BIOMARKERS OF LUNG EPITHELIAL DAMAGE AFTER INHALATION INJURY

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ABSTRACT

Rachel Elaine Vaughan: Biomarkers of Lung Epithelial Damage After Inhalation Injury
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Burn and inhalation injury affects almost half a million people in the United States each year and it is one of the most complicated forms of trauma to treat. Further research is needed in order to better understand the precise mechanisms behind both burn and inhalational injury, as well as means by which to more accurately stratify patients by injury severity in order to care for them more effectively. In this study, I examine two potential biomarkers in mice with possible translational relevance that could be of use in quantifying injury severity in hospitalized patients. IL-33 and 8-isoprostane may be indicators of acute and long-term damage, respectively. IL-33 is elevated at twenty-four hours post-injury in mice with inhalation injury, while 8-isoprostane is elevated in mice with combined burn + inhalation injury at two weeks post-injury. These data may begin to provide a method for better patient evaluation and improved outcome.

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LIST OF ABBREVIATIONS

ALI	acute lung injury
ANOVA	analysis of variance
BALF	bronco-alveolar lavage fluid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immune-sorbent assay
IL	interleukin
IL-1RAcP	Interleukin-1 receptor accessory protein
MPO	myeloperoxidase
Myd88	Myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
NKT	natural killer T cell
PBS	phosphate-buffered saline
ROS	reactive oxygen species
SEM	standard error of the mean
TBSA	total body surface area
TNF-α	tumor necrosis factor alpha
TH2	T helper cell type 2
WL	whole lung

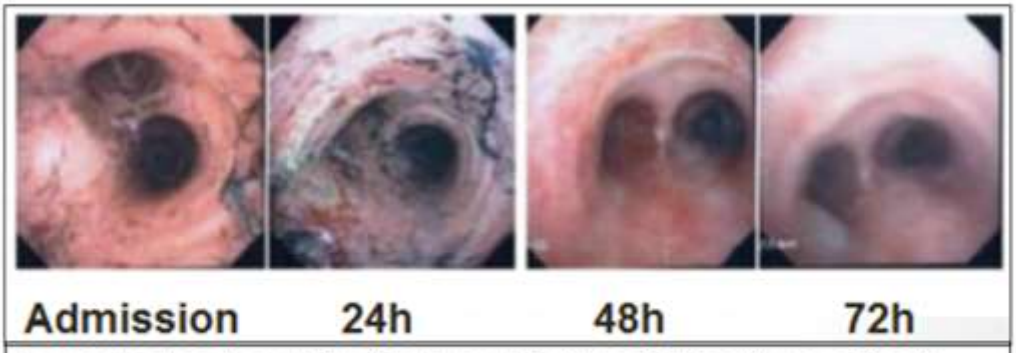
CHAPTER 1: INTRODUCTION

Burn injury affects approximately 450,000 individuals in the United States per year, of which around 50,000 are hospitalized[1]. This comes at a cost of roughly $7.5 billion dollars and presents with a complex pathology affecting multiple organs and systems[1,2]. Burn injury, however, frequently presents with other comorbidities, most commonly inhalational injury from smoke[2]. Therefore, it is necessary to understand the pathophysiology underlying inhalational injury alone, as well as in conjunction with burn in order to better understand how best to treat hospitalized patients. I hypothesize that smoke inhalation causes increased damage to the lung epithelial cells, resulting in the release of cytokines. These biomarkers can then be used to assist in stratifying hospitalized patients by injury severity, thereby allowing for improved treatment efficacy.

Inhalational injury has changed along with the smoke toxicity over the past few decades as increasing numbers of products are manufactured with synthetic materials, causing them to burn hotter, faster, and to release more chemicals into the air that can potentially be inhaled and cause further damage[2]. This heterogeneity of combustion products complicates both research into and treatment of inhalation injury. Previously, animal models have included mice, rats, sheep, and pigs, however the most commonly used source of smoke in these studies is cotton, which does not adequately recapitulate the conditions of a human exposed to a housefire or similar environment[2–4].

Inhalation injury in humans is characterized by possible thermal injury to and/or chemical irritation limited to the upper airway[5]. Toxic compounds present in the combustion products (e.g. aldehydes, carbon monoxide, hydrochloric acid, oxides, etc.) may cause damage to the lung parenchyma and interfere with gas exchange in the lung, further complicating treatment[5]. In the lower airways, thick mucous collects and lung epithelial cells slough off of the basement membrane **(Figure 1)**[5]. Neutrophils infiltrate into the alveolar spaces and release reactive oxygen species (ROS), which may cause further damage to the lung tissue[3,5]. Severe inhalation injury or burn may also lead to acute lung injury (ALI), in which a patient suffers from impaired gas exchange, reduced airway compliance, increased pulmonary vascular resistance, edema, and neutrophil sequestration in the lung tissue[6].

Figure 1: Bronchoscopy image of human lower trachea and bronchi



Lower trachea branching into bronchi of hospitalized burn patients. Sooty debris seen from admission through 24 hours. Inflammation and mucous appear at 48-72 hours.

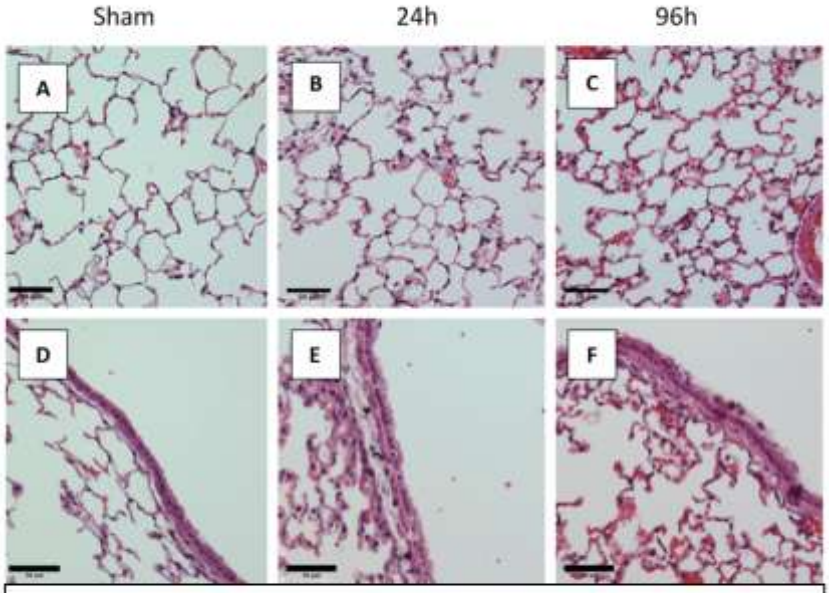
There is currently no standardization for severity of lung injury or biomarkers to identify patients with a greater degree of lung tissue damage. Most treatment centers rely heavily on the use of a bronchoscope to visually evaluate the large airways, looking for debris

accumulation, mucous buildup, and epithelial damage, such as can be seen in **Figure 1**. Additionally, the Baux score—a system used to predict mortality from burn injury based on the percent of total body surface area (TBSA) burned and the patient's age—immediately adds 17 for inhalation injury. A score of 140 is considered to be unsurvivable. While these measures are of some benefit, a more precise method for determining injury severity and patient outcomes would be of enormous use in targeting treatment for individual patients.

Thus far, animal models have been unable to completely mimic the human pathophysiology and so we developed a mouse model of inhalation injury that more closely approximates the human inhalation injury in order to better understand the underlying mechanisms of injury. This allows for a more focused effort at identifying potential biomarkers of lung injury as well as evaluating possible interventions to improve patient outcome.

Despite the obvious differences in scale and differences in the nasal passages between mice and humans, they share key similarities that make mouse models a viable option for burn and inhalation injury. These include similarities in cytokine and chemokine production, and leukocyte activation[7]. From histological images of the alveolar space and large airways, we know that inhalation injury in mice causes the same type of damage as that seen in humans—a disrupted epithelial layer, detached basement membrane, vacuolization, cell death, and the presence of debris and inflammatory infiltrate **(Figure 2)**.

Figure 2: Lung histology of sham and smoke-exposed mice



Distal lung (A-C) and large airway (D-F) images from sham-treated and 24 and 96 hour post-smoke inhalation mice (Dunn)

One possible biomarker that could be of use in indicating injury severity is IL-33, a nuclear cytokine in the IL-1 family that is expressed in epithelial barrier tissues and lymphoid organs[8]. It has been observed in humans and mouse models of asthma and activates Myd88-dependent signaling pathways in target cells expressing ST2/IL-1RAcP receptor complex[8,9]. Such target cells include natural helper cells, innate helper 2 cells, mast cells, basophils, eosinophils, TH2 cells, NKT and NK cells[8]. In a mouse model of ALI, IL-33 pretreatment decreases the survival rate of the mice, increases proinflammatory cytokines TNF-α and IL-6, and increases myeloperoxidase (MPO) levels in lung tissue[9]. Furthermore, IL-33 pretreatment destroys adherens junctions, thereby causing increased pulmonary capillary barrier damage and pulmonary edema[9].

8-isoprostane may also be a good indicator of oxidative stress in lung tissues and elevated levels have been observed in breath condensate, plasma, sputum, and urine in cystic fibrosis, inflammatory airway diseases, and other cases of lung injury[10–12]. Oxidative damage to lipids leads to the production of isoprostanes, including 8-isoprostane[11]. These isoprostanes have frequently been used in other models as a reliable indicator of oxidative stress, as they are structurally stable, produced *in vivo*, and are a common feature of respiratory disease[11].

CHAPTER 2: METHODS

Female C57BL/6 mice from 8-10 weeks old and weighing 18-21 g were obtained from Taconic Farms and housed in pathogen-free facilities. All protocols were approved by the University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee and were verified to follow guidelines from the National Institute of Health concerning use of vertebrate animals in research.

Inhalation protocol

Mice were anesthetized with tribromoethanol (avertin) (475 mg/kg body weight; Sigma-Aldrich). Their backs were then closely shaved to expose the skin and they were given a subcutaneous injection of morphine sulfate (3 mg/kg body weight; Westward). They were each given an unique ear tag identifier and ophthalmic ointment was applied to their eyes. Mice were then placed on an intubation platform and intubated with a catheter (22G x 1", Exel) into the trachea following visualization with a laryngoscope. Mice were secured to a platform in a supine position with Velcro straps placed loosely across the chest and placed into an animal induction chamber (Stoelting NC9296517). Approximately 50g of 2.5 cm x 8 cm sections of particle board were placed into a side-arm flask on a heat block set to 500C, causing the wood to smolder and emit smoke. Additional wood was added as necessary to maintain visual smoke density. Air was pumped through the flask and into the induction chamber for three exposures of two minutes each with a one minute break between exposures. The entire inhalation

apparatus was set up and maintained in a dedicated fume hood. Mice were resuscitated with an intraperitoneal injection of lactated Ringer's solution (1 mL/kg body weight; Baxter Healthcare Incorporated) and allowed to recover on a heating pad. Mice were maintained for the duration of the experiment on morphinated water available at all times in their cages. Sham mice were treated identically, except for the lack of smoldering wood in the flask; instead pure air was pumped through the system. At time of tissue harvest, mice were euthanized with gaseous isofluorane.

Burn protocol

Burned mice were prepared in exactly the same manner as the inhalation mice *(above)*, except that instead of being intubated, they were given a 20% TBSA burn with four applications of a copper rod heated in a 100C water bath and dried immediately prior to application to avoid scald injury. They were resuscitated and maintained on morphinated water identically to the inhalation injury mice.

Broncho-alveolar lavage sample acquisition and processing

Broncho-alveolar lavage fluid (BALF) was collected by inserting a 22G x 1" catheter into the trachea connected to a syringe containing 1 mL 0.6 mM EDTA in PBS. .7 mL was flushed into the lungs, massaged through the lungs by chest palpitations, and then withdrawn. This same 1 mL fluid was flushed a total of three times through the lungs to obtain a primary wash. Typical recovery was 0.75 mL. This procedure was repeated with two more syringes of fluid and combined to form a secondary wash with a recovery of around 1.8 mL. Cells were pelleted out and stored at -80C and the cell-free supernatant from the primary wash was stored at -20C

for later analysis via enzyme-linked immune-sorbent assay (ELISA) for IL-33 (R&D) and 8-isoprostane (Cayman Chemical) according to the manufacturer's instructions.

Whole lung acquisition and processing

Lungs were removed from mice after the BALF was extracted. Lungs were suspended in 0.5 mL PBS, 0.005% BHT, and bullet blender beads (Next Advance). Lungs were then placed in the Bullet Blender (Next Advance) for 5 minutes. Homogenized tissue was then centrifuged to pellet out solid material and the supernatant was extracted and stored at -20C for later analysis via ELISA for IL-33 (R&D) and 8-isoprostane (Cayman Chemical).

Statistical analysis

GraphPad Prism was used to analyze data via Student's t-test, one-way analysis of variance (ANOVA) with Tukey post-test, as appropriate. Data are represented as mean +/- standard error of the mean (SEM). Statistical significance is indicated as * $p<0.05$ and ** $p<0.005$.

CHAPTER 3: RESULTS

8-isoprostane in BALF is increased after burn + inhalation injury at two weeks post-injury

Following burn, inhalation injury, and combined burn + inhalation injury, total 8-isoprostane levels in whole lung (WL) homogenate supernatant remains unchanged at 500 pg/mL to 600 pg/mL from twenty-four hours through two weeks post-injury **(Figure 3)**. However, levels of 8-isoprostane in the BALF were significantly increased ($p<0.05$) in the combined burn + inhalation treated mice **(Figure 4)**. No differences were observed at twenty-four or ninety-six hours post-injury.

Figure 3: Total 8-isoprostane in WL homogenate supernatant is unchanged after injury

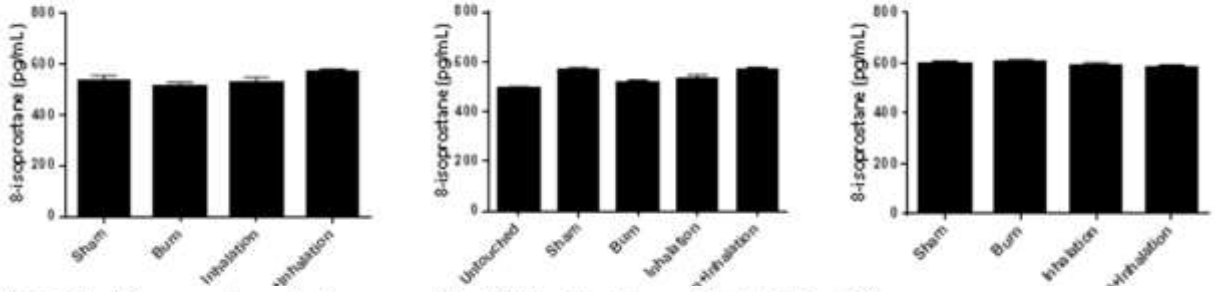
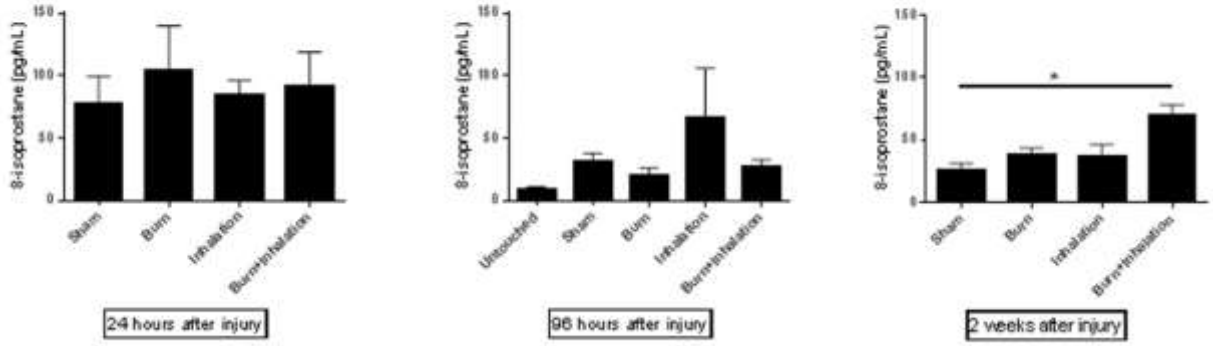


Figure 4: 8-isoprostane is increased in BALF after burn + inhalation injury



IL-33 in BALF is increased in inhalation injury at 24 hours post-injury

Total IL-33 in WL homogenate supernatant also remains unchanged throughout the duration of the experiment **(Figure 5)**. Levels of IL-33 remain fairly consistent between 500 pg/mL to 600 pg/mL. BALF levels of IL-33 indicated a greater sensitivity to injury than the WL homogenate supernatant and there is a significant increase in BALF IL-33 at twenty-four hours post-injury ($p<0.005$) in the inhalation treated mice and an increase ($p<0.08$) at two weeks post-injury in the burned mice **(Figure 6)**. Time points at less than twenty-four hours indicated no significant differences between groups.

Figure 5: IL-33 in WL homogenate supernatant is unchanged after injury

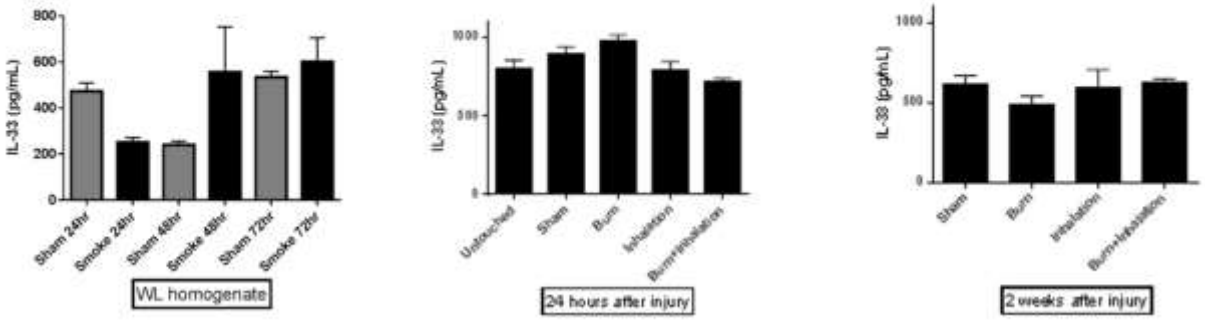
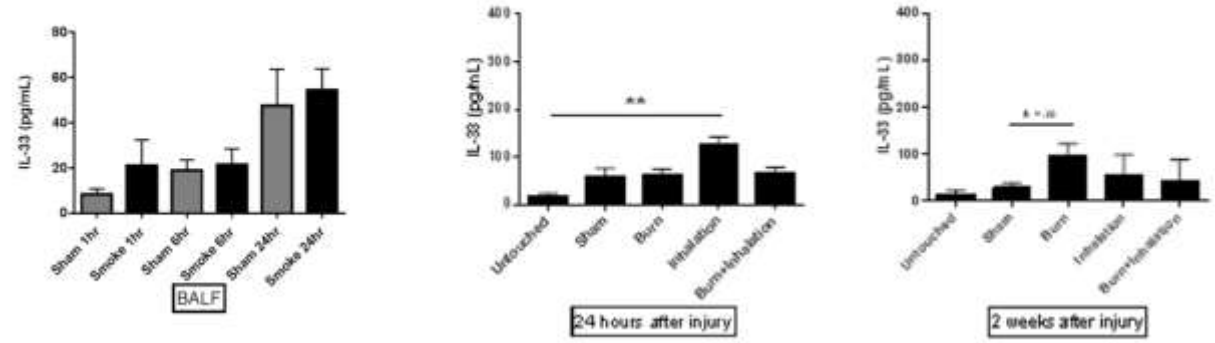


Figure 6: IL-33 is elevated in BALF after injury



CHAPTER 4: DISCUSSION, LIMITATIONS, AND FUTURE DIRECTIONS

From these data, 8-isoprostane appears to be a possible indicator of late damage following burn + inhalation injury. This may be due to oxidative damage from neutrophil infiltration and subsequent release of ROS into the lung tissues. The released ROS may be causing further damage to compromised lung tissue, thereby triggering the release of 8-isoprostane into the BALF.

We further conclude that IL-33 may be an indicator of acute damage to the lung epithelium following the inhalation injury. This may be due to direct injury from particulate matter in the smoke and/or damage from toxic chemicals released from the particle board during combustion. The rise in IL-33 in burn at two weeks post-injury may be an indicator of secondary systemic effects of the burn as the body becomes immune-compromised and cannot continue to adequately support normal lung tissue maintenance.

In addition to its well-documented role as a pro-inflammatory cytokine and alarmin, IL-33 may also play a role in driving an early anti-inflammatory response that promotes healing, similar to that seen in IL-10 in human studies of burn trauma[13]. This mechanism may involve sequestration of NF-κB, thereby reducing NF-κB-triggered gene expression and dampening proinflammatory signaling[14]. Such dampening of the protective pro-inflammatory responses shortly after injury may result in an increased susceptibility to later infection, a common serious complication of burn injury[13].

This study is limited by the inability to provide major supportive care following injury to the mice and the ethical limitations on the severity of injury that can be studied. Mice could not be mechanically ventilated or have blood gases monitored and those with the most severe injuries were euthanized prior to the completion of the study. This limits the relationship to human burn patients who have access to such advanced supportive care following injury. A larger sample size at an increased number of timepoints may also improve the precision of the results and provide greater insights into the mechanism of the injury.

Future studies will include the assessment of serum as another easily available means by which to look for possible biomarkers to evaluate patient injury severity, as well as expanding the screen for other potential candidates. We also plan to evaluate human samples and compare them directly with the results seen in mice in order to ensure that any good biomarker candidates are translationally relevant. Additional studies to determine the level of ROS in the lung following injury and then blocking neutrophil infiltration and re-testing the level of ROS may be useful in determining the cause of damage, particularly at later time points. Finally, a precise chemical analysis of the smoke produced from combustion of the particle board could provide insights into the most relevant toxins that may be inhaled and allow for better patient stratification based on the source of combustion materials in inhalation and combined injury. We hypothesize that the combustion of synthetic products and chemicals will result in an increase in mortality and hospital stay.

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