at least 3 days prior to use with a 12 hour light/dark cycle. Food and water were provided ad-libitum throughout the studies. All experiments were approved by the Institutional Animal Care and Use Committees of Boston University.

TBI Model

Isoflurane anesthetized animals are positioned on a foam-padded platform underneath the impactor rod with the point of impact located in the midline, halfway between the interauricular and interorbital lines overlying the frontal lobes. Once positioned, the rod is raised 7 cm and released, producing approximately 5 kg/cm² of impact force. A felt pad on the end of the impactor prevents rebound impacts and damage to the overlying tissues. Immediately after impact, animals receive an injection of buprenorphine and are allowed to recover on a warming bed until capable of righting themselves from a supine position at which point they are returned to their cage. Sham injury animals undergo the same procedure but the impactor is not dropped. TBI animals require 5 to 10 minutes of recovery before they are able to right themselves as opposed to 30-45 seconds in sham animals.
Pneumonia Model

Pneumonia was induced by anesthetizing 8 mice with isoflurane and administering $5 \times 10^7$ CFU of *Pseudomonas aeruginosa* (ATCC strain Boston 41501) in 50 microliters of Hank’s balanced salt solution via hypopharyngeal administration as previously described (48). A group of 8 mice received 50uL normal saline as a negative control. Eighteen hours later all mice were sacrificed for further analysis.

Whole body plethysmography

Respiratory parameters were measured using a whole body plethysmography system (Buxco Research Systems, Wilmington, NC) prior to TBI or sham injury and repeated at 4, 24, and 48 hours after injury. Measurements included respiratory rate, tidal volume, time of inspiration and expiration, and peak inspiratory and expiratory flow. Mice were individually placed into the chamber of the plethysmograph and left to acclimate for 10 minutes as previously described (56). Mice were conscious and unrestrained, and they showed normal exploratory behavior. Respiratory measurements were also collected at baseline and 18 hours after intervention for mice receiving normal saline or *P. aeruginosa*.

Blood and Lung Sampling

At 4, 24, and 48 hours post TBI or sham injury, 20 µL of blood was collected by facial vein puncture as previously described (27) for a complete blood count with
differential using a Hemavet instrument (CDC Technologies, Oxford, CT).

Following whole body plethysmography measurements at 48 hours, the mice were anesthetized with a solution of 87 μg/g Ketamine and 13 μg/g Xylazine in normal saline. Mice receiving *P. aeruginosa* or normal saline IT were sacrificed after 18 hours. Blood was collected by carotid artery cutdown in both groups.

**Arterial Blood Gas**

48 hours after TBI or sham injury or 18 hours after administration of *P. aeruginosa* or normal saline, mice were anesthetized as described above. Using a dissecting microscope, a midline ventral neck incision was made from the mandible to sternal notch. The fascia and strap muscles were bluntly dissected until the carotid artery was exposed, and it was clamped distally to engorge the vessel with blood. The carotid artery was punctured with a 30G heparin rinsed syringe and 100uL blood was collected for arterial blood gas (ABG). An iSTAT analyzer (Abbott Laboratories, Princeton, NJ) with CG4+ cartridges was used for ABG measurement and arterial oxygen saturation. The remainder of the blood volume was collected in a separate syringe and mixed with 50uL of 169mM EDTA.

**Single lung bronchoalveolar lavage (BAL)**

The thorax was opened with a midline incision through the sternum to expose the heart and lungs. The left lung hilum was clamped closed. Right-sided BAL was performed by exposing the trachea and cannulating it with a polyethylene
catheter and then the right lung was lavaged with a total of 2.5mL of warm Hanks' Balanced Salt Solution (HBSS, Mediatech, Inc., Herndon, VA). 100μl of the first 0.5mL was serially diluted for bacterial colony forming units. The remaining 400μl and two subsequent 1mL aliquots were centrifuged separately and the supernatant from the first wash was stored at -80°C for later measurement of cytokines. Bacterial counts in BAL fluids and lung homogenates were determined 12 hours after samples were plated in triplicate on sheep blood agar plates. The supernatant from the second wash was discarded and the 3 pellets were resuspended in HBSS and combined. A total cell count was performed with a Beckman-Coulter particle counter model ZF (Coulter Electronics Inc., Hialeah, FL) and a differential count was obtained by counting 300 cells on cytospin slides stained with Diff-Quick (Baxter, Detroit, MI).

Following lavage, the right hilum was clamped and tied off distally with 4-0 silk. The right lower lobe was used to determine the wet/dry ratio and the remainder of the right lung was stored for the myeloperoxidase assay. Next, a syringe filled with 10% buffered formalin at 25 cm water pressure was connected to the tracheal catheter and the left lung clamp was released to insufflate the lung for 2 minutes. The left lung was removed and placed into 10% formalin for fixation and histological analysis.
Histopathology

Formalin fixed lung and brain tissue was embedded in paraffin and sectioned at 5 μm for routine histology. Slides were stained with hematoxylin and eosin and edema, hyperemia, congestion, neutrophil margination and tissue infiltration, were evaluated to establish the presence or absence of lung injury as previously described (8).

Myeloperoxidase assay

The myeloperoxidase (MPO) assay was performed as previously described (123).

Enzyme-linked immunosorbent assay

An aliquot of the 1:10 diluted plasma collected at 24 hours from all the mice was used to determine the IL-6 concentration by ELISA as previously described (95). A separate plasma aliquot was stored and used to measure a variety of pro and anti-inflammatory cytokines using the sequential ELISA technique (102). This analysis was also performed on the supernatant of the BAL fluid.

Statistical Analysis

Column analysis was performed using two-tailed T-tests. Serial measures were analyzed using 2-way ANOVA with Bonferroni post-hoc test to determine significance between groups.
Results

Post-Injury Animal Coma Scale

To model human moderate TBI in mice, we needed an injury assessment scale to ensure that our model produced a consistent injury with similar neurological findings between animals. The human Glasgow Coma Scale (GCS) served as a basis for our scale with modifications to make it relevant for animals (63). Our modifications are noted in table 1. Mice were evaluated after returning to sternal recumbence following injury. As moderate TBI corresponds to a score of 9-12 in the human GCS, we only used mice that scored in that range on our mouse modified coma scale (MMCS) and showed no signs of focal neurological deficit (paralysis, side neglect, deviation to one side). These would be assessed in mice that had righted themselves to sternal recumbency for at least two minutes after anesthesia had worn off.
Table 1: Human Glasgow coma scale and mouse modified coma scale. Mice were evaluated on observed resting posture, response to sound (snapping fingers by each ear), and response to being lifted and suspended by the tail.

<table>
<thead>
<tr>
<th>Human Glasgow Coma Scale</th>
<th>Mouse Modified Coma Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eye Opening</strong></td>
<td><strong>Posture</strong></td>
</tr>
<tr>
<td>Spontaneous 4</td>
<td>Moving about cage 4</td>
</tr>
<tr>
<td>To command 3</td>
<td>Hunched 3</td>
</tr>
<tr>
<td>To pain 2</td>
<td>Dazed sternal recumbence 2</td>
</tr>
<tr>
<td>None 1</td>
<td>Prone 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Verbal Response</strong></th>
<th><strong>Response to Sound</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Oriented 5</td>
<td>Turns to investigate 5</td>
</tr>
<tr>
<td>Confused, disoriented 4</td>
<td>Moves away 4</td>
</tr>
<tr>
<td>Inappropriate words 3</td>
<td>Startle 3</td>
</tr>
<tr>
<td>Incomprehensible sounds 2</td>
<td>Wincing (no body movement) 2</td>
</tr>
<tr>
<td>None 1</td>
<td>No response 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Motor Response</strong></th>
<th><strong>Tail Lift Response</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Obey commands 6</td>
<td>Extension and reaching 6</td>
</tr>
<tr>
<td>Localizes pain 5</td>
<td>Extension without reaching 5</td>
</tr>
<tr>
<td>Withdraws 4</td>
<td>Lifts head flexion 4</td>
</tr>
<tr>
<td>Abnormal flexion to pain 3</td>
<td>Flexion no head lift 3</td>
</tr>
<tr>
<td>Abnormal extension to pain 2</td>
<td>Deviates to one side 2</td>
</tr>
<tr>
<td>None 1</td>
<td>No response 1</td>
</tr>
</tbody>
</table>

Best total score 15

As our goal was to study the immunological consequences of TBI, we needed a mouse model of injury that would reflect the types of injuries sustained in mild to moderate TBI patients without confounding immune responses due to skin incisions or craniotomy. The mice also needed to be injured to a level that produced a measurable physiological effect but did not render them comatose or in need of ventilation. An impact force had to be chosen that would result in gross brain parenchymal damage without depressed skull fracture or lethal spreading.
hematoma. For these reasons we chose to use a modified weight-drop injury model (79). We constructed a device consisting of a 180 gram steel rod that slides with minimal friction through a guide sleeve supported in a ring stand (Figure 1A). The rod was raised to different heights to achieve higher or lower impact force as needed. Impact was calibrated using a suspended scale with a deadfall needle that registers peak force.

Figure 1: Weight drop injury device. A) Device with animal platform removed. B and C) views from above and side of animal positioning. Note neck restraint to limit extraneous motion and maintain consistent head position.
Trials in live and dead animals found that an impact of about 5 kg/cm² force (impactor released from 7 cm height) generated the ideal level of both pathological injury and neurological impairment (MMCS ~12-9, table 1) in 90% of the mice. The other 10% were more impaired, died, or displayed focal neurological deficit and were excluded from further study. Formalin-fixed brains from TBI mice were examined for gross and microscopic injury (Figure 2). Injury was found to be confined to the inferior surface of the frontal and temporal lobes and consisted of intraparenchymal bleeding surrounding the middle cerebral and striate vessels. This is consistent with a combination of contracoup and skull-base gliding contusions seen in frontal impacts in humans (133, 137).

The location of the injuries caused by this model has some definite effects on mouse behavior and physiology assessed by the MMCS. The area injured contains the optic nerves and chiasm, the hypothalamus, amygdala, and other elements of the limbic system. Damage to these areas could impair visual function and the fear response, both of which would change the mouse posture and responses to sound and tail-lift (53, 64). Damage to the hypothalamus could change thermal regulation and the fever response which could alter both immune function and the general illness behavior of the mouse (64). We did note aggressive behavior in the mice after injury with a hunched defensive posture and sensitivity to sound and physical stimulus.
Figure 2: Pattern of injury 24 hours after modified weight drop injury TBI. Cerebral contusions bilaterally on the inferior surface of the frontal and temporal lobes corresponding to a contracoup and skull floor washerboard injury mechanism. A) Gross image of inferior surface. B) Frontal section through injury magnified 4X. C) Same section magnified 10X.
Acute Immunological Impact of TBI

Complete blood count and cytokine analysis were performed on samples collected from TBI and sham mice by facial vein bleed (Figure 3). TBI mice had a significant increase in blood neutrophils 4 hours after injury and a decrease in blood lymphocytes. These changes did not affect the total white blood count and both returned to baseline by 24 hours (Figure 3A). The plasma cytokines IL-6, IL-1ra, and MIP-2 followed a similar pattern and returned to baseline by 48 hours (Figure 3B). Interestingly, IL-10 levels were below the limit of detection (0.09868 ng/mL) in the plasma of TBI mice (data not shown) which is contrary to current literature describing increased IL-10 levels following TBI (138). We also did not see the prolonged drop in lymphocyte counts described in previous studies (111, 112).
Figure 3: Blood cell counts (A) and plasma cytokines (B) in TBI and sham injury animals 4, 24, and 48 hours after injury. TBI mice have increases in neutrophil counts accompanied by transient rises in inflammatory cytokines and a transient fall in lymphocyte count. *p<0.05 TBI vs sham Bonferroni post-hoc test.

Respiratory Physiology

Physiological respiratory parameters were measured using whole body plethysmography and the values obtained prior to injury for the mice undergoing TBI as well as values for the sham mice were within published normal ranges for all the parameters measured (130). TBI mice demonstrated significant decreases in respiratory rate accompanied by increases in time of inspiration and expiration (Figure 4). This is in keeping with respiratory depression seen in other models of moderate brain injury (6, 79, 109). By 24 hours these values had returned to
match those of sham animals with the exception of a transient decrease in tidal volume.

![Graphs showing respiratory rate, tidal volume, time inspiration, and time expiration for sham and TBI conditions.](image)

*Figure 4: TBI transiently depresses respiration. Buxco WBP at baseline and 4, 24, and 48 hours post TBI or sham injury demonstrating decreased respiratory rate and increased times of expiration and inspiration. *p*<0.05 TBI vs sham Bonferroni post-hoc test.*

Intra-tracheal pneumonia challenge in non-TBI mice resulted in significant decreases in respiratory rate and tidal volume while increasing times of inspiration and expiration (Figure 5). These changes are similar to those seen in the TBI mice at the 4 hour time point but in pneumonia or lung injury, these changes are due to airway obstruction and increased work of breathing as evidenced by the decreased tidal volume. This differs from the direct brainstem-
mediated slowing of respiratory rate seen in TBI without accompanying changes in tidal volume (6, 79).

Figure 5: Bacterial pneumonia causes significant disturbances in respiratory function. Buxco WBP data 18 hours after IT administration of normal saline or $5 \times 10^7$ CFU of *P. aeruginosa*. *p<0.05

**Hypoxemia**

The most important pulmonary function is performing the gas exchange necessary for maintaining the supply of $O_2$ to the tissues and eliminating $CO_2$. Arterial oxygen saturation was measured from the carotid artery of mice immediately prior to sacrifice at 48 hours in mice subjected to TBI or sham injury and at 18 hours for non-TBI mice receiving *P. aeruginosa* or normal saline. As
seen in Figure 6, the arterial oxygen saturation was maintained in TBI mice and was no different compared to normal saline controls, but gas exchange was markedly impaired in mice with bacterial pneumonia. Maintenance of a normal arterial oxygen saturation indicates that TBI mice do not develop hypoxemia.

![Arterial Oxygen Saturation](image)

Figure 6: Pneumonia but not TBI results in decreased arterial oxygen saturation. Arterial oxygen saturation obtained by carotid cutdown and ABG 48 hours after TBI or sham injury and 18 hours after IT administration of normal saline or $5 \times 10^7$ CFU of *P. aeruginosa*. *p<0.05

**Spontaneous Pneumonia**

Previous studies in a mouse model of ischemic stroke had demonstrated the development of spontaneous pneumonia after ischemic injury to the brain (110). As the pathology demonstrated in the brains of our TBI mice could result in ischemic damage, we assessed the presence of spontaneous pneumonia in our TBI mice using the pneumonia animals as a positive control (137). We cultured samples of BAL supernatant and lung homogenate from these animals for
bacterial growth. The pneumonia mice had high levels of recoverable bacteria in both BAL and homogenate as expected but our TBI mice failed to demonstrate any bacteria (Figure 7). This indicates that our moderate brain injury does not result in the development of spontaneous pneumonia.

Figure 7: TBI does not result in spontaneous pneumonia. Lung homogenate and BAL supernatant bacterial counts demonstrating bacterial growth only in pneumonia positive control mice. BDL—below detection limit.
Lung Damage

Accumulation of neutrophils in the lungs may cause severe degradation of the lung tissue through release of proteases and formation of reactive oxygen species (8, 127). To determine their presence in the alveolar space, lung parenchyma and circulation, neutrophil counts were measured in sacrificed mice. Photomicrographs of representative cytospin slides demonstrate only alveolar macrophages in BAL recovered from TBI, sham, and normal saline mice (Figure 8 A,B,C). In contrast, bacterial pneumonia (Figure 8 D) showed a predominance of neutrophils with an increase in red blood cells. Quantitatively, BAL fluid at 48 hours after TBI showed no influx of neutrophils in the airways (Figure 8 E) versus bacteria. Additionally, there were similar numbers of alveolar macrophages in all mice sampled (Figure 8 F).
Figure 8: TBI does not induce significant cell recruitment to the airways. Representative cytospins (A-D) and differential counts (E and F) of cells recovered by BAL in sham, TBI, normal saline, and pneumonia mice respectively.
Myeloperoxidase Assay

Myeloperoxidase (MPO) is frequently used to measure the presence of neutrophils in tissues (2, 96). MPO activity did not increase in TBI mice compared to sham or normal saline (Figure 9). However, non-TBI mice receiving bacteria had a significant increase in lung MPO compared to the non-TBI normal saline control. The data presented in figures 5 and 6 indicates that there is no significant recruitment of neutrophils to the lungs of the TBI mice that could lead to lung injury.

![Lung MPO Graph](image)

Figure 9: TBI does not result in neutrophil recruitment to the lung. Myeloperoxidase activity in lung homogenates from TBI and sham mice 48 hours after injury and mice 18 hours after receiving IT administration of normal saline or $5 \times 10^7$ CFU of *P. aeruginosa*. 
Lung Histopathology

To confirm the lack of neutrophil recruitment and edema formation in the lungs of the TBI mice, microscopic examination of H&E stained lung sections from all groups was performed. Figure 10 shows representative images from 48 hour TBI and sham injury, as well as a mouse given normal saline or bacteria. There were no visible neutrophils in the alveoli and interstitial spaces of either the TBI or sham mice confirming the results from the BAL cytospin slides. No indication of protein leakage into the alveolar space was found with no detection of hyaline substance accumulation or edema fluid but TBI mouse lungs did demonstrate some dilated blood vessels and hyperemia. Conversely, mice given bacteria exhibited significant edema, hyperemia, congestion, neutrophil margination and infiltration, hemorrhage, and the presence of debris. All of these parameters demonstrate that there is no significant lung injury 48 hours after moderate TBI.
Figure 10: TBI does not result in histologically evident lung injury. Representative microscopic images of sectioned lungs from sham, TBI, normal saline, and pneumonia mice at 10, 20, and 40X magnification. NS- normal saline IT, PA- *P. aeruginosa* IT.
**Lung Inflammation**

Finally, the presence of pulmonary inflammatory mediators was tested by measuring the local concentration of cytokines in the BAL fluid of the mice sacrificed at 48 hours. The BAL fluid concentration of 5 cytokines including IL-6, KC, MIP-2, TNF-α, and IL-1ra was similar in the TBI, sham, and normal saline mice as seen in Figure 11. Importantly, the bacterial pneumonia caused a significant increase in each of the BAL cytokines. These results indicate TBI does not induce inflammatory cytokine production in the airways corresponding to lung injury.

![BAL Cytokines](image)

**Figure 11:** Pneumonia but not TBI induces inflammatory cytokine production in the lung. Cytokine levels in BAL supernatants of mice 48 hours post TBI or sham injury and 18 hours after IT administration of normal saline or $5 \times 10^7$ CFU of *P. aeruginosa*.
Discussion

The development of respiratory dysfunction following TBI is a serious clinical problem that can affect the management and outcome of brain injured patients. To date, no clinical or basic science studies have investigated respiratory function in moderate TBI. In this study we have shown that our mouse model of moderate traumatic brain injury produces a consistent and clinically relevant level of brain injury without invasive manipulation. This level of brain injury does not result in lung injury through neurogenic pulmonary edema or development of spontaneous pneumonia as determined through detailed analysis of lung function and pathology. Our model of *P. aeruginosa* pneumonia did cause significant lung injury that was well-demonstrated by our analytical parameters and served as an appropriate positive control.

Many animal models of TBI require invasive measures like craniotomy to ensure a reproducible injury pattern. While they may generate a specific injury, the mechanism is not as clinically relevant to the brain injury population as closed head injury (145). Our model replicates a commonly encountered mechanism of injury (impact acceleration) and reproducibly induces cerebral contusions in a pattern seen in frontal impacts (57). The level of neurological deficit following injury, when assessed with a modified coma scale, is consistent with those seen in moderate TBI patients. A limitation of our model is the severity of parenchymal injury. To induce more severe injury, the impact required would result in a skull
fracture that would both confound our immunological parameters and most likely result in the death of the animal.

Hematological changes following TBI have been well-documented in the basic science literature and showed marked immunosuppression with sustained drops in blood lymphocytes and inflammatory cytokine production (111). While we did see a brief decrease in lymphocytes, they returned to normal levels by 24 hours after injury and most likely represented the well-documented apoptosis that follows a traumatic injury (67). Inflammatory cytokine production did not seem to be impaired as we did see a brief spike in IL-6, MIP-2, and IL-1ra production after TBI. Importantly we did not detect an increase in IL-10 at any point in our TBI animals which has been suggested to cause lymphocyte apoptosis and decreased inflammatory cytokine production after TBI (113, 138, 140). This may be due to the lesser severity of our injury model.

Severe brain injury has been shown to result in lung injury through neurogenic pulmonary edema and bacterial pneumonia in animal models and humans (55, 59, 149). In our model we did not find evidence of either mechanism of injury. We did observe a brief decrease in respiratory function immediately after TBI with plethysmography. These results were consistent with respiratory depression seen in human and animal TBI mediated by brainstem respiratory centers rather than impaired mechanics of respiration from airway inflammation (31, 130, 147). These parameters returned to baseline levels by 24 hours and gas exchange
was not affected. A more severe injury might induce more hemodynamic shifts to induce the hydrostatic damage of lung vasculature with subsequent inflammatory response due to neural inputs. Interestingly we did see histological evidence of hyperemia and vasodilation in TBI mouse lungs. This may represent tachykinin release into the lungs after TBI (5, 36, 76). Our injury may cause low level substance P release that, in the absence of hydrostatic tissue damage or infection, will induce vasodilation of pulmonary vasculature without edema or recruitment of inflammatory cells.

We did not recover any bacteria from the lungs of our TBI mice and the cytokine and cell recruitment profiles did not reflect infection especially when compared to our pneumonia positive control. This does not mean that the pulmonary immune response to infection will not be altered by TBI and could be explained by better respiratory toilet when compared to the more severe models of injury that found spontaneous pneumonia (110). Further investigation with bacterial challenge of TBI mice will be required to elucidate any effects on the pulmonary immune response to pneumonia that may follow TBI and increase susceptibility to infection.
Chapter 3: Traumatic Brain Injury Enhances Resistance to Bacterial Pneumonia

Introduction

Traumatic brain injury (TBI) is a major cause of death and disability in both in civilian and military trauma patients. While early mortality is primarily attributed to non-survivable head injury, delayed deaths following head injury are often secondary to non-neurologic organ dysfunction as a result of infectious and inflammatory processes. Pneumonia is the most common infectious complication associated with TBI, occurring in 20-60% of head injured patients, rates that are thought to be increased compared to non-head injured patients (55, 58). Risk factors for the development of pneumonia after TBI include post-injury immunosuppression and prolonged mechanical ventilation (19).

Immunosuppression after TBI has been demonstrated predominantly via a decline in the adaptive immune system (86). CD4 and CD8 cell types are decreased after TBI for a prolonged period of time (111, 138, 140). However, both adaptive and innate immunity both play important roles in controlling infection. Neutrophils and macrophages of the innate immune system are vital for appropriate bacterial clearance. Neutrophil recruitment is regulated by multiple factors, including CXCR2 chemokines KC and MIP-2, leukotrienes, and substance P (28, 80). Enhanced recruitment of neutrophils to a site of infection has been shown to significantly improve bacterial clearance and survival (27).
In the present study, we sought to determine the relationship between TBI and pulmonary infection. Our unexpected results indicate that patients suffering TBI have significantly lower rates of pneumonia than non-head injured trauma patients. However, this observation was only found using propensity score matching, underlining the importance of appropriate data analysis. Our clinical data is supported by an animal model of TBI and *P. aeruginosa* pneumonia. We found TBI mice had improved survival following bacterial challenge compared to sham injury animals. TBI mice demonstrated increased neutrophil recruitment to the airspaces in the acute phase of pneumonia which is critical for increased bacterial clearance and reduced pneumonia morbidity and mortality.
Methods

National Trauma Data Bank

The National Trauma Data Bank 7.2 (NTDB; American College of Surgeons) was queried to investigate the clinical association between TBI and pneumonia. The analysis for this study was from the most recently updated data, reporting hospital admissions for 2010. Our study included patients with a hospital length of stay longer than two days, an age greater than 18 at admission, and a blunt mechanism of injury.

Data was analyzed for patients with blunt traumatic injuries and those with traumatic brain injuries, with identification of the frequency of pneumonia as a complication. Head injured patients were identified as those individuals with at least one ICD-9 code consistent with traumatic brain injury. These individuals were further grouped, using the Glasgow Coma Score (GCS), into mild (13-15), moderate (9-12), and severe (<9) TBI categories. The blunt traumatic injury group consisted of patients whose diagnosis codes did not include either a head or neck injury.

Demographics, baseline vital signs, injury severity score, length of stay, and requirement for mechanical ventilation were summarized descriptively and analyzed statistically using SAS Version 9.3. Because of the significant differences observed in the demographic and vital signs data (Table 1), and the potential impact of these differences on incidence of pneumonia, propensity
scoring was used to more precisely match each TBI cohort to the blunt trauma cohort. Propensity scores were generated using multivariate logistic regression with group membership as the dependent variables and a variety of baseline variables, their squares, and interactions as explanatory variables. In all matched data, patients with chest injuries were excluded due to the synergistic factors contributing to the development of pneumonia.

**Animals**

Female ICR mice (Harlan-Sprague Dawley, Indianapolis, IN) were used for all experiments. All mice were 8-10 weeks of age, weighed 24-30 grams, and were housed for at least 3 days prior to use with a 12 hour light/dark cycle. Food and water were provided ad-libitum throughout the studies. All experiments were approved by the Institutional Animal Care and Use Committees of Boston University.

**Murine Models of Moderate TBI and Pneumonia**

Under isoflurane anesthesia, mice were placed in a prone position on a plexiglass bed with their head resting on a foam pad under a weight drop impact device. To induce the TBI, a 170 gram steel rod within a guide tube was released from 5.2cm to a point in the midline, halfway between the interauricular and interorbital lines, producing approximately 5 kg/cm² of impact force. Rebound impact was prevented and the mouse was immediately removed from the device and given an i.p. injection of 0.05mg/kg buprenorphine in 1mL of normal saline.
Mice are placed supine on a warming bed and returned their cages after righting themselves to sternal recumbence. Sham mice underwent similar sedation and analgesia without TBI.

**P. aeruginosa** Pneumonia

Pneumonia was induced 48 hours post-TBI by anesthetizing mice with isoflurane and administering 1x10^7 CFU or 5x10^7 CFU of *Pseudomonas aeruginosa* (ATCC strain Boston 41501) in 50 ul of Hank's balanced salt solution via hypopharyngeal administration (48). Mice were allowed to recover and were monitored for up to 7 days. An additional group of animals was sacrificed four hours after infection to obtain pulmonary bacterial loads by bronchoalveolar lavage (BAL) and lung homogenates.

**Whole Body Plethysmography**

Respiratory parameters were analyzed as described in chapter 2 at baseline, 4, 8, 24, and 48 hours after bacterial challenge. All were compared to a group of mice 48 hours post TBI with no pneumonia.

**Plasma and Tissue Analysis**

Plasma samples were obtained by cardiac puncture at the time of sacrifice under ketamine/xylazine anesthesia. To determine whether this increased bacterial clearance was due to local cell recruitment, we analyzed the cellular content of the BAL fluid. BAL was performed with 5mL of warm Hank's balanced salt
solution (HBSS) in 1mL aliquots. 100µl of the first aliquot was retained for bacterial count. The supernatant from the remainder of the first aliquot was used for cytokine analysis. The cell pellets of all aliquots were combined and counted using a Beckman Coulter particle counter (Coulter Electronics, Danver, MA). Differential counts of these cells were performed by counting 300 cells on cytospin slides stained with Diff-Quick (Baxter, Detroit, MI). After lavage, the lungs were sterilely transferred to 3mL of HBSS and homogenized. Bacterial counts in BAL fluids and lung homogenates were determined 24 hours after samples were plated in triplicate on sheep blood agar plates. Cytokines and chemokines (IFN-γ, IL-1ra, IL-6, IL-10, KC, MIP-2, TNF-α) were measured by sandwich ELISA using matched antibody pairs (R&D Systems) (95).

**Statistical Analyses**

The predicted probabilities of group membership among the patients (propensity scores) in conjunction with a SAS 1:1 matching macro were used to create matched comparison groups. The groups were then compared using chi-square and rank-sum statistics to gauge whether any residual differences remained. Survival studies were analyzed with Kaplan-Meier analysis. Other analyses were completed with T-tests. Serial measures were analyzed using 2-way ANOVA with Bonferroni post-hoc test to determine significance between groups at each time point.
Results

Unadjusted NTDB Data Shows Increased Pneumonia Rates in TBI Patients

The NTDB for the year of 2010 included a total population of 722,836 patients. Table 1 shows demographic and arrival vital signs data for 209,056 patients included in our control cohort, blunt trauma patients without head or neck injuries, as well as 56,528 patients with a blunt trauma and TBI. Statistical differences were found in all demographic variables analyzed.

Table 1. Demographics of all blunt traumatic injuries and traumatic brain injuries in the National Trauma Database for 2010. Excluded were patients in the hospital for <2 days and <18 years of age.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Blunt Trauma w/o TBI (n=208,993)</th>
<th>Blunt Trauma w/ TBI (n=56,528)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Sex, male, }%$</td>
<td>56</td>
<td>64.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{Patient age}$</td>
<td>56.2 ± 21.1</td>
<td>52.5 ± 21.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>ISS</td>
<td>9.8 ± 7.2</td>
<td>16.6 ± 9.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Temperature</td>
<td>36.4 ± 2.0</td>
<td>36.4 ± 1.8</td>
<td>0.0107</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>140.8 ± 27.0</td>
<td>140.7 ± 28.3</td>
<td>0.48</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>86.5 ± 19.0</td>
<td>89.9 ± 20.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Median (interquartile range)</td>
<td>0 (0-2)</td>
<td>2 (0-4)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>ICU Length of stay, days</td>
<td>5 (4-8)</td>
<td>6 (4-10)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

The TBI patients were further divided into mild, moderate, and severe TBI to evaluate rates of pneumonia. The incidence of pneumonia in control, mild, moderate, and severe TBI groups is shown in Figure 1. While patients with mild
TBI had a similar incidence of pneumonia as controls, patients with moderate TBI had a higher incidence of pneumonia than either of these groups. Furthermore, patients with severe TBI had a significantly higher incidence of pneumonia compared to all three other groups.

![Bar chart showing incidence of pneumonia](image)

Figure 1: Incidence of pneumonia from the National Trauma Database for 2010 for blunt traumatic injuries (n=209,056), mild TBI (n=42,802), moderate TBI (n=3,163), and severe TBI (n=8,285). Blunt traumatic injuries have a rate of pneumonia of 3.3%, mild 3.4%, moderate 10.3%, and severe 19.7%. *p<0.05

Because mechanical ventilation is a primary risk factor for acquiring pneumonia, we evaluated the number of patients in each group requiring mechanical ventilation, and the relationship of duration of time on the ventilator with incidence of pneumonia. Whereas 10.5% of the control blunt injury cohort required mechanical ventilation, 25.2% of all TBI patients required mechanical
ventilation. Not surprisingly, the mechanical ventilation needs of head injured patients increased with injury severity, with 12.1% of mild, 48.2% of moderate, and 85.3% of severe TBI patients requiring mechanical ventilation. The relationship between the time on the ventilator and incidence of pneumonia was examined. As expected, the rate of pneumonia increased with greater time spent on mechanical ventilation in each group (Table 2).

Table 2. The incidence of pneumonia in blunt traumatic injury, mild TBI, moderate TBI, and severe TBI for patients ventilated for 1-5 days, 6-15 days, 16-30 days, or >30 days.

<table>
<thead>
<tr>
<th>Total Ventilator Days</th>
<th>Control</th>
<th>Mild TBI</th>
<th>Moderate TBI</th>
<th>Severe TBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 Days</td>
<td>7.45</td>
<td>7.33</td>
<td>5.73</td>
<td>6.19</td>
</tr>
<tr>
<td>6-15 Days</td>
<td>33.6</td>
<td>31.09</td>
<td>30.14</td>
<td>32.29</td>
</tr>
<tr>
<td>16-30 Days</td>
<td>49.98</td>
<td>45.97</td>
<td>49.33</td>
<td>49.26</td>
</tr>
<tr>
<td>&gt; 30 days</td>
<td>60.25</td>
<td>61.81</td>
<td>50</td>
<td>56.85</td>
</tr>
</tbody>
</table>

**Propensity Score Matching Demonstrates That TBI Patients Have Significantly Lower Risk of Pneumonia**

Patients were divided into subgroups for propensity score analysis based on need for mechanical ventilation. Patients were then matched based on age, injury severity score, admission heart rate, respiratory rate, systolic blood pressure, and temperature. Demographic and vital signs data for these groups are shown Table 3a and b. While there were no differences in injury severity score or admission vital signs amongst ventilated patients, other parameters including number of days on mechanical ventilation reached statistical significance.
Table 3. A.) Demographics for control and mild TBI requiring mechanical ventilation matched with propensity scoring. n=2311. B.) Demographics for control and moderate TBI requiring mechanical ventilation matched with propensity scoring. n=694. C.) Demographics for control and severe TBI requiring mechanical ventilation matched with propensity scoring. n=2524

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=2311)</th>
<th>Mild TBI (n=2311)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56.1 ± 20.3</td>
<td>57.0 ± 20.6</td>
<td>0.11</td>
</tr>
<tr>
<td>Sex, male</td>
<td>72.48%</td>
<td>69.67%</td>
<td>0.035</td>
</tr>
<tr>
<td>ISS</td>
<td>17.9 ± 8.3</td>
<td>17.5 ± 8.4</td>
<td>0.32</td>
</tr>
<tr>
<td>Heart rate</td>
<td>89.9 ± 23.3</td>
<td>90.0 ± 21.6</td>
<td>0.92</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>19.4 ± 6.4</td>
<td>19.2 ± 4.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>143.7 ± 29.5</td>
<td>144.5 ± 29.4</td>
<td>0.32</td>
</tr>
<tr>
<td>Temperature</td>
<td>36.3 ± 1.5</td>
<td>36.3 ± 2.1</td>
<td>0.28</td>
</tr>
</tbody>
</table>

| Ventilator days           | 3 (2-9)          | 3 (1-8)           | 0.007   |
| ICU days                  | 6 (3-12)         | 6 (3-12)          | 0.68    |
| Hospital length of stay   | 11 (6-20)        | 11 (6-20)         | 0.81    |

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=694)</th>
<th>Moderate TBI (n=694)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>49.6 ± 19.8</td>
<td>50.3 ± 20.3</td>
<td>0.54</td>
</tr>
<tr>
<td>Sex, male</td>
<td>71.76%</td>
<td>70.03%</td>
<td>0.48</td>
</tr>
<tr>
<td>ISS</td>
<td>17.9 ± 8.5</td>
<td>17.6 ± 8.9</td>
<td>0.53</td>
</tr>
<tr>
<td>Heart rate</td>
<td>92.9 ± 24.9</td>
<td>93.8 ± 24.0</td>
<td>0.48</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>18.4 ± 7.5</td>
<td>18.7 ± 6.3</td>
<td>0.39</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>142.0 ± 30.3</td>
<td>146.9 ± 33.4</td>
<td>0.34</td>
</tr>
<tr>
<td>Temperature</td>
<td>36.2 ± 1.6</td>
<td>36.1 ± 2.8</td>
<td>0.51</td>
</tr>
</tbody>
</table>

| Ventilator days           | 4 (2-10)         | 3 (1-8)              | 0.017   |
| ICU days                  | 6 (3-12)         | 5 (3-11)             | 0.1     |
| Hospital length of stay   | 11 (6-20)        | 10 (5-19)            | 0.014   |

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=2524)</th>
<th>Severe TBI (n=2524)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>45.0 ± 19.4</td>
<td>45.4 ± 19.6</td>
<td>0.55</td>
</tr>
<tr>
<td>Sex, male</td>
<td>75.40%</td>
<td>74.37%</td>
<td>0.4</td>
</tr>
<tr>
<td>ISS</td>
<td>18.2 ± 9.3</td>
<td>18.2 ± 9.3</td>
<td>0.87</td>
</tr>
<tr>
<td>Heart rate</td>
<td>96.0 ± 24.8</td>
<td>95.4 ± 24.2</td>
<td>0.43</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>12.5 ± 8.8</td>
<td>12.2 ± 9.3</td>
<td>0.16</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>143.4 ± 32.2</td>
<td>143.8 ± 31.4</td>
<td>0.87</td>
</tr>
<tr>
<td>Temperature</td>
<td>35.1 ± 2.6</td>
<td>36.1 ± 2.7</td>
<td>0.73</td>
</tr>
</tbody>
</table>

| Ventilator days           | 3 (1-9)          | 3 (2-8)             | < 0.0001 |
| ICU days                  | 5 (3-12)         | 5 (3-11)            | 0.061    |
| Hospital length of stay   | 11 (6-20)        | 10 (5-18)           | 0.047    |
Analysis of pneumonia incidence rates in matched cohorts of ventilated patients shows that those patients with TBI had significantly lower rates of pneumonia than their blunt trauma controls (Figure 2). Pneumonia rates in each cohort of TBI patients were reduced by approximately 20% compared to their matched controls.

![Graph showing pneumonia rates](image)

Figure 2. Propensity scoring was used to match patients in the control group to the mild, moderate, and severe TBI group. The rate of pneumonia for each matched group was then calculated. Mild TBI vs. Control n=2311 in each group. Control 20.0%, Mild TBI 15.8%. Moderate TBI vs. Control n=694. Control 21.3%, Moderate TBI 16.6%. Severe TBI vs Control n=2524. Control 20.4%, Severe TBI 15.6% *p<0.05

Similar results were found when non-ventilated patients were matched using propensity scoring. There were no differences in injury severity score or respiratory rate, but due to the robust sample sizes, all other parameters reached statistical significance. Patients with mild TBI had a significantly lower incidence of pneumonia than controls (1.3% vs. 0.8%, TBI vs control, p <0.0001).
Moderate TBI Confers a Survival Advantage in Mice with Pneumonia

To explore the biological mechanism by which TBI alters the susceptibility to pneumonia, we employed a murine model of moderate TBI with intrapulmonary infection with *P. aeruginosa*. TBI mice demonstrated a reduced mortality rate following the induction of post-injury pneumonia. All of the TBI mice survived the LD$_{50}$ dose of *Pseudomonas* while 40% of sham animals died by 7 days (Figure 3A). To assess whether this effect would be present with a greater infectious load, a second experiment was performed using 5x10$^7$ CFU *P. aeruginosa* (Figure 3B). Again, mice with TBI had significantly improved survival over sham injury animals. Whole body plethysmography showed that TBI mice were able to maintain a normal tidal volume and recovered their time of inspiration faster than sham injury mice with pneumonia (Figure 4).
Figure 3: TBI mice are more resistant to infectious challenge than sham-injury mice. A, day 2 sham and TBI mice received intratracheal instillation of $1 \times 10^7$ CFU of *P. aeruginosa*. B, day 2 sham and TBI mice received intratracheal instillation of $5 \times 10^7$ CFU of *P. aeruginosa*. Each group was followed for survival for 7 days. N=10 in each group. *p* value determined with Log Rank test.
Figure 4: TBI mice recover respiratory parameters faster than sham injury with pneumonia. WBP data showing maintenance of tidal volume and recovery of time inspiration in day2 TBI mice with pneumonia compared to day 2 sham injury animals with pneumonia. *p<0.05 TBI pneumonia vs sham + pneumonia by Bonferroni post-hoc test.

**Moderate TBI Increases Pulmonary Bacterial Clearance**

As shown in Figure 5, TBI mice had significant reductions in bacterial counts in both BAL fluids and lung homogenates. In addition, TBI mice had significantly increased cell recruitment to the lungs 4 hours after the induction of pneumonia (Figure 6A). Differential counts of representative cytospins prepared from the BAL fluid showed significant increases in both neutrophils and macrophages (Figure 6B). Interestingly, the increased cell recruitment was not associated with elevated local or systemic pro-inflammatory cytokine and chemokine production. In fact, TBI mice had significantly lower levels of the cytokine IL-6 and the
chemokines MIP-2 and KC in both BAL fluids and plasma 4 hours after infection (Figure 7). This differs from burn and blunt trauma models which are accompanied by increased pro-inflammatory cytokines with increased macrophages and neutrophils after bacterial pneumonia.

Figure 5: Traumatic brain injury mice have improved bacterial clearance after IT P. aeruginosa challenge. Bacterial counts recovered from bronchoalveolar lavage and whole-lung homogenates from day 2 sham injured and TBI mice 4 hours after intratracheal administration of $1 \times 10^7$ CFU of P. aeruginosa. *p<0.05
Figure 6: TBI mice recruit more cells to the alveoli compared to sham injury mice 4 hours after IT administration of *P. aeruginosa*. Day 2 sham-injured and TBI mice received IT inoculation of $1 \times 10^7$ CFU of *P. aeruginosa* and bronchoalveolar lavage was performed 4 hours later. Bronchoalveolar cell counts measured by Beckman-Coulter particle counter and differential count from representative cytospins. *p<0.05
Figure 7: Traumatic brain injury mice produce lower levels of proinflammatory cytokines and chemokines in the lung and plasma in response to *P. aeruginosa* pneumonia. Bronchoalveolar lavage and plasma levels of proinflammatory cytokines from day 2 sham-injured and TBI mice 4 hours after i.t. administration of $1 \times 10^7$ CFU of *P. aeruginosa* as measured by ELISA. N=10 in each group. *p<0.05
Discussion

To the best of our knowledge, the current study is the first to use propensity score matching to directly evaluate the risk of developing post-injury pneumonia in TBI patients. Using the NTDB, these results are powered by a large population of trauma patients from multiple institutions. Furthermore, our study is unique in that we evaluated mild traumatic brain injury patients as well as severe TBI. Previous studies have shown high rates of pneumonia, as high as 60%, in populations with brain injury (14, 42, 51, 125, 132, 149). However, these studies focus only on severe TBI patients who require prolonged courses of mechanical ventilation. Our study reveals the contrary, showing significantly reduced rates of pneumonia in all groups of brain-injured patients when matched to non-brain-injured blunt trauma patients.

In our study, patients were comparatively matched to appropriate injury severity, length of hospital stay, age, race, vital signs, and days ventilated. Many studies compare a subset of severe TBI patients to non-trauma patients or the general population of trauma patients (23, 117). This limits the validity of these studies due to the differences in mechanism of injury, severity of injury, and time mechanically ventilated. The groups in our study focused only on trauma patients with or without head injury induced by a blunt mechanism. Blunt trauma was chosen because it is the most common mechanism of TBI. This mechanism
of trauma is known to cause a robust inflammatory response and changes in immune response (103, 107, 121, 126).

The use of a national database has inherent limitations, including the inability to capture all desired data points and control for all possible patient variables. One such variable is the unknown history of tracheostomy, which has been shown to reduce the risk of subsequent ventilator-associated pneumonia if performed early in the hospital course (4, 17, 49, 68). Another limitation is the number of institutions involved in data input into the NTDB. This results in a robust sample population, however the inconsistency in data entry and limited definitions for certain entries can result in inaccuracies. Further, pneumonia is not a clearly defined diagnosis but is coded as a complication, therefore the criteria to establish and record the diagnosis of pneumonia may vary from one institution to another, using definitions by radiographic, BAL, or clinical score findings. However, this dataset is strengthened by its robust numbers and the use of propensity scoring to appropriately match patient populations.

Since our clinical results are contrary to current dogma, we examined an animal model of head injury with subsequent pneumonia to determine the causes of reduced risk of pneumonia. To date, there have not been any comprehensive studies of bacterial pneumonia after TBI in animal models. Our studies demonstrate an increased resistance to P. aeruginosa pneumonia following traumatic brain injury. We attribute this enhanced resistance to increased
neutrophil recruitment to the airways resulting in faster bacterial clearance that allowed better respiratory function and improved survival. The neutrophil responses against bacteria in the lungs largely determine the outcome of infection, and insufficient neutrophil recruitment can lead to an inability to clear the infection (27, 28). Similar studies performed after burn and blunt trauma showed enhanced resistance to bacterial infection after injury due to increased neutrophil recruitment and bacterial clearance but are accompanied by significant increases in pro-inflammatory cytokines due to increased toll-like receptor signaling in macrophages and neutrophils (60, 84). Interestingly, there was a significant decrease in pro-inflammatory cytokine production during the early phase in our model. Because our model has increased neutrophil recruitment and reduced pro-inflammatory response, the observed resistance to infection is likely due to a different mechanism specific to TBI. Much of the basic science literature on immune function after TBI demonstrates serious immune depression (21, 40). These studies, however, focus mainly on the adaptive immune system. A proposed mechanism of the observed immune depression was catecholamine and glucocorticoid stimulated release of IL-10 from circulating monocytes inducing an anti-inflammatory state in peripheral immune cells (138). Although we did not detect any increase in IL-10 levels in the plasma or airways, it is possible that this brain injury model is not severe enough to produce this level of immune suppression.
Pulmonary inflammation after TBI has been described in both the clinical and basic science literature as neurogenic pulmonary edema (NPE) (10, 36, 76). Multiple mechanisms have been implicated in the pathophysiology of post-TBI NPE including catecholamine storm, hydrostatic pressure increases, leukotriene B4, and substance P (65, 108, 124). Further investigation into the mechanism of the increased neutrophil recruitment will take into account these possible contributing factors.

Increased vagus nerve signaling after TBI has been shown to initiate a systemic anti-inflammatory response (71, 73). While our study demonstrates improved neutrophil recruitment and bacterial killing, the vagal anti-inflammatory pathway could account for the decreased levels of pro-inflammatory cytokines despite the presence of more inflammatory cells in the lung. The afferent and efferent pathways of the vagus nerve reduce the inflammatory process in stroke and brain injury models (73, 74). Efferent stimulation of the vagus nerve results in a drastic anti-inflammatory process (16, 134). This could serve to moderate the immune response resulting in less collateral damage in the lung parenchyma and improved survival.
Chapter 4: Substance P is Responsible for Increased Neutrophil Recruitment in Response to Bacterial Pneumonia Following Traumatic Brain Injury

Introduction

The immune system can be “primed” by injury to respond to subsequent insults in an exaggerated or more effective manner. Priming has been demonstrated in a variety of animal models of traumatic injury with subsequent “second-hit” infectious or inflammatory challenges (120). Priming classically results in increased recruitment of activated neutrophils and inflammatory cytokine production in response to the second-hit (104). Toll-like receptor 4 (TLR4), an important sensor of bacterial lipopolysaccharide (LPS), is upregulated on macrophages after injury and has been shown to mediate much of the priming response in burn and contusion injury models (60, 84, 91). TLR4 upregulation results in more receptors being activated by LPS and thus increased activation of NF-κB signaling downstream. This increases macrophage proinflammatory cytokine production and subsequent neutrophil activation in response to lower levels of LPS than would be required to elicit the same response in an unprimed animal (91).

Another important priming mediator is the neuropeptide substance P. Released by sensory neurons in response to injury or noxious stimuli, substance P binds the neurokinin-1 receptor (NK-1R) on vascular endothelium, macrophages,
neutrophils, and mast cells (98). NK-1R activation causes vasodilation, increased vascular permeability and adhesion molecule expression, and increased responsiveness to chemokine signaling (92, 106, 129). Importantly, substance P release alone does not induce an inflammatory reaction but requires another inflammatory input like bacterial products or tissue damage to activate cell recruitment and increase vascular permeability (20). Substance P priming is mediated through multiple signaling mechanisms but the best characterized involve potentiating of chemokine receptor signals. In one arm, NK-1R activation increases NF-kB mediated expression of CXC chemokine receptors on the surfaces of neutrophils and increases inflammatory cytokine production in macrophages (106, 129). In an NF-kB-independent mechanism, substance P acts through the NK-1R to enhance intracellular calcium release in response to chemokine binding in neutrophils (32, 106). Interestingly, this pathway does not change neutrophil activity or recruitment in the absence of chemokine stimuli which may explain the necessity for a second inflammatory stimulus in substance P priming (20, 32).

Our previous studies in a mouse model of moderate traumatic brain injury (TBI) and *Pseudomonas aeruginosa* pneumonia found brain injury had a protective effect characterized by increased neutrophil recruitment and bacterial clearance in the lung resulting in improved survival compared to sham injury animals. Through propensity score matching, we also found human TBI patients were less likely to develop pneumonia than matched non-neurological blunt trauma
patients. These findings were contradictory to the prior clinical and basic science literature that described profound immune suppression and susceptibility to infection following TBI (55, 86). While TBI increased neutrophil recruitment to the lungs, we found significantly decreased levels of pro-inflammatory cytokine production both in the blood and lung that differs from the increases seen in other injury-primed responses (104). This enhanced recruitment with decreased cytokine production may stem from the multiple neuroimmune pathways activated by TBI that may result in priming through a mechanism distinct from other injuries. In this study we characterize the priming effect of TBI on the immune response to bacterial pneumonia and identify substance P signaling through NK-1R as the mechanism of increased neutrophil recruitment and bacterial killing observed in TBI mice with pneumonia.
Methods

TBI and Pneumonia Models

As previously described in Chapter 3.

Lung sampling and Wet/Dry ratio

BAL and histology samples were obtained and analyzed as described in chapter 2 with the addition of wet/dry weight ratio. The right lower lung from day 2 TBI or sham injury animals 4 hours was collected and weighed (wet weight). After 24 hours of drying in an incubator at 65°C, the weight was determined again (dry weight) and the wet/dry weight ratio was calculated.

Blood collection

Blood was collected by facial vein bleed for differential count and cytokine analysis as described in chapter 2 from day 2 TBI or sham injury mice 4, 24, 48, and 96 hours after bacterial challenge with $1 \times 10^7$ CFU of *P. aeruginosa*. Arterial oxygen saturation was determined as described in chapter 2.

*Ex-vivo* stimulation of alveolar macrophages

48 hours after TBI or sham injury, mice were sacrificed and alveolar macrophages were collected by bronchoalveolar lavage. Cells were counted by Coulter Counter and macrophages were isolated by culturing 250,000 alveolar cells for 90 min in a 24-well culture plate. Cell cultures were washed 3 times with
sterile PBS to remove non-adherent cells. Isolated, adherent macrophages were then stimulated in RPMI 1640 media supplemented 10% fetal calf serum for 18 hours with media alone, LPS at 10 ng/mL or 100 ng/mL, or live *Pseudomonas aeruginosa* at a ratio 1:100 cells/bacteria. Supernatants were collected and stored at -80°C for future cytokine analysis.

**KC chemokine recruitment and CXCR2 analysis**

48 hours after TBI or sham injury, mice received 500 ng of purified KC chemokine (Peprotech) or normal saline by intratracheal administration. 4 hours later they were sacrificed and cells were collected by BAL for differential count and cytoospin preparation as previously described in chapter 2.

CXCR2 was measured in blood neutrophils collected by cardiac puncture from day 2 TBI and sham injury mice. CXCR2 levels were measured using Per-CP labeled anti-CXCR2 antibodies in GR1+ CD11b+ cells and expressed as geometric mean of fluorescence intensity.

**Neurokinin-1 Receptor antagonist/agonist protocol**

Mice were treated with intraperitoneal injections of 25 mg/kg of the NK-1R antagonist CJ-12,255 (Pfizer) or 1 mg/kg of the NK-1R agonist GR73632 (Tocris Biosciences) in normal saline every 12 hours with the first dose administered 1 hour prior to TBI or sham injury and the last dose one hour prior to IT administration of 5×10⁷ or 1×10⁷ CFU of *P. aeruginosa* 48 hours after injury (29,
Mice receiving $5 \times 10^7$ CFUs were followed one week for survival and those receiving $1 \times 10^7$ CFU were sacrificed one hour later for BAL collection of cells and bacteria as previously described in chapter 3.

**Statistical Analysis**

Column analysis was performed using two-tailed T-tests. Serial measures were analyzed using 2-way ANOVA with Bonferroni post-hoc test to determine significance between groups.
Results

Hematological analysis

Our prior studies found TBI resulted in improved neutrophil recruitment to the lung compared to sham injury animals at 4 hours after intratracheal administration of *Pseudomonas aeruginosa*. To determine whether this was due to more rapid mobilization of white blood cells into the circulation we performed blood count and cytokine analysis on facial vein blood samples obtained 4, 12, 24, 48, and 96 hours after bacterial challenge from day 2 TBI or sham injury animals (Figure 1). We found a significant increase in white blood cells 48 hours after infection in TBI mice composed primarily of neutrophils. However, at 4 hours after infection TBI and sham injury animals had similar white blood cell counts and differential counts. TBI mice also had markedly lower plasma levels of IL-6 and MIP-2 up to 24 hours after infection compared to sham injury animals. This indicated that the increase in neutrophils recruited to the lung at 4 hours in TBI mice with pneumonia was not the result of more neutrophils in the circulation but more efficient recruitment out of the circulation to the site of infection without increased pro-inflammatory cytokine production systemically.
Figure 1: TBI mice have more circulating neutrophils 48 hours after bacterial challenge but not at 4 hours. Hemavet counts of blood neutrophils, lymphocytes, and total white blood cells in facial vein blood samples from day 2 TBI and sham injury mice 4, 24, 48, and 96 hours after IT administration of $1 \times 10^7$ CFU of *P. aeruginosa*. *p<0.05 TBI vs sham by Bonferroni post-hoc test.

**Lung Injury**

Neutrophil recruitment is critical to the clearance of bacterial infection in the lungs but, as they accumulate in the pulmonary vasculature, they can also cause significant tissue damage that can progress to acute lung injury characterized by protein-rich edema fluid in the alveoli, atelectasis, and compromised gas exchange (38, 52, 82). As TBI increases the number of neutrophils recruited to the lung in response to bacterial pneumonia, there was the potential for increased damage to the lung tissue. We therefore analyzed similar parameters to those in chapter 2 to analyze the effect of TBI on the development of lung injury.
injury in bacterial pneumonia. We collected arterial blood, lungs, and BAL fluid from day 2 TBI and sham injury mice 4 hours after IT administration of $1 \times 10^7$ CFU of *P. aeruginosa* to detect the presence of protein-rich edema fluid, gross tissue damage, atelectasis, and impaired gas exchange. Histological sections of lungs from the TBI mice with pneumonia demonstrated significantly less atelectasis, alveolar infiltration and consolidation, and tissue disruption compared to those from sham injury animals with pneumonia (Figure 2A). Gas exchange was not significantly different as shown by arterial oxygen saturation from ABG (Figure 2B). BAL fluid from both groups showed similar protein content indicating that despite the presence of more neutrophils in the airways of TBI mice with pneumonia, there was not an increase in the protein-rich exudate that usually accompanies neutrophil accumulation in the lungs (Figure 2C). However, wet/dry weight ratios of lung tissue from TBI mice with pneumonia were significantly increased compared to sham injury animals with pneumonia indicating an increase in total lung water reflecting increased vascular permeability (Figure 2D). This indicates fluid collection in the interstitium but not the airspaces in TBI mice with pneumonia (83). As gas exchange and protein content of the BAL fluid were unchanged between groups, TBI mice have reduced lung damage by histology but no difference in lung injury.
Figure 2: TBI results in decreased lung damage and increased vascular permeability from bacterial pneumonia. Representative H&E stained sections of day 2 sham injury (A) and TBI (B) mouse lungs 4 hours after IT administration of $1 \times 10^7$ CFU of *P. aeruginosa*. (C) Arterial oxygen saturations, (D) Total BAL protein content, and (E) Wet/Dry lung weight ratios for TBI and sham injury mice 4 hours after IT administration of $1 \times 10^7$ CFU of *P. aeruginosa*. *p<0.05
Ex-Vivo LPS and Bacterial Stimulation of Alveolar Macrophages

To determine whether alveolar macrophages are primed by TBI to respond to subsequent bacterial pneumonia we collected alveolar cells from day 2 TBI and sham injury animals by BAL. We isolated macrophages by adhering them to tissue culture plates and stimulated them in culture media with different concentrations of purified LPS or live bacteria for 18 hours and measured the concentration of IL-6, MIP-2, KC, and TNF-α in the media supernatant. We found no significant differences in the levels of these cytokines and chemokines produced in response to LPS (Figure 3) or bacterial stimulus (Figure 4). As our prior in-vivo studies had found TBI mice produced lower levels of inflammatory cytokines in response to bacteria in the lung, these ex-vivo stimulation studies suggest an interaction in the lung microenvironment is responsible for decreased cytokine production by alveolar macrophages in TBI mice. Once removed from the lung, cytokine production in response to bacterial stimulus is no longer inhibited in alveolar macrophages from TBI mice.
Figure 3: Traumatic brain injury does not affect the response to LPS in alveolar macrophages.

Levels of IL-6, TNF-α, MIP-2, and KC in culture supernatants of alveolar macrophages isolated from day 2 TBI or sham injury mice stimulated for 18 hours with media alone, 10 ng/mL, or 100 ng/mL of LPS.
Figure 4: TBI does not affect cytokine production by alveolar macrophages stimulated ex-vivo with live bacteria. Levels of IL-6, TNF-α, MIP-2, and KC in culture supernatants of alveolar macrophages isolated from day 2 TBI or sham injury mice stimulated for 18 hours with live *P. aeruginosa*.

**KC Chemokine Recruitment**

CXC chemokines are the predominant mediators of neutrophil chemotaxis. Our previous work found TBI mice were able to recruit more neutrophils to the lung in response to bacterial pneumonia despite lower levels of the CXC chemokines KC and MIP-2. This suggested TBI could have a sensitizing effect on neutrophil responsiveness to chemokine stimulus. To test this hypothesis we administered...
purified KC chemokine or normal saline directly into the airways of day 2 TBI or sham injury mice. These mice were sacrificed four hours later at the peak of neutrophil recruitment and alveolar cells were collected by BAL for total and differential cell counts (Figure 5). In response to IT administration of KC, TBI mice recruited significantly higher numbers of cells to the airways composed primarily of neutrophils. This indicated that TBI results in increased neutrophil sensitivity to KC.

Figure 5: TBI increases KC-mediated neutrophil recruitment to the lung. Representative cytospins of BAL fluid from day 2 sham injury (A) or TBI mice (B) 4 hours after IT administration of purified KC. C) BAL cell counts from day 2 TBI and sham injury animals 4 hours after IT administration of normal saline or purified KC chemokine. *p<0.05

To determine whether this enhanced responsiveness was due to increased expression of the receptor for KC, CXCR2, we analyzed blood neutrophils from day 2 TBI and sham injury mice by flow cytometry. As shown in Figure 6, there was no significant difference in CXCR2 between TBI and sham injury animals indicating enhanced signaling without receptor upregulation.
Figure 6: TBI does not increase neutrophil surface expression of CXCR2. Flow cytometry gating (A) and quantification (B) of CXCR2 expression on blood neutrophils from day 2 TBI or sham injury animals.

**NK-1R Antagonist Treatment**

To investigate the role of substance P signaling through the neurokinin-1 receptor in the observed improvement in survival, increased neutrophil recruitment, and bacterial clearance in TBI mice with pneumonia we treated mice with the NK-1R antagonist CJ-12,255 or vehicle for 48 hours beginning one hour prior to TBI or sham injury with the final dose one hour prior to IT administration of $5 \times 10^7$ (for survival Figure 7) or $1 \times 10^7$ CFU (neutrophil recruitment and bacterial clearance Figure 8) of *P. aeruginosa*. CJ-12,255 treatment abolished the survival advantage in TBI mice with pneumonia and reduced neutrophil recruitment and bacterial clearance to levels similar to drug and vehicle treated sham injury animals.
Figure 7: NK-1R blockade abolishes the protective effect of TBI against bacterial pneumonia challenge. Day 2 sham and TBI mice treated with either vehicle or neurokinin-1 receptor antagonist CJ-12255 (Pfizer) were followed for survival after intratracheal administration of $5 \times 10^7$ CFU of *P. aeruginosa*. P-value between TBI and TBI + CJ-12255 treated mice. N=10 in each group. P value determined with Log Rank test.
Figure 8: NK-1R blockade decreases bacterial clearance and neutrophil recruitment in TBI mice but not sham mice. Cell counts and CFUs in BAL fluid from vehicle or CJ-12,255 treated day 2 sham and TBI mice 4 hours after intratracheal instillation of $1 \times 10^7$ CFU of *P. aeruginosa*. N=10 in each group *p<0.05
**NK-1R Agonist Treatment**

To further confirm the effect of NK-1R signaling on neutrophil recruitment and bacterial clearance we treated sham injury animals with the NK-1R agonist GR73632 following the same dosing schedule as the antagonist treatment group to mimic the sustained release of substance P following TBI (36, 76). NK-1R agonism increased neutrophil recruitment and bacterial killing to the levels observed in TBI mice with pneumonia (Figure 9). This did not result in lower levels of MIP-2 or KC in the BAL fluid at 4 hours after pneumonia indicating NK-1R stimulation is not responsible for the observed decreases in chemokine production seen in TBI mice with pneumonia (Figure 10). Taken together, these results indicate a pivotal role for substance P signaling through NK-1R in the enhanced pulmonary immune response to bacterial pneumonia following TBI.
Figure 9: NK-1R stimulation increases neutrophil recruitment to the lung and bacterial killing in sham injury animals. A.) Cell counts and B.) CFUs in BAL fluid from day 2 sham injury mice, day 2 TBI mice, and GR73632-treated day 2 sham mice 4 hours after intratracheal instillation of $1\times10^7$CFU of *P. aeruginosa*. 
Figure 10: NK-1R agonism does not affect chemokine production in the lung in sham injury animals with pneumonia. MIP-2 and KC in BAL fluid from day 2 sham injury mice, day 2 TBI mice, and GR73632-treated day 2 sham mice 4 hours after intratracheal instillation of $1 \times 10^7$CFU of *P. aeruginosa*. 
Discussion

The basic science and clinical literature surrounding TBI and immune function has long held that TBI causes profound immune suppression resulting in increased susceptibility to infection and higher rates of pneumonia in TBI patients compared to non-neurotrauma patients (55, 86, 140). In chapter 3 we showed in both human patients and an animal model of moderate TBI and bacterial pneumonia that prior TBI exerts a protective effect reducing the susceptibility to infection when compared to non-neurotrauma patients and sham injury animals respectively. In this study we investigated the mechanism behind the observed improvement in survival over sham injury animals following bacterial challenge in our animal model of moderate TBI and bacterial pneumonia. We found that substance P signaling through NK-1R plays a critical role in TBI-induced neutrophil priming that leads to increased recruitment to the lung and bacterial killing.

In models of non-neurological injury, a priming effect has been demonstrated that results in a more robust immune response to a second insult like bacterial infection (7, 84, 104). A common pattern is observed in these studies after injury consisting of increased circulating neutrophils, increased pro-inflammatory cytokine production by primed macrophages, and improved bacterial killing capacity (60, 84). In our model of moderate TBI followed by bacterial pneumonia while we did observe increased neutrophil recruitment, and increased bacterial
killing in TBI mice with pneumonia, we found a significant decrease in inflammatory cytokine production compared to sham injury mice with pneumonia. Through *ex-vivo* alveolar macrophage stimulation studies we found that macrophages isolated from TBI mice are only suppressed in cytokine production within the lung microenvironment. Outside of the lung, they respond to bacterial or TLR4 stimulation to the same level as those from sham injury animals. A possible explanation for this finding could be acetylcholine stimulation from TBI-induced increases in vagal tone (73). Acetylcholine suppresses cytokine production by alveolar macrophages through the α7 nicotinic receptor that mediates an inhibitory effect on NF-KB through c-fos activation (105, 134). As it is rapidly degraded, continuous stimulation with acetylcholine is required to sustain this effect (119). By removing the macrophages from the lung, and thus cholinergic stimulation, we may have relieved this inhibition allowing a return to cytokine production at similar levels to cells from sham injury animals.

In this study we showed that this resulted in less lung tissue injury despite the presence of more neutrophils in the lung. In acute lung injury, a build-up of neutrophils in the pulmonary vasculature has been shown to result in premature neutrophil degranulation and markedly increased lung injury with progression into acute respiratory distress (1, 82). In our study we found increased pulmonary vascular permeability in our TBI mice with pneumonia over sham animals that may allow for more efficient egress of neutrophils from the vasculature into the airspaces that may prevent premature degranulation in the vasculature and result
in less tissue damage. Substance P release in the lungs has been demonstrated after TBI in association with the development of neurogenic pulmonary edema (36, 76, 77). Its release is triggered by increased efferent vagal signaling following TBI that cross-activates afferent sensory terminals to release substance P (77). Substance P is a potent vasodilator that may also explain the increased vascular permeability seen in our TBI mice with pneumonia.

Through administration of purified KC chemokine directly into the airways of day 2 TBI and sham injury mice we demonstrated that TBI increases neutrophil responsiveness to chemokine signals in a mechanism independent of increased CXCR2 expression. As NK-1R blockade abolished the increase in neutrophil recruitment in response to pneumonia observed in TBI mice and NK-1R stimulation recapitulated the increase in sham injury mice, substance P release following TBI is most likely responsible for this effect. NK-1R stimulation has been shown to increase sensitivity to chemokine signals in two distinct ways. The first is increasing CXCR2 expression on neutrophils through NF-KB activation (129). We found no difference in CXCR2 expression between TBI and sham injury animals and, coupled with our other findings, this suggests that TBI has an inhibitory effect on NF-KB, likely through simultaneous vagal cholinergic stimulation, that may also account for the observed decreases in inflammatory cytokine production (72, 73). Substance P signaling can also increase neutrophil sensitivity to chemokine stimulation through its NF-KB-independent amplification of chemokine receptor calcium signaling (32, 54). This effect enhances sensitivity
to lower chemokine levels without increasing the amount of chemokine receptors expressed on neutrophils.

The findings in this study identify substance P and NK-1R as potential therapeutic targets to prevent or treat bacterial pneumonia. Further investigation of the anti-inflammatory arm of this response is necessary to elucidate the entire mechanism of this observed effect of TBI on the immune response to bacterial pneumonia. Clinical studies using agents that increase substance P levels have been shown to reduce the incidence of aspiration pneumonia in stroke patients (97). This study offers evidence that NK-1R agonism has potential for use in improving treatment modalities for nosocomial pneumonias like *P. aeruginosa*. 
Chapter 5: Discussion

Traumatic brain injury is an important and complex clinical problem with detrimental effects beyond the nervous system (21). It is therefore important to understand the interactions between the injured brain and the peripheral organ systems it controls. Of particular concern in the acute management of the hospitalized TBI patient are the consequences of TBI on the respiratory system that, beyond the obvious nervous control of respiratory drive, may be compromised by injury from neurogenic pulmonary edema and bacterial pneumonia (6, 55, 148). The findings detailed in the preceding studies are contradictory to the current basic science and clinical literature surrounding the effects of traumatic brain injury on the immune system and the lungs.

The prevailing wisdom held that, through neuroimmune interactions, TBI resulted in profound immune suppression that left TBI patients impaired in their defense against pneumonia. In a 1988 landmark study, Helling and colleagues found 50% of 82 head injured patients in their intensive care unit (ICU) developed at least one nosocomial infection during their hospitalization (55). Pneumonia represented 80% of these infections and the severity of head injury was correlated to the risk of developing pneumonia. Studies by Hsieh and Rodriguez found head injury to be an independent risk factor for the development of pneumonia in surgical ICU patients (61, 118). Piek and colleagues expanded on this study to analyze records from 734 patients with an admission Glasgow coma
score ≤8 from the Nation Institute of Neurological Disorders and Stroke Traumatic Coma Data Bank (109). They found pneumonia occurred in 40.6% of all severe brain injury patients. Berrouane and co-workers compared 565 patients from their ICU with and without neurotrauma and found mechanically ventilated TBI patients had almost double the risk of pneumonia by day 3 of their hospitalization compared to ventilated non-neurotrauma patients (20/1000 ventilator days vs 10.2/1000 ventilator days) (11). With so many studies demonstrating an increased risk of pneumonia in TBI patients, neuroimmune interaction was implicated in an immunosuppressive effect of TBI. Woiciechowsky and co-workers looked for a mechanism to explain these findings and used both neurosurgery patient blood samples and a rat model of TBI to demonstrate a catecholamine-induced release of IL-10 from monocytes following injury that he suggested was responsible for the observed increase in infectious complications (138). Kox, meanwhile, correlated EKG evidence of increased vagus nerve signaling to decreased inflammatory cytokine production and innate immune dysfunction in TBI and intracranial hemorrhage patients (73). With all of these studies indicating immune suppression after TBI, we expected to find an increase in susceptibility to bacterial pneumonia in our TBI model. Instead, we have demonstrated in both human patients and an animal model a protective effect of TBI on the development and resolution of bacterial pneumonia. Our findings, however, involve many of the neuroimmune interactions suggested by the previous literature to be immunosuppressive and injurious. Through
discussion of our findings in the context of the primary literature, we can integrate these concepts to arrive at a better understanding of TBI and the pulmonary immune response to infection.

In our initial studies, we developed a mouse model of moderate TBI produced by impact acceleration. This was similar in severity, mechanism, and pattern of injury to moderate TBI seen in human patients. Our model also circumvented the need for skin and bone incisions found in other models of TBI. This distinction is important as previous studies that had demonstrated lung injury from TBI used models of TBI requiring skin incision and craniotomy (80, 138). These have been shown to induce a significant inflammatory response even without brain injury that could cause lung injury through the release of damage-associated molecular patterns (DAMPs) (22, 24, 75, 146). Our model avoids these confounding factors while still producing relevant brain and overlying soft tissue injury seen in frontal impacts in human patients and allows for study of the immune consequences of isolated brain injury. Our avoidance of invasive manipulation may contribute to the absence of lung injury we found in our mice with TBI.

Another possible factor in the absence of lung injury in our TBI mice could be that our brain injury was not severe enough to cause the profound hemodynamic changes associated with the development of neurogenic pulmonary edema after TBI (47). Likely due to a catecholamine release shortly after injury, pulmonary vasoconstriction in NPE results in significant vascular endothelial injury that
serves to initiate a subsequent inflammatory response (124). We did not find evidence of this response in our TBI mice nor did we find elevations in IL-10 that have been shown to follow a catecholamine surge after injury (138). Through our later studies with NK-1R antagonism, we suspect the neuropeptide substance P was released in our TBI mice and substance P release has been associated with the inflammation and lung injury in NPE (76, 108). While we did not measure substance P directly, the NK-1R blockade and activation studies suggest its presence mediates the increased vascular permeability and neutrophil recruitment. However, without significant vascular endothelial injury from catecholamine release, the release of substance P could not induce an inflammatory response as it requires a second inflammatory input to recruit neutrophils (20). Perhaps a more severe injury would have resulted in catecholamine release and subsequent lung injury but we ran the risk of causing skull fracture and excess mortality if we increased the force of impact in our model.

In chapter 3, we examined clinical data from the National Trauma Database from over 200,000 trauma patients and compared the rates of bacterial pneumonia among those with TBI and those with blunt non-neurological injuries. We found, as had been shown in other clinical studies, that the TBI group had a significantly higher incidence of pneumonia than non-neurotrauma patients (11, 55, 109). This comparison, however, ignored differences between the patient groups that may also have contributed to the development of pneumonia. We were comparing two
groups of patients as if the only variable between them were TBI or non-neurological injury. In fact these two groups had significant difference in the severity of their injuries, admission vital signs, age, sex, and the amount of time spent in the hospital and intensive care units. Each of these variables could individually influence whether a patient would develop pneumonia during a hospitalization and could obscure the actual effect of TBI. Rodriguez showed significant influence of injury severity, mechanism of injury, and admission vital signs on the duration of hospitalization, mechanical ventilation, and most importantly incidence of hospital acquired pneumonia (118). We therefore matched our groups on the aforementioned demographic and clinical variables, excluding outliers until we found no significant difference between the groups in each category. With the confounding variables removed, we found that TBI patients had lower rates of pneumonia. The clinical studies by Helling, Hsieh, and Berrouane all used small patient groups within their respective hospitals to demonstrate increases in pneumonia among TBI patients (11, 55, 61). These small cohorts would not allow for the propensity score matching we performed and could result in biased findings.

Of particular interest to us was matching based on injury severity score (ISS). In an injured patient, the severity of injury is scored in different body regions on a scale from 1-6 with 1 being minor injury and 6 being unsurvivable. The sum of the squared scores of the three most injured body regions is the ISS (90). In matching patients solely on the presence or absence of brain injury, other clinical
studies did not take into account the severity of injury as a factor that predisposes to the development of secondary infection (11, 55, 109). A more severe injury, neurological or non-neurological alike, results in longer hospital and ICU stays, longer periods of mechanical ventilation, and thus increased rates of hospital-acquired infection (41, 100, 118). Studies like those performed by Berrouane and Rodriguez comparing brain injury of different severity to blunt injury of indeterminate severity are simply not comparing similar patients (11, 118). Even the database studies performed by Piek only organized patients by severity of brain injury and did not take into account the ISS of the patients (109). Without more complete characterization of the patient injury, an accurate representation of the risk of pneumonia is not possible. By matching patients using ISS in addition to demographic and vital sign data, we compared patients that differed only in the nature of their injury (neurological versus non-neurological) allowing for a more accurate demonstration of the effect of TBI on the development of pneumonia.

We also demonstrated a protective effect of TBI in our animal model of TBI and P. aeruginosa pneumonia. Our TBI mice had improved survival over sham injury animals following bacterial challenge in both groups. This was mediated by an increased neutrophil recruitment to the lungs in the acute phase (4 hours) after bacterial challenge that resulted in increased bacterial clearance with decreased lung damage in TBI mice with pneumonia. The TBI mice also produced significantly lower levels of pro-inflammatory cytokines in response to bacterial
pneumonia compared to sham injury animals with pneumonia. Decreased cytokine production in response to bacterial challenge after TBI has been suggested in the literature to be demonstrative of immune suppression. Wolach and Kox have demonstrated decreased production of pro-inflammatory cytokines by monocytes collected from peripheral blood samples of TBI patients (73, 141). Quattrochi and colleagues demonstrated in several seminal publications on T-lymphocyte function after TBI a profound reduction in IFN-γ and IL-2 production by lymphocytes isolated from TBI patients (111, 112). Each of these groups concluded that these reductions must indicate increased susceptibility to infection. Our findings demonstrate that decreased cytokine production in TBI mice with pneumonia does not necessarily correspond to a deficit in immune function or increased susceptibility to infection. In our model, lower levels of inflammatory cytokines were associated with less lung damage and may represent a moderation of the inflammatory response that allows efficient bacterial clearance without collateral tissue damage. The observed decrease in inflammatory cytokine production in our study was not accompanied by an increase in production of the anti-inflammatory cytokine IL-10 as had been described in Woiciechowsky's studies of immune function after TBI (138, 140). Again, this may be due to the lesser severity of brain injury and the lack of a significant catecholamine release to induce the monocyte IL-10 production described in the literature (138).
Early neutrophil recruitment is important to the clearance of an infection and has been shown to improve survival in intra-abdominal sepsis (27). In the lungs, neutrophils are also critical to bacterial clearance but they can also cause significant lung injury (1, 38). Neutrophil-mediated lung injury is increased when neutrophils accumulate in the pulmonary vasculature before migrating into the airways (38). This results in early activation and degranulation that damages the surrounding tissues rather than the bacteria in the airways (52). In our model, we found that TBI mice with pneumonia had less lung damage than sham injury animals with pneumonia despite increased neutrophil recruitment. Our data suggests an increase in vascular permeability coupled with an enhanced responsiveness to chemokine signaling allows for more neutrophils to be recruited from the circulation with less accumulation in the lung vasculature. This prevents early degranulation and reduces tissue damage. Other studies of innate immune function have cited reduced neutrophil degranulation and reactive oxygen species release as evidence of immune suppression (141). Here again we demonstrate that this does not necessarily correspond to increased susceptibility to infection or an impaired immune response to bacterial infection.

An important consideration would be whether the enhanced neutrophil recruitment is the result of a neutrophil or vasculature-mediated effect. Increased vascular permeability could result in more neutrophils gaining access to the lung without the need for any increased signaling or sensitivity to chemokines. Chemotaxis assays using neutrophils isolated from the peripheral blood of TBI or
sham injury mice could demonstrate differences in neutrophil migration without the added confounder of vasculature present in our KC instillation studies. A more detailed analysis of lung vascular permeability by measuring BAL albumin, IgM, and Evan's blue extravasation could further determine how much permeability is actually changed. Furthermore, depletion of neutrophils using anti-Ly6G antibody treatment could demonstrate whether neutrophils are critical to mediating the enhanced bacterial clearance at all (143).

Substance P is a member of the tachykinins, a family of neuropeptides that are released in response to noxious stimuli by sensory nerve terminals (54). Substance P is a potent vasodilator and neutrophil chemoattractant that represents a mechanism for both the sensation of and response to painful or noxious injury. Through treatment of TBI animals with a neurokinin-1 receptor antagonist we found that substance P plays a critical role in the increased recruitment of neutrophils to the lung in response to bacterial pneumonia following TBI. Treated TBI mice with pneumonia died at a similar rate to sham injury mice with pneumonia. This decreased survival corresponded with a decrease in neutrophil recruitment to the lungs indicating substance P was responsible for the observed TBI-induced enhancement in neutrophil recruitment. The source of substance P in the TBI mice was likely the afferent sensory terminals of the vagus nerve. In a study of neurogenic pulmonary edema after TBI, Levasseur and colleagues found that blockade of substance P releasing neurons in the lung or vagotomy abolished the inflammatory arm of NPE (76).
Increased vagal efferent signaling has been demonstrated in TBI by Kox and colleagues and in their study they implicated as a potential cause of immune suppression in TBI patients as they correlated vagal activity to decreased cytokine production in cells of the innate immune system in TBI patients (71, 73). However, an important side-effect of increased efferent signaling is increased substance P release from the afferent limb of the vagus as was demonstrated by Li and colleagues using nerve stimulator electrodes to selectively stimulate efferent fibers of the vagus (77). The substantial substance P release they found in the lung linked an increase in efferent signaling to stimulation of afferent signaling in the vagus.

In our initial studies of lung injury following TBI alone, substance P may have caused some mild vasodilation in the lungs of TBI mice but we did not detect any injurious effects. This is likely due to the lack of a secondary inflammatory stimulus in those animals like bacteria or tissue injury (20, 32). Once we administered bacteria into the lungs of our TBI mice, the secondary stimulus was provided and substance P priming of the neutrophils and vasculature generated a more robust and efficient neutrophil recruitment to the lungs. Our experiments with IT administration of purified KC chemokine indicated that TBI mice will recruit more neutrophils than sham animals to the same amount of chemokine. With the vasculature rendered more permeable by substance P, the recruited neutrophils were able to cross into the airspaces and clear the bacteria there more efficiently and with less collateral tissue damage. In treating sham injury
animals with an NK-1R agonist followed by bacterial challenge, we were able to reproduce the substance P priming seen in TBI mice. Agonist-treated sham injury animals with pneumonia demonstrated similar levels of neutrophil recruitment and bacterial killing to those found in TBI mice with pneumonia further proving substance P release is responsible for the effects of TBI on neutrophil recruitment in response to bacterial pneumonia.

The findings detailed here illustrate an important divergence in the study of TBI and the immune system. Many studies have been geared toward limiting inflammation after TBI to prevent further neurological damage and improve functional outcome after injury (35, 36). Of particular import has been the use of NK-1R antagonists to reduce cerebral edema and tissue damage following injury (26). These drugs are already FDA approved for use as anti-emetics and anti-depressants and trials in TBI patients as a neuroprotective agent have been suggested in some studies (26, 33). While neuroprotection after TBI is an important area for therapeutic intervention, our findings suggest that the use of NK-1R antagonists could be potentially dangerous in TBI patients. Inhibition of substance P signaling after TBI could remove an important element of the immune response to secondary infection and leave patients at risk of developing pneumonia or other infections (37, 85). A balance needs to be struck in preventing neuroinflammation but maintaining peripheral immune competence following TBI.
NK-1R agonism already has some promising clinical applications particularly in pneumonia after neurological disease. Substance P has been shown to be critical to cough reflex and pulmonary toilet as well as the early immune response to bacteria in animal models of aspiration pneumonia (66, 85). Nakagawa and colleagues found that decreased levels of substance P in sputum samples from elderly patients were associated with the development of aspiration pneumonia (93). Arai and colleagues demonstrated that prior stroke significantly reduced plasma and airway levels of substance P and those patients with lower substance P levels were at higher risk for developing aspiration pneumonia (5). Nakashima and colleagues investigated the use of nicergoline, an ergotoid drug that has been shown to increase serum and airway levels of substance P, to prevent aspiration pneumonia in 60 elderly stroke patients through improved swallowing reflex and pulmonary toilet (94). This study demonstrated significant reduction (~30%) in aspiration pneumonia and nicergoline was found to be equally effective as the current regimen of angiotensin converting enzyme inhibitor medications used to reduce aspiration in elderly stroke patients. While the study cites improved swallowing and cough reflex as potential reasons for the decrease in pneumonia, they did not investigate any possible immune effects of increased substance P that could also contribute to their findings.
Future Work

An important aspect of our findings that requires further investigation is the anti-inflammatory effects of TBI on the pulmonary immune response to bacterial pneumonia. The observed improvement in neutrophil recruitment and bacterial clearance is accompanied by a moderation of the immune response manifested by decreased inflammatory cytokine production and decreased tissue damage. Treatment with the NK-1R agonist did improve neutrophil recruitment in sham injury animals with pneumonia but had no effect on cytokine production. Substance P release has been associated with lung injury and our findings in our TBI model suggest a compensatory anti-inflammatory element that limits the injurious effects of substance P allowing for a robust but controlled response to bacterial pneumonia following TBI (18, 35, 98).

A possible mechanism of the decreased cytokine production observed in our TBI mice with pneumonia could be cholinergic stimulation of alveolar macrophages and neutrophils from increased vagal parasympathetic tone following TBI (71, 73). The vagus has been implicated as a source of immune suppression in TBI patients particularly of the innate immune system (71). Cholinergic immune modulation mediated through the α7-acetylcholine receptor is characterized as decreased pro-inflammatory cytokine production (IL-6, TNF-a, chemokines) without a corresponding rise in IL-10 production (16, 105). This is the result of NF-KB inhibition through cfos activation that prevents transcription of
inflammatory cytokine mRNA (3, 15). Importantly, we must also consider that the decrease in cytokine production could be due simply to the faster clearance of bacteria requiring a less robust inflammatory response. Examining earlier time points could further identify the contributions of this possibility.

In our model, beyond the decrease in cytokine production we also found that substance P signaling through NK-1R in TBI mice was not able to increase neutrophil surface CXCR2 expression. This is under NF-KB control and suggests the presence of an inhibitory signal that does not prevent the enhanced calcium flux also induced by NK-1R stimulation that occurs independent of NF-KB activity (3, 32, 129). We also demonstrated that the suppression of cytokine production in alveolar macrophages from day 2 TBI mice was not present if macrophages were stimulated outside of the lung. The rapid degradation of acetylcholine could account for this finding and supplementation of the media with acetylcholine would likely have resulted in suppression of cytokine production in the macrophages. Further investigation of the role of cholinergic signaling following TBI is necessary to adequately explain these observations. Our findings suggest investigation of combined substance P and vagal cholinergic therapy could yield therapeutic targets for preventing infection and improving outcomes in TBI and non-TBI patients alike.
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