INVESTIGATIONS IN ASTHMA HETEROGENEITY: THE ROLES OF *ASPERGILLUS FUMIGATUS*-DERIVED EICOSANOID SYNTHASES AND OCCUPATIONAL EXPOSURES TO GRAIN DUSTS ON THE DEVELOPMENT OF FUNGAL ALLERGIC ASTHMA

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By
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ABSTRACT

Allergic asthma is an inflammatory syndrome of the respiratory system which changes the airway wall architecture. Using an aeroallergen, murine model of *A. fumigatus*-mediated asthma, the two studies herein examine the development of asthma in the contexts of host-allergen interactions via *A. fumigatus* knock-outs of eicosanoid synthases and occupational exposures to corn and soybean dusts. The lack of difference between control and treatment groups seen in post-methacholine airway responses, goblet cell metaplasia, peribronchial inflammation, and fibrosis in the first study show that fungus-derived eicosanoid synthases are dispensable in the development of fungal allergic asthma. However, the same set of respiratory parameters in the grain dust study reveals an increase in BAL neutrophilia and serum IgE titer. The study also underscores a need for modifications of dust exposure times and of time-points of data analysis. These two studies represent unique perspectives on asthma pathogenesis and emphasize the heterogeneity of the syndrome.

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FRONTISPIECE

“Have some wine,” the March Hare said in an encouraging tone. Alice looked all round the table, but there was nothing on it but tea.

“I don't see any wine.” she remarked.

“There isn't any,” said the March Hare.

“Then it wasn't very civil of you to offer it,” said Alice angrily.

“It wasn't very civil of you to sit down without being invited,” said the March Hare.

“Speak English!' said the Eaglet. “I don't know the meaning of half those long words, and I don't believe you do either!”

- *Alice in Wonderland*, Lewis Carroll.

PREFACE

This disquisition comprises two separate manuscripts united by a common Abstract, General Introduction, Literature Review, General Discussion, and a master list of References that can be found at the end of the document. Each manuscript, however, has its own detailed Abstract, Introduction, Materials and Methods, Results, and Discussion sections.

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AHR .....airway hyperresponsiveness

AM .....alveolar macrophage

AMCase .....acidic mammalian chitinase

ASM.....airway smooth muscle

BAL.....bronchoalveolar lavage

BALF .....bronchoalveolar lavage fluid

BHR .....bronchial hyperresponsiveness

BMCP .....basophil mast cell progenitor

CCL.....CC chemokine ligand

CCR.....CC chemokine receptor

CD .....cluster of differentiation

COPD.....chronic obstructive pulmonary disease

CPB .....CREB-binding protein

CRTH2.....chemoattractant receptor-homologous molecule

CSK.....C-terminal src kinase

CXCL.....C-X-C motif ligand

DC .....dendritic cell

DP .....D-type prostanoid receptor

ECP .....eosinophil-derived cationic protein

ELISA .....enzyme-linked immunosorbent assay

EMTU .....epithelial mesenchymal trophic unit

FAP .....facilitated antigen presentation

FGF .....fibroblast growth factor

GMCSF .....granulocyte macrophage colony stimulating factor

GPCR .....G-protein coupled receptor

H&E .....hematoxylin and eosin stain

HLA .....human leukocyte antigen

ICAM .....intracellular adhesion molecule

IFN .....interferon

Ig .....immunoglobulin

IL.....interleukin

ITAM .....immunoreceptor tyrosine-based activation motif

JAK .....janus kinase

LT.....leukotriene.

MBP .....major basic protein

MDP.....macrophage and dendritic cell precursor

MMP .....matrix metalloproteinase

NFAT .....nuclear factor of activated T cells

NF-κB .....nuclear factor- κ-light-chain-enhancer of activated B-cells

PAS .....periodic acid-Schiff stain

pDC.....plasmacytoid dendritic cell

PG .....prostaglandin

SCF .....stem cell factor

SH2 .....src homology 2

ST-2.....suppression of tumorigenicity-2

STAT.....signal transducer and activator of transcription

TGFβ.....transforming growth factor β

Th .....T-helper

TNFα.....tumor necrosis factor α

TSLP .....thymic stromal lymphopoietin

VCAM.....vascular cell adhesion molecule

VEGF .....vascular endothelial growth factor

1. LITERATURE REVIEW

1.1. General Introduction to Fungal Allergic Asthma

Breathing is important. In the case of protists, gas exchange is accomplished via simple diffusion; however, in more complex mammalian organisms a specialized system of connective tissue, airways, and respiratory muscles ventilates the body with oxygen and, in exchange, removes harmful carbon dioxide in an energy-efficient manner (Hlastala 2001). Inhaling foreign particles present in the air is an 'occupational hazard' that the lung encounters in this task. While mechanisms for filtering or removing these particulates are present, not all are removed. Some of these foreign particles can be deleterious to the lung, and impair the process of gas exchange. In the case of allergic asthma, foreign particles bearing allergens are sampled by resident pulmonary dendritic cells, which then process and present the allergen to T cells. In the presence of Th2 cytokines such as IL-4, CD40L on T cells binds CD40 on B-cells initiating allergen-specific IgE synthesis. IgE binds to its high and low affinity receptors, FcεRI and FcεRII, respectively, which are found on mast cells, basophils, eosinophils, macrophages, and lymphocytes. Subsequent inhalation of the allergen cross-links IgE molecules on these cells' surfaces, which causes the degranulation of mast cells and eosinophils and the consequent release of cytokines and inflammatory mediators that may cause bronchoconstriction and vasodilation (Barnes 2008, Holgate 2008, Busse 2001).

Asthma patients present with dyspnea and wheezing due to obstructed pulmonary airflow. This occurs as a result of the rapid constriction of the respiratory muscles in response to the mediators released from mast cells and eosinophils. As a consequence, one of the chief

features of asthma is an increased bronchospasmic response known as airway or bronchial hyperresponsiveness (AHR/BHR). AHR is accompanied with mucus hypersecretion as a result of goblet cell metaplasia in the pulmonary epithelium. These acute features comprise the early or immediate-phase response in asthma. The late-phase response is an amplification of the early-phase response but also consists of cytokine and chemokine release which leads to further inflammatory cell infiltration into the airway as well as the initiation of airway remodeling events (Murdoch 2010, Galli 2008).

In most cases, early airway dysfunction is reversible, but persistent allergenic stimulation may lead to the establishment of the so-called Epithelial Mesenchymal Trophic Unit (EMTU) where innate-type inflammatory cells and adaptive immune cells are resident. The constant tissue-damage and repair responses lead to alterations in the architecture of the airways that manifest as airway smooth muscle hypertrophy and peribronchial fibrosis, which reduce the diameter of the airway lumen (Murdoch 2010, Holgate 2010, Camoretti-Mercado 2009, Galli 2008). The impact of these changes is obvious: tenacious AHR and severely reduced lung function.

Perhaps the most compelling aspect of the disorder is that a wide variety of allergens and irritants may lead to the cardinal features of the disease. Where the allergic manifestation of asthma is dominated by a Th2-type, IgE-dependent immune response; the non-allergic version of the disease may be induced by atmospheric pollution, ozone, cigarette smoke, and exercise. Non-allergenic, or intrinsic, asthma does not rely solely on a Th2-type immune response as is the case with allergic, or extrinsic, asthma does. Innate-type cells, coupled with Th2 cells, cytokines, and a local increase in IgE (compared to a systemic upregulation, as in allergy), forms a unique

immunopathogenic pathway of its own that, eventually, leads to the same symptoms (Kim 2010, Humbert 1999). In addition, "gene-environment interactions" (Holgate 2008) and other contributions that are associated with the allergen, such as proteases or toxins for example, add further complexity to the pathogenesis of the disease. While a review of human asthma-susceptibility genes goes beyond the scope of this disquisition, several excellent reviews are available on this topic (Bhakta 2011, Akhabir 2011).

The two main themes of this disquisition are host-fungus interactions and the involvement of environmental exposures to grain dust in a model simulating occupational asthma as induced in agricultural settings. First, the role of *Aspergillus fumigatus*-derived eicosanoid synthases was investigated using our murine model of fungal allergic asthma as an example of host-fungus interactions, and a description of the pathogenic and allergenic potential of *A. fumigatus* is presented. The project unites animal modeling with host-allergen interactions to explore a putative mechanism by which allergic inflammation may be perpetuated in asthma. In a second experimental study, we modeled occupational asthma in an agricultural setting. This is of particular importance in an agricultural state such as North Dakota with 31,600 farming operations and the average farm size spanning 1,253 acres (USDA Agricultural Research Service 2012). The grain dust studies model occupational agricultural exposures to evaluate the effect of grain dust on the asthmatic individual with the intent that this information will provide background to formulate therapeutic intervention strategies. Despite the disparate natures of the two chapters in this disquisition, they are united in the fact that they exemplify the heterogeneity of asthma and the power of animal models of allergic disease to explore the same in different

contexts. The subsequent section will describe prevalent animal models of asthma, with special emphasis on the model developed in our laboratory and its associated read-outs.

1.2. Animal Models of Fungal Allergic Asthma

Animal models present a powerful means to study the immunologic phenomena that characterize acute and chronic inflammation in asthma. So far, mice, rats, guinea pigs, ferrets, cats, dogs, sheep, pigs, horses, and non-human primates have been candidate animals for modeling asthma (Isenberg-Feig 2003). However, only murine modeling shall be discussed for the purposes of this disquisition.

Both acute and chronic murine models of asthma exist and the schemata of allergen sensitization and challenge remain consistent. Acute models explore the initial inflammatory and obstructive events of the disease such as airway hyperresponsiveness, increased levels of IgE, airway wall inflammation and the magnitude of goblet cell metaplasia. In acute models, the animal is sensitized both systemically and locally with the allergen of choice in the presence of an adjuvant. The adjuvant helps boost the immunogenicity of the allergen and prime the Th2-type response (Nials 2008). 2-4 weeks post-sensitization, the animal is subjected to either single or multiple allergen challenges. This may be accomplished via intratracheal (IT), intranasal (IN) (Shin 2009) or, as in our case, inhalational (IH) means of allergen delivery. While the inflammatory parameters produced in acute models are ephemeral, they do offer a means of observing mechanisms that establish pulmonary inflammation.

Chronic models of asthma aid in understanding airway remodeling events such as goblet cell hyperplasia, peribronchial fibrosis, airway smooth muscle hypertrophy, angiogenesis and

persistent AHR (Shin 2009). Chronic models rely on long-term, low-level allergen exposures followed by challenge and analysis at pre-determined time-points (Nials 2008). Chronic models allow for the observation of immunologic interactions in a pre-established milieu of inflammation and so are more faithful to clinical cases of human asthma (Nials 2008, Zosky 2007).

The outcomes observed from mouse models depend on factors such as the choice of mouse-strain, choice of allergen and whether or not an adjuvant was used during the sensitization phase. Commonly used adjuvants include alum, Freund's adjuvant and aluminum hydroxide. While an adjuvant is an immunopotentiator, adjuvant-free protocols have been developed. These, however, require more numerous allergen exposures and carry the risk of inducing tolerance to allergen (Isenberg-Feig 2003).

The advantages of using mice as a model organism are multifarious: they are cheap to purchase and maintain (Isenberg-Feig 2003), we have a complete understanding of their genetics, and the use of transgenic technologies enables us to interrogate differential molecular aspects of the disease (Elias 2003). Further, housing them in specific pathogen-free facilities enables us to rule out the pathology stemming from etiological agents other than those employed in the study. Of the many commercially available laboratory mouse-strains, the two that are relevant to this disquisition include the BALB/c and the C57BL/6 strains. The Th2-skewed BALB/c mice develop high titers of allergen-specific IgE, are hyperresponsive to methacholine provocation, and display increased levels of IL-4 and IL-13 compared to the Th1-skewed C57BL/6 strain (Gueders 2009, Fukushima 2006). However, higher CCL11 levels in C57BL/6 mice enable a greater magnitude of BAL eosinophilia compared to BALB/c's (Gueders 2009). As ideal as the

BALB/c genetic background is for asthma studies, the majority of transgenic/knock-out animals are made using the C57BL/6 background (Shin 2009). The need of the hour, therefore, is an increase in developing transgenic/knock-out BALB/c mice.

One of the prime limitations of murine modeling is the difference between mouse and human airway physiology. The mouse tracheobronchial unit subscribes to a monopodial branching pattern compared to the dichotomous branches seen in humans (Hyde 2006). These differences may account for why allergen-induced pathology extends to the parenchyma and visceral pleura of the murine lung but is restricted to the conducting airways in human beings (Isenberg-Feig 2003). Further, mice are not known to naturally acquire asthma and neither do they exhibit spontaneous AHR. The pulmonary musculature of the murine lung is modest compared to that of the human lung and, thus, may be a reason for the lesser degree of AHR seen in mice (Shin 2009, Hyde 2006). Admittedly, asthma is a "uniquely human disease" influenced by lifestyle and genetic predispositions (Holmes 2011) and those cannot be modeled with high fidelity in mice. Despite these limitations, the ability to manipulate murine biology enables the use of mouse models as predictors of interventionist and therapeutic strategies to control human asthma.

Ovalbumin (OVA) is the most commonly used allergen in mouse models of asthma despite being quite irrelevant to human allergic asthma. However, more pertinent allergens, such as house dust mite (HDM), cockroach and ragweed pollen antigens, and *Aspergillus fumigatus* conidia are becoming more commonplace (Zosky 2007). The allergenic and pathogenic features of *A. fumigatus* are highlighted in a subsequent section, but some comparative features of our laboratory's model of *A. fumigatus*-induced asthma with other models are presented.

The primary difference between our laboratory's allergic asthma model and other established protocols is the means of allergen challenge: the IH challenge, compared to the IT challenge (Hogaboam 2000), is non-invasive, allows for multiple challenges, realistically mimics allergen exposure, maintains the antigenic integrity of the conidia, and results in the characteristic pathologic features of the disease, as discussed previously (Samarasinghe 2011).

Ultimately, the strengths of our model of allergic asthma are manifold. It uses a clinically relevant allergen and can be used to study both acute and chronic features of asthma. It is, thus, a tool to delineate host-allergen interactions and can also be adapted to study occupational asthma in both chronic and acute contexts.

1.3. *Aspergillus fumigatus*: In Atopy and Invasive Disease

The filamentous molds that make up the genus *Aspergillus* are of the oldest known molds described by humans. Discovered in 1729 by the priest Pier Antonio Micheli, the genus was named for the resemblance of the conidiophore to the aspergillum—a device used in Catholic churches to sprinkle holy water. The *Aspergilli* are united in terms of morphology, physiology, genomics, and a well-recognized association with humans as industrial agents, pathogens, allergens, and tools of research in basic science (Bennett 2010).

A. fumigatus was not considered a pathogen until recent times. Rather, it was considered a saprophytic, soil-dwelling mold, existing on organic substrata with major functions in carbon and nitrogen cycling (Latgé, 1999). Indeed, in 1939, one of the earliest reports evaluating the pathogenic potential of *Aspergillus fumigatus* said that "in man and the mammals, such infections are so rare as to be of little practical importance" (Henrici 1939). Given the increase in

immunosuppressed populations due to solid-organ transplants (Pappas 2010), hematopoietic stem cell transplants, and individuals living with HIV/AIDS (Clark 2002) *A.fumigatus*-mediated mycoses are now commonplace (Steinbach 2012, Brenier-Pinchart 2011).

A. fumigatus thrives in a variety of different environments (Pringle 2005) and is widely distributed (Engelhart 2003). *A. fumigatus* conidia are highly hydrophobic, which aids in their dispersal in air currents. (Linder 2005). Moreover, the ability to grow at temperatures as high as 50ºC and to thrive at 37ºC facilitates *A. fumigatus*' function as an important organism in composting and as an opportunistic pathogen of human beings, respectively (Bhabhra 2005). The mold also bears features that help it evade the immune response. Its chitinous cell wall resists the complement-assembled membrane attack complex (Latge 2007), and it also produces a soluble inhibitor of the complement cascade (Kozel 1996). *A. fumigatus* can also produce catalases that help neutralize host cell derived reactive oxygen species (Calera 1997). Other immunomodulatory and immunoresistant features of *A. fumigatus* are not described in this disquisition but have been reviewed elsewhere (Osherov 2012, Abad 2010).

Aspergillus-associated mycoses are a result of an impairment or alteration of the immune response, for example, due to the propensity towards allergy or neutropenia because of immunosuppressive therapy. While the clinical spectrum of diseases caused by *A. fumigatus* is extensive, the two syndromes that are relevant to this disquisition, which will be expanded upon here, are: Invasive Pulmonary Aspergillosis (IPA) and Allergic Bronchopulmonary Aspergillosis (ABPA).

The average diameter of a human alveolus is 200 μM: large enough that *A. fumigatus* conidia can enter. Normally, alveolar macrophages and peripheral blood neutrophils are capable of destroying inhaled conidia and hyphae (Osherov 2012). However, under immunosuppressive circumstances, these cells are unable to eliminate the fungus and infection ensues. In the case of IPA, inhaled *A. fumigatus* conidia germinate within the alveoli, and the hyphal tube can invade the blood stream (Osherov 2012, Dagenais 2009). Clincally, those afflicted with IPA present with fever, cough, dyspnea, bronchopneumonia, and occasionally hemoptysis and/or lung collapse (Soubani 2002). The diagnosis of IPA is challenging and requires a thorough examination of the patient's medical history followed by evaluation of sputum for fungus and fungal culture, high-resolution chest CT, measurement of levels of galactomannan in the sera, and bronchoalveolar lavage or bronchoscopy (Zaas and Alexander 2009).

By contrast, the immunosuppressed population that is afflicted by ABPA is, largely, chronic asthmatics. The syndrome is characterized by Type I, Type III, and Type IVb hypersensitivity responses to *A. fumigatus* antigens (Agarwal 2009). The immunopathogenesis of ABPA implicates fungus-derived proteases in the desquamation of pulmonary epithelial cells. The resulting morphological changes to the airway epithelium cause an inflammatory response driven by IL-6 and IL-8. Consequently, enhanced sampling and presentation of *Aspergillus* antigens by resident dendritic cells to Th2-cells results in a Th2-skewed immune response (Kauffman 2000). The inflammatory and remodeling events that follow are similar to that of asthma (Knutsen 2011, Kurup 1991).

While only 7-9% of cystic fibrosis sufferers and 1-2% of asthmatics develop ABPA (Knutsen 2011), the allergenic potential of *A. fumigatus* is demonstrated in that 20-25% of

asthmatics are sensitized to *A. fumigatus* (Denning 2006). The prime difference between ABPA and *A.fumigatus*-mediated allergic asthma is that, in the former, the fungus actually invades the pulmonary epithelium, germinates and initiates a hypersensitive reaction. This does not happen in fungal allergic asthma. Instead, conidial antigens are processed by dendritic cells and presented to T cells to initiate a Th2-type immune response which directs the immunopathology seen in asthma (Holgate 2010). The subsequent section shall elaborate the role of airway epithelium-fungi interactions in the establishment of allergic disease.

1.4. The Airway Epithelium

The airway epithelium is the first host tissue that inhaled fungi encounters. It is a source of Th2-type cytokines (Bartemes 2012) and provides important protective barrier and immunoregulatory functions. As a physical barrier, the epithelium prevents the entry of allergens by sealing the paracellular spaces via tight junctions. The tight junctions comprised of protein-protein interactions between zona occludens proteins 1-3 (ZO 1-3), claudins 1-5, occludin, and adhesion proteins such as E-cadherin and β-catenin. Proteases associated with the allergen are often involved in the breakdown of these tight junctions, thereby allowing the allergen access to dendritic cells (Matsumura 2012, Wan 1999).

The expression of ICAM-1 (Intracellular Adhesion Molecule-1) and JAM-C (Junctional Adhesion Molecule-C) are upregulated in an inflammatory milieu, increasing both granulocyte adhesion and inflammation (Yang 2005, Zen 2003). The epithelium is also equipped with enzymes that metabolize arachidonic acid to eicosanoid mediators that effect both inflammation and airway remodeling in the lung. Cyclooxygenase expression is upregulated in response to

cytokines and histamine (Redington 2001). The prostaglandins produced by these cyclooxygenases are bronchoconstrictors but also exert protective roles, such as the attenuation of AHR and airway inflammation (Gauvreau 1999, Johnston 1995).

The leukotrienes are produced by the 15-lipooxygenase pathway. Their roles include bronchoconstriction and amplification of airway eosinophilia and collagen deposition (Chu 2002). Further, the sensitized epithelium is a source of endothelin, a vasoconstrictive, bronchoconstrictive and pro-fibrotic peptide mediator (Zietkowski 2008).

1.5. Cytokine Signaling in Allergic Asthma

The development of the Th2 phenotype and, by extension, the pathophysiological hallmarks of asthma requires well-timed signaling by a suite of multi-tasking cytokines. Interleukin-4 (IL-4) is critical for the differentiation of Th0 cells to the Th2 phenotype (Kelly-Welch 2005b). It is produced by Th2 cells, activated basophils, and mast cells. By driving Th2 differentiation, IL-4 incites T cell production of IL-5, IL-9, IL-13, and additional IL-4 (Barnes 2008). IL-4 modulates isotype switching to IgE in s and stimulates mast cells to produce leukotriene C4 (LTC4) (Barnes 2011a). With regard to epithelial cells, IL-4 encourages goblet cell hyperplasia and upregulates adhesion molecules such as VCAM-1 on endothelial cells, driving inflammatory cell accretion (Corren 2011)

IL-4 and IL-13 may be thought of as partner cytokines. They share receptor chains and may perform similar effector functions (Kelly-Welch 2005a), The gene that encodes IL-13 is in the same cluster of the genes that encode other Th2 cytokines (Wills-Karp 2004). However, the functions of IL-13 have more to do with changes to airway wall architecture than Th-cell fate.

IL-13 induces airway hyperresponsiveness and works in concert with TGFβ1 to induce the deposition of Type I and Type II collagen in the peribronchial regions of the lung (Hamid 2008). It also upregulates MMP-1 to induce the degradation of Type III collagen and, thus, diminishes the contractile properties of the airway (Hamid 2008, Hogan 2008). The cytokine also contributes to mucus hypersecretion by upregulating the transcription of mucin-encoding genes such as *MUC5AC* and *MUC5B* (Wills-Karp 2004). Finally, IL-13's ability to recruit eosinophils, via induction of eotaxin genes, is aided by the enzyme AMCase (Zhu 2004).

IL-9 is associated with the Th2 phenotype, but is produced by Th9 and Th17 cells which are involved in mast cell (Xing 2011) and neutrophil trafficking (Iwakura 2008) to the allergic lung, respectively. IL-9 production by the Th9 subset is governed mainly by IL-25. However, TGFβ1 and IL-4 are also essential for IL-9 production (Angkasekkwinai 2010). In allergic asthma, IL-9 is involved in airway remodeling and inducing airway hyperresponsiveness. It also increases expression of VEGF genes from mast cells in a STAT3 dependent manner (Sismanopoulous 2012). Moreover, IL-9 enhances FcεRI expression on mast cell surfaces and also induces IL-6 production by the same, thereby modulating mast cell differentiation and survival (Sismanopoulous 2012, Gullickson 2010, Kearley 2010).

The cytokines IL-5, IL-3, and GM-CSF are linked by a common receptor β-chain and a cytokine-exclusive α-receptor subunit (Broughton 2012). The three cytokines have a common effect of regulating eosinophil differentiation, survival, and recruitment from the bone-marrow. IL-5 is produced by $CD4^+$ T cells, eosinophils, and mast cells, while IL-3 and GM-CSF are produced by these cells in addition to alveolar macrophages and epithelial cells (Broughton 2012, Hamid 2009, Kouro 2009). In eosinophils, receptor engagement by this group of cytokines

activates the JAK2-STAT5 (Buitenhuis 2003) and the Ras-ERK signaling circuits, turns on anti-apoptotic genes and ensures eosinophil survival (Kouro 2009, Martinez-Moczygemba 2003, Adachi 1998).

Apart from eosinophilia, IL-3 and GM-CSF are essential for the development of dendritic cells (van Rijt 2005), mast cells (Mazzoni 2006), and basophils (Asquith 2008). Indeed, ablation of IL-3 leads to a significant decrease in the basophilic contribution of IL-4, while GM-SCF depletion causes dendritic cell numbers to diminish (Asquith 2008, van Rijt 2005). The three main barrier cytokines implicated in the development of allergic asthma include TSLP, SCF, and IL-33. TSLP acts on T cells, smooth muscle cells, dendritic cells, basophils, and mast cells. The receptor for TSLP consists of the α-subunit of IL-17R and an exclusive TSLPR. TSLP upregulates IL-13 production by NK T cells and increases the magnitude of AHR (Zhu 2011, Kashyap 2011). The production of TSLP by dendritic cells creates an elegant autocrine system wherein the cytokine acts upon its source cell to upregulate OX40L (Ito 2005). Interactions of OX40L with OX40, a T-helper cell costimulatory molecule, contributes to the polarization of the immune response in the Th2 direction. Further, TSLP causes an expansion of eosinophil, mast cell and basophil numbers and induces Th2 cytokine production from these cells (West 2012, Kashyap 2011, Kim 2010). SCF is produced by pulmonary epithelial cells, fibroblasts, myofibroblasts, mast cells, and eosinophils. SCF's cognate receptor, c-kit, is found on both mast cells and eosinophils. SCF-induced MAPK signaling enhances mast cell survival, chemotaxis and degranulation. SCF also induced the Th2-cell-recruiting chemokines CCL2, CCL22, and CCL 17 (Reber 2006).

The third barrier-derived cytokine is IL-33, which is a product of epithelial cells necrotizing in response to allergen-mediated inflammation (Lüthi 2009). The IL-33 receptor, ST2, is found on eosinophils, basophils, macrophages, natural killer cells, fibroblasts, and T cells. Signaling by IL-33 is via MAPK pathways and terminates in the activation of NF-κB (Borish 2011).. IL-33 drives Th2 prevalence by upregulating B7-2 and MHC-II on dendritic cells, both of which increase antigen presentation and T cell activation. In mast cells, IL-33-bound ST2 can heterodimerize with c-kit and lead to mass production of proinflammatory cytokines, chemokines, and prostaglandins and leukotrienes. This suggests a collaborative relationship between SCF and IL-33 (Borish 2011, Lynch 2003).

The immunomodulatory cytokines involved in allergic asthma include IFNγ, IL-10, and cytokines of the IL-12 family. The effects of IFNγ in the asthmatic lung counteract those of the typical Th2 cytokines: IFNγ causes isotype switching in s from IgE to IgG2 and also induces apoptosis in metaplastic epithelial cells, effectively resolving mucus hypersecretion, and counteracting the effects of IL-13 (Gough 2008, Lynch 2003). However, when concomitantly administered, IFNγ and IL-13 enhance each other's effects, given the sudden increase in NK cells, dendritic cells and IL-6 production (Ford 2001). This goes to show that the timing of cytokine production is vital in the orchestration of immune responses.

The importance of IL-12 to IFNγ production and its lowered levels in the BAL of asthmatic patients suggest that the main role of the cytokine is to countermand Th2 prevalence. (Barnes 2008a, Lynch 2003). However, IL-27, a member of the IL-12 family, has paradoxical effects on different Th subsets. It inhibits the Th2 and Th17 subsets at the transcriptional level by inhibiting the transcription factors GATA-3 (the "master regulator" of the Th2 phenotype)

(Yoshimoto 2007) and RORγT (and consequently the Th17 phenotype) (Hunter 2012). The cytokine's inhibition of Th1 expansion occurs via its negative regulation of IL-12 production (Hunter 2012). IL-27 upregulates the production of IL-10 from T cells in a MAPK-dependent pathway and solidifies its role as not merely a suppressive cytokine but also an immunomodulatory one (Saraiva 2010). Physiologically, abrogation of IL-27rα in mice leads to increased eosinophila, IgE levels, AHR, and goblet cell metaplasia in models of asthma (Hunter 2012).

The roles of IL-10 in allergic asthma are, at best, complex. The cytokine suppresses both Th1 and Th2 subsets and, in murine models of allergic asthma, diminishes the production of Th2-type cytokines and eosinophilic infiltration. However, IL-10 increases AHR, despite its anti-Th2 program (Lynch 2003, Schuh 2003). Post-allergen sensitization, pulmonary dendritic cells produce IL-10, which shapes the development of IL-10-producing-regulatory T cells in the lung. Adoptive transfer of Tregs diminishes AHR in a mechanism that relies upon TGFβ1 (Akbari 2002) and is revealing of the context-dependent immunomodulatory nature of IL-10.

Ultimately, the cytokine signaling suite that drives and typifies the pathophysiology of asthma relies on several repeated motifs in terms of cytokine structure, function, mode of action, redundancy, alliance, autonomy, and competition. This illustrates the multifactorial nature of asthma—a syndrome, which presents several therapeutic targets, but no single "correct" answer.

1.6. Immunoglobulin E: Structural and Functional Considerations

In atopy, Immunoglobulin E (IgE) is the arbiter of mediator release from basophils, mast cells, and eosinophils and is vital to the immunopathology of allergic disease. IgE is associated

with the Th2 response, which evolved as a repair response against other metazoan parasites, notably helminths (Allen 2011). In this project, however, the Th2 response is associated with allergies. The roles of IgE in this context, particularly in that of allergic asthma, are presented.

Macroscopically, the structure of IgE does not differ from other antibody isotypes in that it consists of two identical light and two identical heavy chains. However, the ε heavy chain of IgE contains four constant domains compared to the three found in the IgG γ chain. This feature restricts the flexibility of IgE since the extra constant domain is found where IgG has a hinge-like region that enables the latter to bend and gain entry to less accessible parts of the body. Crystallographic analyses reveal the constant domains, Cε3 and Cε4, to be linked by disulfide bridges causing the antibody to bend at an angle of 62º. (Wan 2002). IgE's bent appearance coupled with the smaller "elbow-angles" made by the κ light chains may facilitate cross-linking of two bound IgE molecules (Niemi 2007).

The receptors bound by IgE include the high-affinity FcεRI receptor; the low affinity receptor, CD23; and Galectin-3, which binds both FcεRI and IgE. FcεRI exists as a tetramer on mast cells and basophils consisting of an α-chain, a β-chain and two γ-chains, and as a trimer of two γ chains and an α chain on dendritic cells, platelets, smooth muscle cells, monocytes and eosinophils (Kinet 1999). The extracellular regions of the α-chain of the receptor are responsible for the binding of IgE, while the intracellular, ITAM-containing tails of the β and γ chains form the signal transduction hub (Garman 2001). The low-affinity IgE receptor, CD23, belongs to the C-type lectin superfamily of proteins but, unlike other members of that family, does not require a carbohydrate moiety to engage IgE. As is typical of C-type lectins, CD23, too, contains a "head" region of lectin-domain trimers held together by a "stalk" region composed of alpha-helices.

CD23 contains binding sites for both IgE and CD21. CD21, also known as Complement Receptor 2, is expressed on dendritic cells and activated B and T cells and is involved in the feedback loops by which CD23 regulates IgE synthesis (Hibbert 2005).

IgE is classically associated with the activation and degranulation of mast cells and basophils. Therefore, IgE mediates both the early and late phases of the allergic reaction. The cross-linking of IgE-FcεRI complexes, initiates mast cell degranulation and prostanoid synthesis. The substances released from the mast cell in the early phase of the allergic response include chemokines, Th2 cytokines, serotonin, proteases and histamine. The effects of these chemicals comprise the late phase of the allergic response (Gould 2003). The degranulation of mast cells is orchestrated by IgE via an elegant signaling arc that is dependent on Src kinases (Pullen 2012, Suzuki-Inoue 2002). IgE also enables Facilitated Antigen Presentation (FAP). Allergen-loaded IgE bound to CD23 on activated s is endocytosed. Following this, allergen derived peptides are processed and loaded onto HLA-DR which is then expressed on the surface of s enabling them to present antigen to T cells (Karagiannis 2001).

1.7. Immunocytes Involved in Asthma

B-Lymphocytes: While B-cells are recruited to the asthmatic lung and also produce allergen-specific IgE (Ghosh 2012, Lindell 2008), the latter is not their primary function in allergic asthma since majority of the local IgE seen in the lung is derived, not from B-cells, but from basophil populations in peripheral blood (Eckl-Dorna 2012). Apart from conventional B-1 cells, a different, newly-discovered population, the Bregs, produces IL-10, diminishes airway inflammation and also recruits Tregs in TGFβ1-dependent manner (Noh 2011).

T-Lymphocytes: The cardinal type of T-lymphocyte implicated in asthma is of the Th2 subset. These are $CD4^+$ cells which, in a milieu rich in IL-4, are presented with a seemingly innocuous antigen (the allergen) by an antigen-presenting cell (APC). Following co-stimulation and IL-4 signaling, the transcription factor GATA-3 shuts down IFNγ and upregulates the transcription of Th2 –type cytokine genes (Romagnani 2004). Recent evidence has shown that the epithelium-derived pioneer cytokines of Th2 differentiation, IL-33 and IL-25, stimulate innate helper cells called nuocytes to produce IL-13 and IL-5 and thus drive the Th2 phenotype (Neill 2010). Th1 cells and their associated cytokines counter-regulate the Th2 phenotype (Szabo 2003), but, in allergy, the relationship between the two Th-subtypes appears to be far more intricate. Since minute quantities of IFNγ reinforce the effects of IL-13, and the Th2 phenotype (Ford 2001), the roles of Th1 lymphocytes and cytokines in asthma are still evolving.

Th9 cells produce prodigious quantities of IL-9, which is a survival factor for mast cells and is produced by eosinophils as well. IL-9 upregulates FcεRI expression by mast cells and is implicated in bronchial hyperresponsiveness. It also has both potentiating and suppressive effects on Th17 cells (Xing 2011, Nowak 2009).

Th17 cells produce IL-17 and are involved in neutrophil infiltration (Ouyang 2008, Iwakura 2008). IL-17 induces mucus hypersecretion in asthma by turning on the *MUC5B* mucin gene in an IL-6-dependent mechanism (Chen 2002). Interestingly, neutrophils themselves produce the Th17-recruiting chemokines, CCL2 and CCL20 (Pelletier 2010).

Th22 cells are a distinct category of Th-cells typified by the presence of CCR10 and copious IL-22 production with no parallel IL-17 production (Duhen 2009). The roles of IL-22 in

allergic diseases are difficult to parse since they appear intertwined with the effects of Th17 cytokines. There is evidence, however, that IL-22 is mainly a remodeling cytokine which induces airway smooth muscle hypertrophy (Chang 2011)—an effect that is also attributed to IL-17 (Al-Alwan 2012). IL-22 is known to have inhibitory effects on IL-13 and airway eosinophilia in the effector phase of asthma, but a stimulatory effect on the Th2 cytokine, IL-25, in the allergic airway (Takahashi 2011, Tamachi 2006). These data propose that while IL-22 is required for the establishment of allergic inflammation, it reduces established inflammation.

The fifth type of Th-subset relevant to allergic asthma is the the T_{reg} subset. These cells resolve inflammation by producing IL-10 (Kearly 2005) and TGFβ1 (Joetham 2007). They also downregulate MHC Class II expression on APCs (Cederbom 2000). The proportion of T_{regs} in the BAL of asthmatic children is comparatively lower than healthy children or asthmatics treated with corticosteroids (Hartl 2007). Further, the T_{reg} phenotype is not maintained in the absence of chronic allergen stimulation (Meiler 2008). Thus, the damage/repair/damage pattern in asthma that leads to airway remodeling is also dependent on the fluctuating populations of T_{regs} along with the inflammatory and reparatory mediators released by the other Th-subsets involved.

A recent study by Lu et al characterized the different coevally generated Th-subsets found in different pulmonary microenvironments allergic asthma. The Th1, Th2, Th17 and T_{reg} cells were described based on the presence of specific transcription factors (Lu 2011). GATA-3 is the definitive Th2 transcription factor: it not only upregulates Th2-type cytokine genes but also impairs DNA engagement by T-bet, the definitive Th1 transcription factor (Robinson 2002). The transcription factors that typify Th17 and T_{reg} cells are RORγT (Cosmi 2011) and Foxp3 (Robinson 2009), respectively. Upon allergen exposure, Th1, Th2, Th17 and Treg subsets all

showed an increment in numbers compared to their basal levels. Not unexpectedly, the $CD4^+$ GATA-3^+ T-lymphocytes (Th2) showed the highest increase and were found in all compartments of the lungs. The RORγT^+ (Th17) cells were largely concentrated in the alveolar regions of the lung while FOXp3^+ (T_{reg}) cells and T-bet$^+$ T cells (Th1) were found in peribronchial and perivascular tissue, respectively. Both populations were extant in the alveolar tissues as well (Lu 2011). Given that the immune response in asthma is of the Th2 type, it is not surprising that GATA-3^+ T cells permeate the whole lung. The presence of the inflammation attenuating and remodeling-associated Th-subsets is also judicious since quiescence of inflammation enables remodeling (Lu 2011).

Dendritic cells: The role classically ascribed to dendritic cells (DCs) is that of antigen capture, processing and presentation to T cells, and the production of instructive cytokines that help drive Th-cell fate (Parham 2005). The large airways are always studded with an impressive intraepithelial network of $MHCII^{hi}CD11c^{hi}$ dendritic cells. Their ability to form tight junctions with the bronchial epithelium allows them to extend their processes into the airway lumen and carry out a sentinel role (Lambrecht 2009). Pulmonary DCs do not migrate into lymphatics unless an insult to the lung has been perpetrated (Jakubzick 2008). Thus, pulmonary epithelium-DC communication is vital to the eventual Th2 immune response seen in allergic asthma.

Apart from Th2 cell priming, DCs are also involved in the effector phase of asthma. In OVA models of allergy, the absence of inflammatory DCs eliminates eosinophilic inflammation goblet cell metaplasia, and bronchial hyperresponsiveness during allergen challenge, (van Rijt 2005, Lambrecht 1998).

In terms of sustaining allergic inflammation DCs are also important sources of chemokines like, CCL17, CCL22, CCL11 and CCL24, which traffic Th2-cells and eosinophils to the lung. DCs can also internalize and present allergen-bound IgE to Th2 cells leading to a more pronounced and potent allergic immune response (Maurer 1998).

Finally, pulmonary DCs also play roles in the resolution of allergic inflammation. Pulmonary pDCs are responsible for immunoregulation of the characteristic features of asthma. These cells do not present antigen to Th2 cells and depleting them abrogates tolerogenic responses to inhaled allergen (de Heer 2004). Mucosal lung DCs prompt IgA class-switching by producing large quantities of IL-6 and TGFβ1 (Naito 2008). The robust IgA response outnumbers IgE and binds the allergen, thus dampening IgE-mediated inflammation (Smits 2009).

Macrophages: As phagocytes and mediators of inflammation, macrophages form the first line of defense against irritants and potential pathogens (Gordon, 2003). Within the lung, there exist three classes of macrophage: bronchial macrophages (BM), interstitial macrophages (IM) and alveolar macrophages (AM) (Balhara 2012). All macrophages originate from circulating blood monocytes that arise from Macrophage and Dendritic cell Precursors (MDPs) in the bone marrow (Moreira 2011, Landsmann 2007). Apart from location, macrophages are also classified by their functions and means of activation. The classically activated M1 macrophage differentiates in response to LPS and Interferon (IFN)-γ, and is adept at instigating inflammation. Phenotypically the M1 macrophage has an IL-12 ^{high}, IL-23 ^{high} and IL-10 ^{low} signature (Montavani 2013). It exhibits anti-tumor capabilities and combats intracellular pathogens via production of reactive oxygen species, nitric oxide synthases and a repertoire of inflammatory

cytokines including IL-1β, TNF-α, IL-6 and IL-17 (Song 2008). In allergic asthma, the pro-inflammatory activities of M1 macrophages may drive tissue damage, mucus hypersecretion and fibroblast activation (Moreira 2011). Given that IFN- γ increases AHR, it is likely that this feature of asthma is also mediated via a mechanism involving M1 macrophages (Balhara 2012). The alternatively activated, or M2 macrophage, differentiates in response to the Th2 cytokines IL-4 and IL-13 (Gordon 2003). In contrast to M1 macrophages, the M2 macrophages express an IL-12 ^{low}, IL-23 ^{low} and IL-10 ^{high} phenotype and promote airway remodeling (Montavani 2013). They contribute to peribronchial fibrosis, airway smooth muscle hypertrophy, the recruitment of inflammatory cells, goblet cell hyperplasia and mucus production, and increased AHR (Montavani 2013, Camoretti-Mercado 2009).

Neutrophils: Neutrophil populations show a marked increase in cases of severe persistent asthma (Jatakanon 1999) in the light of increased CXCL1, IL-8 and Leukotriene B4 (LTB4) production by the airway epithelium and expanded eosinophil and mast cells populations (Barnes 2011). Neutrophils are the principle source of MMP-9 (Matrix Metalloproteinase-9), one of the prime mediators of asthma (Cundall 2003). MMP-9 is a Type IV collagenase whose substrates collagen types IV, V, XI and XVII, elastin, fibronectin, entactin, fibrin and aggrecan (Atkinson 2002). The enzyme also works in conjunction with the growth factor VEGF to induce angiogenesis (Bergers 2000).

Neutrophil elastase, a serine protease, affects mucus hypersecretion and goblet cell hyperplasia by upregulating and increasing the stability of *MUC5AC* mRNA in epithelial cells. It also impairs the mucociliary elevator via its proteinolytic activities (Voynow 1999).

Neutrophil-derived α-defensins—cationic proteins that are an innate immune offensive against microbial invaders—also contribute to airway inflammation by causing damage to the airway epithelium, stimulating the release of CXCL8 from epithelial cells, instigating degranulation of other local neutrophils, neutralizing local serpins and preventing the degradation of neutrophil elastase, and acting as chemotactic factors for T cells. All of these defensin functions are mobilized in an allergen-dependent manner (Vega 2011).

Neutrophils are capable of producing the inflammatory cytokines TNFα and IL-1β, the granulocyte survival cytokines, IL-3 and IL-6, and their own chemotactic factor, IL-8. Direct neutrophil influence on Th-subsets is via the production of IFN-γ, which leads to skewing of the immune response in the Th1 direction and the activation of M1 macrophages (Amulic 2012). Neutrophils indirectly resolve inflammation by manipulation of cytokine networks. Neutrophils undergoing apoptosis are taken up by macrophages and inhibit IL-23 production from the latter. The lack of IL-23 diminishes IL-17 production and thus the expansion and maturation of neutrophil precursors (Nathan 2006). Fifty percent of asthma cases exhibit pathology that is neutrophil-mediated, while the remainder subscribe to an eosinophilic mechanism (Douwes 2002).

Eosinophils: Classically, eosinophils have been known for their potent cytotoxic effector functions against helminthic parasites (Wegmann 2011, Shamri 2010), but in the mid-1980s eosinophils were revealed as major arbitrators of allergic inflammation (Ghosh 2013, Sampson 2001). Normally, eosinophils comprise of ~1-4% of the circulating leukocytes in the blood, their numbers proliferate in response to Th2-type cytokines and allergenic perturbation (Wenzel 2009). Eosinophils synthesize and release both Th1and Th2-type cytokines, and they do so in a

paracrine, juxtacrine and autocrine fashion (Wegmann 2011, Akuthota 2011, Shamri 2010, Akuthota 2008). The mode of cytokine production and dissemination is markedly different in eosinophils compared to T cells in that, in eosinophils, cytokines are pre-synthesized and stored in secretory vesicles. This allows for rapid mobilization of cytokines upon eosinophil activation (Hogan 2008).

In the context of asthma, eosinophil-derived products mediate airway inflammation, hyperresponsiveness and remodeling. Of these, Major Basic Protein (MBP) causes bronchoconstriction by enhancing acetylcholine production once it binds the M2 muscarinic receptor in parasympathetic nerves (Kariyawasam 2007). Moreover, MBP activates basophils, mast cells and neutrophils (Hogan 2008). The eosinophil-derived cationic protein (ECP) has functions similar to MBP. Though an RNase by nature, it causes degranulation of mast cells and basophils by forming pores in the cell surface membranes of these cell-types (Kariyawasam 2007). Finally, eosinophil peroxidase catalyzes the peroxidative oxidation of halides found in the plasma to produce hypohalous acids which are damaging cells of the airway epithelium (Shamri 2011, Hogan 2008).

Eosinophils are potent producers of IL-4. Not only does this polarize the immune response towards the Th2 phenotype, but it also encourages IL-5 production from Th2-differentiated cells (Leckie 2000). Eosinophils influence eosinophil expansion more directly by producing IL-25 (Revital 2011, Akuthota 2008, Hogan 2008,). They are also implicated in airway remodeling given their propensity to produce IL-13, TGFβ_1, and growth factors such as, angiogenin, VEGF, and FGF (Fibroblast Growth Factor) (Revital 2011, Venge 2010, Kariyawasam 2008, Hogan 2008).

The ability to produce prostanoids and the presence of the prostanoid receptors DP1, DP2, and CRTH2 on eosinophils help further establish the multi-tasking potential of the cell in allergy. Their ligands are cyclooxygenase products, notably Prostaglanin D2 (PGD2) (Wegmann 2011). Eosinophils also bear receptors for Cysteinyl-Leukotrienes (cys-LTs). While the cys-LTs are known to be potent spasmogens and inducers of mucus hypersecretion, their interactions with eosinophils reveals that they impact airway remodeling by causing airway smooth muscle (ASM) hypertrophy (Halwani 2012).

Basophils: In allergic asthma, basophils are a potent source of histamine and cysteinyl leukotrienes (cys-LTs). Histamine brings about bronchoconstriction and vasodilation by binding to histamine receptors on smooth muscle cells and endothelial cells respectively, is a chemotactic factor for mast cells and inhibits Th1 cytokine production (Thurmond 2008).

Unlike mast cells and eosinophils, basophils exclusively produce IL-4 and IL-13 (Sullivan 2009). Moreover, there is strong evidence to show that basophils may be the initial source of the IL-4 that primes the Th2 response in asthma (Oh 2007). Furthermore, it has recently been shown that basophils, and not B-cells, may be the major source of allergen-specific IgE (Eckl-Dorna 2012).

2. *ASPERGILLUS FUMIGATUS*-DERIVED EICOSANOID SYNTHASES DO NOT EXACERBATE THE IMMUNOPATHOLOGY OF ALLERGIC ASTHMA

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2.1. Abstract

Allergic asthma is characterized by acute episodes of airway hyperresponsivesness (AHR) and bronchoconstriction caused, in part, by the local action of leukotrienes and prostaglandins. *Aspergillus fumigatus*, a ubiquitous mold and trigger of allergic asthma, possesses eicosanoid synthases that may function similarly to those of its mammalian hosts. Therefore, we hypothesized that fungal-derived eicosanoid synthases may contribute to an increase in host prostaglandin and leukotriene synthesis and thus exacerbate allergic inflammation. We tested our hypothesis via an inhalation model of fungal allergic asthma using spores from mutated strains of *A. fumigatus* in which either three cyclooxygenase-like genes (*ppoABC*) had been silenced or two putative lipoxygenase (l*oxAB*) genes had been silenced. We found that fungal-derived eicosanoid synthases are dispensable for the development of peribronchovascular inflammation or fibrosis in *Aspergillus*-mediated allergic asthma.

2.2. Introduction

The dysregulation of lipid signaling may have drastic consequences for the host, leading to chronic inflammation, metabolic disorders, and tumorigenesis (Wymann 2008, Funk 2001).

Of the three classes of signaling lipids—phosphoinositides, sphingolipids, and eicosanoids—the eicosanoids are of special importance to the pathogenesis and the pathophysiology of allergic asthma (Barnes, 2011). They are the products of the oxygenation of polyunsaturated, long chain fatty acids, such as arachidonic acid (Wymann 2008, Luo 2006, Barnes 2011). Generally, eicosanoids are not stored, but are synthesized and released upon cytokine signaling or mechanical trauma, acting upon target receptors in both an autocrine and a paracrine fashion. They have powerful physiological effects even in nanomolar quantities (Nebert 2008, Wymann 2008, Harizi 2008).

Prostaglandins (PGs) and cysteinyl-leukotrienes (cys-LTs) are both eicosanoids. In allergic asthma, they contribute to bronchoconstriction, peribronchial fibrosis, eosinophilic inflammation, mucus hypersecretion, and neutrophil and Th2 cell recruitment (Harizi 2008, Miller 2006). The biosynthesis of both prostaglandins and leukotrienes begins with the hydrolysis and release of arachidonic acid from the cell surface membrane by cytosolic phospholipase 2 (cPLA2) (Kanaoka 2004).

The synthesis of prostaglandins is mediated by the cyclooxygenase, or COX, pathway. Of the two isoforms of COX, COX-1 is constitutively expressed while COX-2 gene expression is induced in the presence of inflammatory cytokines (Pang 1998). Cyclooxygenases utilize molecular oxygen to convert arachidonic acid to the endoperoxide prostaglandin G2 (PGG2). PGG2 is then oxidized by COX to produce prostaglandin H2 (PGH2). This unstable product of the COX pathway is converted into specific prostanoids by tissue-associated synthases. In allergic asthma, the key prostanoid is prostaglandin D2 (PGD2) which is released prodigiously by mast cells (Nebert 2008,Luo 2006, Funk 2001, Barnes 1998).

The pathway responsible for cys-LT synthesis utilizes the enzyme 5-lipooxygenase (5-LO), which oxidizes arachidonic acid to the stable 5(S)-hydroperoxy-6-trans-8,11,14-eicosatetranoic acid (5-HPETE). Following oxygenation, 5-HPETE is dehydrated to an unstable epoxide leukotriene A4 (LTA4) form (Rådmark 2009, Peters-Golden 1998). The fate of LTA4 differs by cell-type: in neutrophils, LTA4 hydrolase converts LTA4 to leukotriene B4 (LTB4), while in mast cells, basophils, macrophages, and eosinophils, LTA4 is converted to leukotriene C4 (LTC4) by LTC4 synthase (Lötzer 2007).

The similarities between mammalian and *Aspergillus* phospholipases, COXs and LOs (Nover 2003), creates the potential for "trans-species" enzymatic activity (Dagenais 2008, Tsitsigiannis 2005a, Oakely 2005), which may augment the production of PGs and LTs as well as the immunopathology seen in asthma. Our central hypothesis, therefore, is that knocking out the COX and LO genes from *A. fumigatus* would lead to an amelioration of allergic inflammation seen in asthma.

The fungal *ppoA*, *B* and *C* genes encode mammalian COX-like enzymes that regulate the timing of conidial sporulation (Tsitsigiannis 2005b), while the *loxA* and *loxB* genes encode enzymes with lipooxygenase activity that is very similar to mammalian 5-LO (Brash 1999, Keller NP, personal communication). The contributions of the *ppo* genes to *Aspergillus fumigatus* virulence in a model of invasive aspergillosis (IA) have been established (Dagenais 2008, Tsitigiannis, 2005a, b). However, the role played by these enzymes in *A. fumigatus*-mediated asthma has not, to our knowledge, been explored. Using an inhalational model of fungal allergic asthma and mutated *A.fumigatus* strains in which *lox* and *ppo* genes had been knocked out, we show that these fungal genes are not critical for the development of fungal

allergic asthma physiopathology nor do they appear to exacerbate the inflammatory response of the host.

2.3. Materials and Methods

2.3.1. Animals

BALB/c mice, purchased from Jackson Laboratories (Bar Harbor, ME), were housed on Alpha-dri paper bedding (Shepherd Specialty Papers, Watertown, TN) in micro-filter topped cages (Ancare, Bellmore, NY) in a specific pathogen-free vivarium and were provided with an *ad libitum* access to food and water for the duration of the study. The methodologies employed in the study were consistent with the guidelines of the Office of Animal Welfare and were approved by North Dakota State University's Institutional Animal Care and Use Committee (IACUC).

2.3.2. *A. fumigatus* antigen and cultures

Soluble *A. fumigatus* antigen was purchased from Greer Laboratories (Lenoir, NC), and the fungal culture stock (strain: NIH 5233) was obtained from the American Type Culture Collection (Manassas, VA). Prior approval of the Institutional Biological Safety Committee at North Dakota State University was obtained for any experiments that utilized *A. fumigatus*.

Lyophilized *A. fumigatus* conidia were reconstituted in 5 ml of phosphate-buffered saline according to ATCC protocol, and 60-µL aliquots were stored at 4ºC until use. For the purposes of inhalational challenge, 25-cm^2 cell culture flasks (BD Falcon ^{TM}, BD Biosciences, San Jose,

CA) containing Saboraud dextrose agar (SDA) were inoculated with a single conidia aliquot. These were incubated at 37ºC for 8 d to allow for growth and maturation.

2.3.3. *lox* and *ppo* mutants of *A. fumigatus*

Strains of *A. fumigatus*, deficient in *lox* and *ppo* genes, were a kind gift of Dr. Nancy Keller of The University of Wisconsin at Madison. The means by which the genes were knocked out are detailed in Tsitsigiannis *et al*'s published manuscript (Tsitsigiannis 2005a). The mutants used in this study are as follows:

Table 1. *Aspergillus fumigatus* strains used in this study.

Fungal Strain	**Genotype**
Wild Type	
TJW 62.2	Δ *ppoABC*-RNAi*:: A. parasiticus pyrG pyrG1*
TJMP 38.2	*ΔloxaA:: A .parasiticus pyrG; pyrG1; A. fumigatus argB; argB1*
TTRD 12	*ΔloxA::A. parasiticus pyrG; pyrG1;ΔloxB:: A. fumigatus argB; argB1*

The mutant strains were cultured in the same manner as described for the wild-type fungus.

2.3.4. Allergen sensitization

The sensitization of the animals was carried out with the method detailed by Ghosh *et al.* (2012). Each mouse was sensitized via sub-cutaneous (SC) and intraperitoneal (IP) injection with

10 µg of *A. fumigatus* antigen (Greer Labs, Lenoir, NC) in 50 µL of normal saline with 50 µL of Imject Alum (Pierce, Rockford, IL).

Two weeks following the SC/IP sensitization, local sensitization of the lungs was achieved via a series of three, weekly intranasal (IN) deliveries of 20 µg of *A. fumigatus* antigen dissolved in 20 µL of normal saline.

2.3.5. Aeroallergen challenge

A week following the third intranasal sensitization, the mice were subject to challenge with live, airborne conidia as per Hoselton's published protocol (Hoselton 2010). A 25-cm^2 cell culture flasks containing 8-day-old *A. fumigatus* culture was affixed to an inoculation chamber bearing three ports which allow for a nose-only exposure to aerosolized conidia. Air was blown onto the fungal cultures in the flasks at 2 psi to release the hydrophobic conidia and allow them to enter the inoculation chamber. Spores in the exhaust were captured in a series of sporicide-containing traps. For the duration of the aeroallergen challenge, the entire apparatus was contained in a Class II biological safety cabinet. Before introducing the mice, the inhalation ports were sealed and conidia were passed through the inoculation chamber for 10 min to coat its interior.

The mice were divided into groups (n=5) for challenge and analysis. The WT group of mice was challenged with wild-type *A. fumigatus*, the TJW 62.2 group was challenged with the triple-*ppo* mutant, the TJMP 38.2 group received the *loxA* mutant while TTRD12 group was subjected to challenge with the double-*lox* mutant. The mice were anesthetized with a cocktail of ketamine (75 mg/kg) and xylazine (25 mg/kg), and each mouse was placed supine with its nose

in an inhalation port to breathe the live conidia for 10 min. The aeroallergen challenge was repeated two weeks later. Separate inoculation chambers were used for each fungal mutant and wild type culture.

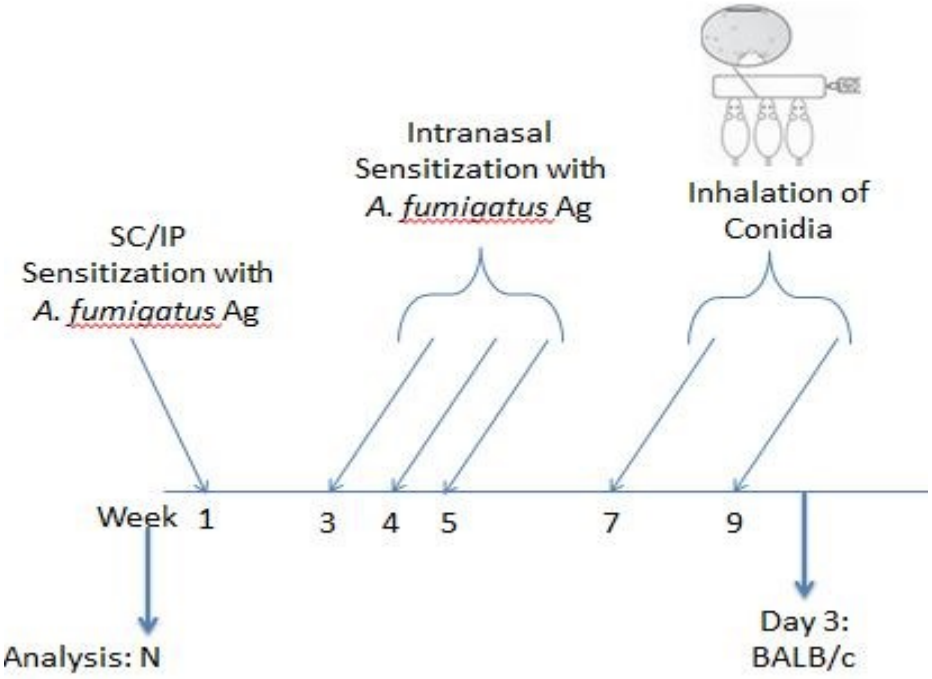

Figure 1. Study timeline. Mice were sensitized with intraperitoneal and subcutaneous injections of A. fumigatus antigen in alum, followed 2 weeks later by 3, weekly intranasal inoculations of the same antigen in normal saline. In weeks 7 and 9, the now allergic mice were challenged with a nose-only inhalation challenge with live A. fumigatus conidia. At prescribed time points after the second conidia exposure, measurements of AHR and sample collection were carried out (BALB/c, day 3).

2.3.6. Measurement of airway hyperresponsiveness (AHR) and tissue harvest

AHR was measured 3 days after the final aeroallergen challenge for BALB/c mice and on day 7 for the C57BL/6 group. These time points have been previously determined to be the peak of inflammation and airway wall remodeling events in the model (Hoselton 2010). Mice

anaesthetized with sodium pentobarbital (Butler, Columbus, OH; 0.1 mg/kg of mouse body weight), intubated, ventilated via a Harvard Pump ventilator (Harvard Apparatus, Reno, NV), and subjected to restrained plethysmography (Buxco, Troy, NY) were used to assess AHR. Baseline airway resistance was measured for each animal, and an intravenous injection of the non-specific spasmogen, acetyl-β-methacholine (420 µg/kg) was administered to determine AHR.

Approximately 500 µl of blood was acquired from each mouse via ocular bleed. The blood was centrifuged for 10 min at 13,000 x *g* to yield serum, which was collected and stored at -20ºC until use. Lungs were then removed from each mouse. Right lungs were snap-frozen in liquid nitrogen for protein analyses, whereas the left lungs were inflated, *ex vivo*, with 1.0 ml of 10% normal-buffered formalin and fixed for 18 h before processing for histology.

2.3.7. Morphometric analysis of leukocytic inflammation in airway lumen

Bronchoalveolar lavage (BAL) fluid was collected by flushing the lungs of each intubated mouse with 1.0 ml of phosphate-buffered saline (PBS). The collected BAL fluid (BALF) was centrifuged at 6,500 x *g* for 10 min to separate cells from the BALF. BALF was collected and stored at -20ºC until use, while the cell pellet was resuspended in 1.0 ml of PBS and immediately cytospun (Shandon Scientific, Runcorn, United Kingdom) onto a glass microscope slide. The cytospun cells were then differentially stained with Diff-Quik (Mercedes Medical, Sarasota, FL). Macrophage/monocytes, neutrophils, eosinophils, lymphocytes, and epithelial cells were counted from five random high-powered fields (1000X) per slide and the mean number of each cell type was then calculated for the sample.

2.3.8. Histological analyses

Lung sections that had been formalin-fixed were embedded in paraffin, and 5-µm sections were cut longitudinally across the coronal plane. The sections were stained with hematoxylin and eosin (H&E) to assess inflammation. Gömöri Trichrome (Richard-Allen Scientific, Kalamazoo, MI) stain was used to visualize the deposition of collagen in the peribronchial regions of each mouse lung. For every specimen, 50 individual points, at ~ 50-µm intervals were measured along the large airways in the histological section. Perpendicular lines were drawn from the points on the basement membrane and extended through the underlying collagen deposits. The mean collagen thickness was tabulated for each specimen, and the mean of those means was tabulated for each of the treatment groups.

2.3.9. Quantification of serum and BAL IgA and IgE, and serum IgG_1, IgG_{2a} and IgG_3

BAL and serum concentrations of IgE and IgA were quantified by ELISA (Bethyl Laboratories, Montgomery,TX). Serum samples were also interrogated for IgG_1, $IgG_{2a.}$and IgG_3 quantities. The dilutions used for ELISA were as follows: IgA: BAL= 1:2; serum =1:500, IgE: BAL= 1:2; serum=1:100, IgG_1, serum= 1:5000; IgG_{2a}, serum=1:5000, IgG_3, serum= 1:50,000. The detection limits for the kits are as follows: IgE= 1.60 ng/ml, IgA= 15.625 ng/ml, IgG_1= 6.25 ng/ml, IgG_{2a}= 3.1 ng/ml, IgG_{2c}= 7.8 ng/ml, IgG_3= 7.8 ng/ml. The assays were run according to the manufacturer's instructions.

2.3.10. Statistical analyses

At each time point, comparisons between the different treatment groups were drawn via an unpaired Student's two-tailed *t*-test with Welch's correction to determine statistically significant differences between antibody titers, AHR, BAL cell numbers, and measurements of peribronchial collagen deposits. GraphPad's Prism Software (San Diego, CA) was used for this purpose.

2.4. Results

Acute AHR is not impacted by the presence or absence of A. fumigatus *eicosanoid genes.*

The mean baseline airway reactivity before methacholine administration was 1.98 $\pm$ 0.10 cm H_20/ml/s. As a consequence of methacholine provocation, there was an increase in AHR in all the groups, but this increase was not different between the animals challenged with the mutated mold spores as compared to wild type *A. fumigatus* (**Fig. 2**).

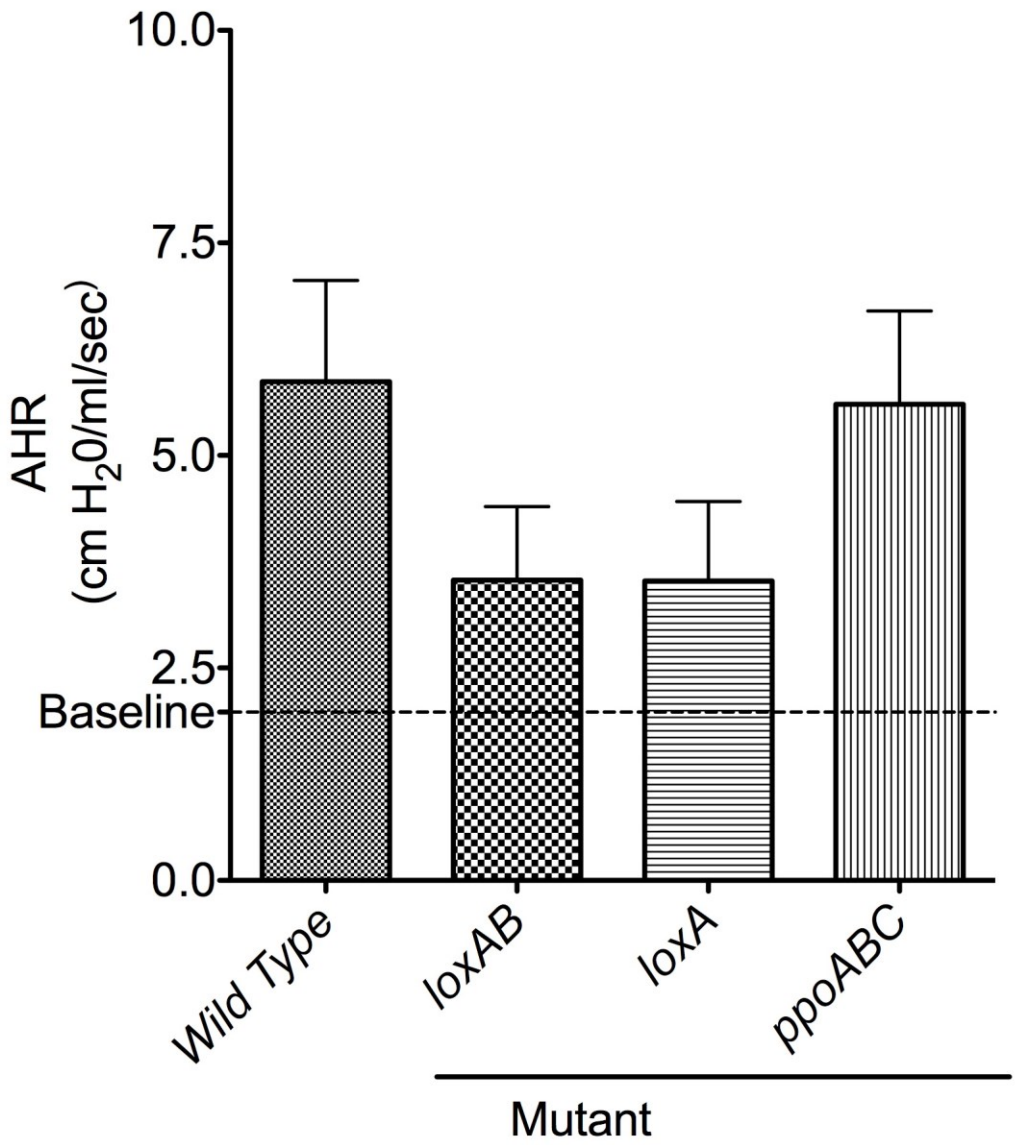

Figure 2. Comparison of AHR elicited for BALB/c mice on Day 3 after allergen challenge. The baseline response (1.98 ± 0.10 cm H_20/ml/s) was recorded before methacholine provocation and is indicated by the dashed line. Peak AHR was recorded after intravenous delivery of methacholine. Data was analyzed using an unpaired Student's *t*-test with Welch's correction. All values are expressed as means ± SEM, n= 4-5 mice per group

There is no difference in the cellularity of the bronchoalveolar lavage acquired from the mutant-challenged and control groups.

Sensitization and challenge with fungus resulted in the recruitment of inflammatory cells into the lung. These included macrophages, eosinophils, neutrophils, and lymphocytes (**Fig. 3**). However, there was no difference in the relative numbers of these cells recruited to the airways of the different treatment groups. The pattern of BAL cellularity was replicated across the groups: the BAL compartment was inundated with macrophages while neutrophils and eosinophils were the second and third most prominent populations, respectively, but only by a small margin. Lymphocytes were the rarest cell type seen in the BAL of all animals.

Antibody profiles after inhalation of A. fumigatus *mutants deficient in eicosanoid genes are unchanged from wild type controls.*

Levels of IgE and IgA were measured in the BAL (data not shown) and sera (**Fig. 4A**, **4B**) of animals challenged with mutant and wild type fungus. IgE production is one of the primary indicators of allergic disease and can be used as a parameter of the extent of allergic sensitization. IgA is produced constitutively and serves as a mucosal barrier: it can also be induced in response to insult to a mucous membrane (Pilette 2004). In the current study, modulation of IgA levels could suggest immunomodulatory aspects of asthma pathogenesis as mediated by the fungus. While IgE production was elevated after fungal challenge with eicosanoid deletion mutant strains of *A. fumigatus*, there was no difference between the quantities of IgE produced by the treatment groups as compared to wild type controls. Similarly, the levels of IgA were similar in the treatment and control groups.

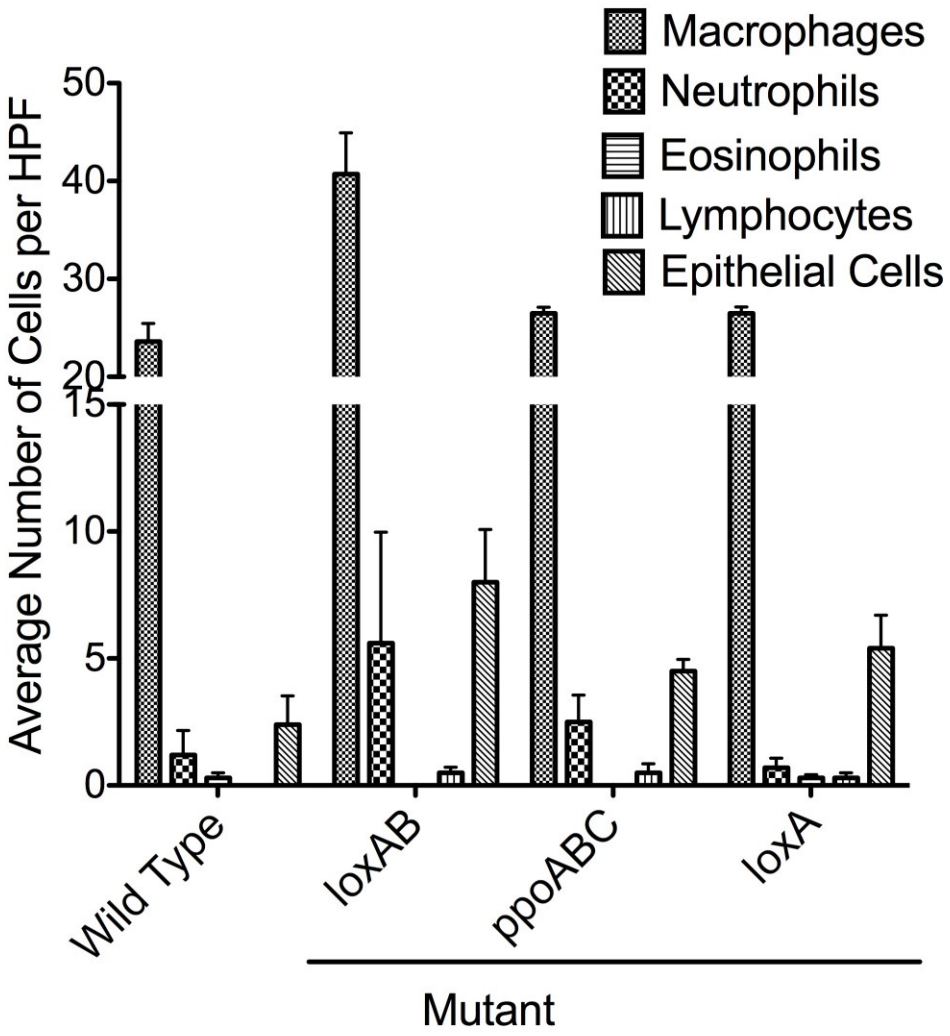

Figure 3. Comparison of BAL cellularity. Lungs of mice challenged with wild-type or mutant fungus were washed with 1.0 ml of PBS. BAL cells were cytospun onto glass slides and stained with the Diff-Quick differential staining system, and cell counts were performed on 5 randomly chosen high-powered fields.The presence of macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells in the BAL are indicative of airway inflammation despite the absence of fungus-derived eicosanoid synthases. Data were analyzed using an unpaired Student's *t*-test with Welch's correction. All values are expressed as means ± SEM and n= 4-5 mice per

Serum IgG_1, IgG_{2a}, IgG_{2c}, and IgG_3 (**Fig. 4C**, **4D**, **4E**). IgG_{2a}, an antibody of the Th1 type, is effective at opsonizing and thus eradicating fungal spores. The same function is attributed to IgG_1 (Th2 type), though to a lesser degree (Mukherjee 1995). An increase in IgG_3 and IgG_{2a} would be suggestive of a Th1-type immune response, if coupled with a decrease in IgG_1 levels. Lowered IgG_3 and IgG_{2a} levels with concomitant IgG1 increase would be more indicative of a Th2 response. While the Th1-type antibodies were less prominent compared to the Th2-type, there were no differences in the individual antibody titers seen across the treatment groups.

Inflammation and peribronchial fibrosis after inhalation challenge with eicosanoid mutant fungus is equivalent to control lungs treated with wild type fungus.

Pulmonary inflammation (**Fig. 5**) was examined via H&E-stained paraffin-embedded lung sections. While all the groups exhibited peribronchovascular inflammation, differences in the degree of inflammation between the control and treatment groups could not be detected visually.

Peribronchial fibrosis, as visualized in Gömöri Trichrome-stained sections to detect peribronchial collagen deposition, was observed in all the lung sections from the study (**Fig 6**). However, there was no discernible difference in the sub-epithelial collagen seen between the treatment and control groups in the study. This was further confirmed by the lack of difference observed in the thickness of peribronchial collagen deposits measured from lung sections acquired from the wild-type controls and the mutant-challenged groups (**Fig 7**).

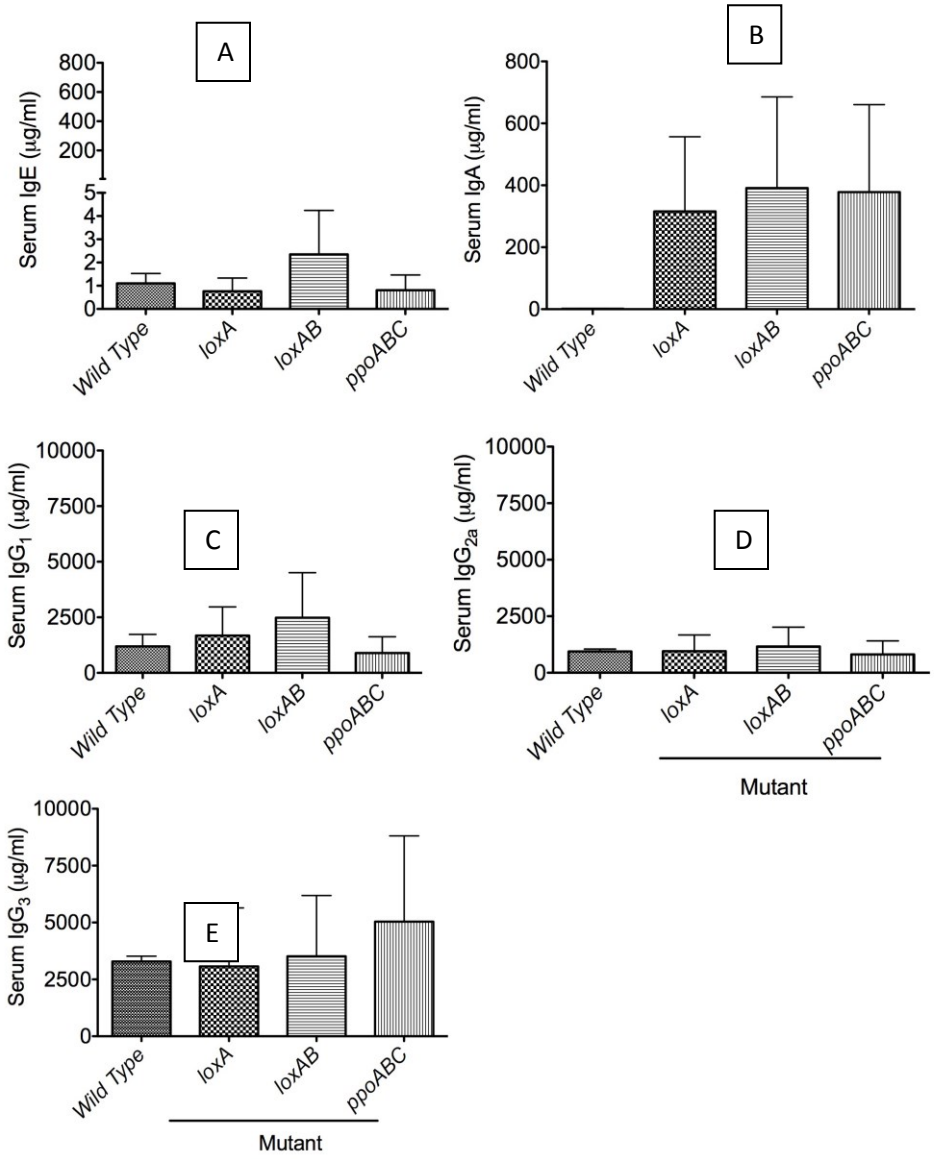

Figure 4. Comparison of antibody titers. IgA (**A**), IgE (**B**), IgG_1 (**C**), IgG_{2a} (**D**), and IgG_3 (**E**) titers in sera drawn from wild type- and mutant-challenged BALB/c mice were measured at Day 3 post-challenge. Serum antibody titers were assessed by ELISA. Data were analysed using an unpaired Student's *t*-test with Welch's correction. All values are expressed as means ± SEM, and n= 4-5 mice per group.

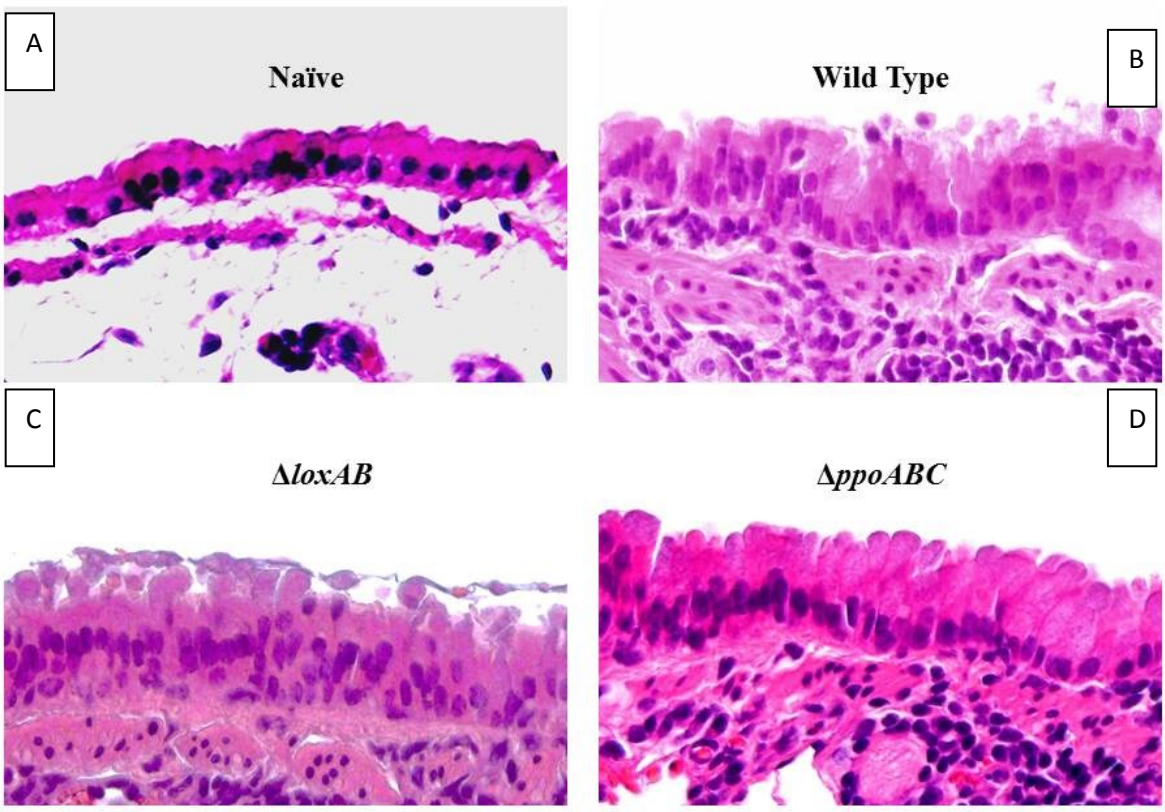

Figure 5. Representative photomicrographs of H&E-stained lung sections of BALB/c mice. Three days post-challenge lungs challenged with inhaled fungal spores exhibited pulmonary inflammation in the wild-type group (**B**), the Δ*ppoABC* group (**C**), and the Δ*loxAB* (**D**) group. A naïve control (**A**) is included for comparison (200X).

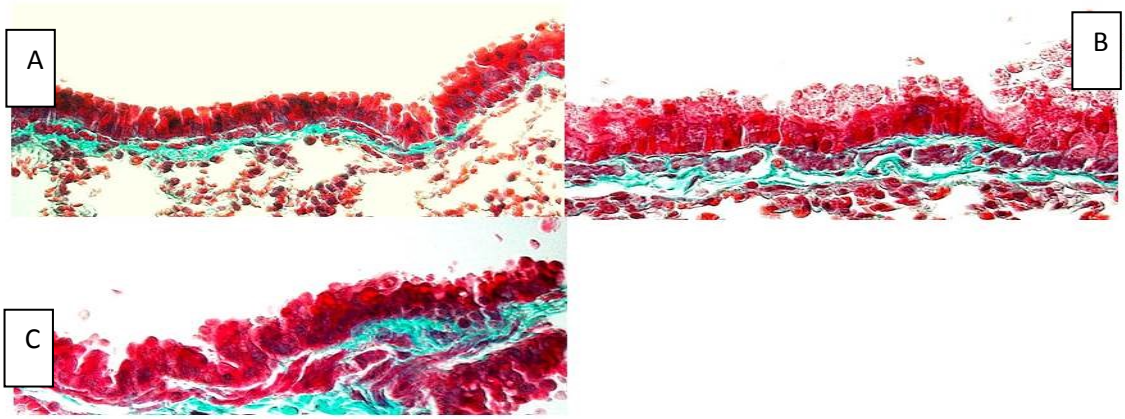

Figure 6. Representative photomicrographs of Gömöri's Trichrome-stained lungs of BALB/c mice. At Day 3 post-challenge, it is seen that there is no visual difference in the magnitude of peribronchial fibrosis in the group challenged with wild-type fungus (**A**) as opposed to the groups challenged with *ΔppoABC* (**B**) and *ΔloxAB* (**C**) (200X).

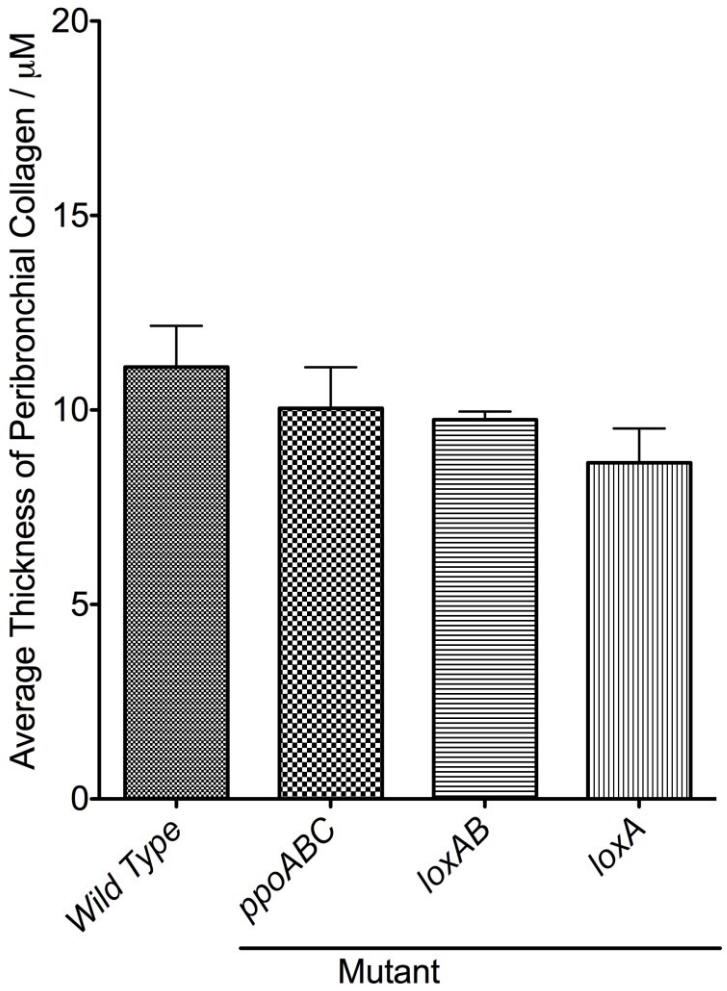

Figure 7. Comparison of the thickness of peribronchial collagen deposits. Fifty discrete measurements were taken along the large airways on a Gömöri Trichrome-stained lung section through the thickness of collagen. All values are expressed as the mean of the means ± SEM, and n= 4-5 mice per group.

2.5. Discussion

In the *Aspergilli*, the *ppoA*, *ppoB*, and *ppoC* genes are important for the maturation of spores (Tsitsigiannis 2005b). The protein products of these genes metabolize oleic, linolenic, and

linoleic acid to produce lipid mediators called psi (precocious sexual inducer) factors which balance the ratio of sexual to asexual spores (Cotier 2011, Tsitsigiannis 2007) during conidiation and also exert control on the secondary metabolite production (Cotier 2011, Kato 2003). In *Aspergillus fumigatus* in particular, *ppoA* encodes an (8R)-dioxygenase with a hydroperoxide isomerase activity at its N-terminus (Garscha 2007) and a highly conserved P450 fold at the C-terminus (Koch 2012). ppoC is a (10R)-dioxygenase (Brodhun 2011), and ppoB regulates ppoA and ppoC activity. (Tsitgiannis 2005b). Comparison of amino acid sequences has revealed proximal and distal heme ligands as well as a tyrosine residue that is vital for a conserved catalysis function across ppoA and mammalian cyclooxygenases (Hornsten 1999). Further, a highly conserved P450 fold found at the C-terminus of ppoA (Koch 2012) is reminiscent of a subclass of eicosanoid-producing enzymes that are involved in rearrangements of fatty acid peroxides rather than the monoxygenase activity credited to ppoA (Brash 2009, Howe 2002).

Aspergillus LOs also carry out roles that are similar to *Aspergillus* COXs. A wheat seed *Aspergillus ochraceus* pathogenic model system reveals that both the timing of conidiation, as well secondary metabolism, are adversely affected in lipooxygenase mutants of the fungus (Reverberi 2010). Studies with *Aspergillus flavus* show that the psi factors produced by the lipoperoxidation reactions mediated by LOX enzymes are required for a quorum sensing mechanism which allows for "decision control" of sporulation or forming overwintering sclerotia (hardened mycelial structure which enables molds to survive the winter and re-sporulate in the spring; analogous to bacterial spore). In fact, deletion of the *lox* gene in *A. flavus* leads to a greater predisposition for sclerotia formation (Horowitz Brown 2008).

The promiscuous nature of enzyme activity (Khersonsky 2006) creates great potential for dynamic host-pathogen communication via lipid intermediaries. The deletion of *ppo* genes from *A. nidulans* in a peanut seed pathogenesis model decreases *lox* gene expression from the host and diminishes fungal colonization (Brodhagen 2008). In humans, binding of cysteinyl leukotrienes to the cysteinyl leukotriene receptor-2 on endothelial cells upregulates the production of endothelial-derived cyclooxygenases in an NFAT/Ca^{2+}-dependent fashion (Lötzer 2007). Fungus-derived leukotrienes may also have the potential to do so in the context of allergic asthma.

This study was intended to address the potential additive effect of the fungal eicosanoid synthases to the developing immune inflammation of the allergic lung. However, our findings show that they appear to make no significant contribution to any aspect of the asthmatic phenotype that was examined. In part, this may be attributed to silencing of the *A. fumigatus ppo* genes by RNAi (Tstitsigiannis 2005a) and also to the variability in sensitizing the mice in the study. The Tsitsigiannis study shows that the triple *ppo* knock-down mutants are hypervirulent, since the lack of oxylipin production fails to stimulate a sufficiently effective immune response to combat invading fungus, suggesting a significant fungal eicosanoid contribution to molecular interactions between host and pathogen (Tsitsigiannis 2005a)

Comparing the quantities of eicosanoid produced by arachidonic acid-fed mutant and wild-type *A. fumigatus* cultures has revealed that the mutants operate at a reduced capacity of eicosanoid production, ~12% less than that of wild-type fungus (Tsitsigiannis 2005a). While this finding is revealing of fungal biology by itself, our study asks the question whether this extent of reduced activity is substantial enough to affect asthma pathogenesis.

A. fumigatus produces pharmacologically relevant concentrations of eicosanoids that are similar to eicosanoids produced by humans. However, the putative fungal COX and LOX enzymes are dispensable for this process. The study was repeated with C57BL/6 mice (data not shown) and, while these mice were sensitized successfully as demonstrated by both pulmonary eosinophilia and elevated IgE titers, the development of allergic inflammation, airway hyperresponsiveness and peribronchovascular inflammation and fibrosis was similarly unaffected by the absence of the eicosanoid genes that were targeted in this study.

Immunocompetent hosts are capable of clearing 99% of *A. fumigatus* conidia within 24 h of inhalation and subsequent exposures do not result in an accumulation of conidia or infection. Rather, subsequent inhalations enable a transitory state of the immune response wherein Th1, Th2, and Th17 responses may develop in tandem or in parallel (Murdock 2011). This explains why both inflammation and airway remodeling events are seen in our model, but these are likely to be a consequence of the immune response to conidia in the airway rather than any mediators produced by the fungus.

Whether or not fungus-derived eicosanoids can bind receptors on mammalian cells and initiate effector functions is not a question that is easily answered, but Kupfahl et al's data (2012) corroborates with ours in that the canonical eicosanoid synthesis pathways are not germane to the host-allergen interactions seen in allergic asthma, since blocking fungal COX and LOX pathways did not affect fungal eicosanoid production (Kupfahl 2012).

2.6. Acknowledgments

We are grateful to Dr. Nancy Keller of The University of Wisconsin at Madison for the gift of *lox* and *ppo* gene knock-down strains of *Aspergillus fumigatus*. We would also like to extend our gratitude to Aaron Mertens, Timothy Miller, Lee Kiederowski, and Breanne Steffan for their assistance in the sensitization of animals in the study and with sample acquisition.

3. INHALATIONS OF SOYBEAN, BUT NOT CORN, DUST RESULTS IN INCREASED NEUTROPHIL RECRUITMENT AND SERUM IMMUNOGLOBULIN E LEVELS IN ALLERGIC C57BL/6 MICE

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3.1. Abstract

Agricultural workers are constantly exposed to grain dust—a complex mixture of particulates that may trigger or exacerbate respiratory distress. While this association is not new, the means by which grain dusts may affect allergic asthma in both the acute and chronic contexts remain unknown. Using our murine, inhalational model of *Aspergillus fumigatus*-mediated asthma, we investigated the impact of repeated corn and soybean dust inhalations on the immunopathogenesis of asthma. Data from this pilot project has revealed increased neutrophilia and serum IgE titers in groups treated with soybean, but not corn dust. The pilot study has, thus, paved the way for future work involving characterization of grain dusts used in the study, as well as the effects of the same on more chronic features of asthma.

3.2. Introduction

Grain dust is a complex amalgam of a diversity of irritating and potentially allergenic materials. These include particles of broken grain pericarp (Chan-Yeung 1992), Gram negative bacteria and endotoxin (Singh 2005), inorganic matter associated with herbicides, pesticides and

fertilizers (Chan-Yeung 1992), animal products, and mites (Newman-Taylor 1994), as well as fungi associated with storage, such as those of the *Aspergillus* and *Mucor* genera and their mycotoxins (Krysinska-Traczyk 2001, Palmgren 1986). Moreover, the composition of grain dust may change due to weather conditions during production, harvest, or storage and with changes in pesticide and herbicide usage.

The association between grain dust and respiratory health has been documented for over three-centuries. In 1713, Bernardino Ramazzini, the acknowledged Father of Occupational Medicine, recorded that "almost all who make a living by sifting or measuring grain are short of breath and cachectic and rarely reach old age…the throat lungs and eyes are keenly aware of serious damage; the throat is choken and dried up with dust, the pulmonary passages become coated with crust formed by the dust, and the result is a dry and obstinate cough." (Skrobonja 2005, Franco 1999, Chan-Yeung 1978). Contemporary interest in pulmonary pathologies driven by grain dust has reinforced and continued Ramazzini's clinical observations. Exposures to grain dust have been linked to hypersensitivity pneumonitis, allergic and irritant rhinitis, infectious pneumonias, asthma and acute asthma-like syndromes, and chronic obstructive pulmonary disease (COPD) (American Thoracic Society 1998).

The range of clinical syndromes caused or exacerbated by grain dust remain relevant given that farming as of 2006, encompassed 2.1 million farming operations exist in the United States and, of these, 37.5% are in the Midwest (Mazurek 2010). The Mazurek study surveyed 12,278 primary farm workers and discovered that 4.9% of them were asthmatic—a percentage that reflects approximately half the national average of asthmatics (8.2%) (Centers for Disease Control 2013) Of these, 24.8% were told by a physician that their asthma had occupational

origins. The use of personal protective equipment (PPE) could certainly help lower the incidence of sensitization, but PPE use in farm workers in the United States is low (Carpenter 2002).

From an immunological standpoint, the pathogenesis of COPD (Spurzem 2005), hypersensitivity pneumonitis (Girard 2004), and asthma (Holgate 2008) are well-described. However, the picture of immunologic response to grain dust in the context of underlying pulmonary disease remains incomplete. So far, it is known that neutrophilia (Von Essen 1995, Von Essen 1994, Deetz 1997) and recruitment of mast cells to the airway, coupled with a surge in IL-8 are possibly involved in bronchoconstriction and lung inflammation observed in asthmatics exposed to grain dust (Palmberg 1998, Park 1998). Studies in grain dust and lung function have relied on nebulizing grain dust extract, interviews with those already afflicted, measurements of lung function via spirometry, and analyses of bronchial biopsies acquired from farm workers (Omland 2002, Park 1998, Post 1998, Von Essen 1995, James 1990, DoPico 1977), rather than modeling inhalational exposures. Further, the potential involvement of preexisting atopic asthma in the sequelae driven by grain dust exposures is also under-investigated. By using an inhalational murine model of fungal allergic asthma, we hope to address these gaps in research pertaining to grain-dust, asthma, and pulmonary health.

3.3. Materials and Methods

3.3.1. Animals

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in micro-filter topped cages (Ancare, Bellemore, NY) and on Alpha-dri paper bedding (Shepherd Specialty Papers, Watertown, TN). The mice had *ab libitum* access to food and water throughout

the study and were contained in a specific pathogen-free facility. The methodologies employed in the study were consistent with the guidelines drawn up by the Office of Animal Welfare and was approved by North Dakota State University's Institutional Animal Care and Use Committee (IACUC).

3.3.2. Fungal antigen and allergic sensitization

Soluble *A. fumigatus* antigen was purchased from Greer Laboratories (Lenoir, NC). The fungal stock culture (strain: NIH 5233) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The lyophilized conidia were resuspended in 5 ml of phosphate-buffered saline, in accordance to the ATCC protocol, and 60-µL aliquots were stored at 4ºC until used.

Allergic sensitization followed the published method described by Ghosh *et al.* (2012), utilizing subcutaneous and intraperitoneal delivery of soluble antigen extract in alum followed by a series of three intranasal exposures to the same antigen in normal saline. Briefly, the mice were subcutaneously and intraperitoneally injected with a 1:2 dilution of *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC) in 100 µL of normal saline and 100 µL of Imject alum (Pierce, Rockford, IL). Two weeks following the subcutaneous and intraperitoneal injections, 3 weekly inoculations of 20 µg of *A. fumigatus* antigen dissolved in 20 µL of normal saline were delivered by intranasal inoculation to locally sensitize the airway.

3.3.3. Conidia culture and aeroallergen challenge

Conidia of *A. fumigatus* were cultured in 25-cm^2 cell culture flasks (BD Falcon™, BD Biosciences, San Jose, CA) containing Saboraud dextrose agar (SDA). These were incubated at 37ºC for 8 d prior to aeroallergen challenge. The aeroallergen challenge itself was carried out in accordance with Hoselton's published protocol (Hoselton 2010). Two challenges with live, airborne conidia, each a week apart, were used for this study.

3.3.4. Grain dust generation

Corn and soybean dusts were provided through a collaboration with the University of Nebraska Medical Center (UNMC). The dusts were collected from the rafters of Prinz Grain and Feed Incorporated commercial grain elevators (Cuming County, NE). The corn and soybean dusts were divided into groups 'A' and 'B' as a means to keep samples separate. For this series of experiments, samples from Corn A and Bean A have been used. The dusts were also sieved before use to remove large artifacts that may obstruct dust delivery. It must be noted that the soybean dust has a finer consistency compared to the corn dust.

Scireq's inExpose system (Montreal, QC, Canada) was used for dust generation. The inExpose is a bench-top inhalational exposure system that allows for nose-only exposure to aerosolized substances. The device has a low-internal volume which allows for the aerosol to be pushed through the device in a fine mist with minimal dilution from the outside environment. The soft restraints that are used to hold the animals in place do not unduly restrict the animal or interfere with temperature homeostasis, thereby eliminating the need to anesthetize the animals prior to exposure and deleterious effects of temperature control. Dust generation entailed affixing

the exposure tower of the inExpose to a 500-ml delivery flask containing 15 g of dust. Air was blown onto the dust at minimal pressure and the flask was agitated for 5 s at 30-s intervals for a total exposure period of 20 min, per exposure. The mice received 3 dust exposures, each 48 h apart and the time-point chosen for analysis was 18 h following the final dust exposure, based on pilot exposures described below.

For the purposes of this study, allergic mice were divided into 3 groups (n=6 per group). The allergy control group received no dust treatment. The sterile control group received dust that was baked at 180ºC for 4 hours so as to control for endotoxin and the influences of bacterial and fungal species.

A group of naïve BALB/c mice (n=5) which received unadulterated dust as well as naïve BALB/c mice (n=5) who were not subject to dust exposures were used for pilot exposures to determine whether or not the dust was being delivered to the airways and also to establish an appropriate time point at which to assess inflammatory parameters and AHR.

3.3.5. Microbiology

Corn and bean dusts in sterile PBS were placed in a stomacher (400 Circulator, Seward, Port St. Lucie, FL) in a 1:10 dilution and were further serially diluted down to 1:1,000,000 Of these, the 1:1000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions were plated onto plates of nutrient agar to reveal the diversity of microbial populations in the dusts. Sterile dusts were subject to the same schemata of dilution and plating to ensure that the dusts had been completely sterilized. The plates were incubated at 37ºC for 48 h after which prominent bacterial colonies were picked and streaked for isolation on plates of nutrient agar. Isolated colonies of the

individual bacterial species were submitted to the NDSU Veterinary Diagnostic Laboratory for identification.

Five-gram samples of corn and bean soybean dusts were submitted to EMSL Analytical Inc. (Denver, CO) for identification of fungal species.

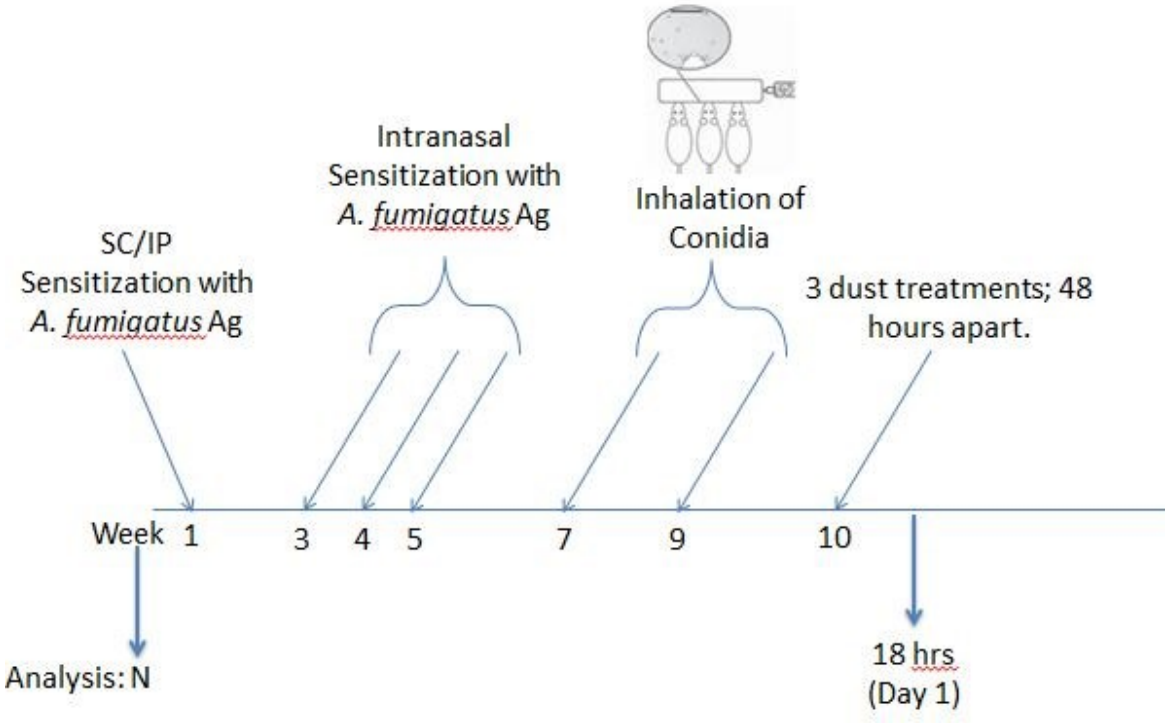

Figure 8. Study timeline. Mice were sensitized with soluble *A. fumigatus* extract in alum intraperitoneally (IP) and subcutaneously (SC) in week 1 and in normal saline in weeks 3-5. The sensitized mice were challenged with live, airborne conidia for 10 min at weeks 7 and 9. The mice were exposed 3 times to grain dust in week 10, each 48 h apart. Tissues were harvested 18 h after the final dust exposure.

3.3.6. Measurement of AHR and tissue harvest

For the current study, AHR was measured 18 h after the final dust treatment, based on the pilot experiments performed on the naïve BALB/c mice. Sodium pentobarbital (Butler, Columbus, OH; 0.1 mg per kg of mouse body weight) was used to anesthetize mice that were then intubated for restrained plethysmography (Buxco, Troy, NY) and ventilated via a Harvard Pump ventilator (Harvard Apparatus, Reno, NV). The value of baseline airway resistance was measured for each animal and averaged for the group. Then, an intravenous injection of the non-specific spasmogen, acetyl-β-methacholine (420 μg per kg), was administered to determine peak airway resistance, a measurement of AHR.

For the acquisition of blood serum, approximately 500 μL of blood was recovered from each mouse via ocular bleed. The blood was centrifuged for 10 min at 13,000 x *g* to yield serum which was collected and stored at -20ºC until used. Right lungs were snap-frozen in liquid nitrogen for protein analyses, and left lungs were fixed in formalin for 18 h and were then processed for histological analyses.

3.3.7. Morphometric analysis of leukocytic inflammation in bronchoalveolar lavage

BAL was performed on each intubated mouse using 1.0 ml of phosphate-buffered saline. The BAL fluid was centrifuged at 6,500 x *g* for 10 min to separate the fluid and the cells. After the fluid was siphoned away from the cell pellet and stored at -20ºC, the cell pellet was resuspended in in 1.0 ml of PBS and cytospun (Shandon Scientific, Runcorn, United Kingdom) onto microscope slides. The cytospun cells were differentially stained via Diff-Quik staining (Mercedes Medical, Sarasota, FL). Five random high-powered fields (1000X) were chosen per

slide and the numbers of different cell types recruited to the lung were counted, and the average numbers of each cell type were then calculated.

3.3.8. Histological analyses

The formalin-fixed and paraffin-embedded lung sections were cut longitudinally across the coronal plane to yield 5-μM sections which were affixed to glass slides. For a qualitative evaluation of the extent of inflammation, hematoxylin-eosin (H&E) staining was used. Goblet cell metaplasia and mucus production were examined via periodic acid-Schiff (PAS) staining.

3.3.9. Measurement of endotoxin levels

GenScript's ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Piscataway, NJ) was used to assess the levels of endotoxin in the corn and bean dusts. Grain dust samples were diluted in the manufacturer's provided endotoxin-free water. For corn dust, dilutions of 1:10,000, 1:100,000 and 1:1,000,000 were used while for bean dust dilutions of 1:10,000,000, 1:100,000,000 and 1:1,000,000,000 were used. These dilutions were determined based on trial runs with a wider range of dilutions aimed to pick the most efficacious dilutions for accurate determination of endotoxin levels. The assay was run based on the manufacturer's instructions.

3.3.10. Measurement of aflatoxin levels

Corn and soybean dust samples were submitted to the Veterinary Diagnostic Laboratory at North Dakota State University for estimation of aflatoxin levels.

3.3.11. Quantification of serum and BAL IgE and IgA

BAL and serum concentrations of IgE and IgA were quantified by ELISA (eBioscience, Vienna, Austria).The dilutions are as follows: IgA: BAL= 1:75; serum =1:2000, IgE: BAL= 1:5; serum=1:500 bean dust group serum= 1:3000. The detection limits for the kits are as follows: IgE= 4 ng/ml, IgA= 0.39 ng/ml. The assays were run according to the manufacturer's instructions.

3.3.12. Statistical analyses

Comparisons between the different treatment groups of mice were drawn via an unpaired Student's two-tailed *t*-test with a Welch's Correction to elicit statistically significant differences between antibody titers, AHR, and BAL cell numbers. GraphPad's Prism Software (San Diego, CA) was used for this purpose.

3.4. Results

At 18 h post dust exposure, AHR elicited in the allergic lung is not altered by grain dust inhalation.

Prior to intravenous methacholine provocation, the baseline AHR values for each groups averaged at 1.45 $\pm$ 0.10 cm H_2O/ml/s. Methacholine administration resulted in an increase in AHR, but there was no difference between the peak AHR values measured for the allergy control group and either of the dust-treated groups (**Fig 9**).

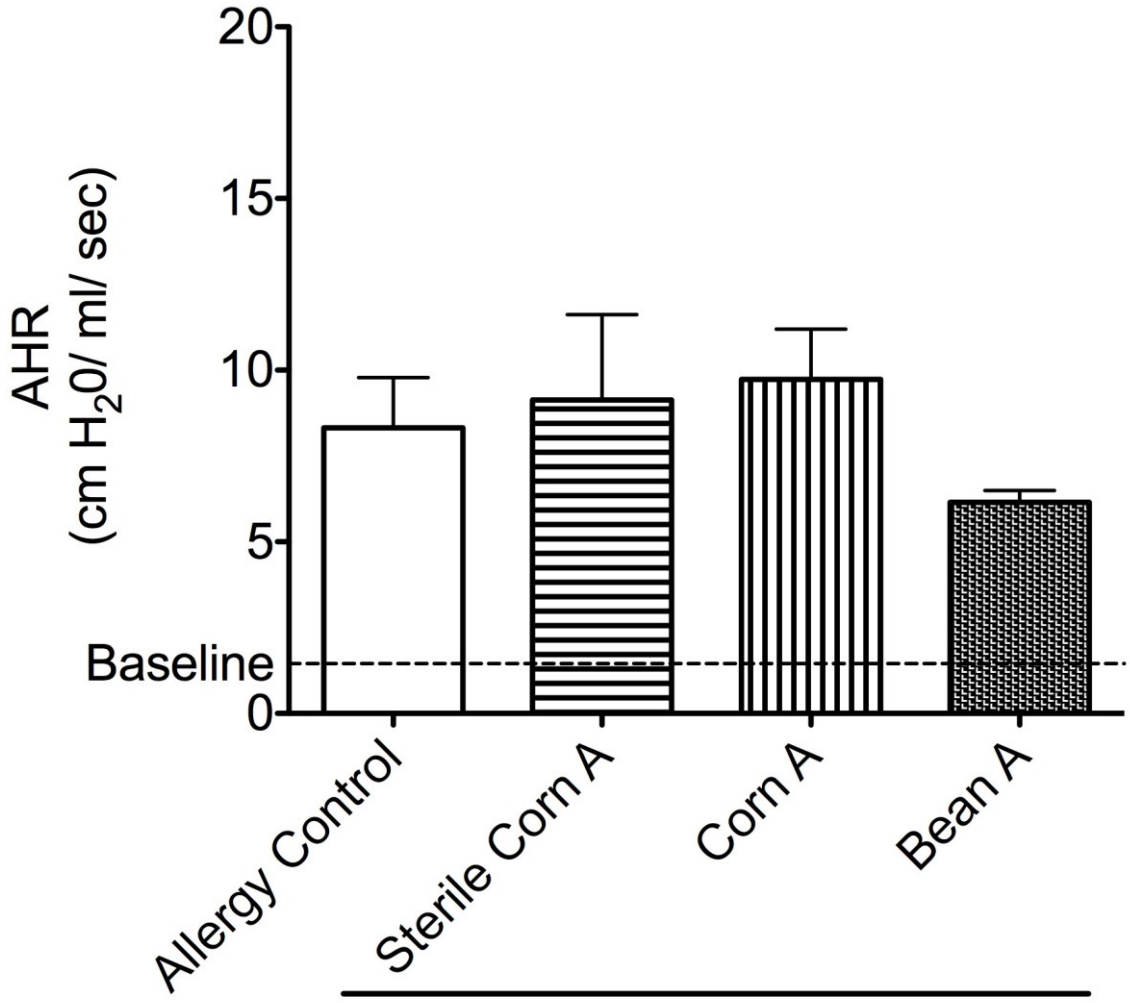

Figure 9. Comparison of AHR values after grain dust inhalation in fungal allergic murine lungs. Airway resistance in C57BL/6 mice that had been sensitized and challenged with *A. fumigatus* conidia was assessed before (baseline, dashed line, 1.45 ± 0.10 cm H_2O/ml/s) and after methacholine injection (420 µg per kg) at a time point 18 h after the third 20-min exposure to sterile corn dust, corn dust, or bean dust or at an equivalent time point for the no dust allergy control. All values are expressed as mean ± SEM, n= 3-6 mice per group.

The relative numbers and pattern of inflammatory cell recruitment to the airway lumen is not changed after inhalation of grain dust as compared to controls; however, more neutrophils were noted after inhalation of soybean dust when compared to corn dust.

The BAL drawn from all groups of mice contained macrophages, neutrophils, eosinophils, and lymphocytes. The relative configuration of BAL cellularity was consistent across the groups. Eosinophils were the most populous cell type, followed by neutrophils, macrophages, and lymphocytes. Treatment groups did not exhibit a significant difference in macrophage (**Fig 10A**), eosinophil (**Fig 10B**), or lymphocyte (**Fig 10C**) numbers as compared to controls. However, soybean dust-treated groups exhibited a statistically significant increase in neutrophil (**Fig 10D**) numbers relative to the corn and sterile corn dust-treated groups, but not to the allergy control.

Spore-forming Gram-positive bacilli are the most prominent microbial species isolated from corn and bean dusts.

The corn dust yielded bacteria of the *Corynebacterium* (**Fig 11B**) and *Bacillus* (**Fig 11A**) genera along with *Micrococcus luteus*. The bean dust yielded three bacilli: an unidentified *Bacillus spp*., *Bacillus cereus* (**Fig 11C**), and *Bacillus thuringiensis* (**Fig 11D**). The bacteria were identified by North Dakota State University's Veterinary Diagnostic Laboratory. The criteria used to ascertain identity included morphological features, Gram character, and biochemical tests that tested the specimen's ability to utilize common metabolites. The sterile dusts yielded no bacteria despite being incubated at 37ºC for 7 d.

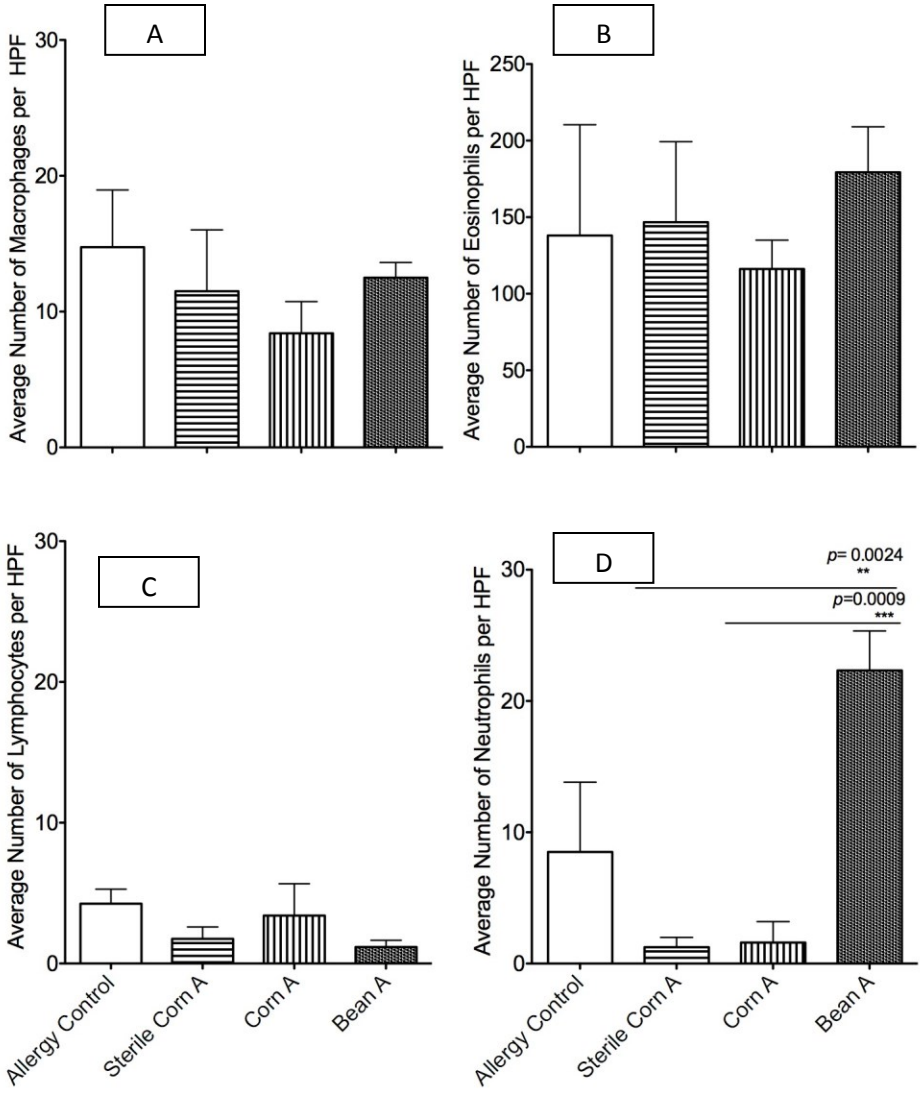

Figure 10. Comparison of BAL cellularity. Cells recruited to the airways of C57BL/6 mice that had been sensitized and challenged with *A. fumigatus* were enumerated for macrophages (**A**), eosinophils (**B**), lymphocytes (**C**), and neutrophils (**D**) from the BAL contents 18 h after the third 20-min dust treatment with sterile corn dust, corn dust, or bean dust or at a comparable time point for no dust controls (allergy control). All values are expressed as the mean ± SEM, and n= 3-6 mice per group. *$p< 0.05$, **$p <0.01$, ***$p< 0.0001$.

The limits of detection in GenScript's ToxinSensor Chromogenic LAL Endotoxin Assay kit were between 0.1-0.01 EU ml^{-1}. The endotoxin levels in the grain dusts were greater the kit's limits of detection.

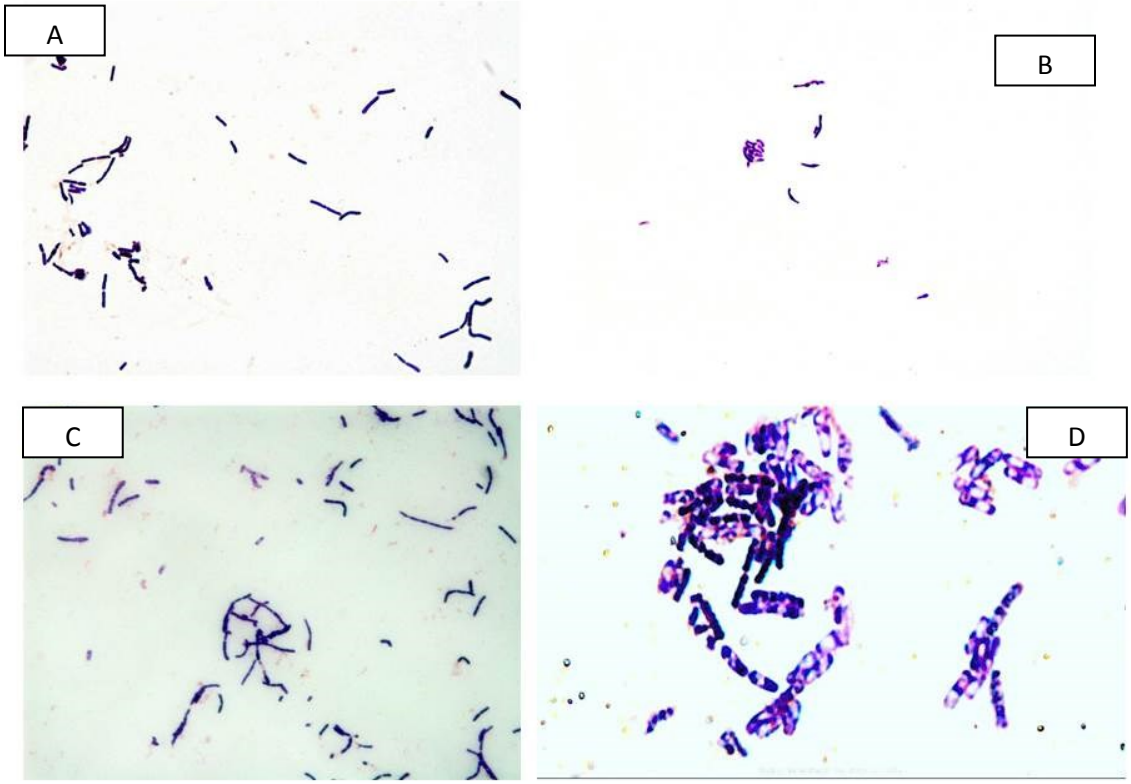

Figure 11. Representative photomicrographs of prominent Gram-stained bacteria isolated from the grain dusts. The corn dust yielded an unidentified *Bacillus spp.* (**A**) (200X) and a bacterium of the *Corynebacterium* genus (**B**) (200X), while *Bacillus cereus* (**C**) (200X) and *Bacillus thuringiensis* (1000X) (**D**) were isolated from the bean

Aspergillus sp. and Penicillium sp. were the most prominent of fungal species isolated from the corn and soybean dusts.

Corn dust yielded a greater diversity of fungal species compared to the soybean dust. However, the majority of the fungi in both dusts were *Aspergillus* and *Penicillium*. There was a greater percentage of *Aspergilli* in the bean dust compared to the corn dust (**Fig 12**).

Test Report: Identification and Enumeration of Culturable Fungi by Bulk (Genus Level ID (EMSL Method M005))

Sample Description	Sample Location	Temp (C)	Sample Measure (g)	Analytical Sensitivity (CFU/g)	Dilution	Fungal Identification	Colony Count	CFUs (CFU/g)	Percent of Total
Corn 1	Field	25	.89	1,120	1000	*Acremonium sp.*	12	13,500	10.8
221301107-0001	**Media:** MEA,&,Cellulose			1,120	1000	*Alternaria sp.*	1	1,120	<1
				1,120	1000	*Aspergillus sp.*	16	18,000	14.4
				1,120	1000	*Cladosporium sp.*	1	1,120	<1
				1,120	1000	*Penicillium sp.*	80	89,900	71.9
				1,120	1000	*Rhizopus sp.*	1	1,120	<1
						Total	**111**	**125,000**	
Bean 2	Field	25	.94	106	100	*Alternaria sp.*	5	532	<1
221301107-0002	**Media:** MEA,&Cellulose			10,600	10000	*Aspergillus sp.*	10	106,000	25.4
				1,060	1000	*Fusarium sp.*	1	1,060	<1
				10,600	10000	*Penicillium sp.*	29	309,000	73.9
				1,060	1000	*Rhizopus sp.*	1	1,060	<1
						Total	**46**	**418,000**	

Figure 12. Screenshot of EMSL fungal identification report. Dust samples cultured on cellulose and malt extract media at 25ºC yielded a variety of fungal species of which *Aspergillus sp.* and *Penicillium sp.* were the most prominent in both dusts.

Aflatoxin levels in the corn and soybean dusts were negligible.

At a detection level of 20 ppb, no aflatoxin was detected in either the corn or the soybean dust samples.

Inflammation and goblet cell metaplasia in the fungal allergic lung are not exacerbated at an early time point after inhalation of corn or bean dust.

Pulmonary inflammation (**Fig 13**) was evaluated based on H&E-stained lung sections acquired from the allergy control and dust-exposed mice. While perivascular and peribronchial inflammation was evident in all of the lungs that had been exposed to conidia, no differences in the overall level of inflammation were visually apparent neither between either of the dust-treated groups and the control group nor between the corn and soybean dust groups.

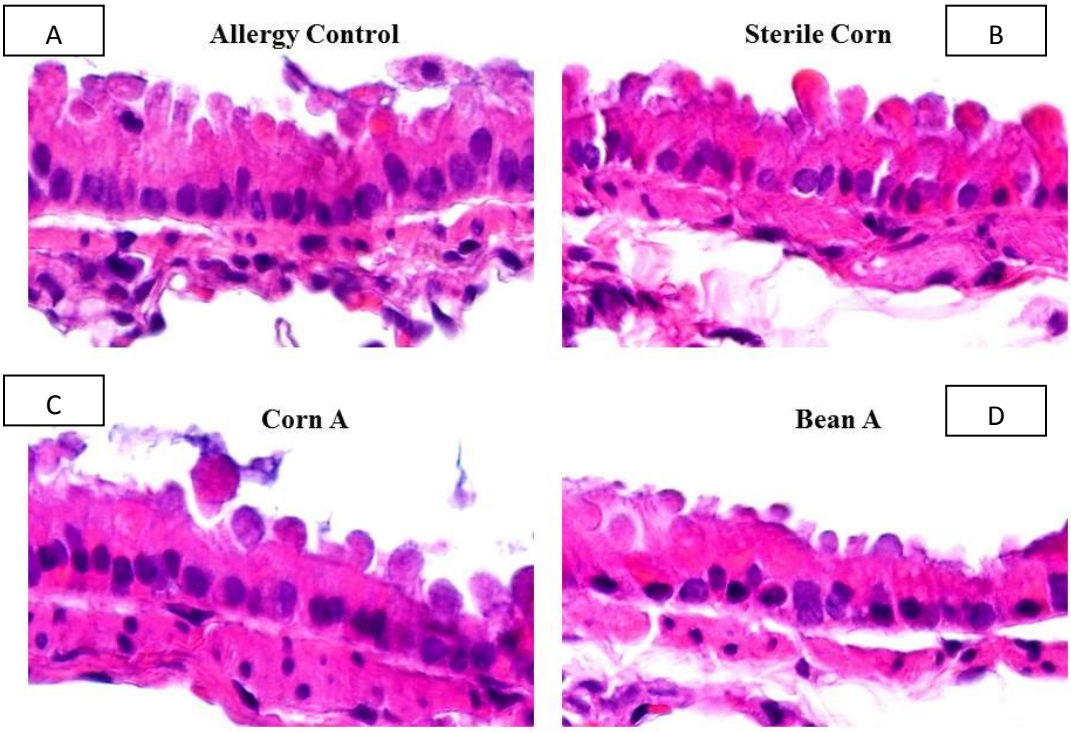

Figure 13. Representative photomicrographs of H&E-stained lung sections from C57BL/6 mice. Post-sensitization and challenge with *A. fumigatus*, lungs show inflammation around blood vessels and airways 18 h after the third dust treatment or comparable time point in allergic, but not dust treated C57BL/6 mice (200X).

Goblet cell metaplasia revealed a similar pattern. While positive staining for mucus (**Fig 14**) is indicative of goblet cell metaplasia, there were no visual differences observed in the pattern of PAS staining between the allergy control and either dust-treated group. Quantification of the number of goblet cells reiterated this finding (**Fig 15**).

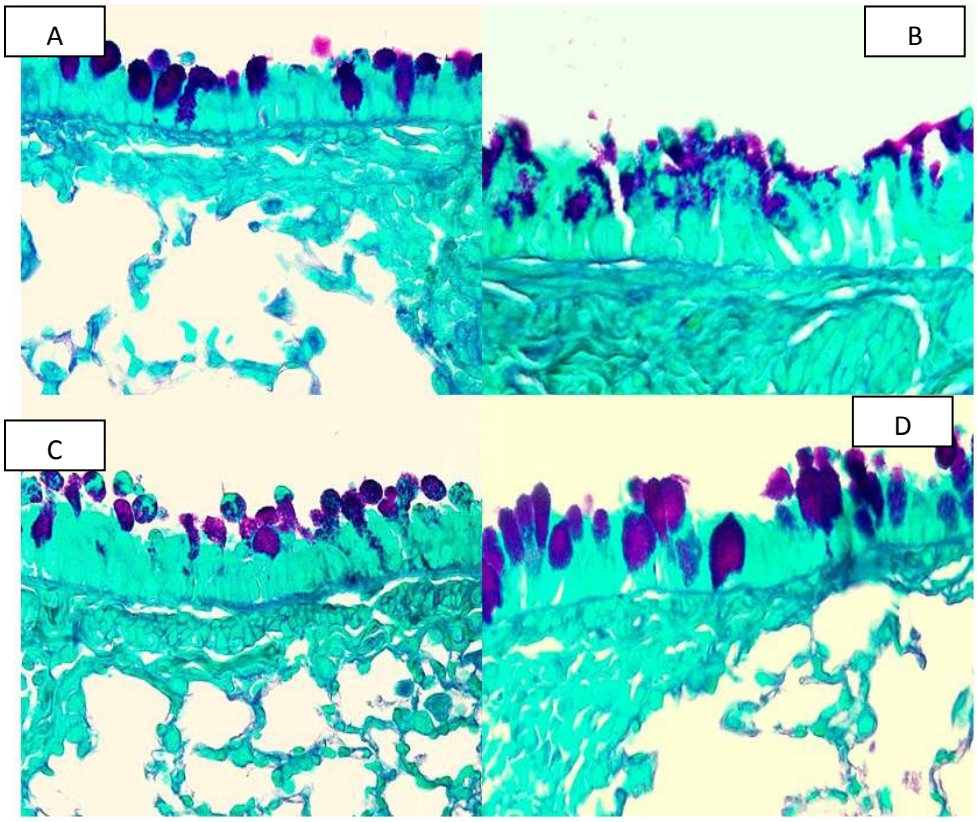

Figure 14. Representative photomicrographs of periodic acid-Schiff-stained lung sections from C57BL/6 mice. These show goblet cell metaplasia (purple) 18 h after the third dust treatment or comparable time point in C57BL/6 mice that had been sensitized and challenged with *A. fumigatus* conidia and treated with no dust A, allergy control), or 3, 20-min exposures to sterile corn dust (**B**), sterile corn dust (**C**), or bean dust (**D**) (200X).

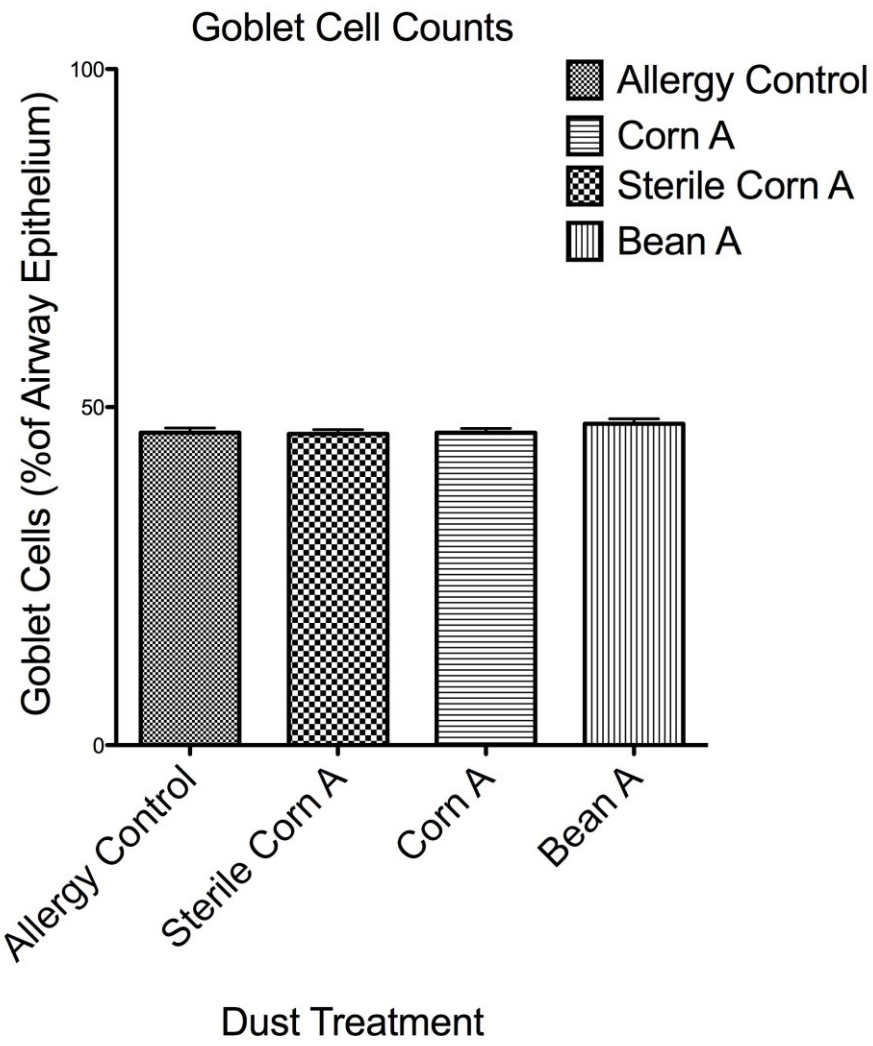

Figure 15. Comparison of goblet cell metaplasia after grain dust inhalation in fungal allergic murine lungs. C57BL/6 mice that had been sensitized and challenged with *A. fumigatus* conidia were treated with no dust (allergy control), or 3, 20-min exposures to sterile corn dust, corn dust, or bean dust. Goblet cell numbers are reported as a percentage of the total epithelial cells in direct contact with the basement membrane of the lateral branches of the conducting airways. Data were analyzed using an unpaired Student's *t*-test with Welch's correction. All values are expressed as means ± SEM, and n= 4-6 mice per group.

BAL IgE and IgA titers in sensitized mice challenged with inhaled fungus are not changed after inhalation of grain dust.

The presence of IgE in the serum is a hallmark of allergy. In this study, elevation of IgE (**Fig 16B**) confirmed successful allergenic sensitization. However, there was no difference between the control no-dust group and any of the dust-treated groups, showing that the inhalation of these agricultural dusts neither exacerbated nor ameliorated this aspect of the allergic phenotype.

IgA elevation (**Fig 16A**) was expected, given that the antibody is secreted in large amounts in response to insult to a mucosal surface. Yet again, the lack of difference between the allergy control and the dust-treated groups in terms of IgA quantities in BAL also shows that the dust treatment did not significantly impact IgA production in the lung.

Serum IgE, but not IgA, is significantly higher in bean dust-treated allergic animals than those treated with corn dusts or in the control group that did not receive dust treatment.

While the levels of serum IgA (**Fig 17A**) were not different among the control and the treatment groups, the soybean dust-treated group showed a significant elevation in IgE (**Fig 17B**) levels compared to that of the allergy control (**p=0.0098), sterile corn dust (*p=0.0306) and corn dust (**p=0.0066) treatments. This pattern was not reproduced in the levels of IgE seen in the BALF (**Fig 16B**), suggesting a potential impact on the systemic sensitization after bean dust inhalation.

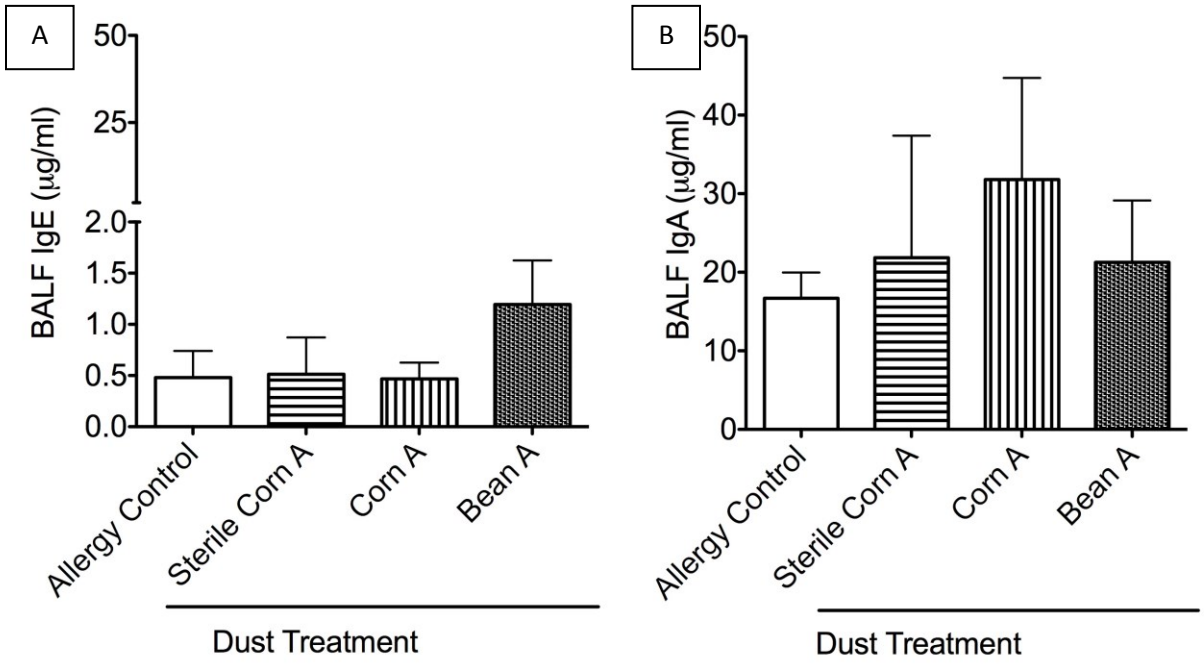

Figure 16. Comparison of BALF antibody titers. BALF IgA and IgE levels after grain dust inhalation in fungal allergic murine lungs. C57BL/6 mice that had been sensitized and challenged with *A. fumigatus* conidia were treated with no dust (allergy control), or 3, 20-min exposures to sterile corn dust, corn dust, or bean dust. IgA (A) and IgE (B) titers were assessed by ELISA in BAL fluid. Data were analyzed using an unpaired Student's *t*-test with Welch's correction. All values are expressed as means ± SEM, and n= 3-5 mice per group.

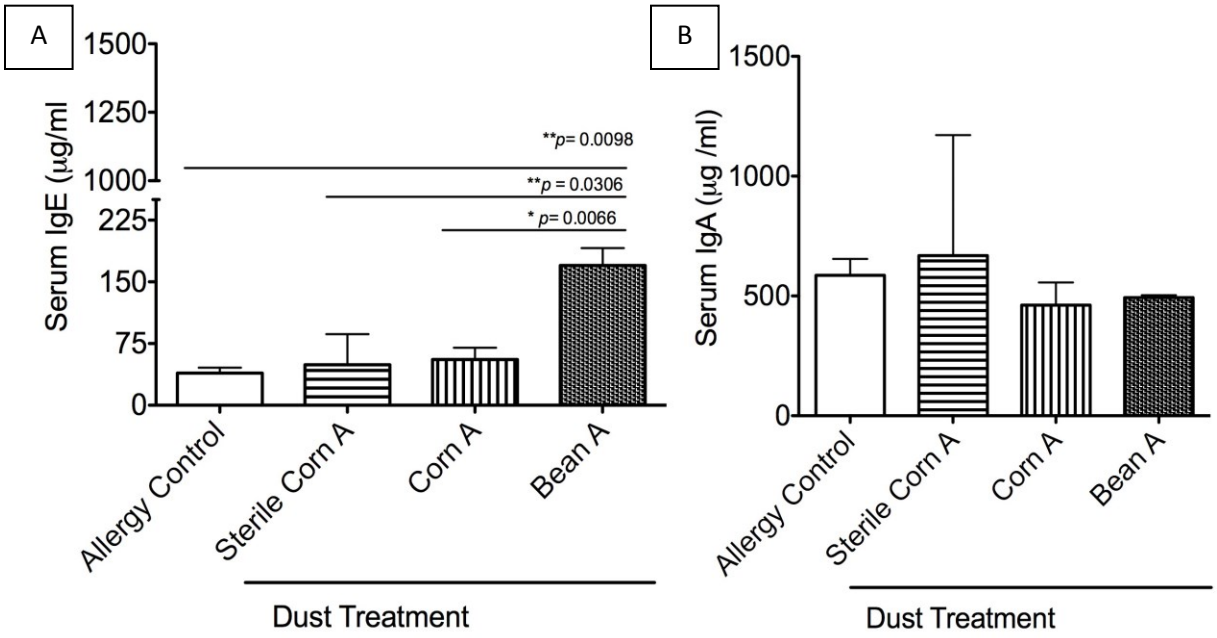

Figure 17. Comparison of serum antibody titers. Serum IgA and IgE levels after grain dust inhalation in fungal allergic murine lungs. C57BL/6 mice that had been sensitized and challenged with *A. fumigatus* conidia were treated with no dust (allergy control), or 3, 20-min exposures to sterile corn dust, corn dust, or bean dust. IgA (A) and IgE (B) titers were assessed by ELISA in BAL fluid. Data were analyzed using an unpaired Student's *t*-test with Welch's correction. All values are expressed as means ± SEM, and n= 3-5 mice per group.

3.5. Discussion

Exposure to grain dust is an occupational health hazard that leads to and, in some cases, exacerbates respiratory and ocular symptoms in both atopic and non-atopic individuals (Liebers 2006, Gripenback 2003, Kimbell-Dunn 2001, Melbostad 2001, Post 1998, James 1990, Olenchock 1986, DoPico 1977). Further complications arise in light of noncompliance in using personal protective equipment (PPE) among agricultural workers (Carpenter 2002) and less inclination towards help-seeking behavior (Hoyt 1997).

From an immunologic standpoint, there have been reports of inflammatory cell recruitment to the airway along with concomitant increases in IL-8 and IL-6 as well as an increase in bronchial hyperresponsiveness (Gripenback 2003, Lemiere 2000, Becker 1999, Palmberg 1998, VonEssen 1995, DoPico 1977) in response to grain dust exposures in both asthmatic and non-asthmatic individuals. Our results do not corroborate with these findings, but there may be reasons why our results do not recapitulate clinical findings.

Two aspects of the current study revealed significant differences from control levels: elevation of serum IgE levels and increased neutrophil recruitment to the airway lumen after treatment with soybean dust. To our knowledge, two studies exist where an elevation of serum IgE was observed in response to grain dust exposures (Park 1998, Park 1998b). Data in both studies was collected from human subjects who worked in agricultural settings. While the studies lacked a histological and a BAL-based readout, AHR in response to methacholine provocation was the same in both the clinical and the animal model study presented here. It is possible that the soybean dust may contain an immunostimulatory component—one that is absent in corn dust—which prompts elevation of specific IgE. The larger presence of *Aspergillus sp* in the soybean dust compared to the corn dust suggests that the IgE increase may be fungus-driven. Secondly, the soybean dust is finer in consistency compared to the corn dust, which may allow the soybean dust-treated animals to have received a greater inoculum of dust in the allotted 20 min, translating to increased neutrophil recruitment that was observed.

In terms of microbial contributions to pathology, the concentration of endotoxin in grain dust dilutions was seen to be too great to be detected by commercial kits. There is, however, no histopathological evidence in this study to support this. It can be concluded, therefore, that

commercial kits are ill-suited to detect levels of endotoxin in samples like grain dust. Further, the lack of difference in pathology between the sterile control and non-sterile dusts suggests that endotoxin may not be involved in the immunopathology seen herein.

The presence of spore-forming Gram-positive microorganisms is not surprising since grain dusts collected from rafters present a harsh, dry environment where spore-formers abound. Both *B. cereus* (Frankard 2004) and *B. thuringiensis* (Ghelardi 2007) are known to cause pneumonia, but these instances are rare and usually in immunocompromised hosts. Again, there is no evidence to suggest pathogenic bacterial processes may be involved in the pathology seen herein.

The 18-h/day-1 time point was chosen based on pilot experiments on naïve mice which showed the advent of neutrophilia 18 h after 20 min of dust treatment. The BAL cellularity of the animals in the study shows that neutrophils and macrophages are prominent cell types in the BAL, but the number of eosinophils greatly outstrips these other innate cell populations. A study by Becker et al (1999) based on non-atopic grain handlers shows that the immune response to nebulized corn dust is compartmentalized. Bronchial epithelial cells contribute to IL-8 production and neutrophilia, while alveolar macrophages produce the IL-1β and IL-6. In addition, polymorphonuclear cells produce the inflammation-dampening sIL-1RA. All of these responses are seen within 6 h of grain dust inhalation and may serve to explain the neutrophilia seen in the pilot experiments while emphasizing the importance of timing and dosage of dust exposures. The attenuated neutrophilia seen in the BAL retrieved at 18 h also calls into question the role of atopy in the kinetics of a grain dust-induced inflammatory response. Indeed, our aeroallergen model of allergy is robust in terms of producing eosinophilic inflammation

(Samarasinghe 2011, Hoselton 2010), so much so that it may overwhelm any additive pathology induced by the dust alone. Future work shall include a non-allergic, dust-treated to account for the above. The use of the inExpose apparatus to deliver grain dust is powerful in that it faithfully mimics the inhalational exposures that an agricultural worker might encounter while working. The primary limitation of this method of delivery is the inability to precisely quantify the amount of dust being delivered to the airway and, by extension, to keep the doses consistent. While these limitations can be overcome by nebulizing grain dust preparations (Von Essen 1995), nebulization in a liquid medium does not recapitulate occupational exposures to grain dust. Further, 20-min exposures may not result in enough dust accumulation in the airway to provoke a distinct immune response. In the absence of dust exposures, the pulmonary pathology, as well as the cellularity of the BAL, imitates that of twice-challenged mice at the same time point (Samarasinghe 2011).

I advocate for amendments to the inhalational model of grain dust delivery given its fidelity to realistic occupational exposures. Also, it is the closest method to the Specific Inhalation Challenge (SIC) test which is "the gold standard" in the diagnosis of occupational asthma (Ortega 2002, Vandenplas 1997). Future work will focus on characterization of the elevated serum IgE in the soybean dust treatment group, as well as longer and more frequent grain dust exposures and analyses at later time points to evaluate changes in airway architecture.

3.6. Acknowledgments

We would like to extend our gratitude to the Central States-Center for Agricultural Safety and Health and our collaborators at The University of Nebraska Medical Center, Omaha, NE for providing the funding for these pilot experiments (CDC/NIOSH/OD).Mr. Adam Fetterman of The Department of Psychology at North Dakota State University for his aid in drawing connections between occupational asthma and help-seeking behavior among farmers and also to Mr. Tanner Barnharst for his assistance with goblet cell counts.

4. GENERAL DISCUSSION

In the end, the facts bespeak the underlying and undeniable importance of asthma research: as of 2011, 18.9 million adults and 7.1 million children in North America currently have asthma. Asthma results in 2.1 million emergency room visits per annum (CDC Faststats) and costs a total of $56 billion per annum in absenteeism, medical expenses, and deaths (American Academy of Allergy, Asthma and Immunology 2013).

The model of fungal allergic asthma that featured in the preceding pages imitates 'real-life'/'every day' fungal exposures as faithfully as possible via aeroallergen challenge and with a degree of versatility that allows one to adapt the system to variants of the allergen—a feature that was successfully exploited in the study presented here that examined the effects of *lox* and *ppo* *A. fumigatus* mutants on asthma progression. The study determined that the *lox* and *ppo* genes cannot be implicated in asthma. In fact, taken with Kupfahl et al's analysis (Kupfahl 2012) of *Aspergillus*-derived eicosanoids, our study reiterates their findings in an *in vivo* context.

By establishing a robust allergic response, the model also finds use as a tool to evaluate interactions between allergic mediators and environmental stimuli. This is exemplified by the corn and soybean dust study presented here. The results from the grain dust study are valuable in that they strongly suggest that the dust is being delivered to the lungs. This technical advancement is critical since it establishes a means to deliver that dust that is in keeping with the model's theme of mimicking 'real-life' exposures.

Finally, given the intricate intermingling of factors that lead to both asthma predisposition and onset, a panacea for the disease seems unlikely. However, asthma can be

managed, and the successful management of asthma is rooted in a research culture that examines and cross-examines the diverse players in the pathogenesis of the disease. This disquisition represents one such attempt.

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