SHORT CHAIN FATTY ACIDS AFFECT NEUTROPHIL EFFECTOR FUNCTIONS AGAINST CYSTIC FIBROSIS RESPIRATORY PATHOGENS

by

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(Under the Direction of Balázs Rada)

ABSTRACT

Cystic fibrosis (CF) lung disease involves chronic bacterial infections, often of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with infiltration of dysfunctional PMNs. Short chain fatty acids (SCFAs) are present at millimolar concentrations in CF sputum as byproducts of anerobic bacterial fermentation. The effect of CF sputum and SCFAs on PMNs has not been well described. The goal of this project was to characterize the effects of CF sputum and SCFAs on the effector functions of PMNs in response to CF clinical isolates of *S. aureus* and P. *aeruginosa*. The results show that CF sputum, following previous observations of inhibiting bacterial killing, increases neutrophil extracellular trap (NET) release. Similarly, SCFAs reduce killing, increase NET release, and inhibit superoxide production. Overall, this is consistent with PMN dysfunction described in the CF lung, characterizes an *in vitro* model to study CF PMNs, and provides a potential explanation for PMN dysfunction occurring in CF sputum.

INDEX WORDS: Cystic fibrosis, PMN, Staphylococcus aureus, killing, sputum, short chain fatty acids, SCFA, respiratory burst, neutrophil extracellular traps

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CHAPTER 1

THE EFFECT OF CF SPUTUM ON PMN EFFECTOR FUNCTIONS

INTRODUCTION

Cystic Fibrosis and Impact

Cystic Fibrosis or CF is a hereditary autosomal recessive disorder and the most common life-limiting genetic disease among Caucasian patients [1]. Many advancements in the treatment of CF have changed this disease from a purely pediatric disease with a short prognosis to one that impacts patients well into adulthood with a median survival age of 46 years[2]. Genetic testing has aided in further understanding the disease and starting early treatment, which has gotten much more effective in recent years. Genetic testing relies on the fact that cystic fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene. The CFTR gene is responsible for coding an epithelial chloride ion channel, allowing for chloride ion flow and regulation [3]. Genetic defects in this chloride channel can be caused by over 2,000 various mutations, affecting disease severity and symptoms [4]. However, the most common mutation is the ΔF508 three base pair deletion. All these CF mutations are able to interfere with the function of the CFTR protein. This can occur through issues with premature transcription termination (Class I), missense mutations causing misfolding that prevents apical membrane integration (Class II), abnormal ATP gating (Class III), reduced chloride conductance from transmembrane mutations (Class IV), and decreased functional protein from splicing errors (Class V) [5]. These mutations lead to defective and dysfunctional CFTR protein that cannot properly

transport chloride and bicarbonate. This is believed to lead to abnormal hydration of airway surface liquid, causing a buildup of thick mucus that cannot be cleared by impaired mucociliary clearance mechanisms. Bicarbonate secretion impairment also leads to mucus issues and pH changes. These mucosal buildups can contribute to pancreatic insufficiency and respiratory problems [6]. Respiratory issues arise from this thick buildup in the airway which contains mucus, DNA, proteins, bacteria, etc. and contributes to respiratory infections and inflammation.

Innate Immune System and CF

The innate immune system is heavily involved in the pathology and host response in cystic fibrosis. A major immune cell that is active in the CF airway is the neutrophil. Neutrophils or polymorphonuclear granulocytes (PMNs) are the most numerous white blood cells in the human body and act as a first line of defense against many pathogens. As a primary line of defense, PMNs often rapidly migrate to areas of damage or infection in response to local cells releasing proinflammatory mediators. These mediators, such as Interleukin 8 (IL-8), allow for PMNs to leave the blood vessels and migrate towards the infected or damaged area. PMNs can then bind to the pathogen though pattern recognition receptors (PRRs), which recognize conserved Pathogen-Associated Molecular Patterns (PAMPs) on microbes and initiate pro-inflammatory cascades that release additional cytokines [7, 8].

Neutrophils employ a variety of effector functions to clear pathogens and contribute to the innate immune response. The most common effector mechanism following PMN binding to pathogens is phagocytosis. With the aid of opsonins on the microbial surface, such as antibodies or complement system components, PMNs are able to engulf the

microbe into a phagosome. Once phagocytosed, the pathogen is then further attacked by the now activated PMN. PMNs are capable of releasing reactive oxygen species or ROS into the environment and also directly into the phagosome to kill bacteria. ROS are partially reduced oxygen species that are used in signaling, but at high local concentrations can oxidize lipids and proteins to destroy pathogens. They consist of the superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl), however, mainly superoxide is produced by PMNs [9]. ROS are released in response to PRR activation, following phagocytosis, or in response to proinflammatory cytokines and mediators. The release of ROS is controlled by the NADPH (Nicotinamide adenine dinucleotide phosphate oxidase/Nox2) oxidase complex. The NADPH oxidase is present in the membranes (cell and phagosome membrane) and in certain granules. This protein complex works to accept electrons from the cytosol and transfer them to O_2 to produce superoxide O_2^-. This unstable superoxide can then kill bacteria through redox reactions on various bacterial targets. While activation of the NADPH oxidase complex leads to the production of superoxide, often following binding, the NADPH complex is not always activated following ligand binding. Priming occurs when the NADPH oxidase is not yet activated but is more susceptible to a stronger oxidative burst following a second signal such as after Toll-like Receptor (TLR), G protein-coupled receptor (GPCR), or Tumor Necrosis Factor receptor (TNFR) stimulation. While superoxide release can be an efficient way for PMNs to eliminate microbes, aberrant superoxide in the extracellular environment can cause significant damage and inflammation to host cells [10, 11].

Another PMN antimicrobial effector mechanism is the release of neutrophil extracellular traps or NETs. NETs are composed of cytosolic and granule proteins, and

histones that assemble on a DNA scaffold. The release of these NETs occurs via nuclear and granule membrane dissolution and subsequent rupture of the cell membrane, allowing for NETs to interact with the intracellular environment and possible pathogens. These NETs then trap and kill pathogens such as bacteria via high local concentrations of antimicrobials [12, 13]. NET release can involve active cell death following membrane permeabilization, but can also occur via a nonlytic mechanism that allows for the rapid release of nuclear or mitochondrial DNA without cell death [14]. During the active cell death mechanism or NETosis, PMNs decondense their chromatin, release it into the cytosol and then can stay in the local extracellular environment for many hours following membrane permeabilization. The process of NETosis is often reliant on ROS release. ROS produced by the NADPH oxidase stimulates myeloperoxidase (MPO), an enzyme that typically catalyzes hydrogen peroxide to halide oxidant reactions and activates Neutrophil Elastase (NE) to translocate from granules into the nucleus. From there, NE can cleave histones for subsequent chromatin decondensation through interactions with MPO. NETs can be measured by the presence of the extracellular DNA (eDNA) that ordinarily does not exist but is key in NET formation [15].

In CF, PMNs have been shown to have increased recruitment to the lungs and dysregulated functions. This increased recruitment to the CF lung is due to the massive cytokine production emanating from the airway. The proinflammatory cytokine IL-8 is one of these overproduced cytokines in CF and mediates PMN infiltration. This excessive IL-8 production is potentially contributed via bacterial stimulation of PAMPs on airway epithelial cells and intrinsic defects in airway epithelium due to the CFTR mutation, further compounded by PMN IL-8 release following recruitment to the lung and exposure to

PAMPs [16]. Additionally, IL-1β is produced by macrophages and acts to also recruit PMNs to the lung [17]. Increased PMN infiltration then leads to a host of issues associated with inflammation and neutrophil effector dysfunction. This is represented by IL-8 and MPO levels in the sputum being used as biomarkers for various stages of CF lung disease [18]. Specifically, IL-8, neutrophil elastase, IL-1b, and PMN counts in the lungs are negatively correlated with FEV_1% (percentage of predicted forced expiratory volume in 1 second), a measure of airway obstruction in lung diseases including CF, suggesting that PMN infiltration and their subsequent activation is detrimental to the CF lung and disease progression [19, 20].

Once recruited to the CF lung, PMNs are inadequate in clearing infections and controlling inflammation. This is first reflected in an impaired ability of CF airway PMNs to phagocytose pathogens. It is still debated whether this defect is due to the airway environment in the CF lung or to an intrinsic defect due to CFTR mutations in the PMNs. CFTR has been shown to be expressed at low levels in PMNs, specifically on membrane-bound intracellular structures. CFTR is obviously absent in CF PMNs. CF PMNs have been demonstrated to have defective chlorination of phagocytosed bacterial proteins, however, the role of intrinsic phagocytic deficiency remains unsettled [21]. Airway PMNs have been confirmed to have decreased phagocytic ability in CF as compared to healthy airway PMNs [22]. However, compared to CF blood PMNs, PMNs isolated from the CF sputum have a decreased ability to phagocytose, suggesting that this is not an intrinsic defect in phagocytosis but a byproduct of the inflammatory CF airway environment [23, 24]. In addition to phagocytosis, CF PMNs have been shown to have dysfunctional reactive oxygen species production. While CF PMNs have been shown to not have defects in intrinsic reactive

oxygen species production, CF airway PMNs do display a reduced respiratory burst. This reduced ROS production has been further associated with reduced lung function [25, 26]. Additionally, NET release by PMNs has also been shown to be increased in the CF airway and sputum [27, 28]. This has been confirmed by reporting increased neutrophil elastase levels in the CF airway and extracellular DNA being associated with decreased lung function and increased airway obstruction [29-31]. The decreased phagocytosis, reduced respiratory burst, and increased NETosis appear to contribute to the reduced killing of pathogens in the CF lung and establishment of chronic infections.

Overall, these effects appear to be specific to CF airway PMNs and not a product simply of CFTR deficiency in PMNs, as CF blood PMNs have been shown to have superoxide and NET formation similar to that of healthy blood PMNs [26, 32]. This suggests that these changes in PMN function in the lung are, at least in part, due to the unique, inflammatory, and dysfunctional CF airway environment [31].

Aerobic Bacteria in CF

The defective CF airway environment consisting of mucosal buildup and dysfunctional immune cells provides a habitat conducive to bacterial infections. Aerobic bacteria have historically caused significant morbidity and mortality in CF patients. While the respiratory microbiome of each CF patient differs significantly, common pathogens include *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus mitis, Haemophilus influenzae,* and *Burkholderia cepacia*. Overall, bacterial infections in CF can have a detrimental effect on the lung function, as measured by FEV_1% and risk of disease progression [33, 34].

Of these bacteria, *Pseudomonas aeruginosa* has historically been the most prevalent pathogen in adult CF patients and often causes chronic infections. Its prevalence has been declining from about 60% in the 1990s to just over 40% in 2019, yet many adult patients are still chronically infected, with 16.9% of *P. aeruginosa*-infected patients colonized with a multi-drug resistant strain [2]. *P. aeruginosa* is an opportunistic gram-negative pathogen that first colonizes CF lungs with a non-mucoid phenotype. Chronic infection is often established and the pathogen progresses to a mucoid form that produces biofilms to evade the host immune response. *P. aeruginosa* is typically cleared by functional epithelial and immune cells in the healthy lung, however, epithelial defects in CF allow for the bacteria to flourish. PMNs, already in high numbers in the CF lung, are able to interact with *P. aeruginosa* via their many defense mechanisms. However, PMNs are unable to clear *P. aeruginosa* due to defective phagocytic capacity. PMNs also attempt to kill *P. aeruginosa* through the release of ROS and NETs, but these mechanisms also fail to clear the infection and can often result in further damage to the lungs and promotion of a proinflammatory cascade. In fact, *P. aeruginosa* can actually utilize pyocyanin, a virulence factor, to increase NET production by PMNs and possibly allow for better survival in a damaged airway [35]. This increased NETosis can also be used by *P. aeruginosa* as a source of ecDNA to aggregate in biofilms and subsequently survive in the CF lung [36].

Another bacterial pathogen that often causes infections of CF patients is *Staphylococcus aureus*. This gram-positive aerobic bacterium is now the most common cause of CF airway infections, with about 70% of CF patients infected. While most *S. aureus* are methicillin sensitive (MSSA), methicillin resistant *S. aureus* (MRSA) has been an ever-increasing pathogen in the general and CF population, with about 25% of CF

patients now infected [2]. Overall, *S. aureus* is often the first pathogen cultured from the lungs of CF children, with some variants associated with a greater decline in lung function than *P. aeruginosa* [37-39]. In addition to being the most prevalent CF bacterial pathogen in children in the United States and worldwide, its incidence and prevalence is also increasing [40]. *S. aureus* has been shown to colonize the CF lung for years and has several mechanisms to survive in the lung and evade the defense of immune cells and PMNs, in particular. *S. aureus* possesses superoxide-dismutases (SODs), SodA and SodM, which function to eliminate superoxide radicals and protect the pathogen from oxidative damage that superoxide released by PMNs can inflict. The expression of these enzymes is additionally upregulated in isolates isolated from CF sputum, likely contributing to their virulence *in vivo* [41]. *S. aureus* also contains nuclease activity that can function to degrade NETs released by PMNs attempting to kill the pathogen in the CF lung. This nuclease activity has been shown to increase in later isolates from the CF sputum and contribute to evasion of PMNs in chronic infection [42]. Similar to *P. aeruginosa*, *S. aureus* can also form biofilms to hide their PAMPs and prevent the immune system from killing the bacteria. These biofilms can contain secreted leukocidins that function to increase NET production by PMNs and function to prevent bacterial clearance [43].

Anaerobic Bacteria in CF

While, historically, aerobic bacteria have been considered the main pathogenic colonizers of the CF lung, there is increasing data to support a much more complex lung environment. The buildup of mucus in the CF airway, stemming from inefficient mucus clearance and the dehydrated airway surface liquid, can contribute to the formation of a mucus plug. These plugs can create areas of the lung that are hypoxic and perfect

environments for anaerobic bacterial growth [44]. These anaerobic bacteria have been detected in the CF sputum and bronchoalveolar lavage (BAL) in numbers similar to other major CF pathogens, such as *P. aeruginosa*, with individual anaerobes even frequently being the dominant taxon in sputum samples. Anaerobes have also been shown to increase in frequency during pulmonary exacerbations with some resistant to antibiotic treatments [45-47]. These anaerobes also interact with the aerobes of the CF lung and are known to be able to increase *P. aeruginosa* growth. Anaerobe *Porphyromonas gingivalis* can also experience increased virulence in the presence of *P. aeruginosa* [48, 49]. Some of the anaerobic bacteria isolated from CF sputum include those of the *Prevotella, Actinomyces, Veillonella*, *Propionibacterium*, and *Streptococcus* genera [50, 51]. These anaerobic communities have been suggested to play a potentially detrimental role in CF lung disease. One study [52], using a Winogradsky column adapted to study CF lung disease, showed that prior to antibiotic treatment during a lung exacerbation, obligate anaerobes were abundant but rapidly decreased in number and were replaced by *Pseudomonas* dominance in the system following antibiotic administration. Anaerobe numbers recovered preceding a second exacerbation and once again displayed a similar trend to antibiotic treatment, suggesting some role for the fermentative anaerobes in CF lung exacerbations.

The CF lung environment or sputum consists of a complex, heterogeneous mixture of mucus, bacteria, bacterial byproducts, host cells, and their secretions. While mucus secretion is increased in CF, mucins in the sputum are often not intact and possibly proteolytically degraded [53]. Host cell inflammation in response to bacteria is common in the CF lung and is represented in sputum through the presence of cytokines downstream of NF-kB signaling such as tumor necrosis factor (TNF)-α, IL-1, IL-6, and IL-8. These can

be released from airway epithelial cells along with granulocyte macrophage colony–stimulating factor (GM-CSF), and granulocyte colony–stimulating factor (G-CSF) to recruit immune cells [54]. Recruited immune cells can include macrophages, dendritic cells, mast cells, T cells and B cells, as well as PMNs. Sputum composition differences have also been linked to differential lung function, with higher neutrophil elastase and cathepsin G levels in the sputum associated with reduced lung function [55].

The unique phenotype of PMNs in the CF airway has previously led to the development of a model to study these PMNs in our laboratory. This model involves the exposure of blood PMNs to the CF sputum supernatant for 3.5 hours to simulate the CF airway. The modulations these PMNs undergo can then be studied and eventually the components of the CF sputum leading to the maladaptive phenotypic changes can be elucidated. Specifically, the effector function of the PMNs under these airway-like conditions has not been studied. My project sought to determine the effect of CF sputum supernatants on the generation of neutrophil extracellular traps and reactive oxygen species from PMNs in response to the common CF clinical isolates of *S. aureus* and *P. aeruginosa*. I hypothesized that the CF Sputum Model would alter PMN effector functions that are seen in the CF lung, including increased NETosis and decreased superoxide release.

METHODS AND MATERIALS

Human subjects

The Institutional Review Board of the University of Georgia approved the human subject study to collect peripheral blood from anonymous volunteers (UGA# 2012-10769-06). Enrolled healthy volunteers were non-pregnant and heavier than 110 pounds without

any infectious disease complication. All adult subjects provided informed consent, and no child participants were enrolled into the study. The studies were performed following the guidelines of the World Medical Association's Declaration of Helsinki.

CF bacterial isolates of *S. aureus* and *P. aeruginosa* and sputum

CF subjects were patients followed at the Adult CF Clinic at Emory University who had signed informed consent to provide sputum or throat swab samples for bacterial isolation and clinical data for the CF Biospecimen Repository in accordance with the Emory University IRB (Emory #00042577). CF diagnosis was tested by pilocarpine iontophoresis sweat testing and/or CFTR gene mutation analysis showing the presence of two disease causing mutations. CF participants undergoing blood draw or sputum collection 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.

Bacterial cultures

Eight clinical *S. aureus* (four MRSA and four MSSA isolates) isolates were obtained from the Emory Cystic Fibrosis Biospecimen Repository (CFBR). First, the bacterial isolates for the assay were prepped and allowed to grow overnight. Surroundings and materials were cleaned with ethanol. *S. aureus* were transferred from frozen aliquots to 3mL of LB Broth medium with an inoculation tube. The clinical isolates of *Staphylococcus aureus* grown were SA-17, SA-19, SA-22, SA-24, SA-25, SA-47, SA-70, SA-74. USA300 and JE-2 were utilized as laboratory control isolates. These isolates were then left to grow at 37°C overnight.

Eight clinical *P. aeruginosa* isolates were also obtained from collaborators at the Massachusetts General Hospital. *P. aeruginosa* was spread on Pseudomonas agar petri dishes (100mL per isolate) and spread with a sterile L-shaped spreader and allowed to grow overnight. Prior to the experiment, the *P. aeruginosa* cultures were transferred to 3mL of LB Broth medium with an inoculation tube and allowed to grow until bacterial isolation (approximately 4 hours). This ensures that *P. aeruginosa* would be in the growth phase and retain its flagellar motility, as this can affect NETosis [56]. The clinical isolates of *P. aeruginosa* grown were 10007, 10014, 10035, 10040, 10063, 10064, 10155, 10091, 10270, and 10094. PA01 was used as a laboratory isolate control.

Bacteria, in their exponential growth phase were pelleted and diluted with HBSS and optical density was measured in a 96 microwell plate. All bacteria were diluted until they reached an optical density of 0.6 adjusted for background which corresponds to 10^9 bacteria/mL. The bacterial samples were no more than ±0.05 from OD0.6.

Following experiments, Colony Forming Unit (CFU) assays were performed to confirm that a 10^9 bacteria/mL concentration was reached, and that no contamination had occurred. 30µL of isolated bacteria was serially diluted to a concentration of $1.0 * 10^{-5}$ to $1.0 * 10^{-7}$, plated on petri dishes and placed in 37°C for 24 hours. Bacterial concentrations were then counted and concentrations between $0.8 * 10^{-9}$ and $1.2 * 10^{-9}$ were considered accepted as 10 MOI (multiplicity of infection).

Human neutrophil isolation

Control human subjects were recruited at the University of Georgia by the Clinical Translational and Research Unit to donate blood for PMN isolation and serum preparation. CF patient blood and serum were collected at Emory University, along with age/gender

matched healthy donors. 20-30 milliliters of blood were drawn into EDTA-coated tubes for PMN isolation. PMNs were isolated using the EasySep™ Direct Human PMN Isolation Kit (Catalog #19666, Stem Cell technologies, Vancouver, BC, CA) based on the manufacturer's protocol. This protocol routinely yields 30-130 x 106 live PMNs with >99.9% red-blood cell depletion, >98% purity, and >99% viability.

Serum isolation

To collect serum, ten milliliters of human peripheral blood were drawn into a silicone-coated tube without anticoagulant and allowed to clot at room temperature for 30 min. Cellular components settled to the bottom while the pinkish supernatant containing some remaining cells was aspirated and then centrifuged twice (10,000 g, 5 min). Cell-free supernatants after centrifugation were defined as serum and removed and aliquoted, while the coagulant was discarded. The serum was kept on ice for same-day experiments and utilized in the preparation of assay medium and for opsonization of the bacterial isolates or frozen at -80 °C for future use.

Sputum Collection

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. Samples were kept on ice at 4°C throughout the isolation process. After collection, sputa were weighed, their volume measured, and 1-2 mLs of ice-cold PBS-EDTA (1xPBS - 5 mM EDTA) were supplemented to every 1.0 gram of sputum samples. Sputum samples were repeatedly and slowly passaged through a sterile 18-gauge needle to homogenize the sputum. Cells were

pelleted via low-speed centrifugation (4°C, 400 g, 10 min). The sputum supernatant was transferred into microcentrifuge tubes and centrifuged (3,000 g, 10 min, 4°C). The clear sputum supernatants were stored in aliquots at -80C until analysis.

Sputum incubation

Following isolation from peripheral blood, PMNs were incubated in a pooled cocktail of CF sputum supernatant that will be referred to as the *in vitro* "sputum model". Sputum supernatant isolated from CF patient donors was pooled into a cocktail consisting of three individual sputa. Healthy blood PMNs (10^7/ml) were pretreated with 30% sputum cocktail for 3.5 h at 37ºC 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 1x HBSS, 10 mM HEPES, 5 mM glucose, and 1% autologous serum to remove extracellular sputum components and to prevent their direct interaction with subsequently added bacteria. Control PMNs not exposed to sputum were also incubated at 37°C in equivalent volume of assay media and processed identically.

Measurement of NET formation

The NET release assay was established previously in the Rada laboratory and was performed according to the original protocol with minor modifications of adding *S. aureus* as stimuli [57]. The assay involves staining with Sytox Orange (Life Technologies, Grand Island, NY, USA) membrane impermeable DNA-binding dye. 250,000 human PMNs were plated per cell and allowed to incubate at 37°C for 30 minutes in a transparent bottom black 96 well plate to allow adherence. Bacteria were diluted with assay media and 0.2% Sytox Orange stain to obtain the desired multipicity of infection (MOI). PMA was used as a positive control to stimulate maximal NET release and unstimulated PMNs were used as

the negative control. Triplicates were used for every condition. Once PMNs and stimulants were mixed, the 96-well plate was placed in a fluorescence microplate reader (Varioskan Flash, ThermoScientific, Waltham, MA, USA) and fluorescence measurements (excitation: 530 nm, emission: 590 nm) were taken for 8 hours every 5 minutes. Relative fluorescence unit (RFU) results were normalized on the signal obtained in PMA-stimulated PMNs and expressed as a percentage.

Measurement of superoxide production

Superoxide production of the cells was tested with the superoxide dismutase-inhibitable cytochrome-c reduction [58]. To measure superoxide production in the extracellular medium, cells (10^6 /ml) were suspended in assay media containing 50 mM cytochrome-c (Sigma, cat#C3131). Aliquots (50uL) of the suspension were added into wells of a 96-well plate and prewarmed at 37∞C for 5 min in a shaking ELISA-reader (Varioskan Flash, ThermFisher). The cells were activated with the requisite stimuli by the addition of 50uL of the stimulus solution containing 10MOI of either *S. aureus* clinical isolates or Zymosan A from Saccharomyces cerevisiae e (Sigma, cat#Z4250) opsonized in 10% autologous serum of the PMN donor. 100nM of PMA was used as a positive control, unstimulated cells and 12.5 mg/ml superoxide-dismutase (SOD) (Sigma, cat#S5395) were used as a negative control. The changes in absorption at 550 nm were recorded for 60 min with two measurements/min at 37ºC with gentle shaking. After subtracting the background values, 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 $\frac{\text{nmol } O_2^{\cdot}}{10^6\ PMNs}$ /hr.

Superoxide production was also measured using the Diogenes-based chemiluminescence kit (National Diagnostics, Atlanta, GA). 250,000 PMNs were allowed to adhere to 96-well solid white plates for 1 hour at 37°C in assay media (previously described). Cells were stimulated by bacterial isolates (10 MOI), PMA (100 nM) or left unstimulated. Chemiluminescence was measured by a Varioskan Flash microplate luminometer (Thermo Scientific, Waltham, MO, USA) for 90 min. 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 (30 min) duration of the measurement. Data was also normalized to the PMA-stimulated control and expressed as a percentage.

Bacterial Killing Assay

For measurement of bacterial killing by PMNs, opsonized *S. aureus* were combined with PMNs at a ratio of 10:1 multiplicity of infection (MOI) in microcentrifuge tubes. These tubes were incubated and mixed regularly for 30 minutes at 37°C to ensure appropriate mixing of bacteria, PMNs, and SCFAs. After 30 min, PMNs were lysed with 1 mg/ml saponin in 1xHBSS (on ice for 5 min). Samples were then diluted in 1xHBSS from 100-fold to 10,000-fold in a separate 96 well plate. The diluted *S. aureus* were then plated on LB blood agar (TSA II 5% SB) at 37°C for 24 hours. *S. aureus* was then counted, and percent killing was calculated by comparing the numbers of surviving bacteria to those at t = 0 with the equation: (1- CFU30min/CFU0min) × 100. Uninfected conditions with SCFAs and PMNs were also plated to ensure contamination did not occur.

RESULTS

The effect of bacterial stimulation on the effector functions on PMNs was tested using the previously optimized *in vitro* CF sputum model. This model is designed to simulate the CF airway environment so that changes that occur in the CF airway can be studied. In this model, human PMNs are isolated from healthy blood and then exposed to pooled supernatants of 30% CF sputum for 3.5 hours at 37°C. This pooled sputum consists of equivalent portions from three individual CF sputum donors. This model has previously been shown to not impair PMN viability while reducing killing of bacteria, as is also described in the CF lung (unpublished results).

First, the effect of the CF sputum was tested on the effector functions of PMNs in response to 10 clinical isolates of *P. aeruginosa*, a pathogen previously established in the laboratory. These clinical isolates were isolated from CF patients recruited at a collaborator's site at the Massachussetts General Hospital and the PA01 lab isolate was used as a control. Clinical isolates consisted of paired donor isolates from early and late time points (Table 1). Most of these isolates were non-mucoid, however, two did have mucoid phenotypes [59]. Measurement of NET release was performed using the Sytox Orange Nucleic Acid Stain and measured as a percent of the average PMA fluorescence, a maximal stimulator of NETs. The clinical isolates were able to induce NETosis, albeit at variable levels between isolates (Figure 1A). Sputum treatment did not affect the release of NETs (Figure 1B). Superoxide production by the PMNs was also measured via the Diogenes chemiluminescence assay. Similarly, CF clinical *P. aeruginosa* isolates were shown to induce the production of variable levels of superoxide (Figure 1C). This superoxide production was not affected by the sputum treatment (Figure 1C).

With PMN effector function being mostly unaffected by sputum treatment in response to *P. aeruginosa,* the effect of the most common CF pathogen, *S. aureus*, was investigated. Eight clinical isolates of *S. aureus* were tested from the Emory CFBR, including 4 MRSA and 4 MSSA isolates (Table 2). These isolates have undergone previous whole-genome sequencing [60]. USA300 was used as a laboratory control strain. Overall, *S. aureus* isolates induced NET release and sputum significantly increased NET release stimulated by SA24, SA22, and SA19 clinical isolates (Figure 2A-B). When examining the aggregation of all clinical isolates, sputum induced significantly more NETosis compared to untreated PMNs (Figure 2C). This increased NET release was consistent among MSSA and MRSA isolates (Figure 2D).

To determine the effect of CF sputum on superoxide production in response to *S. aureus*, both a Diogenes chemiluminescence assay and a superoxide dismutase-inhibitable cytochrome c reduction assay were performed. PMA was used as a positive control and superoxide dismutase (SOD) as a negative control (Figure 3C). Superoxide production in response to *S. aureus* was not affected by sputum treatment (Figure 3B). This data was confirmed with the more sensitive Diogenes chemiluminescence assay (Figure 3A). However, superoxide production in response to opsonized zymosan, an inert fungal particle, was slightly but significantly increased following a sputum incubation (Figure 3D).

DISCUSSION

In this chapter, a previously established CF sputum supernatant model that reduces bacterial killing was tested for its effects on PMN effector functions, specifically NET

release and the production of ROS. This was examined in the context of the CF airway, with clinical isolates of *P. aeruginosa* and *S. aureus* isolated from the CF lung used to simulate the CF airway. It was hypothesized that the effects seen under this CF sputum model would be similar to those seen in airway PMNs in CF.

PMNs are key immune cells in the response to bacterial infections and are present in the CF lung in elevated numbers correlating with declined lung function [19, 20]. Bacterial infections are common in CF and can contribute to the worsening of CF lung function. While the neutrophil response in CF has been shown to be increased, it is still uncertain if the PMNs are simply unable to clear infection, actively contribute to a more damaged airway, or both [19, 20, 30]. However, it is clear that defects in PMN function are occurring in the CF lung and that these defects prevent proper bacterial killing and eventual clearance, resulting in chronic infection. Similarly, while increased NET release has been noted in the CF lung, these NETs are not able to clear the bacterial infections and may even contribute to lung damage. Modulations on PMN superoxide production in the CF lung are less certain. While some have demonstrated a decreased superoxide production capacity for CF airway PMNs, others have shown no change in superoxide production [25, 26]. In order to test whether sputum can modulate healthy PMNs to exhibit similar effector functions as seen in the CF lung PMNs, the CF sputum supernatant model was utilized.

It was first demonstrated that *P. aeruginosa* clinical isolates were able to induce NET release from healthy PMNs and that sputum treatment did not affect NET release. Similarly, clinical isolates were also shown to induce superoxide production by healthy PMNs and sputum did not significantly change the superoxide production seen. This confirms that the clinical *P. aeruginosa* isolates we utilize are relevant to study PMN

effector functions. More importantly, this suggests that CF sputum and its components are not key in the effector changes that occur during *P. aeruginosa* infection.

With *P. aeruginosa* not affected by sputum treatment, *S. aureus*, the most common CF pathogen, was subsequently evaluated for changes due to sputum treatment. Previous data in the lab showed significantly decreased killing of *S. aureus* following sputum treatment. Additionally, sputum treatment has been shown to have no effect on the viability of PMNs. Consistent with what is observed in the CF lung, sputum treated PMNs had increased NET release that was consistent among MRSA and MSSA isolates. *S. aureus* is able to increase NET release from PMNs via production of leukocidins, but also has DNAse activity that can degrade NETs and protect the bacteria from this method of killing [43]. The ability for *S. aureus* to degrade NETs has also been linked to clinical isolates present in the CF lung for a longer period of time, making the use of clinical *S. aureus* isolates in the experiments more relevant to the CF airway [42]. Interestingly, DNAse is a common and somewhat effective treatment in CF to degrade NETs, and the ability for *S. aureus* to increase NET release while also evading these NETs has been linked to NET-mediated airway damage [61]; this could be occurring in the sputum treatment condition, with the increased NETosis and decreased killing.

Superoxide production by sputum exposed PMNs were shown to not be significantly different from non-sputum treated PMNs. This is also somewhat consistent with the reported findings in the CF airway. While some studies report reduced respiratory burst in CF sputum PMNs, there are no reports to our knowledge of increased superoxide production [25, 26]. Opsonized zymosan particles exposed to sputum treated PMNs led to a slightly increased superoxide production, confirming that the NADPH oxidase assembly

or activation was not impaired by the sputum. If superoxide production is indeed reduced in the CF airway, it is possible that the diluted sputum supernatant treatment is not sufficient to elicit this response *in vitro*. Additionally, if a specific component of the CF sputum is responsible for reduced superoxide production, perhaps it would cause this change in higher concentrations and with more direct treatment.

The changes seen with sputum treatment suggest that a component or multiple components in the CF sputum might be leading to the changes observed in the CF lung. It is already documented that the dysfunction of CF PMNs occurs following infiltration into the lung and is not due to intrinsic defects in their function while in the blood [26, 32]. To our knowledge, this study is the first confirmation that this inflammatory CF sputum has the potential to cause this phenotype *in vitro*. Additionally, the use of healthy human PMNs further illuminates the inflammatory nature of the sputum on causing dysfunction in non-autologous and otherwise healthy PMNs. In the CF lung, this sputum could be mediating the host-pathogen interactions that lead to chronic infections and further inflammatory processes, resulting in lung disease.

While *P. aeruginosa* exposed cells were unaffected by sputum treatment, *S. aureus* did contribute to significant changes in PMN effector functions. It remains unclear why CF sputum supernatants were able to cause changes in the presence of the later bacteria but not with the former. Future studies could evaluate these differences between bacteria to determine the applicability of this model to pathogens beyond *S. aureus*. Additionally, differences in NETosis and superoxide production were observed between clinical isolates, and future experiments could evaluate a genetic basis for these variations. While this chapter evaluated effector functions, a mechanism of action of the sputum remains unclear,

especially with the complexity of components present in the sputum. I*n vitro,* these PMNs are isolated, yet in the CF lung, the effects of sputum are widespread and could significantly alter other immune cells that also contribute to the establishment of chronic bacterial infections.

Overall, the effector function changes seen in the *S. aureus* sputum model are consistent with the CF lung environment: increased NETs, decreased killing, and unchanged superoxide production. This helps establish this CF sputum model as a viable *in vitro* model to study the CF airway PMNs and to evaluate what components of the CF sputum could be leading to the changes seen in this model and the lung.

Table 1. Clinical CF *P. aeruginosa* isolates and strains used in this project.

Strain	Patient	Culture Type	Age at Culture (year)	Early/Late Isolate	Early-Late Interval (year)	Mucoidy	Adundance (CFU/gm)
10007	103	Sputum	14.8	Early		Non-mucoid	7.20E+07
10014	103	Sputum	18.7	Late	3.9	Non-mucoid	4.00E+05
10035	110	Sputum	1.1	Early		Non-mucoid	2.00E+04
10040	110	Sputum	13.2	Late	12.1	Non-mucoid	2.00E+08
10063	109	Sputum	6	Early		Mucoid	1.00E+09
10064	109	Sputum	13.1	Late	7.1	Non-mucoid	1.50E+08
10155	108	Sputum	15.1	Early		Non-mucoid	2.80E+02
10091	108	Throat	25.4	Late	10.3	Non-mucoid	
10270	111	Sputum	9.1	Early		Non-mucoid	1.20E+08
10094	111	Throat	19.6	Late	10.5	Mucoid	

Table 2. Clinical CF *S. aureus* isolates and strains used in this project.

Name	Referred to	Type
CFBR 24Sa	SA24	Methicillin-resistant S. aureus strains isolated from Cystic Fibrosis patients MRSA
CFBR 25Sa	SA25	
CFBR 47Sa	SA47	
CFBR 74Sa	SA74	
CFBR 70Sa	SA70	Methicillin-sensitive S. aureus strains isolated from Cystic Fibrosis patients MRSA
CFBR_EB_17Sa	SA17	
CFBR_EB_19Sa	SA19	
CFBR_EB_22Sa	SA22	
JE2	JE2	Common Laboratory Strains (Not clinical isolates)
USA300	USA300	

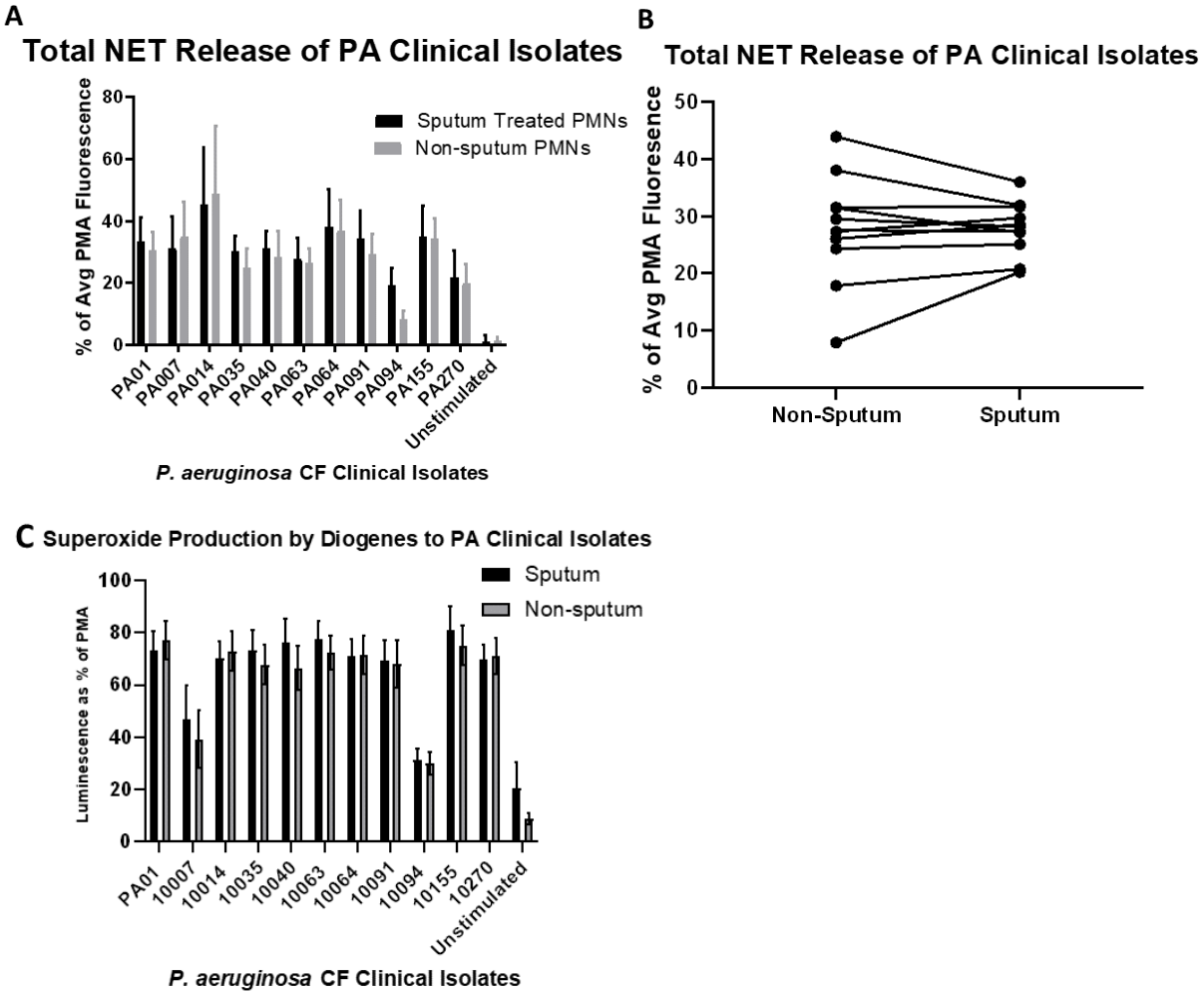

Figure 1. Neutrophil extracellular trap (NET) release in response to clinical CF *P. aeruginosa* isolates following CF sputum treatment.

Healthy human blood PMNs were first treated with 30% (v/v) CF sputum supernatant cocktail and then exposed to the indicated clinical isolates of *P. aeruginosa* (10 MOI). Extracellular DNA (ecDNA) release was measured for 8 hours in the presence of Sytox Orange, a membrane impermeable, DNA-sensitive fluorescent dye and superoxide production was measured with the Diogenes-based chemiluminescence for 1 hour. (A) EcDNA release in *P. aeruginosa*-stimulated PMNs after 8 hours measured as increase in fluorescence. The *P. aeruginosa* induced ecDNA signal difference from 80min to 400min was normalized on the average signal obtained by PMA stimulation (100nM). Mean±S.E.M., n=6. (B) The aggregate effect of CF sputum treatment on ecDNA release in PMNs exposed to clinical isolates of *P. aeruginosa*. n=10 clinical isolates. (C) Superoxide production in *P. aeruginosa*-stimulated PMNs after 1 hour measured as increase in chemiluminescence and normalized to the PMA control (performed by Rachel Fricker). Mean±S.E.M., n=7. Two-tailed, paired Students' t-test.

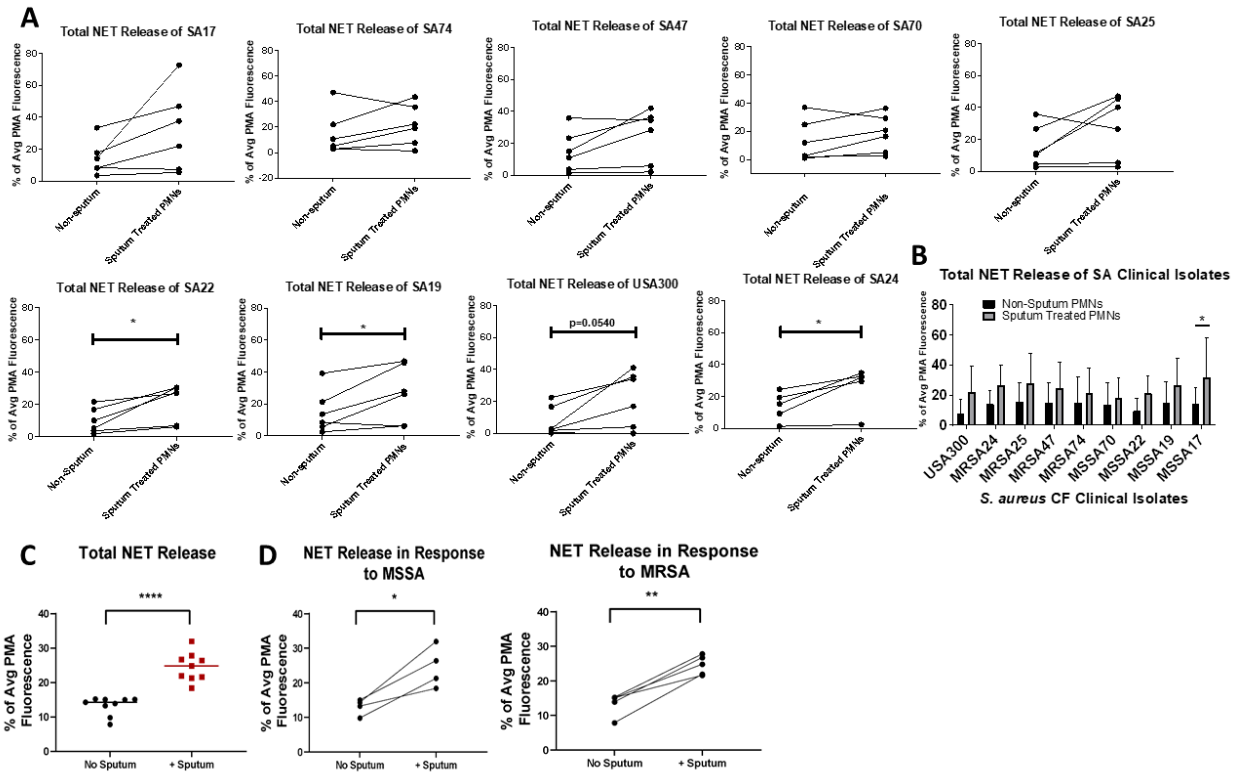

Figure 2. Neutrophil extracellular trap (NET) release increased in response to clinical CF *S. aureus* isolates following CF sputum treatment.

Healthy human blood PMNs were first treated with 30% (v/v) CF sputum supernatant cocktail and exposed to the indicated clinical isolates of *S. aureus* (10 MOI). Extracellular DNA (ecDNA) release was measured for 8 hours in the presence of Sytox Orange, a membrane impermeable, DNA-sensitive fluorescent dye. (A) EcDNA release in *S. aureus* clinical isolate stimulated PMNs after 8 hours measured as increase in fluorescence. The *S. aureus* induced ecDNA signal difference from 80min to 400min was normalized on the average signal obtained by PMA stimulation (100nM). n=5-7. (B) The effect of CF sputum treatment on ecDNA release in PMNs exposed to clinical isolates of *S. aureus*. Mean±S.E.M, n=5-7. (C) The aggregate effect of CF sputum treatment on ecDNA release in PMNs exposed to clinical isolates of *S. aureus*, (D) clinical MSSA isolates, and (E) MRSA clinical isolates. n=10 isolates, 4 MRSA, 4 MSSA. Two-tailed, paired Students' t-test. 2 way-ANOVA, Sidak's multiple comparisons test. *, $p<0.05$; **, $p<0.01$; ***,$p<0.001$; ****, $p<0.0001$. Ns, not significant.

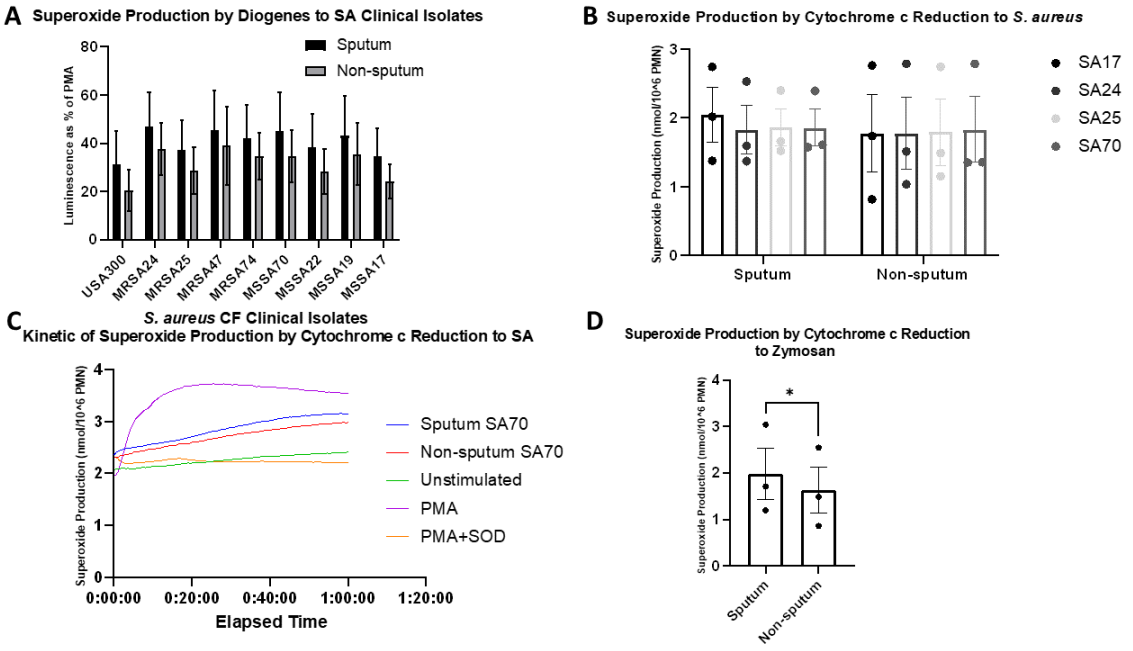

Figure 3. PMN superoxide release in response to clinical CF *S. aureus* isolates following CF sputum treatment.

Healthy human PMNs were first treated with 30% (v/v) CF sputum supernatant cocktail and then exposed to the indicated opsonized clinical isolates of *S. aureus* (10 MOI). Superoxide release was measured by Diogenes-based chemiluminescence and cytochrome-c reduction assay. (A) Superoxide production in *S. aureus*-stimulated PMNs after 1 hour measured as increase in chemiluminescence and normalized to the PMA control (performed by Rachel Fricker). Mean±S.E.M., n=7. (B) Superoxide production in *S. aureus*-stimulated PMNs after 1 hour was measured as $\frac{\text{nmol } O_2^{\cdot}}{10^6\ PMNs}$ using the cytochrome c reduction assay. Mean±S.E.M, n=3. (C) Representative kinetics of *S. aureus*-stimulated PMN superoxide release curves (60 min) (n=3). 100 nM PMA was used as a positive control and 12.5 mg/ml superoxide-dismutase (SOD) was used as a negative control. (D) Comparison of superoxide production by sputum-treated PMNs versus untreated following exposure to zymosan (10 MOI, opsonized). n=3. Two tailed, paired Students' t-test. *, $p<0.05$. Ns, not significant.

CHAPTER 2

THE EFFECT OF SHORT CHAIN FATTY ACIDS ON PMN EFFECTOR FUNCTIONS

INTRODUCTION

CF Sputum

The "CF sputum model" was established as a modulator of PMN effector function, causing increased NETosis, and inhibited killing (unpublished results) in response to clinical *S. aureus* isolates. These effector functions are similar to what is seen in the CF lung, mainly increased NETosis with an inability to clear chronic infections. However, the component of CF sputum supernatant that is causing this change is unknown. While the previous studies focused on the role of PMNs in response to aerobic bacteria, anaerobes are also present in large numbers in the CF lung and could be contributing to the effect seen.

The anaerobes in the CF lung can contribute to the environment most prominently through production of copious byproducts as part of their fermentation. Anaerobes rely on the fermentation of carbohydrates, mucins, and amino acids to produce ATP and produce short chain fatty acids (SCFAs) as a byproduct of this process [47].

Short Chain Fatty Acids

Short chain fatty acids are produced by anaerobic bacteria as byproducts of anaerobic fermentation. They are small carboxylic acids ranging from 1 to 6 carbons in their hydrocarbon chain. They are abundantly produced in mucosal locations with anaerobic bacterial growth, including in the gastrointestinal tract, oral cavity, and in the CF

airway, where anaerobes have been shown to thrive [62]. The most common of these SCFAs are acetate (2 carbon backbone), propionate (3 carbon backbone), and butyrate (4 carbon backbone) and they have been implicated in several inflammatory diseases.

In general, SCFAs have been shown to activate intestinal epithelial cells, leading to the production of chemokines and cytokines that could potentially contribute to inflammation [63]. In the oral cavity, SCFAs are able to stimulate leukocyte cytokine release, while inhibiting gingival epithelial and endothelial proliferation [64]. An anti-inflammatory role for SCFAs has also been described. Acetate is known to ameliorate sepsis-induced acute kidney injury via histone deacetylase inhibition to decrease ROS signaling in T cells [65].

These SCFAs, acetate, propionate, and butyrate, are all seen in the CF lung and CF sputum in high concentrations. SCFAs are produced in millimolar concentrations (up to 15 mM) by the anaerobes present in the CF lung and are also found in the CF sputum in millimolar concentrations. Clinically stable CF patients have an average concentration of these SCFAs of about 2 mM in the sputum, with antibiotic treatment reducing these levels in patients. PMN numbers are correlated with total SCFA concentrations in the sputum. IL-8 has also been shown to correlate with acetate levels in the BAL. SCFAs are also known to affect cells of the CF airway. SCFAs can increase secretion of GM-CSF, IL-1α, and IL-6 in A549 alveolar basal epithelial cells. CF bronchial epithelial cells treated with propionate secrete increased levels of IL-6 and acetate treatment increases IL-8, which is important in granulocyte recruitment [51, 66].

In addition to effects on airway epithelial cells, SCFAs are able to also act directly on PMNs in a variety of inflammatory diseases, including CF. PMNs contain two G

protein-coupled receptors, GPR41 and GPR43, that can sense SCFAs. Activation of these receptors by SCFAs leads to IP_3 formation, intracellular calcium release, ERK1/2 activation and cAMP inhibition. Activation of GPR43 is shown to cause PMN chemotaxis and could explain the correlation between SCFA concentration and PMN numbers in the CF sputum [62, 66-68].

SCFAs have also been described to affect effector functions of PMNs such as reactive oxygen species production, NET release, phagocytosis, and killing, albeit in mixed and uncertain ways. One study with mouse bone marrow-derived PMNs showed that SCFAs induced ROS release, a response they did not see in GPR43 knockout mice, suggesting a GPR43-dependent mechanism of ROS production [69]. However, other studies have demonstrated an inhibition of ROS production by SCFAs. At pH 5.5, butyrate was able to inhibit ROS production in a dose-dependent manner while acetate at pH 6.5 increased ROS production in bovine PMNs [70]. However, another study demonstrated that butyrate inhibited superoxide production of rat and human PMA-stimulated PMNs, while acetate did not affect PMA-stimulated cells but did increase superoxide in unstimulated rat PMNs. Additionally, in rat PMNs stimulated with fMLP that were preincubated with acetate, propionate, or butyrate, reduction in superoxide production was observed [71]. Overall, the modulation of superoxide production of PMNs by SCFAs is unclear, with studies showing varying results depending on cell types, SCFA concentrations, and experimental conditions; some of this variation may be due to potential differences in the oxygen tension between these experiments and some dependent on variable effects in different systems such as the gastrointestinal tract and lung. Still, little is known about how human PMNs superoxide production is affected by SCFAs.

SCFAs also are able to affect NET formation, but their effects are also unclear. Human PMNs treated with acetate have been shown to have decreased NET release in the presence of PMA, however, another, more extensive study with human PMNs demonstrated increased NET release by 20 mM of acetate, propionate, or butyrate. Furthermore, this effect was dependent on GPR43 and NADPH oxidase complex and did not occur at low pH [72, 73]. Bovine PMNs also have showed increased NET release with butyrate following zymosan stimulation, without affecting cell viability [74]. Overall, there is no consensus on whether NET release is increased by SCFAs, and little is known in a CF-specific context.

Phagocytosis is another PMN effector function that has shown to be affected by SCFAs. Butyrate is able to decrease phagocytosis of *C. albicans* by rat PMNs, and propionate and butyrate are able to inhibit phagocytosis of *S. aureus* by human PMNs [71, 75]. Additionally, *C. albicans* killing is also inhibited by butyrate [71].

In addition to these effector functions, SCFAs can also modulate the secretion of cytokines directly from PMNs. Acetate, propionate and butyrate are able to decrease TNF-α production by LPS-stimulated PMNs [76, 77]. A published abstract has also noted decreased IL-10 release in response to butyrate [78].

With SCFAs already being implicated in several inflammatory conditions, their presence in millimolar concentrations in the CF sputum, as a byproduct of abundant CF anaerobes, suggests they may be contributing to the inflammation that is well-described in CF. SCFAs have already been shown to affect CF bronchial epithelial cells and alveolar epithelial cells to release proinflammatory cytokines. These cytokines can act on PMNs to alter their effector functions. Additionally, SCFAs can also increase the chemotaxis of

PMNs to the airway. However, the direct role of SCFAs on PMNs in the CF airway is unknown. Thus, this project determined the effect of SCFA treatment on human PMN effector functions, such as NETosis, ROS production, phagocytosis, and bacterial killing. I hypothesized that the SCFAs would induce PMN effector functions similar to those seen in the CF lung and in the CF sputum model previously discussed. This includes increased NETosis, decreased bacterial killing, and decreased superoxide release. I additionally explore how PMN antibacterial effector functions change with SCFAs under "CF airway-like" conditions. I hypothesized that SCFAs will exacerbate the changes that PMNs undergo once they infiltrate the CF lung and that are concurrently seen in the CF sputum model.

METHODS

Short Chain Fatty Acid Preparation

Methods from Chapter 1 were used according to the same procedures with the addition of SCFAs when relevant. Short chain fatty acids were prepared by dissolving sodium acetate (Sigma cat#S5636), sodium propionate (Sigma cat#P5436), or sodium butyrate (Sigma cat#P5436) in HBSS with 1% HEPES to form a pH 7.4 solution. Solutions of HBSS with 1% HEPES without the SCFAs were used as experimental controls. These solutions were then sterilized and stored at 4°C for future experiments. For NET release, superoxide production, and bacterial killing assays, SCFAs were added concurrently with bacteria and PMNs and remained in the solution for the duration of the assay. For sputum spiking experiments, PMNs were preincubated with the SCFAs and the sputum supernatants, as previously described.

Phagocytosis

Phagocytosis of *S. aureus* to PMNs in the presence of SCFAs was assessed by flow cytometry. Phagocytosis was measured in PMNs that were purified and treated with the 10mM short chain fatty acids as described above. The CF isolate MRSA25 was stained with 5 mM pHrodo™ iFL green STP 280 Ester (Thermofisher, cat#P36012) for 1 hour at 37°C, protected from light. After the staining MRSA25 was opsonized by the addition of 10% autologous serum of the PMN donor and added to PMNs at MOI of 10. MRSA25/PMN co-cultures were incubated for 1 hour 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 1:5,000 for 15 mins at room temperature. PMNs were then washed and resuspended in eBioscience™ flow cytometry staining buffer (Invitrogen, 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 minutes, protected from light. PMNs were washed and resuspended in BD stabilizing fixative. For zombie red detection, the yellow laser at 561nm was used with the 615/20 filter, the red laser at 637nm with the 660/20 laser was used for CD66B AlexaFluor 647 detection, and the blue laser at 488nm with the 530/30 laser was used for pHrodo green detection. To assess phagocytosis of *S. aureus*, the data was 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.

PMN Viability

Human PMNs were incubated for 3.5 hours in 1.5 ml microcentrifuge tubes at a concentration of $1x10^6$ cells/100 µl assay medium (HBSS+1% autologous serum + 5 mM glucose + 10 mM HEPES) with 10mM SCFAs. 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; cat# 423102) at a dilution of 1:10,000 for 15 minutes at room temperature, protected from light. Cells were collected by centrifugation and washed with 1X PBS containing 1% BSA. The granulocyte marker CD66b conjugated to PerCP-Cy5.5 fluorochrome (Biolegend, San Diego, CA; cat# 305108) was used to identify PMNs in the cell suspension. Cells were suspended in 225 µl BD Stabilizing Fixative (BD) and analyzed at the University of Georgia College of Veterinary 226 Medicine Cytometry Core Facility on an LSRII Flow Cytometer (BD, San Jose, CA), 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). Trypan blue staining was also used to confirm results.

RESULTS

With the CF airway well established as a modulator of PMNs and their effector functions, the CF sputum was shown to similarly modulate neutrophil effector functions. Next, the effect of SCFAs, which are documented to be present in millimolar concentrations in the CF sputum, on PMNs was evaluated. The previous data on the sputum model revealed clinical *S. aureus* isolates to be an efficient stimulator of these PMNs and

thus these isolates were utilized for the rest of the experiments (Table 2). Specifically, MRSA isolate SA25 was often utilized due to its potent stimulation of PMN effector functions.

First, the influence of SCFA stimulation on NET release in response to SA25 was evaluated. Physiologically relevant doses of acetate, propionate, and butyrate were chosen (100 uM to 10,000 uM) and NET release was evaluated via the Sytox Orange Nucleic Acid stain (Figure 4A). Data was normalized to the HBSS control. Increasing doses of acetate seemed to cause a trend towards increased NET release, albeit not significant. Propionate did not increase the release of NETs. Butyrate, however, did cause a dose dependent increase in NET release as concentration increased, with the 10 mM dose having significantly greater NETosis as compared to the HBSS control. This positive association between SCFAs and NETosis was consistent for both acetate (R^2=0.52) and butyrate (R^2=0.95), but did not exist for propionate (R^2=0.03) (Figure 4B). Without SA25 stimulation, 10 mM doses of the SCFAs had no effect on NETosis (Figure 4C). Additionally, there were no interactions between the SA25 and SCFAs to affect the Sytox stain (Figure 4D), nor did SCFAs increase the growth of *S. aureus*, as they have been noted to do to *P. aeruginosa* (Figure 4E).

Next, superoxide production was measured with SA25 stimulation and SCFA addition. Superoxide production was measured via the Diogenes Chemiluminescence assay, which was successfully utilized with high sensitivity in previous experiments. Data were reported as the percentage of the luminescence signal achieved by the HBSS control. PMNs were treated with physiologically relevant SCFA doses ranging from 100 uM to 10,000 uM. Acetate treatment led to a dose-dependent inhibition of superoxide production,

with 10 mM and 7.5 mM doses being significantly lower compared to the HBSS control and lower acetate doses (Figure 5A). Similarly, propionate also led to a dose-dependent inhibition of superoxide production with the 10 mM dose being significant as compared to the HBSS control and doses up to 5mM. Butyrate also inhibited superoxide in a dose-dependent manner; 10 mM butyrate treatment led to superoxide production significantly lower than the control and 100 uM to 1000 uM doses. This inhibition of superoxide by SCFAs was also observed with PMNs not stimulated with SA25. Unstimulated cells had much lower overall basal superoxide production, and this was further inhibited by 10 mM doses of butyrate (Figure 5B). With the effect of SCFAs on healthy PMNs elucidated, CF PMNs were also tested. Due to low supply of CF blood, most experiment were done with healthy PMNs, as previously established in the sputum model. CF PMNs treated with 10 mM doses of SCFAs were shown to have a similar superoxide production as healthy PMNs in response to the opsonized clinical SA25 isolate (Figure 5C). While significance was not achieved with either healthy nor CF PMNs in this experiment due to limited donors, there was a similar trend for CF and healthy PMNs showing an inhibition of superoxide production. This trend was consistent with previous data and was also similar when cross-opsonization (CF PMNs exposed to healthy serum opsonized SA25 and vice versa) was performed (Figure 5C).

Next, the phagocytosis of the PMNs in the presence of SCFAs was investigated. Flow cytometry was used to evaluate the effects of 10mM doses of the SCFAs on the phagocytosis of the SA25 isolate labelled with a 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 6A. The SCFAs did not have an effect on the phagocytosis of the SA25 clinical isolate (Figure 6B). Cell viability in the presence of the SCFAs was also assessed with flow cytometry to confirm that the 10mM doses of SCFAs were not killing the 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 6C. The SCFAs do not influence the surface expression of the PMN marker CD66b as shown in Figure 6D. This was further confirmed with a trypan blue staining, which also yielded no changes in viability (results not shown). These results confirm that the SCFAs do not impact the viability of human PMNs and subsequent observations do not result from PMN plasma membrane damage.

Following the investigation of the effects of SCFAs on PMN effector functions, the ability for PMNs to kill bacteria, their primary role in these instances, was evaluated. Based on the previous data showing significant alterations of PMN functions with 10 mM treatments of SCFAs, this dose was used in testing *S. aureus* killing. 4 clinical isolates of *S. aureus*, including 2 MRSA and 2 MSSA isolates were treated with acetate, propionate, and butyrate (Figure 7A). There was a trend towards inhibition of killing to the SA17 and SA24 isolates, however, this was not statistically significant. SA25 killing was shown to be inhibited by propionate and butyrate. Similarly, SA70 killing was significantly reduced by propionate. Overall, when the clinical isolates are aggregated together, 10 mM of

acetate, propionate, and butyrate all significantly reduced killing as compared to the HBSS control (Figure 7B).

With both the sputum model and SCFA stimulation now shown to modulate the effector functions of PMNs, the effect of SCFAs in the sputum model was evaluated. Acetate, propionate, and butyrate were spiked into the sputum model at 10 mM concentrations. PMNs were incubated with the CF sputum supernatant for 3.5 hours as before with the 10 mM of SCFAs. First, superoxide release was evaluated with the Diogenes chemiluminescence assay in response to SA25 stimulation. Sputum treatment did not significantly change the superoxide production, although there was a trend towards increased superoxide production (Figure 8A). Acetate, propionate, and butyrate treatment did not change the production of superoxide. Bacterial killing of SA25 was also evaluated (Figure 8B). Independently, sputum treatment or 10 mM butyrate treatment had a trend towards decreased killing, although this was not significant. Together, spiking 10 mM butyrate into sputum did not further reduce killing.

DISCUSSION

With the CF sputum model previously established to modulate PMN effector functions, this chapter sought to investigate the potential cause of these changes. SCFAs have been previously well described in other inflammatory conditions and are produced copiously as byproducts of anaerobic bacterial fermentation. Anaerobes are present in a vast majority of CF patient's airways and SCFAs are consistently described to be present in millimolar concentrations in the CF sputum. Their effects on the CF epithelial cells have been described as pro-inflammatory and they are shown to act as PMN chemo-attractants

and correlate with PMN numbers in the CF lung [51, 66]. However, their direct effects on PMNs are less established, especially in the context of CF. Thus, in this chapter, the effects of SCFAs on the effector functions of PMNs were evaluated in the presence of clinical isolates of *S. aureus*, which were previously shown to cause changes in the CF sputum model similar to that of the CF airway. It was hypothesized that SCFAs would induce the PMN effector functions seen in the CF lung. This includes increased NETosis, decreased bacterial killing, and decreased superoxide release. It was additionally hypothesized that SCFAs would exacerbate the changes seen in the CF sputum model.

First, SCFAs were shown overall to increase NET production, with a clear dose dependent increase for butyrate. This increased NETosis was also seen in the sputum model and is consistent with the CF lung phenotype [27, 28]. While SCFAs have previously been shown, in some studies, to induce NETosis in bovine and human PMNs, these studies only examined the direct effect of SCFAs in relatively high concentrations [72, 74]. These effects were shown to be partially mediated via the GPR43. Our study establishes the ability of butyrate to modulate NET release in the presence of bacteria—specifically to increase NETosis in the presence of CF clinical isolates of *S. aureus*. These changes were dependent on bacterial presence, as there were no differences in unstimulated cells treated with SCFAs. Because SCFAs are produced by anaerobic bacteria in the CF lung, they would be expected to be found and exert their effect in parallel with bacteria. Additionally, SCFAs did not increase the growth of *S. aureus* as they are documented to do for *P. aeruginosa* [66]. Thus, it is likely the effect of the SCFAs is directed at the PMNs, with *S. aureus* serving to activate them. It is possible that this effect is partially mediated via GPR43 as previous studies show or could be due to another pathway.

Acetate, propionate, and butyrate were all able to inhibit superoxide production of healthy PMNs in a dose dependent manner. This effect was partially independent of bacterial stimulation, as butyrate significantly inhibited superoxide production without SA25 stimulation, albeit to a lesser degree. The literature is mixed on the effect of SCFAs on superoxide production, with some studies reporting increased production [69] and others reporting inhibition [70, 71]. However, this is the first study demonstrating the effect of SCFAs on human PMNs in response to CF clinical *S. aureus* isolates. The clear inhibition provides important information to the effects of SCFAs overall but more importantly points to their importance in CF and airway PMN dysfunction.

These experiments were performed with healthy PMNs due to a lack of CF blood availability. Many of the changes observed in CF PMNs occur following lung infiltration and previous data with the CF sputum model confirmed this, by inducing dysfunction in healthy PMNs following CF sputum exposure. The limited set of CF PMNs evaluated for superoxide production in response to SCFAs did not show significant inhibition of superoxide, however, this was also consistent with the healthy PMNs in these experiments. Both healthy and CF PMNs had similar, non-significant inhibitions of superoxide production. It is expected that with a greater number of blood donors, healthy PMNs would have achieved significant inhibition, as before, and CF PMNs would likely also experience significant inhibition. Cross-opsonization of the bacterium with either the CF or healthy serum showed no difference in superoxide production, suggesting that opsonin-mediated phagocytosis and subsequent superoxide production is not dependent on donor specific factors. Similarly, there were no significant changes seen in the phagocytosis of *S. aureus* by SCFA treated PMNs, somewhat consistent with previous findings in the sputum model

that showed no inhibition of phagocytosis by sputum treatment. Overall, the data with CF PMNs validates the use of healthy PMNs in the experiments and confirms previously noted trends in the CF sputum model. The effect of inhibited superoxide production is also consistent with the decreased superoxide production some studies document in the CF lung [25, 26]. Other studies show no effect on superoxide production with our sputum model also not causing any difference in superoxide production in the presence of sputum. However, this incongruency between the CF sputum model and the data seen with SCFAs could be due to the concentrated effect of SCFAs on the PMNs. The concentrations of SCFAs in our sputum are unknown, however, addition of 10mM of the SCFAs could have had a more potent effect on the PMNs that was simply not observed due to lower levels in the CF sputum and dilution in the CF model.

In addition to NETosis and superoxide release being significantly affected by SCFAs, acetate, propionate, and butyrate were able to significantly inhibit killing of *S. aureus*. This change in killing was not due to changes in the PMN viability, as SCFAs at the highest 10mM dose were confirmed to not decrease viability of the PMNs. The ability for PMNs to kill pathogens describes their overall function, encompassing the changes in other effector functions. This inhibited killing is consistent with the inhibition in the CF lung and also in the CF sputum model. While another study showed that SCFAs can decrease killing of *C. albicans* [71], there is little already known about their effect on bacterial killing. To our knowledge, this is the first study to show inhibition of bacterial killing by SCFAs. This is significant for CF, as these SCFAs showed inhibition of killing of the *S. aureus* isolates present in CF patients airways and infecting patients chronically due to lack of PMN clearance. SCFAs could be a vital component in the CF sputum leading to the

inhibition of killing previously established. In addition to CF, SCFAs are present in a number of inflammatory conditions. The reduced bacterial killing could also play a role in these inflammatory diseases where bacterial infections contribute to pathology, such as in the gut [79].

With SCFAs presumably present in our CF sputum samples, it was hypothesized that adding more SCFAs would increase the effects seen by the SCFA and the sputum independently. However, when the superoxide production and bacterial killing was tested with SCFAs spiked in, there were no significant changes seen. There are several possibilities for this effect. Previously, SCFAs were added to PMNs and their effector functions were measured while the SCFAs were present in solution. In the CF sputum model, a 3.5-hour preincubation was performed and then sputum and SCFAs were discarded prior to measuring effector functions of the PMNs. It is possible that a synergistic effect could have been seen had the SCFAs remained in the solution during measurement. Additionally, if the SCFAs and the sputum are acting on the same mechanism in the PMNs, it is possible that this pathway is saturated with signaling and addition of another component that signals via the same pathway does little to enhance signaling. This lends support for SCFAs and sputum acting in similar manners *in vitro.* Overall, it is still unknown whether the SCFAs act on PMNs through the GRP43 receptor or some other pathway. The literature shows that SCFAs may exert their effects through the GPR43 receptor, but can also affect histone deacetylation to change gene expression more directly [79]. Further studies are still needed to determine the mechanism of action of the SCFAs on PMNs in general and specifically in the context of CF.

While most of the data was limited to healthy PMNs, other studies and our own data suggest that CF PMNs behave relatively normal until they infiltrate the lung. While there were variations between *S. aureus* isolates and different effects observed with the different SCFAs, this is expected in a relatively complex *in vitro* model that utilized clinical bacterial isolates, human PMNs, and clinical CF sputa. This chapter focused on the effector functions of PMNs, but future studies could potentially attempt to elucidate the mechanism of action of the SCFAs. This is difficult to accomplish due to practical challenges in inhibiting or isolating the SCFAs in the heterogenous sputum, but these experiments would yield important data on the overall mechanisms affecting PMN effector functions in this system. Overall, *in vivo* observations of the CF lung show inhibited bacterial killing, increased NETosis, and decreased superoxide production. Our experiments with SCFAs mimicked this effect and could be contributing to the phenotype observed in the CF model and in the CF airway.

CONCLUSIONS

This thesis focused on the modulation of PMN effector functions that occurs when they infiltrate the CF lung and its inflammatory environment. While CF PMNs behave mostly without defect in the circulation, changes have been noted to occur when they chemotax to the CF lung in response to -the often chronic- bacterial infections. These changes involve the increase in NET release, decrease in superoxide production, and inhibition of bacterial killing [25-28]. It is currently unknown what causes these changes in PMNs, however, the identification of a component responsible for these effects would allow for potential targeting in therapeutics aiming to increase PMN effectiveness against

these damaging infections. One component type present in the CF sputum in very high concentrations are SCFAs, especially acetate, propionate, and butyrate. They are produced by anaerobic bacterial fermentation and accumulate in millimolar concentrations in the CF sputum [51, 66].

To determine the effect of CF sputum on PMNs *in vitro*, a CF sputum supernatant model was previously developed that involves the incubation of healthy PMNs in 30% bacterial-free CF sputum supernatant for 3.5 hours to simulate the CF airway environment. Following the observation of reduced *S. aureus* killing in this model and no effect on PMN viability, the effect on NETosis and superoxide production was examined. CF clinical isolates of *S. aureus* were shown to induce increased NETosis and no change in superoxide production, consistent with descriptions of PMNs in the CF lung.

SCFAs, which are present in the sputum in millimolar concentrations and possess some inflammatory roles were then examined for their effects on PMN effector functions. SCFAs were shown to increase NETosis, inhibit superoxide, and inhibit killing of *S. aureus*. This is consistent with what is described in the CF lung. Although there was no synergistic effect seen with SCFAs spiked into the CF sputum, it is possible they are acting on the same mechanism to modulate PMNs. To the best of our knowledge, this data, for the first time, shows that the CF sputum model mimics the environment of the CF lung and creates similar effector functions *in vitro*. Moreover, for the first time, SCFAs were shown to directly impact the effector functions of human PMNs in response to *S. aureus*, with the changes in bacterial killing, superoxide production, and NET release demonstrated. The use of clinical *S. aureus* isolates further contextualized this study to CF, where SCFAs are present in significant concentrations and have been shown to affect airway epithelial cells

in a pro-inflammatory manner, but were not previously investigated for their direct effects on PMNs. This project establishes SCFAs as direct modulators of PMN effector functions in response to the clinical *S. aureus* isolates and potentially serve a detrimental role in the clinical course of CF lung disease. The exact role of SCFAs in CF lung disease will be important to study clinically, with future studies evaluating the mechanism of action of these SCFAs key in elucidating their exact effects in the complex lung environment.

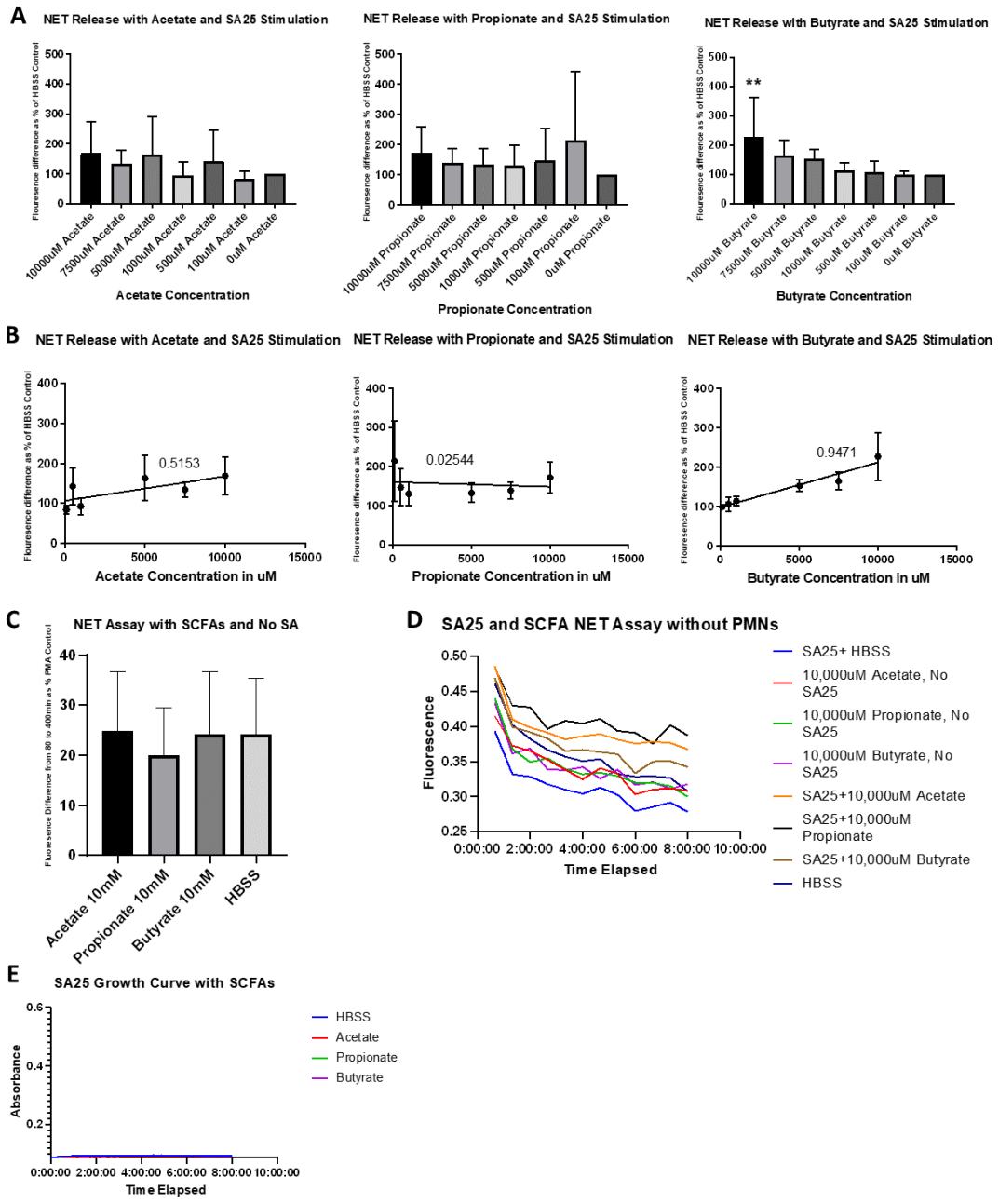

Figure 4. SCFA treatment increases NET release in a dose dependent manner.

Healthy human blood PMNs were exposed to the indicated opsonized clinical isolates of *S. aureus* (10 MOI) and SCFAs (acetate, propionate, butyrate, or HBSS control, with 1.0% HEPES). Extracellular DNA (ecDNA) release was measured for 8 hours in the presence of Sytox Orange, a membrane impermeable, DNA-sensitive fluorescent dye. (A) EcDNA release in *S. aureus* clinical isolate stimulated PMNs after 8 hours measured as increase in fluorescence. The *S. aureus* induced ecDNA signal difference from 80min to 400min was normalized on the HBSS control. Mean±S.E.M, n=6. (B) EcDNA release in *S. aureus* clinical isolate stimulated PMNs after 8 hours measured as increase in fluorescence with

correlation to SCFA concentration. n=6, r^2 value displayed. (C) EcDNA release in unstimulated PMNs exposed to SCFAs after 8 hours measured as increase in fluorescence 80min to 400min and normalized on the PMA control. Mean±S.E.M, n=3. (D) Kinetics of Sytox Orange DNA dye in presence of SA25 and SCFAs without PMNs. (E) Growth curve of SA25 with 10mM doses of the SCFAs in the HBSS assay media over 8 hours. One-way ANOVA with multiple comparisons to the HBSS control and 100uM SCFA condition. *, $p<0.05$; **, $p<0.01$. Ns, not significant.

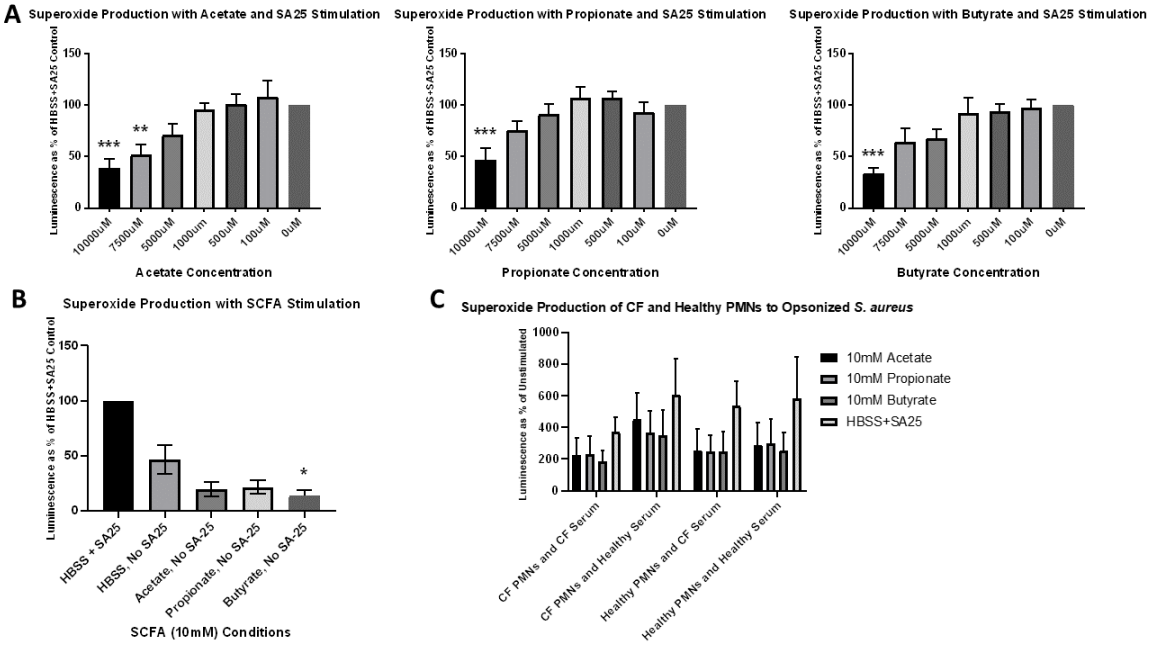

Figure 5. SCFAs inhibit superoxide production in a dose dependent manner.

Healthy human PMNs were exposed to opsonized SA25 (10 MOI) and SCFAs, and superoxide production was measured with the Diogenes-based chemiluminescence and normalized to the HBSS control (A-B) or to unstimulated cells (C). (A) Acetate, propionate, and butyrate inhibited superoxide production by PMNs stimulated with SA25 in a dose-dependent manner. Mean±S.E.M, n=5 (B) Acetate, propionate, and butyrate (10mM) inhibited superoxide production by unstimulated PMNs. Mean±S.E.M, n=5. (C) CF and healthy PMNs had similar, nonsignificant inhibition of superoxide production in response to SA25 and SCFA stimulation. Mean±S.E.M, n=4. One-way ANOVA with Tukey's multiple comparisons *, $p<0.05$; **, $p<0.01$. Ns, not significant.

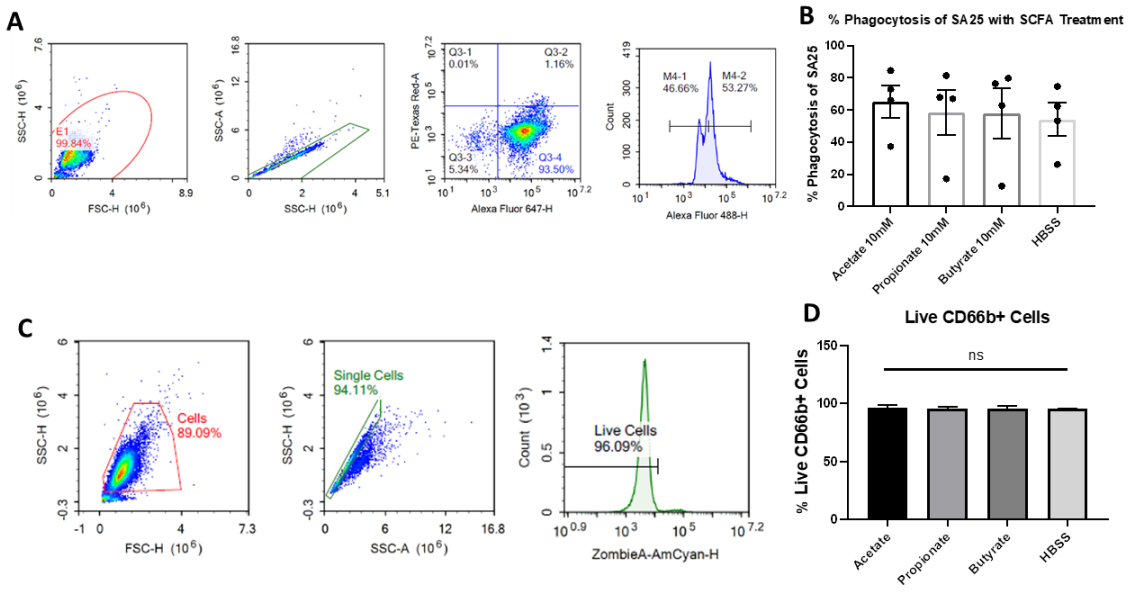

Figure 6. SCFAs do not affect PMN bacterial phagocytosis nor PMN viability.

PMNs were isolated from healthy donors and exposed to SCFAs. To measure bacterial phagocytosis, SA25 CF isolate was labelled with the pH-sensitive dye pHrodo, opsonized, and exposed to PMNs. (A) Representative images of the gating strategy used to determine the percent of SA25 phagocytosed by PMNs for each condition. (B) Comparison of SA25 phagocytosis by PMNs that were treated with SCFAs, n=4. PMN purity and viability following isolation from blood and subsequent 3.5 hour-SCFA incubation was measured by flow cytometry using the Zombie Aqua Cell Viability Kit™. (C) Representative images of the gating strategy used to determine the percent of viable PMNs (CD66b+ /Zombie Aqua-) for each condition tested are shown. (D) Flow cytometric analysis showed no significant difference in the percent of PMNs and CD66 surface expression among the SCFA conditions tested, n=2 One-way ANOVA, Ns, not significant.

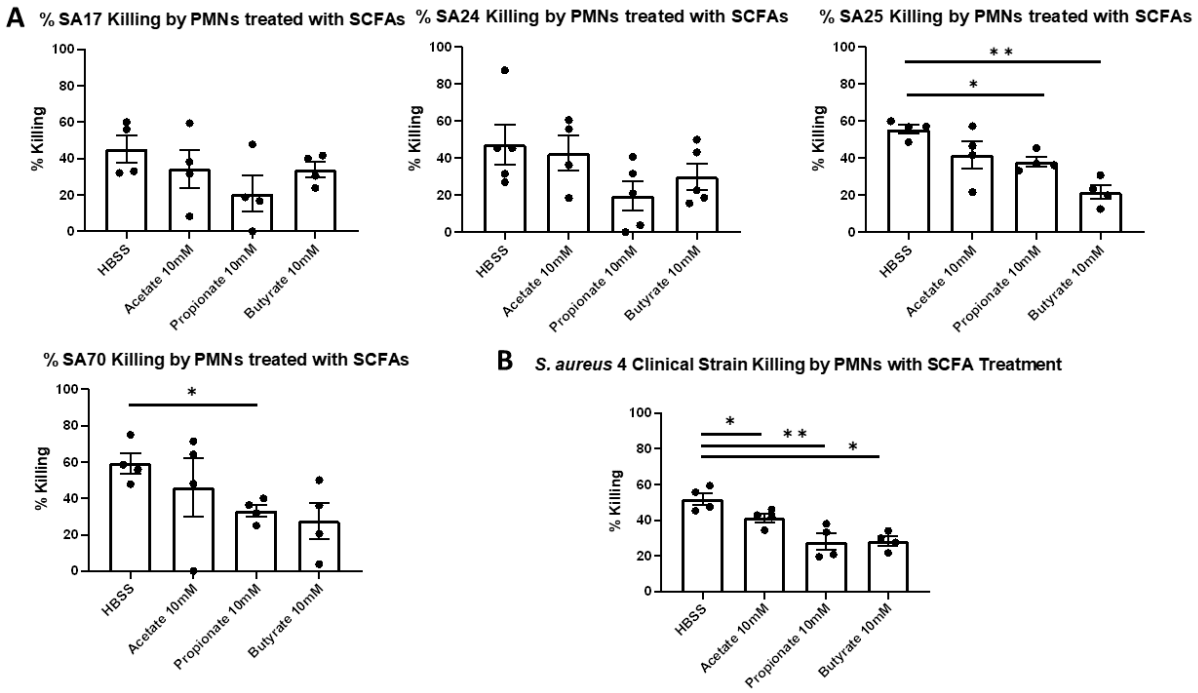

Figure 7. SCFAs inhibit clinical *S. aureus* isolate killing by PMNs.

Healthy PMNs were exposed to SCFAs (10mM) and opsonized clinical *S. aureus* isolates (10 MOI), and bacterial killing was measured with an agar plate-based assay. (A) SCFAs did not significantly affect killing of SA17 and SA24 clinical isolates but significantly inhibited killing of SA25 and SA70 clinical isolates. Mean±S.E.M, n=4-5. (B) Aggregated *S. aureus* killing was inhibited by acetate, propionate, and butyrate. Mean±S.E.M, n= 4 clinical isolates. One-way ANOVA with Dunnett's multiple comparisons test. *, $p<0.05$; **, $p<0.01$. Ns, not significant.

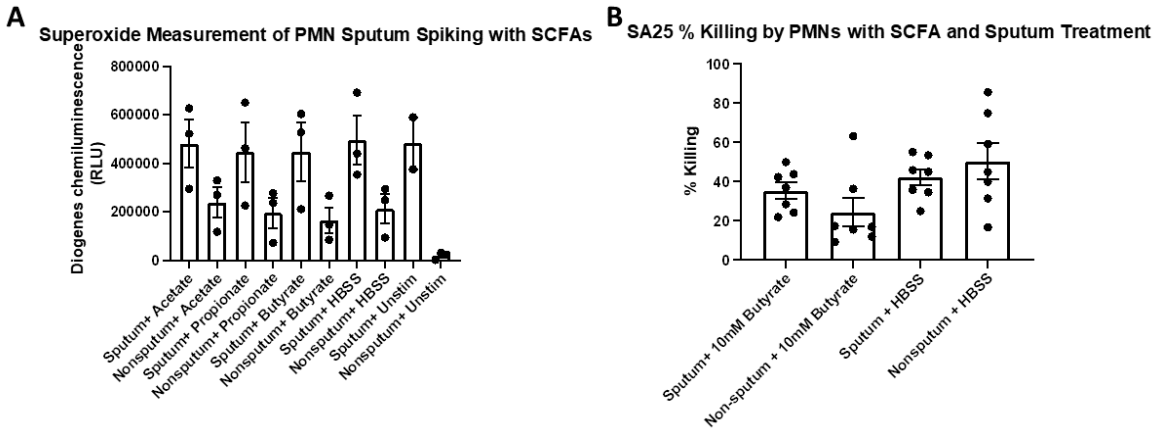

Figure 8. Superoxide production and *S. aureus* killing are unaffected by SCFA spiking into sputum.

Healthy human blood PMNs were first treated with 30% (v/v) CF sputum supernatant cocktail and 10mM acetate, propionate, butyrate, or HBSS control. Then PMNs were exposed to opsonized SA25 (10 MOI) and superoxide production and bacterial killing were measured. (A) Superoxide production was measured with the Diogenes-based chemiluminescence and reported as relative luminescence units (RLU). SCFAs and sputum independently, or in combination, did not affect superoxide production by PMNs in response to SA25. Mean±S.E.M, n=3. (B) Bacterial killing was measured with an agar plate-based assay. Butyrate and sputum independently, or in combination, did not affect SA25 killing. Mean±S.E.M, n=7. One-way ANOVA. Ns, not significant.

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