NEUTROPHIL EFFECTOR RESPONSES IN CYSTIC FIBROSIS LUNG DISEASE

by

KAYLA M. FANTONE

(Under the Direction of Balázs Rada)

ABSTRACT

Cystic Fibrosis (CF) lung disease is responsible for most of the morbidity and mortality in these patients. CF airway disease is characterized by decreased mucociliary clearance, chronic, polymicrobial infections and robust, neutrophil-dominated inflammation. Progression of lung disease is attributed to chronic inflammation by neutrophils and persistence of bacterial infections. *Staphylococcus aureus* (*S. aureus*) is the most prevalent infectious agents in the respiratory tract of CF patients and neutrophils represent the most important immune cell type to fight this pathogen. Furthermore, the CF lung is abundant with inflammatory markers released from increased activation of neutrophils, including neutrophil elastase (NE) that can further damage the lung tissue in these patients. NE contributes to worsened lung damage and inhibition of NE is an important target to combat further lung damage in these patients. We hypothesized that the CF airway environment impairs *S. aureus* killing by neutrophils and the NE inhibitor, ecotin inhibits NE to prevent CF lung pathology without compromising neutrophil-mediated bacterial clearance. Our results show that PMNs exposed to the CF lung environment are impaired in *S. aureus* killing and this impairment is caused by the deficiency of phagolysosome fusion of neutrophils. Our results also show that ecotin is an

effective NE inhibitor in CF airway samples. Altogether, we have discovered a novel pathway in which CF neutrophils in the airways are defective in killing *S. aureus*. We have also uncovered a new potential therapeutic to improve lung damage by NE, as ecotin shows promising effects of NE inhibition.

INDEX WORDS: Cystic Fibrosis, Neutrophils, *S. aureus*, NE

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOPSHY

ATHENS, GEORGIA

2023

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May 2023

DEDICATION

This work is dedicated to all the CF patients who have lost their lives from this disease while awaiting a cure. I would also like to dedicate my thesis to those patients who donated their samples to our research. The major accomplishments made to better understand this disease would not have been done without their generous donations of their biospecimen samples. I would also like to dedicate this work to my grandparents who passed away during my PhD journey. They were my biggest support system and always dreamed of seeing me complete my PhD. I know they are with me in writing this dissertation and I could not have started this process without them. Lastly, I would like to dedicate this work to my two cats, Buddy and Sativa. They have been my biggest emotional support as they have given me so much love during this dissertation. All of these individuals have helped make this work happen and deserve to have this work dedicated to them.

ACKNOWLEDGEMENTS

I would like to first acknowledge my PhD mentor, Dr. Balázs Rada who took me into his lab as a first year with open arms and has supported me throughout this process. We did not expect this work to go into the direction it did, and I am so thankful for his support to study new exciting things and allow me to grow as a scientist in his lab. I would also like to acknowledge my committee members, Dr. M. Stephen Trent, Dr. Wendy Watford, Dr. Eric Lafontaine, and Dr. Joanna Goldberg for support, input, and advice on this work and for my future career.

This work would not have the relevance it does without our collaborators at Emory University, Dr. Arlene Stecenko and Dr. Joanna Goldberg. I would like to thank them for all the support and advice on this project and for providing us with the clinical CF samples. With that, I would like to acknowledge Chris Driggers for helping with the CF sample processing and pickup, making this a smooth, easy process. This work could not have been done without them. I would also like to thank all the human donors for their clinical sample donations to help allow this work to continue. For the ecotin project in particular, I would like to acknowledge our collaborators, Dr. Christine Szymanski and Dr. Harald Nothaft for the generation of ecotin, as this work could not have been done without them as well.

Lastly, I would like to acknowledge the lab members who have been my biggest support system and whom I have made lifelong friends with. These individuals are Demba, Sam, Arthur, Yeongseo, Faris, Ruchi, and Nathalie. To Demba and Sam

especially for always pushing me to be the best I can be in and outside of the lab. Finally, I would like to thank my friends and family for supporting my dreams and being their when I needed you.

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CHAPTER 1

INTRODUCTION

Cytstic Fibrosis (CF) is a genetic disease that affects 80,000 individuals worldwide[1]. The CF Foundation Patient Registry (https://www.cff.org/Research/Researcher-Resources/Patient-Registry/) estimates about 1,000 newly diagnosed patients every year and about 30,000 people in the United States (U.S.) have this disease[2]. The life expectancy of CF is 53 years old, which although has substantially jumped from 38 in recent years, the disease has yet to be cured[3]. CF originates from mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene and affects many organs, but CF morbidity and mortality are primarily due to lung disease and complications with polymicrobial lung infections[4,5]. The CF airway is characterized by polymorphonuclear neutrophil (PMN)-mediated inflammation that begins early in life and worsens over time. The CF airway is also abundant with polymicrobial infections such as, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*)[6–8]. *S. aureus* is one of the earliest pathogens recovered in the airways of CF children, it persists throughout the life of CF patients, and over a decade ago, surpassed *P. aeruginosa* as the most common airway pathogen in CF[6–8]. Therefore, *S. aureus* is the most prevalent pathogen in the respiratory tract of CF patients and the large neutrophil numbers present in their airways make them the most important immune cell to fight *S. aureus*. However, in CF, neutrophils are unable to kill bacterial pathogens

present in the lung and neutrophil infiltration and inflammation cause more severe lung damage to these patients[9].

Neutrophils' primary killing mechanisms against *S. aureus* involve intracellular killing through phagocytosis and extracellular killing by neutrophil extracellular traps (NETs). NETs contain many antimicrobial proteins including neutrophil elastase (NE) that can further damage the lung tissue in these patients. NE enzymatic activity is abundant in the CF lung and NE inhibitors have been shown to attenuate lung injury in animal models[10]. Many studies have shown that using an NE inhibitor to combat respiratory diseases results in a decrease in neutrophil numbers, enhanced clearance of bacteria, reduced lung injury, and higher survival in animal models[10,11]. However, the NE inhibitors that have been developed so far have been withdrawn due to the lack of efficacy, toxicological problems, or side effects in these patients[10]. Our previous research shows that ecotin, a microbial inhibitor of serine proteases broadly targets NE[12]. Therefore, our goal was to assess the effect of the CF airway environment on *S. aureus* killing by neutrophils and whether ecotin efficiently inhibits NE in CF airway samples. Altogether we aimed to describe CF neutrophil effector responses to *S. aureus* clinical isolates and to identify strategies to improve bacterial killing and prevent lung damage in CF patients.

Specific Aims

Our central hypothesis is that the CF airway environment impairs *S. aureus* killing by neutrophils and the NE inhibitor, ecotin, inhibits NE to prevent CF lung pathology without compromising PMN-mediated bacterial clearance.

Specific Aim 1: Describe neutrophil effector responses to CF clinical isolates of *S. aureus* in the CF lung environment.

Specific Aim 2: Determine the mechanism by which the CF lung environment impairs *S. aureus* killing by neutrophils.

Specific Aim 3: Determine the effects of ecotin, an NE inhibitor, on NE activity in different compartments of the CF sputum and on anti-staphylococcal mechanisms of neutrophils.

CHAPTER 2

NEUTROPHIL INFLAMMATION IN CYSTIC FIBROSIS LUNG DISEASE

Cystic Fibrosis Genetic Disease

CF is a life-limiting autosomal, recessive genetic disease that primarily affects Caucasians, although it has been reported in other ethnicities[13]. In 1989, a team of researchers discovered the CFTR gene, which caused a significant breakthrough for genetic diseases as this was the first-disease causing gene to be discovered[14–17]. The disease is caused by a mutation in the CFTR gene on chromosome 7, with F508del (ΔF508) being the most common, affecting two thirds of the population[15–18]. The CFTR functions as a chloride channel regulated by cyclic AMP-dependent phosphorylation and allows for ion transport into and out of cells[15–18]. The CFTR protein is present in the apical membrane of epithelial cells in the respiratory tract, pancreas, liver, gallbladder, intestines, sweat glands, and the reproductive tract[19]. The chloride channel is essential for the osmotic balance of mucus and regulates other ion channels such as the epithelial sodium channel (ENaC) that has a primary role in salt absorption[19]. The CFTR is also expressed in innate immune cells compromising the immune system and inflammation resulting in reduced mucociliary clearance, harming many of the bodies' mucus-producing organs[19]. Damage of these organs causes morbidity and mortality in these patients, with lung disease being the most detrimental.

Almost 2,000 variants have been reported to the Cystic Fibrosis Mutation Database and have been grouped into 6 functional classes, 40 percent (%) are predicted to cause a substitution of a single amino acid, 36% alter RNA processing, 3% cause large

rearrangements of the CFTR, 1% affect the promoter region, 14% are neutral variants, and 6% remain unclear[13,20]. The degree of alteration of the ion transport is determined by the effect of each disease-causing variant and the quantity/function of the CFTR[13]. CFTR is composed of three major motifs: domains that interact with ATP, known as nucleotide-binding domain 1 and 2 (NBD1 and NBD2 respectively); regions that anchor the protein in the membrane known as the membrane spanning domain 1 and 2 (MSD1 and MSD2 respectively); and a site containing numerous sites for phosphorylation known as the regulatory domain (R domain)[13]. The quantity of the CFTR protein produced in the apical cell membrane is based upon the amount of RNA transcribed, the efficiency of RNA splicing, the fraction of correctly folded protein, and the stability of the protein in the membrane[13]. Class 1 mutations also known as protein production mutations, produce premature termination signals by splice site abnormalities, frameshifts due to insertions or deletions, or nonsense mutation affecting NBD1, NBD2, and MSD1. Mutations in this class produce little to no full-length protein causing a complete loss of the chloride channel function in affected cells[20]. Class II also called protein processing mutations, affect protein processing as they fail to traffic to the correct cellular location, affecting NBD1 and NBD2[20]. The most common mutation is class II in which the CFTR containing mutation fails to mature to the fully glycosylated form and the protein is degraded[20,21]. This mutation is also called the gating mutation because the nascent protein folds incorrectly and is recognized as abnormal causing complete degradation of the protein rather than movement to the plasma membrane[20,21]. Class III mutations are mutations in NBD1 and NBD2 affecting the regulation of the opening of the CFTR chloride channel[20]. Intracellular ATP regulates the opening of the chloride channel

through binding of the NBDs and this mutation affects the binding site of the NBD[20]. Class IV mutations also known as conductance mutations, affect the MSDs because they contribute to the channel pore and a number of CF-associated missense mutations have been identified here[20]. This mutation affects the arginine residues located in the putative MSD sequences and the protein is fully processed and present in the plasma membrane, however the shape of the channel changes making it difficult for chloride to move through easily[20]. Class V mutations also known as insufficient protein mutations result in a reduced amount of CFTR in the apical cell membrane[22–24]. These mutations cause mRNA mis-splicing or inefficient splicing of exons interfering with the promoter activity, meaning that mRNA translation is halted and CFTR protein is not produced[22–24]. Lastly, Class VI mutations also called protein instability mutations, reduce CFTR expression by facilitating the channel removal from the plasma membrane[23,25]. It is caused by truncation of the C-terminal by a frameshift mutation or premature stop codons influencing the channel gating[23,25].

Although the multiple genetic variants render this disease as complex to cure, several CFTR modulator therapies have been developed which have made a tremendous impact on the disease. These modulators are designed to correct malfunctioning protein produced by the CFTR mutations and include ivacaftor, lumacaftor, tezacaftor, and elexacaftor. Ivacaftor helps CF patients that have the G551D gating mutation as it binds to the defective protein at the cell surface and opens the chloride channel allowing for the free flow of chloride ions into and out of the cells [26]. Lumacaftor itself was shown to have no significant improvement in lung function in CF patients, therefore Orkambi® was generated which is a lumacaftor/ivacaftor combination therapy to help patients that have

two copies of the ΔF508 mutation[27,28]. Lumacaftor is known as the corrector which helps the CFTR protein form the right shape, traffic to the cell surface and stay longer, and ivacaftor helps hold the gate open for chloride to flow through[27,28]. Tezacaftor acts in the same way as lumacaftor and Symdeko® was created as a combination therapy of ivacaftor and tezacaftor[29]. The difference between Orkambi® and Symdeko® is that it has been shown to have fewer side effects and is approved for patients that only have a single copy of the ΔF508 mutation[29]. The newest modulator is Trikafta® which is a triple combination therapy of elexcaftor, tezacaftor, and ivavaftor. Elexcaftor is also a CFTR corrector that works on alternative binding sites than tezacaftor on the CFTR protein to facilitate the functionality of the protein to the cell surface[30]. The triple combination therapy increases the function of the ΔF508 mutated CFTR protein at the cell surface resulting in increased chloride transport into and out of the cell[30]. Elexacaftor corrects an addition flaw in the formation of the ΔF508-CFTR protein, it helps the CFTR protein perform better and has helped a greater number of patients[30]. CFTR modulators estimate to benefit about 80% of CF patients if given early in life to help the restoration of CFTR function. However, 20% of patients with CF are not eligible for CFTR modulator therapy due to more rare mutations and new approaches are needed for these patients[31–33]. Also, many patients have succumbed to irreversible lung damage as therapies were not previously available, suggesting that therapeutics to target lung complications are also needed.

Cystic Fibrosis Pathophysiology

CF is a chronic progressive disease affecting multiple organ systems and the most common clinical signs include chronic cough, diarrhea, and malnutrition[34]. The most common organ systems affected in CF patients are the respiratory tract and the digestive tract, with respiratory tract complications leading to death in 90% of patients[34–36]. Gastrointestinal disorders are the earliest clinical manifestations shown in these patients, and within the digestive tract, a common symptom is exocrine pancreatic insufficiency resulting in chronic diarrhea and malnutrition[34,37]. This is due to the decrease in secretion of sodium bicarbonate which reduces the efficacy of pancreatic enzymes and the precipitation of bile salts[34,37]. As previously mentioned, the disruption of chloride ions moving freely into and out of epithelial cells causes the overproduction of mucus which prevents the release of enzymes into the duodenum in the intestines, resulting in poor digestion of fat, protein, and carbohydrates[34,37]. The endocrine pancreas is also affected, resulting in diabetes related to glucose intolerance and decreased insulin secretion[37,38]. Pancreatic exocrine insufficiency is the leading cause for intestinal malabsorption and affects about 90% of patients[34,39]. The liver is also affected in these patients from the overproduction of thick biliary secretion causing cirrhosis, accounting for about 2.5% of mortality in these patients[40–42]. About 5%-10% of CF patients develop multilobular cirrhosis during their first ten years of life leading to more complications of hypertension and variceal bleeding overtime[40–42]. Altogether digestive tract complications significantly compromise nutrition, leading to numerous symptoms and organ deterioration throughout their lifetime.

Although digestive tract complications have a major negative impact on CF patients, pulmonary insufficiency is the number one cause of death. The CF airways undergo structural changes early in life with associated chronic airway inflammation. Efficient mucociliary clearance is essential for respiratory health and is dependent on proper hydration of the airway surface liquid[43]. When mucociliary clearance is impaired as shown in CF lung disease, abnormal epithelial fluid absorption will raise osmotic pressures in the mucus layer that depletes the airway surface liquid leading to dehydrated mucus, impaired mucus transport, and mucus adhesion to airway surfaces[44,45]. The increase in mucin secretion causes endobronchial mucus plaques and plugs leading to air-flow obstruction and chronic airway infections[46,47]. The key purpose of airway mucus is to clear pathogens and to provide a protective barrier against toxic endogenous and exogenous products[46]. As mentioned earlier, CFTR deficiency also fails to inhibit the ENaC channel opening, causing the accumulation of chloride and sodium ions inside the cell resulting in dehydration of the airway surface liquid[48,49]. CFTR is vital to defining the changing properties of the ASL and mucus layer, therefore the high prevalence of mucus plugging is associated with air trapping and abnormal lung perfusion due to hypoxic pulmonary vasoconstriction with signs of inflammation in the bronchioles early in life[47,50,51].

Inflammation in the CF airways is powered by constant recruitment of immune cells, predominantly by neutrophils. Neutrophils are the first cells migrating to the respiratory tract where they cause significant lung tissue damage by activation, releasing their various oxidants and proteases. The abundant amount of neutrophils migrating to the airways may be caused by the abnormal secretion of chemoattractants by respiratory

epithelial cells and lymphocyte T-helper (Th) response[52]. Bacterial products in the lung can activate macrophages and airway epithelial cells to produce Interluekin-8 (IL-8), TNF-α, and IL-1β which are key mediators for neutrophil activation and recruitment[52]. The CF airways contain large amounts of pro-inflammatory cytokines causing a hyperactive response of neutrophils propagating a severe cycle of inflammation both locally and systemically in the respiratory tract[52]. Chronic alveolar hypoxia, progressive destruction of pulmonary vascular structure, and persistent increase in cardiac output results in lung function decline overtime[53]. In these patients, lung transplantation may be their only means of survival with lung transplantation increasing in adults from a few in the late 1980s to 1,645 by 2021[2].

Neutrophil Biology

Neutrophils are a major innate immune cell, representing about 70% of all leukocytes in the peripheral blood of healthy humans and more than one hundred billion cells are produced every day in the bone marrow, but have a half-life of about eight hours[54,55]. They develop in the bone marrow from hematopoietic stem cells that locate there by perivascular cells that express membrane-bound stem cell factor and chemokine CXCL12[56,57]. The hematopoietic stem cells then differentiate into multipotent progenitor cells which are transformed into lymphoid-primed multipotent progenitors to eventually become granulocyte-monocyte progenitors[58]. Granulocyte-monocyte progenitors then devote to becoming neutrophils by turning into myeloblasts instructed by the production of granulocyte colony-stimulating factor[58]. From there, myeloblasts begin the maturation process to become a neutrophil including the stages of promyelocyte, myelocyte,

metamyelocyte, and band cell[59]. During this differentiation, the nucleus undergoes changes from a round shape into a lobulated morphology and various receptors begin to be expressed on the cell surface[58,60]. The chemokine receptor, CXCR4 (on myeloid cells) binds to CXCL12 and delivers retention signals which gradually disappears as myeloid cells mature into neutrophils, whereas CXCR2 transmits release signals and increases overtime to eventually progress myeloid cells out of the bone marrow[58,60]. Dendritic cells are responsible for the distribution of neutrophils from the bone marrow to peripheral organs and the blood, as they control the production of growth factor granulocyte colony-stimulating factor and other chemokines responsible for the signals that are needed to initiate stem cells to become neutrophils[61]. Neutrophils exit from the bone marrow by signals from granulocyte colony-stimulating factor and reduction in CXCR4 surface expression to circulate in the blood as they wait for signals to drive them to tissues where they are needed[58,60].

Mature neutrophils contain granules and secretory vesicles that hold important antimicrobial enzymes to destroy pathogens. The goal of neutrophils is to kill invading pathogens as they are known to be the first responders to any site of infection, flooding the area in large numbers. Alveolar macrophages, bronchial epithelial cells, and fibroblasts synthesize IL-8 which drives neutrophils to the lung tissue[52]. In CF airways, epithelial cells produce significantly higher levels of IL-8, leading to the increased influx of neutrophils seen in this disease[62]. Neutrophils must migrate across the endothelium, the extracellular matrix, and the alveolar epithelium from the alveolar capillary bed and postcapillary venules[9]. This recruitment involves complex interactions between cytokines, cell adhesion molecules, and chemoattractants[9]. First, tethering of neutrophils

to the endothelial surface occurs by the binding of selectins causing rolling adhesion and strong adhesion to the vessel wall which is mediated by activated β2 integrins[9]. Migration between the endothelial cells is facilitated by CD11b (on the neutrophil) and intracellular adhesion molecule 1 (ICAM-1)[9]. Lastly, migration through the extracellular matrix occurs along the fibroblasts and eventually through the alveolar epithelial cells which is initiated by CD47 signal regulatory protein α[9]. In CF, it has been shown that soluble ICAM-1 is upregulated in the serum of these patients and lung tissue collected from transplantations displays overexpression of ICAM-1 on the epithelium surface[63,64]. The upregulated ICAM-1 in these patients suggests a role for the increased influx of neutrophils to the airways in these patients. Additionally, neutrophils taken from the blood of CF patients have reduced CXCR4 expression, therefore CF neutrophils show increased release from the bone marrow contributing to the chronic inflammation observed in the lungs[65].

In CF, the increased neutrophil infiltration to the airways is sought to be induced by chronic polymicrobial infections, however, neutrophil inflammation in the lungs is seen in young CF babies/children with sterile lung environments. One hypothesis behind this is that the hypoxic environment brought on by the accumulation of mucus in the airways, leads to hypoxic necrosis of airway epithelial cells, causing the release of IL-1α from dying cells[50]. IL-1α is known to stimulate the production of IL-8 in airway macrophages, the predominant chemoattractant for neutrophil recruitment[50]. IL-1 signaling also stimulates IL-1β production in alveolar macrophages, causing epithelial mucin expression, hypersecretion of mucus, and neutrophil recruitment[50]. Therefore, prior to infection in CF patients, neutrophils are recruited and primed to release their

antimicrobial properties due to the hypoxic environment caused by the deficiency of the CFTR chloride channel, leading to persistent chronic lung inflammation.

Additionally, many pro-inflammatory markers such as IL-8 and IL-1 are produced to aid in neutrophil recruitment during bacterial infections present in the CF lung. Reduced mucociliary clearance allows for opportunistic pathogens to thrive in the lung, leading to more inflammation in the airways. The signals from bacterial peptides recognized by the airway epithelial cells, resident macrophages, and recruited neutrophils generates increased production of pro-inflammatory cytokines to continue to recruit new neutrophils to the airways to fight infections. The positive feedback loop from these signals induces chronic activation and recruitment of neutrophils which is the main source of chronic inflammation in these patients leading to lung function decline overtime and ultimately death.

Neutrophil Effector Responses

Neutrophils are the main drivers of bacterial killing during infection as they have many antimicrobial defense mechanisms. However, neutrophil homeostasis is important to reduce the amount of tissue damage during an infection. The three major defense mechanisms neutrophils undergo to combat bacterial infections are degranulation, NETosis, and phagocytosis. All three mechanisms have powerful properties that target harmful bacterial species in the lung. In addition to targeting bacteria, these processes may also damage lung tissue contributing to the ongoing lung function decline seen in CF patients.

Degranulation

Neutrophils degranulate to release a diverse assortment of antimicrobial proteins and enzymes to kill extracellular bacteria and recruit more leukocytes to the area of infection. Neutrophils have four types of granules that are trafficked to compartments inside and outside the cell that include primary or azurophilic granules, secondary granules, tertiary granules and secretory vesicles[66,67]. The primary granules contain the first line of defense, carrying the most harmful enzymes such as NE, myeloperoxidase (MPO), cathepsins, and defensins[66,67]. The secondary and tertiary granules contain lactoferrin and matrix metalloprotease 9[66,67]. The secretory vesicles contain serum albumin and a variety of receptors to generate a more robust inflammatory response, and some of these vesicles may also contain defensins[66,67]. All granules contain lysozyme, known to attack the peptidoglycan present in the bacterial cell wall[66]. Granules are contained inside the cytoplasm of the neutrophil and receptors that become activated on the plasma membrane or phagosome membrane send signals to initiate the movement of these granules to the cell surface or the phagosome[67]. The release of granule-derived mediators from granulocytes leads to exocytosis by receptor coupling mechanisms. In order for exocytosis to take place, actin cytoskeleton remodeling and microtubule assembly occurs, followed by vesicle tethering and docking which allows for the outer surface of the lipid bilayer membrane of the granule to attach to the inner surface of the target membrane (plasma membrane or phagosome membrane)[67]. Complete granule fusion allows for a fusion pore to form, leading to the release of the granular contents either outside the cell or into the phagosome[67]. Calcium, phospholipids, and Src kinases all play a role in the activation of granules[67]. Inflammatory cytokines and chemokines

that bind to neutrophil receptors increase intracellular calcium which promotes activation of ATP and kinases to act by phosphorylating downstream effector molecules to cause the reorganization of the actin cytoskeleton[67].

Neutrophil degranulation is heightened in CF patients, contributing to the robust inflammation and tissue damage seen. NE released during neutrophil degranulation is abundant in the CF lung compared to other host enzymes which impairs the host defense, injures the bronchial epithelial cells, and destroys the lung extracellular matrix[68]. As mentioned above, NE is released from the primary granules, but it has also been shown to be released from neutrophil-derived exosomes which are able to bind to the extracellular matrix, allowing for the degradation of collagen and elastin[69]. NE also upregulates the gene expression of mucins by degradation of junctional proteins allowing access of inflammatory cytokines to stimulatory receptors in bronchial epithelial cells[70,71]. Therefore, the increased release of NE from neutrophils contributes to the destruction of lung tissue in these patients by airway remodeling[72]. The primary function of NE is to directly kill bacteria which are predominant in the CF lung through the cleavage of the outer membrane protein-A, however bacteria have derived mechanisms which inactivate killing by NE[73]. Both predominant pathogens, *P. aeruginosa* and *S. aureus* have known mechanisms to inhibit killing by NE, suggesting that NE is ineffective in the CF lung. Altogether excessive NE release enhances mucus production, alters cellular differentiation and cellular fate, activates pro-inflammatory signaling, and impairs innate immunity, which all contributes to worsened lung function in CF patients overtime[70]. Many therapeutics against NE have been developed but so far none have been effective in CF patients, suggesting that new improved therapies are needed.

NETosis

Neutrophils expel neutrophil extracellular traps (NETs) as an extracellular killing mechanism to trap and kill bacterial species[74]. NETosis is a form of cell death in which the neutrophil releases its nuclear and granular content such as chromatin containing histones and proinflammatory granule proteins[75]. During NETosis, the nucleus of neutrophils loses its lobed shape and the chromatin decondenses which is potentiated by enzymes, NE and MPO[75]. NETosis is induced by many agonists such as bacterial pathogens that induce reactive oxygen species (ROS) in the neutrophil, activating different sets of kinases[76]. Activation of kinases causes chromatin decondensation leading to histone modifications that is catalyzed by peptidyl deaminase 4 (PAD4) to convert arginine residues to citrulline[75,76]. In the final stages of NETosis, the nucleus disintegrates, and the cell membrane ruptures, releasing granular proteins such as NE, citrullinated histones, MPO, defensins, and cathelicidins, which help kill off pathogens in the airways[75,76].

Neutrophils release NETs in response to various stimuli, however various mechanisms of NET formation are known which include Nox-dependent NET formation, Nox-independent NET formation, transcriptional firing, and histone modification[76]. Nox-dependent NETosis is typically induced by LPS found in Gram-negative bacteria, such as *P. aeruginosa* which binds to TLR4 on the surface of neutrophils to cause ROS production through the Nox enzyme[76,77]. During Nox-dependent NET release, ROS generated through the Nox enzyme induces disintegration of membrane granules and the nucleus, allowing for NE and MPO to interact with the nucleus and cleave histones to cause chromatin decondesation[75,76,78]. On the other hand, calcium ionophores can induce

NET formation in a Nox-independent manner[79]. PAD4 is abundantly present in the cytosol of neutrophils and can bind to calcium from the influx of ionophores and translocate into the nucleus[76,80]. The PAD4 enzyme deaminates histone arginine residues into a neutral citrulline causing chromatin decondensation[76,80]. There is also further evidence to suggest that the calcium-activated potassium channel of small conductance (SK3 channel) and mitochondrial ROS is required to induce Nox-independent NET formation[81]. The calcium ionophore stimulates mitochondrial ROS through influx via the SK3 channel to induce Nox-indepdent NETs[81]. Another important component discovered to induce NET formation is transcriptional firing at promoter regions that help to promote DNA decondensation[76,82]. It has been found that transcription of extracellular-signal-regulated kinase (Erk), activated serine-threonine kinase (Akt), p38 mitogen-activated protein kinase (p38 MAPK), and cSrc (tyrosine protein kinase) -regulated genes are the primary drivers of Nox-dependent NET formation, whereas transcription of Akt, p38 MAPK, cSrc, PyK2 (a tyrosine protein kinase), and c-Jun N-terminal kinase (Jnk)-regulated genes drive Nox-independent formation[76,82]. Lastly, histone modifications are important components for NET formation in neutrophils. Histone acetylation plays an important role in NET formation as well as histone deamination mentioned earlier. Histone acetylation causes neutralization of the positive charge on the histone, which impairs the negatively charged DNA to promote decondensation, which is necessary for NET formation[76,83]. All of these pathways are driven by pro-inflammatory signals released during host recognition of bacteria in the airways.

In CF, chronic infections trigger inflammation by a massive neutrophil influx to the lungs with subsequent release of NETs leading to persistent lung injury through the

accumulation of degranulating neutrophils containing extracellular DNA and antimicrobial cytotoxic peptides such as MPO, lysozyme, lactoferrin, NE, defensins, gelatinase, cathelicidins, and cathepsins. Neutrophils undergo NETosis in response to certain infectious agents, however the CF sputum and bronchioalveolar lavage have high levels of neutrophil granular components without infection, and these high levels correlate with the severity of lung disease in CF patients[84–87]. The massive influx of neutrophils and release of neutrophil DNA in the bronchioles of the CF lungs aggravate mucus viscosity, providing a suitable environment for the colonization of infectious bacteria[84–87]. The enhanced mucus production in the CF airways not only contributes to the increase of bacterial infections, but also contributes to the clogging of airways resulting in disease pathology and tissue damage. As mentioned above, the neutrophil enzymes released destroy healthy lung tissue and these enzymes are also released on the NETs contributing to the destruction of the healthy lung tissue in these patients' overtime. Common chronically colonized bacteria in the CF lung are *S. aureus* and *P. aeruginosa* that are known to be strong NET inducers[88–90]. The dysregulation of NET formation and/or clearance is associated with the severity of the lung disease and the onset is not entirely clear. However, lung pathogens promote NETosis and this poses a threat to CF patients as they are high-risk for chronic infections[88–90]. The DNA released from neutrophils may promote more bacterial colonization and biofilm formation causing more resistance to NET-mediated killing and more clogging of the airways[91]. Some CF patients receive ivacaftor as treatment to reduce neutrophil inflammation and NETosis, reducing disease exacerbation[92–94]. Ivacaftor reduces thick mucus buildup in the lungs which reduces neutrophil inflammation[92–94]. Therefore, understanding the mechanisms of

NETosis in CF could help identify new possible therapeutics to reduce the amount of neutrophil DNA released preventing worsened lung disease, especially those patients unable to take the CFTR modulators available.

Phagocytosis

Phagocytosis is a specialized cellular process that only special innate immune cells undergo to ingest foreign particles such as microbes[95]. Neutrophils are considered a professional phagocytic cell that are supplied with specialized receptors to recognize, internalize, and eventually kill target microbes[96]. There are four phases to phagocytosis by neutrophils: detection, activation of the internalization process, formation of the phagosome, and phagosomal maturation[96]. Phagocytes detect microbes through receptor signaling by two types of receptors, non-opsonic and opsonic receptors[96]. Non-opsonic receptors recognize pathogen associated molecular patterns (PAMPs) which when bound, initiate phagocytosis[96]. Opsonic receptors detect host-derived proteins that bind to target molecules on a pathogen through a process called opsonization[96]. Opsonins are host-derived proteins that include antibodies, fibronectin, complement, and mannose-binding lectin, which label the pathogen as a target for phagocytosis[97]. Fc receptors and complement receptors on the neutrophils bind to these opsonins attached to the pathogen to initiate phagocytosis[97]. In particular, Fc receptors bind to the Fc portion of IgG and IgA antibodies, whereas complement receptors such as C5aR and C3aR will bind to activated complement components such as iC3b bound to the pathogen[96]. Numerous receptors present on the neutrophil membrane must interact with several IgG or complement proteins on the opsonized microbe for effective recognition and detection[96]. Once a pathogen is detected through specific receptor-protein or antibody binding,

several signaling pathways are activated to initiate the actual engulfment of the microbe inside the cell. Changes in the cell membrane and reorganization of the actin cytoskeleton will occur causing a depression of the membrane or a phagocytic cup[96]. The detection through the receptor binding causes activation of GTPase Rac which helps regulate actin cytoskeleton rearrangement[96]. Pseudopods will form around the pathogen until the membrane surrounds the pathogen to form a vesicle containing its own membrane inside the cell, called the phagosome[96]. This signaling process is dependent upon the type of receptor bound, to initiate the activation. Fcγ receptor crosslinking by IgG which is bound to the pathogen induces the activation of Src kinases which phosphorylate tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) sequence within the cytoplasmic tail of the receptor[96,98]. The spleen tyrosine kinase (Syk) will associate with phosphorylated ITAMs and cause downstream phosphorylation and activation of a signaling complex such as phospholipase C (PLCγ)[96,98]. Phosphorylation of PLCγ produces inositoltriphosphate (IP3) and diacylgercol (DAG) which cause calcium release and activation protein kinase C (PKC) leading to the activation of ERK and p38[96,99]. The activation of Syk and its downstream effectors help initiate the phagocytic cup and also regulates Rac and contractile proteins in the cytoskeleton[96]. However, complement receptor signaling of phagocytosis contains a different set of activation processes. The CR3 will bind to the complement molecules iC3b which attach to the pathogen during opsonization and will activate GTPase Rho[96]. This active Rho will induce actin polymerization by phosphorylated and activated myosin II leading to assembly of the phagocytic cup[96,100]. Rho will also promote accumulation of mammalian diaphanous-related formin 1 (mDIA1) which activates linear actin polymerization[96,100]. These

processes, initiate the changes in the cytoskeleton to form the phagosome containing pathogen inside the cell. The phagosome formation requires changes in lipid composition and remodeling of the actin cytoskeleton to generate membrane protrusions that will surround the pathogen and eventually fuse together[96]. Once the pathogen is engulfed inside the phagosome, the final step of phagocytosis takes place which is phagosome maturation.

The phagosome matures when lysosomes fuse to the phagosome membrane transforming into the phagolysosome. Phagolysosomes are microbicidal vesicles that produce ROS and contain various cathepsins, proteases, lysozymes, and lipases to target the pathogens inside[96,101]. The process occurs in three stages: early, late, and phagolysosome[96,101]. Early endosomes fuse with the phagosome membrane through the phosphorylation of GTPase Rab5 that recruits early endosome antigen 1 (EEA1)[96,102–104]. EEA1 recruits Rab7 and once Rab7 binds to the membrane, Rab5 gets recycled[96,102–104]. Rab7 mediates the fusion of late endosomes to the phagosome and V-ATPase molecules which are responsible for the acidification by the translocation of protons into the lumen[96,102–104]. At this stage, lysosomal-associated membrane proteins (LAMPs), cathepsins, and hydrolases are released inside[96]. Lysosomal fusion is the last step of maturation in which the NADPH oxidase assembles to produce ROS and superoxide anions[96]. The production of hydrogen peroxide by the NADPH oxidase will react with chloride ions to form hypochlorous acid which is a very powerful antimicrobial[105,106]. The phagolysosome will also contain several enzymes such as NE and MPO.

The chloride deficiency in CF patients may impair phagosomal mechanisms in neutrophils in the CF lung. A recent study found that CF neutrophils are not impaired in

phagocytosis of *S. aureus*, a predominant CF pathogen but are impaired in phagosomal killing[107]. This suggest that phagosome maturation may be impaired. Also, CF neutrophil phagosomes are less acidic due to less V-ATPase fusing to the membrane which suggests a possible defect in phagolysosome fusion. The lack of chloride in the phagosome may also affect bacterial killing in CF neutrophils by causing reduced hypochlorous acid production[108,109]. Not much research has been done on neutrophil phagosome maturation in the CF lung but a defect in any of the mechanisms described above could lead to the deficient bacterial killing in the CF lung.

Bacterial Infections in CF

Infections in the lower airways of CF patients remain a substantial contributor to morbidity and mortality, regardless of the multiple CFTR modulator therapies that correct CFTR channel function[110]. The CF lung microbiome consists of diverse microbial populations and persistence/survival of each species depends on age, disease progression, and acute clinical events[111]. These patients experience recurrent and chronic infections throughout their lifetime and the infections most common are *S. aureus*, *P. aeruginosa*, *Haemophilus influenzae* (*H. influenzae*), *Stenotrophomonas maltophilia* (*S. maltophilia*), *Achromobacter*, *Burkholderia cepacian* complex (Bcc) respectively. Patients may experience co-cultures of species in the lung at any given time, for example *P. aeruginosa* and *S. aureus* are known to colonize together, as *S. aureus* initiates changes in the lung that are beneficial for *P. aeruginosa* survival[112]. *P. aeruginosa* is one of the most common bacteria found in CF patients however prevalence and percentage of patients with a positive culture has continued to decline due to eradication strategies at

the time of initial acquisition[2,113]. As of 2021, *P aeruginosa* is known to infect older adult CF patients as only 16.8% of patients younger than 18 are infected (compared to 44.6% in 2001)[2]. However, it remains a predominant pathogen given that about 50% of patients older than 35 years have *P. aeruginosa* positive cultures[2]. On the other hand, in 2021 more than half of CF patients had at least one *S. aureus* positive culture[2]. *S. aureus* is most prevalent in CF children and reaches the highest prevalence of 80% being infected between the ages of 6 to 11[2]. This pathogen persists throughout the lifetime of patients and about 35% of patients at the age of 45 still culture positive in the respiratory tract[2]. Another disconcerting characteristic of both *P. aeruginosa* and *S. aureus* is that they have emerged to become antibiotic resistant. Antibiotics are the primary treatment for bacterial infections in CF patients and the options for treatment routes are oral, inhaled, or injected intravenously depending on the severity of infection[114]. Even though, *P. aeruginosa* has evolved to be multidrug-resistant, prevalence of multidrug-resistant *P. aeruginosa* (MDR-PA) is still on the decline due to major focus and research on this pathogen[2,113]. MDR-PA infects mostly older adolescents and adults with CF and reflects constant exposure to antibiotics early in life[2]. Overall infection rates of MDR-PA have dropped from 4.2% to 3.5% in 2021, however of the 16.8% of patients that had *P. aeruginosa* positive cultures, 12.3% of those were reported to be MDR-PA[2]. While MDR-PA declines in CF patients overtime, methicillin-resistant *S. aureus* (MRSA) is on the rise and attributes to chronic *S. aureus* infection in patients[2]. Peak methicillin-sensitive *S. aureus* (MSSA) occurs in patients younger than 10 years whereas MRSA prevalence occurs in patients between 10 to 20 years of age, suggesting antibiotic use in young patients causes resistance later in life[2]. About 30-40% of total *S. aureus* positive patients

under the age of 30 have MRSA positive cultures which rises to about 50% or more in patients older than 30, showing the prevalence of this pathogen overtime[2].Altogether, *S. aureus* and MRSA infection in CF patients is on the rise and more research is needed to understand why this pathogen is emerging and persists throughout their lifetime to ensure that infection rates begin to decline such as *P. aeruginosa*.

Staphylococcus aureus

S. aureus is a Gram-positive bacterium known to colonize 60% of humans[115,116]. Not only is *S. aureus* the number one pathogen infecting CF patients, it is also the leading cause of bacterial infections worldwide, resulting in about 35,000 deaths per year in the United States according to the Center for Disease Control and Prevention[117]. It is an opportunistic pathogen given that it is naturally a part of the nasal commensal microbiota in many humans but can cause mild to life-threatening infections when disseminated, affecting any organ of the body. In CF patients, infections in the lungs causing *S. aureus* pneumonia are the main concern. *S. aureus* has many virulence factors to aid in its survival and as mentioned above has become antibiotic resistant. The transition from MSSA to MRSA is due to changes in the *mecA* gene which is located on a mobile genetic element known as the staphylococcal cassette chromosome *mec*[118]. The *mecA* gene is acquired through horizontal gene transfer and encodes a transpeptidase known as penicillin binding protein (PBP). MSSA contains PBP1-4 that readily binds to methicillin and other β-lactam antibiotics which causes the inhibition of peptidoglycan and cell wall synthesis of the bacteria. However, MRSA contains PBP2a which has a low affinity for β-lactam, so cell wall synthesis is not inhibited.

S. aureus is also known for its virulence and immune evasion molecules against neutrophils, which contributes to its persistence in the CF lung despite the large neutrophil numbers present. *S. aureus* prevents complement and immunoglobulin recognition of neutrophils by blocking the binding of these receptors to inhibit phagocytosis. The generation of a polysaccharide capsule present in about 75% of all clinical isolates of *S. aureus* are known to hide the antigenic or immunogenic proteins on the bacterial cell wall surface, allowing it to go "unnoticed"[119]. Also *S. aureus* contains proteins that bind to the complement receptor C5aR and serine proteases that degrade C3 convertase which blocks C3b from binding to the bacterial surface. The blocking of these complement components inhibits opsonization and ultimately prevents phagocytosis. It also prevents phagocytosis by its numerous surface molecules that bind to host proteins to reduce the recognition by neutrophils. For example, the *S. aureus* protein A (SpA) binds the Fc-region of IgG, which prevents antibody binding with neutrophil Fc receptors and this blocking is shown to reduce opsonization and phagocytosis. *S. aureus* also produces toxins such as leukocidins (Luk) that cause leukocyte cell lysis. These toxins assemble in the host's cell membrane that disrupt cell homeostasis leading to cell death. If phagocytosis of *S. aureus* is successful by neutrophils, they also have mechanisms to cause phagosomal membrane lysis to evade intracellular killing. Toxins such as alpha-hemolysin (Hla) and LukGH/LukAB are known to interact with surface receptors to form cytolytic membrane pores causing lysis of the phagosomal membrane and the plasma membrane allowing for the bacteria to escape. *S. aureus* also possess genes that protect itself from killing by ROS released inside the phagosome of neutrophils as well as outside the cell by NETs. CF clinical isolates of *S. aureus* display high nuclease activity

and high levels of superoxide dismutase A and M (sodA and sodM)[120,121]. High nuclease activity allows the bacteria to resist NET-mediated killing by neutrophils and the superoxide dismutase production reduces oxidative stress in the CF airways to prevent bacterial killing[120,121].

The CF clinical isolates of *S. aureus* differ from isolates cultured from infections of non-CF individuals. *S. aureus* genome alteration rates are much higher in CF isolates than from healthy individuals and the phenotypical heterogeneity of *S. aureus* populations in CF patients are exaggerated by differences in gene expression[122,123]. The bacteria tend to adapt their phenotypic changes towards being persistent and colonizing to strive in the CF airways, rather than disseminating to other organs such as in an aggressive *S. aureus* infection in a healthy individual[123]. CF *S. aureus* isolates suppress their virulence genes to express proteins for adhesion and biofilm formation to protect themselves from the already primed neutrophils and antibiotic therapy[124,125]. The changes in gene expression are regulated by quorum sensing and the accessory-gene-regulator system (*agr*)[123]. Quorum sensing is a signaling system for bacterial cell-to-cell communication, permitting the transfer of information about environmental conditions and bacterial density to regulate gene expressions[123]. The *agr* pathway effects the secretion of virulence factors, predominantly their toxins which are highly immunogenic[123]. CF clinical isolates of *S. aureus* have been shown to have an inactive *agr*-system, which show decreased secretion of toxins to permeate a chronic, localized infection[126,127]. Another unique feature found in the CF lung, are the phenotypic changes of *S. aureus* into small colony variants which have slow growth rates, persist intracellularly, resist antibiotics, and associate with more severe lung disease[128–132].

Many CF clinical isolates have been extensively studied, and there is not one mechanism for which a CF *S. aureus* isolate changes its genome to become persistent, evade immune recognition, and antibiotic treatment[123]. Therefore, developing new strategies to eradicate this pathogen from the CF airways is a daunting challenge but more research to understand the neutrophil-*S. aureus* interactions in the airways are needed to initiate a decline of infection in these patients.

Concluding Remarks

Altogether, CF is a genetic disease that causes severe pulmonary function decline overtime. These patients suffer through a lifetime of chronic inflammation by neutrophils and polymicrobial infections. Neutrophils release their intracellular content through many mechanisms described above in their attempt to kill the pathogens present in the lung which contribute to the worsened lung function. *S. aureus* is the most predominant pathogen in these patients and antibiotic resistance strains are on the rise, making this pathogen harder to combat. *S. aureus* is also resistant to neutrophil-mediated killing through degranulation, NETosis and phagocytosis, suggesting that new therapies to lessen infections are needed. Although CFTR modulators give relief to many patients and have been shown to reduce lung disease severity, there are patients resistant to this treatment. Overall, understanding the mechanisms of *S. aureus* and neutrophil interactions are needed to combat the severity of infection in these patients and new therapeutics are needed to alleviate CF lung disease, and to eventually lead to a cure.

CHAPTER 3

CYSTIC FIBROSIS SPUTUM IMPAIRS THE ABILITY OF NEUTROPHILS TO KILL *STAPHYLOCOCCUS AUREUS* [133]

[133] Fantone K, Tucker SL, Miller A, *et al*. Cystic Fibrosis Sputum Impairs the Ability of Neutrophils to Kill Staphylococcus aureus. 2021:1-22. Reprinted with permission of the publisher.

Abstract

Cystic fibrosis (CF) airway disease is characterized by chronic microbial infections and infiltration of inflammatory polymorphonuclear (PMN) granulocytes. *Staphylococcus aureus (S. aureus)* is a major lung pathogen in CF that persists despite the presence of PMNs and has been associated with CF lung function decline. While PMNs represent the main mechanism of the immune system to kill *S. aureus*, it remains largely unknown why PMNs fail to eliminate *S. aureus* in CF. The goal of this study was to observe how the CF airway environment affects *S. aureus* killing by PMNs. PMNs were isolated from the blood of healthy volunteers and CF patients. Clinical isolates of *S. aureus* were obtained from the airways of CF patients. The results show that PMNs from healthy volunteers were able to kill all CF isolates and laboratory strains of *S. aureus* tested in vitro. The extent of killing varied among strains. When PMNs were pretreated with supernatants of CF sputum, *S. aureus* killing was significantly inhibited suggesting that the CF airway environment compromises PMN antibacterial functions. CF blood PMNs were capable of killing *S. aureus*. Although bacterial killing was inhibited with CF sputum, PMN binding and phagocytosis of *S. aureus* was not diminished. The *S. aureus*-induced respiratory burst and neutrophil extracellular trap release from PMNs also remained uninhibited by CF sputum. In summary, our data demonstrate that the CF airway environment limits killing of *S. aureus* by PMNs and provides a new in vitro experimental model to study this phenomenon and its mechanism.

Keywords: cystic fibrosis, PMN, *Staphylococcus aureus*, killing, sputum, respiratory burst, neutrophil extracellular traps

Introduction

Cystic fibrosis (CF) airway disease is characterized by decreased mucociliary clearance, chronic, polymicrobial infections and robust, neutrophil-dominated inflammation. Pulmonary disease is the leading cause of morbidity and mortality in CF patients, due to the progression of chronic respiratory infections and host inflammation[4]. *Staphylococcus aureus* (*S. aureus*) is the most prevalent infectious agent in the respiratory tract of CF patients[134]. According to the United States' Cystic Fibrosis Foundation's annual reports in 2018 and 2019, 70% of CF patients were infected with *S. aureus*, including 25% with methicillin-resistant *S. aureus* (MRSA). *S. aureus* is one of the earliest pathogens recovered in the airways of CF children; it persists throughout the life of CF patients, and over a decade ago, surpassed *P. aeruginosa* as the most common airway pathogen in CF[6–8]. MRSA is associated with accelerated decline in lung function, increased hospitalization and mortality in CF[135,136]. For these reasons, it is of great importance to study *S. aureus* in CF to better understand CF lung disease[6,134].

One of the earliest abnormalities in CF airway disease is the recruitment of polymorphonuclear neutrophil granulocytes (PMNs) to the lungs. PMNs are an important component of the airway's antimicrobial defense[136]. PMNs' primary killing mechanisms against pathogens involve intracellular killing through phagocytosis and extracellular killing by neutrophil extracellular traps (NETs)[137,138]. PMNs represent the most important immune cell type fighting *S. aureus* including MRSA[119,139–141]. Antibody- or complement-enhanced phagocytosis and the associated respiratory burst generating reactive oxygen species (ROS) represent the main mechanisms by which PMNs kill *S. aureus*[119,139,142]. PMN-mediated killing of *S. aureus* in CF airways is obviously impaired

since *S. aureus* is a major respiratory pathogen in CF[6–8]. The reason for this remains unclear, as *S. aureus* has been detected inside PMNs in CF airways indicating that phagocytosis occurs to some extent[128].

The persistence of *S. aureus* infections in the presence of PMNs in the CF lung indicates that anti-staphylococcal PMN killing mechanisms fail. We hypothesized that the CF airway environment impairs PMN-mediated clearance of *S. aureus*. The goal of our study was to determine the potential effects of the CF airway environment on antibacterial effector functions of PMNs against *S. aureus*.

Materials and Methods

Human Subjects

All the human subject studies were performed by following the guidelines of the World Medical Association's Declaration of Helsinki.

Control Subjects

Control human subjects recruited at the University of Georgia (UGA) provided informed consent before blood donation for PMN isolation according to the protocol UGA# 2012-10769-06. Healthy subjects were chosen to match the sex and age distributions of CF patients. While control subjects did not suffer from CF based on self-report, they could have theoretically included CFTR heterozygous subjects that are represented in the general population with an approximate 1:25 frequency and are otherwise healthy. Two healthy subjects were recruited at Emory University to donate sputum samples following induction of sputum through inhalation of a 7% hypertonic saline solution.

CF Patients

CF subjects were patients recruited at the Adult CF Clinic at Emory University. CF patients signed informed consent to provide blood and sputum samples (IRB00042577). CF diagnosis was confirmed by pilocarpine iontophoresis sweat testing and/or CFTR gene mutation analysis showing the presence of two disease causing mutations. CF participants were selected for blood draw or sputum collection only if they were clinically stable and on no new medications within the previous three weeks of the clinic visit. Sputum cultures were taken on the day of the clinic visit when the blood was drawn, and the presence or absence of *S. aureus* as identified by the clinical microbiology laboratory was noted. Baseline lung function was defined according to the guidelines of the CF Foundation Patient Registry which is the average of the best percent predicted forced expired volume in one second (FEV_1) for each quarter of the calendar year. Blood was drawn into a silicone-coated tube and processed as above until shipped to UGA for analysis.

PMN and Serum Isolation

Control human subjects were recruited at UGA by the Clinical Translational and Research Unit to donate blood for PMN isolation and serum preparation. PMNs and serum were also isolated from the peripheral blood of CF patients along with age/gender-matched healthy donors at Emory University. 20–30 milliliters of blood were drawn into EDTA-coated tubes and PMNs were isolated using the EasySep™ Direct Human PMN Isolation Kit (Catalog #19666, Stem Cell technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. This protocol routinely yields 30–130 × 10^6 live PMNs with >99.9% red blood cell depletion and ~99% purity of the isolated

cells. Additional ten milliliters of blood were drawn into a separate, silicone-coated tube without anticoagulant and allowed to clot at room temperature for 30 min. The tube was centrifuged twice (1300× *g*, 5 min), the resulting supernatant (serum) was collected, and the coagulant was discarded. The serum was kept on ice for same-day experiments or frozen at −80 °C for future work.

Sputum Collection and Processing

All human studies involving sputum collection from CF patients and healthy controls were approved by the Emory University Institutional Review Board and were in accordance with institutional guidelines. All donors gave consent before sputum collection. Sputum samples were processed and modified from the previously published method[143]. Briefly, samples were kept on ice at 4 °C throughout all experimental procedures. After collection, sputa were weighed, their volume measured, and 3 mLs of ice-cold PBS-EDTA (1 × PBS–5 mM EDTA) were supplemented to every 1.0 g of sputum samples. Sputum samples were repeatedly and slowly passaged through a sterile 18-gauge needle to homogenize the sputum. Cells were pelleted after low-speed centrifugation (4 °C, 800× *g*, 10 min). The sputum supernatant was transferred into microcentrifuge tubes and centrifuged (3000× *g*, 10 min, 4 °C). The clear sputum supernatants were stored in aliquots at −80 °C until analysis.

In vitro "CF Sputum Model"

To test whether the CF airway environment alters PMN effector functions, an in vitro model was established using blood PMNs isolated from healthy donors and exposed to CF sputum supernatants. Sputum supernatants were pooled from three individual CF patients and called "sputum cocktails". Five different sputum cocktails were tested.

Healthy blood PMNs (10^7 /mL) were pretreated with 30% sputum cocktail for 3.5 h at 37 °C resulting in a final concentration of 1.5 mM EDTA in the sputum. At the end of incubation, PMNs were washed twice with the assay medium consisting of 1 × HBSS, 10 mM HEPES, 5 mM glucose, and 1% (*v/v*) autologous serum to remove extracellular sputum components and to prevent their direct interaction with subsequently added bacteria. Additional control experiments showed that these two washes were sufficient to avoid a potential direct contact of any, minimal, residual, carryover CF sputum and bacteria. PMNs used as control samples not exposed to CF sputum were kept in undiluted assay medium without CF sputum for the same amount of time.

Bacteria

Eight *S. aureus* isolates recovered from the respiratory tract culture of CF patients were obtained from the Emory Cystic Fibrosis Biospecimen Repository (CFBR). Four MRSA and four MSSA isolates were used in this work. The age of the four CF patients donating the MSSA strains ranged between 4 and 22 years while those of the MRSA donors ranged between 20–53 years. The antibiotic sensitivity profile of the isolates has been determined. USA300, was used as a laboratory reference strain[144,145].

The *S. aureus* strain JE2 expressing red fluorescence protein (SA-rfp) was constructed by isolating pHC48 from RN4220[146,147], a restriction-deficient *S. aureus* strain, and electroporating it into JE2. Briefly, electrocompetent cells of JE2 were made by using a modified procedure from Lofblom et al.[148]. The restriction system was subsequently inactivated at 56 °C and bacteria were resuspended in 10% glycerol + 500 mM sucrose. Finally, the pHC48 plasmid was electroporated using a pulse at 2.1 kV and cells were recovered in TSB + 500 mM sucrose before plating on TSA with 10 μg/mL

chloramphenicol to select for cells that had successfully accepted pHC48. All the clinical isolates and strains of *S. aureus* used in this work are listed in Table 3.1.

For all the experiments, *S. aureus* was grown on blood agar (TSA II 5% SB) at 37 °C. The following day, bacteria were cultured from the agar plates in optimal growth medium (2 mL LB medium) at 37 °C shaking for 2–3 h. The bacteria were harvested and centrifuged at 10,000× *g* for 5 min, supernatants were discarded, and the bacterial pellets were washed twice with 1 × HBSS. After the last wash, the bacterial pellets were resuspended in 1mL 1 × HBSS and optical density was measured in a 96-well microplate at 600 nm in Varioskan flash™ microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Based on previous experience, bacterial optical density values of 0.6 were determined to correspond to 1.0×10^9/mL bacterial concentration[149]. Calculated bacterial concentration values were confirmed in every experiment by colony counting. The bacteria were then opsonized with 10% (*v/v*) autologous serum of the PMN donor (healthy control or CF patient) for 20 min at 37 °C. Following opsonization, the bacteria were washed by centrifugation at 10,000× *g* for 5 min and resuspended in assay medium.

Cell Viability

Human PMNs were incubated for 3.5 h in 1.5 mL microcentrifuge tubes at a concentration of 1×10^6 cells/100 µL assay medium (HBSS + 1% autologous serum + 5 mM glucose + 10 mM HEPES) with or without 30% of the CF sputum cocktail. Following incubation, cells were washed and resuspended in 1X PBS. Cell were then stained with a Zombie Aqua Fixable Viability Kit (Biolegend, San Diego, CA, USA; cat# 423102) at a dilution of 1:10,000 for 15 min at room temperature, protected from light. Cells were collected by centrifugation and washed with 1 × PBS containing 1% BSA.

PMN viability was also assessed by flow cytometry in some experiments. The granulocyte marker CD66b conjugated to PerCP-Cy5.5 fluorochrome (Biolegend, San Diego, CA, USA; cat# 305108) was used to identify PMNs in the cell suspension. Cells were suspended in 500 µL BD Stabilizing Fixative (BD) and analyzed at the University of Georgia College of Veterinary Medicine Cytometry Core Facility on an LSRII Flow Cytometer (BD, San Jose, CA, USA), using the violet laser the 525/50 filter for Zombie Aqua detection, and the 488 nm blue laser with the 695/40 filter for PerCP/Cy5.5 detection. Data were analyzed with BD FACsDiva™ software (BD Biosciences, San Jose, CA, USA).

High Throughput Bacterial Killing Assay

To assess bacterial killing, a high throughput 384-well microplate-based assay was used that is the improved and expanded version of our previously described, 96-well microplate-based protocol to assess bacterial survival[150]. Briefly, purified human PMNs and opsonized bacteria were mixed at a ratio of 10:1 multiplicity of infection (MOI, *S. aureus*: PMN)) in microcentrifuge tubes. The tubes were incubated and mixed regularly for 30 min at 37 °C to ensure appropriate mixing of bacteria and PMNs. For assessing the killing of bacteria by PMNs treated with sputum, untreated PMNs were used as control. After 30 min, PMNs were lysed with 1 mg/mL saponin in 1 × HBSS (on ice for 5 min). Samples were then diluted in 1 × HBSS 100-fold in a separate microcentrifuge tube on ice. The 384-well microplate was placed on ice and 80 µL of LB broth was placed in each well. 20 µL volumes of each experimental sample were pipetted into corresponding wells in quadruplicates. As a negative control, saponin dissolved in corresponding HBSS/LB medium mix was placed in wells to ensure detection of potential external

bacterial contamination. For absolute quantitation, a standard curve was established with known bacterial concentrations for each bacterial strain or isolate tested that were applied in every experiment. The standard curves of each CF isolate were highly reproducible between experiments. To determine the starting bacterial inoculum (0 min), bacteria were added to PMNs and immediately (few seconds later) lysed with saponin. In our experience, this is a more appropriate time zero control than using only bacteria because the sample also includes PMNs. Once all samples were added to the microplate, the plate was placed in the incubator at 37 ℃ for 15 min to warm up evenly and then placed in a microplate spectrophotometer Varioskan Flash (Thermo Scientific, Waltham, MO, USA). The microplate reader measured bacterial growth in each well at 600 nm absorbance every 4 min for 16 h with constant heating at 37 ℃ and 5 s of shaking prior to each read to prevent bacterial settlement. After the measurement, growth curves were generated, the initial bacterial concentrations in each sample were determined and bacterial killing efficiencies were assessed and expressed as decrease in surviving bacteria over time[150].

Attachment and Phagocytosis

Attachment/binding and phagocytosis of *S. aureus* to PMNs was assessed by flow cytometry. Bacterial attachment to PMNs was quantified using the *S. aureus* strain JE-2 expressing red fluorescence protein (SA-rfp). Healthy human PMNs were isolated and incubated with or without 30% sputum supernatant as previously described. PMNs were then washed twice to remove the sputum and were resuspended in assay medium. Since infecting PMNs with SA-rfp alone will not be able to distinguish between *S. aureus* being attached to the outside of the cells or taken up inside the phagosome, human PMNs were pretreated with the cytoskeleton inhibitor, cytochalasin-B (10 µM, 30 min at 37 °C), to

prevent phagocytic uptake of *S. aureus*. SA-rfp was opsonized with 10% autologous serum of the PMN donor and added to PMNs at MOI of 10. To assess attachment/binding to PMNs, SA-rfp/PMN co-cultures were incubated with frequent mixing for 30 min at 37 °C, then washed twice with cold 1 × PBS and resuspended in PBS. Cells were stained with a Zombie Aqua™ Fixable Viability Kit (Biolegend, San Diego, CA, USA, cat#423102) at a dilution of 1:5000 for 15 min at room temperature, protected from light. PMNs were then washed and resuspended in 1 × PBS containing 1% BSA and stained with the PMN marker CD66b conjugated to PercP-Cy5.5 fluorochrome (Biolegend, cat#305108) at a final concentration of 1 µg/mL for 30 min, protected from light. PMNs were washed and resuspended in BD stabilizing Fixative and analyzed by the NovoCyte Quanteon 4025. For zombie aqua detection, the violet laser at 405 nm with 525/50 filter was used, the blue laser at 488nm with the 695/40 filter was used for PerCP/Cy5.5 detection, and the yellow laser at 561nm with the 586/20 filter was used for rfp.

Phagocytosis was measured in PMNs that were purified and treated with the CF sputum as described above. The CF isolate MRSA24 was stained with 5 mM pHrodo™ iFL green STP Ester (Thermofisher, cat#P36012) for 1 h at 37 °C, protected from light. After the staining MRSA24 was opsonized by the addition of 10% autologous serum of the PMN donor and added to PMNs at MOI of 10. MRSA24/PMN co-cultures were incubated for 1 h at 37 °C protected from light with consistent mixing. PMNs were then washed with cold 1xPBS twice and resuspended in PBS to be stained with a Zombie Red™ Fixability Kit (Biolegend, cat#423109) at a dilution of 1:5000 for 15 min at room temperature. PMNs were then washed and resuspended in eBioscience™ flow cytometry staining buffer (Thermo Fisher Scientific, Waltham, MA, USA, cat#00-4222-26). PMNs

were then stained with the granulocyte marker CD66b AlexaFlour 647 (Biolegend, cat#561645) at a final concentration of 1 μg/mL for 30 min, protected from light. PMNs were washed and resuspended in BD stabilizing fixative. For zombie red detection, the yellow laser at 561 nm was used with the 615/20 filter, the red laser at 637 nm with the 660/20 laser was used for CD66B AlexaFluor 647detection, and the blue laser at 488 nm with the 530/30 laser was used for pHrodo green detection.

To assess attachment and phagocytosis of *S. aureus*, all the data were analyzed at the University of Georgia College of Veterinary Medicine Cytometry Core Facility on a NovoCyte Quanteon 4025 with NovoSamplerQ utilizing NovoExpress software v.1.4.1, Agilent, Santa Clara, CA, USA.

NADPH Oxidase Activity Measurements

ROS production was measured using the Diogenes-based chemiluminescence kit (National Diagnostics, Atlanta, GA) as before[88,149,151,152]. Shortly, 250,000 PMNs were allowed to adhere to 96-well solid white plates for 1 h at 37 ℃ in assay medium (previously described). Cells were stimulated by *S. aureus* isolates (10 MOI), PMA (100 nM) or left unstimulated. Chemiluminescence was measured by a Varioskan Flash microplate luminometer (Thermo Scientific, Waltham, MA, USA) for 90 min. ROS production data are shown as kinetics of representative curves (relative luminescence units, RLU) or integrated superoxide production by analyzing accumulated luminescence for the entire (60 min) or partial (15 min) duration of the measurement and normalizing it on the PMA-stimulated signal as 100%. Superoxide generation of PMNs was specifically tested by the superoxide dismutase-inhibitable cytochrome-c reduction assay as described earlier[150,153]. To measure extracellular superoxide production, PMNs were suspended in

assay medium containing 50 μM of cytochrome-c (Sigma, cat#C3131). The cell suspension was added into a 96-well plate and incubated at 37 °C for 5 min in a shaking microplate spectrophotometer Varioskan Flash (ThermoScientific). PMNs were activated with 100 nM PMA, indicated *S. aureus* CF clinical isolates or Zymosan A particles from *Saccharomyces cerevisiae* (Sigma, cat#Z4250) opsonized in 10% of autologous serum of the PMN donor at MOI of 10. The increases in absorption at 550 nm were recorded for 60 min with two measurements/min at 37 °C. Superoxide production was calculated with the use of an absorption coefficient of 21 mM^{-1} cm^{-1} for cytochrome-c according to the Lambert-Beer law and expressed as nmol $O_2^{\cdot -}/10^6$ PMNs/hr.

NET Release

DNA release from human PMNs was quantitated as described[154]. Briefly, 250,000 PMNs/well were seeded on 96-well black transparent bottom plates and incubated for 30 min at 37 °C. Next, 0.2% Sytox Orange (Life Technologies, Grand Island, NY, USA) membrane-impermeable DNA-binding dye was added to PMNs and PMNs were infected with 10:1 MOI of the indicated *S. aureus* isolates. Fluorescence (excitation: 530 nm, emission: 590 nm) was recorded for up to 8 h in a fluorescence microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. Relative fluorescence unit (RFU) results were normalized on the signal obtained in PMA-stimulated PMNs and expressed as its percentages. Unstimulated PMNs were used as the negative control.

Citrullination of PMN histone H3 protein was also used as a measure of NET formation. Purified PMNs were incubated for 3.5 h with or without 30% CF sputum supernatants, as described above. Following the incubation, PMNs were washed twice

with 1x PBS to remove the sputum and resuspended in PBS. Cells were stained with a Zombie Aqua™ Fixable Viability Kit (Biolegend, cat#423102) at a dilution of 1:5000 for 15 min are room temperature, protected from light. PMNs were washed with PBS/1% BSA, and then fixed and permeabilized with the Fix and Perm/ Cell Fixation and Permeabilization Kit (Abcam, Cambridge, MA, USA) following manufacturer's instructions. All subsequent cell processing was performed on ice, protected from light. The primary anti-histone H3 (citrulline R2 + R8 + R17) (Abcam, Cambridge, MA, USA) antibody was incubated with the cells for 30 min. Cells were washed with PBS/ 1% BSA followed by incubation with goat-anti-rabbit-FITC (BD PharmingenTM, Franklin Lakes, NJ, cat#554020) for 30 min. Cells were washed with PBS/ 1% BSA and stained with the granulocyte marker CD66b conjugated to PercP-Cy5.5 fluorochrome (Biolegend, cat#305108) for 30 min. PMNs were washed with PBS/1% BSA and resuspended in 500 µL Stabilizing Fixative (BD Biosciences, San Jose, CA, USA), and stored at 4 °C until analysis. Samples were read at the University of Georgia College of Veterinary Medicine Cytometry Core Facility on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) within 24 h of staining. Data were analyzed with the BD FACsDiva™ software (BD Biosciences, San Jose, CA, USA).

DNAse Activity Measurement

DNAse activity was measured by a fluorometric assay kit (BioVision, Milpitas, CA, USA; cat #: K429-100) detecting DNAse enzyme activity by cleavage of a DNA probe to yield a fluorescent product. The *S. aureus* isolates were prepared in molecular biology-grade water using filter tips to prevent DNA contamination. A DNA probe standard curve was generated following the protocol ranging from 0–20 pmol/well. 50 µL

of the DNA probe standards and of the bacterial samples were added to a white, 96-well plate in triplicates and 50 µL of the reaction mix were added directly after. Fluorescence was measured in kinetic mode every 30 s for 90 min at 37 °C. RFU values were applied at each time point to the standard curve equation generated to determine pmol of DNA cleaved at each reaction point (pmol/min).

To confirm results produced by the DNAse I fluorometric assay, Remel™ DNASE test agar was also used (Thermo Fisher Scientific, cat #: R453252). The test agar was suspended in demineralized water and autoclaved for sterility. *S. aureus* isolates were grown overnight to generate single colonies. Three colonies from each isolate were taken and smeared in a single-straight line in the middle of the agar dish (one isolate per Petri dish). The test agar dishes were incubated overnight at 37 °C. The next day, the test agar with grown *S. aureus* cultures were flooded with 1N HCL for 5 min. The 1N HCL was aspirated and the DNA clearing zones were measured with a ruler in mm.

Apoptosis

Apoptosis of human PMNs was measured by flow cytometry using the Apotracker(TM) Green (Biolegend, San Diego, CA; cat# 423102) apoptosis probe in combination with propidium iodide (Sigma Aldrich, St. Louis, MO, USA) staining. Cells were stained with Apotracker(TM) Green following manufacturer's protocol, protected from light. PMNs were collected by centrifugation, washed twice with 1X PBS containing 1% BSA, resuspended in 300 uL 1 × PBS containing 1% BSA. Propidium iodide was first diluted at a 1:1 ratio in 2 × PBS and then added to the cells at a 1:10 dilution. Samples were immediately analyzed at the UGA Veterinary Medicine

Cytometry Core Facility on a NovoCyte Quanteon 4025 with NovoSamplerQ utilizing NovoExpress software v.1.4.1 (Agilent, Santa Clara, CA, USA).

Statistical Analysis

Results of multiple bacterial isolates obtained by the high-throughput killing assay were analyzed by one-way ANOVA and Tukey's multiple comparison test. Results between PMNs treated with sputum and no sputum were analyzed by two-tailed, paired Student's t-test. Results between two patient cohorts were analyzed by Mann–Whitney test. Correlation between two parameters was evaluated with Spearman's rank-order correlation. Data are expressed as mean plus-minus standard error of mean (SEM). The correlation coefficient (r) was calculated. Statistically significant differences were considered as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistical analysis was carried out with GraphPad Prism version 6.07 for Windows software (GraphPad Software, San Diego, CA, USA).

Results

In vitro Model of CF Airway-Like Conditions

To investigate whether the CF airway environment affects PMNs' ability to kill bacterial pathogens, we established a new in vitro experimental model. In this model, human PMNs isolated from the blood of healthy volunteers are exposed to pooled supernatants of sputum samples collected from CF patients to mimic the CF airway environment. This treatment referred to as "CF sputum model" throughout the manuscript has been optimized and involves a 3.5 h-incubation of human PMNs with 30% (*v/v*%

diluted in assay medium) CF sputum supernatant pooled from three independent CF patients in equal proportions.

CF Sputum Exposure Does Not Impair PMN Viability

It is relevant to confirm that the CF sputum model does not affect the viability of PMNs. Viability was defined as maintenance of membrane barrier function of PMNs that is a widely accepted measure in any cell. Viability was measured based on the general principle of active dye extrusion of living cells using the Zombie Aqua dye. A representative of the flow cytometry gating strategy used to assess the viable percentage (Zombie Aqua-negative) of purified PMNs (CD66b-positive cells) is shown in Figure 3.1A. The sputum treatment does not influence the surface expression of the PMN marker CD66b (Figure 3.1B). There was no significant difference in cell viability between healthy blood PMNs exposed to the CF sputum cocktail and those incubated in assay medium only. In contrast, PMNs treated with 100 nM PMA for 30 min showed a significant and expected reduction in viability to an average of 74% when compared to sputum-supernatant treated or untreated PMNs (Figure 3.1C). These results confirm that the CF sputum exposure does not impact the viability of human PMNs and subsequent observations do not result from PMN plasma membrane damage.

CF Sputum Does Not Induce Apoptosis in Human PMNs

The exposure of human PMNs to the CF sputum supernatant could also lead to the induction of apoptosis. To address this, human PMNs were exposed to CF sputum for 3.5 and 16 h and apoptosis was assessed by flow cytometry to identify the following cell populations: viable, early apoptotic, necrotic and late apoptotic. The CF sputum treatment did not increase the proportion of early or late apoptotic cells (Figure 2). On the contrary,

there was a trend, although not significant, towards the CF sputum delaying spontaneous apoptosis and increasing the proportion of viable PMNs after 16 h of ex vivo incubation (Figure 3.2). CF sputum did not cause PMN necrosis even after 16 h of incubation (Figure 3.2). These results confirm that the CF sputum does not lead to necrotic or apoptotic death of PMNs.

CF Isolates of *S. aureus* Are Killed by Human PMNs

Next our goal was to explore the effect of CF sputum treatment on PMNs' ability to kill CF respiratory pathogens. Table 3.1 displays the *S. aureus* CF clinical isolates from 8 different CF subjects that were used in this study. Four MRSA isolates and four MSSA isolates obtained from CF patients were selected from Emory's CFBR. Whole-genome sequences are available with the assembly metrics for all *S. aureus* isolates[155].

Killing of *S. aureus* CF clinical isolates by healthy PMNs was measured by the 384-well microplate-based killing assay[150]. USA300 was used as a laboratory reference strain (Table 3.1)[144,145]. Our result show that all CF isolates of *S. aureus* could be killed by healthy PMNs (Figure 3.3A). To explore whether CFTR deficiency of human blood PMNs would affect their ability to kill *S. aureus*, the eight *S. aureus* CF isolates previously studied were also subjected to measure their killing by CF PMNs using the microplate-based killing assay. As results in Figure 3.3A show there were no significant differences in PMN-mediated MRSA or MSSA clearance between non-CF and CF PMNs. Interestingly, only killing of the laboratory strain USA 300 was significantly impaired in CF PMNs compared to healthy cells (Figure 3.3A). Therefore, we conclude that human normal and CF blood PMNs are competent in killing *S. aureus*.

To further confirm this with classical methodology, the high-throughput killing assay results were repeated by agar plate-based colony counting assays in case of four CF isolates (two randomly picked MRSA, and 2 MSSA isolates) (Figure 3.3B). Results generated with both methods reveal the same extent of killing with no significant differences between the results. To further characterize the microplate-based killing assay, we show representative data for USA300 and MSSA22 indicating a tight correlation between initial bacterial concentrations and incubation time values (Figure 3.3C). Moreover, additional control experiments confirm that the use of saponin under the conditions of the killing assay does not interfere with *S. aureus* growth (Figure 3.3D). To exclude the possibility that some residual sputum will be left with PMNs (despite extensive washes) that could affect *S. aureus*, we exposed human PMNs to CF sputum for 3.5 h, washed the cells subsequently twice with PBS according to the protocol and collected the supernatants of the last wash buffer. As shown in Figure 3.4E, this wash buffer did not have any inhibitory action on the exponential growth of *S. aureus* MRSA24. Thus, the CF sputum treatment or steps of the killing assay protocol do not interfere with *S. aureus* growth further confirming that the 384-well plate-based, high-throughput killing assay described here represents an efficient and reliable way to measure *S. aureus* killing, equivalent to the classical colony-counting method.

CF Sputum Compromises the Killing of *S. aureus* Clinical Isolates by PMNs

To determine whether the CF sputum inhibits PMN-mediated *S. aureus* killing, PMNs from healthy donors were pretreated with CF sputum supernatant and bacterial killing was measured using all eight *S. aureus* CF isolates simultaneously via the microplate-based killing assay. Each trend line connecting the points from ‘no sputum’ to

'sputum' represents one experiment. When occasionally the bacteria were not killed more than 20% by healthy PMNs (in the absence of sputum treatment), the data were excluded as a strong enough baseline of bacterial killing by PMNs was not achieved. Seven out of the eight CF *S. aureus* isolates tested showed a significant reduction in PMN-mediated killing upon sputum treatment (Figure 3.4A). PMNs were infected with a MOI of 5 with MSSA70 and MSSA17, as both isolates were highly resistant to killing at MOI of 10. Interestingly, clearance of only one *S. aureus* isolate, MRSA74, by PMNs was not compromised by the sputum treatment (Figure 3.4A). Killing of the *S. aureus* control strain, USA 300, was significantly inhibited by the CF sputum (Figure 3.4B). To address whether the observed inhibitory effect of the CF sputum is unique to CF, we also tested sputa collected from two healthy controls. As the results in Figure 4C show, killing of the tested *S. aureus* CF isolate (MRSA24) was only inhibited by CF sputum, not by sputa from control subjects. Altogether, we provide evidence to show that CF sputum inhibits the killing of *S. aureus* clinical isolates by PMNs.

CF Sputum Does Not Inhibit PMN Attachment and Phagocytosis of *S. aureus*

Phagocytosis is the main mechanism by which PMNs kill *S. aureus*. Phagocytosis involves two steps, bacterial attachment to the PMN surface that is followed by uptake into the phagosome. To determine whether the first step, bacterial binding to PMNs, is affected by the CF sputum, flow cytometry was utilized. A *S. aureus* strain JE-2 expressing red-fluorescence protein (SA-rfp) was generated and used to quantify the binding of *S. aureus* by PMNs in presence of cytochalasin B to inhibit bacterial uptake. A representative of the flow gating strategy used to assess the percent of *S. aureus* attached to live PMNs (CD66b-positive, zombie-negative cells) is shown in Figure 3.5A. The

same number of PMNs were tested in each condition. Our results found that there was no impairment of *S. aureus* binding to PMNs by the CF sputum (Figure 3.5B). On the contrary, a small but significant increase in binding of *S. aureus* to PMNs was observed (Figure 3.5B). Phagocytosis was quantified using flow cytometry by staining the CF isolate MRSA24 with the pH-sensitive dye pHrodo to detect bacteria only inside the phagosome. A representative flow gating strategy used to assess the percent of *S. aureus* inside phagosomes of purified, live PMNs (CD66b-positive, zombie-negative cells) is shown in Figure 5C. Similar to attachment, there is enhanced phagocytosis of *S. aureus* by PMNs incubated with CF sputum compared to those incubated with assay medium alone (Figure 3.5D). Altogether, our data show that the CF sputum supernatant does not inhibit *S. aureus* binding to or phagocytosis by PMNs.

CF Sputum Does Not Inhibit PMN Superoxide Production in Response to *S. aureus*

Oxidative killing mechanisms of human PMNs are essential to kill S. aureus, both in vitro[150] and in vivo[156]. We therefore hypothesized that the CF sputum decreases ROS production in PMNs stimulated with CF isolates of *S. aureus*. ROS generation by the PMN NADPH oxidase was stimulated by each of the *S. aureus* isolates used in the study (Figure 3.6A). *S. aureus* exposure stimulated an ROS signal that was comparable to that obtained by the positive control PMA (Figure 3.6B). No substantial differences could be observed between CF sputum-treated and untreated PMNs after *S. aureus* stimulation (Figure 3.6C). To confirm these results with a classical detection method specific to superoxide anions, the superoxide dismutase-inhibitable cytochrome-c reduction assay was used. These results further confirmed that there is no significant difference in the superoxide output between CF sputum-treated PMNs vs. untreated PMNs after infection

by *S. aureus* (Figure 3.6D). No inhibition of superoxide production was observed either when a nonliving particle, zymosan, was used in PMNs (Figure 3.6E). Overall, these results indicate that CF sputum pretreatment does not impair PMNs' ability to generate ROS in response to S. aureus.

CF Sputum Does Not Inhibit NET Release of PMNs in Response to *S. aureus*

Neutrophil extracellular traps (NET) represent an extracellular, antimicrobial trapping and killing mechanism of PMNs[138]. NETs are released from PMNs in presence of *S. aureus* and have been detected in CF airways[138,157]. Therefore, we hypothesized that NET formation of PMNs induced by *S. aureus* CF isolates is also detectable in our experimental system. We also aimed at exploring whether *S. aureus*-stimulated NET extrusion is affected by the CF sputum treatment. PMNs released NETs in response to all *S. aureus* isolates tested (Figure 3.7A). CF sputum pretreatment of PMNs did not lead to decreased NET release in the case of any of the bacteria (Figure 3.7B,C). Consistently, each line represents one experiment with a different healthy PMN donor. On the contrary, sputum exposure resulted in significantly enhanced NET release in case of three of the eight CF *S. aureus* isolates (Figure 3.7C). This was further confirmed by an independent method, measuring histone H3 citrullination by flow cytometry on uninfected PMNs treated with the CF sputum cocktail (Figure 3.7D). Overall, NET formation was not inhibited by CF sputum treatment in PMNs stimulated with *S. aureus*.

***S. aureus* CF Clinical Isolates Possess DNAse I Activity**

Since we observed two types of responses among the CF isolates with regard to their NET release (NET formation was either enhanced or unaffected by CF sputum), and long-term survival of *S. aureus* in CF airways has been associated with its DNAse

activity[121], we decided to determine whether differences in NET formation can be related to DNAse activities of the *S. aureus* CF isolates. DNAse activities were measured using two independent methods, a fluorescence-based enzymatic activity protocol and an agar plate-based assay. As results in Figure 3.8A,B show, DNAse activities of the utilized *S. aureus* isolates spread across a range. Data obtained via the two independent methods strongly correlated with each other (Figure 3.8C). There was, however, no correlation between NET release induced by *S. aureus* isolates and their nuclease activity, suggesting that the DNAse activity expressed by the *S. aureus* isolates does not influence the amount of detectable NETs released from PMNs in vitro (Figure 3.8D,E).

Conclusions

We established an *in vitro* model to mimic the CF airway environment to study PMN effector functions. We hypothesized that PMNs from healthy individuals are capable of killing *S. aureus* isolates from CF patients, but bacterial killing would be inhibited by the CF airway environment. Our study aids to show that this simple, in vitro model mimics the impaired killing of *S. aureus* by PMNs observed in the airways of CF patients.

Chronic polymicrobial infections and PMN recruitment to the airways contribute to progressive lung disease in CF[158]. *S. aureus* is the most prevalent airway pathogen in CF and its resistance to antibiotics is increasing among adult patients[134]. PMNs are the first responders to the airways to fight infection; however, PMNs in the CF lung are inefficient at clearing *S. aureus*, leading to chronic infections. Therefore, understanding

why PMNs are defective in combating *S. aureus* infections in CF is relevant to potentially reduce disease morbidity and mortality.

First, we showed that the CF sputum supernatant pretreatment does not affect the integrity of the PMN cell membrane. Therefore, the effect of inhibition on bacterial killing is not due to the loss of PMN cell viability. This was important to establish as normal PMN effector functions require live PMNs with intact plasma membrane[134]. Even PMN cytoplasts, ghost cells generated from human peripheral PMNs by experimental removal of their nuclei and most of their granules, are capable of motility, superoxide production and phagocytosis and maintain an intact plasma membrane.

The "sputum cocktail" is a mixture of sputum supernatants from patients to demonstrate that this effect occurs across the board in CF disease. Individual sputum supernatants will be tested in the future using this model that could help further explain the variability amongst CF patients with regard to their PMNs' impaired *S. aureus* killing. Elborn et al., also observed an upregulation of matrix metalloproteinase-8, annexin I, and nicotinamide phosphoribosyl transferase in CF sputum compared to healthy controls which has been associated with delayed apoptosis and clearing of inflammatory cells[159–162]. There is evidence to suggest that IL-8 and myeloperoxidase (MPO) are biomarkers in the CF sputum at different stages of lung inflammation, such that IL-8 correlates with exacerbations, and MPO and IgG degradation correlates with lower FEV_1% score[163]. Disruption of the PMN cytoskeleton, delayed apoptosis, and upregulated cytokines in the CF sputum could all alter PMNs' function in the lungs[164]. Understanding and further investigating these pathways using this *in vitro* model could

lead to a novel discovery of a critical dysregulated pathway for PMNs to efficiently kill bacteria in the lungs of CF patients.

A clinically relevant question is whether the inhibitory effect of human sputum on *S. aureus* killing by PMNs described here is unique to CF. Sputum obtained from healthy controls did not show any inhibition. While these results suggest that it is a specific feature of the CF sputum, more studies are needed to further confirm this as only a limited number of non-CF control subjects could be studied in this work.

PMNs in CF airways have been shown to co-localize with some bacteria (most likely phagocytosed) but CF blood PMNs were suggested to be impaired in intracellular bacterial killing[108,165,166]. Studies suggest that CFTR dysfunction may affect chloride supply to the phagolysosomes and impaired hypochlorous acid production, impacting NADPH oxidase and MPO-chlorinating activities[108,165–167]. A caveat to these studies is that bacterial killing was measured with CF blood PMNs and PMNs in the airways were not examined, which is where chronic infection takes place. Only very sporadic data are reported related to *P. aeruginosa* phagocytosis or killing by CF airway PMNs or PMNs under CF airway-like conditions[168,169]. Interestingly, our data show unimpaired phagocytosis of *S. aureus* when PMNs are exposed to the CF airway environment. While this assay is a combined measure of bacterial uptake and phagosomal pH changes, and the results suggest that phagocytosis of *S. aureus* by PMNs and subsequent phagosomal acidification is not affected by the CF airway environment, it has only been investigated in our study using one of the eight *S. aureus* isolates. Our data showed no difference in the overall ROS production between sputum-treated and untreated PMNs that is in contrast with a clinical study showing that sputum PMNs in CF patients display reduced

respiratory burst[170]. Assessing the oxidative output in PMNs in the CF airways is a complex task that can partially depend on the experimental and detection systems used[171,172]. Superoxide production initiated by opsonized zymosan particles was not impaired by the CF sputum pretreatment of PMNs. This further confirms that the assembly and activation of the NADPH oxidase is not affected by the CF sputum pretreatment. As previously mentioned, the CFTR mutation has been proposed to impair chloride supply to the phagolysosomes and to affect PMN killing mechanisms in the phagosome[173]. A prior study reported that CF heterozygous PMNs have increased MPO activity[173]. While this could indeed have, theoretically, manifested in some of the variability in our control subject cohort, the same study reported that CF heterozygous PMNs had normal NADPH oxidase activity, MPO release and their microbial killing was directly not assessed. Therefore, due to these and the relatively rare occurrence of CFTR heterozygous patients in the normal population (1:25), it is unlikely to change the major conclusions in this work.

Parts of the CF lung are nutrient rich and optimal for *S. aureus* growth. Based on a transcriptomic analysis of *S. aureus* grown in sputum, *S. aureus* can take up oxygen and is able to acquire iron in the CF lung[174]. *S. aureus* cultures isolated from the CF lung expressed virulence and metabolism genes that differed from *S. aureus* isolated from human joint infection and chronic wound infection[174]. The *S. aureus* isolates had a distinct metabolic profile, and it has been shown that metabolic status of bacteria impacts the efficacy of antibiotics, as well[174,175]. Our study also displays that the *S. aureus* isolates express DNAse I activity suggesting an evasion of NET-mediated killing by the bacterium. Although, NET release is not inhibited by the CF sputum, the bacterial

isolates express DNAse activity to degrade the NETs being released and these data suggest that NETs being released by PMNs in the CF lung are ineffective in killing *S. aureus* and contribute to lung tissue damage. It has been shown that DNAse expression by *Streptococcus* allows the bacterium to escape NET-mediated killing by degradation, as the NETs were able to trap bacteria but not kill them[176–178]. DNAse I is an endonuclease that cleaves DNA phosphodiester bonds, targeting single-stranded DNA, double-stranded DNA, and chromatin DNA in a non-specific manner and has been used as a therapeutic for CF patients to degrade DNA and reduce sputum viscosity[179]. Therefore, patients prescribed DNAse I may display more resistance to NET-mediated bacterial killing. Altogether, PMNs in the CF lung generate NETs and ROS, however the CF sputum along with the virulent *S. aureus* affects the PMNs' ability to efficiently kill. Further analysis of whole-genome sequences for all the *S. aureus* clinical isolates used in this study may help to interpret the discrepancies seen[155]. *S. aureus* is known to express multidrug-resistant genes and efflux transporters that are able to pump out toxic molecules[180,181]. The *S. aureus* resistance to killing is supported by the DNAse I activity expressed by the CF isolates in this study and a study that identified *S. aureus* CF clinical isolates display high nuclease activity that is protective against NET-mediated killing[121]. An abundance of NETs in the CF sputum correlated with chronic *S. aureus* infection in CF patients, suggesting that *S. aureus* may induce NET release by PMNs in the airways but deploy high nuclease activity to evade NET-mediated killing[121]. This abundance of NETs in the sputum is characteristic of the CF lung. Measurements of free DNA in CF patients' sputum, an indirect indicator of NET release in the airway, show an association of declined lung function with increased NET presence in the lung[86]. The increased NET

release from sputum-treated PMNs exposed to some of the *S. aureus* isolates is consistent with this increased NET presence in the CF lung.

This is the first study to investigate the capacity of PMNs to kill *S. aureus* CF clinical isolates. Our data demonstrate that *S. aureus* killing by PMNs is decreased by prior exposure to CF sputum. Attachment of bacteria to PMNs, their uptake into PMNs and PMN-mediated ROS production and NET formation are, however, not inhibited. Several other potential mechanisms could hide behind these observations that will be further investigated in the future. Steps of the PMN oxidative cascade downstream of the NADPH oxidase could be inhibited by the sputum environment. Patients who suffer from the inherited disorder, chronic granulomatous disease, characterized by mutations in proteins of the NADPH oxidase enzyme complex, are susceptible to *S. aureus* infections[106,119,182,183]. Published data suggest that downstream ROS such as MPO-derived HOCl are mainly responsible for direct killing of *S. aureus* and not initial members of the oxidative cascade, superoxide or H_2O_2[106,119,182,183]. Inhibition of MPO enzymatic activity could lead to lower HOCl production in the phagolysosome and to impaired intracellular bacterial killing, in presence of unaltered phagocytosis and NADPH oxidase activity. The somewhat enhanced NET formation induced by the CF sputum detected in case of some of the *S. aureus* isolates could also lead to depriving critical components of the intracellular killing machinery, such as MPO and neutrophil elastase. Decreased primary granule fusion with the phagosome could clearly deliver suboptimal amounts of MPO and elastase leading to diminished bacterial killing[184,185]. *S. aureus* has been described to survive in PMN phagosomes and even lyse human PMNs in vitro[186,187]. While the CF sputum treatment alone does not affect PMN viability, it could

prime PMNs for an enhanced or accelerated lysis by subsequent challenge by *S. aureus* that would ultimately result in impaired bacterial killing.

Overall, our study demonstrates the usefulness of a novel, in vitro experimental model to mimic the CF airway environment and its effect on the antimicrobial effector functions of PMNs. We are the first to investigate bacterial killing of several *S. aureus* CF clinical isolates by human PMNs and to identify the difference in PMN killing of *S. aureus* clinical isolates following PMN exposure to CF and healthy sputum. The data suggest a CF sputum-dependent inhibition of PMN killing of *S. aureus.* This model will be important for future studies elucidating the dysfunction of PMNs present in the airways of CF patients.

Acknowledgments

CF human subject samples were provided by the CF Biospecimen Registry at the Children's Healthcare of Atlanta and Emory University CF Discovery Core. We also thank all the control subjects and CF patients to provide specimens for this study.

Author Contributions

K.F. performed most experiments, designed research protocols, wrote and revised the manuscript; S.L.T., A.M., R.Y., E.E.B. and R.F. performed experiments and proofread the manuscript; A.A.S. designed research protocols, supervised collection of samples from CF patients, reviewed the manuscript and acquired funding; J.B.G. designed research protocols and reviewed the manuscript; B.R. conceptualized the idea, designed research protocols, reviewed the manuscript, acquired funding and supervised the study. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by grants provided by the National Institutes of Health (5R01HL136707 and R21AI154343 to B.R. and UL1TR002378 to A.S.), as well as the Cystic Fibrosis Foundation (MCCART15R0 to A.S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Cystic Fibrosis Foundation.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Boards of the University of Georgia (2012-10769-06, 05-04-2012) and Emory University (00042577).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Tables

Identifier in this work	CF sample name	Methicillin sensitivity	Strains or isolates	CF patient ID	References
MRSA24	CFBRSa24	resistant	Cystic Fibrosis clinical isolates	CFBR-219	*Bernardy* et al.[155]
MRSA25	CFBRSa25	resistant		CFBR-134	
MRSA47	CFBRSa47	resistant		CFBR-105	
MRSA74	CFBRSa74	resistant		CFBR-201	
MSSA17	CFBR_EB_Sa117	sensitive		CFBR-280	
MSSA19	CFBR_EB_Sa119	sensitive		CFBR-309	
MSSA22	CFBR_EB_Sa122	sensitive		CFBR-322	
MSSA70	CFBRSa70	sensitive		CFBR-171	
USA 300	-	resistant	Reference strain	-	*S. aureus* subsp. aureus (ATCC® BAA1717 ™)
SA-rfp	-	-	JE2 background	-	This work

Table 3.1: *S. aureus* CF clinical isolates and reference strains used in this study.

Figures and Figure Legends

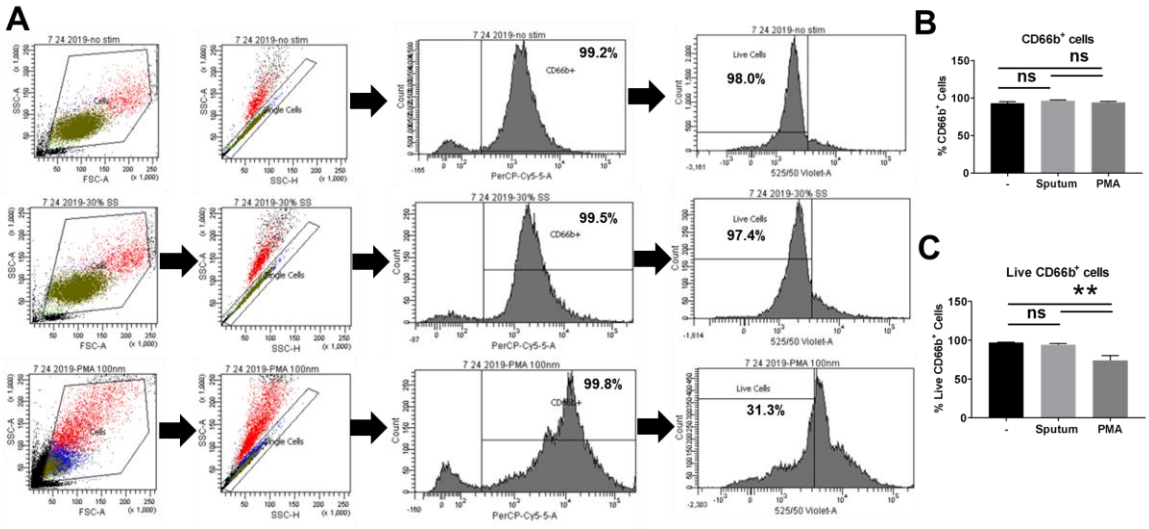

Figure 3.1: CF sputum supernatant treatment does not affect PMN viability. PMN purity and viability following isolation from blood and subsequent 3.5 h-CF sputum incubation was measured by flow cytometry using the Zombie Aqua Cell Viability Kit™. (A) Representative images of the gating strategy used to determine the percent of viable PMNs ($CD66b^+$/Zombie $Aqua^-$) for each condition tested are shown. (B) Flow cytometric analysis showed no significant difference in the percent of PMNs and CD66 surface expression among the conditions tested (n = 15). (C) CF sputum treatment does not impair PMN viability under the conditions used in this study. Treatment with 100 nM PMA for 30 min, however, results in a significant decrease in PMN viability compared to both the sputum supernatant-treated and untreated PMNs (n = 15). One-way ANOVA, Tukey's multiple comparison test. **, $p < 0.01$; ns, not significant. PMA, phorbol 12-myristate 13-acetate; CF SS; Cystic fibrosis sputum supernatant.

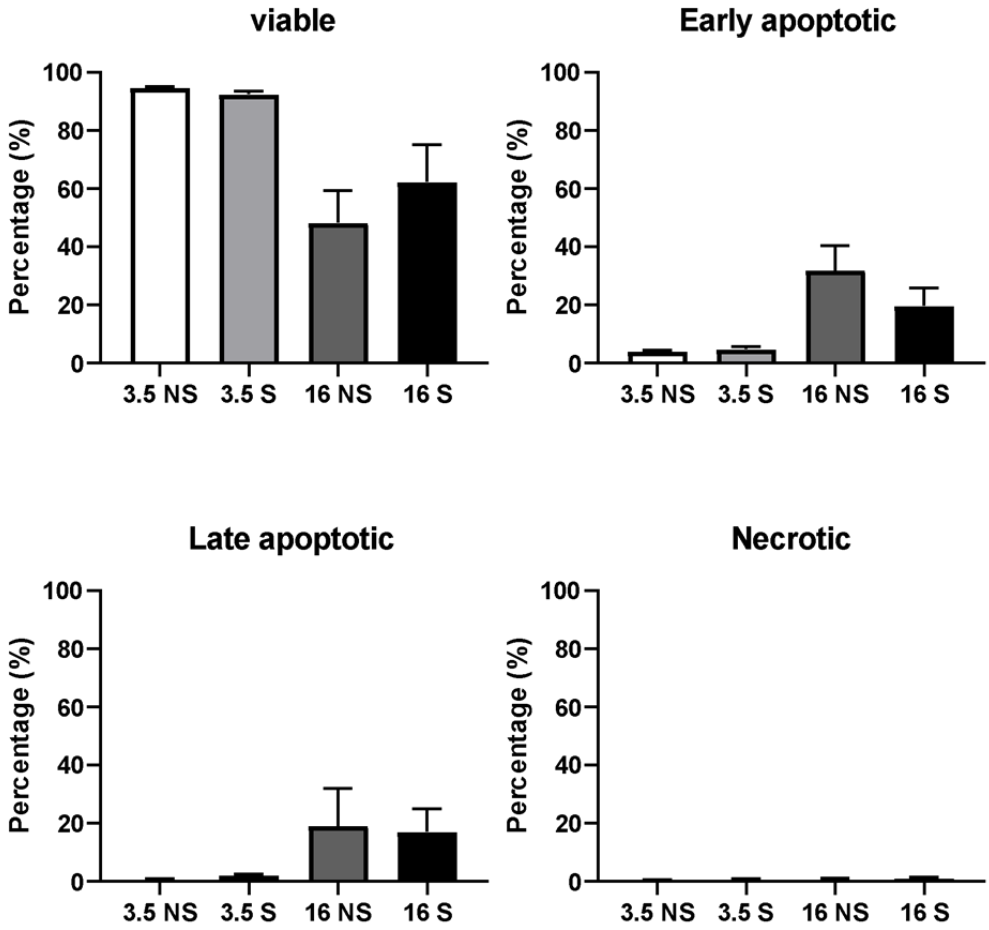

Figure 3.2: CF sputum supernatant does not induce apoptosis or necrosis in human PMNs. Human blood PMNs were incubated in CF sputum supernatants for 3.5 or 16 h prior to fluorescence staining with Apotracker (TM) Green apoptosis probe and propidium iodide (PI) viability dye. The following cell populations were identified by flow cytometry: viable (double negative), early apoptotic (PI-negative, Apotracker-positive), necrotic (PI-positive, Apotracker-negative) and late apoptotic (double positive). Results are expressed as mean ± S.E.M (n = 6). One-way ANOVA, Dunn's multiple comparison test. ns, not significant.

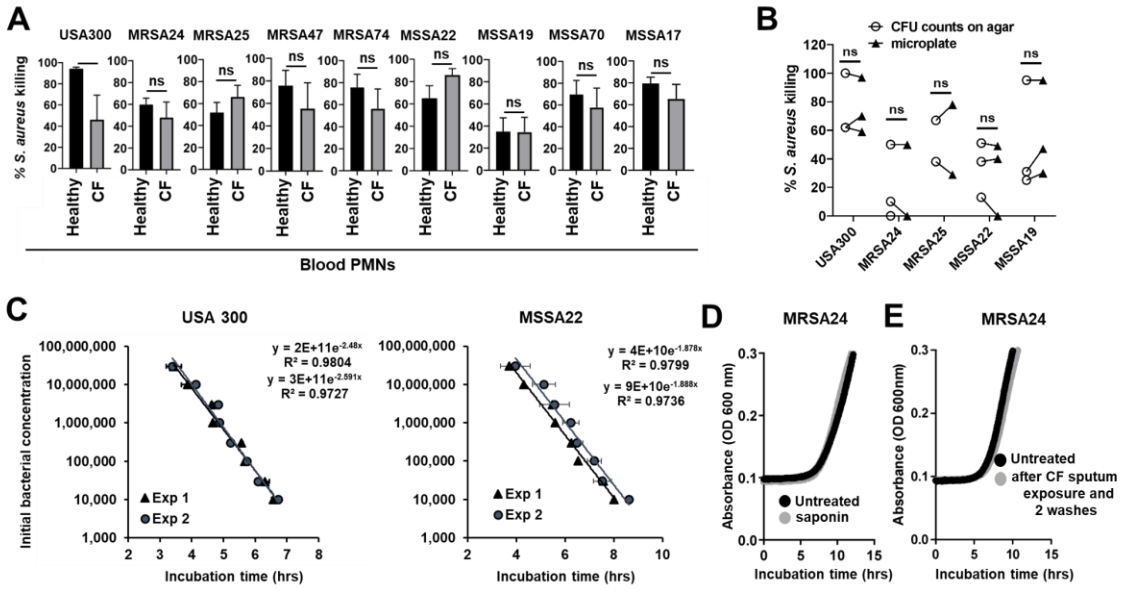

Figure 3.3: Healthy and CF human blood neutrophils kill CF isolates of *S. aureus* in vitro. (A) Healthy and CF human blood PMNs were infected with the *S. aureus* reference strain (USA300), 4 MRSA isolates, and 4 MSSA isolates collected from CF patients. All isolates were infected at MOI of 10 except MSSA70 and MSSA17 that were infected at MOI of 5. Bacterial killing was measured by the high-throughput microplate-based assay. Mean ± S.E.M, $n = 6$. Two-tailed, paired Students' *t*-test. (B) Comparison of PMN-mediated *S. aureus* killing by two methods: high-throughput microplate-based assay and agar plate based colony counting assay. Lines connect data derived from the same, individual experiment and same samples via the two different methods. Experiments were repeated three times on three independent human donors' neutrophils. (C) Standard curves of USA 300 and MSSA22 growth calibrations are shown as examples indicating the tight correlation between initial bacterial concentrations (*y* axis) and the incubation time values (*x* axis). Two representative curves for each strain are shown in the same graph with trend lines, the equation and R^2 values. Mean ± S.D. (D) Representative growth curves of MRSA24 with or without saponin treatment that is used in the killing assay ($n = 3$). (E) Representative growth curves of MRSA24 without any treatment or in medium that was used to resuspend human PMNs after CF sputum exposure for 3.5 h and two washes according to the protocol of the killing assay. These data show that two washes of human PMNS are sufficient to ensure that the CF sputum exposure of PMNs does not interfere with subsequent growth of tested bacteria. CFU, colony-forming unit; ns, not significant; OD, optical density.

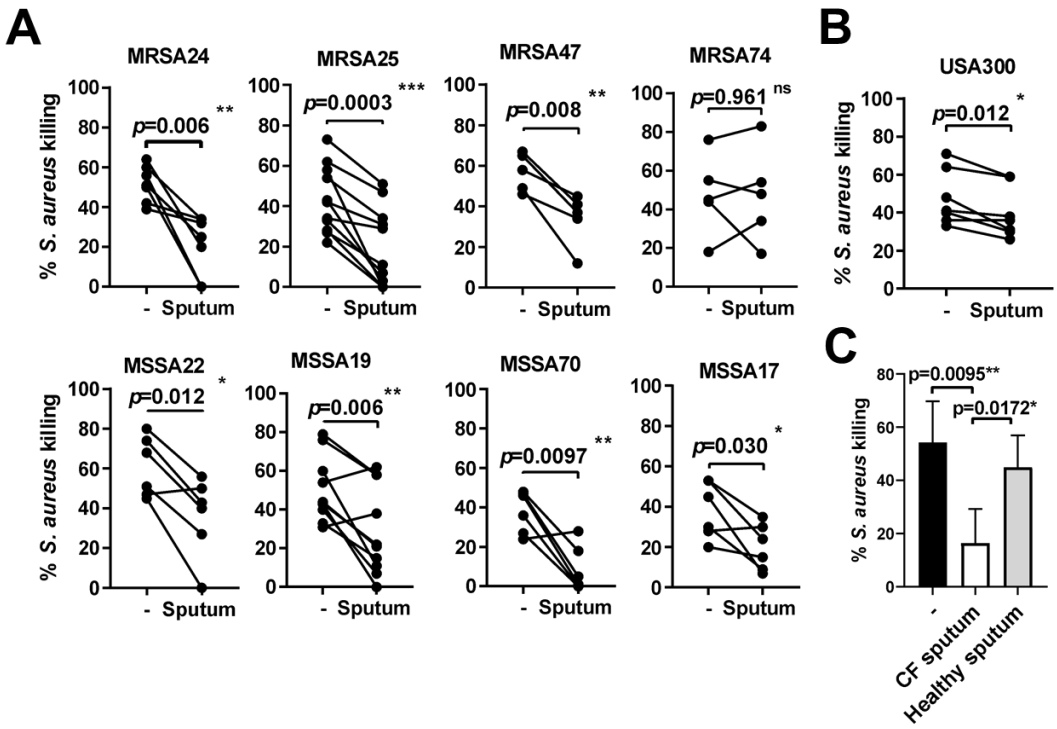

Figure 3.4: CF sputum treatment impairs neutrophils' ability to kill *S. aureus*. PMNs were first treated with 30% (*v/v*) CF sputum cocktail and then infected with the *S. aureus* lab strain (USA300), 4 MRSA isolates and 4 MSSA isolates collected from CF patients. Bacterial killing was measured by a high-throughput microplate-based assay. (**A**) The effect of CF sputum pretreatment on PMN-mediated killing of *S. aureus* CF clinical isolates (n = 5–11) or (**B**) USA 300 (n = 7) is shown. All isolates were infected at MOI of 10 except MSSA70 and MSSA17 were infected at MOI of 5. Mean ± S.E.M. Data were analyzed by Wilcoxon matched-pairs signed rank test. (**C**) The effect of pretreatment with sputum cocktails isolated and pooled from CF patients or healthy controls (n = 2) on PMN-mediated killing of MRSA24 (n = 4). Mean ± S.E.M. Two-tailed, paired Students' t-test (**A**, **B**) or one-way ANOVA test (**C**) were used. Statistically significant differences were considered as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Ns, not significant.

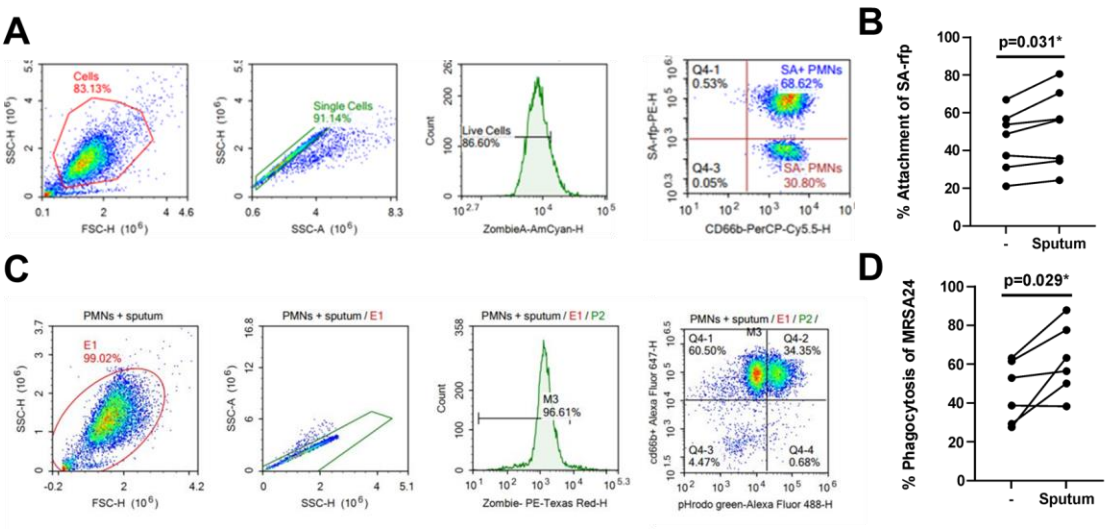

Figure 3.5: CF sputum treatment does not impair *S. aureus* binding and phagocytosis by PMNs. PMNs were isolated from healthy donors and exposed to CF sputum supernatant. To measure bacterial attachment, PMNs were infected with fluorescently labeled, opsonized *S. aureus* (SA-rfp) (10 MOI). (**A**) Representative images of the gating strategy used to determine the percent of SA-rfp attached PMNs ($CD66b^+$/Zombie $Aqua^-$) for each condition. (**B**) Comparison of the attachment of SA-rfp to PMNs untreated or treated with CF sputum supernatant (n = 7). (**C**) To determine phagocytosis, MRSA24 CF isolate was labelled with the pH-sensitive dye pHrodo, opsonized and exposed to PMNs. Representative images of the gating strategy used to determine the percent of MRSA24 phagocytosed by PMNs for each condition. (**D**) Comparison of MRSA24 phagocytosis by PMNs that were untreated or treated with CF sputum supernatant (n = 6). Two-tailed, paired Students' t-test. *, $p < 0.05$.

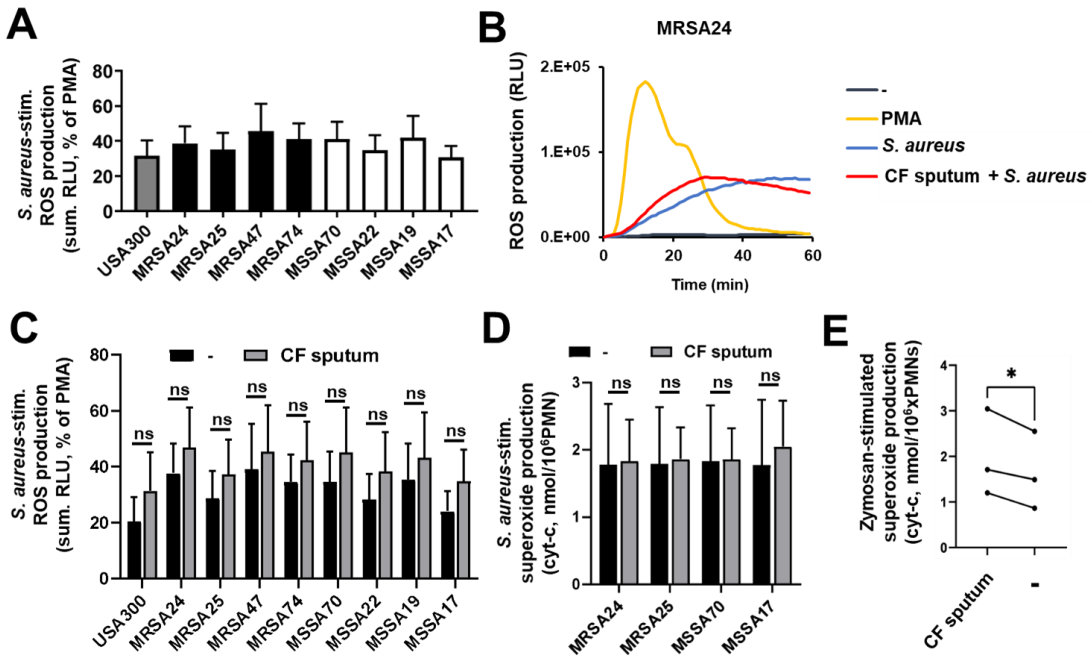

Figure 3.6: CF sputum does not impair PMN superoxide production in response to CF isolates of *S. aureus*. Human blood PMNs were exposed to the indicated isolates of *S. aureus* (10 MOI) and ROS production was measured by Diogenes-based chemiluminescence (**A**–**C**) or cytochrome-c reduction assay (**D**–**E**). (**A**) PMNs respond to the presence of CF bacterial isolates with robust ROS production (mean ± S.E.M, n = 6–7). (**B**) Representative kinetics of *S. aureus*-stimulated PMN ROS release curves (60 min) (n = 7). In total, 100 nM PMA was used as a positive control. The effect of CF sputum exposure on (**C**) ROS production or (**D**) extracellular superoxide generation in human PMNs exposed to the indicated isolates of *S. aureus* is shown. ROS production was calculated for 60 min (n = 6–7) by Diogenes-based chemiluminescence while superoxide production was measured for 60 min by the cytochrome-c reduction assay (n = 3). Mean ± S.E.M. Data were analyzed by Wilcoxon matched-pairs signed rank test. (**E**) Comparison of superoxide production by sputum-treated PMNs vs. sputum-untreated cells following exposure to zymosan (10 MOI, opsonized). Two-tailed, paired Students' *t*-test. Statistically significant differences were considered as *, $p < 0.05$. Ns, not significant.

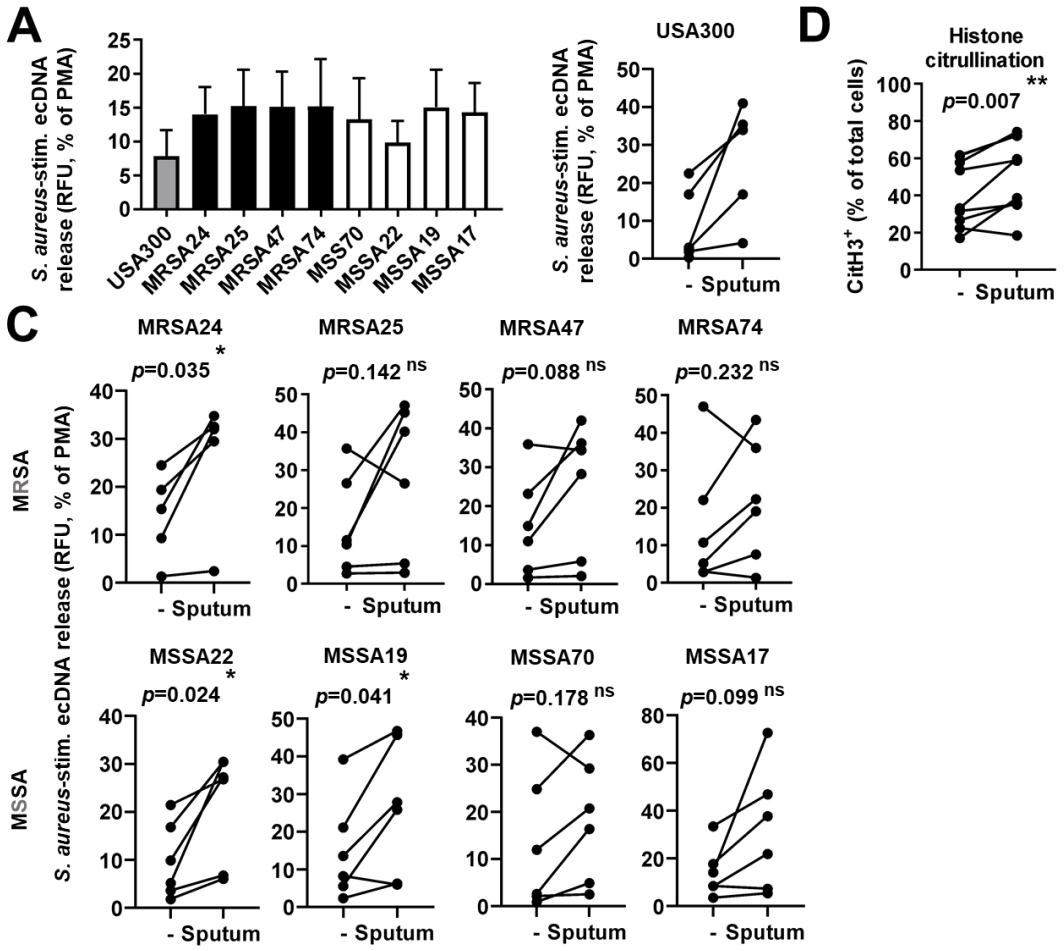

Figure 3.7: NET formation triggered by CF isolates of *S. aureus* is not compromised by CF sputum. Human blood PMNs were exposed to the indicated isolates of *S. aureus* (10 MOI) and extracellular DNA (ecDNA) release was measured for up to 8 h in presence of the membrane-impermeable, DNA-sensitive fluorescent dye, Sytox Orange. (**A**) EcDNA release in *S. aureus*-stimulated PMNs after 8 h measured as increase in fluorescence. The signal by unstimulated PMNs was subtracted and the *S. aureus*-induced ecDNA signal was normalized on the signal obtained by PMA stimulation (100 nM). Mean ± S.E.M, n = 5–6. (**B**) The effect of CF sputum treatment on ecDNA release in PMNs exposed to USA300. Mean ± S.E.M, n = 6. (**C**) The effect of CF sputum treatment on ecDNA release in PMNs exposed to the indicated CF isolates of *S. aureus*. Mean ± S.E.M, n = 6. (**D**) Histone H3 citrullination in PMNs exposed to the CF sputum in the absence of bacterial stimulation measured by flow cytometry (n = 9). Two-tailed, paired Students' *t*-test. Statistically significant differences were considered as *, $p < 0.05$; **, $p < 0.01$. Ns, not significant.

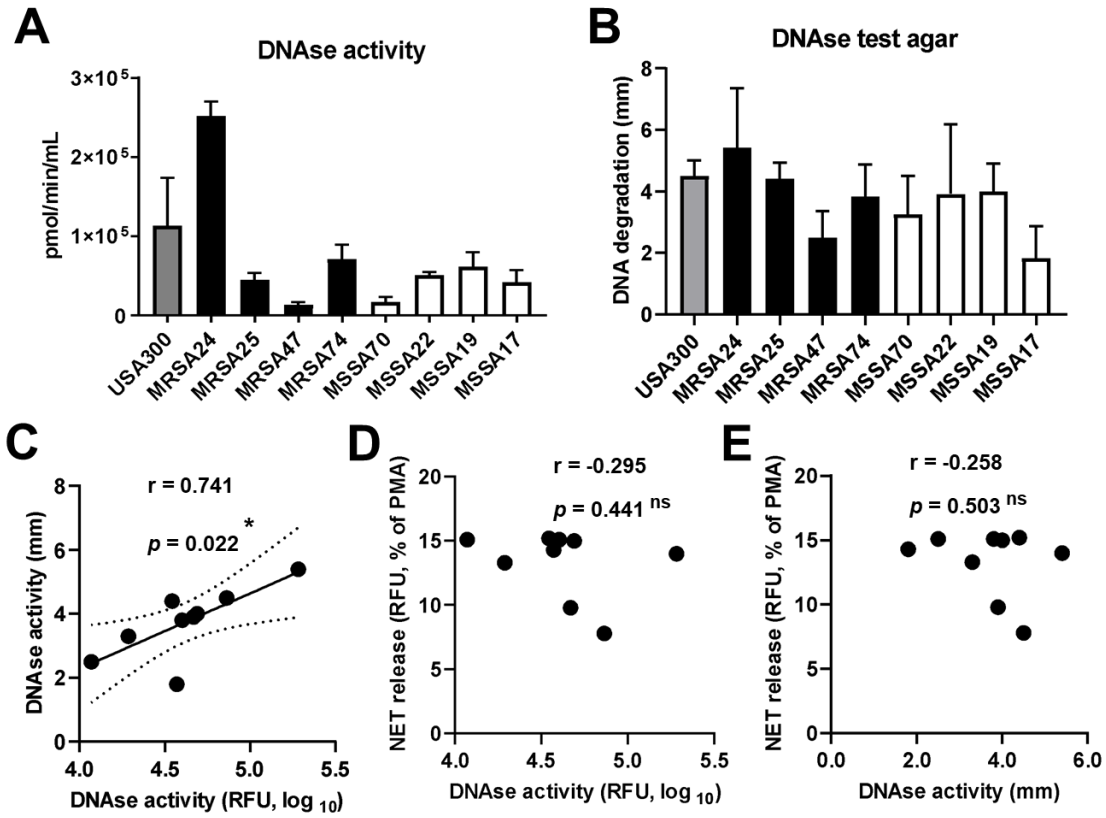

Figure 3.8: DNAse activity of CF clinical isolates of *S. aureus*. DNAse activity was measured in the indicated CF isolates of *S. aureus* by (**A**) a fluorescent enzymatic activity assay (n = 3–6), or (**B**) a DNAse I agar plate-based assay (n = 3). Mean ± S.E.M. Correlation analysis is shown between (**C**) the two DNAse activity measures, (**D**) NET release and results of the fluorescent DNAse activity assay, and ® NET release and results of the plate-based DNAse activity assay. Pearson's correlation coefficient(r). Statistically significant differences were considered as *, $p < 0.05$. Ns, not significant.

CHAPTER 4

CYSTIC FIBROSIS SPUTUM IMPAIRS NEUTROPHIL-MEDIATED KILLING OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS BY DIMINSHING PHAGOSOMAL PRODUCTION OF REACTIVE OXYGEN SPECIES

[1]Fantone K., Goldberg J., Stecenko A., and Rada B., to be submitted to *Cellular and Molecular Immunology*

Abstract

Cystic fibrosis (CF) airway disease is characterized by chronic polymicrobial infections and infiltration of neutrophils (PMNs). In CF, PMNs are unable to kill and clear the main lung pathogen affecting these patients, *Staphylococcus aureus (S. aureus).* Healthy PMNs kill *S. aureus* primarily through intracellular mechanisms such as phagocytosis and reactive oxygen species production. Why PMNs are unable to kill *S. aureus* in CF remains largely unknown. Our published data proposed that the CF airway environment significantly impaired the ability of human PMNs to kill CF isolates of *S. aureus*. CF blood PMNs could kill *S. aureus* to the same extent as healthy blood PMNs, suggesting a less important role of CFTR deficiency in *S. aureus* killing by PMNs. Our published data also reported that the CF sputum did not affect *S. aureus* phagocytosis or NETosis in response to the pathogen. Therefore, we hypothesized that the CF airway environment inhibits phagolysosome fusion in PMNs during MRSA infection, a critical step for effective bacterial killing. Human PMNs were isolated from healthy donors and CF patients and infected with CF clinical isolates of MRSA after exposure to pooled CF sputum supernatants to mimic the milieu found in the CF airway environment. Our results show that all the *S. aureus* CF clinical isolates are mainly killed by phagocytosis rather than extracellular killing mediated by NET formation. Additionally, we found that the CF airway environment impairs phagosomal ROS generation during MRSA infection. Interestingly, CF sputum exposure increased, not reduced, ROS production in another intracellular vesicle compartment, endosomes that formed after the uptake of IgG immune complexes. We also discovered that co-localization of primary granule markers, myeloperoxidase (MPO) or cathepsin D (CatD) with MRSA in the phagosome was

significantly impaired by the CF sputum. Co-localization of neutrophil elastase (NE), another azurophilic granule marker, remained however unaffected. Among the opsonin receptors tested (Fcγ and complement receptors), the CF sputum did not affect surface expression of any of the Fcγ receptors, only reduced that of complement receptor 5a. Altogether our data show that the CF airway environment inhibits the ability of healthy and CF human PMNs to kill *S. aureus*. Our results also indicate that the fusion of the phagosome with primary granules is not directly affected by the CF sputum. More likely, ROS-dependent release of MPO and CatD into the phagosome or the distribution of NADPH oxidase subunits or secondary granules among intracellular vesicle compartments could rather be directly impacted by the CF sputum and ultimately leading to impaired *S. aureus* killing.

Keywords: Cystic Fibrosis, PMN, MRSA, Phagocytosis

Introduction

Cystic Fibrosis (CF) is a life-threatening, inherited disease that affects mucus-producing cells, causing severe damage and inflammation in the lungs. Lung disease in CF patients is characterized by reduced mucociliary clearance leading to chronic inflammation and polymicrobial infections starting at infancy[1,3]. Lung disease is the leading cause of morbidity and mortality in these patients due to the progression of chronic respiratory infections and inflammation dominated by polymorphonuclear neutrophil granulocytes (PMNs)[4,5]. PMNs are crucial for the clearance of bacterial pathogens through various mechanisms. However, despite the high numbers of PMNs found in the CF lung, they are unable to kill persistent bacterial infections present.

According to the United States' Cystic Fibrosis Foundation's annual report in 2021, *Staphylococcus aureus* (*S. aureus*) remains the most prevalent pathogen in CF, infecting about 80% of children under the age of 12 and about half of the total CF patients have *S. aureus* colonized in their lung[2]. While, *S. aureus* is a threat to CF patients, antibiotic resistant is on this rise and methicillin-resistant *S. aureus* (MRSA) attributes to many chronic *S. aureus* infections in these patients[2]. About 30-40% of patients under the age of 30 that have tested positive for *S. aureus* in their lung contain MRSA positive cultures and this number rises to about 50% or more in patients over the age of 30[2,6]. Therefore, MRSA is a prevalent pathogen in these patients throughout their lifetime and the tolerance to antibiotics contributes to the difficulty in finding treatment[6].

As previously mentioned, PMNs are the main drivers in killing pathogens such as *S. aureus* however they are inefficient in doing so in the CF lung. *S. aureus* is an opportunistic pathogen, and our previous research demonstrates that PMNs from the

whole-blood of healthy individuals and CF patients are capable of killing CF clinical isolates of *S. aureus* and MRSA[133]. However, once healthy PMNs are exposed to the CF sputum, they are less able to kill CF clinical isolates of *S. aureus*[133]. Therefore, our goal was to understand how PMNs become ineffective in killing MRSA in the CF airways. We previously described that the CF airway environment does not negatively affect extracellular killing of *S. aureus* through NETosis[133]. *S. aureus* is predominantly killed by PMNs through phagosomal ROS generation following phagocytosis. Therefore, our goal was to further investigate this mechanism and to understand how the CF airway environment affects interactions of phagosomes with MRSA during infection of PMNs[139,142,188]. The final stages of phagosomal maturation include phagolysosome fusion where granules containing antimicrobial enzymes such as neutrophil elastase (NE), myeloperoxidase (MPO), and cathepsin D (CatD) are released into the phagosome to target and kill pathogens. Therefore, we hypothesized that the CF airway environment impairs phagosomal maturation in PMNs during MRSA infection. The goal of this study was to determine a mechanism for which the CF airway environment impairs PMN-mediated killing of MRSA.

Materials and Methods

Human Subjects

All the human subject studies were performed by following the guidelines of the World Medical Association's Declaration of Helsinki.

Healthy Subjects

Healthy human subjects were recruited at the Clinical and Translational Research Unit (CTRU) at the University of Georgia (UGA) and provided informed consent before blood donation for PMN isolation according to the protocol UGA# 2012-10769-06. Healthy subjects were chosen to match the sex and age distributions of CF subjects. Healthy subjects were defined as individuals that did not suffer from CF or an autoimmune disease based upon self-report.

CF Patients

CF subjects were patients recruited at the Adult CF Clinic at Emory University. CF patients signed informed consent to provide blood and sputum samples (IRB00042577). CF diagnosis was confirmed by pilocarpine iontophoresis sweat testing and/or CFTR gene mutation analysis showing the presence of two disease causing mutations. Sputum cultures were taken on the day of the clinic visit when the blood was drawn, and the presence or absence of bacterial infection was identified by the clinical microbiology laboratory. Baseline lung function was defined according to the guidelines of the CF Foundation Patient Registry which is the average of the best percent predicted forced expired volume in one second (FEV_1) for each quarter of the calendar year. Blood was drawn into EDTA-coated tubes and one silicone-coated tube and processed the same day.

PMN and Serum Isolation

PMNs and serum were isolated from the peripheral blood of healthy donors or CF patients. 20–40 milliliters of blood were drawn into EDTA-coated tubes and PMNs were isolated using the EasySep™ Direct Human PMN Isolation Kit (Catalog #19666, Stem

Cell technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. This protocol routinely yields 30–130 × 10^6 live PMNs with >99.9% red blood cell depletion and ~99% purity of the isolated cells. An additional ten milliliters of blood were drawn into a separate, silicone-coated tube without anticoagulant and allowed to clot at room temperature for 30 min. The tube was centrifuged twice (1300× *g*, 5 min), the resulting supernatant (serum) was collected, and the coagulant was discarded. The serum was kept on ice for same-day experiments or frozen at −80 °C for future work.

CF sputum collection and processing

All human studies involving sputum collection from CF patients were approved by the Emory University Institutional Review Board and were in accordance with institutional guidelines. All donors gave consent before sputum collection. Sputum samples were processed and modified from the previously published method[133,143]. CF sputum cocktails were made as previously published to test the "CF Sputum Model"[133]. Briefly, Sputum supernatant from five CF patients were pooled and PMNs were exposed to 30% (*v/v*) CF sputum cocktail for 3.5 hours at 37ºC. Following incubation, PMNs were washed twice with 1 x HBSS and resuspended in assay media (1 x HBSS, 10mM HEPES, 5mM glucose, and 1% (*v/v*) autologous serum).

Bacteria

Four methicillin-resistant *S. aureus* (MRSA) isolates recovered from the respiratory tract of CF patients were obtained from the Emory Cystic Fibrosis Biospecimen Repository (CFBR). The age of the four CF patients of the MRSA donors ranged between 20–53 years. USA300, was used as a laboratory reference strain[144,145]. Table 1 lists the isolates used in this study.

For all experiments, *S. aureus* was grown on blood agar (TSA II 5% SB) at 37 ºC overnight and cultured the following day in optimal growth medium (3 mL LB medium) at 37 ºC shaking for 2-3 hours. The bacteria were collected, resuspended in 1 x HBSS and optical density was measured in a 96-well microplate at 600 nM in Varioksan flash™ microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). As previously published, optical density value of 0.6 were determined to correspond to 1.0 x 10^9 CFU/mL bacterial concentration. For all experiments, bacteria were opsonized with 10% (*v/v*) autologous serum of the PMN donor (healthy control or CF patient) for 20 mins at 37ºC. Following opsonization, there bacteria were washed by centrifugation and resuspended in assay medium prior to infecting PMNs.

High throughput bacterial killing assay

To assess bacterial killing, a high throughput 384-well microplate-based assay was used based upon our previously published method[133]. Briefly, purified human PMNs from healthy control or CF patients, age and gender matched were either untreated or treated with CF sputum cocktail as described above. Following pretreatment of CF sputum, opsonized bacteria were mixed at a ratio of 1:1 multiplicity of infection (MOI, MRSA: PMN) in microcentrifuge tubes. The tubes were incubated at 37ºC for 30 min which mixing every 5-7 mins. After 30 mins, PMNs were lysed in 1mg/mL saponin in 1 x HBSS on ice for 5 mins and diluted in 1 x HBSS by 100-fold. The samples were transferred to a 384-well microplate containing LB broth at a 100-fold dilution in quadruplicates. The microplate was read in a microplate spectrophotometer Biotek Epoch 2™ (Agilent, Santa Clara, CA, USA). Bacterial growth was measured with an absorbance reading at 600 nM every 4 mins for 16 hours with constant heating at 37ºC. After the

measurement, growth curves were generated, and bacterial killing was expressed as a decrease in surviving bacteria over time.

Drug treatment of PMNs for inhibition of phagocytosis or NETs

To assess the primary mechanism for MRSA killing by PMNs from healthy donors, phagocytosis of PMNs was inhibited by 10µM cytochalasin-B (MedChem Express, cat#14930-96-2) and NETosis of PMNs was inhibited by 10µg/mL of DNAse I (Sigma-Aldrich, cat#AMPD1-1KT). After PMN isolation from blood of healthy donors, PMNs were either untreated, pretreated with 10uM cytochalasin-B, or 10ug/mL DNAse I for 20 mins at 37ºC. Following pretreatment, PMNs were infected with opsonized MRSA isolates as described above. Control samples were added with bacteria and drug alone (no PMNs), to assess whether drug affects bacterial growth itself.

Phagocytosis

Phagocytosis of MRSA24 by PMNs from healthy donors with/without CF sputum cocktail treatment was determined by the imaging cytometer, Amnis® ImageStream®-X Mark II™ (Millipore Sigma, Burlington, MA, USA). Phagocytosis of MRSA24 by PMNs from healthy donors and CF donors, and from CF sputum-treated CF PMNs was performed by flow cytometry on the Novocyte Quanteon 4025™ (Aligent, Santa Clara, CA, USA). MRSA24 was first stained with 5mM pHrodo™ iFL green STP Ester (Invitrogen, cat#P35369) for 45 mins at 37ºC, protected from light. After staining, pHrodo-stained MRSA24 was opsonized with 10% autologous serum for 15mins and added to PMNs at MOI of 1. PMN-MRSA24 cocultures were incubated for 1 hour at 37ºC, protected from light. For negative control, PMNs were placed on ice and protected

from light after addition of pHrodo-stained MRSA24. After 1 hour, PMNs were washed with 1 x PBS and resuspended in 1 x PBS.

For ImageStream® analysis, PMNs were fixed with stabilizing fixative (BD Biosciences, cat#338306) at a 1:3 dilution for 15 mins. After fixation, PMNs were washed and resuspended with 1 x PBS, and stained with Dapi nucleic acid staining (Sigma-Aldrich, cat#28718-90-3) at 300nM for 15 mins at room temperature. After Dapi staining, PMNs were washed and resuspended in 100µl 1 x PBS and analyzed on ImageStream® at 40X resolution. For MRSA24 (pHrodo green) detection, the blue laser at 488 nm was used and read at channel 2 with the 480-560 nm filter. For Dapi detection, the violet laser at 405 nm was used and read at channel 7 with the 430-505 nm filter. Data were analyzed with the IDEAS® 6.2 ImageStream® software at the University of Georgia Coverdell Biomedical Microscopy Core.

For flow cytometry analysis of phagocytosis of MRSA24, PMNs were stained with Zombie Aqua™ Fixable Viability kit (Biolegend, cat#423102) at a dilution of 1:500 for 15 mins after washing, following infection. PMNs were then washed and resuspended in 1% BSA in 1 x PBS. PMNs were stained with the granulocyte marker, CD66b, AlexaFluor 647 (Biolegend, cat#561645) at a final concentration of 1ug/mL for 30mins. PMNs were then washed and resuspended in BD stabilizing fixative and analyzed on the Novocyte Quanteon™. Zombie Aqua was detected by the violet laser at 405 nm with the 525/50 filter, MRSA24 (pHrodo-green) was detected by the blue laser at 488 nm with the 530/30 filter, and CD66b was detected by the red laser at 637 nm with the 660/20 filter. Data were analyzed at the University of Georgia College of Veterinary Medicine Cytometry Core Facility with the NovoSamplerQ utilizing NovoExpress software v.1.4.1.

Neutrophil Elastase Activity Assay

To measure Neutrophil Elastase (NE) activity, PMNs were either untreated or treated with CF sputum cocktail as described above. Following, pretreatment PMNs were either placed on ice or infected with MRSA24 at MOI of 1 for 30 mins at 37ºC. For a positive control, 2µM of a PMN stimulator, fMLP was added. Following infection, uninfected and infected PMNs were centrifuged at 450g for 5 mins at 4ºC to pellet PMNs. The supernatant was transferred to new microcentrifuge tubes and kept at 4ºC. The PMN cell pellet was resuspended in 100µl of 1 x PBS and sonicated for 30 seconds to lyse the cells. After cell lysis, PMNs were centrifuged at 10,000g for 5 mins at 4ºC to remove cell debris. The supernatant was collected into new microcentrifuge tubes and labeled as lysates. NE activity of these samples was determined by following the manufacturer's protocol for the fluorometric Neutrophil Elastase Activity Assay Kit (Cayman Chemical, cat#600610). A standard curve was generated with human neutrophil elastase diluted in cell assay buffer ranging from a concentration of 0.156 mU/ml to 10mU/ml. Substrate solution diluted in DMF was added to the samples, standards, and blanks. Enzymatic activity was detected with a fluorometric microplate reader, Varioskan Flash™ (Thermo Scientific, Waltham, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Fluorescence kinetics were measured every 2 mins for 2 hours at 37ºC. The average relative fluorescence units (RFU) were taken and neutrophil elastase concentration (mU/ml) of the samples were calculated upon the standard curve equation generated.

Phagolysosome fusion by ImageStream Cytometry

To measure phagolysosome fusion of NE, MPO, and CatD with MRSA24, ImageStream® analysis was performed. PMNs were isolated from healthy donors and/or CF patients, and either untreated or treated with CF sputum cocktail as described above. MRSA24 was stained with pHrodo green as previously described and PMNs were infected with pHrodo-stained MRSA24 for 30 mins at 37ºC at MOI of 1, protected from light. For a negative control, pHrodo-stained MRSA24 was added to PMNs and immediately placed on ice, protected from light. Following infection, PMNs were washed and resuspended in BD stabilizing fixative at a 1:3 dilution for 15 mins at room temperature. After fixation, PMNs were washed with 1 x PBS and resuspended in blocking buffer (5% BSA in 1 x PBS containing 10% horse serum and 0.1% Triton-X100). PMNs were blocked for 30 mins at room temperature, protected from light. After blocking, PMNs were washed with 5% BSA and resuspended in 1% BSA in 1 x PBS with 5% horse serum. Primary antibodies were added at a 1:500 dilution overnight at 4ºC. The antibodies used were neutrophil elastase rabbit polyclonal antibody (Thermo Scientific, cat#PA5-87158), myeloperoxidase rabbit polyclonal antibody, unconjugated (Thermo Scientific, cat#PA5-16672), and cathepsin D rabbit polyclonal antibody Proteintech, cat#2137-1-AP). The next day, PMNs were washed with 1% BSA in 1 x PBS and resuspended in 1% BSA with 1% horse serum and stained with secondary antibody for 1 hour at room temperature. The secondary used was VectaFluor™ horse anti-rabbit IgG, Dylight® 594 antibody at a 1:200 dilution. Following incubation with the secondary antibody PMNs were washed with 1% BSA and resuspended in 1 x PBS and stained with 300 nM Dapi nuclear stain for 15 mins at room temperature. Following Dapi

stain, PMNs were washed and resuspended in 100µl 1 x PBS and quantified on ImageStream® at 40X resolution. For MRSA24 (pHrodo green) detection, the blue laser at 488 nm was used and read at channel 2 with the 480-560 nm filter. For NE, MPO, and CatD detection, the yellow laser at 561 nm was used and read at channel 4 with the 595-640 nm filter. For Dapi, the violet laser at 405 nm was used and read at channel 7 with the 430-505 nm filter. The data were analyzed with the IDEAS® 6.2 ImageStream® software with the colocalization wizard.

MPO activity assay

MPO activity was measured with the Myeloperoxidase Chlorination Fluorometric Assay Kit (Cayman Chemical, cat#10006438), following the manufacturer's protocol. PMNs were prepared as described above in the Neutrophil Elastase Activity Assay. A standard curved was generated with the fluorescein standard diluted in MPO assay buffer ranging from 5 nM to 200 nM. Initiator solution was added to the samples, standards, and blanks which consisted of MPO assay buffer with 16.4% chlorination substrate, and 8.2% of 5mM hydrogen peroxide. Detection of MPO chlorination was detected with a fluorometric microplate reader, Varioskan Flash™ at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence kinetics were measured every min for 1 hour. The average RFUs were taken for all samples and standards and MPO activity concentration (nM) of each sample was calculated with the standard curve equation generated.

Cathepsin D activity assay

Cathepsin D Activity was measured with a fluorometric assay. PMN samples were prepared as previously described in the Neutrophil Elastase Activity Assay. A

standard curve was generated with recombinant human cathepsin D protein, carrier-free (R&D Systems, cat#1014-AS) ranging from 3.125 ng/mL-200ng/mL. The standards and substrate were diluted in assay buffer (0.1 M NaOAc, 0.2 M NaCl, pH 3.5). 50µl of substrate diluted to 60µM in assay buffer was added to the samples, standards, and blanks. Detection of Cathepsin D activity was detected with the fluorometric microplate reader, Varioskan Flash™ at an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Fluorescence kinetics were measured every min for 1 hour. The average RFUs were calculated for all samples and standards and Cathepsin D activity concentration (ng/mL) was calculated based upon the standard curve equation.

Phagosomal ROS by ImageStream Cytometry

To measure phagosomal ROS production by PMNs after exposure to CF sputum cocktail, ImageStream® analysis was performed. For all experiments, PMNs were immediately placed on ice after administration of MRSA24 and/or OxyBURST/Fc OxyBURST for the negative control. To measure ROS production, PMNs were isolated from healthy donors and either untreated or treated with CF sputum cocktail as previously described. Following sputum exposure and washing, PMNs were given 10µM of OxyBURST™ Green H2DCFDA, SE (Invitrogen, cat#D2935) for 1 hour at 37ºC. After 1 hour, PMNs were washed with 1 x PBS and resuspended in 100µl of 1 x PBS and placed on ice. Live PMNs were analyzed on ImageStream®. To measure phagosomal ROS, after sputum exposure, PMNs were given 120ug/reaction of Fc OxyBURST™ Green Assay Reagent (Invitrogen, cat#F2902) for 1 hour at 37ºC. After 1 hour, PMNs were washed, resuspended in 100µl of 1 x PBS and placed on ice. Quenching of green fluorescence on the outside of the cell was done with the addition of 0.4% Trypan Blue.

Live PMNs were analyzed on ImageStream®. For detection of OxyBURST and Fc OxyBURST positive PMNs, PMNs were analyzed at 40X resolution and the green laser at 488 nm was used with channel 2 (480-560 nm filter).

To measure colocalization of OxyBURST and MRSA24 in the phagosome, PMNs were isolated from healthy donors and either untreated or treated with CF sputum cocktail. MRSA24 was stained with 5mM pHrodo™ Red, succinimidyl ester (Invitrogen, cat#P36600) for 45 mins at 37ºC. After, pHrodo Red staining, MRSA24 was washed with 1 x HBSS and stained with 1mM of OxyBURST for 45 mins at 37ºC. Following OxyBURST stain, the pHrodo-oxyBURST-stained MRSA24 was opsonized with 10% (*v/v*) autologous serum for 15mins at 37ºC. The pHrodo-oxyBURST-stained MRSA24 was washed and resuspended in assay media and added to PMNs at MOI of 1 for 1 hour at 37ºC. After 1 hour, PMNs were washed, resuspended in 1 x PBS and live PMNs were analyzed on ImageStream® at 40X resolution. To measure colocalization of Fc OxyBURST and MRSA24, MRSA24 was first stained with pHrodo Red for 45 mins at 37ºC and then opsonized with 10% (*v/v*) autologous serum for 15 mins at 37ºC. PMNs were then infected with pHrodo-stained MRSA24 (red) at MOI 1 and administered 120ug/reaction of Fc OxyBURST for 1 hour at 37ºC. After 1 hour, PMNs were washed, resuspended in 1 x PBS and placed on ice. Live PMNs were analyzed with ImageStream® at 40X resolution. For detection of OxyBURST and Fc OxyBURST, the green laser at 488nm was used at channel 2 (480-560 nm filter). For the detection of pHrodo-red MRSA24, the yellow laser at 561 nm was used and read at channel 3 with the 560-595 nm filter. The data were analyzed with the IDEAS® 6.2 ImageStream® software with the colocalization wizard (Supplemental Figure 1).

Flow Cytometry of PMN Cell Surface Receptors

For flow cytometry analysis of PMN cell surface receptors, PMNs were either untreated or treated with CF sputum cocktail and stained with Zombie Aqua™ Fixable Viability kit (Biolegend, cat#423102) at a dilution of 1:500 for 15 mins after washing. PMNs were then washed and resuspended in 1% BSA in 1 x PBS. Two panels were generated for detection of the Cell surface receptors. In the first panel (panel 1), PMNs were stained with 1ug/mL FITC CD66b (Biolegend, cat#305104) and 2ug/mL of PE anti-human IgG FcγR (Biolegend, cat#410707) for 1 hour. For a negative control, 1µl of human FcR blocking reagent (Miltenyi Biotec, cat#130-0590901) was given 10mins prior to adding antibodies. For the second panel (panel 2), PMNs were stained with 1ug/mL of PerCP/Cy5.5 CD66b, 2ug/mL APC anti-human C3AR antibody (Biolegend, cat#345805), 2ug/mL of Alexa Fluor® 700 anti-human CD88 (C5aR) antibody (Biolegend, cat#344313), 2ug/mL of FITC anti-human CD64 (FcγRI) antibody (Biolegend, cat#305005), 2ug/mL of PE anti-human CD32 (FcγRII) antibody (Biolegend, cat#303205), and 2ug/mL of Brilliant Violet 421 mouse anti-human CD16 (FcγRIII) antibody (Fisher Scientific, cat#BDB562878) for 1 hour. PMNs were then washed and resuspended in BD stabilizing fixative and analyzed on the Novocyte Quanteon™. Zombie Aqua was detected by the violet laser at 530 nm with the 525/50 nm filter. For panel 1, CD66b was detected by the blue laser at 488 nm (530/30 nm filter) and IgG FcγR was detected with the red laser at 660 nm (670/30 nm filter). For panel 2, CD66b was detected with the blue laser at 695nm (695/40 nm), C3AR was detected with the red laser at 660nm (660/20 nm filter), C5AR was detected with the red laser at 725 nm (720/40 nm filter), CD64 was detected with the blue laser at 488 nm (530/30 nm filter),

CD32 was detected with the yellow laser at 860 nm (585/40 nm filter), and CD16 was detected with the violet laser at 445 nm (450/50 nm filter). Data were analyzed at the University of Georgia College of Veterinary Medicine Cytometry Core Facility with the NovoSamplerQ utilizing NovoExpress software v.1.4.1.

Statistical Analysis

Results between PMNs treated with and without CF sputum were analyzed by two-tailed, paired Student's t-test. Data are expressed as mean plus-minus standard error of mean (SEM). Statistically significant differences were considered as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ^{ns}, not significant. Statistical analyses were carried out with GraphPad Prism version 9.0 for Windows software (GraphPad Software, San Diego, CA, USA).

Results

CF Sputum Treatment Impairs CF PMNs' ability to kill MRSA

Our previously published data demonstrated that PMNs isolated from healthy donors and exposed to CF sputum cocktail had impaired killing of CF clinical isolates of MRSA[133]. Our next goal was to explore the effect of the CF sputum cocktail on CF PMNs and their ability to kill MRSA. To confirm our results that the CF sputum impairs PMN-mediated killing of MRSA, CF PMNs were exposed to CF sputum cocktail and infected with CF clinical isolates of MRSA and our lab reference strain USA300. Our results show that CF PMNs exposed to CF sputum are impaired in killing 3 out of the four clinical isolates tested (Figure 4.1A). Each trend line represents one experiment and one CF donor. Killing of MRSA24 and MRSA47 are significantly impaired by CF PMNs

exposed to CF sputum, whereas MRSA74 is not (Figure 4.1A). This is consistent with our previous data where we did not see a significant difference in killing of MRSA74 by PMNs untreated or treated with CF sputum[133]. Although MRSA25 is not significantly impaired in killing by CF PMNs treated with CF sputum shown by a p-value of 0.0624, it is trending towards significance and does show an overall impairment (Figure 4.1A). Additionally, USA300 is significantly impaired by CF PMNs exposed to CF sputum which is also consistent with our previously published data[133] (Figure 4.1B).

We also showed previously that there was no difference in bacterial killing of MRSA CF clinical isolates by PMNs isolated from the peripheral blood of healthy donors vs. CF patients[133]. We obtained similar results with new CF patients further strengthening our prior observation (Figure 4.1B)[133]. Additionally, we noticed the same effect on the lab reference strain USA300 where CF PMNs are significantly impaired in killing USA300 compared to PMNs from healthy donors (Figure 4.1B). Altogether, we provide evidence that the CF PMNs are competent to kill CF clinical isolates of MRSA, however CF sputum impairs the capability of CF PMNs to kill.

MRSA killing by PMNs is predominantly mediated by phagocytosis, not NET release

Our next goal was to identify how MRSA is predominantly killed by healthy PMNs. PMNs kill microorganisms through two major mechanisms, intracellular killing following phagocytosis and extracellular killing mediated by NETs. To determine which mechanism CF clinical isolates of *S. aureus* are killed by in our experimental system, phagocytosis was impaired by cytochalasin-B (blocks the formation of contractile microfilaments and inhibits PMN phagocytosis of bacteria[189,190]), while formed NETs

were destroyed by the treatment of DNAse I. Our data demonstrate that killing of three out of the four MRSA clinical isolates were significantly impaired when phagocytosis was inhibited in healthy PMNs (Figure 4.2). The *S. aureus* CF clinical isolates whose killing was impaired by cytochalasin-B treatment correspond to the same isolates whose killing by PMNs were compromised by the CF sputum. MRSA74 killing by PMNs was not impaired by the CF sputum or by inhibition of phagocytosis (Figure 4.2). Killing of none of the *S. aureus* CF clinical isolates was inhibited when NETs were degraded (Figure 4.2). Additionally, healthy PMNs were significantly impaired in killing USA300 when phagocytosis was inhibited but not when NETs were destroyed (Figure 4.2). Overall, we propose that MRSA is predominantly killed via phagocytosis-initiated intracellular mechanisms in healthy PMNs.

Since we observed that phagocytosis is the main mechanism by which PMNs kill MRSA, we measured whether phagocytosis was impaired in CF PMNs or by CF sputum exposure. Our previously published data demonstrated that phagocytosis of *S. aureus* was not impaired by healthy PMNs treated with CF sputum[133]. To further confirm this evidence, we measured phagocytosis by an independent method, ImageStream analysis which also demonstrated that healthy PMNs exposed to CF sputum were not impaired in phagocytosing MRSA24 (Figure 4.3A, B). CF PMNs exposed to CF sputum also did not show a difference in phagocytosis of MRSA24 when compared to untreated PMNs (Figure 4.3C). Additionally, there was no significant difference in phagocytosis of MRSA24 by untreated PMNs from healthy vs CF donors (Figure 4.3D). Therefore, our results suggest that the impairment of bacterial killing of MRSA by CF sputum exposure

is not due to an inhibition of phagocytosis and CFTR deficiency does not lead to reduced phagocytic abilities in PMNs.

CF sputum impairs phagosomal ROS in MRSA-phagocytosing human PMNs

ROS is an important component of PMNs to target and kill *S. aureus.* Therefore, we investigated whether phagosomal ROS production was impaired by the CF sputum. This was done by surface-labelling MRSA24 with two fluorescent stains, pHrodo to detect *S. aureus* in the phagosome, and OxyBurst to detect H2O2 production close to the bacterial surface. Co-localization of pHrodo with Oxyburst identifies phagosomal ROS production. Interestingly, CF sputum inhibited phagosomal ROS production in MRSA24-phagocytosing human PMNs (Figure 4.4A, B). To explore how CF sputum treatment affected the total PMN H2O2 output, the effect of CF sputum on OxyBurst-labelled human PMNs was measured that surprisingly revealed significant increase in total ROS output (Figure 4.4C-D). These results suggest that CF sputum limits the phagosomal delivery of ROS to MRSA24 to impair downstream bacterial killing. Impairment of phagosomal ROS production is not a general effect in the cell derived from overall ROS output changes as CF sputum increased, not reduced, total ROS generation.

CF sputum reduces co-localization of MPO with phagocytosed MRSA

Primary granules containing MPO also fuse with the phagosome in *S. aureus*-engulfing PMNs, therefore the effect of CF sputum on co-localization of MPO with MRSA was assessed.

MPO trafficking to the phagosome was measured in untreated healthy (Figure 4.5A) and CF (Figure 4.5B) PMNs infected with MRSA24. First, we show that there is a significant decrease in MPO trafficking to MRSA in CF blood PMNs compared to

healthy blood PMNs (Figure 4.4C). Also, CF sputum pretreatment resulted in decrease in MPO co-localization with MRSA in healthy PMNs (Figure 4.5D). This effect of the CF sputum was absent in CF blood PMNs (Figure 4.5D). CF sputum did not affect the MPO fluorescence values (Figure 4.5E) excluding the possibility that reduced co-localization of MPO with MRSA is an indirect consequence of decreased total MPO levels. MPO co-localization with DNA was not changed by CF sputum (Figure 4.5F), indicating that hijacking MPO by DNA (to likely promote NET release) is not the reason for concomitant decreased levels of MPO associated with the bacterium.

To observe if CF sputum affects MPO activity, we determined MPO activity using a MPO chlorination activity. Uninfected PMNs exposed to the CF sputum showed no difference in MPO activity compared to untreated PMNs (Figure 4.5G). MRSA24 infected PMNs exposed to CF sputum also did not show differences in MPO activity inside the cells and released from the cells compared to untreated PMNs (Figure 4.5G).

These data suggest that 1) CFTR could promote MPO delivery to *S. aureus* in the human PMN phagosome, 2) CF sputum reduces MPO-MRSA co-localization in healthy PMNs via targeting CFTR, and 3) CF sputum does not affect overall MPO protein levels or enzymatic activities in PMNs.

CF sputum does not affect NE co-localization with phagocytosed MRSA

Phagosomal maturation is a crucial component to ensure bacterial death inside the phagosome of PMNs. Reduced MPO-MRSA co-localization in PMNs by the CF sputum could be due to impaired fusion of MPO-containing primary granules with the MRSA-containing phagosome. Primary, azurophilic granules containing neutrophil elastase (NE) will fuse to the phagosome during phagolysosome fusion, the last step of phagosomal

maturation[191]. We aimed to determine whether CF sputum impairs phagosomal maturation by measuring phagolysosome fusion in MRSA-infected healthy PMNs exposed to the CF sputum. Co-localization of NE to MRSA24 (present in the phagosome) was performed by ImageStream and no significant difference executed by the CF sputum was observed (Figure 4.6A-B). Total NE levels in PMNs were not affected by the CF sputum (Figure 4.6C). Additionally, NE can also travel to the nucleus to mediate the process of NET formation[192]. Our results suggest that the CF sputum does not impair NE trafficking to the nucleus in MRSA24-infected PMNs (Figure 4.6D). Altogether, CF sputum does not affect NE/MRSA24 co-localization and therefore it is unlikely to impair fusion of phagosomes with primary granules.

CF sputum reduces CatD co-localzation with phagocytosed MRSA

To further explore the delivery of the primary granule content to MRSA24 in the phagosome, we measured the co-localization of MRSA with cathepsin D (CatD), a PMN aspartyl protease, that has also been detected in primary granules and whose release from the granules into the phagosome has been reported to be ROS-dependent. Healthy PMNs treated with CF sputum were significantly impaired in trafficking CatD into the phagosome during MRSA24 infection compared to untreated PMNs (Figure 4.7A, B). CatD levels in PMNs remained unaffected by CF sputum (Figure 4.7C). Interestingly, CatD enzymatic activities in PMNs were also reduced by CF sputum (Figure 4.7D) while no difference was observed in CatD/MRSA co-localization between healthy and CF PMNs (Figure 4.7E). Overall, we conclude that the CF sputum impairs CatD trafficking to the phagosome and catD activity in MRSA-infected PMNs.

CF sputum enhances ROS production in response to IgG immune complexes

To explore if reduction of phagosomal ROS release by the CF sputum was specific to this intracellular compartment, ROS production in endosomes formed after the uptake of IgG immune complexes was measured. We utilized the Fc OxyBurst reagent, an immune complex covalently linked to H2DCF which will oxidize to the fluorescent DCF upon exposure to H2O2. Fc OxyBurst measures endosomal ROS production in response to IgG immune complex uptake in PMNs. Interestingly, CF sputum significantly enhanced endosomal ROS production in uninfected PMNs after IgG immune complex stimulation (Figure 4.8A-B). When infected with MRSA at the same time as IgG IC stimulation, co-localization of the endosomal ROS signal and MRSA was observed that was inhibited significantly by the CF sputum (Figure 4.8C-D). These interesting results suggest that CF sputum differentially affects ROS generation in the phagosomal and endosomal compartments in PMNs and also likely influences their fusion.

CF sputum reduces C5a receptor expression without affecting Fcγ receptor expression in PMNs

Phagocytosis is mediated through the interaction of opsonins and antibodies bound to pathogens to complement or Fcγ receptors on the PMN surface. Since we observed that *S. aureus* phagocytosis was not impaired by the CF sputum and to see whether the observed enhancement in IgG IC-stimulated endosomal ROS generation was due to higher receptor expression, we first measured the surface expression of the complement receptors, C3a and C5a, after CF sputum treatment by flow cytometry. We saw that the C3a receptor displays no difference compared to untreated PMNs, while we

did observe a significant reduction of C5a receptor expression CF sputum treated PMNs compared to untreated (Figure 4.9A-B). Additionally, we examined surface expression of Fcγ receptors known to bind IgG ICs. There are multiple Fcγ receptors present on the PMN surface, so we examined CD64 (FcγRI), CD32 (FcγRII), and CD16 (FcγRIII). We observed that CD64 and CD16 did not show enhanced or reduced cell surface expression after exposure to the CF sputum (Figure 4.9B). Although not significant, we did notice a trend toward enhanced cell surface expression of CD32 following CF sputum exposure (Figure 4.9B). Our data suggest that altered Fcγ receptor surface expression on PMNs by CF sputum is unlikely the reason for increased endosomal ROS generation. Quantitative changes in the levels of the NADPH oxidase complex or their redistribution among intracellular vesicular compartments in PMNs could rather be responsible for the observed results.

Conclusions

In conclusion, our data demonstrate a unique mechanism to explain the impaired killing of MRSA by PMNs in the CF airway. Ultimately, we confirm that CF PMNs are capable of killing CF clinical isolates of MRSA until they are exposed to the CF airway environment which impairs PMN-mediated killing. In more than half of CF patients, lung disease is related to persistent *S. aureus* infections that disregard the abundant numbers of PMNs in the airways. This suggests that the CF airway environment impacts PMNs' killing capabilities and we hypothesized that the CF airway environment impairs phagosomal maturation in MRSA-infected PMNs.

One of the earliest abnormalities in CF airway disease is the recruitment of PMNs to the lungs. PMNs' primary killing mechanisms against pathogens including *S. aureus* involve 1) intracellular killing through phagocytosis and 2) extracellular killing by neutrophil extracellular traps (NETs)[137,138]. While NETs are abundant in CF airways[86,193,194] and *S. aureus* induces NET release in human PMNs[90,195], *S. aureus* is fairly resistant to NET-mediated killing[90,121,196–198], suggesting that NETs released in the CF lung do not likely mediate elimination of *S. aureus*. *S. aureus* has been detected inside PMNs in CF airways indicating that phagocytosis of *S. aureus* occurs to some extent in vivo[128], confirming that uptake of *S. aureus* inside PMNs occurs in CF airways. Antibody- or complement-enhanced phagocytosis and the associated respiratory burst generating reactive oxygen species (ROS) represent the main mechanisms by which PMNs kill *S. aureus*[119,139,142].

One of the proposed mechanisms behind impaired bacterial killing of CF PMNs suggests that CFTR is expressed in healthy PMNs and plays an important role in supporting phagocytic killing of microbes. CFTR is proposed to transport chloride into the phagosome for the formation of bactericidal HOCl and for establishing an optimal ionic and pH environment[107,199]. In CF patients' PMNs this CFTR-mediated chloride transport would be deficient and phagosomal bacterial killing would be compromised. CFTR gene expression and protein localization to secretory granules was reported in human PMNs[165]. Intraphagosomal chloride transport and in vitro killing of the *P. aeruginosa* laboratory strain PAO1 have been shown to be impaired in human CF PMNs[165,166]. *Cftr*-deficient mice (global or myeloid-specific) exhibit defective clearance of laboratory strains or a mucoid clinical isolate of *P. aeruginosa* and suboptimal

resolution of inflammation in an in vivo zymosan model[200–202]. While these data indicate that endogenous CFTR promotes bacterial killing in PMNs, the most significant differences were observed in mice that have several limitations to study CF lung disease[203,204]. Only two manuscripts reported somewhat impaired in vitro killing of *P. aeruginosa*[166] or *Burkholderia cenocepacia*[205] by human CF PMNs. There are no indications in the medical literature showing impaired killing of *S. aureus* by human CF PMNs. On the contrary, we reported normal *S. aureus* killing in CF PMNs in vitro[133] and independent investigators published that in vitro killing of *P. aeruginosa* by CF blood PMNs is not impaired[206]. To our knowledge, we are the first ones to use CF bacterial isolates to address this question, and not only one but eight different CF isolates of *S. aureus* were used[133]. Interestingly, killing of none of the 8 CF *S. aureus* isolates was impaired in CF PMNs in our study, only the killing of the standard strain, USA300[133]- indicating the importance of testing several CF isolates of *S. aureus*. We conclude that the topic remains controversial and additional studies are required.

The CF airway environment is a very complex, inflammatory milieu that contains bacteria, immune cells, epithelial cells, mucins, exosomes, antibodies, DNA, NETs, cytokines, lipids and soluble molecules of both, host and microbial origin. PMNs transmigrating to the airways enter this hostile environment in CF. CF airway PMNs have reduced ability to produce ROS compared to matched, blood PMNs[170]. Studies comparing CF blood and airway PMNs described functional and signaling changes during airway homing including increased surface expression of CD11b, CD66b and CD63, decreased expression of CD16 and CD14, important phagocytic receptors[143]. It is very challenging though to reliably purify CF airway PMNs without activation and flow

cytometry remains the only main method allowing their studies. To perform biologically more meaningful studies (beyond surface marker exploration), several groups developed in vitro models where PMNs develop a CF airway-like phenotype[133,169,207,208].

Our CF sputum model was previously established to mimic the CF airway environment which included exposing healthy PMNs to pooled supernatants of CF sputum samples[133]. This study is the first to demonstrate the effect of the CF sputum on CF PMNs in which we show that it impairs bacterial killing of MRSA (CF clinical isolates and USA300), which further confirms that our model mimics the conditions characteristic of the CF airway environment. We also further confirmed that the CF airway environment does not impair phagocytosis of a CF clinical isolate of MRSA and there is no defect of *S. aureus* phagocytosis in CF PMNs. Another recent study also demonstrated that CF PMNs are not impaired in phagocytosis of *S. aureus*[107]. However, our data showing that MRSA killing is significantly impaired by the addition of cytochalasin B indicate that CF clinical isolates of MRSA are primarily killed through phagocytosis in healthy human neutrophils. Cytochalasin B is known to inhibit cytokinesis, single cell movement, and uptake of bacteria, thus impairing phagocytosis[189,190]. Furthermore, it has also been shown to impair the translocation of granule containing MPO to the phagosome in human neutrophils, which also eludes to the impairment of phagolysosome fusion by the CF sputum as a possible explanation for impaired killing[189]. It is well-established that phagosomal maturation which includes the release of antimicrobial enzymes, ROS, and HOCl production are crucial in killing *S. aureus* inside the phagosome of neutrophils[106,107,119,139,188,209,210]. MPO uses H2O2 dismutated from superoxide anions produced by the NADPH oxidase in the phagosome

that reacts with native MPO to generate compound I, which is a potent oxidant to mediate electron oxidation of Cl- to produce HOCL[109,211–214]. It is speculated that CF PMNs may be impaired in killing *S. aureus* in the phagosome due to the lack of normal CFTR function which may affect chloride supply and hypochlorous acid production, impacting NADPH oxidase and MPO-chlorinating activities[108,109,165–167]. It was discovered that CF PMNs have about 2.7-fold lower Cl- in their phagosomes than normal PMNs, indicating that the production of HOCL is also lower[215]. However, CFTR is not the only chloride channel described on the phagosomal membrane that help to influx Cl- into and out of the phagosome[214]. Therefore, this study focused on the effect of the CF airway environment on phagolysosome fusion and phagosomal ROS generation during MRSA infection.

The phagosome matures when lysosomes (neutrophil granules) fuse to the phagosomal membrane transforming into the phagolysosome. Phagolysosomes are microbicidal vesicles that produce ROS through NADPH oxidase assembly and contain proteases, lysozymes, and lipases[96,101]. NE, MPO, and CatD are contained in primary, azurophilic granules that fuse to the phagosome membrane during phagolysosome fusion. In this study, we examined the presence of NE, MPO, and CatD in the phagosome of CF sputum treated PMNs after MRSA24 infection in which the presence of these proteins illustrates that phagolysosome fusion occurred to enable their release inside the phagosome to colocalize with MRSA. We first identified the presence of the active enzymes inside and released from PMNs exposed to the CF sputum. Interestingly, we found that NE and CatD activity was significantly reduced inside uninfected CF sputum treated PMNs, suggesting that the CF sputum promotes degranulation prior to infection. However, once infected with MRSA, there were no differences found in the active forms

of these proteins inside or released from the PMNs. NE and MPO activity are found in the CF sputum at high quantities, confirming that release of these enzymes from PMNs are not impaired[216,217]. However, the colocalization NE to MRSA in the phagosome was not impaired by the CF sputum, however MPO and CatD were impaired. It was also discovered that CF PMNs prior to exposure of the CF airway environment were significantly impaired in colocalizing MPO to MRSA in the phagosome, suggesting that CF PMNs have defective MPO trafficking to the phagosome. This observation is novel and also suggest an endogenous role of CFTR in neutrophil-mediated killing of *S. aureus*. Based on our results, a functional CFTR is needed in neutrophils to ensure sufficient localization of MPO to *S. aureus*-containing phagosomes. Additional work using more CF patients' neutrophils is needed to strengthen this exciting observation.

Additionally, we discovered that phagosomal ROS was shown to be impaired during MRSA infection. It has been previously discovered that CF clinical isolates express high levels of superoxide dismutase genes which aid in protection against human PMNs by neutralizing superoxide radicals[120]. Differences in superoxide dismutase activities among the isolates could be partially responsible for their susceptibility to neutrophilic killing and also for differences seen by the CF sputum. The reduced phagosomal ROS in MRSA infected PMNs exposed to the CF sputum may further explain the discrepancy between our results that include NE colocalizing to MRSA in the phagosome is not impaired, however MPO and CatD are impaired despite their presence in primary granules. It has been previously described that MPO and CatD release into the phagosome are dependent on ROS in PMNs[142,218–220]. Therefore, it is possible that the primary granules are fusing to the phagosome however MPO and CatD are awaiting

signals from the phagosomal ROS to be released from the granules into the phagosome whereas NE is not dependent on ROS. Studies show that CF macrophages infected with USA300 have an upregulation of CD63 on the phagosome membrane and that healthy PMNs infected with MRSA had 97-98% CD63 enrichment on their phagosomes, indicating that following MRSA ingestion, granule-phagosome fusion occurs[221,222].

Despite the inhibition of MPO and CatD colocalizing with MRSA inside the phagosome, we did not see an inhibition of MPO and CatD activity in CF sputum treated PMNs. Primary granules containing NE, MPO, and CatD are not only trafficked to the phagosome but are trafficked to the nucleus to induce NETosis and released outside the cell. Also, our previously research that NETosis was not impaired in MRSA infected PMNs exposed to the CF sputum[133]. Therefore, we further investigated whether trafficking to the nucleus was impaired by the CF sputum. We did not see impairment of colocalization of NE and MPO to the nucleus in MRSA infected PMNs treated with CF sputum.

Furthermore, we investigated the PMN phagocytic receptors. Phagocytosis of MRSA is receptor-mediated and occurs through two pathways, complement-mediated and Fcγ-mediated. Complement receptors and Fc receptors are opsonic receptors that detect host-derived proteins that bind to target molecules on *S. aureus*[97]. In particular complement receptors such as C3a and C5a will bind to activated complement components such as iC3b bound to the pathogen, whereas Fc receptors bind to the Fc portion of IgG and IgA antibodies[96]. Our study involves an opsonization step with autologous serum prior to infection of MRSA and any of these mechanisms are plausible for inducing phagocytosis in our system. In uninfected PMNs exposed to the CF sputum

we found that there was no difference in expression of the C3a receptor and an inhibition of the C5a receptor. Interestingly, it was discovered that C5a and C3a are correlated with worsened lung function in CF and that serine proteases are capable of cleaving C5aR to inactivate the C5a signaling capability[223,224]. Therefore, the abundance of serine proteases, such as neutrophil elastase present in the CF sputum may cleave these receptors and affect this signaling pathway[224,225]. No changes were detected on the surface levels of CD16, CD32 and Cd64, main Fcγ receptors.

Overall, our study demonstrates a unique mechanism in which the CF airway environment compromises MRSA killing by PMNs. We are the first to describe that impaired killing of CF clinical isolates of MRSA by CF PMNs associates with reduced co-localization of the bacterium with primary granule components, MPO, CatD, and ROS generation inside the phagosome. The data suggests a CF sputum-dependent effect on phagosomal bacterial killing in PMNs during MRSA infection. Future studies will aim to identify the components of the sputum responsible for this effect on the PMNs.

Acknowledgements

CF human subject samples were provided by the CF Biospecimen Registry at the Children's Healthcare of Atlanta and Emory University CF Discovery Core. We would also like to acknowledge the Clinical and Translational Research Unit at University of Georgia for the collection of all control subjects. We would like to thank all the control subjects and CF patients that provided specimens for this study. We would also like to acknowledge Jamie Barber for training and use of the University of Georgia College of Veterinary Medicine Cytometry Core Facility, and Julie Nelson for training and use of the University of Georgia Coverdell Biomedical Microscopy Core Facility.

Author Contributions

K.F. performed all experimentation, designed research protocols, wrote, and revised the manuscript. A.A.S. designed research protocols, supervised collection of samples from CF patients, reviewed the manuscript and acquired funding. J.B.G designed research protocols and reviewed the manuscript. B.R. conceptualized the idea, designed research protocols, revised, and reviewed the manuscript, acquired funding, and supervised the study.

Funding

This study was supported by the following NIH grants: R21AI154343 (NIAID, PI: Rada), R01HL136707 (NHLBI, PI: Rada).

Tables

Identifier in this work	CF sample name	Methicillin sensitivity	Strains or isolates	References
MRSA24	CFBRSa24	resistant	Cystic Fibrosis clinical isolates	*Bernardy* et al.[155]
MRSA25	CFBRSa25	resistant		
MRSA47	CFBRSa47	resistant		
MRSA74	CFBRSa74	resistant		
USA 300	-	resistant	Reference strain	*S. aureus* subsp. aureus (ATCC® BAA1717 ™)

Table 4.1: MRSA isolates.

Figures and Figure Legends

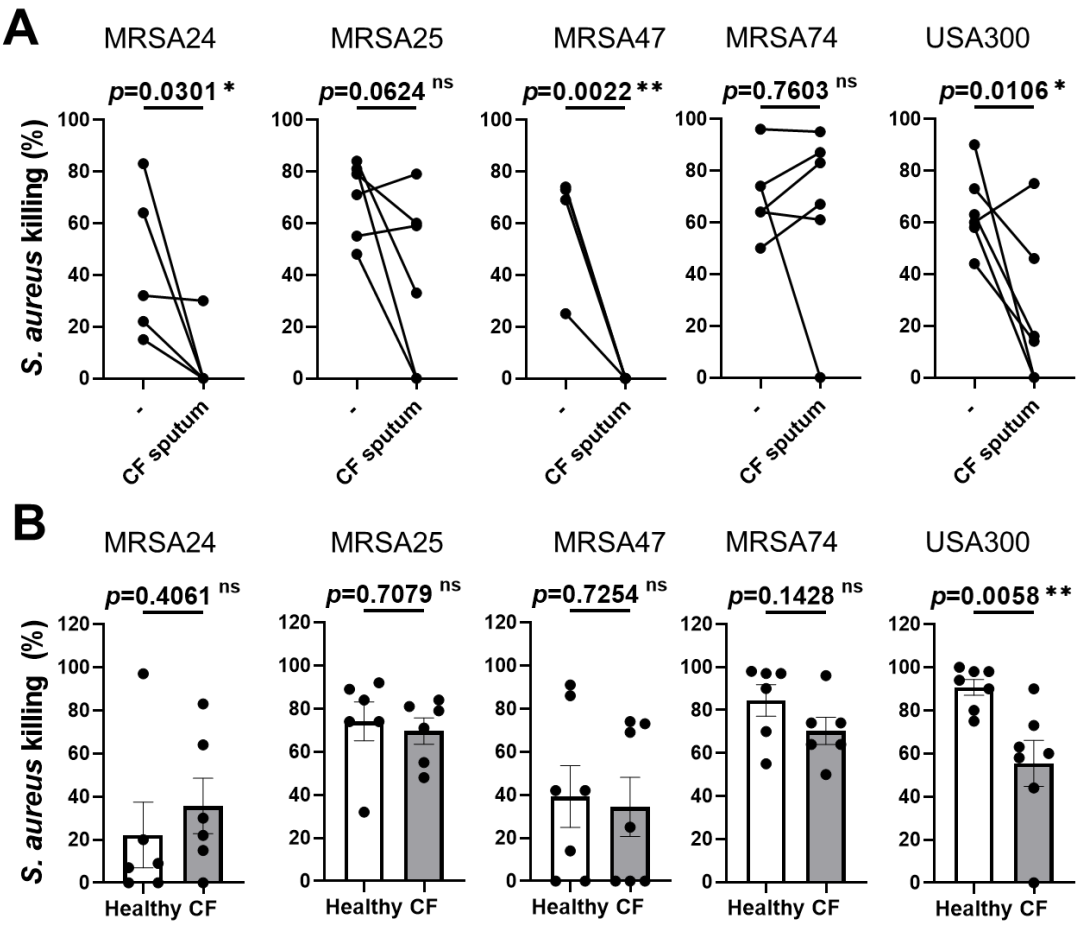

Figure 4.1: CF sputum treatment impairs the ability of CF PMNs' to kill MRSA. (A) CF PMNs were isolated from the whole-blood of CF subjects and either untreated or treated with 30% (*v/v*) CF sputum supernatant cocktail and then infected with 4 MRSA clinical isolates and the MRSA lab strain (USA300) at MOI of 1 for 30 mins (n=4-7). **(B)** PMNs were isolated from the whole-blood of CF subjects or healthy donors and infected with 4 MRSA clinical isolates and USA300 (n=6-7). Bacterial killing was measured by the high-throughput microplate-based assay. Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

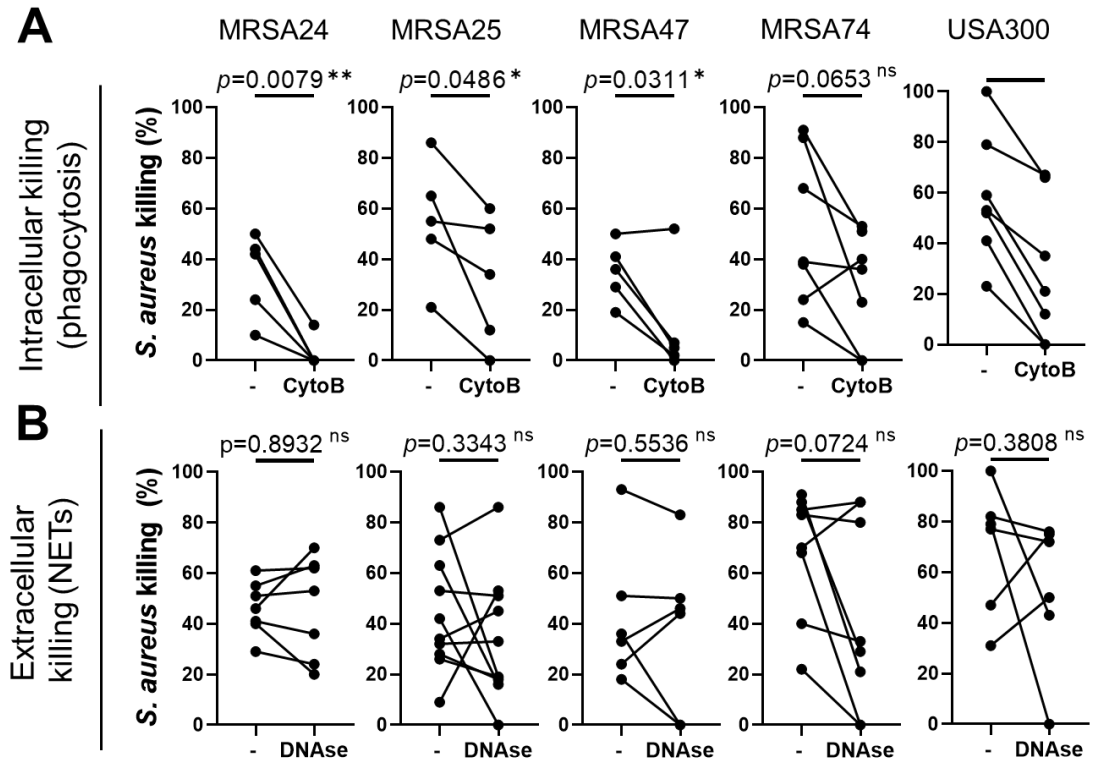

Figure 4.2: Intracellular killing initiated by phagocytosis is required for MRSA killing by human PMNs. PMNs were isolated from healthy donors and untreated or treated with **(A)** 10 µM Cytochalasin B (CytoB) (n=5-7) or **(B)** 10 U/ml of DNAse (n=5-7) for 30 mins and infected with 4 MRSA clinical isolates and the lab reference strain (USA300) at MOI of 1 for 30 mins. Bacterial killing was measured by the high-throughput microplate-based assay. Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

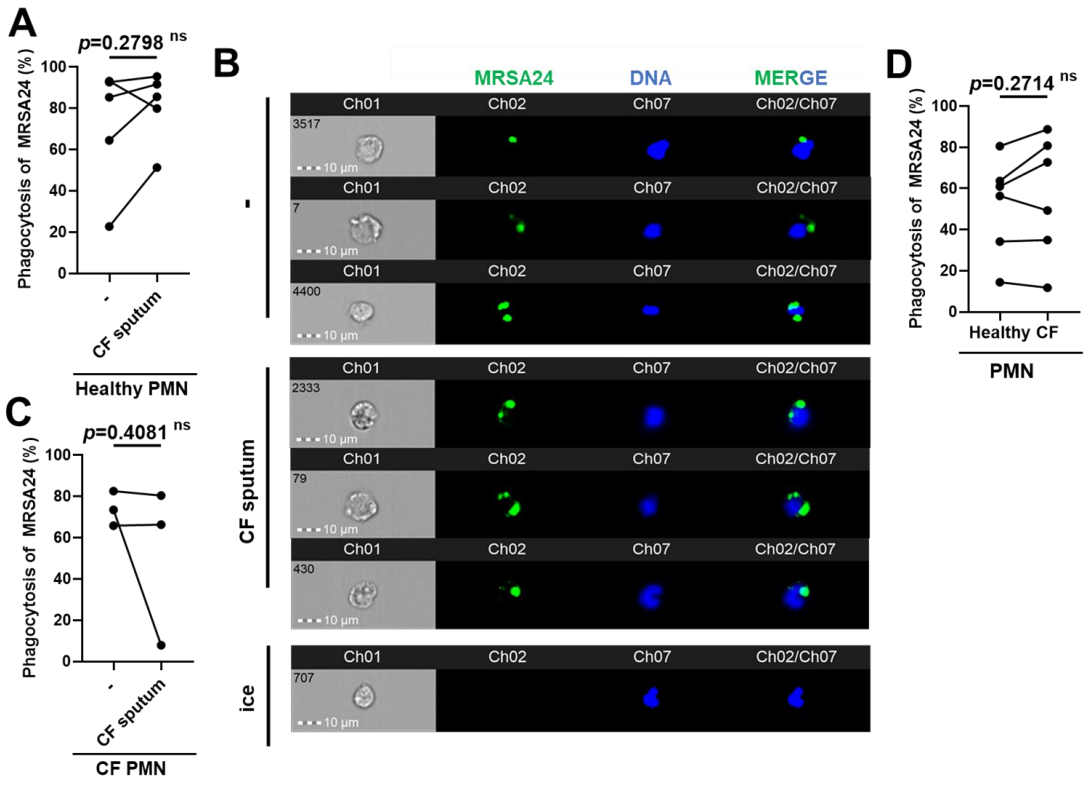

Figure 4.3: CF sputum does not inhibit MRSA phagocytosis by healthy or CF PMNs. (A) PMNs from healthy donors were isolated and were either untreated or treated with CF sputum and infected with pHrodo-stained MRSA24 for 1 hour. PMNs were place on ice as a negative control. Phagocytosis was measured with ImageStream cytometry (n=5). **(B)** Representative images of (A), DNA (blue), MRSA24 (green). **(C)** Phagocytosis of pHrodo-stained MRSA24 from PMNs isolated from CF subjects and either untreated or treated with CF sputum measured with ImageStream cytometry (n=3). **(D)** PMNs were isolated from healthy donors or CF subjects and infected with pHrodo-stained MRSA24 and phagocytosis was measured with flow cytometry (n=6). Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

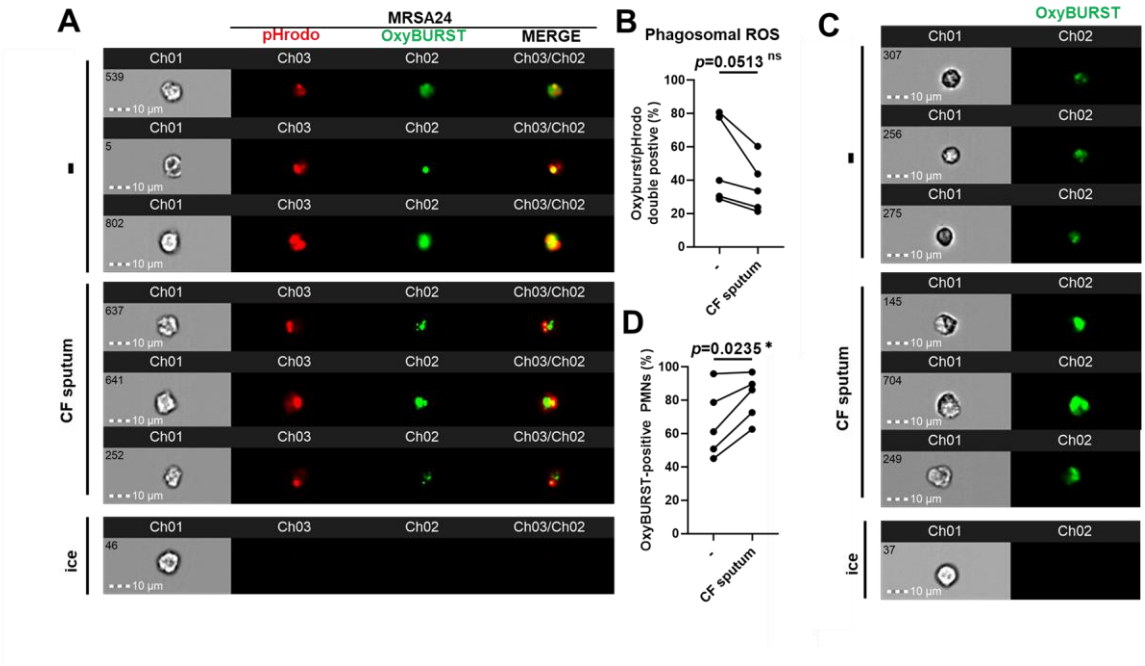

Figure 4.4: CF sputum impairs phagosomal ROS production in MRSA-phagocytosing human PMNs. PMNs were isolated from healthy donors and either untreated or treated with CF sputum. PMNs were placed on ice for the negative control. **(A)** MRSA24 was stained with pHrodo-red for 45 mins than with OxyBurst green for 45 mins prior to infection. PMNs were then infected with pHrodo-OxyBurst-stained MRSA24 following CF sputum treatment for 1 hour at a MOI of 1. Representative images are shown, pHrodo (red), OxyBurst (green). **(B)** Double positive PMNs for OxyBurst and pHrodo were analyzed by ImageStream cytometry (n=5). **(C)** Following CF sputum treatment of PMNs, 10 µM of OxyBurst green was added for 1 hour. Representative images are shown, OxyBurst (green). **(D)** OxyBurst positive cells were analyzed by ImageStream cytometry (n=5). Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

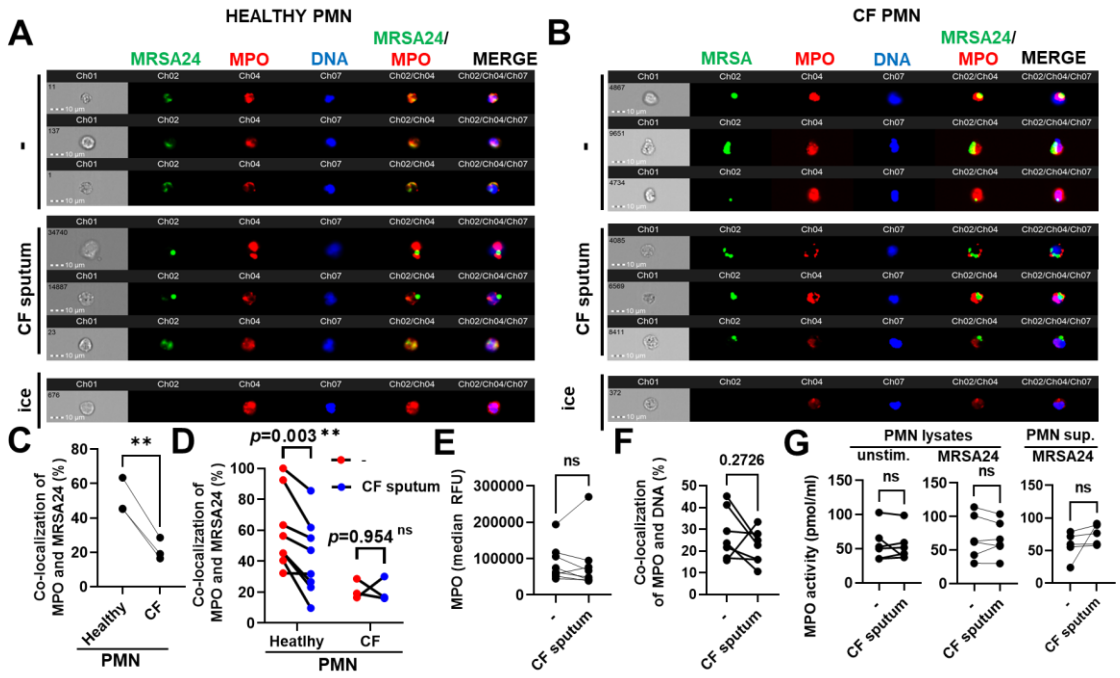

Figure 4.5: CF sputum reduces co-localization of MPO with phagocytosed MRSA. **(A)** PMNs were isolated from healthy donors and were either untreated or treated with CF sputum and infected with pHrodo green-stained MRSA24 for 30 mins at MOI of 1. PMNs were then fixed and stained with a MPO antibody. Neutrophils were place on ice as a negative control. Representative images are shown performed by ImageStream, MRSA (green), MPO (red), DNA (blue). **(B)** PMNs were isolated from CF subjects and either untreated or treated with CF sputum and infected with pHrodo green-stained MRSA24 for 30 mins at MOI of 1. PMNs were then fixed and stained with a MPO antibody. Representative images are shown, MRSA24 (green), MPO (red), DNA (blue). **(C)** Co-localization of MRSA24 and MPO in PMNs from healthy donors and CF subjects not treated with CF sputum, performed by ImageStream cytometry analysis (n=3). **(D)** Co-localization of MRSA24 and MPO in PMNs from healthy donors (n=8) and CF subjects (n=3) untreated (red) and CF sputum treated (blue). Analysis was performed by ImageStream cytometry. **(E)** Median fluorescence intensity of MPO in healthy PMNs untreated or treated with CF sputum was measured with ImageStream cytometry (n=8). **(F)** Co-localization of MPO and DNA in healthy PMNs either untreated or treated with CF sputum was measured by ImageStream cytometry (n=8). **(G)** Healthy neutrophils were either untreated or treated with CF sputum. PMNs were then either lysed or infected with MRSA24 at MOI of 1 for 30 mins. After infection, PMN supernatants were collected or lysed. MPO activity was measured using a fluorometric quantification (n=5-6). Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

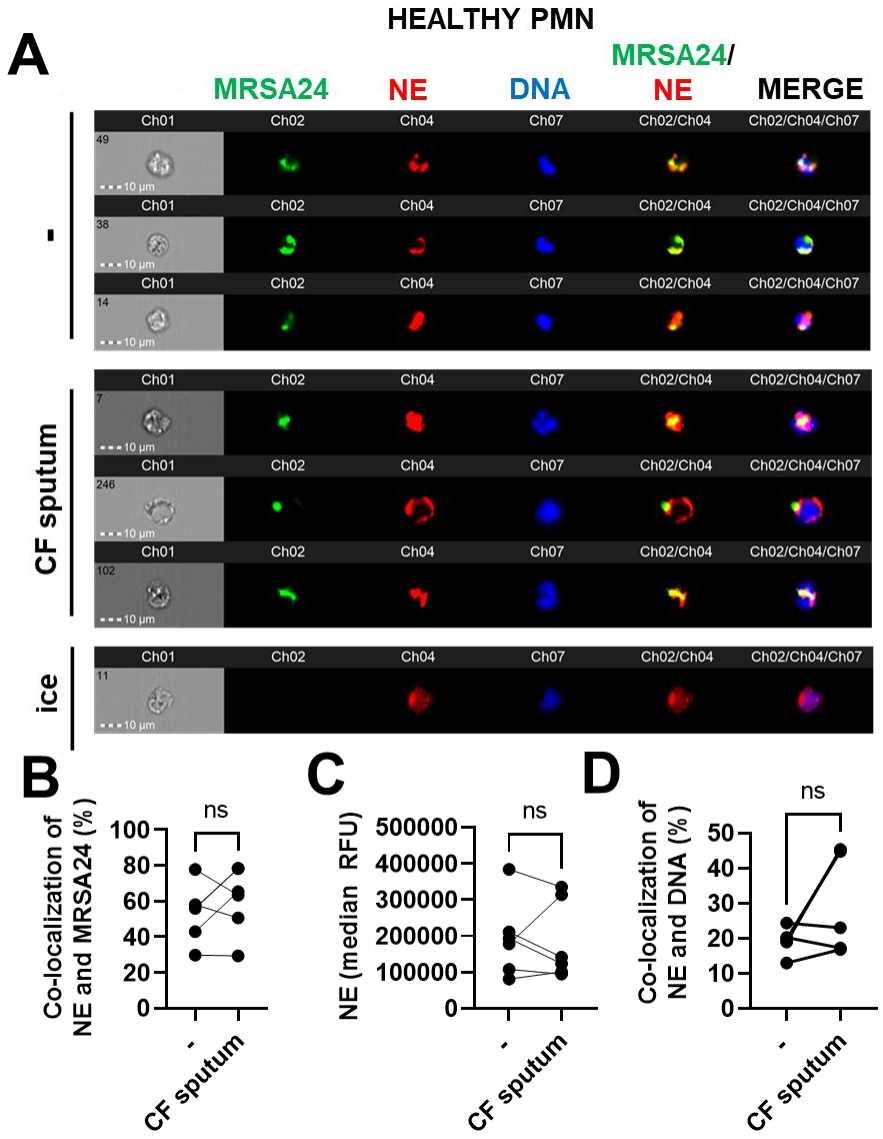

Figure 4.6: CF sputum does not affect neutrophil elastase (NE) co-localization with phagocytosed MRSA. **(A)** PMNs were isolated from healthy donors and were either untreated or treated with CF sputum and infected with pHrodo green-stained MRSA24 for 30 mins at MOI of 1. PMNs were then fixed and stained with a NE antibody. PMNs were place on ice as a negative control. Representative images are shown, performed by ImageStream, MRSA (green), NE (red), DNA (blue). **(B)** Co-localization of MRSA24 and NE in PMNs from healthy donors untreated or treated with CF sputum was performed by ImageStream cytometry analysis (n=6). (C) Median fluorescence intensity of NE in healthy PMNs untreated or treated with CF sputum was measured with ImageStream cytometry (n=6). (D) Co-localization of NE and DNA in healthy PMNs either untreated or treated with CF sputum was measured by ImageStream cytometry (n=4). Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

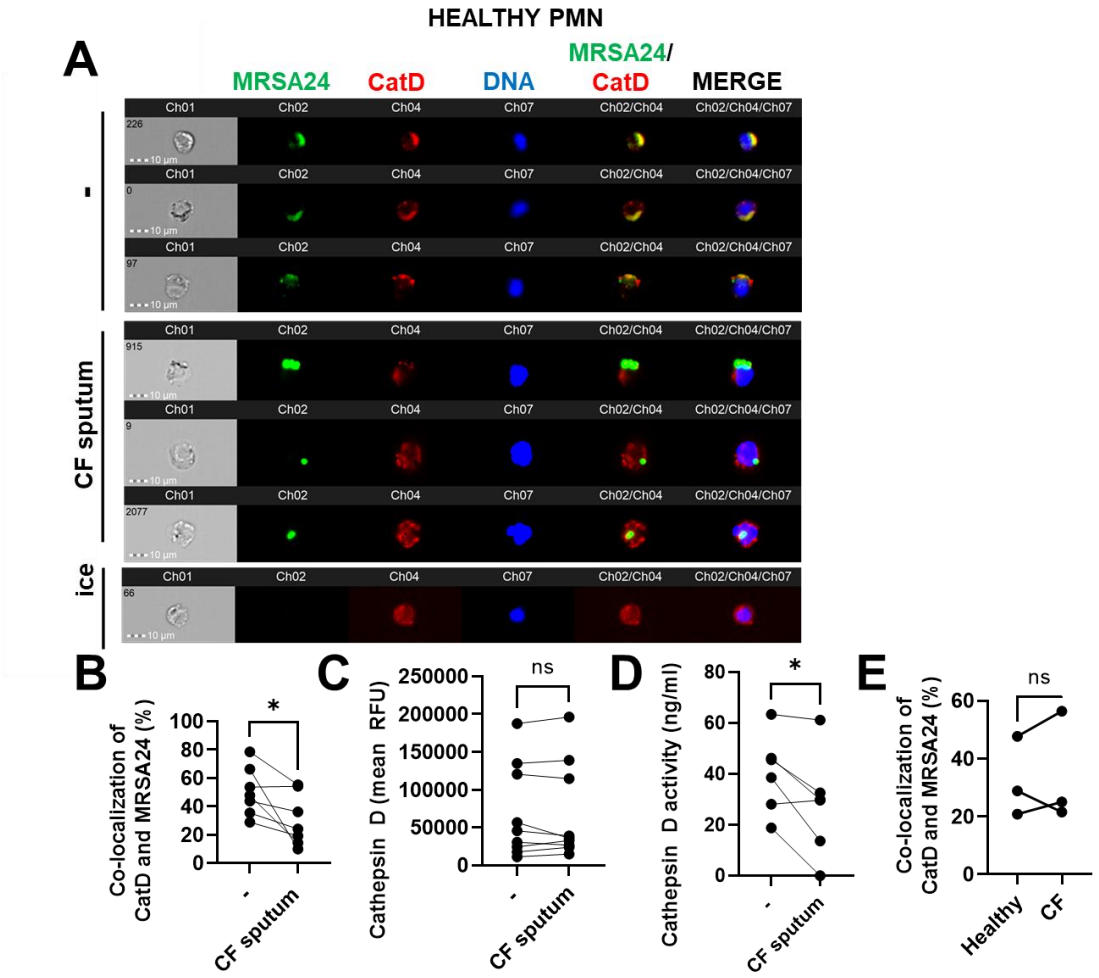

Figure 4.7: CF sputum reduces Cathepsin D (CatD) co-localization with phagocytosed MRSA. (A) PMNs were isolated from healthy donors and were wither untreated or treated with CF sputum and infected with pHrodo green-stained MRSA24 for 30 mins at MOI 1. PMNs were then fixed and stained with a CatD antibody. PMNs were placed on ice as a negative control. Representative images are shown, performed by ImageStream, MRSA24 (green), CatD (red), and DNA (blue). **(B)** Co-localization of MRSA24 and CatD in PMNs from healthy donors untreated or treated with CF sputum was performed by ImageStream cytometry analysis (n=7). **(C)** Median fluorescence intensity of CatD in healthy PMNs untreated or treated with CF sputum and measured by ImageStream cytometry (n=7). **(D)** Healthy PMNs were either untreated or treated with CF sputum and lysed. CatD activity was measured using a fluorometric quantification (n=6). **(E)** PMNs were isolated from healthy donors and CF subjects and infected with pHrodo-stained MRSA24. Co-localization of CatD and MRSA24 was measured by ImageStream cytometry (n=3). Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

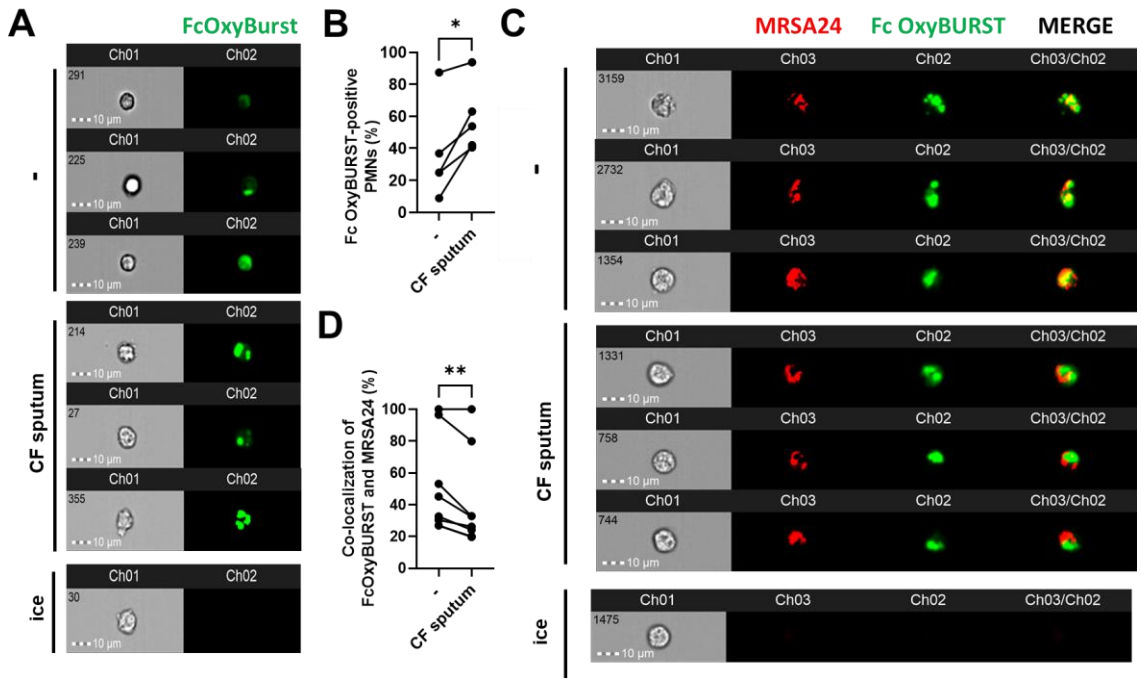

Figure 4.8: CF sputum enhances endosomal ROS production in response to IgG immune complexes. PMNs were isolated from healthy donors and either untreated or treated with CF sputum. PMNs were placed on ice for the negative control. **(A)** After sputum exposure, 120 µg/ml of Fc OxyBurst green was added to PMNs for 1 hour. Representative images are shown, Fc OxyBurst (green). **(B)** Fc OxyBurst positive neutrophils were analyzed with ImageStream cytometry analysis (n=5). **(C)** MRSA24 was stained with pHrodo-red and added to untreated PMNs and sputum treated PMNs at MOI of 1 for 1 hour. At the same time as infection with MRSA24, 120 µg/ml of Fc OxyBurst green was added to the PMNs for 1 hour. Representative images are shown, MRSA24 (red), Fc Oxyburst (green). **(D)** Co-localization of Fc Oxyburst and MRSA24 in healthy PMN untreated or treated with CF sputum was analyzed by ImageStream cytometry analysis (n=7). Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

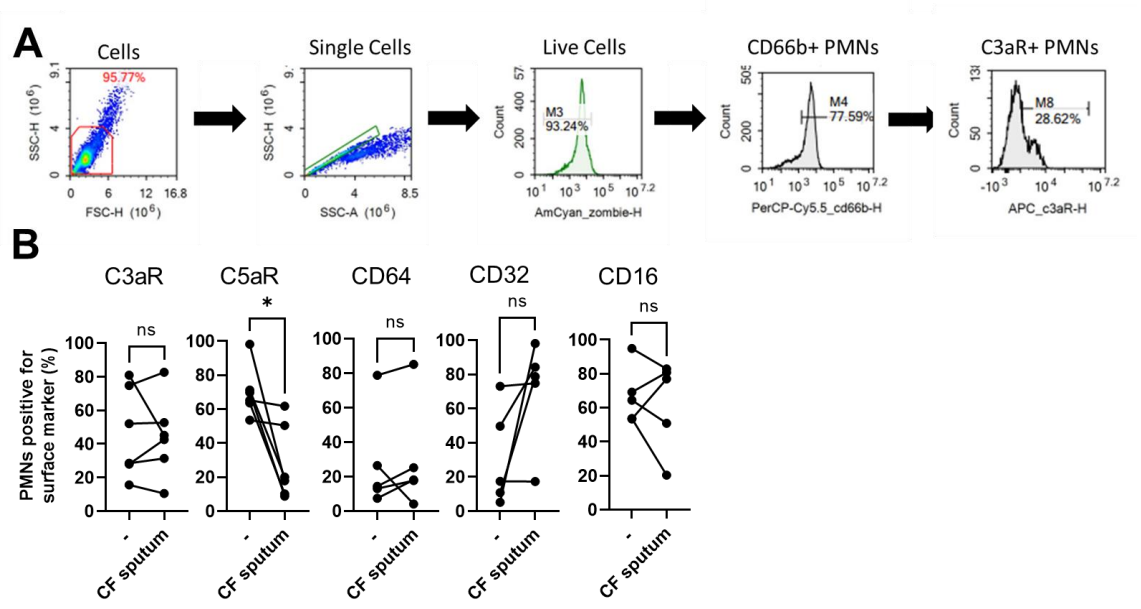

Figure 4.9: CF sputum reduces C5a receptor expression on human PMNs. PMNs were isolated from healthy donors and either untreated or treated with CF sputum. **(A)** Representative images of the gating strategy used to determine the percent expression of each cell surface marker for live PMNs (CD66b+/ Zombie Aqua^{-}). **(B)** The comparison of untreated vs. CF sputum treated PMNs for surface expression of complement receptors, C3aR and C5aR, and Fc receptors, CD64, CD32, and CD16. Mean ± S.E.M. Data were analyzed by two-tailed paired student's t-test. Statistically significant difference were considered as ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

CHAPTER 5

THE BACTERIAL SERINE PROTEASE INHIBITOR ECOTIN INHIBITS NEUTROPHIL ELASTASE ENZYMATIC ACTIVITY IN CYSTIC FIBROSIS SPUTUM

[1]Fantone, K., Nothaft , H., Crippen, C., Stecenko, A., Szymanski, C., and Rada, B., to be submitted to *PLOS ONE*

Abstract

Cystic Fibrosis (CF) airway disease is characterized by impaired mucociliary clearance, chronic, polymicrobial infections and robust, neutrophil-dominated inflammation. Pulmonary disease is the leading cause of morbidity and mortality in CF patients, due to the progression of chronic respiratory infections and host inflammation dominated by neutrophils. One of the earliest abnormalities in CF airway disease is the recruitment of neutrophils to the lungs which, upon activation, release their intracellular content perpetuating proinflammatory signals as well as causes lung tissue damage via release of neutrophil elastase (NE). Our goal is to characterize the potential beneficial effect of the bacterial neutrophil elastase inhibitor, ecotin, in the CF airway. Our results indicate that ecotin from three Gram-negative bacterial species not thought to be CF pathogens (*Campylobacter. rectus*, *Campylobacter showae*, and *Escherichia. coli*) inhibit NE activity in CF sputum in a dose-dependent manner, with *Campylobacter* ecotins being the most effective. In contrast, *P. aeruginosa* ecotin were relatively weak in inhibiting NE. Importantly, ecotins did not impair human neutrophils' ability to kill the major CF respiratory pathogens, *P. aeruginosa* and *S. aureus*, in vitro. Overall, we demonstrate that bacterial ecotins inhibit NE activity in CF sputum without compromising the neutrophils' ability to kill CF pathogenic bacteria. Thus, these data suggest that bacterial ecotins could represent a novel anti-protease therapeutic strategy.

Introduction

Cystic Fibrosis (CF) is a recessive genetic disease affecting about 80,000 people worldwide with progressive airway disease causing the majority of the morbidity and mortality[1]. Progression of CF lung disease is primarily due to chronic inflammation partially mediated by neutrophils and to the persistence of polymicrobial infections. Persistent inflammatory signals in the CF airways cause activation of newly recruited neutrophils which contributes to lung tissue damage overtime[84]. Neutrophils contain many antimicrobial proteins and enzymes to help fight off infections through intracellular mechanisms of phagocytosis and extracellular mechanisms of degranulation and neutrophil extracellular trap (NET) formation[73,138,185]. However, despite the high number of neutrophils in the airways of CF patients, eventually a select group of bacteria persist that cannot be eliminated. Thus, these activated neutrophils not only release toxic intracellular cargo causing lung tissue damage, but they also fail to clear bacteria which enhance and perpetuate this dysregulated inflammatory response.

Neutrophil elastase (NE) is a serine protease that is required for the normal antimicrobial function of neutrophils[226]. Specifically, extracellular NE degrade interstitial collagen fibers enabling neutrophil cell migration and intracellular NE kills bacteria within the phagosome[226]. In CF, however, NE is released from neutrophils in the airways in large amounts and causes harm to host by targeting host proteins of the extracellular matrix of the lung tissue and irreversible lung damage over time[46,70,227–229]. NE is one of the most ample host enzymes found in the CF lung and degrades elastin, fibronectin, and collagen found in the epithelial lung tissue[69]. It has also been shown to impair other host defenses by injuring bronchial epithelial cells and destroying the lung extracellular

matrix[70,71]. The chronic release of NE by neutrophils causes airway remodeling contributing to worsened lung damage and function in these patients[70,72]. Several studies underscore the importance of the neutrophil and neutrophil-derived products driving CF airway disease progression. For example, the number of neutrophils and the levels of DNA, myeloperoxidase (MPO), NE, and polymorphonuclear granulocytes (PMN) chemoattractants in CF sputum all correlate with CF lung disease severity[86,217,225,230–237]. NE is elevated in the broncheoalveolar fluid of CF infants and toddlers, and is the best predictor of future bronchieactasis[51,238].

There are only a few clinical studies using NE inhibitors in CF and the results are variable. For example, AZD9668, a reversible and selective NE inhibitor, significantly reduced sputum inflammatory biomarkers but it failed to achieve the primary end points of reduction in sputum PMN counts, decreased NE activity, and improved lung function[239]. Another phase II clinical trial using inhaled alpha-1-antitrypson (A1AT) in CF showed no impact on lung function but did diminish free NE activity, PMN count, *P. aeruginosa* load and IL-8 levels[240]. Thus, the potential efficacy of NE inhibitors in CF still needs to be explored, particularly since the recently developed highly effective modulator therapies (HEMT) are not very effective in controlling the inflammatory response and many believe that HEMTs will require adjunct anti-inflammatory therapy[241].

Bacteria have also evolved mechanisms to resist antimicrobials properties of neutrophils. Specifically, Gram-negative bacteria developed strategies to evade harm by immune cell serine proteases, such as NE through the production of a protein called ecotin[242,243]. Ecotin is a small, homodimeric protein of 38 kDa resistant to boiling and

stable at pH 1.0 demonstrating exceptional stability[244]. In bacteria expressing ecotin, the protein is translocated into the periplasmic space and protects the bacterium from human host proteases. We have recently found and characterized ecotin homologs in *Campylobacter rectus* and *Campylobacter showae*[12]. We showed that ecotin inhibits free or NET-bound NE in a dose-dependent manner[12]. The recently resolved structure of the ecotin-NE complex reveals a heterotrimeric complex (Fig. 1)[245].

Ecotin was first discovered in *E. coli* as a protease inhibitor that translocates to the periplasmic space for protection[243,246]. Specifically ecotin exhibits a broad range of activity against exogenous serine proteases such as trypsin, chymotrypsin, factor Xa, kallikrein, urokinase, factor XII, and NE[12,243,246–249]. Since the first discovery in *E. coli*, ecotin homologs have been observed in over 300 organisms including *P. aeruginosa*, which is a dominant pathogen in CF airways[12,243,250,251]. In *P. aeruginosa*, ecotin was shown to escape the cell and bind to biofilm matrix exopolysaccharides that protect bacterial communities from antimicrobial enzymes. Ecotin has also been shown to enhance its persistence and survival in biofilms which are also commonly found in the CF airways[252].

Therefore, in this study we explored ecotin from four bacterial species and its inhibitory action of NE in CF airway samples. We constructed ecotin from two *Campylobacter* species, *C. rectus* and *C. showae* as our previous research showed that ecotin from both species were able to inhibit recombinant human NE and rescue bacterial killing by neutrophils[12]. We also constructed ecotin from *E. coli* as our previous research also shows that *E. coli* ecotin inhibits recombinant human NE. Lastly, we constructed *P. aeruginosa* ecotin which has been shown to protect *P. aeruginosa* in the CF airways. We

hypothesized that ecotin will effectively inhibit NE enzymatic activity in CF sputum samples without compromising neutrophil-mediated killing of CF respiratory pathogens.

Materials and Methods

Human subjects

All human subject studies were performed following the guidelines of the World Medical Association's Declaration of Helsinki. Healthy human subjects were recruited at the University of Georgia (UGA) and provided informed consent for blood donation for neutrophil isolation according to the protocol UGA# 2012-10769-06.

CF patients

CF subjects were recruited from the Adult CF Clinic at the Children's Healthcare of Atlanta and Emory University CF Care Center and provided informed consent to obtain sputum samples (IRB00042577). All human studies involving sputum collection from CF subjects were approved by the Emory University Institutional Review Board and were in accordance with institutional guidelines. CF diagnosis was confirmed by pilocarpine iontophoresis sweat testing and/or CFTR gene mutation analysis to show the present of two disease causing mutations. CF subjects were selected for sputum collection only if they were clinically stable and on no new medications within the previous three weeks of the study visit.

Sputum processing

Sputum samples were processed as described earlier[133]. The sputa were kept on ice at 4°C throughout the processing procedure. The sputum was weighed and for every 1.0 grams of sputum, 3 mLs of cold PBS-EDTA (1xPBS with 5 mM EDTA) were added.

Sputum was then repeatedly passaged through a sterile 18-gauge needle to homogenize the sputum and then centrifuged at 800xg for 10 mins at 4°C to pellet the cells. To ensure removal of cells, mucus, and bacteria, the sputum supernatant was transferred into new tubes and centrifuged at 3,000xg for 10 mins at 4°C. The clear sputum supernatants were stored at -80°C and used in this study.

Bacterial strains, plasmids, and growth conditions for the expression of ecotin proteins

The bacterial strains used in this study are listed in Table 5.1. *E. coli* was grown on LB agar or in LB broth at 37°C with shaking overnight. *Campylobacter* strains, *C. rectus* and *C. showae* were grown on Blood Heart Infusion (BHI) agar/broth supplemented with 5% horse blood under anaerobic conditions, *P. aeruginosa* was grown on LB agar/broth. The antibiotics added to growth medium for selection were ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The plasmids for the expression of ecotin from *E. coli*, *C. rectus* and *C. showae* used in this study have been described in our prior publication[12]. The expression plasmid for ecotin from *P. aeruginosa* was constructed as follows: The full length ecotin gene was amplified from gDNA of *P. aeruginosa* PAO1 with oligonucleotides PAO1-eco-NdeI-F-

(5'- AAGGAGATATACATATGAAAGCACTACTGATCGCCGCCGGCGTTG-3') and PAO1-eco-SalI-R-

(5'-ATACTCGTGTCGACTTCGCTGACCGCTTTCTCGACCTTTTCG-3'), introducing NdeI and SalI restriction sites, respectively. The obtained PCR product was inserted into plasmid pET24b after restriction with NdeI and SalI and candidate plasmids isolated from colonies obtained after transformation of the ligation reaction in o *E. coli* DH5alpha were

analyzed by restriction digests; one positive clone that carries the PAO1 ecotin-his6 gene under the control of the T7 promoter was used as a template to amplify the PAO1 ecotin-His6 encoding sequence (using oligonucleotides ecotin-temp-pET22b-BamHI-F: (5'-ATATGGATCCGGCGTAGAGGATCGAGATCTCG-3) and ecotin-temp-pET22b-SalI-R-(5'-TAGCAGTCGACTCAGCTTCCTTTCGGGCTTTGTTAGC-3') that was subsequently inserted into the EcoRV restriction site on pBBR1-MCS4. One candidate plasmid the expresses the PAO1 ecotin under the control of the plac promoter was transferred into the overexpression strain *E. coli* BL21 *eco*::*kan*. Expression and purification of ecotin from PAO1, *E. col*i, *C. rectus* and *C. showae* was carried out as described previously, and ecotin activity was confirmed in trypsin serine protease inhibition assays as described previously[12].

Neutrophil elastase activity assay

Neutrophil elastase (NE) enzymatic activity in CF sputum supernatants were measured with the NE activity kit following the manufacturer's protocol (Cayman Chemical, cat #600610). CF sputum was diluted to a measurable range based upon the NE bulk standard which consisted of 18 U/ml of human NE. The standard curve was generated with human NE ranging from 10 mU/ml to 0 mU/ml in NE activity assay buffer. CF sputum and standards were added to a 96-well fluorometric microplate and ecotin was added to the CF sputum at varying doses. Ecotin from *C. rectus*, *C. showae*, and *E. coli* were added at a range from 0-1666.67 nM whereas ecotin from *P. aeruginosa* was added at a range from 0-555.56 nM due to its limited availability. Sivelestat (MedChemExpress, cat#127373-66-4), a known NE inhibitor [239,240,244,245,253–256], was used as a reference and was added at a final concentration of 5 µM. The NE-specific substrate

(Z-Ala-Ala-Ala-Ala) 2Rh110 is selectively cleaved by NE to yield the highly fluorescent compound R110. Fluorescence was read at 485/525 nm (excitation/emission wavelengths) every 2 minutes for 2 hours in a Varioskan Flash™ fluorescent microplate reader (Thermo Fisher Scientific, Waltham, MA). For analysis, the average relative fluorescence unit (RFU) values were recorded for each well, the blank was subtracted, and NE activity (mU/ml) was calculated based upon the equation generated with the help of the standard curve: NE (mU/ml) = [(RFU – y-intercept) / slope] x dilution factor.

Bacterial killing

Bacterial killing measurement of *P. aeruginosa* and *S. aureus* by neutrophils was performed with a reference lab strain and a CF clinical isolate for each bacterial species as shown in Table 5.1. The *P. aeruginosa* strains were streaked on Pseudomonas isolation agar while the *S. aureus* bacterial strains were streaked on blood agar (TSA II, 5% Sheep blood) and grown at 37ºC overnight. Colonies were picked the following day and grown in LB broth for about 3 hours with shaking at 37ºC. The bacteria were harvested, washed twice with 1xHBSS and absorbance was measured at 600nm in a 96-well microplate using the Varioskan Flash™ microplate photometer. As previously published, optical density of 0.6 of the bacterial suspension corresponds to 1.0 x 10^9 CFU/ml[133]. The concentration of bacteria was confirmed for each experiment with serial dilution and colony counting. Prior to adding bacteria to neutrophils, bacteria were opsonized with 10% (v/v) autologous serum for 20 mins at 37ºC. After opsonization, serum was removed from bacteria by centrifugation at 10,000xg for 10 mins and the bacterial pellet was resuspended in assay medium[133,149,150] (previously described).

Neutrophils were isolated from the whole-blood of healthy controls as previously described and resuspended in assay media ($1x10^7$ cells/ml)[133,149,150]. Next, neutrophils were either untreated or treated with 555.56 nM of ecotin and at the same time, bacteria were added to neutrophils at a MOI of 1 (1:1). Neutrophils were then incubated at 37ºC for 30 mins with shaking every 2-3 mins to ensure proper mixing of bacteria and neutrophils. After 30 minutes of incubation, the neutrophils were lysed in saponin (1 mg/ml in 1xHBSS) for 5 mins and diluted 100-fold into1xHBSS as previously published[3]. For a bacterial input control, bacteria were added to neutrophils and immediately lysed to enumerate the bacteria added to neutrophils at time=0 (input). Serial dilutions of each bacterial strain were generated in LB broth in each experiment to generate standard curves and placed in a 384-well microplate. Bacterial growth was measured over time in the Varioskan Flash™ microplate photometer at 600 nm absorbance every 4 min for 16 hours with constant heating (37ºC) and shaking. For quantification of bacterial killing by neutrophils, growth curves for each strain were generated, the initial bacterial concentrations in each sample were determined, and bacterial killing was assessed as the decrease in surviving bacteria overtime as described[133,150]. To measure the effect of ecotin on bacterial survival/growth, 555.56 nM of ecotin was added to $1x10^7$ CFU/ml bacteria (that corresponds to the concentration of bacteria when co-cultured with neutrophils) and incubated for 30 mins at 37ºC. These samples were diluted 100-fold and added to LB broth on the 384-well microplate – in an exact same manner as the neutrophil-containing samples.

Statistical analysis

Results for assessing inhibition of NE by ecotin in the CF sputum were analyzed by one-way ANOVA and Dunnet's multiple comparison test (comparison of each ecotin concentration to untreated control). Results for assessing the differences in inhibition by ecotin between each CF sputum was analyzed by two-way ANOVA and Tukey's multiple comparison test. The correlation between two parameters was analyzed with Spearman's rank-order correlation and the correlation coefficient (r) was calculated. Lastly, the assessment of ecotin on neutrophil-mediated bacterial killing was analyzed by one-way ANOVA and Tukey's multiple comparison test. Data are expressed as mean ± S.E.M. Statistically significant differences were considered as *ns*, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All statistical analyses were performed with GraphPad Prism version 9.01 for Windows software (GraphPad Software, San Diego, CA, USA).

Results

Neutrophil elastase enzymatic activities vary in the CF sputum

To assess whether we can detect NE activities in CF sputa, sputum supernatants from seven CF patients were assessed for NE activity (mU/ml) using a fluorescent microplate-based kit as done previously[257,258]. Figure 5.1A demonstrates representative kinetics of NE enzymatic activities in one CF sputum, in recombinant NE solution and in assay buffer as a control over a time period of 2 hours. There is a positive correlation of NE activity as evidenced by the rise in fluorescence representing the increased concentration of the fluorescent NE cleavage product of 2Rh110 (Figure 5.1A). The

CFBR185 sputum was diluted 500-fold. Figure 1B displays the NE activity levels observed in the sputum supernatants of the seven CF subjects. Overall, there is a range of NE activity present in the sputum from CF adults.

***C. rectus* ecotin inhibits NE in the CF sputum**

Our primary goal was to determine whether ecotin can inhibit NE present in the CF sputum and to what extent. We have previously cloned and expressed recombinant ecotins from three Gram-negative bacteria: *Campylobacter rectus*, *C. showae* and *E. coli*[12]. All three ecotins inhibited the enzymatic activities of purified human NE in a dose-dependent and identical fashion[12]. Based on these observations we planned to test these recombinant ecotins for their potential NE-inhibitory action in CF sputa. First, we tested ecotin isolated from *C. rectus*, a bacterium that is typically present in the human oral flora, can cause oral abscesses but is not associated with CF lung disease in any way[259]. *C. rectus* ecotin was added to the sputum supernatants of four CF subjects in increasing doses (0-1.67 μM) and percent inhibition of NE activity was determined relative to ecotin-free sputum. *C. rectus* ecotin significantly inhibited NE enzymatic activities in all four CF sputa in a dose-dependent manner (Figure. 5.2A). *C. rectus* ecotin displayed complete NE inhibition (100%) at doses higher or equal to 166.6 nM in all four CF sputum samples (Figure 5.2A). There is also complete inhibition (100%) by sivelestat, a known NE inhibitor (5 μM), for all sputa tested which represents our positive control in this study (Figure 5.2A)[260]. The inhibitory curves allowed us to determine IC_{50} values for *C. rectus* ecotin in all four CF samples and represented a relatively wide range (23.7-726.7 nM) as indicated in Figure 5.2B. Altogether, *C. rectus* ecotin can achieve complete

inhibition of NE activity in the CF sputum and the dose needed for 50% inhibition varies from subject to subject.

***C. showae* ecotin inhibits NE in the CF sputum**

C. showae is a species closely related to *C. rectus* and, also produces ecotin that inhibits the activities of human NE in an identical fashion *in vitro*[12,261]. Next, we tested whether *C. showae* ecotin is also a potent inhibitor of NE in CF sputum. *C. showae* ecotin also significantly inhibited NE enzymatic activities in the sputa collected from the indicated three CF subjects (Figure 5.3A). *C. showae* ecotin displays complete inhibition (100%) of NE at the high dose of 1666.67 nM in each of the three sputa (Figure 5.3A). *C. showae* ecotin still shows a close-to-complete inhibition of NE activity in the sputum from subject number CFBR185 at a dose as low as 55.56 nM (Figure 5.3A). For sputum samples CFBR 614 and 185, *C. showae* ecotin significantly inhibits NE at a concentration as low as 16.67 nM, and NE activity is significantly inhibited in all three sputa at the dose of 55.5 6 nM (Figure 5.3A). The level of inhibition of NE by *C. showae* ecotin does differ between the three sputa tested as it is also reflected in the IC50 values (16.1, 46.5 and 87.5 nM) (Figure 5.3B). Overall, *C. showae* ecotin inhibits NE in CF sputa and is slightly more effective than *C. rectus* ecotin.

***E. coli* ecotin is less inhibitory on NE in CF sputum than *Campylobacter* ecotins**

Ecotin was first described in *E. coli*[243]. We compared the efficacy of *Campylobacter* ecotins on NE activity in the CF sputum to that of *E. coli* ecotin. *E. ecoli* ecotin was much less effective at inhibiting NE compared to *Campylobacter* and only reached statistical significane in three of the four CF sputum supernatants tested and only at the highest doses (Figure 5.4A). Although not significant, there is a positive trend of

percent NE inhibition as the *E. coli* ecotin concentration increases for CFBR614 (Figure 5.4A). Sivelestat displays a complete inhibition for all four sputa tested (Figure 5.4A). In addition, *E. coli* ecotin does not completely inhibit NE in the CF sputum at any tested dose, unlike what was seen in the case of ecotins from *Campylobacter* species (Figure 5.4A-B vs. 5.3A-B). The differences in the NE-inhibitory effect of the *E. coli* ecotin among the four CF sputa tested is also reflected in the IC_{50} values. The *E. coli* ecotin was least effective in the CFBR 683 sputum where no IC_{50} value could be calculated and the level of inhibition of NE activity had not reached 50% (Figure. 5.4B). Similar NE-inhibitory profiles were observed in case of CFBR308 and CFBR614, but it was less impressive in CFBR 185 (Figure 5.4B). Overall, *E. coli* ecotin has only modest inhibitory effect on NE in the CF sputum and is much less effective than the *Campylobacter* ecotins.

***P. aeruginosa* ecotin inhibits NE in the CF sputum**

Ecotin from *P. aeruginosa* was found to inhibit NE *in vitro*[252] . Therefore, we cloned *P. aeruginosa* ecotin and explored its effect on NE activities in CF sputum. *P. aeruginosa* ecotin significantly inhibited NE activity in a dose-dependent manner in only one of the three CF sputa tested (Figure 5.5A). *P. aeruginosa* showed a positive correlation between percent inhibition of NE in the sputum from CFBR208, however no clear trend was observable in sputa of patients CFBR185 and CFBR308 (Figure 5.5A). In the CFBR208 sputum, *P. aeruginosa* ecotin was able to significantly inhibit NE at concentrations as low as 1.67 nM and higher (Figure 5.5A). While *P. aeruginosa* ecotin significantly inhibited NE in the sputum from CFBR185 at a concentration of 16.67 nM, at higher ecotin concentrations it did not display significant inhibition and a clear dose-

dependent trend could not be confirmed (Figure 5.5A). *P. aeruginosa* was also unable to inhibit NE in the sputum of patient CFBR308. P. aeruginosa ecotin could not inhibit NE enzymatic activities by more than 50% in any of the CF sputa tested, hence no IC_{50} values could be determined (Figure 5.5B). Sivelestat, on the other hand, displayed complete inhibition of NE in all three sputa tested (Figure 5.5A). Altogether, *P. aeruginosa* ecotin is the weakest NE inhibitor in CF sputum tested in this study.

Ecotins from *Campylobacter* species have the strongest inhibitory effect on NE in the CF sputum

To compare the inhibitory actions of the tested ecotins on NE activities in the CF sputum, we aligned the dose-dependent results for all four ecotins in case of the CF sputum sample that could be tested using all ecotins, CFBR185. As results of Figure 5.6A show, ecotins from each of the two *Campylobacter* species effectively inhibited NE at high concentrations. *P. aeruginosa* ecotin was unable to inhibit CF sputum NE activities at any of the concentrations tested (Figure 5.6A). E. coli ecotin displayed an inhibitory action only at the highest dose (Figure 5.6A).

To further characterize the NE-inhibitory action of ecotins, IC50 values for each ecotin in each CF sputum supernatant have been compiled (where available). As it is shown in Figure 5.6B, *C. showae* ecotin displayed the lowest average IC_{50} values for NE inhibition in CF sputum, while *C. rectus* ecotin was the second-best, and followed by *E. coli* ecotin. *P. aeruginosa* ecotin was not included since no IC50 values could be determined (Figure 5.5). Altogether, these data suggest that while all ecotins showed at least some sign of NE inhibition in CF sputum, *C. showae* and *C. rectus* ecotins outperformed *E. coli* and *P. aeruginosa* ecotins, revealing an unexpected complexity of

the NE inhibitory pattern among ecotins in the CF sputum, not seen when purified human NE was used.

Ecotin does not impair neutrophil-mediated killing of *P. aeruginosa* and *S. aureus*

Data showcased earlier indicate that some ecotins are potent NE inhibitors in the CF sputum whereas others are ineffective at inhibiting NE (Figures 5.2-5.6). Furthermore, the bacteria with the lowest ecotin NE inhibitory activity was the most pathogenic in CF. To investigate further this variation in biologic activity, we next addressed whether these four ecotins impact neutrophil-mediated bacterial killing to a varying degree. Therefore, we tested the effects of ecotins on neutrophil-mediated killing of the two predominant CF pathogens, *P. aeruginosa* and *S. aureus*. Ecotin was added to neutrophils from healthy donors and killing of opsonized bacteria was assessed. Two *P. aeruginosa* strains (PAO1 and a CF isolate, PA10007) and two *S. aureus* strains (USA300 and a CF isolate MRSA24) were tested. First, bacterial killing of *P. aeruginosa* by neutrophils was measured after treatment with ecotin at a dose that significantly inhibits NE activity in CF sputum (555.6 nM) in case of three bacterial species. Overall, none of the ecotin species impaired neutrophil-mediated killing of *P. aerguionsa* (Figure 5.7A). Interestingly, *E. coli* ecotin significantly increases neutrophil-mediated killing of PAO1, whereas there was no difference in killing of the CF clinical isolate 10007 by any of the ecotins (Figure 5.7A). Next, bacterial killing of *S. aureus* by neutrophils after ecotin treatment was measured with two strains, the lab reference stain USA300 and a CF clinical isolate MRSA24. As shown in Figure 5.7B, there was no significant effect of any of the ecotins on *S. aureus* killing by neutrophils.

To ensure that ecotins do not interfere with bacterial growth, ecotin and bacteria alone were incubated (without neutrophils), and the growth of indicated bacterial strains were measured overtime following absorption. As shown in Figure 5.7C and D, when compared to untreated bacteria, there were no differences in the growth kinetics of bacteria overtime influenced by ecotins. Altogether, ecotin does not impair neutrophil-mediated killing or growth of *P. aeruginosa* and *S. aureus*.

Conclusions

In this study, we have shown ecotin's effectiveness to inhibit NE present in human CF sputum samples. This study describes the ability of ecotin to inhibit NE enzymatic activity in the CF sputum while not compromising overall bacterial killing of two predominant CF pathogens, *P. aeruginosa* and *S. aureus.* The ecotin homologs from *C. rectus*, *C. showae*, *E. coli*, and *P. aeruginosa* showed a surprisingly variability in the capacity to inhibit NE activity in the CF sputum whereas none compromised bacterial killing and/or bacterial growth. *C. rectus* and *C. showae* ecotin displayed a strong inhibitory effect of NE activity in the CF sputum, with complete inhibition at the higher doses tested. In contrast, *E. coli* and *P. aeruginosa* were both weak inhibitors of NE activity and in some instances, failed to show any inhibitory action.

The NE-inhibitory patterns of tested ecotins in the CF sputum is different from the one reported by us using purified human NE where no differences were seen[12]. The reason for this could hide in ecotins' structural differences. Our previous data show that there is low conservation between proteins at the amino acid level between ecotin orthologs of the *Campylobacter* species versus *E. coli*[12]. *C. rectus* ecotin has 27% identity

and *C. showae* has 33% identity to the *E. coli* ecotin[12]. Based on protein sequence alignment, *P. aeruginosa* ecotin is similar to *E. coli* ecotin with about 60% identity. *P. aeruginosa* ecotin is also dissimilar from the *Campylobacter* ecotins with 25% identity to *C. rectus* and 27% to *C. showae*[12]. In the *Campylobacter* homologs, two cysteines are conserved (Cys50 and Cys87) which form an intra-subunit disulfide bond in *E. coli* ecotin[12]. Also, the substrate binding pocket residues are different compared to *E. coli*[12]. The methionine at the P1 binding site only present in the *C. showae* ecotin whereas *C. rectus* contains a leucine in this position [12]. Altogether, these structural differences may play a role in their ability to bind and inhibit NE active sites.

The CF sputum contains NE in multiple components such as free NE released from neutrophils, NETs, and exosomes[69,217,262]. Therefore, the inhibition of NE by ecotin gave insight to whether ecotin can overall reduce the amount of NE activity present in the CF sputum samples. The differences between the ecotin species and their ability to inhibit NE in the total sputum versus NETs could be due to the efficacy of ecotin to inhibit NE in the different compartments. Diving further into the components of the CF sputum and the affinity for each ecotin to inhibit NE could help explain this result. Additionally, CF patients infected with *P. aeruginosa*, may contain its ecotin in their lung environment, which has been shown to bind to Ps1 on the CF biofilm exopolysaccharide matrix[252]. It is suggested that *P. aeruginosa* ecotin is secreted and binds to the outer layer of the biofilm matrix to protect *P. aeruginosa* from neutrophil-mediated death[252]. Therefore, *P. aeruginosa* ecotin displaying robust inhibition of NE on NETs could be due to its high affinity to DNA.

Despite, *P. aeruginosa* ecotin being present in the CF lung, NE remains to cause severe damage of lung tissue, therefore *P. aeruginosa* ecotin may only be efficient in protecting biofilms from NE. Our data suggests that ecotin inhibits NE in the CF sputum while not compromising neutrophil-mediated killing of CF clinical isolates in vitro, displaying a potential for ecotin to be used as an NE-targeting therapeutic to prevent further lung damage. There are currently no approved NE inhibitors for human use. Sivelestat was used a positive control in this study as it has been shown to reduce NE and corresponding lung injury, however clinical trials deemed this drug ineffective in humans[255]. A meta-analysis analyzing the clinical trials performed with Sivelestat on patients with acute respiratory distress syndrome (ARDS) discovered that overall it did not improve mortality and did not alter the duration of mechanical ventilation[263]. One study was even halted early due to an increase of mortality in patients with administered Sivelestat and the reason for the increased mortality remains unknown[264]. However, it has been shown that the inhibition of NE by Sivelestat did not enhance infection, suggesting that NE inhibitors will overall benefit lung function improvement and not worsen infections[255,264–266]. Therefore, new inhibitors against NE are needed. Ecotin from *C. rectus* and *C. showae* showed the same inhibitory effect as Sivelestat, with complete NE inhibition in the CF sputum. Also, the significant inhibition by ecotin was shown at a low concentration of 555.56 nM versus 5μM of Sivelestat, suggesting much lower concentrations of ecotin are needed to inhibit NE in the CF lung.

Altogether, our study characterizes the effect of four ecotin homologs on the CF sputum and neutrophil effector responses. This is the first study to characterize *P. aeruginosa* ecotin and its poor ability to inhibit NE in the CF sputum. This is also the first

study to test ecotin as a NE inhibitor for CF airway disease. Our in vitro data suggest that ecotin from *C. rectus* and *C. showae* have the potential to significantly reduce NE activity in the CF airways, however more studies are needed to determine the effect on the reduction of lung injury. Future studies will test ecotin in the βENaC-Tg CF mouse model[267,268] with a neutrophilic and NE-mediated, CF-like lung disease. Our team has characterized this mouse model which displayed significant lung inflammation mediated by neutrophils[268]. The addition to ecotin in vivo to measure its effect on NE activity and lung injury will further confirm that ecotin has potential for therapeutic use in CF lung disease. Lung function decline is the main cause of morbidity and mortality and CF patients, with NE correlating to decreased lung function. NE has been a target in these patients for years with no successful approved therapeutics thus far. Our future work will aim to better characterize ecotin's effect on inhibiting NE and reducing lung disease in CF.

Acknowledgements

The authors would like to acknowledge the CF Biospecimen Repository at the Children's Healthcare of Atlanta and Emory University CF Discovery Core for providing CF human samples. We would also like to thank all the healthy subjects for their blood donations and the staff of the UGA Clinical and Translational Research Unit. Lastly, we would also like to thank Dr. Joanna Goldberg at Emory University for her contribution of providing the CF *S. aureus* clinical isolate and Dr. Samuel Moskowitz (Massachusetts General Hospital, currently: Vertex Pharmaceuticals) for providing the CF *P. aeruginosa* clinical isolate.

Author Contributions

K.F. performed most experiments, provided formal analysis, investigation, methodology, and wrote and revised the manuscript. H.N. performed conceptualization, methodology, formal analysis, supervision, and validation. C.C. and B.Z. performed methodology. A.S. supervised the collection of specimens from CF patients. C.S. provided conceptualization, investigation, resources, and project administration. B.R. conceptualized the idea, designed methodology, acquired funding, reviews the manuscript, and supervised the study.

Funding

This work was funded by the National Institutes of Health awards (R01HL136707-01A1 to B. Rada) and the Cystic Fibrosis Foundation (003303I221 to B. Rada). This work was also supported by the Georgia CTSA Training Grant, TL1 TR002382 and UL1TR002378. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Tables

Species	Strain	Characteristic	Reference
E. coli	DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ$^-$	Invitrogen
E. coli	BL21(DE3)	*E. coli* str. B F- *ompT gal dcm lon hsdS$_B$*($r_B^- m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S)	Invitrogen
E. coli	C600(RK212.2)	*leu thr thi lacy supE44 tonA;* pRK212.2, Amp^R, Tet^R	*Figurski* et al.[269]
C. rectus	Wildtype strain, ATCC 33238	Human periodontal pocket	*Tanner* et al.[270]
C. showae	Wildtype, CCUG 30254	Human gingival crevice	*Etoh* et al.[271]
P. aeruginosa	Wildtype, PAO1	Water and soil	*LaBaer* et al.[272]
S. aureus	Wildtype, USA300	Methicillin-resistan	*S. aureus* subsp. Aureus (ATCC® BAA1717™)
S. aureus	MRSA24	Cystic Fibrosis clinical isolate	*Bernardy* et al.[155]
P. aeruginosa	10007	Cystic Fibrosis clinical isolate	Yoo et al.[88]

Table 5.1: Bacterial strains used in this study.

Figures and Figure Legends

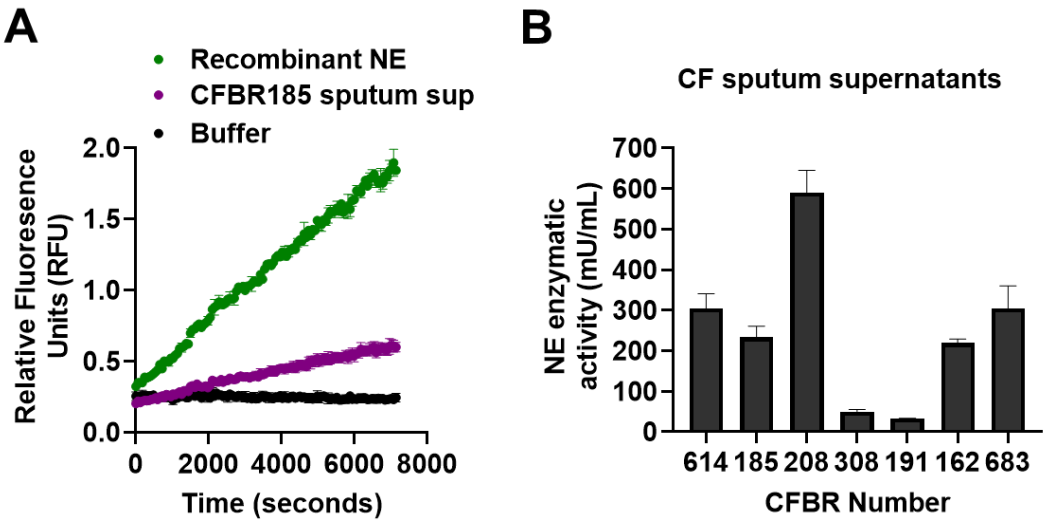

Figure 5.1: Neutrophil elastase activities in sputum supernatants of CF subjects. Neutrophil elastase (NE) enzymatic activity measurement was performed in CF sputum supernatants obtained from seven CF subjects with a fluorometric assay kit and fluorescence was measured in a microplate reader (Ex/Em = 355/530nm). **(A)** A representative kinetic curve of NE activity obtained using the CFBR185 sputum. Relative fluorescence units (RFU) of NE activity are shown in diluted (1/500) CF sputum over time compared to recombinant NE and buffer alone (n = 3). Mean ± S.E.M. **(B)** NE activity from each of the CF patients' sputum samples. Results are expressed as mean ± S.E.M (n=3-11).

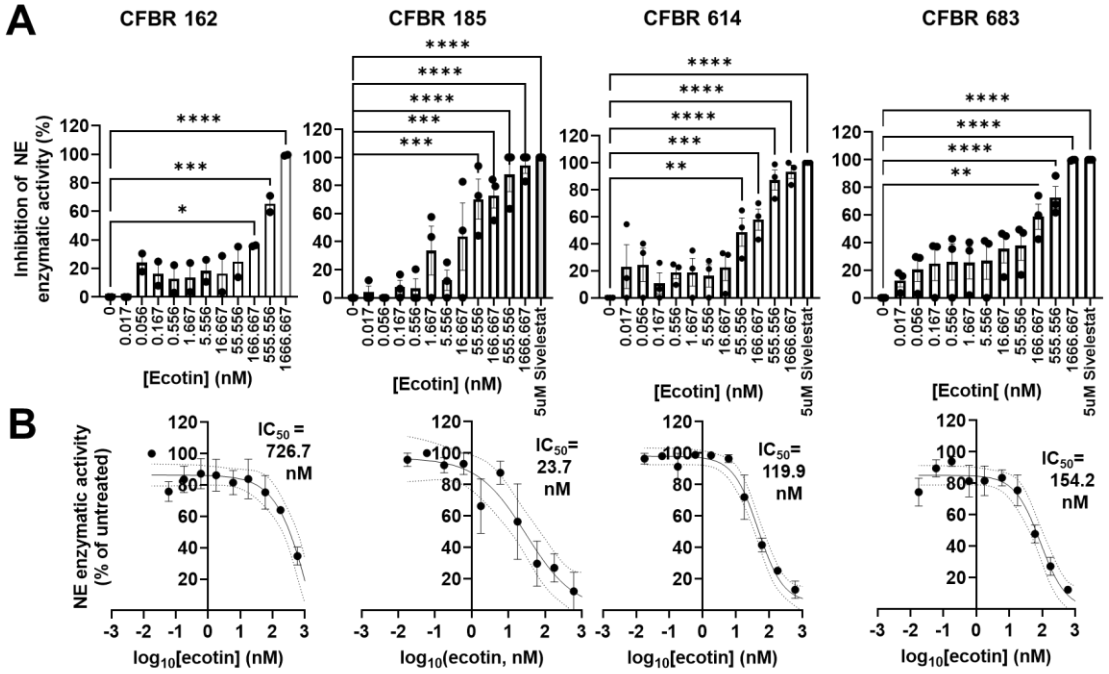

Figure 5.2. *C. rectus* ecotin inhibits NE in the CF sputum. (A) *C. rectus* ecotin was added in increasing concentrations (0-1666.7 nM) to the sputum supernatant from four CF subjects and NE activity was measured (mU/ml). 5 µM of sivelestat was used as a positive control. Percent inhibition of NE activity for each sputum sample was determined by the quantification of NE activity (mU/ml) at each dose of ecotin over the quantification of NE activity without ecotin, represented as 0 nM on the graph (n=2-3). Mean ± S.E.M. Data were analyzed by one-way ANOVA, Dunnett's multiple comparison test. **(B)** Representative graphs for the IC_{50} values (nM) for *C. rectus* ecotin on its inhibitory effect of NE activity, represented as percent of untreated CF sputum (n=3). IC_{50} values for each CF sputum were determined by interpolation of standard curves, Sigmoidal, 4PL, X is log(concentration) with 95% confidence interval bands. Mean ± S.D. Statistical differences were considered as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

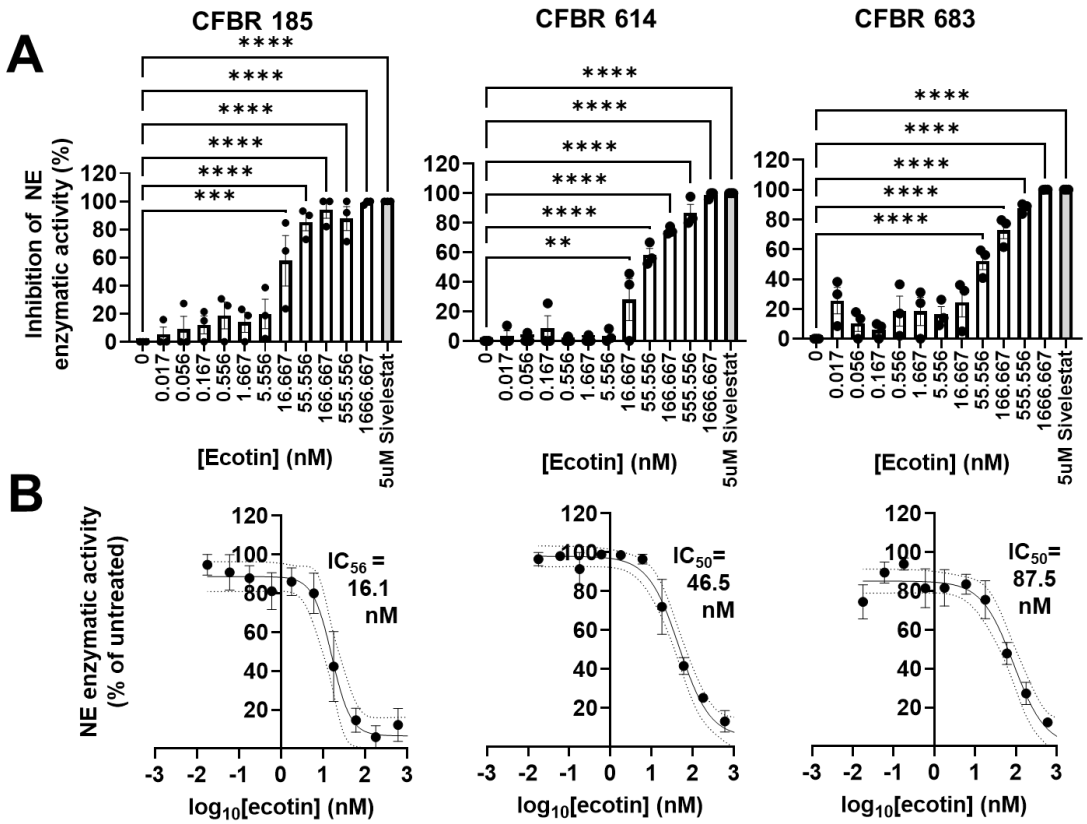

Figure 5.3. *C. showae* ecotin inhibits NE activity in the CF sputum. (A) *C. showae* ecotin was added in increasing concentrations (0 -1666.7 nM) to the sputum supernatant of three CF subjects and NE activity was measured (mU/ml). 5 µM of Sivelestat was served as the positive control for NE inhibition. Percent inhibition of NE activity for each sputum sample was determined by the quantification of NE activity (mU/ml) at each dose of ecotin over the quantification of NE activity without ecotin, represented as 0nM on the graph (n=3). Mean ± S.E.M. Data were analyzed by One-way ANOVA, Dunnett's multiple comparison test. **(B)** The summary data for percent inhibition of NE activity by the addition of increasing concentrations of *C. showae* ecotin to each CF sputum (n=3). Each dot represents the mean of percent inhibition for each ecotin dose for that given sputum (represented by the dot color). Differences in inhibitory effect of ecotin between the three CF sputa was determined by Two-way ANOVA, Tukey's multiple comparison test. Statistical differences were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

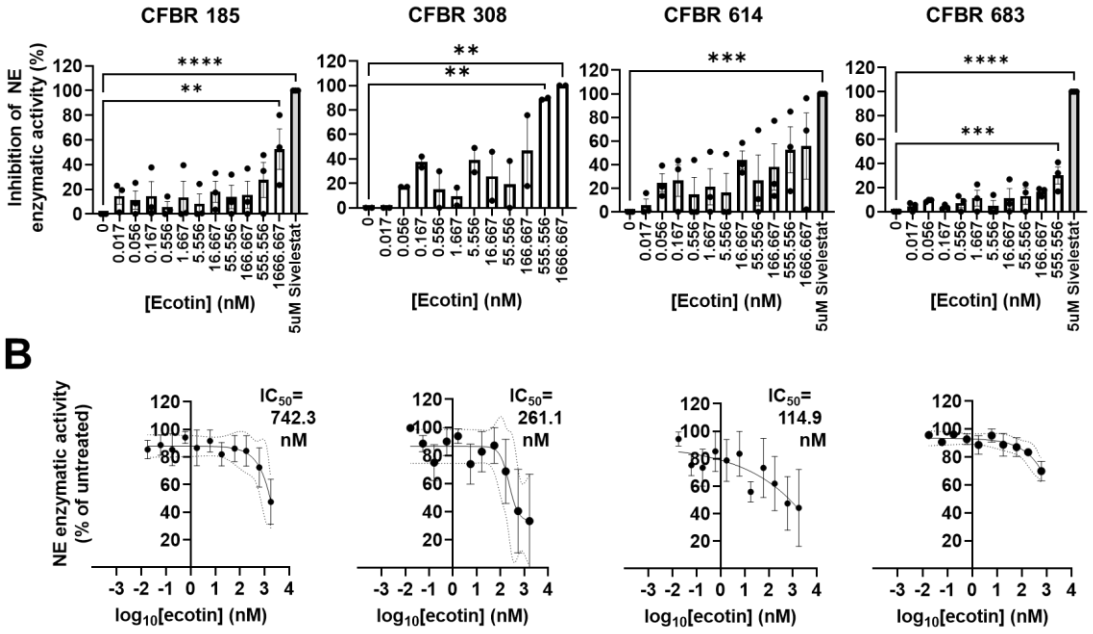

Figure 5.4. *E. coli* ecotin inhibits NE in the CF sputum. (A) *E. coli* ecotin was added in increasing concentrations (0 -1666.7 nM) to the sputum supernatants of four CF subjects and NE activity was measured (mU/ml). 5 µM of sivelestat served as positive control. Percent inhibition of NE activity for each sputum sample was determined by the quantification of NE activity (mU/ml) at each dose of ecotin over the quantification of NE activity without ecotin, represented as 0nM on the graph (n=3). Mean ± S.E.M. Data were analyzed by One-way ANOVA, Dunnett's multiple comparison test. **(B)** The summary data for percent inhibition of NE activity by the addition of increasing concentrations of *E. coli* ecotin to each CF sputum (n=3). Each dot represents the mean of percent inhibition for each ecotin dose for that given sputum (represented by the dot color). Differences in inhibitory effect of ecotin between the three CF sputa was determined by Two-way ANOVA, Tukey's multiple comparison test. Statistical differences were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

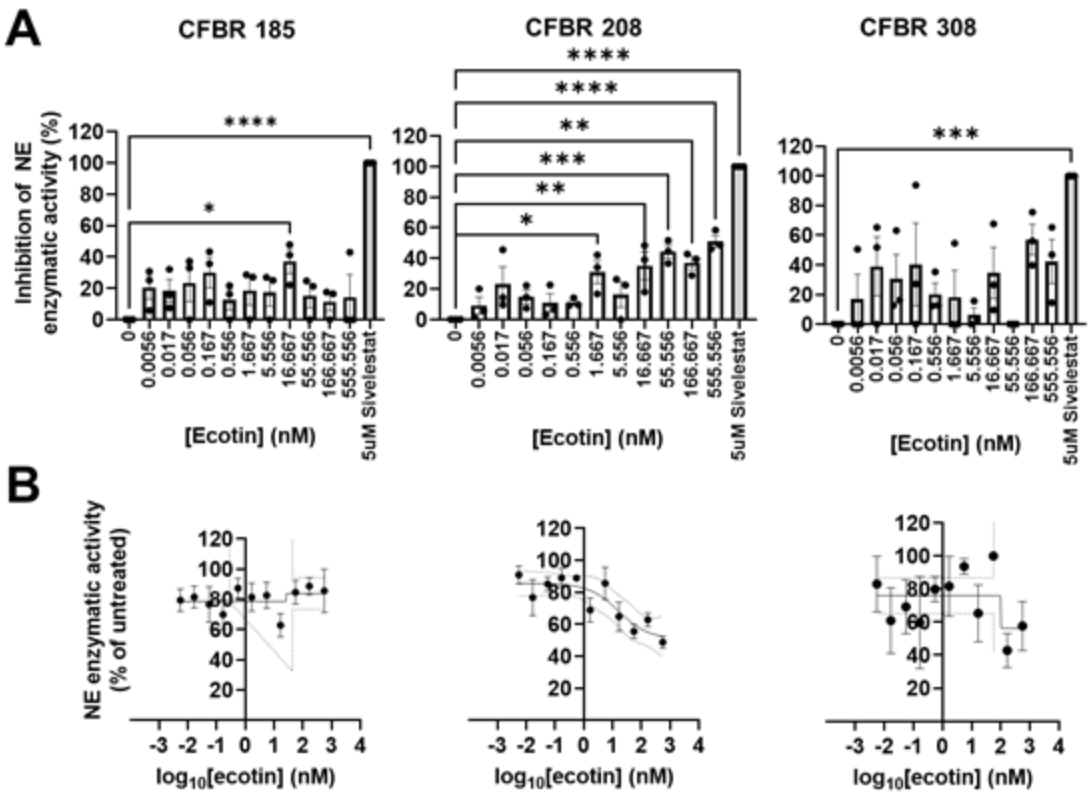

Figure 5.5. *P. aeruginosa* ecotin is a weak inhibitor of NE activity in the CF sputum. **(A)** *P. aeruginosa* ecotin was added in increasing concentrations (0-555.56 nM) to the sputum supernatant from three CF subjects and NE activity was measured (mU/ml). 5 µM of sivelestat was added for a positive control. Percent inhibition of NE activity for each sputum sample was determined by the quantification of NE activity (mU/ml) at each dose of ecotin over the quantification of NE activity without ecotin, represented as 0nM on the graph (n=3). Mean ± S.E.M. Data were analyzed by One-way ANOVA, Dunnett's multiple comparison test. **(B)** The summary data for percent inhibition of NE activity by the addition of increasing concentrations of *P. aeruginosa* ecotin to each CF sputum (n=3). Each dot represents the mean of percent inhibition for each ecotin dose for that given sputum (represented by the dot color). Differences in inhibitory effect of ecotin between the three CF sputums was determined by Two-way ANOVA, Tukey's multiple comparison test. Statistical differences were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

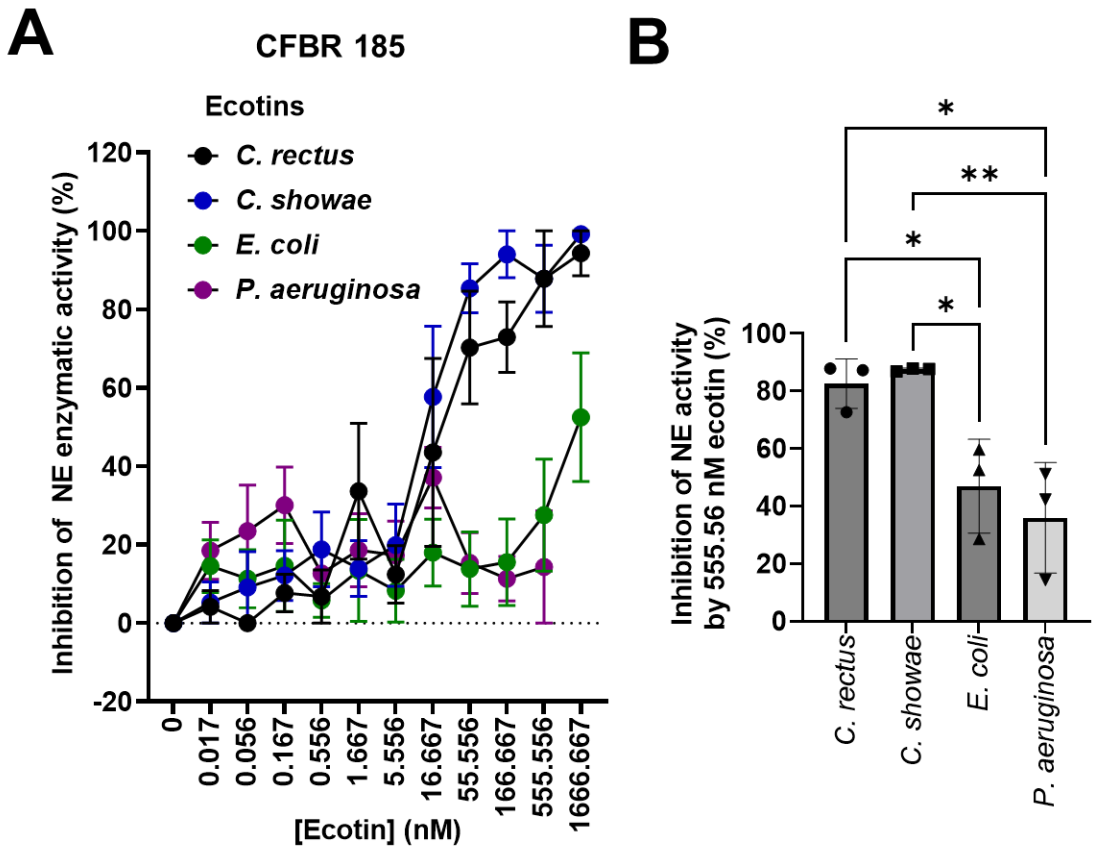

Figure 5.6. Ecotin from *Campylobacter* species has the most robust NE-inhibitory effect in CF sputum. (A) The comparison of each ecotin and its effect on percent inhibition of NE activity in the sputum at increasing concentrations for CF subject CFBR185 (n=3). Each dot represents the mean of percent inhibition by each ecotin (represented by dot/line color) for subject sputum CFBR185. Mean ± S.E.M. **(B)** Each ecotin's IC_{50} (nM) values in each CF sputum are displayed. Each dot in the bar graph represents a different CF sputum sample. Mean ± S.E.M.

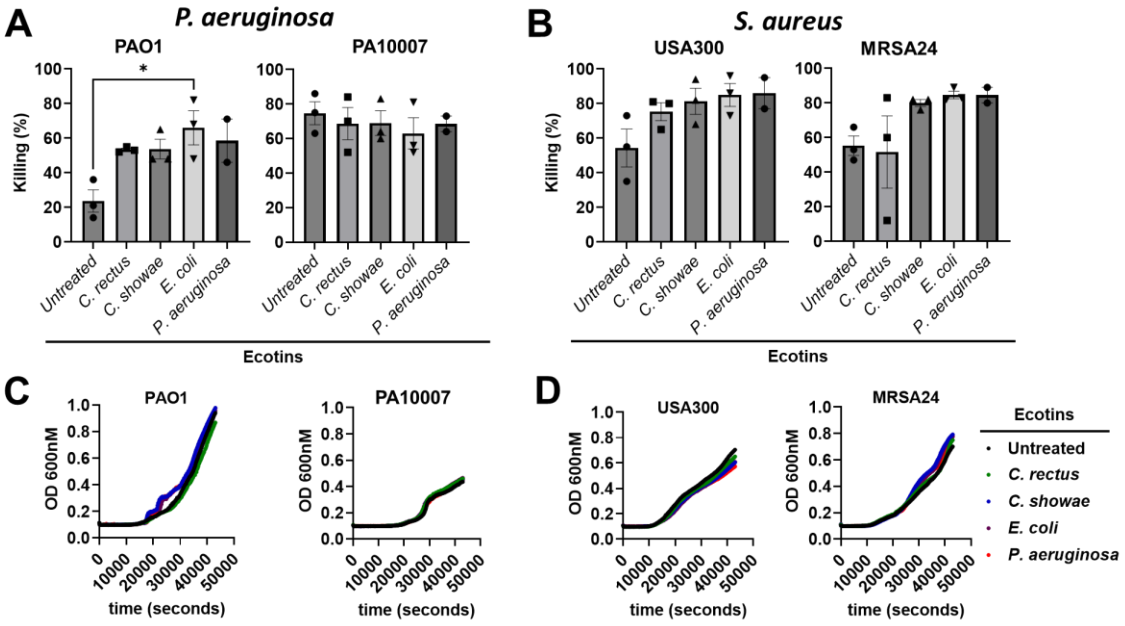

Figure 5.7. Ecotin does not impair neutrophil-mediated bacterial killing of *P. aeruginosa* and *S. aureus*. (A) The results of bacterial killing of *P. aeruginosa* by neutrophils with or without ecotin. Two *P. aeruginosa* strains (PAO1 and the CF clinical isolate, 10007) were tested. Neutrophils were either untreated or treated with 555.56nM of the indicated ecotins and infected with *P. aeruginosa* at MOI of 10 (n=3). Bacterial killing was measured by a microplate-based assay. Each dot in the bar graph represents one neutrophil donor and its corresponding percent killing of *P. aeruginosa*. Mean ± S.E.M. Comparison of percent killing by ecotin-treated neutrophils to untreated neutrophils was analyzed by One-way ANOVA, Tukey's multiple comparison test. Statistical differences were considered as ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. **(B)** The results of bacterial killing of *S. aureus* by neutrophils with or without treatment of ecotin. Two *S. aureus* strains were tested, USA300 and the CF clinical isolate, MRSA24. Neutrophils were either untreated or treated with 555.6 nM of each ecotin and infected with *S. aureus* at MOI 10 (n=3). Mean ± S.E.M. Data were analyzed as described in (A). **(C)** Kinetic growth curves of *P. aeruginosa* bacterial strains in the presence of 555.56nM ecotin (without neutrophils). **(D)** Kinetic growth curves of the *S. aureus* bacterial strains in the presence of 555.6 nM ecotin (no neutrophils).

CHAPTER 6

CONCLUDING REMARKS

Despite new CFTR modulator therapies to improve the function of the CFTR protein, CF remains uncured. Pulmonary function decline and bacterial infections such as *P. aeruginosa* and *S. aureus* lead to decreased patient survival[135,273–275]. It was found that patients with *P. aeruginosa* lung infection are 2.6 times more at risk of death and patients with MRSA are 1.27 times more at risk of death than CF patients who did not have either lung infection present[135,273]. The CF airway environment is complex, and contains several inflammatory proteins, enzymes, antibodies, and innate immune cells. Also, *S. aureus* is the number one predominant pathogen in the CF airway infecting more than half of the patients. Therefore, the goal of this thesis was to better understand the CF airway environment and its effect on neutrophils during *S. aureus* infection. Our hypothesis was that the CF airway environment impairs *S. aureus* killing by neutrophils, and the NE inhibitor ecotin, inhibits NE without compromising PMN-mediated bacterial clearance. We answered this hypothesis in three aims represented by chapters 3-5 respectively.

Our first aim was to describe the neutrophil effector responses to CF clinical isolates of *S. aureus* in the CF lung environment. In this aim, we established an in vitro model to mimic the CF airways which is crucial to study PMN effector functions. Our model consisted of pooling CF sputum supernatant from several CF patients and exposing PMNs derived from the whole-blood of healthy donors to this sputum for 3.5 hours prior to infecting with *S. aureus*. This model mimicked the CF airways during *S.*

aureus infection which describes, impairment of bacterial killing, uncompromised superoxide production, and enhanced NET release. In this aim we also described that the CF airway environment did not impair phagocytosis and that CF neutrophils from the whole-blood are not impaired in *S. aureus* killing of CF clinical isolates. This was the first study to measure multiple CF clinical isolates of *S. aureus* which included methicillin-sensitive strains and methicillin-resistant strains. We also described that the CF clinical isolates used in this study had high DNAse I activity levels, suggesting a resistance toward NET-mediated killing. Future work could investigate the *S. aureus* clinical isolates and their virulence factors/genes to help explain their persistence in the CF lung. Our next objective was to further identify the mechanism in which the CF airway environment impairs neutrophil-mediated killing of *S. aureus* which was investigated in aim 2.

Aim 2 further explored the effect of the CF airway environment on neutrophils during MRSA infection. In this aim, we focused on MRSA, as we did not see a discrepancy in aim 1 between MSSA and MRSA isolates and MRSA is harder to combat in the CF lung with little data exploring its relation to neutrophils in CF. In this aim we were able to confirm data from aim 1 with neutrophils from CF patients in that CF neutrophils are not impaired in killing MRSA until they are exposed to the CF airway environment. We also confirmed that phagocytosis is not impaired by the CF sputum in healthy and CF neutrophils. This led us to further investigate the mechanism of phagocytosis by neutrophils in the CF airway environment during MRSA infection. With this, we discovered that the CF airway environment impaired phagosomal ROS and the presence of MPO and cathepsin D inside the phagosome was impaired during MRSA

infection. This aim was the first to identify a potential dysregulated pathway that impairs neutrophils to efficiently kill bacteria in the CF lung. Our future goals for this work would be to dive into this mechanism further. Investigating the pathways needed to produce phagosomal ROS would help explain the reason for the inhibition seen. NADPH oxidase is a complex that involves the activation and membrane translocation of $p47^{phox}$ to $p67^{phox}$ and $p40^{phox}$ to the phagosome membrane to produce superoxide anions[276]. Superoxide anions are enzymatically converted to H_2O_2 which is then converted to HOCL by MPO released from granules reacting with chloride ions in the phagosome[276]. Therefore, future studies would include investigating the formation of the NADPH oxidase complex and HOCL production inside the phagosome of both healthy and CF neutrophils exposed to the CF airway environment. The lack of ROS, MPO, and Cl^- inside the phagosome would result in significantly reduced HOCL and further explain the impaired killing of *S. aureus*. Also, in this aim we measured the presence of enzymes trafficked through primary granules so investigating enzymes present in secondary granules and late lysosomes such as lactoferrin would also be beneficial to conclude whether phagolysosome fusion is truly impaired in the CF airway during MRSA infection. Additionally, our model has two major players that affect the neutrophils' phagosomal response which is the CF airway environment and the CF clinical isolates of MRSA. MRSA could be playing a role in the reduction of phagosomal ROS and assembly of the NADPH oxidase in the phagosome. Therefore, further identifying virulence factors such as superoxide dismutase in our CF isolates could potentially explain a manipulation of the phagosomal response. Lastly, identifying components of the CF sputum that affects the neutrophils' ability to kill MRSA would be crucial for

identifying new targets for therapies. The components of the CF sputum can be fractionated by size which would help uncover the component of the CF sputum affecting neutrophils effector responses to MRSA. Our data along with these future studies will lead to a detailed mechanism of impaired neutrophil effector responses by the CF airways. Altogether aim 1 and 2 have identified a crucial model to study PMN effector responses and have advanced the understanding of why neutrophils are inefficient in killing *S. aureus* in the CF lung.

Our goal of aim 3 was to characterize the NE inhibitor, ecotin in the CF sputum and its effect on anti-bacterial mechanisms of neutrophils. Ecotin is a known serine protease inhibitor produced by Gram-negative bacteria and NE is a serine protease released by neutrophils that is abundant in the CF lung and contributes to worsened lung pathology over time. Our results in aim 2 also confirmed that NE is not impaired by the CF airway environment and does not assist in killing *S. aureus*. Therefore, NE is detrimental to the airway causing substantial damage and does not perform its purpose in targeting bacteria. The data in this aim displayed significant inhibition of NE enzymatic activity in the CF sputum after treatment of ecotin while not compromising bacterial killing of the two major CF pathogens, *P. aeruginosa* and *S. aureus*. The importance of aim 3 was to lay out the foundation for ecotin as a potential therapeutic treatment against NE in the CF airways. NE in the CF lung has been a target for more than a decade, with no inhibitors approved by the FDA thus far. Ecotin is a very stable protein, and its therapeutic use should be investigated further. Exploring whether ecotin can inhibit NE on the surface of neutrophils or inside the neutrophil would amplify its use in the CF airway and determine whether ecotin can prevent NE release from neutrophils. The CF

sputum contains high levels of extracellular vesicles and activated neutrophils release these vesicles that can carry NE into the small compartments of the lung to cause damage[69,277]. Therefore, identifying whether ecotin can inhibit NE carried on extracellular vesicles in the CF airways would be beneficial for its use in CF patients. Future studies should also strive to identify the effect of the inhibition of NE by ecotin in our in vitro CF airway model described in aim 1 and 2. For example, our data in chapter 4 showed significant inhibition of the C5a receptor expression on neutrophils exposed to the CF sputum and NE has been shown to cleave C5a, therefore the inhibition of NE in the sputum may show differences in downstream neutrophil effector responses[224]. This data would be important to determine ecotin's effect on neutrophils in the CF airways. Lastly, future studies are needed in an in vivo mouse model to determine its immunogenicity and effect in an animal model. Previous work has characterized a CF mouse model, in which the CF airway environment replicates what is seen in CF human patients and our preliminary work shows enhanced NE activity in bronchoalveolar lavage fluid in this mouse model (data not shown)[267,268,278]. Thus, future research will include the administration of ecotin in the CF mouse model to further identify its use as a therapeutic in patients.

Altogether this thesis uncovered a new mechanism to describe the effect of the CF airway environment on neutrophils and a novel explanation for why *S. aureus* survives in the airway despite the abundance of neutrophils. We are also the first to utilize a bacterial protein, ecotin, for therapeutic use against NE. The results of this thesis have helped further CF research by identifying a partial explanation for *S. aureus's* chronic survival in the lung and identifying new strategies for therapeutics to ultimately help CF patients live

a healthier life. Our future work will aid to identify the factor responsible for inhibiting neutrophil's ability to kill *S. aureus* which will further help identify new therapeutic strategies. Our future work will also dive into the clinical aspects and use for ecotin to prevent lung damage by NE. The mission of the CF foundation is to give CF patients the opportunity to lead long, fulfilling lives through funded research, and this thesis and all the contributing authors have added to CF research in hopes to fulfill this mission.

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