GENERATION OF CLINICALLY RELEVANT MODELS OF BURN-ASSOCIATED COMORBIDITIES FOR ANALYSIS OF IMMUNE DYSFUNCTION AFTER BURN INJURY

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ABSTRACT

Laurel Brianne Kartchner: Generation of clinically relevant models of burn-associated comorbidities for analysis of immune dysfunction after burn injury
(Under the direction of Bruce A. Cairns and Robert Maile)

Burn injury is a significant form of trauma that leads to alterations in the functionality of multiple body systems. One vital system that promotes both healing and protection from invasive pathogens is the immune system. After burn injury the immune system is severely impeded. However, models of burn injury are unable to successfully recapitulate phenotypes seen among burn-injured patient populations. Studies indicate that presence of burn-associated comorbidities can greatly improve the translatability of models and that study of comorbidities is essential for improving treatment of patients.

Here we report the development of a model of repeated bacterial exposure after burn injury. This model is able to recapitulate immune cell recruitment and alterations in cytokine production that mimic those seen among patient populations. Namely, we found that repeated infections lead to increased bacterial burden after burn injury, and the loss of a lab-derived burn "protection" phenotype that is common in the literature late after burn injury. We found that this phenotype corresponds with burn-dependent alterations in pulmonary innate immune cell numbers and function. We believe that these cells represent a potential target for therapeutic intervention.

Additionally, we have worked to establish a model of inhalation injury to examine inhalation-dependent alterations in the immune profile that take place both independently and concomitant with burn injury. We have found that inhalation and burn injury independently contribute to damage in our murine model of inhalation. We examined the pulmonary compartment and found that burn and inhalation independently affect the recruitment of neutrophils to either the airspace or the lung tissue. We also found that inhibition of nitric oxide production can ameliorate damage that takes place after inhalation injury, representing a potential target for therapeutic intervention among the patient population.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

RONS	Reactive oxygen and nitrogen species
NOS	Nitric oxide species
PAMP	Pathogen associated molecular pattern
DAMP	Damage associated molecular pattern
IL	Interleukin
SIRS	Systemic inflammatory response syndrome
CARS	Compensatory anti-inflammatory response syndrome
MARS	Mixed antagonistic response syndrome
CFU	Colony forming units
DHR	Dihydrorhodamine
FBS	Fetal bovine serum
LB	Luria broth
MFI	Mean fluorescence intensity
NO	Nitric oxide
PAK	*Pseudomonas aeruginosa* PAK strain
TBSA	Total body surface area
TLR	Toll-like receptor
CXCL	CX chemokine ligand
CCL	C chemokine ligand
PBMC	Peripheral blood mononuclear cell
ARG	arginase

CHAPTER 1: INTRODUCTION

Each year approximately 486,000 individuals experience a burn injury requiring medical treatment [1]. These patients have an extended hospital stay relative to other forms of trauma, resulting in a treatment that is not only difficult and lengthy, but costly [2]. The average treatment cost per burn patient can exceed $88,000 USD [3]. One leading cause of lengthened hospital stay after burn injury is the presence of a burn-associated comorbidity. Patients who present with comorbidities of burn injury often require additional therapeutic intervention and experience complicated clinical outcomes relative to other patients [4].

When a patient experiences a significant burn injury, the wound tissue releases damage-associated molecular patterns (DAMPS) that can alter many systems throughout the body [5]. One major compartment affected by DAMPS is the immune system. Burn injury is known to lead to significant systemic immune dysregulation [5-8]. DAMPS released into the body lead to an overall systemic inflammatory response syndrome (SIRS), in which the immune system becomes activated in response to damage [9, 10]. Immune dysregulation and activation after burn injury is often typified by the presence of altered levels of cytokines, chemokines, and immune cells [11].

In the case of significant trauma such as burn injury, damaged tissue causes the release of a cytokine storm, which not only activates the SIRS response, but also triggers an overall compensatory anti-inflammatory immune response (CARS) [12]. Historically the SIRS and

CARS responses were thought to be biphasic in nature, with a SIRS phenotype arising early after injury and a CARS response characterizing the late response to injury [13]. However, recent developments in the field indicate that typical injury results in the simultaneous initiation of both the SIRS and CARS response, with a different phenotype overriding the overall response at any given time. The ability to detect simultaneous presence of both pro- and anti-inflammatory mediators of immunity has been designated a mixed antagonist response syndrome (MARS) [14-21]. Although the MARS phenotype more fully encompasses the immune responses that take place after injury, it is possible to detect overriding immune responses characterized as being largely SIRS or CARS at any given timepoint after injury. However, it is important to emphasize the complexity of the immune response during the treatment and recovery of patients due to the presence of both pro-and anti-inflammatory signals.

As previously mentioned, immediately after burn injury there is an overwhelming shock response that is characterized by the presence of DAMPS, cytokine storm, and abnormal immune cell profiles which are characteristic of SIRS. In order to study this early response and improve interventions available for burn patients many researchers have generated animal models that characterize the SIRS response early after burn injury [10, 22-26]. However, very few studies are able to generate an animal model that recapitulates the CARS phenotype seen among the human population. In fact, many researchers have actually generated data indicating that late after injury, burned animals are more capable of mounting a pro-inflammatory response and clear a bacterial infection than their sham counterparts [27-33]. This phenomenon can be ablated through elimination of various immune cell compartments [27, 29], however, this phenomenon represents a significant flaw in the application and use of animal models for the study of the immune response after significant trauma. It is essential that scientists utilize animal models that

are successfully recapitulate phenotypes seen among the patient population, and to that end modifications of models currently utilized are necessary.

There are major markers that have been identified as being key and typical of patient responses after injury. Specifically, we have previously demonstrated that aberrant production of IL-10 from several cell types, including macrophages and neutrophils, are likely a major factor contributing to the immunopathology responsible for susceptibility to infection [16, 22, 34, 35], whereby our group has indicated the prognostic potential of IL-10/IL-12 protein ratios within bronchial washes on predicting lung and systemic infections following severe burn trauma (Figure 1.1, [16, 17, 22]). Recent gene expression analysis of peripheral blood mononuclear cells (PBMCs) from burn patients has shown a strong correlation between increased IL-10, increased arginase 1 (ARG1), reduced IL-12 and reduced nitric oxide synthase 2 (NOS2) expression with burn severity and susceptibility to bacterial infection. As burn injury generates numerous inflammatory stimuli including DAMPs that activate innate immune cells [17, 36], inducing/regulating pro- and anti-inflammatory responses, our group has also defined a profile of DAMPS that can predict the immune suppression observed [17] .

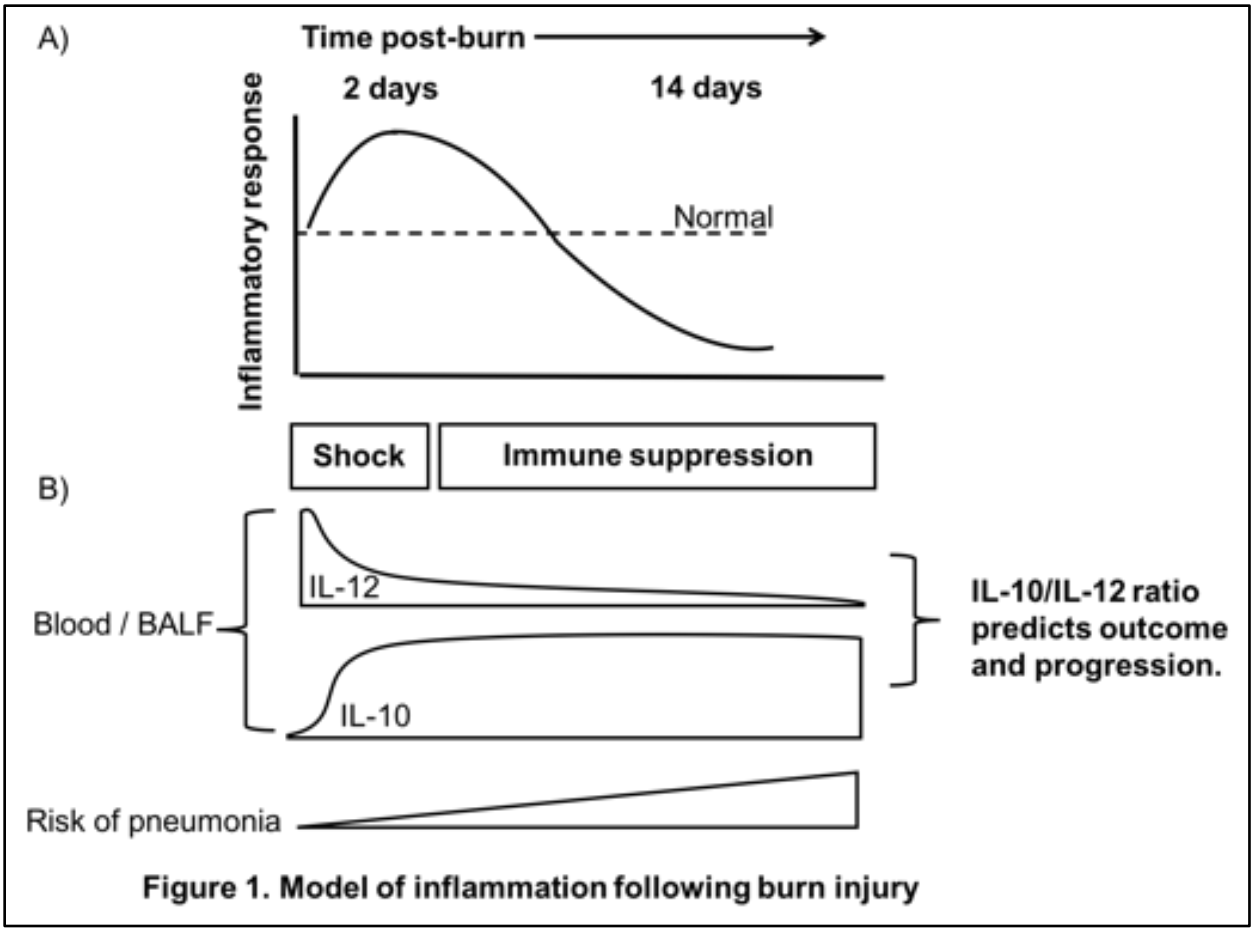

Figure 1. Model of inflammation following burn injury

When patients present at the hospital with a burn injury, their treatment is often complicated due to the presence of a burn-associated comorbidity. Multiple studies have shown that the presence of a burn-associated comorbidity further alters the innate and adaptive immune responses to burn injury [37-43]. Additionally, research indicates that inclusion of comorbidities associated with burn injury eliminates the burn-mediated "protective" response late after injury and more closely recapitulates phenotypes seen among the human population [37, 38, 44]. Research focused on human patients ensures the translational relevance of any results found and is important for understanding the variables that lead to disease progression. However, animal models are essential for understanding the underlying cellular and molecular mechanisms of

immune-mediated disease progression. Animal models permit the study of systems that are inaccessible in human patients and also allow for testing of various potential therapeutic targets to improve overall outcomes of disease and/or injury. Therefore, improved models, such as those that include comorbidities of burn injury are necessary in order to improve understanding of burn-mediated immunopathology.

Burn and Inhalation injury

Approximately 10-20% of all burn patients present to the hospital with a burn-associated inhalation injury [45]. Presence of an inhalation injury can cause a 20% increase in mortality among burn patients, significantly lengthen hospital stay, and result in increased incidence of bacterial infection [46-48]. In fact, inhalation injury is a leading cause of burn-associated mortality [49, 50]. Many models of inhalation injury have been generated, however few improvements in treatment of patients with inhalation injury have been achieved [45]. Additionally, while multiple studies have examined the immune-mediated effects of inhalation injury [51-53], few have examined the combined effects of burn and inhalation injury in spite of the fact that combined injury results in damage more severe than can be attributed to each injury alone [39].

Studies have indicated that presence of severe inhalation injury leads to increased levels of multiple immune-related cytokines in collected bronchoalveolar lavage fluid (BAL), specifically increased levels of interleukin-8 (IL-8), IL-4, IL-6, IL-9, IL-15, interferon-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte chemotactic protein-1 (MCP-1) [54]. Additionally, multiple studies have demonstrated that presence of inhalation injury is strongly associated with increased numbers of neutrophils collected from

BAL [54-58]. Presence of neutrophils in BAL after inhalation injury has additionally been associated with overall immune dysfunction and susceptibility to bacterial infection [59-61]. Neutrophil presence has additionally been associated with protein deposition in the airway, and associated pulmonary damage [60, 62, 63].

Neutrophils present in the lungs after inhalation injury release proteases, oxidants, and their downstream products such as nitrate and nitrite, which can lead to significant damage to the lungs [64, 65]. Multiple studies have detected indicators of oxidative stress in the pulmonary compartment after inhalation injury [45, 66, 67], and that inhibition of molecules involved in production of oxidants such as nitric oxide synthase (NOS) can lead to improved outcome after inhalation injury [68, 69]. Indeed, inhibition of inducible NOS (iNOS) has been proposed and tested as a potential therapeutic treatment of inhalation injury in ovine models [70, 71]. It has been reported that multiple studies are currently examining the therapeutic potential of inhibition of NOS, modulators of reactive nitrogen species, and antioxidants in treatment of inhalation injury [72]. These studies are important in order to be able to generate potential improvements in treatment of inhalation injury.

Burn and infection

Patients who are admitted to the hospital generally experience a shift in their overall microbiota and develop a profile similar to that of both the nurses who treat them as well as the hospital environment [73, 74]. Due to the nature of burn injury, burn patients experience barrier dysfunction resulting in wounds that commonly become infected [75]. Burn wounds also lead to significant immunosuppression in patients that can allow infections to develop into sepsis, central line infections, and pneumonia [8, 11, 76-79]. In fact, the leading cause of mortality

among burn patients is the presence of a bacterial pneumonia [80], and studies have indicated that repeated infectious episodes significantly increase mortality among patients [81]. In order to decrease infection-associated mortality among burn patients it is essential that further studies be conducted.

Many studies have been conducted using mouse models to examine burn-dependent changes in the immune system and how these changes alter the response to bacterial infection [22, 82-84]. Animal models have been successful at recapitulating immunosuppression and susceptibility to bacterial infection early after burn injury [85-91]. Various labs have sought to identify potential vaccines and treatments that could generate bacterial-specific immune responses to improve outcomes in the face of infection. Multiple groups have applied bacterial byproducts such as flagellin or PilA from *Pseudomonas aeruginosa* to generate vaccines to decrease bacterial burden and associated mortality after burn injury [92-94]. Additional groups have focused instead on altering host responses in order to promote a response that will result in increased clearance of bacterial presence and improve outcomes. Burn injury leads to significantly altered metabolism among patients [76, 86], which is known to alter the immune profile [95, 96]. In order to improve the overall immune response some labs have targeted host metabolic responses through insulin therapy to decrease overall systemic inflammation and promote bacterial clearance [97, 98]. Other labs have chosen to more directly target the immune response by administering granulocyte colony-stimulating factor (G-CSF) to improve survival after injury and promote clearance of bacteria [29].

Although murine models of burn injury are able to recapitulate immunosuppression early after burn injury, many labs have found that late after burn injury mice are protected from bacterial infection and have improved clearance relative to their sham counterparts [27, 28, 30,

31, 33]. Some labs postulate that this protection is largely due to the recruitment of neutrophils to the pulmonary vasculature after burn injury [27, 28], and have demonstrated that elimination of the neutrophil population using an antibody against Ly6G results in loss of this protective phenotype. Additional studies have indicated that the presence of burn and an additional comorbidity of burn injury results in the loss of the burn-associated protective phenotype as well [37, 38, 99].

Burn and alcohol

Ingestion of alcohol is known to impair judgement and is often associated with experiencing a trauma [100]. Therefore, patients who present to the burn center have a high probability of being intoxicated. Studies have indicated that intoxicated patients who present to the hospital with a burn injury have significantly increased mortality, frequent intubations, delayed healing, increased infectious complications and lengthy hospital stays relative to non-intoxicated patients [101-107]. Burn injury is known to alter metabolism of patients, an effect that is further exacerbated with alcohol consumption [108].

Analysis of the patient population indicates that intoxicated patients who present with burn injury span both chronic alcohol abusers and acute binge drinkers [109-112]. This indicates that animal models of both chronic and acute alcohol exposure are necessary to accurately reflect clinical outcomes. Animal studies have demonstrated that intoxication at the time of burn injury additionally leads to significant immune dysfunction within the splenic [113], intestinal [102, 114-116], adipose [117, 118], skeletal [108], pulmonary [119-121], vascular [122] and hypothalamic-pituitary-adrenal compartments [123]. Due to the systemic effects of alcohol intoxication it is thought that immune consequences of intoxication during burn injury are

systemic as well and lead to loss of protection and an inability to fight bacterial infectious agents. Alcohol exposure is known to alter levels of reactive oxygen and nitrogen species, glutathione metabolism, bone formation and wound healing [108], each of which is known to be involved in regulation of immune cell activity [124-127]. It is therefore not surprising that intoxication leads to alteration of the immune compartment through cellular mechanisms involving each of these pathways. Some groups postulate that modulation of these pathways through use of compounds such as glutathione precursor supplementation may improve negative consequences of burn injury during intoxication [108]. The immune compartment is a potentially powerful therapeutic target to promote improved healing and decreased mortality after burn injury while intoxicated [128].

Burn and ageing

Recent studies indicate that burn injury of the aged population is associated with worsened physical conditioning and quality of life after injury, and increased mortality [129-131]. Treatment strategies for older patients who have experienced a burn injury have not led to significant changes in length of hospitalization or mortality [132].

It has long been noted that elderly patients experience altered immune responses relative to young patients due to immunosenescence [133], a condition further complicated by the presence of a burn injury [134]. Elderly patients commonly have elevated levels of inflammatory cytokines, and upon experiencing a burn injury, aged patients will generate a stronger pro-inflammatory cytokine response than their younger adult-aged counterparts [134].

Animal studies comparing immune responses in mice of differing ages indicated that murine models are able to recapitulate age-dependent differences in cytokine responses to burn

injury [135-137]. These differences are related to alterations in immune cell phenotype and function and play an important role in cellular polarization in response to burn injury [138]. Age-dependent alterations in cytokine responses are associated with delays in wound healing, and imply that studies should examine therapies focused on decreasing the expression of these cytokines in order to promote healing [139, 140]. Multiple groups have found that in aged mice, alterations in the cytokine response to burn injury may lead to deficiency in some, but not all immune cell migration to the wound site, potentially altering progression of wound healing and preventing a typical healing response [141, 142], a response that leads to increased susceptibility to bacterial infection in aged mice [143]. Additional studies have indicated an important role of multiple molecular immune markers in promoting a healing response after burn injury, such as hypoxia inducible factor-1α (HIF-1α) which mediates migration of immune cells from the bone marrow to the site of injury to promote the healing response [142]. It is possible that immune mediators such as HIF-1α would be viable targets of immunotherapeutic treatment after burn injury. Additional studies indicate that cytokine therapy may potentially be successful in the treatment of elderly burn patients [144], however it is important to note that in an aged population cells do not respond as robustly to cytokines present [143].

Discussion

Patients who require treatment for burn injuries experience substantial immune dysregulation. Presence of a concomitant injury or complicated event associated with the burn injury can significantly disturb immune homeostasis and result in altered healing and poor outcomes among patients. Many studies have focused on burn-associated immune dysregulation, and the field is currently expanding to include research on additional comorbidities of burn

injury. In addition to the comorbidities discussed here, additional research is needed to study the combined effects of burn and radiation [41, 145, 146], the effects of sex in responses to burn [147, 148], burn associated with diabetes [85, 149, 150], and the presence of multiple simultaneous comorbidities [44].

The innate arm of the immune system is altered after burn injury, and comorbidities commonly promote further dysregulation of innate immunity. Multiple studies have indicated that immune cells of myeloid lineage are dysregulated in response to burn injury. Burn injury leads to altered recruitment of neutrophils to the pulmonary compartment, as well as altered hematopoiesis. Neutrophils are a powerful cell type that is responsible for protecting the body from bacterial insult[151]. Studies have shown that neutrophils are both necessary and sufficient to clear multiple bacterial pathogens [152, 153], and dysfunction of the myeloid arm of the immune system is commonly associated with comorbidities of burn injury.

In Chapters 2-4 we discuss unique models that we have generated in our laboratory to study burn and its associated comorbidities of inhalation injury and infection. We have utilized these models to assess potential molecular mechanisms involved in burn-associated diseases, focusing on the role that macrophages and neutrophils play in recovery after burn injury. In the future, we plan to utilize these models to test potential therapeutics for improved outcome after burn injury.

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CHAPTER 2: ONE-HIT WONDER: LATE AFTER BURN INJURY, GRANULOCYTES CAN CLEAR ONE BACTERIAL INFECTION BUT CANNOT CONTROL A SUBSEQUENT INFECTION[2]

Overview

Objective: Many studies have described the acute immune response to burn injury in both human and animal models, yet few studies are able to recapitulate long term immune suppression and bacterial susceptibility seen in burn patients. We hypothesized that patients suffer immune exhaustion due to repeated microbial challenge, therefore single challenge in animal models will not recapitulate this clinical phenotype. Herein we describe a novel mouse model of repeated infection after burn injury, which reveals immune suppression resulting in decreased pulmonary clearance of bacteria.

Methods: Wildtype mice receiving a full-thickness contact burn were infected with *Pseudomonas aeruginosa* 14 days and/or 17 days after burn or sham injury. Pulmonary macrophages and neutrophils were enumerated and evaluated for immune activation and

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function.

Results: In the double infection model, burn mice were unable to clear bacteria compared to sham injured or singly infected burn mice. After a second infection, neutrophils and macrophages recruited to the airways exhibited 1) reduced production of anti-bacterial reactive oxygen and nitrogen species, and 2) increased production of the anti-inflammatory cytokine interleukin-10 in the macrophage population compared to sham or singly infected burn mice.

Conclusions: In our model, burn mice exhibited immunosuppression with specific perturbations in their pulmonary innate cell populations. This phenotype is similar to previously reported clinical data and will allow further investigation into immune susceptibility of burn patients.

Introduction

Burn injury causes significant lengthy hospital stays among patients. Every year 486,000 patients in the United States seek medical attention for burn injury [1]. Burn injury commonly creates a significant wound that is slow to heal and causes significant immune dysregulation and immunosuppression. Patients that experience this immunosuppression often acquire additional associated complications such as colonization with nosocomial bacteria. Infection of burn patients is also commonly associated with increased morbidity and mortality; patients who have more than one incidence of bacterial infection during the course of their hospital stay can experience a 42% increase in mortality [2].

Bacterial pneumonia is a leading cause of mortality among burn patients [3]. One common source of bacterial pneumonia within the burn patient population is *Pseudomonas aeruginosa*, a gram-negative opportunistic infection [4, 5]. Full clearance of pseudomonal infections requires the activation and integration of the immune system [6, 7]. Studies have demonstrated that neutrophils are the primary innate immune cells responsible for preventing and clearing bacterial infections, and that a neutrophil response is both necessary and sufficient for clearance of *P. aeruginosa* infections [8].

Immediately after burn injury patients experience a general activation of the immune response, with one model defining this as early systemic inflammatory response syndrome (SIRS) [9-11]. This response is typically associated with cytokine storm, immune cell proliferation and systemic immune cell recruitment [12]. Continual activation of the immune system leads to activation of a late compensatory anti-inflammatory response syndrome (CARS) [13]. Although many studies have demonstrated and examined the SIRS response in both human and animal models [14-19], few studies are able to recapitulate the CARS response seen in the

human population using animal models. In fact, multiple studies in animal models indicate that after injury, burn mice are more capable of responding to infection than their sham counterparts [20-26]. Recent studies indicate that the SIRS/CARS paradigm may not accurately represent the complex immune response in burn patients because pro- and anti- inflammatory mediators are often detected simultaneously [27-32] and patients experience a mixed antagonist response syndrome (MARS) at all time points. We retain the SIRCS/CARS terminology for this study to define the "early" and "late" phases after injury and the immune bias of the MARS response after burn injury.

Additional studies indicate that SIRS and CARS among burn patients leads to release of immune cytokines and alterations in the immune profile, and that poor outcomes following infection within patient populations can be predicted by production of the cytokines interleukin 10 (IL-10) and interleukin 12 (IL-12) [30, 33-36]. Additionally, murine studies have indicated that these cytokines play an important role in burn-associated responses to bacterial infection [14, 34, 37, 38]. Researchers have demonstrated that treatments resulting in decreased IL-10 production after burn injury lead to increased bacterial clearance and improved outcome [14, 39, 40] and that current therapeutic targets exist capable of altering cytokine production after burn injury [36]. These findings indicate that IL-10 and IL-12 are important markers and potential targets for therapeutic interventions.

It is evident that there is an increased neutrophil presence in the lung vasculature early and late after burn injury [20-22]. Late after injury these neutrophils are part of the overall heightened immune response and can result in improved outcome in burn mice following single infection [20, 21], an effect that is lost with the elimination of the protective neutrophil population using anti-Ly6G antibodies [22]. These results present a unique case in which data

obtained from patients late after burn injury is not reflected in the animal model. We have additionally demonstrated that in the presence of burn-associated comorbidities such as irradiation or smoke exposure, infection with bacterial exposure can result in a loss of the protective effect of burn injury [41, 42].

In a nosocomial environment, the skin microbiota of patients commonly changes to match that of their environment and the nurses with whom they commonly interact [43, 44]. Burn wounds represent a disrupted barrier to the environment, and burn patients have a high incidence of infection (39% in our burn unit) due to large burn wounds and necessary surgery, resulting in subsequent immune dysregulation [45-47]. In contrast, mice utilized in experimentation are housed in specific-pathogen free environments and are protected from bacterial exposure. We hypothesized that the pulmonary neutrophil population present after burn injury creates a protective environment in which mice are prepared to respond to an initial pulmonary infection with *P. aeruginosa*. However, we believe that this population represents a finite resource and that once activated to respond to a bacterial insult this short-lived population is unable to replenish and mount continual protection to repeated exposure to bacteria. We additionally predict that these cells may play an important role in the production of IL-10 and IL-12 *in vivo*.

Material and methods

Animals

Female C57BL/6 mice aged eight to twelve weeks old, weighing >18 grams were purchased (Taconic Farms) and used for this study. Mice were shaved dorsally, given a subcutaneous injection of morphine (3mg/kg body weight, West-Ward Pharmaceuticals) and underwent a 20% total body surface area burn as previously described [14, 41, 48, 49]. A full-thickness contact burn was achieved using a 65 gram copper rod (1.9 cm in diameter) that had been heated to 100°C and applied to the dorsum/flank for four applications, each lasting 10 seconds. Animals were then placed in individual cages, given food and water ad libitum and monitored twice daily. All sham animals underwent the same procedure with the exception of the application of the burn injury. All animals were housed in a specific pathogen-free environment and all procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill in accordance with NIH guidelines for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Bacterial Inocula preparation and infection

A wildtype strain (PAK) of *P. aeruginosa* was utilized for all infections as previously described [14]. Bacteria from frozen cultures were grown overnight in Luria Broth (LB). The following morning cultures were diluted 50x and grown for approximately two hours until mid-log phase growth was achieved (OD_{600}=0.6-1.2). Cultures were then centrifuged at 14000 rpm for 30 seconds, and the pellet was washed using 1mL PBS+1% Protease Peptone solution (PBS+1%PP). Bacterial pellets were re-suspended and diluted to the desired concentration and

verified by optical density at 600nm. Bacterial concentration was then confirmed by serial dilution and plating on LB agar plates.

Mice infected intraperitoneally received a 1mL injection of *P. aeruginosa* at a concentration of $5x10^5$ CFU/mL. Uninfected mice were given an intraperitoneal injection with 1mL of PBS+1%PP as a control. Mice infected intratracheally were anesthetized with an intraperitoneal injection of Avertin (0.475mg/g body weight: Sigma-Aldrich). Mice were then placed on an intubation platform, and infected by visualization of the vocal cords with a laryngoscope (Model LS-2, Penn Century Inc.), and inserting a MicroSprayer® Aerosolizer (Model 1A-1C and FMJ-250 High-Pressure Syringe Penn Century, Inc.) through the vocal cords, after which a 50uL volume was aerosolized into their lungs using either a bacterial innocula ($2x10^7$ CFU/ML in PBS+1%PP) or vehicle (PBS+1%PP).

Enumeration of bacteria

At time of sacrifice, the left lobe of the liver, the lungs, and the spleen were removed and placed in 0.5mL of LB broth on ice. Tissues were homogenized using a BulletBlender (Next Advance) and three 3.2mm stainless steel beads per tube of tissue. Tissue homogenate was serially diluted and plated on LB agar for quantification. Plates were incubated overnight at 37°C.

BAL and Whole Lung tissue collection

Bronchoalveolar lavage collection was performed on mice to obtain cells collected from the airway as previously described [50-52]. Mice were killed using administration of isoflurane

and a catheter (22G x 1", Exel) was placed into the trachea and tied off. A syringe with 1mL 0.6mM EDTA in PBS was connected to the catheter and 0.6 mL of the fluid was flushed into the lungs, the lungs were massaged, and then the fluid was withdrawn into the syringe to obtain a primary wash. This procedure was repeated three times. Two additional washes were performed. Samples were spun down for cellular analysis and the supernatant was collected for assay via Bradford assay and enzyme-linked immune-sorbent assay (ELISA). Lungs were removed from the animals and minced using sterile razor blades. Lungs were then placed in 4mL of PBS supplemented with 10% Fetal Bovine Serum (PBS+FBS), 0.1µg/mouse DNase, 1500 u/mouse collagenase and shaken at 250rpm at 37°C for 1h for digestion of tissue as previously described [14]. Samples were then filtered using a 100µm cell strainer and then pelleted. Pelleted cells then underwent ACK lysis for removal of red blood cells and then samples were washed and resuspended in PBS+FBS for staining for flow cytometric analysis. Cells collected from BAL and whole lung tissue were counted using a hemocytometer with 0.01% trypan blue viability dye.

Flow cytometric analysis

Cells were incubated with anti-mouse CD16/32 Block (eBiosciences) to block Fc receptors as previously described [14, 49]. Cells were then stained with antibodies against CD45, CD11c, CD11b, Ly6G, F4/80 and/or NOS2. Cells were then fixed in 1% paraformaldehyde and examined using a Dako CyAn (Beckmann-Coulter) and then data was analyzed using Summit software (Beckman-Coulter). Initial exclusion of CD45- cells was conducted, and then neutrophils (CD45+CD11b+CD11c-Ly6G+) and macrophages (CD45+CD11c+Ly6G-) were examined. To determine the potential of the neutrophils to produce reactive oxygen and nitrogen

species (RONS), analysis was performed using dihydrorhodamine-123 (DHR123). Samples were stained using fluorochrome-conjugated antibodies as previously stated. Prior to fixation in paraformaldehyde, the samples were resuspended in DHR123 (1.875 mg/mL, Invitrogen). The samples were then split into stimulated and unstimulated samples. Samples were stimulated using 98nm Phorbol myristate acetate (PMA) for 30 minutes at 25˚C in the dark. All samples were then fixed in a 1% paraformaldehyde solution, and analyzed using flow cytometry. Reactive Oxygen and Nitrogen Species (RONS) expression was determined for each cell population present, as previously described [53].

Statistical Analysis

All data were visually displayed in GraphPad Prism Version 5.0 for Windows and analyzed by Student's t-Test or One-Way Analysis of Variance (ANOVA) with a Tukey post-test. Data are represented as mean+/- standard error of the mean (SEM). Statistical significance is indicated as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

Results

Repeated infection leads to susceptibility to bacterial dissemination in a murine model of burn injury.

Hospital stays frequently result in changes in the microbiome of patients and these changes commonly lead to development of hospital acquired infections [43, 45, 46]. Animal models of bacterial exposure often depend on a single exposure to bacteria, which is not representative of a nosocomial environment. To more closely mimic clinical conditions with

repeated bacterial exposure, we employed an infection strategy in which mice were initially infected (14 days after burn injury) intratracheally (IT) with *P. aeruginosa*, and then received a high-dose intraperitoneal (IP) infection with *P. aeruginosa* (17 days after burn injury). After a single IP infection, sham injured mice exhibited significant mortality and high, disseminated bacterial burden in the lungs and distal organs (Figure 2.1A). Administration of an initial IT infection 3 days prior to the IP infection reversed this phenotype and protected the sham mice from death (Figure 2.1A). In contrast, after a single IP infection, burn-injured mice experienced burn "priming" protection against a dose of bacteria that was fatal to sham counterparts, as predicted by earlier studies [20-22, 25, 26, 49] with significantly reduced bacterial dissemination (Figure 2.1B). However, burn mice that had an initial IT infection were unable to clear a secondary IP infection and demonstrated increased mortality and systemic bacterial burden (Figure 2.1C). This suggests that burn "priming" is transient, and a secondary infection causes burn-mediated immune protection to collapse in comparison with sham mice.

Repeated infection leads to increased neutrophil and macrophage numbers in the lungs late after burn injury.

To examine the potential underlying mechanism of burn-dependent alterations in immune response to bacterial infection in our double infection model, we examined changes in immune cells present in the infected tissue. We focused on the lungs because pulmonary infections are a common comorbidity and cause of mortality after burn injury. First, we examined the initial intratracheal (IT) response in isolation. 14 days after administration of sham or burn injury, mice were infected IT with *P. aeruginosa*. Live cells were enumerated from either BAL or whole lung

tissue to characterize burn-associated responses to a single IT bacterial infection 24 hours after infection; we observed a significant increase in the number of live cells in whole lung in sham and burn mice after IT infection (Figure 2.2A). Using specific flow cytometric quantification of innate immune cells in the lung (representative staining shown in Figure 2B), we found that there were no burn or infection-dependent changes in the total number of macrophages present in the BAL or whole lung tissue (Figure 2.2C and 2.2D). However, infection led to a marked increase in the numbers of neutrophils present in BAL collected from both sham and burn-injured mice (Figure 2.2E). Additionally, we found that infection led to an increased number of neutrophils present in the whole lung tissue collected in mice, a phenotype augmented by burn-injury (Figure 2.2F). These data agree with earlier studies which demonstrate that an increased number of neutrophils are responsible for the burn "priming" after burn [14, 20, 22].

In order to identify the cellular mechanism responsible for burn-mediated susceptibility to repeated bacterial exposure in our mouse model, we examined changes in innate cellular compartments after repeated bacterial infection. Burn or sham mice were initially infected (14 days after burn injury) intratracheally (IT) with *P. aeruginosa*, and then received a high-dose intraperitoneal (IP) infection with *P. aeruginosa* (17 days after burn injury). We analyzed cells present in BAL and found that there was an infection-dependent increase in the number of neutrophils, and that the number of neutrophils was significantly higher in burn mice than in sham treated animals (Figure 2.3A). We additionally found that while infection of sham-treated animals did not alter macrophage levels in the BAL, burn injured mice experienced increased macrophage recruitment to BAL after priming with intratracheal infection (Figure 2.3B). These data collectively imply that both neutrophils and macrophages are recruited to the airways of animals that have been infected twice.

Neutrophils from burn mice are not able to be activated after secondary infection to increase RONS production.

In addition, we sought to examine immune changes that took place in the actual tissue of the lungs. Enumeration of single cell suspensions generated from whole lung tissue found that there was an infection-dependent increase in the total number of neutrophils present in the tissue (Figure 2.3C). We additionally noted that there was an infection-dependent and injury-dependent change in the total number of macrophages present in the tissue of the whole lung, implying that macrophages could potentially mediate burn-dependent differences in response to infection (Figure 2.3D). We then examined macrophage and neutrophil function to determine their microbicidal potential. We utilized DHR123 to quantify the levels of RONS present in cells collected from whole lung tissue. We found that burn injury alone causes increased neutrophil RONS production as characterized by mean fluorescent intensity (Figure 2.4A&B). Additionally, we found that after burn animals are infected, basal RONS generation decreases, with doubly-infected animals generating significantly decreased RONS relative to uninfected burn counterparts. We therefore hypothesized that diminishment of RONS production is the source for lost burn-mediated protection. We found nominal changes in macrophage RONS production (Figure 2.4C&D). Loss of RONS activity in neutrophils also correlates with changes in intracellular expression of inducible nitric oxide synthase (NOS2) (Figure 2.4E). Upon infection, sham control mice exhibit reduction of NOS2 intracellular expression compared to burn mice, which is partially recovered upon secondary infection. In contrast, NOS2 expression was not changed in burn mice after an initial infection but was decreased upon subsequent infection.

Neutrophil and macrophage IL-10 and IL-12 are differentially expressed after single and double infections in burn mice.

We have previously demonstrated that aberrant production of IL-10 and reduced IL-12 cytokines is likely a major factor contributing to the immunopathology responsible for susceptibility to infection in burn patients [14, 30, 33, 34, 36]. We examined production of anti-inflammatory cytokine IL-10 and pro-inflammatory IL-12 in the macrophage and neutrophil populations in our model of double infection after burn injury. We collected BAL from animals 24 hours following single or double bacterial infection. Using flow cytometry, we found that intracellular IL-10 levels in neutrophils were significantly increased after burn injury or single infection compared to sham animals (Figure 2.5A) with a significant decrease after double infection in burn mice compared to single infection. In contrast, only double infection led to an increase in production of IL-10 in the macrophage population in burn-injured mice (Figure 2.5B). When we examined intracellular levels of IL-12 we found that neutrophils from singly infected burn mice exhibited a significant increase in IL-12 compared to sham controls (Figure 2.5C), which may act as a cellular mechanism for burn "priming" (Figure 2.1). This expression was abolished on double infection (Figure 2.5C). There was no increase after burn alone, single infection or double infections in burn macrophages (Figure 2.5D). Taken together, these data highlight a new finding; that there is differential expression of pro- and anti- inflammatory cytokines by macrophage and neutrophil populations on secondary bacterial challenges. These data recapitulate and provide a possible cellular mechanism for our previous findings that elevated IL-10 and decreased IL-12 predict increased infection rates, and poor clinical outcome in patients [30, 36].

Discussion

Murine models are frequently utilized to examine molecular and cellular mechanisms of burn-associated immune dysfunction [14, 49, 54-56]. However, clinically relevant animal model of injury should recapitulate phenotypes seen among human patients. Multiple studies have indicated that current murine models of burn injury are insufficient to elicit immunosuppression late after burn injury [20, 57]. In our model burn mice required repeated exposure to bacterial insult in order for burn-mediated immunosuppression to become apparent. This phenotype is similar to previously reported clinical data. Previous studies have indicated that mortality is only significantly increased among the burn population after a secondary bacterial challenge [2]. This clinical result in conjunction with our data implies that the immune compartment is sufficient to protect patients against a single bacterial insult, but that a second infection will result in immunosuppression.

We hypothesized that immune cells that respond to repeated infection would exhibit immune cell exhaustion and that they would be therefore unable to mount appropriate immune responses to subsequent infection. To characterize immune exhaustion, we examined immune cells present as well as their cellular function. We found that burn injury resulted in increased numbers of neutrophils present in whole lung tissue collected from mice. Neutrophils are recruited to the lungs after injury, and represent a pool of innate immune cells that could rapidly respond to any bacterial insult in the lung compartment. Neutrophils function by production of multiple mediators, such as RONS, in order to destroy bacterial pathogens and have been found to be necessary and sufficient to resolve infection with *P. aeruginosa* [6, 8]. We postulated that the recruited neutrophil population would represent a finite resource, and that after initial immune activation and use of the neutrophil reservoir any innate immune cells later recruited to

the compartment would experience immune cell exhaustion. Upon initial examination we found that despite the presence of neutrophils in whole lung tissue, a single intratracheal infection resulted in comparable recruitment of cells to the airspace in both sham and burn-treated animals. We also found that intratracheal infection resulted in recruitment of cells to the whole lung tissue, with slightly elevated numbers of neutrophils present in the whole lung tissue of burn-treated animals. Increased neutrophil numbers in the lungs correlated with improved bacterial clearance in burn mice after a single infection when compared to their sham-injured counterparts. Upon subsequent infection, sham mice "catch up" by recruiting more neutrophils to combat infection; functionally we do not observe any difference between RONS or cytokine production between the first and second infection. In contrast, the neutrophils recruited to combat a second infection late after burn injury produce less RONS and IL-12 than those that were recruited after the first bacterial hit. These results support our hypothesis that despite burn-induced "priming" the immune system is unable to mount a successful immune response to a secondary bacterial exposure.

Upon infection of a host, bacterial agents such as *P. aeruginosa* will release pathogen associated molecular patterns (PAMPS) that are able to alter the immune response. Multiple studies have indicated that the presence of PAMPS in a host is sufficient to result in altered immune cell signaling, cytokine production and changes in RONS-associated machinery [58, 59]. Studies have additionally indicated that burn injury leads to increased RONS and pro-inflammatory cytokine production in immune cells which can mediate additional tissue injury [60-63]. Some studies have indicated that it is possible to decrease RONS activity shortly after burn injury and decrease damage to lungs while still permitting successful clearance of subsequent infection [62], and other studies have implicated neutrophils as the only innate cell

type responsible for burn-mediated hyperactivity [20, 22]. In this study we report increased total numbers of macrophages and neutrophils present in the whole lung tissue after burn injury repeated infection. We additionally found that burn injury increased basal RONS and pro- and anti- inflammatory cytokine activity of neutrophils, but that upon repeated infection this was lost. We also found that upon each incidence of infection these cells decreased their baseline production of RONS. Repeated incidence of infection would cause cells to be repeatedly exposed to PAMPS and result in changes in intracellular signaling leading to changes in RONS production. We have previously demonstrated that increased production of IL-10 and reduced IL-12 cytokines from several pulmonary and systemic cell types, including macrophages and neutrophils, is a major factor contributing to the immunopathology responsible for susceptibility to infection in burn patients [14, 30, 33, 34, 36]. We have also demonstrated the prognostic potential of IL-10/IL-12 protein ratios within bronchial washes on predicting lung and systemic infections following severe burn trauma [14, 30, 36]. We report here that that there is differential expression of pro- and anti- inflammatory cytokines by macrophage and neutrophil populations which are significantly different on secondary bacterial challenges. Although macrophages from burn mice did not have any apparent change in RONS production after burn or infections, they exhibited a significant increase in IL-10 production after a secondary bacterial infection. These data suggest that macrophages might "guide" subsequent immune responses in the pulmonary micro-environment and warrants further investigation. Multiple studies have indicated that cytokines play an important role in patient immune responses, and we have found that levels of IL-10 and IL-12 are predictive of outcomes after burn injury [30, 36]. Our study indicates that burn and infection alter innate immune cell production of IL-10 and IL-12. Moreover, our preliminary investigations suggest a relationship between peripheral blood IL-10/IL-12 and

arginase 1 gene (ARG1)/NOS2 gene expression ratios and indicate that they have a powerful predictive ability for susceptibility to infection in burn patients. When we examined neutrophil levels of NOS2 we found that infection of sham-injured mice resulted in decreased NOS2 expression. This phenotype was not found in burn injured-mice after a single exposure to *P. aeruginosa*, however loss of NOS2 levels took place after repeated infection, potentially indicative of exhaustion arising in burn-injured mice and associated loss of burn-mediated protection.

We and others have previously reported that cells from burn-injured animals exhibit altered signaling of toll-like receptors (TLRs), the receptors responsible for detection of PAMPS and subsequent activation of the immune system [14, 34, 47, 64, 65]. Alteration of TLR function after burn injury have additionally been shown to lead to alterations in cytokine production in a model of burn injury [66]. It is possible that alterations in innate immune cell TLR levels result in alterations in the immune response, production of cytokines, and subsequent response to bacterial infection. Future studies will establish the link between TLR signaling and antimicrobial activity in our model of burn injury and repeated infection.

FIGURES

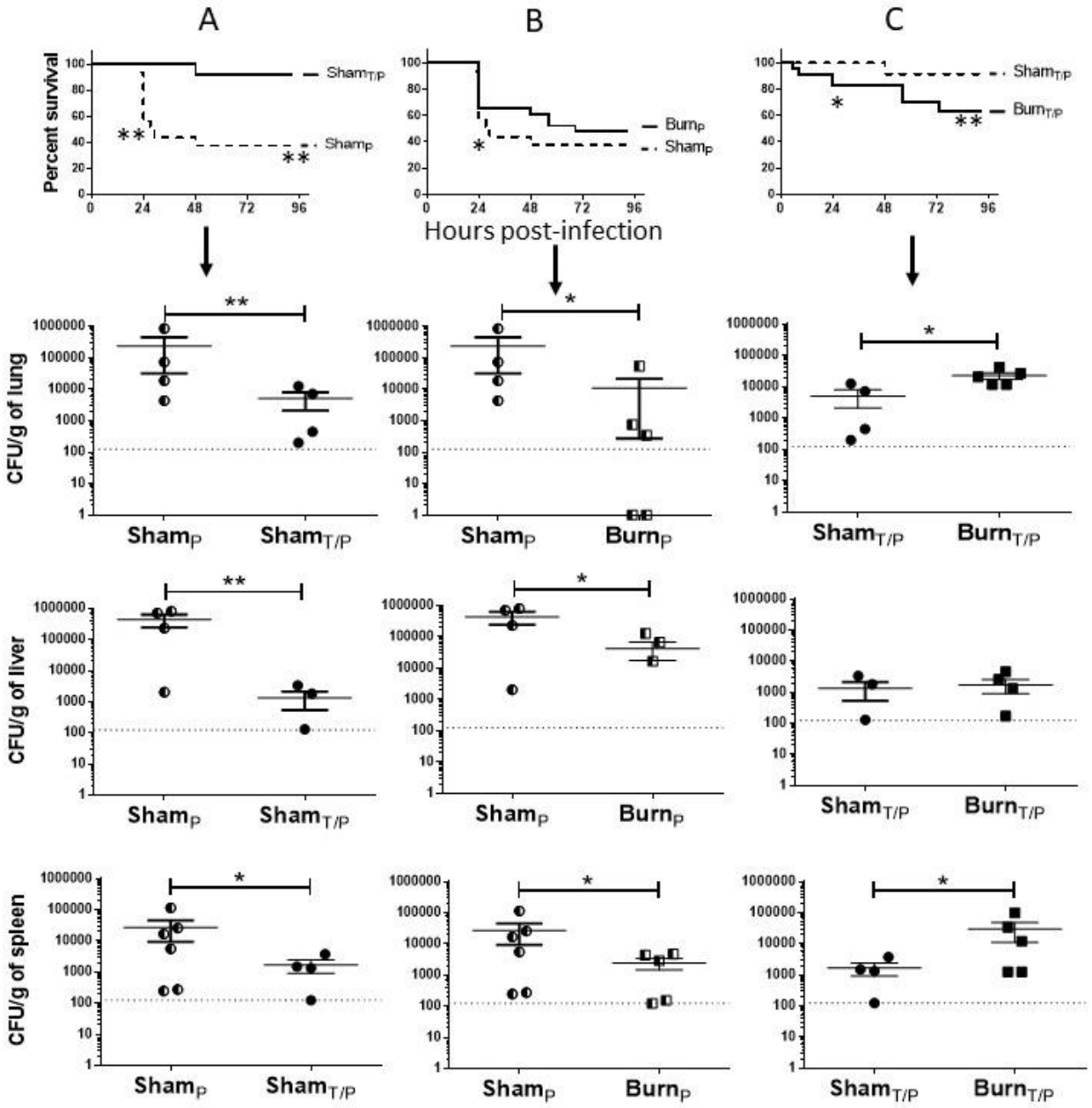

Figure 2.1: Repeated, but not single infection, leads to susceptibility to bacterial infection in a murine model of burn injury. Mice (n=4-6 per group) underwent sham or burn injury and were infected with either A) intraperitoneal (IP) infection with *P. aeruginosa* 17 days after injury (Sham$_P$); or intratracheal (IT) inoculum of *P. aeruginosa* 14 days after injury and a subsequent a IP infection with *P. aeruginosa* 17 days after injury (Sham$_{T/P}$); B) IP infection with *P. aeruginosa* 17 days after injury (Sham$_P$ or Burn$_P$); or C) IT with *P. aeruginosa* 14 days after injury, followed by IP infection with *P. aeruginosa* 17 days after injury (Sham$_{T/P}$ or Burn$_{T/P}$). Survival was monitored for up to 96 hours after final infection. Lungs, liver and spleen were

harvested from surviving mice for *P. aeruginosa* quantification by colony forming unit (CFU) analysis. Dashed line represents lower limit of detection for the CFU assay. Data shown are +/- SEM. *p<0.05, **p<0.01, and representative of three repeated experiments. Numbers of initial mice in representative figures; survival plots A-C, $Sham_P$, n=9; $Sham_{T/P}$, n=9; $Burn_P$, n=10; $Burn_{T/P}$, n=10.

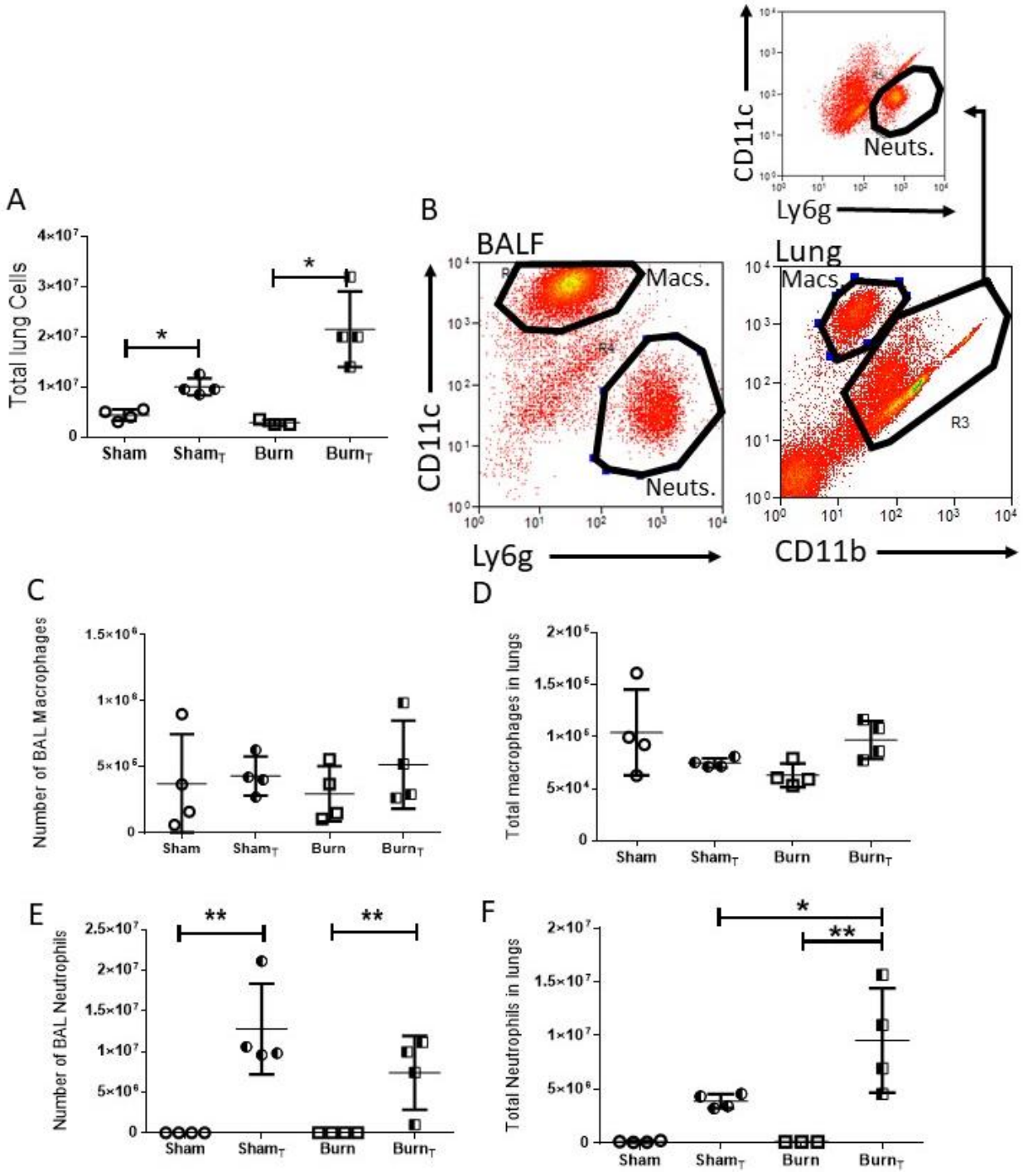

Figure 2.2: Single IT infection leads to increased neutrophil numbers in the lung BAL late after burn injury. Mice (N=4-6 per group) underwent sham or burn injury and were infected (14 days after burn injury) IT with *P. aeruginosa* ($Sham_T$ or $Burn_T$) or uninfected (Sham or Burn) and lungs harvested 24 hours later; A) total cells enumerated counted on a haemocytometer, B) representative flow cytometry of BAL or whole lung tissue used to identify neutrophils and macrophages; cells are shown after gating on live singlets and common leucocyte CD45+ marker; C-F) number of macrophages or neutrophils from either BAL or whole lung were quantified for each injury and infection group. Data shown are +/-SEM. *p<0.05, **p<0.01, and representative of three repeated experiments. Numbers of mice in representative figures; A, C-F), Sham, n=4; $Sham_T$, n=4; Burn, n=4; $Burn_T$, n=4.

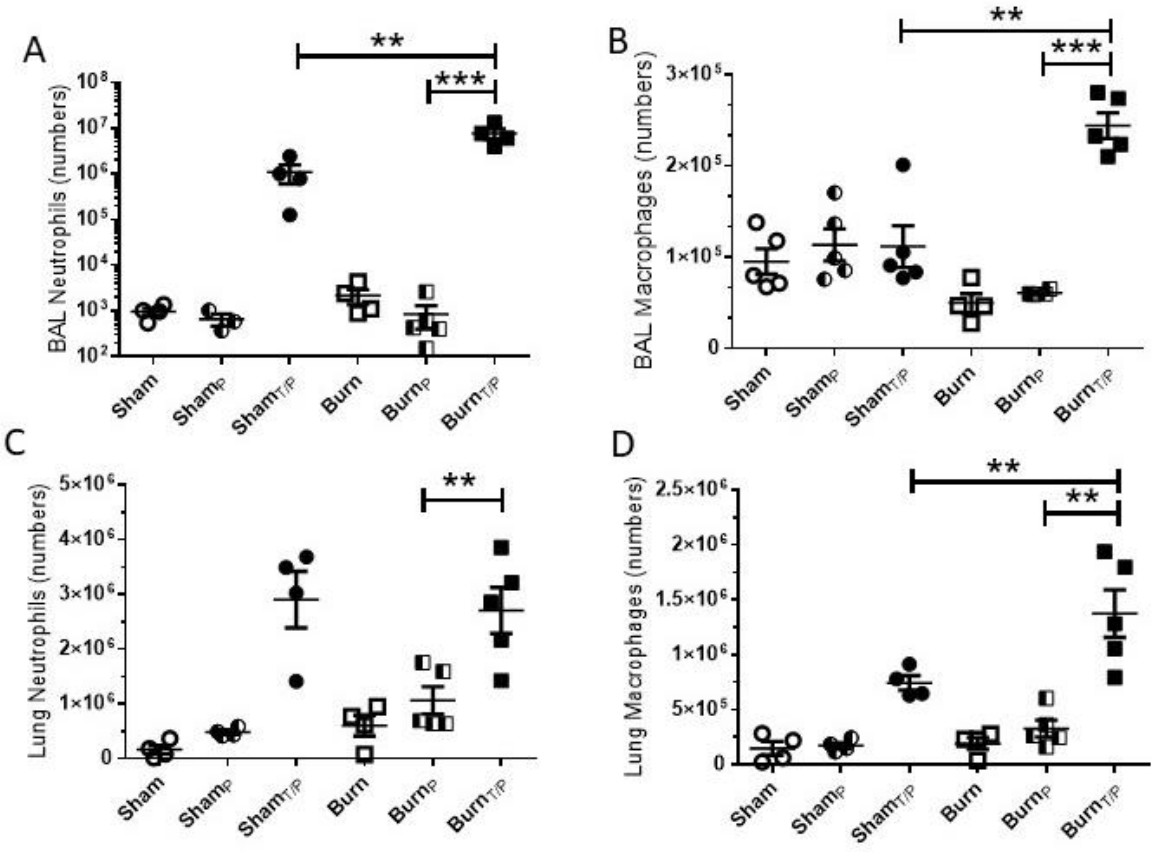

Figure 2.3: Double infection leads to increased neutrophil and macrophage numbers in the lung late after burn injury. Mice (N=4-6 per group) underwent sham or burn injury and were given IT inoculation with PBS+1%PP or with *P. aeruginosa* ($Sham_{T/P}$ or $Burn_{T/P}$) 14 days after injury, followed by IP infection IP with *P. aeruginosa* ($Sham_P$ or $Burn_P$) at 17 days after injury, or left uninfected (Sham or Burn). Lungs were harvested 24 hours later; A-D) number of macrophages or neutrophils from either BAL or whole lung were quantified for each injury and infection group. Data shown are +/-SEM. *p<0.05, **p<0.01, ***p<0.005 and representative of three repeated experiments. Numbers of mice in representative figures; A-D), Sham, n=5; $Sham_P$, n=5; $Sham_{T/P}$, n=5; Burn, n=5; $Burn_P$, n=5; $Burn_{T/P}$, n=5.

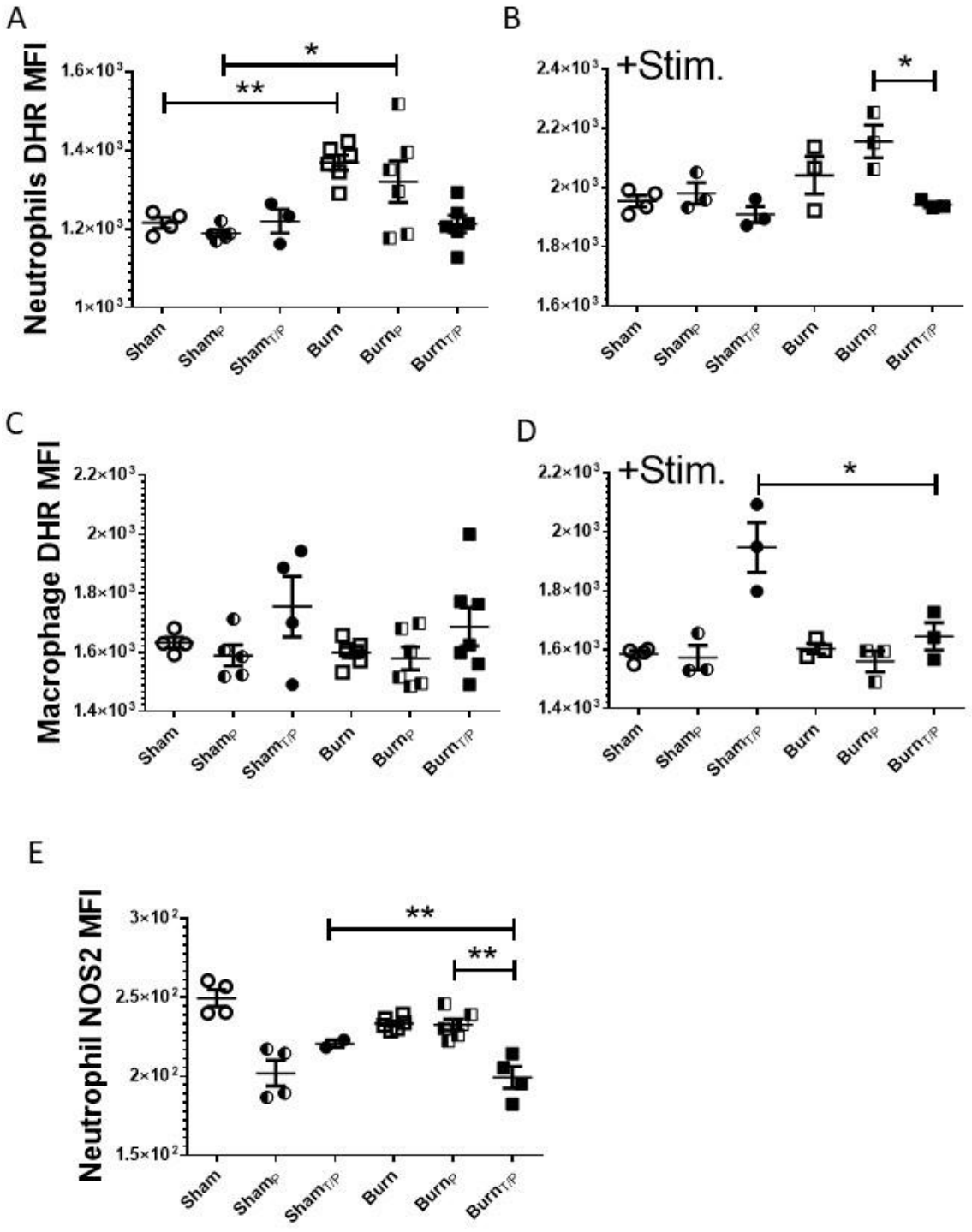

Figure 2.4: Neutrophils from burn mice are not able to be activated after secondary infection to increase RONS production. Mice (N=4-6 per group) underwent sham or burn injury and were infected (17 days after injury) either IP with *P. aeruginosa* (Sham$_P$ or Burn$_P$) or IT with *P. aeruginosa* 14 days after injury followed by IP infection at 17 days after injury (Sham$_{T/P}$ or Burn$_{T/P}$), or left uninfected (Sham or Burn). Lungs were harvested 24 hours later; A-C) reactive oxygen / nitrogen oxidative (RONS) ability per cell was measured by flow cytometry

in the absence or presence of Phorbol myristate acetate (PMA) stimulation ("+Stim."), D) NOS2 expression per cell was quantified in neutrophils using flow cytometry. Data shown are +/-SEM. *p<0.05, **p<0.01, and representative of three repeated experiments. Numbers of mice in representative figures; A-E), Sham, n=4; $Sham_P$, n=5; $Sham_{T/P}$, n=5; Burn, n=6; $Burn_P$, n=6; $Burn_{T/P}$, n=6.

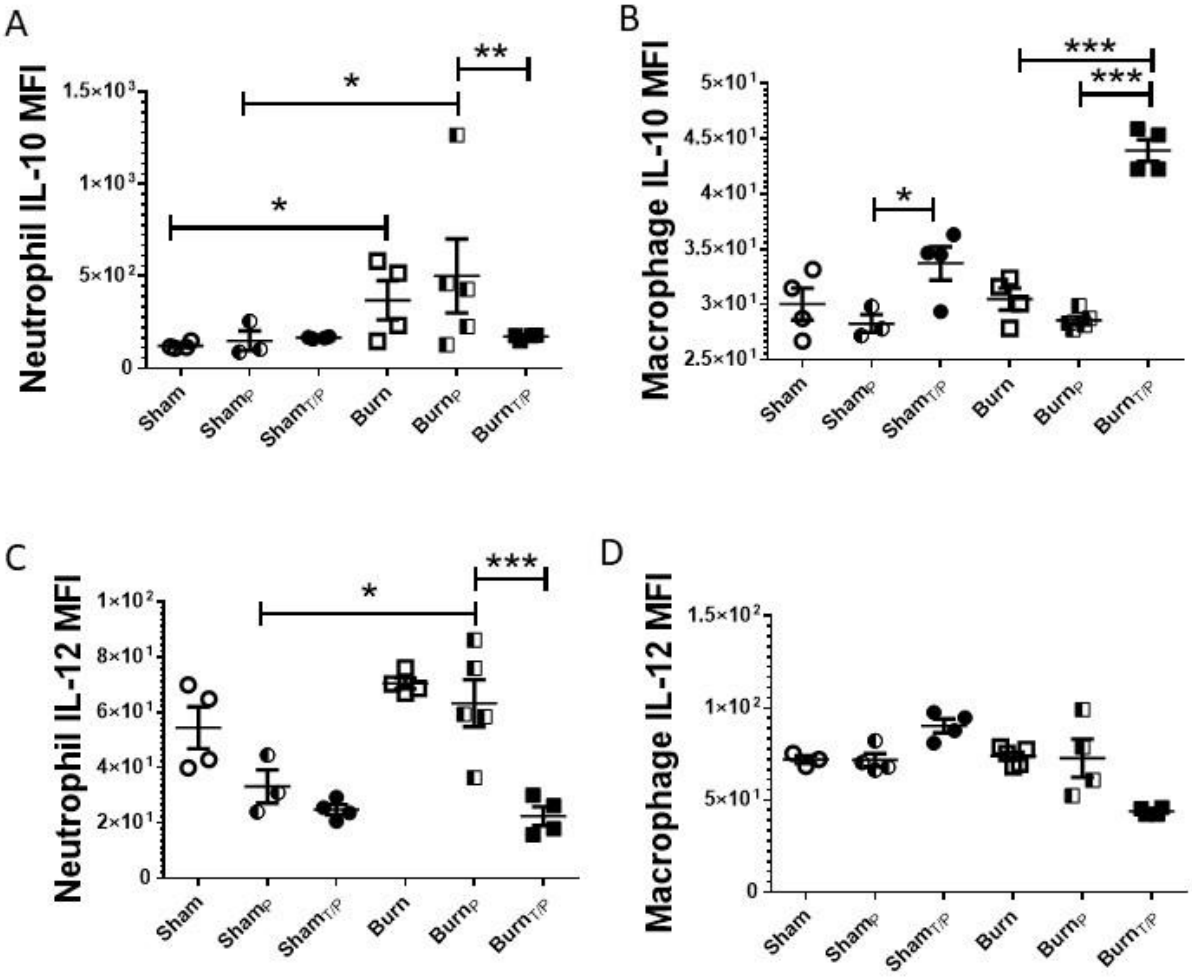

Figure 2.5: Neutrophil and macrophage IL-10 and IL-12 are differentially expressed after single and double infections in burn mice. Mice (N=4-6 per group) underwent sham or burn injury and were infected (17 days after injury) either IP with *P. aeruginosa* ($Sham_P$ or $Burn_P$) or IT with *P. aeruginosa* 14 days after injury followed by IP infection at 17 days after injury ($Sham_{T/P}$ or $Burn_{T/P}$), or left uninfected (Sham or Burn). Lungs were harvested 24 hours later; A-D) intracellular IL-10 and IL-12 expression per cell was measured by flow cytometry (MFI). Data shown are +/-SEM. *p<0.05, **p<0.01, ***p<0.005 and representative of three repeated experiments. Numbers of mice in representative figures; A-D), Sham, n=4; $Sham_P$, n=5; $Sham_{T/P}$, n=4; Burn, n=4; $Burn_P$, n=5; $Burn_{T/P}$, n=5.

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CHAPTER 3: DEVELOPMENT AND METHODOLOGY OF A CLINICALLY-RELEVANT MURINE MODEL OF INHALATION INJURY[3]

SUMMARY

Inhalation injury represents a significant comorbidity of burn injury that currently remains understudied. In order to assess the inhalation-dependent alterations that take place after burn injury, our lab has created a murine model of inhalation injury. Herein we describe the use of clinically-relevant particle board as a smoke source to create a laboratory-controlled system that can be utilized to assess many different scientific questions. In this chapter of the thesis we describe in detail how to utilize and replicate this model for use in other systems, focusing on the overall methodology and requirements involved.

[3] This chapter contains methodology that is intended to be utilized for submission in the future. This is the author's original work. The current authors, in order: Kartchner LB, Mac M, Shyng BJ, Dunn JLM, Maile R, Cairns BA.

INTRODUCTION

Burn injury represents a severe form of trauma that leads to lengthy hospital stays and slow healing among patients. Patients who present to the hospital with burn injuries often experience significant systemic immune dysregulation [1-4]. Large wounds present on epithelial tissue lead to a systemic release of damage-associated molecular patterns (DAMPS) that are known to markedly impair the immune compartment and lead to activation of the immune system as characterized by altered levels of cytokines, chemokines, and immune cells in patients [5]. Burn injury leads to an overwhelming shock response that is characterized by the presence of DAMPS, cytokine storm, and abnormal immune cell profiles.

Immune dysregulation in burn patients is often complicated by the presence of a burn-associated comorbidity. For example, approximately 10-20% of burn patients are diagnosed with a burn-associated inhalation injury [6], which can result in a 20% increase in mortality relative to patients who experience a burn injury alone [7-9]. This has led to inhalation injury being a leading cause of burn-associated mortality [10, 11]. Over time, many animal models of inhalation injury have been created to examine inhalation-mediated alterations in the immune profile [12-14], however few advances in the treatment of inhalation injury have been generated [6]. This could potentially be due to the lack of models that examine the combined effects of burn and inhalation injury [15], or due to a lack of clinically relevant smoke sources as many inhalation injury models depend on smoke generated from cotton [16, 17].

Here, we describe a novel model for the study of inhalation injury, wherein we use clinically relevant sources for the generation of wood smoke. We additionally describe the steps necessary to create and utilize this model for application of study of inhalation injury.

Protocol

The following protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and comply with current standards for animal care and use as defined by the National Institutes of Health. All mice were purchased from Taconic Farms, aged 8-10 weeks old, and housed in specific pathogen free facilities.

1. *Creation of woodsmoke apparatus*
    1. Purchase particle board in a lot size large enough for the completion of all experiments necessary.
    2. Cut the particle board into segments that are approximately 2.5cm x 8cm in size, excluding any pieces that have residual paint for consistency. Store any extra particle board in a sealable storage bag to prevent humidification of wood.
    3. Obtain a hot plate stirrer that can be heated to 500°C (VWR, #12365-382), a side-arm flask (Fisher Scientific, #FB3002000), rubber tubing (Fisher Scientific #14-178D), 2mL serological pipette tip (Corning, #4486) with cotton filter removed, one-hole rubber stopper (Fisher Scientific, #14135N), induction chamber (Stoelting Co., #50216), a small/flat plastic platform approximately 8.5cm x 13cm and Velcro strips.
    4. Place Velcro strips on the small plastic platform to create a device meant to hold an anesthetized mouse in place.
    5. Place the hot plate in a designated fume hood and place the flask on the hot plate.
    6. Insert the 2mL serological pipette into the one-hole rubber stopper and place the stopper on the flask.

7. Use rubber tubing to connect the 2mL serological pipette to the airflow valve of the hood.
8. Using approximately 40cm of rubber tubing, connect the side arm of the flask to the clear induction chamber utilized, with the chamber turned on its long side.
9. Final setup of the apparatus should reflect that shown in figure 3.1.

2. Preparation of mouse for exposure.
    1. Mice are initially weighed and anesthetized with tribromoethanol (475mg/kg; Sigma-Aldrich).
    2. In order to provide consistency for comparison of experiments where a burn injury takes place, shave the dorsum of the mouse and then administer a subcutaneous injection with morphine sulfate (3mg/kg; Westward Pharmaceuticals).
    3. Place a cannula (22G x 1”, Exel) on a blunted and shortened needle to provide structural support.
    4. Place mice in a supine position on an intubation platform (Penn Century).
    5. Visualize the trachea with a laryngoscope (Penn Century).
    6. Insert the previously prepared cannula through the vocal chords and into the trachea. Remove the blunted needle from the catheter.
    7. Carefully remove mouse from intubation platform without displacing the cannula while maintaining supine orientation.
    8. Secure the mouse in place using the Velcro-strip platform previously made in order to provide support to the mouse.
3. Performing Inhalation Injury

1. Preheat the flask to 500°C.
2. Weigh approximately 18g of previously charred particle board and approximately 30g new particle board and place into flask while air is applied pumped into the system. Weigh any new wood that is placed into the system to determine the final weight of wood used for the experiment.
3. Keep air pressure applied to the system constant throughout the experiment.
4. Once smoke has reached appropriate density, disconnect tubing to the induction chamber and open the chamber door to allow full clearance of smoke in the chamber.
5. Place the previously intubated mouse into the induction chamber.
6. Close the door and reconnect the tubing to the chamber.
7. Expose the mouse to smoke for two minutes.
    i. As smoke is applied, visually assess the exposure. Breathing of mice should continue throughout the exposure. Breathing should also create what appear to be “inverse smoke rings,” where air being breathed out from the mouse is cleared of smoke.
    ii. If necessary, to ensure consistency of smoke density it is acceptable to slightly vent the chamber. Ideal exposure will result in smoke creating a visual obstruction at 1-1.5 inches from the chamber door, allowing for visualization of the mouse head.
8. Disconnect tubing from the chamber and open the chamber door to allow full clearance of smoke in the chamber and expose the mouse to fresh air for one minute.

9. Repeat steps 7 and 8 two more times, in order to allow three exposures total.
10. After exposure of the mouse, assess the breathing of the mouse. If assistance is necessary, continual movement of the mouse in the hands of the experimenter will improve respiration.
11. Following procedure place the mouse on a heating pad to ensure maintenance of body temperature while under anesthesia.
12. After the mouse has woken, administer an intraperitoneal injection with lactated Ringer's solution (1mL/kg body weight; Baxter Healthcare Incorporated), and place the mouse in a cage for short-term monitoring prior to return to the animal facility.
13. Mice can be given morphine water in their drinking water ad libitum for the duration of the experiment in order to ensure similarity with treatment of burn-injured animals, and also ability to compare results found in these studies with those of patients who receive inhalation injury.
14. Monitor mice throughout the course of the experiment.

Discussion

Patients who experience an inhalation injury experience significant deterioration of their health and have worsened overall outcomes when compared to healthy individuals. Additionally, inhalation injury causes marked increases in mortality among burn patients and represents an important area of study. In this chapter of this thesis we describe a fully-developed model of inhalation injury for study using a murine model. This model will allow for further studies focusing on the metabolic, immune, pulmonary, and other systems that are affected by inhalation injury. In chapter 4 of this thesis we describe the use of this model to characterize the pulmonary

immune response to a combined burn and inhalation injury model. These models are critically important in the discovery of mechanistic underpinnings of inhalation injury-mediated disease, as well as a powerful tool for testing of potential therapeutics that can be used in clinical studies in the future.

FIGURES

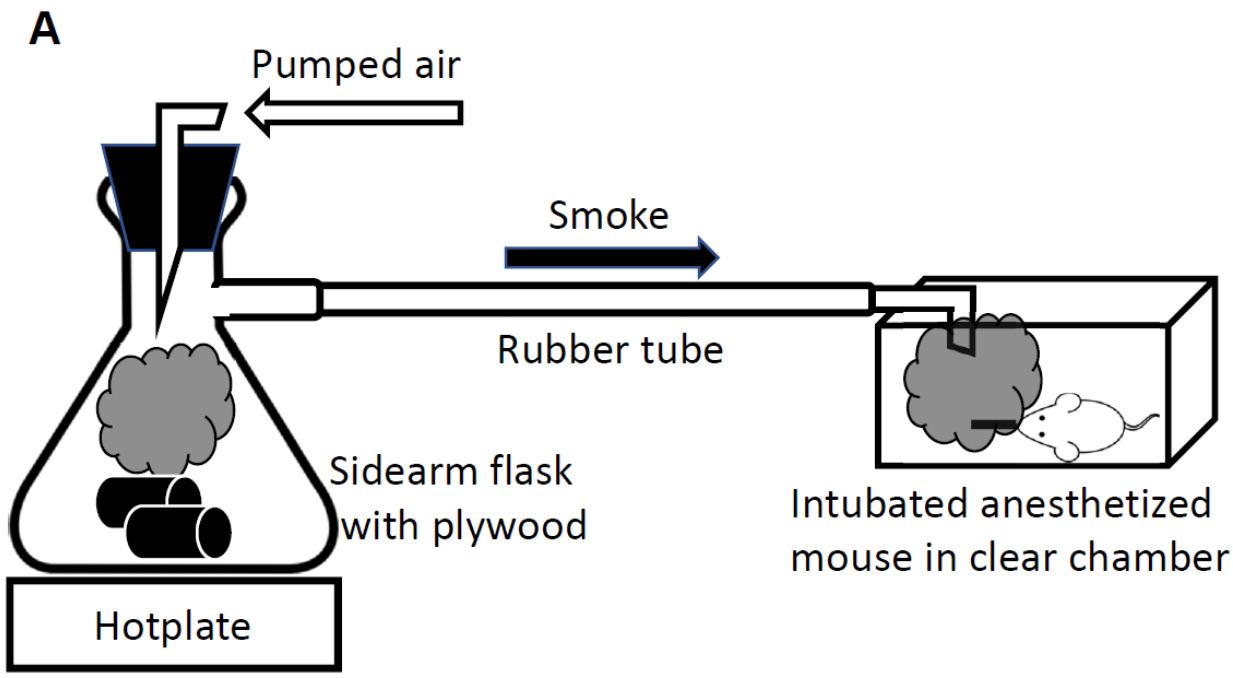

Figure 3.1: Experimental setup for successful generation of inhalation injury of murine animals. Mice are anesthetized, shaved, given morphine, incubated and placed on platform in chamber where they are exposed to smoke generated from particle board sections that smolder in a side-arm flask on a hotplate. Each mouse receives three exposures to the smoke, with each exposure lasting two minutes followed by one minute of exposure to normal air.

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CHAPTER 4: IMMUNE-MEDIATED MECHANISMS OF PULMONARY DAMAGE AFTER BURN AND INHALATION INJURIES INDEPENDENTLY LEAD TO DAMAGE GENERATED[3]

Overview

Inhalation injury is commonly associated with burn injury and leads to significant increases in morbidity and mortality among burn patients. In this study we utilize a murine model of burn and inhalation injury to examine the individual and combinatory effects of each individual injury and their effects on the pulmonary innate immune system. We found that combined injury, but not inhalation or burn injury alone, resulted in significantly increased mortality. Inhalation injury led to an increase in the total number of cells present in the airways of animals, and that increases in cells also corresponded with increases in protein concentration. Increased numbers of cells in the airways was largely attributable to neutrophil influx. We discovered inhalation-injury dependent alterations in levels of cytokines and chemokines present in the bronchoalveolar lavage obtained from injured mice, which correlated with functional changes in the levels of nitric oxide synthase in macrophages. Finally, we examined the potential therapeutic use of a nitric oxide inhibitor L-NAME and found that administration of L-NAME at the time of injury resulted in decreased total neutrophils present in airways, as well as decreased

[4] This chapter contains a manuscript that is currently in preparation for submission. This is the author's original work. The current authors, in order: Kartchner LB, Dunn JLM, Mac M, Shyng BJ, Glenn LI, Vaughan RJ, Maile R, Cairns BA.

protein levels. These findings could help guide future studies focused on treatment of patients afflicted with an inhalation injury.

INTRODUCTION

Burn injury results in significant loss of productivity among trauma patients [1]. Burn patients experience one of the most severe forms of trauma, often resulting in lengthy treatment and hospital stay [2]. Recovery of patients with a burn injury is often complicated by many factors such as total body surface area (TBSA) of injury, infection, presence of inhalation injury, modality of burn injury, concomitant trauma as well as many other comorbidities of injury [3-5]. Burn injuries are also known to lead to significant immune dysregulation that is commonly thought to contribute to the difficulties patients experience in overcoming their injury [6-8]. One significant comorbidity frequently found among burn patients is presence of inhalation injury, which is commonly associated with an increase in hospital length of stay, bacterial infections and mortality [9-15].

Treatment of patients with a burn injury has significantly advanced over recent years, however inhalation injury has proven difficult to treat and remains associated with increased mortality and morbidity [16, 17]. Inhalation injury and burn injury have independently been shown to mediate significant immune dysregulation [18-24]. Studies of patient populations indicate that each injury independently causes immune dysregulation, and that their combined effects result in damage in excess of that expected due to individual contributions from each injury [25]. Although combined injury is known to cause significant complications and lead to worsened outcome, few models have been generated that examine the immune-mediated effects of combined burn and inhalation injury [26, 27].

Studies have indicated that either burn injury or inhalation injury alone can lead to increased neutrophil presence in the pulmonary compartment [27-31]. Neutrophils present in the pulmonary vasculature and alveolar spaces often lead to production of reactive oxygen and nitrogen species, resulting in subsequent damage to the pulmonary compartment [30, 32-34]. Studies have also indicated that pulmonary damage due to smoke inhalation is associated with increased oxidative stress and damage [32, 35], which often occur in the presence of neutrophils. Additionally, presence of neutrophils in the bronchial spaces has been associated with immune dysfunction and increased susceptibility to bacterial infection [36-38]. Altered production of oxidants, as well as alterations in the levels of nitric oxide have been show to contribute to pathology after burn injury as well [39, 40]. Some labs have indicated that inhibition of inducible nitric oxide synthase (iNOS), a molecule involved in production of nitric oxide, is a potential therapeutic with effective treatment potential in ovine animal models [41, 42].

In this study we utilized a recently developed murine model of combined burn and inhalation injury to examine alterations in early and late immune responses after injury. We hypothesized that a combined inhalation and burn injury would lead to immunosuppression similar to that seen in human populations. We additionally examined the therapeutic potential of antioxidant-focused therapies in treatment of animals in our model to improve outcome after combined injury.

Methods

Animals

Eight to twelve-week-old female C57BL/6 mice weighing between 18-21g were purchased from Taconic Farms for use in this study. Additional mice deficient in gp91phox machinery or NOS2 were utilized for a subset of experiments. Mice were anesthetized using tribromoethanol (475mg/kg body weight, Sigma-Aldrich) and then shaved dorsally and given a subcutaneous injection of morphine (3mg/kg body weight, West-Ward Pharmaceuticals). A subset of mice were administered a 20% total body surface area burn injury as previously described [43-45]. A 65-gram copper rod (1.9cm in diameter) was heated to 100°C in a hot water bath and then held to the dorsum/flank of the animal for four separate applications lasting 10 seconds each to achieve a full-thickness contact burn. Inhalation injury was then applied to a subset of mice as previously described in chapter 3. Briefly, following burn or sham procedure all mice were then placed on an intubation platform (Penn Century) and their trachea were visualized using a laryngoscope. A catheter (22Gx1", Exel) was then placed between the vocal chords and mice were secured to a platform and placed in an animal induction platform (Stoelting NC9296517). A side-arm flask was placed on a hot plate in a dedicated fume hood and set to 500°C. Approximately 50g sectioned plywood (2.5cm x 8cm, Lowe's Item #12206 Model # 776391100000) was heated to induce smoldering and generate smoke. Air was then pumped into the flask at constant pressure, resulting in flow of smoke into the induction chamber. Smoke density in the chamber was visually assessed and considered appropriate when visual obstruction was obtained at a depth of 1-1.5inches from chamber wall. If thinning of smoke was necessary the chamber door was vented to maintain a consistent thickness of smoke. Each animal was exposed to the smoke three times for exposures lasting two minutes each, followed by one

minute of air exposure (fully venting the chamber). Animals were allowed to recover on a heated surface until anesthesia subsided and then were given an intraperitoneal injection with lactated Ringer's solution (0.1 mL/g body weight: Hospira, Lake Forest, IL). Mice were then placed in individual cages and given food and water ad libitum and monitored twice daily. All Sham animals underwent all interventions with the exceptions of application of the cooper rod and while in the induction chamber were exposed to air only rather than smoke particles.

All animals were housed in the UNC Chapel Hill Department of Comparative Medicine's specific pathogen-free animal housing facilities and all protocols and procedures were approved by the University of North Carolina's Institutional Animal Care and Use Committee in accordance with NIH-specified guidelines.

BAL and Whole lung tissue isolation

Mice were killed through exposure to isoflurane after which Bronchoalveolar lavage was conducted to collect cells from the airway as previously described [46-48]. Dissection was performed to expose the trachea and a catheter (22G x 1", Exel) was inserted into the trachea and tied off with a thread. A syringe containing 1mL PBS with 0.6mM EDTA was attached to the end of the cather, and 0.6mL of solution was washed into the lungs, after which the lungs were massaged. The same syringe was used for two more washes which were then collected into a tube for primary BAL washes. A second and third syringe were then used to repeat the collection procedure, resulting in nine washes with 3 mL solution used for each mouse. Samples were then centrifuged (5 min, 12,000 RCF) and supernatants were collected and stored (-20°C) for further analysis at a later date. Cells pelleted were then used for further analysis. Identification of protein content of BAL supernatant was determined using a Bradford colorimetric protein assay according to manufacturer's instructions (Bio-Rad, #5000006). Addtionally, BAL supernatant

was utilized to characterize cytokines present in the airways. Samples were processed for analysis on a Bio-plex mouse chemokine panel 33-plex (Bio-Rad #12002231) according to manufacturers protocol. Samples were washed using the Bio-Plex Pro wash station (Bio-Rad #30034376) and analyzed using the Bio-Plex MAGPIX Multiplex reader (Bio-Rad #171015001).

Lung tissue was also removed from animals and physically separated using sterile razor blades. Lung tissue was then placed in 4mL PBS+10% Fetal Bovine Serum (PBS+FBS) supplemented with 0.1µg/mouse DNase and 1500 u/mouse collagenase. Samples were then shaken at 250rpm at 37°C for 1h to digest tissue and obtain a single cell suspension as previously described (insert Julia references if applicable here) [43]. Digested lung sample was then filtered with a 100µm cell strainer and then pelleted (5min, 300g). Red blood cells present in the pellet were then lysed using ACK lysis (2 minute exposure) after which samples were washed and suspended in PBS+FBS for further analysis and use. Cells obtained from BAL washes and whole lung tissue processing were then counted using a hemocytometer with 0.01% trypan blue viability dye.

Flow Cytometry

Fc receptors on cells were blocked using anti-mouse CD16/32 (eBiosciences) as previously described [43, 44]. Antibodies against CD45, CD11c, CD11b, Ly6G, F4/80 and/or NOS2 were then utilized to stain cells. Cells were then washed twice and then fixed with 1% paraformaldehyde. Stained samples were examined on a Dako CyAn (Beckmann-Coulter) and data obtained was analyzed using Summit software (Beckman-Coulter). Cells identified as CD45+ were than examined to identify neutrophils (CD45+CD11b+CD11c-Ly6G+) and macrophages (CD45+CD11c+Ly6G-).

Statistical Analysis

All data were examined in GraphPad Prism Version 5.0 for Windows and analyzed using a Student's t-Test or One-Way Analysis of Variance (ANOVA) with a Tukey post-test. Data are displayed as mean+/- standard error of the mean (SEM). Statistical significance is indicated as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

Results

Combined burn and inhalation injury result in significantly increased mortality that is absent in the presence of either injury alone.

Mice underwent either sham, burn, inhalation, or combined (burn and inhalation) injury in an apparatus as previously described in chapter 3. Single injury resulted in nominal mortality, however combined injury resulted in approximately a 40% mortality within two weeks of injury (Figure 4.1A). Inhalation injury is known to significantly alter lung physiology, therefore we assessed levels of protein present in BAL collected from animals at early (96 hour, Figure 4.1B) and late (14 days, Figure 4.1C) timepoints after injury. We found that independent of the presence of a concomitant burn injury, inhalation injury resulted in increased protein concentration within the airways. In order to determine if changes in protein concentration were associated with alterations in the cellular composition of the lungs we examined the total number of cells present in both the BAL washes and in single cell suspensions obtained from the whole lung tissue. We found that there was an inhalation-dependent increase in total cells present in the BAL (Figure 4.1D), and a burn-dependent increase in total cells collected from the whole lung

tissue (Figure 4.1E), a phenotype that persisted until 14 days after injury (Data shown is representative of 14 days after injury.

Burn and inhalation injury result in alterations in the innate immune composition present in the lungs.

We then utilized flow cytometric analysis to determine if alterations in numbers of cells were related to alterations in immune cells compositions. We found that there was an inhalation-dependent increase in the numbers of neutrophils present in the BALF early after injury (Figure 4.2A), but no alterations in the number of macrophages (Figure 4.2B). We additionally found that this phenotype was replicated at the late timepoint, in spite of the short-lived nature of neutrophils (Figures 4.2C and 4.2D). When we analyzed the cells collected from whole lung tissue 14 days after injury we found that there was a bun-dependent increase in the total number of neutrophils, a phenotype that was heightened in animals that had additionally experienced an inhalation injury (Figure 4.2E). We also found that there was a burn-dependent increase in the total number of macrophages in the whole lung tissue (Figure 4.2F).

To further characterize the overall immune response taking place in the lungs, BAL supernatant was analyzed on a Bio-Rad 33-plex array to identify various cytokines that had been secreted into the airways. We found that there were significant inhalation-dependent increases in the levels of the following cytokines in the airways: IL-1β, IL-6, IL-16, CCL7, CCL17, CCL19, CCL22, CCL24 and CXCL10 (Figure 4.3A-I). Many of these cytokines function as chemokines that regulate recruitment of both innate and adaptive immune cells to a given area, indicating activation of the overall immune response.

Mice who experienced an inhalation injury had increases in both total neutrophils and a corresponding increase in total protein in the BAL. We postulated that cells present in the BAL

may be leading to increased production of oxidants such as nitric oxide. Using flow cytometry we found that there was an increase in intracellular nitric oxide synthase 2 (NOS2) in macrophages from inhalation-treated animals, but not in neutrophils (Figure 4.4A and 4.4B). *Inhibition of production of oxidants results in decreased pulmonary damage and decreased neutrophil recruitment after inhalation injury.*

NOS is involved in the intracellular processes involved in utilizing L-Arginine to produce L-Citrulline and nitric oxide. Nitric oxide is then utilized by the cell and can cause damage to its surrounding environment. In order to decrease pulmonary damage and regulate the immune response we administered an inhibitor of nitric oxide production called L-Nitroarginine methyl ester (L-NAME) intranasally immediately after inhalation injury. We found that administration of L-NAME after inhalation injury resulted in decreased protein concentration in the BAL (Figure 4.4C) as well as a decreased total number of neutrophils collected from the BAL (Figure 4.4D).

Discussion

Many labs utilize murine models to identify cellular mechanism and potential therapeutic targets for the treatment of burn-associated immune dysfunction [43, 44, 49-51]. In this manuscript we utilize a model that has been previously developed in our lab (insert Julia reference here) to examine the combined effects of burn and inhalation injury. Our model successfully demonstrates immune dysregulation found in patients who are admitted to the hospital due to inhalation injury, as is characterized by increased mortality, numbers of neutrophils present in the airway and protein present in the airway [52, 53]. Our murine model allows us to uniquely examine alterations in the lung tissue, as this is an organ that cannot be utilized for study in humans.

Multiple studies have indicated that after burn and inhalation injury there is an increase in neutrophils present in the airway, and that these neutrophils are associated with an increase in oxidants and associated oxidative damage of the pulmonary tissues [32, 35, 54-58]. Additionally, preliminary studies have been conducted that indicate that after burn injury inhibition of production of reactive oxygen species can lead to decreased damage to the lungs, or increased clearance of subsequent bacterial infections [56]. Additional studies have demonstrated that inhibition of nitric oxide production after inhalation injury resulted in decreased systemic indicators of oxidative injury [30]. Our study examined alterations in the lung that took place after a combined burn and inhalation injury, and then identified a nitric oxide inhibitor as a potential therapeutic to downregulate the immune response after inhalation injury in order to prevent excessive immune-mediated damage to the pulmonary system.

Additionally, our studies found that multiple cytokines are secreted into the airway after injury, including immunomodulatory cytokines such as IL-1β, IL-6, and IL-16. These cytokines are commonly associated with the events of shock and have been found to be elevated in patients who are being treated for burn injury [8, 16]. Additionally, studies have indicated that levels of IL-1β and IL-6 correlate with poor clinical outcomes among patients [59]. Each of the other cytokines and chemokines that were elevated in the BAL are involved in the recruitment of T cells, monocytes, macrophages, dendritic cells and neutrophils. Recruitment of these cells would lead to increased cell numbers, and overall immune activation to take place at the site of injury, potentially leading to worsened damage of the lungs.

Previous data in our lab has demonstrated that inhalation injury of mice deficient in NOS2 do not experience the inhalation-dependent increase in numbers of neutrophils or in protein concentration present in the BALF of wildtype mice. This led us to believe that NOS

could be a viable target for therapeutic intervention to decrease inhalation-dependent pulmonary damage. Clinical trials with non-selective NOS inhibitors in the treatment of shock resulted in increased mortality among patients [60]. Indeed, preliminary studies in our lab additionally indicated that NOS2 deficiency results in increased mortality late after inhalation injury (Figure 4.5A). This phenomenon appears to be location-dependent within the body as well, as recent studies have demonstrated that intravenous administration of a nitric oxide agonist can improve vascularization and response after burn injury [61]. This data combined indicates that specific responses need to be targeted, where full ablation of NOS activity results in increased mortality, but that moderate inhibition of NOS signaling can prevent damage that takes place after inhalation injury. Among patients, ideal treatment strategies would prevent overactivation of the immune response that often takes place when patients experience a major shock such as a burn injury. However, while it is important to prevent hyperimmunity, it is also essential to maintain the integrity of the immune system in order to allow the patient to fight potential pathogens they may encounter in order to maintain ideal health. This is why it is essential to identify therapeutics that would allow for slight immunomodulation rather than complete polarization of the immune response.

FIGURES

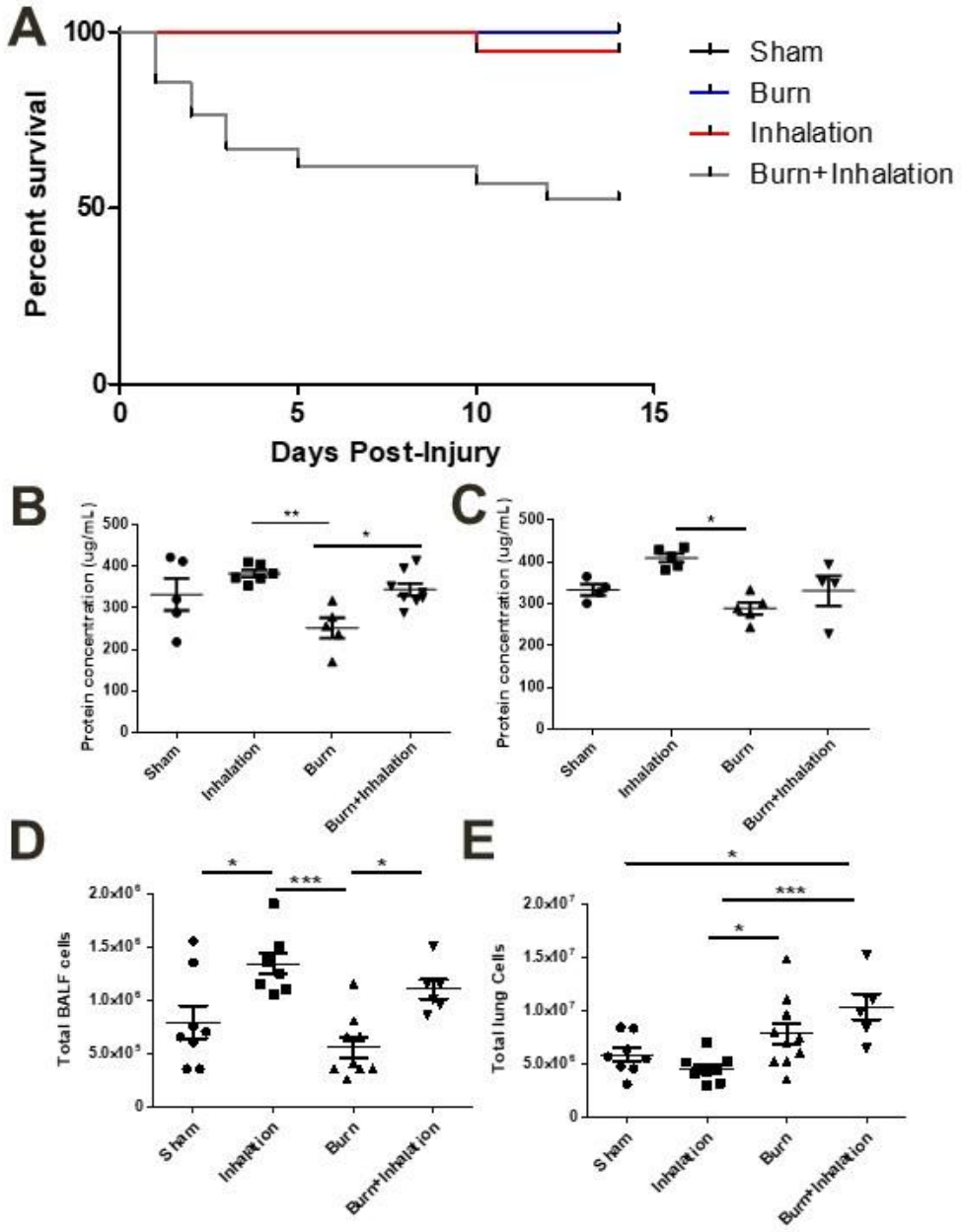

Figure 4.1: Combined injury results in increased mortality over time, while major markers of pulmonary damage are elevated in an inhalation-dependent manner. Mice underwent sham, burn, inhalation or combined injury A) Mice were examined over the course of 14 days and mortality was recorded; B) Protein levels detected in BAL primary washes 96hrs after injury.; C) Protein levels detected in BAL primary washes 14days after injury.; D) Total number of cells that were isolated from the BAL primary and secondary washes 14 days after injury.; E) Total number of cells obtained from single cell isolation of lung tissue 14 days after injury. Data shown are +/-SEM. *p<0.05, **p<0.01, and representative of three repeated experiments.

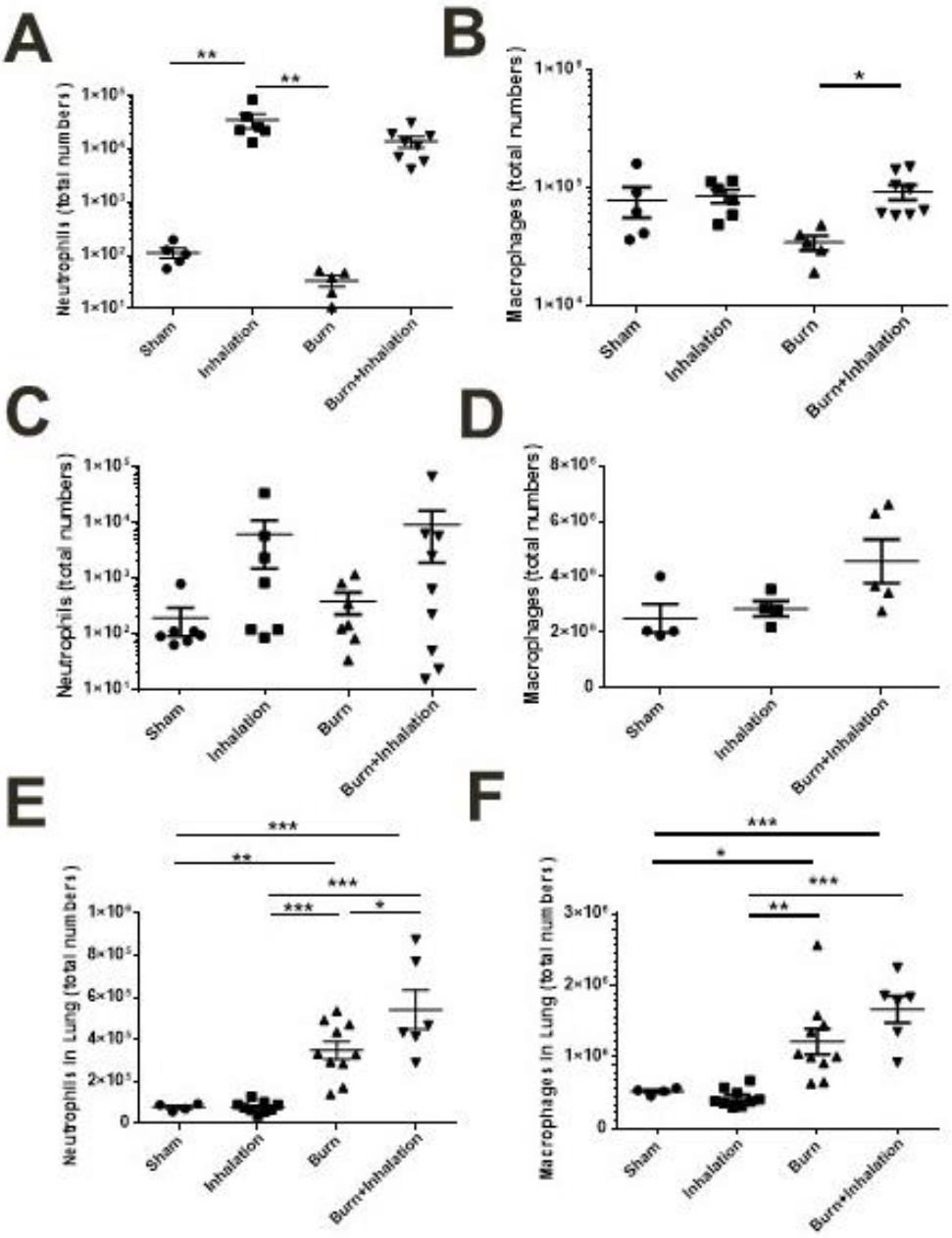

Figure 4.2: Innate immune cells are recruited to the airspace and to the pulmonary tissue in an injury-dependent manner. Mice underwent sham, burn, inhalation or combined injury, and cells isolated were examined via flow cytometric analysis A) Neutrophil numbers collected from BAL were found to be increased in an inhalation-dependent manner 96hrs after injury; B) No detectable changes in the total number of macrophages collected from BAL were detected 96hrs after injury.; C) Neutrophil numbers collected from BAL increased in an inhalation-dependent manner 14days after injury; D) No detectable changes in the total number of macrophages in BAL were detected 14days after injury.; E) Total neutrophils obtained from single cell isolation of lung tissue 14 days after injury were increased in a burn and combined

injury-dependent manner.; F) Total macrophages obtained from single cell isolation of lung tissue 14 days after injury were increased in a burn-dependent manner. Data shown are +/-SEM. *p<0.05, **p<0.01, and representative of three repeated experiments.

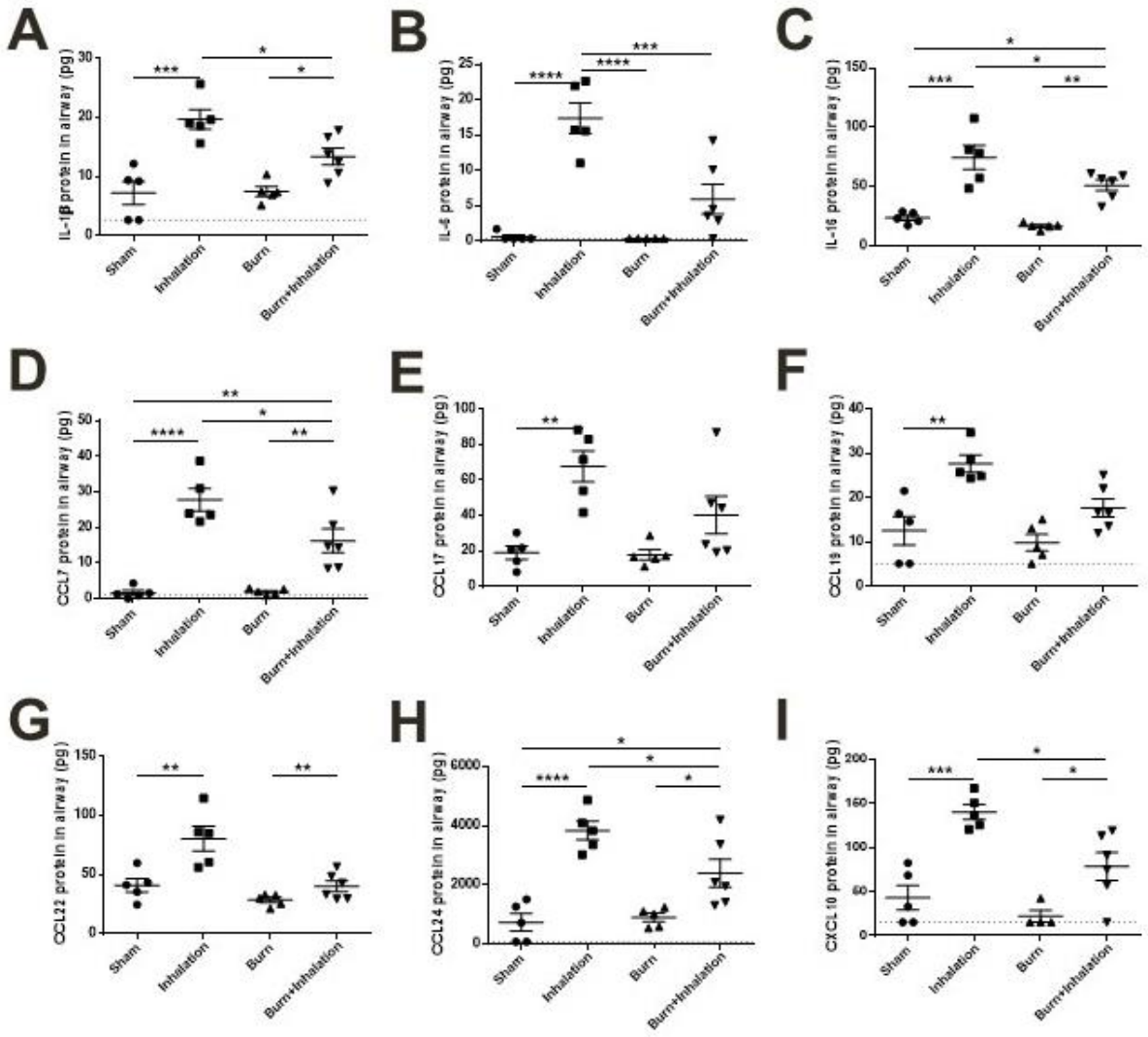

Figure 4.3: Multiple cytokines and chemokines are elevated in an inhalation-dependent manner 96hrs after inhalation injury. Mice underwent sham, burn, inhalation or combined injury, and supernatant collected from BAL washes was analyzed using a Bio-plex mouse chemokine panel 33-plex. Inhalation dependent increases in amount of A) IL-1β, B) IL-6, C) IL-16, D) CCL7, E) CCL17, F) CCL19, G) CCL22, H) CCL24, I) CXCL10 were detected. Data shown are +/-SEM. *p<0.05, **p<0.01, and representative of three repeated experiments.

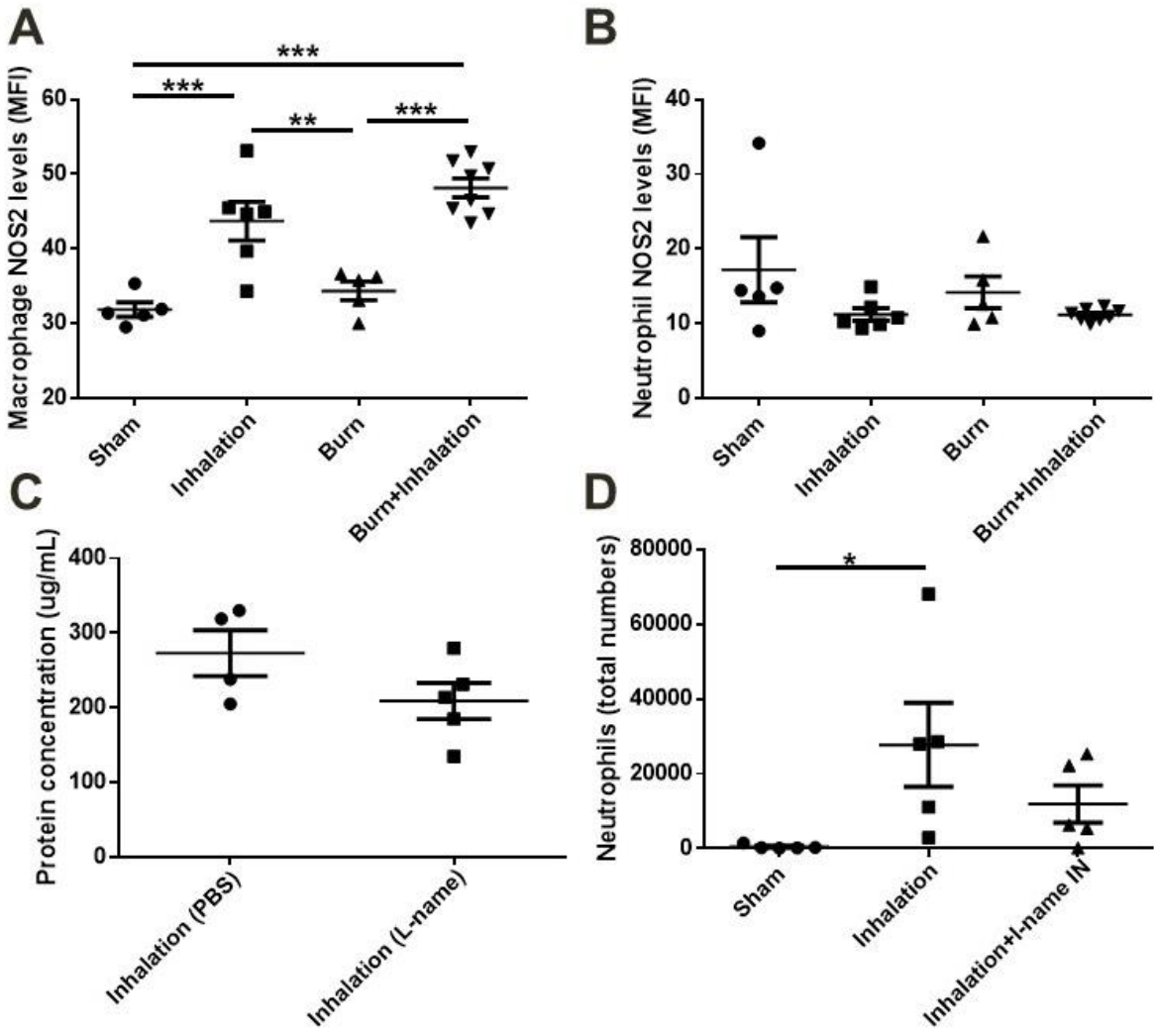

Figure 4.4: Nitric oxide generation is involved in pathology of inhalation injury. Mice underwent sham, burn, inhalation or combined injury, and cells isolated were examined via flow cytometric analysis A) Macrophages present in BAL were found to have an inhalation-depndent increase in NOS2 levels as detected by intracellular staining. B) Inhalation injury did not result in alterations in NOS2 levels in neutrophils in BAL. C) Intranasal administration of L-NAME at the time of injury resulted in slight decrease in protein concentration collected in the BAL after inhalation injury. D) Intranasal administration of L-NAME at the time of inhalation injury resulted in decreased numbers of neutrophils collected from BAL. Data shown are +/-SEM. *p<0.05, **p<0.01, and representative of three repeated experiments.

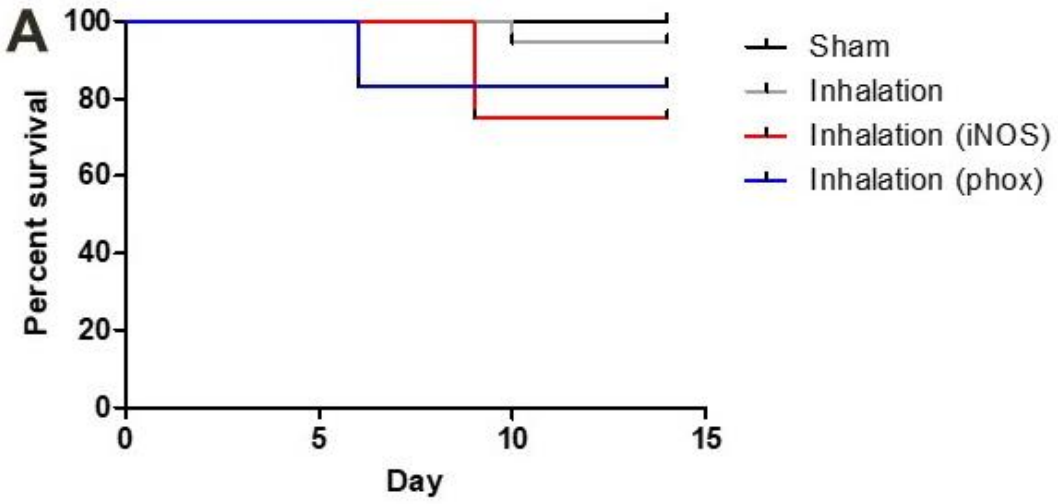

Figure 4.5: Complete ablation of nitric oxide machinery results in increased mortality after inhalation injury. Mice underwent sham or inhalation injury, and were monitored over time A) Mice deficient in nitric oxide and Reactive oxygen and nitrogen machinery experienced increased mortality after inhalation injury relative to sham and inhalation injured wildtype controls.

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CHAPTER 5: DISCUSSION

Concluding remarks

Improvement in the treatment of traumatic burn injury is necessary, however currently there are many barriers to burn-associated drug discovery. In order to find and test therapeutics, there needs to be a significant improvement in animal models of burn injury that are utilized. Any models utilized need to recapitulate the various major markers of disease that are indicated among the human patient population. Burn injury causes significant immune dysregulation among patients, which leads to increased susceptibility to infection as well as additional injury-associated complications [1, 2]. Testing of treatments of burn injury will need to address the immune dysfunction that takes place, and to improve overall immune responses.

Many models of burn injury are unable to fully replicate the immune-mediated damage that can arise throughout the course of initial injury followed by treatment and recovery. In order for a model to successfully capture the complex nature of burn injury it should exhibit similar etiology and molecular mechanisms to those seen among humans. Largely, models should be able to successfully detect alterations in levels of cytokines, chemokines, damage-associated molecular patterns and immune cells present in various tissues that are compromised in the human population [3-7].

In an effort to create effective models of burn injury, our laboratory has recently focused on examination of burn models that take into account multiple burn-associated comorbidities, such as concomitant infection and/or radiation exposure [8-10]. Our studies indicated similar

findings to those of other groups, namely that early immune dysfunction after injury is relatively easy to recapitulate [9, 11], however, late after injury burn-treated animals often experience heightened immune responses that are protective against bacterial challenge, a phenotype that does not reflect what takes place among human counterparts [12, 13]. We have additionally found that this phenotype is overcome if burn is administered in the presence of an additional burn-associated comorbidity, such as a radiation injury [8, 10].

Models generated

In this dissertation we examine two novel models of burn-associated comorbidities and their respective effects on the immune system. We noted that previous literature indicates that following burn injury, a single incidence of infection does not lead to increased mortality [14]. However, repeated bacterial exposures led to approximately a 36% increase in mortality. Skin represents a significant barrier that protects individuals from the outside environment, and a burn wound represents a significant disruption to that barrier that could potentially allow patients to be exposed to additional bacteria from their environment. It is reasonable to believe that patients would experience multiple bacterial exposures during the course of their treatment of a burn injury. Therefore, we generated the model discussed in chapter 2 of this thesis, in which animals were infected with *Pseudomonas aeruginosa* twice in order to elicit immune responses that may reflect those seen among burn patients.

In chapters 3 and 4 of this thesis we focus on the development of a model of inhalation injury. One significant byproduct of burning compounds is smoke generated. Many patients who experience a flame burn arrive at the hospital with an associated inhalation injury, resulting in approximately 10-20% of all burn patients treated at hospitals having an inhalation injury [15].

Presence of a burn-associated inhalation injury leads to increased mortality, complicated treatment, and lengthened hospital stays for patients [16-18]. Many animal models have sought to uncover the underlying mechanism of inhalation-dependent alterations of the immune system [19-21], however few groups have examined the combinatory effects of burn and inhalation injury [22] in spite of the fact that studies indicate that damage after burn and inhalation injury combined is more significant than is attributable to each injury alone [22]. We created a model that utilizes particle board, a compound commonly found in building materials, to create a clinically-relevant model of study of inhalation injury that we have utilized to examine the effects of inhalation injury independently as well as associated with a burn injury.

Commonalities between models of burn injury.

One commonality between studies of patients and burn-associated models is that there is alteration in the immune profile after burn injury. Largely, immediately after burn injury the wound causes significant damage to the immune compartment that is characterized by the generation of damage-associated molecular patterns (DAMPS) [1, 3, 4]. DAMPS generated lead to alterations in the cytokine profile and immune cell phenotypes found systemically. Among burn patients, a leading cause of mortality is burn-associated pulmonary damage and associated infections and pneumonia [23]. Due to the importance of the pulmonary system to survival and the relative susceptibility of burn patients to pulmonary complications, we chose to focus our research on the pulmonary compartment in examination of both our models of burn-associated comorbidities.

We found that in both models, burn injury caused the recruitment of neutrophils to the pulmonary tissue. These neutrophils were not present in the airway of the lungs unless there was

some additional stimulus to promote the recruitment of neutrophils to the airspace. For example, infection of animals with an airway infection resulted in increased numbers of neutrophils to be present in the airspace. Additionally, in our model of inhalation injury we found that the presence of an inhalation injury resulted in neutrophil recruitment to the airspace, however, this phenotype occurred independently of the burn injury. We have additionally shown that recruitment of these cells corresponds with damage to the area, potentially creating an environment in which bacteria could thrive (depicted in figure 5.1).

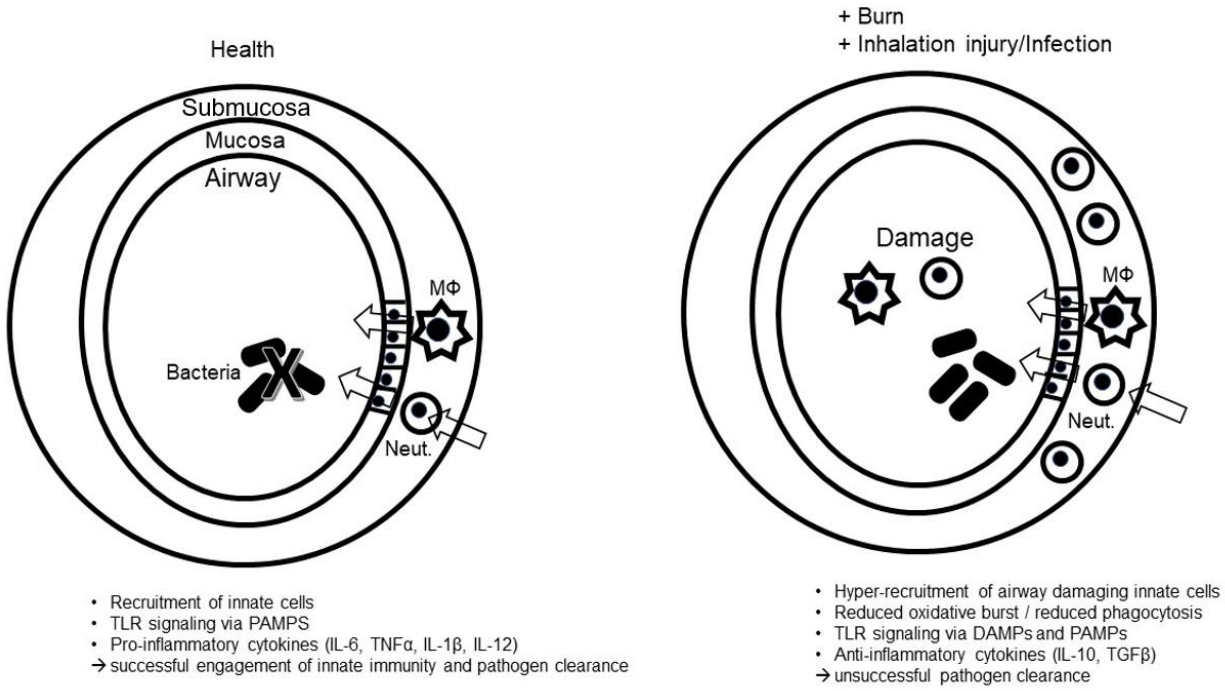

Figure 5.1: Comparison of a healthy and burn+comorbidity environment. After burn injury, various DAMPS will be released, however it is only when there is an additional stimulus in the airways that cells are recruited into the airspace, potentially contributing to increased bacterial burden and worsened outcomes after experiencing a burn+comorbidity.

We also found that neutrophil intracellular levels of reactive oxygen and nitrogen species (RONS) were significantly altered after burn injury. Burn injury resulted in increased levels of RONS. We also found that inhalation injury resulted in alterations of levels of nitric oxide synthase. This corresponds with previous data that has been published indicating that regulation of oxidative stress after burn injury is significantly altered and represents a potential target for improved healing [24, 25]. We additionally found that it is possible to alter levels of RONS, and that targeting nitric oxide could prove a successful potential therapeutic for treatment after inhalation injury.

Additionally, we found that burn injury and its associated comorbidities lead to alterations in the production of immune-mediated cytokines and chemokines. Indeed, injury-associated cytokine production led to production of chemokines that are commonly associated with communication and regulation between the adaptive and innate immune systems. Alterations in levels of cytokines such as IL-6, IL-10 and IL-12 indicate that wound-induced damage in our models is able to generate significant alterations in the immune compartment that are similar to changes seen in the human population. After inhalation injury we also found increased levels of a number of chemokines associated with recruitment of dendritic cells, B cells and T cells. It would be important for future studies to focus on examining how these additional cell types are altered by these injuries and characterize how to further improve the immune response after injury.

Conclusion

Comorbidities of burn injury are known to result in significant increases in mortality. The models that have been generated during the course of this thesis have allowed for improved

probing of the immune responses. These models also represent a powerful tool that can be utilized to answer many clinical questions and address variables that significantly affect burn injury. For example, studies have indicated that severity of inhalation injury is dependent on the source utilized to generate smoke particles. Using our model, we could study various sources of smoke to examine both universal and source-dependent differences in immune consequences of inhalation injury. Additionally, these models could be used to examine multiple pre-clinical treatments in order to discover therapies that could significantly improve outcome after burn injury and promote recovery. Finally, we could also study the effects of additional comorbidities that are involved in driving responses after burn injury, such as age, BMI, smoker status, and presence of alcohol in the system at the time of burn injury in order to further replicate factors that affect clinical outcomes. These studies are necessary and essential in order to improve patient treatment and survival after burn injury.

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