INTERACTIONS OF *ASPERGILLUS FUMIGATUS* AND *PSEUDOMONAS AERUGINOSA* CONTRIBUTE TO RESPIRATORY DISEASE SEVERITY AND DEATH

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DOCTOR OF PHILOSOPHY

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ABSTRACT

The lung was recently identified to consist of a complex microenvironment made up of microorganisms that interact with one another and the host cells via direct and indirect interactions. As a result, understanding the dynamic of the microbiome in chronic respiratory diseases has become the focus of pulmonary researches. In cystic fibrosis (CF), chronic infections are a comorbidity associated with the genetic disorder. Recently, it was noted that the interactions of the fungus, *Aspergillus fumigatus*, and the bacterium, *Pseudomonas aeruginosa* together contribute to more severe disease outcomes in CF patients. *In vitro* co-cultures show that *P. aeruginosa* and *A. fumigatus* can affect one another's growth and pathogenicity, but very few studies have attempted to model interactions of these microorganisms *in vivo*. Based on clinical and basic research, we developed a co-exposure model in which we could compare non-allergic and allergic animals co-exposed to *Pseudomonas aeruginosa* and *Aspergillus fumigatus*. While both groups had significant neutrophilia and production of acute phase response cytokines and chemokines, the allergic co-exposed group had a greater mortality with 34.8% of the animals expiring by 24h in comparison to 12.5% for the non-allergic co-exposed animals and 100% survival in the controls. A contributing factor to the more severe disease outcomes in the allergic co-exposed group is the increase in eosinophilic inflammation and IL-17A production, which only occurs when both microorganisms are viable. In addition, it was found that viable *P. aeruginosa* but not *A. fumigatus* causes interstitial inflammation, significant neutrophilia, and even death during co-exposures. The decline in health of animals co-exposed to the fungus and bacteria could be attributed not only to the host's inflammatory response, but also to the spatial and temporal co-localization in the lung. To address this, we performed *in vitro* studies finding

an aggregation of the microorganisms that could also be identified *in vivo.* This current research emphasizes the need for *in vivo* studies on polymicrobial interactions.

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DEDICATION

To my mom, who instilled the curiosity of the scientific world early in my life and encourages creativity and hard work every day. She has been my sounding board and rock throughout my scientific career. When times were tough, she listened and when they were exciting, she shared in the excitement.

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LIST OF ABBREVIATIONS

1-HP 1-Hydroxyphenazine

ABPA .... Allergic bronchopulmonary aspergillosis

AHR .... Airway hyperresponsiveness

ALI .... Acute lung injury

AprA.. Alkaline protease

ARDS .... Acute respiratory distress syndrome

ATP .... Adenosine triphosphate

BAL.. Bronchoalveolar lavage

BALF.. Bronchoalveolar lavage fluid

cAMP .... Cyclic adenosine monophosphate

CCL .... Chemokine (C-C motif) ligand

CD .... Cluster of differentiation

CF.. Cystic fibrosis

COPD .... Chronic obstructive pulmonary disease

CXCL .... Chemokine (C-X-C motif) ligand

DNA .... Deoxyribonucleic acid

ECP .... Eosinophil cationic protein

EDN.. Eosinophil derived neurotoxin

EETs.. Eosinophil extracellular traps

EIA .... Enzyme immunoassay

EPO .... Eosinophil peroxidase

H&E .... Hematoxylin and eosin

HIES.. Hyper-IgE Syndrome

HPF .... High-powered field

ICAM .... Intracellular adhesion molecule-1

IFN .... Interferon

IH .... Inhalational

IL .... Interleukin

IN .... Intranasal

IP .... Intraperitoneal

ITS .... Internal transcribed spacer

LasB .... Elastase B

LPS .... Lipopolysaccharide

MBP .... Major basic protein

MCP-1 .... Monocyte chemoattractant protein-1

MPO .... Myeloperoxidase

NADH .... Nicotinamide adenine dinucleotide + hydrogen

NADPH .... Nicotinamide adenine dinucleotide phosphate + hydrogen

NETs .... Neutrophil extracellular traps

NLRP3 .... NACHT, LRR and PYD domains-containing protein 3

OTUs .... Operational taxonomic units

PBS .... Phosphate buffered saline

PCA .... Phenazine 1-carboxylic acid

Pch .... Pyochelin

PCN .... Phenazine 1-carboxamide

Pvd .... Pyoverdine

PYO .... Pyocyanin

PRR .... Pattern recognition receptor

rRNA .... Ribosomal ribonucleic acid

ROS .... Reactive oxygen species

SC.. Subcutaneous

SEM.. Scanning electron microscopy

SO.. Superoxide formation

TEM .... Transmission electron microscopy

TLR .... Toll-like receptor

TNF .... Tumor necrosis factor

VOCs .... Volatile organic compounds

1. GENERAL INTRODUCTION

1.1. Research Significance

Asthma is a chronic respiratory disease that affects over 300 million people worldwide (1-3). Many of these individuals suffer from allergic asthma, which is triggered by allergens, including molds, pollens, and animal dander. Individuals that are allergic to pet dander and food can control their symptoms as they can better avoid interactions with their allergens. Allergies to molds tend to result in poorly controlled responses as the microorganisms are ubiquitous in both indoor and outdoor environments making avoidance difficult (4-7). Poorly controlled severe asthma with fungal sensitization (SAFS) makes up approximately 10% of asthma cases globally (8). Noninvasive colonization with fungi, including *Aspergillus fumigatus*, is a major characteristic of this asthma subclassification (8,9).

A. fumigatus is a type of mold that is typically found growing on decaying organic materials (saprophytic), that has small conidia that are inhaled deep into the lung. To most that inhale the fungal spores, the innate immune response will adequately clear the spores and the individuals do not exhibit any signs or symptoms of exposure or respiratory disease. However, in patients with atopic asthma or impaired immune responses exposure to fungal spores can lead to a number of respiratory diseases, both noninvasive and invasive. While asthma is the main disease caused by *A. fumigatus*, this fungi is also responsible for invasive aspergillosis, which causes 40-90% mortality in immunocompromised individuals (10), and allergic bronchopulmonary aspergillosis (ABPA), where noninvasive colonization with atopy results in a complication of asthma as well as cystic fibrosis (CF) (11, 12). Interactions of *A. fumigatus* with other common respiratory microorganisms have been identified within the last few decades (13,14). However, it was not until the identification of the lung microbiome in 2010 (15-17), that

the interactions among microorganisms in respiratory disease gained consideration. Altered bacterial composition in the lung, specifically with an increase in proteobacteria, in respiratory diseases has resulted in a closer exploration of how microorganisms could be contributing to disease severity and mortality (15, 18-21).

Many *in vitro* and clinical studies have been published recently exploring the interactions of *A. fumigatus* with the bacterium, *Pseudomonas aeruginosa. P. aeruginosa* is an opportunistic, Gram negative bacterium of the proteobacteria family. It is most well-known for its colonization of CF lungs (22) and its role in hospital-acquired infections, such as ventilator-associated pneumonia (VAP) (23, 24). For several years, co-colonization of the lungs with *A. fumigatus* and *P. aeruginosa* has been recognized as increasing morbidity for patients, which contributes to an increased economic burden and (25-28). Yet, it has been relatively recently that *in vitro* studies have focused on the growth (29-31) and virulence (30, 32) of these microorganisms together and there have been few publications focusing on *in vivo* interactions of these two microorganisms (30, 33, 34).

1.2. Experimental Design

In this work, we sought to study the interactions of two common opportunistic pathogens of the human lung—*A. fumigatus* and *P. aeruginosa*—to begin to characterize how the complex interaction, not only between the pathogen and host but also among the microbiota of the lung affect the initiation and maintenance of a chronic disease, in this case allergic asthma. We utilized antigenic extracts from *Aspergillus fumigatus* to sensitize animals to the fungus followed by two nose-only inhalational exposures to the live conidia of *A. fumigatus.* Intranasal exposure to *P. aeruginosa* directly followed the second inhalational exposure to the conidia. We also conducted experiments on non-allergic animals that were not sensitized to *A. fumigatus* antigens

but had the same acute exposures to the live fungal conidia and bacteria. ***Our central hypothesis is that the spatial and temporal co-exposure to* Aspergillus fumigatus *and* Pseudomonas aeruginosa *results in microbial synergy that damages lung tissue or causes immune dysfunction resulting in increased morbidity***.

To address this hypothesis, we established the following three aims:

- Aim 1: Establish a co-exposure model and identify the extent to which allergy contributes to increased disease severity (Chapter 3).
- Aim 2: Determine whether microbial viability is required to initiate inflammatory responses and the extent to which microbial viability impacts disease outcomes (Chapter 4).
- Aim 3: Characterize the physical interactions between the microbial populations *in vitro* and determine if similar interactions occur *in vivo.*

1.3. Project Impact

Colonization with a combination of *A. fumigatus* and *P. aeruginosa* are recognized as contributory to more respiratory disease severity, particularly in CF, where ABPA and chronic infections by *P. aeruginosa* are co-morbidities (25). However, very few models have been established to study the interaction of these microorganisms *in vivo* (30, 33, 34). While the experimental model used in this research project is not a CF model, valuable information may still be gained from this work regarding the interactions of these microorganisms in pulmonary disease. This includes insight into why allergy to *A. fumigatus* antigens with subsequent co-exposure to live *A. fumigatus* and *P. aeruginosa* organisms results in such severe disease outcomes in CF. The versatility of this model will also provide avenues for future polymicrobial

studies. Clinically, the outcomes of these studies may inform current health care practices and establish better treatment options.

1.4. Organization of the Dissertation

This dissertation highlights microbe-microbe interactions as well as the influence that polymicrobial exposures have on host inflammatory responses. The literature review provides the reader with our current understandings of the lung microbiome/mycobiome as well as the pathogenesis of *A. fumigatus* and *P. aeruginosa* in respiratory disease. This dissertation also includes clinical and basic research accounts of interactions of *A. fumigatus* and *P. aeruginosa* and their role in disease severity. Granulocytic impacts on disease severity, specifically acute lung injury, are also included in the literature review section.

Subsequently, an account of the individual research projects and the discoveries made are reported in the format of journal manuscripts. Each manuscript is a stand-alone document that contains an introduction, methods, results, discussion, and references. At the end of the dissertation is a combined outlook on the research findings and their subsequent influence on future research projects.

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2. LITERATURE REVIEW

2.1. Microbial Composition of the Lung Changes in Disease

The microbiological community is driven to identify the microorganisms that can be naturally found in all body systems and to identify potential changes that can occur in disease. The Human Microbiome Project motivated researchers to identify the microbial communities of the body at a genetic level, also known as the microbiome, and culturing microorganisms to determine the living microbiota present in specific locations, like that of the skin, urogenital tract, and the gastrointestinal tract (1, 2).

Initial efforts of the Human Microbiome Project did not include an identification of the microbiome of the lower respiratory tract as it was considered a sterile environment. This Project has since become more inclusive to include the lung and identify fungal microorganisms, classified as the mycobiome (3-5). As our understanding of this topic grows, so does our understanding of disease development and progression.

2.1.1. Challenges in Identifying the Lung Microbiome

One of the biggest challenges associated with accurately characterizing the human lung microbiome is the sampling methods used to isolate microbial populations. Sampling of the lower respiratory tract requires that equipment be passed either through the nasal or oral passage, both of which have distinct populations of microorganisms that could contaminate the lower respiratory sample (6-11). Some of the most common methods include sputum collection, protected specimen brushing, and bronchoalveolar lavage (BAL). While sputum collection is still believed to be an inaccurate means of sample collection, minimal contamination of BAL and protected specimen brushings by the upper respiratory tract has been found (9, 11). The method of sample collection isn't the only avenue for specimen contamination, as some research groups

found that bacterial genetic material could still be sequenced from reagents that were "sterile" (12, 13). Redundancy and appropriate controls are required for this work, and research groups with the lung microbiome as their focus use multiple methods to validate their results.

Another difficulty associated with identifying lung microbiota involves the characterization of viable, but not culturable, bacteria. While most research groups sequence 16S rRNA, there are some that still rely on culturable methods to determine or validate microbial populations. The inability to culture live organisms from lung samples have been reflected in some publications as evidence that the microbiome is not associated with disease (14). However, most current literature regarding this subject refutes its credibility using genetic sequencing of various lung samples from the healthy to the diseased, proving the presence of specific microbial genetic components (6, 15, 16).

As researchers delve even further into attempting to understand the diversity of the lung microenvironment, they have also started to look in the mycobiome to explore the fungal makeup of the lung. Fungal organisms are often more difficult to extract genetic information from so accuracy of the common citizens of the mycobiome and their frequency is still somewhat questionable. The current mycobiome studies involve validation of methods in addition to the identification of fungal phyla (5, 17-22).

2.1.2. Identifying the Microbial Composition of the Respiratory Tract

The lung has a complex microenvironment consisting of microorganisms and host cells, in addition to numerous proteins, carbohydrates, lipids, and nucleic acids. Changes in this microenvironment, especially that of the microbiome, can have impacts on disease susceptibility or be the result of diseases. Whether changes to the microbiome contribute to the establishment

of disease or vice versa, the bacterial and fungal microbiomes are associated with a shift from health to disease states in the lung (6, 7, 15-17, 23-26).

Foundational research on the lung microbiome found that both healthy children and adults harbor both Bacteroidetes (*Prevotella* spp.) and Firmicutes (*Streptococcus* spp.) as the most highly represented proportion of phyla in the lung (6) . The formation of the respiratory microbiome can be explained through a neutral model that looks at the dispersal of species from the surroundings, including the oral and nasopharyngeal tracts, resulting in ecological drift. This neutral model found that the upper respiratory tract, especially the nasopharyngeal region, is essential to the development of the lung microbiome (27). Redundant sampling techniques, including BAL and protected specimen brushings were used to show that the upper respiratory regions were distinctly different than the samplings of the lower respiratory tract. This led to the conclusion that BAL and protected specimen brushing methods minimally impact the results (11, 13). These studies reveal that healthy lungs have a diverse population of bacteria, and the phylum Bacteroidetes is necessary for maintaining a healthy lung (6, 28).

In disease states, there appears to be an imbalance, or dysbiosis, of the phyla represented in the lung and reduced diversity (6, 7, 9, 15, 16, 29). Asthmatic and COPD patients tend to have a shift from predominantly Bacteroidetes to Proteobacteria, which has major repercussions on the frequency of exacerbations and disease severity (6, 7, 9, 15, 16, 24-26, 30, 31). For instance, it was found that patients that have asthma and a shift to a Proteobacteria-dominant microbiome have increases in airway hypersensitivity responses and bacterial burden (15). Changes in the microbiome may occur prior to the development of asthma, potentially as early as infancy, with primary changes to the nasopharyngeal region resulting in an ecological shift of potentially pathogenic bacteria to the lungs (8).

While the discovery and appreciation of the human lung microbiome is important as a means in which to study and identify lung disease, few studies have addressed the appropriateness of the microbial lung composition of rodents as an experimental model for human disease. One such study does show a variability in the mouse versus human lung microbiomes. In this work, the dominant phyla in the mouse lung include Proteobacteria and Firmicutes (32). In comparison, Proteobacteria is more commonly associated with human disease when present in large quantities, while Bacteroidetes are usually found in healthy lungs (6, 7, 15, 16, 24-26, 30, 31, 33). With the lungs of mice closely reflecting the bacterial makeup of the diseased human lung, they may be an ideal system in which to research human respiratory illnesses as they pertain to microbial interaction and host-pathogen-pathogen interactions.

With the growing understanding of the implications of the lung microbiome in disease, we must consider other microbial communities, including that of fungal organisms. Fungal microorganisms have been implicated in respiratory disease conditions including *Aspergillus fumigatus* in severe allergic asthma and CF (34-40). However, are certain fungal populations needed for the normal functioning and health of the lung? Current understandings of the mycobiome in respiratory diseases are based off of small sample sizes though they provide a foundational account of both healthy and diseased respiratory mycobiomes (5, 17-19, 21, 22). Using samples collected via oral wash, induced sputum, and BAL, an initial mycobiome of the healthy lung has been identified with 90% of the internal transcribed spacer (ITS) reads represented by *Candida,* a member of the phylum Ascomycota. It was also identified that many operational taxonomic units (OTUs) were shared between the oral and lung communities, though there were some fungal species that were more abundant in the BAL or induced sputum samples in comparison to the oral washes (19).

The progress in identifying the mycobiome during different disease states is in its infancy. Many of the current studies available on this topic have focused on techniques for enhancing the mycobiome detection. Redundant testing of oral washes, induced sputum, and BAL were done to determine if there is a risk of the oral mycobiome contaminating the lung mycobiome samples (51). Another study tested propidium monoazide pretreatment of the samples to eliminate dead or damaged cells. This study found that the most active fungus is during acute exacerbations of CF, which led them to conclude that the mycobiome is made up of environmental fungi and is a transient population (18). The concept of a transient mycobiome was also supported in the work by Kramer et al. in which a cohort of CF patients under constant antibiotic treatment versus those that are stable (nonexacerbation) showed high fungal fluctuations over time (17). A more recent study utilized sputum samples that were culture positive or negative for *Blastomyces dermatitidis/gilchristii* to validate their next generation sequencing techniques for mycobiome studies (22). This study shows great promise as the technique could also detect other common fungal organisms, including the genus *Aspergillus.*

As mentioned previously, *Aspergillus* has species that are associated with allergic airway diseases and a co-morbidity during CF (8-12). Therefore, characterization of the phyla Ascomycota, which includes *Aspergillus*, was completed using sputum samples from CF patients. characterization of the fungal communities was completed showing Ascomycota dominating the sputum samples (17, 23). It was found that there were only 2.7% positive samples for *Aspergillus* (17). Unfortunately, neither of these studies compared their samples with a healthy mycobiome (17, 23). There are still many facets of this topic that have not been well addressed. With many new articles on techniques for differentiating the lung mycobiome, hopefully a more distinguished mycobiome can be identified and characterized (21, 22).

2.2. The Pathogenesis of *Pseudomonas aeruginosa*

As we have recently discovered, the lung is not a sterile environment making it quite unlikely that infections occur with just one microorganism involved. Yet much of the research up to this point focuses on the impact of individual microorganisms on respiratory function and disease. Building on our knowledge of the lung microbiome and its dysbiosis in disease, we explored the effect of the common respiratory microorganism and pathogen *P. aeruginosa* on the dysbiosis of the lung and the impact on disease severity.

P. aeruginosa, a Gram negative, rod-shaped bacterium is associated with chronic colonization in CF and nosocomial infections of the lung. As a member of the phylum Proteobacteria, this microorganism has been found as a part of the lung microbiome in diseased states (7, 10, 24). Though infrequently mentioned at a species level in microbiome research, studying this microorganism in a changing lung microenvironment is of upmost importance because this bacterium has numerous virulence factors and immune evasion techniques, is multidrug resistant, and it has a well-known role in respiratory disease (41, 42).

2.2.1. Factors That Contribute to Disease Severity in *P. aeruginosa* Infections

From the production of secondary metabolites to immune evasion techniques, *P. aeruginosa* has adapted several virulence factors that have been found to damage the lung microenvironment and perpetuate the infection. These virulence factors facilitate cell-to-cell communication, otherwise known as quorum sensing, impairing innate immune barriers and cellular functions resulting in systemic tissue damage (41, 42). Studies focusing on these virulence factors have implemented both *in vitro* and *in vivo* research to characterize their effect on disease states.

2.2.1.1. Phenazines and the Lung Microenvironment

P. aeruginosa secretes numerous chemicals and proteins involved in its pathogenesis (41, 42). Some of the most well studied secreted factors are the phenazines. Phenazines, including pyocyanin (PYO), phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), and 1-hydroxyphenazine (1-HP), are quorum sensing molecules produced by *P. aeruginosa* as virulence factors that interact with mammalian cells in numerous ways (Figure 2.1). PYO is the most characterized phenazine produced by *P. aeruginosa* and is a redox molecule that when reduced, produces no pigment. Upon oxidation, PYO forms superoxide radicals and is blue pigmented (43, 44). In clinical accounts of *P. aeruginosa* infections, the presence of these phenazines are associated with disease severity, especially that of PYO (45, 46).

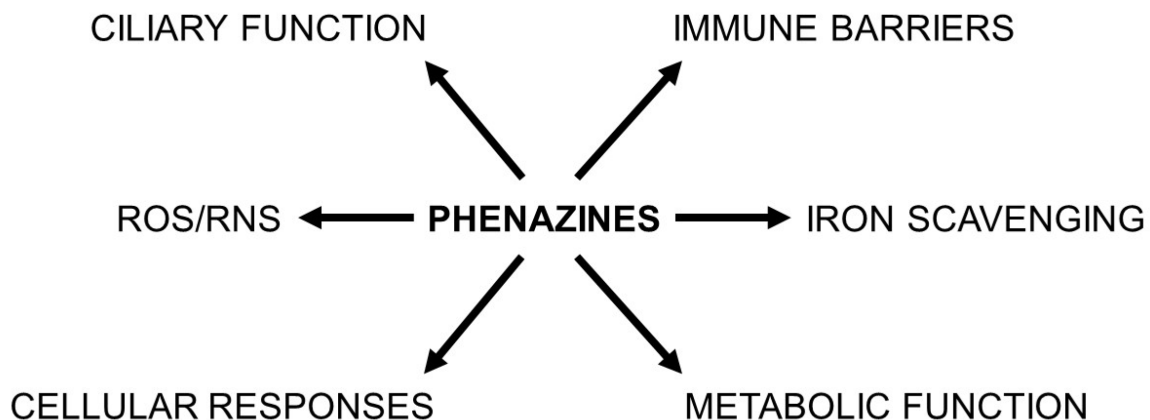

Figure 2.1. Phenazine interactions with mammalian hosts
Phenazines have roles in numerous host functions including immunomodulatory and cellular functions.

PYO is small (210 g/mol) and able to cross host membranes allowing for its interaction with NADH and NADPH where it accepts and donates electrons (47, 48). The result is the formation of reactive oxygen species (ROS), which can damage the host tissue through oxidative stress and perpetuate the survival of the bacterium in oxygen-poor environments (49). The location of superoxide (SO) formation by PYO was recently found to be primarily at the

mitochondrial level of the host cell though there was some evidence that suggested SO production could occur in the cytosol of cells likely mediated by PYO's oxidation of NADH or NADPH (48). Both the cytosol and the mitochondria contribute to ATP production via the citric acid cycle and the electron transport chain and is necessary for cellular survival. PYO is able to inhibit aconitase activity and reduce ATP production resulting in cellular death (50). The impact of oxidative stress by PYO on the host has been studied extensively, with implications in inhibiting the innate host response and the death of neutrophils (51).

The stimulation of IL-8 production by PYO is important as it is a chemoattractant for the neutrophil, which is an innate immune cell. While one may consider the recruitment of the neutrophil to the area of infection to be a disadvantage for the sustainability of a *P. aeruginosa* infection, PYO will trigger an accelerated apoptosis in the neutrophils, making them ineffective at clearing the microorganisms (51, 52). Death of neutrophils by PYO occur through the production of ROS in the mitochondria mediating the release of cytochrome *c* and requires mitochondrial acid sphingomyelinase (48). Clearance of these apoptotic neutrophils is also impaired by phenazine production as there is a reduction in phagocytic activity of macrophages, which can be shown both *in vitro* and *in vivo*. This can be attributed to PYO's ROS production stimulating an increase in the activity of Rho, which inhibits the phagocytosis of apoptotic cells (53). The implication of being unable to clear apoptotic neutrophils is the release of DNA and the leaking of proteases, like neutrophil elastase, that could contribute to increased host tissue injury and allow for an unchecked spread of the bacteria into the tissue.

Mucus in the lung will trap microorganisms and expel them with the movement of the cilia up and out of the lower respiratory tract. Inhibition of ciliary beat is of great importance to understanding the pathogenicity of *P. aeruginosa*. Damaging this mechanism offers an initial

evasion of the host's innate immune response and allows for the bacteria to persist in the lung. PYO and 1-HP are involved in the inhibition of ciliary function (45, 54, 55). Acute PYO exposure results in a dose-dependent gradual disruption of ciliary beat with no recovery, while acute exposure to 1-HP results in a dose-dependent rapid decrease in ciliary function though ciliary beat which may be recovered with time (54).

The reduction of ciliary function may contribute to the initial establishment of bacterial infection in the lung, but PYO may also act to impair the inflammatory response by limiting the host neutrophilic response (56-58). Interleukin-8 (IL-8) is a chemokine produced by airway epithelial cells that is released in response to bacterial detection or tissue damage. It quickly recruits neutrophils from the bone marrow as a first line of cellular innate immunity. As expected, an increase in IL-8 is associated with increased exposure to the bacterial product PYO (56-58). However, while levels of PYO between 5-100 μM were associated with increased IL-8 levels, concentrations exceeding 100 μM had the opposite effect, resulting in lower levels of IL-8. Interactions of PYO with tumor necrosis factor (TNF) may also contribute to the inhibition of the macrophage/lymphocytic chemokine (C-C motif) ligand 5 (CCL5), previously known as RANTES (56). Later, it was also found that PYO and PCA can coordinate the increased production of IL-8 and the cluster of differentiation 54 (CD54) otherwise known as intercellular adhesion molecule 1 (ICAM-1), both of which are essential in the emigration of cells during the inflammatory response (58). Another aspect of the inflammatory response that may be impacted by PYO production is the regulation of inflammatory cytokines, like IL-1β, by the Nod-like receptor protein 3 (NLRP3) inflammasome (59, 60).

Inflammasomes, which recognize danger signals, can trigger proinflammatory activities by the production of caspase-1 and -5 that proteolytically cleave cytokines to their active states.

The NLRP3 inflammasome is activated by a diverse array of stimuli making it one of the most versatile inflammasomes and is homologous in mice and humans (59). As previously discussed, PYO triggers the production of ROS and it can also induce reactive nitrogen species (RNS). When exploring the impact that these reactive species have on the inflammasome formation, Virreira-Winter and Zychlinsky found that induction of excessive ROS/RNS production silenced the NLRP3 inflammasome activity (60). By affecting the chemokine and cytokine production, the cellular response of the innate immune system may likewise be impacted. This is especially the case as it seems that by producing PYO, *P. aeruginosa* directs the immune response to be predominantly neutrophilic.

While most of the studies that have focused on phenazine production have utilized set concentrations of PYO, *P. aeruginosa* strains produce phenazines under different durations of time and result in different concentrations (61-63). A recent study compared the genome and transcriptome of ATCC 27853 with a variety of *P. aeruginosa* strains, including clinical isolates and commonly used research strains like PAO1. When considering phenazine biosynthesis in ATCC 27853 and PAO1, this study found that the genes associated with these pathways are similar, but the gene clusters are preceded by different genomic architecture that could contribute to the processing of the phenazines. When testing productions of phenazines between the two strains, the ATCC 27853 strain has a higher level of PYO production in comparison to PAO1 (61). The higher PYO level and other genomic variations in the ATCC 27853 strain suggests that it is more virulent than PAO1, which has historically been used to study infections by *P. aeruginosa* and may support the study of the ATCC 27853 clinical isolate in more disease models.

Currently, PA14 and PAO1 are the most frequently used strains of *P. aeruginosa* when studying disease. They have varying virulence in mammalian hosts, with PA14 displaying higher virulence, which was found to be due in part to a mutation in *ladS* . The result is an increase in the type three secretion system and a decrease in biofilm formation (64). The variability in these strains demonstrates a need to study more than just one strain when attempting to understand the progression of respiratory diseases.

2.2.1.2. Protease Activity of **P. aeruginosa** *and Immune Dysfunction*

The production of proteases by *P. aeruginosa* has implications in immune dysfunction and damage to the lung microenvironment that allows for establishment and dissemination of *P. aeruginosa*. While effective on their own, in some cases the protease will work in concert with other virulence factors, including other proteases or phenazines, to have even greater host damaging effects.

Elastase B (LasB) and alkaline protease (AprA) are zinc-dependent metalloproteases that cleave numerous human proteins during infection including the cytokines interferon-ɣ (IFN-ɣ), TNF-α, IL-6, and IL-8 (65-68). These cytokines are key to the initiation of the host immune response to invading microorganisms. They are involved in the fever response, vasodilation, neutrophil chemotaxis, and overall regulation of the immune cell response. By eliminating these cytokines from the region of infection, *P. aeruginosa* can fly under the radar and establish itself. Additionally, Las B has been found to degrade the pulmonary surfactant protein-A, which plays a role in the opsonization of *P. aeruginosa* thus resulting in diminished phagocytosis of the bacterium (69). In addition to modulating the host's immune response by cleaving cytokines and barrier proteins, these proteases also have a role in host evasion by degrading flagellin (70).

Flagellin is a building block of flagella that is released by the bacterium during times of exponential growth as the tail of the flagellum is sensitive to damage. Toll-like receptor 5 (TLR5), a host pattern recognition receptor (PRR), is specifically activated by flagellin and leads to a strong inflammatory response that results in the elimination of the bacteria from the host (71, 72). In order to prevent detection by the host's immune response, *P. aeruginosa* has adapted its proteases to degrade the monomeric flagellin. Both AprA and LasB have been implicated in the manipulation of the bacterium's flagella resulting in an evasion of the host's immune response (70, 73). While degradation of the flagellin aids in the initial evasion of the host immune response, complete degradation of the flagellum is not beneficial to the bacterium. Polymerized flagellin that compose the flagellar filaments cannot be cleaved by AprA, which was further substantiated by depolymerizing the filaments and showing the degradation of the resulting monomeric flagellin (73). These findings show that the flagellum can remain intact and the microorganism can still evade host immune response if the potent immune stimulator, monomeric flagellin, is degraded.

While these functions of protease action on host cytokines and bacterial flagellin have similar roles in aiding the evasion of the host immune response, AprA and LasB will also cause significant structural damage in the host tissue allowing for the spread of the microorganisms past immune barriers. The epithelium lining the respiratory tract is one of the first barriers that the bacterium will encounter. LasB weakens the physical barrier by disrupting the tight junctions and increasing permeability between lung epithelial cells, allowing for *P. aeruginosa* invasion (74). Continual protease production by *P. aeruginosa* may alter cell migratory paths and proliferation processes dampening or even preventing the host's ability to repair respiratory tissue damage (68, 75, 76). The study by Ruffin et al used knockout strains of *P. aeruginosa* or a

LasB protease inhibitor that revealed bacterial proteases, including LasB, inhibit tissue repair. When directly exposed to the protease, LasB, damaged cells have a reduced ability to stimulate their repair. However, the repair function was restored when the same cells were also exposed to the quorum-sensing inhibitor associated with LasB (76).

Along with the abilities of the proteases themselves causing significant tissue damage, they can also stimulate the production of other secondary metabolites like phenazines that will damage the host (77). Production of the phenazine, PYO, may be attributed to the function of AprA. Initial studies examining the interaction of PYO and AprA found that AprA did not adversely impact PYO production and in fact, overexpression of AprA resulted in increased PYO perhaps associated with the release of peptides and amino acids that result when proteins are cleaved (77).

2.2.1.3. Iron Acquisition

Bacteria often require iron for growth. One way that the host counters the spread of the bacteria is to limit the bacterium's access to iron (78, 79). As such, successful bacterial systems have acquired means by which to survive environments that have little access to free iron including siderophore production, stealing other microbial siderophores, and the use of phenazines (79-91).

Iron is essential for the virulence of *P. aeruginosa*, making it more invasive and lethal (79, 81, 82). *P. aeruginosa* has numerous mechanisms by which it can access iron during infection with the most common being the production of the siderophores, pyochelin (Pch) and pyoverdine (Pvd) (83, 84). In the host, most iron is bound by heme or transport molecules like transferrin or lactoferrin (85). The production of siderophores is highly regulated via *fur* (80, 86). When the environment is iron limiting, siderophores will be produced and released to sequester

and solubilize iron. They vary in their specificity, depending on the iron form, and in their affinity for iron (87). The high affinity for iron that Pvd has even leads to the displacement of iron molecules from host transferrin. Pch has lower affinity to iron in comparison to Pvd and is not as well studied. However, it is important to note that the role of Pch as a redox molecule is in causing oxidative tissue damage and may have a synergistic relationship with PYO as they cause even more damage when together (87). When the siderophores are unavailable or the situation is unfavorable for their production, *P. aeruginosa* can use other microbial siderophores, termed xenosiderophores, providing an advantage when in polymicrobial environments as it does not need to expend energy to produce siderophores, rather the energy can be used to produce other virulence factors (88).

In advanced infections, such as may be the case in CF lungs, some *P. aeruginosa* strains will lose their ability to make Pvd and Pch (89). To survive, *P. aeruginosa* uses phenazines for the acquisition of iron (79, 90). Wang et al. show that the phenazine, PCA, is able to reduce Fe^{3+} to Fe^{2+} in order to alleviate iron-limiting conditions allowing for the bacterium to be able to utilize the iron (90). While this is a great example as to how the phenazines can contribute to iron scavenging directly, they are also able to induce siderophore production by *A. fumigatus* during polymicrobial interactions (91).

2.3. The Pathogenesis of *A. fumigatus*

We likely inhale thousands of fungal spores daily, including *A. fumigatus* (92). While considered an opportunistic pathogen, *A. fumigatus* has been associated with severe respiratory diseases including severe allergic fungal asthma, invasive aspergillosis, and is often found as a chronic colonizer in CF (35, 38, 40, 93-98). If we look back on the mycobiome of several disease states, we can find an increase in the phylum, Ascomycota, of which *A. fumigatus* is a member

(17, 23). *A. fumigatus* has adapted to producing several different toxins and proteases that can cause significant tissue damage to the host and can evade host immune detection by modulating immune responses (93, 99).

2.3.1. Manipulation of Host Immune Recognition by *A. fumigatus*

Recognition of *A. fumigatus* by the host is essential for mounting an effective immune response. Modifying its spores or dampening the PRRs responsible for detection, including TLR2, TLR4, or dectin-1, of *A. fumigatus* are quite effective at allowing the fungus to evade initial recognition (100-102).

2.3.1.1. Interactions with PRRs

One mechanism by which *A. fumigatus* can modulate host recognition is by impairing signaling response that occurs when the PRR is triggered. Specifically, when TLR2 and TLR4 are activated by *A. fumigatus,* cytokines are produced the stimulate the initiation of the inflammatory response, including IL-6, TNF-α, and IL-1α/β (125). In the work by Chai et al., they tested the effect that *A. fumigatus* conidia and hyphae have on the production of the cytokines IL-6, TNF-α, and IL-1β when stimulating TLR2 and TLR4 responses. It was found that the production of IL-6 and IL-1β are significantly reduced when compared to controls, but there was no effect on TNF-α. The conidia were the most effective at modulating this response. The greatest effect was shown with the highest concentration of *A. fumigatus* when stimulating TLR4 (100).

The same group later substantiated their results by also testing dead conidia, in which they saw a return of cytokine expression (101). During this study, they also sought to determine which cell wall components were responsible for this effect on the cytokines and it was found that β-glucan and galactomannan were most effective against the TLR4-induced response, while

α-glucan was effective against both TLR2 and TLR4 responses. Chitin had a minimal impact on modulating the PRR signaling (101).

2.3.2. Proteases and Toxins Impair the Innate Immune Response to *A. fumigatus*

Like *P. aeruginosa*, *A. fumigatus* produces numerous toxins and proteins that can suppress the host's immune response, whether through damaging immune barriers and cells or cleaving cytokines and chemokines (93). Understanding the fungal interaction with the host provides insight into mechanisms that the fungus produces to protect itself in adverse environments.

2.3.2.1. Protease Effect on Epithelial Barrier

Numerous proteases are produced by *A. fumigatus* that influence the initiation of the inflammatory response in the lung including triggering the production of cytokines and chemokines by epithelial cells or in physically damaging the epithelial barrier. Initial studies regarding the protease production examined the effect of culture filtrate on epithelial cell lines. What they discovered was that the culture filtrate caused an increase in the cytokines IL-6, IL-8, and the monocyte chemoattractant protein-1 (MCP-1) in a dose dependent manner. When the culture filtrate concentration exceeded 200-µg/mL, IL-6 levels fell significantly lower than the negative control. However, IL-8 production remained elevated over the negative control even at high concentrations, though the cells did begin detaching from the culture surface. When the samples were exposed to serine protease inhibitors, cell detachment was prevented and there was reduced cytokine production (104). While the culture filtrate had some impact on the epithelial cells, some groups wanted to determine if the type of tissue infected by protease-producing *A. fumigatus* mattered in the effects that would occur.

Farnell et al. used different protein substrates including casein, porcine lung, and porcine gastric mucin to determine if *A. fumigatus* produced different proteases on different substrates. They found that growth of *A. fumigatus* was slower on the porcine substrates and the pH increased during the growth of *A. fumigatus* for all substrates, though in a time-dependent manner. The most secretion of proteases occurred with the porcine lung substrate and they determined the proteases to be predominantly serine proteases using protease inhibitors (105). As much of the focus of *A. fumigatus* infection and colonization is respiratory, it is important to determine what impact that these serine proteases have on the pathogenesis of the organism in the lung.

The serine proteases produced by *A. fumigatus* can disrupt epithelial adhesion to the basement membrane. In the *in vitro* study by Kogan et al., the epithelial cells (A549) were exposed to either fungal conidia or culture filtrate and the impact on fibers associated with epithelial cell interaction with the extracellular matrix, including actin, tubulin, and intermediate filament cytoskeleton fibers. The integrity of the tubulin and intermediate filament cytoskeleton fibers was not compromised upon exposure to the fungal components. However, actin fibers were disrupted and by 24h was completely depolymerized. In addition, focal adhesion was compromised resulting in cell detachment from the surface. This was mitigated with the addition of serine protease inhibitors (106). This further supports the work by Ladarola et al, which found that there is substantial lower respiratory tract destruction associated with the *A. fumigatus* serine protease. They found that the serine protease was more effective at degrading elastin than neutrophil elastase. It also degrades collagen (Types I and III) and fibronectin contributing to further destruction of the epithelial barriers (107).

2.3.2.2. Gliotoxin and Phagocytic Cells

Phagocytic cells of the innate immune response, including neutrophils and macrophages, are involved in the clearance of *A. fumigatus* (108-110). However, to counteract their effect, *A. fumigatus* has developed means by which it can inhibit or kill phagocytic cells. This includes the production of the mycotoxin, gliotoxin.

When an individual breathes in *A. fumigatus* spores, they are so small and hydrophobic that they plunge deep into the airways. The host depends on the efficient clearance of these spores by either the mucocilliary escalator or phagocytic cells (111). Initial recognition of the fungus is usually through PRRs including dectin-1, TLR2, and TLR4, by identifying differences in resting and germinating conidia and cell wall components of the fungus to initiate the appropriate immune response (100-102). Unfortunately, most individuals that develop infections by *A. fumigatus* are usually immunocompromised in some way (112-114). For instance, individuals taking immunosuppressive chemotherapy, as in autoimmune diseases or cancer patients, are at a greater risk of infection as the phagocytic cells are either not present or in low numbers (112-115). In other cases, the immune cells may be present, but not adequately functioning due to other microbial insults affecting their actions or changes to the microenvironment that are not conducive to effective actions, like in CF (38, 95, 98, 116).

The resting conidia (spores) of *A. fumigatus* are considered metabolically inert (117). It is only upon germination that they begin to produce secondary metabolites (118, 119). The metabolites, especially gliotoxin, can impair phagocytic function (120).

Macrophages and neutrophils are both phagocytic cells that respond to fungal insults differently. In an article by Knox et al., it was found that the macrophage was the most effective at phagocytosing conidia and the neutrophil required hyphae to stimulate their immune response.

If you depleted the macrophage population, there was an increase in invasive disease (121). During the initiation of the inflammatory response to *A. fumigatus* in the lung, residential alveolar macrophages are likely some of the first phagocytic cells to interact with the fungal spores. In an article by Schlam et al., gliotoxin exposed macrophages were assessed for their binding to and phagocytic activity of opsonized particles. They found that gliotoxin exposure reduced the binding of the macrophage to the particle and subsequently inhibited phagocytosis in a dose-dependent manner. This was further tested by this group with serum-coated or uncoated fungal particles that showed decreased engagement by macrophages for both sets, though the decreased activity was more exaggerated with the unopsonized fungal particles and they confirmed that the macrophages were still viable. Protrusions from the macrophage as a means to interact with the surface and fungal particles do get lost upon exposure to gliotoxin, identifying a potential mechanism of the mycotoxin on the macrophage's cell adhesion. Further testing also showed a decrease in F-actin in the macrophages, which was attributed to a decrease in phosphatidylinositol 3,4,5-triphosphate production by gliotoxin (122). Gliotoxin also has an impact on the macrophage precursor, the monocyte (123).

In the article by Stanzani et al, blood lymphocytes and monocytes were exposed to gliotoxin and their populations were assessed before and after gliotoxin exposure using flow cytometry. They found a reduced number of monocytes, but similar numbers of lymphocytes following exposure to gliotoxin. When further considering the cause of the decrease in monocytes they found an increase in caspase-3, which is associated with apoptosis (123). As macrophages play a major role in the clearance of the fungal conidia, the loss of functioning macrophages results in immune evasion by the conidia and provides an opportunity for the

germination and dissemination of the fungus. This is further buoyed by the effect that gliotoxin has on the other phagocytic cell of the innate immune response, the neutrophil.

Not unlike gliotoxin's effect on the macrophage, it also impaired F-actin reorganization and inhibits the neutrophil's ability to phagocytose the fungus (124). To determine if gliotoxin acts of secondary messenger, Comera et al. tested cAMP inhibitors and agonists as well as exogenous arachidonic acid on the effect of gliotoxin. It was found that inhibition of cAMP activity had no effect on gliotoxin's function. In fact, gliotoxin activity is enhanced with the presence of cAMP agonists. However, arachidonic acid restored the neutrophil's phagocytic activity after gliotoxin exposure (124).

2.3.2.3. Gliotoxin and Other Microorganisms

While there is no doubt that gliotoxin is effective at impairing the host's phagocytic response, it is also important to recognize the effect of this mycotoxin against other microorganisms. First and foremost, gliotoxin is a defense mechanism produced by *A. fumigatus* to damage other microorganisms competing for the same resources. As such, Svahn et al decided to see if specific bacteria-associated molecules could stimulate the production of gliotoxin (125).

Svahn et al tested LPS, peptidoglycan, and lipoteichoic acid to determine if there was an increase or decrease in gliotoxin levels at different concentrations. Their findings were that the peptidoglycan increased gliotoxin the most (65%) followed by LPS (37%) and lipoteichoic acid (35%) (125). This finding is important because as we showed previously, the lung is not a sterile environment (3, 5-7, 9, 10, 13, 19, 22, 25, 26, 28-31, 126-129). Therefore, *A. fumigatus* is likely encountering at least one of these bacterial-associated molecules and this could be enhancing its virulence and may give the fungal organism an advantage. One microorganism that is often found with *A. fumigatus* is *P. aeruginosa* (40, 95, 96, 116, 130, 131).

2.4. *Aspergillus fumigatus* and *Pseudomonas aeruginosa* Interactions in Clinical and Basic Research

As we continue to identify changes in the microbial populations from healthy individuals to those with chronic respiratory diseases, there has also been a notable shift in the methods in which we study respiratory disease. Specifically, we have shifted our methods from studying individual microorganisms associated with respiratory diseases to a more complex approach that focuses on multiple microorganisms found in disease.

2.4.1. Clinical Accounts of Co-infection by *A. fumigatus* and *P. aeruginosa*

Clinically, *A. fumigatus* and *P. aeruginosa* are individually implicated in numerous diseases with varying degrees of severity (34, 36, 39, 41, 132-135). When together though, *A. fumigatus* and *P. aeruginosa* can lead to worsened disease prognoses in several chronic diseases, like cystic fibrosis, and even in immune disorders, like Hyper-IgE Syndrome (35, 40, 95, 131, 136, 137).

2.4.1.1. Cystic Fibrosis

Cystic Fibrosis (CF) is a genetic disorder that affects approximately 70,000 people worldwide and is associated with mucosal secretions that are thick and difficult to clear from the lungs (138, 139). Individuals with CF are inundated with respiratory pathogens and frequently hospitalized due to chronic reoccurring infections, with *P. aeruginosa* being one of the main culprits with 60-80% of the adult CF population chronically colonized (140-142). While *P. aeruginosa* is a major contributing factor to decreased quality of life in CF patients, *A. fumigatus* further complicates CF disease outcomes with an overall prevalence that ranges from 5.9 to 57% in CF patients (35, 40, 95, 134, 136, 143).

A hypersensitivity reaction to *A. fumigatus*, allergic bronchopulmonary aspergillosis (ABPA) occurs in approximately 10% of CF patients and results in a significantly poorer outcome for those with CF (38, 95-98, 116, 143). Therefore, many of the current clinical studies on this topic focus on determining the onset of chronic *P. aeruginosa* infections and *A. fumigatus* sensitization.

In an early study from the nineties, it was found that colonization of *P. aeruginosa* in the lungs of those with CF was delayed if there was a positive *Aspergillus* skin test for allergies (96). A later retrospective study from the nineties looked at adolescents with CF and determined that the onset of *P. aeruginosa* colonization began in early adolescence and ABPA occurred later, suggesting that *P. aeruginosa* colonization favors sensitization to *A. fumigatus* (38). This was further supported in a study that identified microorganisms that are commonly colonized together in CF and their impact on lung function. It was found that *P. aeruginosa* and *A. fumigatus* have a synergistic harmful effect in CF lung disease and colonization by *A. fumigatus* increased the risk of *P. aeruginosa* and vice versa (136).

Recently, a study by Reece et al. showed that *A. fumigatus* was found most commonly in pre-adolescents and adolescents. In the Irish population tested in this study, it was found that 3.1% were co-colonized with *A. fumigatus* and *P. aeruginosa.* This study also showed that the lung function was lower in co-colonized lungs and that there were more hospital admissions per person. Patients co-colonized also had an increase in respiratory exacerbations per person with a 64% increase in comparison to those intermittently colonized with just *P. aeruginosa* and they are more frequently prescribed with antimicrobials (40).

2.4.1.2. Hyper-IgE Syndrome

Hyper-IgE Syndrome (HIES) is a rare primary immunodeficiency that results in increased IgE and recurrent infections. Many of these infections are acute pneumonias that result in pneumatocoeles, an air-filled lung cavity, which can become superinfected with opportunists like *P. aeruginosa* and *A. fumigatus* (131, 137). Between 1998 and 2003 there were six female patients that died of HIES in early to mid-adulthood. *Aspergillus* and *Pseudomonas* pneumonia followed one another in close succession several years after the first cases of pneumonia for most of these reported cases (131).

Two of the patients died suddenly of massive pulmonary hemorrhage with either *P. aeruginosa* or *A. fumigatus* at autopsy and one of the patients died from progressive respiratory failure from a combination of *Pseudomonas* and *Aspergillus.* The other remaining patients died of complications associated with either *P. aeruginosa* and/or *A. fumigatus* (131).

While this study has a small sample size and is limited to those with HIES, there are some aspects that are like CF. Including that the incidence of *A. fumigatus* pneumonia seems to coincide with *P. aeruginosa* pneumonia incidences. Additionally, the death of these individuals occurred for most early in life (131).

2.4.2. *In vitro* Studies

In order to address the potential antagonistic or synergistic interactions of *P. aeruginosa* and *A. fumigatus,* there have been multiple studies that have examined their impact on one another's growth and pathogenicity in *in vitro* modeling systems that vary from simple growth assays to more complex modeling systems, like organotypic lung models (91, 130, 144-155).

2.4.2.1. Chemical Secretions by **P. aeruginosa** *Alters the Function of* **A. fumigatus**

Secondary metabolites of *P. aeruginosa* inhibit the growth of *A. fumigatus* (144, 146, 148). Researchers have approached this concept by studying the growth of *A. fumigatus* under various conditions including in biofilms and hyphal growth. In a study by Mowat et al., researchers studied both direct and indirect influences of *P. aeruginosa* on the ability of *A. fumigatus* to initiate biofilm formation as well as their impact on preformed biofilms. When incubated with live *P. aeruginosa* prior to forming biofilms, *A. fumigatus* showed a reduction in overall fungal biomass during this study. In comparison, pre-formed biofilms produced by *A. fumigatus* were not impaired by direct exposure to *P. aeruginosa. A. fumigatus* conidia that were exposed to live *P. aeruginosa* quorum-sensing mutants resulted in better growth of the *A. fumigatus,* which was also reproduced in the indirect cultures (144).

In a similar study, Shirazi et al. sought to identify the mechanism by which *P. aeruginosa* inhibits *A. fumigatus* biofilm formation using culture filtrates from planktonic cultures (149). Contrary to the previously mentioned study, the preformed biofilm of *A. fumigatus* was significantly inhibited by the *P. aeruginosa* culture filtrates (149). This may be due in part to the microorganism strains as some of these studies have identified differences to be strain-dependent (152). Mucoid *P. aeruginosa* strains cultured from CF patients were less inhibitory to the *A. fumigatus* biofilm in comparison to non-mucoid CF strains. Additionally, membrane-damage was detected in *A. fumigatus* biofilms exposed to both types of CF *P. aeruginosa* stains with a greater effect associated with the non-mucoid CF strains. Shirazi et al. used a TUNEL assay to show that there is more apoptosis occurring in the *A. fumigatus* biofilms associated with all *P. aeruginosa* culture filtrates. Apoptotic initiation was also studied in the context of reactive oxygen species production, mitochondrial potential, and caspase activity. The initiation of

apoptosis was found to be greater in *A. fumigatus* biofilms treated with the *P. aeruginosa* culture filtrates (149).

Phenazines are likely found in the culture filtrates that inhibit *A. fumigatus* growth and biofilm development. Briard et al. tested four different phenazines and found that PCA was the least inhibitory against *A. fumigatus* mycelial development, which may be associated with structural characteristics of the chemical and hyphal cells. However, mycelial development was reduced in the presences of 1-HP and PYO and absent with PCN. For the phenazines to begin penetrating the fungal cells and target the mitochondria, conidia need to be germinating (91). As previously mentioned, targeting the mitochondria will limit the availability of ATP for the fungus (50). Subsequent ROS production occurs in the mitochondria and *A. fumigatus* requires superoxide dismutases, like Sod2p, for protection. Free oxygen radicals that result from the production of ROS are also induced in fungal cultures exposed to phenazines (91).

Zheng et al. found that *A. fumigatus* conidiation is also an area that can be impacted by *P. aeruginosa* phenazines (147). Conidia are an important reproductive cell involved in the distribution and survival of the fungal organism (39, 93, 156). Phenazine interactions with the spores could contribute to the overall survival of the fungus in respiratory disease. Over production of PYO and methylated PCA results in an increase in conidiation. Cultures that secreted PCN and other enzymatically modified PCA results in repressed conidiation. Further investigation into this topic by Zheng et al. found that the methylated PCA is the chemical that induced the enhanced conidiation. It was also identified that the conidiation is likely occurring due to oxidative stress regulation in the fungus (147).

As we learned earlier, phenazines can also be used by *P. aeruginosa* to access iron (79, 90). Using *A. fumigatus* mutant strains that allow for the study of iron chelation, Briard et al.

found that PYO, PCN, and PCA show iron-independent effects on growth inhibition of the fungus. In fact, low levels of these phenazines resulted in an improved iron acquisition of *A. fumigatus.* Alternatively, 1-HP induces iron starvation in *A. fumigatus,* and was found to chelate iron (91).

Access to iron during interactions of *A. fumigatus* and *P. aeruginosa* is a driving factor in the synergistic relationship of these microorganisms and contributes to growth impairment in *A. fumigatus*. Sass et al. implemented several different *P. aeruginosa* mutant culture filtrates. In particular, they focused on the role of Pvd, a siderophore produced by *P. aeruginosa*, as a potential cause for growth inhibition in the fungus (79-84, 86, 87, 151). As some of the culture filtrates from mutants associated with Pvd were no longer able to inhibit *A. fumigatus* biofilm formation and growth, they concluded Pvd limits the access of free iron for *A. fumigatus.* This was further supported through the restoring of inhibition by adding pure Pvd (151). In a more recent study by Sass et al., it was found that *A. fumigatus* has adapted in co-cultures with *P. aeruginosa* to out compete the bacteria for access to iron. They found that by exposing the bacteria to fungal supernatant during growth lead to a bacterial culture filtrate that was less inhibitory to *A. fumigatus* biofilms during their development or maintenance (157). When *A. fumigatus* supernatants were combined with pure Pvd, inhibitory effects previously noted by the siderophore were reduced (151, 157). Sass et al. identified hydroxamate siderophores as the protective molecule found in the *A. fumigatus* supernatant by showing a *sidA* mutuant, which lacks all four hydroxamate siderophores, cannot provide the same protection in the culture supernatant as the wild-type fungal supernatant(157). By the production of these siderophores, *A. fumigatus* may not be at such a disadvantage when it comes to survival during interactions with

P. aeruginosa, research is limited in *A. fumigatus* growth advantages when it comes to co-culturing with *P. aeruginosa.*

By studying secondary metabolites, we have begun to understand the potential relationship between *A. fumigatus* and *P. aeruginosa* in terms of limiting or inhibiting fungal growth and in some contexts the growth of the bacteria. However, both microorganisms are known to produce other chemicals, called volatile organic compounds (VOCs), that may lead to interkingdom communication that could also affect the growth of the organisms (148, 153, 155, 158-161). VOCs have been increasingly studied for their role in microbial communication though those reported to be produced by *P. aeruginosa* and *A. fumigatus* can differ across journal articles (148, 155, 160, 161). As VOCs are considered a means of communication between microorganisms and have been shown to change when different microorganisms interact (148, 153, 155, 158-161), it begs to question whether *A. fumigatus* and *P. aeruginosa* produce specific compounds within the presence of one another.

Therefore, Neerincx et al. developed a culture system in which they could identify VOCs produced individually by *A. fumigatus* and *P. aeruginosa* and assess changes that occur in the chemicals produced when there is a co-culture of the microorganisms (155). Independent of time, Neerincx et al. found that six VOCs are produced only when *A. fumigatus* and *P. aeruginosa* are co-cultured, including 2-ethyl-benzenamine, 2-hexanone, 3-methyl-3-penten-2-one, 4-mehtyl-3-penten-2-one, 1-propanal, and 2-octanone. It was also identified that the co-culture and the mono-culture resulted in some common chemical productions of 1-undecene and 3-methyl-1H-pyrrole (155). Three common VOCs were produced with the co-cultures and mono-cultures of *A. fumigatus*, including 2-tridecanone, 2-undecanone, and 2-nonanone (155). As they monitored these chemicals over various timepoints, changes to the microenvironment of

the culture can also impact the production of these VOCs and could account for some of the different chemicals identified. Another aspect that can alter the production of these VOCs is whether these microorganisms are in direct or indirect contact with one another (144, 155).

In a study by Mowat et al., it was found that direct contact with *P. aeruginosa* cells and the more indirect method of supernatant exposure both led to inhibition of *A. fumigatus* biofilm formation. They identified the quorum-sensing networks, LasI and LasR are involved in restricting *A. fumigatus* biomass by using knockout strains. Further characterization of VOCs produced by *P. aeruginosa* showed octanoic acid, decanol, decanoic acid, and dodecanol as effective at reducing *A. fumigatus* biomass (144).

Another system by Briard et al. utilize solid media with separation of *P. aeruginosa* and *A. fumigatus* from one another to mimic an indirect communication between *A. fumigatus* and *P. aeruginosa*, which allowed them to not only identify a potential role *P. aeruginosa* has in enhancing the growth of *A. fumigatus*, but they were also able to identify VOCs that either were similar or differed slightly from the previously mentioned VOCs identified in co-culture (148).

These VOCs included dimethyl sulfide (DMS), dimethyl disulfide (DMDS), 2,5-dimethylpyrazine (2,5-DMP), 1-undecane, 2-nonanone, 2-undecanone, and 2'-aminoacetophenone (2'-AAP)(148). Stimulation of *A. fumigatus* growth was further assessed with individual VOCs and it was determined that DMS had the highest stimulatory effect on *A. fumigatus* perhaps due to the sulfur content which is a nutrient source for the fungus. The authors hypothesize that VOCs role is in the initial invasion of the lung, but once colonization occurs, a more antagonistic response occurs between the fungus and bacteria (148). While this is a potential mechanism, there is a need to test this *in vivo* as there are many complexities of the lung that are not accounted for in either of these VOC studies. One such aspect is the potential of

the effect that these bacterial and fungal VOCs have on host responses, which has not been well characterized (115, 162).

While most studies focus on *P. aeruginosa* inhibition of *A. fumigatus*, some have shifted this focus onto the impact that *A. fumigatus* has on *P. aeruginosa* growth. Manavathu and Vazquez characterized culture supernatant from *A. fumigatus* growth, and found a heat-stable compound that inhibited but did not kill *P. aeruginosa* (145). To further the understanding of what this compound could be, Reece et al. used culture supernatants from *A. fumigatus* and *P. aeruginosa(152)*. Similar to Manavathu and Vazquez, they found that *A. fumigatus* culture supernatant inhibited *P. aeruginosa* biofilm development (145, 152). Gliotoxin was identified as the inhibitory compound during the Reece et al. study (152).

2.4.2.2. Enhancing Pathogenicity

The growth implications of co-culturing *A. fumigatus* and *P. aeruginosa* have been well established with evidence that the bacteria is able to inhibit or enhance the fungal growth dependent on the chemicals being produced by *P. aeruginosa* (79-84, 86, 87, 151) . It has also been well established that *A. fumigatus* and *P. aeruginosa* co-inhabit the same lung in diseases like CF and this results in more severe disease outcomes (35, 40, 95, 131, 136, 137). Therefore, there have been some studies that have focused on the impact that these two microorganisms have on one another's pathogenicity (154).

Elastase is a protease produced by *P. aeruginosa* to aid in the breakdown of host tissue resulting in the invasion of the bacteria. It can also be effective at damaging *A. fumigatus.* In the study by Smith et al., because *A. fumigatus* growth was inhibited when near *P. aeruginosa*, elastase production was tested for and found to be enhanced in the presence of the fungus, likely due to induced expression of *lasB*, which encodes elastase, in *P. aeruginosa* by *A. fumigatus*.

The elastase produced as a result of this co-culture of *P. aeruginosa* and *A. fumigatus* proved to be detrimental to a lung epithelial cell line (154). This enhanced pathogenicity could account for some of the tissue damage associated with co-colonization of these microorganisms in CF patients.

2.4.2.3. Impact of Co-exposure on Host Immune Responses in* In Vitro *Studies

While focuses of most *in vitro* studies relate to microbe-microbe interactions, some studies have recognized the importance of identifying the impact that theses microorganisms have on the host immune responses. Acute individual exposures to either *A. fumigatus* or *P. aeruginosa* results in a pro-inflammatory response. This pro-inflammatory response includes an increase in IL-6 and IL-8, and in co-cultures its stimulation is dependent on the strains of *A. fumigatus* and *P. aeruginosa* as only a few combinations of these microorganisms result in an enhanced response in CF epithelial cells (152).

In order to study the host immune response in the context of a more accurate representation of the lung microenvironment, Barkal et al. developed an organotypic model of the lung including human cells to form bronchioles with the airway, vascular, and extracellular matrix compartments included. While primarily developed for the study of *A. fumigatus* pathogenesis, the researchers did utilize this model to approach the impact that VOC communication has on the host immune response (153).

In order to explore this communication, an insert was created to promote VOC production and indirect contact between the bacteria and fungus. The co-culturing of *A. fumigatus* and *P. aeruginosa* induces IL-8 and GM-CSF increases that are overall greater than in individual cultures and is attributed to VOCs. There are also increases in IL-6 and IFN-ɣ with co-cultures though they are diminished when using a mutant that lacks a global regulatory of

secondary metabolite production in *A. fumigatus.* Il-1β production is elevated under all scenarios tested (153).

2.4.3. *In vivo* Studies

Clearly, *P. aeruginosa* and *A. fumigatus* interactions can have an important impact on our understanding of clinical diseases (40). While *in vitro* models provide valuable information regarding the synergistic and sometimes antagonistic relationships between *A. fumigatus* and *P. aeruginosa* (91, 130, 144, 148, 149, 151, 152, 154, 157), they lack the complexity of the host lung. To our knowledge few *in vivo* models of this type of co-exposure have been developed (152, 163, 164).

In the early 21st century, the first *in vivo* modeling system of *A. fumigatus* and *P. aeruginosa* co-colonization was established using immune suppressed mice. These mice were initially inoculated with *A. fumigatus* then immune suppressed with cortisone acetate, which was followed by an intranasal inoculation of *P. aeruginosa* nearly two weeks later. They found that mice infected *A. fumigatus* had a survival rate of 30%. Adding *P. aeruginosa* to lungs infected with *A. fumigatus* resulted in a slight increase in survival to 50%. Animals exposed to *P. aeruginosa*-only exhibited the greatest survival at 90% (163). It is important to note that while the animals were immunosuppressed, cortisone acetate does not typically reduce granulocytic inflammation, but instead reduces lymphocytic responses (165). Therefore, the host response would still adequately function to phagocytose invading microorganisms via its granulocytes.

A few years later, this same group published a similar model in which they were studying the combinations of antimicrobial therapeutics in an acute model of co-infection using immunosuppressed mice. Unlike the previous study, they inoculated anesthetized mice simultaneously with both the bacteria and conidia via intranasal inoculations. They show that all

the mice in the *P. aeruginosa*-only and the co-infected groups died within three days of infection, while the *A. fumigatus*-alone group had 50% survival on day 14. Also, the research group identified the combination therapy of amphotericin B and pazufloxacin mesilate as effective at preventing tissue necrosis and hyphae proliferation and resulted in an improved survival of co-exposed animals (164). Both models were great foundations to studying co-infections of *A. fumigatus* and *P. aeruginosa* in the complex host lung. However, to our knowledge no other *in vivo* models with mice have been attempted, though a recent model did use *Galleria mellonella,* wax worms, to study an acute co-infection with both *P. aeruginosa* and *A. fumigatus* (152).

In this model, the researchers determined the LD_{50} for each of the individual microbial cultures. Then, they used non-lethal inoculations of *A. fumigatus* to infect the wax worms 24h prior to inoculating with *P. aeruginosa*. They monitored the larvae and determined the LD_{50} for the combined microorganisms. It was found that most combinations of *P. aeruginosa* and *A. fumigatus* resulted in a decreased LD_{50}. This included the *P. aeruginosa* strain, ATCC 27853, with the *A. fumigatus* strain, AF1 (152).

These are the only *in vivo* models that have explored the topic of *A. fumigatus* and *P. aeruginosa* co-exposures and yet they have not delved into the factors associated with the interactions between these microorganisms and the progression to more severe outcomes for respiratory issues like that of CF (152, 163, 164). While much of the research using *in vitro* modeling systems have been beneficial in expanding our understanding of the microbe-microbe interactions between *A. fumigatus* and *P. aeruginosa*, it is not enough to determine a mechanism for the more severe disease progression in individuals that are co-colonized with these

microorganisms. The impact that the immune response can have on lung function inflammatory responses can be just as damaging as the microbial insults.

2.5. Granulocytes In Disease: Are They Helpful or Harmful?

As the first inflammatory cells present at the site of an injury or infection, granulocytes, including neutrophils and eosinophils, undoubtably have important roles in protecting the host from damage(166). However, there is just as much evidence that the granulocytes contribute to greater damage to the host as they can cause oxidative stress and tissue damage from granular contents (166-168). Above, we mentioned the role of granulocytes, specifically the neutrophil, in the host's immune response against *A. fumigatus* and *P. aeruginosa* individually. Here, we will discuss the effector functions of the granulocytic cells and how they can negatively impact the host outcome during a response to microorganisms.

2.5.1. Neutrophil Effector Functions

The neutrophil is a key cell in the innate immune response to invading microorganisms (166). These cells kill microorganisms through phagocytosis, the production of ROS and cytotoxic proteins, the release of granules, and in the formations of neutrophil-extracellular traps (NETs)(110, 166, 169-172). The most well recognized and studied function is the phagocytosis of microorganisms and their subsequent death in the phagosome (167). One of the major advantages of the phagocytic function of the neutrophil is the fact that it is much quicker than the macrophage in engulfing the microorganism and fusing the preformed granules with the phagocytic vacuole to form the phagosome (173). Part of the phagocytic process is activation of the NADPH oxidase, which results in ROS (174). Myeloperoxidase (MPO) is one such NADPH oxidase that is a primary granule of the neutrophil and is critical in killing bacteria and fungi, including *A. fumigatus* (168).

As mentioned above, neutrophils have pre-formed granules that can contain cytotoxic proteins (166-168). While these granules are typically going to fuse with the phagocytic vacuole, if the granule fuses with the plasma membrane or there is leakage of the phagosome, it results in the extracellular release of the granules and neutrophil activation. This can be advantageous for neutrophils as it promotes the recruitment of cells and helps breakdown barriers in their emigration to the site of infection (168). Some of the peptides in the granules may also promote survival of the neutrophils and induce cytokine production (175). Neutrophil survival can occur through hypoxia as well (176).

Cytokine production by neutrophils is another area in which the inflammatory cell can direct the host response. Neutrophils produce a wide variety of cytokines and chemokines, including acute phase response cytokines and chemokines and IL-17A. Production of these proteins can stimulate the egress of other inflammatory cells to the site of infection, or it can stimulate effector functions in cells already present, including other neutrophils (177). The production of the cytokine IL-17A in association with the neutrophil is usually to severe asthma phenotypes, which result in corticosteroid resistance(129, 178, 179).

One of the newest areas of research in neutrophil effector functions is in the formation of NETs. NETs are complexes of extracellular DNA, MPO, and neutrophil elastase that extrude outward from the cell and apprehend invading microorganisms (171, 172, 180-183). In comparison to phagocytosis, it does take a little more time to form them, but NETs are able to localize and capture numerous microorganisms of varying sizes and expose them to high concentrations of cytotoxic and antimicrobial proteins (182, 184). It has also been found that anuclear neutrophils are still able to carry out effector functions following the release of extracellular DNA when NETs are formed (181).

Unfortunately, NETs can also be detrimental to the host microenvironment and elicit further inflammatory responses. The release of extracellular DNA in the host tissue during NET formation can be pro-inflammatory as it is considered a damage-associated molecular pattern (DAMP)(185). Histones are involved in endothelial and epithelial cell death, which in addition to the breakdown of the extracellular matrix by MPO and elastase, can contribute to decreased lung integrity (186, 187).

2.5.2. Eosinophil Effector Functions

Eosinophils are the hallmark innate inflammatory cell of helminth infections and allergic responses. Like the neutrophil, the eosinophil is a granulocyte that contains granules comprising of major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil derived neurotoxin (EDN) (188). Release of these granules is the major effector function of the cell and can occur either by classical exocytosis, in which granular contents fuse with one another and are all released at the same time, or piecemeal degranulation, where more focused secretion occurs of specific granular proteins and not all are released (189).

Degranulation can be a powerful tool for host protection. An example of eosinophilic degranulation can be found in the study by Yoon et al, where eosinophils were lethal against *Alternaria alternaria,* likely recognizing the β-glucan of the surface of the spore and degranulating (190). Unfortunately, the same cytolytic properties associated with damaging or killing fungal organisms can also contribute to tissue damage. MBP damages the bronchial epithelium resulting in altered mucocilliary function (191, 192). Increased vascular permeability can also be an effect of the granular proteins (193).

Another function of the eosinophil is in the production of cytokines and chemokines. Like the neutrophil, the eosinophil can produce numerous cytokines and chemokines that can

facilitate both the innate and adaptive immune responses, including the production of IL-17A. In a study by Guerra et al, eosinophils drove inflammatory responses to the Th17 axis, which is typically considered a more severe asthma phenotype (194, 195).

The newest function of eosinophils to be studied is the formation of eosinophil extracellular traps (EETs). Like NETs, EETs contain extracellular DNA components and granular proteins and they function in much the same way. Asthma research is one area in which this concept has been studied. It was found that EETs correlate with reduced pulmonary function and marked increases in ROS resulting in sloughing of the airway epithelium (196).

2.5.3. Acute Lung Injury

Eosinophils are the hallmark innate inflammatory cell of helminth infections and allergic responses. Like the neutrophil, the eosinophil is a granulocyte that contains granules comprising of major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil derived neurotoxin (EDN) (188). Release of these granules is the major effector function of the cell and can occur either by classical exocytosis, in which granular contents fuse with one another and are all released at the same time, or piecemeal degranulation, where more focused secretion occurs of specific granular proteins and not all are released (189).

Degranulation can be a powerful tool for host protection. An example of eosinophilic degranulation can be found in the study by Yoon et al, where eosinophils were lethal against *Alternaria alternaria,* likely recognizing the β-glucan of the surface of the spore and degranulating (190). Unfortunately, the same cytolytic properties associated with damaging or killing fungal organisms can also contribute to tissue damage. MBP damages the bronchial epithelium resulting in altered mucocilliary function (191, 192). Increased vascular permeability can also be an effect of the granular proteins (193).

Another function of the eosinophil is in the production of cytokines and chemokines. Like the neutrophil, the eosinophil can produce numerous cytokines and chemokines that can facilitate both the innate and adaptive immune responses. This includes cytokines associated with the acute phase response during early immune response to the production of IL-17A. In a study by Guerra et al, eosinophils drove inflammatory responses to the Th17 axis, which is typically considered a more severe asthma phenotype (194, 195).

The newest function of eosinophils to be studied is the formation of eosinophil extracellular traps (EETs). Like NETs, EETs contain extracellular DNA components and granular proteins and they function in much the same way. Asthma research is one area in which this concept has been studied. It was found that EETs correlate with reduced pulmonary function and marked increases in ROS resulting in sloughing of the airway epithelium (196).

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3. PAPER 1: GRANULOCYTIC INFLAMMATION, ACUTE LUNG INJURY, AND INNATE IMMUNE DYSFUNCTION IN AN *IN VIVO* NON-ALLERGIC AND ALLERGIC MURINE MODEL OF *ASPERGILLUS FUMIGATUS* AND *PSEUDOMONAS AERUGINOSA* CO-EXPOSURE[1]

3.1. Abstract

The lung consists of a dynamic microenvironment that impacts mechanical and immunological pulmonary functions. Changes in the bacterial and fungal populations found in the lung may lead to severe respiratory diseases associated not only with host-microbe interactions, but also microbe-microbe interactions. Conventional mono-culture *in vivo* studies do not address the impact that microorganisms can have on one another. To that end, we utilized a mixed exposure model in which allergic or non-allergic mice were treated with a non-lethal dose of *Aspergillus fumigatus* fungal spores and intranasal *Pseudomonas aeruginosa* bacteria. At 24h after inoculation, fungal and bacterial control animals were ambulatory. In contrast, the health of animals receiving both fungus and bacteria steadily declined with 12.5% non-allergic and 34.8% allergic co-exposed animals dead and the remaining morbund within 24-h after exposure. The co-exposed animals appeared hunched and lethargic. Histology revealed interstitial neutrophilia and pronounced septal thickening accompanied by pulmonary hemorrhage. Allergic co-exposed animals had significantly more eosinophils and IL-17A production. This model of co-exposure is intended to provide greater insight into polymicrobial exposures and lung disease.

[1] The material in this chapter was co-authored by Breanne N. Steffan, Scott A. Hoselton, and Jane M. Schuh. Breanne N. Steffan had primary responsibility for collecting samples, preforming experiments, and analyzing results. Breanne N. Steffan was the primary developer of the conclusions that are advanced here and revised all versions of this chapter. Scott A. Hoselton processed and cut tissue sections for histology and checked the math and statistical analysis conducted by Breanne N. Steffan. Jane M. Schuh served as proofreader and was consulted in the developing of conclusions by Breanne N. Steffan.

3.2. Introduction

Worldwide, over 300 million people have asthma and approximately 100,000 of these individuals die each year (1). Some of the most severe cases of asthma include those triggered by fungal organisms, including the mold *A. fumigatus (2).* It is a ubiquitous spore-forming fungus that is found in very diverse environments and has a well-recognized role in allergic bronchopulmonary aspergillosis (ABPA), invasive aspergillosis, and severe allergic fungal asthma, as well as in noninvasive colonization and/or allergy in Cystic Fibrosis (CF)(3-5). Recent work on the lung microbiome discovered an increase in proteobacteria in the lungs of asthmatic patients (6). *P. aeruginosa* is a member of the family, proteobacteria, and is an opportunistic pathogen implicated in numerous respiratory diseases, including CF (7) and ventilator-associated pneumonia (8, 9). In clinical accounts of CF, a more severe clinical outcome results when individuals have evidence of both *A. fumigatus* and *P. aeruginosa* colonization with some evidence that suggests allergy to *A. fumigatus* leads to even more severe disease outcomes (10).

In vitro co-culture models using *A. fumigatus* and *P. aeruginosa* have identified secreted microbial factors that may affect the growth and virulence of one microbe on the other (11-15). For example, phenazines produced by *P. aeruginosa* inhibit growth and biofilm formation by *A. fumigatus* (12-14). Additionally, *A. fumigatus* may stimulate *P. aeruginosa*'s elastase production, potentially exacerbating host tissue damage (15). Building upon the foundation that *in vivo* monoculture and *in vitro* co-culture models have provided, the current study addresses the impact that the co-exposure of *A. fumigatus* and *P. aeruginosa* have on the pulmonary immune response in non-allergic and allergic airway disease.

For this study, we used the clinical *P. aeruginosa* strain, ATCC 27853, that has been predominantly studied *in vitro* in the context of biofilms and used as a control lab strain (11, 16-18). This strain is nonmucoid which is more susceptible to phagocytosis and host immune protection, suggesting a *P. aeruginosa* involved in early environmental exposure (19).. When this strain has been used for *in vivo* models, typically the host has been immunocompromised (11, 20, 21). This strain has been used in limited research studies to explore the *in vitro* (19) and invertebrate *in vivo* (11) interactions with *A. fumigatus* to gain a better understanding of the relationship between the two microorganisms. The lung is a complex microenvironment that cannot be solely replicated with *in vitro* studies. We generated this pulmonary *in vivo* model to study the dual exposure of the lung with this strain of *P. aeruginosa* and a clinical strain of *A. fumigatus* in both allergic and non-allergic hosts.

3.3. Materials and Methods

3.3.1. Experimental Animals

BALB/c mice (6-8 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed on Alpha-dri paper bedding (Shepherd Specialty Papers, Watertown, TN) in microfilter-topped cages (Ancare, Bellmore, NY) in a specific pathogen-free facility with *ad libitum* access to food and water. All research protocols were carried out under the guidelines of the Office of Laboratory Animal Welfare (OLAW) and in compliance with North Dakota State University's Institutional Animal Care and Use Committee (IACUC).

3.3.2. Fungal and Bacterial Cultures

The single lyophilized culture of *A. fumigatus* (ATCC 13073) was reconstituted in 5-mL PBS, and 60-µl aliquots of the suspension were stored at 4°C until use. Each 25-cm^2 vented-cap

culture flask with Sabouraud dextrose agar (SDA) was inoculated with a 60-µl aliquot of *A. fumigatus* and grown at 37˚C for 8d.

P. aeruginosa (ATCC 27853) was cultured at 37˚C with agitation in tryptic soy broth (TSB) to mid-logarithmic phase. The bacteria were stained with 10% trypan blue, counted by hemocytometer, and resuspended in normal saline to 1.5 x 10^9-mL. The hemocytometer counts were confirmed with plate counts on TSA. All protocols using microorganisms were approved by North Dakota State University's Institutional Biosafety Committee (IBC).

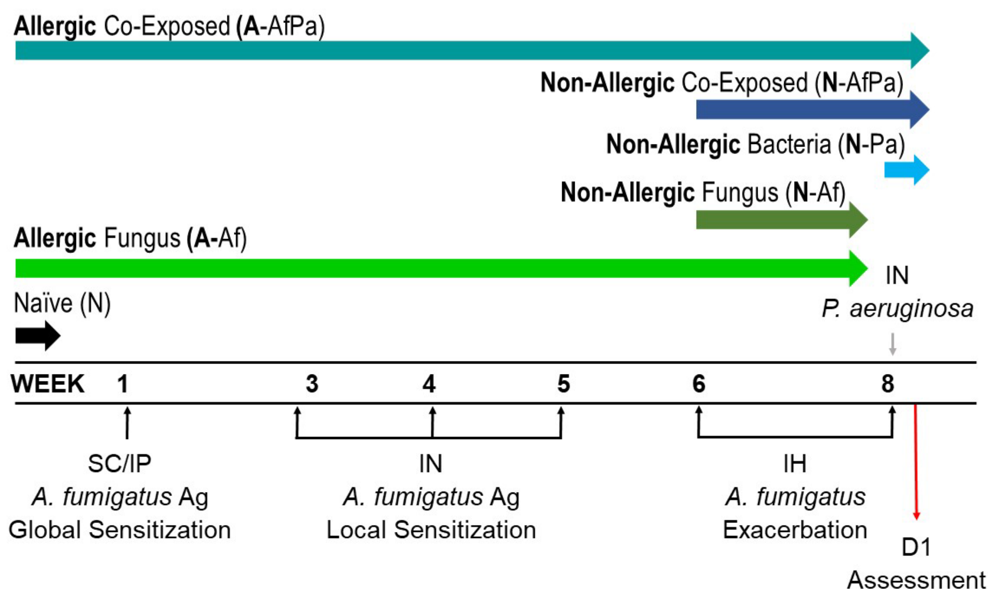

Figure 3.1. Co-exposure model
Timeline of the allergic fungal asthma model demarcated with arrows representing when the treatments take place for the respective groups studied. Allergy is induced by subcutaneous (SC) and intraperitoneal (IP) injections of *A. fumigatus* antigen (Ag), which stimulate global sensitization followed by intranasal exposures to *A. fumigatus* Ag to localize the inflammatory response in the respiratory tract. Inhalational (IH) exposures to live *A. fumigatus* spores follow allergic sensitization to exacerbate the airways. Treatment groups in this study include: naïve (N), non-allergic bacteria (**N**-Pa), non-allergic fungus (**N**-Af), allergic fungus (**A**-Af), non-allergic co-exposed (**N**-AfPa), and allergic (**A**-AfPa).

3.3.3. Allergic Sensitization to *A. fumigatus*

A. fumigatus antigen extract (Greer Laboratories, Lenior, NC) was combined with phosphate buffered saline (PBS) at a 1:1 ratio with the adjuvant, Imject Alum to a total

concentration of 10 μg/mL. Animals were injected with 0.1-mL subcutaneously and 0.1-mL intraperitoneally to initiate the global sensitization. After two weeks, local sensitizations were completed by diluting the same *A. fumigatus* antigen extract to 200 μg/mL and 20 μL intranasally (IN) once per week for three weeks. Following the third IN, mice undergo the inhalational exposures described below.

3.3.4. Fungal Inhalation and Bacterial Exposure

A three-mouse nose-only inhalation chamber was fitted with an in-line 25-cm^2 fungal culture flask. The apparatus was housed in a Class II biological safety cabinet for procedures. In order to liberate the hydrophobic spores, 2-psi of air was delivered across the flask allowing for the spores' delivery through the inhalation chamber. Exhaust air containing conidia was collected into two serial traps containing the sporicide, SporGon (Decon Labs, King of Prussia, PA). Mice were anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) and placed supine with their noses in the ports and exposed to a 10-min nose-only inhalational (IH) of live *A. fumigatus* conidia. This process was repeated two weeks later for a total of two 10-min *A. fumigatus* exposures. Allergic-*A. fumigatus* controls were included (n=13). Allergic co-exposed (n=23) and non-allergic co-exposed (n=16) animals were inoculated intranasally (IN) with 3 x 10^7 *P. aeruginosa* bacteria in 20-μl of PBS immediately after the second inhalation of live *A. fumigatus* conidia.

A control group that received *A. fumigatus*-only (n=9) was used to assess baseline parameters of fungal challenge. Mice in the bacteria-only group (n=10) were anesthetized before exposure to 3 x 10^7 *P. aeruginosa* bacteria, a dose that proved to be non-lethal in these immunocompetent animals as shown with our bacterial control. Naïve controls (n=14) were not exposed to mold or bacteria.

3.3.5. Blood Culture

To determine bacteremia in the co-exposed animals, 100-µL of blood was aseptically collected via cardiac puncture, spread onto tryptic soy agar plates, and incubated at 37°C for 24h. *P. aeruginosa* species identification was verified using a Bruker MALDI-TOF Biotyper and MALDI Biotyper Compass software, version 4.1.80 (North Dakota Veterinary Diagnostic Laboratory Fargo, ND).

3.3.6. Morphometric and Histological Analysis

Following tracheostomy, 1.0 mL sterile PBS was used to lavage the bronchoalveolar space. Resulting BAL fluid was centrifuged at 3,000 x *g* for 10 min to separate cells from the fluid. Samples were resuspended in sterile PBS and diluted for cytospin (Shandon Scientific, Runcorn, U.K.). Slides were differentially stained for morphometric identification (Quick-Dip stain; Mercedes Medical, Sarasota, FL). To estimate the inflammation in the lumen of each mouse, the average number of inflammatory cells in ten random, high-powered fields were counted under oil immersion (1000X) and the average for each group is reported.

Formalin-fixed, paraffin-embedded lungs were cut longitudinally across the coronal plane in 5-µm sections, mounted on glass slides, and stained with hematoxylin and eosin (H&E) to assess gross pathology and airway inflammation. Immunohistochemical (IHC) staining was used to assess spatial localization of neutrophil elastase. Antigen retrieval was enhanced by heating histological specimens in a microwave pressure cooker with citric acid (pH 6) for approximately 10 min. Samples were then stained using the Cell and Tissue Staining HRP-AEC kit (R&D Systems, Minneapolis, MN, #CTS006) per the manufacturer's protocol. Tissue sections were stained using a 1:200 dilution of a neutrophil elastase primary antibody (Abcam #ab68672) and incubated overnight in a humidity chamber at 4°C.

All images were captured with a DP74 color camera using cellSens software on the Olympus BX53 upright microscope (Waltham, MA).

3.3.7. Genetic Expression Analysis

Total RNA was extracted from lung tissue using TRIzol and quantified on a NanoDrop (Wilmington, DE). Reverse transcriptase and random primers (iScript^{TM} Reverse Transcription Supermix for RT-qPCR Cat #1708841; Bio-Rab Laboratories, Inc.; Hercules, CA) were used to generate cDNA from 1 µg of RNA. Real-time reactions contained 8 µL of cDNA template (1:16 dilution), 10 µL of 2x QuantiFast SYBR Green master mix ([Cat #204054], Qiagen; Valencia, CA) and 2 µL of 10x QuantiTect primer assays specific for cDNA for a total of 20 µL. Primers included the following targets: *Tlr2* (Cat# QT00129752), *Tlr4* (Cat# QT00259042), *Tlr5* (Cat# QT00262549), *Cxcl1* (Cat# QT00115647), *Cxcl2* (Cat# QT00113253), *Il6* (Cat# QT00098875), *Il1a* (Cat # QT00113505), *Il1b* (Cat # QT01048355), *Tnfa* (Cat # QT00104006), *Il17a* (Cat# QT00103278), and *Hprt* (Cat # QT00166768) (all from Qiagen; Valencia, CA). The CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, CA) was used for the qPCR reaction with the following parameters: a 15-min at 95˚C hot start followed by cycles of 94˚C for 15-sec, 55˚C for 30-sec, and 72˚C for 30s for a total of 40 cycles. Gene expression was normalized to naïve samples and the average fold change per treatment group is presented on relative mRNA expression graphs.

3.3.8. Protein Analysis of Cytokines

Cytokine production was assessed by EIA using the Invitrogen^{TM} Ready-SET-Go! Uncoated ELISA kits. BAL fluid samples were diluted 1:5 for IL-17A (Cat# 88-7371-88) 1:25 for IL-1α (Cat # 88-5019-88), IL-1β (Cat # 88-7013-88), and TNF-α (Cat # 88-7324-88) and

1:80 for IL-6 (Cat # 88-7064-88) following manufacturer's protocol (Invitrogen, Thermo Fisher Scientific; Waltham, MA).

3.3.9. Statistical Analysis

Using GraphPad Prism version 8.2.0 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com), statistical differences across all groups used an unpaired Student's *t* test with a Welch's correction. The co-exposed group is compared with individual exposure controls and statistical significance is represented as different letters. Samples not detected are denoted as n.d. in place of bar. A log-rank (Mantel-Cox) test was used for the comparison of survival curves.

3.4. Results

3.4.1. Fungal Allergy Results in Greater Mortality When Co-Exposed to *A. fumigatus* and *P. aeruginosa*

All animal subjects were monitored closely during the 24h post exposure. Naïve animals and those that just received fungus (with or without allergy) exhibited no signs of distress, were ambulatory, and consumed food and water normally post-anesthesia and throughout the study. Four out of ten animals exposed to just the bacteria exhibited initial signs of disease (up to 12h post exposure), including lethargy, ruffled fur, and hunched posture, but were still ambulatory and appeared to be recovering at 24h post exposure.

Allergic and non-allergic mice that were co-exposed to *A. fumigatus* and *P. aeruginosa* awoke in the normal time frame from anesthesia. However, early after the co-exposure, the animals entered a precipitous decline in health with physical signs of ruffled fur, hunched posture, labored breathing, and lethargy. By 24h, two of the non-allergic (12.5%) and eight of the allergic (34.8%) co-exposed animals had expired, and all the remaining animals had entered

moribund state. A survival comparison was completed by determining the expected survival of all the groups, followed by quantification of overall discrepancies between the observed and expected survivals in each individual group. Then, the trend between the discrepancies and the different groups are assessed (22). This statistical analysis shows that there was a significant decline in survival for the co-exposed animals with a p=0.0030.

Blood cultures of the co-exposed animals grew bacterial colonies with similar morphology and later identified as P. aeruginosa. No bacteria were found in the blood of P. aeruginosa-only animals. The study was halted at 24h post exposure in accordance with our IACUC protocol and NDSU's humane endpoint guidelines to allow for consistent analysis of experimental parameters.

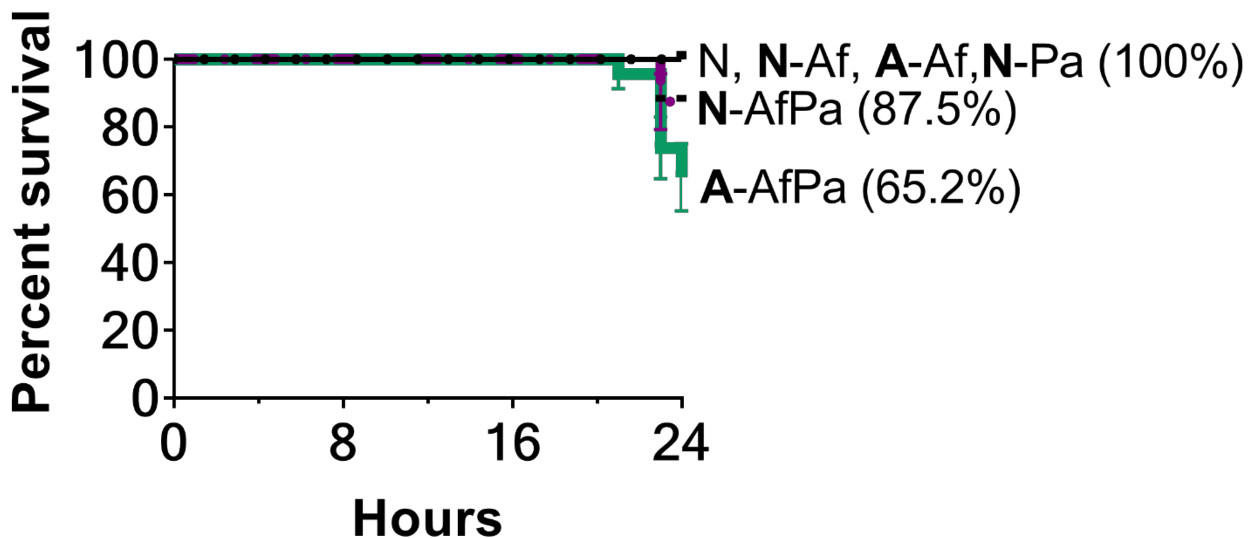

Figure 3.2. Fungal allergy followed by co-exposure to *A. fumigatus* and *P. aeruginosa* results in increased mortality
Animals in the following groups were monitored for 24-h: naïve (N, black), non-allergic *A. fumigatus* (**N**-Af, black), allergic *A. fumigatus* (**A**-Af, black), *P. aeruginosa* (**N**-Pa, black), non-allergic co-exposed (**N**-AfPa, purple), and allergic co-exposed (**A**-AfPa, green). Percent survival is listed for all groups and the survival curves were compared using a Log-rank (Mantel-Cox) test with p=0.0030.

3.4.2. Granulocytic Recruitment in Non-Allergic and Allergic Co-Exposed Animals Differ

As expected, resident macrophages were the principal immune cell retrieved from naïve lungs. Animals non-allergic and allergic exposed only to the fungus exhibited inflammation similar to the naïve group at this early timepoint, with a few eosinophils beginning to egress to the airways in allergic mice. In animals just exposed to the bacteria, there was a significant increase in neutrophilia (Figure 3.3B), though macrophages and eosinophils remain similar in number to the naïve group (Figure 3.3 A & C). Blood and bacteria can be found in the airways of four out of ten bacteria-only animals (data not shown). However, gross appearance of the lung remained normal (Figure 3.4A).

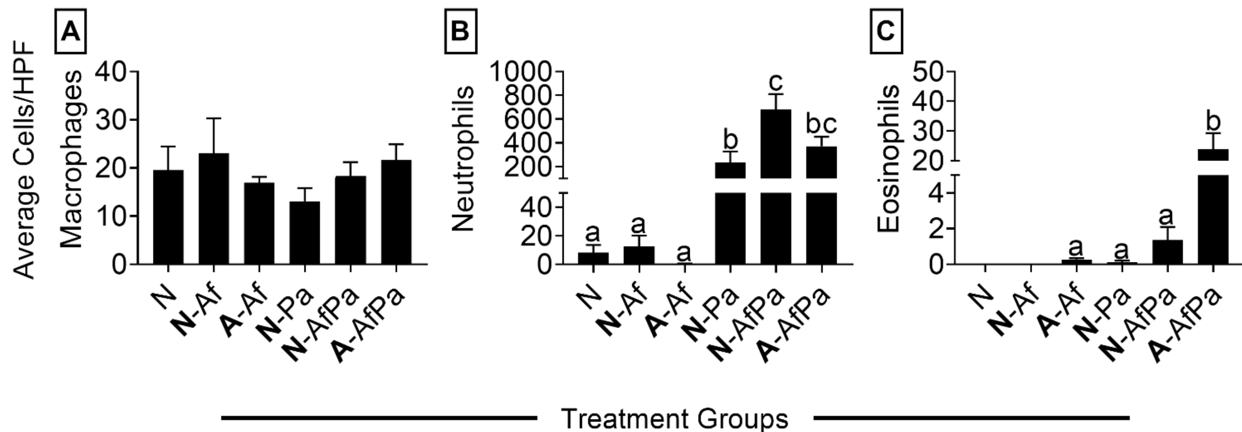

Figure 3.3. Granulocytic inflammation differs in co-exposed animals
Average cells (macrophages, neutrophils, and eosinophils) per high-powered field (HPF) are reported for: naïve (N), non-allergic *A. fumigatus* (**N**-Af), allergic *A. fumigatus* (**A**-Af), *P. aeruginosa* (**N**-Pa), non-allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**N**-AfPa), and allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**A**-AfPa). A Student's *t* test was completed with Welch's correction. Statistical significance is represented as different letters.

Non-allergic and allergic co-exposed animals both exhibited robust neutrophilia. Non-allergic co-exposed animals recruited nearly 2x as many neutrophils to the airways in comparison to the *P. aeruginosa*-only group. While sensitized co-exposed groups recruited significant numbers of neutrophilia, they also had 10x more eosinophils than the *Aspergillus*-

only groups at this timepoint (Figure 3.3 B&C). In addition to the considerable granulocytic inflammation in the co-exposed animals, blood and bacteria can also be found in the airways, sometimes to a point of overshadowing inflammatory cells (not shown). Correspondingly, numerous red blood cells were observed in the lungs of co-exposed animals, which was noted in the dark red appearance of the whole lung upon dissection (Figure 3.4 B & C).

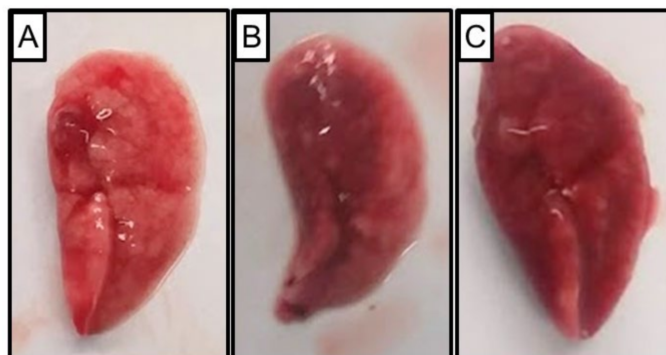

Figure 3.4. Gross anatomy of the lung upon dissection.
Upon dissection, images were taken of *Pseudomonas*-only (A), nonallergic *A. fumigatus* and *P. aeruginosa* (B), and allergic *A. fumigatus* and *P. aeruginosa.*

3.4.3. Interstitial Inflammation is Associated with Bacterial Dissemination Throughout the Co-exposed Lung, Accompanied by Edema and Pulmonary Hemorrhage

Hematoxylin and Eosin (H&E) staining of the fungus-only (non-allergic and allergic) samples revealed no change from naïve animals (Figure 3.5 G-I). The bacteria-only samples had minimal peribronchial inflammation, but none in the lateral branches (Figure 3.5 J). Septal thickening, associated with interstitial inflammation, was noted in the co-exposed samples (Figure 3.5 K&L). Additionally, several small airways were partially of completely obstructed by cellular debris in the co-exposed lungs. Pulmonary edema was noted in the histological evaluation of the co-exposed lung (Figure 3.5 K & L). Although difficult to quantify due to the influx of inflammatory cells in the tissue, bacteria were evident in the pulmonary tissue of the

co-exposed mice upon histological analysis and dissemination verified to be *P. aeruginosa* with blood cultures previously mentioned.

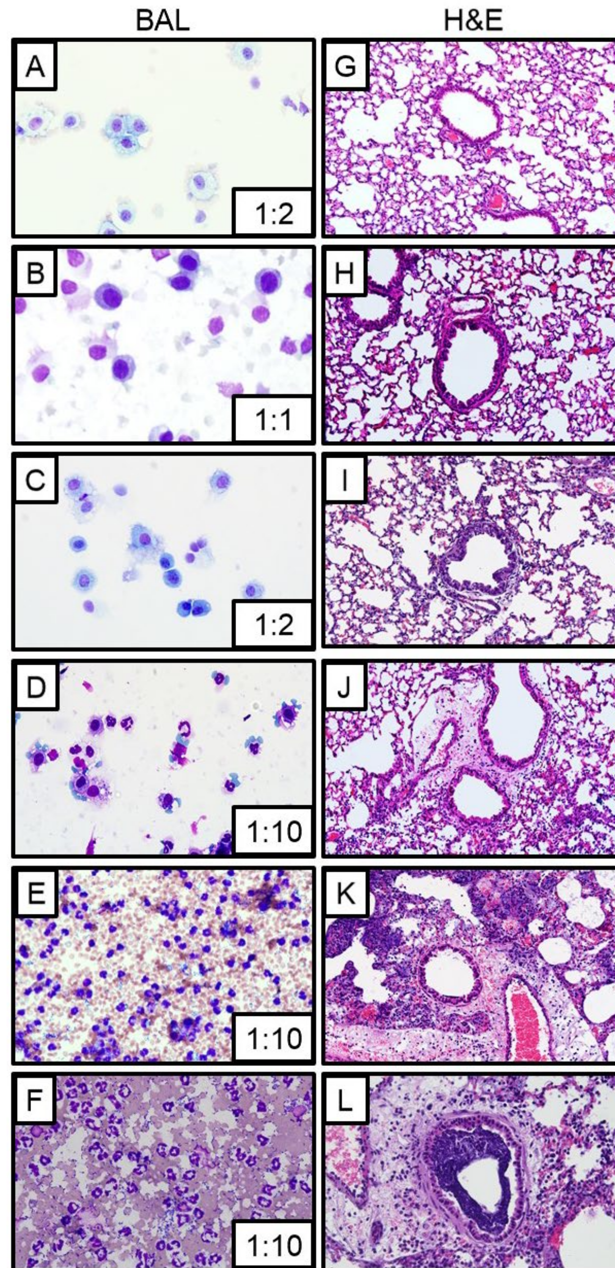

Figure 3.5. Airway and tissue inflammation associated with exposures to *A. fumigatus* and *P. aeruginosa* under allergic and non-allergic conditions
Representative photomicrographs of bronchoalveolar lavage (BAL) of airways at 600x total magnification (A-F) and hematoxylin and eosin stained tissue (G-L) at 200x total magnification are shown for naïve (A & G), non-allergic *A. fumigatus* (B & H), allergic *A. fumigatus* (C & I), *P. aeruginosa*-only (D & J), non-allergic *A. fumigatus* and *P. aeruginosa* (E & K), and allergic *A. fumigatus* and *P. aeruginosa* (F & L). Dilution factors for BAL washes are reported for each individual group in the bottom right corner.

Neutrophil elastase is produced by neutrophils in response to interactions with either *A. fumigatus* or *P. aeruginosa* (23, 24). Immunohistochemical staining of neutrophil elastase (punctate red staining) in pulmonary sections identified a robust recruitment of these cells to the pulmonary tissue in the co-exposed groups (Figure 3.6 B&C) and in the *P. aeruginosa*-only group (Figure 3.6 A).

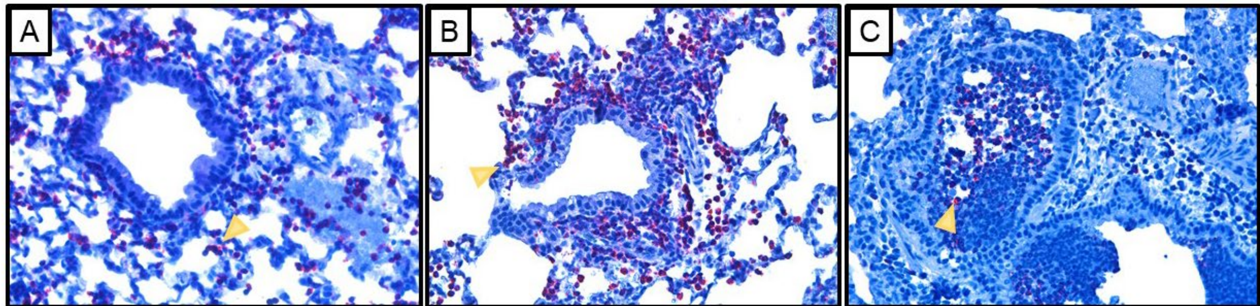

Figure 3.6. Tissue inflammation by neutrophils
Photomicrographs at 400x magnification of immunohistochemical staining of neutrophil elastase (punctate red staining—indicated by yellow arrowhead) for the *P. aeruginosa*-only (A), non-allergic *A. fumigatus* and *P. aeruginosa* (B), and allergic *A. fumigatus* and *P. aeruginosa* (C).

3.4.4. Elevated Granulocytic Chemokine Gene Expression in Co-Exposed Animals

The relative mRNA expression of the neutrophil chemokines *cxcl1* and *cxcl2* did not vary greatly between the allergic and non-allergic co-exposed animals (Figure 3.7 A&B). However, the expression is significantly greater (over 500- and 300-fold respectively) when comparing with controls. While not as substantial, mRNA expression for the eosinophilic chemokine, *ccl11*, resulted in about a 4-fold increase for both co-exposed groups (Figure 3.7 C).

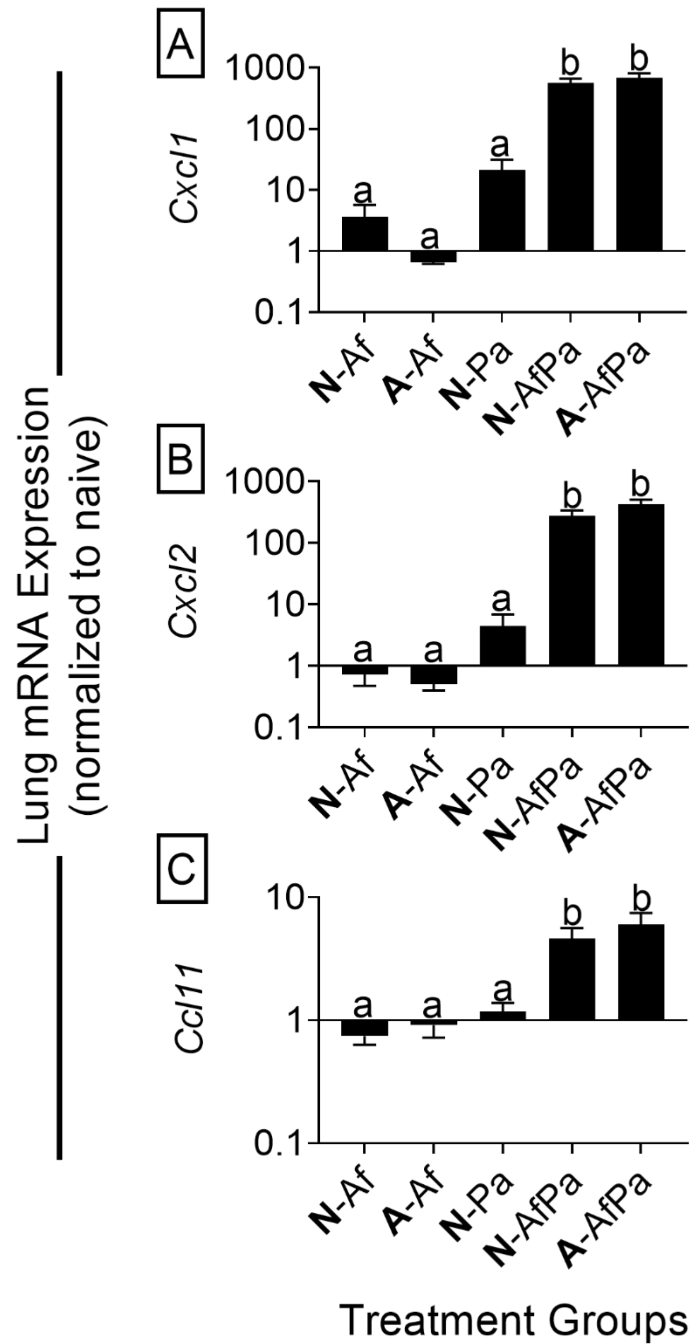

Figure 3.7. Lung mRNA chemokine expression following *A. fumigatus* and *P. aeruginosa* co-exposure

Gene expression of neutrophilic chemokines (*cxcl1* and *cxcl2*) and the eosinophilic chemokine (*ccl11*) was measured in the following groups: non-allergic *A. fumigatus* (**N**-Af), allergic *A. fumigatus* (**A**-Af), *P. aeruginosa* (**N**-Pa), non-allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**N**-AfPa), and allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**A**-AfPa). Samples are reported as average ± SEM. A Student's *t* test was completed with Welch's correction. Statistical significance is represented by different letters.

3.4.5. Altered *Tlr2/4/5* Expression During Polymicrobial Exposure

Expression of pattern recognition receptors (PRRs), including toll-like receptors (TLRs), provide valuable insight into the initial recognition of microorganisms and the resultant inflammatory response. As such, we characterized the gene expression of the receptors TLR2, TLR4, and TLR5. TLR2 and TLR4 are known to recognize structural components of *A. fumigatus* and *P. aeruginosa*, like chitin and lipopolysaccharide (LPS)(25, 26). TLR5 is most well-known in recognizing flagella (26), though there have been some reports that it can be elevated during *A. fumigatus* infections (27). *Tlr2* gene expression was significantly elevated in both allergic and non-allergic co-exposed groups in comparison to non-allergic fungus-only (p=0.0068, p=0.0015), allergic fungus-only (p=0.0076, p=0.0016), and bacteria-only (p=0.0146, p=0.0029). However, the expression of *tlr2* did not vary significantly between both co-exposed groups (p=0.4185) (Figure 3.8A).

We also assessed the gene expression of *tlr4* in which the non-allergic co-exposed group had significantly higher gene expression in comparison to the non-allergic fungus-only (p=0.0016), allergic fungus-only (p=0.0013), and bacteria-only (p=0.0135) controls, but not in comparison to the allergic co-exposed (p=0.1033) group. The allergic co-exposed group showed an increased expression in comparison to the allergic fungus-only (p=0.0072) and the non-allergic fungus-only (p=0.0071), but not in comparison to the bacteria-only control (p=0.1509) (Figure 3.8B). It is important to note that there were limited samples in which we were able detect gene expression for the non-allergic fungus control (n=3) and the bacteria-only control (n=3).

The final TLR that we analyzed was TLR5. The greatest *tlr5* gene expression was in the bacteria-only control. When compared with the co-exposed groups, the non-allergic group there

was a slight decrease in expression, but it was not statistically significant (p=0.0619). The bacteria-only and allergic co-exposed animals did not have any statistical difference (p=0.2582) (Figure 3.8C). The allergic fungus control had the lowest *tlr5* expression, which was significant in comparison to all groups.

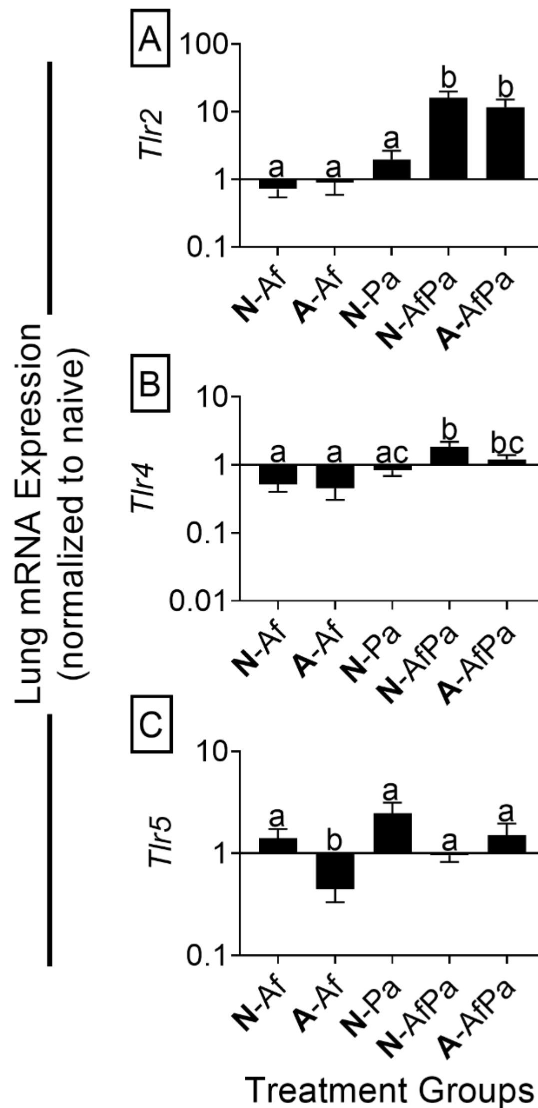

Figure 3.8. Toll-like receptor expression following polymicrobial exposure
Gene expression of toll-like receptors (TLR2/4/5) were measured in the following groups: non-allergic *A. fumigatus* (**N**-Af), allergic *A. fumigatus* (**A**-Af), *P. aeruginosa* (**N**-Pa), non-allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**N**-AfPa), and allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**A**-AfPa). Samples are reported as Average ± SEM. A Student *t* test was completed with Welch's correction. Statistical significance is represented by different letters.

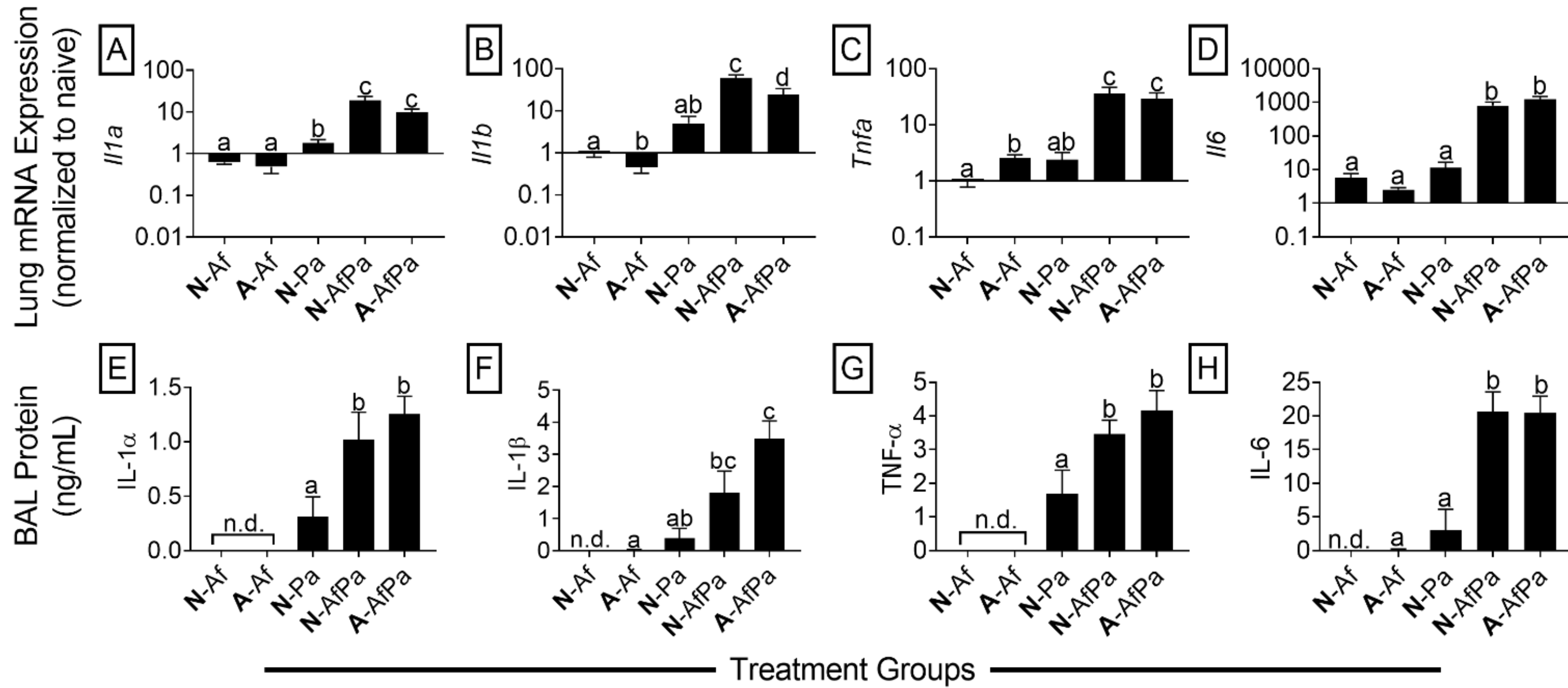

Figure 3.9. Acute phase response cytokines produced during fungal and bacterial co-exposure
Acute phase cytokines, including IL-1α (A&E), IL-1β (B&F), TNF-α (C&G), and IL-6 (D&H), lung mRNA expression (normalized to naïve) (A-D) and BAL protein expression (ng/mL) (E-H) are shown as averages ± SEM for the following groups: non-allergic *A. fumigatus* (**N**-Af), allergic *A. fumigatus* (**A**-Af), *P. aeruginosa* (**N**-Pa), non-allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**N**-AfPa), and allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**A**-AfPa). A Student's t test was completed with Welch's correction. Statistical significance is represented by different letters and samples not detected are indicated (n.d.).

3.4.6. Assessment of the Acute Phase Response in Co-Exposed Animals

With such an acute timeline to death in the co-exposed animals, we sought to see if there were elevated levels of acute phase response cytokines that are associated with vascular permeability and sepsis (28, 29). The cytokines that we assessed via gene expression (Figure 3.9 A-D) and protein analysis (Figure 3.9 E-H) included IL-1α, IL-1β, IL-6, and TNF-α.

The first set of cytokines we looked at included those in the IL-1 family. IL-1α is a pyrogen that has two forms, cleaved and non-cleaved, both of which are biologically active (30). When tissue is damaged, it will leak cytosolic IL-1α*,* which acts as an alarmin and triggers a pro-inflammatory response, including the production of chemokines for the recruitment of innate immune cells (30, 31). Likely due to the significant tissue damage, *Il1a* gene expression showed significantly elevated expression in both allergic and non-allergic co-exposed animals in comparison to non-allergic fungus-only (p=0.0023, p=0.0012), allergic fungus-only (p=0.0003, p=0.0012) and bacteria-only (p=0.0010, p=0.0020) groups (Figure 3.9A). Though the expression was not significantly different when comparing gene expression in both co-exposed groups (p=0.0798). The bacteria-only group had a slightly elevated gene expression of *Il1a.* Upon analysis of protein expression (Figure 3.9E), the allergic and non-allergic fungus groups were not detected (n.d.) at this dilution, this makes sense as it is only released upon infection or tissue injury (30). As this is such an early timepoint, it is not likely that we would find much evidence of this occurring in these animals. The bacteria-only group had a slightly elevated protein concentration; however, it was statistically lower than the non-allergic co-exposed (p=0.0319) and allergic co-exposed (p=0.0009) groups. The co-exposed groups were not statistically different in protein production of IL-1α (p=0.4436).

Like IL-1α, IL-1β is proinflammatory though it must be cleaved in order to be biologically active (31). When active, IL-1β can induce increased IL-17 production and Th17 differentiation that is associated with neutrophilic inflammation and more severe diseases, including severe asthma (32). As a marker for severe asthma, we characterized the production of IL-1β in our animals to gauge disease severity. *Il1b* is significantly elevated in the allergic and non-allergic co-exposed animals in comparison to non-allergic fungus-only (p=0.0180, p=0.0003), allergic fungus-only (p=0.0161, p=0.0003) and bacteria-only (p=0.0475, p=0.0006) groups (Figure 3.9B). Between the two co-exposed groups, there was also a significant decrease in gene expression from the non-allergic co-exposed in comparison to the allergic co-exposed (p=0.0323) animals. IL1β was at the limit of detection in the allergic-fungus and the bacteria-only groups, but the protein was not detected in the non-allergic fungus-only group at this dilution (Figure 3.9F). The control groups, allergic-fungus-only and bacteria-only, contained significantly less protein in comparison to the allergic co-exposed group (p<0.0001, p<0.0001). The non-allergic co-exposed had significantly more IL1β (p=0.0183) when compared with the allergic fungus-only group, but not the bacteria-only group (p=0.0694). The allergic co-exposed group has a similar concentration of IL1β when compared to the BALF of the non-allergic co-exposed animals (p=0.0624).

We also looked at the gene expression of the pro-inflammatory cytokine TNF-α (Figure 3.9C). TNF-α is another pyrogen that mediates neutrophil migration by promoting the expression of adhesion molecules (33), suppresses appetite (34), and high levels can induce shock-like symptoms (35). Because of the steady decline in our co-exposed groups over a short time frame and the increased migration of neutrophils to the lung, we decided to characterize the presence of TNF-α in the lungs. The controls all had fold changes roughly between one and three for *tnfa*.

The bacteria-only group did not have any statistical difference with the non-allergic fungus-only (p=0.1378) and the allergic fungus-only groups (p=0.8472), though the allergic (p=0.0064) and the non-allergic (p=0.0055) co-exposed groups had significantly greater *tnfa* expression. The two fungal controls, non-allergic and allergic, were different when comparing gene expression with the allergic having a slight increase in *tnfa* expression over the non-allergic group (p= 0.0041). The allergic and non-allergic co-exposed animals had substantially greater *tnfa* expression when compared with the non-allergic fungus-only (p=0.0045, p=0.0041) and the allergic fungus-only (p=0.0066. p=0.0056) groups. The two co-exposed groups did not differ statistically (p=0.5889). Upon protein analysis (Figure 3.9H), both fungus-only groups were not detected at the dilution. However, there were increases in BALF TNF-α for the bacteria-only and both co-exposed groups. The allergic and non-allergic co-exposed groups did not differ statistically in TNF-α (p=0.3259) but were significantly higher in concentration in comparison to the bacteria-only group (p=0.0135, p=0.0471).

The final pro-inflammatory pyrogen of the acute phase response that we tested for was IL-6. Il-6 can reduce serum iron levels, limiting access to invading microorganisms (36). There is an enhanced vascular permeability associated with increases in IL-6, which facilitates inflammatory cell migration to the lungs (28). Also, IL-6 is indispensable in the differentiation of IL-17-producing Th17 cells (37). We found both allergic and non-allergic co-exposed groups had nearly a 1000-fold increase in *il6* expression, though they were not statistically different from one another (p=0.2383) (Figure 3.9D). They did have statistically greater expression in comparison to non-allergic fungus-only (p=0.0007, p=0.0015), allergic fungus-only (p=0.0007, p=0.0015), and bacteria-only (p=0.0008, p=0.0016) groups. This high *il6* expression was also represented in the protein concentration in the BALF of the co-exposed animals with over 20

ng/mL for both groups and they were not statistically different from one another (p=0.6245) (Figure 3.9G). Both the allergic fungus-only and the bacteria-only groups had slight production of IL6, though they were not statistically significant in comparison to one another (p>0.9999). When compared with the allergic and non-allergic co-exposed groups, the allergic fungus-only (p<0.0001, p<0.0001) and the bacteria-only (p=0.0001, p=0.0003) BALF were significantly less concentrated with IL6. The non-allergic fungus-only group did not have any detectable IL6 at this dilution. Because of the significant increase in IL-6 production by the co-exposed animals, we decided to test for a Th17-associated cytokine, IL-17A.

3.4.7. IL-17A Production in Animals Co-Exposed to *A. fumigatus* and *P. aeruginosa*, Especially with Fungal Allergy

The production of the pro-inflammatory cytokine, IL-17A, was assessed using mRNA (not shown) and protein analysis (Figure 3.10). Naïve, non-allergic fungus-exposed, and allergic fungus-exposed animals did not have any mRNA or protein detectable at the respective dilutions. Bacteria-only mRNA for IL-17A was not detectable at the current dilution, however, there was a slight increase in protein levels of IL-17A. Animals that were co-exposed to *A. fumigatus* and *P. aeruginosa* exhibited mRNA (not shown) and protein expression that was substantially greater than the controls. Allergic co-exposed animals produced significantly more IL-17A than the non-allergic co-exposed counterparts (p=0.0254).

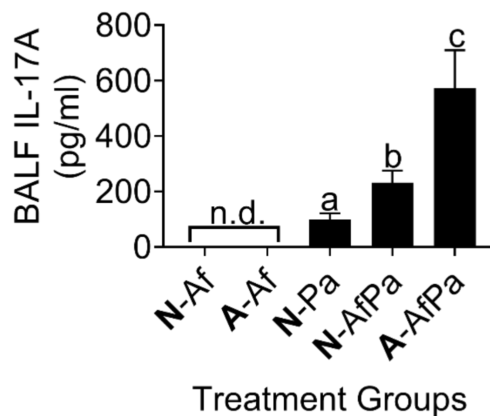

Figure 3.10. IL-17A in animals co-exposed to *A. fumigatus* and *P. aeruginosa*
IL-17A protein production was measured in the following groups: non-allergic *A. fumigatus* (**N**-Af), allergic *A. fumigatus* (**A**-Af), *P. aeruginosa* (**N**-Pa), non-allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**N**-AfPa), and allergic co-exposed *A. fumigatus* and *P. aeruginosa* (**A**-AfPa). Samples are reported as Average ± SEM. A Student's *t* test was completed with Welch's correction. Statistical significance is represented by different letters and samples not detected are indicated (n.d.).

3.5. Discussion

In this current study, we show that non-allergic and allergic mice co-exposured to *A. fumigatus* and *P. aeruginosa* results in significant inflammation, bacteremia, profound morbidity, and death within 24h. The precipitous decline of co-exposed animals is associated with a spike in acute phase response cytokines and chemokines, granulocytic inflammation, and a potential shift in immune recognition through TLRs. While the allergic and non-allergic co-exposed animals had similar responses, the allergic mice had an earlier and greater percent mortality when compared with the non-allergic. Major differences between these two groups included the eosinophilic response and increased IL-17A, both of which could contribute to the more severe disease phenotype.

The innate immune response uses PRRs to recognize general characteristics of microorganisms. For instance, TLR5 is primarily associated with the recognition of the bacterial flagellin (26) and is also upregulated in the presence of *A. fumigatus*, though the ligand is not

currently known (27). TLR2 and TLR4 are associated with structural components of the mold (38) and the lipopolysaccharide associated with *P. aeruginosa* (26). Incidentally, *tlr2* and *tlr4* are increased in the co-exposed animals. A previous article looking at post-septic mice and their survival with *P. aeruginosa* pneumonia found that knocking out *tlr2* resulted in a lower risk of death, which was also true of using *tlr2*$^{-/-}$*tlr4*$^{-/-}$ mice. Knocking out *tlr5* did not improve the survival of the animals (39). Other studies have shown that TLR5 is required for the clearance of *P. aeruginosa* (40, 41). Our results similarly show that when there is an increase in both *tlr2* and *tlr4* mRNA expression, we see more severe morbidity and even death. This is likely due to the initiation of the host immune response by the activation of the host TLRs and the subsequent inflammation.

The co-exposed animals have significant neutrophilia occurring in the interstial spaces of the lung. Neutrophils function to control the growth and spread of both fungal and bacterial microorganisms via phagocytosis in addition to their production of cytokines and granules containing toxic proteins(23, 24, 42, 43). The shear volume of neutrophils emigrating into the lung tissue, on top of the acute response cytokines already produced to aid in their extravasation, are likely responsible for the vascular leakage and pulmonary hemorrage that occurs in the co-exposed animals (44). While both the non-allergic and allergic co-exposed animals had high neutrophilia, the significant influx of eosinophils only occurred with the allergic co-exposed group and correlated with a higher mortality.

Like neutrophils, eosinophils produce pro-inflammatory cytokines and contain granular proteins, which they can release once they reach their target. The granules consist of two ribonucleases, a peroxidase, and the eosinophil major basic protein (MBP-1). Their release into the lumen and tissues of the lung can result in significant damage to the host by disrupting the

epithelium and lysing alveolar epithelial cells (45). The presence of the eosinophils is not entirely unexpected for the allergic animals, however, the significant influx into the airways is a bit early when we compare to the controls, including the allergic fungus-only control. While its function in the death of the allergic co-exposed animals is not identified yet, it is likely associated with the acute injury characterized in these animals.

In addition, both the eosinophils and neutrophils may be implicated in the production of IL-17A (46, 47). Both co-exposed groups have significantly higher IL-17A production in comparison to controls, with the allergic co-exposed group exhibiting the highest concentration. IL-17A can be produced by numerous cell types, including neutrophils, macrophages, and innate-like lymphoid cells. Its production can lead to increases in cytokines like IL-6, IL-1β, and TNF-α. Recently, eosinophils were implicated in the production of IL-17A during a pulmonary fungal infection study. In this study, researchers looking at acute pulmonary aspergillosis found that *A. fumigatus* conidia elicit eosinophils to produce IL-17a as early as 8h following exposure (46). Because the allergic co-exposed group had significantly more eosinophilia and IL-17a, this could be an explanation as to this phenomenon.

Although a small number of studies using immunocompromised/suppressed animals/insects have been done (48-50), this study represents the first research utilizing an immunocompetent mammalian host in order to characterize interactions of *P. aeruginosa* and *A. fumigatus* with the host immune response. In a study by Yonezawa et al., the researchers infected *A. fumigatus* via intratracheal (IT) inoculation and suppressed the immune response with cortisone acetate prior to intranasal infection with *P. aeruginosa* two weeks after the *A. fumigatus* infection (48). The use of cortisone acetate reduces lymphocytic inflammation, but not granulocytic responses (51). Although 30% of *A. fumigatus*-only group survived to 14 days after

pseudomonal infection (26d post *Aspergillus* infection), the addition of *P. aeruginosa* increased the survival of the animals to 50%, likely due to its ability to inhibit *A. fumigatus* (12, 14, 48, 50). Animals just infected with *P. aeruginosa* had a 90% survival (48). This same group later published an additional study that focused on therapies to prolong the survival of immunocompromised mice co-infected with *A. fumigatus* and *P. aeruginosa* (49). No other models in a mammalian host have been published on these microbial interactions.

This model of co-exposure to *A. fumigatus* and *P. aeruginosa* may provide evidence based direction on the way we treat respiratory diseases. We show that even in healthy animals, a co-exposure with both of these microorganisms can have disastrous consequences. Co-infections with both of these microorganisms can lead to diminished health of CF patients in the clinical settings and their prognosis is even worse if there is an allergy to *A. fumigatus* confounding their disease (10). Our initial results support this clinical account and we have promising data to suggest that eosinophilia and the production of IL-17A could be likely contributors to increased mortality in this treatment group. Our next step is to determine if all microorganisms need to be viable to stimulate the same inflammatory response and to delve more into the microbe-microbe-host dynamic of this type of respiratory disease. The versatilty of this model may even provide us with the ability to study other microbial interactions in the host lung.

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4. PAPER 2: *PSEUDOMONAS AERUGINOSA*, BUT NOT *ASPERGILLUS FUMIGATUS*, MUST BE VIABLE TO CAUSE DEATH IN CO-EXPOSED ALLERGIC ANIMALS[2]

4.1. Abstract

Aspergillus fumigatus is an opportunistic pathogen that causes severe fungal airway diseases, including severe asthma with fungal sensitization (SAFs) and allergic bronchopulmonary aspergillosis (ABPA). Studying the interaction of *A. fumigatus* with other common respiratory microorganisms, like *Pseudomonas aeruginosa*, is imperative as the lungs are not a sterile environment and many times ABPA is a comorbidity to a more troublesome respiratory disease, like in cystic fibrosis (CF). Previous work by our research group found that upon co-exposure to *A. fumigatus* and *P. aeruginosa,* allergic animals had significantly more severe disease outcomes including death (34.8%) in comparison to controls. In this current study, we gamma-irradiation killed *A. fumigatus* and/or *P. aeruginosa* prior to co-exposing allergic animals. Animals that were only given live *A. fumigatus* and *P. aeruginosa* served as our control. When *P. aeruginosa* was gamma-irradiated the animals had some inflammation occur, including neutrophilia, but all the animals survived and were in good health regardless of the viability of *A. fumigatus.* However, animals exposed to gamma-irradiated *A. fumigatus* but live *P. aeruginosa* had similar disease profiles as the co-exposed live control, with substantial inflammation, high expression and production of acute phase response cytokines/chemokines, and lung injury with hemorrhage. The survival for these animals was at 90% though they had fewer eosinophils and IL-17A production in comparison to the viable fungus and bacteria co-exposed group.

[2] The material in this chapter was co-authored by Breanne N. Steffan, Scott A. Hoselton, and Jane M. Schuh. Breanne N. Steffan had primary responsibility for collecting samples, preforming experiments, and analyzing results. Breanne N. Steffan was the primary developer of the conclusions that are advanced here and revised all versions of this chapter. Scott A. Hoselton processed and cut tissue sections for histology and checked the math and statistical analysis conducted by Breanne N. Steffan. Jane M. Schuh served as proofreader and was consulted in the developing of conclusions by Breanne N. Steffan.

4.2. Introduction

Globally, there are over ten million cases of allergic fungal airway disease, including both allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS), with *Aspergillus fumigatus* as the main culprit (1). The structure of *A. fumigatus* conidia is such that it can penetrate deep into the airways to the alveolar spaces (2). Unfortunately, for those that are allergic to *A. fumigatus,* avoidance techniques are difficult as the mold is ubiquitous in nature. This results in reduced productivity for adults and children, increased emergency department visits, and a high propensity for hospitalizations (3-5).

In some cases, ABPA is a co-morbidity for other respiratory diseases, like Cystic Fibrosis (CF)(6, 7). CF is a genetic disease in which the cystic fibrosis transmembrane conductance regulator (CFTR) gene is mutated and the result is thick and sticky mucosal secretions (8, 9). While CF affects numerous regions of the body, its effect on the function of the lung is well known and a major contributor to the morbidity and mortality of the disease (9). Those with CF tend to have chronic lung infections caused by both bacterial and fungal culprits with *Pseudomonas aeruginosa* and *A. fumigatus* as frequent colonizers (6, 10). Chronic colonization with *P. aeruginosa* correlates with increased chances of colonization with *A. fumigatus* and vice versa (11, 12). In addition, CF patients that have ABPA and *P. aeruginosa* tend to have more severe disease outcomes (12-14). As such, there has been a shift to study both *A. fumigatus* and *P. aeruginosa* together to identify their interactions with one another and how it affects the host.

Recently, our group began developing a model system that would allow us to study this complex interaction of fungus-bacteria-host. Our initial study utilized non-allergic and allergic animals exposed to *A. fumigatus, P. aeruginosa,* or both. At 24h, individually exposed mice were still in good health with little to no tissue damage. However, the co-exposed animals had an 18%

(non-allergic) and 34.8% (allergic) mortality at 24h with the remaining animals reaching moribund status. These animals had diffuse tissue inflammation and were hemorrhagic. Taking this information, we sought to determine whether viability of the both microorganisms was required for the progression of severe disease. Our current study used our previously established model of allergy and co-exposed allergic mice to combinations of viable and non-viable *A. fumigatus* and *P. aeruginosa.*

4.3. Materials and Methods

4.3.1. Experimental Animals

BALB/c mice (6-8 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed on Alpha-dri paper bedding (Shepherd Specialty Papers, Watertown, TN) in microfilter-topped cages (Ancare, Bellmore, NY) in a specific pathogen-free facility with *ad libitum* access to food and water. All research protocols were carried out under the guidelines of the Office of Laboratory Animal Welfare (OLAW) and in compliance with North Dakota State University's Institutional Animal Care and Use Committee (IACUC).

4.3.2. Fungal and Bacterial Cultures

An aliquot of *A. fumigatus* (ATCC 13073) was inoculated in a 25-cm^2 vented-cap culture flask with SDA, as previously described, at 37°C for 8d. *P. aeruginosa* (ATCC 27853) was cultured with agitation in tryptic soy broth at 37°C to a mid-logarithmic phase. Using a hemocytometer, bacteria were counted and resuspended in PBS to 1.5 x 10^9 per mL. All protocols using microorganisms were approved by North Dakota State University's Institutional Biosafety Committee (IBC).

4.3.3. Allergic Sensitization to *A. fumigatus*

As previously described, the *A. fumigatus* antigen extract (Greer Laboratories, Lenior, NC) is combined with phosphate buffered saline (PBS) and this solution is at a 1:1 ratio with the adjuvant, Imject Alum to a total concentration of 10 µg/mL. Animals are injected with 0.1-mL subcutaneously and 0.1-mL intraperitoneally to initiate the global sensitization. After two weeks, we begin local sensitizations by diluting the same *A. fumigatus* antigen extract to 200 µg/mL. Then, mice are given 20 µL intranasally (IN) once per week for three weeks. Following the third IN, mice will undergo the inhalational exposures described below.

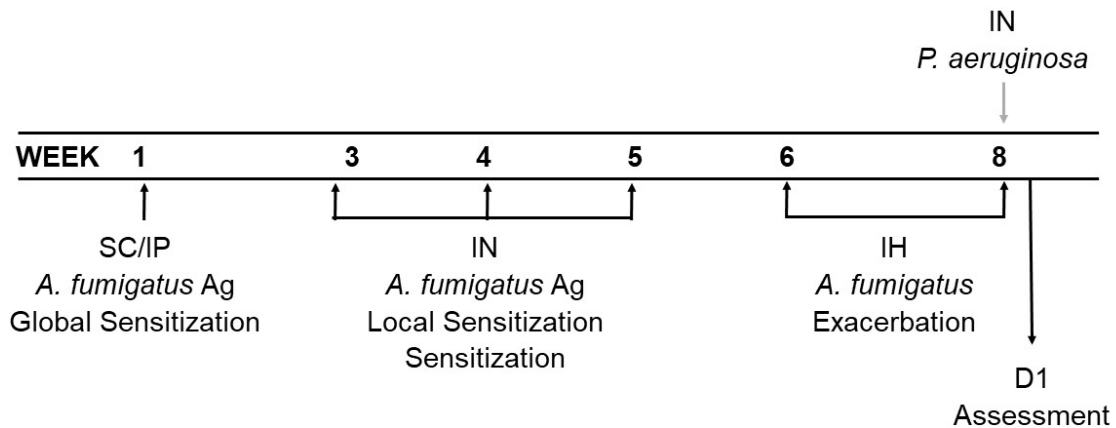

Figure 4.1. Allergy and co-exposure timeline
BALB/c animals were sensitized to *A. fumigatus* (weeks 1-5) and exposed to live *A. fumigatus* (weeks 6 & 8) and live *P. aeruginosa* (week 8). Animals were sacrificed at 24-h or once they reached moribund status.

4.3.4. Inhalational Exposure to *A. fumigatus* and/or *P. aeruginosa*

The inhalational exposure to *A. fumigatus* is as previously described, briefly, mice were anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) and placed supine with their noses in the ports and exposed to a 10-min nose-only inhalational (IH) of live *A. fumigatus* conidia. This process was repeated two weeks later for a total of two 10-min *A. fumigatus* exposures. Allergic co-exposed treatment group animals were inoculated intranasally

(IN) with 3 x 10^7 *P. aeruginosa* bacteria in 20-µl of PBS immediately after the second inhalation of live *A. fumigatus* conidia (AfPa). The experiment was repeated three times (n=23).

For the studies requiring non-viable conidia and/or bacteria, a ^{137}Cs gamma irradiator (Radiation Machinery Corporation, Parsippany, NJ, USA) was used subjecting the abovementioned cultures to a lethal dose of gamma radiation (8 kGy). The experiments were staggered so that the live bacteria used was the same stock for the dead *P. aeruginosa.* The live/dead experiments were repeated twice for live fungus and dead bacteria ($AfPa^{\gamma}$) (n=10), dead fungus and live bacteria ($Af^{\gamma}Pa$) (n=10), and dead fungus and bacteria ($Af^{\gamma}Pa^{\gamma}$) (n=10).

4.3.5. Morphometric and Histological Analysis

Following tracheostomy, one mL sterile PBS was used to lavage the bronchoalveolar space. Resulting BAL fluid was centrifuged at 3,000 x *g* for 10 min to separate cells from the fluid. Samples were resuspended in sterile PBS and diluted for cytospin (Shandon Scientific, Runcorn, U.K.). Slides were differentially stained for morphometric identification (Quick-Dip stain; Mercedes Medical, Sarasota, FL). To estimate the inflammation in the lumen of each mouse, the average number of inflammatory cells in ten random, high-powered fields were counted under oil immersion (1000X) and the average for each group is reported.

Formalin-fixed, paraffin-embedded lungs were cut longitudinally across the coronal plane in 5-µm sections, mounted on glass slides, and stained with hematoxylin and eosin (H&E) to assess gross pathology and airway inflammation. All images were captured with a DP74 color camera using cellSens software on the Olympus BX53 upright microscope (Waltham, MA).

4.3.6. Genetic Expression Analysis

Total RNA was extracted from lung tissue using TRIzol and measured on a NanoDrop (Wilmington, DE). Reverse transcriptase and random primers (iScript^{TM} Reverse Transcription

Supermix for RT-qPCR Cat #1708841; Bio-Rab Laboratories, Inc.; Hercules, CA) were used to generate cDNA from 1 µg of RNA. Real-time reactions contained 8 µL of cDNA template (1:16 dilution), 10 µL of 2x QuantiFast SYBR Green master mix ([Cat #204054], Qiagen; Valencia, CA) and 2 µL of 10x QuantiTect primer assays specific for cDNA for a total of 20 µL. Primers included the following targets: *Cxcl1* (Cat# QT00115647), *Cxcl2* (Cat# QT00113253), *Il6* (Cat# QT00098875), *Il1a* (Cat # QT00113505), *Il1b* (Cat # QT01048355), *Tnfa* (Cat # QT00104006), and *Hprt* (Cat # QT00166768) (all from Qiagen; Valencia, CA). The CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, CA) was used for the qPCR reaction with the following parameters: a 15-min at 95˚C hot start followed by cycles of 94˚C for 15-sec, 55˚C for 30-sec, and 72˚C for 30s for a total of 40 cycles. Gene expression was normalized to naïve samples and the average fold change per treatment group is presented on relative mRNA expression graphs.

4.3.7. Protein Analysis of Cytokines

Cytokine production was assessed by EIA using the Invitrogen™ Ready-SET-Go! Uncoated ELISA kits. BAL fluid samples were diluted 1:5 for IL-17A (Cat# 88-7371-88) 1:25 for IL-1α (Cat # 88-5019-88), IL-1β (Cat # 88-7013-88), and TNF-α (Cat # 88-7324-88) and 1:80 for IL-6 (Cat # 88-7064-88) following manufacturer's protocol (Invitrogen, Thermo Fisher Scientific; Waltham, MA).

4.3.8. Statistical Analysis

Using GraphPad Prism version 8.2.0 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com), statistical differences across all groups used an unpaired Student *t* test with a Welch correction. The co-exposed group is compared with individual exposure controls and statistical significance is represented as different letters. Samples not detected are

denoted as n.d. in place of bar. A log-rank (Mantel-Cox) test was used for the comparison of survival curves. A log-rank test determines the expected survival of all the groups, followed by quantification of overall discrepancies between the observed and expected survivals in each individual group. Then, the trend between the discrepancies and the different groups are assessed (15).

4.4. Results

4.4.1. *P. aeruginosa* Must Be Viable to Induce Mortality in Animals Co-Exposed with *A. fumigatus*

All animals were monitored closely over 24h for signs of distress including lethargy, hunched posture, ruffled fur, labored breathing. The co-exposed treatment groups that received gamma-irradiation killed bacteria ($AfPa^{\gamma}$ and $Af^{\gamma}Pa^{\gamma}$) woke up from anesthesia in normal time and by 24h were ambulatory, active, and looked normal. In co-exposed animals that received gamma-irradiation five out of ten were moribund and 10% expired at 24h. The remaining animals were ambulatory and active at 24h. As previously reported, the AfPa treatment group had a progressive decline in health early following exposure with approximately 34.8% death at 24h and the remaining animals were moribund (Figure 4.2). In the treatment groups with live bacteria, *P. aeruginosa* was able to be retrieved from blood of animals that appeared sick. There was a significant difference upon comparing the survival curves using a log-rank (Mantel-Cox) test (p=0.0116).

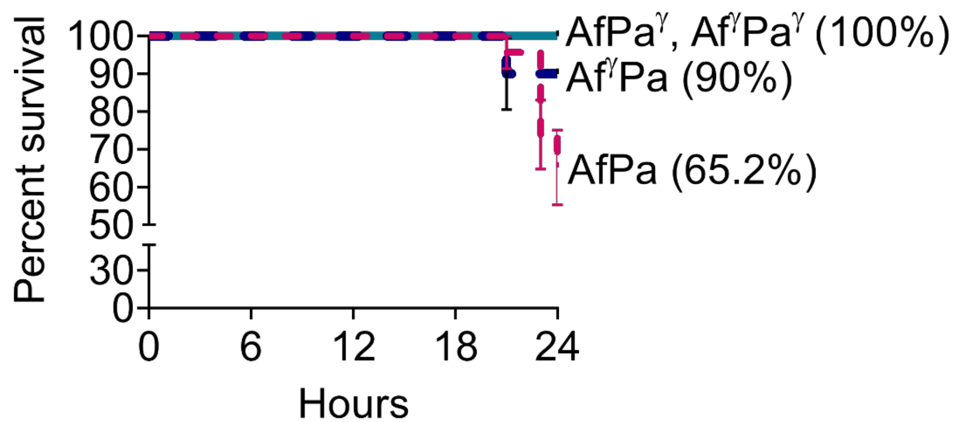

Figure 4.2. Survival of animals co-exposed to viable or non-viable fungus and bacteria
Percent survival is shown for the first 24-h post exposure to live *A. fumigatus* and *P. aeruginosa* (AfPa, pink; 65.2%), gamma-irradiated *A. fumigatus* and live bacteria ($Af^{\gamma}Pa$, blue; 90%), live *A. fumigatus* and gamma-irradiated *P. aeruginosa* ($AfPa^{\gamma}$, teal; 100%), and gamma-irradiated *A. fumigatus* and *P. aeruginosa* ($Af^{\gamma}Pa^{\gamma}$, teal; 100%).

4.4.2. Animals Co-Exposed to Bacteria and Fungus Exhibit Robust Neutrophilia Regardless of Microbial Viability

The neutrophil was the main inflammatory cell found in the airways of all the treatment groups (Figure 4.3B). While the groups exposed to live bacteria had the greatest accumulation of neutrophils in their airways, $AfPa^{\gamma}$ and $Af^{\gamma}Pa^{\gamma}$ still had an average of 100 neutrophils/HPF. $Af^{\gamma}Pa$ did not have statistically more neutrophils than $AfPa^{\gamma}$ (p=0.0753) or AfPa (p=0.6260) but did have more than groups in which more than one microorganism was gamma-irradiated (p=0.0397). The animals co-exposed to live microorganisms had significantly more neutrophils when compared with $AfPa^{\gamma}$ (p=0.0409) and $Af^{\gamma}Pa^{\gamma}$ (p=0.0145).

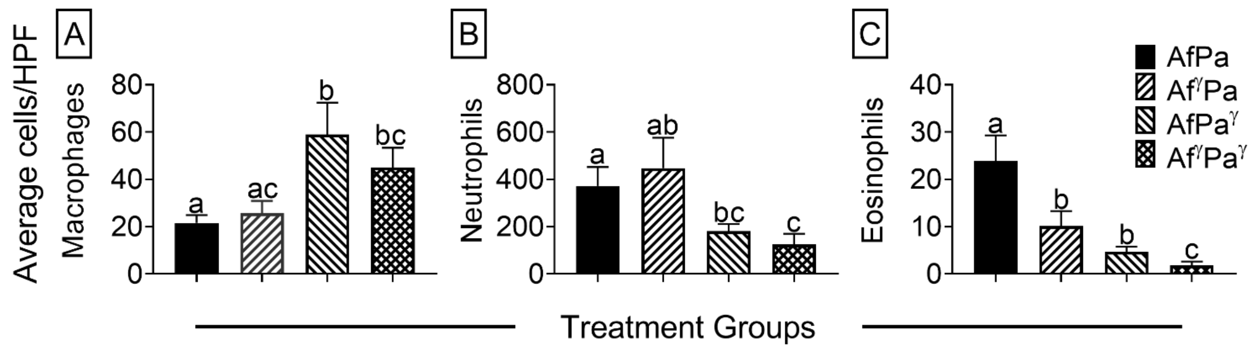

Figure 4.3. Granulocytic inflammation following co-exposure to viable and non-viable microorganisms
Cells were differentially counted as macrophages (A), neutrophils (B), and eosinophils (C) under a high-powered field (HPF; oil-immersion). Groups averages ± SEM are listed for allergic animals exposed to live *A. fumigatus* and *P. aeruginosa* (AfPa; solid black), gamma-irradiated *A. fumigatus* and live bacteria (Af$^{\gamma}$Pa; forward hashes), live *A. fumigatus* and gamma-irradiated *P. aeruginosa* (AfPa$^{\gamma}$; backward hashes), and gamma-irradiated *A. fumigatus* and *P. aeruginosa* (Af$^{\gamma}$Pa$^{\gamma}$; cross hashes). Using an unpaired Student's *t* test with Welch's correction statistical differences are indicated by different letters.

Animals exposed to live *A. fumigatus* and gamma-irradiated *P. aeruginosa* had similar numbers of macrophages as compared with Af$^{\gamma}$Pa$^{\gamma}$ (p=0.3982) but had roughly twice as many macrophages egressing to their lungs in comparison to AfPa (p=0.0233) and AfPa$^{\gamma}$ (p=0.0420). AfPa had fewer macrophages in comparison to the animals co-exposed to non-viable fungus and bacteria (p=0.0215), though AfPa$^{\gamma}$ (p=0.3982) did not differ from Af$^{\gamma}$Pa$^{\gamma}$ (Figure 4.3A).

All animals had been sensitized to *A. fumigatus* so we would expect eosinophilia, as a hallmark cell of allergic disease progression. However, AfPa had over 2x more eosinophils than Af$^{\gamma}$Pa (p=0.0409), 5x more eosinophils than AfPa$^{\gamma}$ (p=0.0038), and 10x more eosinophils than Af$^{\gamma}$Pa$^{\gamma}$ (p=0.0013). When compared with one another Af$^{\gamma}$Pa and AfPa$^{\gamma}$ showed no difference in eosinophil numbers (p=0.1370) but did differ significantly when compared to the group with both gamma-irradiated microorganisms (p=0.0312; p=0.0490) (Figure 4.3C).

4.4.3. Lung Inflammation Only With Viable Bacteria During Co-Exposure

As with our previous study, animals with the live bacteria (regardless of *A. fumigatus* viability) had interstitial inflammation with septal thickening and edema. Macroscopic (not shown) and microscopic indications of pulmonary hemorrhage can also be seen in the AfPa and Af$^{\gamma}$Pa treatment groups (Figure 4.4 A & B). There was interstitial inflammation occurring in the AfPa$^{\gamma}$ but very little edema and no hemorrhaging (Figure 4.4C). Animals exposed to both gamma-irradiated microorganisms showed little evidence of tissue inflammation (Figure 4.4D).

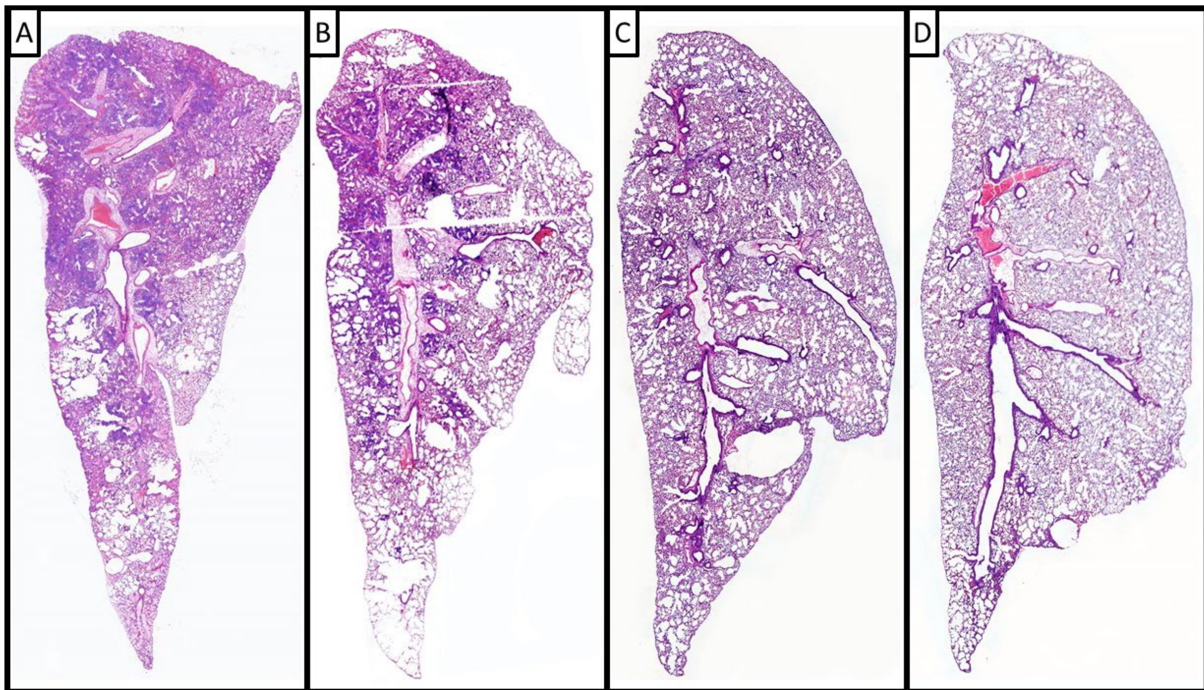

Figure 4.4. Increased tissue inflammation with viable bacteria for the co-exposure
H &E stained whole lung photomicrographs were taken using multiple image alignment and are shown at 200x total magnification for live *A. fumigatus* and *P. aeruginosa* (AfPa; A), gamma-irradiated *A. fumigatus* and live *P. aeruginosa* (AfɣPa; B), live *A. fumigatus* and gamma-irradiated *P. aeruginosa* (AfPaɣ; C), and gamma-irradiated *A. fumigatus* and *P. aeruginosa* (AfɣPaɣ, D).

4.4.4. Expression of Granulocytic Chemokine Expression is Associated with Viable Bacteria

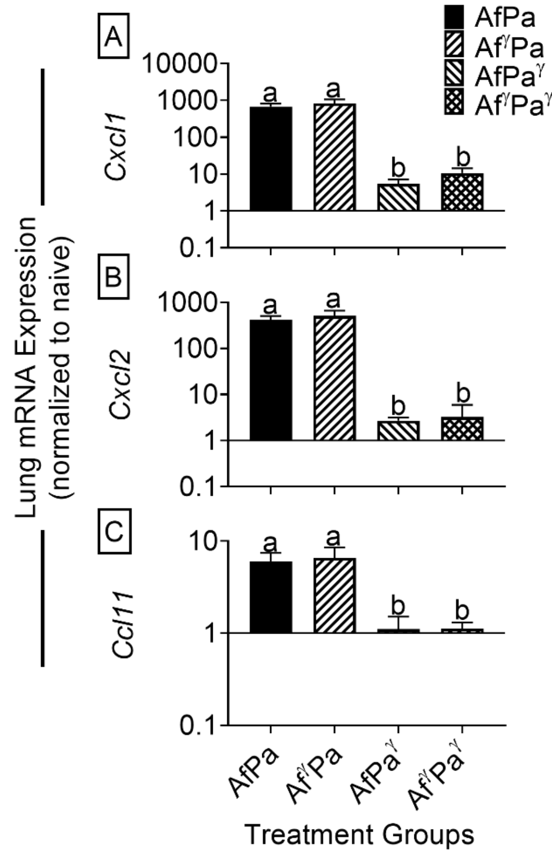

Figure 4.5. Chemokine expression following exposure to viable or non-viable microorganisms Lung mRNA expression normalized to naïve of *cxcl1*(A), *cxcl2* (B), and *ccl11*(C) for the following groups: live *A. fumigatus* and *P. aeruginosa* (AfPa; solid black), gamma-irradiated *A. fumigatus* and live bacteria (Af$^{\gamma}$Pa; forward hashes), live *A. fumigatus* and gamma-irradiated *P. aeruginosa* (AfPa$^{\gamma}$; backward hashes), and gamma-irradiated *A. fumigatus* and *P. aeruginosa* (Af$^{\gamma}$Pa$^{\gamma}$; cross hashes). Samples are represented as average ± SEM. Using an unpaired Student's *t* test with Welch's correction statistical differences are indicated by different letters.

We examined the gene expression of *cxcl1, cxcl2,* and *ccl11* to determine differences between animals exposed to viable and/or nonviable microorganisms when compared to AfPa. The first two chemokines, *cxcl1* and *cxcl2* are associated with neutrophil recruitment. Expression of *cxcl1* and *cxcl2* in AfPa$^{\gamma}$ (p=0.0001, p=0.0004) and Af$^{\gamma}$Pa$^{\gamma}$ (p=0.0002, p=0.0004) groups were significantly lower than AfPa. Af$^{\gamma}$Pa expression of *cxcl1* and *cxcl2* was not lower (p=0.5545,

p=0.6420) than AfPa (Figure 4.5 A & B). Clearly, *P. aeruginosa* viability is important for the signals recruiting the neutrophils to the airways.

The eosinophilic chemokine gene expression was also similar with AfPa having higher *ccl11* expression when compared with AfPa$^{\gamma}$ (*p*=0.0048) and Af$^{\gamma}$Pa$^{\gamma}$ (*p*=0.0045), but Af$^{\gamma}$Pa was not lower (*p*=0.8197) (Figure 4.5 C).

4.4.5. Bacterial Viability Was Necessary to Stimulates the Highest Level of Acute Phase Response Cytokines in Co-Exposed Animals

The lungs were tested for the mRNA expression of acute phase response cytokines IL-1α, IL-1β, IL-6, and TNF-α. protein levels in the BAL fluid. Consistently, the samples that were exposed to live *P. aeruginosa* (AfPa and Af$^{\gamma}$Pa) consistently produced more acute phase cytokines that those exposed to gamma-irradiated bacteria (Figure 4.6 A-D). This was also found to be the case when assessing the concentration of these cytokines (Figure 4.6 E-H). The cytokine levels in the samples that were exposed to gamma-irradiated bacteria were not detectable for both IL-1α/β and the dual gamma-irradiated bacteria and fungus treatment groups did not have detectable TNF-α.

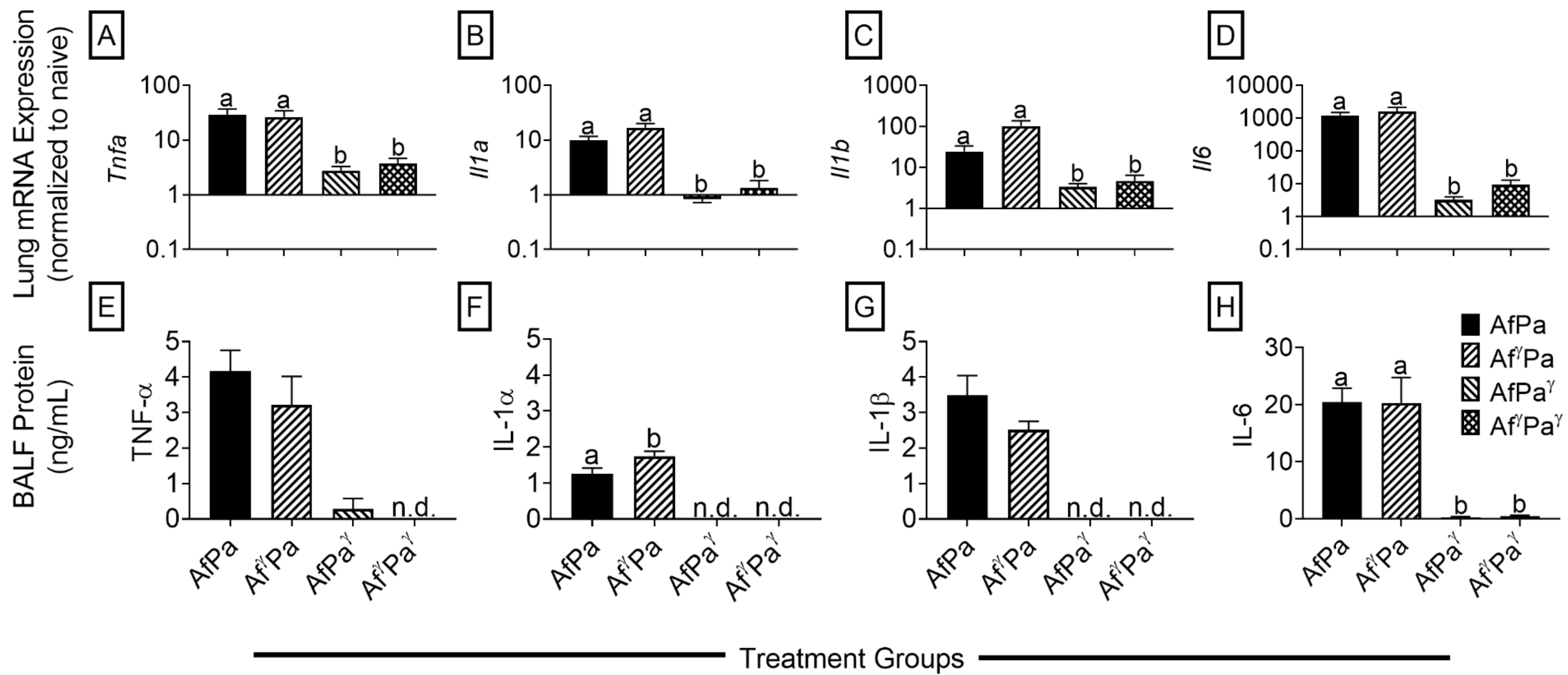

Figure 4.6. Viable *P. aeruginosa* was necessary to stimulate acute phase response cytokines following fungal/bacterial co-exposure Acute phase cytokines TNF-α (A&E), IL-1α (B&F), IL-1β (C&G), and IL-6 (D&H) lung mRNA expression normalized to naïve (A-D) and protein production in ng/mL (E-H) are reported for the following groups: live *A. fumigatus* and *P. aeruginosa* (AfPa; solid black), gamma-irradiated *A. fumigatus* and live bacteria (Af$^{\gamma}$Pa; forward hashes), live *A. fumigatus* and gamma-irradiated *P. aeruginosa* (AfPa$^{\gamma}$; backward hashes), and gamma-irradiated *A. fumigatus* and *P. aeruginosa* (Af$^{\gamma}$Pa$^{\gamma}$; cross hashes). Samples are represented as average ± SEM. Using an unpaired Student's *t* test with Welch's correction statistical differences are indicated by different letters. Samples not detected are represented by n.d. in place of a bar.

4.4.6. IL-17A Expression Was Impacted By Bacterial Viability

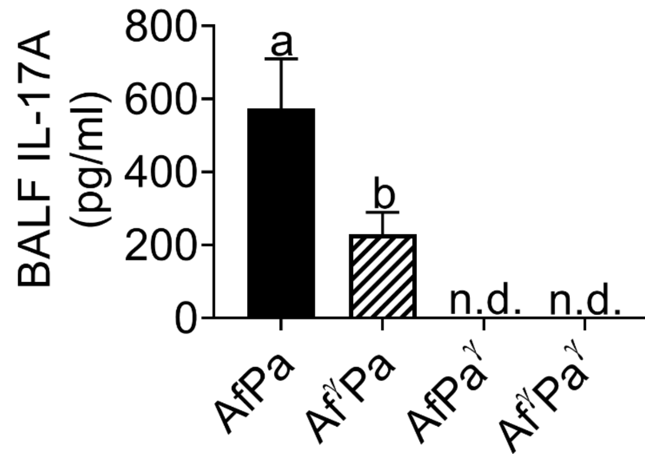

Figure 4.7. IL-17A production following co-exposure occurs only with viable *P. aeruginosa* IL-17A concentration in the BAL fluid (BALF) in pg/mL is reported as the average ± SEM for the following allergic groups: live *A. fumigatus* and *P. aeruginosa* (AfPa; solid black), gamma-irradiated *A. fumigatus* and live bacteria ($Af^{\gamma}Pa$; forward hashes), live *A. fumigatus* and gamma-irradiated *P. aeruginosa* ($AfPa^{\gamma}$; n.d.), and gamma-irradiated *A. fumigatus* and *P. aeruginosa* ($Af^{\gamma}Pa^{\gamma}$; n.d.). Statistical differences are indicated by letter differences. Samples are indicated by n.d. if not detected.

As we observed in the previous study on the co-exposure to *A. fumigatus* and *P. aeruginosa*, the allergic co-exposed animals had a greater concentration of the pro-inflammatory cytokine, IL-17A, when compared to controls. Therefore, we wanted to test to see if the same is true of the viable and non-viable pairings of the bacteria and fungus. The only other group that produced IL-17A was the group with gamma-irradiated fungus but live bacteria ($Af^{\gamma}Pa$). When compared to group AfPa, the concentration of IL-17A $Af^{\gamma}Pa$ was significantly lower (p=0.0291) (Figure 4.7).

4.5. Discussion

The purpose of this study was to determine the impact of viability on the host immune response to *A. fumigatus* and *P. aeruginosa*, loss of lung integrity associated with interstitial inflammation and edema, an/or the outcome of death. Our findings suggest that the bacterial

viability had a significant impact on the disease outcomes, but fungal viability did not. When animals were exposed to live bacteria, pulmonary inflammation progressed with significant neutrophilia, elevated acute phase cytokines and chemokines and lung injury associated with interstitial inflammation and vascular leakage. Animals exposed to live bacteria and irradiation killed fungus had a 90% survival in comparison to live fungus and bacteria at a 65.2% survival, which may have been impacted by higher IL-17A and eosinophilic inflammation. Animals that were co-exposed to gamma-irradiated bacteria ($AfPa^{\gamma}$; $Af^{\gamma}Pa^{\gamma}$) exhibited neutrophilia and cytokine/chemokine production, but at a much lesser extent than the animals exposed to live bacteria.

P. aeruginosa and *A. fumigatus* individual exposures/infections are known to result in both IL-17A and eosinophilic recruitment (16-18). IL-17A is a major cytokine that is produced by numerous cells, including neutrophils and eosinophils (16, 19, 20). Severe asthma, characterized by neutrophilia and IL-17A, results in reduced lung function and patients are more likely to have asthmatic symptoms resistant to corticosteroid (21, 22). IL-17A has been associated with airway hyperresponsiveness (AHR) in asthmatic patients, with tissue injury, not unlike what we see in our current study, and has been implicated in facilitating pathogenic inflammation (22-24). Eosinophils can also contribute AHR and tissue injury in asthma, as well (25, 26).

Unfortunately, airway function was not readily gauged in the co-exposed animals as their lung integrity was severely impaired making AHR tests difficult to perform. We would likely need to complete an earlier timepoint in order to obtain an airway function reading. However, if both the eosinophils and IL-17A contribute to increased AHR (22, 25, 26), it would make sense that our the allergic co-exposed group may be more prone to death as their airways were already

more sensitive to bronchoconstriction. While the host immune response is expected to be a major contributor to the disease outcomes, it is also important to understand that microbial interactions are an important predictor of disease outcomes as the group that received killed fungus with live *P. aeruginosa* that had similar neutrophilic inflammation and acute phase cytokines and chemokines in comparison to the group exposed to both live fungus and bacteria.

This study suggests that it is likely a protein or other compound produced when both *A. fumigatus* and *P. aeruginosa* are viable that stimulates both the eosinophilic response and the production of IL-17A. Previous studies looking at *A. fumigatus* and *P. aeruginosa* have found that not only are volatile organic compounds differentially produced when these microorganisms are cultured together (27-29), but the bacteria and fungus can both produce cytotoxic proteins (30) and toxins (31) that will not only damage one another but also the host. Alternatively, the bacteria may use the viable or nonviable spore as scaffold for its biofilm development which allows for an evasion of the host response (32).

Aspergillus-Pseudomonas interactions are common, and their study has accelerated over the last ten years(11, 12, 14, 27-31, 33-37). These studies have shown that *A. fumigatus* and *P. aeruginosa* show that they can impair one another’s growth and affect pathogenicity (14, 27-31, 33-37). *P. aeruginosa* is known to produce biofilms (38) and its dissemination into the blood in our study could be facilitated by its interaction with *A. fumigatus* by evading the host and/or accelerating growth. One method that the bacteria and/or the fungus could use to evade detection from the host the through the formation of biofilms (39), which could lead to proliferation of the bacteria resulting in dissemination of the microorganism and decreased survival of the host.

4.6. Acknowledgements

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5. PAPER 3: MACROSCOPIC AND MICROSCOPIC INTERACTIONS OF *ASPERGILLUS FUMIGATUS* AND *PSEUDOMONAS AERUGINOSA*[3]

5.1. Abstract

The study of fungal and bacterial interactions provides insight into many different fields including agricultural and clinical settings. Fungal-bacterial interactions can be as disorganized as fungi and bacteria sharing the same environment to actual physical interactions of bacteria forming biofilms on the surface of fungal conidia. Of interest to our research group is the interactions between *A. fumigatus* and *P. aeruginosa* in respiratory disease. Previous studies have shown that growth of *A. fumigatus* is impaired by *P. aeruginosa* but we don't know how these microorganisms directly interact with one another and how this could contribute to the disease phenotype that we have identified. Our previous work has identified a severe disease phenotype when allergic animals are co-exposed to live *A. fumigates* and *P. aeruginosa*. Therefore, this study takes a microscopic approach to studying the interactions between these two microorganisms. What we have found is the production of fungal-bacterial aggregates early in co-culture that grow into large masses by 720 min (12h). Inside these masses at 1440 min (24h) it appears that conidia are germinating and forming hyphae. *In vivo,* we found that the bacteria and fungus still aggregate together, but at not to the same large mass extent that we see *in vitro*.

[3] The material in this chapter was co-authored by Breanne N. Steffan, Scott A. Hoselton, and Jane M. Schuh. Breanne N. Steffan had primary responsibility for collecting samples, preforming experiments, and analyzing results. Breanne N. Steffan was the primary developer of the conclusions that are advanced here and revised all versions of this chapter. Scott A. Hoselton assisted in analysis of electron micrographs. Jane M. Schuh served as proofreader and was consulted in the developing of conclusions by Breanne N. Steffan.

5.2. Introduction

From the production of microbial compounds (1-3), bioremediation (4), and the pathogenicity of microorganisms (5, 6), microbial communities are impacting the way we approach a variety of fields including agricultural and clinical settings. In agricultural and clinical settings, numerous studies have been conducted that focus on the physical complexes that occur between fungal and bacterial microorganisms. Typical descriptions of microbial communities involve alpha diversity, where the species richness and diversity are identified. For studies that want to compare different environments, beta diversity is also employed (7). For instance, Hilty et al characterized the lung microbiome of a healthy individual by identifying operational taxonomic units (OTUs) to determine the microbiome diversity in the lung. After identifying the healthy lung microbiome, they compared the OTUs present for different disease states including asthma and chronic obstructive pulmonary disease (8). While microbiome studies are important in identifying microorganisms that interact with one another, they do not provide information regarding the complexities of these communities when it comes to their structure and development.

Biofilms are highly structured and result in the development of macromolecules that maintain the structure of the community (9). Bacterial-fungal biofilms can exist as mixed complexes or bacterial biofilms can form on the biotic surfaces of the fungi (10). In some agricultural and clinical studies, it has been found that the bacteria can have an endosymbiotic relationship, with the bacteria occupying the cytoplasm of the fungal hyphae or spores (11-13). The study of these fungal-bacterial interactions are necessary in clinical diseases including CF, where the combined colonization of the lungs by *Pseudomonas aeruginosa* and *Aspergillus fumigatus* contribute to more severe disease outcomes (14).

In vitro studies that have co-cultured these microorganisms with one another have found that *P. aeruginosa* impairs the growth of *A. fumigatus* by preventing conidial germination, hyphal growth, and biofilm development (15-18). However, few studies have defined the type of physical interactions that result at the microscopic scale. Upon the *in vitro* co-culturing of *A. fumigatus* and *P. aeruginosa,* we show that a fungal-bacterial precipitate forms with the bacteria surrounding the spore surface. Similar formations can be found in the bronchoalveolar lavages of mice dually exposed to the microorganisms.

5.3. Materials and Methods

5.3.1. *Aspergillus fumigatus*

The single lyophilized culture of *A. fumigatus* (ATCC 13073) was reconstituted in 5-mL PBS, and 60-µL aliquots of the suspension were stored at 4°C until use. Each 25-cm^2 vented cap culture flask with Sabouraud dextrose agar (SDA) was inoculated with a 60-µL aliquot of *A. fumigatus* and grown at 37°C for 8d. In a BSL-2 Safety Cabinet, the 8d *A. fumigatus* conidia were collected into PBS Tween 80 to prevent clumping. Samples were counted with a hemocytometer, centrifuged at 600 x *g* for 10-min at 4°C, and resuspended to 10^9 conidia/mL in PBS. All work using *A. fumigatus* was approved by North Dakota State University's Institutional Biosafety Committee (IBC).

5.3.2. *Pseudomonas aeruginosa*

The freeze-dried ATCC 27853 *P. aeruginosa* strain was reconstituted with 5-mL of tryptic soy broth (TSB) as per manufacturer's instructions. The bacterial culture was streaked for isolation on tryptic soy agar (TSA). Isolated colonies were inoculated into TSB and allowed to grow to mid-log at 37°C and constant agitation at 150 rpm. Mid-log cultures were resuspended in freezing media and stored at -80°C until use. Upon reconstitution, the freezer stock was

resuspended in TSB and grown to mid-log at 37°C with constant agitation at 150 rpm. Samples were then streaked for isolation of TSA and the isolated colony was used to inoculate TSB and be propagated as previously described. Mid-log cultures were counted using Trypan Blue and a hemocytometer. Samples were centrifuged at 2,000 x *g* for 10-min at 4°C and resuspended to 10^9 bacteria/mL in TSB. All work using *P. aeruginosa* was approved by North Dakota State University's Institutional Biosafety Committee (IBC).

5.3.3. Co-culturing *A. fumigatus* and *P. aeruginosa*

To co-culture these microorganisms, we took one mL of 10^9 conidia and one mL of 10^9 bacteria and combined them in a 50 mL conical tube with TSB to a total volume of 10-mL. We also had individual controls that were inoculated with just bacteria or conidia. All cultures were grown at 37°C with shaking at 150 rpm for up to 1440 min (24h).

5.3.4. Experimental Animals

BALB/c mice (6-8 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed on Alpha-dri paper bedding (Shepherd Specialty Papers, Watertown, TN) in microfilter-topped cages (Ancare, Bellmore, NY) in a specific pathogen-free facility with *ad libitum* access to food and water. All research protocols were carried out under the guidelines of the Office of Laboratory Animal Welfare (OLAW) and in compliance with North Dakota State University's Institutional Animal Care and Use Committee (IACUC).

5.3.5. Co-Exposure to *A. fumigatus* and *P. aeruginosa*

A three-mouse nose-only inhalation chamber was fitted with an in-line 25-cm^2 fungal culture flask with 8d conidia inoculated as previously described. The apparatus was housed in a Class II biological safety cabinet for procedures. In order to liberate the hydrophobic spores, 2-psi of air was delivered across the flask allowing for the spores' delivery through the inhalation

chamber. Exhaust air containing conidia was collected into two serial traps containing the sporicide, Spor-Gon (Decon Labs, King of Prussia, PA). Mice were anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) and placed supine with their noses in the ports and exposed to a 10-min nose-only inhalational of live *A. fumigatus* conidia. Co-exposed treatment group animals were inoculated intranasally (IN) with $3x10^7$ *P. aeruginosa* bacteria in 20-µL of PBS immediately after the inhalation of live *A. fumigatus* conidia. Mice that just received the IN of $3x10^7$ *P. aeruginosa* bacteria were also anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) prior to exposure to keep experimental conditions consistent. Samples were collected at 12h post exposure.

5.3.6. Microscopy

Using an inverted microscope with a $MoticamX^2$ camera (Motic, British Columbia, Canada), images were obtained of the co-cultures at timepoints between 0-1440 min (24h) incubations. Additionally, 100-uL of each sample was dried onto a microscope slide and stained with a differential stain (Quick-Dip stain; Mercedes Medical, Sarasota, FL).

Samples from time-points in the *in vitro* and *in vivo* studies were fixed using glutaraldehyde and transported to NDSU's Electron Microscopy Center for scanning electron microscopy (SEM), field-emission scanning electron microscopy (FESEM), and transmission electron microscopy (TEM). Procedures were performed using the JEOL JSM-6490LV variable-pressure SEM, the JEOL JSM-7600F FESEM, and the JEOL JEM-100CX II TEM by qualified scientists.

5.4. Results

5.4.1. Formation of Fungal-Bacterial Aggregate

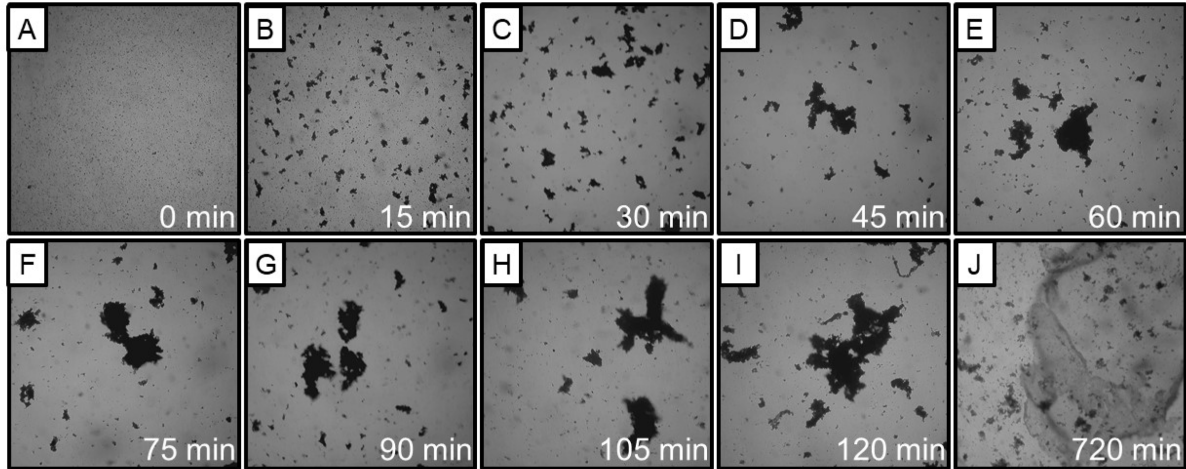

Figure 5.1. *In vitro* aggregation of *Aspergillus fumigatus* and *Pseudomonas aeruginosa* Interactions of *A. fumigatus and P. aeruginosa* were tracked from 0-120 min (A-I) and at 720 min (J) using inverted microscopy at 40x total magnification.

Numerous studies that have focused on the direct and indirect interactions of *A. fumigatus* and *P. aeruginosa* and the impact on the microbial growth (14, 17, 19-21), yet little is known about the actual physical interactions that occur between these microorganisms at a microscopic level. In Figure 5.1, we show a time course of *A. fumigatus* and *P. aeruginosa* interactions, in which aggregates of the fungus and bacteria develops and gets progressively larger as time passes. Initially, the bacteria and the fungus are separate entities (Figure 5.1A). At 15 min of co-culturing, we begin to see small direct physical interactions between the fungal spores and bacteria microscopically (Figure 5.1B). These interactions are not able to be seen at the macroscopic level until 30 min of co-culturing (macroscopic observations not shown).

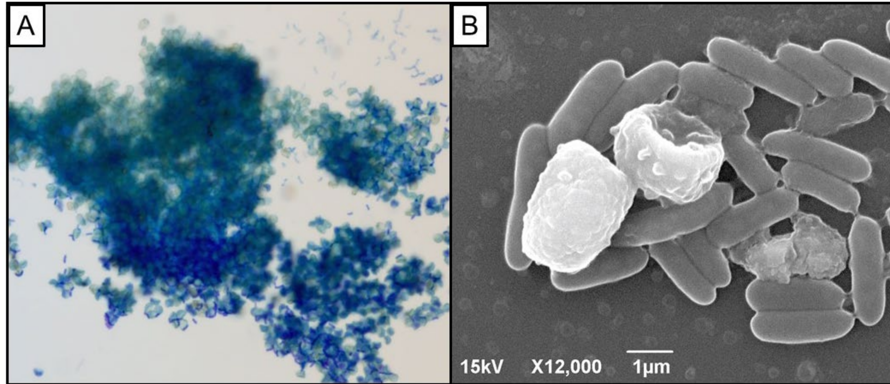

Figure 5.2. *In vitro* aggregate of *A. fumigatus* and *P. aeruginosa* at 120 min (2h) Photomicrographs of the *A. fumigatus* and *P. aeruginosa* aggregates using bright-field microscopy (A) at 1000x total magnification and by scanning electron microscopy (SEM) at a total magnification of 12,000x (B).

At 120 min (2h) of co-culturing the microorganisms, the aggregates were visible macroscopically (not shown) and microscopically (Figure 5.1I; Figure 5.2) and would not go back into solution. In contrast, the fungus-only cultures started to grow at approximately 240 min (4h), fell out of solution, but were easily suspended again upon gentle agitation. At this time-point we were also able to obtain a SEM photomicrograph (Figure 5.2.B) that shows the fungal spores surrounded by the bacteria. Some of the fungal spores appeared to be dimpled or damaged. While we know that the interactions result in a large aggregate of cells by 120 min (2h), we also wanted to see if the structures remained at 720 min (12h) (Figure 5.1J) and 1440 min (24h) (Figure 5.3) of co-culturing. At 720 min (12h) a more film-like structure is present that is much larger than previous aggregates (Figure 5.1J). This structure breaks down a bit by 1440 min (24h) (Figure 5.3.A) but is still quite large.

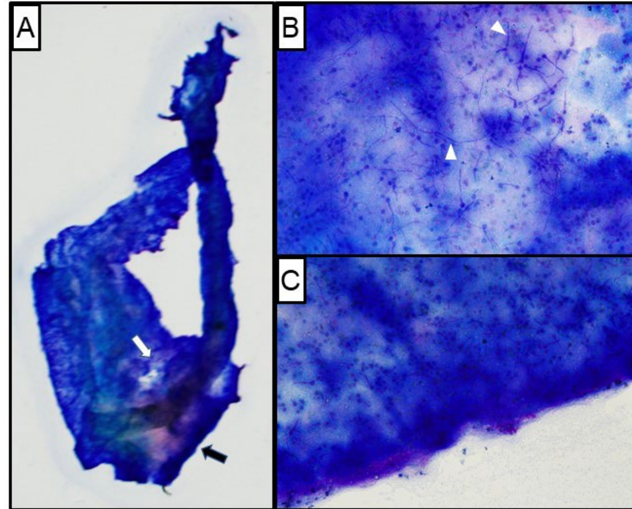

Figure 5.3. Photomicrograph of *in vitro* fungal-bacterial aggregate at 1440 min (24h) Aggregation of *A. fumigatus* and *P. aeruginosa* at 20x total magnification (A) and 600x total magnification (B&C). The white arrow indicates where the upper photomicrograph (B) was taken and the black arrow indicates the region that the bottom photomicrograph was taken. The white arrow heads are pointed to hyphal growth (B).

At 1440 min (24h) following co-culturing *A. fumigatus* and *P. aeruginosa,* macroscopic aggregates are clearly visible in the culture media. Upon microscopic examination (Figure 5.3), the mass contains both fungal and bacterial organisms with what appears to be hyphal growth occurring in the center of the structure (white arrow heads, Figure 5.3B). This observation is important in that current research suggests that *A. fumigatus* does not grow in the presence of *P. aeruginosa* (17, 19, 20). Clearly bacterial growth is also evident inside and outside of the structure (Figure 5.3C).

5.4.2. Absence of Flagella Upon Direct Interactions with Fungal Spores

One of the questions that developed upon carrying out our *in vivo* studies was whether *P. aeruginosa* was masking its flagella when co-exposed with *A. fumigatus.* To address this, we co-cultured our samples and took photomicrographs using TEM of the bacterial-fungal interactions at 2h (Figure 5.4A) and 24h (Figure 5.4.B) co-incubations to visualize the organisms.

When examining bacteria in direct contact with the fungal spores at 2-h of co-culturing (Figure 5.4.A) the bacteria seems to lack flagella. However, the flagella appear to return to the bacteria at 24-h of co-culturing (Figure 5.4.B).

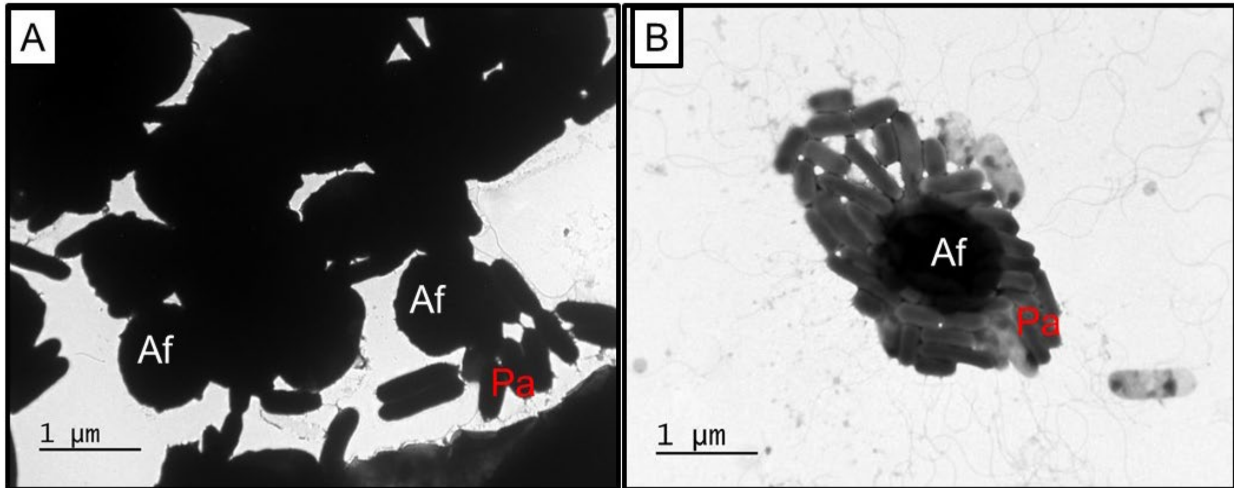

Figure 5.4. Transmission electron micrographs (TEM) of *A. fumigatus* and *P. aeruginosa* interactions
TEM photomicrographs of *A. fumigatus* (Af; white letters) and *P. aeruginosa* (Pa; red letters) at 120 min (2h) (A) and 1440 min (24h) (B) co-culturing.

5.4.3. *In vivo* Direct Interactions of *P. aeruginosa* and *A. fumigatus*

Animals were exposed to both *P. aeruginosa* and *A. fumigatus* to determine their interactions with one another *in vivo*. We chose to take a BAL sample at 720 min (12h) post co-exposure as the animals were in a precipitous decline in health at this stage and we saw a distinct aggregate formed *in vitro* at this timepoint. We looked at samples from both female (Figure 5.5.A) and male (Figure 5.5.B) with no difference. In both there appears to be a direct interaction of both microorganisms in the lung. We did not recover any structures as large as the aggregates that formed *in vitro*, but this could be due to many reasons including the shear force during sample retrieval, aggregation formation timing may differ in the host, or perhaps the larger aggregates are bound or stuck in the airway.

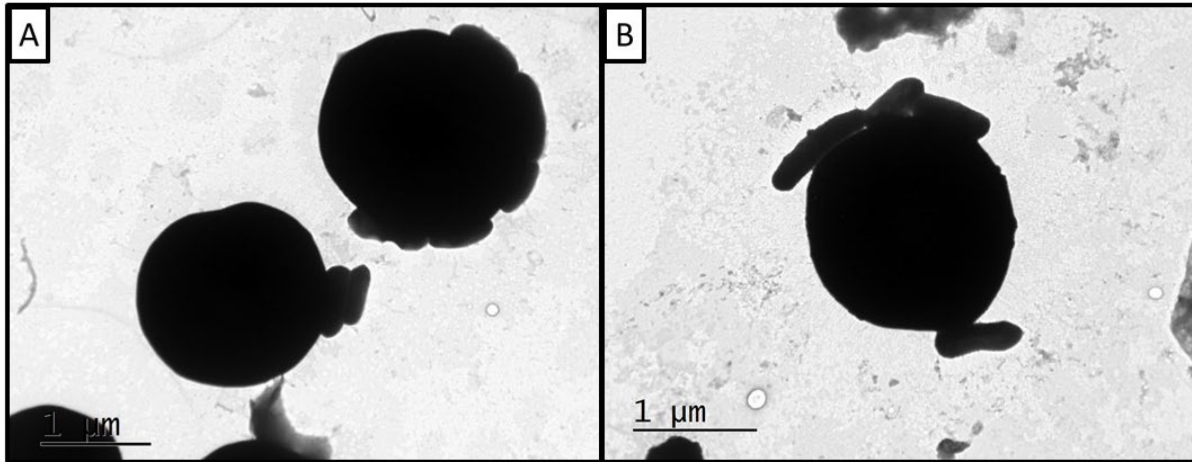

Figure 5.5. *In vivo* interactions of *A. fumigatus* and *P. aeruginosa*
Representative scanning electron photomicrographs of bronchoalveolar lavage (BAL) samples from female (A) and male (B) animals 720 min (12h) following co-exposure to *A. fumigatus* and *P. aeruginosa*.

5.5. Discussion

Fungal-bacterial communities have been identified for years in agricultural and clinical settings (1-6). Clinically, we have identified microbial communities for numerous disease states (8). However, the aggregation of microorganisms is not well characterized in current literature. Physical interactions aid microorganisms in their survival and colonization in adverse environments (10, 22). We were curious as to the type of interactions that take place between *A. fumigatus* and *P. aeruginosa* as our previous *in vivo* studies show that co-exposure to both microorganisms leads to significant morbidity and death. When we rendered the bacteria and/or fungus non-viable, we still had a similar inflammatory response, but the outcome of death was reduced or eliminated. Therefore, we decided to focus more on the interactions of the two microorganisms with one another and found that the bacteria will interact with the fungus to form a large aggregate during co-culturing. A similar interaction can be found *in vivo*.

Fungal organisms have been identified as self-aggregating or aggregation with other microbial organisms. In a study by Klotz et al, the agglutinin-like sequence (ALS) adhesin was

identified as an integral protein involved in the aggregation of the yeasts *Candida* or *Saccharomyces* with one another or other yeast and bacteria (23). *A. fumigatus* can also form aggregates with itself and other microorganisms. When the conidia swell during germination, α1-3glucan chains are exposed and facilitates aggregation and growth (24). Though, interactions between *A. fumigatus* and *P. aeruginosa* show fungal germination and growth is impaired by the bacterium.

Staphylococcus aureus is known to adhere *A. fumigatus* spores which resulted in changes to the conidial structure, minimal hyphal development, and lysis of the spores. The cytoplasmic material released upon spore lysis was a material in which the bacteria could bind. Both microorganisms had a reduced biofilm formation (25). We too see the *P. aeruginosa* adhere to the external surface of the spore and changes to the conidia structure following the co-culture of the microorganisms together.

This physical interaction could have an impact on the pathogenicity of both microorganisms as many of the *in vitro* studies with *P. aeruginosa* and *A. fumigatus* have found impaired iron chelation or increased virulence factors. Future work on this project entails transcriptome research on both the fungus and bacteria to see if there are specific virulence genes that are upregulated as a result of this interaction.

5.6. Acknowledgements

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is solely the responsibility of the authors and does not represent the official views of the ND Agricultural Experiment Station or the National Institutes of Health.

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6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

This current work addresses initial questions into the interactions of *A. fumigatus* and *P. aeruginosa* in pulmonary disease. We show that non-allergic and allergic animals co-exposed to *A. fumigatus* and *P. aeruginosa* have a profound increase in morbidity and mortality in comparison to fungal and bacterial controls. Allergic animals co-exposed to the fungus and bacteria had the most severe disease out of all the groups with a 34.8% mortality at 24h and the remaining animals were in a moribund state. The major differences that this group had in contrast to the other groups were its significant eosinophilic inflammation and the increase in IL-17A production. This remained the case when we tested whether viability of the microorganisms was necessary for the disease outcomes that occurred in this allergic co-exposed group. Our research showed bacterial but not fungal viability results in neutrophilia and acute phase response cytokines and chemokines in allergic animals, which was similar to that of the allergic animals co-exposed to live fungus and bacteria. Increased eosinophilia and IL-17A were specific for the most severe phenotype, the allergic animals co-exposed to live *A. fumigatus* and *P. aeruginosa*.

Because the co-exposure to live *A. fumigatus* and *P. aeruginosa* resulted in the most severe cases, we decided to characterize the physical interactions that the microorganisms have with one another both *in vitro* and *in vivo*. When co-cultured, the microorganisms begin to aggregate within fifteen minutes of interaction. This aggregate is not easily disassociated and upon looking microscopically at the center of the aggregate using bright-field microscopy, we were able to see the bacteria surrounding the fungal spores and hyphal growth in the center of the mass. TEM images of BAL samples from co-exposed animals shows a similar interaction, where

the bacteria are surrounding fungal spores. This physical interaction may be driving host evasion methods by hiding flagella from host detection or by forming a biofilm that makes it difficult for the host to effectively clear from the airways.

Together, this evidence suggests that both host and microbial factors play a role in the decline in health of animals co-exposed to these microorganisms. Allergy may contribute to the eosinophilia and IL-17A expression resulting in the pathology witnessed in our model.

6.2. Future Directions

Currently, we are running transcriptome analysis on the co-cultures of *A. fumigatus* and *P. aeruginosa* at early and late timepoints in the development of the aggregates to identify factors that induce aggregate formation as well as to determine if the interaction results in the production of different virulence factors by the fungus and bacteria as a means of antibiosis. One such class of virulence factors that we are interested in is the production of volatile organic compounds (VOCs).

We are also interested in whether this is a strain specific response, so we are looking into options of using different *P. aeruginosa* strains (both mucoid and non-mucoid) and if this is allergy specific. There are numerous models of respiratory allergy, including the house dust mite, ovalbumin, and other fungal models. This project has multiple directions that can be taken to answer numerous questions on polymicrobial interactions and the immune response.