6-4-2024

The Role of MicroRNAs in Lung Inflammation After Alcohol and Burn Injury

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THE ROLE OF MICRORNAS IN LUNG INFLAMMATION AFTER ALCOHOL AND BURN INJURY

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

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CHICAGO, IL

MAY 2024

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Mashkoor Choudhry for his guidance and expertise, which has empowered me to pursue science with confidence. I would also like to thank Dr. Xiaoling Li and Dr. Caroline Herrnreiter for their expertise, assistance, and patience throughout my graduate studies. Moreover, I want to express my gratitude to all the members of the Choudhry Lab at Loyola University Chicago, with whom I have had the opportunity to collaborate with during my two quick years on campus. My interaction with everyone in this lab has truly made my time at Loyola an enjoyable and unforgettable experience. I would also like to express my appreciation to all my friends and family for their unwavering support throughout this process. Also, a special thank you to the Alcohol Research Program and the Burn and Shock Trauma Research Institute, as well as my current program director, Dr. Brian Mounce, and the Infectious Disease and Immunology Program. Lastly, I would like to say thank you to my thesis committee members for their insight, expertise, and support, as well as the National Institutes of Health for their funding.

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ALI	Acute Lung Injury
ANOVA	Analysis of Variance
ARDS	Acute Respiratory Distress Syndrome
COPD	Chronic Obstructive Pulmonary Disorder
CXCL1	Chemokine Ligand
DAMP	Damage Associated Molecular Pattern
ELISA	Enzyme-Linked Immunosorbent Assay
ICAM-1	Intracellular Adhesion Molecule-1
IECs	Intestinal Epithelial Cells
IL	Interleukin
i.p.	Intraperitoneal
KC	Keratinocytes-Derived Chemokine (Also denoted as CXCL1)
LPS	Lipopolysaccharide
Ly6G	Lymphocyte Antigen 6 Family Member G
MiRISC	MiRNA Induced Silencing Complex
MOF	Multiple Organ Failure
MPO	Myeloperoxidase
PAMPs	Pathogen Associated Molecular Patterns
RT-qPCR	Reverse Transcription-Quantitative Polymerase Chain Reaction

SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
TBSA	Total Body Surface Area
TJ	Tight Junction Protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
UTR	Un-Translated Region
ZO	Zonula Occludin

CHAPTER 1

INTRODUCTION

Burn injuries affect around half a million people in the United States annually, resulting in 40,000 hospitalizations[1]. The pathophysiological consequences of severe burn injury are systemic and complex[2]. Specifically, severe burn patients are at an increased risk of infection, multiple organ failure (MOF), and sepsis, which significantly contributes to mortality[3,4]. About half of reported burn injuries occur while a patient is under the influence of alcohol[5]. Alcohol is a major confounding factor in the pathophysiology of burn injuries. Patients intoxicated at the time of burn injury exhibit increased susceptibility to infections, and an increased risk of sepsis, MOF, and higher mortality rates compared to patients who did not consume alcohol prior to sustaining an injury[6]. Furthermore, these patients are at an increased risk for pulmonary infections and complications because of their suppressed immune state[7]. Respiratory failure, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and pulmonary infections are the leading causes of post-burn morbidity and mortality[8]. Prior studies have shown an association between prolonged lung inflammation and oxidative stress after combined alcohol intoxication and burn injury with ARDS and MOF, but the underlying pathophysiologic mechanisms are not fully understood[3,6-8].

Our laboratory investigates the effect of alcohol and burn pathophysiology through the use of a mouse model of acute ethanol intoxication and burn injury. In this particular model, each mouse is administered a single dose of 0.4 mL of 25% ethanol via oral gavage, which results in

a blood alcohol content (BAC) of 90-100 mg/dL four hours after administration. Next, each mouse receives a scald burn injury that covers approximately 12.5% of their total body surface area (TBSA) on their shaved dorsum. This mouse model holds clinical relevance since it reflects the occurrence of traumatic injuries in individuals under the influence of alcohol[9]. It is also important to realize that we are dealing with a relatively minor injury model that exhibits significant pathological changes when alcohol intoxication precedes a burn injury. Through this model, our laboratory, in conjunction with others, has demonstrated that alcohol intoxication and burn injury has significant effects on various organ systems in the body, such as the gastrointestinal tract, liver, and lungs[8,10-12]. Specifically, the combined effect of alcohol and burn injury has been found to induce pulmonary edema, neutrophil infiltration, and increased susceptibility to infection, surpassing the individual effects of either insult alone, with no direct injury to the lungs themselves[2].

The lungs are particularly susceptible to damage from this combined insult due to their intricate structure, various resident inflammatory cells, and complex vasculature[8]. Experimental evidence has shown that alcohol intoxication prior to burn injury leads to pulmonary dysfunction, resulting in poor clinical outcomes[13]. Furthermore, alcohol causes a surge of pro-inflammatory cytokines (e.g., IL-6) to enter the systemic circulation, which can quickly accumulate in and negatively impact the pulmonary vasculature[6,8,14,15]. Clinical evidence has shown a significant correlation between elevated IL-6 levels and an increased risk of mortality in burn patients[16]. Whereas the inhibition or knockout of the IL-6 gene confers protection against uncontrolled pulmonary inflammation caused by a combined injury of alcohol and burn[8]. These findings suggest that IL-6 could be driving this aberrant response. Therefore, gaining insight into

the source and underlying factors behind the production of IL-6, as well as other pro-inflammatory mediators like CXCL1, MPO, and Ly6G, could provide new insights into how alcohol affects the pulmonary response to an indirect injury, potentially leading to pulmonary infections and complications.

Alcohol consumption has been found to have detrimental effects on the intestinal barriers and gut microbiota, leading to the development of various post-burn comorbidities such as sepsis and MOF[2,17-19]. The presence of acute intestinal inflammation, characterized by the release of pro-inflammatory cytokines like IL-6 and IL-17, as well as an influx of neutrophils, contributes to gut barrier dysfunction following alcohol intoxication and burn injury[20-22]. This combined injury of alcohol and burn allows gut-bacteria and pro-inflammatory pathogen-associated molecular patterns (PAMPs) to enter the mesenteric lymphatics, resulting in local and systemic inflammatory responses and distant organ damage[18,20,23].

Despite decades of research, there are currently no effective therapeutic strategies available that can improve the survival of patients with pulmonary complications (e.g., ALI and ARDS) following a combined alcohol and burn injury[25-30]. Over the past few years, microRNAs (miRNAs) have emerged as a new area of biomedical research due to their key regulatory roles in post-transcriptional gene expression in various biological and pathological processes, including ALI and ARDS[25]. MiRNAs are small, single-stranded, non-coding RNA molecules that recognize and bind to specific messenger RNAs (mRNAs) through complementary base-pairing to the 3' untranslated region (UTR) of the mRNA; this interaction leads to post-transcriptional gene silencing or mRNA degradation[-26]. It has been estimated that there are about 2,588 miRNAs in humans, which are responsible for regulating more than 60% of the human

genome. Remarkably, a single miRNA can target and regulate multiple mRNA molecules[26-27]. Therefore, miRNAs play a critical role in regulating various signaling pathways within cells, highlighting their importance as a key regulatory mechanism. Specifically, miRNAs are essential for maintaining pulmonary barrier function and lung homeostasis. Aberrant changes in miRNA expression have been associated with lung inflammation and pulmonary barrier dysfunction. Consequently, these changes contribute to the pathophysiological progression of various inflammatory disorders affecting the lungs, including asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis[28-30]. Despite growing appreciation that miRNA expression can be influenced by traumatic injury and alcohol intoxication, the precise mechanism by which they contribute to excessive lung inflammation and pulmonary barrier dysfunction remain poorly understood[28-30].

Overall, our data suggests that reduced miR-146a expression may have a significant impact on the pathogenesis of excessive lung inflammation and pulmonary barrier dysfunction after alcohol and burn injury. This, in turn, could lead to serious systemic complications like sepsis and MOF. Through the use of our lab's well-established mouse model of acute ethanol intoxication and burn injury, we observed a significant reduction in the expression of anti-inflammatory miR-146a in small intestinal epithelial cells (IECs) of ethanol-burn mice compared to sham vehicle ($p < 0.05$). *In vitro* studies were then carried out to investigate whether reduced miR-146a expression had an effect on intestinal inflammation and gut barrier disruption following alcohol and burn injury. These studies revealed that miR-146a expression has a critical role in regulating the expression of pro-inflammatory cytokines in IECs induced by lipopolysaccharide (LPS) by modulating p38 MAPK signaling and targeting TRAF6.

Therefore, our central hypothesis is that reduced miR-146a expression drives pulmonary inflammation following alcohol and burn injury. In order to test this hypothesis, we developed two specific aims. Aim 1 determines the impact of alcohol and burn injury on miR-146a and other anti-inflammatory miRNA expression in the lungs after alcohol and burn injury. Aim 2 evaluates the effects of *in vivo* administration of the miR-146a mimic on lung inflammation following an alcohol and burn injury.

First, histopathological analysis of hematoxylin and eosin (H&E) stained lungs one day after alcohol and burn injury indicated that alcohol intoxication prior to burn injury exacerbated lung inflammation, characterized by increased pulmonary congestion and reduced alveolar space compared to vehicle controls. Additionally, the expression levels of various pro-inflammatory mediators, such as ICAM-1, CXCL1, CXCR2, MPO, and Ly6G, were evaluated using RT-qPCR and were found to be significantly upregulated in lung tissue one day after alcohol and burn injury. Biological sex did not seem to have an effect on this pathophysiological process. Next, lung tissue expression levels of miR-146a and various anti-inflammatory miRNAs were examined by RT-qPCR, revealing a significant decrease in the expression levels of miR-146a and miR-671 one day after a combined injury of alcohol and burn.

To investigate the therapeutic potential of targeting reduced miR-146a expression in lung tissue to prevent uncontrolled lung inflammation one day after an alcohol and burn injury, a miR-146a mimic was administered via intraperitoneal (i.p.) injection one day before injury, resulting in increased miR-146a expression in lung tissue. This study demonstrated that the upregulation of miR-146a in lung tissue led to a significant reduction in lung inflammation one day after alcohol and burn injury. Overall, this study shows the significant impact that miR-146a

downregulation has in exacerbating lung inflammation following alcohol and burn injury, which can result in serious complications such as sepsis and MOF. These findings shed light on the role of dysregulated miRNAs in lung inflammation and identify miR-146a as a promising therapeutic target in the treatment and management of patients with alcohol and burn injuries.

CHAPTER 2

LITERATURE REVIEW: MICRORNAS ARE KEY REGULATORS OF LUNG INFLAMMATION AND BARRIER HOMEOSTASIS AFTER ALCOHOL INTOXICATION AND BURN INJURY

Introduction to MicroRNAs

The discovery of miRNAs as important regulatory molecules in the nematode *Caenorhabditis elegans* marked a major scientific breakthrough[31]. Further studies have revealed that miRNAs are highly conserved in all animal model systems, indicating that they play a fundamental role in regulating target mRNAs in normal physiology across various animal species[31,32]. Through complementary base pairing, a single miRNA can control the expression of multiple target mRNAs, and a single mRNA can be targeted by multiple miRNAs[31-33]. Over 60% of the human genome is regulated by at least one miRNA, which indicates that even small changes in miRNA expression can have a significant impact on cellular processes[33,34]. Aberrant miRNA expression has been implicated in various pathological conditions, including cardiovascular diseases, metabolic and neurodegenerative disorders, inflammatory diseases, and cancer[35].

The biogenesis of miRNAs is a carefully controlled, multistep process that begins with the transcription of miRNA by RNA polymerase II in the nucleus. This transcriptional process results in the formation of a long stem-loop structure that contains one or more mature miRNAs, known as the primary miRNA transcript (pri-miRNA)[32,33]. A microprocessor complex,

consisting of Drosha and DGCR8, recognizes and cleaves the pri-miRNA, transforming it into a precursor-miRNA (pre-miRNA)[32-34]. The hairpin-shaped pre-miRNA is then transported to the cytoplasm through Exportin 5, where it is processed by Dicer to generate a miRNA duplex[32,36,37]. The miRNA duplex is then dissociated, giving rise to an active "guide strand" (designated miR-) that binds with Argonaute to form a miRNA-induced silencing complex (miRISC) and a passenger strand (designated miR*-) for degradation[36,37]. Recent reports suggest that only one strand is preferentially loaded into Argonaute, resulting in the adoption of the miRNA-5p and -3p nomenclature to indicate whether the final mature miRNA originated from the 5' or 3' strand of the miRNA duplex[36-38].

The miRNA in the miRISC complex recognizes and binds to the 3' UTR of the target mRNA through its seed sequence, which consists of 2-8 nucleotides at the 5' end. This complementary base pairing, along with the presence of GW182 proteins in the miRISC complex, leads to either mRNA degradation or post-transcriptional silencing to reduce target gene expression[32-34,36-38]. In the case of miRNA-based mRNA degradation, GW182 proteins recruit de-adenylase complexes following miRNA:target mRNA interaction, causing shortening of the 3' poly-A tail[32,36]. Decapping enzymes are then recruited to these target mRNAs, causing them to be degraded from the 5' to 3' direction by a cytoplasmic exoribonuclease. Similarly, certain miRISC complexes facilitate the post-transcriptional silencing of their target mRNAs. This occurs when the 5' methylated cap dissociates from the 3' poly-A tail, leading to the unwinding of the target mRNA and the inhibition of translation initiation[32,36,37].

Functional mature miRNA in cells are tightly regulated throughout their biogenesis so that they can maintain control over their expression and regulation of various biological

processes. This tightly regulated process is important because dysregulated miRNA expression has been linked to various pathological conditions. Over the past couple of years, it has become evident that transcription factors have a key regulatory role in miRNA transcription. More specifically, these regulatory proteins control the expression of miRNAs in response to cellular signals and create feedback mechanisms, which in turn causes the miRNAs to target the transcription factors that govern their activity[39,40].

Post-transcriptional modifications to biogenesis factors and effector proteins can influence the regulation of miRNA expression activity by altering their activity and specificity[40]. More specifically, Drosha and Dicer are two key enzymes that control the efficiency with which pre-miRNA are processed into mature miRNA during miRNA biogenesis. Changes in the protein levels of either of these two enzymes can affect miRNA processing. Furthermore, RNA-binding proteins can bind to miRNA transcripts and/or accessory proteins, regulating the processing efficiency of Drosha and Dicer in response to physiological stimuli or external signals[32,36,39,41]. The nuclear export of pre-miRNA to the cytoplasm can be regulated by adjusting the activity of Exportin 5; this process requires the hydrolysis of its cofactor Ran-GTP, which allows for the release of the pre-miRNA and other associated components into the cytoplasm[42]. Although Argonaute proteins are not primarily involved with the processing of miRNA, the specific mechanisms involved in the selection and loading of a miRNA strand into Argonaute are not fully understood[32,36]. Furthermore, the stability of the miRNA duplex and the degradation of the passenger strand by nucleases seem to be largely influenced by interactions with Argonaute and target mRNAs[36,41].

MicroRNAs and Lung Homeostasis

MiRNAs have important regulatory roles in the respiratory system, particularly in lung homeostasis and the proper functioning of the pulmonary epithelial barrier. Under normal conditions, pulmonary epithelial cells actively contribute to lung homeostasis by maintaining the integrity of the pulmonary epithelial barrier, which serves as a physical barrier between the outside environment and the delicate inner tissues of the lung. The respiratory system can be functionally divided into the conducting (nose to bronchioles) and respiratory (alveolar duct to alveoli) zones. Within the conducting airways, the epithelium is arranged as a densely packed pseudostratified layer made up primarily of ciliated, secretory, and basal cell types. These epithelial cell populations vary along the proximal-distal axis. For example, the total number of secretory cells tends to increase when going from the larger to smaller conducting airways. Thin type I epithelial cells (AT1) and cuboidal type II epithelial cells (AT2) are found in the alveolar regions, where they actively participate in gas exchange and produce surfactants. The main cell types with progenitor potential in the pulmonary epithelium are basal cells, secretory cells, and AT2 cells[43,44].

To maintain a healthy pulmonary epithelial barrier, basal stem cells in the proximal airways self-renew and differentiate into different types of epithelial cells during lung homeostasis and after an injury. These cells are typically dormant, but they can proliferate and differentiate in response to physical insults, chemical damage, or pathogen infections. This intricate process requires strict control over the proliferation, differentiation, migration, and apoptosis of pulmonary epithelial cells[45]. Furthermore, pulmonary epithelial cells form intercellular junctions that regulate paracellular and alveolar-capillary permeability. Tight

junction (TJ) proteins, including transmembrane proteins (i.e., claudin and occludin) and intracellular accessory proteins (i.e., zona occluden proteins), are essential for maintaining the integrity of paracellular and alveolar-capillary permeability[44].

The primary focus of early research on miRNA function in the pulmonary epithelium was on specific miRNAs that were found to have important regulatory roles in the development of lung cancer. The Let-7 family was the first group of miRNAs that were found to be downregulated in lung cancer compared to normal lung. After further testing, the Let-7 family was discovered to have tumor suppressor activity in this pathophysiological process. Eventually, studies were conducted to identify some of the critical regulatory roles that miRNAs have in normal lung development as well as in lung homeostasis[46]. Due to the Dicer enzyme's indispensable role in the miRNA biogenesis pathway, early studies used a Dicer knockout model in the mouse lung epithelium to examine the impact of miRNAs on lung development. In one study, Harries et al. found that the knockdown of Dicer in lung epithelial cells of Cre/loxP mice resulted in abnormal apoptosis and airway branching during lung development[46,47].

As it became clear that miRNAs have important regulatory functions in the proliferation and differentiation of pulmonary epithelial cells, numerous studies shifted their focus to the potential role of miRNAs in ALI and its more severe form, ARDS[48]. ALI and ARDS are two severe forms of respiratory failure caused by uncontrolled inflammation and damage to the lungs' endothelial and epithelial barriers[49]. This serious pathological condition can be further characterized by the breakdown of alveolar-capillary integrity, increased neutrophil infiltration, excessive cytokine and chemokine production, and fluid accumulation in the lungs. Pulmonary

edema reduces gas exchange in the lungs, leading to hypoxemia and eventually lung failure[44,48,49].

Some studies have reported changes in the expression of specific miRNAs in the lungs of patients with ALI/ARDS, which has been shown to influence the inflammatory response and tissue repair mechanisms during these two pathophysiological processes[48,49]. Additionally, the uncontrolled release of pro-inflammatory mediators into systemic circulation has been shown to affect the expression and distribution of TJ proteins in lung tissue, resulting in pulmonary barrier dysfunction[44,49]. On the other hand, some miRNA have been shown to prevent cellular damage and repair damaged airway epithelia during ALI/ARDS by targeting specific molecules and reducing the acute phase immune response[49]. Therefore, miRNAs could potentially be a novel class of biomarkers and therapeutic agents for ALI/ARDS since they are actively involved in the maintenance of lung homeostasis and pulmonary barrier function.

MicroRNA Involvement in Lung Inflammation and Barrier Disruption

The activation of the pulmonary epithelial cells are supported by the presence of pattern recognition receptors (PRRs). These receptors can identify both endogenous damage-associated molecular patterns (DAMPs) from dying or damaged cells and exogenous PAMPs. When these ligands bind to their cognate PRRs, downstream inflammatory signaling pathways are activated, which leads to the activation of the NF-κB signaling pathway and the release of various pro-inflammatory mediators (e.g., IL-1β, IL-6, and TNF-α) to counteract the threat. Nevertheless, dysregulated activation of the immune system can disrupt immunological homeostasis and contribute to the onset of pulmonary inflammatory diseases. MiRNAs play a crucial role in regulating the immune response, making them an integral part of this process. They accomplish

this by regulating the expression of toll-like receptors (TLRs) and other essential components of the TLR-signaling pathway, such as transcription factors and signaling proteins. This is pertinent because TJ integrity, apoptosis, and pulmonary epithelial cell proliferation are all regulated by the TLR-signaling pathway, which in turn maintains the function of the pulmonary epithelial barrier[44,47,50].

In the respiratory epithelium, various types of TLRs are affixed to endosomes and the cell membrane. The most important TLR for identifying inflammatory signals induced by LPS is TLR4[50-53]. The development of ALI/ARDS is closely associated with the TLR4-mediated NF-κB signaling pathway. Previous studies have demonstrated that TLR4 signaling and NF-κB activation can be inhibited by knocking out the key adaptor protein myeloid differentiation primary response 88 (MyD88) gene. This can lead to a decrease in pro-inflammatory cytokine and chemokine production, ultimately lowering the severity of ALI/ARDS[50-53]. On the other hand, when TLR4 expression and activity are elevated, it can negatively affect the pulmonary barrier by stimulating the production of pro-inflammatory cytokines and chemokines. This acute inflammatory response causes a breakdown of pulmonary epithelial TJ proteins and the actin cytoskeleton, resulting in pulmonary edema and lung injury[44,47]. The dysregulated activation of TLR4 signaling, which initiates pulmonary inflammation, has been shown to contribute to the development and progression of chronic respiratory disorders like asthma, COPD, and cystic fibrosis[47,51-53].

The crosstalk between pulmonary epithelial cells and immune cells, whether they are resident or recruited, is essential in various aspects of airway immunity[54]. Pulmonary epithelial cells are capable of producing both pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α,

and anti-inflammatory cytokines, like IL-10 or TGF-β. These cytokines can affect the composition and behavior of immune cell populations. The presence of anti-inflammatory cytokines is particularly important in that they promote immune tolerance and help reduce inflammation in the lungs. Whereas pro-inflammatory cytokines can stimulate the recruitment of innate immune cells and alter the function of T cells, therefore worsening lung inflammation. One example of this is the increased expression of IL-6 in the inflamed pulmonary epithelium, which can contribute to the development of pulmonary hypertension via excessive neutrophil recruitment to the lungs[55]. Moreover, M2 macrophages polarize and secrete IL-6 as well as Th2 cell-produced IL-4 and IL-13, which leads to increased extracellular matrix deposition and worsening pulmonary fibrosis. These elevated levels of IL-6 have been linked to decreased lung function[54-56]. In short, studies have shown that pulmonary barrier dysfunction is a distinguishing characteristic in a number of inflammatory lung diseases, emphasizing the crucial link between inflammatory signaling and the deterioration of pulmonary barrier integrity[44,47,50-56].

The majority of studies that have investigated miRNAs' control of important inflammatory signaling pathways have been done in relation to immune cell signaling. Several studies suggest that miRNAs are essential for maintaining homeostasis between lung inflammation and pulmonary barrier function, despite the paucity of knowledge regarding miRNA regulation of inflammation within the pulmonary epithelium. As was previously mentioned, the knockout of Dicer within pulmonary epithelial cells leads to abnormal lung development and cellular responses. These genetic mouse models have been used in other studies that have shown increased lung inflammation, which is characterized by a greater influx of immune cells into lung tissue and an increased release of pro-inflammatory cytokines[46,47].

Similarly, some studies have found abnormal miRNA expression patterns in inflamed lung tissues of COPD patients[57,58]. Therefore, miRNA expression profiles can be used to monitor the progression of COPD and the inflammatory status of these patients[57]. The high incidence of lung cancer in individuals with COPD has led to the emergence of miRNAs as potential biomarkers and prognostic tools[57,58]. Therefore, gaining a more profound understanding of aberrant miRNA expression and their association with target genes could provide valuable insights into the potential therapeutic applications of miRNA[46,57].

Clinical Implications of MiRNAs

Swift progress has been made to expand our knowledge of the roles of miRNAs and their mechanisms of action in biological pathways since their discovery in 1993[53,57]. Since miRNAs are known to be involved in both development and disease, they are a particularly appealing target for new therapeutic and diagnostic approaches. Even more so, the many benefits that this novel class of molecules offers make them excellent choices for biomarkers in a wide range of diseases. One benefit of using miRNAs is that they are relatively stable in harsh environmental conditions when compared to other RNA types, which makes it easy to identify their expression patterns[46,48,49]. Secondly, miRNAs are easily isolated and found in a variety of bodily fluids, including urine, saliva, and blood[46,60]. Third, miRNAs have distinct expression patterns that correlate with various disease types and change based on how the disease progresses or how well a patient responds to treatment; this is particularly important when trying to determine a patient's prognosis and therapy response. Fourth, a majority of miRNAs show a remarkable degree of specificity towards either a specific tissue or biological condition(s), despite their conservative expression across species[46,48,49,60]. Therefore, the development of a non-invasive and highly

sensitive panel of miRNAs has garnered significant interest for facilitating early diagnosis and prognosis of diseases; this is especially important in conditions such as cancer, where early detection greatly impacts patient prognosis.

Since their discovery in 2008, extracellular miRNAs have been extensively studied, primarily due to their stable expression and high-level of detection in bodily fluids[49,61]. Previous studies have suggested that miRNAs can be secreted from cells through two main mechanisms: either by forming complexes with proteins like high-density lipoprotein, nucleophosmin (NPM1), and Argonaute (AGO2), or by being enclosed in extracellular vesicles (EVs; i.e., exosomes, microvesicles, or apoptotic bodies). The contents of these extracellular miRNA are then internalized by other cells and released into these target cells. Despite the fact that the exact mechanism of miRNA packaging and secretion is still unclear, it remains widely known that extracellular miRNAs have a significant effect on intercellular communication, which influences the progression of numerous diseases[49,61-63].

As the discovery of miRNAs associated with various diseases continues to expand and our proficiency in detecting extracellular miRNAs advances, a wide variety of miRNA-based diagnostic tools have been developed and put into use in clinical practice. These tools include assays that accurately and effectively assess extracellular miRNAs as early indicators of disease, as well as assays that evaluate tumor biopsy miRNA expression to characterize malignancy. Many miRNA-based diagnostic panels are now available for use in clinical settings. Among these panels, the *miRviewTM Mets* panel helps identify neoplasms with unknown or indeterminate primary sites. While the *RosettaGX Reveal* panel provides a differential analysis of thyroid nodules, distinguishing between benign and malignant ones; this panel has been

instrumental in substantially reducing unnecessary surgical procedures. Another notable cancer-related panel is *ThyraMir*, a miRNA classifier, which has been crucial in the detection of thyroid carcinoma. Beyond oncological applications, miRNA biomarkers have prompted the development of diagnostic tools for other medical conditions. For instance, the *CogniMIR* panel is currently undergoing clinical evaluation for its potential to facilitate early detection of Alzheimer's disease. In addition, the *OsteomiR* panel is currently being used to assess the fracture risk in people with type-2 diabetes and in post-menopausal women who are osteoporotic. Finally, *thrombomiR* is being used to determine platelet functionality, showcasing the multifunctionality of miRNA-based diagnostics across various fields of medicine[63].

Although extracellular miRNAs are promising candidates for a new class of biomarkers for diagnosing and predicting many diseases, further research is still needed to gain a better understanding of their role in the pathophysiology of diseases. Thus, the focus of some studies has shifted to defining the functional effects of aberrant miRNA expression on the pathophysiology of disease. Furthermore, numerous studies have shown that there is typically a positive correlation between the expression of extracellular miRNAs in bodily fluids and tissue-specific miRNA expression[64]. Therefore, extracellular miRNAs allow researchers to study the mechanistic role of miRNAs in disease pathogenesis and to easily detect changes in miRNA expression in diseases and correlate them with changes in tissue expression. For example, miR-320c has emerged as a potential therapeutic biomarker exclusively for patients with COPD owing to its high expression levels in the plasma of COPD patients when compared to healthy individuals[65,66]. Consistent with these findings, an alternative study observed a significant increase in the expression levels of miR-320c in bronchoalveolar lavage samples collected from

patients diagnosed with COPD. Altogether, these results imply that miR-320c may be a useful COPD diagnostic biomarker[66].

The role of miRNAs in disease has sparked interest in therapeutic targeting of abnormal miRNA expression. MiRNA restoration and miRNA inhibition-based therapies are currently the two main strategies for miRNA therapy. MiRNA mimics are used in restoration-based therapies to increase the expression of beneficial miRNAs that have been downregulated when a disease is present. These artificial double-stranded RNA molecules mimic the mature miRNA duplex. These mimics function by utilizing the guide strand to specifically target and bind to mRNA molecules, while the passenger strand is degraded. However, there can be unintentional mRNA targeting and serious side effects due to strand bias in the mimic. Natural miRNAs have limited therapeutic efficacy because they are easily degraded by RNAses and can activate TLRs, which in turn stimulates the innate immune system. To overcome these challenges, chemical modifications can be made to the RNA phosphodiester or ribose sugar backbones of the synthetic miRNA mimics. One promising modification site is the 2'-OH position of the ribose sugar, which is unnecessary for miRNA function and prone to degradation by various nucleases. Two common chemical modifications at this site include locked nucleic acids (LNA) and 2'-O-methyl (2'-OMe). Further modifications to the RNA phosphodiester backbone include the addition of one or two terminal phosphorothioate linkages, which improve its stability and reduce innate immune responses. Chemical modifications are also made to the passenger strand in miRNA mimics to prevent off-target effects; the passenger strand of a miRNA mimic is split into two shorter segments, each of which is too short to interact with target mRNA molecules. This

approach lowers the possibility of inadvertent mRNA targeting while increasing the specificity of the miRNA mimic[63.67].

Conversely, the main objective of miRNA-based inhibition therapies is to target specific overexpressed miRNAs that contribute to the development of different diseases through the use of synthetic inhibitors. MicroRNA inhibitors, also known as antagomirs, and LNA miRNA inhibitors work by selectively binding to the miRNA to prevent it from interacting with the target mRNA. This binding between the miRNA inhibitor and its target miRNA prompts the creation of RNA duplexes, which ultimately leads to the degradation of the miRNAs by RNAse H. Like miRNA mimics, miRNA inhibitors also need to be chemically modified in order to increase their stability and thwart immunological reactions. As anti-sense molecules, antagomirs and LNA miRNA inhibitors have a high-degree of complementarity with their target miRNAs. The 2'-O and 4'-C atoms of the ribose ring are joined by a methylene link in the LNA miRNA inhibitors, which limits the ring's flexibility and promotes a rigid conformation; the binding affinity of miRNA inhibitors to their target miRNAs is strengthened, and nuclease resistance is conferred by these modifications[63,67,68].

Despite rapid advancements in the field of miRNA-based therapeutics, there are still no approved miRNA-based drugs that are commercially available at this time. However, there are a number of miRNA-based therapies that have entered clinical trials. One of these treatment options is MRG-110, a miR-92a inhibitor developed jointly by MiRagen Therapeutics and Servier[69,70]. This antagomir targets miR-92a, a miRNA that is essential for inhibiting angiogenesis and the healing process in various organ systems. Thus, by inhibiting miR-92a, MRG-110 promotes angiogenesis and the healing of wounds in patients suffering from

cardiovascular disease (e.g., heart failure). MRG-110 underwent a phase I clinical trial in 2019 that proved it to be safe and effective in human subjects[70]. Likewise, MRG-201, known as Remlarsen, is a synthetic RNA molecule that functions similarly to the miR-29 family (miR-29a/b/c), renowned for its ability to inhibit the synthesis of collagen and other key proteins involved in scar formation. For this reason, a phase II clinical trial is currently evaluating this miRNA-based therapy to treat keloid and fibrous scar formation in multiple organ systems, such as the lungs[63,69-71]. All in all, these studies demonstrate the remarkable potential that miRNA-based therapeutics can have in the treatment of various diseases.

Acute Alcohol Intoxication and Burn Injury

Burn injuries are a unique form of trauma that results from skin contact with heat, cold, chemical, radioactive, friction, or electrical sources[18,72]. In 2023, 450,000 Americans sought medical attention for burn injuries alone; about 40,000 of these patients were hospitalized as a result of their injuries[1,73]. Moreover, approximately 86% of these hospitalizations were due to thermal burn injuries. These burns usually result from contact with hot liquids/objects, steam, flames, or electricity[73]. Every year, around 3,400 individuals lose their lives as a result of burn injuries or associated complications (e.g., infections, smoke inhalation, and organ failure)[73].

In addition to identifying the cause of the burn injury, the severity of the injury can be classified based on the depth and extent of the wound. Superficial or first-degree burns only impact the outermost layer of the skin, called the epidermis, resulting in dry and reddened skin with transient pain that diminishes over time. Partial thickness or second-degree burns affect both the epidermis and the underlying dermis, resulting in red, moist, and painful skin, often characterized by the formation of blisters filled with fluid. Surgical excision and skin

transplantation are usually required for proper treatment of the burn site, depending on the severity of this burn injury. Skin that has sustained a full-thickness or third-degree burn is dry, rough, and discolored because the burn penetrates through the epidermis, dermis, and subcutaneous layers. Due to damage to the nerve endings, these injuries usually do not cause pain and require surgical intervention and careful management to prevent infection. Last but not least, fourth-degree burns cause local necrosis and loss of the affected area by penetrating from the epidermis to the subcutaneous layer, affecting muscles and bones. Similar to third-degree burns, fourth-degree burns are characterized by the absence of pain and usually require surgical intervention and careful management to prevent infection. Medical professionals consider not only the depth of the wound but also the TBSA affected in order to assess the burn's severity. Minor burns are defined as those that encompass less than 10% of the TBSA. On the other hand, severe burns are categorized based on specific criteria: >30% TBSA for children, >20% TBSA for adults, and >10% TBSA for elderly patients[18,74].

Therefore, it is imperative to promptly refer and triage patients after assessing the severity of their burn injury. This step holds immense significance because burns that cover at least 15% of the patient's TBSA trigger an immediate local and systemic immune response[74]. Consequently, this leads to extensive systemic inflammation and multiple-organ dysfunction. As a result, the patient's recovery can be significantly compromised due to these systemic responses, resulting in severe harm. Severe burn injuries usually lead to distributive shock, a condition where fluid leaks from the capillaries into the interstitial space, leading to severe edema and fluid accumulation in the tissues. This also causes hypovolemia, resulting in hypotension and end organ hypoperfusion with resultant organ dysfunction and damage[18,72,74].

The lungs are particularly susceptible to injury from this insult due to their intricate structure, diverse array of resident inflammatory cells, and extensive vasculature[8]. If the lungs sustain damage, the leaking fluid can disrupt gas exchange between the alveoli and the capillaries. This fluid shift can occur rapidly and profoundly, resulting in the onset of ALI and ARDS[75].

Additionally, the systemic circulation is rapidly and continuously flooded with catecholamines, glucocorticoids, acute-phase proteins, and pro-inflammatory cytokines and chemokines immediately after a severe burn injury. During this acute inflammatory phase, there is a significant release of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α. These cytokines can disrupt the integrity of distant blood vessels, leading to vascular leakage and eventually MOF. This unregulated inflammatory response and excessive cytokine release can lead to the development of systemic inflammatory response syndrome (SIRS), which is characterized by an overactive immune system. This uncontrolled inflammatory response contributes to distant organ damage, thus exacerbating the initial injury[74.75]. Furthermore, increased neutrophil infiltration and inflammation are early indicators of SIRS. Studies have shown that IL-6 levels in the plasma of burn patients rapidly rise and reach their highest point within 6 hours after the burn injury. Hence, higher levels of IL-6 may be indicative of a more severe burn and an increased risk of death. Therefore, it is of utmost importance to effectively manage and decrease the intensity of the immune response during this phase to prevent serious complications[76].

Most burn injuries that occur are not standalone events and are typically accompanied with additional factors, such as the consumption of alcohol and/or drugs by the individual[19]. It is worth noting that almost half of reported burn injuries occur when patients are under the

influence of alcohol, resulting in more adverse clinical outcomes compared to those who did not consume alcohol prior to being hospitalized[5]. Burn patients who were intoxicated tend to experience a higher rate of infection, prolonged hospital stays, and a greater reliance on ventilator support than non-drinking burn patients[77]. The consumption of alcohol alone poses a significant risk for morbidity and mortality on a global scale[78].

Studies have shown that alcohol exposure prior to a burn injury leads to intestinal barrier dysfunction and increased bacterial translocation outside the gut compared to burn injury alone. This, in turn, leads to elevated levels of IL-6 in the liver, resulting in increased lung inflammation[77]. Ethanol exposure has also been found to impede the innate and adaptative immune responses through the inhibition of the $NLRP_3$ inflammasome pathway and the reduction of cytokine production[79,80]. Therefore, when alcohol is present during a burn injury, it causes significant harm to vital organs such as the lungs, liver, and gastrointestinal tract, leading to extensive tissue damage[5,19,77-80].

Pulmonary Complications Following Alcohol and Burn Injury

Complications in remote highly vascularized organs, such as the lungs, kidneys, gastrointestinal tract, and bone marrow, have been extensively studied in cases of SIRS following alcohol and burn injury. Among these organs, the lung is the most likely to fail due to its extensive vasculature, which makes it more prone to injury after a burn. This vulnerability arises from the fact that all the blood pumped by the heart must pass through the lungs. Thus, burns cause an uncontrollably high level of pro-inflammatory mediators to be released into the lungs and systemic circulation, which causes neutrophils to be recruited, activated, and retained in lung tissue[81,82].

Neutrophils are an essential component of the innate immune response and exhibit a wide range of functions aimed at quickly eliminating invading pathogens and necrotic cells from the site of injury; these functions include phagocytosis, the formation and release of extracellular traps, and the release of reactive oxygen species and nitric oxide. These functions work together synergistically to defend against and kill pathogens[83,84]. However, when neutrophil apoptosis is delayed and inflammation persists, it can result in significant injury to pulmonary epithelial cells; this causes swelling of the pulmonary endothelial cells and widening of the intercellular junctions, ultimately disrupting the alveolar-capillary barrier. When this barrier is compromised, capillary fluid quickly leaks into the lung parenchyma and causes extensive lung damage[85,86]. Increased permeability of the alveolar-capillary interface to fluids and proteins is a characteristic feature of ARDS[87].

Furthermore, patients who have experienced alcohol and burn-related injuries may develop indirect ARDS due to a gastrointestinal tract infection from their own microbiota. Numerous studies have presented evidence supporting the concept that hypoperfusion and acute intestinal inflammation, observed in both patients and animals shortly after experiencing a burn injury, lead to gut barrier disruption and an increase in intestinal permeability. This allows gut bacteria to translocate and enter the portal venous system, liver, and lungs[18]. Hence, this process triggers an extensive local and systemic inflammatory response, leading to the development of SIRS and multiple organ dysfunction syndrome. Similarly, gut-derived DAMPs can be released into the mesenteric lymphatics and subsequently transported to the lungs and systemic circulation following a combined alcohol and burn injury. This process causes alveolar-capillary barrier disruption, resulting in the development of indirect ARDS. In short, the gut plays a

critical role as a pro-inflammatory organ, contributing to damaging effects in distant organs like the lungs through the release of DAMPs, even in the absence of systemic bacterial translocation[18,82,88]. Overall, the etiology of indirect lung injury resulting from an infection at a distant location is complex and poorly understood.

Standard of Care for Alcohol Intoxication and Burn Injuries

The standard of care for burn injuries requires prompt and appropriate fluid resuscitation to counteract the rapid onset of hypovolemia within the first 24 hours after a burn injury. Various formulas have been proposed for fluid resuscitation, but the Parkland formula is the most widely used approach. During the first 24 hours after the burn injury, Ringer's lactate (RL) solution is used at a rate of 4 mL/kg/% TBSA when using the Parkland formula. To be exact, the first 8 hours are spent giving 2 mL/kg/% TBSA, and the remaining volume is given over the next 16 hours. Intravenous administration is typically preferred for fluid resuscitation; however, in situations where resources are limited, the oral and enteral routes can also be used. The main goal of fluid resuscitation is to restore hemodynamics by providing fluid and electrolytes to improve tissue perfusion and oxygen delivery, thereby preventing worsening edema, organ failure, and airway complications like ALI/ARDS[89-91]. On the contrary, over-resuscitation can lead to "fluid creep," which occurs when the total volume of fluid administered surpasses 6 mL/kg/% TBSA, leading to complications such as abdominal compartment syndrome, reduced wound perfusion, and respiratory and organ failure[89,92,93].

Colloids and crystalloids are the two main types of fluids used for resuscitation after a burn injury. Colloid solutions are comprised of large insoluble proteins (e.g., hydroxyethyl starches) in a crystalloid solution, which have the ability to increase the intravascular volume. On

the other hand, crystalloid solutions (e.g., RL and normal saline) are composed of water and electrolytes, which are used to increase the intravascular volume and facilitate rapid re-distribution into the interstitial tissues[94,95]. Although crystalloid solutions are commonly used in clinical practice, recent studies have suggested that fluid resuscitation with RL solution could cause neutrophil activation after hemorrhage. While the administration of saline in high volumes has been linked to nephrotoxicity[94,96]. Comparably, the administration of colloid solutions has been shown to cause anaphylactic reactions, while the use of hydroxyethyl starches in critically ill patients has been linked to a higher risk of mortality and renal failure[97,98]. As a result, there is a great deal of variation in how healthcare professionals manage burn patients' fluids. Therefore, further studies are needed to better understand how fluid therapy affects the lungs, and to determine the best method for managing fluids in individuals with ALI/ARDS[99].

Although the administration of fluids plays a vital role in the management of burn injuries, it is important to realize that it does not completely restore organ function. As a result, burn patients may still experience systemic complications that require close monitoring and treatment. In mouse burn models, pro-inflammatory cytokine levels remained consistently elevated for several days following a burn injury, mirroring findings in pediatric burn patients. Although increasing evidence suggests that suppressing excessive inflammation may help reduce alcohol and burn induced pulmonary complications and MOF, there are currently no treatment options available that specifically target this acute inflammatory phase[89,94,100,101]. To address this, it is imperative to explore potential therapeutic strategies that restore the integrity of the pulmonary barrier and alleviate lung inflammation after alcohol and burn injuries. By doing so,

we may be able to offer additional treatment options to burn patients, ultimately reducing the risk of severe complications such as sepsis and MOF.

Conclusion

Numerous studies have shown that when alcohol intoxication precedes a burn injury, it can result in acute lung inflammation, disruption of the alveolar-capillary barrier, and progression to severe complications such as ALI and ARDS. To improve patient outcomes, it is important to investigate potential therapeutic strategies that can prevent acute lung inflammation following alcohol and burn injuries. MiRNAs are crucial for maintaining lung homeostasis, but their impact on pulmonary dysfunction post-alcohol and burn injury is not well understood. Therefore, it is crucial to study changes in miRNA expression in lung tissue and understand how these changes contribute to the rapid onset of lung inflammation and subsequent lung barrier damage following alcohol and burn injury. These studies have the potential to advance our understanding of how miRNAs regulate lung homeostasis in disease, while also identifying potential therapeutic targets for improving lung function in various diseases characterized by lung inflammation.

CHAPTER 3

INVESTIGATING THE IMPACT OF ALCOHOL AND BURN INJURY ON MIR-146A AND OTHER ANTI-INFLAMMATORY MIRNA EXPRESSION IN LUNGS AFTER ALCOHOL AND BURN INJURY

Abstract

Previous studies conducted in our lab using a mouse model of acute ethanol intoxication and burn injury revealed that there was a significant downregulation of anti-inflammatory miR-146a in small IECs in ethanol-burn mice compared to the sham vehicle ($p < 0.05$). *In vitro* studies showed that the expression of miR-146a controls the expression of pro-inflammatory cytokines induced by LPS in IECs by controlling p38 MAPK signaling and by targeting TRAF6. In this study, we sought to determine the impact of alcohol and burn injury on miR-146a and other anti-inflammatory miRNA expression in lungs after alcohol and burn injury. The aim of this study was to gain a deeper understanding of their role in pulmonary inflammation following alcohol intoxication and burn injury. We discovered that alcohol and burn injury exacerbated pulmonary inflammation and pathology using a well-established mouse model of acute ethanol intoxication and burn injury in our lab. Interestingly, we observed that biological sex did not seem to have an effect on this pathophysiological process. We also found that ethanol-burn mice had significantly lower expression levels of anti-inflammatory miR-146a and miR-671 than the sham vehicle ($p < 0.05$). Additionally, there was a noticeable downward trend in the expression of miR-150 and miR-194. Collectively, our findings from this study indicate that alcohol and

burn injury alters the expression of multiple miRNAs, which can result in lung inflammation and could potentially contribute to pulmonary barrier dysfunction.

Introduction

Close to half a million people in the United States are affected by burn injuries each year, leading to approximately 40,000 hospitalizations[1]. Almost half of reported burn occur while a patient is under the influence of alcohol[5]. Patients who are intoxicated at the time of a burn injury demonstrate increased susceptibility to infections, an elevated risk of sepsis, MOF, and higher mortality rates compared to those who did not consume alcohol before sustaining the injury[6]. These patients are also particularly susceptible to pulmonary infections and complications due to their suppressed immune state[7]. Disruption of immunological homeostasis and subsequent emergence of pulmonary inflammatory diseases can be attributed to prolonged activation of the immune system and pulmonary barrier dysfunction[44,47,50]. Pulmonary epithelial cells play a vital role in maintaining the lung's physical barrier. They are responsible for regulating the TJ proteins, which ensure proper fluid balance and gas exchange. They also prevent exogenous PAMPs and endogenous DAMPs from entering both the local and systemic circulation[44,47,50]. Previous studies have shown that circulating DAMPs can stimulate TLR4 and activate the NF-κB signaling pathway following a severe burn injury, resulting in excessive lung inflammation[102]. Increases in the activation of the NF-κB pathway can result in increased levels of IL-6, CXCL-1, and ICAM-1 expression. This signifies the recruitment and infiltration of neutrophils into the lung tissue, occurring within a span of hours to a day after the injury. The occurrence of acute lung inflammation drives pulmonary barrier dysfunction, leading to the

accumulation of fluid in the lung parenchyma, local and systemic inflammation, and multiple organ dysfunction syndrome[6,7,102].

The biogenesis of miRNAs is a multi-step, carefully controlled process that begins with RNA polymerase II transcription in the nucleus to generate pri-miRNA molecules. Following their cleavage by Drosha and DGCR8, these pri-miRNA molecules become pre-miRNA molecules, which are then translocated to the cytoplasm via Exportin 5 and are further processed by Dicer into a miRNA duplex. This miRNA duplex consists of two strands of RNA that are complementary to each other. From this duplex, one strand is selectively taken up and loaded into RISC with the help of Argonaute[36,37]. Once loaded into RISC, the selected miRNA strand becomes a functional mature miRNA that can bind to the 3'-UTR of target mRNAs, resulting in post-transcriptional silencing or mRNA degradation. MiRNAs have important regulatory functions in the maintenance of lung homeostasis and pulmonary barrier function[43,44]. Variations in the expression of miRNA have been linked to the development of various inflammatory lung diseases, including asthma, pulmonary fibrosis, and COPD[28-30]. However, the impact of abnormal miRNA expression in lung tissue after trauma, particularly in cases of alcohol intoxication and burn injury, have not been extensively studied.

In this study, we hypothesized that downregulation of miR-146a and other key anti-inflammatory miRNAs would promote excessive lung inflammation after a combined alcohol and burn injury. To investigate this, we used our lab's mouse model of acute ethanol intoxication and burn injury to determine the effect of alcohol and burn injury on the expression of miR-146a and other anti-inflammatory miRNAs in the lungs. Furthermore, we determined if biological sex had any effect on this pathophysiological process.

Materials and Methods

Laboratory Mice

Male and female C57/BL6 mice were acquired from Charles River Laboratories and kept in conventional laboratory housing at the Loyola University Medical Center Comparative Medicine facility in Maywood, Illinois, USA. The mice were housed for a duration of two weeks prior to the start of the experiment, which caused their age to range from 8-10 weeks when the study began. During the experiment, all mice had a weight within the range of 23-31 grams. All animal experiments conducted were done in compliance with the Institutional Animal Care and Use Committee (IACUC) in Loyola University Chicago's Health Sciences Division.

Murine Model of Acute Ethanol Intoxication and Burn Injury

Male C57/BL6 mice were randomly distributed among four experimental groups: sham injury and vehicle treatment (referred to as sham vehicle), sham injury and ethanol treatment (referred to as sham ethanol), burn injury and vehicle treatment (referred to as burn vehicle), or burn injury and ethanol treatment (referred to as ethanol-burn). In a separate experiment, male and female C57/BL6 mice were divided into two groups according to their biological sex: sham vehicle and ethanol-burn.

In both experiments, mice were gavaged with 400 μL of 25% ethanol in water (2.9 g/kg) or saline vehicle on the day of injury at a concentration intended to raise the BAC to 90-100 mg/dL four hours after ethanol exposure. This is marginally higher than the legal limit of 80 mg/dL (0.08%) and similar to the BAC observed in hospitalized patients[77]. Mice received 1 mg/kg of buprenorphine subcutaneously three hours after the gavage as a pain reliever. Four

hours after the gavage, the mice were given an i.p. injection of ketamine hydrochloride (~ 80 mg/kg) and xylazine (~ 1.2 mg/kg) to induce unconsciousness, and their dorsums were shaved.

After that, the mice were put into a plastic template exposing ~12.5% of their TBSA, as determined by Meeh's formula[103]. Animals in the burn group were submerged in a water bath set at ~85°C for ~7 seconds to induce an insensate, full-thickness scald burn injury. Sham animals, on the other hand, spent an equivalent amount of time in a water bath at room temperature (37°C). After being gently dried, the mice were given an i.p. injection of 1.0 mL of saline to resuscitate them. The animals were then put back into their cages, which were placed on heating pads to help the mice maintain their body temperature, and monitored to make sure they recovered from the anesthesia. From there, the mice were put back into their regular housing and given full access to food and water. In order to eliminate potential confounding factors associated with circadian rhythms, all experiments were conducted between 12 and 1 pm. The animals were euthanized 24 hours after the burn injury. At that time, lung tissue was harvested, and the left lobe was snap-frozen in liquid nitrogen for RNA and protein analysis.

Total RNA Isolation

Total RNA was extracted from homogenized lung tissue for miRNA or mRNA analysis using the MirVana miRNA isolation kit (Invitrogen) as per the manufacturer's instructions. The concentration and purity of RNA was evaluated using a Nanodrop 2000 spectrophotometer (ThermoScientific).

RT-qPCR Analysis of MiRNA Expression

Total RNA was isolated using the MirVana miRNA isolation kit, which was then reverse transcribed and amplified into cDNA using the miRCURY LNA RT Kit (Qiagen). Then, using

the miRCURY LNA SYBR Green PCR Kit and target-specific primers for the miRCURY LNA PCR Assay (Qiagen), the relative expression of the miRNAs were measured by quantitative real-time PCR (qPCR). Target miRNA C_t values for each sample were normalized to the housekeeping control SNORD68, and the $\Delta\Delta C_T$ method was used to calculate relative expression.

RT-qPCR Analysis of mRNA Expression

Total RNA was isolated using the MirVana miRNA isolation kit, which was then reverse transcribed and amplified into cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems). Then, using Taqman Fast Advanced Master Mix and FAM Taqman primer probes (Life Technologies) tailored to each target gene, qPCR was used to measure the relative expression of the target genes. The C_t cycle values of the target genes in each well were standardized to the C_t values of GAPDH housekeeping control using VIC TaqMan primer probes within the same reaction. The $\Delta\Delta C_T$ method was used to calculate the relative expression.

ELISA Analysis

Lung tissue was sonicated and analyzed for the presence of IL-6, CXCL1, and MPO (R&D Systems) using their respective ELISA kits, following the manufacturer's instructions. The levels of these pro-inflammatory mediators were quantified and reported as pg per mg of total protein.

Histopathologic Examination of the Lungs

Following the extraction of lung tissue from the male C57/BL6 mice, the entire right lobe was inflated with 10% formalin and fixed overnight. Following a 24-hour fixation period, the tissue was then transferred to a solution of 70% ethanol. These samples were then sent to AML

Labs (St. Augustine, FL, USA), where they were coronally sectioned at 5 mm and stained with H&E. To evaluate these samples, a Nikon Eclipse E100 LED microscope and Motic Images Plus 3.0 ML software (Kowloon, Hong Kong) were utilized.

All histological images were captured blindly at 10x and carefully examined for any discernible signs of lung inflammation, such as thickening of the alveolar walls, pulmonary congestion, and increased tissue cellularity. Additionally, previous studies have reported a correlation between elevated tissue cellularity and alveolar wall thickening with a reduction in alveolar airspace and increased pulmonary congestion[77]. To obtain a comprehensive view of the entire right lobe of the lung from each animal, a total of six low-power field images were taken in a blinded manner at 10x magnification. Then, one image was randomly selected from each group to represent that treatment group; scale bar is 410.5 µm.

Statistics

Data was reported as mean values ± standard error of the mean (SEM). Statistical analysis was conducted using GraphPad Prism 10 software. For experiments that had two groups, a student's t-test was used for analysis. Experiments with more than two groups were subjected to two-way ANOVA and compared by Tukey's post-hoc test, with an exception to the four-group experiment that profiled four different miRNAs; a student's t-test was used to analyze this data to accurately depict if there was a statistical difference between the sham vehicle and ethanol-burn mice. P-values or adjusted p-values were considered statistically significant when $p < 0.05$.

Results

Alcohol intoxication and burn injury can cause uncontrolled lung inflammation, which contributes to pulmonary barrier disruption and the development of serious post-burn complications, such as ALI, ARDS, and MOF[3,6-8]. To determine if miRNAs might be contributing to the increase in lung inflammation, we identified four miRNAs that exhibit decreased expression levels in lung tissue after alcohol and burn injury. Following previous findings in our lab, four anti-inflammatory miRNAs were selected for RT-qPCR analysis: miR-146a, miR-150, miR-194, and miR-671. **Figure 1** shows the preliminary analysis of miRNA expression in lungs from male mice one day post-injury, which reveals a significant decrease in expression of miR-146a, miR-194, and miR-671 in ethanol-burn mice compared to sham vehicle ($p < 0.05$); there was also a downward trend in miR-150 expression ($p = 0.09$).

In parallel, we also determined lung inflammation and the findings as shown in **Figure 2**, suggest there was a significant upregulation in the expression of neutrophil chemoattractant CXCL1 and neutrophil chemoattractant receptor CXCR2 in the lungs of male ethanol-burn mice compared to the sham vehicle one day after alcohol and burn injury ($p_{adj} < 0.05$). Additionally, there was a clear upward trend in pro-inflammatory cytokine IL-6 ($p_{adj} =0.30$) and pulmonary endothelial/epithelial ICAM-1 ($p_{adj} = 0.06$) expression in lung tissue after combined injury.

We also determined IL-6, CXCL1, and MPO protein levels in the lung tissue. The results as summarized in **Figure 2** shows significantly increased levels of CXCL1 in the lungs of male mice post-alcohol and burn injury ($p_{adj} < 0.05$), while IL-6 and MPO levels were not significantly different. **Figure 3** reveals histopathological changes in the lungs of male mice post-alcohol and burn injury via H&E staining. There was minimal to no inflammation in the

lungs of sham vehicle mice one day after the sham injury. However, mice that were given ethanol prior to sham injury had increased pulmonary inflammation compared to sham vehicle mice. In contrast to the sham groups, there was an increase in cell infiltration and this was associated with a decrease in alveolar airspace and an increase in pulmonary congestion in mice subjected to burn injury alone. Ethanol-burn mice exhibited increased tissue cellularity and thickening of alveolar walls in the lungs compared to mice that only received a burn injury.

Biological sex did not appear to have an effect on this pathophysiological process in the lungs. As shown in **Figure 4**, alcohol and burn injury increased pulmonary inflammation in both males and females, characterized by significantly increased ICAM-1 (Male: $p < 0.05$; Female: $p < 0.01$), CXCL1 (Male: $p < 0.05$; Female: $p < 0.01$), MPO (Male: $p < 0.01$; Female: $p < 0.05$), and Ly6G (Male: $p < 0.001$; Female: $p < 0.05$) expression relative to sham vehicle; IL-6 expression also trended upwards for both males ($p = 0.08$) and females ($p = 0.16$).

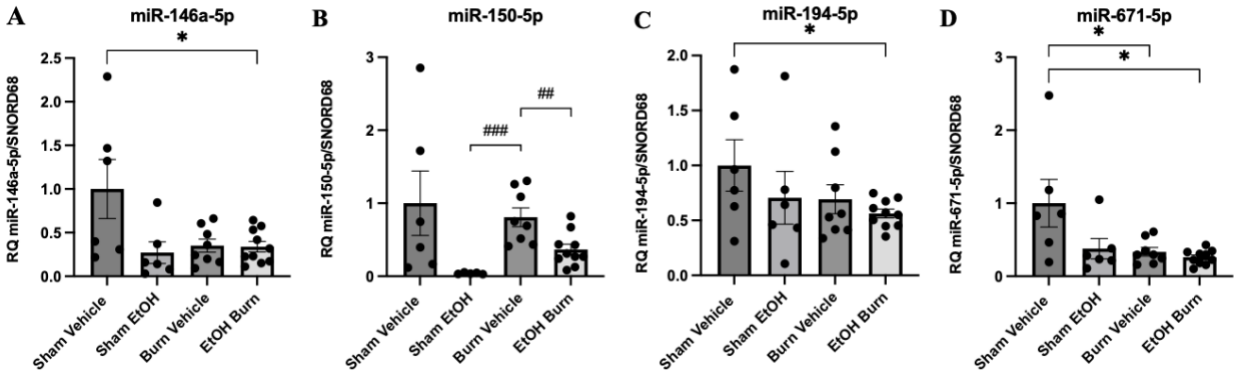

Figure 1. Profiling the Expression of Anti-Inflammatory miRNAs in Lung Tissue One Day After Acute Ethanol Intoxication and Burn Injury. Lung tissue was collected from male mice one day after combined insult and total RNA was isolated for RT-qPCR analysis. Primers specific for (A) miR-146a-5p, (B) miR-150-5p, (C) miR-194-5p, and (D) miR-671-5p were used to assess the relative expression of these miRNAs. Graphs are expression relative to average sham vehicle control, with mean ± SEM (n = 5-10 mice per group). SNORD68 was used as endogenous control. Statistical analysis via student's t-test with significance depicted as: * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$ as compared to sham vehicle; # $p < 0.05$, ## $p < 0.01$, ### $p \leq 0.001$ as compared to burn vehicle.

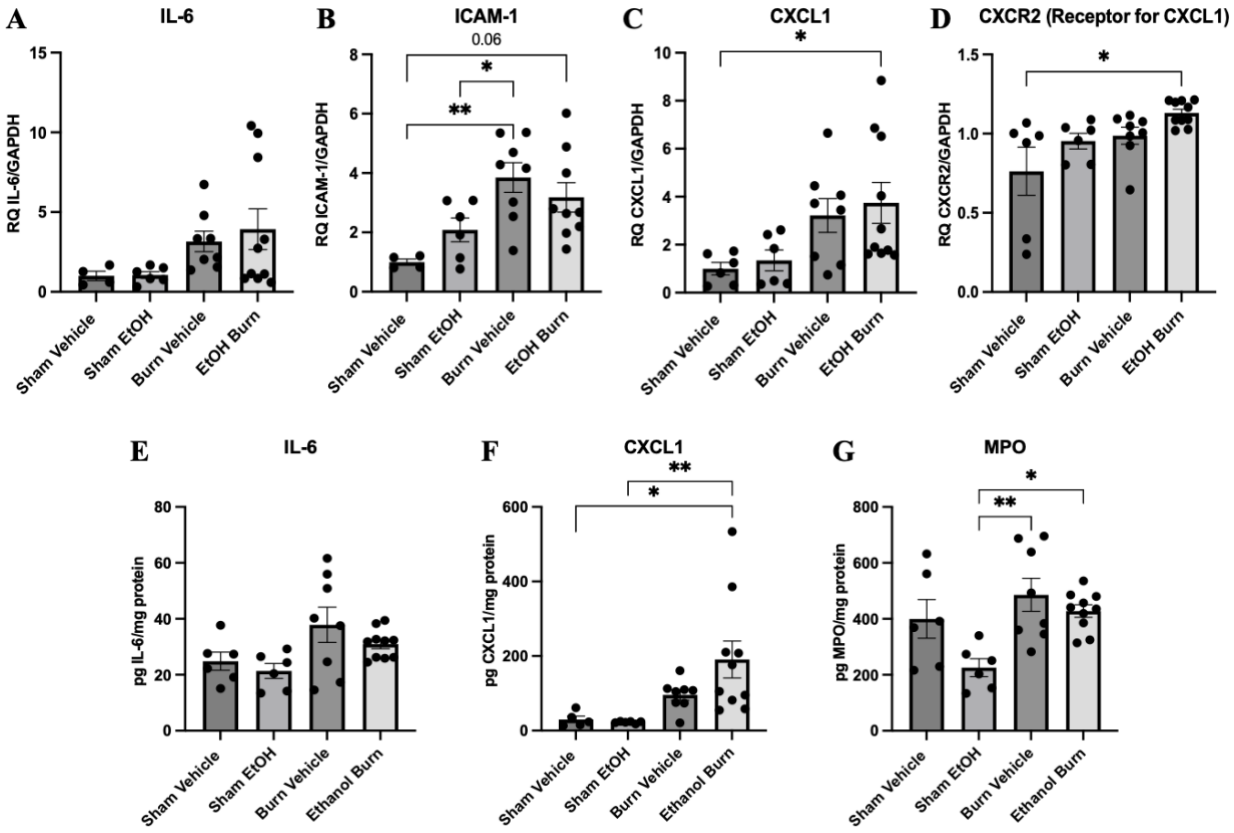

Figure 2. Pulmonary Inflammation Worsened One Day After Acute Ethanol Intoxication and Burn Injury. Lung tissue was collected from male mice one day after alcohol and burn injury and total RNA was isolated for RT-qPCR analysis of gene expression using primers specific for (A) IL-6, (B) ICAM-1, (C) CXCL1, and (D) CXCR2. Graphs are expression relative to average sham vehicle samples, with mean ± SEM (n = 4-10 mice per group). GAPDH was used as endogenous control. Statistical analysis via two-way ANOVA. Significance (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$) and shown as compared by Tukey's multiple comparisons test. Lung tissue was homogenized via sonication and ELISAs were preformed to quantify the concentrations of (E) IL-6, (F) CXCL1, and (G) MPO. Concentrations were reported in pg/mg total protein. Graphs are mean ± SEM (n = 5-10 mice per group). Statistical analysis via two-way ANOVA. Significance (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$) and shown as compared by Tukey's multiple comparisons test.

Further RT-qPCR analysis was carried out on these samples to confirm our findings from the preliminary analysis of miRNA expression in lungs one day post-injury, which revealed a significant decrease in expression of miR-146a, miR-194, and miR-671 after alcohol and burn injury (**Figure 1**). **Figure 5** shows that miR-146a and miR-671 expression were significantly decreased in lung tissue from male mice following alcohol and burn injury compared to the sham vehicle ($p < 0.05$), while miR-194 expression was not significantly changed but trended

downwards. Interestingly, miR-146a expression was found to be significantly increased in the lungs of female ethanol-burn mice compared to sham vehicle ($p < 0.01$). However, the expression of miR-671 was found to be significantly decreased in the lungs of female ethanol-burn mice compared to the sham vehicle ($p < 0.01$), while miR-194 expression showed no statistically significant difference but trended downwards.

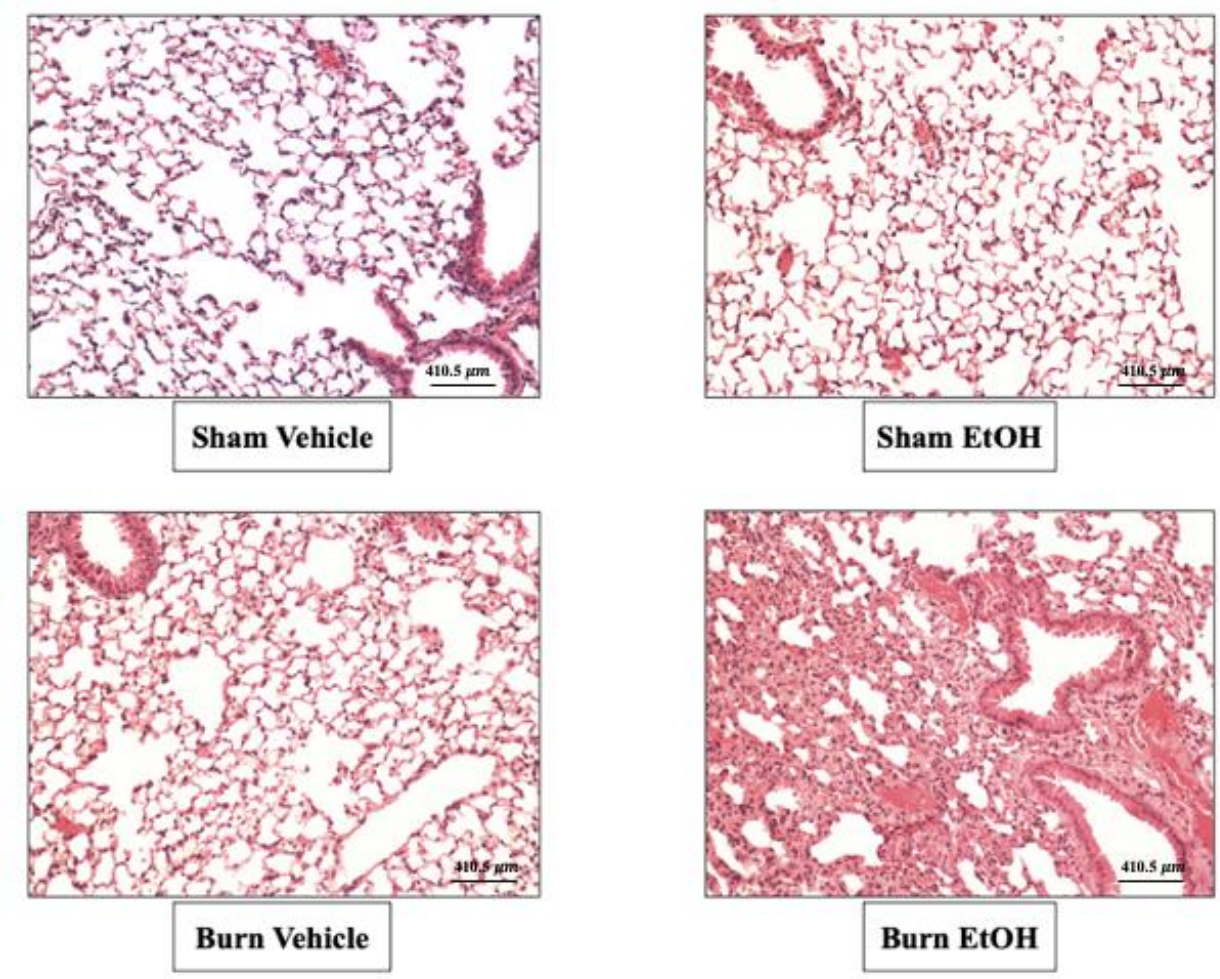

Figure 3. Pulmonary Inflammation One Day After Acute Ethanol Intoxication and Burn Injury. Coronally cut lung sections from each treatment group (n = 4 male mice per group) were examined for histological changes associated with pulmonary inflammation one day after vehicle or ethanol exposure and sham or burn injury via H&E staining. To obtain a comprehensive view of the entire right lobe of the lung for each animal, a total of six low-power field images were taken in a blinded manner at 10X magnification. Then, one image was randomly selected from each group to represent that treatment group; scale bar is 410.5 μm.

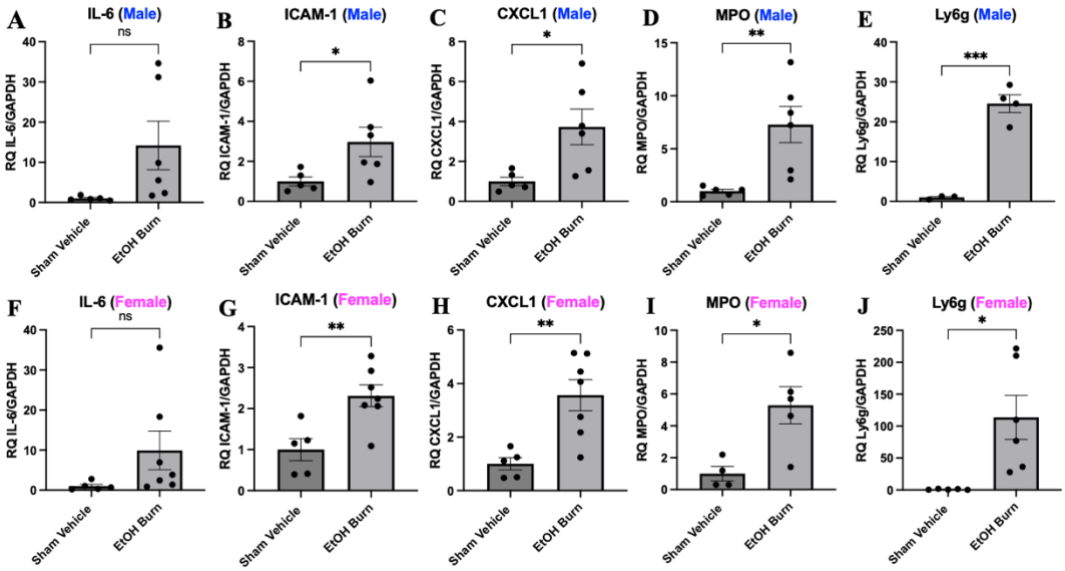

Figure 4. The Pathogenesis of Pulmonary Inflammation Post Alcohol and Burn Injury was Not Influenced by Biological Sex. Lung tissue was collected from both male and female mice one day after alcohol and burn injury and total RNA was isolated for RT-qPCR analysis of gene expression using primers specific for IL-6 (A: Male; F: Female), ICAM-1 (B: Male; G: Female), CXCL1 (C: Male; H: Female), MPO (D: Male; I: Female), and Ly6g (E: Male; J: Female). Graphs are expression relative to average sham vehicle samples, with mean ± SEM (n = 3-7 mice per group). GAPDH was used as endogenous control. Statistical analysis via student's t-test with significance depicted as $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$.

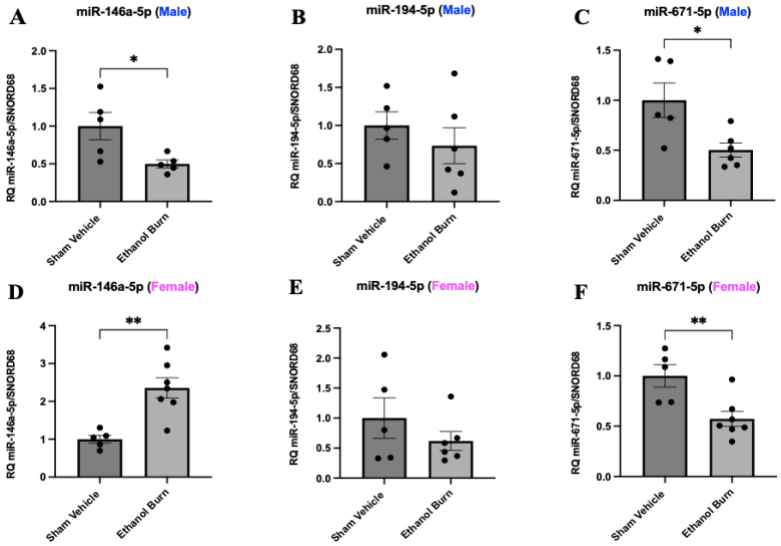

Figure 5. Profiling the Expression of Anti-Inflammatory miRNAs in Lung Tissue From Both Male and Female Mice One Day After Acute Ethanol Intoxication and Burn Injury. Lung tissue was collected from both male and female mice one day after combined insult and total RNA was isolated for RT-qPCR analysis. Primers specific for miR-146a-5p (A: Male; D: Female), miR-194-5p (B: Male; E: Female), and miR-671-5p (C: Male; F: Female) were used to assess the relative expression of these miRNAs. Graphs are expression relative to average sham vehicle samples, with mean ± SEM (n = 5-7 mice per group). SNORD68 was used as endogenous control. Statistical analysis via student's t-test with significance depicted as * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$ as compared to sham vehicle.

Summary

In this study, we analyzed the expression of anti-inflammatory miRNAs in lung tissue and investigated their potential role in increasing lung inflammation after an alcohol and burn injury. Through the use of our lab's mouse model of acute ethanol intoxication and burn injury, we found that the expression of miR-146a and miR-671 in the lungs of male mice were significantly downregulated one day after the combined injury. The downregulation of these two miRNAs were found to be associated with increased lung inflammation and pathology in male mice one day after the combined injury, characterized by increased ICAM-1, CXCL1, CXCR2, MPO, and Ly6G expression, as well as increased levels of CXCL1 in male ethanol-burn mice relative to the sham vehicle. The expression of these pro-inflammatory mediators were also found to be significantly upregulated in the lungs of female ethanol-burn mice compared to sham vehicle, which indicated that biological sex does not have an effect on this pathophysiological process. In short, our results suggest that altered miR-146a expression following alcohol intoxication and burn injury may be driving lung inflammation and ultimately pulmonary barrier disruption. This study also highlights the benefits of using miRNAs as a potential therapeutic target following a combined injury, as they play important regulatory roles in inflammation.

CHAPTER 4

ASSESSING THE IMPACT OF *IN VIVO* MIR-146A MIMIC ADMINISTRATION ON LUNG INFLAMMATION FOLLOWING ALCOHOL AND BURN INJURY

Abstract

MiRNAs are small, endogenous, non-coding RNA molecules that downregulate target genes and act as key regulators in the maintenance of lung homeostasis. Various studies have shown that acute alcohol intoxication and burn injury can lead to acute lung inflammation and pulmonary barrier dysfunction. However, the specific impact of changes in miRNA expression on this pathophysiological process has yet to be explored. The aim of this study was to determine if *in vivo* restoration of miR-146a expression in the lungs could reduce lung inflammation after an alcohol intoxication and burn injury. In this study, male C57BL/6 mice were administered either 50 ug scramble or miR-146a mimic through an i.p. injection using *in vivo*-jetPEI/5% glucose (Polyplus Transfection) solution; this injection was given one day before subjecting the mice to alcohol and burn injury. One day after the combined insult, lung tissue was collected and processed to obtain total RNA. Our results demonstrate that restoration of miR-146a expression significantly reduced lung inflammation one day after alcohol and burn injury, characterized by reduced expression of IL-6 and neutrophil markers (CXCL1, Ly6G, and MPO), as well as reduced levels of IL-6. These results suggest that miR-146a may have a central role in driving lung inflammation after alcohol and burn injury, and more importantly, they highlight the therapeutic potential of targeting miRNAs to restore lung homeostasis after a combined injury.

Introduction

MiRNAs are small, highly-conserved, non-coding RNA molecules that recognize and bind to the 3'-UTR of target mRNAs via complementary base-pairing, resulting in mRNA degradation or post-transcriptional silencing[25-26]. A single miRNA is often capable of recognizing and regulating the expression of various target mRNAs via complementary base pairing[31-33]. Over 60% of the human genome is regulated by at least one miRNA, which indicates that even small changes in miRNA expression can have a significant impact on cellular processes[33,34]. Moreover, abnormal miRNA expression has been linked to a wide range of pathological conditions, such as cardiovascular diseases, neurodegenerative and metabolic disorders, inflammatory diseases, and cancer[35]. The growing knowledge of miRNA-related pathophysiology has opened a new field of biomedical research that focuses on targeting aberrant miRNA expression for therapeutic purposes. Common therapeutic strategies include administering exogenous miRNA mimics to counteract the downregulation of important miRNAs, as well as miRNA inhibitors that suppress miRNA function, resulting in the upregulation of target mRNA translation[63,67,68]. Several studies have demonstrated the remarkable potential that miRNA-based therapeutics can have in the treatment of different diseases, leading to a widespread acknowledgement of the therapeutic potential of miRNAs. Nevertheless, the field remains relatively nascent, with only a limited number of miRNA-based therapeutics progressing to pre-clinical development or clinical trials.

Lung inflammation is associated with numerous pulmonary complications and is a significant contributor to increased post-burn morbidity and mortality[7,8]. Alcohol intoxication before a burn injury worsens lung inflammation, disrupts the pulmonary barrier, and leads to

poor clinical outcomes like sepsis, ALI, ARDS, and MOF[3,4,6-8,13]. Clinical studies have demonstrated that inhibition of pro-inflammatory cytokines, such as IL-6, confers protection against uncontrolled pulmonary inflammation post-alcohol and burn injury[8]. Additionally, it has been shown that lung inflammation after a combined injury can lead to extensive lung tissue damage and pulmonary barrier dysfunction via recruitment, activation, and retention of neutrophils in the lung tissue[81,82]. Within lung tissue, miRNAs have important roles in the maintenance of pulmonary barrier function and lung homeostasis. Abnormal changes in miRNA expression have been associated with lung inflammation and pulmonary barrier disruption, therefore driving the pathophysiological process of various inflammatory disorders of the lungs such as COPD, asthma, and pulmonary fibrosis[28-30]. Although trauma and alcohol intoxication are known to alter miRNA expression, less is known about their role in promoting lung inflammation and lung barrier disruption following alcohol and burn injury[28-30].

We previously demonstrated that miR-146a expression was significantly downregulated in lung tissue one day after alcohol and burn injury, which may be contributing to the pathogenesis of excessive lung inflammation and pulmonary barrier dysfunction. Recent findings from our lab have shown that *in vivo* administration of miR-146a mimic into male mice significantly reduced inflammation in small IECs following alcohol and burn injury, characterized by reduced expression of IL-6 and reduced levels of neutrophil markers (CXCL1 and Ly6G).

To explore the therapeutic potential of miR-146a restoration in the lungs, we administered an *in vivo* ready miR-146a mimic (via i.p. injection) in our mouse model. Our results demonstrated that the overexpression of miR-146a in lung tissue significantly reduced

lung inflammation one day after alcohol and burn injury. All in all, this study shows that miRNA expression is important for lung homeostasis and could be targeted for a new therapeutic approach to manage lung inflammation.

Materials and Methods

Laboratory Mice

Male C57/BL6 mice were acquired from Charles River Laboratories and kept in conventional laboratory housing at the Loyola University Medical Center Comparative Medicine facility in Maywood, Illinois, USA. The mice were housed for a duration of two weeks prior to the start of the experiment, which caused their age to range from 8-10 weeks when the study began. During the experiment, all mice had a weight within the range of 23-25 grams. All animal experiments conducted were done in compliance with the IACUC in Loyola University Chicago's Health Sciences Division.

Murine Model of Acute Alcohol Intoxication and Burn Injury

Male C57/BL6 mice were randomly distributed among four experimental groups: sham vehicle + scramble mimic (referred to as scramble SV), sham vehicle + miR-146a mimic (referred to as miR-146a SV), ethanol-burn + scramble mimic (referred to as scramble EB), and ethanol-burn + miR-146a mimic (referred to as miR-146a EB). Each mouse was administered 50 ug of either scramble or miR-146a mirVana mimic (ThermoFisher Scientific, HPLC purified, *in vivo* ready) with 6 uL *in vivo*-jetPEI reagent in 250 μL 5% glucose (Polyplus Transfection) one day before the combined injury. Mice were then gavaged with 400 μL of 25% ethanol in water (2.9 g/kg) or saline vehicle on the day of injury at a concentration intended to raise the BAC to 90-100 mg/dL four hours after ethanol exposure. This is marginally higher than the legal limit of

80 mg/dL (0.08%) and similar to the BAC observed in hospitalized patients[77]. Mice received 1 mg/kg of buprenorphine subcutaneously three hours after the gavage as a pain reliever. Four hours after the gavage, the mice were given a single i.p. injection of ketamine hydrochloride (~ 80 mg/kg) and xylazine (~ 1.2 mg/kg) to induce unconsciousness, and their dorsums were shaved.

After that, the mice were put into a plastic template exposing ~12.5% of their TBSA, as determined by Meeh's formula[103]. Animals in the burn group were submerged in a water bath set at ~85°C for ~7 seconds to induce an insensate, full-thickness scald burn injury. Sham animals, on the other hand, spent an equivalent amount of time in a water bath at room temperature (37°C). After being gently dried, the mice were given an i.p. injection of 1.0 mL of saline to resuscitate them. The animals were then put back into their cages, which were placed on heating pads to help the mice maintain their body temperature, and monitored to make sure they recovered from the anesthesia. From there, the mice were put back into their regular housing and given full access to food and water. In order to eliminate potential confounding factors associated with circadian rhythms, all experiments were conducted between 12 and 1 pm. The animals were euthanized 24 hours after the burn injury. At that time, lung tissue was harvested and snap-frozen in liquid nitrogen for RNA and protein analysis.

Total RNA Isolation

Total RNA was extracted from homogenized lung tissue for miRNA or mRNA analysis using the MirVana miRNA isolation kit (Invitrogen) as per the manufacturer's instructions. The concentration and purity of RNA were evaluated using a Nanodrop 2000 spectrophotometer (ThermoScientific).

RT-qPCR Analysis of miR-146a Expression

Total RNA was isolated using the MirVana miRNA isolation kit, which was then reverse transcribed and amplified into cDNA using the miRCURY LNA RT Kit (Qiagen). Then, using the miRCURY LNA SYBR Green PCR Kit and a miR-146a-specific primer for the miRCURY LNA PCR Assay (Qiagen), the relative expression of miR-146a was measured by quantitative real-time PCR (qPCR). miR-146a C_t values for each sample were normalized to the housekeeping control SNORD68, and the $\Delta\Delta C_T$ method was used to calculate relative expression.

RT-qPCR Analysis of mRNA Expression

Total RNA was isolated using the MirVana miRNA isolation kit, which was then reverse transcribed and amplified into cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems). Then, using Taqman Fast Advanced Master Mix and FAM Taqman primer probes (Life Technologies) tailored to each target gene, qPCR was used to measure the relative expression of the target genes. The C_t cycle values of the target genes in each well were standardized to the C_t values of GAPDH housekeeping control using VIC TaqMan primer probes within the same reaction. The $\Delta\Delta C_T$ method was used to calculate the relative expression.

ELISA Analysis

Lung tissue was sonicated and analyzed for the presence of IL-6, CXCL1, and MPO (R&D Systems) using their respective ELISA kits, following the manufacturer's instructions. The levels of these pro-inflammatory mediators were quantified and reported per mg of protein.

Statistics

Data was reported as mean values ± SEM. Statistical analysis was conducted using GraphPad Prism 10 software. All experiments in this study with more than two groups were subjected to a two-way ANOVA and Tukey's post-hoc test. P-values or adjusted p-values were considered statistically significant when $p < 0.05$ and denoted as * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, and **** $p < 0.0001$.

Results

To validate the upregulation of miR-146a following *in vivo* mimic administration, the expression of miR-146a was assessed in lung tissue one day after alcohol and burn injury. As shown in **Figure 6**, mice treated with miR-146a mimic showed an increase in miR-146a expression in lung tissue following ethanol-burn injury; however, this was not found to be different from mice treated with scrambled control ($p = 0.120$). However, the treatment of ethanol-burn mice with miR-146a mimic significantly reduced lung inflammation as assessed by decreased IL-6 ($p < 0.01$), CXCL1 ($p < 0.001$), and Ly6G ($p < 0.01$) gene expression compared to those treated with scrambled control (**Figure 7A-C**). MPO gene expression was also found to be reduced in ethanol-burn mice treated with miR-146a mimic, but it failed to reach significance ($p = 0.059$; **Figure 7D**). Moreover, there was no statistical difference in ICAM-1 expression in ethanol-burn mice treated with the miR-146a mimic compared to mice treated with scrambled control ($p > 0.05$; **Figure 7E**).

To further evaluate lung inflammation and neutrophil infiltration after alcohol and burn injury, the protein levels of pro-inflammatory cytokine IL-6 and neutrophil markers CXCL1 and MPO were assessed via ELISA analysis. As shown in **Figure 8A**, restoration of miR-146a in

ethanol-burn mice significantly reduced IL-6 levels in lung tissue following alcohol intoxication and burn injury ($p < 0.01$). CXCL1 levels were also found to be trending downward in ethanol-burn mice treated with miR-146a mimic but failed to reach significance ($p = 0.120$; **Figure 8B**). However, there was no statistical difference in MPO levels in the lungs of ethanol-burn mice treated with the miR-146a mimic compared to mice treated with scrambled control ($p > 0.05$; **Figure 8C**).

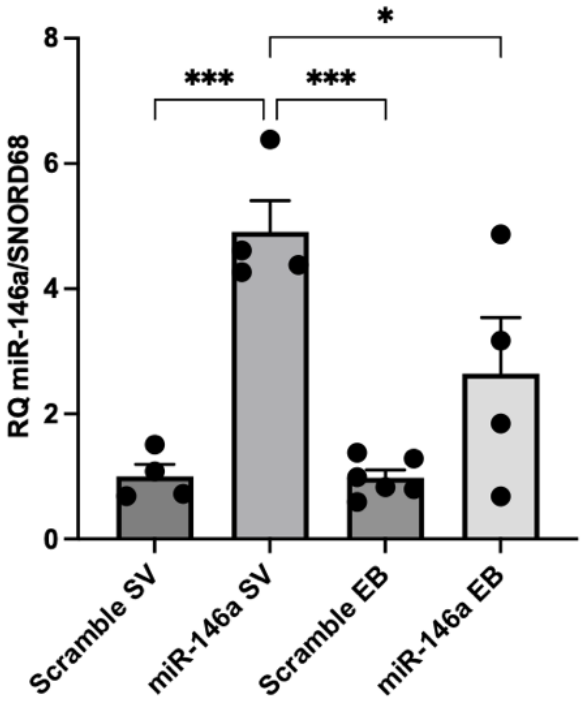

Figure 6. *In Vivo* miR-146a Mimic Administration Successfully Overexpressed miR-146a in Lung Tissue. Lung tissue was collected from male mice one day after combined ethanol and burn injury and total RNA was isolated for RT-qPCR analysis. A primer specific for miR-146a-5p was used to assess the relative expression of miR-146a-5p. Graph includes miR-146a expression relative to average scramble SV samples, with mean ± SEM (n = 4-6 mice per group). SNORD68 was used as endogenous control. Analysis via two-way ANOVA. Significance (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$) and shown as compared by Tukey's multiple comparisons test.

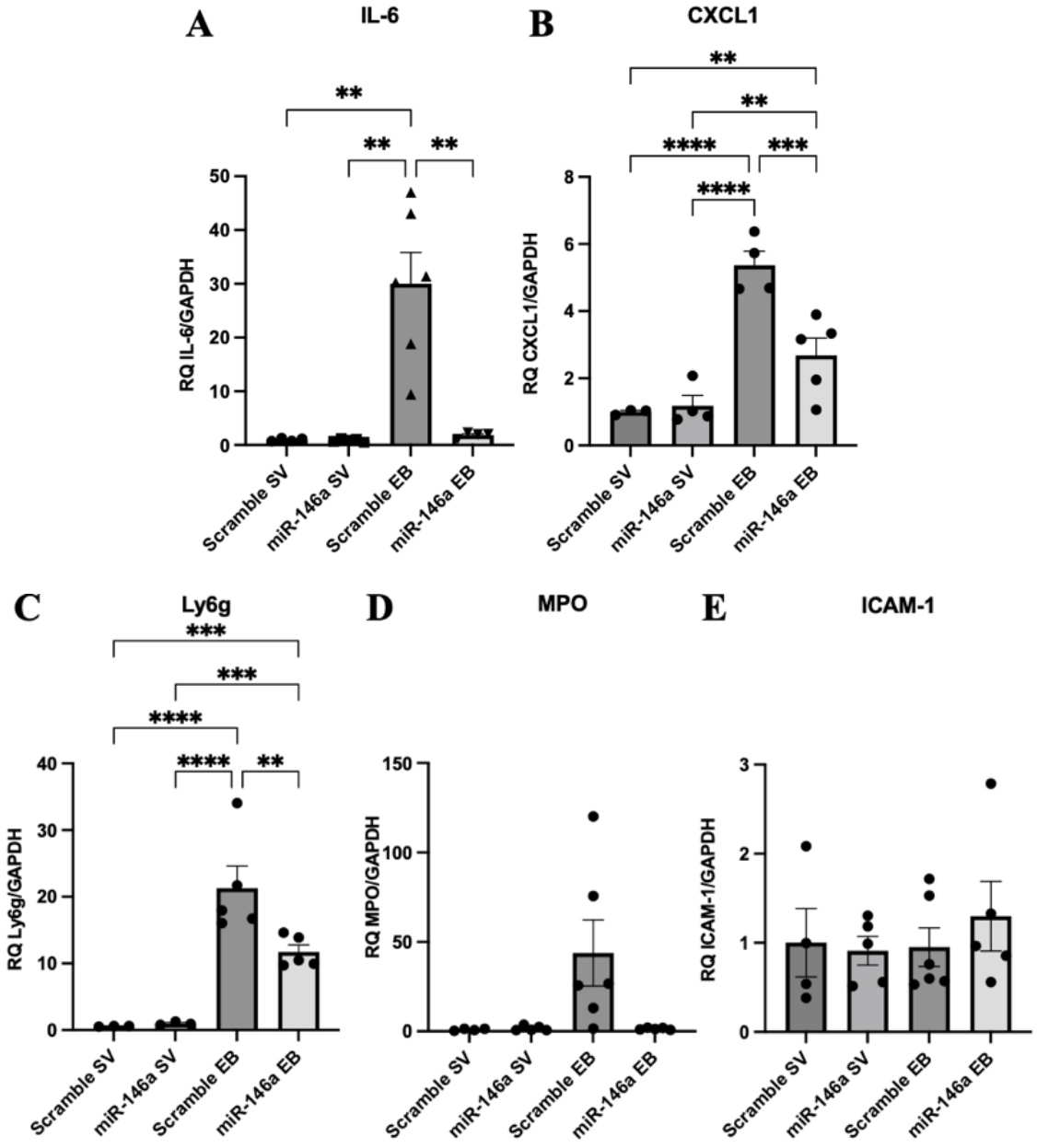

Figure 7. Restoration of miR-146a Reduced Lung Inflammation After Alcohol and Burn Injury. Lung tissue was collected from male mice one day after combined ethanol and burn injury and total RNA was isolated for RT-qPCR analysis of target gene expression using primers specific for IL-6, CXCL1, Ly6g, MPO, and ICAM-1. Graphs are relative expression to average scramble SV samples, with mean ± SEM (n = 3-6 mice per group). GAPDH was used as endogenous control. Analysis via two-way ANOVA. Significance (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$) and shown as compared by Tukey's multiple comparisons test.

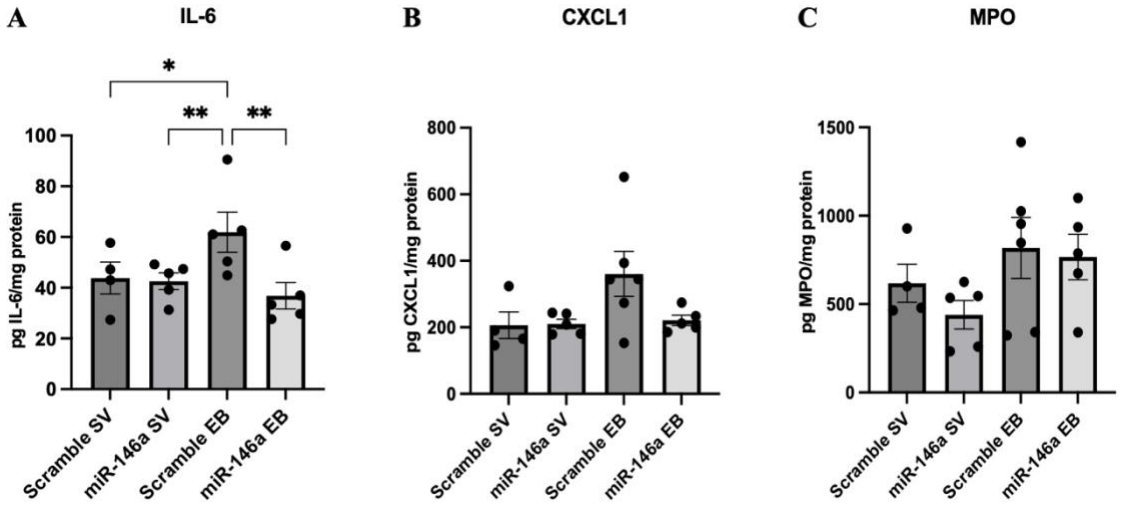

Figure 8. Restoration of miR-146a Reduced Levels of Pro-Inflammatory Mediators in Lungs Post Alcohol and Burn Injury. Lung tissue was homogenized via sonication and ELISAs were preformed to quantify the concentrations of (A) IL-6, (B) CXCL1, and (C) MPO. Concentrations were reported in pg/mg total protein. Graphs are mean ± SEM (n = 4-6 mice per group). Statistical analysis via two-way ANOVA. Significance (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$) and shown as compared by Tukey's multiple comparisons test.

Summary

The purpose of this study was to further investigate the impact that decreased miR-146a expression had on lung homeostasis and to evaluate the therapeutic potential of targeting miR-146a expression to reduce lung inflammation after alcohol and burn injury. We found that miR-146a mimic administration into mice successfully overexpressed miR-146a expression in lung tissue following alcohol intoxication and burn injury. As a result, this significantly reduced lung inflammation following alcohol and burn injury, characterized by reduced expression of IL-6 and neutrophil markers (CXCL1, Ly6G, and MPO), along with reduced levels of IL-6. Overall, our findings suggest that restoration of miR-146a expression in lung tissue successfully reduced lung inflammation one day after combined injury. Hence, this study shows that downregulation of miR-146a expression in lung tissue is a potential therapeutic target to prevent excessive lung inflammation following alcohol intoxication and burn injury.

CHAPTER 5

DISCUSSION

New Contributions to the Field of Alcohol and Burn Injury

Lungs are usually the first organ to fail following a traumatic injury, such as severe burns, and are known to cause various systemic complications (e.g., sepsis and MOF) that can ultimately result in death[2-4,8,81,82]. Alcohol intoxication is a major confounding factor in the pathology of burn injuries, with around half of reported burn injuries occurring when patients are under the influence of alcohol[5]. Studies have shown that when alcohol intoxication precedes a severe burn injury, patients become particularly susceptible to pulmonary infections and complications because of their suppressed immune state[7]. As a result, respiratory failure, ALI, ARDS, and pulmonary infections are the leading causes of post-burn morbidity and mortality[8]. Despite decades of research, there are currently no effective therapeutic strategies available that can improve the survival of patients with pulmonary complications (e.g., ALI and ARDS) following a combined alcohol and burn injury[25-30]. MiRNAs have been found to be key regulators in lung homeostasis; however, their role in lung inflammation following alcohol intoxication and burn injury has yet to be explored. The overall purpose of this thesis project was to investigate if reduced miR-146a expression drives lung inflammation following alcohol and burn injury in the hopes that this specific miRNA could be a potential therapeutic target to reduce lung inflammation following alcohol intoxication and burn injury. Furthermore, this project sought to identify other anti-inflammatory miRNAs, such as miR-150, miR-194, and

miR-671, that are downregulated following an alcohol and burn injury and could be contributing to excessive lung inflammation after this combined injury.

Previous studies have revealed that miR-146a has a key regulatory role in immune cell signaling during an inflammatory response and may have a similar role in lung tissue[104-107]. Our results demonstrate a significant downregulation of miR-146a and miR-671 expression in lungs one day after alcohol and burn injury. The significant downregulation of these two miRNAs were found to be associated with increased lung inflammation and pathology one day after the combined injury. Upon further examination, biological sex did not appear to have an effect on this pathophysiological process. However, it is important to note that miR-146a expression in lung tissue from female ethanol-burn mice was significantly upregulated when compared to sham vehicle; whereas the inverse was found in the lung tissue from male ethanol-burn mice. The reasoning behind this inverse relationship in miR-146a expression in lung tissue from male vs. female mice after this combined insult remains unclear and warrants further investigation in future studies.

Nevertheless, the significant downregulation of miR-146a expression in lung tissue from male ethanol-burn mice validates previous findings in our lab in which miR-146a expression was significantly decreased in small IECs isolated from male mice one day after alcohol and burn injury. Therefore, male mice were solely used to investigate the impact of *in vivo* miR-146a mimic administration on lung inflammation following an alcohol and burn injury. In this study, *in vivo* overexpression of miR-146a significantly decreased lung inflammation and could potentially be a therapeutic target that promotes lung homeostasis after an alcohol and burn injury.

Aberrant miR-146a Expression and Lung Inflammation After Alcohol and Burn Injury

Although aberrant miRNA expression is linked to an array of pathological conditions, such as cardiovascular diseases, metabolic disorders, and inflammatory diseases, the impact of miRNA expression to lung inflammation after alcohol and burn injury has yet to be explored[3,6-8,35]. Within lung tissue, miRNAs are key regulators in the maintenance of lung homeostasis and pulmonary barrier function. Abnormal changes in miRNA expression have been shown to be associated with the pathogenesis of various diseases involving lung inflammation and pulmonary barrier dysfunction[28-30]. However, there is limited understanding of how miRNAs affect lung inflammation following alcohol intoxication and burn injury. Through our lab's mouse model of acute ethanol intoxication and burn injury, we previously assessed the expression of four anti-inflammatory miRNAs (miR-146a, miR-150, miR-194, and miR-671) that were found to be associated with the regulation of inflammation in small IECs following ethanol and burn injury. From these four miRNAs, the expression of miR-146a and miR-150 were found to be significantly downregulated ($p < 0.05$) in IECs isolated from ethanol-burn mice compared to sham vehicle. *In vitro* studies were then performed to investigate the mechanism(s) by which reduced expression of miR-146a may play in the development of intestinal inflammation and intestinal barrier disruption following alcohol and burn injury. These studies demonstrate that miR-146a expression has a key regulatory role in LPS-induced inflammatory cytokine expression in IECs by modulating p38 MAPK signaling and targeting TRAF6.

Based on these findings, we sought to determine whether reduced expression of miR-146a and other anti-inflammatory miRNAs (miR-150, miR-194, and miR-671) promote excessive lung inflammation after alcohol and burn injury. Using our lab's well-established

mouse model of acute ethanol intoxication and burn injury, we found a significant decrease in miR-146a and miR-671 expression in lung tissue from male mice one day after alcohol and burn injury. Our results are believed to be the first reported findings that assess the expression of miR-146a and miR-671 in lung tissue from male mice after this combined injury. Therefore, the role and corresponding expression level of miR-671 in this pathophysiological process are not well understood and require further investigation. However, one previous study demonstrated that reduced miR-671 expression increased neuroinflammation via activation of the NF-κB signaling pathway in an acute ischemic stroke mouse model[104]. Therefore, reduced miR-671 expression in lung tissue could be a potential contributor to excessive lung inflammation following an alcohol and burn injury.

Additionally, our results validate another study that reported a significant decrease in miR-146a expression in burn-induced ALI rats, which led to heightened lung inflammation through increased activation of the TLR4/NF-κB signaling pathway[105]. Our results are consistent with these findings, as we observed a significant decrease in miR-146a expression in lung tissue from male mice following alcohol intoxication and burn injury. This decrease in miR-146a expression was found to be associated with increased lung inflammation and pathology, evident one day after the combined injury. Excessive lung inflammation was characterized by a significant increase in expression of pro-inflammatory mediators (ICAM-1, CXCL1, CXCR2, MPO, and Ly6G), as well as significantly increased levels of CXCL1, in lung tissue from male mice one day after alcohol and burn injury. These pro-inflammatory mediators induce lung inflammation and neutrophil recruitment post alcohol and burn injury. In addition to these findings, examination of H&E-stained lung tissue revealed an increase in tissue cellularity,

pulmonary edema, and alveolar wall thickness in mice that were intoxicated with alcohol prior to sustaining a burn injury. All in all, our results suggest that alcohol intoxication at the time of injury is sufficient to exacerbate post-burn lung inflammation. More importantly, the downregulation of miR-146a expression in lung tissue one day after alcohol and burn injury potentiates lung inflammation.

Therapeutic Potential of miR-146a Mimic Administration for Lung Inflammation

The discovery of miRNAs association with various diseases is continuously expanding, which has prompted the exploration of targeting miRNA expression as a potential therapeutic approach. Although not officially approved by regulatory bodies, several miRNA-based treatments have demonstrated effectiveness in human clinical trials. Several studies have demonstrated the potential of miRNA-based treatments in regulating the overactivation of inflammatory signaling pathways, particularly in conditions linked to lung inflammation. Ongoing clinical trials are investigating the effectiveness of MRG-201 (Remlarsen) in managing pulmonary fibrosis through the use of a synthetic RNA molecule that inhibits the synthesis of collagen and other key proteins involved in scar formation[63,69-71].

Therefore, therapeutic targeting of miRNAs associated with lung inflammation could potentially be a novel therapeutic approach to reduce lung inflammation and prevent serious post-burn complications following a combined injury of alcohol and burn, such as SIRS, sepsis, ALI, ARDS, and MOF. Through the use our lab's mouse model of acute ethanol intoxication and burn injury, we found that miR-146a expression was significantly downregulated in lung tissue one day after the combined injury. The downregulation of this miRNA was associated with increased pulmonary inflammation and pathology one day after alcohol and burn injury.

In various inflammatory cells, miR-146a is induced by TLR signaling and acts as an essential negative feedback regulator in innate immune responses by targeting MyD88 adaptor proteins (i.e., IRAK1 and TRAF6) to prevent uncontrolled activation of inflammatory signaling pathways (i.e., NF-κB and MAPK pathways)[106]. By impairing these MyD88-dependent signaling pathways, miR-146a is able to suppress the production of various pro-inflammatory mediators (e.g., IL-6, CXCL1, and ICAM-1) in many cell types, including macrophages, neutrophils, and endothelial/epithelial cells. Therefore, miR-146a has an important regulatory role in TLR-mediated innate immunity, autoimmunity, and the development of endotoxin-induced tolerance[105-109].

In this regard, overexpression of miR-146a should impair MyD88-dependent signaling pathways and suppress the production of pro-inflammatory mediators in many cell types, including bronchial epithelial cells. Following *in vivo* miR-146a mimic administration into our mouse model of acute ethanol intoxication and burn injury, our results showed that miR-146a mimic administration successfully overexpressed miR-146a in lung tissue one day after combined insult. This overexpression of miR-146a significantly reduced the expression of pro-inflammatory mediators and neutrophil markers in the lung, indicating that miR-146a mimic administration successfully prevents lung inflammation following a combined insult. Although it was not investigated in this study, our findings suggest that miR-146a expression may indirectly affect pulmonary barrier function by reducing inflammatory signaling or directly regulating pulmonary epithelial cell proliferation or TJ protein expression. Further studies are needed, both *in vitro* and *in vivo*, to get a better understanding of these mechanisms and how they may impact the function of the pulmonary barrier after alcohol intoxication and burn injury.

Although our lab uses a relatively minor injury model (~12.5% TBSA scald burn) that does not cause pulmonary complications unless alcohol intoxication precedes burn injury, the expression level of ICAM-1 (**Figure 2.B**) and protein levels of IL-6 and MPO (**Figures-2.E** and **-2.G**) have been shown to increase solely due to the burn injury. However, when alcohol is present, the expression levels of various pro-inflammatory mediators (IL-6, CXCL1, and CXCR2) are noticeably higher compared to burn injury alone (**Figure 2**). This combined insult of alcohol and burn injury has been found to induce pulmonary edema, neutrophil infiltration, and increase susceptibility to infection, surpassing the individual effects of either insult alone, with no direct injury to the lungs themselves[2]. One previous study has shown that the inhibition or knockout of the IL-6 gene confers protection against uncontrolled pulmonary inflammation caused by a combined insult of alcohol and burn[8]. In line with these findings, our studies demonstrate that restoration of miR-146a expression in lung tissue significantly reduced lung inflammation, as shown by reduced IL-6 and neutrophil marker expression (CXCL1 and Ly6G), after alcohol and burn injury.

It is important to acknowledge one major limitation in the conclusions drawn from this study regarding the effects of miR-146a expression in lung tissue on lung inflammation following alcohol and burn injury. When a miRNA mimic is administered in an animal model through i.p. injection, it can have indirect effects and result in an increase in miRNA expression in various organs and cell types. Therefore, we are unable to determine the extent to which miR-146a expression increased in immune cells in this study, since they are found in both the bloodstream and lung tissue, as opposed to pulmonary epithelial cells. Moreover, we cannot determine the effect that this may have on decreasing lung inflammation following a combined

injury of alcohol and burn. Both inflammatory immune cell infiltration (i.e., neutrophils) and inflammatory cytokine production have been implicated in the pathogenesis of lung inflammation. While our findings support the claim that restoration of miR-146a expression in lung tissue reduces lung inflammation and prevents the recruitment of neutrophils to the lungs, it is likely that miR-146a expression in immune cells also influences the observed outcomes. To get a better understanding of the effects of miR-146a expression in various cell types, further studies are needed to assess the restoration of miR-146a expression in immune cells and microvascular endothelial cells in the lungs.

Final Conclusions

Our studies have only just begun to establish the framework for addressing a critical gap in our current knowledge regarding the mechanisms that lead to pulmonary dysfunction in patients after alcohol intoxication and burn injury. In our studies, we have identified two miRNAs that have key regulatory roles in the maintenance of lung homeostasis, providing insight into their potential involvement in pulmonary dysfunction post-alcohol and burn injury. More specifically, we observed a significant decrease in expression of miR-146a in lung tissue one day after combined injury, and we have revealed how this reduction in miR-146a perpetuates lung inflammation, potentially impacting pulmonary barrier function. To determine if *in vivo* restoration of miR-146a expression in the lungs could reduce lung inflammation after an alcohol intoxication and burn injury, we administered a miR-146a mimic into our mouse model of acute ethanol intoxication and burn injury. Our results from this study demonstrated that restoration of miR-146a expression significantly reduced lung inflammation one day after alcohol and burn injury. These results suggest that miR-146a may have a central role in driving lung inflammation

after alcohol and burn injury, and more importantly, they highlight the therapeutic potential of targeting miRNAs to restore lung homeostasis after a combined injury. Future studies will need to be conducted to further investigate the impact of altered miRNA expression on pulmonary dysfunction, and to explore the therapeutic potential of using these miRNAs to reduce lung inflammation and improve patient outcomes who are affected by this combined insult.

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VITA

Connor Guzior was born in Munster, IN, on February 8, 1997, to Steve and Pam Guzior. He attended Loyola University Chicago, where he earned a Bachelor's of Science in Chemistry in May 2020 and his Master's degree in Public Health with a concentration in Epidemiology in May 2022. Shortly afterwards, Connor matriculated into the Loyola University Chicago Stritch School of Medicine's Infectious Disease and Immunology Master's Program under the mentorship of Dr. Mashkoor Choudhry in August 2022.

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