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Short-Term Second-Hand Smoke Exposure Predisposes the Lung to Inflammation, Elastic Fiber Injury, and Pulmonary Emphysema

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SHORT-TERM SECOND SMOKE EXPOSURE PREDIPOSES HE LUNG TO INFLMMATION, ELASTIC FIBER INJURY, AND PULMONARY EMPHYSEMA

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ABSTRACT

SHORT-TERM SECOND SMOKE EXPOSURE PREDIPOSES HE LUNG TO INFLMMATION, ELASTIC FIBER INJURY, AND PULMONARY EMPHYSEMA

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To determine whether cigarette smoke exposure predisposes lungs to secondary injury, our laboratory developed a hamster model using lipopolysaccharide (LPS) to induce pulmonary inflammation following exposure to second-hand smoke. With this model, inflammatory effects of LPS on smoke-exposed hamsters was measured by various morphological and biochemical parameters. Additional experiments studied the effects of exogenous ET-1 and BQ123, which modulate the adherence of inflammatory cells to lung capillary endothelium. Total bronchoalveolar lavage fluid (BALF) leukocytes, percent BALF neutrophils, and elastin-specific desmosine and isodesmosine (DID) crosslinks (free and peptide-bound) in BALF and whole lungs were measured 24 hours (hrs) after LPS treatment. Morphometric changes were evaluated with the mean linear intercept (MLI) method which measures airspace enlargement, and a disease index was used to quantify the thickness of interstitial walls and the intensity of inflammatory infiltrates 24 hrs post-LPS treatment. Animals treated with smoke and LPS showed a significant increase in BALF neutrophils (15.0% vs 5.1%, respectively) and free lung DID (359 vs 93.1 ng/mg wet lung) compared to the room-air/LPS group. There was also a significant increase in the disease index in the smoke/LPS group compared to room air/LPS controls (1.6 vs 0.9 respectively). As a measure of irreversible lung injury, MLI was significantly elevated in the smoke/LPS group compared to room air/LPS controls

(83.6 vs 55.7 um, respectively). Conversely, animals treated with BQ123 before smoke/LPS administration showed a significant decrease in BALF neutrophils compared to smoke/LPS alone (8.5% vs 15.8% respectively). While BALF neutrophils were significantly increased in room air-exposed animals given ET-1 compared to room air alone (13.9% vs 5.2%, respectively), the addition of ET-1 to animals given smoke and/or LPS had anti-inflammatory effects, suggesting ET-1 counteracts the proinflammatory activity of these other agents. The results of these studies demonstrate that the combination of cigarette smoke and secondary inflammation induced by LPS causes severe acute lung injury that degrades lung elastic fibers and enlarges airspaces. Treatment with BQ123 reduced this inflammatory process, suggesting it may have a therapeutic role in reducing the risk of lung damage in smokers with pneumonia or other types of secondary injury.

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CHAPTER 1: INTRODUCTION

1.1 Cigarette and Secondhand Smoke

According to the Center for Disease Control and the US Surgeon General, tobacco smoking is the leading preventable cause of death in the United States that causes more than 480,000 deaths each year (United States Department of Health and Human Services, 2014). Smoking has caused more deaths each year than HIV and illegal drug use. It has also been well established that smokers are more susceptible to chronic diseases such as periodontal diseases, hypertension, chronic obstructive pulmonary disease (COPD), impotence, and osteoporosis. However, smokers are more susceptible to bacterial infections than are non-smokers and such infections are life-threatening. Both active smokers and people exposed to secondhand smoke toxins are at increased risk of bacterial infections. Smoking continues to be a major risk factor without having COPD because it makes individuals susceptible to bacterial infections such as invasive pneumococcal disease. This disease involves any infection caused by bacteria called *Streptococcus pneumoniae* and has been shown that smoking is the strongest risk factor for this disease. In secondhand smoking, exposure to it has been associated with an increased risk of meningococcal disease and it is a significant risk factor for children attributed to maternal smoking. As such, cigarette smoke can rapidly induce the production of several inflammatory mediators that recruit inflammatory cells to the lung. Therefore, even short-term exposure to second-hand smoke might predispose the lung to secondary injury by other agents. About humans, the model provides an opportunity to determine the relationship between smoking and lung infection.

1.2 Secondhand Smoking and Bacterial Infections

Tobacco smoking continues to be a prevalent long-term problem in both the United States and the world. A global study was created called the *Global Burden of Diseases* in which it looked at the major causes and factors of deaths across the world and smoking was ranked two on the major cause of deaths. The study has stated that more than 8 million people died prematurely because of smoking in 2017 and 7 million people died from smoking tobacco alone. Specifically, secondhand smoking is a concurrent issue as it causes large health impacts (Naghavi et al., 2017). It has been estimated 1.2 million people died prematurely from secondhand smoke. Since 1964, approximately 2.5 million nonsmokers have died from health problems caused by exposure to secondhand smoke (United States Department of Health and Human Services, 2014). When put in comparison to other causes of death secondhand smoke kills more people than road accidents globally. Globally, the US is one of the many countries in which smoking is considered a major cause of death in the nation. According to the CDC, nearly 14 of every 100 U.S adults aged 18 years or older currently smoke cigarettes. However, there has been a decline in smoking since 2005 from 21 of every 100 adults to 14 of every 100 adults. With the decline of cigarette use, cigarette smoke-related issues continue to persist in those individuals and the people who are being exposed unintentionally to cigarette smoke. This unintentional exposure is called secondhand smoking in which the cigarette smoke comes from the burning end of the cigarette bud and is breathed out by the smokers. In recent years, there has been a decrease in the number of nonsmokers being exposed to secondhand smoke due to the changes in the rules for smoking in public and indoors. There are also growing changes in the lifestyle where smoking is becoming less

socially acceptable. It continues to be a problem as it causes numerous health problems in infants and children, including more frequent and severe asthma attacks, respiratory infections, and sudden infant death syndrome. Secondhand smoke has been known to cause lung cancers in adults who have never smoked, and secondhand smoke causes more than 7,300 lung cancer deaths among U.S non-smokers each year. Primarily, this laboratory is studying the effects of secondhand smoking and a bacterial infection stimulated by lipopolysaccharides. It is well known that smokers are more susceptible to nonsmokers to a wide range of chronic diseases. However, smokers become more susceptible to multiple bacterial infections than nonsmokers and such infections can be dangerous.

There are many bacterial infections associated with tobacco smoking such as nasopharyngeal, respiratory tract infections, bronchitis, and COPD (Bagaitkar et al., 2008). There are potential mechanisms on why there is an increased susceptibility to bacterial infections such as tobacco-induced physiological and structural changes in humans, increase in bacterial virulence, and the dysregulation of the immune function. These mechanisms are not mutually exclusive, and they can all occur simultaneously (Bagaitkar et al., 2008). There have been instances where tobacco smoke exposure may play a direct role in bacterial colonization of the respiratory tract by hindering the clearance of bacteria. Cigarette smoke may also have physiological and structural changes in vasculature and respiratory tract such as inducing vasoconstriction of the peripheral arteries but vasodilation of the cerebral blood vessels.

1.3 Emphysema - COPD

Pulmonary emphysema is a progressive lung disease that is a categorized form of COPD. It is often caused by chronic and significant exposure to noxious gases and most commonly caused by cigarette smoking. There are also possible causes for emphysema such as biomass fuels and environmental pollutants such as sulfur dioxide and particulate matter (Pahal P, Avula A, 2020). The manifestations of emphysema are the consequences of damage to the airways and bronchioles which includes alveolar sacs and the alveoli. The damage causes abnormal permanent dilation of the airspaces and the destruction of the alveoli walls due to the proteinases such as elastase (Pahal P, Avula A, 2020). As elastin is an important component of the extracellular matrix that is required to maintain the lung parenchyma and small airways, the elastase and anti-elastase imbalances increase the susceptibility for the lung to be damaged which can lead to airspace enlargement (Pahal P, Avula A, 2020). This allows to conduct a method called mean linear intercept on the lung airspace diameter as cigarette smoking and secondhand smoking can increase your risk of emphysema and COPD (Thurlbeck, 1967). The measurement of the lung airspace will help us determine whether the combination of secondhand smoke and a bacterial infection can cause enlarged airspace in the lung.

1.4 Neutrophils and Cigarette Smoke

Neutrophils are the main concern in this experimental model as it is one of the most abundant types of white blood cells and forms the essential part of the innate immune system. This becomes important as cigarette smoking has been strongly associated with multiple respiratory infections such as *Streptococcus pneumoniae* and *Legionella pneumophila* and chronic diseases such as COPD and asthma (Thulborn et al.,

2019). Neutrophils are an important biomarker as they are the most abundant leukocyte in the human body and are considered the first line of defense in the innate immune system. They can destroy invading foreign bodies through phagocytosis and intracellular degradation. They are also considered mediators of inflammation. These infections and diseases are often the major cause of mortality across the world. The influx of pulmonary neutrophils often occurs in response to bacterial infection and a common feature of chronic inflammatory lung diseases. A theory has been proposed by the American Thoracic Society that compromised anti-bacterial nature in neutrophils may be a major cause of increased susceptibility to infectious diseases in smokers (Guzik et al., 2011).

1.5 Lipopolysaccharides

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. Lipopolysaccharide is localized in the outer layer of the membrane and is exposed to the cell surface. The general structure of LPS is a hydrophobic lipid section that is responsible for the toxic properties, a hydrophilic core polysaccharide chain, and a repeating hydrophilic O-antigenic oligosaccharide side chain that is specific to the bacterial serotype. The LPS used in the studies is from Escherichia coli O26:B6 (L8274) sourced from Sigma-Aldrich. The function of LPS is to protect the bacterium against the action of bile salts and lipophilic antibiotics. LPS are heat-stable endotoxins and have been recognized as a factor in septic shock in humans and more generally for inducing a strong immune response in normal mammalian cells. LPS has been widely used for lung inflammation models to simulate chronic lung diseases such as COPD and emphysema. Lipid A is the main structure where all the endotoxic activity

occurs. In our study, LPS is being used to simulate a condition of a secondary lung infection after a chronic smoker contract a respiratory disease or infection. This is due to the fact smoking is a factor in increasing the risk for respiratory infections and exacerbating chronic lung diseases.

1.6 Endothelin-1 and Cigarette Smoke

Endothelin or ET is a family of 21 amino acid peptides that have a wide range and overlapping functions in the lung and its associated diseases. There are three different isoforms of endothelin, ET-1, ET-2, and ET-3. ET-1 is one of the most abundant isoforms and better chartered and the only difference between the three is the number of amino acids in each one. The lung has the highest levels of ET-1 secreted by endothelium, smooth muscle, airway epithelium, and a variety of other cells. ET-1 circulates in the plasma and the normal lung, it localizes to the vascular endothelin, airway, and vascular smooth muscle cells. There is less ET-1 in the lung epithelium. ET-1 has been implicated in a long list of lung diseases such as ischemia pulmonary edema, acute respiratory distress syndrome, sepsis, asthma, chronic obstructive pulmonary disease, pulmonary fibrosis. ET-1 is not stored in cells, but it is processed and transported through the cell in vesicles which results in direction secretion toward the smooth muscle, and it allows for ET-1 to act in a paracrine or autocrine manner. This secretion into the circulation allows for the ET-1 to act as a hormone (Kowalczyk et al., 2015).

There are currently two distinct human endothelin receptors called endothelin A (ET_A) and endothelin B (ET_B) receptors. ET_A specifically has a higher affinity for ET-1 and ET-2 and the ET_A receptors are in great abundance on the vascular and airway

smooth muscle of the lung. ET_B is found mostly on the lung endothelium and the clearance of ET-1 is mediated by the ET_B receptor primarily in the lung but also in the kidney and liver. The activation of both receptors causes the ET_A on the smooth muscle cells to constrict while the ET_B causes bronchoconstriction (Kowalczyk et al., 2015).

Endothelin-1 is the focus of our experiments as it is an important biomarker for lung injury and inflammation. Since ET-1 is produced in large amounts in the endothelium, there are often large amounts of it in the bronchoalveolar lavage fluid and indicates that there is inflammation or some sort of injury in the lungs. Experimental lung injury of many different types increases circulating ET-1, BAL ET-1, and lung tissue ET-1. These levels are also increased in cases of sepsis, burns, acute lung injury, and acute respiratory distress syndrome (Kowalczyk et al., 2015).

1.7 Endothelin Antagonists and BQ-123

Since the endothelin family has powerful vasoconstrictor properties, it has been implicated in the pathogenesis of pulmonary diseases and COPD. These studies allow for the introduction of endothelin receptor antagonists. Endothelin receptor antagonists are a type of target therapy used to treat people with pulmonary hypertension and have been shown to slow the progression of pulmonary hypertension and may reverse some of the damage to the heart and lungs. This is done by reducing the amount of endothelin in the blood which would limit the harm of excess endothelin in the body. There are several different types of antagonists such as BQ-123, FR 139317, TAK-044, and BQ788. Our focus on our experiments is the use of the cyclic pentapeptide BQ-123. The structure of BQ-123 is a D-Asp-L-Pro-D-Val-L-Leu-D-Trp- peptide isolated from the Streptomyces

misakiensis (Ihara et al., 1991). The role of BQ-123 is that it is a potent and selective antagonist for ET_A and it also inhibits ET-1 receptor binding and blocks Ca^{2+} mobilization, cellular contraction, and MAP kinase activation. The inhibition of ET-1 and ETA-R using BQ-123 is done by reversing the established contractions to the receptor, displacing the peptide from it. It has been studied for potential therapeutic effects in preventative lung injury such as emphysema and COPD (Chen et al., 2010). It can also be a viable treatment for such diseases, and it may be possible to be used in the treatment of other chronic diseases such as pulmonary hypertension (Hanasato et al., 1999).

1.8 Desmosine and Isodesmosine

Elastin cross-linking in the lung consists of two amino acids unique to mature elastin; desmosine and its isomer isodesmosine (DID) which can be used as a biomarker of smoked induced emphysema and COPD (Luisetti et al., 2008). Specifically, free desmosine has been used as a measure for smoke-induced damage and COPD. Desmosine in several matrices such as urine, plasma, and sputum have been used to determine whether patients have COPD or emphysema (Ma et al., 2011). Liu et al. reported an increased level of free DID when hamsters were exposed to secondhand cigarette smoke for 2h/day, 5 days/week for a period of 3 months (Liu et al., 2015). Another study by Churg et al showed a consistent increase in desmosine and hydroxyproline, both degradation products of the extracellular matrix, in BALF of smoke-exposed animals after 6 and 24 hours (Wright et al., 2002).

Improvements have followed when it comes to measuring desmosine such as the use of quantitative high-performance liquid chromatography with tandem mass

spectrometry. Generally, the measurement of desmosine is designed for total desmosine amounts and requires hydrolysis of plasma and urine samples in concentrated HCl to unbind the biomarkers. Instead, the use of a deuterium labeled internal standard was added directly to a matrix sample which allows for improved accurate and precise reading of the free desmosine levels (Miliotis et al., 2013).

1.9 Experimental Design

Our laboratory has created an animal model designed to look at the relationship between acute exposure to cigarette smoke and secondary lung injury induced by injecting lipopolysaccharide (LPS) intraperitoneally into hamsters. This is done by using a TE-10 smoking machine which exposes the hamsters to secondhand cigarette smoke. The inflammation induced by LPS mimics bacterial pneumonia, which is often worse in cigarette smokers due to increased sequestration of inflammatory cells in the lung vascular compartment. The cigarette smoke will expose hamsters to a 3-day regimen of second-hand smoke inhalation to determine if this procedure is sufficient to recruit inflammatory cells to the lung, the hamster will be injected intraperitoneally with 0.1ml of 0.2mg/ml of LPS. In addition to LPS, other agents are being tested which include BQ123 and exogenous endothelin-1. Exogenous endothelin-1 has been used in our laboratory which has proven to modulate the adherence of inflammatory cells to endothelium in the lung capillary network (T. Bhavsar et al., 2008). If BQ123, an FDA-approved drug, is found to inhibit inflammatory cell influx, it may serve as a potential therapeutic agent for the prevention of lung injury due to cigarette smoke.

Using this model, we will test our primary hypothesis that short-term exposure to cigarette smoke predisposes the lung to secondary injury by increasing the inflammatory response to LPS. Parameters that will be evaluated include histopathological changes in the lung, cell content in bronchoalveolar lavage fluid (BALF), and the amount of free and peptide-bound desmosine in BALF. In addition, the effect of LPS on BALF leukocyte chemotaxis will be tested in vitro.

Our laboratory has previously tested the use of endothelin-1 which caused preferential recruitment of neutrophils in acute lung inflammation induced by either LPS or cigarette smoke (T. Bhavsar et al., 2008). When ET-1 was injected into the smoked-exposed animals, it caused an increased number of neutrophils in the BALF but a reduced total number of BALF leukocytes. In our experiment, we hypothesize that injection of ET-1 increases the neutrophil response in the lung to the combination of both cigarette smoke and LPS.

Our laboratory has also tested drugs in dealing with inflammation of the lungs, secondary infections, and possible treatments in COPD. Certain substances such as phosphoramidon and HJP272 have been tested as viable drugs to block the formation of inflammatory mediators such as endothelin-1 (Patel et al., 2014). Phosphoramidon is a known zinc metalloproteinase inhibitor that blocks the formation of ET-1 and it has shown to be a valuable tool in the study of hypertension, stroke, and diseases of the kidney (T. M. Bhavsar et al., 2008). It has shown to cause a reduction of acute inflammation in the LPS-induced lung injury. In our experiments, we tested the viability of BQ-123 as another drug in the treatment of LPS-induced lung injury. It is a known ET_A receptor antagonist and has been tested to prevent the development of pulmonary

emphysema in rats. BQ-123 has been proven to prevent the development of cigarette smoke extract-induced emphysema, block the expression of ET_A receptors, and inhibit pulmonary apoptosis. In our experiments, we hypothesize that BQ-123 can block endothelin-1 activity to decrease the neutrophil response toward cigarette smoke or the combination of cigarette smoke and LPS.

To test these hypotheses, hamsters will be exposed to cigarette smoke for 3 days, and then treated with lipopolysaccharide (LPS) to induce secondary lung injury. It is anticipated that animals exposed to smoke will have a significantly greater reaction to LPS than those treated with LPS alone. The relationship between brief (second-hand) cigarette smoke exposure and lung injury remains unclear. We have studied the effect of lipopolysaccharide (a proinflammatory mediator present in tobacco) on this process to determine this relationship. Other agents to be tested include BQ123 and endothelin-1, which both drugs can modulate the adherence of inflammatory cells to endothelium in the lung capillary network. If BQ123, an FDA-approved drug, is found to inhibit inflammatory cell influx, it may serve as a potential therapeutic agent for the prevention of lung injury due to cigarette smoke.

The first experiment that has been performed was the pilot study on the viability of intraperitoneal injection of LPS after the hamsters were exposed to cigarette smoke for 2 hours in a period of 3 days. The concentration of the LPS injected into the hamsters would be 0.2mg in 0.1 ml of saline. This is done to affirm that IP injection of LPS can simulate the bacterial infection of the lungs after the hamsters were exposed to cigarette smoke. The low dose of LPS is to confirm that it will not cause any noticeable to the

hamsters. The purpose of this experiment is to show that short-term exposure to cigarette smoke can cause inflammation and simulate a bacterial infection in the lungs.

In the second set of experiments, the smoke exposure will be increased to 4 hours for 3 days from 2 hours. The pilot study allowed us to establish that short-term exposure caused some inflammation in the lungs. In this experiment, we would look at the possibility of increasing the exposure time to elicit more inflammation in the lungs and see if the LPS will cause a higher response.

In the third set of experiments, we added another compound called BQ-123. BQ-123 has been proven to be a useful therapeutic agent against inflammation and lung injury. It can then be tested against cigarette smoke and LPS to see whether it will reduce the amount of inflammation occurring in the lungs and decrease the number of leukocytes. The dose of the BQ-123 is 200 ug in 0.1ml of saline. The smoking protocol has stayed the same to keep the consistency in the previous experiment.

In the fourth set of experiments, ET-1 was used exclusively and no LPS nor BQ-123 was used in this experiment. Since ET-1 is a powerful vasoconstrictor, a low dose of 5ug in 0.1 ml of saline is required for this experiment. The rationale of this experiment is to see whether ET-1 will cause an increase or decrease in inflammation after the hamsters were exposed to cigarette smoke.

In the fifth set of experiments, ET-1 and BQ-123 were used in this experiment. These two drugs will be combined with LPS to gauge the inflammatory response. This would determine whether BQ-123 or ET-1 can decrease the number of leukocytes or neutrophils arriving in the BALF. We would also be looking at the disease index of the

group to determine whether these drugs affect the thickness of the interstitium in the lungs and if these drugs can decrease the thickness.

1.10 Possible Outcomes from Experiments

There are possible outcomes that can be found in these experiments. An outcome is that the exposure protocol may be too short to cause any major inflammation or damage to the lung and may not affect them as much as we think. Another outcome is that the short exposure to cigarette smoke and the addition of LPS may have an increased effect of inflammation in the lungs and cause slight thickening in the interstitial walls.

In the experiment using BQ-123, the introduction of an antagonist may probably decrease or increase the immune response towards the LPs and the Cigarette smoke. It is also possible that the BQ-123 will not affect the immune response as this type of experiment is not a well-studied treatment method, specifically in the lung.

In experiments including the use of ET-1 and LPS, we might see an increase of leukocytes and inflammation in the lungs as it is well-known ET-1 is an important indicator for lung injury in the BALF and as “free ET-1”. Although due to ET-1 being a powerful vasoconstrictor, it may decrease the number of leukocytes entering the lung to respond to LPS and cigarette smoke.

1.11 Summation

The effects of acute secondhand smoking and bacterial infections are still unclear. This model will allow us to observe the effects and allow us to determine the relationship between secondhand smoking and bacterial infections. In order to simulate such an event,

our laboratory has created a hamster model that allows us to replicate secondhand smoking using a smoking machine and injecting LPS to cause a bacterial infection in the lungs. Compared to previous models, we will be using a minimum amount of LPS and using other agents to look for therapeutic agents to provide an alternative treatment to secondhand smoke inhalation and bacterial infections

1.12 Objectives, Hypothesis, Specific Aims

1.12.1 Objectives

From these discussions, the effects of acute cigarette exposure and its damage to the lungs are not well studied. The discussion that usually occurs when it comes to smoking tobacco products is usually for chronic damage that occurs in long-term smokers and those who are exposed to secondhand smoke for a long period. With these studies, we can look at whether short periods of secondhand smoking can predispose the lung to secondary injury.

The objective of this study was to explore the effects of short-term secondhand smoke exposure and the immune response from LPS treatment. This study also looks at a possible treatment involving an endothelin receptor antagonist, BQ123.

1.12.2 Hypotheses

- Second-hand smoke can cause short-term inflammation and can enhance secondary injury caused by LPS.
- The combination of smoke and LPS may cause damage to lung elastic fibers, resulting in airspace enlargement.
- The addition of BQ-123 can reduce the secondary inflammatory activity of LPS.

- The addition of ET-1 may increase the secondary inflammatory activity of LPS.

1.12.3 Specific aims

- To examine the effects of short-term, secondhand cigarette smoke and the effects of a simulated bacterial infection via LPS in a hamster model on various factors of inflammation such as lung histology, BAL neutrophils, desmosine levels, and neutrophil chemotaxis
- To observe the effects of BQ-123 on LPS and cigarette smoke as a possible treatment for smoke-related secondary lung injury
- To evaluate the effects of exogenous ET-1 on lung inflammation induced by cigarette smoke and LPS.

Aim 1: To test the first hypothesis, we needed to determine the effects of short-term secondhand cigarette smoke on LPS-induced infiltration of inflammatory cells into the lungs, the damage on the lung parenchymal, and the elastic fiber injury. These were evaluated by the following parameters.

a) Total leukocyte count in BALF
b) Percent neutrophil count in BALF
c) Total and free DID content in BALF
d) Average Thickness of interstitial walls

Rationale: The number of leukocytes and neutrophils in the BALF following the treatment of LPS and secondhand cigarette smoke demonstrates the extent of inflammation in the lungs that would help us understand the effects of a bacterial infection in the lungs after being exposed to cigarette smoke. The thickness of the

interstitial walls in the lung can help us understand the damage caused by both the cigarette smoke and the LPS. This may help explain the deterioration of lung function when two inflammatory agents are present in the lung.

Aim 2: To test the second hypothesis, the procedures for the smoke inhalations and injections are the same. However, we added an additional substance to test the efficacy of a possible treatment for the damage caused by both the bacterial infection and the cigarette smoke called BQ-123. It has been used previously in a study to find the efficacy of inhibiting cigarette smoke-induced endothelin receptor expression in pulmonary arteries. These were evaluated by the following parameters.

a) Total leukocyte count in BALF
b) Percent neutrophil count in BALF
c) Total and free DID content in BALF

Rationale: The reason for this experiment is to find treatments for cigarette-induced bacterial infections as smoking increases your risks of bacterial infections such as tuberculosis and pneumonia. By testing a substance with known properties of decreasing the damage of cigarette smoke and bacterial infections, we may be able to provide evidence that BQ-123 can be an effective treatment for such issues.

Aim 3: To test this hypothesis, the procedure for the smoke inhalation is the same but the procedure for the injections is different. We introduced a standalone substance called endothelin-1 or ET-1 This will be used to determine whether the introduction of an inflammatory mediator will increase the recruitment of leukocytes into the lung after exposure to cigarette smoke.

Rationale: The reason for such a treatment is that the lab has previously experimented with ET-1 to demonstrate that the introduction of ET-1 can causes preferential recruitment of neutrophils. In this experiment, we want to determine whether the introduction can reduce the inflammation and recruitment of neutrophils after being treated with cigarette smoke.

Chapter 2: Materials and Methods

2.1 Materials and Methods

2.1.1 Cigarette Source

The cigarettes were provided by the Center for Tobacco Reference Products (CTRP), part of the University of Kentucky, College of Agriculture. The specific cigarette we used is the 3R4F. The 3R4F Kentucky reference cigarettes have been widely used as monitor cigarettes for mainstream smoke analysis and in vitro and in vivo toxicological data of cigarettes and novel tobacco products. The 3R4F product is then shipped to the CTRP where the cases are placed in plastic bags and stored in a cold room at approximately 3.3°C and 50-60% relative humidity. This type of cigarette is the ideal product for the experimental model and has been used in different experiments such as studies for COPD and secondhand smoking.

These cigarettes will be used in the TE-10 Teague Enterprises smoke machine. It is a microprocessor-controlled cigarette smoking machine that produces either side-stream or mainstream smoke or a combination of the two from filtered, non-filtered, or standard size research cigarettes. One to ten cigarettes can be smoked at a time. Cigarettes are loaded into a magazine where they are automatically pushed into a wheel and lighted. The cigarettes are puffed according to the FTC method and automatically ejected after a certain number of puffs. Expended butts are extinguished in water. Smoke is captured in a chimney and transported to a collecting and mixing chamber for use in exposure studies. The size of the smoking chamber that the animals will be contained is 28 x 19 x 15in.

The average total smoke particulates are measured using an AME Volumetric dry gas meter combined with a timed filter sampler which has a diaphragm pump and a timer. The air mover is a linear pump with a maximum sample flow rate of 17 LPM. The outflow from the pump can be connected to a dry gas meter to measure volume sampled. A 0.45um EMD Millipore filter paper was used to weigh the smoke particles. The filter paper is first weighed before it is inserted into the in-line filter holder and the meter number is read before it is activated to vacuum the smoke into the filter paper. After 4 hours, turn off the meter, read the measurement on the meter, and remove the filter and weight the filter. To calculate the smoke particulate matter, take the difference of the gravimetric measurements before and after it is read and the weight of the filter paper before. Then divide the difference of the paper weight by the gravimetric difference. This is expressed in mg/m^3. The average total smoke particulates were $15.7mg/m^3$ at a SEM of 5.6.

2.1.2 Animals

Female Golden Syrian hamsters, weighing 90-100 grams, were purchased from Envigo. (Somerset, NJ) were used throughout the study. 2-3 animals were housed per cage with food and water available ad-libitum for at least 24 hours before any experimentation. All protocols were approved by St. John's University Institutional Animal Care and Use Committee.

2.2 Experimental Design

2.2.1 Preliminary Model

A preliminary experiment was conducted to check the efficacy of performing an intraperitoneal injection of LPS to induce inflammation in the lungs. The smoking procedure is exposing the animals to secondhand cigarette smoke for 2 hours per day for a period of 3 days. The animals were then injected with 200ug of LPS in 0.1ml saline and left to rest for 24 hours. They were then euthanized via CO2 asphyxiation.

2.2.2 Model 1 - LPS

In the first experiment, the smoking duration was increased from 2 hours to 4 hours to simulate the effects of acute secondhand smoke. The animals were divided into two groups, Group 1 was exposed to the smoke chamber and Group 2 was exposed to the room air in the Animal Care Center. After being exposed to the smoke and left to rest for 24 hours, all the groups will be injected with 200ug of LPS in 0.1ml NaCl solution and left to rest. They were then euthanized after 24 hours via CO_2 asphyxiation.

2.2.3 Model 2 - BQ-123

In the second experiment, the smoking protocol remained the same, but the injection protocols were modified. We decided to test the efficacy of an antagonist called BQ-123 to look at it as a possible treatment for smoke-induced emphysema. Group 1 was exposed to cigarette smoke while group 2 was exposed to room air. After they were exposed and were left to rest for 24 hours, Group 1 was injected with 200ug of BQ-123 in 0.1ml of saline. The animals were left to rest for 30 minutes and then all groups were injected again with LPS. They were then euthanized after 24 hours via CO_2 asphyxiation.

2.2.4 Model 3 - ET-1

In the third experiment involving ET-1, four groups were created: Group 1 was exposed to room air, Group 2 was exposed to cigarette smoke, group 3 was exposed to

smoke and injected with ET-1, and group 4 was exposed to room air and injected with ET-1. The dose of ET-1 will be 10ug in 0.1ml of saline. They will then be euthanized after 24 hours via CO_2 asphyxiation.

2.2.5 Model 4 – BQ-123, ET-1, and LPS

In the fourth experiment, it involves the use of all three substances. Four groups were created: Group 1 were exposed to cigarette smoke, injected with BQ123, and then injected with LPS 30 minutes later; Group 2 were exposed to cigarette smoke, injected with LPS, and then injected with ET-1 30 minutes later; Group 3 were exposed to cigarette smoke and injected with LPS; Group 4 were exposed to room air and injected with PBS. They were all euthanized after 24 hours via CO_2 asphyxiation.

A total of 120 animals were used in both sets of experiments. All procedures involved in these studies were reviewed and approved by the IACUC at St. John's University.

2.3 Extraction of Bronchoalveolar Lavage Fluid and Lung Samples

All animals used in this study were euthanized by CO_2 asphyxiation. The lower thoracic area of the animals was cleansed with 70% alcohol and surgically exposed to cut the diaphragm, ensuring complete lung collapse. An incision was made in the cervical area and two muscle layers (cleidomastoideus and digastricus) were cut to expose the trachea. A 16-gauge needle was inserted into the trachea (BD Precision Glide Needle) attached to a 5ml syringe. BALF was obtained by injecting and withdrawing phosphate-buffered saline (PBS) 3 times at 4ml, 3ml, and 3ml volumes to reach a final amount of 10ml for each animal. The extraction of BALF is done slowly to prevent damage to the

neutrophils from the suction of the syringe. After lavage, lungs were removed from the chest and separated from extrapulmonary structures, then stored at -20 degrees Celsius for further studies. BALF photomicrographs were taken using a Nikon Eclipse Ts2R inverted research microscope at a magnification of 200x. The software used to take the photos is a NIS-Elements Viewer.

2.4 Microscopic Studies

2.4.1 Histological Evaluation of the Lungs

After euthanasia, the lungs were fixed using 10% neutral-buffered formalin for 2 hours at a pressure of 20 cm of mercury, then removed from the chest and immersed in a centrifugal tube filled with formalin for 48 hours. The extrapulmonary structures were removed from the lungs and the samples were cut into random pieces and placed in cassettes for histological processing. The process of creating the blocks is done by using an STP 120 from Thermo Fisher. The machine is programmed to create the blocks by dipping them in solvents and preservatives. The cassettes were passed through graded concentrations of ethanol, ranging from 35%, 70%, 95%, and 100%, for 5-minute intervals, followed by repeated immersion in clean 100% xylene for 15, 25, and 40 minutes. The samples were then sequentially incubated in xylene-paraffin mixture (1:1) for 1 hour, 100% paraffin for 1 hour, and fresh 100% paraffin for 2 hours. They were then embedded in blocks of paraffin wax, using a Leica 1160 tissue-embedding machine. The resulting tissue blocks were stored in a refrigerator before processing with a microtome. Tissue sections were cut with a standard rotary microtome (American Optical Company) at a thickness of 5um, placed gently in a water bath at 35 degrees Celsius, then

placed carefully on a Silane™ coated slides, and incubated at 55 degrees Celsius overnight for future analysis.

2.4.2 Hematoxylin and Eosin Staining (H&E)

After the tissue sections were dried of all moisture, they were taken for staining. The tissue sections were deparaffinized in three changes of xylene for 5 minutes in each staining jar, then they were rehydrated in decreasing concentrations of ethanol (ETOH) from 100%, 95%, 70%, and 30% at 2-minute intervals. The sections were then washed in distilled water for 1 minute and stained with hematoxylin for 10 minutes. They were then immersed in distilled water for 2 minutes, then in a solution mixture of ammonium chloride (NH_4CL) and ETOH for 1 minute, washed in tap and distilled water for 1 minute each, and dehydrated in 70% ETOH for 2 minutes. The sections were then counterstained in eosin solution for 2 minutes and then dehydrated in alcohol as follows: 95% ETOH for 2 minutes and 3 changes of 100% ETOH for 2 minutes each. Finally, sections were cleared in 3 changes of xylene 2 minutes each mounting with coverslips using Permount.

Following euthanasia, the lungs were fixed in situ for several hours with 10% neutral-buffered formalin at a pressure of 20cm H2O. The lungs were removed from the thorax as a single block and fixed in formalin. After removing the extrapulmonary structures, the lungs were cut into random pieces and processed for histological studies. The solvents used are xylene, ethanol, formaldehyde, and paraffin. The blocks are then filled with paraffin after the tissue processor has completed its run. Slide sections were cut at 0.5um using an American Optical Company Microtome with Accublade microtome blades. The sections are then placed on a hot water bath and taken in by a Silane™-coated slide. The slides are then placed on a heating pad to have the water evaporated

from the slides. After the slides are dry, the sections are stained in hematoxylin and eosin. The thickness of the interstitial walls was determined by a score from 1-5. Tissue sections were graded for injury (inflammatory index), using the following scale based on previously published criteria: 0 = no reaction in alveolar walls; 1 = diffuse reaction in alveolar walls, with no thickening of the interstitium; 2 = diffuse presence of inflammatory cells in alveolar walls with a slight thickening of the interstitium; 3 = moderate interstitial thickening accompanied by inflammatory cell infiltrates; 4 = interstitial thickening involving more than half of the microscopic field. Results were expressed as an average of 25 microscopic fields. This type of scoring has been used previously to evaluate the interstitial damage to the lung and observe inflammatory cell infiltrates (Patel et al., 2014). The purpose of such analysis is that exposure to cigarette smoke and intraperitoneal injection of LPS can increase the thickness of the interstitial walls and the accumulation of various leukocytes.

2.4.3 Mean Linear Intercept of the Lung Airspace Diameter

Another measurement was conducted on the lung sections by doing the mean linear intercept of the lung airspace diameters. This was done using ImageJ in which horizontal lines were displayed across each lung section image. For each line, the points where the line intersects with an alveolar wall are counted, skipping every other line for a total of 4 lines per image. After they are counted, divide the measured length of the image by the average numbers of intersecting points per line. This will give the average distance between the alveolar walls. The pictures were taken and measured at a magnification of 100x (Thurlbeck, 1967). This measurement was conducted to determine whether the

combination of second-hand smoke exposure and secondary inflammation in the lung can cause enlargement of the lung airspace.

2.5 Analysis of BALF

Total leukocyte counts were determined using a hemocytometer (Hausser Scientific, Horsham, PA) at a magnification of 400x. The BALF was then centrifuged for 3 minutes at 5 shelves, the supernatant was removed and stored at -20 degrees Celsius for further analysis. Differential cell counts were performed by resuspension of the pellet in 1ml PBS, followed by plating of the cells onto microscope slides with a cytospin (Shandon Inc., Pittsburgh, USA) for 7 minutes at a speed of 800 rpm. The slides were air-dried, fixed in methanol for 2 minutes, treated with Giemsa-Wright stain at a ratio of 3:2, and a coverslip was added using Cytoseal. At least 200 cells were identified using standard morphological criteria to determine percent neutrophil in BALF.

2.6 Measurement of BALF Isodesmosine and Desmosine

For measurement of free DID, aliquots of cell-free lavage fluid were concentrated by evaporation to a volume of 0.5ml and combined with 10% trifluoroacetic acid to precipitate proteins, which were discarded after centrifugation. The supernatants were mixed with a solution of butanol, glacial acetic acid, and water (4:1:1), then applied to a CF1 column to separate the free DID.

To determine total DID, aliquots of cell-free lavage fluid were mixed with 37% HCl and hydrolyzed at 110°C for 24 hours. The samples were then dried, suspended in butanol, glacial acetic acid, and water (4:1:1), and filtered through a CF1 column to isolate DID.

Separation of the crosslinks was performed on a 2x100mm dC18 column (waters Corporation, Milford, MA) using 2 mobile phases: (A) 7mM heptafluorobutyric acid (HFBA) and 5mM ammonium acetate in 80% acetonitrile. Following elution with a programmed gradient, the crosslinks were analyzed with a TSQ discovery electrospray tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA), using selected reaction monitoring of mass to charge ratio transitions. A d4-labeled desmosine standard was used to quantify the results.

Another method, utilizing LC-MS-MS, was also used to determine free desmosine in BALF and whole lungs. The lungs were first weighed and added with an appropriate volume of ultra-pure water to produce a 1:2 dilution (e.g., 2 mL of water to 2 g of the lung). The samples were then homogenized using a Brinkmann Homogenizer. 1 mL of the resultant homogenate was directly aliquoted into a separate tube and fortified with desmosine-D4. 1 mL of BALF samples were directly aliquoted and fortified with desmosine-D4.

Both desmosine and desmosine-D4 were extracted from lung and BALF samples by cationic exchange solid phase extraction (SPE). The cation exchange sorbent serves to retain desmosine and desmosine-D4. First, 3 mL of 0.1N HCl (pH ~ 1.0) was added to 1 mL BALF or 1 mL of lung homogenate, followed by a vortex and centrifugation for 10 min at 3000 rpm prior to column loading. The SPE cartridges were conditioned with 4 mL of methanol followed by 3 mL of 0.1N HCl. Subsequently, the samples (3 mL of the pre-added 0.1N HCl and 1 mL of BALF or lung homogenate containing desmosine and desmosine-D4) were loaded into each designated pre-conditioned column and were allowed to drip with gravity flow. A series of wash steps were performed on each column

in the order of 3 mL of 0.1N HCl and 4 mL of methanol. The columns were then allowed to dry at full vacuum for 30 seconds. Elution of desmosine and desmosine-D4 was utilized with 3 mL of a solvent prepared fresh as a mixture of 95% methanol and 5% concentrated ammonium hydroxide. Eluents were evaporated to dryness under vacuum using a Rotovap, and the residues were reconstituted in 200 µL of a 95:5 mixture of water and methanol and injected into the chromatographic system.

2.7 Chemotaxis Assay

BALF leukocytes from untreated animals were centrifuged and suspended in Dulbecco's culture medium. They were gently mixed to ensure uniformity and placed in the upper wells of Boyden chambers with a 3 µm pore size membrane (Sigma-Aldrich, St Louis, MO) and exposed to either LPS or no LPS. The lower wells were filled with additional media to cover the base of the upper wells. The same volume of media was used for each well. Additional chambers only contained culture medium in both upper and lower wells and were used for background measurements. Following incubation at 37 degrees Celsius for 24 hours, the cells were carefully removed from the top side of the insert. The contents of the lower wells were gently mixed to evenly distribute the cells. Cell migration through the membrane was determined with a CyQuant® cell assay kit (Thermo Fisher, Waltham, MA) which uses a fluorescent dye that binds to DNA. The treated cells were transferred to a 96-well microplate and a combination of fluorescent dye solution and lysis buffer was added to each well. The samples were incubated at room temperature for 15 minutes and fluorescence emission was measured with a fluorescent plate reader using a 480/520 nm filter set.

2.8 Statistical Analysis

One-way analysis of variance (ANOVA) and Bonferroni post-hoc tests were used to determine statistically significant differences among three or more treatment groups. When only two groups were compared, a two-tailed t-test was utilized. A p-value of less than 0.05 was considered statistically significant. Results were expressed as mean ± standard error of the mean (SEM).

CHAPTER 3: RESULTS

3.1 Model 1 – The effect of smoke on LPS-induced Inflammation

Syrian Golden hamsters were exposed to cigarette smoke for 4 hours per day over a 3-day period, then given IP LPS 24 hours later. Bronchoalveolar lavage was performed 24 hours after LPS administration. As shown in Figure 1, animals treated with smoke and LPS showed a significant increase in percent BALF neutrophils compared to those given LPS alone (15.0 vs 5.1 percent, respectively, $p<0.01$). In contrast, the total BALF leukocytes did not show a significant difference between the groups (Figure 2). As shown in Figure 3, animals treated with LPS and smoke had a significantly higher disease index and showed thicker interstitial walls and more intense neutrophil infiltrates compared to LPS alone (1.6 vs 0.9, respectively; $p<0.05$).

BALF DID was also measured 24 hrs after LPS administration. The amount of free and total BALF desmosine was not significantly greater in the animals treated with both smoke and LPS compared to those given LPS alone (Fig. 5, Fig. 6). Furthermore, there was no correlation between percent neutrophils and either total or free BALF desmosine (Figures 8 and 9). However, as shown in Figure 7, there was a significant correlation between free BALF desmosine and total BALF desmosine, indicating that free BALF DID responds to inflammatory injury in the same manner as total BALD DID, and may act as a surrogate for total BALF DID.

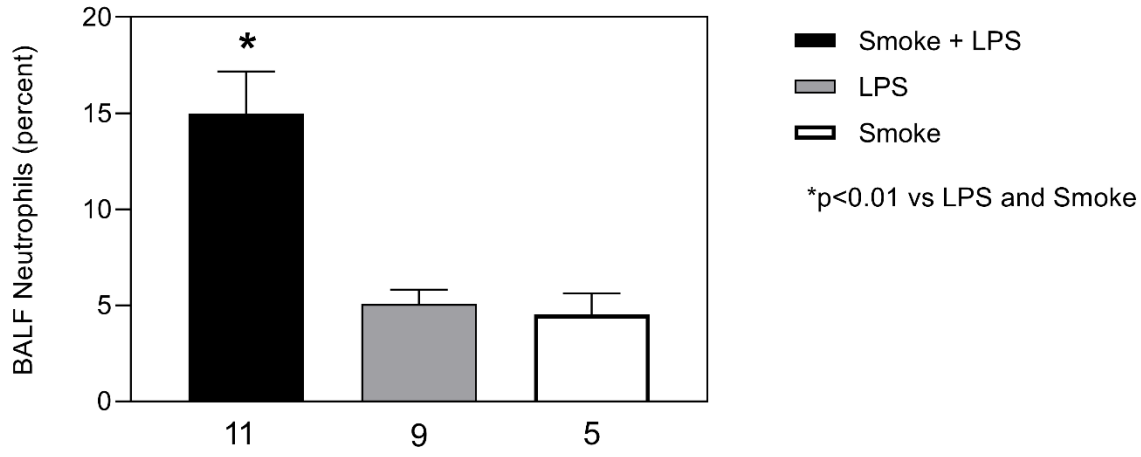

Figure 1. Percent BALF Neutrophils Content in Smoked Only Hamsters. Graph showing the percent BALF neutrophil content. The animals were treated with either smoke or room air, then given IP LPS 24 hours later and euthanized 24 hours after LPS treatment. The group receiving smoke + LPS had significantly increased BALF neutrophils compare to the other groups (one-way ANOVA with Bonferroni post-test). The results were expressed as mean ± SEM ($p=0.0003$). The numbers under each bar represent the sample size (N).

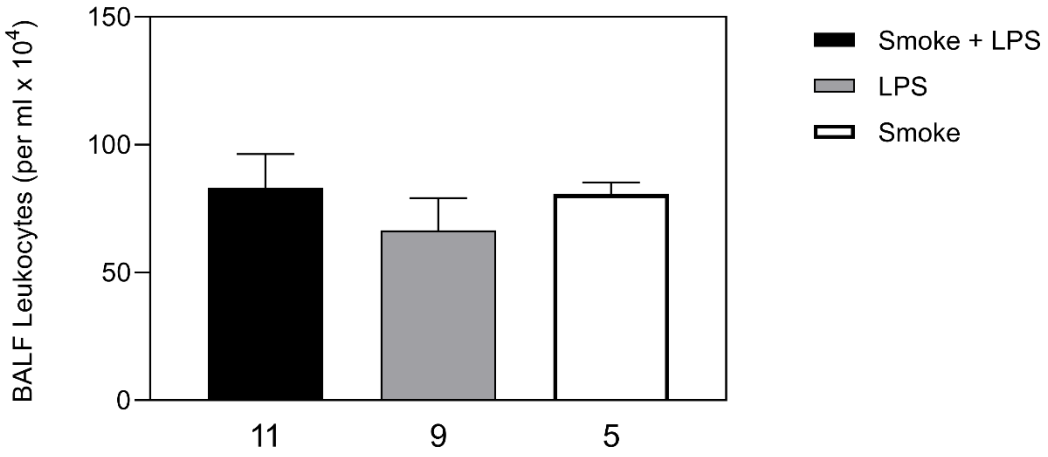

Figure 2. Total BALF Leukocyte Count in Smoked Only Animals. Graph showing the total BALF leukocyte counts. The animals were treated with either smoke or room air, then given IP LPS 24 hours later and euthanized one day later. There were no statistically significant differences among the groups. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

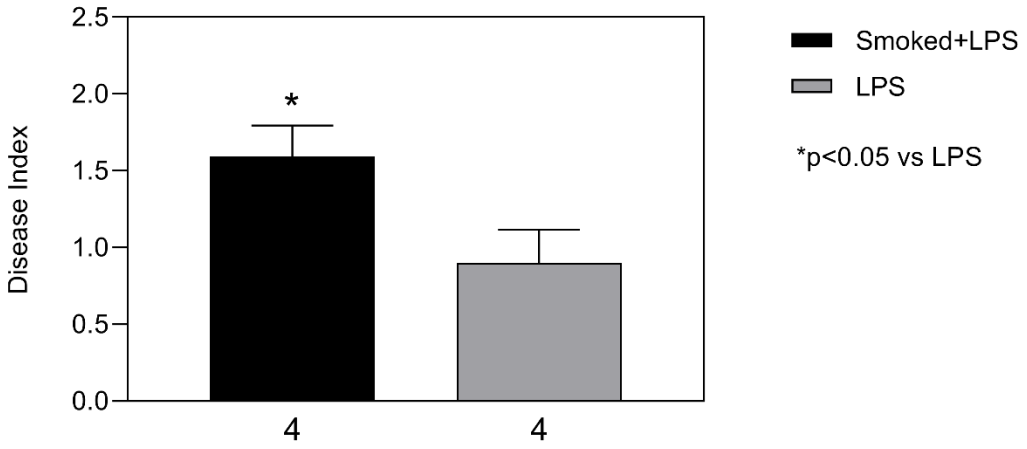

Figure 3. Disease Index of Smoked and Room Air Animals. Graph showing the disease index of both smoked and control animals. Animals treated with smoke and LPS had a significantly higher index than those remaining in room air prior to LPS. The animals were exposed to either smoke or room air, then given IP LPS 24 hours later. The animals were euthanized 24 hours after LPS treatment. The results were expressed as mean ± SEM. Statistical analysis was performed with the two-tailed Mann Whitney test ($p=0.0561$). The numbers under each bar represent the sample size (N).

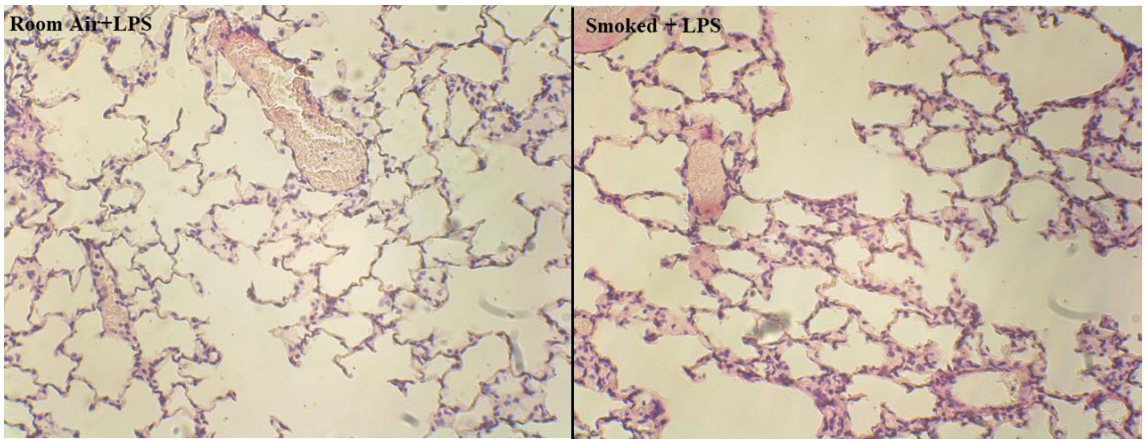

Figure 4. Room Air and Smoke Lung Histology. Photomicrograph of paraffin-embedded sections from hamster lungs stained with hematoxylin and eosin. The left photomicrograph represents the lung of an animal exposed to only room air and injected with IP LPS. The right photomicrograph represents the lung of an animal exposed to only cigarette smoke for 4 hours per day for 3 days. The right photograph shows significantly thicker interstitium walls and more infiltrates compared to the left photograph. The animals were euthanized 24 hours after IP treatment. The photomicrographs were taken at 100x magnification.

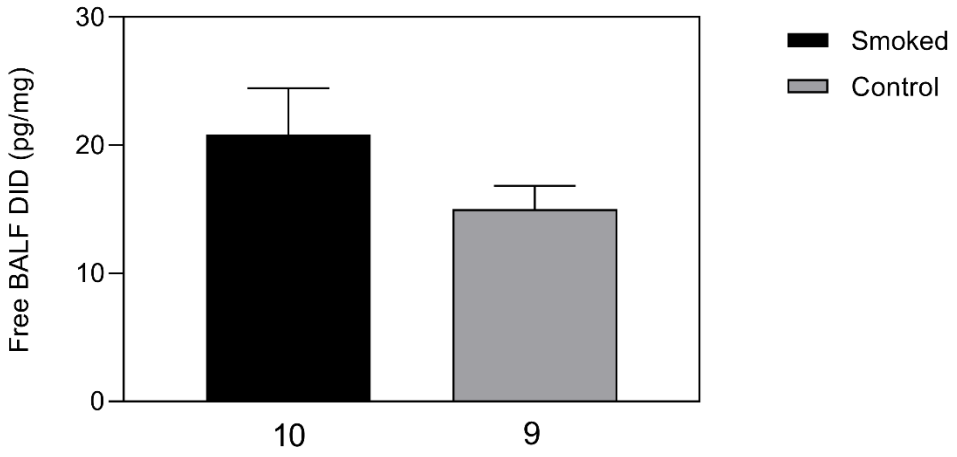

Figure 5. Total amount of Free BALF DID in Smoke and Control. Graph showing the total amount of free BALF DID in both Smoked and Control. There was no significant difference between the two groups. The animals were exposed to either smoke or room air, then treated with IP LPS. There was no statistically significant difference between the groups (two-tailed t-test). The animals were euthanized 24 hours after IP treatment. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

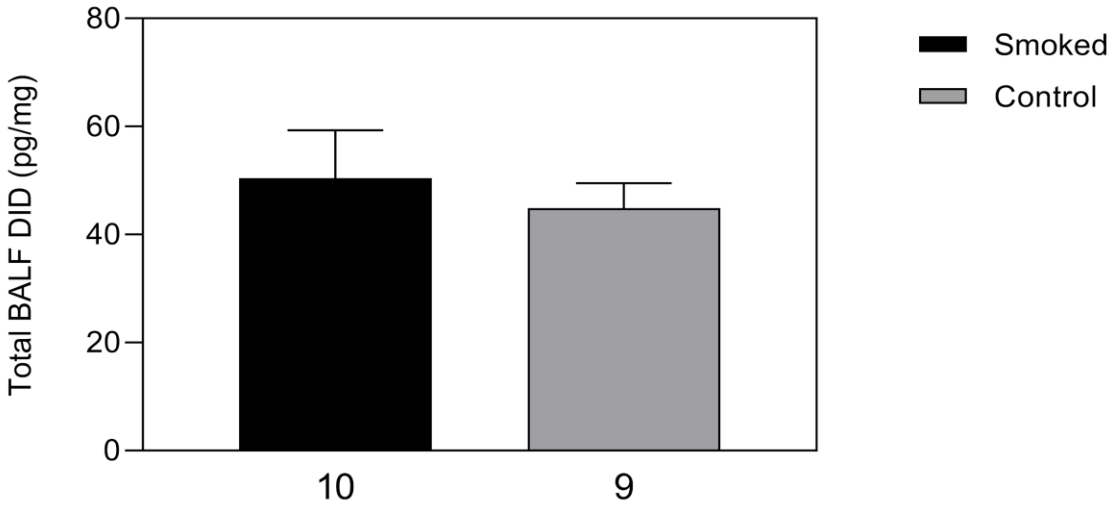

Figure 6. Total amount of Total BALF DID in Smoke and Control. Graph showing the total amount of total BALF DID in both Smoked and Control. There was no significant difference between the two groups. The animals were exposed to either smoke or room air, then treated with IP LPS. There was no statistically significant difference between the groups (two-tailed t-test). The animals were euthanized 24 hours after IP treatment. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

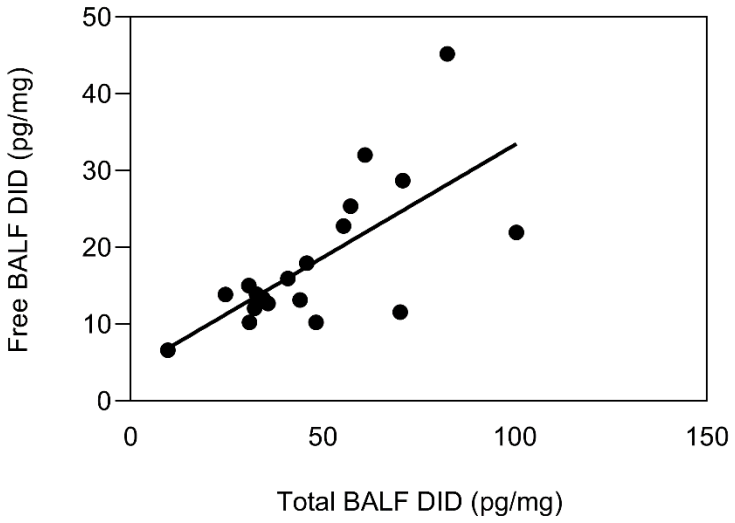

Figure 7. Comparison of BALF Free DID and Total DID. Graph showing significant correlation between free BALF DID and total BALF DID (r^2=0.4728, $p<0.01$). The animals were exposed to either smoke or room air, then treated with IP LPS. The animals were euthanized 24 hours after IP treatment. The results were expressed as a simple linear regression at 95% confidence level (p=0.0011).

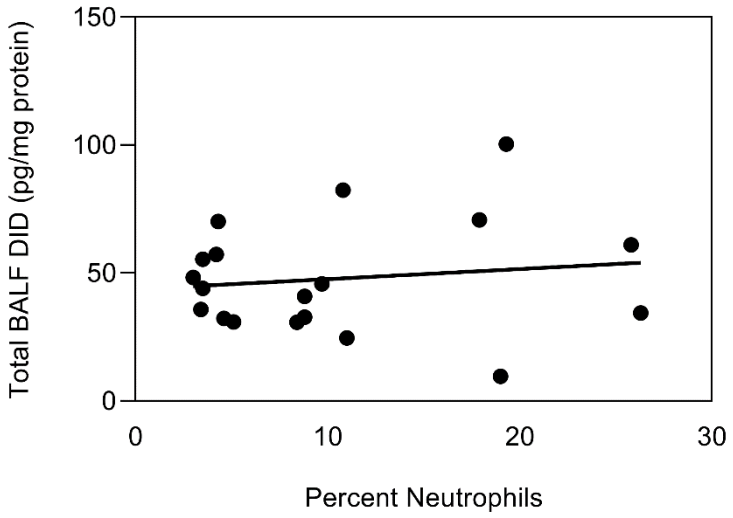

Figure 8. Linear Regression of Percent Neutrophils and Total BALF DID. Graph showing the relationship between Total BALF DID and percent BALF neutrophils. There was no significant correlation between these parameters (r^2= 0.02, $p<0.05$) The animals were exposed to either smoke or room air, then treated with IP LPS. The results were expressed as a simple linear regression at 95% confidence level (N of 19 on both X and Y axis, p=0.5775).

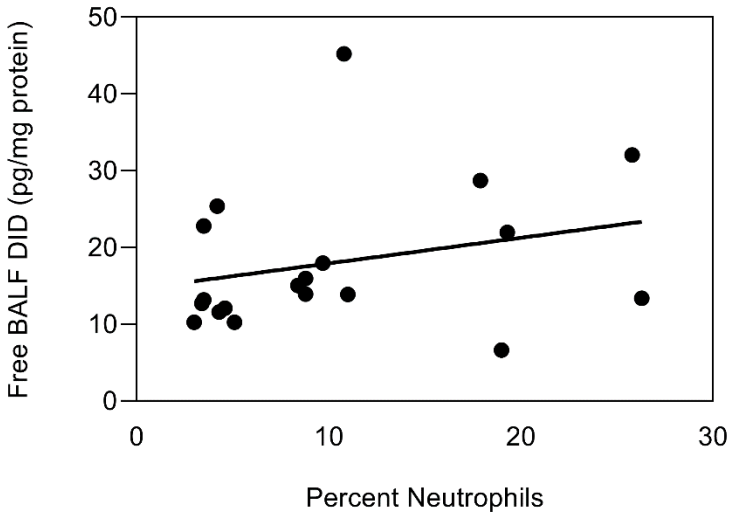

Figure 9. Linear Regression of Percent Neutrophils and Free BALF DID. Graph showing the relationship between free BALF DID and percent neutrophils. There was no significant correlation between these parameters (r^2=0.07, $p<0.05$). The animals were exposed to either smoke or room air, then treated with IP LPS. The results were expressed as a simple linear regression at 95% confidence level (N of 19 on both X and Y axis, p=0.2634).

3.2 Model 2 – The effect of BQ-123 on smoke-enhanced LPS Inflammation

An endothelin receptor inhibitor was utilized to determine the potential role of endothelin in smoke-enhanced LPS-induced inflammation. As shown in Figure 11, the hamsters were exposed to cigarette smoke for 4 hours per day over a 3-day period, then treated with IP BQ123 prior to LPS administration, there was a significant decrease in percent BALF neutrophils with animals given BQ123 compared to LPS alone. (8.5 vs 15.8 percent, respectively, $p<0.05$). However, there was no significant difference in the BALF leukocytes between the groups (Fig. 10).

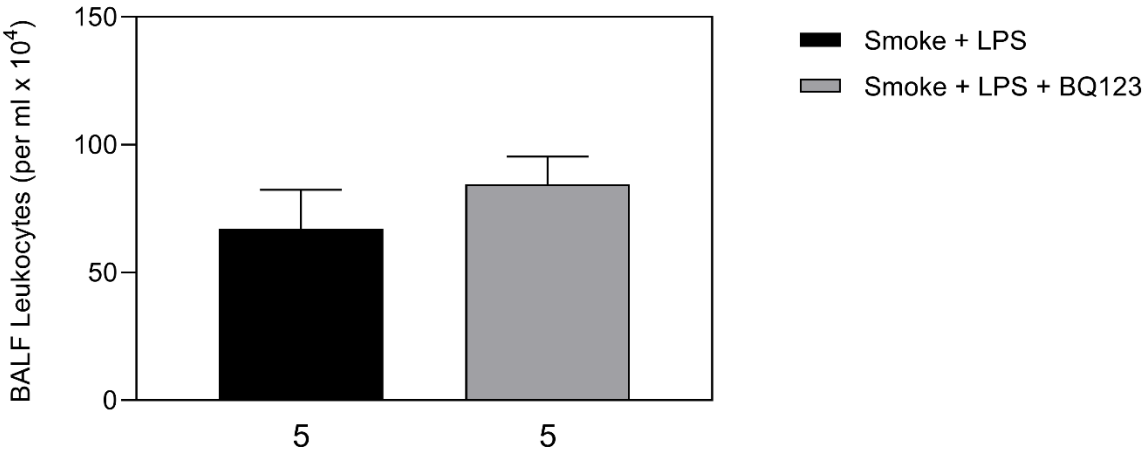

Figure 10. BALF Leukocytes for Both LPS and BQ123. Graph showing the total BALF leukocyte counts. The animals were exposed to smoke and treated 24 hours later with either IP LPS or IP BQ-123 and IP LPS 30 minutes later. All animals were euthanized 24 hours after IP treatment. There was no statistically significant difference between the groups (two-tailed t-test). The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

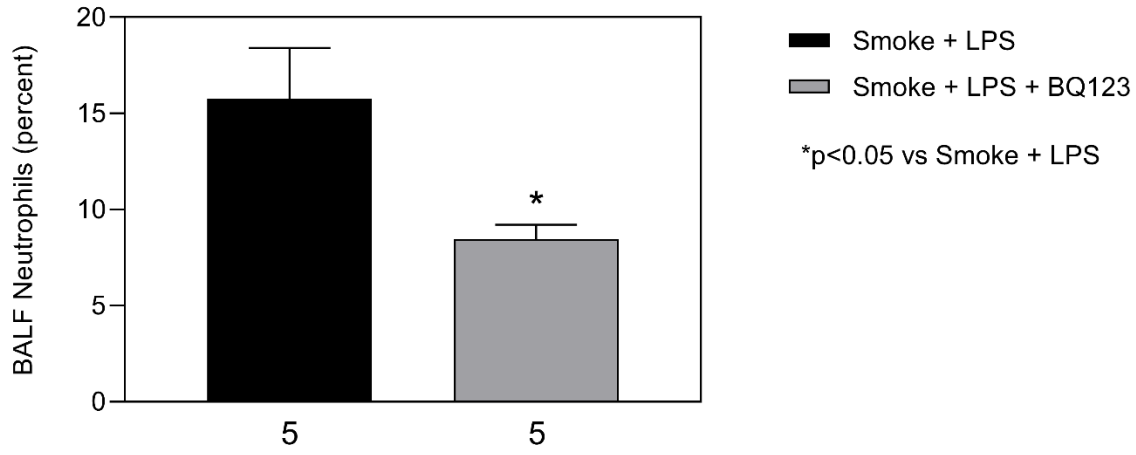

Figure 11. BALF Neutrophils for both LPS and BQ123. Graph showing the percent neutrophil content. The animals were exposed to smoke and treated 24 hours later with either IP LPS or IP BQ-123 and IP LPS 30 minutes later. All animals were euthanized 24 hours after IP treatment. There was a statistically significant difference between the groups ($p<0.05$; two-tailed t-test). The results were expressed as mean ± SEM ($p=0.0289$). The numbers under each bar represent the sample size (N).

3.3 Model 3 – The effect of ET-1 on smoke-enhanced Inflammation

The use of exogenous administration of ET-1 was utilized to show whether ET-1 can influence the number of neutrophils entering the lungs. The hamsters were exposed to cigarette smoke for 4 hours per day over a 3-day period, then the animals were treated with IP ET-1. Another group of hamsters was exposed to room air only, then given IP ET-1. As shown in Figure 12, total BALF leukocytes were significantly decreased in smoke exposed animals receiving ET-1 compared to animals only treated with smoke (23.5 vs 60.4 x 10^4, $p<0.01$). There was also a significant decrease in BALF leukocytes in room air exposed animals given ET-1 compared to those left untreated (26.2 vs 67.0 x 10^4, $p<0.001$). As shown in Figure 13, there was a significant increase in percent neutrophils in the room air/ET-1 group compared to room air only controls (12.5 vs 5.2, respectively, $p<0.01$). These findings suggest that ET-1 may preferentially increase neutrophil migration from the vascular compartment while suppressing overall leukocyte diapedesis.

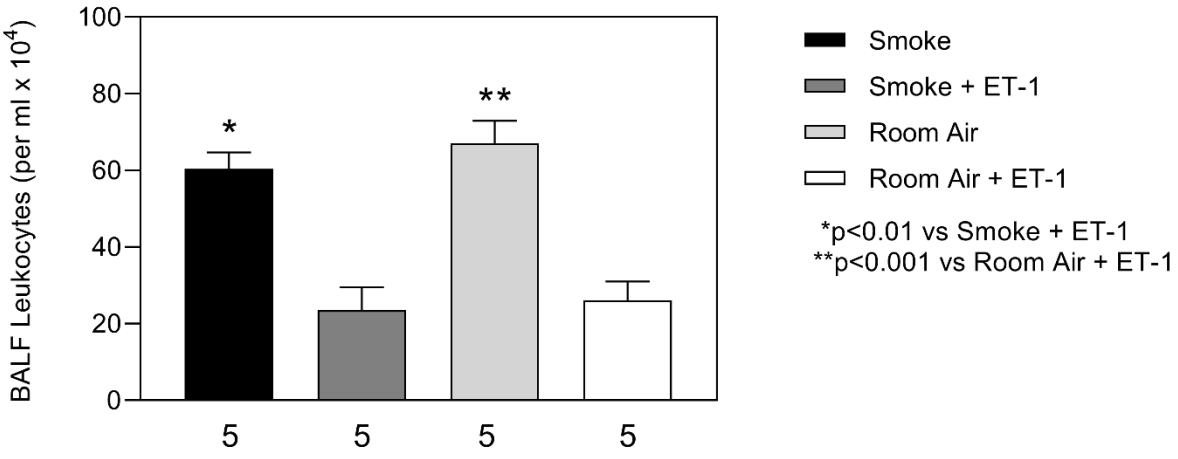

Figure 12. Total Leukocyte Content for ET-1 with Smoked and Room Air Animals. Graph showing BALF leukocyte content. All animals were exposed to cigarette smoke or room air prior to the IP injection protocol. The animals were treated with either IP ET-1 or PBS 24 hours post-smoke exposure and euthanized one day later. For both smoke and room air-exposed animals, ET-1 significantly decreased BALF leukocytes and compared using one-way ANOVA with Bonferroni post-test. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N). The numbers under each bar represent the sample size (N).

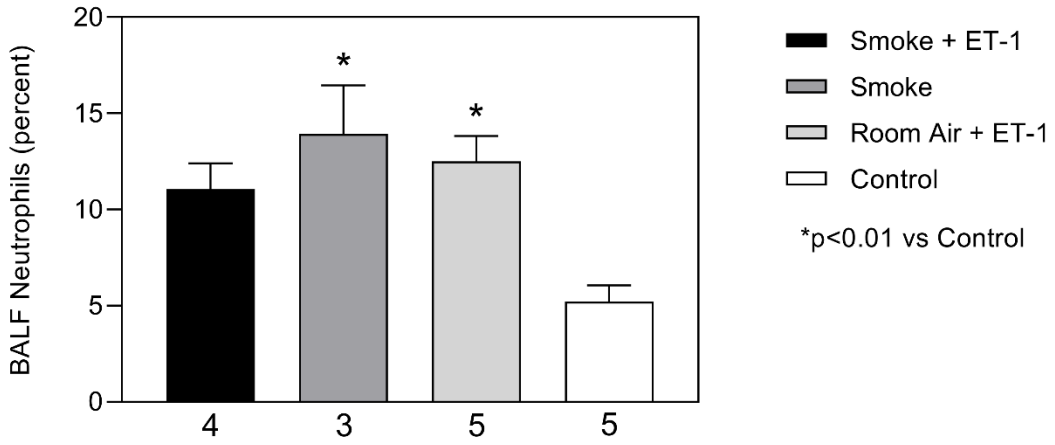

Figure 13. Percent Neutrophils Content for ET-1 with Smoked and Room Air Animals. Graph showing BALF neutrophil content. All animals were exposed to cigarette smoke or room air prior to the IP injection protocol. The animals were treated with either IP ET-1 or PBS at 24 hours post-smoke exposure and euthanized one day later. Both the smoke + ET-1 and ET-1 only groups had significantly increased BALF neutrophils compared to controls exposed to room air and injected with PBS and compared using one-way ANOVA with Bonferroni post-test. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

3.4 Model 4 – The effect of ET-1 and BQ-123 on smoke-enhanced LPS Inflammation

Both ET-1 and BQ-123 were used to look at their effect on LPS-enhanced inflammation in smoke-exposed animals. As shown in Figure 15, there was a significant increase in percent BALF neutrophils in the Smoke+LPS group compared to room air controls (9.3 vs 1.5 percent, respectively; $p<0.01$), which was abrogated by both BQ123 and ET-1. Similarly, as show in Figure 14, there was a significant increase in total BALF leukocytes in the Smoke+LPS group compared to room air controls, which was reversed with ET-1 and BQ123 (84.40 vs 19.85 vs 25.95 x 10^4, respectively; $p<0.0001$). These changes in BALF neutrophil populations are shown in Figure 16.

Animals treated with Smoke + LPS and Smoke + LPS + ET-1 had a significantly higher disease index than room air controls that were not treated with these agents (1.8 and 1.9 vs 0.8, respectively; $p<0.0001$; Figures 17 and 18). These findings suggest that ET-1 may cause thickening of the alveolar wall and increases in the disease index, despite limiting the influx of leukocytes into the lung. Such an effect is possible if ET-1 induced interstitial edema by enhancing vascular permeability.

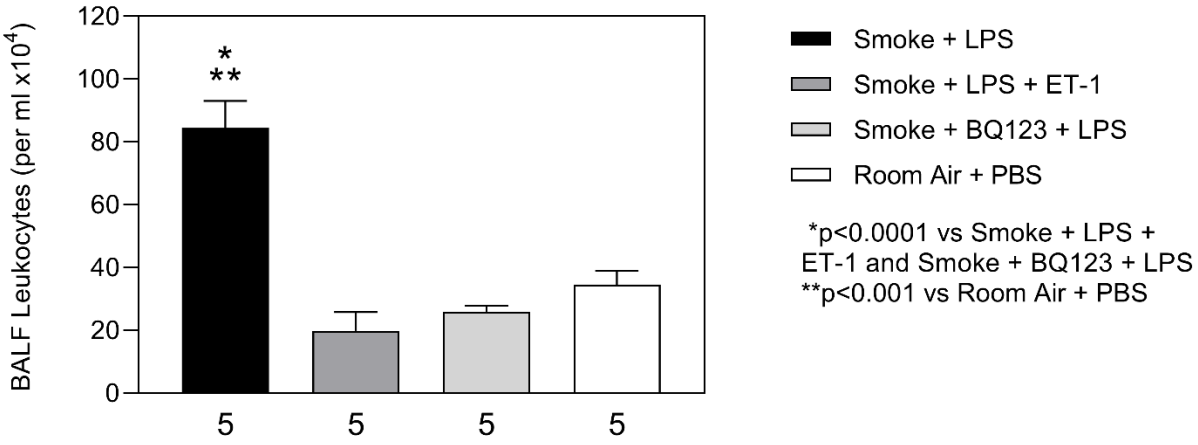

Figure 14. Total Leukocyte Content for LPS, ET-1 and BQ-123. Graph showing BALF leukocyte content. All animals were exposed to cigarette smoke or room air prior, then given IP injections of either BQ123 or ET-1 at 24 hours post-smoke exposure. Animals receiving BQ123 were first treated with this agent and then given LPS 30 minutes later. Animals receiving ET-1 were first treated with LPS and then treated with ET-1 30 minutes later. All animals were euthanized 24 hours after IP treatment. The group receiving smoke + LPS had significantly increased BALF leukocytes compared to all other groups using one-way ANOVA with Bonferroni post-test. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

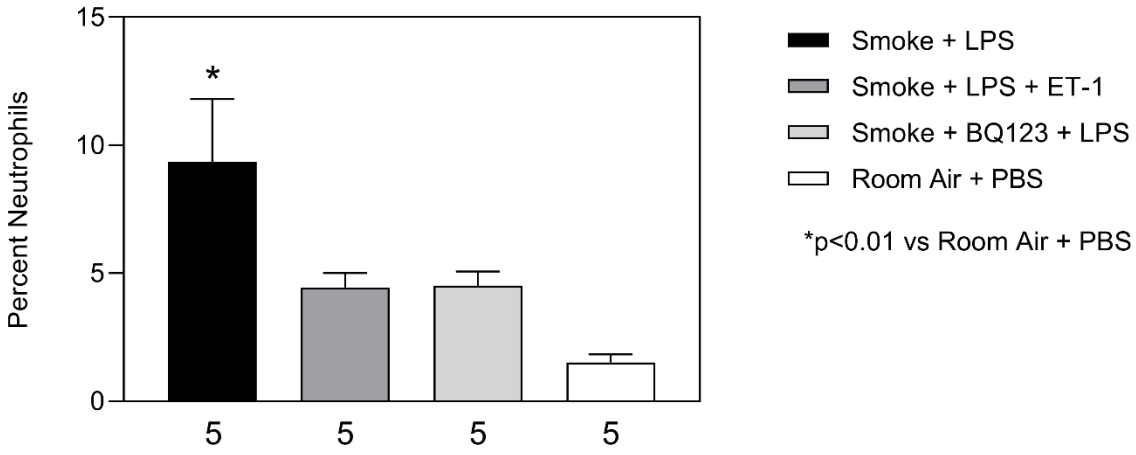

Figure 15. Percent Neutrophil Content for LPS, ET-1 and BQ123. Graph showing BALF neutrophil content. All animals were exposed to cigarette smoke or room air prior, then given IP injections of either BQ123 or ET-1 at 24 hours post-smoke exposure. Animals receiving BQ123 were first treated with this agent and then given LPS 30 minutes later. Animals receiving ET-1 were first treated with LPS and then treated with ET-1 30 minutes later. All animals were euthanized 24 hours after IP treatment. The group receiving smoke + LPS had significantly increased BALF neutrophils compared to the room air + PBS group and compared using one-way ANOVA with Bonferroni post-test. The results were expressed as mean ± SEM. The numbers under each bar represent the sample size (N).

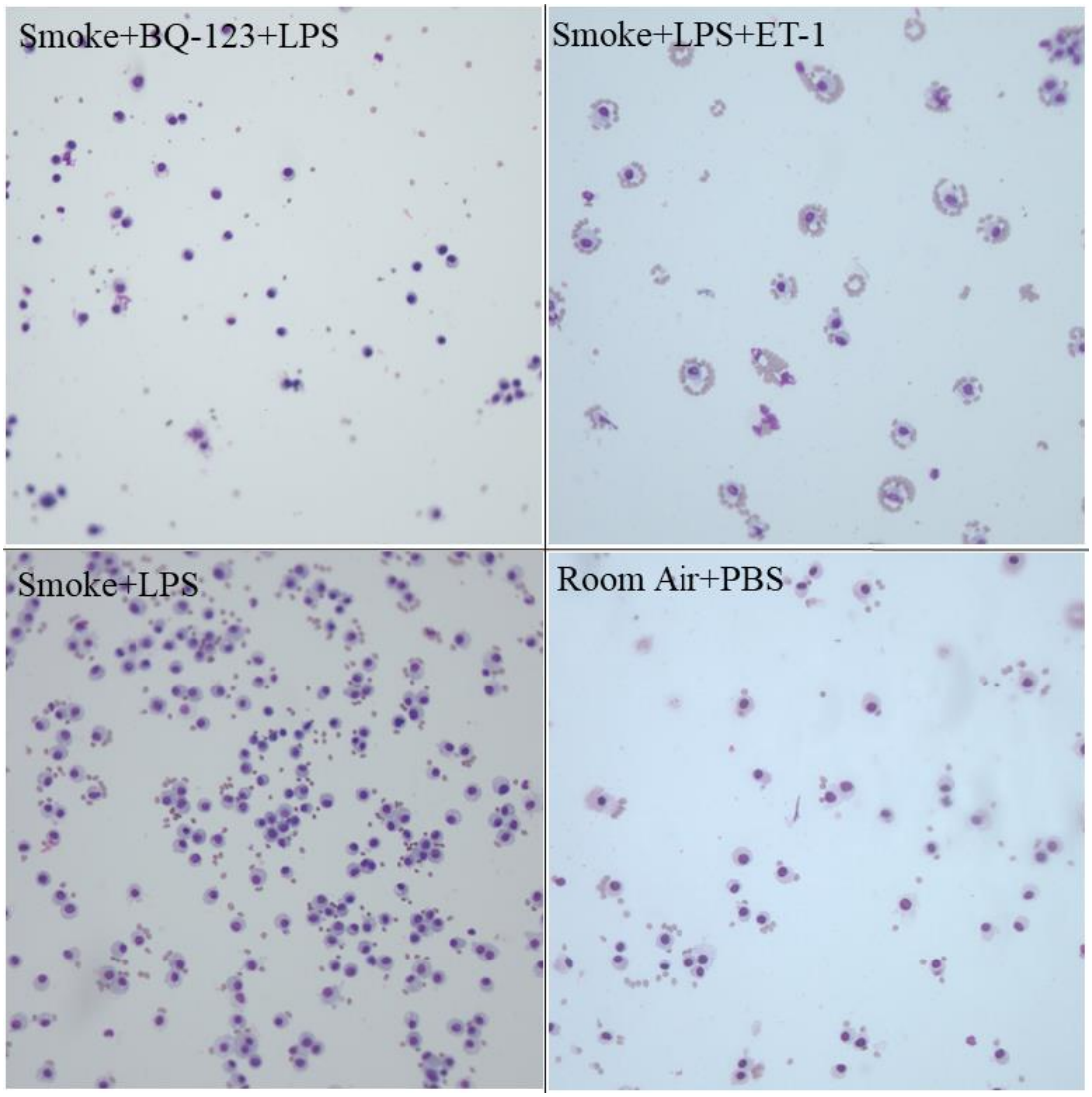

Figure 16. BALF Photomicrographs – BQ-123, ET-1, LPS, and PBS.

Photomicrographs showing neutrophil content with animals exposed to BQ-123, LPS, and ET-1 taken at 200x. On the top left, there is significantly less neutrophils when BQ-123 is used compared to LPS. On the top right, there is significantly less neutrophils when ET-1 is used compared to LPS. The groups involving BQ-123, LPS, and ET-1 were exposed to smoke and the control was only exposed to room air.

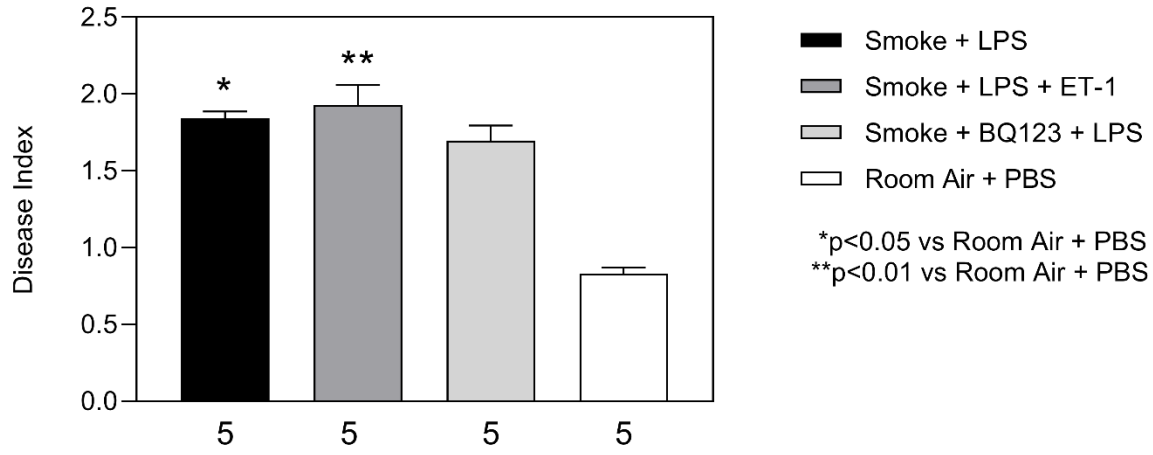

Figure 17. Disease Index of ET-1, BQ-123, and LPS with Smoked animals. Graph showing the disease index of LPS, ET-1+LPS, BQ123+LPS and Room Air+PBS. Animals treated with smoke and treated with IP LPS+ET-1 and LPS had a significantly higher index than those remaining in room air prior to LPS ($p<0.0001$). The animals were exposed to either smoke or room air, then given IP LPS 24 hours later. The animals were euthanized 24 hours after LPS treatment. Statistical analysis was performed with a one-way ANOVA test. The results were expressed as mean ± SEM ($p<0.0001$). The numbers under each bar represent the sample size (N).

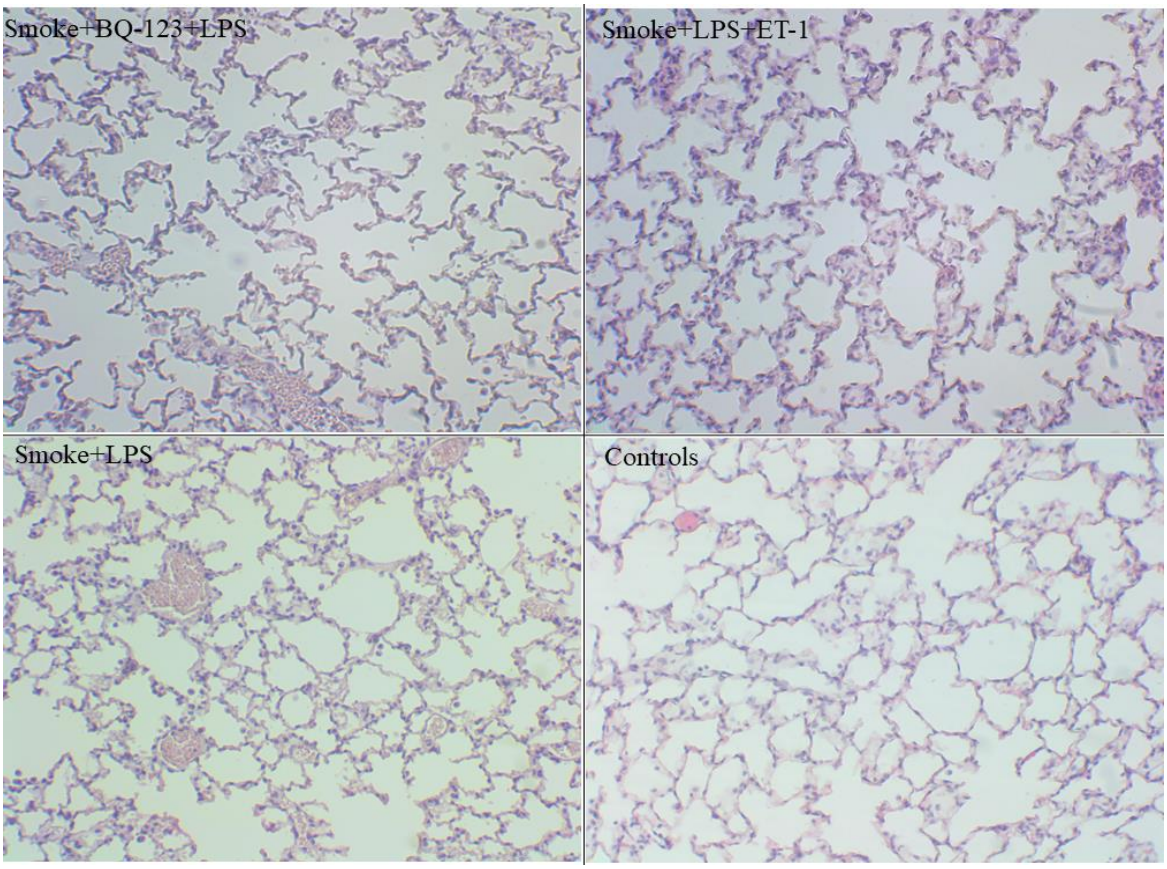

Figure 18. Lung Histology of BQ-123, ET-1, LPS, and PBS Photomicrographs of paraffin-embedded slide sections from lungs treated with smoke and injected with either ET-1, LPS, or BQ-123. Controls were exposed to room air and given PBS which had thicker interstitial walls and more infiltrates when compared to the control. The photomicrograph on the top right shows an animal smoked and treated with LPS + ET-1 which had thicker walls and more infiltrates when compared to the control. The animals were euthanized 24 hours after IP treatment. The photomicrographs were taken at 100x magnification.

3.5 Chemotaxis Assay

A Boyden chamber assay was used to measure the chemotactic activity of LPS in smoked hamsters. As shown in Figure 19, BALF leukocytes from hamsters exposed to smoke and did not show increased chemotaxis for LPS dissolved in culture media compared to media alone (72.0 vs 46.8 fluorescence units per 10^4 cells). Similarly, BALF leukocytes from room air exposed animals showed no enhancement in chemotaxis for LPS (83.1 and 39.9 fluorescence units per 10^4 cells, respectively). These results suggest that the effect of LPS on leukocyte migration into the lung is not directly related to the chemotactic activity of LPS but may instead depend on secondary release of proinflammatory cytokines by vascular endothelium or other lung interstitial cells.

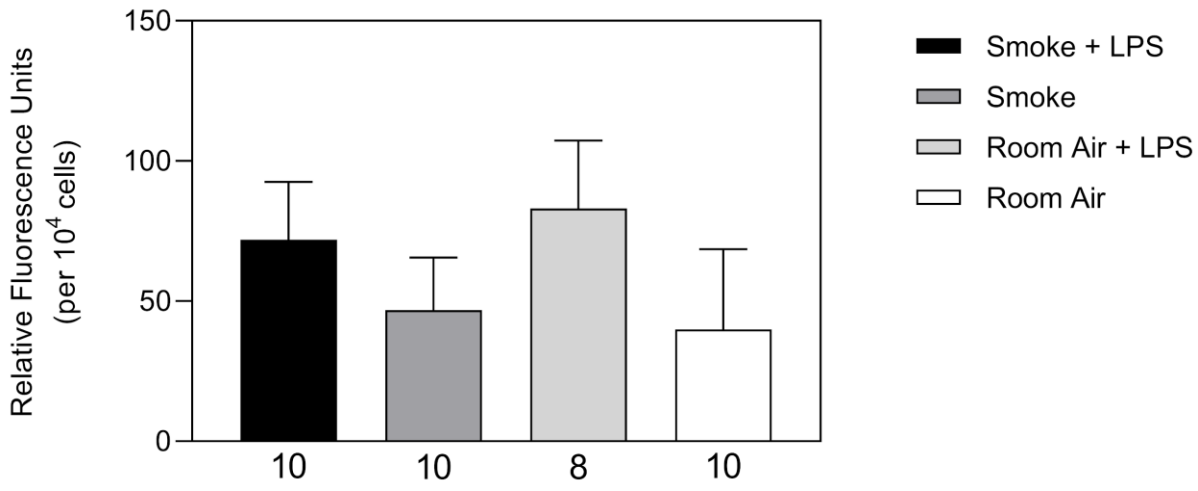

Figure 19. Chemotaxis Assay. Graph showing the chemotaxis assay results. There was no significant difference between the animals exposed to smoke and room air. BALF leukocytes from both smoked and room air animals were exposed to LPS in a Boyden chamber (chemotaxis) assay. Relative fluorescence emission of leukocytes was measured at a fluorescent plate reader using a 480/520 nm filter set. Results were expressed as mean ± SEM. Comparison among groups was done using one-way ANOVA followed by Bonferroni multiple comparison post-hoc test. The numbers under each bar represent the sample size (N).

3.6 Mean Linear Intercept

Measurement of the Mean Linear Intercept was performed 24 hours after LPS treatment to determine whether short-term exposure to cigarette smoke combined with secondary inflammation induced by LPS can induce emphysematous changes. As shown in Figure 20, animals treated with Smoke+LPS had a significantly greater MLI compared to animals treated with room air and PBS (83.60 vs 55.7 respectively; $p<0.0001$). These findings suggest that even brief exposure to second-hand cigarette smoke can result in irreversible lung damage when associated with pneumonia or other forms of secondary inflammation.

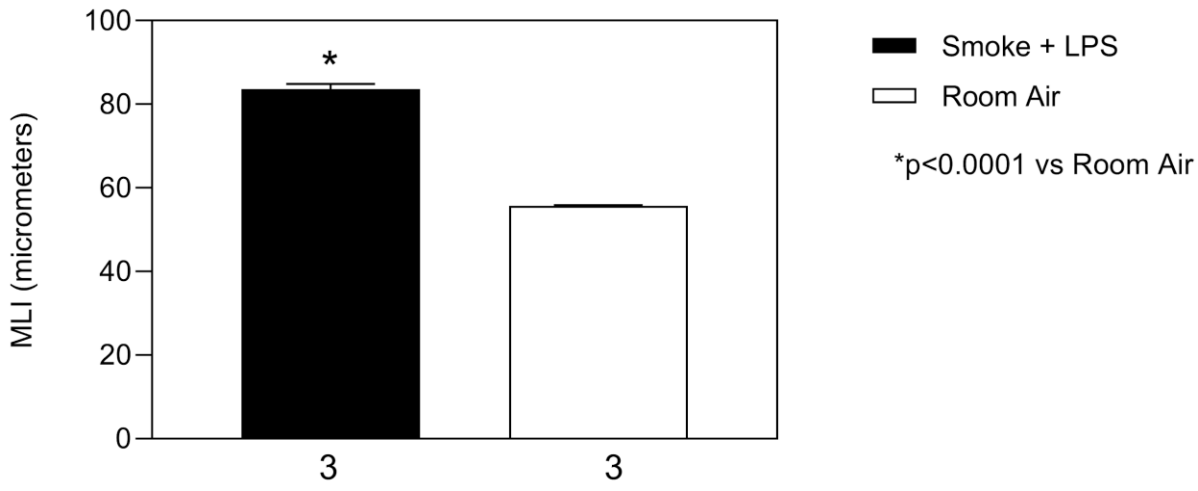

Figure 20. Mean Linear Intercept on Lung Airspace Diameter. Graph showing a significant difference in mean linear intercept on the lung airspace diameter. Animals treated with Smoke+LPS had a significantly larger airspace compared to animals treated with room air and PBS. The first group was exposed to smoke for 4 hours per day during a period of 3 days and injected with IP LPS. The second group was only exposed to room air and PBS. The animals were then euthanized 24 hours after IP administration. The numbers under each bar represent the sample size (N)

3.7 Free Lung Desmosine

Measurements of free lung desmosine were conducted 24 hours after IP LPS treatment to determine whether the combination of smoke exposure and secondary inflammation can cause increased elastic fiber injury and if this can be a better biomarker for elastic fiber damage compared to using free BALF desmosine. As shown in Figure 21, free lung desmosine was significantly higher in the group exposed to Smoke+LPS compared to the other groups treated with smoke only, room air+LPS, and room air only (359.1 vs 95.05 vs 93.07 vs 0.000 respectively). This suggests that the combination of cigarette smoke and LPS acts synergistically regarding elastic fiber injury and that free lung desmosine may be a better indicator of damage to these fibers than free BALF desmosine. These results in combination with the MLI measurements support the hypothesis that short-term cigarette smoke increases the susceptibility of the lung to severe injury from secondary sources of inflammation such as bacterial infections.

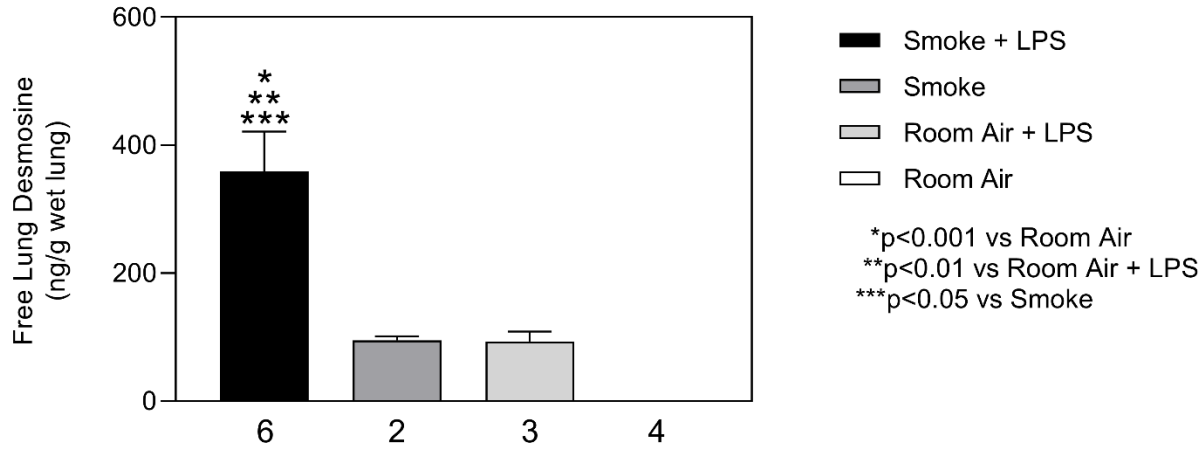

Figure 21. Free Lung Desmosine. Graph showing significant difference in Free Lung Desmosine. Animals treated with Smoke+LPS have a significantly higher amount of free desmosine in the lung compared to the Smoke, Room-Air+LPS, and Room air. The animals were exposed to either smoke or room air, then given IP LPS or PBS 24 hours later. The animals were euthanized 24 hours after treatment. Statistical analysis was performed with one-way ANOVA. The numbers under each bar represent the sample size (N).

CHAPTER 4: DISCUSSION

4.1 Smoke Exposure and Secondhand Smoke Exposure

Cigarette smoking and secondhand cigarette smoke exposure continue to be a major issue in the U.S. According to the CDC and US Surgeon General, tobacco smoking has contributed to more than 480,000 deaths per year in the United States, including more than 41,000 deaths resulting from secondhand smoke exposure (United States Department of Health and Human Services, 2014). Smoking has been known to cause cancer, heart disease, lung diseases, COPD, emphysema, and chronic bronchitis. Secondhand smoke has also been a contributing factor in smoke-related deaths as secondhand exposure can increase the risk of a stroke. In children, exposure to secondhand smoke increases their risk of ear infections, severe asthma attacks, respiratory infections, and a greater risk of sudden infant death syndrome. In adults, non-smokers exposed to secondhand smoke can cause heart disease, lung cancer, and stroke.

4.2 Cigarette Smoke and Bacterial Infections

As it is well known that cigarette smoke can cause a variety of issues, bacterial infections are one of the most critical problems affecting smokers and nonsmokers. Active smokers and those exposed to secondhand smoke are at increased risk of bacterial infections. Cigarette smoke exposure increases the susceptibility to respiratory diseases and infections in the lung such as tuberculosis and pneumonia. This can cause smokers and nonsmokers to be more vulnerable to multiple bacterial infections which can be life-threatening for both active smokers and nonsmokers. They can be exposed to bacteria such as pneumococcus and meningococcus as they are more commonly occurring

bacterial infections in both active and second-hand smokers than in nonsmokers. Lipopolysaccharides (LPS) are used in our experiments to stimulate bacterial infections such as meningococcus due to it being a gram-negative bacterium capable of producing endotoxins similar to LPS (Rouphael & Stephens, 2012). Other studies have shown that cigarette smoke can drastically increase air concentrations of endotoxins with each inhalation containing 17.4 pmol of endotoxin per smoked cigarette (Larsson et al., 2004).

4.3 Management of Smoking and Prevention of Acute Lung Injury

With cigarette smoke being a major contributor to deaths in the US, there are methods to stop smoking that can decrease your risk of respiratory diseases and bacterial infections. The most common is smoking cessation. According to the US Surgeon General cessation of cigarette smoking is beneficial at any age as it improves health status and enhances the quality of life. It reduces the risk of many adverse health effects such as respiratory diseases, COPD, and bacterial infections (United States Department of Health and Human Services, 2014). Other methods of smoke cessation have been used such as nicotine replacement therapy or smoke cessation medications (Feldman & Richards, 2018). There are FDA-approved smoking cessation drugs such as varenicline and bupropion to prevent you from continuing smoking. There are also more commercial and common products such as nicotine gum and patches. However, there are little to no therapeutic agents that have been approved to prevent or reduce acute lung injury. There are some methods such as aerosolized high dose systemic corticosteroids that can prevent acute lung injury, but it is still under clinical trial and have been associated with worse outcomes in patients with sepsis shock (Litell et al., 2011).

4.4 Current Treatments for Bacterial Infections

Since smoking is one of the main risk factors for respiratory diseases, the cessation of smoking can decrease the risks of both bacterial infections and respiratory diseases such as COPD and emphysema. For the treatment of bacterial infections, the use of antibiotics and sometimes antifungal drugs are used. An example would be the use of macrolide or tetracycline have been recommended for patients with no risk factors for drug-resistant *Streptococcus pneumoniae* (Feldman & Richards, 2018). Bacterial meningitis is a common infection in both smokers and second-hand smokers with a two-to-fourfold higher risk of getting the infection. The standard treatment for meningitis is ampicillin as smokers and secondhand smokers are considered immunocompromised by the CDC (United States Department of Health and Human Services, 2014).

4.5 LPS Causes Sequestration of Neutrophils in the Alveolar Wall Capillaries

Secondhand smoke exposure is well known to cause inflammation in the lungs. Our results have shown that secondhand smoke exposure and intraperitoneal injection of LPS causes an increased number of neutrophils in the lungs and increased thickness in the alveolar walls. In addition, LPS-treated lung tissue saw an increased movement of neutrophils into the alveolar wall, causing accumulation in the interstitium and alveolar walls (Figure 4). Other studies have shown that cigarette smoke exposure causes polymorphonuclear leukocytes, causes an increased number of PMN in the circulation, and a degree of deterioration in lung function. This type of accumulation can be caused by the deformability of PMN following smoke exposure which may be a cause in their retention in the pulmonary capillaries (Terashima et al., 1999).

In our histology, we saw that LPS can cause diapedesis of the sequestered neutrophils which then accumulate in the alveoli, contributing to the increased inflammation and thickness of the interstitium (Figure 4). Animals treated with cigarette smoke and LPS had a significantly higher disease index compared to the control (Figure 3). There were significantly more infiltrates and thicker interstitium walls indicating that both cigarette smoke and LPS increased inflammation in the alveolar walls. This occurs when the inflammatory response goes through a process of reaching the site of inflammation and moves through the endothelial surface towards the tissue. The infiltration of activated neutrophils into the lung compartments is one of the indicators in acute lung injury and acute respiratory distress syndrome. Cigarette smoke has been known to be associated with an increased incidence of acute respiratory distress syndrome (Siew et al., 2016). Other studies have shown that the administration of aerosolized LPS can cause LPS-induced PMN transendothelial migration into the interstitial space and alveolar airspace. (Basit et al., 2021).

4.6 Use of Endothelin Receptor Antagonists and Blockage of Endothelin Receptors

Endothelin Receptor antagonists (ERAs) play an important role in the treatment of pulmonary arterial hypertension and have been tested for their protective effects in different models of acute lung injury (ALI). Our laboratory and other studies have used ERAs to prevent the development of ALI and ET-1-induced lung edema. In previous laboratory experiments, Patel et al used a novel endothelin antagonist called HJP272 which resulted in attenuated LPS-induced acute lung injury in hamsters in which HJP272 was able to significantly reduce the number of neutrophils and reduced lung injury. Other

studies have used nonselective ERAs such as Bosentan and Tezosentan. Chen et al have used bosentan and other ERAs in determining that ET-1 plays an important role in the pathogenesis of emphysema. The use of bosentan and BQ123 prevented the development of cigarette smoke extract-induced emphysema and blocked the expression of the ET_A receptor (Chen et al., 2010).

In our results, we have shown that blocking endogenous ET-1 may counteract the effects of LPS in the alveolar and inflammatory response with the use of ERAs. In Figure 11, there was a significant decrease in neutrophil content in the group treated with BQ123 compared to a group treated without BQ123. It is also shown in Figure 11 and 15, there was a significant decrease of neutrophils in a group injected with BQ123 compared to a group treated only with smoke+LPS. Since BQ-123 is known to be a selective antagonist for the ETA receptor and can competitively block the receptor, reducing the likelihood of ET-1 from binding. It is possible that when BQ-123 blocks ETA receptors which prevents endogenous ET-1 from binding to the receptors which reduce the level of inflammatory response against LPS and resulting in lower neutrophil counts compared to the animals treated with LPS or cigarette smoke. Other studies have shown that BQ-123 has protective effects against LPS-induced oxidative stress in the Wistar rat lungs and concluded that BQ-123 was able to prevent lung edema development and decrease LPS-induced oxidative stress (Piechota et al., 2011). Another study conducted by Sampaio et al showed that intratracheal instillation of BQ-123 inhibits neutrophil and eosinophil accumulation in LPS-induced pleurisy (Sampaio et al., 2004).

4.7 Exogenous ET-1 can induce neutrophil diapedesis in unsmoked animals.

Our results have shown that external use of ET-1 can cause neutrophils to migrate towards the lung and sequester into the alveolar walls in animals not treated with either LPS or smoke. As shown in Figure 12, there was a significantly higher number of leukocytes in the smoke and room air compared to animals treated with Smoke+ET-1 and Room Air+ET-1. This indicates that there are possible vasoconstrictions on the airways and parenchyma that reduce the number of leukocytes entering the lung. A study done by Admaicaza et al showed that the use of exogenous ET-1 can induce significant changes in the mechanical properties of the airways and parenchyma in guinea-pig lungs (Adamicza et al., 2001). In Figure 13, there was a significant increase in neutrophils in groups treated with ET-1 and cigarette smoke compared to animals exposed to room air and smoke+ET-1. This may be due to the fact that many endothelial cells produce endogenous ET-1 which includes leukocytes and macrophages (Kowalczyk et al., 2015). ET-1 also stimulates the synthesis of TNF-α which further enhances the inflammatory response by stimulating the chemotaxis and phagocytosis of macrophages, monocytes, and neutrophils. It is possible that the exogenous ET-1 may cause the neutrophils to sequester into the lungs due to it being attracted to ET-1 as it is a proinflammatory mediator. Other studies have shown that ET-1 induces neutrophil recruitment in adaptive inflammation via TNAF-***α*** and CXCL1/CXCR2 in mice (Zarpelon et al., 2012).

4.8 Exogenous ET-1 may cause vasoconstriction in smoked-induced and LPS-induced, reducing the effects of neutrophil diapedesis.

Exogenous ET-1 may cause vasoconstriction in smoked-treated animals. Cigarette smoke has been well known to cause vasoconstriction in the pulmonary system due to

increased endogenous ET-1 from cigarette smoke exposure. Cigarette smoke can induce ET-1 synthesis to respond to the smoke-induced inflammation. As shown in Figure 15, there was a significant increase of neutrophil content in animals treated with smoke+LPS compared to the group treated with smoke+LPS+ET-1. It is possible that adding exogenous ET-1 to already existing endogenous ET-1 may cause the blood vessels to constrict further towards the lungs, resulting in fewer neutrophils in the alveoli. Other studies have shown that ET-1 levels are elevated in heavy and light smokers and patients who have COPD and pulmonary hypertension (Lu et al., 2018). They also have shown that cigarette smoke also elevates ET-1 levels and causes increased expression of ET_A receptors (Milara et al., 2012). With cigarette smoke already increasing the levels of endogenous ET-1, an additional amount of exogenous ET-1 entering the body can cause an increased effect on vasoconstricting which can decrease the number of neutrophils entering the lung.

4.9 Exogenous ET-1 may reverse LPS-induced vasodilation, counteracting its effect on neutrophil diapedesis.

Exogenous ET-1 may reverse LPS-induced vasodilation in the alveolar walls which can counteract the effects of neutrophils diapedesis. LPS is known to cause vasodilation when injected into animals, which increases the inflammatory response of neutrophils (Farias et al., 2002). Our results have shown that when the animals were exposed to cigarette smoke and then injected with LPS it increased the number of leukocytes and neutrophils entering the lung compared to a group treated with Smoke+LPS+ET-1 (Figure 14, 15). As shown in both Figures 14 and 15, the pretreated

Smoke+LPS animals were injected with IP exogenous ET-1, the number of leukocytes and neutrophils significantly decreased compared to the group treated with smoke and LPS. It is possible that the ET-1 is constricting the blood vessels that are already dilated from the endotoxin, reducing neutrophil diapedesis. Our results have also indicated that there is rapid neutrophil turnover in the lungs as the exogenous ET-1 has a vasopressor effect on the BALF neutrophil counts by lowering the cumulative positive effect of smoke exposure (Figure 15). It is possible that there are a lower number of BALF neutrophils due to exogenous ET-1 inducing vasoconstriction in the blood vessels, resulting in fewer neutrophils in smoke-treated animals. However, the mechanism on how ET-1 can reverse LPS-induced vasodilation in the alveolar walls is uncertain.

4.10 Short-term Smoke Exposure and Elastin Breakdown

Short-term exposure to second-hand cigarette smoke increased the number of lung neutrophils but it was not enough to cause significant elastin breakdown. Desmosine and isodesmosine are used as a biomarker for elastin breakdown and are found in different matrices such as the lung and the sputum (Luisetti et al., 2008). Our results have shown that animals exposed for 4 hours daily for a period of 3 days had an increased number of neutrophils and leukocytes in the lung but there was no significant correlation in elastin breakdown and percent neutrophils in the BALF. However, there was a correlation between the free BALF desmosine and total BALF desmosine levels (Figure 7).

As shown in Figure 20, the lung airspace size was measured, and it showed a significant increase in airspace size compared to the control group. This is consistent with the increased free lung desmosine as shown in Figure 21. Figure 21 also shows that free

lung desmosine is a better indicator of lung damage compared to BALF desmosine. In the group exposed to Smoke+LPS (Fig.21), there was a significantly higher amount of free desmosine compared to the other groups. This may indicate that the combination of short-term cigarette smoke and secondary inflammation caused by LPS may cause significant elastin damage in the lung. Cigarette smoke and LPS appear to interact synergistically which can result in more damage to the lung. This type of result is similar to patients diagnosed with emphysema and a common feature of COPD (Wollmer et al., 2014). Our laboratory has previously shown that smoke-induced elastin breakdown may take 1 to 2 months of exposure. When the animals were exposed to cigarette smoke for 3 months, the percent lung surface area decreased and the amount of free BALF desmosine increase significantly during the second month of exposure (Cantor et al., 2018). Other studies have shown that desmosine can be a reliable biomarker for COPD and pulmonary emphysema that can be found in urine, plasma, sputum, lung, and BALF matrices. (Luisetti et al., 2008) (Cantor et al., 2018)

4.11 Conclusion

The results obtained from the cigarette smoke/LPS model suggest that many secondhand smokers can become increasingly vulnerable to secondary infections that cause more damage to their lungs. Our results show that acute exposure to cigarette smoke and simulating a secondary infection using LPS causes enlargement of airspace size that is irreversible due to elastic fiber degradation and rupture of alveolar walls. The use of endothelin receptor antagonist may help decrease the inflammatory response and possibly prevent further acute lung injury, based on our finding that BQ-123, a potent selective ERA, decreases neutrophil influx into the lung. In contrast, exogenous ET-1

increased the proportion of BALF neutrophils in animals exposed to room air while lowering total BALF leukocyte content, consistent with its previously demonstrated proinflammatory effect on neutrophils. However, in animals pretreated with both smoke and LPS, exogenous ET-1 showed anti-inflammatory activity, suggesting the possibility that its vasopressor effect on the capillary bed compromises blood flow to the lung, thereby reducing leukocyte influx into the lung. Further investigation of the effects of ET-1, bacterial infections, and ERAs on smoke-related lung injury may lead to more effective approaches to preventing exacerbation of elastic fiber damage and emphysematous changes following pneumonia or other types of secondary inflammation.

BIBLIOGRAPHY

1. Adamicza, Á., Peták, F., Asztalos, T., Tiszlavicz, L., Boros, M., & Hantos, Z. (2001). Endothelin-1-induced airway and parenchymal mechanical responses in guinea-pigs: The roles of ETA and ETB receptors. *European Respiratory Journal*, *17*(5), 975–981. https://doi.org/10.1183/09031936.01.17509750
2. Bagaitkar, J., Demuth, D. R., & Scott, D. A. (2008)use increases susceptibility to bacterial infection. *Tobacco Induced Diseases*, *4*(1), 12. https://doi.org/10.1186/1617-9625-4-12
3. Basit, A., Galkina, E. V, Ley, K., Basit, A., & Galkina, E. V. (2021). *Sequential recruitment of neutrophils into lung and bronchoalveolar lavage fluid in LPS-induced acute lung injury*. *1394*, 807–815. https://doi.org/10.1152/ajplung.00477.2004.
4. Bhavsar, T., Liu, X. J., Patel, H., Stephani, R., & Cantor, J. O. (2008). Preferential recruitment of neutrophils by endothelin-1 in acute lung inflammation induced by lipopolysaccharide or cigarette smoke. *International Journal of COPD*, *3*(3), 477–481. https://doi.org/10.2147/copd.s2837
5. Bhavsar, T. M., Cerreta, J. M., Liu, M., Reznik, S. E., & Cantor, J. O. (2008). Phosphoramidon, an endothelin-converting enzyme inhibitor, attenuates lipopolysaccharide-induced acute lung injury. *Experimental Lung Research*, *34*(3), 141–154. https://doi.org/10.1080/01902140701884430
6. Cantor, J., Ochoa, A., Ma, S., Liu, X., & Turino, G. (2018). Free Desmosine is a Sensitive Marker of Smoke-Induced Emphysema. *Lung*, *196*(6), 659–663. https://doi.org/10.1007/s00408-018-0163-1

7. Chen, Y., Hanaoka, M., Droma, Y., Chen, P., Voelkel, N. F., & Kubo, K. (2010). Endothelin-1 receptor antagonists prevent the development of pulmonary emphysema in rats. *European Respiratory Journal*, *35*(4), 904–912. https://doi.org/10.1183/09031936.00003909
8. Farias, N. C., Borelli-Montigny, G. L., Fauaz, G., Feres, T., Borges, A. C. R., & Paiva, T. B. (2002). Different mechanism of LPS-induced vasodilation in resistance and conductance arteries from SHR and normotensive rats. *British Journal of Pharmacology*, *137*(2), 213–220. https://doi.org/10.1038/sj.bjp.0704850
9. Feldman, C., & Richards, G. (2018). *Appropriate antibiotic management of bacterial lower respiratory tract infections [version 1 ; referees : 2 approved] Referee Status : 7*(0), 1–12. https://doi.org/10.12688/f1000research.14226.1
10. Guzik, K., Skret, J., Smagur, J., Bzowska, M., Gajkowska, B., Scott, D. A., & Potempa, J. S. (2011). Cigarette smoke-exposed neutrophils die unconventionally but are rapidly phagocytosed by macrophages. *Cell Death and Disease*, *2*(3), 1–11. https://doi.org/10.1038/cddis.2011.13
11. Hanasato, N., Oka, M., Muramatsu, M., Nishino, M., Adachi, H., Fukuchi, Y., & Mura-, M. (1999). *Attenuates Hypoxic Pulmonary Hypertension in Rats*. 225–232.
12. Ihara, M., Fukuroda, T., Saeki, T., Nishikibe, M., Kojiri, K., Suda, H., & Yano, M. (1991). An endothelin receptor (ETA) antagonist isolated from Streptomyces misakiensis. *Biochemical and Biophysical Research Communications*, *178*(1), 132–137. https://doi.org/10.1016/0006-291X(91)91789-F
13. Kowalczyk, A., Kleniewska, P., Kolodziejczyk, M., Skibska, B., & Goraca, A.

(2015). The role of endothelin-1 and endothelin receptor antagonists in inflammatory response and sepsis. *Archivum Immunologiae et Therapiae Experimentalis*, *63*(1), 41–52. https://doi.org/10.1007/s00005-014-0310-1

14. Larsson, L., Szponar, B., & Pehrson, C. (2004). Tobacco smoking increases dramatically air concentrations of endotoxin. *Indoor Air*, *14*(6), 421–424. https://doi.org/10.1111/j.1600-0668.2004.00290.x
15. Litell, J. M., Gong, M. N., Talmor, D., & Gajic, O. (2011). Acute lung injury: Prevention may be the best medicine. *Respiratory Care*, *56*(10), 1546–1554. https://doi.org/10.4187/respcare.01361
16. Liu, X., Ma, S., Liu, S., Liu, M., Turino, G., & Cantor, J. (2015). The Ratio of Free to Bound Desmosine and Isodesmosine May Reflect Emphysematous Changes in COPD. *Lung*, *193*(3), 329–334. https://doi.org/10.1007/s00408-015-9712-z
17. Lu, Q., Gottlieb, E., & Rounds, S. (2018). Effects of cigarette smoke on pulmonary endothelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *314*(5), L743–L756. https://doi.org/10.1152/ajplung.00373.2017
18. Luisetti, M., Ma, S., Iadarola, P., Stone, P. J., Viglio, S., Casado, B., Lin, Y. Y., Snider, G. L., & Turino, G. M. (2008). Desmosine as a biomarker of elastin degradation in COPD: Current status and future directions. *European Respiratory Journal*, *32*(5), 1146–1157. https://doi.org/10.1183/09031936.00174807
19. Ma, S., Turino, G. M., & Lin, Y. Y. (2011). Quantitation of desmosine and isodesmosine in urine , plasma , and sputum by LC – MS / MS as biomarkers for

elastin degradation. *Journal of Chromatography B*, *879*(21), 1893–1898. https://doi.org/10.1016/j.jchromb.2011.05.011

20. Milara, J., Gabarda, E., Juan, G., Ortiz, J. L., Guijarro, R., Martorell, M., Morcillo, E. J., & Cortijo, J. (2012). Bosentan inhibits cigarette smoke-induced endothelin receptor expression in pulmonary arteries. *European Respiratory Journal*, *39*(4), 927 LP – 938. https://doi.org/10.1183/09031936.00021411
21. Miliotis, T., Lindberg, C., Semb, K. F., van Geest, M., & Kjellström, S. (2013). Quantitative high-performance liquid chromatography-tandem mass spectrometry method for the analysis of free desmosines in plasma and urine. *Journal of Chromatography A*, *1308*, 73–78. https://doi.org/10.1016/j.chroma.2013.06.063
22. Naghavi, M., Abajobir, A. A., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abera, S. F., Aboyans, V., Adetokunboh, O., Ärnlöv, J., Afshin, A., Agrawal, A., Kiadaliri, A. A., Ahmadi, A., Ahmed, M. B., Aichour, A. N., Aichour, I., Aichour, M. T. E., Aiyar, S., Al-Eyadhy, A., … Murray, C. J. L. (2017). Global, regional, and national age-sex specifc mortality for 264 causes of death, 1980-2016: A systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*, *390*(10100), 1151–1210. https://doi.org/10.1016/S0140-6736(17)32152-9
23. Pahal P, Avula A, S. S. (2020). *Emphysema*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK482217
24. Patel, S., Liu, X., Liu, M., Stephani, R., Patel, H., & Cantor, J. (2014). HJP272, A novel endothelin receptor antagonist, attenuates lipopolysaccharide-induced acute lung injury in hamsters. *Lung*, *192*(5), 803–810. https://doi.org/10.1007/s00408-014-9628-z

25. Piechota, A., Polańczyk, A., & Goraca, A. (2011). Protective effects of endothelin-A receptor antagonist BQ123 against LPS-induced oxidative stress in lungs. *Pharmacological Reports*, *63*(2), 494–500. https://doi.org/10.1016/S1734-1140(11)70516-3

26. Rouphael, N. G., & Stephens, D. S. (2012). Neisseria meningitidis: Biology, microbiology, and epidemiology. *Methods in Molecular Biology*, *799*(3), 1–20. https://doi.org/10.1007/978-1-61779-346-2_1

27. Sampaio, A. L. F., Rae, G. A., & Henriques, M. das G. M. O. (2004). Effects of endothelin ET A receptor antagonism on granulocyte and lymphocyte accumulation in LPS-induced inflammation . *Journal of Leukocyte Biology*, *76*(1), 210–216. https://doi.org/10.1189/jlb.1003504

28. Siew, E. D., Janz, D. R., Bernard, G. R., May, A. K., Jacob, P., Havel, C., Benowitz, N. L., & Lorraine, B. (2016). *Cigarette Smoke Exposure and the Acute Respiratory Distress Syndrome*. *43*(9), 1790–1797. https://doi.org/10.1097/CCM.0000000000001089.Cigarette

29. Terashima, T., Klut, M. E., English, D., Hards, J., Hogg, J. C., & Van Eeden, S. F. (1999). Cigarette smoking causes sequestration of polymorphonuclear leukocytes released from the bone marrow in lung microvessels. *American Journal of Respiratory Cell and Molecular Biology*, *20*(1), 171–177. https://doi.org/10.1165/ajrcmb.20.1.3276

30. Thulborn, S. J., Mistry, V., Brightling, C. E., Moffitt, K. L., Ribeiro, D., & Bafadhel, M. (2019). Neutrophil elastase as a biomarker for bacterial infection in COPD. *Respiratory Research*, *20*(1), 170. https://doi.org/10.1186/s12931-019-

1145-4

31. Thurlbeck, W. M. (1967). Internal surface area and other measurements in emphysema. *Thorax*, *22*(6), 483–496. https://doi.org/10.1136/thx.22.6.483
32. United States Department of Health and Human Services. (2014). The Health Consequences of Smoking—50 Years of Progress A Report of the Surgeon General. *A Report of the Surgeon General*.
33. Wollmer, P., Jacobson, J., Andersson, T., & Löndahl, J. (2014). The airspace dimension test – A new way to diagnose emphysema? *European Respiratory Journal*, *44*(Suppl 58), P2134. http://erj.ersjournals.com/content/44/Suppl_58/P2134.abstract
34. Wright, J. L., Farmer, S. G., & Churg, A. (2002). Synthetic serine elastase inhibitor reduces cigarette smoke-induced emphysema in guinea pigs. *American Journal of Respiratory and Critical Care Medicine*, *166*(7), 954–960. https://doi.org/10.1164/rccm.200202-098OC
35. Zarpelon, A. C., Pinto, L. G., Cunha, T. M., Vieira, S. M., Carregaro, V., Souza, G. R., Silva, J. S., Ferreira, S. H., Cunha, F. Q., & Verri, W. A. J. (2012). Endothelin-1 induces neutrophil recruitment in adaptive inflammation via TNFα and CXCL1/CXCR2 in mice. *Canadian Journal of Physiology and Pharmacology*, *90*(2), 187–199. https://doi.org/10.1139/y11-116
36. Larsson, L., Szponar, B., & Pehrson, C. (2004). Tobacco smoking increases dramatically air concentrations of endotoxin. Indoor Air, 14(6), 421–424. https://doi.org/10.1111/j.1600-0668.2004.00290.x

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