IMMUNE DYSFUNCTION IN CYSTIC FIBROSIS

BY

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LIST OF ABBREVIATIONS

α-SMA……	Alpha Smooth Muscle Actin
BAL……..	Bronchoalveolar Lavage
CARD……	Caspase Activation and Recruitment Domains
CCL……..	Chemokine (C-C Motif) Ligand
CD……	Cluster of Differentiation
CF…….	Cystic Fibrosis
CFPE……	Cystic Fibrosis Pulmonary Exacerbation
CFTR……	Cystic Fibrosis Transmembrane Conductance Regulator
EGF…….	Epidermal Growth Factor
ELISA……..	Enzyme Linked Immunosorbent Assay
FERV……	Forced Expiratory Reserve Volume
FEV_1……	Forced Expiratory Volume, One Second
FEV_{100}…….	Forced Expiratory Volume at 100 Milliseconds
FMLP……..	Formyl-Methionyl-Leucyl-Phenylalanin
FRC……..	Forced Residual Capacity
IC……..	Inspiratory Capacity
ICS……	Inhaled Corticosteroids
ICU……..	Intensive Care Unit
IFN-γ……..	Interferon Gamma
IHC……	Immunohistochemistry
IκBα…….	Inhibitor of κB
IL……..	Interleukin
iNOS……	Induced Nitric Oxide Synthase

LPS……..	Lipopolysaccharide
LTB4……	Leukotriene B4
MAPK……..	Mitogen-Activated Protein Kinase
MPO……..	Myeloperoxidase
mRNA……	Messenger Ribonucleic Acid
NE……..	Neutrophil Elastase
NF-κB……..	Nuclear Factor Kappa-Light Chain-Enhancer of Activated B Cells
NGS……..	Next Generation Sequencing
NLRC4……	Nod-Like Receptor CARD-Containing Protein 4
NLRP3……..	Nod-Like Receptor Protein 3
NO……	Nitric Oxide
NSAID……	Nonsteroidal Anti-Inflammatory Drugs
OTU……..	Operational Taxonomic Units
P2X7R……	Purinergic 2X7 Receptor
PBMC……..	Peripheral Blood Mononuclear Cells
PFA……..	Paraformaldehyde
PFT……..	Pulmonary Function Tests
PMN……	Polymorphonuclear Leukocytes
rRNA……..	Ribosomal Ribonucleic Acid
TGF-β……..	Transforming Growth Factor Beta
TLC……	Total Lung Capacity
TNFα……..	Tumor Necrosis Factor Alpha
UPR……..	Unfolded Protein Response
VEGF……..	Vascular Endothelial Growth Factor

ABSTRACT

Cystic Fibrosis (CF) is one of the most widespread life-shortening genetic diseases. CF is often diagnosed at birth; there is no cure, and many CF patients die from chronic lung disease at a young age. Patients with CF experience declining pulmonary function related to chronic airway infection, inflammation and scarring. While the mechanism connecting abnormal CFTR function to chronic bacterial infection and inflammation of the airway has not been fully elucidated, recent evidence suggests that CF patients may have defects in their mucosal immunity, resulting in a predisposition to infection and production of a disproportionate inflammatory response to microbial stimuli. Studies show increased cytokine expression in sputum and bronchoalveolar lavage fluid samples in CF patients. To determine if this is secondary to the chronic inflammatory environment of the lungs or innate to CF immune cells itself, leukocytes were isolated from peripheral blood and stimulated with LPS and compared to healthy donors. Our hypothesis is that there will be an increase in cytokine expression in blood leukocytes as well, with a further increase in expression after LPS stimulation. This will demonstrate that the chronic inflammatory response is inherent to the CF mutation itself, as blood is sterile and devoid of infection.

Our study demonstrated that there was increased NF-κB mRNA gene expression in CF compared to healthy controls. However, NF-κB protein level was decreased when assessed by ELISA, even after stimulation with LPS, which is contradictory to what we hypothesized. Furthermore, there was decreased neutrophil elastase, IL-1β, CCL-2, IL-6, and IL-10 protein levels after LPS stimulation. There were also differences between stable and CFPE samples as well, with the CFPE group demonstrated a decreased gene expression in NF-κB after LPS stimulation. These findings suggest that there is an innate immune dysfunction in CF which is

independent of chronic infection and inflammation in the lungs, resulting in impaired cytokine secretion, and a further dysfunction which may lead to the pathogenesis of CFPE.

Currently, the only treatment option for end-stage pulmonary disease in CF is lung transplantation. There are no therapies that can reverse pulmonary fibrosis once established, leading to a permanent and irreversible decline in function. As such, anti-fibrotic therapies are an attractive option to restore lung function and decrease morbidity and mortality. In this study, we have successfully established a murine pulmonary fibrosis model, using bleomycin as our causative agent. We were able to demonstrate a decrease in inspiratory capacity, forced vital capacity, forced expiratory volume, and total lung capacity, which is consistent with the pathophysiology of pulmonary fibrosis. Furthermore, our study demonstrated increased Ashcroft scores in our bleomycin group compared to saline group, corresponding to an increased amount of fibrosis in the bleomycin samples. Establishment of a murine pulmonary fibrosis will enable us to test new agents in hopes of developing a new treatment modality for CF.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (1). Pulmonary disease is the major cause of morbidity and mortality in CF, and is hallmarked by chronic bacterial infections, persistent inflammation, and mucus plugging of small airways causing obstruction and bronchiectasis (2, 3). The CFTR gene encodes a chloride channel expressed in numerous cell types, including various epithelial cell lines, and non-epithelial cell lines such as fibroblasts and immune cells (4). Recent data has suggested that normal CFTR expression is necessary for the proper function of immune cells such as neutrophils (5-7), macrophages (8-12), lymphocytes (13-15), and dendritic cells (16). As a result, mutations or deletions in the CFTR gene, has been linked to dysfunction of immune cells when compared to healthy controls. For example, high levels of IL-8 have been demonstrated in sputum (17), BAL (18), and primary epithelial cells of CF patients (19). It was previously thought that the hyper-inflammatory response was a feedback mechanism to the chronic inflammatory environment of the lungs secondary to chronic infection (20). However, recent studies suggests that chronic inflammation in CF occurs prior to birth in CF independent of infection (21), and may be inherent to the CFTR mutation itself. For example, increased neutrophil counts and IL-8 levels were found in bronchoalveolar lavage (BAL) samples that had no growth on bacterial cultures (22). CF primary epithelial lung cells demonstrated enhanced Nuclear Factor Kappa-Light Chain-Enhancer of Activated B Cells (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) signaling, which resulted in increased levels of IL-1β-induced IL-8 production (23). Epithelial cells from CF patients exposed to LPS derived from *Pseudomonas aeruginosa* induced higher levels of IL-6 expression than epithelial cells from healthy controls (24). In addition, blood polymorphonuclear leukocytes (PMNs) isolated from CF patients have a decreased chemotactic response to leukotriene B4 (25) and decreased

chlorination of phagocytosed bacteria (6). Gene expression defects have also been reported, such as decreased expression of FcγRIII and L-selectin upon stimulation with either IL-8 or formyl-methionyl-leucyl-phenylalanin (FMLP) (26). CF fetal lungs have a higher concentration of NF-κB when compared to healthy controls (24). Furthermore, neutrophil elastase (NE) expression and activity are elevated in CF sputum, associated with reduced levels of inhibitory anti-proteases such as secretory leukoproteinase inhibitor and α_1-antitrypsin (27, 28). NE damages airway walls by increasing mucus secretions, generating chemoattractants, and digesting structural proteins such as elastin, which leads to bronchiectasis (27). Elevated levels of neutrophils in the lungs are also correlated with increased myeloperoxidase activity. Furthermore, BAL-derived macrophages isolated from CF patients also display a predominantly M1-proinflammatory phenotype, with decreased M2 anti-inflammatory phenotype (11). There are also increased T-cells found in CF airways, which produce high levels of inflammatory cytokines (29). In addition, a further imbalance between pro-inflammatory and anti-inflammatory T-cell subpopulations may lead to further dysregulation of the inflammatory response (14). However, the pathophysiology of the immune imbalance remains to be elucidated. It is still unclear whether this imbalance is secondary to the CFTR gene mutation itself, or impaired cell function leading to inadequate bacterial clearance that results in a chronic pro-inflammatory response (30). Consequently, other investigators have suggested that the abnormal airway inflammation is a direct result of the altered airway physiology and chronic microbial infection in the lungs. In healthy patients, the mucus layer and the air-surface-liquid interface serves as a barrier for protection, trapping and clearing pathogens via the mucociliary escalator. However, in CF, loss of CFTR function leads to dehydration of the air-surface-liquid interface, resulting in a dysfunctional mucus layer that is dense and adhered tightly to the lung epithelium (30). This impairment leads to susceptibility to chronic infections from opportunistic

microorganisms such as *Pseudomonas* (30). Furthermore, vulnerability to infections in CF patients occur only in the lungs, as opposed to multiple organs if a systemic immune defect was truly present (31). Moreover, study of immune cell dysfunction in the lung environment presents limitations as well. It is difficult to isolate confounding effects that the infectious environment of the lungs has on immune cells. Additionally, others have reported that studies using airway cells isolated from BAL have revealed technical problems associated with the presence of infection and mucus contamination that interferes with assay techniques, in particular flow cytometry (13). To overcome these limitations and determine if immune dysfunction is secondary to the CFTR mutation itself, we propose to study total leukocytes isolated from the blood of CF patients and compare the effects of inflammatory stimuli *in vitro* compared to healthy leukocytes. We will be using lipopolysaccharide (LPS) as our inflammatory stimulant, then measure the gene expression of seven inflammatory cytokines and acute phase reactants to determine if differences exist. Statistically significant differences will be confirmed by measuring protein expression levels using enzyme-linked immunosorbent assay (ELISA).

Immune Dysfunction and Pulmonary Exacerbations

A dysfunctional immune system with impaired cytokine response is likely to contribute to the pathophysiology of cystic fibrosis pulmonary exacerbations (CFPE). Although there are currently no consensus diagnostic criteria for CFPE, it is generally defined as a clinical event where patients present with changes in cough, sputum production, dyspnea, decrease energy level, anorexia, weight loss, and a decrease in pulmonary function (32). CFPE are major contributors to the overall morbidity and mortality of cystic fibrosis, with each exacerbation demonstrating a negative linear relationship in 5-year survival rates equal to subtracting 12% from the measured FEV_1 value (33). Moreover, CFPE events tend to increase with age and pulmonary impairment. As the majority of CF patients will succumb due to accumulation of

multiple episodes of acute respiratory decompensation caused by CFPE, approaches to reduce or predict their occurrence would have a significant impact on the health and life expectancy of CF patients. Currently there are currently no biomarkers available to predict or diagnose CFPE (34). Much research has been dedicated to studying CFPE, however, little is currently known about its pathophysiology (32). It is thought that the predominantly neutrophilic inflammatory response during CFPE are activated by the NFκB pathway, which results in activation of pro-inflammatory cytokines by both bronchial and respiratory epithelial cells (35). Increases in pro-inflammatory cytokines such as IL-8, IL-6, IL-1β, tumor necrosis factor alpha (TNFα), leukotriene B4 (LTB4), and free NE has been detected during CFPE, with subsequent decreases in expression levels after administration of antibiotics (28, 36-40). However, it is currently unknown whether this increase in cytokine expression is beneficial or detrimental in resolution of the CFPE. Recent microbiome studies have suggested that CFPE may be due to a clonal expansion of existing, less-prevalent strains rather than acquisition of new virulent strains (41), suggesting an inability of the immune system to control existing infection. A dysfunctional immune system with impaired cytokine response could therefore contribute to the pathophysiology of CFPE. Previous studies have demonstrated a correlation between cytokine expression and CFPE in bronchial mucosal biopsies. CFPE patients demonstrated a significantly decreased TGF-β and IFN-γ expression level when compared to stable CF patients, with the highest expression levels found in CF patients with mild pulmonary disease and history of infrequent exacerbations (42). However, since immune cells were isolated from bronchial mucosal biopsies, it is unclear whether the cytokine dysfunction has an infectious etiology, or secondary to the CFTR mutation itself. Our hypothesis is that the cytokine dysfunction is secondary to the CFTR mutation, and this dysfunction is a significant contributor to the pathogenesis of CFPE. To test this hypothesis, we used leukocytes isolated from the peripheral circulation, of CF patients experiencing CFPE, and compared to CF

patients who have been clinically stable for at least 6 months. We expect to see that there is decreased cytokine response in CFPE samples compared to stable samples in the peripheral leukocytes at baseline as well as post stimulation.

The Role of the Pulmonary Microbiome in Cystic Fibrosis

There appears to be a relationship between inflammation, the pulmonary microbiome and clinical status in CF. However, studies that aim to examine the correlation between lung microbiota, airway inflammation, and clinical presentation are limited and lacking (43). How community diversity changes during different clinical states, such as during CFPE, is currently unclear. As such, additional research in this area is warranted. Chronic polymicrobial colonizations in the CF airways are common, and thus can rarely be eradicated with antibiotic therapy. Previously, identification of organisms in CFPE sputum collections were challenging, as few microbiology labs have expertise in handling the viscosity of CF sputum due to hydration secondary to the CFTR defect. In addition, different organisms in the specimen may have different growth requirements, which increases the difficulty to isolate pure bacterial cultures for identification and susceptibility testing (32). The advent of molecular sequencing approaches, most notably 16S ribosomal ribonucleic acid (rRNA) sequencing, has expanded the species of bacteria that can be identified in sputum samples. This technique has identified species that have not been cultured previously in CF sputum samples and not thought to play a role in the pathophysiology of CF pulmonary disease. In addition, it showed that standard bacterial cultures were only able to distinguish a small fraction of total species in CF samples. Next generation sequencing (NGS) also allows discrimination of bacterial community structures, such as assessment of species richness (the number of different species present) and evenness (the relative abundancies of each species) in each sample (44). One commonly utilized index used to characterize species diversity is the Shannon index, in which the proportion of species

relative to the total number of species is calculated, multiplied by the natural logarithm of this proportion, then summed across species (45). Studies using NGS have demonstrated that the lung microbiome of infants and young children are typically dominated by species such as *Streptococcus* and anaerobic bacteria such as *Veillonella* and *Prevotella (46)*. Furthermore, anaerobic species often exists in abundances equal to or greater than typical CF pathogens such as *Pseudomonas aeruginosa* (47, 48), suggesting that anaerobes may play a greater role in the pathophysiology of CF pulmonary disease than previously thought (49). For example, studies have demonstrated that the relative abundance of *Prevotella* was associated with increased lung function and decreased markers of airway inflammation (50). Furthermore, several facultative anaerobic species, such as *Gemella* (51)*, Rothia* (52)*,* and *S. milleri* (53) has been associated with CFPE in adults. Bacterial diversity then increases during the first decade of life, followed by a decrease during adolescence and persisting into adulthood (54-57). This decrease mirrors a similar decline in lung function, suggesting a correlation between bacterial diversity and lung function (47, 51, 58). On the contrary, greater community diversity during adulthood is positively correlated with less severity and decreased progression of pulmonary disease (55, 59, 60). Although there seems to be a positive correlation between community diversity and pulmonary function, the etiology of this relationship has not been elucidated. This is further complicated by patients receiving antibiotic treatments, such as during a CFPE. Microbiomes with high community diversity can exhibit major perturbations in community diversity after initiation of antibiotic therapy (51, 55). However, these variations tend to be temporary, with microbiomes quickly returning to their pre-antibiotic diversities once therapy is stopped (47, 57, 61-63). Conversely, patients with low community diversity, such as patients with end stage pulmonary disease, experience little to no change in diversity after initiation of antibiotic therapy (47, 51, 64). In addition, prolonged antibiotic use has been associated with decreased

microbiome richness (65), and is the primary predictor of decreased diversity in adults (55). Recent studies have suggested that CFPE may be due to a clonal expansion of existing, less-prevalent strains rather than acquisition of new virulent strains (41). For example, one study of CF patients with *Pseudomonas*-dominate samples experienced a decrease in both the relative and absolute abundances of *Pseudomonas* during CFPE (51). Patients with sputum samples demonstrating dominance of a CF pathogen-associated genera (defined as ≥60% of all sequences within the community) had increased airway inflammation (66). Furthermore, the total bacterial density remains constant during a CFPE (44). Currently, antibiotic therapies given as maintenance and during CFPE are aimed at the most prevalent species in CF, namely *Pseudomonas* and *Staphylococcus*. NGS can potentially identify less-prevalent causative species before CFPE, and target antibiotic therapy to eradicate these pathogens before patients experience clinical deterioration and symptoms. Our hypothesis is that there is a significant altercation in the pulmonary microbiome during CFPE, associated with a decrease in pulmonary function and increase in pro-inflammatory cytokine expression. Using NGS, we will be able to identify differences in microbiome between stable and CFPE samples, and correlate this to pulmonary function and cytokine expression using logistic regression analyses. To our knowledge, this will be the first study analyzing the relationship between the lung microbiome, lung function, and cytokine expression. Furthermore, as we will be measure cytokine expression in both sputum and blood, we will be able to determine whether blood cytokine levels correlate with sputum levels, and whether an innate defect in immune cells secondary to the CFTR mutation exists, independent of infection. We expect that the pulmonary microbiome will have prognostic implications in prediction of CFPE, and that this method can be incorporated into clinical practice to better direct antibiotic therapy to target causative agents and maintain pulmonary function. We also expect that diversity is inversely proportional to inflammation,

with a lower Shannon Index associated with an increase in pro-inflammatory cytokine expression.

Preventing Pulmonary Fibrosis

Although life expectancy for patients with cystic fibrosis has improved dramatically in the last 4 decades (67), the majority of patients will succumb due to accumulation of multiple episodes of CFPE (33). Unfortunately, the average life expectancy of CF patients reaching adulthood is 37.5 years (68). Lung transplantation remains the only option for treatment of end-stage disease. Although post-transplantation outcomes have continued to improve, 10-year survival rates are still below 50% (69). As inflammation is the hallmark of cystic fibrosis pulmonary disease and pulmonary complications are the leading cause morbidities and mortalities in CF, anti-inflammatory therapies that aim to ameliorate the inflammatory response and promote healing are warranted. However, while anti-inflammatory therapies may prevent further destruction of pulmonary parenchyma, it does not have any effect on tissues already damaged from previous inflammation. Ultimately, loss of lung function is due to the replacement of healthy lung parenchyma with fibrosis, secondary to the chronic inflammatory response in an attempt to clear infection (70). As such, therapies aimed to reverse fibrosis and restore healthy lung tissue is an important adjunct in the treatment of CF. Currently approved therapies for idiopathic pulmonary fibrosis (IPF) have only demonstrated efficiency in delaying pulmonary decline, but has not been able to reverse the decline in lung function. Recently, a synthetic homolog of signaling peptide acALY18 (Naclynamide™) has shown anti-fibrotic properties *in vitro*, abrogating fibrosis in disease scleroderma cells and resolving a benign canine acral granuloma (71). It is postulated that acALY18 covalently binds to transcription factor XBP1, thus modulating the unfolded protein response (UPR), NLRP3 inflammasome signaling, and the innate immune response (71). Activation of the NLRP3 inflammasome leads to secretion of pro-

inflammatory cytokines such as IL-1β and IL-18 in chronic lung diseases, including CF (72). *In vitro* studies have demonstrated that IL-1β can stimulate collagen expression in a dose-dependent manner (73). Overexpression of IL-1β in airway epithelial cells also leads to release of TNF-α, IL-6, TGF-β, and platelet derived growth factor, which results in deposition of collagen in the lungs (74). As such, drugs that target the NLRP3 inflammasome may be able to ameliorate the collagen deposition in the lungs secondary to a dyregulated inflammatory response and promote tissue repair and recovery. Therefore, our hypothesis is that Naclynamide™ may be able to prevent fibrosis in the lungs, resulting in improvement of pulmonary function and median survival rate. As such, a murine model of pulmonary fibrosis was utilized to evaluate the anti-fibrotic efficacy of Naclynamide™ *in vivo*.

STATEMENT OF PURPOSE

In this study, we compared expression levels of pro-inflammatory cytokine between healthy donors (HD) and CF patients using leukocytes isolated from blood. Our hypothesis is that there is a dysregulation in CF immune cells secondary to CFTR mutation, resulting in a sustained expression of pro-inflammatory cytokines. We will be analyzing leukocytes isolated from the blood to distinguish if any differences if present are due to CFTR mutation or the chronic inflammatory response due to infection in the lungs. In addition, we will be comparing the cytokine expression response between stable and CFPE patients. As CFPE may be due to a clonal expansion of existing, less-prevalent strains rather than acquisition of new virulent strains, we hypothesize that the pathogenesis of CFPE is related to a cytokine expression defect resulting in an inability of the immune system to control current infection. Finally, as the end result of chronic inflammation and CFPE is a permanent decline in pulmonary function due to replacement of healthy lung parenchyma with fibrotic tissue, the development of therapies aimed to prevent and reverse fibrosis is in dire need. In order to accomplish this, a pulmonary fibrosis murine model using bleomycin as our causative agent was developed. Using this model, an NLRP3 inhibitor, Naclyamide™ as tested to determine its anti-fibrotic properties. We hypothesize that Naclynamide™ will result in improved pulmonary function and decreased amounts of fibrosis in lung tissues when compared to saline controls. At the conclusion of this study, we aim to answer the following questions:

1) What is the difference in inflammatory cytokine expressions between healthy subjects versus CF patients in circulating immune cells?
2) Is there a difference in cytokine expressions between different CF disease states, i.e. clinical stability versus pulmonary exacerbation?

3) Are there changes in the pulmonary microbiome that are associated with changes in disease state?
4) What is the association between pulmonary microbiota diversity and inflammation?
5) Are inflammatory cytokine levels in the blood correlated with levels in the lungs?
6) Does Naclynamide™ have anti-fibrotic properties *in vivo*?

METHODS

Patient Selection

This study including chart reviews, and sample collections were approved by the Wake Forest Baptist Medical Center Institutional Review Board (Study ID#IRB00029036) and informed consent was obtained from all participants. Blood samples from 37 patients confirmed to have CF through sweat chloride tests and/or genetic sequencing were collected during routine medical care at the Adult CF Center at Wake Forest Baptist Medical Center. CFPE was defined according to the 1994 Cystic Fibrosis Foundation Microbiology Consensus Conference as the presence of at least 3 of 11 clinical symptoms: increased cough, sputum production, fever, weight loss, school or work absenteeism, increased work of breathing, decreased excise tolerance, or a deterioration in the respiratory exam, chest radiograph, FEV_1, or hemoglobin saturation (41). Samples were collected prior to initiation of antibiotics and anti-inflammatories if indicated. Chronic suppressive antibiotics started prior to CFPE onset, such as azithromycin or tobramycin, were continued as indicated. Copies of all signed forms were given to the patient, and a copy was placed in the medical record for reference. Inclusion criteria includes: adults 18 years or older with a diagnosis of CF, and healthy adults 18 years or older to serve as controls. Exclusion criteria includes: current oral or IV antibiotic use (except for suppressive therapy), current use of anti-inflammatory medications, and history of blood dyscrasia. Data collected on each subject includes age, gender, race, ethnicity, current medications and illnesses and, for CF subjects only, CF genotype, current CF severity/lung function and history of sputum cultures.

Blood and Sputum Collection

Approximately 15 mL of blood were collected into sodium heparin coated collection tubes, and transferred to our lab for further processing as detailed below. Sputum was collected using the Norgen Biotek Sputum DNA Collection, Preservation, and Isolation Kit™

(Norgen Biotek, Thorold, Canada). Briefly, approximately 2 mL of sputum was expectorated into the collection tube, and the contents of the preservation ampule emptied into the collection tube. The tube was shaken well for 10 seconds, then stored at room temperature. Furthermore, blood was collected in a similar manner from 24 healthy volunteers. All *in vitro* assays were performed at our lab located at the Wake Forest Institute of Regenerative Medicine.

Isolation of Nucleated White Blood Cells

After blood collection, total human leukocytes were isolated from buffy coat using HetaSep™ (StemCell Technologies, Vancouver, Canada) gravity sedimentation as described by the manufacturer. All leukocytes were included to better understand the interactions between different cell subsets. Briefly, 1 part HetaSep™ was added to 5 parts whole blood. The sample was centrifuged at 90 x *g* at room temperature for 3 minutes and incubated for 40 minutes. The leukocyte-rich supernatant was collected, and washed 3 times with Roswell Park Memorial Institute (RPMI) 1640 + 1% bovine serum albumin (BSA). Ten mL of red blood cell (RBC) lysis buffer was added to ensure complete lysis of RBCs. Cell count and viability was determined using Trypan Blue exclusion. Isolated cells were separated into two groups: a control group that did not receive any stimulation, and a stimulation group that received 10ng/mL LPS to mimic infection. A total of 2 x 10^6 cells per well were plated onto 12-well plates using Roswell Park Memorial Institute (RPMI) 1640 medium + 1% bovine serum albumin (BSA) for each group. Cells were incubated in triplicates at 37^{o}C with 5% CO_2 for 18 hours. After incubation, cells and supernatants were harvested for further analyses.

mRNA Isolation, cDNA Creation, and RT-qPCR

After incubation, mRNA was isolated from pelleted cell preparations using Trizol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, cells were

harvested and centrifuged, and cell supernatant removed for further analyses. One mL TRIzol reagent was added to each sample for cell lysis. After addition of 0.2 mL of chloroform for homogenization, the samples were centrifuged at 12,000 x g for 15 at 4^{o}C. The aqueous phase of the samples were isolated by angling the tube at 45^{o} and pipetting the solution out. RNA precipitation and wash was performed using isopropanol and ethanol, respectively. Quantification of mRNA after isolation was assessed using a NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE). cDNA were generated from previous mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as described by the manufacturer. RT-qPCR was performed using Taqman® Gene Expression Primers (Applied Biosystems, Foster City, CA) and TaqMan Fast Advanced Master Mix (Applied Biosystems, Austin, TX) according to the manufacturer's instructions. ΔΔCT analyses will be performed using one healthy sample as baseline for comparison. A review of the literature revealed 28 cytokines that were associated in the pathophysiology of the inflammatory response in CF (Table One).

Table One: List of cytokines implicated in the pathophysiology of CF

IL-8 (75, 76)	EGF (77)	NLRP3 (78)
Neutrophil Elastase (79)	TGF-β (80)	IL-13 (81)
NF-κB (82)	IL-2 (83)	CD163 (84)
TNF-α (82)	IL-4 (82)	CCL-2 (85)
IL-17A (82)	CD80/86 (86)	IL-10 (77)
IL-1β (82)	Arginase (82)	NO/iNOS (87)
VEGF (88)	IL-12 (77)	Mannose Receptor (89)
IL-6 (82)	MPO(90)	P2x7r (91)
CCL-18 (77)	IFN-γ (81)	IL-18 (78)
NLRC4 (78)		

Only cytokines that had a significant positive response after stimulation with LPS at this time-point was used in subsequent studies. Seven cytokines were measured using RT-qPCR: IL-8, IL-1β, neutrophil elastase (NE), IL-6, NF-κB, IL-10, and CCL-2.

Nuclear Extraction and NF-κB ELISA

Total human leukocytes were collected after incubation and centrifuged at 500 x *g* for 5 minutes. The cell pellets were used for nuclear and cytoplasmic extractions using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Briefly, cells were harvested by centrifugation at 500 x g for 5 minutes and washed with phosphate buffer solution (PBS). The packed cell volume for each sample was estimated to be 20 µL. Cytoplasmic and nuclear extractions were performed using the reagents supplied in the kit. Both extracts were stored at -80^{o}C until use. Next, nuclear extracts were used to perform the NF-κB ELISA using the Human NF-κB p50 Transcription Factor Assay Kit (abcam, Cambridge, MA) according to the manufacturer's protocol, with the exception that 75 µL of nuclear extracts were used instead.

Neutrophil Elastase ELISA

Cell supernatants were collected after incubation as described above. ELISA was performed using the Human PMN Elastase Platinum ELISA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

IL-1β, IL-6, IL-8, and CCL-2 ELISA

Cell supernatants were collected after incubation as described above. Sputum samples were collected using the Norgen Biotek Sputum Kits as described above. ELISA was performed using the respective Boster ELISA kits (Boster Biological, Pleasanton, CA) according to the manufacturer's protocol.

16s rRNA Sequencing

Sputum samples collected in the Norgen Biotek Sputum DNA Collection, Preservation, and Isolation Kit™ as described above, and sent to LC Sciences (Houston, TX) for 16s rRNA sequencing. Briefly, 338F and 806R primers were used to target the V3 and V4 regions of the 16S gene, which creates single amplicons that are approximately 469 base pairs in length. Library preparation was performed using the Nextera XT DNA Library Preparation Kit™ (Illumina, San Diego, CA). All sequences from all samples were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarities. Microbiome analysis from raw DNA sequencing data was performed using QIIME.

Murine Pulmonary Fibrosis Model

Six to eight weeks old male C57BL/6 mice weighting approximately 20 to 25 grams were obtained from Charles Rivers Laboratories. Mice were randomly assigned into four groups: saline control (n = 18), Bleomycin + saline only (n = 16), Bleomycin plus Naclynamide™ (4 μg/kg, n = 7) administered starting at day 0, and Bleomycin plus Naclynamide™ (n = 2) administered starting at day 9 (delayed) (Figure 1). Day 9 was selected as previous experiments have demonstrated the beginning of fibrosis establishing between days 7 to 10. Therefore, administration of Naclynamide™ after initial establishment of fibrosis would demonstrate efficiency of therapy to reverse fibrosis. Mice were anesthetized on day 0 with isoflurane and Buprenorphine SR (0.5-1 mg/kg) given for analgesia. Bleomycin (2.5 U/kg) or an equivalent volume of injectable grade saline was given via intratracheal injection (1 μL/g). Subsequently, mice were fed a wet chow diet, weighted daily, and given 1 mL subcutaneous saline injections twice daily to maintain hydration. Injectable grade saline or Naclynamide™ was administrated via retro-orbital injection every three days from day 0 to day 18 except for the delayed Naclynamide™ group, where retro-orbital Naclynamide™ injections commenced starting at day

9 instead. At day 21, mice were anesthetized using a combination of isoflurane, ketamine (110 mg/kg), and xylazine (10 mg/kg). Tracheostomy was performed using an 18-gauze tracheostomy cannula. Pulmonary function tests (PFT) were performed using the Buxco® pulmonary function testing system (Wilmington, NC). Mice were subsequently euthanized using CO_2 and lungs harvested for further analyses.

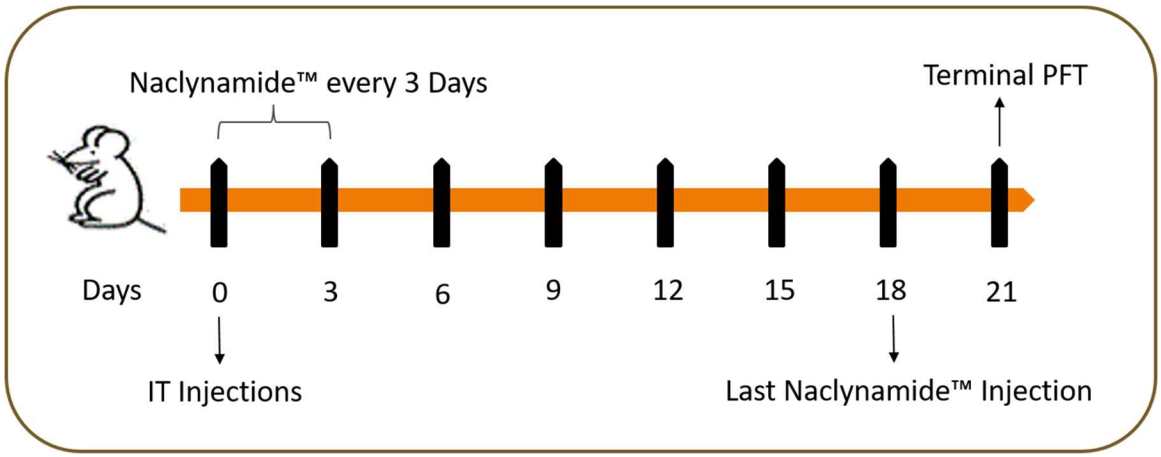

Figure One. Timeline for Murine Pulmonary Fibrosis Model

Ashcroft Scoring

After PFT, the left lung was tied off using Silk 3-0 sutures, and the right lung inflated with 4% paraformaldehyde (PFA) and submerged in PFA for at least 24 hours. The left lung was flash-frozen in liquid nitrogen and stored in -80^{o}C for subsequent RNA and protein analyses. The right lung was embedded in paraffin and sectioned for H&E staining following standard protocols. Pulmonary fibrosis was assessed using a numerical scale developed by Ashcroft *et al* (92). Scoring was performed using the Olympus® BX63 microscope by one blinded researcher. Briefly, each sample was systemically scanned in the microscope using the 10x objective. Fifteen fields were randomly chosen from each lobe of the lung and allotted a score between 0 and 8. Two lobes from each sample was scored, and the mean scores of all fields was designated as the fibrosis score for that sample.

Statistical Analyses

Experimental design and post-experimental data analyses were performed with assistance from the Wake Forest Clinical and Translational Science Institute. GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA) was used to analyze cytokine data. Differences between mean values were analyzed using an analysis of variances (ANOVA), with post-hoc Tukey HSD tests conducted on all possible pairwise comparisons. A p-value less than 0.05 was determined as statistically significant. Pulmonary function tests were analyzed via ANOVA with post-hoc Bonferroni tests conducted using SPSS version 25 (IBM, Armonk, New York). A p-value less than 0.05 was determined as statistically significant.

RESULTS

Patient Population

Thirty one CF (25 males and 13 females) were included in this study. Mean FEV_1 was 2.41L, which was 54.5% of predicted. In addition, 31 (22 males and 9 females) donors were recruited as healthy controls. The demographic data of CF patients versus healthy controls are shown in Table 2, and CF non-exacerbating vs. CF exacerbating patients shown in Table 3.

Table Two: CF Patients vs. Healthy Controls Demographics

	CF (n=37)	**Healthy Control (n=31)**
Age, years	31.0 (18-54)	35.8 (23-57)
Male/Female	25/13	22/9
FEV_1, liters[1]	2.41 (0.46-4.50)	N/A
FEV_1, % predicted[1]	64.5 (11.0-1140)	N/A

Data are presented as median and (range).
[1] Lung function during same clinic visit when blood was collected.

Table Three: CF Non-Exacerbating vs. CF Exacerbating Patient Demographics

Characteristic	**Non-Exacerbating (n=23)**	**Exacerbating (n=14)**
Age, year		
Mean	30.4 ± 10.8	33.1 ± 9.7
Range	18 - 54	18 - 52
Gender (%)		
Male	14 (61%)	10 (71%)
Female	9 (39%)	4 (29%)
FEV_1, L		
Mean	2.50 ± 1.13	2.34 ± 1.03
Range	0.46 - 4.47	1.35 - 5.69
FEV_1, % predicted		
Mean	67.30 ± 28.00	60.62 ± 26.23
Range	11.00 - 114.00	19.40 - 109.00
FVC, L		
Mean	3.32 ± 1.17	3.61 ± 1.33
Range	1.15 - 5.25	0.66 - 4.50
FVC, % predicted		
Mean	80.39 ± 24.71	74.96 ± 22.38
Range	22.00 - 130.00	31.00 - 109.00
Orkambi Use (%)	12 (52%)	4 (28%)

Gene expression of in-vitro CF peripheral leukocytes compared to healthy controls

Several studies have suggested that CF patients with chronic infections demonstrate a hyper-inflammatory immune cell activity in sputum and BAL samples. To investigate whether this response is present and innate to the circulating immune cells themselves, we isolated total nucleated white blood cells from the peripheral blood, stimulated them with LPS, and measured the cytokine levels of seven pro-inflammatory cytokines (Figure 2).

There was a significant increase in gene expression levels after LPS stimulation, compared to unstimulated for IL-1β (1.5-fold unstimulated vs. 23-fold stimulated for healthy, $p = 0.0062$, 1.5-fold unstimulated vs. 19-fold stimulated for CF, $p = 0.0117$), IL-6 (0.8-fold unstimulated vs. 110-fold stimulated for healthy, $p = 0.0131$, 0.8-fold unstimulated vs. 106-fold stimulated for CF, $p = 0.0047$), IL-10 (2.6-fold unstimulated vs. 74-fold stimulated for healthy, $p = 0.0001$, 2.6-fold unstimulated vs. 47-fold stimulated for CF, $p = 0.0102$), and CCL-2 (0.7-fold unstimulated vs. 4-fold stimulated for healthy, $p = 0.0037$, 0.7-fold vs. 5-fold stimulated for CF $p = 0.0007$) for both the healthy and CF groups. In addition, there was a significant difference in NF-κB expression in the CF group after LPS stimulation, which was not detected in the healthy samples. NF-κB was the only measured gene that demonstrated significant differences between CF and healthy samples. Furthermore, there were no differences in expression levels for IL-8 or NE following LPS stimulation.

Although CF samples have similar baseline NF-κB levels when compared to healthy controls, there was a significant increase after LPS stimulation in CF samples compared to healthy ($p < 0.01$). Subsequently, we tested whether the observed increased NF-κB gene expression also resulted in increases in nuclear translocated NF-κB transcription factor activation. During the inactive state, NF-κB remains bound to the inhibitor protein IKB in the cell cytoplasm. After activation of the NF-κB pathway, it dissociates from IKB and translocate into

the nucleus, where it binds to sequence specific promotor regions of its targeted genes to initiate transcription of pro-inflammatory cytokines (93). There was a statistically significant decrease in NF-κB protein level for CF stimulated ($p = 0.0483$, Figure 3f) groups compared to healthy donors, suggesting a hypo-activation response of the NF-κB pathway after LPS stimulation.

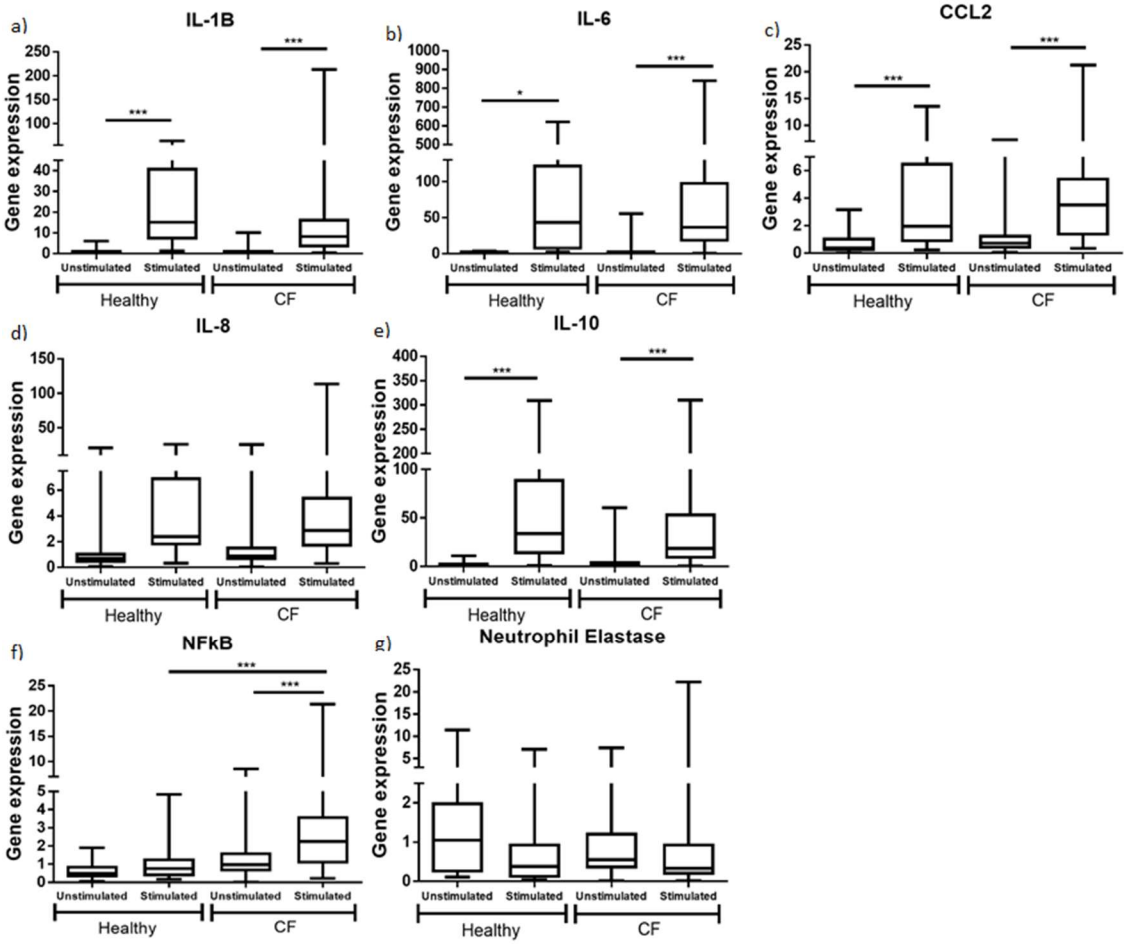

Figure Two. Acute Phase Reactants gene expression in peripheral blood derived total white cells of healthy and CF patients, both at baseline and following stimulation with 10ng/ml LPS. Gene expression was determined as fold-change using ΔΔCT analysis from one healthy sample as baseline for comparison. (a) IL-1β. (b) IL-6. (c) CCL-2. (d) IL-8. (e) IL-10. (f) NF-κB. (g) Neutrophil Elastase. * = $p < 0.05$. *** = $p < 0.01$. Cystic Fibrosis (CF). Lipopolysaccharide (LPS).

Protein translation of in-vitro CF peripheral leukocytes compared to healthy controls

Neutrophil Elastase (NE) gene expression showed similar levels between healthy controls (1.9-fold unstimulated vs. 0.9-fold stimulated) and CF patients (1.9-fold unstimulated

vs. 1.6-fold stimulated), regardless of whether unstimulated or stimulated by LPS. (Figure 2g). This is contrary to what has been found in BAL-derived neutrophils and macrophages from CF patients, which found increased neutrophil elastase activity following LPS stimulation (94). One possible explanation for this difference is the prolonged incubation time in our study before gene expression analysis, which was optimized for a larger range of inflammatory factors. One of the challenges looking at gene expression for multiple acute-phase reactants is determination of the optimal incubation time to analyze gene expression levels. Earlier studies on cytokine expression levels have suggested maximal expression times between 24 to 48 hours (13). For our study, cells were incubated with or without LPS for 18 hours prior to mRNA isolation. Previous studies have suggested that the optimal time of stimulation that induces maximal release of NE was 25 minutes (95). Similarly, Aggarwal et al. reported a significant difference between control and LPS stimulated cells after 5 hours (96), albeit at twice the LPS dose used in our study. Finally, Gupta et al. demonstrated no difference in NE expression in sputum supernatant 6 hours post inhalation of LPS (97). However, when we measured NE protein level secreted into the culture media, CF patient cells showed significantly decreased amount of NE when compared to healthy controls after LPS stimulation (Figure 3). Although previous papers have observed increased levels of NE in CF BAL fluids, it is uncertain whether this increase represents an adequate response to the chronic infectious stimulus in the lungs. In addition, levels of NF-κB and IL-1β, CCL-2, IL-6, and IL-10, cytokines which are under the regulation of the NF-κB pathway, were decreased as well. Lastly, we also observed decreased CF baseline protein expression of CCL-2, which is responsible for recruitment of macrophages, monocytes, memory T cells, and dendritic cells to the sites of inflammation (98, 99).

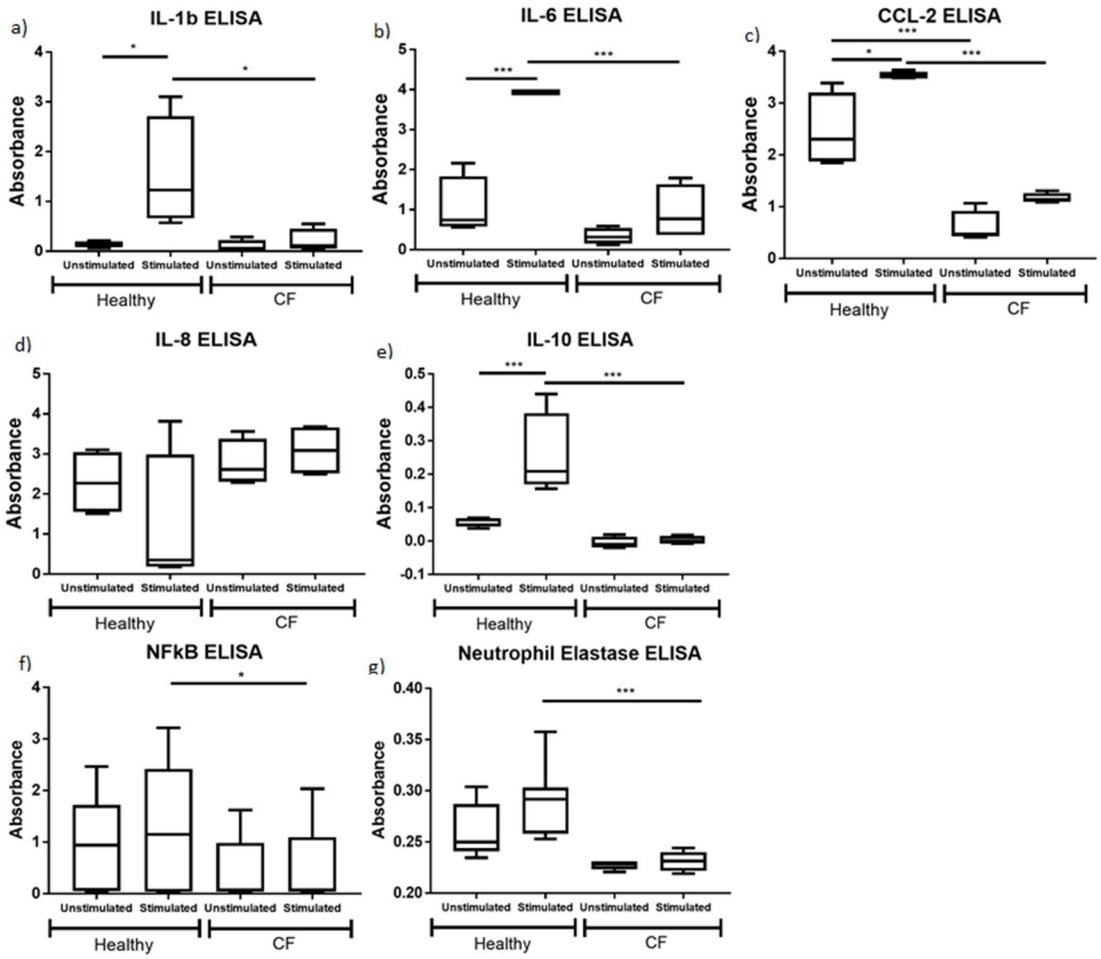

Figure Three. Acute phase reactants protein expression between healthy and CF patients. (a) IL-1β. (b) IL-6. (c) CCL-2. (d) IL-8. (e) IL-10. (f) NF-κB. (g) Neutrophil Elastase. * = p-value < 0.05. *** = p-value < 0.01. CF = cystic fibrosis. LPS = lipopolysaccharide

Gene expression and protein levels in stable CF patients compared to CFPE

To test the hypothesis of whether disease status had any influence on cytokine expression, samples were collected from patients that were clinically stable, and patients that were experiencing a CFPE at time of blood draw. Although both groups had similar levels of NF-κB expression at baseline, the CFPE group had a lower NF-κB expression level after stimulation with LPS when compared to the clinically stable group (p = 0.0395, Figure 4f). There were no differences in the other cytokine expression levels. It is also interesting to note that there were no significant differences between the unstimulated and stimulated groups in the CFPE samples, versus significant differences in NF-κB, IL-6, IL-10, and CCL-2 for stable CF patients. In addition,

we also see differences from stimulation in IL-1β, IL-6, IL-10, and CCL-2 in healthy samples. Part of the reason why we did not see significance in some cytokines could be due to variations between samples; we had a smaller sample size for CPFE due to limited numbers of exacerbating patients able to be recruited for the study. It is possible that additional samples would be required to detect any significance differences. However, as we do see differences in several cytokines for stable CF patients, this suggests that immune cells during CFPE have an increased dysfunction in protein expression when compared to their stable counterparts.

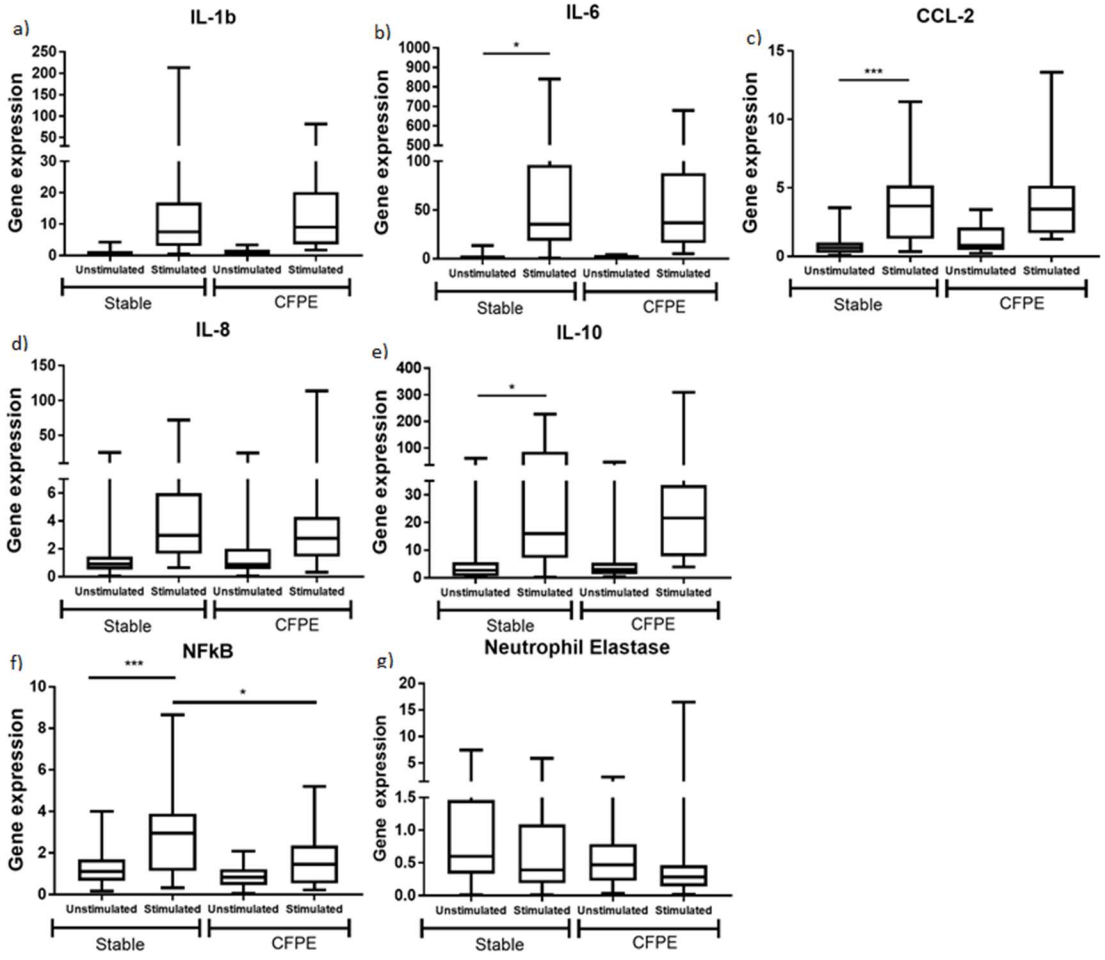

Figure Four. Acute phase reactants gene expression between stable and CFPE patients. Stimulated group: Stimulation with 10 ng/mL lipopolysaccharide. Gene expression was determined as fold-change using ΔΔCT analysis from one healthy sample as baseline for comparison. (a) IL-1β. (b) IL-6. (c) CCL-2. (d) IL-8. (e) IL-10. (f) NF-κB. (g) Neutrophil elastase. * = p-value <0.05. *** = p-value < 0.01. CFPE: cystic fibrosis pulmonary exacerbation.

Microbiome Analyses

To test our hypothesis that the microbiome was associated with inflammation and clinical status, sputum samples were sent for 16s rRNA sequencing and sputum cytokine protein levels detected using ELISA kits as described. In addition to confirmation of species identified via traditional culture methods, 16s rRNA sequencing also identified species that were not usually associated with CF (Figure 5). Table four demonstrates the preliminary exploratory data for three CF patients, one of which is experiencing a CFPE. Our preliminary data seems to suggest that lower Shannon indexes (and therefore decreased species evenness and richness), was associated with increased levels of sputum cytokines, in particular IL-1β and CCL-2. Furthermore, there may be an association between lower Shannon indexes and predicted FEV_1 and FVC. Figure 5 outlines the most prevalent bacterial species in each sample, along with relative abundances. Two of the three samples were positive for *Pseudomonas aeruginosa*, which is consistent with traditional culture results. It is interesting to note that although present, *Pseudomonas* only represented a small fraction of the microbiome, most likely due to the effectiveness of chronic suppressive antibiotic therapy. In addition, previous studies have reported an association between facultative anaerobic species such as *Gemella* and *Rothia* to CFPE in adults. Indeed, we do see both species isolated from our CFPE sample, suggest a pivotal role of these species in the pathophysiology of CFPE, and that future studies aiming to eradicate anaerobic species during CFPE are warranted.

Table Four: Correlation between Shannon Indexes to Pulmonary Function and Sputum Cytokine Levels

ID	Gender	Age	Shannon Index	FEV1 (%)	FVC (%)	FEV1 (L)	FVC (L)	IL-1β*	IL-6*	CCL-2*	TNF-α*	CFPE
a	M	25	1.00	41	61.7	1.84	3.38	1.4132	0.0593	2.9711	0.1277	No
b	F	26	3.38	92.6	105.2	2.91	3.85	N/A	N/A	N/A	N/A	Yes

c	**M**	**37**	**4.21**	**46.4**	**66.4**	**1.79**	**3.12**	**0.1509**	**0.0203**	**0.1648**	**0.0018**	**No**

*** = Units are** mean absorbance values for each sample

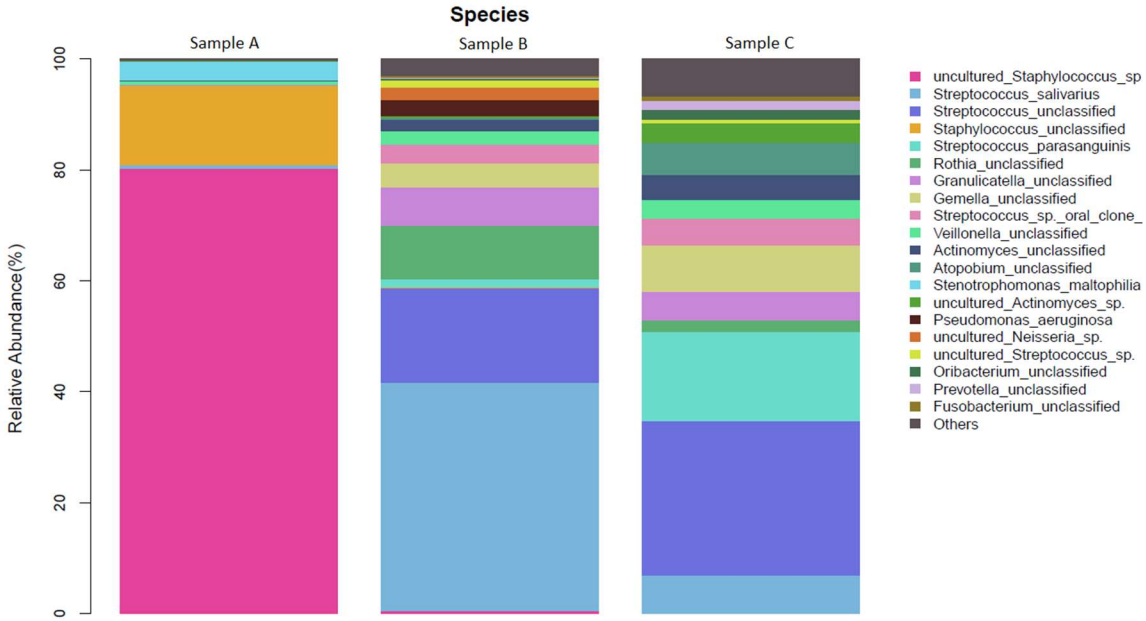

Figure Five. Relative abundance by species for each sample.

Murine Pulmonary Fibrosis Model

In order to develop a novel anti-fibrotic therapy for CF, a murine pulmonary fibrosis model using bleomycin was developed. Our hypothesis is that Naclynamide™ will have significant anti-fibrotic properties via its inhibition of the NLRP3 inflammasome. Bleomycin was administered on day 0 via intratracheal injections. Naclynamide™ was administered every three days starting either on day 0 or day 9. On day 21, pulmonary function tests were performed, and lung tissue harvested for histology. Figure 6 shows the average daily mice weights separated by each group. The average starting weight for each group was: 23.8 ± 2.2 g for saline, 23.5 ± 1.5 g for Bleomycin + saline, 23.5 ± 0.9 g for Bleomycin + Naclynamide™, and 22.7 ± 2.8 g for Bleomycin + delayed Naclynamide™. Day 21 average weights for each group was: 24.3 ± 1.9 g for saline, 23.1 ± 2.1 g for Bleomycin + saline, 22.5 ± 2.3 g for Bleomycin + Naclynamide™, and 21.7 ± 2.8 g for Bleomycin + delayed Naclynamide™. Saline group mice lost

weight on day one after the initial surgery and saline injection, but steadily gained weight since. Conversely, groups that received Bleomycin on day 0 exhibited continuous weight lost until approximately day 9, when weights slowly increased.

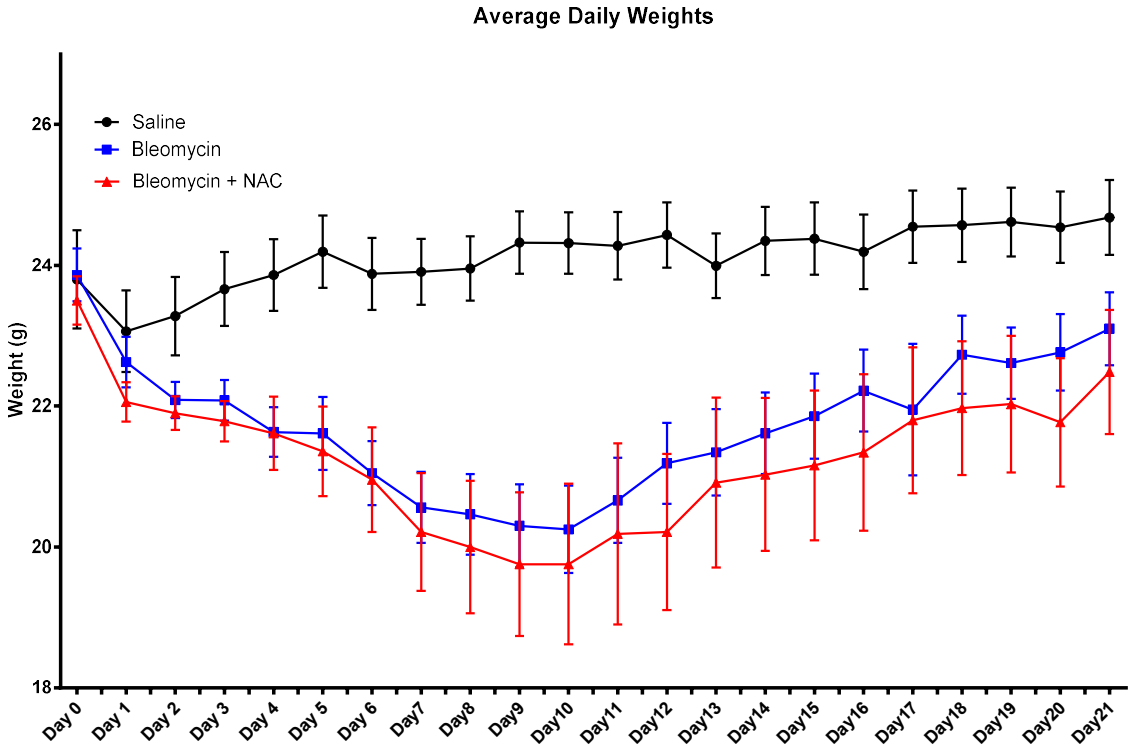

Figure Six. Graphical representation of daily mice weights by groups. Error bars represents means with standard error of the mean (SEM)

Differences in Pulmonary Function Parameters

Figure seven through figure thirteen outlines the results for different pulmonary function parameters. There is a non-statistically significant reduction in average inspiratory capacity (IC), average force vital capacity (FVC), forced expiratory reserve volume (FERV), and total lung capacity (TLC) in mice that received Bleomycin injections. In addition, the FEV_{100}/FVC ratio is preserved (Figure 13). This is in concordance with what is expected, as pulmonary fibrosis is a restrictive disease hallmarked by a reduction in FVC, with a FEV_1/FVC ratio greater than 80% in humans (100). A loss of lung elasticity due to replacement of healthy lung parenchyma with fibrosis will lead to an inability of the lung to expand appropriately during

inhalation, resulting in a decreased IC. Subsequently, decreased IC leads to decreased FVC, FERV, and TLC. There were no appreciable trends in the other pulmonary function parameters. Again, this is expected as pulmonary fibrosis is a restrictive disease. As such, expiratory volume and flow would be expected to remain normal. There was also no discernible differences between the Bleomycin + saline group versus the Bleomycin + Naclynamide™ groups. We would have expected to see improved pulmonary function parameters in our Naclynamide™ groups compared to the Bleomycin group; ideally, IC, FVC, FERV, and TLC in the Bleomycin + Naclynamide™ groups should parallel the saline control group. Indeed, we see that the lC, FVC, and FERV is improved in the Bleomycin + Naclynamide™ group when compared to the Bleomycin + saline group. One of the reasons that we did not see a significant difference is that there are variations in lung function between individual mice. As tracheostomy and insertion of a cannula for PFT is invasive, each mice could not serve as its own control. Therefore, we would need additional samples to decrease the amount of variation to enable detection of any significant differences.

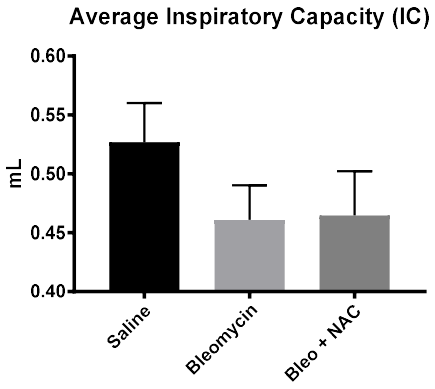

Figure Seven. Average Inspiratory Capacity (IC). Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

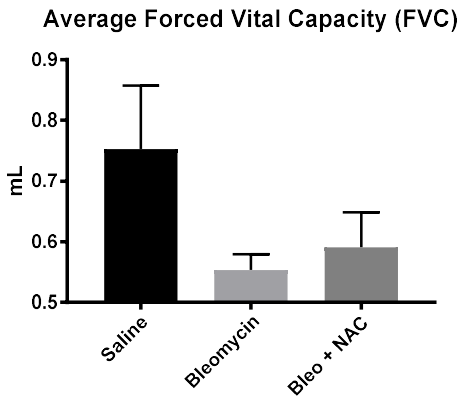

Figure Eight. Average Forced Vital Capacity (FVC). Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

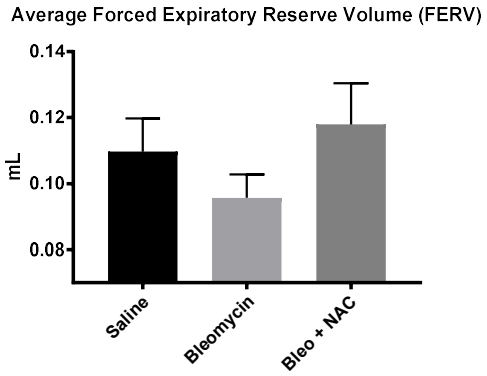

Figure Nine. Average Forced Expiratory Reserve Volume (FERV). Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

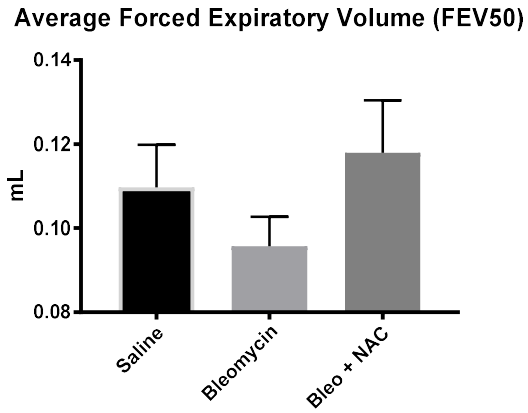

Figure Ten. Average Forced Expiratory Volume at 50 milliseconds (FEV_{50}). Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

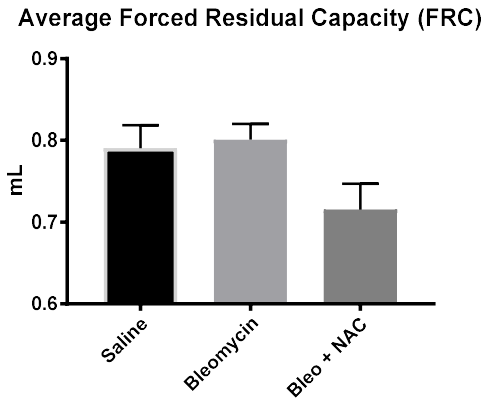

Figure Eleven. Average Forced Residual Capacity (FRC). Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

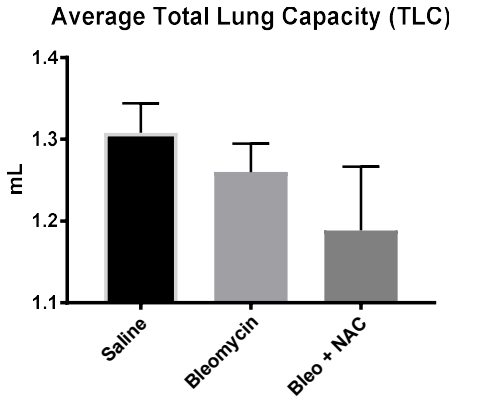

Figure Twelve. Average Total Lung Capacity (TLC). Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

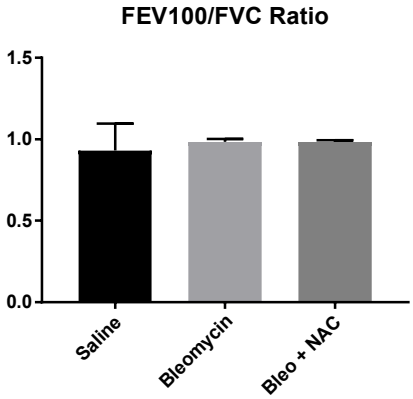

Figure Thirteen. Average FEV_{100}/FVC. Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

Histological Analyses and Ashcroft Scores

Figure 14 demonstrates representative images from our four groups: 1) saline only, 2) Bleomycin + saline, 3) Bleomycin + Naclynamide™, and 4) Bleomycin + delayed Naclynamide™. The average Ashcroft score for each group was: 1.467, 2.2866, 2.0134, and 1.533 (Figure 15). Bleomycin + saline group had the highest Ashcroft score, associated with the most severe fibrosis. On average, approximately two-thirds of the lung lobes had minimal fibrosis in the Bleomycin + saline group, with patches of intense fibrosis encompassing approximately the remaining one-third of the lobe. Lobes from the saline group had little to no fibrosis throughout the lung, with any signs of fibrosis appearing near the periphery, and likely scored above 0 due to under-inflation of the lungs, making the alveoli near the periphery thicker than normal, rather than fibrosis. Finally, lobes in the Bleomycin + Naclyamide™ had less patches of fibrosis compared to the Bleomycin + saline group, averaging one-fourth of the lung.

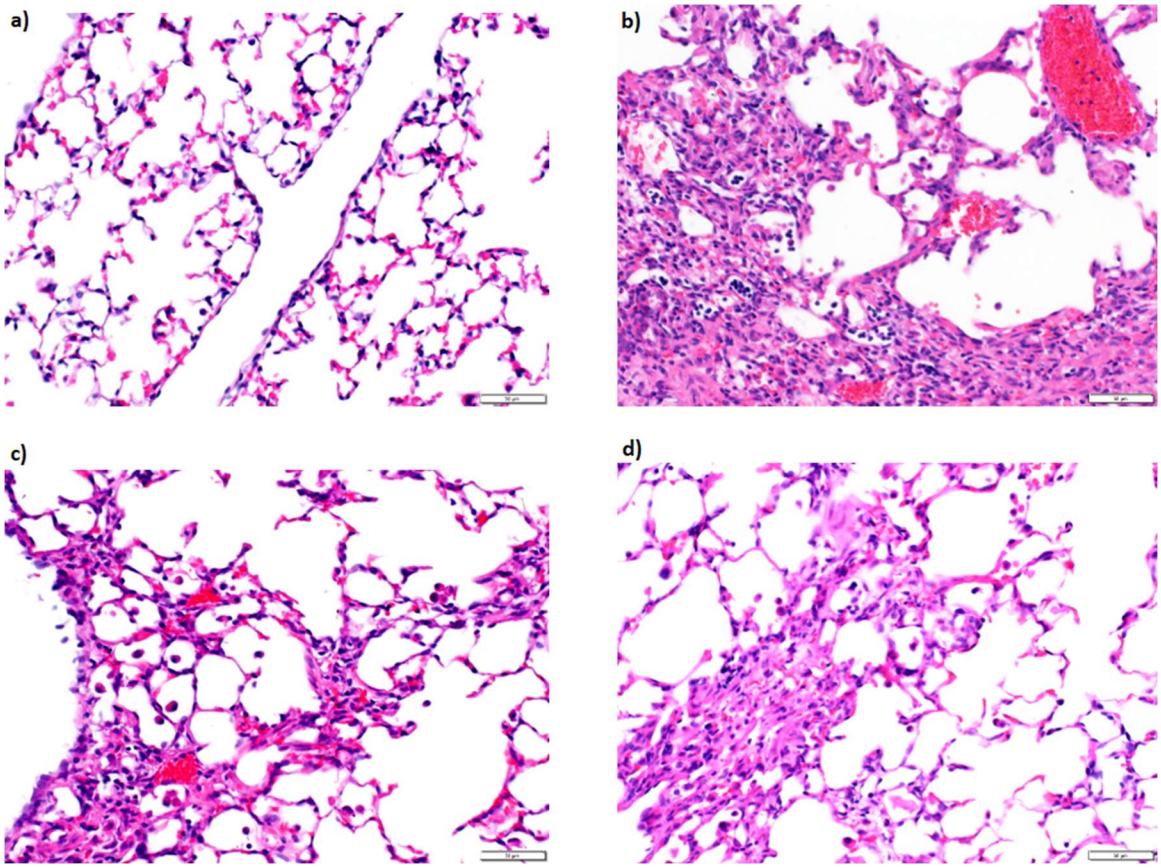

Figure Fourteen. Representative H&E slides for each group: a) saline only, b) Bleomycin + saline, c) Bleomycin + Naclynamide™, and d) delayed Bleomycin + Naclynamide™. The most intense fibrotic response was seen in the Bleomycin + saline group as expected. Saline group had little to no fibrosis, while Bleomycin + Naclyamide™ group had an intermediate fibrotic response.

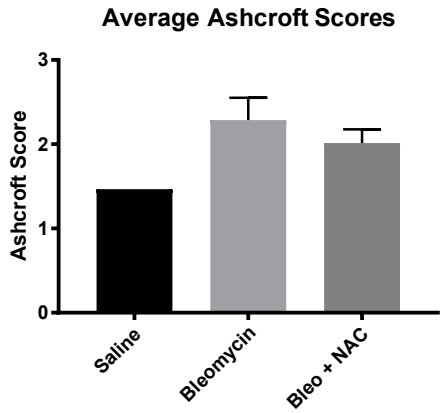

Figure Fifteen: Average Ashcroft scores for each group. Error bars represents means with standard error of the mean (SEM). Saline (n = 18), Bleomycin (n = 16), Bleomycin + Naclynamide™ (n = 7)

DISCUSSION

The overall aim of this study was to determine if there was an immune dysfunction that exists in peripheral leukocytes of CF patients, due to the *CFTR* mutation. Peripheral leukocytes instead of BAL-derived cells were used to isolate the possible confounding effects of the infectious environment of the lungs. Both gene and protein expression levels were analyzed to confirm any differences that were detected. Furthermore, we hypothesized that there was an association between the lung microbiome, clinical status, and cytokine expression, and that this relationship may have prognostic implications in targeting antibiotic therapy and maintenance of pulmonary function. Finally, current therapies for CF only aim to prevent further damage in the lungs; there are currently no available therapies to reverse fibrosis and restore lung function once damage has occurred. As such, anti-fibrotics are an attractive class of therapies that can improve the quality of life and life expectancy of CF patients.

Gene expression of in-vitro CF peripheral leukocytes compared to healthy controls

This study is the first study to the authors' knowledge that evaluates inflammatory cytokine expression levels in circulating from blood leukocytes of stable CF patients, and CF patients experiencing pulmonary exacerbations and control non-CF subjects. Previous studies examining inflammatory cytokines have used samples collected from bronchoalveolar lavages (BAL) or sputum. However, by evaluating cells isolated from an a chronically infective environment, results can be confounding due to inability to distinguish effects between potentially innate cell dysfunction and the unique chronic inflammatory environment of the CF lungs secondary to chronic bacterial infection and colonization. Sputum samples also have unclear sensitivity and specificity for lower airway tract infection due to inter- and intra-patient variations in sputum expectoration. Furthermore, regional differences in disease severity exist within the CF lung. Induced sputum likely comes from the proximal oropharynx, which may not

be representative of the most diseased segment of the lung (39). This limits their applicability to measure cytokine levels accurately. Blood samples are ideal since collections are standardized, readily accessible, and can be obtained from subjects in any age range and disease severity. By evaluating immune cells isolated directly from the peripheral blood, without exposure to the chronically infected lung environment, this study is directly address the question of whether CF immune cells have an innate dysfunction, or whether observations of abnormal cell functions are caused by exposure to the CF lung microenvironment. This is a key question regarding the pathophysiology of the chronic inflammatory response seen in CF. One hypothesis is that there is an innate dysfunction of immune cells in CF and imbalance of pro-inflammatory versus anti-inflammatory responses, leading to chronic and unrelenting inflammation independent of infection. Conversely, other investigators have suggested although immune cells in CF function normally, altered airway physiology and chronic microbial infection in the lungs result in abnormal and sustained airway inflammation. This study aimed to utilize leukocytes isolated from blood to distinguish whether or not there is an innate dysfunction of CF immune cells, or whether this dysfunction is secondary to the chronic inflammatory and infectious environment of the lungs.

This study demonstrated that leukocytes from CF patients have a higher baseline mRNA expression of NF-κB compared to healthy controls. In addition, CF leukocytes also continued to have an elevated NF-κB response after stimulation with LPS. It was previously thought that the increased NF-κB expression seen in CF was secondary to bacterial response by immune cells; bacterial LPS binding to toll-like receptors (TLR) and calcium accumulation in the endoplasmic reticulum leading to NF-κB pathway activation (101, 102). However, we demonstrated elevated NF-κB expression levels in cells in the peripheral circulation, confirming that NF-κB pathway activation occurs systemically, and not just within the infective lung microenvironment. This is

in accordance with previous studies, which suggests that localization of CFTR to lipid rafts are critical for regulation of NF-κB mediated immune responses via responsive signaling complexes to regulate cytokine expression (103). Wild type CFTR also binds to Tumor Necrosis Factor Receptor Type 1-Associated Death Domain Protein (TRADD), a key adaptor molecule involved in Tumor Necrosis Factor-alpha (TNF-α) signaling, which activates the NF-κB pathway downstream (104). CFTR inhibition of TRADD requires a functional CFTR protein. Mutations in the CFTR protein would thus lead to dysregulation and sustained activation of NF-κB. However, although our study showed that NF-κB gene expression was elevated, this did not result in an increased translational response. Inactive NF-κB binds with inhibitor IKB in the cytoplasm. After activation, NF-κB dissociates from IKB and translocate into the nucleus, where it binds to sequence specific promotor regions of its targeted genes to initiate transcription of pro-inflammatory cytokines. In our study, we noted that mRNA gene expression and protein translation do not necessarily correlate, as there are many regulatory pathways and modifications that occur after transcription and translation. The actual amount of NF-κB protein found active in the nucleus after LPS stimulation was significantly less when compared to healthy controls. The discrepancy between NF-κB gene and protein expression could be a result of decreased protein expression secondary to immune cell dysfunction, leading to loss of inhibitory feedback and increased gene transcription to stimulate more protein expression. For example, NF-κB activation results in transcription of inhibitors such as inhibitor of κB (IκBα) that works in a negative feedback loop mechanism (105). Decreased NF-κB would result in a decrease in inhibitors as well, leading to a sustained gene expression response. Furthermore, we also detected a significant decrease in NE, IL-1β, CCL-2, IL-6, and IL-10 protein levels in CF supernatant after LPS stimulation when compared to healthy control samples. IL-1β, CCL-2, IL-6, and IL-10 are directly modulated by the NF-κB pathway (23, 106-108); therefore, decreased

activation of the NF-κB pathway would result in decreased secretion of these cytokines. In addition, NE release from neutrophils is dependent on both TNF-α and IL-8 (109), which are also under the regulation of the NF-κB pathway. Looking at our IL-8 mRNA data, we noticed a significant variation in the healthy stimulated sample, which may be a reason why no significance was detected. Due to this variation, there was also no significant differences between the healthy unstimulated and stimulated groups. Further samples should be collected to determine if there is also a decrease in IL-8 protein levels after stimulation. Taken together, these findings suggest that the immune response observed in CF might not necessarily be due to hyper-inflammation but rather to immune cell dysfunction and exposures to bacterial infections. Specifically, there appears to be a reduced activation NF-κB as well as downstream inflammatory proteins such as NE, IL-1β, CCL-2, IL-6, and IL-10, suggesting a suppressed immune signaling and activation response. Previously studies have also suggested an impaired innate response in CF. BAL-derived neutrophils and macrophages showed decreased phagocytic activity, decreased antigen presenting capabilities, and decreased lymphocyte activation compared to non-CF controls (110). This data suggests that these observations may be a result of upstream defects in key inflammatory regulatory pathways.

In addition to an impaired innate response in CF, several studies have suggested functional abnormalities in the immune system of CF patients, such as defective apoptosis of immune cells (111-113). CF neutrophils demonstrate delayed apoptotic responses to the normal early pro-apoptotic effects of TNF-α, yet remain fully responsive to the anti-apoptotic effects of GM-CSF (111). This phenomenon can lead to the development of dysfunctional or exhausted immune cells, similar to dysfunctional T cells that are characteristic of chronic viral infections and cancer (114-117). Exhausted CD8 T-cells have been demonstrated to progressively lose the ability to produce inflammatory cytokines such as IL-2, TNF, and IFN-γ

(114, 117-119). As such, CF leukocyte exhaustion could also lead to dysfunction in NF-κB activation and cytokine secretion. CF leukocytes are especially prone to exhaustion due to chronic exposure to stimuli secondary to chronic bacterial colonization, in addition to an impaired apoptotic response, which prolongs the life of CF immune cells. In support of this, CF $CD4^+$ T-cells have also been shown to exhibit a markedly reduced IFN-γ secretory response after stimulation with anti-CD3, which normally induces a maximal IFN-γ in healthy controls (13). Furthermore, CF PBMC and BAL derived leukocytes also express significantly less IL-18, a NF-κB-regulated pro-inflammatory cytokine that stimulates IFN-γ production in T-helper type 1 cells, which we speculate may also be due to immune cell exhaustion (120). Decreased neutrophil apoptosis and functional exhaustion is also associated with a reduced respiratory burst, with a higher proportion of apoptotic neutrophils associated with better lung function (121). In addition, studies have indicated that the severity of CF lung disease is correlated with significant heritability, independent of the CFTR genotype (122). A genome-wide single nucleotide polymorphism scan revealed interferon-related development regulator–1 (IFRD1), a histone deacetylase dependent transcriptional co-regulator expressed during terminal neutrophil differentiation, as a modifier of CF lung disease severity (122). Subsequently, an IFRD1-/- mouse model demonstrated a significant decrease in NF-κB nuclear translocation and activation in LPS-stimulated neutrophils (123). Our study is in concordance with these studies, and suggests that IFRD1 deficiency in CF may also lead to a decrease in NF-κB activation and secretion of pro-inflammatory cytokines. Lastly, studies have proposed that lymphocyte Cl^- channels play a pivotal role in regulation of Ca^{2+} influx and subsequent cell activation and signal transduction (124). Therefore, mutations in the CFTR gene leading to translation of a defective Cl^- channel may result in cytokine expression dysfunction secondary to chloride flux alternations. This

presents as a potential target for development of new therapies aimed to increase cytokine expression to control or ameliorate infection and prevent clinical deterioration.

Gene expression and protein levels in stable CF patients compared to CFPE

Interestingly, CF patients experiencing a CFPE have a further decrease in NF-κB gene expression after LPS stimulation when compared to stable CF patients. This supports our hypothesis that CFPE may be due to an immune dysfunction, and that this dysfunction may be due to immune exhaustion. Although previous studies have demonstrated elevated levels of acute phase reactants and pro-inflammatory cytokines such as C-reactive protein, IL-6, and NE in BAL fluids during CFPE, it is currently unknown whether this elevation is an adequate response to resolve CFPE. In addition, previous studies have also suggested decreased cytokine expression in BAL samples from CFPE patients. Expression of TGF-β and IFN-γ were drastically reduced in CFPE patients; conversely, high levels of TGF-β and IFN-γ were associated with mild pulmonary disease and history of infrequently pulmonary exacerbations (42). These studies suggest that immune cell function may be further compromised during CFPE, leading to an inability to mount an effective response to resolve CFPE and the clinical symptoms that are the hallmark of CFPE.

Microbiome Analyses

The microbiome and presence of specific species of bacteria may be important in the pathophysiology of CFPE. It has been shown that decreased diversity with prevalence of a pathogen-dominated airway is associated with an increase in inflammation and subsequent structural lung damage (125). However, studies that aim to examine the correlation between lung microbiota, airway inflammation, and clinical presentation are limited and lacking. As such, our aim is to analyze the relationship between the lung microbiome, lung function, and cytokine expression. Furthermore, as we will be measure cytokine expression in both sputum and blood,

we will be able to determine whether blood cytokine levels correlate with sputum levels. Although the study sample for microbiome analyses is small, our exploratory data suggests several interesting findings that merit continuation of our analyses in a larger scale pilot study. First, there appears to be an inverse relationship between the Shannon index , which accounts for both species abundance and evenness in a community, with lower predicted pulmonary functions and cytokine levels. To the author's knowledge, this is the first study that examines the correlation between Shannon indexes and sputum inflammatory cytokine levels. Previously studies have demonstrated that decreased diversity is associated with decreasing lung function; patients with end stage lung disease often demonstrate a marked constriction in community diversity (44, 59, 126). Using 16s rRNA sequencing, a more accurate analysis of the microbiome can be performed and tracked over time. Sequencing performed during clinical stability can serve as a baseline to compare subsequent studies. Microbiome diversity and specific species populations can be monitored over time to detect any shifts in evenness, which may indicate the early onset of CFPE. This would provide an optimal opportunity to initiate treatment before the clinical deterioration in pulmonary function that is the hallmark of CFPE.

Murine Pulmonary Fibrosis Model

A murine pulmonary fibrosis model was developed to test the anti-fibrotic properties of the NLRP3 inhibitor Naclyamide™. Bleomycin was used to create pulmonary fibrosis, and this was administered directly into the lungs via intratracheal injection. Our hypothesis is that Naclynamide™ may be able to prevent fibrosis in the lungs, resulting in improvements in pulmonary function and median survival rate. First, our study demonstrated that weight can be a reliable surrogate for a successful intratracheal Bleomycin injection. The saline group lost weight only on day 1, with subsequent recovery in weight soon after (Figure 4). This is most likely due to the stress of the initial surgery on day 0. Conversely, mice that have received

Bleomycin continued to lose weight until approximately day 9. After this time period, mice begin to gain weight. This most likely represents a self-limiting response to the Bleomycin that begins to resolve itself after this time period. Furthermore, the relatively short induction time to fibrosis (2—4 weeks with intratracheal injection method) makes Bleomycin the most utilized model (127).

Although our murine pulmonary fibrosis model did not demonstrate any statistical differences in pulmonary function, several trends were noted on the PFTs that mirror PFTs in patients with pulmonary fibrosis. In particular, we noticed a decrease in IC, FVC, and FERV, with a preserved FEV_1/FVC ratio, which is indicative of a restrictive pulmonary pattern that is consistent with disease caused by pulmonary fibrosis (128). One explanation for the non-statistical significance between PFTs is the difference in lung function between individual mice. As demonstrated by the error bars in Figures 5-10, there are significant variations in pulmonary functions between individuals in each group. Genome-wide association studies have identified multiple loci that are related to pulmonary function, including loci that are associated with clinical phenotypes of airflow obstruction, COPD, or asthma (129). Ideally, to circumvent this issue, each mouse would serve as its own control. Unfortunately, insertion of a tracheostomy tube for PFT measurements is highly traumatic, and there is a high mortality and morbidity associated with this procedure. As such, each mouse is tracheostomized only once at the end of the study, then euthanized once PFTs are completed. As such, we will continue to recruit additional mice in both the saline control and Bleomycin + saline groups to establish a difference. It is currently planned to have 30 samples in each group, as the sampling distribution should approach a normal bell curve. Subsequently, we can test our drug to see if it does have anti-fibrotic properties. By increasing the number of samples in each group, the variation in pulmonary function should decrease, and we would expect to find significant

differences in IC, FVC, FERV, and TLC. As we currently do not have significant differences between our two control groups (saline only and Bleomycin + saline), we cannot determine if Naclynamide™ has any effects on fibrosis. However, it does appear on histology that there were less areas of fibrosis compared to the Bleomycin + saline group, along with a non-significant decrease in Ashcroft scores. Further studies will need to be conducted to investigate this trend. Finally, because we are administering Naclynamide™ using a retro-orbital injection technique, we cannot be certain if the drug has reached the pulmonary parenchyma. Pharmacokinetic studies will need to be performed and lung tissues analyzed to determine the optimal route, dosage, and timing of administration.

Limitations and Future Directions

Several weaknesses exist in our study that warrant discussion. First, we only used one stimulus to activate leukocytes, with LPS acting via the LPS receptor signal transduction pathway. LPS was chosen due to the fact that it is the most common stimulus of immune cell activation in CF, especially for leukocytes in the lungs where it is chronically exposed to bacterial colonization. This will also allow us to compare how LPS-activated peripheral immune cells behave in comparison to previous studies of BAL-derived immune cells. Other pathways of activation that were not studied include: phorbol 12-myristate 13-acetate (PMA)—anti CD3 activation of T cells through ligation of the T-cell receptor, and PMA—ionomycin activation of T cells through extracellular Ca^{2+} influx (13). In reality, immune system activation is most likely achieved through activation and feedback from multiple pathways working in synergy, and study of one specific pathway represents an oversimplified approach to this mechanism. However, it is interesting to note the reduction in inflammatory protein expression after LPS stimulation found in this study, and thus further studies are needed to confirm these findings with other stimuli as well. Second, we only examined one time point after LPS stimulation. The

time for maximal cytokine expression differs between different cytokines and stimulants, with studies reporting times varying from 6 to 72 hours (130-133), depending if the cytokine is an early, intermediate, or late response protein. In the future, running a time course to map cytokine expression kinetics would be useful to determine if there are differences with different time points. Third, the ability to collect adequate sputum samples decrease with age. Induced sputum collection is very difficult in infants and children, and because of the invasive nature of BAL they are not considered standard of care. Therefore, further research examining to relationship between immune cell dysfunction and contribution of infection is warranted to identify specific cell subsets or released proteins that can be targeted in a time- and inflammation-specific manner in CF lung disease. Fourth, most of the CF patients are chronically taking azithromycin, a macrolide antibiotic that has been shown to demonstrate anti-inflammatory properties (134). Experiments using an LPS-induced lung neutrophilia mouse model have demonstrated that azithromycin can inhibit production of GM-CSF and IL-1β by macrophages (135). Furthermore, azithromycin has been shown to inhibit NF-κB nuclear translocation, with a resultant reduction in pro-inflammatory cytokine levels such as TNF-α, G-CSF, and CCL-2 in BALs (136). However, other cytokines regulated by NF-κB such as IL-1β and IL-6, were not affected (136). As such, additional studies are warranted to investigate the molecular mechanism of the anti-inflammatory properties of azithromycin, and to determine whether this is contributing to the immune dysfunction that is inherent in CF immune cells. Fifth, we were not able to finish all of the sample collections for our study. We were only able to collect 3 sputum samples for 16s rRNA sequencing and microbiome analyses. Interestingly, our preliminary data suggests that there is an inverse relationship between the Shannon index with lower predicted pulmonary functions and cytokine levels. Additional samples are merited to confirm these findings and assess their usefulness in clinical applications. Furthermore, our

current study does not provide longitudinal data for individual patients. However, our current preliminary data demonstrates that 16s rRNA sequencing is cost-effective and that microbiome analyses may have clinical implications that can guide therapy. As such, longitudinal data would ideally be collected as part of a future study. Finally, we did not establish significance in pulmonary function in our murine pulmonary fibrosis model, most likely due to inadequate sample sizes. However, our model was able to demonstrate important trends, such as decreases in IC, FVC, FERV, and TLC that correspond to a restrictive lung disease, such as pulmonary fibrosis. Additional samples will be needed to establish significance and to determine if Naclynamide™ has anti-fibrotic properties and is able to prevent or reverse fibrosis.

CONCLUSION

This study is the most extensive study to date that examines cytokine and inflammatory protein expression in peripheral blood cells of CF patients. In addition, this is the only study to the author's knowledge that examines the relationships between cytokine expression, pulmonary exacerbations, microbiome constituents, and lung function. The origin of inflammation in CF is controversial; and out study was designed to evaluate whether inflammatory changes in CF precedes and occurs independently of infection, and may be secondary to a basal defect in CF leukocytes. Our study demonstrated that CF leukocytes do in fact demonstrate impaired cytokine regulation and expression, reflecting a systemic effect that is independent of exposure of immune cells to the chronic infectious environment of the lungs. Specifically, although there was increased NF-κB mRNA transcription, there was a decrease in protein expression of downstream regulatory cytokines, such as NE, IL-1β, CCL-2, IL-6, and IL-10. These findings may have widespread clinical applications that could alter therapy options for treatment of CF. Currently, a mainstay of therapy in CFPE is anti-inflammatory drugs, such as NSAIDs and corticosteroids. If a dysfunction inflammatory protein response is associated with the pathophysiology of CFPE, then anti-inflammatory drugs may further exaggerate this dysfunction and result in a detrimental response. Conversely, agents that can increase immune cell responses should be studied. One of our speculation is that immune dysfunction could be due to decreased apoptosis in immune cells, leading to cell exhaustion. Therapies that can increase apoptosis may be able to restore immune cell function and prevent CFPE. Furthermore, how the pulmonary microbiome contributes to inflammation and its correlation to clinical status is currently unknown. Restoration of homeostasis and diversity within the microbiome may be a key to prevention and treatment of CFPE.

Lastly, Naclyamide™ is a novel approach and potentially important adjunct to treatment for CF. There are currently no agents that can restore lung function once damage has occurred. It is well recognized that inflammation is an inciting event that leads eventually to fibrosis, through secretion of cytokines such as TGF-β, IL-4, IL-13, among others (137). Therefore, Naclyamide™ as an inhibitor of NLRP3, may represent a new class of medication that can be effective in restoring pulmonary function in CF.

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CURRICULUM VITAE

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EDUCATION

Wake Forest University Graduate School of Arts and Sciences — August 2016–Present
Winston-Salem, NC
Masters of Science Candidate
Molecular Medicine & Translational Science

University of Queensland/Ochsner Clinical School — Jan. 2010–Dec. 2013
Brisbane, Australia/New Orleans, LA
Bachelor of Medicine, Bachelor of Surgery

University of California, San Diego — July 2007–June 2009
La Jolla, CA
Bachelor of Science, *Cum Laude*, Human Biology
GPA: 3.76/4.0
Honors: UCSD Provost's Honors (Fall 2007 – Spring 2009)

Mount San Antonio College — July 2005–May 2007
Walnut, CA
General Studies
GPA: 3.23/4.0
Honors: Dean's List (Fall 2006-Spring 2007)

POST-GRADUATE EDUCATION AND TRAINING

University of Florida School of Medicine — July 2015–June 2016
Department of General Surgery
Gainesville, FL
General Surgery Residency

Mayo Clinic School of Graduate Medical Education — July 2014–June 2015
Department of General Surgery
Rochester, MN
General Surgery Internship

CERTIFICATIONS

Advanced Cardiovascular Life Support, American Heart Association
Issued: 2017. Status: Active

Advanced Trauma Life Support, ACS Committee on Trauma
Issued: 2014. Status: Active

Basic Life Support, American Heart Association
Issued: 2017. Status: Active

MEDICAL LICENSURE

Physician License, Medical Board of California
#A153154. Issued: December 2017. Status: Active

PROFESSIONAL AND SOCIETY MEMBERSHIPS

- National Postdoctoral Association, Affiliate Member 2017–Present
- American Association for the Advancement of Science, Member 2017–Present
- American College of Surgeons, Resident Member 2012–Present
- The Society of Thoracic Surgeons, Pre-Candidate Member 2012–Present
- Minnesota Medical Association, Resident Member 2014–2015
- Zumbro Valley Medical Society, Resident Member 2014–2015
- American Medical Association, Student Member 2010–2013
- American College of Physicians, Student Member 2010–2013
- Alpha Epsilon Delta Pre-Medical Honor Society, UCSD Chapter 2007–2009

HONORS AND AWARDS

- 3rd Place, Wake Forest Institute of Regenerative Medicine 2018 Retreat Poster Competition January 2018
- 2nd Runner-Up, Committee on Trauma and Acute Care Surgery Resident Paper Competition, American College of Surgeons NC/SC Chapter Meeting July 2017
- Best Poster Award, The Future of Regenerative Medicine Congress May 2017
- UCSD Provost's Honor 2007-2009
- Mount San Antonio College Dean's List 2006-2007

GRANT SUPPORT

- Christopher L. Moseley Foundation Grant for Stem Cell Research 2017–Present
- Cystic Fibrosis Foundation Research Grant 2016–2017
- T32 Ruth L. Kirschstein National Research Service Award (NRSA) Research Training Grant 2016–2018

PROFESSIONAL ACTIVITIES

- Journal Reviewer for: Nature Medicine 2018–Present
- Journal Reviewer for: Stem Cells Translational Medicine 2016–Present
- Journal Reviewer for: Bioprinting 2016–Present

RESEARCH PROJECTS

Wake Forest Institute of Regenerative Medicine 2016–Present
Winston-Salem, NC
Mentors: Sean V. Murphy, PhD, Antony Atala, MD. Investigated the role of inflammation and etiology of pulmonary exacerbations in patients with cystic fibrosis. Performed assays such as PCR, RNA extraction, ELISA, and flow cytometry.

Wake Forest Institute of Regenerative Medicine 2016–Present
Winston-Salem, NC
Mentors: Sean V. Murphy, PhD, Cara Clouse, DVM, Antony Atala, MD. Established cystic fibrosis model in ferrets and tested stem cells as therapy to decrease pulmonary inflammation.

Wake Forest Institute of Regenerative Medicine 2016–Present
Winston-Salem, NC
Mentors: Sean V. Murphy, PhD, John Jackson, PhD, Cara Clouse, DVM, Antony Atala, MD. Established lung fibrosis model in mice and tested drugs aimed to reverse/improve lung fibrosis and inflammation.

Wake Forest Baptist Health, Department of Surgery 2016–Present
Winston-Salem, NC
Mentors: Daniel Couture, MD, J. Jason Hoth, MD/PhD. Retrospective study on variables that predict worsening repeat head CT after mild traumatic brain injury, and the need to consult neurosurgery.

Wake Forest Baptist Health, Department of Surgery 2016–Present
Winston-Salem, NC
Mentors: Jason Halvorson, J. Jason Hoth, MD/PhD. Retrospective study on efficacy and outcome of orthopedic trauma residents responding to all traumas.

Mayo Clinic College of Medicine, Department of Cardiac Surgery 2014-2015
Rochester, MN
Mentor: Joseph Dearani, MD. Analyzed the pearls and pitfalls of congenital cardiovascular operations for genetic syndromes.

Ochsner Clinical School, Department of Cardiothoracic Surgery 2013–2017
New Orleans, LA
Mentor: Patrick E. Parrino, MD. Retrospective analysis of concomitant mitral and tricuspid valve operations.

Ochsner Clinical School, Department of Cardiology 2013–2016
New Orleans, LA
Mentor: Sangeeta Shah, MD. Established exercise and diet guidelines for patients that underwent congenital heart surgery.

Ochsner Clinical School, Department of OB/GYN 2012–2012
New Orleans, LA
Mentor: Robert C. Moore, MD. Retrospective study on variables that predict latency in patients with preterm premature rupture of membranes

University of California, San Diego, Department of Biology 2008–2009
La Jolla, CA
Mentor: Dmitri Nusinow, PhD. Analyzed genes that control circadian rhythm in plants.

PUBLICATIONS

PEER-REVIEWED PUBLICATIONS

1. **Leung ST**, Khoury O, Barrios C, Ortega VE, Murphy SV. Immune Exhaustion and Cytokine Dysfunction in Cystic Fibrosis. The Journal of Cystic Fibrosis. Submitted.
2. **Leung ST**, Luo TD, Parrino PE. Comparison between Mitral Valve and Concomitant Mitral with Tricuspid Valve Operations: A Retrospective Analysis. Wake Forest Journal of Science and Medicine. Volume 3: Issue 1, Fall 2017, ISSN 2380-3991.
3. Rosenthal TM, **Leung ST**, Ahmad R, Young T, Lavie CJ, Moodie DS, et al. Lifestyle Modification for the Prevention of Morbidity and Mortality in Adult Congenital Heart Disease. Congenital heart disease. 2016;11(2):189-98.

BOOK CHAPTERS

1. **Leung ST,** Overschmidt O, Allickson J, Cetrulo K, Taghizadeh RR, Couto PS, Atala A, Murphy SV. Review of Processing Technology and Techniques for Perinatal Stem Cells Banking and Clinical Applications. Perinatal Stem Cells: John Wiley & Sons, Inc.; 2017. In Revision.

ABSTRACTS

1. **Leung ST**, Luo TD, Parrino PE. Reduced Aortic Cross-clamp Time May Reduce Morbidity during Concomitant Valvular Operations. Case Reports in Surgery and Invasive Procedures. Volume 1: Issue 3, October 2017.
2. **Leung ST**, Khoury O, Murphy SE. Immune Dysregulation during Cystic Fibrosis Pulmonary Exacerbations. Poster Session Abstracts. Pediatric Pulmonology. 2017;52(S47):S214-S516.

POSTERS AND ORAL PRESENTATIONS

- The Wake Forest Repeat Head CT Rule for Mild TBI with Intracranial Hemorrhages. Poster at American Association of Neurosurgical Surgeons. New Orleans, LA. April 2018.
- Reprogramming Cell Therapy for Pulmonary Fibrosis. Poster at the 2018 Wake Forest Institute for Regenerative Medicine Retreat. Pinehurst, NC. January 2018
- Perinatal Stem Cells for the Treatment of Inflammatory Lung Diseases. Oral presentation at the World Stem Cell Summit. Miami, FL. January 2018.
- Immune Dysfunction during Cystic Fibrosis Pulmonary Exacerbations. Poster at North American Cystic Fibrosis Conference. Indianapolis, IN. November 2017.
- Comparison between Mitral Valve and Concomitant Mitral with Tricuspid Valve Operations: A Retrospective Analysis. Poster at International Surgery Conference. Toronto, CA. October, 2017.
- Variables that Predict Radiographic Progression after Mild Traumatic Brain Injury. Oral presentation at American College of Surgeons, NC/SC Chapter Meeting. Pinehurst, NC. July 2017.
- Perinatal Cell Therapy for the Treatment of Inflammatory Lung Disease. Poster at the Future of Regenerative Medicine Congress. Teaneck, NJ. May 2017.
- Necessity of Repeat Head CT after Head Injury with ICH. Wake Forest University Surgical Research Day. Winston-Salem, NC. October 2016.

EXTRACURRICULAR

Wake Forest Graduate School 2017–Present
Student Representative, Molecular Medicine and Translational Sciences Executive Committee

Wake Forest Graduate School Association 2016–Present
Secretary

Ochsner Health System Institutional Review Board 2012–2014
Permanent Member

University of California, San Diego 2008-2009
Teaching Assistant, Department of Biology

PERSONAL

Fluent in Cantonese Chinese. Interests include badminton, tennis, hiking, fishing, cooking, and attending music and food festivals.