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Alterations in Pathogenicity Factors of *Pseudomonas aeruginosa* in Response to Cigarette Smoke Exposure

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

Sae Jeong Ahn

Committee in Charge:

Laura Crotty Alexander, Chair
Eric Allen, Co-Chair
Ella Tour

2016

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Co-Chair

Chair

University of California, San Diego

2016

DEDICATION

I dedicate this thesis to my amazing father, mother, and grandparents,

without whom I would not be who I am today.

Thank you for your unconditional love, support, prayers, and encouragement.

EPIGRAPH

"It's better to know how to learn than to know."

Dr. Seuss

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First and foremost, I would like to express my deepest appreciation to my committee chair, Dr. Laura Crotty Alexander, for giving me the opportunity to join her lab and learn from the best. I am grateful to have such an amazing mentor and role model. Without her guidance and support, this dissertation would not have been possible.

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ABSTRACT OF THE THESIS

Alterations in Pathogenicity Factors of *Pseudomonas aeruginosa* in Response to Cigarette Smoke Exposure

by

Sae Jeong Ahn

Master of Science in Biology

University of California, San Diego, 2016

Laura Crotty Alexander, Chair

Eric Allen, Co-Chair

Cigarette smoking is one of the leading preventable causes of death in the United States. It is well recognized that cigarette smoking alters the human defense mechanism, and increases susceptibility to bacterial infections and diseases, such as pneumonia and chronic obstructive pulmonary disease. However, the effects of

cigarette smoke on the pathogenic aspect of this human-pathogen dynamic remains poorly understood. Therefore, we sought to determine the effects of cigarette smoke on the pathogenicity properties of *Pseudomonas aeruginosa* (PSA), a prominent airway pathogen in adult smokers. Here, we show that while cigarette smoke exposure slows PSA growth in a dose-dependent manner, it increases certain pathogenicity factors. PSA exposed to cigarette smoke extract (CSE) showed increased resistance to killing by reactive oxygen species, suggesting that cigarette smoke may aid in the survival of PSA in phagolysosomes, a principal innate immune antimicrobial mechanism. When human neutrophils were infected with PSA, cigarette smoke exposure increased resistance to neutrophil killing, with 100% of CSE-PSA surviving, while 20% of control PSA was killed. Furthermore, we discovered that a long-term CSE exposure causes a more dramatic inhibition in PSA growth, compared to PSA with one time CSE exposure; this suggests that the PSA population in long-term smokers may possess different virulence properties than their sporadic/non-smoker counterparts. We conclude that cigarette smoke-induced resistance phenotypes in pathogens may be an important contributor to the vulnerability of cigarette smokers to infectious airway diseases. We hope our findings will improve our understanding of the effects of cigarette smoke on human health.

INTRODUCTION

Cigarette Smoking

Cigarette smoking is one of the leading preventable causes of death, disease, and disability worldwide. Direct and second hand exposure are responsible for approximately 6 million deaths per year, of which 480,000 occur in the United States alone[1]. In the U.S., over 16 million individuals suffer from a disease caused by smoking[1]. Such diseases consist of cancer, heart disease, various lung diseases and chronic obstructive pulmonary disease (COPD), including emphysema and chronic bronchitis.

Impact of Cigarette Smoke on Human Health

Cigarette smoke breaches host defense mechanisms by causing direct changes in the human body that impede its resistance to bacterial colonization[2]. Although the exact mechanisms are not completely understood, some of these changes are thought to increase susceptibility to upper and lower respiratory tract infections[3]. The mechanisms by which cigarette smoke predisposes individuals to infection include alterations in the respiratory structure and immune responses.

The risk of infections can be increased through cigarette smoke induced structural changes in the respiratory tract[3]. Such alterations include disrupted airway epithelial cells, and thus reduced mucociliary clearance of the respiratory system[4], increased bacterial adherence to epithelial cells[3], mucus hypersecretion, impeded epithelial elastic properties, and impaired phagocytic activities[5]. For instance, previous studies have indicated that elements that constitute cigarette smoke, such as nitric

oxide, acrolein, acetaldehyde, formaldehyde, and free radicals in the smoke, can lead to structural disruptions in the respiratory epithelium[6,7]. These structural changes are noteworthy as they promote bacterial colonization and exacerbate airway inflammation, which further impair host immunity and facilitate chronic inflammation and bacterial colonization[3]. Furthermore, during a study with stable chronic bronchitis patients, Hill et al observed a positive correlation between airway bacterial abundance and markers of airway inflammation, such as neutrophil activation and number[8]. This suggests that increased airway bacterial colonization is interconnected with increased airway inflammation.

Another mechanism by which cigarette smoking can increase susceptibility to infections is through alterations in the host innate and adaptive immunologic responses[3,9], both of which play a significant role in preventing airway infections. Cigarette smoke impedes various functions of monocytes, neutrophils, macrophages, and dendritic cells, which are key players of the human innate immune system[10]. For example, while examining the effects of cigarette smoke on the differentiation and key effector functions of neutrophils, Xu and his team observed that neutrophils exposed to nicotine and other cigarette smoke constituents during differentiation displayed suppressed bacterial killing ($p < 0.01$) and oxidative burst ($p < 0.001$)[11]. Oxidative burst is an important mechanism where neutrophils release reactive oxygen species like H_2O_2 to eliminate internalized pathogens during phagocytosis[10]. An impeded generation of reactive oxygen species, therefore, directly hinders the ability to kill bacterial cells. Similarly, a study by Dunn et al also showed that cigarette smoke treatments reduced the production of intracellular and extracellular reactive oxygen

species by human neutrophils[12]. Taken together, these findings may help explain the association between cigarette smoking and increased susceptibility to bacterial infections. Cigarette smoke has a negative impact on dendritic cells as well. Their key function is to connect the innate and adaptive immune responses by processing and presenting inhaled environmental antigens to the adaptive immune cells[9,13]. Cigarette smoke not only reduces the number of dendritic cells in the lungs, but also suppresses their maturation in the lymph nodes, which subsequently inhibits the expression of antigen presenting and costimulatory molecules[13].

Current studies indicate that the adaptive immune responses are also suppressed by cigarette smoke exposure. While there is a limited understanding of the repercussions of cigarette smoke on lymphocyte functions, smokers exhibit reduced levels of anti-bacterial IgG ($p < 0.001$) than their non-smoking counterparts[14]. This is confirmed by the findings of Quinn and his team, who showed a striking association between cigarette-induced decrease in serum IgG2 levels and increase in periodontal inflammation[15]. This may help illustrate the mechanisms of increased bacterial infection and amplified inflammatory responses observed in smokers. Furthermore, several groups have shown that cigarette smoke suppresses T and B cell proliferation and function [16–18].

Impact of Cigarette Smoke on Bacterial Pathogenicity

Several studies have examined the association between cigarette smoke and pathogenic bacteria. Sustained cigarette smoke exposure has been shown to not only

affect the human defense responses, but also facilitate pathogenic proliferation and persistence[19] and modify the normal nasopharyngeal microflora composition in the respiratory tract[5]. Furthermore, cigarette smoke can increase bacterial virulence by enhancing its binding to epithelial cells[20] and altering the bacterial gene expression of certain virulence factors. In particular, when the effect of cigarette smoke on bacterial adherence was assessed by El Ahmer et al, multiple bacterial species in smokers – including, but not limited to, *Neisseria meningitides, Streptococcus pneumonia, Bordetella pertussis, Staphylococcus aureus* – exhibited significantly higher binding to epithelial cells than did those in nonsmokers[20]. In addition, the modification in bacterial gene expression and phenotype as a direct result of cigarette smoke exposure is exemplified in the upregulation of fimbrial proteins in *Porphyromonas gingivalis* [21]. Fimbrial proteins play a crucial role in the initial bacterial adherence to junctional epithelial cells, and therefore are important virulence factors[21]. Overall, however, the mechanistic understanding of the impact of cigarette smoke on bacterial phenotype and their pathogenic properties is lacking in literature.

On a pathological level, cigarette smoking is a major cause of Chronic Obstructive Pulmonary Disease (COPD)[19]. COPD patients suffer from abnormal lung inflammations as a response to cigarette smoke, and subsequently experience tissue destruction and airflow limitation[22]. In their study, Gally et al revealed that Fatty Acid Binding Protein 5 (FABP5) was down regulated in the airway epithelium of smokers with COPD versus those without COPD. Additionally, their results showed that FABP5 levels in normal human bronchial epithelial cells increased following bacterial inoculation with *P. aeruginosa*, a Gram-negative bacterium, but decreased upon

cigarette smoke exposure[19]. This understanding of FABP5 is significant – the down regulation of FABP5 expression leads to amplified *P. aeruginosa* bacterial colonization, whereas FABP5 overexpression exerts a protective downstream effect against *P. aeruginosa* infection in airway epithelial cells. This suggests that cigarette smoke-induced decrease in the airway epithelial FABP5 expression should increase individuals' susceptibility to infections by pathogens like as *P. aeruginosa*; this notion is, in fact, consistent with the results of Gally et al[19].

Multiple groups have shown that *P. aeruginosa* plays a prominent role in the progression of chronic lung diseases. In COPD patients, for example, it causes more frequent infections[19] and diseases, like pneumonia[23], as the severity of COPD advances. In addition to its function in the development and exacerbation of COPD[23], *P. aeruginosa* infections are also common in patients with cystic fibrosis and bronchiectasis. In severe cases, *P. aeruginosa* infection can be resilient and difficult to eliminate, and often leads to early mortality in cystic fibrosis patients[24]. When comparing bronchiectasis patients with and without *P. aeruginosa* colonization in their respiratory tracts, those with *P. aeruginosa* displayed a higher severity in the level of disease progression and impairment of pulmonary functions[25]. For this reason, it is critical to understand the interconnection between *P. aeruginosa* and cigarette smoking in order to interpret the current presumption that smoking facilitates lung infections and diseases [26].

All in all, cigarette smoke has been widely associated with increased susceptibility to infection and disease. Current understanding of its impact on the host-

pathogen paradigm, however, is limited to the human defense and inflammatory mechanisms in the respiratory tract, and fails to acknowledge the pathogenic side of the host-pathogen dynamic. In previous work, our lab has analyzed the effects of cigarette smoke on the virulence properties of *Staphylococcus aureus*, in particular methicillin-resistant *S. aureus* (MRSA). Cigarette smoke exposure increased MRSA's ability to adhere to and invade epithelial cells and altered its surface charge, thereby increasing its resistance to killing by antimicrobial peptides (AMPs)[24]. The prominent role of *P. aeruginosa* in pneumonia, COPD, and other major pulmonary diseases make this pathogen an important one to understand. The goal of our present study is to examine the cigarette smoke-induced changes in the virulence properties of *P. aeruginosa* via changes in bacterial growth, hydrophobicity, biofilm, resistance to reactive oxygen species, surface charge, resistance to killing by cells of host defense, adherence to and invasion of lung epithelial cells (A549s) and virulence *in vivo*. We hypothesize that cigarette smoke exerts great stress on *P. aeruginosa*, leading to changes that enhance their aggressiveness and evasion from host immune responses.

RESULTS

Cigarette smoke exposure increases PSA resistance to neutrophil killing.

Neutrophils, or polymorphonuclear leukocytes (PMNs), play a key role in the innate immune system by serving as the first line of defense against foreign microorganisms. Previous studies have shown that mice with depleted neutrophils[25] or impaired neutrophil function[26] have enhanced susceptibility to bacterial and fungal infections. To examine whether cigarette smoke exposure affects PSA's resistance to neutrophils, PSA was exposed to CSE *in vitro* (CSE-PSA) or grown in control MBM media (control PSA), and their susceptibility to killing by neutrophils was measured (Fig 1A). CSE PSA showed increased survival compared to control PSA ($p < 0.05$), indicating that PSA becomes more resistant to killing by human neutrophils upon cigarette smoke exposure.

This observation could be due to a number of reasons. It has been previously demonstrated that neutrophils rapidly congregate at sites of injury or infection. Here, neutrophils combat invading microorganisms by producing neutrophil extracellular traps (NETs) - a protein-bound chromatin used to target extracellular pathogens - and exposing them to reactive oxygen species (ROS) and antimicrobial peptides (AMPs)[27–29]. To determine whether NETs are the mechanism of killing that CSE PSA have become resistant to, we subjected CSE PSA and control PSA to a NET-specific killing assay and enumerated the surviving bacteria. Upon plating and enumerating the surviving PSA, we found no apparent difference in the percentage of survival against NET-induced killing between CSE PSA and control PSA (Fig 1B), suggesting that CSE PSA are not resistant to this modality of neutrophil antimicrobial activity.

Another mechanism neutrophils use to kill and digest invading microorganisms is via oxidative burst, a process by which neutrophils expose internalized bacteria to reactive oxygen species, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and hypochlorous acid (HClO)[30]. Based on our hypothesis that cigarette smoke exposure would increase PSA pathogenicity, we predicted that CSE-PSA would display an increased resistance to killing by H_2O_2 relative to control-PSA. To investigate this hypothesis, we exposed CSE- and control-PSA to various concentrations of H_2O_2 to mimic phagolysosome conditions with ROS and quantified surviving PSA at 60 minutes. The CSE-PSA showed consistent survival over the course of 60 minutes, while their control counterparts were rapidly killed overtime; this was consistent with our hypothesis that cigarette smoke would increase PSA resistance to killing by H_2O_2. This trend was evident during both 1% and 3% H_2O_2 treatments. For example, it took over 60 minutes for control-PSA to be killed during the 1% H_2O_2 treatment, but only 30 minutes to be killed by the 3% H_2O_2 treatment. In both cases, however, the CSE-PSA maintained a consistent CFU/mL (no cells killed Fig 1C). This suggests that PSA exposed to cigarette smoke is more resistant to ROS, and therefore, less susceptible to killing via oxidative burst, one of the primary mechanisms by which neutrophils kill bacteria in the phagolysosomes.

To confirm that the differences seen in Figs 1A and 1C were, in fact, a result of CSE-PSA resistance to oxidative burst, we repeated the neutrophil killing assay with the addition of DPI, an inhibitor of oxidative burst (Fig 1D). As seen in Fig 1D, CSE-PSA showed significantly higher survival against neutrophil killing than did control-PSA; however, the inhibition of oxidative burst via DPI treatment of neutrophils

produced no difference in the CSE- (DPI treated CSE-PSA) and control-PSA (DPI treated PSA) survivals. This further confirms that the increased survival of CSE-PSA observed in Fig1A is a result of higher resistance to killing by ROS during oxidative burst. This resistance to neutrophil killing suggests that cigarette smoke exposure confers increased virulence to PSA, by helping the bacteria resist host defenses.

Cigarette smoke exposure does not affect PSA surface characters.

Changing surface charge is one virulence mechanism by which bacteria avoid interactions with harmful agents like AMPs[24]. For instance, our lab has previously shown that cigarette smoke exposures alters the surface charge of methicillin-resistant *Staphylococcus aureus* (MRSA) to become more positive and hydrophobic, which may explain why AMPs are less able to kill CSE exposed MRSA compared to control-MRSA[24]. To assess whether cigarette smoke exposure causes surface changes of PSA, a hydrophobicity assay was performed. The results showed no significant difference between the hydrophobicity of control- and CSE-PSA (Fig 2), suggesting that the increased pathogenicity observed in CSE-PSA in Fig 1A is not a result of altered surface properties of PSA.

Cigarette smoke exposure does not alter PSA biofilm formation.

Recent studies have revealed that PSA communicates via a cell-to-cell signaling system called Quorum sensing to respond to signals from different microbial

species[31]. PSA utilizes this system in order to control the expression of virulence genes that aid in biofilm production.[32] It is frequently reported that bacteria within biofilms exhibit strong resistance to host defense mechanisms and result in persistent infections, categorizing biofilm formation as a virulent property.[33] Because cigarette smoke contains many chemicals and toxins, which create a stressful environment, we predicted that PSA would try to protect itself via increasing production of biofilm. We hypothesized that cigarette smoke exposure would increase the virulence level of PSA by inducing increased biofilm production, and expected to observe a greater amount of biofilm produced by CSE PSA in relation to control PSA. As seen in Fig 3, however, we did not find consistent results and our hypothesis was not supported. There are a number of reasonable ways to interpret this data. It is possible that biofilms are not crucial in causing resistance against neutrophils. Alternatively, PSA *in vivo* may use alternative pathways to produce different amounts of biofilm in comparison to PSA *in vitro.*

Cigarette smoke exposure acts as a stressor and leads to dose-dependent inhibition of PSA growth rate.

In order to confirm that the increased survival of CSE-PSA observed in the neutrophil killing assay (Fig 1A) is not a mere reflection of increased growth of the smoke exposed pathogen, PSA was cultured in mammalian base media and increasing concentrations of CSE (Fig 4A). A_{600nm} of CSE-PSA and control-PSA were measured over time. Growth curves revealed that CSE PSA had inhibited growth, in a dose-

dependent manner, in relation to control PSA. This was similar to what was seen in our work with MRSA. This indicates that the increased resistance of CSE-PSA to neutrophil killing (Fig 1A) is not caused by increased bacterial growth. In addition, we believe the cigarette smoke acts as a great environmental stressor for the bacteria, causing it to shift its metabolism toward defensive maneuvers rather than growth and replication[24]. Furthermore, to evaluate whether the CSE-induced inhibition in PSA growth is permanent and possibly heritable, PSA was subjected to multiple CSE exposures (Fig 4B). More specifically, PSA was exposed to CSE once and transferred to MBM overnight, and this process was repeated until PSA was exposed to CSE for a total of three times; we refer to this as CSE x 3. As in Fig 4A, the cultures were agitated at 37 degrees Celsius and their absorbance was measured at 600nm. The (CSE x 2) in MBM – PSA that was exposed to CSE twice, then had 1mL of it transferred into MBM - showed no difference compared to Cx2 in MBM – control PSA that was transferred into fresh MBM two times, then had 1mL of it transferred into new MBM for the third time. This shows that the changes in PSA caused by CSE exposure, as seen by the impeded growth in CSEx3, may not be permanent; in fact, CSE-PSA grows similar to control PSA once transferred back into MBM. However, there was a notable difference in the growth rate of CSEx3 and CSEx1. This shows that multiple CSE exposure inhibits PSA growth to a greater extent than does a one-time CSE exposure, demonstrating that changes induced by once daily exposure to CSE are present on the third (many generations after the original CSE exposures) exposure. This supports our belief that CSE exposure causes heritable changes, which can still be detected after the stress is removed even if requiring the re-application of the

stressor to be detected.

Cigarette smoke exposure increases PSA virulence in a mouse model of pneumonia.

In order to examine whether the cigarette smoke induced changes observed *in vitro* convey increased virulence, we used a well-established mouse model of pneumonia. CD-1 female mice were infected intranasally with control PSA and CSE-PSA. An equal initial inoculum of bacteria ($5x10^6$ CFU / 75uL) was administered into the left nare for both control PSA- and CSE-PSA-infected mice to ensure the initial bacterial counts in the lungs were equal between the two groups (confirmed by serial dilution and plating of the initial inoculum). Within 72 h of infection, 50% of CSE-PSA-infected mice died, while 0% of control PSA-infected mice died (n=7 for control group, n= 8 for CSE group) (Fig 5). By day 6 post-infection, 100% of CSE-PSA-infected mice were dead, while 28% of the control mice continued to live past day 10 after infection ($p = 0.045$). This difference in mortality confirms that CSE exposure makes PSA more aggressive than control PSA, ultimately supporting our hypothesis that cigarette smoke exposure increases the virulence of airway colonizer and pathogen PSA.

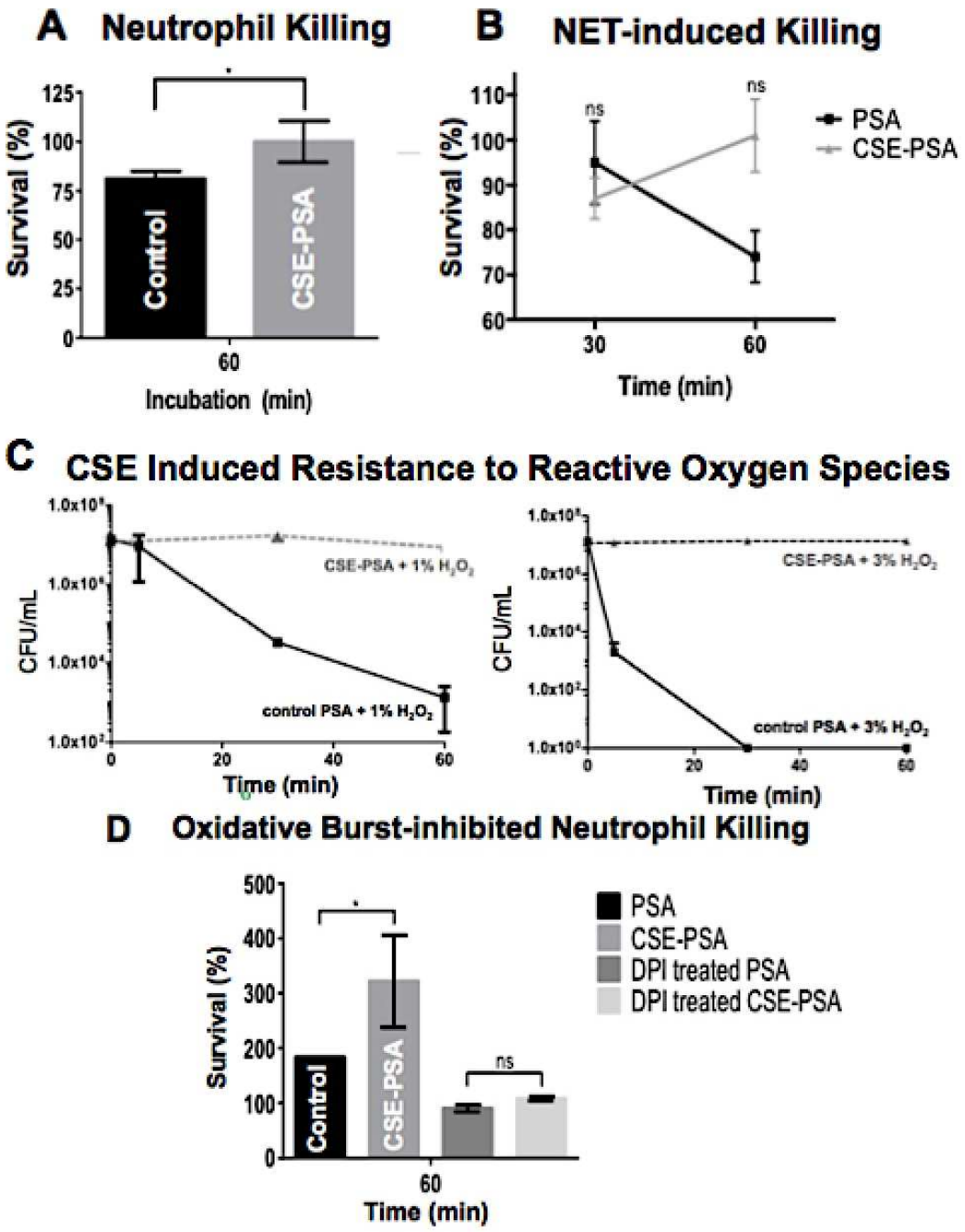

Figure 1. PSA becomes more resistant to killing via oxidative burst by human neutrophils after cigarette smoke exposure. (A) CSE-PSA resisted killing by neutrophils as seen by the increase in survival by CSE-PSA compared to Control-PSA. (B) This phenomenon was not due to higher resistance to killing by neutrophil extracellular traps (NETs). (C) H_2O_2 assays was performed with 1% and 3% H_2O_2 treatments, respectively. Treatment with 75% CSE increased PSA survival in the presence of reactive oxygen species (ROS). (D) Inhibition of oxidative burst via DPI treatment of neutrophils produced no difference in PSA and CSE-PSA survivals. This suggests that the increased survival of CSE-PSA observed in Fig. 2A is a result of higher resistance to killing by ROS.

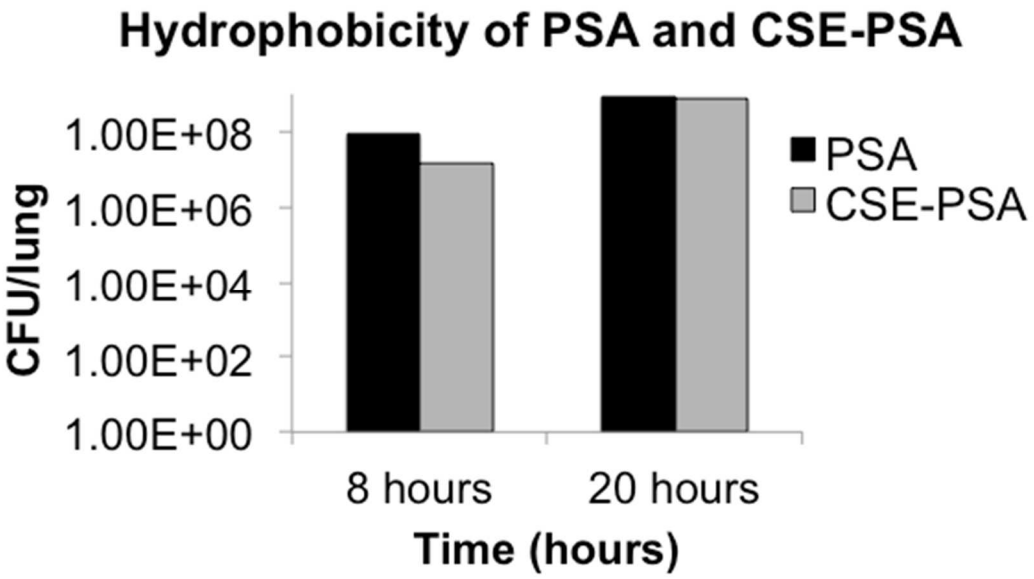

Figure 2. Cigarette smoke exposure does not induce changes in PSA hydrophobicity.

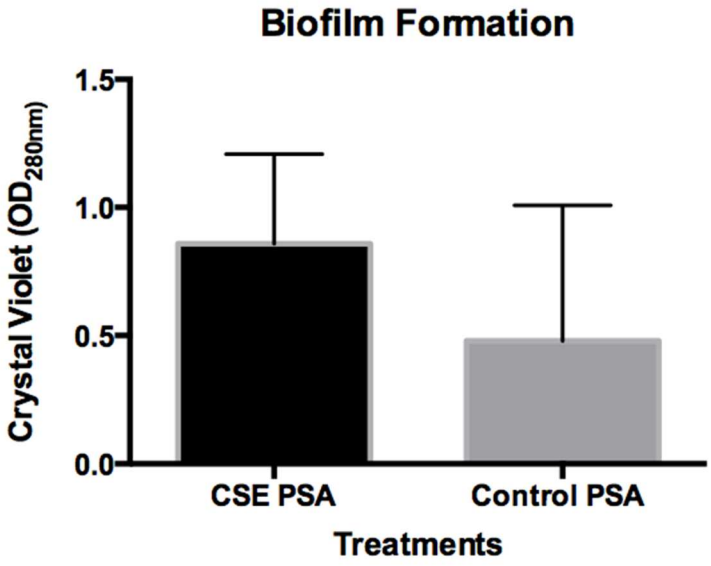

Figure 3. Cigarette smoke exposure does not alter PSA biofilm formation.

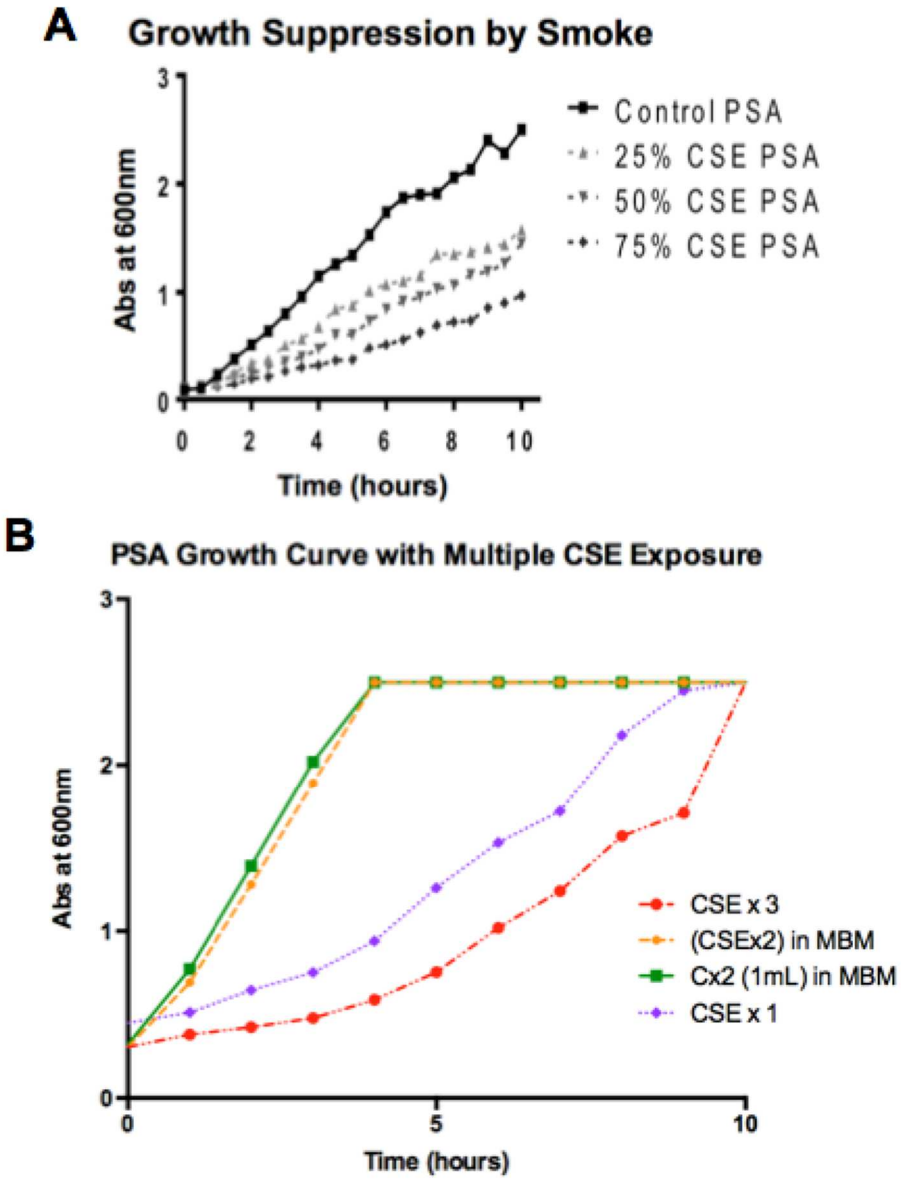

Figure 4. Cigarette smoke exposure acts as an environmental stressor and leads to dose-dependent inhibition of PSA growth. (A) PSA was cultured in MBM (Control PSA) or in MBM with increasing concentrations of cigarette smoke extract (CSE-PSA). Following agitation at 37ºC, A_{600nm} of control-PSA and CSE-PSA were measured every hour for 10 hours. (B) Three day long, multiple CSE exposure growth curve. PSA was exposed to CSE once (CSEx1), twice and transferred to MBM (CSEx2 in MBM), or three times (CSEx3) and grown with agitation at 37ºC. Cx2 indicates control PSA that was transferred into fresh MBM twice. On the day of the growth curve measurement, 1mL of control PSA (Cx2 1mL in MBM) was transferred into fresh MBM and grown with agitation at 37ºC.

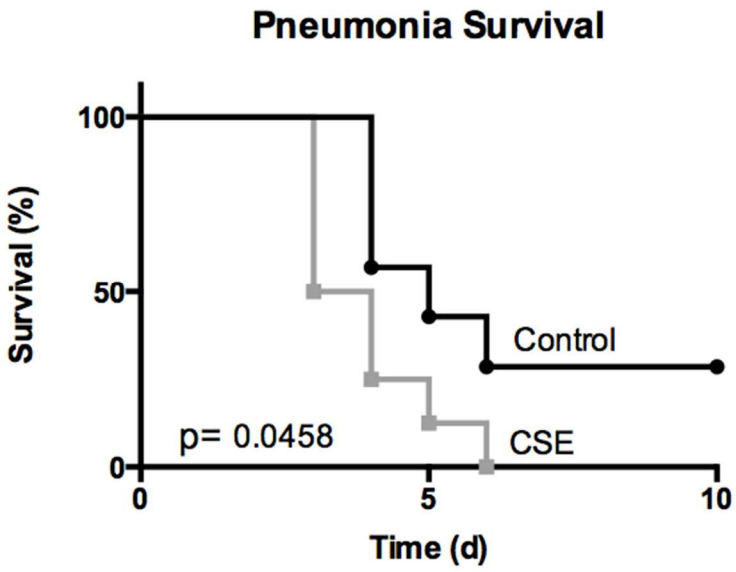

Figure 5. PSA exposed to cigarette smoke displays increased virulence in a mouse model of pneumonia. In a mortality model of pneumonia, 100% of mice infected with CSE-PSA died within 6 days, while 28% of mice infected with control PSA survived (n=7 for control group, n= 8 for CSE group) (p = 0.0458).

DISCUSSION

Our studies show that cigarette smoke exposure increases PSA resistance to neutrophil killing. Furthermore, by subjecting PSA to NETs, H_2O_2, and DPI, we were able to confirm that this observation was specifically due to the increased resistance to ROS during oxidative burst (Fig1). It is well recognized that cigarette smoke impedes innate immune responses, and consequently facilitates pathogenic proliferation and persistence in the airways of patients with pulmonary diseases like COPD[19]. Taken together, this may explain why PSA is difficult to clear in the airway of smokers – one of the main mechanisms used by neutrophils to kill and clear bacteria is inhibited, making them less able to eliminate PSA colonies in the airway.

Several things could be happening to the PSA defense mechanisms to change its resistance to ROS. There are a number of experiments to perform to follow up on the results obtained thus far, to better understand the specific mechanisms by which PSA becomes more resistant to oxidative burst. Our studies indicate that the increased resistance to neutrophil killing observed in CSE-PSA is not a result of changes on the bacterial surface, as seen by the unchanged hydrophobicity between CSE-PSA and control-PSA in Fig 2, but rather of the internal changes in PSA. Understanding the cigarette smoke induced changes on the genetic level will be essential to confirm this belief. Past studies have shown that cigarette smoke induced alterations in gene expressions can enhance the virulence of pathogenic bacteria. Our lab has previously discovered that cigarette smoke increases the expression of certain genes associated with cell surface changes in MRSA. For example, MRSA exposed to CSE displayed upregulated *mprF*, a gene responsible for encoding membrane proteins that facilitate

the modification of staphylococcal surface charge from negative to positive. This may account for the increased MRSA resistance to killing by AMPs upon CSE exposure[24]. In addition, Kulkarni and his team found that cigarette smoke exposure potentiates the upregulation of crucial virulence genes, such as those encoding surface adhesins (i.e. fnbA, fnbB, clfB) and proteins that help evade bactericidal functions of the host immune responses (i.e. staphylocoagulase, nuclease, Staphylococcal protein A). Such alterations augment MRSA resistance to phagocytosis and NET-mediated killing[34]. In analyzing our theory that the observed CSE-PSA resistance to neutrophil killing is caused by internal changes rather than changes on the bacterial surface, we hope to use qRT-PCR to observe which genes are up- or down-regulated as a result of cigarette smoke exposure, and ultimately validate the genes consistent with the altered CSE-PSA phenotypes in Fig 2. We expect to see an upregulation of ROS-scavenging enzymes, such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx)[35,36], in cigarette smoke exposed PSA.

Furthermore, a bacterial metagenomic study by Sapkota and her team discovered that cigarettes contain 15 different classes of bacteria and a wide variety of potential pathogens, including *Pseudomonas aeruginosa*[37]. In addition, consistent exposure to cigarette smoke has been linked with altered microfloral populations in the nasopharynx, which promotes colonization by opportunistic pathogens, such as *Streptococcus pneumonia, Haemophilus influenza*, and more[38]. A future goal of our study could be to look further into the differences in the airway microbiome of smokers and nonsmokers, such as the differences in the colonization rates of the different bacterial strains present in the two subject groups.

To take this further, we can explore ways to determine which specific components of the cigarette smoke are responsible for driving the bacteria to become more resistant to ROS and neutrophil killing. One way to address this question is by trying to isolate and remove such component(s) during the cigarette smoke exposure process, and check for altered phenotypes in PSA thereafter. For instance, cigarette smoke consists of over 4,000 chemicals and toxins; more specifically, it contains and generates populations of radical oxygen species like hydrogen peroxide and hydroxyl radicals[39]. It is possible that the ROS inside the cigarette smoke is responsible for inducing changes within PSA that make it more impervious to neutrophil killing. To test this, we can infuse CSE with antioxidants, or reducing agents that neutralize free radicals by donating electrons to inhibit them from becoming oxidized[40], before inoculating it with PSA. If this changes the phenotype of CSE-PSA to become more similar to that of control-PSA, it would suggest that the ROS present in cigarette smoke may also play a role in protecting the bacteria against neutrophil killing. Other tests to determine which components of cigarette smoke drive the changes seen in CSE-PSA are necessary in understanding *how* the bacteria are changing to become more resilient again ROS.

In this study, we concluded that cigarette smoke exposure *does* lead to significant changes in PSA pathogenicity and virulence phenotypes. One caveat to our experiment is that our cigarette smoke exposures were all done in tissue culture. The most ideal system would be able to expose the bacteria to cigarette smoke in a physiological setting. This is challenging, however, as we cannot affect the bacteria without affecting and stimulating the host defenses as well, making it difficult to attest

whether any potential differences induced by CSE exposure are a result of changes within the host or pathogen defense mechanisms. One method by which we can attempt to account for this is by collecting PSA strains from the airways of smokers and nonsmokers, and examining them *in vitro* via the methodologies described in this thesis. Assessing whether PSA from smokers exhibit increased resistance to ROS relative to their nonsmoker counterparts, for instance, could further substantiate the conclusions of this study.

In conclusion, the aforementioned studies and future experiments will help elucidate the mechanisms underlying the increased virulence observed in PSA exposed to cigarette smoke, and more specifically, its resistance to killing by human neutrophils.

Material presented in this Master's thesis contains information currently being prepared for submission for publication in Ahn, Sae Jeong; Hwang, Johnny; Crotty Alexander, Laura.

MATERIAL AND METHODS

Preparation of CSE- and control-PSA cultures.

10mL of control mammalian base media (MBM) (RPMI + 10% FBS + 20% LB) and 10mL of 75% CSE (7.5mL of 100% CSE made in MBM + 2.5mL MBM) were prepared. 75% CSE was inoculated with overnight PSA (PA01 strain) at 1:10 (9mL of 75% CSE + 1mL overnight PSA culture), and control tubes at 1:20, 1:50, and 1:100. Tubes were incubated at 37° C with shaking (225rpm) until grown to mid-log phase (OD_{600} = 1.2-1.4). A control tube with OD closest to that of CSE was chosen.

To prepare the bacterial slurry, CSE-PSA and control PSA were transferred into their respective 50mL conical tubes, washed with PBS, then centrifuged at 3200rpm for 8 minutes (4° C). This step was repeated. After the second wash, the supernatant was discarded from each tube, and each pellet was resuspended in 400uL PBS; this is the bacterial slurry. Two small test tubes were filled with 3mL PBS and both slurries were added to their own tube until OD_{600} = 1.2-1.4.

Neutrophil killing of bacteria.

CSE- and control-PSA subcultures, and respective bacterial slurries (OD = 1.0) were prepared as previously described. To isolate neutrophils, 25mL venous blood was drawn using a 30mL heparinized syringe. The 25mL blood in the syringe was layered on top of 25mL Polymorphprep™ in a 50mL Falcon tube without mixing the layers. The blood was run slowly down the side of the tube, then centrifuged at 1,600rpm for 30 min at room temperature. The remaining plasma and mononuclear

cells were drawn out and discarded. The PMN layer was removed and transfered into a 50mL Falcon tube and the volume was brought up to 50mL with 1X DPBS and centrifuged at 1,600rpm for 10 min. After discarding the supernantant, 5mL of molecular grade water was added and mixed via pipetting up and down to lyse red blood cells (RBCs). The volume was then immediately brought up to 50mL with 1X DPBS and centrifuged at 1,600rpm for 10 min. If the resulting pellet was pink in color, the RBC lysing process was repeated once more. Once the pellet was off-white in color, the supernatant was aspirated and discarded, and the pellet was resuspended in 1000uL 1X DPBS by pipetting up and down.

For hemocytometer count, PMN was diluted 1:20 (10uL PMN + 90uL 1X DPBS + 100uL trypan blue). A clean cover slip was placed over the clean hemocytometer and 10uL of PMN + trypan blue mixture was transferred into the groove. After calculating PMN/mL, neutrophils were prepared at 5 x 10^6 PMN/mL. 50uL/well was added to row A of a flat-bottom 96-well plate and 50uL RPMI+10%FBS to empty wells as growth control. Phorbol myristate acetate (PMA – activator of PMN antimicrobial pathways, including NETs) was prepared at 2X (1:320 dilution of freezer stock in RPMI+2%FBS). 50uL were added to all wells. The plate was 1) incubated for 20 min at 37°C with 5% CO_2, *or* for a pure-NET killing assay, 2) incubated for 3 hours at 37°C with 5% CO_2.

Bacterial slurry of OD = 0.7 was prepared in 3mL PBS as describe previously. This was diluted in RPMI 10% FBS to obtain 5 x 10^6 CFU/mL and centrifuged at 1,600rpm for 5 min. The supernatant was pulled off from cells/growth control wells and infected with 50uL bacteria. This was centrifuged at 1,600rpm for 10 min to

increase bacteria-PMN contact and incubated for 30 and 60 min at 37°C with 5% CO_2. 5uL of .25% Triton X 100-PBS was added to each well and the PSA:PMN mixture was serially diluted. 25uL of the bottom 3 dilutions were streaked out on LB plates and incubate at 37°C overnight.

H_2O_2 sensitivity assay.

CSE- and control-PSA subcultures were grown to OD_{600} = 1.2-1.4, as described previously.Two PBS-washes were performed with spins (3200rpm x8min) in between. Cacterial pellets were resuspended in 3mL of LB until OD = 0.7-1.0, and 200uL of resuspended pellets were added per well in a round-bottom 96 well plate, in triplicate for each peroxide dose. Appropriate concentrations of H_2O_2 were and placed on orbital shaker/rocker for 7, 14, and 21 minutes at room temperature. At each time point, multichannel pipettes were used to take 25uL from each well and transfer into a plate for serial dilution. Plate was placed back on shaker. Surviving CFU were enumerated by serial dilution and plating on LB. To plate, 10uL of sample was taken out from each well, plated on LB, and the plates were placed in an incubator (37°C) overnight.

Bacterial hydrophobicity assay.

Bacterial slurries of CSE- and control PSA were prepared with PBS as described. Slurries were added to two small test tubes containing 3mL LB to an OD_{600} of 1.2-1.4. 1mL of each was transferred into separate sterile 1.5mL Eppendorf tubes. N-hexadecane was added for final concentration of 20% (1mL bacteria + 250uL 100% n-hexadecane), vortexed for 2 minutes, and incubated at room temperature (on bench)

for 30 minutes. The lower aqueous (PBS) layers of CSE and control were transferred into fresh tubes, vortexed, and 25ul x3 of each was transferred into row (A) of a 96 well round-bottom plate containing 225uL PBS (3 wells of 25uL CSE aqueous layer + 3 wells of 25uL control aqueous layer). This was serially diluted down to the final row and plated on LB to enumerate the bacteria.

Biofilm formation assay.

On the first day of the experiment, CSE- and control-PSA subcultures were prepared. Bacterial slurries were made by resuspending the pellets in 400uL LB. Two small test tubes were filled with 3mL LB each, and bacterial slurry was added until absorbance ~ 0.7. This was diluted directly 1:100 in LB and 200 µl per well was transferred into 6 wells in the middle of a 96-well flat-bottom plate (one row of control PSA, one row of CSE-PSA). Outer rows of the plate were filled with PBS to minimize evaporation. The plate was incubated at 37°C with shaking for 24h.

On the second day, the cultures were aspirated and the wells were washed three times with 250 uL sterile PBS. Once the wells were dry, 200µl of 0.2% aqueous crystal violet solution was added into each well. The plate was incubated at room temperature for 15 min. PBS wash was performed three times. The crystal violet bound to the biofilm was extracted using an 80:20 (v/v) mixture of ethanol and acetone; 200uL of this mixture was added to each well and incubated for 15 min. After incubation, absorbance was measured at 280nm with a plate reader.

Bacterial growth curves.

Overnight PSA cultures were diluted 1:10 in 0%, 25%, 50%, and 75% CSE and incubated with shaking (37°C). Absorbance was measured at 600nm at 60 min intervals until the control reached the plateau phase.

For the multiple CSE exposure growth curve, 75% CSE and control MBM were prepared and inoculated with 1:10 overnight PSA culture in the morning. The cultures were allowed to grow throughout the day, then 1mL of both CSEx1 (PSA exposed to CSE once) and Cx1 (control PSA) were transferred into MBM and grown overnight. On the second morning, CSEx1 was transferred into fresh 75% CSE, and Cx1 into fresh MBM. The cultures were allowed to grow during the day and transferred into fresh MBM at night. In addition, fresh overnight PSA was prepared for a new CSEx1 for the next morning. This process was repeated again until CSEx3 was made on the third morning. The samples prepared for the multiple exposure growth curve include:

1) CSEx3: PSA exposed to CSE three times

2) CSEx1: PSA exposed to CSE one time

3) CSEx2 in MBM: PSA exposed to CSE two times, then transferred into fresh MBM on the third day

4) Cx2 in MBM: Control PSA transferred into fresh MBM each day

The samples were agitated at 37°C and OD_{600} were measured at 60 min intervals.

Murine pneumonia infection model.

Ketamine-xylazine was used to sedate 5-7 months old female CD-1 mice (Charles River). Mice were infected intranasally with 5 x 10^6 CFU PSA in 75uL. Mice were

kept upright for 1 min to ensure that the bacteria were taken in. They were recovered on heating pads. Mice were weighed every 24 h and their mortality rate was measured.

Statistical analyses.

All *in vitro* are representative of at least three replicate experiments, each of which was performed in triplicate. All averages, significance values (p value), t tests, etc. were analyzed using Prism. One-way analysis of variance (ANOVA) was used to analyze hydrophobicity and neutrophil killing. Some growth curves were analyzed with the Friedman test while others were graphed with Microsoft Excel. Resistance to H_2O_2 was analyzed with the Mann-Whitney test. Mouse pneumonia survival analysis was done via a Kaplan Meier survival curve.

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