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Thesis

MILD TRAUMATIC BRAIN INJURY ALTERS PNEUMONIA-INDUCED COAGULOPATHY IN MICE

by

EVAN C. CATUDAL

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Approved by

First Reader ______________________________

Deborah J. Stearns-Kurosawa, Ph.D.
Associate Professor of Pathology and Laboratory Medicine

Second Reader ______________________________

Shinichiro Kurosawa, M.D., Ph.D.
Associate Professor of Pathology and Laboratory Medicine

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MILD TRAUMATIC BRAIN INJURY ALTERS PNEUMONIA-INDUCED COAGULOPATHY IN MICE

EVAN C. CATUDAL

ABSTRACT

Traumatic brain injury (TBI) is a major cause of death among trauma patients and is associated with a high rate of mortality due to complications such as bacterial pneumonia, sepsis, and subsequent coagulopathies. While severe TBI is positively associated with the development of pneumonia, mild traumatic brain injury (mTBI) results in a paradoxical decrease in mortality following bacterial pneumonia via an unidentified mechanism.

New evidence suggests that mTBI stimulates vagus nerve signaling resulting in an anti-inflammatory state that is mediated by neurotransmitters (NT) such as acetylcholine (ACh) and substance P (Sub P). This anti-inflammatory state induced by mTBI has been correlated with an increased resistance to pneumonia (PNA) in mice and has been shown to be mediated in part by increased bacterial clearance in the lungs via enhanced neutrophil recruitment. However, it has not been investigated whether this reduced mortality is due to alterations in the coagulation system and if they have any effect on either the severity or occurrence of disseminated intravascular coagulation (DIC), a common sequelae of pneumonia-induced sepsis.

Our study investigates whether mTBI prior to pneumonia challenge in mice impacts the onset or severity of DIC. We assess and define DIC in our mouse models by

changes in plasma coagulation parameters including fibrinogen, D-dimer, and plasminogen activator inhibitor-1 (PAI-1)

Our study expanded on the prior results showing that mTBI prior to pneumonia significantly decreased mortality in mice challenged intratracheally with high concentrations of *Pseudomonas aeruginosa* (*Psd.)*. We found that mTBI administration prior to pneumonia rescued fibrinogen levels with increased plasma D-dimer levels and a trend to decreased PAI-1, suggesting a compensated fibrinolytic state and amelioration of DIC. Circulating neutrophil and absolute leukocyte counts were also increased in mTBI/pneumonia models compared to those with pneumonia alone, supporting previous evidence implicating mTBI-induced enhanced bacterial clearance in lungs. Taken together, these data raise the possibility that novel neuroimmune pathway(s) exist that are protective and impact coagulation.

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LIST OF ABBREVIATIONS

ACh .... Acetylcholine

ALC .... Absolute Lymphocyte Count

APC .... Activated Protein C, *also* Antigen-Presenting Cell

APTT .... Activated Partial Thromboplastin Time

BBB .... Blood-Brain Barrier

CBC .... Complete Blood Count

CDC .... Centers for Disease Control and Prevention

CFU .... Colony Forming Unit

DAMP(s) .... Damage-Associated Molecular Pattern(s)

DIC .... Disseminated Intravascular Coagulation/Coagulopathy

EPCR .... Endothelial Protein C Receptor

FDP .... Fibrin Degradation Product

FFP .... Fresh Frozen Plasma

HBSS .... Hanks' Balanced Salt Solution

HMGB1 .... High Mobility Group Box 1 Protein

IFN-γ .... Interferon-gamma

LPS .... Lipopolysaccharide

MCP-1 .... Monocyte Chemoattractant Protein 1

mTBI .... Mild Traumatic Brain Injury

NET(s) .... Neutrophil Extracellular Trap(s)

NK (cell) .... Natural Killer (cell)

NT(s) .... Neurotransmitter(s)

PAI-1 .... Plasminogen activator inhibitor-1

PAMP(s) .... Pathogen-Associated Molecular Pattern(s)

PC .... Protein C

PNA .... Pneumonia

PRR .... Pathogen-Recognition Receptor

Psd. .... *Pseudomonas aeruginosa*

PT .... Prothrombin Time

SIRS .... Systemic Inflammatory Response Syndrome

Sub P .... Substance P

TBI .... Traumatic Brain Injury

TCT .... Thrombin Clotting Time

TF .... Tissue Factor

TMB .... 3,3’,5,5’-Tetramethylbenzadine

TNF-α .... Tumor necrosis factor alpha

WBC .... White Blood Cell

INTRODUCTION

Traumatic brain injury (TBI) is a leading public health concern in the United States. More than 2.8 million people are hospitalized with injury each year in the U.S. which results in an annual economic loss of $406 billion in medical costs and lost productivity.[8] The effects and severity of TBI range broadly and can result in psychiatric disturbances including alterations in sensation, language, and emotion and is also known to cause infectious complications, most notably pneumonia. Pneumonia affects 20-60% of patients with TBI, many of whom die from sequelae such as severe sepsis complicated by Disseminated Intravascular Coagulation (DIC).[1,3,7,11,15] Until recently it was unknown whether the *severity* of TBI correlated with either the likelihood of acquiring pneumonia or mortality due to pneumonia.

Mild TBI Confers Increased Resistance to Pneumonia

TBI severity is classified by the United States Centers for Disease Control and Prevention (CDC) as either “mild” (a brief change in mental status or consciousness) or “severe” (an extended period of unconsciousness or amnesia after the injury).[21] A recent meta-analysis of patient hospitalization data from The National Trauma Data Bank revealed that patients who experienced *mild* TBI (mTBI) had a rate of pneumonia occurrence five times lower than patients who experienced *severe* TBI.[9] Pneumonia occurrence following mTBI was also similar in frequency to that of patients who experienced “blunt” injuries, i.e., injuries not involving the head or neck. After taking into consideration the contribution of mechanical ventilation to pneumonia rates it was

discovered that patients with TBI, either mild or severe, had pneumonia rates 25% lower than that of patients who experienced a blunt injury of similar severity.[9] Since patients with mTBI had a significantly lower chance of acquiring pneumonia compared to blunt trauma patients it was thought that mTBI modulates a physiologic mechanism that is able to confer a decreased susceptibility to pneumonia. It was thought that this mechanism was in some way related to changes in inflammation.

Subsequent mouse experiments showed that TBI increased neutrophil recruitment to the airways resulting in more effective bacterial clearance and improved survival.[32] It was found that this increased clearance was likely not mediated by an inflammatory response since levels of pro-inflammatory cytokines were significantly decreased following bacterial inoculation. It was postulated that substance P, a known inducer of neutrophil activation, may be mediating the neutrophil recruitment in the lungs independent of inflammation. Administration of the substance P receptor antagonist CJ-12255 was found to abrogate the survival benefit of mTBI to lethal pneumonia.[32] The results of this experiment and related studies suggested that TBI induces the release of substance P in the lung and promotes leukocyte recruitment and clearance.[29,32]

This novel, non-inflammatory mechanism of increased resistance to pneumonia led researchers to believe that efferent stimulation of the vagus nerve following mTBI may explain the absence of pro-inflammatory cytokines ordinarily seen during an acute infection, possibly conferring resistance to pneumonia by reducing the severity of sepsis and DIC.[1,15,32]

Sepsis, Inflammation, & Coagulation

Sepsis is a potentially deadly clinical condition caused by dysregulated immune and physiologic responses to an infection. Bacterial pneumonia is the most common infectious inducer of sepsis, being implicated as the cause in approximately 47% of sepsis cases.[17] The exact mechanism of sepsis pathogenesis in still unknown. However, one can begin to unravel its secrets by first understanding its epidemiology and clinical complications.

The stages of sepsis progression, in order of severity, are referred to as: sepsis, severe sepsis, and septic shock. Sepsis is characterized by identified or suspected infection, along with two or more features of Systemic Inflammatory Response Syndrome (SIRS) which include changes in temperature, heart and respiratory rate, and circulating leukocyte levels.[26] Severe sepsis is defined by dysfunction of at least one organ. Septic shock, the final and most deadly form of sepsis, is defined by hypotension due to severe sepsis. Mortality due to severe sepsis with shock has been estimated at around 50% with higher mortality found in patients with comorbidities like diabetes, congestive heart failure, and renal insufficiency. These comorbidities are of particular concern because they have an additive effect on the organ failure that occurs during severe sepsis.[13]

Infection induces rapid activation of lymphocytes, neutrophils, and macrophages which leads to enhanced phagocytosis of bacteria as well as necrosis of healthy cells and tissues.[13] Leukocyte activation induces inflammation and cytokine production which feedback to amplify the leukocyte activation. Cytokines are also produced when pathogen

recognition receptors (PRRs) on antigen presenting cells (APCs) such as macrophages and dendritic cells recognize either opsonization proteins on the surface of microbes or unique molecular patterns on, in, or made by the microbe. These unique molecular patterns are termed pathogen-associated molecular patterns (PAMPs) and can include lipopolysaccharides (LPS) found in the cell wall of gram-negative organisms such as *Pseudomonas spp.*, as well as lipoproteins and nucleic acids.[13]

Cytokines such as TNFα, IL-1β, IL-6, and CXCL-8 released by inflammatory cells stimulate synthesis and expression of adhesion molecules on endothelial cells, allowing for leukocyte migration into tissues.[5,15,26,28] Il-12 is produced by APCs, activated T cells and neutrophils in response to antigenic stimulation. IL-12 is involved in the differentiation of naive T cells into Th1 cells and also stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells. IL-12 mediates enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes.[26]

Effector CD4+ T cells secrete the cytokine interferon (IFN)-γ which activates phagocytic cells. Activated phagocytic cells now can better kill phagocytized microbes and interact with B cells. These activated B cells now produce antibodies which further opsonize microbes. Despite this considerable lymphocyte activation during sepsis patients often have decreased T-cell counts due to apoptosis. It is thought that this apoptosis is a result of up regulation of CTLA-4 and concurrent down regulation of CD86 co-stimulatory molecules on APCs.

IL-6 is a major cytokine involved with the pathogenesis of sepsis. IL-6 is secreted mainly by macrophages in response to PAMPs and by fibroblasts and endothelial cells.[2,23,24,33] It is responsible for stimulating the production of acute phase proteins from hepatocytes and for stimulating the production of neutrophils in the bone marrow. IL-6 supports the maturation of B cells into antibody-secreting plasma cells and is antagonistic to regulatory T cells. IL-6 also mediates fever by initiating synthesis of PGE_2 in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilization that also leads to increased body temperature.

Levels of the cytokine IL-6 are so closely linked to sepsis that they have been shown to be proportional to the rate of mortality following sepsis. Cuschieri *et al* analyzed patient data from the *Inflammation and the Host Response to Injury Large Scale Collaborative Program* and showed that early elevation in IL-6 to a level greater than 350 pg/mL had a strong correlation with subsequent mortality following severe injury.[2] This group also saw that patients with greater than 350 pg/mL IL-6 were more likely to receive massive blood transfusions, an independent predictor of higher mortality. A related mouse study from Remick *et al* showed that IL-6 levels greater than 2000 pg/mL six hours after cecal ligation and puncture predicated mortality three days later.[24]

Pro-inflammatory cytokines play a major role in the pathogenesis of disseminated intravascular coagulopathy (DIC), a disruption of the body’s coagulation system that results in simultaneous development of systemic thrombosis and hemorrhage. Due to the dynamic and complex nature of the coagulation system, DIC is difficult to treat and often fatal.

Sepsis Induces Disseminated Intravascular Coagulopathy

Disseminated intravascular coagulopathy is a *consumptive coagulopathy* or a *consumptive thrombo-hemorrhagic disease* and is characterized by the triad of hypercoagulability, loss of anticoagulant capability, and impaired fibrinolysis.[13]

DIC may be catalyzed by any disease or insult that injures the endothelium or inappropriately activates the coagulation cascade. Tissue factor is generally accepted as the initiator of coagulation in the context of severe sepsis. [14,28] In DIC, coagulation activation is mirrored by inhibition of the body's anticoagulant mechanisms by altering antithrombin levels and reducing the activity and functionality of the anti-coagulant protein C-protein S- thrombomodulin system as well as by impairment of fibrinolysis.

Tissue damage from insults like trauma, surgery, infection, or snake bites release thrombogenic tissue factor into the circulation which may induce coagulation by itself.[12,14] Alternatively, neoplasms that produce large quantities of circulating cells or that have high cellular turnover may also activate coagulation. Leukemia, characterized by high levels of circulating leukocytes, is known to be especially thrombogenic due to the presence of tissue factor on many of these cells. Leukemia is also considered an inflammatory state. The inflammatory state, found is numerous diseases including sepsis, is another key mediator of DIC.

Pro-inflammatory cytokines including IL-1, IL-6, IL-12, and TNF-α induce the expression of thrombogenic tissue factor (TF) on several cell types, decrease levels of antithrombin, inhibit protein C, and impair fibrinolysis.[14,25] Apoptotic cells, often seen in inflammation, can release tissue-factor containing micro particles into systemic

circulation. Tissue factor activates the extrinsic coagulation pathway and ultimately the formation of the prothrombinase complex comprised of factors Xa, Va, and calcium. The prothrombinase complex can form on these micro particles as well as endothelial cells, bacterial cell walls, or on any anionic phospholipid cell surface.[13] This complex catalyzes the conversion of prothrombin to thrombin which results the conversion of fibrinogen to fibrin and the formation of fibrin clots.

Inflammation induces adhesive platelet-neutrophil interactions that can result in release of pro-coagulant DNA fibers and neutrophil extracellular traps (NETs) as well as histones which serve to amplify the inflammatory response.

These hemostatic alterations lead to thrombi formation in microvasculature which result in ischemic necrosis, multi-site bleeding, and ultimately multi-organ failure. DIC is diagnosed on the basis of both clinical signs and symptoms and laboratory values. Patients with DIC will have a decrease in platelets and fibrinogen levels, elevated D-dimer (a marker of fibrinolysis), and a prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT), both of which are measurements of functionality of various aspects of the coagulation cascade.[13] Peripheral blood smears will often contain *schistocytes*, red blood cells that have sheared into pieces as they travel through the fibrin mesh of thrombi in the microvasculature under high pressure. These patients with DIC will also bleed from one or more sites such as the eyes, nose, or mucous membranes.

A D-dimer is the most common protein fragment in a class known as fibrin degradation products (FDP). FDPs are released into circulation during dissolution of a fibrin clot, the primary function of tertiary hemostasis. The protein is called a “D-dimer”

because it contains two cross-linked D fragments of the fibrin protein. D-dimer concentrations in plasma or serum above the normal reference range indicate that a fibrin clot (thrombosis) has recently or is currently in the process of being broken down.

D-dimer is a significant diagnostic and prognostic marker of DIC. It has been shown that there is a strong linear correlation between increasing D-dimer levels and mortality following DIC.[18,22] This is because D-dimer levels indicate the extent of systemic thrombosis and therefore the severity of the DIC. One study showed that patients with community-acquired pneumonia (CAP) who had D-dimer levels between 256 and 1000 ng/mL had a two to three fold higher 90-day mortality compared to CAP patients with normal D-dimer levels ($\leq$256ng/mL).[18] Patients with D-dimer levels greater than 1000ng/mL had more than a five-fold higher mortality 90 days post-pneumonia compared to the group with normal D-dimer levels. Alternatively, the presence of an elevated D-dimer may also indicate that the body's tertiary hemostatic machinery is working and therefore may serve as a positive prognostic indicator in some situations.

A lesser measured parameter during a coagulation crisis is plasminogen activator inhibitor-1 (PAI-1). PAI-1 is the primary inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the activators of plasminogen and therefore fibrinolysis. Thus, PAI-1 is an inhibitor of fibrinolysis and has an endogenous function of ensuring that clots are not degraded before tissue healing has occurred. Several studies have investigated the diagnostic and prognostic significance of this protein and have shown that there is a strong correlation between PAI-1 levels and the severity of sepsis and DIC making it a poorer prognostic marker in these cases.[18] Higher PAI-1 levels during these disease states

indicate that thrombi are not being dissolved adequately which will exacerbate the microvascular hemolysis and tissue ischemia that is already occurring. PAI-1 is also an inflammatory mediator and is seen in higher concentrations in cancer, obesity, and metabolic syndrome (all of which are considered inflammatory states) and is thought to contribute to the increased occurrence of thrombosis in patients with these conditions.[13]

Hyperfibrinolysis during sepsis or trauma has been attributed to inactivation of PAI-1 by activated protein C (APC).[31] Neutrophil elastase is also known to degrade PAI-1 as well as fibrin and fibrinogen, further potentiating fibrinolysis.[31] Thus, fibrinolysis due to PAI-1 concentrations is directly influenced by at least two separate mechanisms during sepsis further illustrating the complexity of the coagulation system.

Current Therapies for DIC

Treating DIC is more of an art than a science. This is because science thus far been unable to produce a treatment that is able to control both the clotting and the bleeding aspects of DIC. Historically, physicians employed heparin as the gold-standard treatment for DIC as they felt that controlling thrombi formation was more important that controlling bleeding.[13] Heparin was often administered along with platelet or fresh frozen plasma (FFP) transfusions in an attempt to restore components of the coagulation cascade that have been depleted.[26] Allogeneic blood products are still used as a treatment for DIC but the advent of recombinant factor concentrates has shown promise in decreasing mortality from DIC without the risk of transfusion-related complications.

Recombinant activated protein C (PC) , has been proposed as a novel viable treatment for DIC.[26] Activated protein C inactivates factors Va and VIIIa thereby reducing thrombin production and preventing clot formation. The protein C pathway starts by the protein C zymogen binding to the endothelial protein C receptor (EPCR) and subsequent interaction with thrombomodulin-bound thrombin. This complex results in activation of protein C (APC) which catalyzes the limited proteolysis of factors Va and VIIIa. Recombinant protein C was brought to market as a therapeutic for severe sepsis and showed promise, but it was discontinued due to bleeding complications and ineffectiveness in patients with less severe sepsis.

Recently, a soluble thrombomodulin therapeutic has been in the works and hopes to modulate coagulation via a mechanism similar to that of activated protein C.[9] Soluble thrombomodulin is less able to contribute to activation of protein C relative to its membrane-bound equivalent, thereby reducing the risk of bleeding in patients. Structurally-modified soluble thrombomodulin has also been developed with the intention of reducing its proteolysis and oxidation in plasma thereby prolonging its half-life in the body.

It has been theorized that mediation of inflammation and by extension, coagulation, may be a primary mechanism by which mild traumatic brain injury lessens the mortality of patients with hospital-acquired pneumonia but so far this mechanism has not been investigated using animal models.

Traumatic Brain Injury Induces Changes in Inflammation and Coagulation

The effects of traumatic brain injury echo across nearly every branch of human physiology. These effects are divided into primary and secondary events.[1,3,29,30] Primary events involve mechanically-induced tissue damage, laceration, and nerve fiber shearing and occur immediately following TBI. Secondary injury involves biochemical and physiological changes that stem from the initial insult and primary events. The effects of secondary injury alter many of the systems that control inflammation and potentially coagulation and are thought to be the cause of much of the mortality seen in TBI patients.

The most significant events that occur during secondary injury are release of neurotransmitters (NT), oxidative stress, and disruption of the blood-brain barrier (BBB). Secondary injury causes excess release of the excitatory neurotransmitters glutamate and aspartate as well as activation of NMDA, Ca^{2+} and Na^{+} channels. Ca^{2+} and Na^{+} influx activated intracellular catabolic processes as well as lipid peroxidases, proteases, caspases, and phospholipases.[3,30] Caspases induce DNA fragmentation, membrane degradation, and ultimately cellular necrosis and apoptosis. Necrotic cells release damage-associated molecular patterns (DAMPs) including high-mobility group box 1 protein (HMGB1), DNA, RNA, S100 molecules, the purine metabolites ATP, adenosine, and uric acid, and finally hyaluronan fragments which all contribute to widespread inflammation thereby activating coagulation.[3]

As stated earlier, inflammatory cytokines are significant induces of tissue factor expression. These include TNF, IL-1α, IL-1β, IL-6, IL-8, leukemia inhibitory factor, IFN-γ, and monocyte chemoattractant protein 1 (MCP-1).[1,3] Anti-inflammatory cytokines

such as transforming growth factor (TGF)-β, IL-4, IL-10 and IL-13 diminish tissue factor expression and may be induced by various stimuli such as LPS, TNF, IL-1, MCP-1 and C-reactive protein (CRP).[3]

The pro-inflammatory cytokines TNF and IL-1 contribute to adhesion of monocytes and upregulation of monocyte tissue factor.[7] Tissue factor synthesis in monocytes requires direct interaction with activated Th1 cells and Th1 cytokines, namely IFN-γ. The Th2 cytokines (IL-4, IL-10 and IL-13) are considered inhibitory of monocyte tissue factor production.[13] Indeed, the dynamic of humoral and cellular immunity is important in regulation of systemic tissue factor activity.

Substance P, a pro-inflammatory mediator, is increased following TBI and is thought to induce a phenomenon known as *neurogenic inflammation*.[29,32] Neurogenic inflammation is characterized by brain edema due, vasodilation, leukocyte migration, and plasma protein extravasation.

As mentioned earlier, some studies have implicated the vagus nerve as part of immunomodulatory reflex by which the autonomic nervous system detects inflammatory stimuli and modulates cytokine production. The vagus nerve normally inhibits systemic inflammation and failure of this system following TBI results in increased systemic inflammation.[1,15,32] This immunosuppression is thought to be the cause of the complicating infections seen in TBI patients.

In respect to immunosuppression, it has been demonstrated that TBI decreases neutrophil superoxide generation, IgG and IgM production, complement production, and circulating levels of T-cells, T-helper cells, T-suppressor cells, and Natural Killer (NK)

cell.[7] These changes show impaired innate and adaptive immune functions. One mechanism of this impairment may be due to hyperactivation of Neutrophils immediately following TBI resulting in a decrease in their number and functionality 18-72 hours following injury.[7]

Cortisol release due to a stress response to tissue injury after TBI is known to also suppress immune functionality. Glucocorticoids such as cortisol are well known to be suppressors of the pro-inflammatory cytokines IL1-β, IL-11, IL-12, IFN-γ, IL-8, and prostaglandins. Several sources propose bacterial translocation from the gut to be the source of opportunistic infections including pneumonia following TBI.[6,16] These infections are thought to occur as a result of this immunocompromised state following TBI. Gautreaux *et al* showed that depletion of CD4+ and CD8+ T-cells in mice increased gut translocation of *E. coli*, *S. typhimurium, Lactobacillus spp. Staphylococcus spp.,* and *P.* mirabilis, thereby showing the importance of T-cell inhibition as a cause of bacterial translocation from the gut and a potential cause of pneumonia following TBI.[6]

Mrakovcic-Sutic *et al* studied humans with TBI and reported a decrease in the numbers of NK cells and Monocytes 7 days after TBI but no decrease in the numbers of circulating B cells.[19] Cytotoxic lymphocytes were also decreased 4-days post injury but recovered by day 7. The decrease in cells with a cytotoxic phenotype seen here is thought be an explanation for increased susceptibility to infection following TBI.

In respect to directly altering coagulation, studies have shown that immediately following TBI there is in initial hypercoagulable state defined by increased soluble fibrin levels.[15] It has also been shown that markers of fibrinolytic activity such as D-dimers and

alpha-2-plasmin inhibitor-plasmin complex are elevated proportional to the severity of injury.[15] Other studies have shown that mTBI immediately stimulates thrombin activity in the brain and induces amnesia via activation of Protease Activated Receptor 1 (PAR-1).[10] Thrombin activity in the brain returned to baseline one hour after trauma and then increased to high levels seventy-two hours later. The levels of thrombin activity were found to correlate directly with the levels of brain PAR-1 levels as well as sensitivity to seizure like activity following injury.

Whether mild traumatic brain injury rescues patients from disseminated intravascular coagulopathy is a quandary that still begs to be investigated. Nevertheless, the information suggesting that TBI has an effect on coagulation is robust and extensive. Traumatic brain injury induces a systemic inflammatory response that directly activates coagulation and suppresses humoral and cellular immunity leading to opportunistic infections and sepsis-induced coagulopathy. TBI also has also been shown to have direct effects on activity of coagulation components within the brain itself. This study looks at coagulation parameters in mouse models of mTBI and pneumonia in an attempt to shed some light on this conundrum.

Specific Aims

Based on the information discussed above, this study aims to:

1. Determine what, if any, changes in hemostasis occur as a result of mild traumatic brain injury in mice

2. Determine what, if any, changes in levels of circulating leukocytes (cellular immunity) or platelets occur as a result of mild traumatic brain injury

We expect our studies will show that:

1. Mild traumatic brain injury alters circulating leukocyte after bacterial pneumonia challenge
2. Mild traumatic brain injury ameliorates pneumonia/sepsis induced disseminated intravascular coagulopathy by sustaining fibrinogen levels in plasma and by facilitating tertiary hemostasis

METHODS

Animals

Female ICR mice (Harlan Sprague Dawley, Indianapolis, IN) were used for all experiments. All mice were 8-10 weeks old, weighed between 24-30g, and were acclimated for at least 3 days prior to experiments. Animals were housed in a temperature and humidity controlled facility with a 12-hour light/dark cycle. Food and water were provided *ad libitum*. The Institutional Animal Care and Use Committees at Boston University approved all experiments. This study was done in collaboration with the laboratory of Dr. Daniel Remick, Chair and Professor of Pathology and Laboratory Medicine. All animal handling and experiments were performed by members of the Remick laboratory and blood samples were provided for this study of coagulation testing.

Murine Models of Mild Traumatic Brain Injury and Pneumonia

The mTBI-pneumonia model was performed essentially as described in Remick, et al.[23] Briefly, mild traumatic brain injury was modeled in mice using a closed-head weight-drop technique. Under isofluorane 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 mild TBI, a 170-g steel rod within a guide tube was released from a height of 5.2 cm to impact a point in the midline of the skull, halfway between the interauricular and interorbital lines. Control mice were subjected to a sham injury which was induced with tail trauma. To induce tail trauma, mice were anesthetized using isoflurane and placed in a prone position on the surface of the mTBI apparatus. The 170g steel rod is released to a height of 8.5cm onto a spot 2.0cm from the base of the tail. Immediately following impact the mouse is removed and given a subcutaneous injection of 0.05mg/kg buprenorphine in 1mL of normal saline. Mice are placed on a warming pad until they are able to right themselves. Both sham and mTBI mice were sacrificed by cervical dislocation under isoflurane anesthesia at 24 hour and 48 hour time intervals post-injury. Mice that did not receive either injury served as a control group, hereby referred to as "Naïve" mice.

Pneumonia (PNA) was initiated 48 hours post-injury to a subgroup of both the sham and mTBI injury groups. A pneumonia-only control group was also tested which did not receive either the sham or mTBI trauma. Under isoflurane anesthesia, pneumonia was induced by administration of either $1x10^7$, $5x10^7$, or $1x10^8$ colony forming units (CFUs) of *Pseudomonas aeruginosa* (ATCC strain Boston 41501) in 50uL of Hank's

balanced salt solution (HBSS) by intratracheal instillation. All PNA mice were sacrificed under isoflurane anesthesia 18 hours post-pneumonia by cervical dislocation. A diagram showing the various experimental groups of this experiment is illustrated in figure 1.

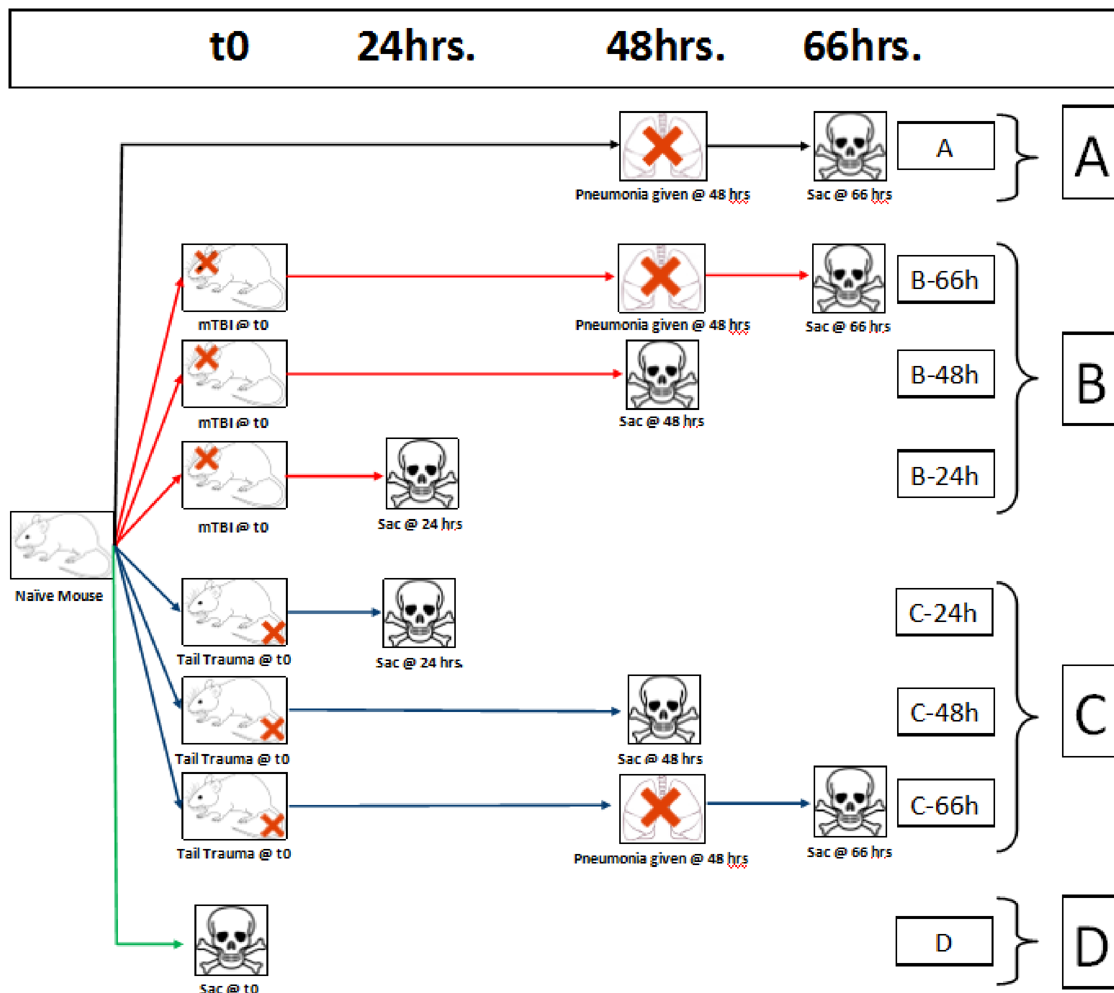

Figure 1. Experimental Timeline. At t0, naïve mice were either given either mild traumatic brain injury (mTBI), tail trauma, were sacrificed, or were placed in a pneumonia-only group. These 4 groups are mTBI (B), TT (C), Naïve (D), and PNA-only (A), respectively. Mice in the mTBI group (B) were either sacrificed at 24 hours (B-24h), sacrificed at 48 hours (B-48h), or given pneumonia at 48 hours and then later sacrificed at 66 hours (B-66h). Mice in the TT group (C) followed the same timeline as the mice in the mTBI group, denoted as (C-24h, C-48h, and C-66h). Mice in the PNA-only group (A) were given pneumonia at 48 hours and then later sacrificed at 66 hours. Mice in the naïve group (D), which served as the most basic control group, were sacrificed at t0.

Specimen Collection

Whole blood specimens were collected terminally via retro-orbital exsanguination. Mice were anesthetized with 99% isoflurane for 30 seconds or until unconscious (Figure 2). Mice were firmly grasped by their neck scruff and the retro-orbital area was wiped with sterile gauze saturated with 3.8% sodium citrate to prevent clot formation. The left eye was then detached from the mouse using sterile tweezers. A 20uL aliquot of whole blood was removed via micropipette directly from the orbital socket and placed in 355uL Hemavet 950 FS Hematology Analyzer diluent (Drew Scientific, Waterbury, CT) for the purpose of performing complete blood counts. The remaining whole blood (approximately 800uL) was collected in an Eppendorf tube containing 100uL of 3.8% sodium citrate for a final dilution of 1:9. Exsanguination did not exceed 2 minutes per mouse. The whole blood and citrate was constantly mixed by pipet during collection to avoid clotting and the retro-orbital area was continuously wiped with a sodium citrate-soaked gauze to prevent clot formation. Mice were euthanized after collection by cervical dislocation.

Within 10 minutes of collection the total whole blood volume collected was quantified using a graduated 1mL sterile syringe with a 22 gauge beveled tip. The whole blood samples were then centrifuged at 3000rpms for 15 minutes at 4°C and then checked for clots. Plasma aliquots were removed from the packed cells with a 200uL pipet and transferred to individual Eppendorf tubes and frozen immediately at -80°C. Care was taken not to remove or disturb the buffy coat which contains platelets that secrete PAI-1.

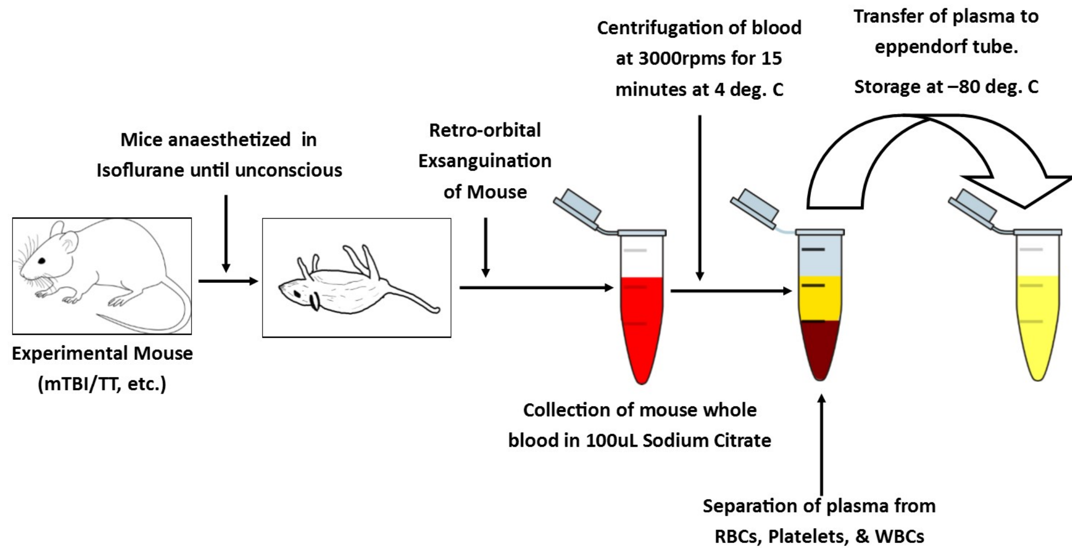

Figure 2. Mouse Plasma Collection Procedure. Mice were anesthetized with Isoflurane for 30 seconds or until unconscious. Whole blood was collected in an Eppendorf tube containing 100uL of 3.8% sodium citrate via retro-orbital exsanguination. Whole blood samples were centrifuged at 3000rpms for 15 minutes at 4°C. Plasma was removed from the packed cells and transferred to individual Eppendorf tubes and frozen immediately at -80°C.

Complete Blood Counts

Complete Blood Counts (CBCs) were performed on a Hemavet 950 FS Hematology Analyzer (Drew Scientific, Waterbury, CT). 20uL of whole blood was diluted in 355uL of manufacturer-purchased reagent diluent and the diluted sample was analyzed per the Hemavet 950 FS standard operating protocol supplied by the manufacturer.

Fibrinogen

Plasma fibrinogen concentrations were determined using a Thrombin Clotting Time (TCT) assay performed on a Sigma Amelung KC4 Delta™ coagulation analyzer (Trinity Biotech, Wicklow, Ireland). The TCT assay measures the time it takes for a clot to form in plasma containing an anticoagulant (3.8% sodium citrate) following incubation at 37° C and addition of excess thrombin (Figure 3).

A fibrinogen standard curve was prepared using pooled plasma standard with a known concentration of 130 mg/dL diluted 1:5, 1:10, and 1:20 in imidazole buffer. The final fibrinogen concentrations of these diluted standards were 26.0 mg/dL, 13.0 mg/dL, and 6.5 mg/dL, respectively. A stock solution of thrombin with a concentration of 2000 U/mL was diluted 1:100 in imidazole buffer for a final concentration of 20 U/mL. 100uL of diluted standard was added to individual KC4 Delta™ Tetravette wells in duplicate and incubated at 37° C for 60 seconds. 50uL of dilute thrombin was then added to each sample well, starting an electronic timer, which stopped upon clot formation. TCTs from

these standards were used to generate a standard curve of fibrinogen concentration in mg/dL (X-axis) vs. TCT in seconds (y-axis).

Unknown mouse plasma samples were diluted 1:10 in imidazole buffer. 100uL of diluted mouse plasma was added to individual KC4 Delta™ Tetravette wells in duplicate and incubated at 37° C for 60 seconds. 50uL of dilute thrombin was then added to each sample well and the time it took for a clot to form was recorded by the electronic timer on the analyzer. Duplicate sample results with greater than 10% difference were rejected and rerun. The TCTs from the unknown samples were compared to the standard curve in order to determine their fibrinogen concentrations

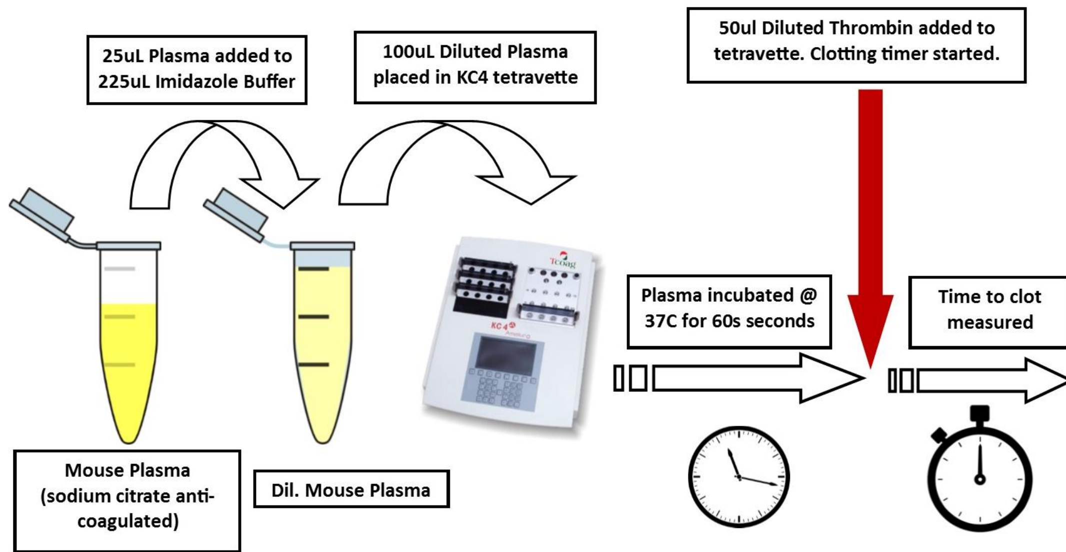

Figure 3. Thrombin Clotting Time Procedure. 25uL of mouse plasma (anticoagulated in 3.8% sodium citrate) was diluted 1:10 in 225uL imidazole buffer. 100uL of diluted plasma was added to KC4 tetravette wells and incubated for 60 seconds. 50uL of diluted thrombin was added and a clot timer was started. TCT was determined by how much time it took for the fibrin clot to stop a metal ball from rotating freely inside the tetravette well.

D-dimer

D-dimer levels were measured using an Asserachrom D-Di (Diagnostica Stago, S.A.S., Asnières sur Seine, France) ELISA Kit and unknown D-dimer values were calculated from the standard curve using the manufacturer-provided human D-dimer standards. Standards, run in duplicate, had a concentration range from 0.714 ng/mL to 45.667 ng/mL. All unknown samples were diluted 1:21 and run in duplicate.

The D-dimer to be measured was captured by mouse monoclonal anti-human D-dimer antibody coated on the internal walls of the plastic microplate wells. Following a washing, peroxidase-coupled rabbit anti-human fragment D antibodies were then added which bound to the remaining antigenic determinants of the well-bound D-dimer. After a second washing, 3,3',5,5'-Tetramethylbenzadine (TMB) substrate was added and is cleaved by the antibody-bound peroxidase producing a blue color. This reaction was then stopped by the addition of 1M H_2SO_4 which produced a yellow color proportional to the blue color and the optical density of the wells was then read at 450nm. The intensity of the yellow color in the wells is proportional to the concentration of D-dimer in the plasma samples.

The ELISA plate was read using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA) and the results were analyzed using SoftMax Pro 5.4 data analysis and acquisition software (Molecular Devices, Sunnyvale, CA).

PAI-1

PAI-1 levels were measured using an Abcam PAI-1 Total Mouse ELISA kit (Abcam plc, Cambridge, United Kingdom). Unknown PAI-1 concentrations were calculated from the standard curve using the manufacturer-provided PAI-1 standards. Nine standards were run with a range of concentrations from 0.1 ng/mL to 50 ng/mL. All unknown samples were run in duplicate, undiluted, but yielded some wells with optical densities greater than what was detectable by the plate reader. These samples were rerun singly, diluted 1:5, and were compared to a new standard curve that was run concurrently with the diluted samples.

Free, latent, and complexed mouse PAI-1 present in plasma is captured on Anti-mouse PAI-1 antibodies coated and dried on the microtiter plate wells. Wells are washed with a blocking buffer containing 3% bovine serum albumin (w/v) in 1X Tris-buffered saline (0.1M Tris, 0.15M NaCl, pH 7.4) and an excess of anti-PAI-1 secondary antibody is added. Excess is washed away and bound antibody, which is proportional to the total PAI1 present in the samples, is then reacted with an anti-rabbit HRP detection antibody. Following an additional washing step, TMB is added which reacts with the HRP producing a blue color. This reaction is then stopped by the addition of 1M H_2SO_4, producing a yellow color proportional to the blue color and the optical density of the wells is then read at 450nm. The amount of color development is directly proportional to the concentration of total PAI1 in the sample.

The ELISA plate was read using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA) and the results were analyzed using SoftMax Pro 5.4 data analysis and acquisition software (Molecular Devices, Sunnyvale, CA).

Statistics

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

RESULTS

Due to the logistical hurdles involved in performing multiple mouse experiments, the experimental groups had varying numbers of mice per group and results for all the experimental parameters could not be obtained for some of the mice due to specimen requirements. In early experiments some of the samples collected clotted and could not be assayed. The blood collection procedure was subsequently modified as described and data from these samples are included.

Complete Blood Counts

Complete blood count parameters that were analyzed included total WBC counts, absolute neutrophil counts (ANC), absolute lymphocyte counts (ALC), and platelet counts. Results for each of these parameters were grouped according to the experimental groups stated in the methods section. A summary of the CBC results is shown in Table 1. Groups that received pneumonia challenge were also separated on the basis of the concentration of *Pseudomonas* they received for some of the statistical analyses.

It was concluded that injury, both tail trauma and mild traumatic brain injury (B & C), had effects on total WBC, ANC, and ALC parameters but did not affect platelet levels (figures 4, 5, 6, & 7). The general trend was that injury increased total WBC counts, ANCs, and ALCs in all mouse groups that were not challenged with pneumonia.

Challenge with *Pseudomonas* pneumonia drastically decreased total WBC and ALC levels both in the presence and absence of trauma (B-66h + C-66h & A, respectively). The decreases in total WBC and ALC were proportional to each other

likely because the majority of circulating WBC are lymphocytes in mice. Pneumonia challenge decreased total WBC and ALC counts the greatest in non-injury mice (A).

The ANC in both the mTBI/PNA (B-66h) and the TT/PNA (C-66h) groups were higher following pneumonia challenge than in mice given only pneumonia challenge (A). It was therefore proposed that injury (mTBI and tail) may afford mice resistance to pneumonia by increasing circulating levels of neutrophils.

	Total WBC Range (K/uL)	ANC Range (K/uL)	ALC Range (K/uL)	Platelet Range (K/uL)
Naïve (t0) (D)	3.18-5.80	0.73-0.97	2.30-4.58	572-657
Tail Trauma-24h (C-24h)	4.56-6.64	0.99-1.24	2.98-5.06	606-757
mTBI-24h (B-24h)	1.68-3.02	0.66-1.12	0.91-1.87	441-803
Tail Trauma- 48h (C-48h)	2.42-9.18	0.60-1.98	1.58-6.95	566-777
mTBI- 48h (B-48h)	4.58-5.94	0.81-1.65	2.56-4.21	712-906
TT/PNA- 66h (C-66h)	1.28-3.60	0.11-1.92	0.40-1,87	601-1066
mTBI/PNA- 66h (C-66h)	1.30-4.26	0.13-2.63	0.67-1.50	653-1064
PNA only- 66h (A)	0.46-1.74	0.06-0.56	0.39-1.36	535-910
	Total WBC Average	ANC Average	ALC Average	Platelet Average
Naïve (t0) (D)	4.49	0.85	3.44	615
Tail Trauma-24h (C-24h)	5.25	1.12	3.76	689
mTBI-24h (B-24h)	2.42	0.83	1.45	653
Tail Trauma- 48h (C-48h)	6.45	1.33	4.53	636
mTBI- 48h (B-48h)	5.28	1.31	3.54	818
TT/PNA- 66h (C-66h)	2.15	0.86	1.17	816
mTBI/PNA- 66h (C-66h)	2.81	1.48	1.13	903
PNA only- 66h (A)	1.41	0.38	0.91	796

Table 1. Summary of CBC Results. Data ranges and averages for the CBC parameters considered in this experiment. Results are shown for each experimental group (n = 2-8/group).

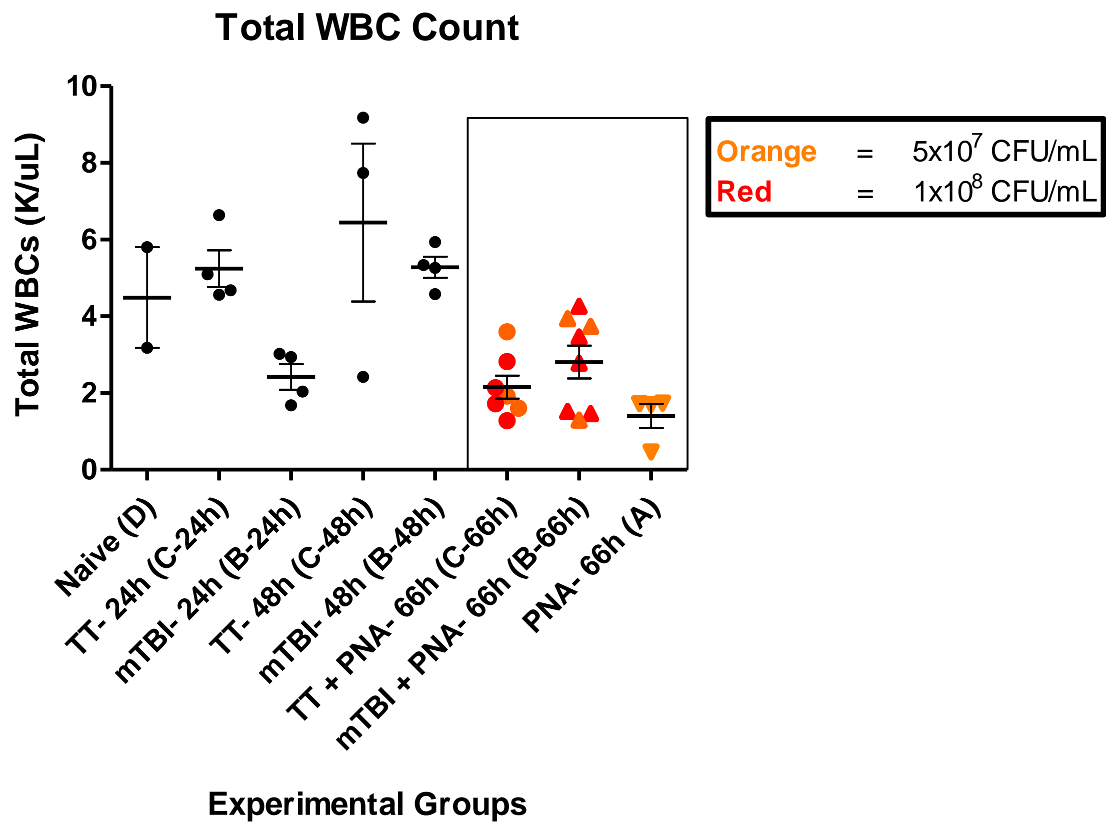

Figure 4. Total WBC Counts. (n = 2-8/group). Neither mTBI (B-24h and B48h) nor TT (C-24h and C-48h) (non-pneumonia groups) affected WBC counts significantly compared to the Naïve group (D) at any time point. All unpaired, two-tailed t-tests- (Naïve vs. TT-24h- p=0.5220), (Naïve vs. mTBI-24h- p=0.0934), (Naïve vs. TT-48h- p=0.5387), (Naïve vs. mTBI-48h- p=0.4317).

Challenge with *Pseudomonas* pneumonia (PNA-66h) (A) in the absence of either type of injury *significantly* decreased WBC counts compared to the Naïve control group (D) (p=**0.0296**).

The (TT + PNA-66h) cohort (C-66h) had WBC counts lower than the Naïve group (D): (**p=0.0245**).

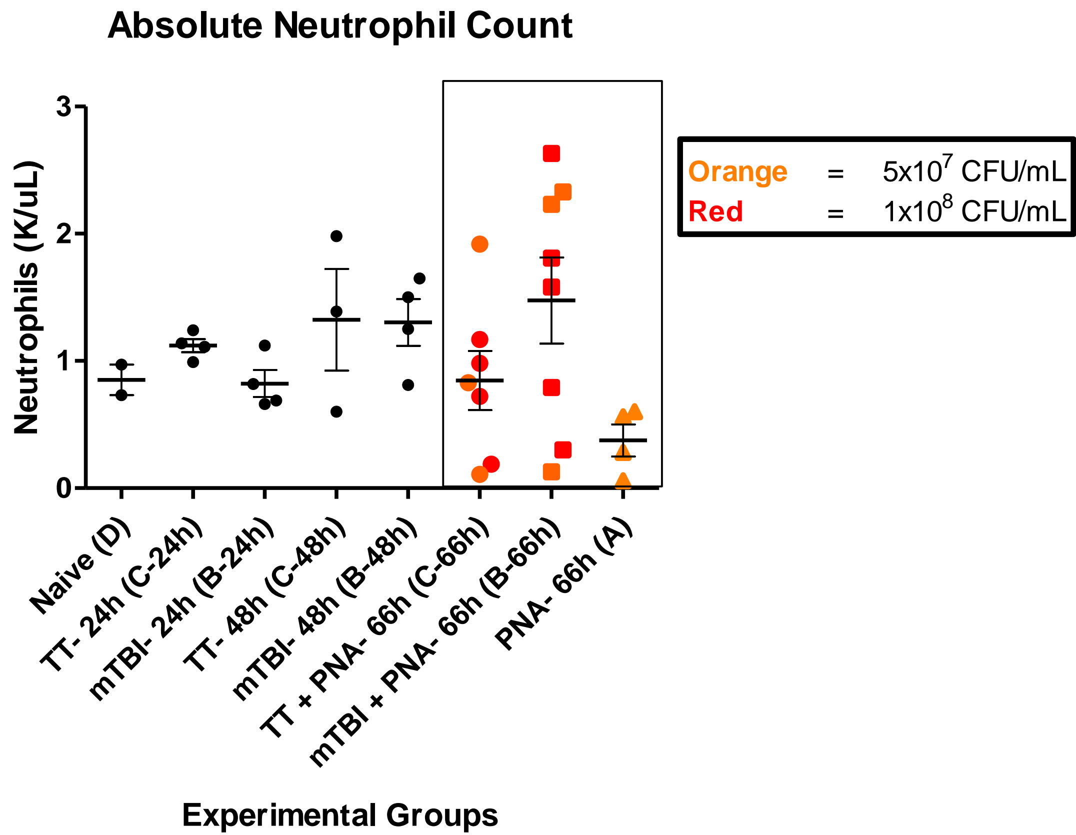

Figure 5. Absolute Neutrophil Counts. (n = 2-8/group). Neither mTBI (B-24h and B48h) nor TT (C-24h and C-48h) (non-pneumonia groups) affected Neutrophil counts significantly compared to the Naïve group (D) at any time point. All unpaired, two-tailed t-tests- (Naïve vs. TT-24h- p=0.0645), (Naïve vs. mTBI-24h- p=0.8820), (Naïve vs. TT-48h- p=0.4328), (Naïve vs. mTBI-48h- p=0.1878).

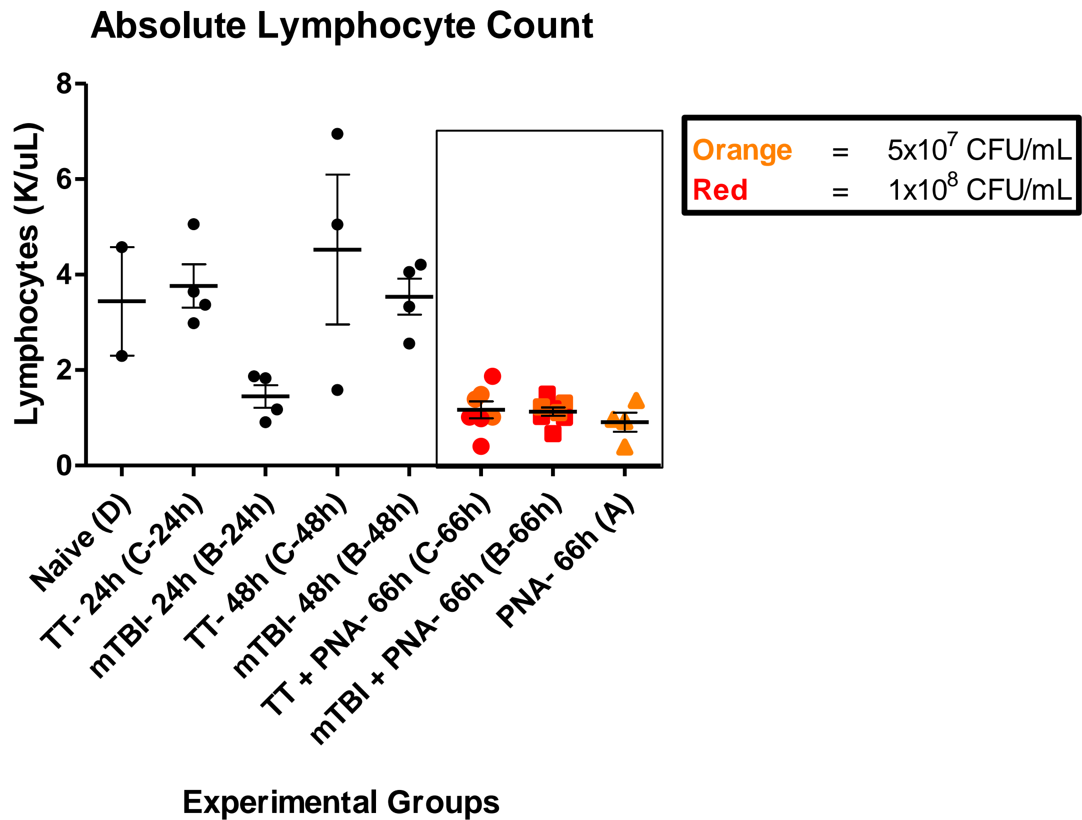

Figure 6. Absolute Lymphocyte Counts. (n = 2-8/group). Mild traumatic brain injury (mTBI) and tail trauma (TT) had varying effects on lymphocyte levels. Neither mTBI (B-24h and B48h) nor TT (C-24h and C-48h) (non-pneumonia groups) affected Lymphocyte counts significantly compared to the Naïve group (D) at any time point. All unpaired, two-tailed t-tests- (Naïve vs. TT-24h- p=0.7573), (Naïve vs. mTBI-24h- p=0.0640), (Naïve vs. TT-48h- p=0.6553), (Naïve vs. mTBI-48h- p=0.9169).

Challenge with *Pseudomonas* pneumonia (PNA-66h) (A) in the absence of either type of injury *significantly* decreased Lymphocyte counts compared to the Naïve control group (D) (p=**0.0291**).

The (TT + PNA-66h) (C-66h) and (mTBI + PNA-66h) (B-66h) groups had Lymphocyte counts *significantly* lower than the Naïve group (D): (p=**0.0068** and p=**0.0014**, respectively). This is consistent with known effects on lymphocyte counts as a result of severe sepsis.

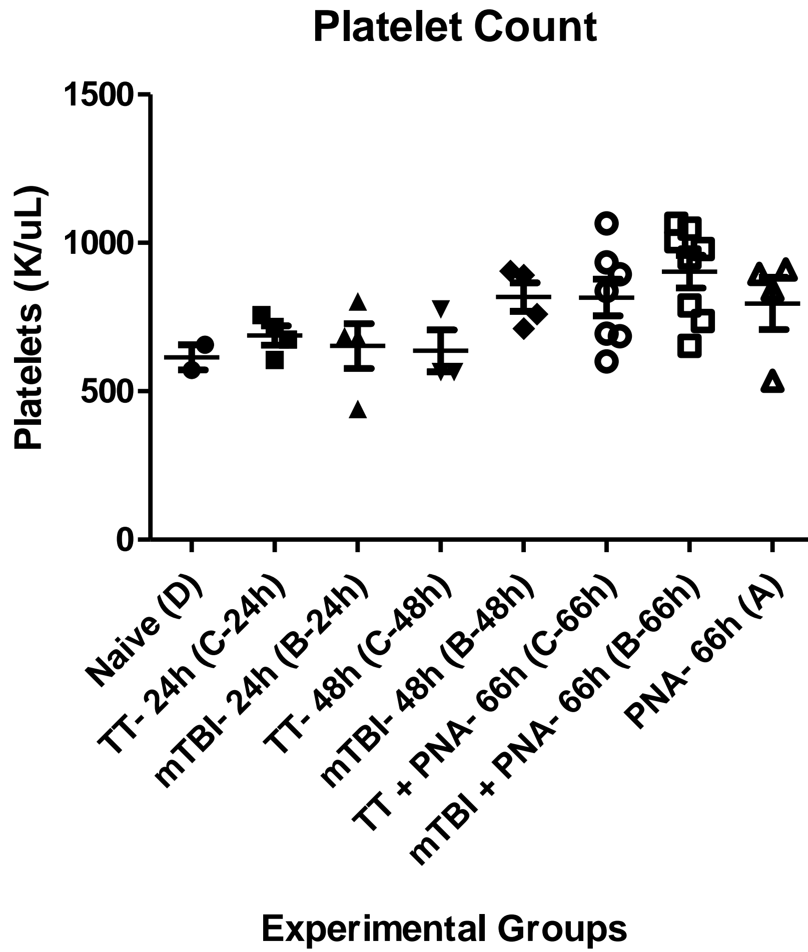

Figure 7. Platelet Counts. (n = 2-8/group). Circulating platelet counts did not change significantly between experimental groups.

Fibrinogen

Fibrinogen concentrations are inversely proportional to the results obtained from thrombin clotting time (TCT) assay. TCT results from the unknown mouse plasma samples were compared to a standard curve obtained by assaying varying dilutions of normal pooled plasma with known fibrinogen concentrations.

Mild traumatic brain injury (mTBI) at both the 24 hour (B-24h) and 48 hour (B-48h) time points increased circulating fibrinogen levels in plasma relative the control (naïve) group (D). Tail trauma (TT) did not significantly increase fibrinogen levels as compared to the control at either the 24 hour (C-24h) or the 48 hour (C-48h) time points.

Challenge with *Pseudomonas* pneumonia alone (A) decreased fibrinogen levels in the low (1x10^7) CFU/mL group but raised fibrinogen levels in the medium (5x10^7) CFU/mL group compared to the naïve group (D). All of the mice in the high (1x10^8) CFU/mL died prior to 18 hour endpoint and samples were unable to be collected from them.

Both the (TT + PNA-66h) (C-66h) and the (mTBI + PNA-66h) (B-66h) groups had fibrinogen levels higher than mice challenged with pneumonia only (PNA-66h) (A). mTBI had the greatest effect on raising fibrinogen levels in mice challenged with the low (1x10^7 CFU/mL) of *Pseudomonas* (B-66h).

Increased fibrinogen levels observed in the mTBI mice during pneumonia (B-66h) may be a result of either an earlier (or better) acute phase response, or possibly due to less coagulation stimulus and therefore less consumption of fibrinogen.

	Fibrinogen Range (mg/dL)	Fibrinogen Average (mg/dL)
Naïve (t0) (D)	160-210	197
Tail Trauma-24h (C-24h)	220-240	230
mTBI-24h (B-24h)	340-510	395
Tail Trauma- 48h (C-48h)	180-230	210
mTBI- 48h (B-48h)	170-410	315
TT/PNA- 66h (C-66h)	310-480	421
mTBI/PNA- 66h (C-66h)	350-845	462
PNA only- 66h (A)	80-590	249

Table 2. Summary of Fibrinogen Results. (n = 3-12/group). Data ranges and averages of the fibrinogen concentrations calculated from the thrombin clotting time (TCT) assay.

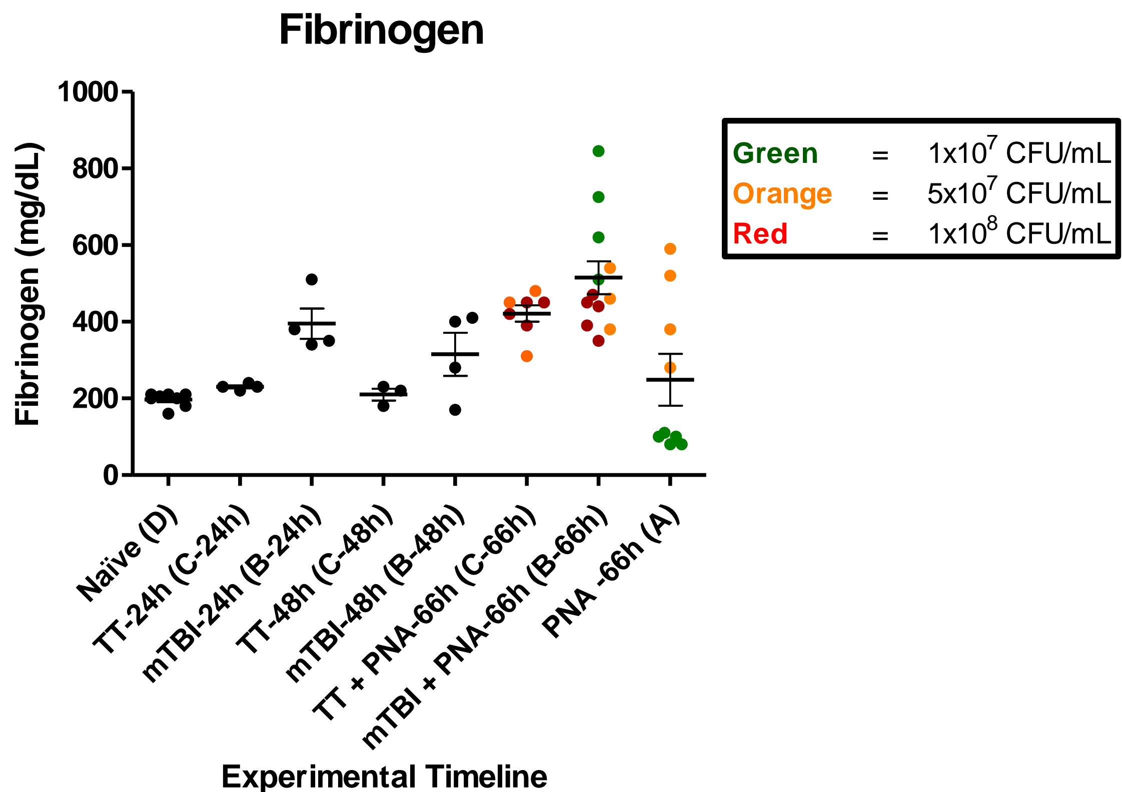

Figure 8. Fibrinogen Concentrations. (n = 3-12/group). Mild traumatic brain injury (mTBI) but not tail trauma (TT) at both the 24h and 48h time points (B-24h and B48h, C-24h and C-48h, respectively) *significantly* raised fibrinogen levels compared to Naïve mice (D): all unpaired, two-tailed t-tests- (Naïve vs. TT-24h- p=0.2241), (Naïve vs. mTBI-24h- p=**0.0019**), (Naïve vs. TT-48h- p=0.3671), (Naïve vs. mTBI-48h- p=**0.0002**).

The (TT + PNA-66h) (C-66h) and the (mTBI + PNA-66h) (B-66h) groups had fibrinogen levels *significantly* higher than the naïve group (D). Unpaired, two-tailed t-tests- (TT + PNA-66h vs. Naïve- p=**0.0077**), (mTBI + PNA-66h vs. Naïve- p=**<0.0001)**

The (TT + PNA-66h) (C-66h) group had fibrinogen levels *significantly* higher than those of the pneumonia-only group (PNA-66h) (A). The (mTBI + PNA-66h) (B-66h) group also had higher fibrinogen levels compared to the (PNA-66h) group (A) but it was not significant. Unpaired, two-tailed t-tests: (TT + PNA-66h vs. PNA-66h- p=**0.0060**), (mTBI + PNA-66h vs. PNA-66h- p=0.3286)

D-dimer

D-dimer is a well-established clinical biomarker of fibrinolysis. Mild traumatic brain injury (mTBI) and tail trauma (TT) at both the 24 hour and 48 hour time points (B-24h and B48h, C-24h and C-48h, respectively) did not significantly affect D-dimer concentrations compared to the control (naïve) group (D).

Challenge with *Pseudomonas* pneumonia alone (A) approximately doubled circulating D-dimer levels in both the low (1x10^7) CFU/mL and medium (5x10^7) CFU/mL groups compared to the control (naïve) group (D). All of the mice in the high (1x10^8) CFU/mL pneumonia-only group (A) died prior to the 18 hour endpoint and samples were unable to be collected from them.

Both mTBI (B-66h) and TT (C-66h) raised D-dimer levels when challenged with pneumonia compared to the pneumonia-only control group (A) and the naïve control group (D)

mTBI may confer increased resistance to pneumonia by a mechanism related to fibrinolysis. It has been proposed that mTBI may do this by preventing excess thrombus formation during pneumonia/septic-induced disseminated intravascular coagulopathies (DIC) by sustaining appropriate levels of fibrinolysis.

	D-dimer Range (ng/mL)	D-dimer Average (ng/mL)
Naïve (t0) (D)	0.831-30.380	12.648
Tail Trauma-24h (C-24h)	2.759-19.224	8.129
mTBI-24h (B-24h)	7.024-23.894	14.855
Tail Trauma- 48h (C-48h)	3.457-10.008	7.723
mTBI- 48h (B-48h)	4.347-16.598	11.409
TT/PNA- 66h (C-66h)	20.713-44.013	33.518
mTBI/PNA- 66h (C-66h)	18.020-81.811	47.377
PNA only- 66h (A)	13.690-58.290	27.321

Table 3. Summary of D-dimer Results. Data ranges and averages of the D-dimer values obtained from the D-dimer ELISA.

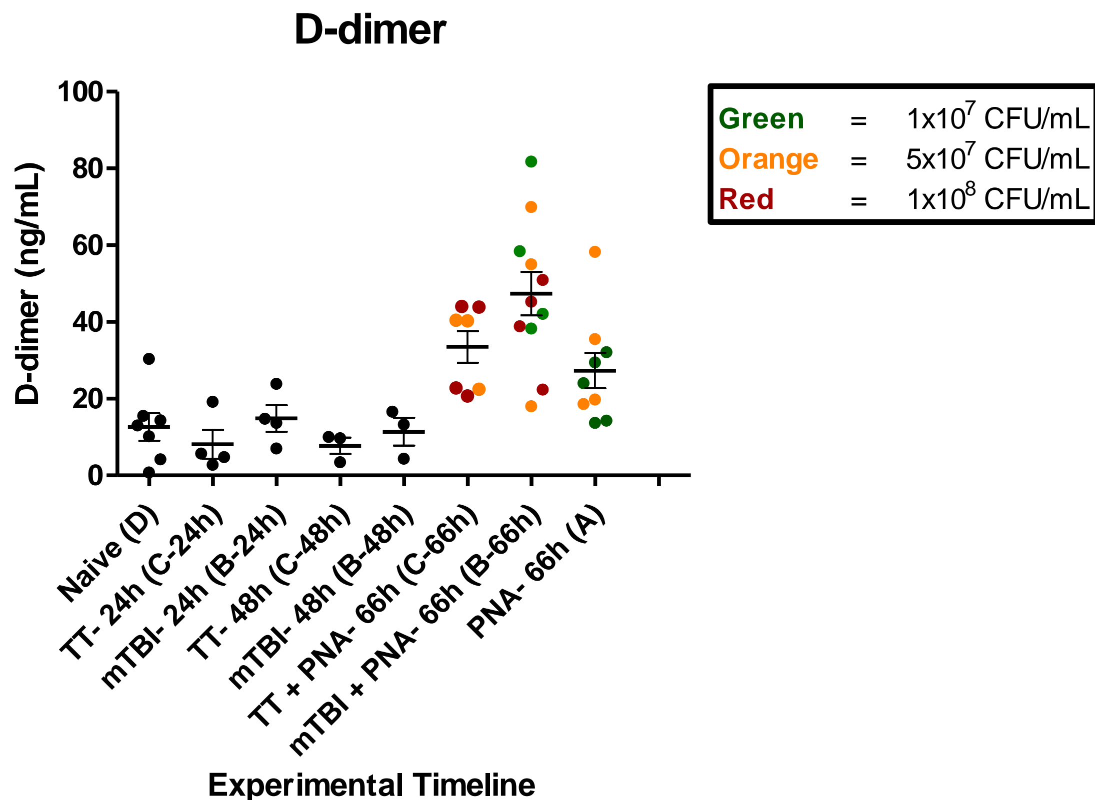

Figure 9. D-dimer Concentrations. (n = 3-11/group). Neither mTBI nor TT (non-pneumonia groups) (B-24h and B48h, C-24h and C-48h, respectively) affected D-dimer concentrations significantly compared to the Naïve group (D) at any time point. All unpaired, two-tailed t-tests- (Naïve vs. TT-24h- p=0.7471), (Naïve vs. mTBI-24h- p=0.6458), (Naïve vs. TT-48h- p=0.2741), (Naïve vs. mTBI-48h- p=0.6779).

Challenge with *Pseudomonas* pneumonia (PNA-66h) (A) in the absence of either type of injury raised circulating D-dimer levels but was not different from naïve control group (D) (p=0.3682).

Both the (TT + PNA-66h) (C-66h) and the (mTBI + PNA-66h) (B-66h) cohorts had D-dimer levels higher than the Naïve group (D): (p=0.7489 and p=0.1071).

PAI-1

Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor (serpin) that serves to proteolytically inhibit tissue plasminogen activator (tPA) and urokinase (uPA). tPA and uPA are both activators of plasminogen, the primary coagulation factor involved in fibrinolysis. Therefore, PAI-1 inhibits fibrinolysis and elevated PAI-1 can be a risk factor for thrombi formation.

During sepsis, levels of PAI-1 are found to be positively correlated with the severity of disseminated intravascular coagulopathy (DIC).[18] High PAI-1 levels indicate less fibrinolysis and that thrombi are not being broken down, potentially resulting in ischemia and microvascular hemolysis. Theoretically, PAI-1 levels are negatively correlated with D-dimer levels since inhibition of fibrinolysis by PAI-1 should decrease the levels of fibrin-degradation products (FDPs). However, since primary, secondary, and tertiary hemostasis are all occurring at much higher levels in DIC, it is likely that D-dimer concentrations would still be elevated even in the presence of high PAI-1 levels. This is a likely explanation for the results obtained in this experiment.

Either type of trauma (TT or mTBI) (C-24h and C-48h, B-24h and B48h, respectively) did not significantly affect PAI-1 levels compared to the naïve control group (D). All three mice groups that were challenged with pneumonia (A, B-66h, and C-66h) had PAI-1 levels significantly higher than the naïve control group (D). Both TT and mTBI along with penumonia (C-66h and B-66h, respectively) reduced PAI-1 levels

compared to pneumonia challenge only group (A). mTBI (B-66h) lowered PAI-1 levels more than TT (C-66h) in the pneumonia challenge groups.

These results indicate that mTBI may be lessening the severity of DIC during pneumonia via a mechanism related to sustaining fibrinolytic pathways.

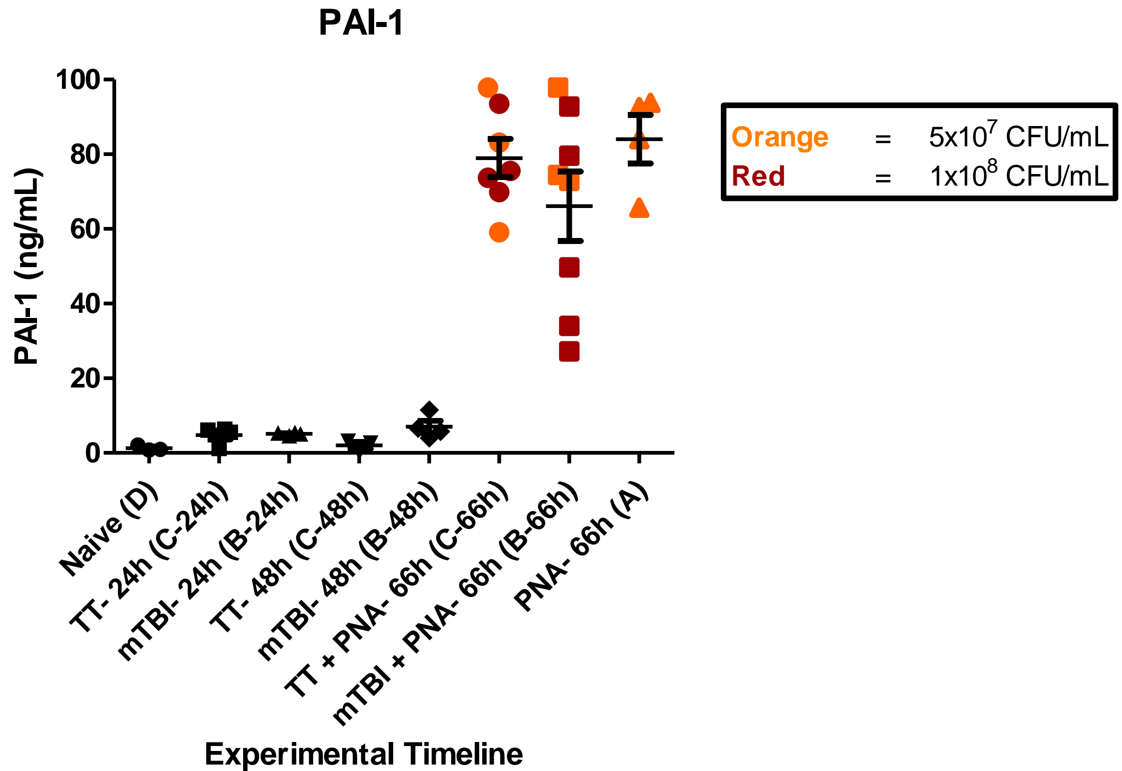

Figure 10. PAI-1 Concentrations. (n = 3-8/group). Neither mTBI nor TT (non-pneumonia groups) (B-24h and B48h, C-24h and C-48h, respectively) significantly affected PAI-1 concentrations compared to the Naïve group (D) at any time point. All unpaired, two-tailed t-tests- (Naïve vs. TT-24h- p=0.1608), (Naïve vs. mTBI-24h- p=0.3417), (Naïve vs. TT-48h- p=0.3244), (Naïve vs. mTBI-48h- p=0.0942).

Challenge with *Pseudomonas* pneumonia (PNA-66h) (A) in the absence of either type of injury *significantly* raised circulating PAI-1 concentrations compared to the Naïve control group (D) (p=**0.0061**).

Both the (TT + PNA-66h) (C-66h) and the (mTBI + PNA-66h) (B-66h) cohorts had PAI-1 levels *significantly* higher than the Naïve group (D): unpaired, two-tailed t-tests- (p=**0.0056** and p=**0.0015**, respectively).

DISCUSSION

Consistent with earlier, published data from the Remick laboratory, mTBI rescued mice from otherwise lethal pneumonia induced with a high concentration of *Pseudomonas aeruginosa*. This model enabled us to characterize the hemostatic changes that occur when mice are subjected to mTBI prior to induction of pneumonia. As a result of mTBI, overall fibrinogen levels and fibrinolysis appeared to be higher, indicating a blunting of the consumptive coagulopathy compared to the pneumonia mice alone. Most of the data showed trends, rather than statistical differences, but were consistent in their overall effect. In particular, PAI-1 values trended toward lower values in the mTBI + pneumonia group (B-66h) (compared to pneumonia or TT + pneumonia, C-66h), even with only 3-4 animals/group. Addition of more animals per group and evaluation of a time course of responses will likely greatly increase data confidence and identify real differences.

It was also shown that both mTBI and blunt trauma were able to sustain circulating Neutrophil concentrations in mice challenged with pneumonia (B-66h and C-66h, respectively) compared to mice challenged with pneumonia only (A). Thus, enhancing innate immune function may be another mechanism by which trauma is able to rescue mice from pneumonia. This is consistent with prior data published with the model.[23]

It was recently shown in mice challenged with pneumonia that mTBI increased neutrophil recruitment to the airways resulting in more effective bacterial clearance and improved survival.[32] It has also been shown that burn injury causes activation of Neutrophils as determined by Mac-1 expression, superoxide generation, and bactericidal activity.[20] Our results may corroborate these findings because in both instances elevated neutrophil levels are correlated with lower mortality from pneumonia. Furthermore, the decrease in mortality seen in mice who were challenged with mTBI prior to pneumonia challenge correlates well with the meta analysis performed by The National Trauma Data Bank which demonstrated that patients who experienced traumatic brain injury has a significantly lower rate of hospital acquired pneumonia following admission.[9]

Our experiment showed that mTBI or injury in general may confer increased resistance to pneumonia by a mechanism related to coagulation. We demonstrated that fibrinogen levels are higher (less consumption) in mice subjected to trauma prior to pneumonia challenge compared to mice only challenged with pneumonia. These findings correlate with what is known about fibrinogen and clotting times as prognostic markers for DIC. The thrombin clotting time (TCT) assay we used involves the direct conversion of fibrinogen to fibrin by the addition of thrombin, thereby forcing in vitro clot formation to depend solely on the concentration of fibrinogen in the sample. Because fibrinogen is the limiting coagulation factor in this reaction a TCT is useful for identifying low levels of fibrinogen (hypofibrinogenemia or afibrinogenemia) and/or abnormal fibrinogen function (dysfibrinogenemia). Therefore, a TCT and the related PT and APTT assays are

useful for diagnosing and prognosticating conditions such as disseminated intravascular coagulation (DIC) where a fibrinogen levels are low due to excessive coagulation.

Other coagulation-related changes demonstrated in this experiment include an elevation in D-dimer following TBI/pneumonia (B-66h) and tail trauma/pneumonia (C-66h) compared to the pneumonia-only mouse group (A). An elevated D-dimer, in the context of DIC, indicates that fibrinolysis is occurring and may offer a positive prognosis due to it being evidence of clot dissolution and exodus from a thrombotic crisis.

Mice subjected to mTBI and pneumonia (B-66h) in this experiment showed a trend toward PAI-1 levels lower than mice subjected to either tail trauma and pneumonia (C-66h) or pneumonia only (A). PAI-1 is a potent inhibitor of fibrinolysis due to its proteolytic activity on tissue plasminogen activator (tPA) and urokinase (uPA), two activators of plasminogen, the primary enzymes of fibrinolysis.[13] It has been shown that PAI-1 levels correlate with a poorer prognosis during DIC because of its inherent ability to prevent clot dissolution. In this experiment, a trend toward lower PAI-1 in both of the injury/pneumonia groups (B-66h and C-66h) compared to the pneumonia-only group (A) suggests that a blunted coagulation response may contribute to the survival benefit in the mTBI mice.

Irrespective of the limitations of this study it is indeed true that trauma has a profound effect on resistance to pneumonia as well as on coagulation; the mechanism of which is still unknown. Investigation of cytokine levels in serum, flow cytometry analysis of leukocyte subpopulations, neutrophil activation, as well as well as histologic analysis

of the brain may shed some light on how much of this phenomenon is related to inflammation or changes in brain structure, respectively. Additionally, investigation of more coagulation parameters including protein C and coagulation factor levels may allow us to glean more insight into what exactly is occurring on the level of coagulation.

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CURRICULUM VITAE

Evan C. Catudal

256 Doyle Avenue, Providence, RI
(401) 339-3530
eccatudal@gmail.com
Date of Birth: July, 1992

EDUCATION

BOSTON UNIVERSITY SCHOOL OF MEDICINE

Master of Science in Pathology and Laboratory Science (2014-2016)

- **Curriculum-** A thesis-based curriculum with coursework focused on advanced immunology and pathology with both clinical and research applications. Coursework also included several classes relating to the regulation of both clinical and pre-clinical biopharmaceutical development as well as a course in computer-based biostatistics.
- **Research**- I investigated how mild traumatic brain injury (mTBI) altered coagulation response to bacterial-induced pneumonia in a mouse model, potentially contributing to a survival benefit. This research was performed in in the laboratory of Dr. Shinichiro Kurosawa and Dr. Deborah Stearns-Kurosawa.
- **Thesis Title-** *Mild Traumatic Brain Injury (mTBI) Alters Pneumonia-Induced Coagulopathy in Mice.*

UNIVERSITY OF MASSACHUSETTS DARTMOUTH

Bachelor of Science in Medical Laboratory Science (2010-2014)

- **Clinical Internship** (Jan. 2014-Apr. 2014) - Completed a semester-long internship and training program in the clinical pathology department Tufts Medical Center in Boston, MA. Rotations included Hematology, Microbiology, Clinical Chemistry, Andrology, Blood Banking, Flow Cytometry, HLA & Tissue

Typing, Cytopathology, Surgical Pathology, and Phlebotomy. *This internship resulted in my being offered a job there.*

- **Senior Case Study Project**- *A Case Study of MDS/MPN Syndrome with AML Progression*

PROFESSIONAL EXPERIENCE

TUFTS MEDICAL CENTER — Boston, MA

Blood Bank, Transfusion, and Stem Cell Laboratory

Medical Technologist — **May 2014-Present**

- Responsibilities include accessioning and testing blood samples and human blood product derivatives. Tests include ABO/Rh typing, direct & indirect antiglobulin testing, RBC phenotyping, antibody elutions and adsorptions, antibody identification, and antibody titers.
- Other responsibilities include blood product processing, storage, preparation, and irradiation, communicating blood bank policy and recommendations to clinical pathology residents, physicians, and nurses, instrumentation QA/QC, writing and implementing new protocols, procedures, and software, and ensuring laboratory compliance with regulatory agencies.
- Assisted in the implementation of a new laboratory information system (LIS) -a multi-year endeavor- and was integral in ensuring a smooth transition from our outgoing system during go-live in August, 2015.

BRISTOL COMMUNITY COLLEGE — Fall River, MA

Learning Resource Center

Library Assistant — **May 2011-July 2014**

- Responsibilities included providing circulation and reference assistance to patrons and college students as well as managing library inventory using complex software.

- Served as a technical resource for students, staff, and guests for programs such as Microsoft Word, Excel, and PowerPoint as well as internet browsers such as Internet Explorer, Mozilla Firefox, and Google Chrome.
- Assisted in the revision of policies and procedures in compliance with appropriate Federal and State mandates.
- Managed the daily operations of the college library in the absence of the reference librarian.

THE COMPUTER DOCTORS, LLC **Tiverton, RI**
Computer Technician **December 2008-2010**

- Responsibilities included building, diagnosing, and repairing customer computers both physically and remotely using *VMware* software.
- Assisted in small business network installation as well as website development and maintenance.

BLACK GOOSE CAFÉ **Tiverton, RI**
Savory Food and Pastry Cook **July 2007-May 2010**

- Responsibilities included preparing large volumes of pastries and savory dishes, operating a moderately sized restaurant kitchen, conducting financial transactions with patrons and suppliers, as well as opening and closing of the restaurant on weekends.

BATTLESHIP COVE **Fall River, MA**
Function Caterer **February 2006-August 2007**

- Responsibilities included the preparation and serving of food for functions and events exceeding 1000 patrons on the Battleship USS Massachusetts.

CERTIFICATIONS & LICENSURE

- Medical Laboratory Scientist (*American Society for Clinical Pathology*)- May 2014
- Phlebotomist (*American Society for Clinical Pathology*)- May 2014

PROFESSIONAL AFFILIATIONS

- American Society for Clinical Laboratory Scientists (*ASCLS*): *ASCLS-CNE* (Central New England) Student Member
- American Society for Clinical Pathology (*ASCP*)- Medical Laboratory Scientist
- College of American Pathologists (*CAP*) – Medical Laboratory Scientist

HONORS & AWARDS

- Chancellor's Merit Scholarship- University of Massachusetts Dartmouth
- Honors Society- University of Massachusetts Dartmouth
- Member of the Delta Mu Chapter of *Lambda Tau,* The National Academic Honor Society of Medical Laboratory Science