

LUNG MUCOSAL RESPONSE TO REPEATED INHALATIONAL INSULTS WITH
IMMUNOMODULATORY AGENTS IN A MURINE MODEL OF FUNGAL ASTHMA:
AIRWAY EPITHELIUM TAKES THE CENTER STAGE

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ABSTRACT

Asthma is a debilitating disease of the lungs affecting 235 million people worldwide. Fungus-associated asthma leads to a particularly severe type of disease, and exposure to environmental fungi and their products is unavoidable due to the ubiquitous nature of fungal species. Besides being allergenic, fungi are opportunistic pathogens, and anti-fungal and/or allergic pathways may be modified through repeated inhalation of immunomodulatory agents, affecting the outcome of fungus-induced asthma.

Our aim in this project was to investigate the extent to which repeated inhalation of immunomodulatory agents influence the lung mucosal responses in a naïve murine host or in one that had been sensitized to fungal proteins (allergic). The immunomodulatory substances chosen hold relevance to human inhalational exposure, and included live or irradiation-killed *Aspergillus fumigatus* (a fungi) spores, deoxyxynivalenol (a mycotoxin), and fluticasone propionate (an inhalationally administered corticosteroid, commonly prescribed for allergic asthma). In a naïve host, inhalation of live *A. fumigatus* spores showed pathological features of fungal asthma. However, in an allergen-sensitized lung, both dead and live *A. fumigatus* spores established fungal airway disease, albeit to different extents. Next, we tested the effect of deoxynivalenol in an allergic host and found that its repeated inhalation did not affect pulmonary disease pathology, but did lead to a dose- and time- dependent increase in mucosal and systemic total IgA. Finally, we tested the effect of fluticasone propionate, and found that it did not influence the development of fungal airway disease, but did induce dynamic changes in lung physiology and antibody titers.

Besides mimicking human inhalational exposures, inhalation ensures direct interaction of the inhaled substances with airway epithelium, which plays an important role in defense against

inhaled substances and in asthma pathophysiology. By analyzing various mechanisms involved in murine lung-mucosal response to the inhaled substances, a critical involvement of airway epithelium as an orchestrator of immune responses is highlighted, and this would inform mechanism-based future studies. In conclusion, this project is likely to aid in establishing evidence based standards for fungus-related exposures and in making informed therapeutic decisions for fungus-associated diseases.

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DEDICATION

To my adorable son for bringing immense joy and laughter into my life, to my mom and dad for their unconditional love and support, to my husband for being my pillar of strength!

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LIST OF ABBREVIATIONS

ABPA.....	Allergic Bronchopulmonary Aspergillosis
AHR.....	Airway Hyperresponsiveness
AMs.....	Alveolar Macrophages
ASM.....	Airway Smooth Muscle
BAL.....	Bronchoalveolar lavage
CD.....	Cluster of Differentiation
DC.....	Dendritic Cell
ECM.....	Extracellular Matrix
GM-CSF.....	Granulocyte Macrophage Colony Stimulating Factor
ICAM.....	Intercellular Adhesion Molecule
ICS.....	Inhaled Corticosteroid
IFN.....	Interferon
Ig.....	Immunoglobulin
IL.....	Interleukin
GC.....	Goblet Cell
PRR.....	Pattern Recognition Receptors
PAMP.....	Pathogen-associated Molecular Pattern
SAFS.....	Severe Asthma with Fungal Sensitivity
TGF.....	Transforming Growth Factor
Th.....	T-helper
TNF.....	Tumor Necrosis Factor
TLR.....	Toll-like Receptors
VEGF.....	Vascular Endothelial Growth Factor

GENERAL INTRODUCTION

Background and significance of the research

Asthma is a chronic inflammatory disease of the lungs, and its symptoms include wheezing, breathlessness, chest tightness, and coughing with variable airway obstruction and airway hyperreactivity. Asthma affects 235 million people worldwide, resulting in a loss of 15 million disability-adjusted life years annually [16]. Each year, 250,000 people die of asthma [16]. It is one of the most common chronic disease among children, with approximately 7 million affected in the U.S. alone [364]. The total incremental cost (direct and productivity costs) of asthma in the U.S. was estimated at \$56 billion [23], thereby suggesting that this disease is associated with a huge socio-economic burden.

One of the clearest risk factors for developing asthma is exposure to inhaled substances and particles that are known to provoke allergic reactions or irritate the airways [16]. In this regard, fungal exposures and fungus-induced asthma have gained significant interest, since they cause a particularly severe type of asthma that can result in multiple hospitalizations [244], intensive care unit admissions [4], and asthma-related deaths [35]. Fungus-induced asthma is difficult to manage therapeutically [67,302] and inhaled corticosteroids (ICS), which are the cornerstone for symptom management in asthma, have shown variable efficacy for this specific subtype [5,131,154,155,305]. Moreover, avoidance strategies are largely ineffective since environmental exposure to fungi is ubiquitous, and viable or non-viable fungi or their components are inhaled in indoor and outdoor environments. Although a number of federal agencies provide guidance to the public on health effects associated with mold exposure and on ways to mitigate it, the United States Government Accountability Office (U.S. GAO) reported a

lack of federally accepted health based standards for safe level of molds [248]. The problem is particularly concerning in post-flooding or post-hurricane situations and in agricultural settings where repeated inhalation of high quantities of mold over an extended period of time is likely [21,68,119,124].

Aspergillus fumigatus is one of the commonly detected fungal species in the flooded indoor environments [54,269,276,281] and in grain dust samples [119,192]. *A. fumigatus* works in the environment to provide carbon and nitrogen turnover in decaying organic matter. However, due to its small size (2-3 μm in diameter) and hydrophobicity, the spores (conidia) may remain suspended in the air for a long time, increasing the likelihood of inhalation deep into the alveolar spaces of human lungs. While invasive aspergillosis is a serious disease that occurs in immunocompromised individuals, most fungal diseases are associated with less mortality, but an ongoing morbidity, as is the case with allergic diseases in humans. Several allergic diseases have been associated with *A. fumigatus*, such as allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitization (SAFS), rhinitis, sinusitis, and hypersensitivity pneumonitis. *A. fumigatus* is a source of 23 listed allergens [328], and a meta analysis study showed a 15-48% prevalence rate for *Aspergillus* sensitization in bronchial asthma [3]. Secreted proteases [168,174,345] and cell wall-associated molecules, such as β -glucan [100,138,325] and chitin [277,350], are produced by *A. fumigatus* and may be important in host-pathogen interaction in the genesis of *A. fumigatus*-induced pulmonary disease. However, the role of these molecules, in context of environmental exposure to dry, aerosolized *A. fumigatus* spores remains unknown.

In addition to being a potent allergen, *A. fumigatus* is an opportunistic pathogen. The extent of colonization by the fungus varies in allergic disorders and a competent immune system

is critical in preventing invasive disease [59]. An alteration of these events, either due to an underlying disease or drug therapy, may affect fungal clearance and/or the outcome of allergic asthma. The lung is exposed to copious amounts of environmental aerosols on a regular basis, many of which have an immunomodulatory potential. While most inhaled substances are efficiently cleared by the lung's defense or homeostatic mechanisms, the acute and chronic effects of repeated inhalational insults may change or damage the lung, thereby altering the outcome of *A. fumigatus* induced asthma.

Since inhalation is a particularly difficult route of exposure to protect in humans, experimental models to study the effects of inhaled substances are absolutely critical in order to establish cause and affect relationships and to elucidate the underlying pathways involved. Such studies are likely to provide proof-of-concept for designing better therapeutics and to establish evidence based standards related to their exposure. *In vitro*, *ex vivo*, and *in vivo* model systems have been utilized to study the effects of commonly inhaled substances [290]. While the *in vitro* models mostly involve tracheobroncheal, bronchial, or alveolar epithelial cells; *ex vivo* modeling involves the use of an isolated perfused lung, and *in vivo* studies employ small and large animals. Although the *in vitro* and *ex vivo* methods are simpler, cheaper, and allow better control of variables for data acquisition, the disposition of the inhaled substance via absorption, mucociliary clearance, phagocytosis, or metabolism cannot be accurately mimicked in these settings. Indeed, *in vivo* studies using animal models are by far the best way to recapitulate normal human exposure to inhaled substances.

In addition to being a superior method to mimic natural human exposure, inhalation ensures direct interaction of the inhaled substance with the pulmonary epithelium, which is the first layer of cells that comes in contact with the inhaled pollutants, allergens, pathogens,

chemicals, or drugs. Pulmonary epithelium has gained significant attention over past years as being central to the pathogenesis of asthma [142]. Under normal circumstances, the epithelium contains ciliated columnar cells, mucus-producing goblet cells (GCs), and surfactant-secreting clara cells that form a highly regulated barrier made possible through the formation of tight junctions localized at the apical end of airway epithelium [141]. In addition to providing a physical barrier, pulmonary epithelium can recognize the microbes via Pattern Recognition Receptors (PRRs), secrete anti-microbial substances, produce pro-inflammatory and anti-inflammatory agents, and regulate IgA synthesis [271,292]. Epithelium takes the center stage in orchestrating the asthma pathogenesis, since repeated inhalation of environmental substances, such as fungal proteases, can damage the epithelium and the barrier [334], thereby leading to increased penetration of noxious agents or allergens into the sub-mucosa. Additionally, by release of various pro-inflammatory mediators and growth factors in response to inhaled substances, epithelium can regulate the cardinal features of asthma, such as airway inflammation, goblet cell metaplasia, remodeling and hyperresponsiveness.

Dr. Stephen Holgate described the epithelial mesenchymal trophic unit (EMTU), which maintains tissue homeostasis but is dysregulated in asthma. According to this concept, repeated damage to the epithelium, due to inhaled biologically active substances, proteases, irritants etc. result in production of signals that act on underlying mesenchyme to propagate and amplify inflammatory and airway remodeling responses in the airways. Genome wide association studies found several epithelial genes to be associated with asthma. Drugs that increase the airways' resistance to inhaled environmental allergens and pathogens, rather than merely suppressing inflammation, may be a more effective new generation of therapies to treat this chronic disease [143,333]. The role of airway epithelium in asthma pathogenesis is depicted in **Figure 1**.

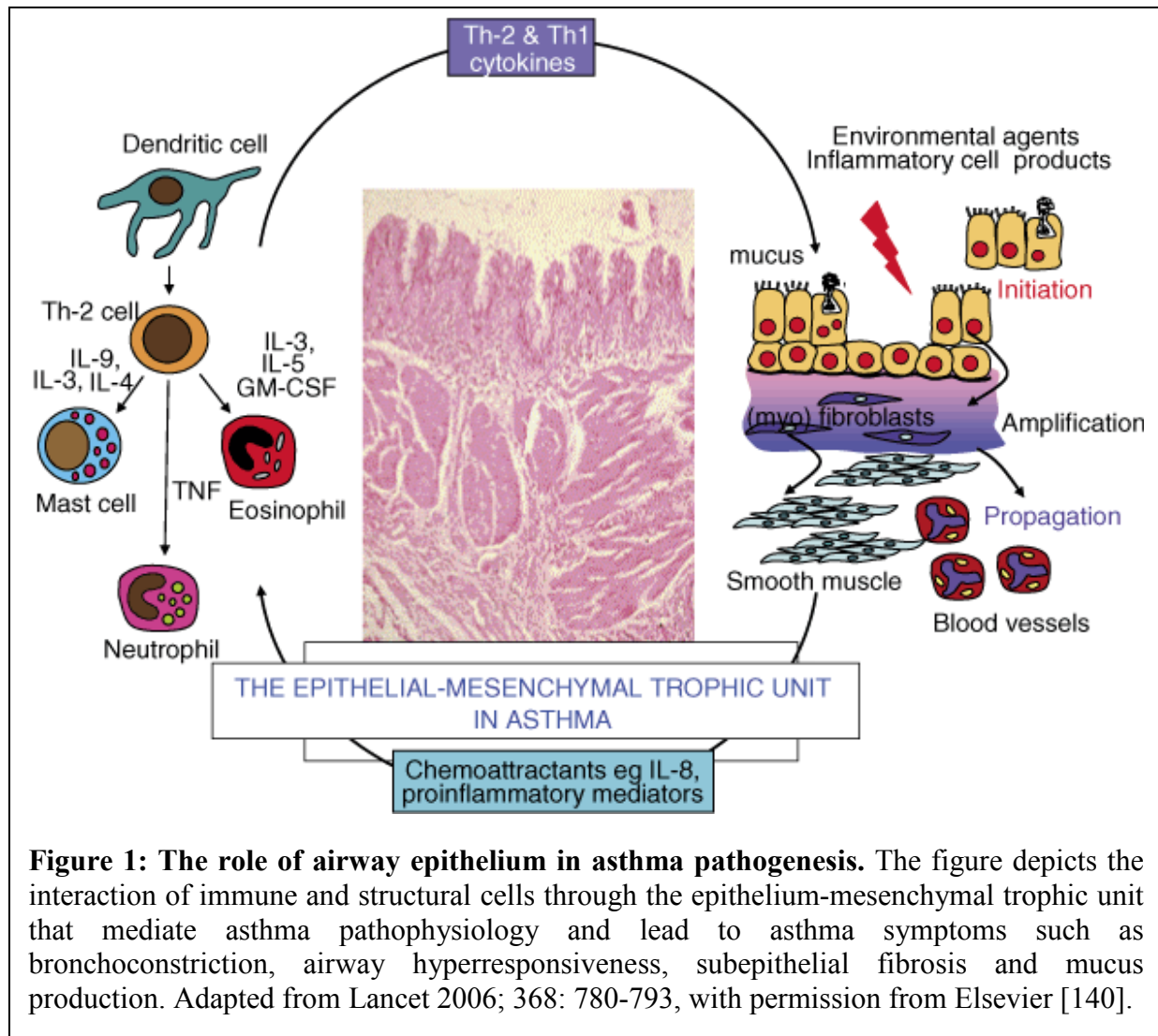


Figure 1: The role of airway epithelium in asthma pathogenesis. The figure depicts the interaction of immune and structural cells through the epithelium-mesenchymal trophic unit that mediate asthma pathophysiology and lead to asthma symptoms such as bronchoconstriction, airway hyperresponsiveness, subepithelial fibrosis and mucus production. Adapted from Lancet 2006; 368: 780-793, with permission from Elsevier [140].

Specific aims of the dissertation project

Our objective in this study was to investigate the ability of inhaled immunomodulatory agents to elicit or affect the symptoms of fungus-induced allergic asthma. For this purpose, we chose substances which hold relevance to human inhalational exposure: 1) Irradiation-killed and viable *A. fumigatus* spores, which differ in their ability to produce fungal proteases, toxins, and other metabolites that are expressed in the cell-wall associated with germination; 2) Deoxynivalenol (DON), a mycotoxin obtained from *Fusarium* species. DON is one of most

prevalent mycotoxin in barley, corn, rye, safflower seeds, wheat, and mixed feeds [266,346], and DON is routinely detected in grain dust released during farm operations, making inhalational exposure in these environments exceedingly possible [118,183,184,190,242]. Additionally, DON is a potent immunomodulatory agent [261], with several demonstrated effects on mediators of allergic asthma. While repeated inhalation of DON in an allergic farm worker is highly likely, the research in this domain is lacking; 3) Fluticasone Propionate, an ICS, which is commonly prescribed in allergic asthma. For delivery of these substances via inhalation in an animal model, we used our newly developed fungal delivery apparatus, which recapitulates human exposure through repeated exposure to dry, aerosolized fungal spores, or a commercially available bench top inhalation exposure system fitted with a nebulizer. The central hypothesis for this work was that direct interaction of pulmonary epithelium with inhaled immunomodulatory agents will influence the symptoms of fungus-induced allergic asthma. The specific aims of this project were:

Specific aim 1

Determine the ability of inhaled dead or live *A. fumigatus* spores to induce allergic pulmonary disease in naïve mice (without prior exposure to fungal antigens). Our working hypothesis was that the allergic asthma symptoms will be elicited by inhalation of live *A. fumigatus* spores, but that dead *A. fumigatus* spores would not be able to establish allergic disease.

Specific aim 2

Assess the extent to which inhaled dead or live *A. fumigatus* spores exacerbate pulmonary symptoms in a previously established allergic state (with prior sensitization to fungal antigens).

Our working hypothesis was that allergic asthma will be exacerbated by inhalation of either dead or live *A. fumigatus* spores.

Specific aim 3

Determine the extent to which repeated inhalation of deoxynivalenol influences the pulmonary response in mice previously sensitized to fungal proteins (allergic). Our working hypothesis was that the inflammatory pulmonary response will be enhanced with inhalation of deoxynivalenol.

Specific aim 4

Determine the extent to which repeated inhalation of fluticasone propionate inhibits allergic asthma symptoms in mice previously sensitized to fungal proteins (allergic). Our working hypothesis was that repeated inhalation of fluticasone propionate will fail to suppress the symptoms of live *A. fumigatus* induced allergic asthma, since topical corticosteroid treatment is often unable to control fungal asthma symptoms clinically.

Organization of the dissertation

The dissertation has been organized to update the reader's background with the pertinent literature that has guided this research. A list of references is included at the end of literature review, which pertains to general introduction and literature review. The subsequent chapters provide a detailed account of the research carried out, specific aims for which have been listed above. Specific Aims 1 and 2 have been combined into chapter#1. Specific Aims 3 and 4 are covered in chapters#3 and 4, respectively. Each chapter is written in the format of journal manuscript and is a stand-alone manuscript, which includes abstract, introduction, material and methods, results, discussion, and references used in that research. The general discussion section

at the end combines the findings of all the research projects. An additional list of references is included at the end which pertains to general discussion section.

LITERATURE REVIEW

The lung

The human lung is a vital organ for respiration. We inhale 10, 000 L of air each day [349], and the lung is designed to optimize the exposure of blood to oxygen. Its architecture is analogous to that of a tree. The trachea, bronchi and bronchioles (collectively termed as airways) form the trunk and branches of a tree, whereas the sac-like alveoli (gas-exchange surface) are comparable to the leaves [255]. The inhaled air passes through the nose and mouth, via larynx and trachea into a rapidly dividing series of about 16 generations of bronchi and bronchioles. The presence of cartilage distinguishes bronchi from the bronchioles. By the time bronchioles branch out to the 17th generation, alveoli begin to appear in their walls. By the 20th generation of airways, the airway wall is entirely made up of alveoli and is referred to as alveolar ducts. The alveolar ducts end into blind sacs called alveolar sacs [78]. The alveoli are made up of broad and thin (~1 μm) alveolar epithelial cells, which are in close contact with pulmonary capillaries and have a large (~140 m^2) surface area [44], thereby allowing for efficient gas exchange at the alveolar surface.

While maintaining the crucial respiratory function, the lung is constantly faced with an important task of discriminating between harmless inhaled substances and those which can alter or damage the lung. The anatomical, physiological and the immune system related components play a critical role in maintaining lung homeostasis. An alteration of these processes may result in a disease, such as allergic asthma and airway remodeling (structural changes in the lung), which has been associated with progressive loss of lung function. This review will discuss the defense mechanisms in a healthy lung, and pathophysiological features of asthma.

Asthma: A disease of the lung

Asthma has been a globally prevalent disease since antiquity. Aazein (Greek term for asthma) first appeared in Homer's epic poem *The Iliad*, composed in the seventh or eighth century BC and described as labored breathing, panting, and gasping induced by exertion. In the works of Hippocrates (father of medicine) and others from the fifth century BC, asthma was recognized not merely as a sign of extreme exhaustion, but a distinct form of pathology with its own symptoms, causes, prognosis, demographics and treatments. References to asthma have been found in the ancient texts by Chinese authors, ayurvedic practitioners of India, Egyptian, Japanese and Korean doctors [158]. The term "asthma" appeared in the English language in 1398 [234].

Significant advances in science have led to increased understanding of asthma and its mechanisms, thereby resulting in improved diagnostic and therapeutic approaches. Although the number of deaths due to asthma has declined, it still remains a huge socio-economic burden. Asthma affects 235 million people worldwide [16]. The World Health Organization has estimated that 15 million disability-adjusted life years are lost annually due to asthma, and 250,000 people die of asthma each year [16]. In the U.S., 25 million people are known to have asthma [364], and there are 497,000 hospitalizations annually [233]. Asthma is one of the most common chronic disease in younger generation [16], and there are around 7 million affected children in the U.S. [364]. The impact of the disease is especially prominent amongst African American and Puerto Rican populations [364]. In 2007, the total incremental cost of asthma (direct costs and productivity costs) to society was estimated at \$56 billion in the U.S.[23]. With prevalence rates increasing globally by 50% every decade [210], the global economic burden of asthma will continue to rise.

Although considerable advances have been seen in understanding the cellular, molecular and genetic basis of asthma, the basic comprehension of the causes and the reasons for increased prevalence rates remain unclear. While asthma was traditionally considered as an atopic (a genetic predisposition toward the development of immediate hypersensitivity reactions against common environmental antigens) disease, epidemiological studies have suggested the role of environment and lifestyle related factors (e.g. exposure to tobacco smoke, pollution, diet and infections) in the etiology of asthma. Asthma is now considered, not as a single disease entity, but a heterogeneous syndrome made up of several phenotypes (**Table 1**). Each phenotype differs in its immunology, pathology, clinical expression, response to treatments and long-term outcomes. The severity of the disease may also differ with the patient's age, sex, genetic background, and environment he/she is exposed to (**Table 1**). With regard to the environmental factors, not only the environment that a person is exposed to during his/her lifetime, but also to which he/she is exposed to prenatally, may influence the outcome of the disease [262].

Table 1: Classification of asthma phenotypes

Basis for classification	Examples of asthma phenotype or triggers
Type of inflammation	Th-2 associated, non-Th-2 associated, neutrophilic, eosinophilic, neutrophilic and eosinophilic or pauci-granulocytic (absence of any overt inflammation)
Host related factors	Obesity, genetic, allergic or non-allergic, atopy
Age of onset	Early (childhood) or late (adult)-onset
Sensitivity to steroids	Steroid-sensitive or steroid-resistant
Symptoms	Stable (well controlled symptoms) or asthma exacerbation (sudden onset of symptoms)
Degree of severity	Mild, moderate, severe
Allergen-induced	House dust mite, pollen, ragweed, cockroach, mold, pet dander
Non-allergen induced	Ozone, cigarette smoke, diesel particles, infection, aspirin, exercise, cold air, occupational asthma (triggered by chemical fumes, gases or dust encountered at work place)
Special types	Allergic bronchopulmonary aspergillosis (ABPA), Severe Asthma associated with Fungal Sensitivity (SAFS), Virus-induced wheeze or bronchiolitis

Therapeutic options available for asthma remain largely symptomatic, and very few new drugs have made it to the clinic in last 50 years [145]. Corticosteroids (non-specific anti-inflammatory) and β_2 -adrenergic (spasmolytic) drugs have been the main-stay of asthma therapy, and work well for mild-to-moderate forms of the disease. However, 5-10% of the asthma population experience more severe forms of the disease, which remain symptomatic despite high doses of conventional inhaled and oral anti-inflammatory drugs [329]. Although this represents a small subset of patients, severe asthmatics account for half of the total healthcare costs and asthma-related deaths [359]. Additional concerns with the current therapeutics include patient-compliance, potential long-term adverse effects, and the need for disease-modifying drugs that go beyond symptomatic relief for all asthmatics.

Since the introduction of β_2 -adrenergic agonists in 1969 and corticosteroids in 1974, only two drugs have been translated into the clinical use: leukotriene inhibitors and anti-IgE antibody, and both of them have shown limited efficacy [145]. This unmet medical need and the associated socio-economic burden drives asthma research. The urgency of the situation was summed up in a 2008 Lancet editorial: “Progress in understanding asthma and its underlying mechanisms is slow; treatment can be difficult and response unpredictable, and prevention or cure is still a pipedream. Asthma, one of the most important chronic diseases, remains a genuine mystery” [76].

Pathophysiology of asthma

In an Expert Panel Report-3, published by the National Heart, Lung and Blood Institute (NHLBI) in 2007, asthma was defined as a common chronic disorder of the airways that is complex and characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation [82]. The symptoms of asthma include

wheezing, breathlessness, chest tightness and coughing. The airflow obstruction in asthma occurs due to a variety of changes to the airways including bronchoconstriction, airway hyperresponsiveness, airway edema, and remodeling [364].

Bronchoconstriction

Bronchoconstriction, also known as asthma exacerbation, involves constriction of the airway smooth muscle (ASM). Bands of smooth muscle circumferentially surround the airway lumen. Like a boa constrictor squeezes its prey, smooth muscle bundles narrow the airway lumen upon constriction [133]. Bronchoconstriction is clinically manifested as shortness of breath and chest tightness, which may occur sporadically or be rather persistent in nature. Host related factors that may underlie bronchoconstriction include genetic make-up, stress, obesity, airway inflammation, airway remodeling, mucus hypersecretion and contractile dysfunction of the ASM itself [82,133,363]. Environmental agents known to trigger bronchoconstriction include allergens (e.g. pollen, pet dander, cockroach antigen, house dust mite antigen), infectious agents (e.g. respiratory viral, bacterial and fungal pathogens), nonsteroidal anti-inflammatory drugs (e.g. aspirin), irritants (e.g. cigarette smoke, air pollution), cold air or exercise. Alternatively, bronchoconstriction may be seemingly spontaneous, especially at night. While allergen-induced bronchoconstriction mostly involves IgE-dependent release of mediators such as histamine, tryptase, leukotrienes and prostaglandins that directly contract smooth muscle, the mechanisms underlying asthma exacerbation triggered by other listed substances remain less defined. Nonetheless, airway inflammation remains a pretty consistent feature across the phenotypes. Since bronchoconstriction is the major symptom involved in identifying asthma patients, it has long been recognized as a therapeutic target, and is relieved by the spasmolytic β_2 -adrenergic

agonists. Other therapeutic agents available for asthma act indirectly to control bronchoconstriction.

Airway hyperresponsiveness (AHR)

AHR is defined as an exaggerated bronchoconstrictor response to a wide variety of stimuli, and occurs in almost every asthmatic patient. While the level of AHR is variable among patients with asthma, and within individuals themselves, it correlates with the severity of asthma [56]. Several mechanisms underlie the development of AHR in asthma patients, including airway inflammation, neuronal dysfunction as well as airway remodeling (discussed later) [43,240]. The contribution of inflammation to AHR remains ill-defined, and several factors such as the location of the cells (i.e. lumen vs. bronchial wall), cell type, and mediators released during lung injury may affect the AHR measurement [104,360]. AHR can be assessed using direct stimuli that act on the airway smooth muscle (e.g. methacholine, histamine) or indirect stimuli that require the presence of airway inflammation (e.g. osmotic stimuli, adenosine monophosphate), and the choice of method may affect the AHR measurement. Lastly, unlike inflammation and lung function, AHR may take longer to resolve with the use of inhaled corticosteroids (ICS). Therefore, AHR has been proposed as a better marker for asthma control with the ICS usage in the clinical settings [38].

Pulmonary edema

Pulmonary edema results from increased fluid accumulation in the airway sacs, which ultimately leads to airflow obstruction. Increased size of the airway wall blood vessels (vascular dilation) and/or increased vascularity in the lung (angiogenesis) have been implicated as the underlying factors leading to pulmonary edema [195,337]. Several inflammatory mediators and growth factors released in an asthmatic lung may lead to increased vascular dilation and

angiogenesis. For instance, increased expression of vascular endothelial growth factor (VEGF) has been associated with enhanced vascularity of bronchial mucosa in asthma patients [149]. However, VEGF is not viewed upon as a therapeutic target, since it is involved in fetal development.

Airway remodeling

Airway remodeling involves structural alteration in the lung, and includes sub-basement membrane thickening, subepithelial fibrosis, airway smooth muscle hypertrophy, vasodilation, angiogenesis, mucus hypersecretion and nerve proliferation. Airway remodeling features are the key events associated with the persistent nature of the disease [82]. The remodeled airways are likely to become more susceptible to environmental insults (for example, biologically active allergens, viruses, air pollutants, certain drugs and chemicals) that lead to accelerated and progressive loss of lung function, and render the patients less responsive to therapy [140]. Historically, remodeling was attributed to the consequences of long-standing and persistent inflammation [63]. However, this view has been challenged in the light of findings in infants and 4 year old children [20,163,257,288,347]. It is now hypothesized that remodeling can occur in parallel, rather than in sequence, with inflammation and might be a component of the natural history of asthma for some patients [73]. The current asthma therapeutics such as ICS, ICS/long acting β_2 -adrenergic agonists combination, anti-IgE or anti-IL5 antibodies, have shown variable efficacy in reducing airway remodeling features in asthma patients [36,117,256,321,385]. Due to the lack of treatment options, development of airway remodeling is associated with disproportionately increased socio-economic burden, even though its expression in individual asthma patients remains erratic in onset and severity. Indeed, clinical management of airway

remodeling will be benefitted by elucidation of specific underlying pathways, and by identifying various asthma phenotypes and associated remodeling features.

Angiogenesis, the formation of new blood vessels from pre-existing ones, was first demonstrated in asthma patients by Dunnill, in 1960. He demonstrated that 20 asthmatic subjects who died of acute attacks of asthma had an enlarged capillary bed in the airway wall [72]. Since then, angiogenesis has been recognized as an important feature of tissue remodeling in asthma. The increase in the number and size of vessels can contribute to the thickening of the airway wall, which in turn may lead to critical narrowing of the bronchial lumen, leading to an amplification of bronchial hyperresponsiveness [228]. Increased blood flow and microvascular permeability may contribute to pulmonary edema, which further contributes to airway wall thickening [291]. VEGF, Angiopoietin-1 and 2 have been shown to play fundamental roles in physiological and pathological forms of angiogenesis, and can serve as biomarkers in the clinical settings [279].

Asthma diagnosis

Asthma mostly starts in early life, and it is the most prevalent chronic disease amongst children. However, there is no conclusive test to diagnose asthma at an early age, because the respiratory symptoms, such as wheeze, cough and shortness of breath are not specific for asthma, and commonly occur during viral respiratory infections or may be prompted by multiple triggers [69]. Recurrent wheezing episodes amongst children are considered to overlap with onset of asthma, often determined retrospectively. The diagnosis and choice of therapeutic regimen for asthma patients is based on reported asthma symptoms, combined with lung function tests to assess reversible airway obstruction and airway hyperresponsiveness [353]. The diagnosis and

management is complicated by the fact that asthma is a heterogenous disease in its onset, course, response to treatment and underlying pathophysiological mechanisms. The symptoms and lung function tests are not reflective of the underlying extent and type of inflammation. While bronchoscopy with biopsies, bronchoalveolar lavage and sputum induction are good methods to glean at the pulmonary inflammation and remodeling, they are too invasive to be used in general clinical practice. Allergic asthma is diagnosed with the help of skin prick tests, but is limited by the lack of specific diagnostic tools, or patient's sensitization to multiple allergens. Indeed, a need for specific, sensitive and non-invasive biomarkers to identify varying asthma phenotypes is strongly recognized [353].

Amongst various proposed biomarkers, one of the closest ones to clinical implementation is periostin, a matricellular protein secreted by bronchial epithelial cells upon stimulation with IL-13 [353]. Serum periostin levels correlate strongly with sputum eosinophilia [164], and patients with higher periostin levels respond better to anti-IL-13 therapy, compared to patients with low levels of periostin [57]. Other biomarkers being investigated include: nitric oxide in exhaled breath, eosinophil cationic protein, hypoxia-inducible factor-1 α , VEGF, IgE, levels in the bronchoalveolar lavage, sputum or serum, fraction of leukotriene E₄ metabolite excreted in the urine [45,332].

Recently, genome wide association studies have provided insight into the unique and common genetic origins of adult-onset and childhood-onset of asthma [69]. Several loci have been associated with asthma risk, including the *ORMDL3* locus, *ADAM33*, and various cytokines and cytokine receptor genes, such as *IL18R1*, *IL33*, *IL2RB*, *IL10*, *TGF β 1* and *IL6R* [226]. Additionally, genomic variations have been associated with medication response [129]. However, clinical utility of genetic approaches is yet to be established due to the lack of

reproducible results. With the advent of newer generation sequencing technologies, this problem is likely to be resolved.

Origins of asthma

Asthma begins early in life and several risk factors have been associated with development of asthma, including atopy, eczema [14,97,132,297], allergic rhinitis [308], parental history of asthma, AHR [132,268,297,327], viral infections [314] and environmental tobacco smoke exposure [225]. Complex interactions between genetic and environmental factors form the underlying basis of asthma expression. Identification of these factors, which make certain people susceptible to asthma symptoms and not others, is an active area of investigation.

Atopy, which is defined as the genetic predisposition to develop IgE-mediated responses to common aeroallergens, is the strongest identifiable predisposing factor to develop asthma [14,82]. Research has provided evidence that an imbalance between Th-1 and Th-2 cytokines established in early life forms the basis for development of atopic diseases. A shift towards a Th-2 cytokine profile characterizes atopy-associated asthma. It may develop either due to over-expression of Th-2 cytokines, or under-expression of Th-1 cytokines since Th-1 cytokines are inhibitory for Th-2 cytokine production. An explanation for the origin of this cytokine imbalance is provided by the popular “hygiene hypothesis”, which assumes that the immune system of the newly born is skewed toward Th-2 cytokine generation, and environmental exposure to infectious agents in early life, are necessary to activate Th-1 responses, to strike a balance between the two. Support for this hypothesis comes from studies showing a reduced asthma incidence in association with infections (e.g. *Mycobacterium tuberculosis*, measles, hepatitis A, and various other bacterial and fungal pathogens), exposure to other children (e.g. older siblings

and early enrollment at the child care centers), less frequent use of antibiotics, direct exposure to raw milk and animals amongst children raised on farms [8,42,75,79-82,98,99,153,304].

Additionally, incidence of asthma and related allergic diseases has increased in Western countries over the past 50 years, and this has been attributed to urbanization and related changes to diet and lifestyle, which prevent the activation of Th-1 associated responses [8]. However, one of the main arguments against hygiene hypothesis stems from the fact that therapeutics targeted against Th-2 cytokines have shown very limited efficacy in the clinical trials, suggesting that the pathophysiology of asthma is more complex than being simply a Th-2/Th-1 cytokine imbalance.

While atopy is considered as a strong risk factor for childhood asthma, it is interesting to note that no association has been found between allergen exposure and asthma [191,202], and avoidance of asthma did not lead to reduction in asthma development [96]. Additionally, the atopy genes that regulate total or specific IgE production and blood eosinophils are not detected as asthma susceptibility genes in genome-wide association studies [69]. Nonetheless, an evidence for genetic basis of the disease has been provided in a large twin study, which estimated that genetic factors account for 34% contribution to the variation in the age of onset of asthma [342].

Genome-wide association studies have linked several genes to development of asthma expression. Most importantly, a locus on chromosome 17q12-21 (encoding *ORMI-like 3* (*Saccharomyces cerevisiae*) *ORMDL3* and *GSDMB*) has been identified as a risk factor predominantly for the childhood-onset of asthma, but not adulthood-onset of asthma in different human races [226,318], except African-Americans [214]. While inheritable component is well recognized for expression of asthma, the linkage of the identified genes to pathophysiological processes is less clear. The proposed function for *ORMDL3* and *GSDMB* genes includes T-

lymphocyte activation [46] and apoptosis [180], respectively. Some of the other genes linked with expression of asthma include *ADAM33*, and various cytokine and cytokine receptor genes, such as *IL18R1*, *IL33*, *IL2RB*, *IL10*, *TGFβ1* and *IL6R* [226].

Environmental factors account for 66% contribution to the variation in the age of onset of asthma [342]. Exposure to allergens and viral respiratory tract infections, at a critical time of immunological and physiological development, are the two major risk factors associated with development, persistence, and severity of asthma [82]. The allergens most commonly associated with asthma include cockroach allergens, pollens, fungi, house dust mite antigens and pet dander. Several reports have been published to establish a relationship between allergen exposure and development of asthma, and some of studies published over last year are mentioned here.

In an interesting report, Santiago et al found that there was an immunological (IgE) cross-reactivity between cockroach allergen, a glutathione-S-transferase and a glutathione-S-transferase of *Wuchereria bancrofti*, a human filarial pathogen. This suggests that molecular mimicry may underlie the pathogenesis of sensitization in allergic diseases [295]. Darrow et al reported that Poaceae (grass) and *Quercus* species (oak) pollen concentrations are associated with emergency department visits for asthma [62]. Ziska and Beggs reported that climate changes associated with increased Earth's surface temperature and accumulation of CO₂, are likely to affect pollen production, and increase the allergen exposure in humans [386]. Reponen et al examined the relationship between indoor fungal exposure and the subsequent development of asthma at age 7 years in a birth cohort study of 289 children. They found that an increased risk for asthma was associated with high score on the Environmental Relative Moldiness Index [278]. However, this study measured the mold concentrations in dust samples, and improved methods

for quantifying mold exposure are necessary [259,272]. With regard to cat exposure and sensitization, birth cohorts have shown that exposure to cats in the home is associated with sensitization to cat allergens. However, in school children cat ownership can have inverse relationship with sensitization, thereby suggesting that early childhood exposure to cats might be associated with lower rates of cat sensitization in adulthood [259]. Lastly, a building literature supports the influence of maternal exposure to environmental pollutants, such as allergens, traffic-related air pollution, tobacco smoke, and organochlorine compounds and social factors on allergic outcomes. However, further research into mechanisms by which prenatal exposures influences allergic responses is necessary [262]. Epigenetics does provide an interesting frontier to explain some of these observations, and is likely to be the focus of asthma research over next couple of years.

It was a long held belief that the respiratory tract below the larynx is sterile. However, this belief has recently been challenged with the identification of lung associated microbiota. The bronchial tree possesses at least, 2,000 bacterial genomes per square centimeter, and the prevalent bacterial species varies in asthmatic and healthy controls [136]. Additionally, epidemiological studies show strong associations between asthma and infection with respiratory pathogens, including common respiratory viruses (e.g. rhinoviruses, human respiratory syncytial virus, adenoviruses, coronaviruses and influenza viruses), as well as bacteria (e.g. *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa*) and fungi (e.g. *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium* and *Didymella* genera) [77].

Rhinovirus infection has been particularly linked with increased predisposition to develop asthma, as rhinovirus-induced wheezing in the first three years of life is the greatest risk factor

for developing asthma at 6 years of age. Rhinovirus infection is believed to be the main cause of asthma exacerbation, in those genetically at risk for asthma [157]. The defect might be related to asthmatic epithelial cells, which respond to rhinovirus by generating increased amounts of pro-Th-2 cytokine TSLP [358]. Lastly, although a conclusive link between sex, sex hormones and asthma has not been established, studies have shown a higher prevalence of asthma amongst boys in early life, and amongst women at puberty [148].

Asthma, an epithelial disease

While atopy is considered as a predominant pre-disposing factor for developing asthma, only about 7-11% of atopics express this as asthma [258]. Furthermore, local sensitization of the lower airways seems to be more important than systemic atopy in the development of asthma [316]. This paradox begs a question, that which important factors translate the atopic phenotype into a lower airways disease. For over two decades now, scientists all over the world, particularly Dr. Stephen Holgate, a Medical Research Council Professor of Immunopharmacology at the School of Medicine, Southampton, UK, have been proponent of a hypothesis that asthma is predominantly an airway epithelial disease. The hypothesis is based on several clinical and experimental studies, compiled in the form of excellent reviews in the field. One of the noteworthy observations is that the asthma susceptibility genes discovered by genome-wide association studies, including ORMDL3, IL-33, SMAD3, are mostly expressed in the epithelium and innate immune pathways, rather than being the genes associated with atopy and serum IgE such as HLA-DQ locus, FCER1A, STAT6 and IL-13 [111,144,226]. Additionally, epithelium's barrier function (described later in the airway epithelium section) is intrinsically defective in people who develop asthma, which is likely to facilitate penetration of inhaled environmental allergens, and pollutant particles into the airway wall [374]. Interestingly, several asthma-related

allergens have intrinsic biological properties to damage the epithelial barrier, which serves to increase their penetration capabilities and activate the DCs. For examples, components of house-dust mite, cockroach, animal and fungal allergens are proteolytic and can disrupt the epithelial tight junctions and activate the protease-activated receptors [159]. Lastly, defective filaggrin production in atopic dermatitis patients may result in epicutaneous allergen transfer [245]. Later is proposed as an alternative route for development of allergic sensitization in asthma patients [219].

Besides asthma, the epithelial hypothesis of the disease has been extended to explain airway remodeling as well. Dr. Stephen Holgate hypothesized that the action of environmental agents on the airway epithelium is translated to the underlying mesenchyme via the epithelial-mesenchymal trophic unit (EMTU). The EMTU is involved in foetal lung growth and branching during development, but appears to become reactivated in asthma, propagating and amplifying pathological inflammatory and remodeling responses in the sub-mucosa [139,145].

Defense mechanisms in a healthy lung

Besides being a source of oxygen, the outside air introduces numerous particles, microorganisms, toxins, and chemicals into the lung. Additionally, the lung is exposed to the contents of the gastrointestinal system via aspiration during swallowing or regurgitation [78]. In a healthy lung, the inhaled substance is either removed via mucociliary escalator, phagocytosed, metabolised or absorbed into the blood or lymphatic circulation [290]. The hair and the mucociliary layer in the nose and the pharynx traps the inhaled particles, which are subsequently removed by the shear and turbulence created by sneezing and coughing. These barriers in the

upper airways prevent the penetration of particles, bigger than 2-3 μm , into the lower airways [238].

The viscoelastic mucus is composed of mucins, including mucoglycoproteins and proteoglycans, which are predominantly the products of two genes; Muc5AC and Muc5B [150,344]. These mucins are packaged into the secretory granules of specialized mucus producing cells called goblet cells. In addition to the goblet cells, submucosal glands can also secrete mucins. While Muc5AC is the main mucin from goblet cells, Muc5B is predominantly secreted by the submucosal glands. Under normal conditions, goblet cells and submucosal glands are present in large airways, becoming increasingly sparse towards lung periphery, with few or none being found in the small airways [156,162].

While the thick mucus entraps the inhaled particles, the ciliary beat propels them towards the oesophagus for removal via coughing and/or swallowing [238]. A ciliated epithelial cell carries around 200 cilia per cell, which beats @ 8-15 Hz. This mucociliary escalator ensures that the insoluble particles are swept out of the lungs within 24 hours. However, the smaller sized hydrophobic particles can bypass the mucociliary escalator to lodge deep into the alveolar spaces, where they might reside for several days [255]. In the alveolar spaces, phagocytic alveolar macrophages perform the housekeeping function [203].

The airway epithelium

This is the first layer of cells in the lung that comes in contact with the inhaled microbes or noxious stimuli, which has escaped the mucociliary barrier. The bronchi and bronchioles are lined with tall and thick (10-60 μm) columnar epithelial cells and mucus producing goblet cells. The alveoli are made up of broad and thin ($\sim 1 \mu\text{m}$) alveolar epithelial cells (AECs), which are classified as type I and II pneumocytes. AEC type I are large flat cells, covering about 95% of

the alveolar surface, which amounts to about 5000 μm^2 . Their main function is to participate in gas exchange, and their thinness allows them to do just that. These cells are very sensitive to injury. Additionally, AEC type I possess the machinery for sodium transport: a process necessary in removing pulmonary fluid, and in lieu preventing the development of pulmonary edema. However, due to the lack of sufficient number of mitochondria inside them, it remains unknown if they can produce sufficient adenosine triphosphate to carry out an active transport. AEC type II covers about 5% of the alveolar surface, which amounts to about 250 μm^2 . They serve as progenitors for alveolar epithelium, helping to regenerate the lung after injury. The turnover of AECs in the normal lung is estimated to be around 2-3 weeks. Additionally, AEC type II secretes surfactants, which help to reduce the surface tension of the respiratory secretions, thereby allowing for efficient gas exchange. Surfactants are composed primarily of phospholipids and proteins designated as SP-A, SP-B, SP-C, and SP-D. The surfactant proteins are collectively termed as collectins. SP-A and D are critical to lung homeostasis, as they have been shown to play dual roles in suppressing or enhancing inflammation depending on their binding orientation [93].

Within the tracheobroncheal epithelium, ciliated columnar epithelial cells, mucus-producing goblet cells, and surfactant producing clara cells are held together to form an impermeable barrier due to the formation of tight junctions localized at the apical surface. These tight junctions comprise of proteins such as ZO-1, occludins, claudins, β -catenin, E-cadherin, and other junction adhesion molecules [121]. The physical barrier provided by tight junction proteins prevents the antigens from accessing the subepithelial mucosa. The epithelium derived peptidases (e.g. aminopeptidase M and neutral endopeptidase) [270], and protease inhibitors (e.g. secretory leukocyte protease inhibitor) [217], degrade the peptides and proteases obtained from

host or pathogen and prevent the onset of inflammation. Additionally, the epithelial cells secrete several antimicrobial substances, for example: β -defensins, lysozyme, lactoferrin, human cathelicidin LL-37, surfactant proteins A and D [271]. These barrier functions performed by the airway epithelium, coupled with the previously discussed mucociliary escalator, prevent the microbes from binding to the epithelial cells.

Apart from being a physical barrier, airway epithelium can orchestrate the inflammatory responses in the lung. They have been shown to possess the pattern recognition receptors (PRRs) [18], that include toll-like receptors (TLRs), NOD like receptors, protease-activated receptors (PARs), and C-type lectins by the virtue of which they can recognize pathogen associated molecular patterns (PAMPs) on microbes and damage associated molecular patterns (DAMPs) on host cells [24,112,115,188]. Epithelial cells can phagocytose antigens [254] and present the antigen, via up-regulation of MHC class I and II in the presence of cytokines, heat shock proteins or allergens [31,61,285,341]. Additionally, upon activation, epithelial cells up-regulate the adhesion molecules such as ICAM-1, and secrete a range of different chemokines for the recruitment of monocytes (e.g. MCP-1), neutrophils (e.g. IL-8), basophils (e.g. MCP-1), eosinophils (e.g. IL-5 and eotaxins), Th-2 lymphocytes (CCL17) etc.

Epithelial cells may also play an essential role in local accumulation, activation, class switch recombination, and immunoglobulin synthesis by B cells in the airways [166]. Specifically, they have been hypothesized to promote IgA secretion in the lung [292]. IgA is the most important antibody isotype in the respiratory secretions, as it provides defense via neutralization, immune exclusion or antigen excretion [33,58]. Epithelium secretes cytokines which are essential for B cell clonal proliferation (IL-2), IgA isotype switch (TGF- β), and differentiation into IgA secreting plasma cells (IL-5, IL-6 and IL-10). Furthermore, epithelial

cells produce a glycoprotein called secretory component (SC), which not only confers stability against proteases on the secretory IgA (S-IgA) but is also an important receptor for the transport of polymeric IgA (pIgA) from the parenchyma into the lumen [292].

Lastly, airway epithelium may down-regulate the inflammatory responses by release of soluble receptors for pro-inflammatory cytokines (e.g. IL-1 receptor, TNF- α receptor), anti-inflammatory mediators (e.g. IL-10, TGF- β , PGE₂) or degrading enzymes for pro-inflammatory mediators (e.g. histamine N-methyltransferase, catalase) [66,95,193,251,322,348].

Dendritic cells (DCs)

DCs are professional antigen-presenting cells (APCs), which continuously patrol the airway lumen for inhaled substances. They extend their branching processes through the epithelial cell layer to sample the inhaled antigens without disrupting the epithelial tight junction proteins [307]. Alternatively, rupture of epithelial tight junctions by proteolytic inhaled substances or induction of innate immune signaling due to engagement of PRRs on epithelial cells activates the epithelial cells to release chemokines for recruitment and/or activation of DCs [122,169]. Recent studies have shown that interaction between the DC community within the lung and pulmonary epithelium is vital for DC activation and migration to lymph nodes [121,160]. The chemokines (e.g. ligands for CCR1 and CCR5) recruit the immature DCs to the lung. Immature DCs possess high endocytic activity and low antigen-presentation capabilities. DCs endocytose the antigen [64,241], process them into small peptides, migrate to the paracortical T-cell zone of draining lymph nodes in the lung, and present the peptides via the major histocompatibility complexes (MHC) I and II for recognition by T cell receptors [284,294]. This process of antigen uptake, migration to draining lymph nodes and antigen presentation is called DC maturation and involves molecular events, such as down-regulation of

CCR1 and CCR5, up-regulation of CCR7 and molecules involved in antigen-presenting such as CD40, CD80 and CD86 [238]. In the T-cell area, mature DCs arrest and select the rare antigen-specific T lymphocytes and form an immunological synapse for 12-24 hour, during which they communicate the nature of antigen to the T cell, thus inducing an optimum type of T-cell response. While some activated T (T follicular) helper cells either migrate to the B cell follicles to help immunoglobulin class switching, others such as Th-1, Th-2 and Th-17 cells are transported back to the site of infection, to help kill the pathogens. Thus, DCs play a very important role in bridging innate and adaptive immunity [187].

There are several different subsets of DCs that have been defined, and it remains unknown if all the functions of DCs are performed by a single or different type of cells. Two subsets of DCs are recognized in humans: Myeloid DCs (or DC1s) and Plasmacytoid DCs (or DC2s). While both subsets originate from CD34⁺ hematopoietic stem cells, the myeloid DCs have a lineage relationship with monocytes and macrophages, and plasmacytoid DCs have a lineage relationship with T cells, as differentiated by their CD markers or growth factor requirements. Blood monocytes (Pre DC1s) can be induced to become myeloid DCs (DC1s) by culturing them for 7 days in GM-CSF and IL-4 or IL-13, or spontaneously as blood monocytes leave the bloodstream and enter the tissues. Pre-DC2s, on the other hand, differentiate into immature DC2s. Upon CD40 ligand activation, immature myeloid DCs undergo maturation and produce large amounts of IL-12, which are able to polarize naïve CD4⁺ T cells into Th-1 cells. On the other hand, plasmacytoid DCs undergo maturation, upon CD40 ligand activation, to produce low levels of IL-12, and polarize naïve CD4⁺ T cells into Th-2 phenotype. The two DC subsets differ in the expression of PRRs, with monocytes preferentially expressing TLR1, 2, 4, 5 and 8, and plasmacytoid preDC2s strongly expressing TLR 7 and 9. The expression of distinct

sets of TLRs allows these subsets to respond to different microbial ligands [165]. In mouse studies, although three major subsets of DCs have been described, their precise lineage relationship is not well understood [147]. The lymphoid DCs cells are found in the lymph nodes and spleen, and induce Th-1 responses by producing IL-12. The myeloid DCs are found in the peripheral tissues, lymph nodes and spleen, and induce Th-2 responses [208]. A murine counterpart of human plasmacytoid DCs has been described in the lymph nodes, spleen and bone marrow, which produces large amounts of IFN- α upon viral infection [15]. Additionally, in a murine asthma model, critical role of plasmacytoid DCs in inducing tolerance against harmless inhaled allergen has been demonstrated [65]. Indeed, identifying different DCs based on their type and anatomical distribution is a huge technical challenge [101,146].

Alveolar macrophages (AMs)

AMs are the long-lived residents of the alveolar and conducting spaces. They represent 85% of the cells retrieved in the bronchoalveolar lavage (BAL) fluid. In response to infection, their number increases, although it is not clear, if that is due to the formation of more cells from the stem cells in the lung or recruitment of additional macrophages from the bone marrow. AMs neutralize the inhaled particles [207], and if unable to control infection, they may release chemokines to recruit neutrophils and mononuclear cells to the site of infection.

Like DCs, macrophages can recognize the PAMPs by virtue of several PRRs including TLRs, mannose receptor or receptors for complement proteins [39,83,182]. AMs perform anti-microbial activities due to their potential to produce lysozyme, defensins, reactive oxygen and nitrogen species, and several components of the complement cascade [120,177]. Under certain, poorly understood circumstances, AMs may acquire antigen presentation capabilities to activate T cells [237,238]. Depending upon the type of T-helper cell function supported, macrophages

can be classified into two types: classically activated M1 macrophages, which promote IL-12 mediated Th1 functions, and alternatively activated M2 macrophages, which support Th-2 effector functions [198]. Once the infection has resolved, they perform house-keeping functions by removing cell debris and exudates.

It is believed that macrophages have dual roles of being pro- and anti- inflammatory in function. They may participate in initiating inflammation, via release of inflammatory cytokines such as IL-1 and TNF- α , or in controlling inflammation, via release of inhibitors of inflammatory cytokines [92] or anti-inflammatory IL-10 [236]. Recently, it was shown that different subpopulations of macrophages account for this dual role. The lung interstitial macrophages (F4/80⁺CD11c⁻), which are distinct from AMs (F4/80⁺CD11c⁺) inhibit DC maturation after TLR stimulation, thereby preventing allergic response to harmless airborne antigen [25]. An interesting set of studies provide information on how lung environment may instruct AM to suppress innate and adaptive immune responses. Takabayshi K et al showed that under baseline conditions, AMs tether to the AECs through a TGF- β inducible integrin α v β 6, which continuously inhibits AM functions. This adherence is lost upon exposure of AMs to microbial products, and enables AMs to carry out phagocytosis and pro-inflammatory functions. Subsequent production of matrix metalloproteinases induces latent TGF- β , thereby reinstating the tonic inhibition of AMs [229,335]. Additionally, tolerance is maintained in the lung, in the absence of microbial exposure, by surfactants SP-A and SP-D, when their globular heads bind to receptor signal regulatory protein- α on the resident cells [93]. Lastly, macrophage activity is kept in check by a negative regulatory receptor (CD200R), which is believed to have higher expression on AMs than systemically derived macrophages. CD200R's ligand is expressed by airway epithelial cells, which demonstrates another level of homeostatic mechanism present in

the lung resident cells, and allows the lung to discriminate between harmless and potentially deleterious antigens [200,319].

Regulatory T cells (Tregs)

Tregs are distinct subtypes of CD4⁺ T cells, which suppress the functions of effector T cells, and therefore, play a major role in maintenance of tolerance during constant exposure to inhaled antigens. T_{regs} can be divided into two subtypes: the thymus-derived CD4⁺ Foxp3⁺ natural T_{regs} (nT_{regs}) and peripherally antigen induced adaptive CD4⁺ T_{regs} (inducible iT_{regs}), which may or may not be Foxp3⁺ [218]. During infection or cancer, the CD4⁺CD25⁺ T cells can convert to CD4⁺CD25⁺Foxp3⁺T cells in the periphery under the influence of TGF-β and retinoic acid [50,230]. A transcription factor called FoxP3 (forkhead-winged helix plays a critical role in the development and function of nT_{regs} in mice and humans [181,275]. Earlier it was thought that Foxp3⁺ T_{regs} mediated suppression by inducing IL-2 mRNA in the target Foxp3⁻ T cells [336,343]. However, now it is known, that this is not the only mechanism, and a combination of cell-cell contact dependent and independent mechanisms are involved in mediating suppression.

One of the cell-cell contact based mechanism involves an interaction of CTLA-4 with CD80 and CD86 on APCs (e.g. DCs) [253,368], which promotes the secretion of indoleamine 2, 3-dioxygenase, a potent suppressive molecule that induces the catabolism of tryptophan into proapoptotic metabolites [113]. Another contact mechanism involves an interaction of ligation lymphocyte activation gene-3 on T_{regs} with MHC class II on APCs, which suppresses DC maturation, and as a consequence, the activation of effector T-cell responses [196]. The contact independent mechanisms involve secreted molecules such as galectin-1 and fibrinogen-like protein [94,309]. In contrast, the iT_{regs} are believed to mediate their suppressor activities in a cell-cell contact independent manner only, and involve secretion of anti-inflammatory cytokines such

as IL-10 [114] and TGF- β [51,87]. For contact based suppressive effects, T_{regs} must be recruited to the lung, and this is believed to be under the influence of chemokine gradient involving CCR4 [200,296].

The importance of T_{regs} population in modulating allergic responses has recently gained considerable attention. Adoptive transfer of antigen-specific CD4⁺CD25⁺ T_{regs} resulted in suppression of the allergic response, including AHR, eosinophilic inflammation and Th-2 cytokine production [172]. Conversely, depletion of CD4⁺CD25⁺ T_{regs} before allergen challenge enhanced the allergic response in the lung [194]. Due to these immunosuppressive actions, induction of T_{reg} response is being viewed as an attractive therapeutic option. For this purpose, products from certain infectious agents, for example *Bordetella pertussis* [37] and *Heligmosomoides polygyrus* [365], has been proposed as a novel immunotherapeutic option for allergic disorders [218].

$\gamma\delta$ T cells

These are the intraepithelial T cells, originally discovered in the epidermal layer of the skin, but later found in all epithelial tissues, including lung, gut, tongue and reproductive tract [9]. Due to their residency at the epithelial borders, $\gamma\delta$ T cells are at the interface of host and environmental interactions. This location not only allows them to interact with the inhaled environmental substances but also the resident cells in the lung, including epithelial cells, AMs and DCs. Therefore, it has been proposed that $\gamma\delta$ T cells play an important role in tissue homeostasis and immune surveillance [128].

The major distinction between $\gamma\delta$ T cells and the conventional T cells is in the protein molecules that make up their T cell receptor (TCR). While $\gamma\delta$ T cell's receptor is made up of γ and δ chains, the conventional TCR is made up of α and β chains. Additionally, while $\alpha\beta$ T cells

express diverse cell receptors, and recognize a vast array of foreign antigens, the $\gamma\delta$ T cells have limited to no receptor diversity. For this reason, $\gamma\delta$ T cells are also referred to as innate like lymphocytes. $\gamma\delta$ T cells are believed to recognize tissue-specific stress-or damage- induced self ligands [243]. While the cognate ligands for $\gamma\delta$ TCR remains an active area of investigation, in humans, soluble proteins such as tetanus toxoid produced by *Clostridium tetani*, Ig gamma light chain and enterotoxin A have been identified in the ligand pool. Additionally, unlike $\alpha\beta$ T cells, epithelial $\gamma\delta$ T cells do not recognize antigen in context of MHC class I or class II molecules, and do not express CD4 or CD8 co-receptors, or the co-stimulatory CD28 molecule [127,130]. It remains controversial, if there are any equivalent molecules required for $\gamma\delta$ T cell activation [370]. After activation, $\gamma\delta$ T cells can be classified into three subsets based on their effector functions: IFN- γ , IL-4 and IL-17 producing $\gamma\delta$ T cells [312]. Besides producing different cytokines, these subsets have shown differences in the type of PRRs they possess [209,280]. At present the precise role of $\gamma\delta$ T cells remains controversial in allergic asthma. The $\gamma\delta$ T cell-deficient mice were found to be naturally hyperresponsive upon airway challenge, which suggested an immunoregulatory role for these cells [186]. However, paradoxically, $\gamma\delta$ T cells have also been shown to promote allergic responses in the airways [387].

NK (natural killer) T (NKT) cells

These cells are a special subset of T cells, which constitute a small proportion of lymphocytes that express markers of both T cells (TCR), and NK cells (e.g. NK1.1, NKG2D) [339]. NKT cells are currently divided into three subsets based on their TCR: Type I, II and II. Out of these, type I NKT cells, also known as invariant NKT (iNKT) cells are the best studied due to the availability of reagents to identify and study them [212]. iNKT cells respond to glycolipid antigens presented in the context of the MHC-class I like molecule called CD1d,

which appears to function as a PRR [170,204,303,323,351]. Upon activation, iNKT cells can produce perforin and granzyme B [171,222,338]. Additionally, they secrete different types of cytokines and chemokines, which activate or suppress cells of innate and adaptive immunity. IL-4 and IL-13 or IL-17 and IL-22 or Foxp3⁺, IL-10 secreting subsets of iNKT cells is currently recognized. Due to the secretion of different types of cytokines by iNKT cell, its role has been implicated in several diseases, including asthma, infectious diseases, malignancy, autoimmune diseases, allograft rejection and atherosclerosis [212].

Several endogenous and exogenous lipids have been shown to activate iNKT cells. The endogenous lipids include: lysosomal glycosphingolipid and cellular phospholipids, the exogenous lipids include those derived from pathogens, such as *Borrelia burgdorferi*, *Leishmania donovani*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *cytomegalovirus*, and *Aspergillus fumigatus* [7,212]. At this point, it is not clear if the ligands can directly activate the NKT cells or indirectly through secretion of cytokines. Cytokines, which have been shown to activate NKT cells include epithelial derived IL-25, IL-33 and Thymic Stromal Lymphopoietin (TSLP) [212].

NKT cells have been shown to play a crucial role in the pathophysiology of asthma, in mouse models. Mice lacking NKT cells failed to develop allergen-induced AHR and showed reduced airway inflammation after challenge with ovalbumin. Subsequent mechanism-based studies showed that NKT cells collaborate with Th-2 cells in the development of AHR [6,197]. Besides ovalbumin, the role of iNKT cells has been demonstrated in ozone [265] and Sendai virus [176] induced AHR in mice. In humans, studies have shown variable number of iNKT cells ranging from 1% of lung lymphocytes in naïve mice to 14% after allergen challenge, suggesting that the number of NKT cells in the lungs may change over time with acute exacerbations or as

asthma symptoms improve [211,212]. However, undisputed evidence for the role of NKT cells in human asthma is yet to be established, which would dictate the therapeutic options available, especially for severe asthma where iNKT cells are likely to play an essential role [211].

Asthma: An immunological disease

Asthma pathophysiology, as depicted in **Figure 1**, involves a dynamic interaction between structural cells in the lung, including epithelium, DCs, airway smooth muscle and fibrocytes, as well as immune cells, such as granulocytes, mast cells, T-helper lymphocytes and B cells (humoral immune response).

Primary sensitization of the airways

Primary sensitization of the airways is the induction of allergic-type asthma. Interaction of the airway epithelium with bacteria, viruses, fungi or pollutants activates the innate signaling pathways, which in turn leads to chemokine secretion and recruitment of immature myeloid DCs from the bone marrow to the mucosal epithelium. DCs respond to danger signals via PRRs, capture allergens that have breached the epithelium or by extending their processes through the epithelial tight junctions, process the antigens into smaller peptides and load these peptides onto MHC class II. These DCs, which are now potent APCs, travel to the T cell zone of draining lymph nodes, and present the antigens to the naïve T cells. The interaction between the mature DCs and naïve T cells involves MHC class II and TCR, and co-stimulatory molecules, which drive differentiation and proliferation of Th-2 cells. These Th-2 cells travel back to the lung to carry out effector functions, which manifest as asthma symptoms. Even in the absence of allergens, memory T cells may reside in the lung. Several epithelial derived cytokines and chemokines, such as IL-33, IL-24, CCL17 and CCL22 play an important role in DC activation

and Th-2 maturation. Uptake of allergens by airway DCs in allergic individuals is facilitated by interaction of the allergen with IgE attached to FcεRI. DCs are not present in the airways at birth. Damage to and/or activation of the respiratory epithelium by microbes and irritants are the main trigger that initiates the recruitment of immature DCs from the bone marrow [144].

The molecular mechanisms involved in Th-2 lymphocyte differentiation from naïve CD4⁺T cells remain partially understood. It is known that an obligatory source of IL-4 is required to activate the transcription factors signal transducer and activator of transcription 6 (STAT6) and GATA-binding protein 3 (GATA-3), to induce Th-2 polarization. After sensitization, IL-4 is one of the major cytokines produced by Th-2 cells. However, before sensitization, the cellular source of IL-4 remains unknown. Two competing ideas put forth, to explain the IL-4 requirement include: 1) Th-2 response may occur by default in the absence of strong Th-1 and Th-17 promoting cytokines or when only a weak MHC class II TCR interaction occurs resulting in IL-4 production from naïve CD4⁺T cells. Alternatively, mast cells and basophils have been proposed as the potential sources of IL-4, to induce Th-2 polarization during allergic sensitization [144].

Inflammatory response in asthma and airway remodeling

Inflammation plays a central role in pathophysiology of asthma, underlying the clinical symptoms of asthma, viz airway obstruction, AHR and airway remodeling. The role of various cell types and of humoral immune response in asthma pathophysiology is discussed in the following section.

Mast cells: Mast cells are strongly linked with the pathophysiology of anaphylaxis and acute allergic reactions. However, a large body of evidence now suggests that mast cells are also key drivers of long term pathophysiological changes and tissue remodeling, which are the

hallmarks of chronic inflammation in asthma [91,106]. Mast cell precursors originate in the bone marrow, which migrate to the peripheral tissues, where they complete their differentiation and maturation. Mature mast cells are long-lived cells and distributed throughout the body, with relatively high numbers occurring near body surfaces, including airway epithelium [47]. Due to their location, they are the first cells that come in contact with inhaled environmental substances.

IgE and mast cells mediate immediate type hypersensitivity response. In an allergic individual, whose tissue mast cells already have IgE bound to the FcεRI, re-exposure to the original or a cross-reactive bivalent or multivalent antigen results in cross-linking and aggregation of adjacent FcεRI bound IgE, which triggers signaling pathways in mast cells, that results in secretion of biologically active compounds. These biologically active compounds include pre-formed histamine, serotonin, tryptase, chymase, carboxypeptidase A₃, heparin, chondroitin sulfate, prostaglandins (e.g. PGD₂), leukotrienes (e.g. LTB₄, LTC₄, LTD₄, LTE₄) and certain cytokines, within minutes of antigen exposure [105]. This is known as early phase response, and if localized at the airways, it leads to increased vascular permeability, contraction of the airway smooth muscle, and enhanced secretion of mucus [91]. Systemically, the early phase response leads to serious and potentially fatal condition called anaphylaxis [282]. Mast cells also release other cytokines, chemokines and growth factors, which are produced in mast cells from new transcripts, and are released several hours after initial mast cell activation. This is known as the late phase response, and marks the second round of inflammation in the airways, which involves recruitment of T cells, DCs, neutrophils, eosinophils and monocytes [90]. The late phase products can also potentially influence airway remodeling. Studies in wild-type, genetically mast-cell deficient and mast-cell-knockin mice indicated that activation of mast cells through the FcRγ chain is required for full development of many features of allergic

inflammation and tissue remodeling (e.g. goblet cell metaplasia and lung collagen deposition) in a model of chronic asthma [89,90]. However, these effects are not specific to mast cells only, and it is unknown if mast cells are directly or indirectly involved in mediating these effects.

In vitro [137,246,252,376] and *in vivo* [26,252,383] evidence has indicated that the extent of antigen and IgE-dependent mast cell activation may be influenced, positively or negatively, by microenvironmental factors such as adenosine, sphingosine-1-phosphate, certain chemokines and cytokines (e.g. IL-4, IL-33, IFN- γ), and bidirectional interaction of mast cells with T cell subsets. Additionally, mast cell mediator secretion can be directly activated by many stimuli in an antigen and IgE independent manner. This includes TSLP [91], IL-33 [137], ligands for TLRs, proteases, products of complement activation and bacterial toxins [89-91]. An evidence of such an IgE-independent mast cell activation based mechanism probably underlies the development of goblet cell metaplasia in a chronic asthma model [382,383]. Interestingly, in a different model of asthma, mice lacking mast cell associated chymase showed diminished airway hyperreactivity, airway inflammation and airway smooth muscle thickening, which suggest a negative regulatory role for mast cells in asthma [356]. Relevance of these findings in humans is currently unknown. In humans, the mast cell numbers tend to vary with asthma severity, with higher number of chymase positive mast cells associated with severe asthma [19]. Nonetheless, therapeutic approaches to target mast-cell associated tyrosine kinases are currently being investigated [70].

Eosinophils: Eosinophils were so named by Paul Ehrlich in 1879 because of the intense staining of its granules with the acidic dye eosin [107]. Eosinophils originate from CD34⁺ hematopoietic stem cells in the bone marrow, under the control of transcription factors, such as GATA-1, PU.1 and CCAAT/enhancer binding protein (C/EBP) [220], and growth and differentiation factors, such as IL-3, granulocyte-macrophage colony stimulating factor (GM-

CSF) and IL5 [293]. Although eosinophils may transiently be found in the blood, they are predominantly tissue-dwelling cells, where they can reside upto 2 weeks, under the autocrine effect of GM-CSF. In healthy individuals, most eosinophils are found in the gut, mammary gland, uterus, thymus and bone marrow, and their levels are regulated by eotaxin-1 (chemokine for CCR3 present on eosinophils) [13,152,224].

The function of eosinophils under baseline conditions may include pregnancy associated changes in the uterus [283], mammary gland development [110], maintenance of epithelial barrier integrity [88] and innate host defenses in the gut [381], and long-term maintenance of plasma cells in the bone marrow [55]. Eosinophils have also been shown to present antigens in context of MHC class II, and can provide co-stimulatory signals for T cell activation [311].

Eosinophil recruitment into the lung is promoted by several cytokines, such as IL-5, IL-3, GM-CSF, and IL-17, chemokines such as eotaxins, MIP-1 α , RANTES, MCP-3, IL-8, lipids such as leukotrienes B₄ and E₄, fungal chitin, and anaphylatoxins such as C3a and C5a [103]. Once recruited, eosinophils are activated as a part of innate or adaptive immune response. Eosinophil activation in response to microbe derived ligands or proteases in innate immune defenses involve TLRs (specifically TLR7) [232], protease activated receptors [215], and β_2 integrin molecule called CD11b [379]. In adaptive immunity, cytokines secreted by Th-2 lymphocytes, and sIgA have been shown to promote eosinophil activation [71]. The role of IgE in mediating eosinophil activation is controversial, given the observation that the number of high affinity receptors expressed on the surfaces of eosinophils from patients with allergic diseases was minimal, and ligation of Fc ϵ RI did not result in detectable degranulation [178,306].

Upon activation, eosinophils release their granule proteins, which include major basic protein (MBP), eosinophil cationic protein, eosinophil peroxidase, eosinophil derived neurotoxin

and β -glucuronidase. These proteins allow eosinophils to exert cytotoxic, helminthotoxic, antiviral, antifungal and neurotoxic functions. These proteins have been linked to human asthma, as well [179]. For instance, concentrations of MBP in the BAL fluids from patients with asthma are correlated with the severity of bronchial hyperreactivity [108]. Additionally, these granule proteins can cause substantial damage to the cells in the lung [267]. Mediators, such as leukotriene C₄ and platelet activating factor, released upon eosinophil degranulation contract airway smooth muscle, promote mucus hypersecretion, alter vascular permeability and induce neutrophil and eosinophil infiltration [179]. Additionally, eosinophil is the major source of MMP-9 [324], which cleaves collagen and is likely required during eosinophil migration [185]. Lastly, eosinophils not only participate in microbe and host damaging activities in the lung, but also carry out tissue repair and airway remodeling processes. While eosinophil-derived TGF- β enhances proliferation and collagen synthesis, TGF- α promotes mucin secretion in the lung. Several growth factors are also derived from eosinophils, such as osteopontin, vascular endothelial growth factor, MMPs and nerve growth factor, which participate in airway remodeling [352]. Through secretion of various cytokines and chemokines, such as IL-4, TNF- α , MIP-1 α , RANTES, APRIL and indoleamine 2,3-dioxygenase, eosinophils can modulate the functions of other immune and structural cells in the lung [179].

Eosinophil recruitment has long been considered as a prominent feature of allergic asthma. Studies of mild to severe asthma have suggested that 50% of the cases are associated with eosinophilic inflammation, the rest being neutrophilic, mixed neutrophilic and eosinophilic or paucigranulocytic (absence of any observable inflammatory process) [126,362,363,373]. For long, they have been considered as immunopathological feature in asthma. However, the focus is

now shifting towards the effector, possibly host-defense functions of eosinophils in fungal and viral induced asthma [103].

Neutrophils: Neutrophils are also known as polymorphonuclear (PMNs) cells because of their multi-lobed nuclei, which aids in their morphological identification. These are the most abundant type of leukocytes in the body. Neutrophils mature in the bone marrow, and represent less than 2% of the cells in the BAL under normal circumstances. However, if the alveolar cells are unable to control the infectious agents, a massive flux of neutrophil occurs, within hours of the infectious challenge. Neutrophils are extremely important for innate immune defense against bacterial and fungal pathogens, and their deficiency leads to severely immunocompromised states in humans [238].

The migration of PMNs into the lungs first involves weak interactions between carbohydrate moieties (e.g. Sialyl Lewis-x moiety) and TNF- α /IL-1 (secreted by macrophages) induced selectins (e.g. P-selectin and E-selectin) on the vascular endothelium. This results in rolling of the PMNs on the vascular endothelium surface. The subsequent strong interactions involve intercellular adhesion molecule (ICAM)-1 on the surface of PMNs and endothelial cell adhesion molecules, which results in extravasation of the neutrophils into the site of infection [238]. The PMN recruitment process is guided by gradients formed by chemokines, such as IL-8 (or its mouse counterparts ENA-78 or KC), GRO- α , and GRO- β in humans, which are secreted by cells residing in the lung, such as DCs, macrophages, endothelial and epithelial cells [354].

Activated neutrophils are phagocytic, cytotoxic, and can form extracellular DNA traps [315], which helps to eliminate bacterial and fungal microorganisms. Additionally, neutrophils secrete a range of other cytokines, such as IL-1, IL-3, IL-6, IL-8, TNF- α , IL-12, IFN- γ , GM-CSF and TNF- α , which orchestrate the immune response through their effects on macrophages, DCs

and T cells [227]. Lastly, experimental evidence suggests that neutrophils may also act as APCs [247,367].

Besides their anti-microbial functions, neutrophils secrete a myriad of molecules, which have been shown to play an important role in asthma pathophysiology. These include elastase, reactive oxygen species, myeloperoxidase, lactoferrin, thromboxane A₂, MMP-8 and 9, IL-8 and eosinophil cationic protein. Recent studies by Monteseirin J's group have found that in asthmatic individuals, these mediators are released through IgE-dependent mechanisms. Neutrophils can be activated through 3 IgE receptors, but primarily through galectin-3 and especially FcεRI [227]. Neutrophilic products, such as elastase, lactoferrin and IL-8 may promote the recruitment and activation of eosinophils [175,199,313,340,388].

Neutrophils have been associated with asthma and severe asthma for several years [161,361]. Although eosinophils were long recognized as an important feature of patients with chronic, stable asthma, studies of mild to severe asthma have suggested that only 50% of the cases are associated with eosinophilic inflammation, and the rest are neutrophilic, mixed neutrophilic and eosinophilic, or paucigranulocytic (absence of any observable inflammatory process) [126,362,363,373]. However, no consensus exists as to what level of neutrophilia should define the neutrophilic asthma. Neutrophilic asthma is usually the corticosteroid-resistant, since corticosteroids have been shown to inhibit neutrophil apoptosis [167], which is the main means of eliminating the neutrophils from the allergic lung. Additionally, IgE-dependent delayed neutrophil apoptosis in allergic patients may also contribute to persistent neutrophilic inflammation in atopic asthma [287]. In affected individuals, lung neutrophilia has been associated with lower lung function, more trapping of air, thicker airway walls, and greater expression of MMPs [41,317,372].

Basophils: Basophils are the least abundant type of granulocytes, which mature in the bone marrow and have a life span of around 60 h under steady conditions in mice [249]. While murine basophils are definitely important effector cells against gastrointestinal helminthes and in chronic allergic inflammation of the skin, their role in allergic asthma remain inconclusive.

The recruitment of basophils in helminth infections is influenced by IL-3 from the T cells [189,310]. Additionally, TSLP promotes basophilia [260], and insulin-like growth factors 1 and 2 regulate recruitment of basophils in nasal polyps. Basophil survival, migration and activation are further regulated by the adipokine Leptin that directly binds to the Leptin receptor on basophils [331]. Like mast cells, basophils contain granules that stain with basophilic dyes, and express high-affinity receptor for IgE (FcεRI) [123]. The effector molecules released by basophils include vasodilators such as histamine and platelet-activating factor, leukotriene C4 and Th-2 associated cytokines IL-4 and IL-13 [300].

Basophils gained a lot of attention recently, as accessory cells producing IL-4, which is necessary for Th-2 polarization. Support for this mechanism comes from studies in interferon response factor 2 [135] and tyrosine kinase Lyn deficient mice [49], which show increased levels of circulating basophils and a Th-2 biased phenotype. Furthermore, basophils were shown to be in direct contact with Th-2 cells in the draining lymph nodes as observed via immunofluorescence microscopy [260,320,380]. This led to the hypothesis that basophils, rather than DCs serve as APCs for inducing Th-2 polarization. However, this hypothesis remains untested, because of the limitation that the anti-FcεRI antibody (MAR-1), which depletes basophils and was used to test this hypothesis [320] , also depletes inflammatory FcεRI DCs [144,355]. In the ovalbumin challenge mouse model of asthma, the eosinophils and Th-2 cells were normal in basophil-depleted Mcpt8Cre mice [250]. Basophils are found in large numbers in

the post-mortem biopsies of fatal asthma [173] and bronchial biopsies of atopic asthma patients [206]. Thus, the role of basophils in human asthma remains less clear.

T-helper lymphocytes: Effector T-helper cells develop from naïve T cells upon activation by pathogen-derived antigens presented by APCs, in particular DCs, in the T cell zone of the lymph nodes. Once activated, the effector T cells migrate back to the site of antigen portal and carry out their functions. Till date, the exact mechanisms which guide the development of these antigen-specific T cells remain unknown. Originally, the effector T cell population was believed to be divided into Th-1 and Th-2 type. However, additional subsets are increasingly being recognized, and now include Th-17, Th-9, Th-22 and T_{reg} (previously discussed) cells.

Asthma is classically considered as an archetypal Th-2 type disease, with elevated circulating IgE levels and eosinophilic inflammation. A large body of experimental data from mouse and human studies documented the expression of Th-2 cytokines; IL-4, 5 and IL-13 in allergic lung. IL-13, in particular, is believed to be critical in driving eosinophilic inflammation, mucus production and airway remodeling. Multiple cycles of allergen-specific Th-2 type inflammation has been proposed to drive a series of pulmonary changes that are akin to wound-healing scenario. However, due to the failure of therapeutics targeting Th-2 type inflammation in clinical trials, the field is challenging the “Th-2 type” paradigm for asthma. The role of other T cell subsets in asthma is a topic of intense investigation [201].

Th-17 cells express IL-17A, IL-17F or both. While mouse studies have shown a pathogenic role for Th-17 cell population, especially in severe asthma [366], abrogation of IL-17 function during allergic sensitization protected mice against asthma development [301]. Thus, the role of Th-17 cells in asthma remains largely unclear.

Th-22 cells are characterized by secretion of IL-22. Although Th-22 cells have not been documented in the human lung, but IL-22 is expressed in the peripheral blood of asthma patients. IL-22 can also be secreted by Th-17 cells [384]. Mechanistic studies in mice have shown that IL-22 is important in initiation of allergic sensitization but might be protective at later stages [32].

Th-9 cells are characterized by secretion of IL-9, but these cells are not yet being considered as a separate T cell subset, due to the lack of defining transcription factor. These cells have been shown to promote allergic responses by increasing the recruitment of mast cells in the lungs [201].

A new, innate type-2 immune effector leukocyte, nuocyte, is being proposed as the missing link between innate and adaptive Th-2 response. Nuocytes expand in number in response to epithelial-derived IL-25 and IL-33, and are an early source of IL-13 [286]. While initially proposed to play a role in parasite infections, nuocytes are now being implicated in allergic airway sensitization and parasite-induced eosinophilia in mouse models [22,377]. Their role in human asthma is yet to be established. It is now being realized that T cells do not become fully committed to a specific subset after antigen exposure, but might retain flexibility, which is dictated by environmental influences, such as allergen exposure, atopy, genetics, age, infection history, pollution or diet. This T cell plasticity may also explain different phenotypes (**Table 1**) observed for asthma in the clinical settings. Thus, understanding the molecular mechanisms behind T cell plasticity in different asthma phenotypes is important to enable design of effective therapeutics [201].

Humoral immune response: Isotype switching of IgM⁺ B cells to IgE producing plasma cells, via IgG1⁺ B cells, is considered as a defining feature of atopy. Molecular mechanisms in class-switching involve germline gene transcription, DNA recombination, and B cell

differentiation into IgE secreting plasma cells. The process involves presentation of MHC class-II associated allergen presentation by Th-2 cells, co-stimulatory signals involving CD40 and CD40 ligand and an obligatory source of IL-4 or IL-13 [10,375]. IgE binding to its high-affinity FcεRI receptor on mast cells and basophils, and cross-linking by allergens triggers mast cell and basophil degranulation to release preformed and newly generated mediators, cytokines, and growth factors, which mediate the key symptoms of asthma [144]. Accordingly, anti-IgE antibody, omalizumab is available as a therapeutic option, but is effective only for a subset of asthma patients.

The sites of IgE production and nature of IgE producing cells has been of great research interest [74]. In the bronchial mucosa of asthmatics, class switching to IgE occurs irrespective of atopic status, providing a possible mechanism for non-atopic and atopic asthma [378]. For long, it was considered that IgE is produced by plasma cells residing in the lung, but is now proposed that IgE producing cells may exist in the nasal and lung mucosa as well [74].

The role of IgE in degranulating eosinophils remains controversial. Instead antigen specific IgA has been proposed to trigger the effector functions of eosinophils in the presence of antigen [231]. But, in murine model of fungal asthma, absence of IgA did not result in any phenotypic differences [102].

Fibrocytes: Fibrocytes were discovered in 1994 by Brucala and associates, as distinct blood-borne cells with fibroblast-like properties, which are involved in tissue repair [40]. Their main function is to promote fibrosis and angiogenesis [125], and therefore contribute to airway remodeling in asthma [40]. Additionally, fibrocytes are potent APCs and can elicit the recruitment and activation of T cells [52]. They can also secrete cytokines, chemokines and

growth factors, which are relevant in mediating fibroproliferation [53]. Fibrocytes are found in a variety of tissues under normal and pathological conditions [223].

The evidence for involvement of fibrocytes in remodeling of the airway in asthma is provided by several studies. Schmidt and associates have shown that there are significant CD34⁺Col I⁺ cells as well as few CD34⁺αSMA (smooth muscle actin)⁺ cells below the basement membrane in the bronchial mucosa of asthmatic patients and that these cells increase dramatically 24 h after exposure to an allergen [299]. While CD34⁺/CD45⁺ and Col I⁺ define fibrocytes, increase in αSMA marks their differentiation into myofibroblasts-like cells, which are involved in wound-healing [1,223,299]. In *in vitro* settings, differentiation of fibrocytes into myofibroblasts occurs in the presence of endothelin-1 [48] and TGF-β1 [151], the same factors which have also been associated with airway remodeling in asthma. While thickening of the airway epithelial basement membrane from fibrosis is often seen in asthmatics, the origin of this fibrosis is not known. Nihlberg and colleagues showed an increase in fibrocytes in close proximity to the basement membrane in patients with mild asthma and that the basement membrane thickness correlated with the number of fibrocytes in the airway [239]. Fibrocyte trafficking is likely to follow similar mechanisms as the trafficking of leukocytes, and involves adhesion molecules, chemoattractants and their receptors [205]. In particular, the biological axis of CXCL12/CXCR4 has been demonstrated to play a major role in mediating the contribution of fibrocytes to pulmonary fibrosis [109,264]. However, the signals that initiate fibrocyte recruitment into the lung are not precisely known. A better understanding of these signals may aid in designing therapeutics for airway remodeling in asthma, which remains unresponsive to currently available therapeutics.

Airway smooth muscle (ASM): In normal airways, ASM contraction regulates airway caliber and bronchomotor tone. In asthmatic airways, ASM has long been considered as the main cell type responsible for bronchial hyperresponsiveness (BHR). Their role in BHR was recognized early and has been the target of β 2-adrenergic drugs, which are spasmolytic agents, and mainstay of asthma therapy. Besides maintaining a contractile tone, ASM also plays a role in inflammatory response and airway remodeling, because of which they have now been implicated in asthma pathophysiology [34]. The role of ASM in 1) BHR 2) airway inflammation and 3) airway remodeling is discussed in the following paragraphs.

A number of studies have shown that sensitization of human ASM with asthmatic serum induces a non-specific increase in smooth muscle responsiveness, demonstrating the existence of mediators in the serum of asthmatic patients that promote airway responsiveness [298]. While the entire list of these mediators remains unknown, some of them include TNF- α , IL-1 β and IL-13. In vitro studies have suggested that a variety of pro-asthmatic signals, such as repeated stretch or exposure to cytokines drastically augment ASM contractile force by altering multiple pathways: i) aberrant activation of contractile and/or impaired function of relaxant receptors ii) alteration of Ca²⁺ regulatory signaling molecules and iii) the activity of elements of the contractile apparatus through Rho-dependent pathways [12,34].

Inflammatory cells, such as T lymphocytes and mast cells, have been shown to infiltrate the ASM, suggesting a direct interaction of the ASM with inflammatory cells, which may orchestrate the asthmatic inflammation. ASM mast cell infiltration is observed in atopic, non-atopic, eosinophilic and non-eosinophilic asthma [11,30,47,289]. Upon activation, mast cells release tryptase and pro-inflammatory cytokines, such as TNF- α , which stimulate the production of TGF- β ₁, and stem cell factor by ASM cells, which in turn induces mast cell chemotaxis [29].

Additionally, ASM may secrete other chemotactic factors including Th-1, Th-2 and pro-inflammatory cytokines and chemokines [34]. Mast cell product tryptase may also contribute to BHR by inducing calcium release [28], and ASM remodeling by inducing ASM proliferation [27].

CD4⁺ T cell localization within the ASM layer has been reported in ovalbumin-sensitized rats [273] and in human asthmatics, and has been related to asthma severity [274]. However, the functional consequences of CD4⁺ T cell and ASM interaction are not well known. Experimental evidence suggests that T cells may enhance ASM contractility to acetylcholine [116], and may induce ASM hyperplasia [273,274]. On the other hand, ASM cells may enhance T cell survival, thus contributing to the perpetuation of bronchial inflammation [273].

Increased ASM mass in the asthmatic airway is one of the prominent features of the structural changes which constitute airway remodeling. This increased muscle mass is considered as the most important reason for exaggerated response to bronchoconstricting stimuli in asthma, resulting in narrowed lumen. It is unknown whether hyperplasia, hypertrophy or a combination of both contributes to an increase in ASM muscle mass, and it is possible that the changes are not homogeneous throughout the airway [34]. One of the theories proposed to explain the increase in muscle mass suggest that it relates to an increase in ECM proteins deposited in and around the muscle bundles and migration of mesenchymal precursors from the peripheral blood into the lung [17]. Additionally, hyperproliferative pathways, such as MAP kinases ERK, p38, PI3 kinases and proliferation markers, such as nuclear antigen are all up regulated, while levels of anti-proliferative prostaglandin E₂ and C/EBP α are decreased in cultures of asthma derived ASM [34].

ASM is now considered not only a target for bronchodilation, but an active player in the pathophysiology of asthma through its ability to modulate inflammation and airway remodeling. Increased understanding of ASM's role would help to design better therapeutic targets.

Models for inhalation studies

Since inhalation of a substance in an environment is largely unavoidable and involves intangible aerosols, models for inhalation studies are absolutely critical to establish cause and effect relationships, and standards related to the inhalation exposure. *In vitro*, *ex vivo* and *in vivo* model systems have been utilized to study the effects of inhaled substances.

Since epithelial cells are the first cells that come in contact with the inhaled substance, *in vitro* studies have utilized lung epithelial cell lines and primary culture of lung epithelial cells. The lung epithelial cell lines have been established from lungs of cancer patients (Calu-1, Calu-3, Calu-6, H441, HBE1, A427, and A549), normal patients (9HTE16o-, 16HBE14o-, 1HAEo-, BEAS-2B, CF/T43 and AK-D) or animals (SOPC1) [326]. Similarly, the primary cultures have been obtained from epithelial cells of human (tracheobronchial, bronchial or alveolar type II epithelial cells) or animal (tracheal or alveolar type II epithelial cells) origin. While primary cultures of columnar epithelial cells from trachea or bronchi are technically feasible [85,213], majority of the studies utilize alveolar epithelial cells. This relates to the ease of obtaining the AECs, which represent 97% of the total lung epithelial surface area [60,290]. The primary culture of type I epithelial cells is still in infancy because of the difficulties involved in harvesting and maintaining these cells with sufficient purity and physiological phenotype [357]. Type I epithelial cells are the terminally differentiated cells with a life span of approximately 120 days [134], and most of the studies investigating type I epithelial cell response have taken

advantage of terminal differentiation of alveolar type II epithelial cells (progenitors cells) into the type I cells. Epithelial cells can be cultured at the air-liquid interface or submerged in the growth media. However, the two models exhibit genetic and phenotypic differences compared to the epithelium in the body [2,84,263]. Studies utilizing lung epithelial cells have been particularly useful in establishing transepithelial mechanisms [326], and host epithelium-pathogen interactions [216]. Compared to an *in vivo* approach, *in vitro* methods are simple, robust, and cheaper and allow better control of variables for data acquisition. However, disposition of the inhaled substance via absorption, mucociliary clearance, phagocytosis or metabolism cannot be accurately mimicked in an *in vitro* setting. Simulating the mucociliary clearance and absorption is technically unfeasible. While epithelial cells might express the metabolic enzymes [86], phagocytosis would require epithelial cell-phagocyte co-culture models. Moreover, because of the phenotypic differences from normal lung epithelium and changes over the culture, an extrapolation of an *in vivo* response from *in vitro* data would require caution.

A refinement over the *in vitro* models is an isolated perfused lung (IPL), where the lung is isolated from the body and housed in an artificial set up under experimental conditions [330]. IPL not only allows investigation of temporal and spatial distribution of the inhaled substance, but can also accommodate the variables leading to disposition of the substance, such as absorption, mucociliary clearance, phagocytosis or metabolism. Although pulmonary circulation can be maintained in an IPL, the tracheobronchial circulation is severed as a result of surgery, thereby providing an inadequate representation of the *in vivo* scenario. Moreover, maintaining an IPL at 37°C for more than 2-3 hours has been a technical challenge [221].

For reasons discussed above, *in vivo* studies using animal models are by far the best way to represent the complex disposition in the intact lung. Small rodents such as rats, guinea pigs

and mice and big animals such as rabbits, dogs, sheep and monkeys are routinely employed for inhalation studies [290]. Cost, research objectives, animal size, time and ease involved in breeding, ease of genetic manipulation, availability of reagents and tools for cellular and molecular analysis and technical expertise dictate the choice of species utilized in different settings. Although a substance may be delivered into the animal lung via intranasal, intratracheal or inhalation route, inhalation represents the natural route of human exposure. For inhalation purposes, nose-only and whole body exposure systems exist. Both the exposure systems eliminate the need for surgery and allow for repeated inhalation of the substance under investigation. However, unlike nose-only exposure, the whole body exposure system requires anesthesia and might allow for absorption of the test substance via skin or ingestion. The inhaled substances not only interact with the components of lung mucosa, but are also likely to be absorbed into the systemic circulation. Several features of the lung such as, large surface area, extremely thin epithelium, relatively less enzymatic activity and rich blood supply, allow for a favorable absorption of chemicals into the circulation [369].

Asthma models

For the reasons mentioned above, an *in vivo* approach is the best to study asthma, which is a pulmonary disease. Since asthma is a complex, multifactorial disease, no single best animal model replicates all of the morphological and functional features of a chronic human disease. Since a medical need for treatment options, especially for severe asthma, continues to persist, models that mimic the exact cellular and biochemical processes underlying chronic inflammation and airway remodeling are necessary. Although the best approach to investigate these processes is to perform studies on human asthmatics, the ethical and technical constraints prohibit the

elucidation of underlying mechanisms. Animals including horses, dogs, rabbits, cats, rats and mice, have been routinely used as models for asthma. However, with the exception of cats (eosinophilic bronchitis) and horses (heaves), none of these animals naturally exhibit asthma like syndrome similar to that in humans [145,235].

The mouse has become the most widely used species for asthma research, because of its small size, ease of breeding and genetic manipulations, and availability of specific reagents and tools necessary to conduct the cellular and molecular analysis. While Balb/c is the most commonly used strain of mouse, as they develop a good Th-2 biased immunological response, other strains such as C57BL/6 and A/J have also been successfully used, although striking differences have been reported within species with different strains of mouse, for the extent to which they develop an allergic response to the same sensitizer [145].

Ovalbumin (OVA), derived from chicken egg, is the most commonly used allergen for inducing asthma in mice. However, OVA is never implicated in human asthma. Newer models using alternative, clinically relevant allergens, such as house-dust mite, cockroach extracts, grass pollen and fungal antigens have been developed. The method of introducing the allergen or drugs into the lung (e.g. intranasal, intratracheal instillation of solutions, inhalation of nebulized solutions or dry powders) can also influence the outcome of the study. For example, OVA if delivered via inhalation, the natural route of human exposure, in the absence of systemic sensitization, lead to tolerance. For inducing allergic sensitization in these models, the allergen is systemically delivered in the presence of an adjuvant, (e.g. aluminium hydroxide), which is known to promote a Th-2 biased immune response. Adjuvant-free protocols or protocols using inhalation as the only route of delivery have also been developed, but these usually require

greater number of exposures to achieve suitable sensitization, and an intrinsic enzymatic activity in the allergen [145,235].

The pulmonary branching pattern differs between rodents and humans, which may have implications for studies using inhalation as the route of delivery for allergens or drugs. While deposition of inhaled aerosols is primarily controlled by their aerodynamic diameter in humans, this does not hold true for small rodents such as mice and rats, which are obligate nose-breathers. In humans, orally inhaled aerosols greater than 5-10 μm are mostly trapped by oropharyngeal deposition, the smaller aerosols $\leq 5 \mu\text{m}$ penetrate into the lung and 1-2 μm sized aerosols reach the alveolar spaces [371]. However, particles within the 1-5 μm can be deposited throughout the tracheobronchial or pulmonary regions of small rodents [198].

The acute models of asthma, mostly utilizing OVA, focus on inflammatory mechanisms pertinent to asthma, and fail to model the etiology and natural history of asthma. Furthermore, the inflammation seen in mouse is not restricted to conducting airways, whereas it is in humans. Mast cells, one of the key cellular players in human asthma, are not recruited into the airway wall or epithelium of mice. The chronic models of asthma have been developed that utilize more clinically relevant allergens and sensitization routes, but are also not devoid of limitations. For example, a large increase in airway smooth muscle, which is a characteristic feature of chronic asthma, is seen in very few models, only after a high level challenge [235]. While animal models have been instrumental in understanding some of the mechanistic pathways linked to various aspects of the disease, study variables make comparisons across studies problematic. These include species, strain, sex and age of the animal, variations in sensitization and challenge schedules, method of introducing the allergen or drugs into the lung (e.g. intranasal, intratracheal instillation of solutions, inhalation of nebulized solutions or dry powders), choice of allergen,

endpoints used to assess asthma or whether acute or chronic aspects of asthma are being modeled.

Due to these limitations, the utility of asthma models to study asthma is constantly debated, and is emphasized by the fact that few new drugs have made it to the clinic during the past 50 years, with many that perform well in preclinical animal models of asthma, failing in humans owing to lack of safety and efficacy. Indeed, chronic models of asthma, using clinically relevant antigens, or natural routes of human exposure are a step forward in improving the animal models, but their clinical utility is yet to be established. Only recently, animal models are being reported, which have considered the environmental factors associated with the disease, such as pollution, obesity, environmental tobacco smoke, mold exposures and viral infections [145].

In an attempt to improve asthma modeling, the National Center for the Replacement, Refinement, and Reduction of Animals in Research in UK has invested £1 million in research projects to develop tissue engineering approaches to model the asthmatic airway using cells taken directly from well-characterized patients with asthma and healthy volunteers, and are likely to incorporate microfluidics into the bioreactors, to examine how environmental insults interact with asthma susceptibility genes. A team of tissue engineers, cell biologists, biomaterials specialists, immunologists, clinicians and regulators has been brought together to drive the development of models that will have the most utility in predicting human asthma [145].

**CHAPTER 1: THE IMPACT OF ASPERGILLUS FUMIGATUS VIABILITY AND
SENSITIZATION TO ITS ALLERGENS, ON THE MURINE ALLERGIC ASTHMA
PHENOTYPE¹²**

Abstract

Aspergillus fumigatus is a ubiquitously present respiratory pathogen. The outcome of a pulmonary disease may vary significantly with fungal viability and host immune status. Our objective in this study was: 1) to assess the ability of inhaled irradiation-killed or live *A. fumigatus* spores to induce allergic pulmonary disease and 2) to assess the extent to which inhaled dead or live *A. fumigatus* spores influence pulmonary symptoms in a previously established allergic state. Our newly developed fungal delivery apparatus allowed us to recapitulate human exposure through repeated inhalation of dry fungal spores in an animal model. We found that live *A. fumigatus* spore inhalation led to a significantly increased humoral response, pulmonary inflammation and airway remodeling in naive mice, and is more likely to induce allergic asthma symptoms than the dead spores. In contrast, in allergic mice, inhalation of dead and live conidia recruited neutrophils and induced goblet cell metaplasia. This data suggests that asthma symptoms might be exacerbated by the inhalation of live or dead spores in individuals with established allergy to fungal antigens, although the extent of symptoms was less with dead spores. These results are likely to be important while considering fungal-exposure

¹ The material in this chapter was co-authored by Sumali Pandey, Scott A. Hoselton and Jane M. Schuh. Sumali Pandey had primary responsibility for designing the study, treating the mice, collecting and processing the murine samples, perform analytical experiments, analyze the data and prepare the manuscript. Scott A. Hoselton cut lung sections for histology and proof-read the manuscript. Jane M. Schuh conceived the idea and proof-read the manuscript.

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assessment methods, and for making informed therapeutic decisions for mold-associated diseases.

Introduction

For centuries, fungi have been associated with asthma and other airway diseases [5]. Humans inhale viable and non-viable fungi or their components in many indoor and outdoor environments, and mold-related exposures can pose a significant concern to human health [11,20,24,85]. Although a number of federal agencies provide guidance to the public on health effects associated with mold exposure and on ways to mitigate it, the United States Government Accountability Office (U.S. GAO) reported a lack of federally accepted health-based standards for safe mold levels [67]. The problem is particularly concerning in post-flooding or post-hurricane situations and in agricultural settings where the repeated inhalation of mold over an extended period of time is likely [9,30,31,51].

Aspergillus fumigatus is one of the commonly detected fungal species in flooded indoor environments [14,35,65,68] and in grain dust [30,48]. *A. fumigatus* has evolved to provide carbon and nitrogen turnover in decaying organic matter. However, due to their small size (2-3 μm in diameter) and hydrophobicity, the spores (conidia) may remain suspended in the air for a long time, increasing the likelihood of inhalation deep into the alveolar spaces of human lungs. Exposure to *A. fumigatus* spores is ubiquitous and symptomless for most people, but it causes a spectrum of diseases in susceptible hosts. While invasive aspergillosis is a serious disease that may occur in immunocompromised individuals, most fungal diseases are associated with less mortality but an ongoing morbidity, as is the case with allergic diseases in humans [15].

Several pulmonary diseases have been associated with *A. fumigatus*, such as allergic bronchopulmonary aspergillosis, severe asthma with fungal sensitization, rhinitis, sinusitis, and hypersensitivity pneumonitis. Although *A. fumigatus* is a source of 23 listed allergens [7], and is an opportunistic pathogen, *A. fumigatus*-induced pulmonary diseases may or may not involve elevated serum IgE or fungal colonization [2,3,44]. A meta analysis study showed 15-48% prevalence rate for *Aspergillus* sensitization in bronchial asthma [1]. Besides being allergenic, research has provided evidence for secreted proteases [41,42,80] and cell wall-associated molecules, such as β -glucan [26,37,75] and chitin [66,81], in orchestrating the host response to inhaled *Aspergillus*.

As our understanding of the host-pathogen interaction in the genesis of an *A. fumigatus*-induced pulmonary disease is emerging, models that mimic natural human exposure are critical. In the environment, humans inhale dry, airborne *A. fumigatus*. To mimic the exposure in experimental animals, invasive and non-invasive methods have been employed, such as intratracheal (IT), intranasal (IN), and inhalational (INH). Depending on the method used, the deposition, clearance [18] and stimulation of host-immune responses to the substance intended for pulmonary delivery varies substantially [70,90]. Besides being invasive, IT delivery results in a concentrated central deposition in the upper respiratory tract, where mucociliary clearance is predominant. Additionally, the IN and IT methods require suspension of the fungal spores in a liquid, which can considerably alter the spore coat, the concentration of soluble fungal antigens, and metabolic activity of the fungus [79]. Detergents such as Tween-80, which is used for the suspension of fungal spores for IT delivery, may influence host-pathogen interaction by damaging the host epithelial cells [54] and/or by influencing fungal properties [8]. In contrast, the non-invasive INH method allows for repeated exposure to the same substance (as would

occur in humans) and results in a dissemination of conidia throughout the lungs. In the INH method, no suspension of the conidia is required.

Inhalation of environmental substances is ubiquitous and an unavoidable phenomenon. While a healthy lung remains remarkably tolerant to inhaled antigens [4,6,42], allergy is a genetic predisposition to develop lung and systemic hypersensitivity reactions to environmental antigens (environmental allergies). This suggests that responses to inhaled substances can substantially vary in healthy and diseased states. Indeed, allergy is one of the strongest risk factors for acquiring asthma [36,61,63].

Our objective in this study was two-fold: 1) to assess the ability of inhaled irradiation-killed or live *A. fumigatus* spores to induce allergic pulmonary disease and 2) to assess the extent to which inhaled dead or live *A. fumigatus* spores influence pulmonary symptoms in a previously established murine allergic state. For this purpose we used an inhalational apparatus that has been developed in our laboratory for the delivery of dry, aerosolized irradiation-killed (moisture-, heat- and pressure- free sterilization method) or live airborne spores [38]. Previous studies comparing host-immune responses to live and dead (typically killed by autoclaving) *A. fumigatus* conidia are not only limited by an unnatural route of human exposure, but also exhibit variable inflammation and pro-allergic responses [4,25,37,64,69]. Moreover, reports comparing the pulmonary histopathological changes associated with live or dead *A. fumigatus* conidia are lacking. Studies, such as the one presented here, are likely to aid in establishing evidence based standards for environmental mold exposures and remediation, as well as informing decisions for mold-associated pulmonary disease diagnoses, prognoses, and therapeutic interventions.

Material and methods

Animals

BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA), and housed in a specific pathogen-free murine colony at Van Es Hall, North Dakota State University (NDSU, Fargo, ND, USA) in micro filter topped cages (Ancare, Bellmore, NY, USA). Murine groups challenged with irradiated or live *A. fumigatus* conidia were caged separately. The study was conducted under the guidelines and approval of the Institutional Animal Care and Use Committee of NDSU.

Inhalation of irradiation-killed or live A. fumigatus in a non-sensitized or allergically sensitized murine host

For protocol A (**Fig. 2A**), mice without prior exposure to fungal antigens (non-sensitized murine host), were challenged with airborne, dry, irradiation-killed or live *A. fumigatus* conidia, using a previously described inoculation chamber and spore delivery method [38]. For *A. fumigatus* (strain NIH 5233; American Type Culture Collection) cultures, fresh fungal culture was spread onto sterile Sabouraud dextrose agar in a 25-cm² culture flask and incubated at 37°C for 8 days. A separate aliquot was used for each fungal culture flask to ensure an equal yield of mature conidia. For dead conidia, the 8-day-old *A. fumigatus* culture flask was subjected to a lethal dose of gamma radiation (8 kGy) in a ¹³⁷Cs gamma irradiator (Radiation Machinery Corporation, Parsippany, NJ, USA). Mice were anesthetized with an intraperitoneal (IP) injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) prior to administration of a 10-min, nose-only inhalation (INH) of dead or live *A. fumigatus* conidia. To mimic repeated INH of an environmental allergen in humans, mice were challenged once a week for three weeks.

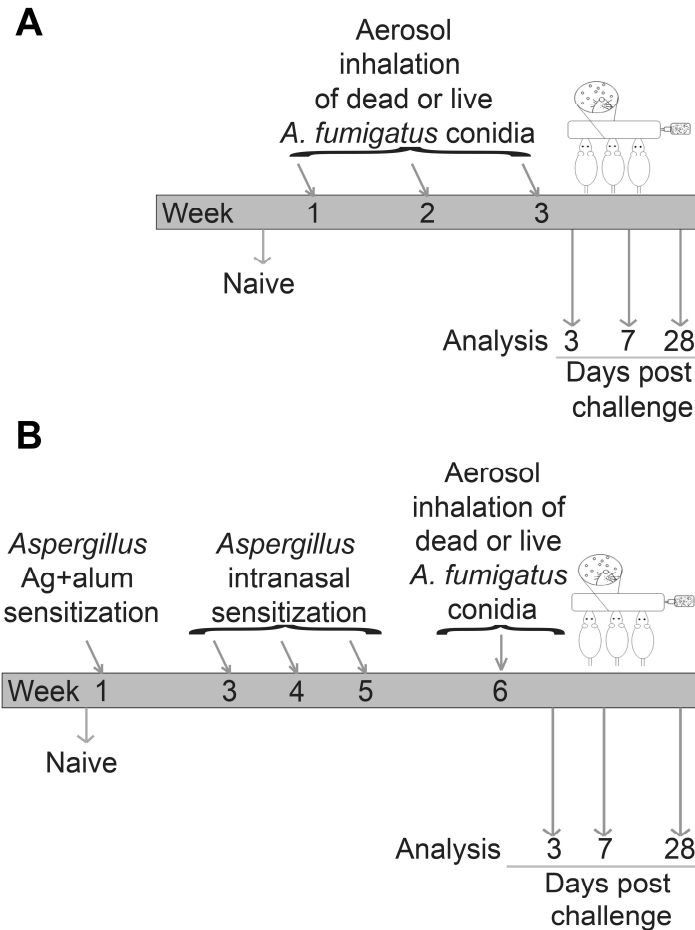


Figure 2: Schematic representation of the inhalation (INH) challenge protocol with irradiation-killed or live *Aspergillus fumigatus* conidia in a non-sensitized (A) or sensitized (B) murine host. For protocol A, mice without prior fungal exposure were anesthetized with an intraperitoneal (IP) injection of ketamine and xylazine and subjected to a 10-min, nose only INH of dry, aerosolized, dead or live *A. fumigatus* conidia, once a week for three consecutive weeks. For protocol B, mice were sensitized to fungal antigens by subcutaneous and IP injection of *A. fumigatus* antigen mixed with alum in PBS. This was followed by three weekly intranasal inoculations of *A. fumigatus* antigen in PBS. A week later, mice were challenged once a week for three consecutive weeks, with dead or live *A. fumigatus* conidia, in exactly the same way as for Protocol A. Challenged mice treated according to protocol A or B were analyzed on days 3, 7 and 28 post-third challenge. Naïve animals were maintained as negative controls.

For protocol B (**Fig. 2B**), mice were sensitized to fungal extracts prior to challenge with dead or live *A. fumigatus* conidia. Mice were sensitized by subcutaneous and IP injection of 10 μ g of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC) suspended in 0.1 ml Imject[®] Alum (Pierce, Rockford, IL, USA) and 0.1 ml PBS. Two weeks after the injections, each mouse

received a series of three, weekly 20- μ g intranasal (IN) inoculations consisting of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) dissolved in 20 μ l PBS. One week after the last IN inoculation, mice were challenged with a 10-min, nose-only INH of dead or live *A. fumigatus* conidia, as in protocol A. Mice were challenged once a week for three weeks and samples were collected at days 3, 7, and 28 post-third fungal challenge. Naïve animals that were neither sensitized to fungal extract nor challenged with dead or live *A. fumigatus* conidia were maintained as baseline controls for the study. All fungal work was conducted in Class II biological safety cabinet, with the prior approval of the Institutional Biosafety Committee of NDSU

Differential cell counts

The trachea was cannulated and 1 ml of sterile PBS was used to lavage the bronchoalveolar space of the mouse. Total and differential cell counting was performed as previously described [38]. Representative photomicrographs were obtained using a Zeiss Z1 AxioObserver inverted microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

Serum and BALF antibody analysis

Serum and BALF samples were obtained as previously described [38]. Mouse isotype specific ELISA kits were used for quantification of IgA, IgG₁, IgG_{2a} (Bethyl Laboratories Inc., Montgomery, TX, USA), and IgE (BD Biosciences, Inc. San Jose, CA, USA) antibody levels as per manufacturers' directions. For protocol A, the serum was diluted as follows: IgA 1:1000, IgG₁ 1: 2500, IgG_{2a} 1:2500, and IgE 1:20, and the BALF was diluted as follows: IgA 1:5, IgG₁ 1:10, IgG_{2a} 1:10, and undiluted for IgE analysis. In protocol B, sensitization protocol resulted in greater antibody production. The serum was diluted as follows: IgA 1:1000, IgG₁ 1: 5000, IgG_{2a}

1:5000, and IgE 1:100, and the BALF was diluted as follows: IgA 1:10, IgG₁ 1:20, IgG_{2a} 1:20, and IgE 1:2.

cDNA synthesis

The inferior and postcaval lobes of the lung were collected at each time point, snap frozen in liquid nitrogen, and stored at -20°C until use. Total RNA extracted from the homogenized lung tissues using TRIzol[®] reagent (Invitrogen Life technologies, Grand Island, NY, USA) was subjected to DNase (Promega, Madison, WI, USA) (1 unit DNase/μg RNA) treatment for 30 min at 37°C. RNA yield was determined by measuring the absorbance of ultraviolet light at 260 nm using a Synergy HT plate-reader (Biotek Instruments Inc., Winooski, Vermont, USA). In order to prime the synthesis of first strand of cDNA by reverse transcription, 0.5 μg of random primers (Promega, Madison, WI, USA) per μg of RNA were allowed to anneal to the DNase treated RNA at 70°C for 5 mins. Up to 2 μg of RNA was used for each reaction. The RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and dNTP mix (Promega, Madison, WI, USA) at 37°C for 1h. The reaction was stopped by heat inactivation at 70°C for 10 mins. The cDNA was used for analyzing the gene expression via quantitative PCR (qPCR), as mentioned below.

qPCR

The expression of *ccl-17*, *tslp* and *hprt-1* (internal control) genes in murine lungs was analyzed by qPCR using SYBR green based master mix and RNA specific QuantiTect primer assays (QIAGEN, Valencia, CA, USA) for mouse. The reaction was set up on ABI 7500 real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA): 95°C for 10 min (activation of

HotStar Taq DNA Polymerase), 95°C for 15 secs (denaturation), 60°C for 1 min (annealing and extension). The denaturation, annealing, and extension cycles were repeated 40 times and the fluorescence data was collected at the end of each cycle. Dissociation curves analysis was performed, and the data was analyzed using $2^{-\Delta\Delta CT}$ method to calculate the relative fold change in the lung, standardized against naïve controls. A four-point dilution curve was established to show the validity of $2^{-\Delta\Delta CT}$ calculations prior to use for analysis, and to determine the appropriate dilution of cDNA to be used for real-time qPCR reaction.

Histological analysis

Whole left lungs were fixed in 10% neutral buffered formalin and paraffin embedded. 5- μ m thick lung sections were affixed to microscope slides and stained with hematoxylin and eosin (H&E) stain (Dako North America Inc., Carpinteria, CA, USA), Periodic acid Schiff (PAS) stain or Gomori's trichrome stain (both from Richard Allan Scientific Inc., Kalamazoo, MI, USA) to assess pulmonary inflammation, goblet cell (GC) metaplasia, and collagen deposition in the lung, respectively. Representative photomicrographs were obtained using a Zeiss Z1 AxioObserver inverted microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

A photometric analysis using Olympus MicroSuite™ software (Olympus America Inc., Center Valley, PA, USA) was employed to analyze the histology images. The PAS-positive mucus-producing GCs were counted in 5 randomly selected 200- μ m segments of basement membrane in the lateral bronchial branches or small airways of mice lungs. The percentage of GC to total columnar epithelial cells was calculated for each group. The H&E-stained sections were used to measure the thickness of columnar epithelial cells lining the airways. At least 50 discrete points at 50- μ m intervals were selected along the second or third lateral (L2 or L3)

bronchial branch and a perpendicular line extending from the basement membrane was drawn through the cell to the height of epithelial cell layer thickness. For subepithelial collagen deposition, at least 100 discrete points at 50 μm intervals were selected along bronchial branch L2 or L3, and a perpendicular line was drawn from a point on the basement membrane through the full thickness of the peribronchial collagen. Group mean was determined by averaging the mean for each mouse in a group.

Immunohistochemistry

Longitudinal lung sections of left lungs affixed to glass slides were deparaffinized and placed in citric acid buffer (10 mM, pH 6.0) and cooked in a microwave pressure cooker (Nordic ware, Minneapolis, MN, USA) for 10 min for antigen retrieval. IgA, IgE and IgG (primary antibodies from Southern Biotech, Birmingham, AL, USA) staining was performed using horseradish peroxidase (HRP)-3-amino-9-ethylcarbazole (AEC) cell and tissue staining kit (R&D Systems, Minneapolis, MN, USA), as per manufacturer's recommended protocol. Red colored precipitates were identified as areas of positive staining in the lung. Representative photomicrographs were obtained using a Zeiss Z1 AxioObserver inverted microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

Statistical analysis

All results are expressed as the mean \pm the standard error of mean. GraphPad Prism 5 software (GraphPad Software, Inc., LaJolla, CA, USA) was used to calculate statistics; differences between groups were tested with two-tailed unpaired student's *t*-test with Welch's correction. In all cases, $p < 0.05$ was considered statistically significant.

Results

*Effect of live and irradiation-killed *A. fumigatus* inhalation on allergy-associated responses*

Our first objective was to assess the extent to which the inhalation of dead or live *A. fumigatus* conidia could induce allergy in a non-sensitized murine host (Protocol A, **Fig. 2A**). In experimental animal models, allergy is typically assessed by elevated antibody levels (IgE and IgG₁). We found that repeated inhalation of dead or live *A. fumigatus* conidia did not result in elevated serum IgE levels, compared to naïve mice (**Fig. 3A**). Similar results were observed in three repeats of the study. However, the serum IgG₁ levels were elevated in mice challenged with live, but not dead conidia, at days 3 and 28 post-third fungal challenge, compared to naïve mice (**Fig. 3B**). The BALF antibody levels were measured to assess the local effect of inhaled conidia on the humoral immune response. Although a decrease was observed at earlier timepoints, by day 28 the BALF IgE levels in mice challenged with live conidia showed a ~ 2 fold increase over naïve levels (**Fig. 3C**). Similarly, an elevation in BALF IgG₁ was observed at days 3 and 28, in mice challenged with live, but not the dead conidia (**Fig. 3D**).

Since allergy is believed to be a Th-2 dominant process, we analyzed the mRNA profiles of two Th-2 associated markers; *ccl17* and *tslp*. At day 7, mice challenged with live, but not the dead conidia showed elevated *ccl17* mRNA levels (**Fig. 3E**). At day 3, the *tslp* mRNA levels showed an unexpected decrease in mice challenged with dead or live conidia, compared to naïve mice (**Fig. 3F**). Although the reason for the later finding is not immediately apparent, the elevation in BALF IgE, IgG₁, and lung *ccl17* mRNA levels with live conidia inhalation suggest that viable conidia have a greater tendency to induce allergy, than the non-viable conidia (**Fig. 3**).

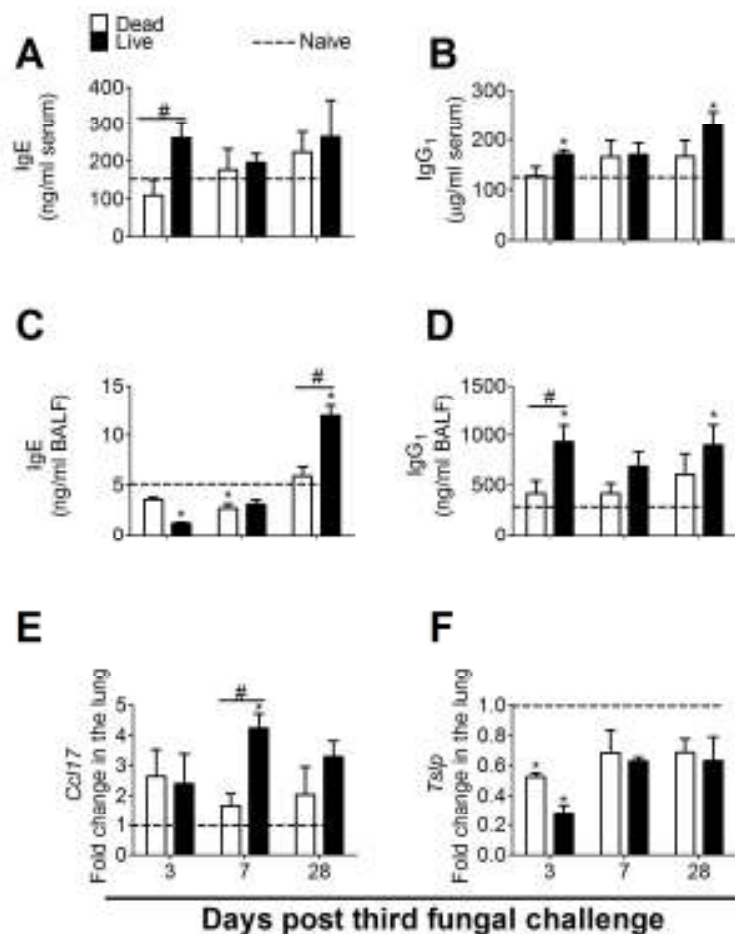


Figure 3: Effect of inhaled dead or live *A. fumigatus* conidia on the development of allergy-associated responses in a non-sensitized murine host. Total IgE (A, C) and IgG₁ (B, D) levels were determined in the serum (A, B) and bronchoalveolar lavage fluid (BALF) (C, D) samples obtained from mice challenged with dead or live *A. fumigatus* conidia, at days 3, 7 and 28 post-third fungal challenge. Commercially available mouse-specific ELISAs were used for this purpose. Ccl17 (E) and *tslp* (F) mRNA levels were determined in the murine lung homogenates, via SYBR green based quantitative-polymerase chain reaction. The fold change in the lung was determined using 2- $\Delta\Delta$ CT method and standardized against naïve levels (dashed line). Bars represent mean \pm SEM, n = 3-5 mice/group. *, #, p value < 0.05, as compared with naïve mice or mice challenged with dead conidia, respectively.

Inhalation of live A. fumigatus conidia elicits mucosal antibody response

Previous study with live and heat-killed *A. fumigatus* conidia assessed the antibody levels in the serum but not in the BALF [69]. Since dissociated trends for serum and BALF IgE and IgG₁ antibody levels were observed with inhalation of live *A. fumigatus* conidia, we investigated

the systemic and local levels of other antibody isotypes in these mice. The naïve mice had undetectable levels of BALF IgA (**Fig. 4A**) and IgG_{2a} (**Fig. 4B**). With the exception of sporadic, low level detection in a few mice (fraction on the graph = no. of positives/total no. of mice in a group), the mice challenged with dead conidia had BALF IgA and IgG_{2a} levels similar to naïve

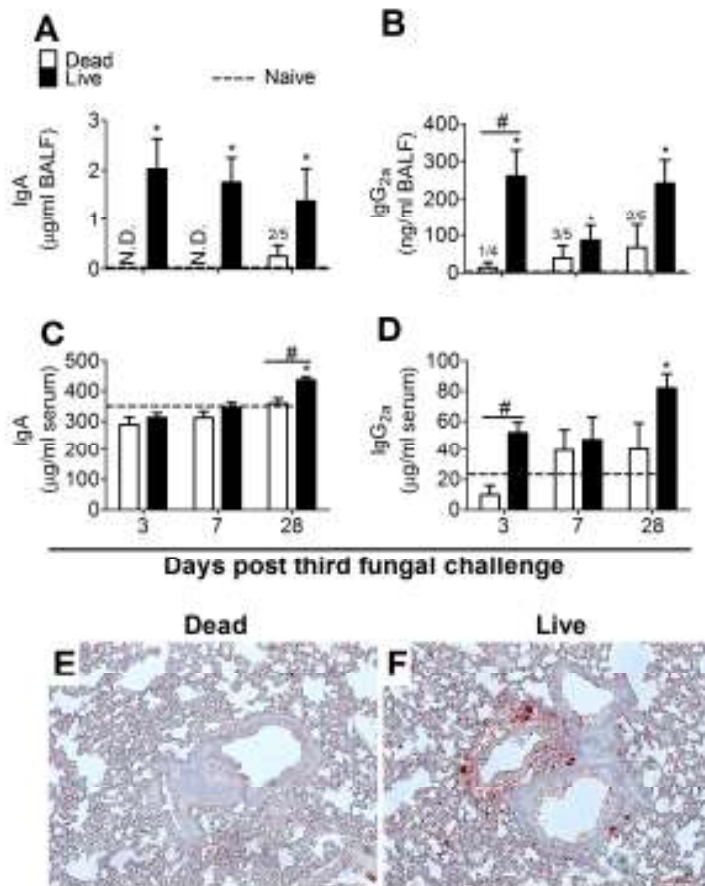


Figure 4: Effect of inhaled dead or live *A. fumigatus* conidia on IgA and IgG_{2a} levels in the serum, BALF and lung sections of a non-sensitized murine host. Total IgA (A, C) and IgG_{2a} (B, D) levels were determined in the BALF (A, B) and serum (C, D) samples obtained from mice challenged with dead or live *A. fumigatus* conidia, at days 3, 7 and 28 post-third fungal challenge. Commercially available mouse-specific ELISAs were used for this purpose. Immunohistochemical staining for IgA antibody localization on lung sections obtained from mice challenged with dead (E) or live (F) *A. fumigatus* conidia, at day 7 post-third fungal challenge showed cell-associated IgA in mice challenged with live *A. fumigatus* conidia (E). IgE and IgG (not shown) positive immunohistochemical staining showed secreted or endothelium associated antibody in these mice. Bars represent mean \pm SEM, n = 4-5 mice/group. * and # designate a p < 0.05, as compared with naive mice or mice challenged with dead conidia, respectively. Scale bar = 100 μ m.

levels (**Fig. 4A and B**). In contrast, the mice challenged with live conidia had significantly increased BALF IgA and IgG_{2a} levels at days 3, 7 and 28 (**Fig. 4A and B**). The mice challenged with dead conidia had detectable levels of serum IgA and IgG_{2a}, and the levels were similar to naïve mice (**Fig. 4C and D**). In contrast, by day 28, the serum antibody levels of IgA (**Fig. 4C**) and IgG_{2a} (**Fig. 4D**) were significantly elevated in mice challenged with live conidia. The effect of live conidia inhalation on the BALF antibody levels was prominent as early as day 3 post-fungal challenge. However, a significant elevation in serum antibody levels was not observed until day 28 post-fungal challenge, suggesting a predominantly local effect, and a delayed systemic effect of inhaled live *A. fumigatus* conidia on the humoral immune response in these mice (**Fig. 4A-D**).

To further assess the spatio-temporal distribution of antibodies, immunohistochemical (IHC) staining was performed on murine lungs challenged with dead or live *A. fumigatus* conidia. The murine lung sections incubated with buffer without primary antibody were maintained as negative controls, and consistently stained negative for red precipitates (not shown). The IHC identified an abundance of cell associated IgA in the parenchyma and the peribronchovascular region of the murine lungs challenged with live conidia (**Fig. 4F**), but not the dead conidia (**Fig. 4E**) at days 3, 7 and 28 post-third fungal challenge. A peak in IgA positive staining was observed at day 7 and is depicted in **Fig. 4E and F**. In contrast to IgA, IgE and IgG positive staining was not cell associated, but mainly appeared as secreted, lining the endothelium of the blood vessels around the large airways (data not shown). While increased IgE positive staining was observed in lung sections obtained from mice challenged with live conidia, IgG positive staining was similar in both the groups. Based on our observations, it would be

reasonable to expect the presence of IgA-producing cells in the lungs and an extrapulmonary source for IgE and IgG.

Live conidia inhalation elicits significantly increased granulocytic pulmonary inflammation

The lung sections stained with H&E or cytopinned BALF samples stained with a differential stain were analyzed to assess total and differential inflammation in the mice challenged with dead or live *A. fumigatus* conidia.

The naïve mice did not show any peribronchovascular inflammation in the H&E stained lung sections (not shown). At all the tested time points, the incidence and severity of pulmonary histopathology were significantly less in mice challenged with dead conidia (**Fig. 5A**) than in mice challenged with live conidia (**Fig. 5B**). Representative pictures at day 3 after challenge with dead or live conidia are depicted in **Fig. 5A and B**, respectively. In the mice challenged with live conidia, the inflammation was most intense at day 3 post challenge (**Fig. 5B**), and although a decrease in inflammation was seen at day 7, it persisted even at day 28 post challenge. In the mice challenged with dead conidia, the inflammation was resolved by day 7. The peribronchovascular inflammation was centered around large-diameter, main axial conducting airway and extended into the more distal, small diameter, terminal bronchioles. At all the time points, the inflammatory influx was comprised predominantly of mononuclear inflammatory cell infiltrate composed mainly of large and small lymphocytes and monocytes.

In the BALF obtained from naïve mice (dashed line in **Fig. 5C, D, G, H**), macrophages were the only cell type detected, and even after challenge with dead or live conidia, macrophages remained the predominant cell type (88% for dead group and 51% for live group) at all time points (**Fig. 5C**). Although both dead (p value < 0.05) and live conidia (p value = 0.06) recruited macrophages at day 3 post-challenge (**Fig. 5C**), distinct differences were noted in the appearance

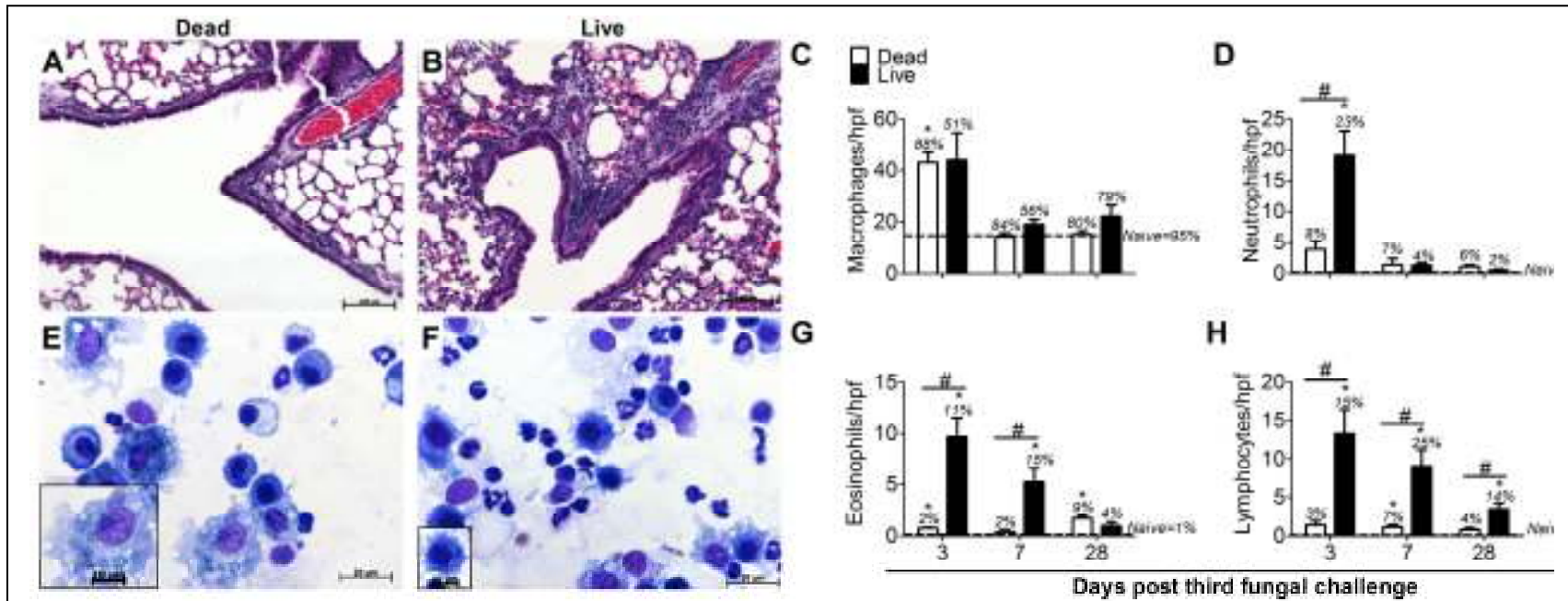


Figure 5: Effect of inhaled dead or live *A. fumigatus* conidia on pulmonary inflammation in non-sensitized mice. Peribronchovascular inflammation observed on hematoxylin and eosin (H&E) stained lung sections peaked at day 3, for mice challenged with dead (A) or live (B) *A. fumigatus* conidia, and is depicted here. BALF samples from naïve mice or mice challenged with dead or live *A. fumigatus* conidia were cytopspun and stained with Quick Dip stain to aid in the analysis of differential cell types based on their morphology and staining pattern. Monocytes/macrophages (C), neutrophils (D), eosinophils (G) and lymphocytes (H) were enumerated for each group at days 3, 7, and 28 post-third fungal challenge. Counts from naïve mice are represented by a dashed line (C,D,G,H). The italicized numbers in (C), (D), (G) and (H) represent the cells quantified as % of total cells. At day 3, significant differences in the number of granulocytes were observed in the mice challenged with dead (E) or live (F) conidia. Additionally, macrophages (shown in the inset) from the two groups appeared morphologically different. The differences in the size of the insets indicate differences in size of the macrophages shown in the insets. Macrophages from dead conidia challenged mice had numerous greenish spherical objects, presumably conidia, inside them (E inset), and these were rarely observed in the macrophages from live conidia challenged group (F inset). Bars represent mean \pm SEM, $n = 4-5$ mice/group. *, #, p value < 0.05 , as compared with naive mice or mice challenged with dead conidia, respectively. Scale bars = 100 μm (A, B), 20 μm (E, F), and 10 μm (E, F insets).

of the macrophages obtained in the cytopinned BAL samples from the two groups (**Fig. 5E and F insets**). The cytoplasm of the macrophages obtained in the BAL of mice challenged with dead conidia looked enlarged, foamier and hypervacuolated as compared with those obtained in the BAL from mice challenged with live conidia. Greenish spherical objects, which are presumably the intact conidia, could be observed more frequently in the macrophages of the dead group. On average, 10 greenish spherical objects per macrophage (range = 0 to 28) were counted from the dead group compared with an average of 1 greenish spherical object per macrophage (range = 0 to 7) observed in the live group. Although the true identity of these greenish spherical objects remain obscure right now, their consistent presence in the macrophages obtained from the BAL of mice challenged with dead *A. fumigatus* conidia is certainly intriguing, and a focus of future investigation.

Mice challenged with live conidia recruited a significantly greater number of neutrophils (**Fig. 5D**), eosinophils (**Fig. 5G**) and lymphocytes (**Fig.5H**), compared to mice challenged with dead conidia. Although mice challenged with dead conidia showed sporadic presence of eosinophils, the number (~1-2) was too low to significantly contribute towards the inflammatory profile at the tested time points (**Fig. 5G**).

Taken together, this data suggests that the inhalation of live *A. fumigatus* conidia results in significantly greater pulmonary inflammation, compared to the inhalation of dead conidia or in the absence of fungal challenge (**Fig. 5**).

*Enhanced airway remodeling is observed with live *A. fumigatus* conidia inhalation*

Airway remodeling is defined as structural change of the airway wall, which arises from injury and repair, and plays an important role in asthma pathophysiology by decreasing the lung function. The naïve levels for remodeling parameters such as GC metaplasia (**Fig. 6A-C**),

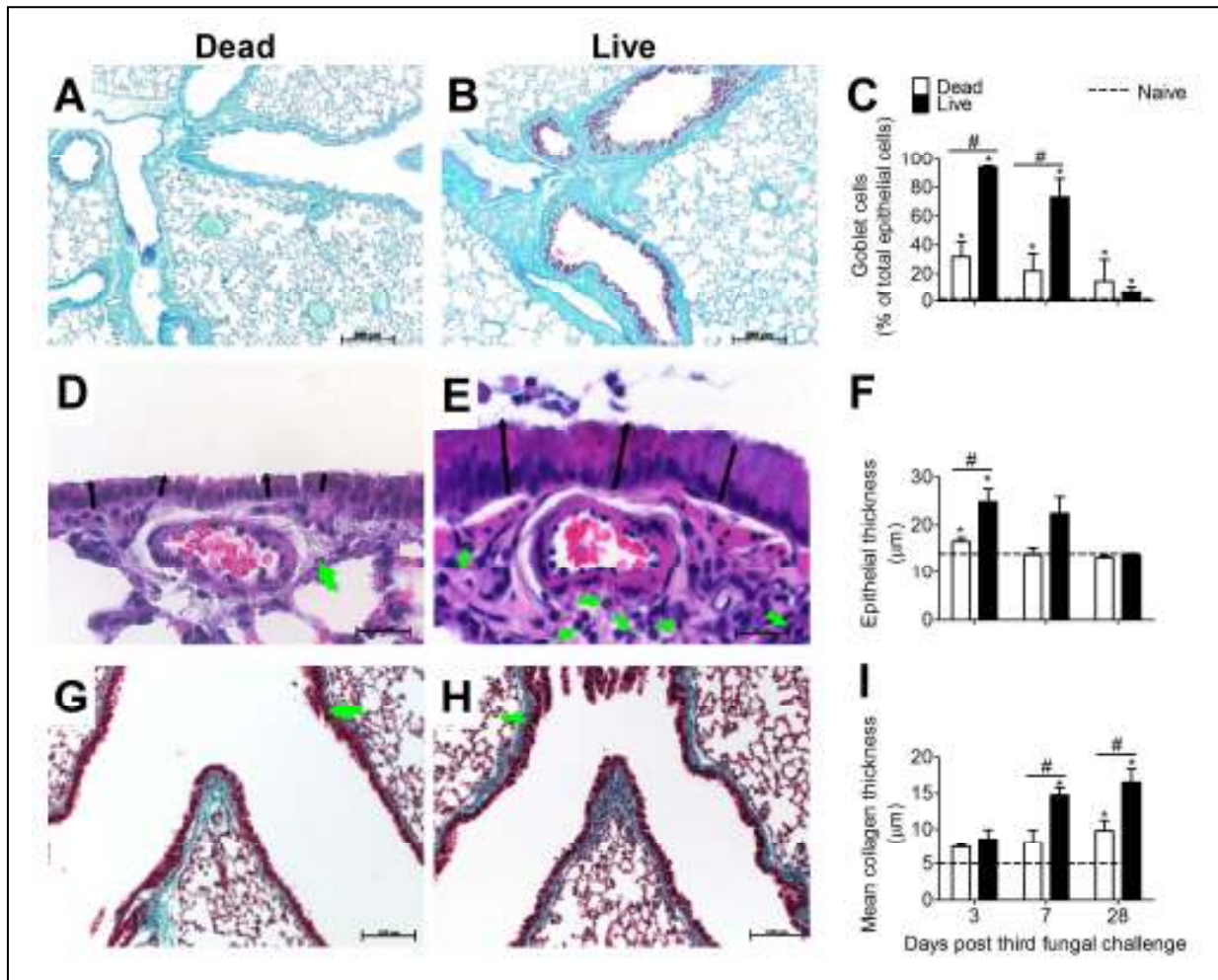


Figure 6: Effect of inhaled dead or live *A. fumigatus* conidia on airway remodeling in non-sensitized murine host. Periodic acid-Schiff (PAS) staining (A, B), H&E staining (D, E), and Gomori's trichrome staining (G, H) were used to measure goblet cell% lining the columnar epithelial cells (C), epithelial layer thickness (F) and subepithelial collagen layer thickness (I), respectively. Green arrows are pointing towards the granulocytes in the peribronchovascular region (D, E) or subepithelial collagen deposition and smooth muscle thickness (G, H). Naïve levels are indicated by the dashed line (C, F, I). Bars represent mean \pm SEM, n = 3-5 mice/group. *, #, p value < 0.05, as compared with naïve mice or mice challenged with dead conidia, respectively. Scale bars = 200 μ m (A, B) and 20 μ m (D, E) and 100 μ m (G, H).

epithelial layer thickening (Fig. 6D-F) and subepithelial collagen deposition (Fig. 6G-I) are shown as a dashed line. Challenge with dead and live conidia led to an increase in GC% over naïve mice at days 3, 7 and 28 post-third fungal challenge (Fig. 6C). However, the increase was significantly greater with live conidia challenge (~3 fold increase at days 3 and 7) than with dead

conidia challenge (**Fig. 6C**). Additionally, live conidia led to significantly increased epithelial thickness at day 3 post challenge, as compared to dead conidia challenge or no challenge (**Fig. 6F**). Furthermore, an increased number of granulocytes were identified based on morphology or red cytoplasmic staining in the subepithelial zone of mice challenged with live conidia at day 3, as compared to mice challenged with dead conidia (green arrows in **Fig. 6D and E**). Correlating with increased granulocyte recruitment in the peribronchovascular region of murine lungs challenged with live conidia, we observed increased subepithelial collagen deposition (green arrows in **Fig. 6G and H**) in these mice at days 7 and 28 (**Fig. 6I**).

Th-2 associated systemic and mucosal humoral immune responses are enhanced following inhalation of live A. fumigatus conidia in allergically sensitized mice

The next question we asked was: does an allergically sensitized murine host, respond differently to dead and live *A. fumigatus* conidia inhalation? For this purpose, we sensitized the murine host to fungal extract antigens (Protocol B, **Fig. 2B**) prior to the three weekly challenges with dead or live *A. fumigatus* conidia. As described in previous studies from our lab, sensitization without challenge with *A. fumigatus* conidia (Day 0 time point) leads to an increased humoral response, but the pulmonary inflammation and GC metaplasia remain similar to naïve mice [27,38]. Due to the prior sensitization of mice to fungal extracts, the systemic and mucosal humoral immune response was significantly elevated in the sensitized mice, compared to naïve mice (**Fig. 7**). To investigate if inhalation of dead or live *A. fumigatus* conidia can influence the established humoral immune response, we analyzed the immunoglobulin levels in the serum (**Fig. 7A, C, E, G**) and BALF (**Fig. 7B, D, F, H**). When compared to the inhalation of dead *A. fumigatus* conidia, exposure to live *A. fumigatus* led to a significant increase in serum IgE levels at days 3 and 7 (**Fig. 7A**), BALF IgE levels at day 3 (**Fig. 7B**), serum IgA (**Fig. 7C**)

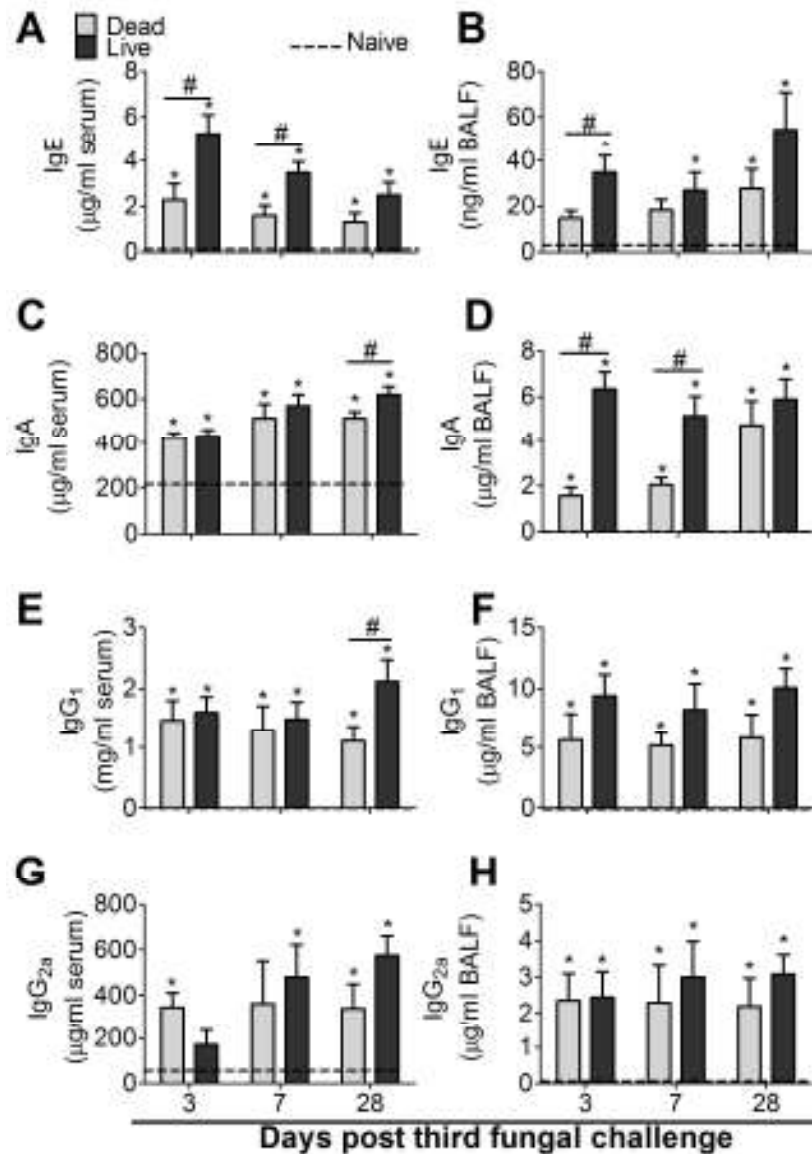


Figure 7: Effect of inhaled dead or live *A. fumigatus* conidia on systemic and mucosal antibody levels in a sensitized murine host. Total IgE (A, B) and IgA (C, D), IgG₁ (E, F) and IgG_{2a} (G, H) levels were determined in the serum (A, C, E and G) and BALF (B, D, F and H) samples obtained from mice challenged with dead or live *A. fumigatus* conidia, at days 3, 7 and 28 post-third fungal challenge. Commercially available mouse-specific ELISAs were used for this purpose. Bars represent mean±SEM, n = 4-8 mice/group. *, #, p value < 0.05, as compared with naive mice or mice challenged with dead conidia, respectively.

and serum IgG₁ (Fig. 7E) at day 28, and BALF IgA at days 3 and 7 (Fig. 7D). In contrast, the BALF IgG₁ (Fig. 7F) and serum and BALF IgG_{2a} (Fig. 7G, H) antibody levels remained similar

in mice challenged with live or dead conidia at all the tested time points. Taken together, our data suggests that compared to Th-1 associated humoral immune response (IgG_{2a}), Th-2 associated humoral immunity (IgE and IgG₁) and mucosal antibody (IgA) is more susceptible to an increase upon inhalation of live, but not the dead, *A. fumigatus* conidia in a sensitized murine host (**Fig. 7**).

Significant neutrophil recruitment and goblet cell metaplasia is observed in allergically sensitized mice challenged with dead and live A. fumigatus conidia

The analysis of differential cell types in the BALF showed that mice challenged with dead *A. fumigatus* conidia had significantly more macrophages compared to naïve mice at day 3 post-third fungal challenge (**Fig. 8A**). The differences in macrophage counts in mice challenged with live *A. fumigatus* conidia were not statistically significant, with respect to naïve mice (**Fig. 8A**). Unlike the morphological differences observed in non-sensitized mice (**Fig. 5E and F**), macrophages appeared the same in allergically sensitized mice challenged with either dead or live conidia.

Compared to naïve mice, the inhalation of dead or live conidia recruited neutrophils by day 3, and there were no differences in neutrophil counts between mice challenged with live or dead *A. fumigatus* conidia (**Fig. 8B**). At day 3 after challenge, mice challenged with dead conidia had elevated numbers of eosinophils (**Fig. 8C**) and lymphocytes (**Fig. 8D**). By day 7, the numbers were reduced to naïve levels. Mice challenged with live conidia had significantly more eosinophils (**Fig. 8C**) and lymphocytes (**Fig. 8D**) at days 3 and 7, as compared to mice challenged with dead conidia and naïve mice.

The GC% was significantly elevated in mice challenged with dead and live *A. fumigatus* conidia compared to naïve mice at days 3, 7 and 28 post-third fungal challenge (**Fig. 8E**). In

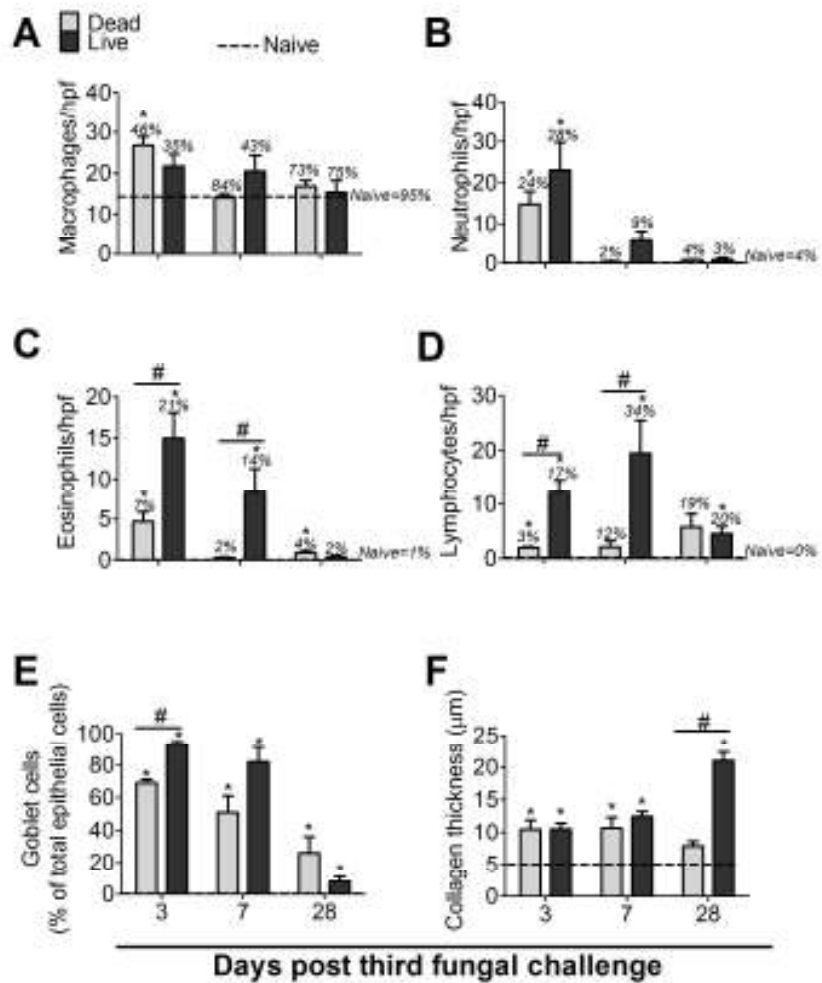


Figure 8: Effect of inhaled dead or live *A. fumigatus* conidia on pulmonary inflammation and airway remodeling in sensitized mice. BALF cells from naïve mice or mice challenged with dead or live *A. fumigatus* conidia were cytopun and stained with Quick Dip stain for differential analysis. Monocytes/macrophages (A), neutrophils (B), eosinophils (C), lymphocytes (D) were enumerated for each group at days 3, 7, and 28 post-third fungal challenge. The italicized numbers in (A-D) represent the cells quantified as % of total cells. PAS staining or Gomori's trichome staining was used to measure goblet cell% lining the columnar epithelial cells (E) or subepithelial collagen layer thickness (F). Naïve levels are indicated by the dashed line. Counts from naïve mice are represented by a dashed line (A-F). Bars represent mean \pm SEM, n = 4-8 mice/group. * and # represent p < 0.05, as compared to naïve mice or mice challenged with dead conidia, respectively.

contrast to non-sensitized mice (**Fig. 6C**), the inhalation of live conidia led to a modest (<1.5 fold) increase in GC%, compared to the inhalation of dead conidia, at day 3, but by day 7, both the groups had similar levels (**Fig. 8E**). The collagen thickness was increased in mice

challenged with dead and live conidia at days 3 and 7, compared to naïve mice (**Fig. 8F**). However, by day 28, a significant (~4 fold) increase in collagen thickness was observed in mice challenged with live conidia, compared to naïve mice and dead conidia challenged mice (**Fig. 8F**). Taken together, this data suggests that compared to the inhalation of dead conidia in a sensitized murine host, inhalation of live *A. fumigatus* conidia results in only a modest difference in GC% at earlier time points, but a significant increase in collagen deposition at day 28 post-challenge.

Discussion

Although previous studies have reported differences in innate and adaptive immune responses to live and dead *A. fumigatus* conidia [4,37,64,69], this is the first comparative study to have utilized inhalation as a route for pulmonary delivery of dry, aerosolized *A. fumigatus* conidia. We showed that in a non-sensitized murine host (Protocol 1A), live but not irradiation-killed *A. fumigatus* conidia inhalation, elicited significantly more pulmonary inflammation, mucosal antibody production, and airway remodeling, which suggests that inhalation of live, but not dead, *A. fumigatus* conidia has a greater tendency to elicit asthma symptoms.

We also report a comparison of host immune response with repeated inhalation of live and dead *A. fumigatus* conidia in a murine host with prior sensitization to fungal extracts (Protocol 1B). In these mice, inhalation of live and dead *A. fumigatus* conidia elicited asthma symptoms, albeit the extent of response to dead conidia was lesser than that for live conidia. As described in previous studies from our lab, sensitization without challenge with *A. fumigatus* conidia (Day 0 time point) leads to an increased humoral response, but the pulmonary inflammation and GC metaplasia remain similar to naïve mice [27,38]. Indeed, as a result of

sensitization with *A. fumigatus* extract and alum, an increased eosinophil recruitment, systemic and mucosal humoral immune responses were observed, suggesting a Th-2 predominant response in sensitized mice as compared with their non-sensitized counterparts. However, striking differences were observed with regard to neutrophil recruitment and GC% in sensitized (Protocol 1B) versus non-sensitized (Protocol 1A) mice challenged with dead *A. fumigatus* conidia. The sensitized mice challenged with dead conidia recruited neutrophils ($24\% \pm 5\%$ of total BAL cells) at day 3 post challenge, which was similar to the sensitized mice challenged with live conidia ($28\% \pm 6\%$), and significantly higher than non-sensitized mice challenged with dead conidia ($8\% \pm 2\%$). Additionally, sensitized mice challenged with dead conidia showed significantly increased GCs ($69\% \pm 2\%$ of the total columnar epithelial cells) in the airways, at day 3 post challenge, compared with $32\% \pm 10\%$ seen in non-sensitized mice challenged with dead conidia.

In experimental settings, allergy is typically assessed by elevated antibody levels (IgE and IgG₁) [36,63]. Although an elevation in serum IgE, with 4 or 8 intranasal challenges of live *A. fumigatus* conidia has been reported previously [53], a significant increase in serum IgE was not observed in our study after three, repeated inhalations of dead or live *A. fumigatus* conidia in non-sensitized mice. In accordance with our study, elevations in serum IgG₁ but not IgE, with live *A. fumigatus* conidia have been observed previously [69]. The differences are likely to be due to the dosing regimen and exposure frequency. It has been previously proposed, that in contrast to T cell responses, antibody-based methods lack sensitivity to detect allergy, at least in mice [62]. The problem is likely to exist in clinical settings as well, since not all asthma patients with fungal colonization, are detected as IgE positive and vice versa [17,44]. Since the diagnosis

may dictate the use of anti-fungal and/or anti-IgE therapy, a need for better diagnostic methods for assessing fungus associated allergy/asthma exists.

Elevated antibody levels in the BALF may play a critical role in host defense against respiratory pathogens and inhaled allergens. The elevated BALF antibody levels, with live, but not the dead, *A. fumigatus* conidia, starting as early as day 3 post challenge, suggests that lung mucosa is the primary target for inhaled *A. fumigatus* conidia. Both extrapulmonary (bone marrow or respiratory lymph nodes) and pulmonary sources of IgE have been identified [19], although no IgE⁺ cells were detected by IHC in the present study. Although the source of antibodies was not immediately apparent, our results are in alignment with studies in human ABPA patients, where elevated IgA, IgG and IgE levels have been detected in the BALF [29]. The IgE levels in the BALF of non-sensitized mice challenged with live *A. fumigatus* conidia showed a ~2 fold elevation over naïve levels at day-28 post-challenge. Although quantitatively the levels were still in nanograms, a couple of things are striking about this observation. Our data suggests, that inhalation of live, but not the dead, *A. fumigatus* conidia might play an important role in the development of allergic disease, in the human population. BALF but not the serum IgE levels showed early elevation, suggesting that the lung mucosa is the predominant site for *A. fumigatus* induced IgE production and/or accumulation, in individuals exposed to live *A. fumigatus*. This would warrant against skin prick test to ascertain fungal sensitization. A similar observation has been previously reported in allergic rhinitis patients, who had allergen specific IgE in nasal secretions, and negative skin prick test response [39,49]. Diagnostic standards related to BALF IgE levels, in order to determine fungal sensitization do not exist. Since an accurate diagnosis dictates the administration of anti-IgE therapy, further research is needed to validate BALF IgE levels in fungal sensitization.

A genetic predisposition to develop allergy is mostly associated with Th-2 predominant response. *A. fumigatus* proteases have been shown to promote a Th-2 type response [42], and it is a source of 23 listed allergens [7]. In previous studies, significant variations in total and Th-2 associated inflammatory responses to live and dead *A. fumigatus* have been observed. Rivera *et. al.* reported that intratracheal (IT) challenge with dead conidia (heat-inactivated conidia; HIC) primed IL-4 and IL-13 producing CD4⁺T cells (pro-allergic) in the draining lymph nodes. In contrast, live conidia recruited IFN- γ producing CD4⁺T (counter-regulatory for Th-2) cells to the airways [69]. Porter *et. al.* reported that prolonged intranasal (IN) administration of dead (paraformaldehyde fixed) *A. fumigatus* conidia promoted significant lung IL-4 and eosinophil (pro-allergic) responses, although reduced compared to responses with live conidia [64]. Similar results were obtained with live and dead (irradiated) *A. niger* species in another study by Porter *et. al.* [62]. In contrast, Amanianda *et. al.* showed that IN delivery of dead (dormant, paraformaldehyde fixed) *A. fumigatus* conidia elicited no inflammation in mice [4]. Hohl T.M. *et. al.* also reported that dead (HIC) conidia were less stimulatory with regard to inflammation [37]. Finally, Murdock *et. al.* reported co-evolution of Th-1, Th-2 and Th-17 responses during repeated IN exposure to *A. fumigatus* conidia [53]. While Th-1 responses are likely to inhibit Th-2 responses, and the role of Th-17 immune response in asthma is still unclear, further investigations that mimic natural human exposure, such as the one presented here, are warranted.

CCL17 promotes the recruitment of Th-2 cells into the airways [58], and fungal proteases have been implicated in up-regulating CCL17 [43]. Moreover, elevated serum CCL17 levels have been proposed as a biomarker of Allergic Bronchopulmonary Aspergillosis [32,47]. Based on these findings, we hypothesized that live, but not the dead, *A. fumigatus* conidia challenge, due to *in-situ* protease production, would induce *ccl17* in non-sensitized mice. Indeed, *ccl17*

mRNA up-regulation with live, but not the dead *A. fumigatus* conidia inhalation provided evidences in support of our hypothesis. Thymic Stromal Lymphopoietin (TSLP) is believed to be a master regulator of Th-2 driven inflammation [34,74] and the allergic asthma phenotype [91]. Additionally, an *in vitro* study demonstrated increased TSLP in epithelial cells, with fungal (*Alternaria*) extracts [45]. This led us to hypothesize that challenge with live *A. fumigatus* conidia would lead to increased *Tslp* gene expression, indicative of a Th-2 dominant response. However, we found an unexpected decrease in *Tslp* mRNA expression with live and dead *A. fumigatus* conidia, at day 3 post-fungal challenge. While the reason for the reduction is not immediately apparent, an *A. fumigatus* specific effect on murine lung might be involved, and is likely to be independent of fungal viability status. Interestingly, PAR-2 (mRNA), which is believed to be important in fungal (*Alternaria*) extract mediated TSLP induction [45], is inhibited in a TLR4 dependent manner by *A. fumigatus* infection in immunocompromised mice [52]. To our knowledge, this is the first documentation of *tslp* mRNA levels in an *A. fumigatus* induced pulmonary disease model, and further investigation is warranted.

While mucus production is largely considered as an innate immune response in healthy people, the Th-2 predominant immune response in asthma patients results in chronic mucus hypersecretion [16,88]. In the present study, a non-sensitized murine host challenged with live *A. fumigatus* conidia showed significantly increased mucus producing goblet cells, compared with dead conidia, which suggested that mucus production was not only an innate immune response to particulate matter, but can be significantly affected with the viability status of the inhaled substance. This raises a question regarding which fungal associated factors might contribute to GC metaplasia in *in vivo* settings? Indeed, proteases produced by *A. fumigatus* can induce a Th-2 dominant response [42,46], and specifically serine protease activity in *A. fumigatus* was found to

be essential to induce the expression of the mucin producing *Muc5ac* gene and mucin secretion in the bronchial epithelial cells [56]. While these studies have established a critical role for proteases in *A. fumigatus* induced mucus secretion, they have either utilized purified protease extracts and/or are *in vitro*. Therefore, the present study demonstrates the importance of factors associated with viable, dry, aerosolized conidia (mimicking natural human exposure), and potentially an *in situ* production of proteases, directly or indirectly promoting GC metaplasia. We also showed that GC metaplasia was significantly enhanced with the inhalation of both dead and live conidia in sensitized mice, suggesting that a pre-existing Th-2 predominant immunity in sensitized mice, may also contribute to GC metaplasia, irrespective of fungal viability status.

Although pulmonary fibrosis is an infrequently observed feature of human asthma, studies have linked the intensity of fibrosis to the severity of the disease [13]. While the immunology of pulmonary fibrosis is still evolving, it is believed to be the end result of chronic inflammatory processes that result in substantial tissue injury. Indeed, the recruitment of macrophages and granulocytes (neutrophils and eosinophils) in the peribronchovascular region can cause substantial lung tissue damage due to the release of reactive oxygen species and toxic granule proteins. In the present study, irrespective of the underlying allergic sensitization, challenge with live, but not dead conidia, led to increased collagen deposition, which correlated with increased granulocyte recruitment in response to live conidia challenge. While the role of neutrophils in collagen deposition is possibly limited to causing tissue injury [59], eosinophils, macrophages and Th-2 lymphocytes are likely to play an important role in pro-fibrotic repair, by regulating the recruitment, proliferation or activation of fibroblasts [89]. Thus, it is possible that waves of leukocytes, with varying functions are recruited in the lung to orchestrate various phases of wound and pro-fibrotic repair after live *A. fumigatus* conidia challenge. Since *ccl17*

mRNA was up-regulated at day 7 post-fungal challenge with live *A. fumigatus* conidia in non-sensitized mice, and neutralization of CCL17 leads to a reduction of bleomycin induced pulmonary fibrosis [10] it is tempting to hypothesize that up-regulation of *ccl17* at day 7 post live *A. fumigatus* challenge, marks the shift in host-immune response from a tissue damaging, acute inflammatory response to a pro-fibrotic repair response, and a time-dependent neutralization of *ccl17* may result in reduced pulmonary fibrosis.

In accordance with the previous study [37], we observed a selective recruitment of neutrophils with inhalation of live *A. fumigatus* conidia, but not the dead conidia, in the non-sensitized murine host. Previous studies have established the importance of β -glucan/dectin-1 and MyD88 dependent signaling pathways in neutrophil recruitment, in response to live *A. fumigatus* challenge in non-sensitized mice [37,75]. In the present study, the recruitment of neutrophils in response to live and dead *A. fumigatus* conidia, in sensitized mice suggest that although neutrophil recruitment requires Pathogen Associated Molecular Patterns, it is not exclusive to conidial swelling or live *A. fumigatus* conidia. Increased antigen load or bystander immune activation, in mice sensitized to fungal extracts and challenged with live or dead *A. fumigatus* may result in neutrophil recruitment. Additionally, recruited neutrophils may contribute to increased goblet cell metaplasia seen in these mice (described above), via secretion of neutrophil elastase [23,72,82].

In the present study, we identified that neutrophils and eosinophils are recruited with inhalation of live *A. fumigatus* conidia, even in the absence of systemic sensitization (elevated serum IgE) to fungal extracts (non-sensitized host), and without any adjuvant. This is in contrast to a clinically irrelevant, proteinaceous ovalbumin, which is used in many asthma-related animal models and leads to tolerance in the absence of systemic sensitization [71,77,78]. While several

studies have reported the recruitment of granulocytes in response to live *A. fumigatus* conidia [22,37,53,64,73], and have implicated the role of β -glucan, chitin and secreted proteases, the present study is the first to demonstrate this phenomenon using a natural route of human exposure to dry, aerosolized *A. fumigatus* conidia. Additionally, both eosinophilia [50,87] and neutrophilia [21,28,40,57,76,86] have been associated with severe asthma. A subset of asthmatics having eosinophilia and neutrophilia identifies patients with the most severe asthma [33], suggesting the importance of identifying environmental triggers that lead to such inflammation. Neutrophils lead to a particularly severe phenotype of asthma, which responds poorly to corticosteroids.

The differences observed with inhalation of live and dead *A. fumigatus* conidia in non-sensitized mice, are pointing towards the emerging conclusion from several different studies that conidial swelling, the first step of germination, is essential in display of immunogenic surface moieties; such as chitin and/or β -glucan for the recruitment of eosinophils and neutrophils [4,37,66,81]. Since we administered dry, resting conidia, the swelling of live *A. fumigatus* conidia most likely occurred inside the murine lungs and triggered the recruitment of granulocytes. Dead *A. fumigatus* conidia, due to their incapability to swell and get rid of the immunologically inert surface rodlet layer, probably provide insufficient antigens and/or PAMPs to trigger the immune response. However, sensitized mice, which are predisposed to launch a Th-2 mediated immune response, are hypersensitive to inhaled dead *A. fumigatus* conidia thereby recruiting neutrophils and inducing goblet cell metaplasia, both of which may lead to asthma exacerbation in humans [12,21,55,83,84].

A limitation of the present work relates to the fact that fungal dose, exposure frequency and mouse strain-dependent differences may lead to variations in the extent of allergic and/or

hypersensitivity pneumonitis response. Nonetheless, *A. fumigatus* exposure in the environment is ubiquitous, and repeated exposures to high and low doses, at varying frequencies, may occur in different settings, such as in mold infested homes, agricultural settings or as isolated outdoor exposures. Animal models that mimic natural human exposure to aerosolized fungal spores are critical in investigating the host-pathogen interaction, which govern *A. fumigatus* induced pulmonary disease. Lack of evidence based risk characterization is the prime reason that despite significant advances in analytical techniques that allow the measurement of cultivable and non-cultivable fungi and their components [60], there is no consensus on methodology for monitoring spore concentrations in the environment. For the same reason, results across studies cannot be compared and guidelines or personal exposure limits cannot be established, for assessing fungal exposure. Indeed, studies, such as the one presented here are likely to aid in establishing evidence based standards for mold-related exposures and in making informed therapeutic decisions for mold-associated diseases.

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**CHAPTER 2: PERSISTENT, DOSE-DEPENDENT INCREASES IN PULMONARY IgA
ACCOMPANY THE INHALATION OF DEOXYNIVALENOL IN A MURINE MODEL
OF ALLERGIC FUNGAL ASTHMA³**

Abstract

Inhalation of grain dust has been associated with allergic disease in farm workers, and grain dust samples are routinely contaminated with Deoxynivalenol (DON), which is a trichothecene mycotoxin. DON is a potent immunomodulatory agent, with demonstrated effects on inflammatory mediators, several of which are involved in the pathophysiology of allergic asthma. Additionally, immune related effects of dietary DON are well established but there is no data available to validate route-to-route (dietary to inhalation) extrapolation. The objective of this study was to assess the extent to which inhaled DON influences the allergic asthma related immune-mediators and phenotype in a murine model of allergic asthma. Beginning at one day post allergen challenge, allergic mice were exposed to 30 or 60 ng of inhaled DON once daily for six days. The dosages were selected to mimic human exposure in agricultural settings. On day 7 and 28 after allergen challenge, serum was analyzed for IgE and IgA, and bronchoalveolar lavage fluid (BALF) was analyzed for IgA levels. Cytokine levels were analyzed in lung homogenates, and goblet cell metaplasia with associated mucus production was visualized by periodic acid Schiff staining of lung histology sections. We found that the inhalation of DON increased serum and BALF IgA levels in a dose- and time-dependent manner. However, serum

³ The material in this chapter was co-authored by Sumali Pandey, Scott A. Hoselton and Jane M. Schuh. Sumali Pandey had primary responsibility for designing the study, treating the mice, collecting and processing the murine samples, perform analytical experiments, analyze the data and prepare the manuscript. Scott A. Hoselton cut lung sections for histology and proof-read the manuscript. Jane M. Schuh conceived the idea and proof-read the manuscript.

IgE, cytokine levels and goblet cell metaplasia were unaffected with the doses of DON used in this study. These results will help to build upon the current body of knowledge in mycotoxin interaction with the lung mucosa to aid in establishing evidence-based standards regulating the exposure to DON inhalation by grain handlers.

Introduction

Mycotoxins are secondary metabolites of filamentous microfungi and are toxic to vertebrates in low concentrations [10]. Discovered by Japanese scientists in the early 1970s, DON is a trichothecene mycotoxin produced by *Fusarium* species [67]. It is one of the most prevalent mycotoxins found in barley, corn, rye, safflower seeds, wheat, and mixed feeds [10]. Changing weather patterns, minimum tillage and crop rotation practices, and increased acreage of susceptible host crops may in some instances change environmental factors to provide more optimal conditions for *Fusarium* infection in grains [39]. Ingestion of *Fusarium*-contaminated grains has been associated with outbreaks of human gastroenteritis, and a chronic low dose exposure to DON has been linked to anorexia, growth impairment, neuroendocrine changes, and immune dysfunction in experimental animals [58].

DON's cellular targets include not only the structural cells, such as epithelial cells [28,42] and fibroblasts [9,30], but may also include macrophages, T cells, B cells [25], and NK cells [11]. DON's effect on the immune system is of particular interest. While a high dose exposure to DON leads to leukocyte apoptosis [78-80], a low dose exposure has been shown to upregulate the mRNA of tumor necrosis factor (TNF)- α , as well as Th-1 and Th-2 cytokines [5,14,17,46,57,73,77]. In experimental infections with bacteria and viruses, DON has been shown to augment or suppress the host immune response [35,36,38,48,68,71,72]. DON's

combined effect can be either immunosuppressive or immunostimulatory depending on the dose, exposure frequency, and timing of the functional immune assay used for detection [55]. This may be related to a differing susceptibility of various cell types to DON, by the type of immune cells that are recruited to the site of DON exposure at low or high doses, by the differences in the acute versus the chronic response to the toxin, or by a combination of these factors.

Oral ingestion of DON significantly increases antibody production. Dramatic elevations in IgA were observed with chronic ingestion of DON in mice [54]. The induced IgA antibody was found to be self-reactive [59-61], and there was a concomitant increase in circulating IgA immune complexes, mesangial IgA deposition, and hematuria [16]. The resulting pathological characteristics resembled that of IgA nephropathy, the most common type of glomerulonephritis in humans [15]. In investigating its impact on serum IgE, a hallmark of allergic disease, significant elevations were observed in mice after feeding DON [52]; however, when tested in a mouse model of allergy, subcutaneous administration of DON did not affect IgE levels [28]. Differences in models or routes of exposure may be responsible for the varying results. Additionally, DON's effect on other allergy related parameters was not investigated in these studies.

Allergic asthma is an inflammatory disease of the lung and involves a complex interplay of several immune system mediators, such as Th-2 cytokines and IgE. Occupational exposure to grain dust may be an underlying cause or an exacerbating agent in allergic diseases that are prevalent in farmers [7] who represent 30% of adults disabled by respiratory illness [22]. DON is routinely detected in grain dust released during grain production, transport, storage and processing, making inhalation exposure in these environments a high probability [23,31,33,34,45]. Due to its immunomodulatory properties, inhalation of DON can exert its

influence on several allergic asthma mediators, and potentially impact the outcome of the disease.

While repeated inhalation of DON in an allergic grain handler can occur, the research in this domain is lacking. Most of the immune related effects of DON have been established using human or mouse cell culture models, or in animal models involving oral ingestion of DON. To our knowledge, there is only one study that has investigated the effects of DON delivered intranasally, and found that DON was more toxic when delivered via nasal route compared to oral gavage [3], which makes it all the more pertinent to test DON's effects when delivered via inhalation.

The purpose of this study was to investigate the extent of the effect of inhaled DON in a murine model of allergic asthma because: 1) feeding studies and *in vitro* experiments have shown that DON regulates various immune mediators of allergic asthma, such as serum IgE and Th-2 cytokines 2) DON inhalation in occupational settings is frequent and unavoidable, and 3) although the immunological effects of dietary DON are well established [13], there are no occupational exposure limits for DON inhalation, despite the concern that respiratory DON exposure may be even more toxic than the oral route [3]. The implications from this study hold relevance for exposure to DON via inhalation amongst grain handlers such as farmers and millers.

Material and methods

Animals

Balb/c mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were bred and housed in a specific pathogen-free murine colony at Van Es Hall, North Dakota State University (NDSU;

Fargo, ND, USA). Mice were placed in micro filter topped cages (Ancare, Bellmore, NY, USA) on Alpha-dri™ paper bedding (Shepherd Speciality Papers Inc., Watertown, TN, USA) and were fed and watered *ad libitum*. Mice treated with deoxynivalenol (DON) or vehicle (solvent for DON) were caged separately. The study was conducted under the guidelines of the Institutional Animal Care and Use Committee of NDSU.

DON, fungal antigen, and conidia

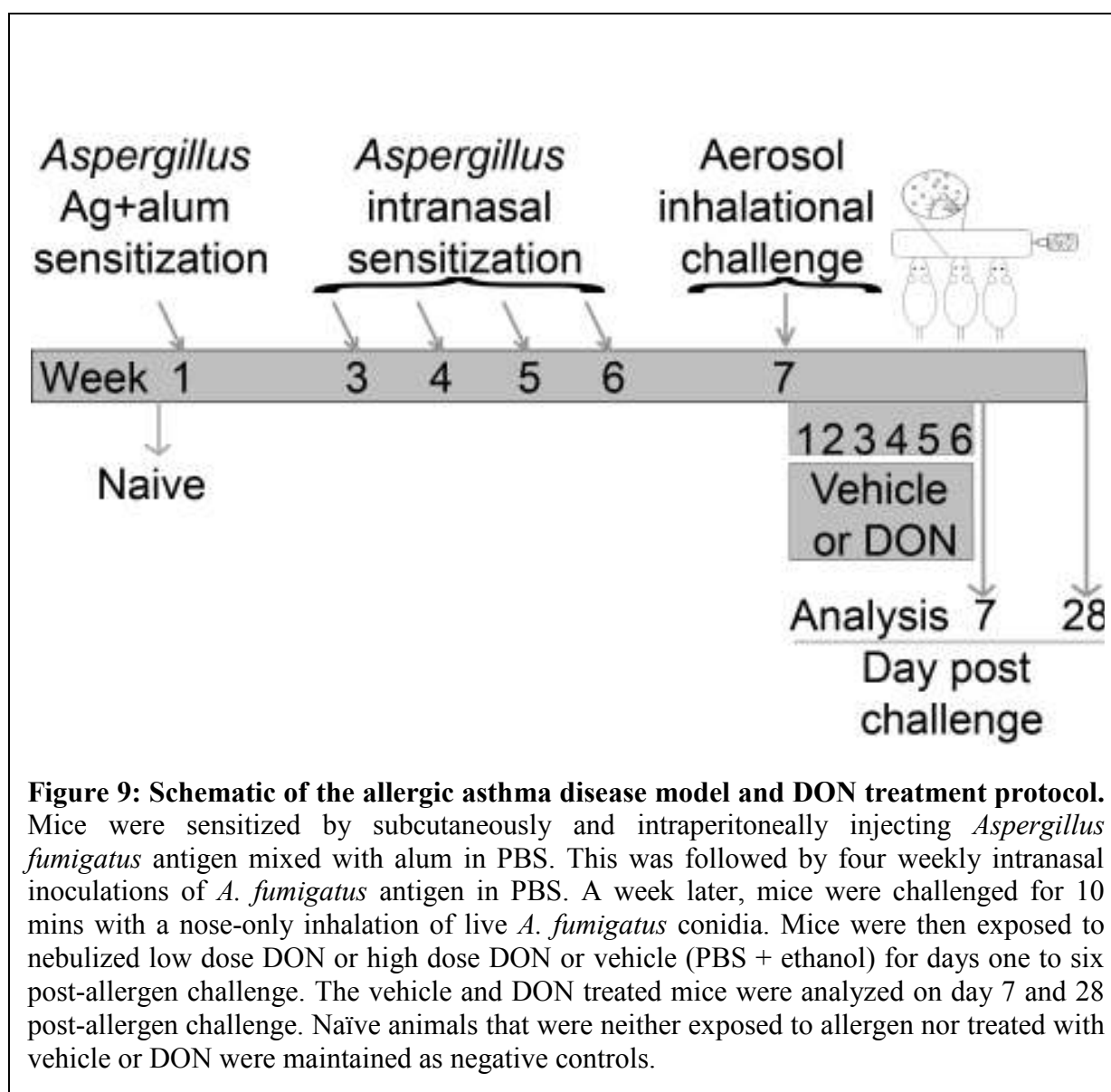
DON was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in biotechnology grade anhydrous ethanol (Amresco LLC, Solon, OH, USA) at a concentration of 10 mg/ml. For inhalation exposures, DON was diluted in phosphate buffered saline (PBS) to obtain the desired concentrations.

Soluble *A. fumigatus* antigen was purchased from Greer Laboratories Inc. (Lenoir, NC, USA), and lyophilized fungal culture stock (strain NIH 5233) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The fungal culture stock strain was reconstituted in 5 ml PBS, and 60 µl aliquots were stored at 4°C in the dark. For *A. fumigatus* cultures, one aliquot was spread onto sterile Sabouraud dextrose agar in a 25 cm² culture flask and incubated at 37°C for eight days. A different flask of *A. fumigatus* culture was used for each set of mice challenged with inhalational *A. fumigatus*, to ensure dose uniformity amongst treatment group. All experimental procedures utilizing *A. fumigatus* were conducted with prior approval from the Institutional Biological Safety Office of NDSU.

Allergic asthma disease model

The schematic of allergic asthma disease protocol used in the present study is depicted in **Figure 9**. Animals were sensitized and challenged as previously described [26]. Briefly, mice were sensitized by subcutaneously and intraperitoneally (IP) injecting 10 µg of soluble *A.*

fumigatus antigen (Greer Laboratories, Lenoir, NC, USA) suspended in 0.1 ml Imject® Alum (Pierce, Rockford, IL, USA) and 0.1 ml PBS. Two weeks after the injections, each mouse received a series of four, weekly 20 µg intranasal (IN) inoculations consisting of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) dissolved in 20 µl PBS. One week after the last IN inoculation, mice were anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) and subjected to a 10 min, nose-only inhalation of live *A. fumigatus* conidia, which constituted the allergen challenge. Mice were euthanized with sodium



pentobarbital (0.001 mg/kg; Butler, Columbus, OH, USA) on days 7 and 28 post allergen challenge. Serum, bronchoalveolar lavage fluid (BALF) and lung tissue samples were collected, as described below.

DON exposure via inhalation

The schematic of the allergic asthma disease and DON or vehicle treatment protocol is presented in **Figure 9**. After allergic sensitization and allergen challenge, mice were randomly divided into three groups and exposed to a nebulized low dose of DON (0.5 µg/ml nebulizer concentration), high dose of DON (1 µg/ml nebulizer concentration), or vehicle (PBS + ethanol) each day for days one through six after allergen challenge. For the nose-only exposure of allergic mice to aerosolized DON or vehicle, a bench top inhalation exposure system called *inExpose* (Scireq, Montreal, QC, Canada) fitted with an Aeroneb Lab Nebulizer Unit (pore size 4-6 µm; Aerogen Limited, Galway, Ireland) was used inside the fume hood. Mice were anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg) and restrained with soft wired restraints for a 10 min exposure to aerosolized DON or vehicle at a nebulization rate of 0.2 ml/min. Air was adjusted to a flow rate of 1000 ml/min as measured by an inline flow meter (Scireq, Montreal, QC, Canada). The inhaled dose was calculated using the following equation [2]: $ID (mg/kg) = C_{chamber} (mg/L) \times RMV (L/min) \times D (min) / BW (kg)$, where ID is the inhaled dose, C is the concentration of the substance in the exposure chamber, BW is the mouse body weight (0.03 kg), RMV is the respiratory minute volume for mice ($0.608 \times BW(kg)^{0.852}$) and D is the duration of exposure (10 min in this study). Using this equation, it was estimated that each mouse in the low and high DON dosage groups received a daily dose of 30 and 60 ng DON, respectively. Naïve animals that were exposed to neither allergen nor treated with vehicle or DON were maintained as negative controls in the study.

Serum and BALF antibody analysis

Approximately 500 μ l of blood was collected from each mouse by ocular bleed and centrifuged at 13 000 \times g for 10 min to yield serum, which was stored at -20°C until use. The trachea was cannulated, and 1 ml of sterile PBS was used to lavage the bronchoalveolar space of the mouse. BAL fluid (BALF) was separated from BAL cells by centrifugation at 7500 \times g for 10 min and stored at -20°C until use. Using a mouse isotype specific ELISA kit (Bethyl Laboratories Inc., Montgomery, TX, USA), serum samples diluted 1:1000 and BALF samples diluted 1:5 from each mouse were analyzed for IgA, per manufacturer's directions. Similarly, serum samples diluted 1:200 from each mouse were analyzed for IgE (BD OptEIA, San Jose, CA, USA), per manufacturer's protocols.

Cytokine analysis from lung homogenates

Superior and middle lobes of mice lungs were dissected, snap frozen in liquid N₂, and stored at -20°C until use. Prior to analysis, the lungs were homogenized in 2 ml of sterile PBS containing double strength complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and centrifuged at 600 \times g for 10 min. The supernatant was collected for cytokine analysis. IL-4, IL-5, and IL-12 levels were determined using commercially available ELISA kits (BD OptEIA, San Diego, CA, USA), as per manufacturer's directions. TNF- α levels were determined using DuoSet ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), as per manufacturer's directions. The detection limits for IL-4, IL-5, IL-12, and TNF- α were 7.8, 15.6, 62.5, and 31.3 pg/ml, respectively. Cytokine levels in the lung homogenates were normalized to total protein levels, which were determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Histological analysis

Whole left lungs were harvested and fixed in 10% neutralized buffered formalin. The lungs were paraffin-embedded and sectioned to 5- μ m thicknesses. These sections were affixed to microscope slides and stained with periodic acid Schiff (PAS) stain (Richard Allan Scientific Inc., Kalamazoo, MI) to assess mucus production and goblet cell metaplasia in the lung. Sections were mounted using xylene based non-aqueous mounting media (Richard Allan Scientific Inc., Kalamazoo, MI, USA) and visualized with bright field microscopy. A photometric analysis package Olympus MicroSuite™ (Olympus America Inc., Center Valley, PA) was used to analyze the histology images. The magenta-colored, PAS-positive, mucus-producing goblet cells were differentiated from normal columnar epithelial cells and percentages were calculated from 5 randomly-selected, 200 μ m segments of basement membrane in the lateral bronchial branches or small airways of mouse lungs for each group. The group mean was calculated from the individual means of each lung section.

Statistical analysis

All results are expressed as the mean \pm the standard error of mean (SEM). GraphPad InStat 3 software (GraphPad Software, Inc., LaJolla, CA, USA) was used to calculate statistics; differences between groups were tested with a two-tailed unpaired student's *t*-test with Welch's correction. In all cases, $p < 0.05$ was considered statistically significant.

Results

Allergic phenotype was unaffected with DON inhalation

Serum IgE is considered a hallmark of allergic asthma. Total IgE levels were determined in the serum of allergic animals exposed to the vehicle (PBS + ethanol), low dose DON (0.5

µg/ml), or high dose DON (1 µg/ml), via mouse isotype specific ELISA (**Fig. 10**). For each time point, every animal that was exposed to the allergen had significantly elevated serum IgE levels compared to the naïve controls (dashed line), reflecting a sustained allergic phenotype in these mice. Mice exposed to the vehicle, low dose DON, or high dose DON produced equivalent amounts of IgE at days 7 and 28 after allergen challenge, indicating that inhaled DON did not have an effect on serum IgE production (**Fig. 10**).

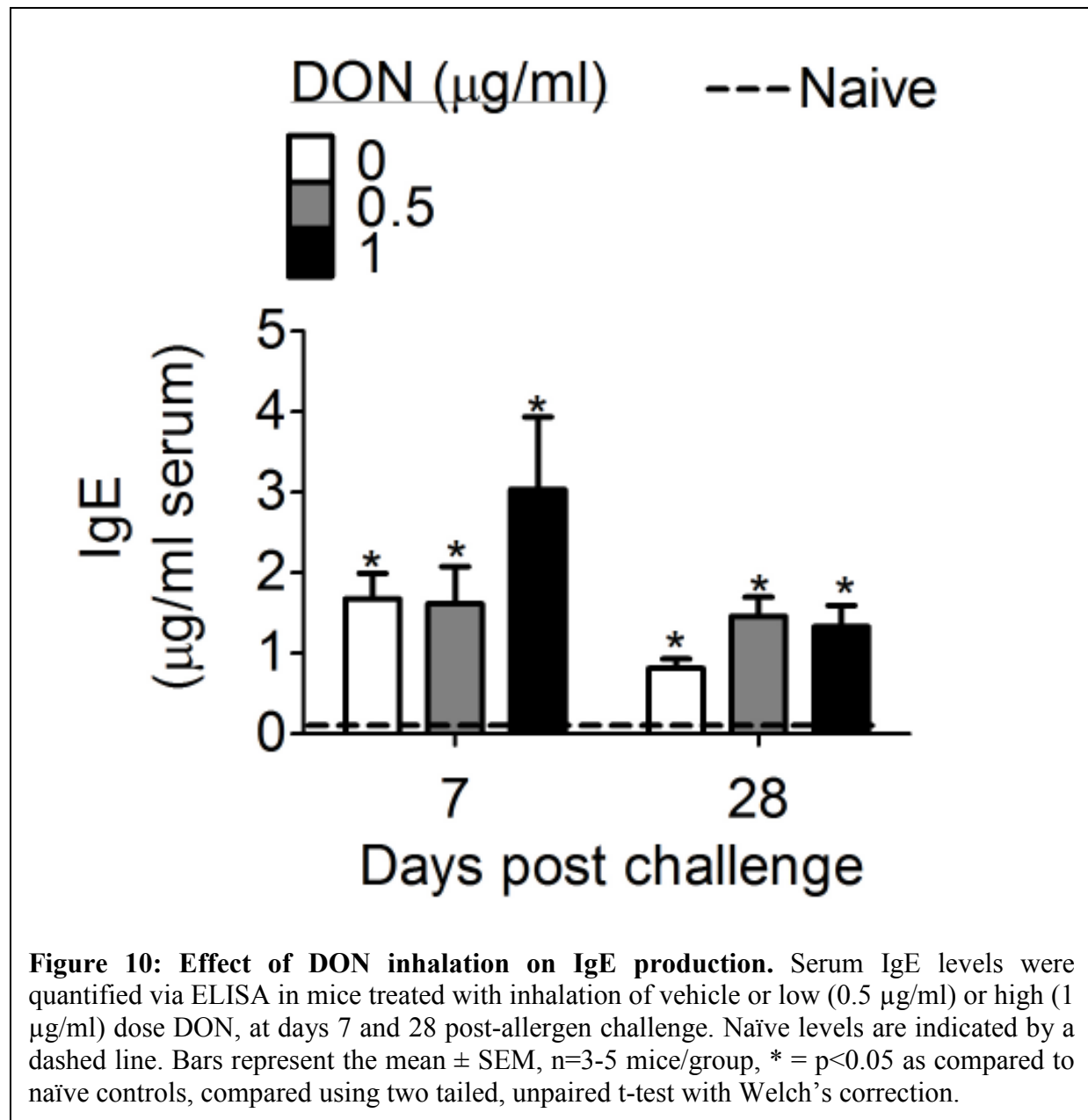
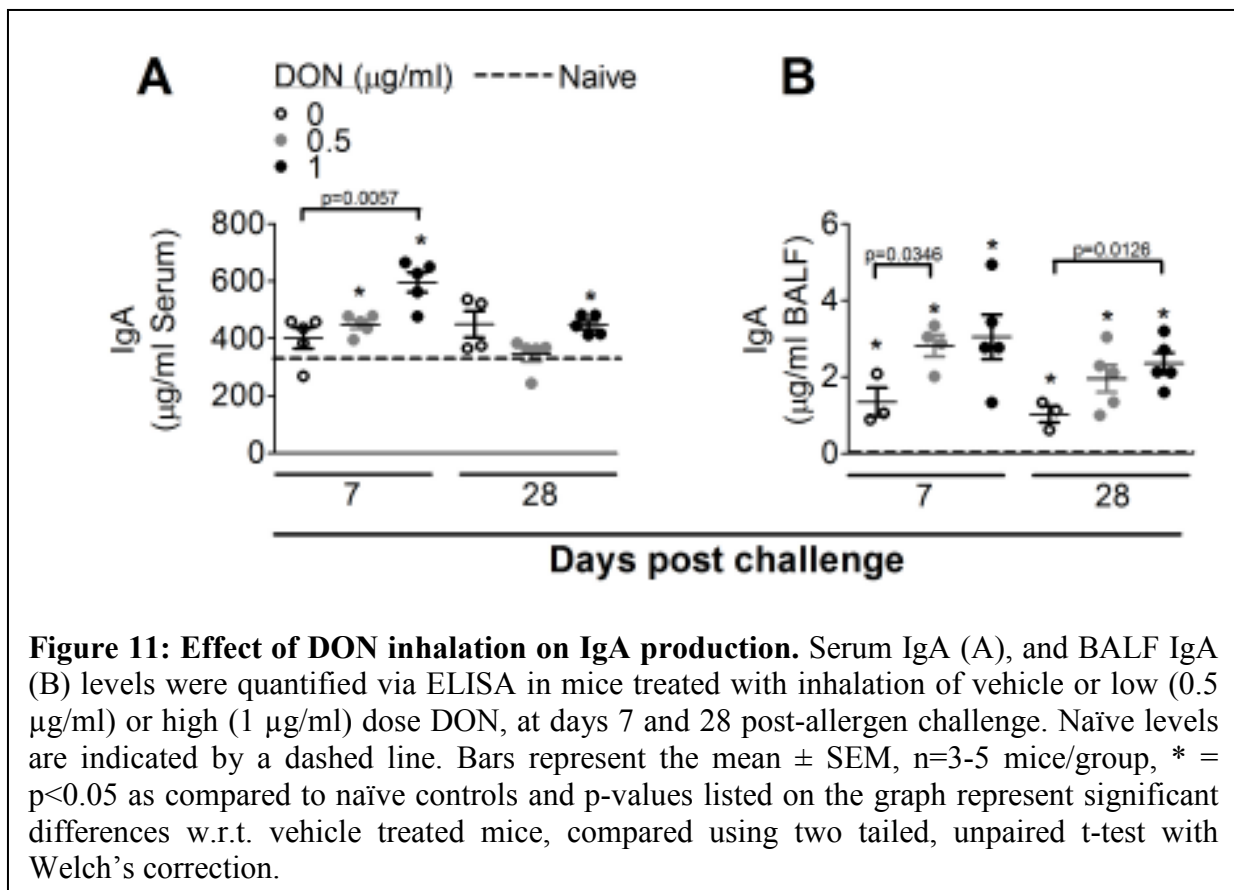


Figure 10: Effect of DON inhalation on IgE production. Serum IgE levels were quantified via ELISA in mice treated with inhalation of vehicle or low (0.5 µg/ml) or high (1 µg/ml) dose DON, at days 7 and 28 post-allergen challenge. Naïve levels are indicated by a dashed line. Bars represent the mean ± SEM, n=3-5 mice/group, * = p<0.05 as compared to naïve controls, compared using two tailed, unpaired t-test with Welch's correction.

DON inhalation leads to an increase in IgA in allergic mice

IgA is the most abundant antibody in mucosal secretions and undergoes transcytosis across the epithelium into the bronchoalveolar space. Dietary DON is known to upregulate serum and mesangial IgA in the mouse [54]. We investigated whether inhaled DON had any effect on IgA levels in the serum (**Fig. 11A**) and the BALF (**Fig. 11B**). While serum antibody levels reflect systemic humoral responses, BALF antibody levels indicate the amount of antibody in the local lung mucosa.



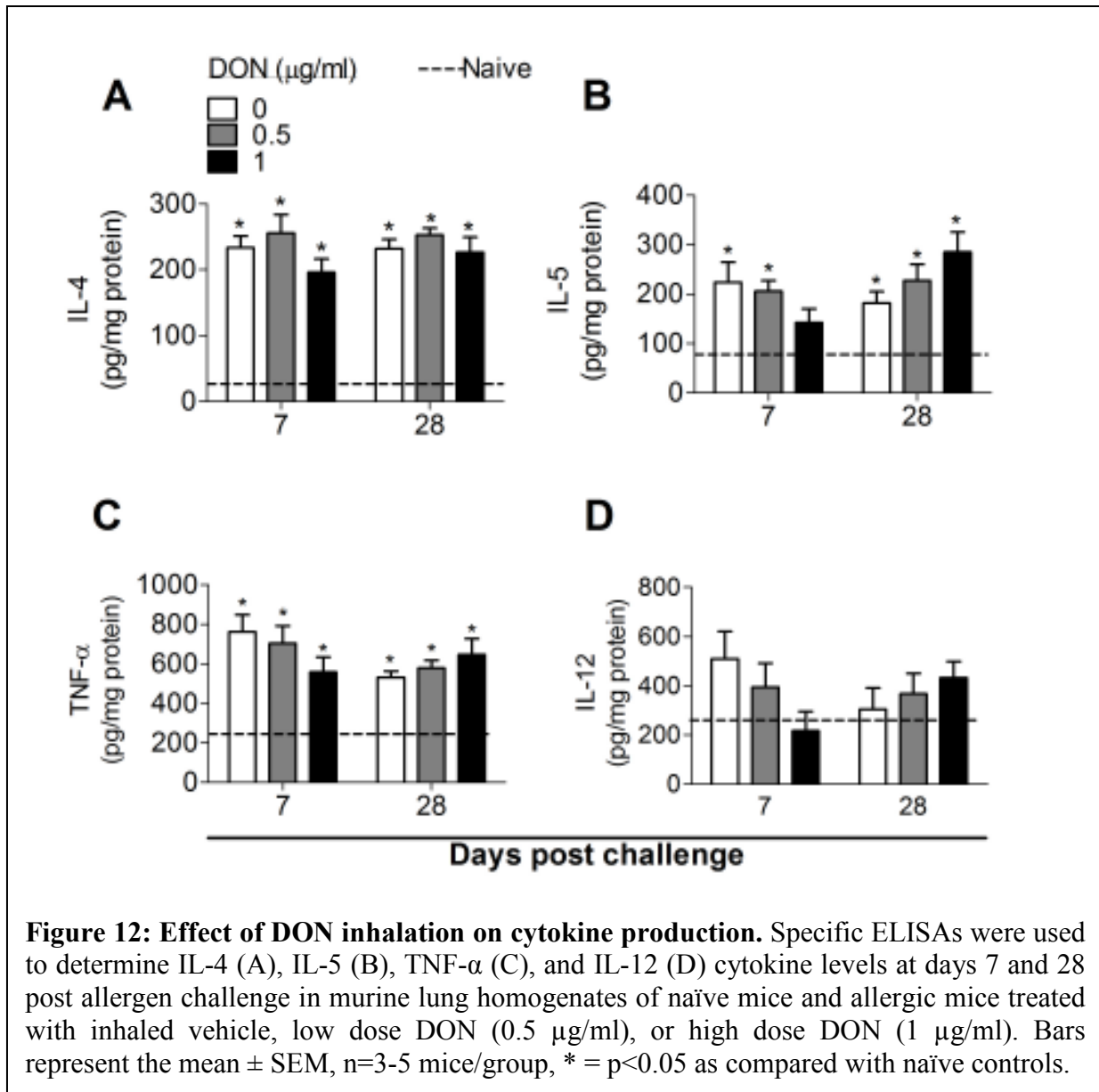
Vehicle treated mice had serum IgA levels comparable to naïve controls at days 7 and 28 post-allergen challenge, which shows that allergic sensitization and allergen challenge did not affect systemic IgA production. With DON inhalation, a dose-dependent increase in serum IgA was observed at day 7 but not at day 28 post challenge. Trend analysis performed using One-way

ANOVA with a post-test for linear trend, showed a significant linear trend for increase in IgA levels with increasing dose at day 7 after challenge ($p = 0.0006$) but not at day 28 (**Fig. 11A**). Mice exposed to a high dose of DON, but not low dose DON, had significantly increased serum IgA levels, compared to naïve mice or vehicle-treated mice at day 7 (**Fig. 11A**). These data suggests that inhaled DON can transiently increase systemic IgA production, in a dose-dependent manner.

Naïve animals had negligible IgA secreted into the BALF (dashed line) (**Fig. 11B**). Allergen sensitization and challenge resulted in increased BALF IgA levels, compared to naïve mice, and this increase was maintained until day 28 post-allergen challenge. Inhalation of DON showed a dose-dependent increase in BALF IgA levels. A significant linear trend was observed for increase in BALF IgA levels with increasing DON dose at days 7 ($p=0.0407$) and 28 ($p=0.0209$) post-allergen challenge as determined using One-way ANOVA with a post-test for linear trend (**Fig. 11B**). When compared with the vehicle exposed mice, BALF IgA levels in mice exposed to low dose DON were significantly increased at day 7, but not at day 28 (**Fig. 11B**). In contrast, mice exposed to high dose DON showed a significant increase in BALF IgA levels at day 28 but not at day 7 post-challenge. Overall, our data provides evidence that inhaled DON, like dietary DON, can lead to a dose-dependent and a sustained increase in local IgA.

DON inhalation did not affect the lung cytokine levels in allergic mice

After allergic sensitization and allergen challenge, vehicle- or DON-treated mice had elevated levels of IL-4 (**Fig. 12A**), IL-5 (**Fig. 12B**), and TNF- α (**Fig. 12C**), as compared to naïve controls (dashed line). An exception was observed in the mice exposed to high dose DON at day 7 post-allergen challenge where the IL-5 levels failed to reach statistically significant difference compared to naïve controls (**Fig. 12B**). The IL-12 levels (**Fig. 12D**) remained unchanged at days



7 and 28 post-allergen challenge. While IL-4 and IL-5 are the prototypical Th-2 cytokines, IL-12 is a necessary growth factor for Th1 cell development. The elevated levels of IL-4 and IL-5 and unchanged IL-12 levels suggest a Th2-dominant disease phenotype in these mice as a result of allergic sensitization and challenge. Compared to the vehicle treated mice, inhalation of low or high dose DON did not alter the cytokine profile established in the lungs of allergic mice (**Fig. 12**).

DON inhalation did not affect goblet cell metaplasia in allergic mice

Goblet cell metaplasia is a prominent symptom of allergic asthma, and mucus clogged airways result in decreased airflow [62]. Recent research has indicated that mucus secretion in response to inhaled antigens requires IL-6 [44], a cytokine elevated in response to DON treatment [57]. Therefore, we investigated the effect of DON inhalation on goblet cell metaplasia in lung sections stained with periodic acid Schiff stain (**Fig. 13**). At day 7 post-allergen

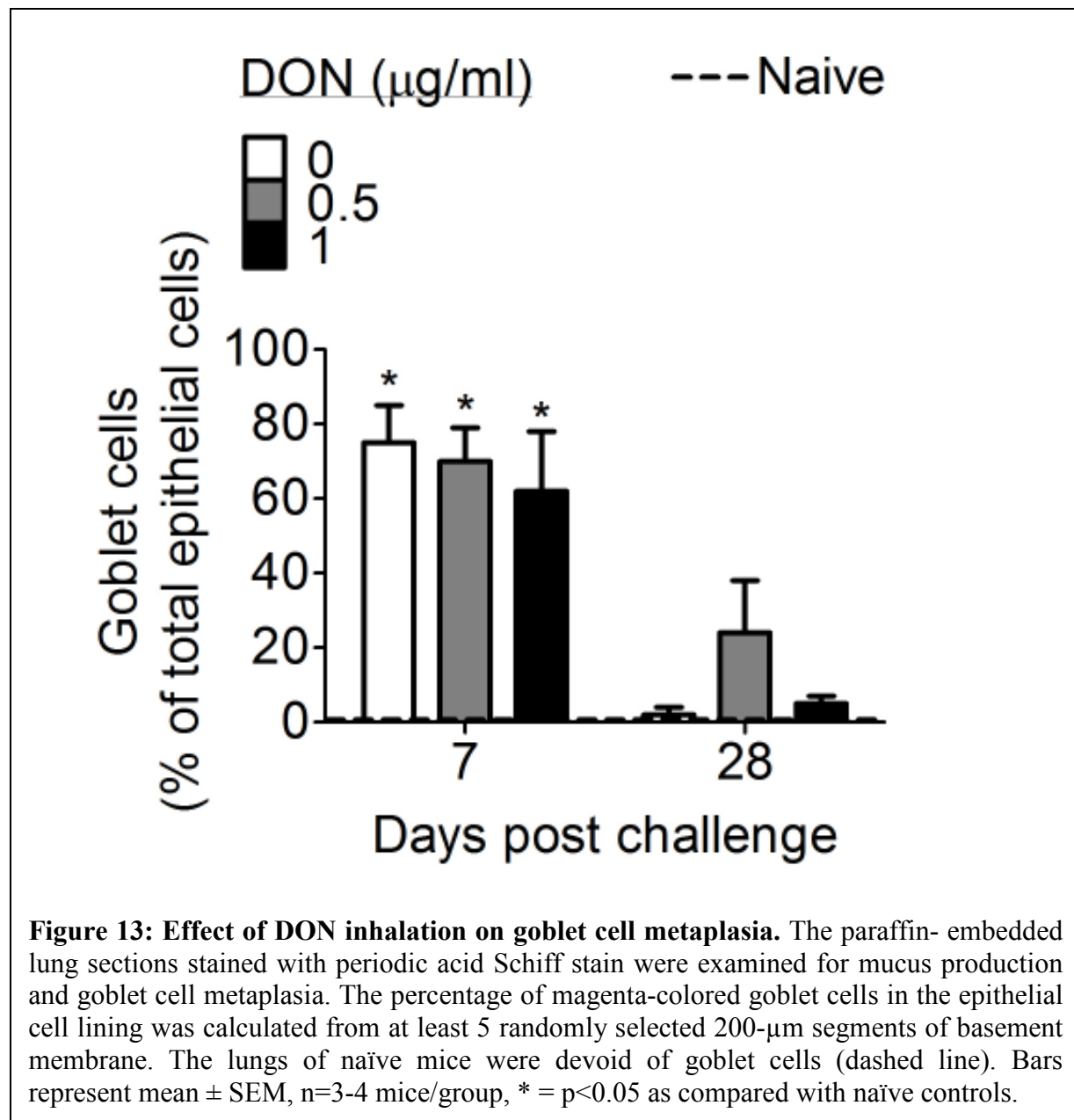


Figure 13: Effect of DON inhalation on goblet cell metaplasia. The paraffin- embedded lung sections stained with periodic acid Schiff stain were examined for mucus production and goblet cell metaplasia. The percentage of magenta-colored goblet cells in the epithelial cell lining was calculated from at least 5 randomly selected 200-µm segments of basement membrane. The lungs of naïve mice were devoid of goblet cells (dashed line). Bars represent mean \pm SEM, n=3-4 mice/group, * = p<0.05 as compared with naïve controls.

challenge, the percentage of cells that had undergone metaplasia from normal columnar epithelial cell to goblet cells was significantly elevated in vehicle- or DON-treated mice. By day 28, the percentage of goblet cells had returned to an equivalent of naïve animals'. Inhalation of low or high dose DON did not have any effect on goblet cell percentage at days 7 or 28 post-allergen challenge (**Fig. 13**).

Discussion

Although the immunological effects of dietary DON have been studied extensively in health and disease, to our knowledge, this is the first report in which the effects of inhaled DON in an allergic asthma model have been documented. The present study shows that the inhalation of DON in an experimental allergic asthma model elicits a dose- and time- dependent increase in local (BALF) and systemic (serum) IgA levels. However, at the tested dosages, inhaled DON did not have any effect on allergic asthma mediators such as serum IgE and cytokine levels or allergic asthma symptom such as goblet cell metaplasia.

Dietary DON was first shown to increase polymeric total serum IgA in the 1980s [21,49]. The gut mucosal lymphoid tissue is believed to be the predominant site of orally administered DON [12,50,51]. In the present study, we demonstrated that inhaled DON leads to a dose- and time- dependent increase in serum and BALF IgA levels. While the elevation in serum IgA levels was short lived, the increased BALF IgA levels persisted until three weeks after withdrawal of DON treatment (at day 28 post-allergen challenge). This suggests that lung associated mucosal tissue is the predominant site of inhaled DON mediated IgA production and/or accumulation. A transient increase in serum IgA at day 7 post-allergen challenge was observed. This could have resulted from spillage of the mucosal IgA into the systemic pool.

Alternatively, systemic absorption of DON via lung perfusion and its effect on Peyer's patch, spleen, or bone marrow could have resulted in increased serum IgA levels. In support of the later hypothesis, Amuzie *et. al.* found that intranasally inoculated DON is absorbed into the blood, and affects cytokine production in the lung and in the distant organs [3]. Furthermore, in an experimental respiratory reovirus infection model, orally administered DON led to an increase in IgA production in the gut, serum and BALF [36]. Taken together, this data suggests that while the mucosal immune system is the principal target site for DON mediated IgA production, the systemic IgA response is also elevated in response to inhalation or oral administration of DON.

DON-mediated IgA regulation was found to be dependent on increased cytokine (IL-2, -4, -5, and -6) production by CD4⁺ T cells [12,51,73] and/or IL-6 production by macrophages [75]. An indispensable role for IL-6 has been demonstrated since a dietary DON induced IgA increase was not observed in IL-6 deficient mice [53]. Although mechanisms involved in dietary DON mediated IgA production are well studied [54], there are no parallel studies for DON induced IgA production in the lung. The structural cells in the lung, such as alveolar macrophages and airway epithelial cells are likely to be a significant source of IL-6. Additionally, airway epithelial cells can secrete other cytokines involved in IgA production, namely IL-2, -4, -5, -10, and TGF- β [63]. Furthermore, epithelial cells produce a glycoprotein called secretory component, which confers stability against proteases to secretory IgA and allows polymeric IgA to be translocated from the basolateral side of epithelial cells into the bronchoalveolar space [63]. In the absence of inflammation in the lung, structural cells are likely to be the key players in regulating IgA production in the lung. By using inhalation as the route of DON administration in the present study, we ensured a direct interaction of DON with the lung mucosa. Therefore, we propose that DON's interaction with the structural cells in the lung play

an essential role in mediating DON's effects on IgA production, and will be the focus of future investigations.

An episode of allergic inflammation is characterized by infiltration of granulocytes and lymphocytes in the lung. Although the key features of allergic asthma, such as serum IgE, lung IL-4, IL-5 and goblet cell metaplasia were significantly elevated in comparison to naïve mice, we did not observe a robust inflammatory influx in the present study. The allergic inflammation was largely resolved by day 7 post-allergen challenge and completely absent by day 28 post-allergen challenge (data not shown). We have previously reported that a single *A. fumigatus* challenge, while sufficient to induce an allergic phenotype, is not sufficient to attain maximum eosinophilia or IgE [64]. Increasing the number of *A. fumigatus* challenges and using C57BL/6 strain of mice leads to a significantly increased inflammatory influx [26,64]. Nonetheless, in the absence of allergic inflammation, the present study highlights the importance of lung structural cells in maintaining increased BALF IgA levels until three weeks after the withdrawal of DON exposure (day 28 post-allergen challenge). Since the half life for producing IgA plasma cells is five days [37], the study suggests a continued effect of DON inhalation on IgA production at the lung mucosal surface.

The elevation in IgA production with inhaled DON is of significance for several reasons. First, IgA provides a first line of defense at mucosal surfaces against infection and protects the systemic immune system from potentially deleterious responses to innocuous antigens, which can otherwise culminate in inflammatory bowel disease or asthma [74]. In allergic individuals, the precise role of IgA remains inconclusive. Through its ability to neutralize aeroallergens, it is likely to protect against the development of asthma [65]. Contrastingly, IgA has also been shown to perpetuate allergic asthma symptoms via eosinophil degranulation [8,43]. Since DON

inhalation in agricultural settings is likely to be unavoidable and frequent, there is an immediate need to assess its potential to affect IgA mediated functions in health and disease. Second, elevations in serum IgA with dietary DON lead directly to immunopathological symptoms of IgA nephropathy [54]. Although mesangial IgA deposition was not examined in our study, our results identify a previously unidentified route of exposure (inhalation), which might result in DON-induced IgA nephropathy at higher dosages. Third, while IgA production in the gut has been well studied, the literature on lung IgA production is limited. Our study provides evidence that DON can be utilized to study *in vivo* IgA production and function at these functionally related but anatomically distinct mucosal sites (the gut and the lung).

Chronic feeding of DON has been shown to increase total serum IgE levels [52]. However, when tested for its adjuvant effect in an allergic asthma disease model, subcutaneously administered DON had no effect on serum IgE levels [28]. The difference in route of DON administration in the two studies suggested that mucosal associated lymphoid tissues might be crucial in mediating DON induced IgE response. Both gut associated lymphoid tissues [4,20] and respiratory mucosa [19,27,29,40] have been shown to contribute to IgE production. We hypothesized that inhaled DON would have a direct action on lung associated lymphoid tissues to influence IgE levels. However, lack of effect of inhaled DON on BALF and serum IgE levels in our study, implicates the exclusive sensitivity of GALT to DON's action in mediating IgE synthesis. The increased sensitivity of GALT to dietary DON's effect could be attributed to several factors including contribution from structural and immune cells present in the GALT or availability of dietary or microbial antigens, enzymes, or hormonal stimulus from the intestinal lumen. Additionally, disparities in dosage and exposure frequency to DON or mouse strain differences cannot be ruled out to account for the differences in IgE response.

DON's ability to regulate the immune response via its effects on the gene expression of cytokines and chemokines is well documented [25,55,57]. Several *in vitro* studies have shown that DON increases the production of the Th-2 cytokines, IL-4 and IL-5, in murine CD4+ lymphocytes or cloned thymoma cells [6,17,46,47,73], although reports from *in vivo* studies are limited. Similarly DON has been shown to upregulate TNF- α [3,14,56] and IL-12 levels [76,77]. However, in our study, we did not observe any change in cytokine levels with DON inhalation. Differences in our model systems (e.g. route, dose and duration of DON exposure or normal vs. allergic mice) may have shifted the window of detection for these cytokines.

The inhalation of large amounts of airborne dust generated during grain farming or processing is likely to be the major source of mycotoxin exposure amongst agricultural workers. Although several studies have reported DON concentrations in the grain dust samples, the concentrations hugely vary depending upon the type of agricultural work, season, methods used for drying the grain samples, time spent by grain samples in storage, extent of mold growth in the samples, and cereal species [45]. The maximum reported DON concentration from these studies ranges between 0.283-2.2 ng/mg grain dust [23,31,33,45] (Table 2).

Table 2: DON amounts likely to be inhaled in agricultural settings.

S.no.	Grain dust samples	DON concentration range in grain dust (ng/mg) [Ref.]	DON concentration at OSHA PEL for total dust (ng/m ³)	DON inhaled by a 70 kg grain handler over an eight-hour shift (ng)
1.	Wheat	0.46 [31]	6.9	25
2.	Barley, oats and spring wheat	0.34 [45]	5.1	18.3
3.	Barley, oats and spring wheat	2.2 [23]	33*	118.8
4.	Rye, barley, oats, buckwheat, corn	0.283 [33]	4.2	15.1

Occupational Safety and Health Administration (OSHA) has prescribed a permissible exposure limit (PEL) for total dust, which is 15 mg/m³ time weighted average over an eight-hour work shift [1]. Assuming that the total dust exposure remains within the OSHA PEL and using the maximum reported DON concentration in the grain dust samples, human tidal volume and respiratory rate of 0.5 L and 15 breaths/min. [69], we estimated that a 70 kg human is likely to inhale 15.1-118.8 ng of DON during an eight-hour shift (**Table 2**). Using the previously described method for calculating the dose delivered via inhalation [2], it was determined that in our study each mouse in the low and high DON dose groups would have received a daily dose of 30 and 60 ng DON, respectively.

Recently, 30 ng/m³ concentration of airborne substances was derived as the concentration of no toxicologic concern that is expected to pose no hazard to humans exposed continuously throughout a 70 year lifetime [18,25]. This concentration establishes the screening level for identifying inhalational hazards for human health. Even in the ideal situations, where the total dust exposure remains within the standards established by OSHA, the DON concentration in the total dust can exceed limits considered to be toxicological (asterisked DON concentration in **Table 2**). Several studies have shown that the mean concentration for total dust in agricultural environments often exceeds the OSHA PEL [24,32,41,70], resulting in DON exposure concentrations which significantly exceed the minimum toxicological levels. In order to establish a proof of concept, we have established the immunological effects of inhaled DON with only two tested DON dosages. Additional studies with higher dosages would be required for exposure specific risk characterization.

The observations of the present study must be viewed in the light of certain limitations, which are typical of inhalation studies. Significant differences occur in the anatomy and

physiology of mice and humans, which may impact the pharmacological outcomes. Most importantly, unlike humans, mice are obligate nose breathers, which may result in deposition and clearance of the inhaled dose in the nasopharyngeal region, besides in the trachea, bronchi and the lungs. Additionally, the inhaled dose measurements are difficult, compared to other routes of administration. The inhaled dose is a function of several parameters related to the delivery apparatus such as; flow rate, the type of nebulizer, nebulization rate and particle size. Due to the same reason, route-to route extrapolation of inhalational doses between studies is not possible. Large inter-subject variability may result from differences in the breathing pattern and lung geometry of individual mice.

Our study demonstrates the effect of inhaled DON on the allergic lung. To our knowledge, this is the first study where DON has been administered via inhalation in an allergic asthma disease model. Although, acute and chronic effects of dietary DON have been extensively studied, there is no data available to validate route-to-route (dietary to inhalation) extrapolation. Therefore, animal studies to investigate the effects of inhaled DON are critical to establish evidence based standards to regulate exposure. Since DON concentrations routinely exceed 30 ng/m³ in agricultural settings, grain handlers fall in the high risk category. Our study suggests that if they have an underlying allergic asthma disease, DON has the potential to influence the disease progression by dysregulating IgA levels. While our study demonstrates the effect of DON inhalation on IgA production in an allergic mice, further studies in non-allergic mice are necessary to establish if DON acts alone, or allergic sensitization and/or *A. fumigatus* challenge play a synergistic role. Epidemiological studies in humans will also play an important role in influencing future study designs.

Since DON is a non-volatile fungal product [66], the exposure occurs by inhalation of mold spores, mycelia fragments or contaminated substrates. Therefore, additional studies would be required to ascertain the synergistic effect of other immunomodulatory substances, such as other mycotoxins, endotoxins and β -glucans present in the moldy grain dust.

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CHAPTER 3: INHALED FLUTICASONE PROPIONATE DOES NOT PREVENT THE DEVELOPMENT OF ASTHMA SYMPTOMS, BUT INHIBITS AN INCREASE IN PULMONARY IgE IN A MURINE MODEL OF ASPERGILLUS FUMIGATUS INDUCED ALLERGIC ASTHMA⁴

Abstract

Inhaled corticosteroids (ICS) are the most commonly used anti-inflammatory drugs for the management of asthma symptoms. However, their clinical efficacy in fungus-associated asthma exacerbation remains variable. Fungus-associated asthma is a particularly severe type of asthma, probably due to the simultaneous activation of allergic and anti-fungal inflammatory pathways. Our objective in this study was to assess the extent of effect of nebulized fluticasone propionate (FP), a commonly prescribed ICS, in a murine model of live *Aspergillus fumigatus*-induced allergic asthma. The mice were sensitized with *A. fumigatus* extract and challenged with two inhalation exposures of live *A. fumigatus* conidia. The once-a-day FP treatment began during the sensitization phase and continued until one day before the pre-determined time point after the second fungal challenge. At the tested dosage, the FP treatment did not significantly reduce the number of inflammatory cells or goblet cell metaplasia. However, dynamic changes were observed in the lung physiology and antibody levels. Compared to the vehicle treated mice, there was a transient increase in AHR, total serum IgE, and IgG₁ in the FP-treated mice at day 3 post-second fungal challenge. However, at day 14 post-second challenge, an increase in

⁴ The material in this chapter was co-authored by Sumali Pandey, Sumit Ghosh, Scott A. Hoselton and Jane M. Schuh. Sumali Pandey had primary responsibility for designing the study, treating the mice, collecting and processing the murine samples, perform analytical experiments, analyze the data and prepare the manuscript. Sumit Ghosh helped with mice sensitization protocol. Scott A. Hoselton injected the mice intravenously for airway hyperresponsiveness analysis, cut lung sections for histology and proof-read the manuscript. Jane M. Schuh conceived the idea and proof-read the manuscript.

bronchoalveolar lavage fluid IgE was inhibited in FP treated mice. In conclusion, our model allowed us to investigate the temporal and spatial changes associated with ICS treatment in a murine model of fungus-induced allergic asthma. The observations are likely to help in making informed decisions in the therapeutic management of fungus-associated asthma.

Introduction

Asthma affects 235 million people worldwide and is associated with a large socioeconomic burden [5]. In the U.S., 56.3% of asthmatics have an underlying atopy with positive skin test reactivity to at least one allergen [4]. The respiratory symptoms in allergic asthmatics are mostly triggered by inhalation of aeroallergens such as: pollen, animal dander, house dust mite, cockroach antigen and fungi. Sensitization to fungi, defined as increased fungus-specific serum IgE and/or fungal colonization, is associated with a particularly severe form of asthma. This specific phenotype, called Severe Asthma with Fungal Sensitization (SAFS) [30], is difficult to manage therapeutically [31,92] and results in multiple hospitalizations [71], intensive care unit admissions [1], and asthma-related deaths [14]. While the relationship between fungal sensitization and severe asthma is poorly understood, it is hypothesized that the anti-fungal defense mechanisms might work synergistically with allergic pathways to result in a severe asthma phenotype [30].

Research has implicated several different fungi in SAFS, including *Aspergillus fumigatus* [72], which is ubiquitously present in the air and is routinely inhaled by humans. In addition to SAFS, *A. fumigatus* is associated with a number of pulmonary disorders such as allergic bronchopulmonary aspergillosis, rhinitis, allergic sinusitis, and hypersensitivity pneumonitis [24]. Besides being a potent allergen, *A. fumigatus* is an opportunistic pathogen. The extent of

colonization by the fungus varies in allergic disorders, and a competent immune system is critical in preventing invasive disease. In the normal lung, inhaled conidia are phagocytosed and subsequently killed by the resident alveolar macrophages (AMs) through oxidative burst mechanisms [54,79]. Neutrophils are recruited to the lungs quickly after and kill the fungi by the release of reactive oxygen, proteases, or granule proteins or through the formation of extracellular traps [17,62]. Neutrophils are absolutely essential in preventing an invasive disease by *A. fumigatus* [42,64,80,91]. Further protection against invasive disease relies on lymphocytes. While CD4 T lymphocytes have been shown to have a definitive function [21,22,45,82], the role of antibodies and B lymphocytes in defense against *A. fumigatus* remains inconclusive [23,43,66,82,100]. Nonetheless, the effective clearance of inhaled fungal spores relies on the activation of multiple pro-inflammatory pathways of the respiratory immune system. An alteration of these events, either due to a disease or drug therapy, may affect the fungal clearance and/or outcome of allergic asthma disease.

Inhaled corticosteroids (ICS) are the mainstay of asthma therapy, and their use has revolutionized asthma symptom management with marked reductions in morbidity and mortality rates. Due to their anti-inflammatory effects, ICS suppress asthma symptoms and prevent exacerbations [11]. The effects of corticosteroids (CS) are mediated through the glucocorticosteroid receptor- α and result in the activation of several anti-inflammatory genes (e.g. IL-10, I κ B- α , etc.) and repression of pro-inflammatory genes (e.g. NF- κ B, AP1, ICAM-1, VCAM-1, etc.). Consequent to decreased production of chemotactic mediators and adhesion molecules or induction of apoptotic pathways, CS reduce the number of inflammatory cells in the airways, including eosinophils, mast cells, T and B lymphocytes, and dendritic cells. Additionally, CS can abrogate the pro-inflammatory functions of structural cells in the lung such

as; macrophages [12,68], epithelial cells, endothelial cells and smooth muscle cells [8,9,28].

Unlike oral corticosteroids, the systemic absorption of ICS and consequent risk of opportunistic infections is low. However, prolonged use can potentially lead to infections with oral or respiratory pathogens. Indeed, the broad-spectrum, non-specific actions of ICS make them pertinent drugs for asthma management.

The role of ICS in fungus-induced allergic inflammatory disease is unclear. The purpose of this study was to investigate the extent of effect of a commonly prescribed ICS, Fluticasone Propionate, using our previously reported murine model of *A. fumigatus*-induced allergic asthma [53]. The model involves sensitization of a murine host to fungal proteins followed by a challenge with viable *A. fumigatus* spores (conidia) via inhalation. The allergic asthma disease model is unique as it mimics the natural route of human *A. fumigatus* exposure and is non-invasive, thereby allowing repeated inhalation of fungal conidia and ICS, as would be the case with humans. The intent of the study was to assess the efficacy of anti-inflammatory ICS treatment in the context of live *A. fumigatus* induced allergic inflammation.

Material and methods

Animals

Age matched C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a specific pathogen-free facility for the duration of the study. Mice were housed on Alpha-dri™ paper bedding (Shepherd Specialty Papers Inc., Watertown, TN, USA) in micro filter topped cages (Anicare, Bellmore, NY, USA) and were fed and watered *ad lib*. The study was conducted under the guidelines of the Institutional Animal Care and Use Committee of North Dakota State University (NDSU; Fargo, ND, USA).

Fungal antigen and conidia

Soluble *Aspergillus fumigatus* antigen was purchased from Greer Laboratories Inc. (Lenoir, NC, USA). The green fluorescent protein (GFP)-expressing *A. fumigatus* conidia, a kind gift by Dr. Margo Moore (Simon Fraser University, Burnaby, BC, Canada), were harvested from an 8-day-old mature culture suspended in phosphate buffered saline (PBS) and stored as aliquots at 4°C in the dark. In order to establish mature *A. fumigatus* cultures for fungal challenge via inhalation protocol, one 60-µl aliquot was spread onto sterile Sabouraud dextrose agar in a 25-ml culture flask and incubated at 37°C for 8 days. A different aliquot was used for each fungal culture to ensure an equal yield of mature conidia in each flask. All experiments utilizing *A. fumigatus* were conducted in a Class II biological safety cabinet and with prior approval from the Institutional Biosafety Committee of NDSU.

Aspergillus fumigatus induced allergic asthma

The schematic of allergic asthma disease protocol used in the present study is depicted in **Figure 14**. Animals were sensitized and challenged as previously described [53]. Briefly, mice were sensitized by subcutaneously and intraperitoneally (IP) injecting 10 µg of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) suspended in 0.1 ml Imject[®] Alum (Pierce, Rockford, IL) and 0.1 ml PBS. Two weeks after the injections, each mouse received a series of four, weekly 20 µg intranasal (IN) inoculations consisting of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) dissolved in 20 µl PBS. One week after the last IN inoculation mice were challenged with live GFP *A. fumigatus* conidia. For this purpose, mice were anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (25 mg/kg), placed supine with their nose in the inoculation port and subjected to 10-min inhalation of live GFP *A. fumigatus* conidia. The challenge was repeated two weeks later with live GFP *A. fumigatus*

conidia. The purpose of using GFP *A. fumigatus* conidia was to enable us to observe fungal outgrowth, if any, in the lungs of the mice treated with FP, using fluorescence microscopy. Pilot studies had indicated no differences in inflammatory profiles with the use of GFP *A. fumigatus* conidia, compared to wild type *A. fumigatus* conidia. A group of animals that was sensitized with *A. fumigatus* extract but neither challenged with live GFP *A. fumigatus* conidia nor treated with fluticasone propionate (FP) or vehicle (solvent for FP) were used as negative controls (day 0 time point).

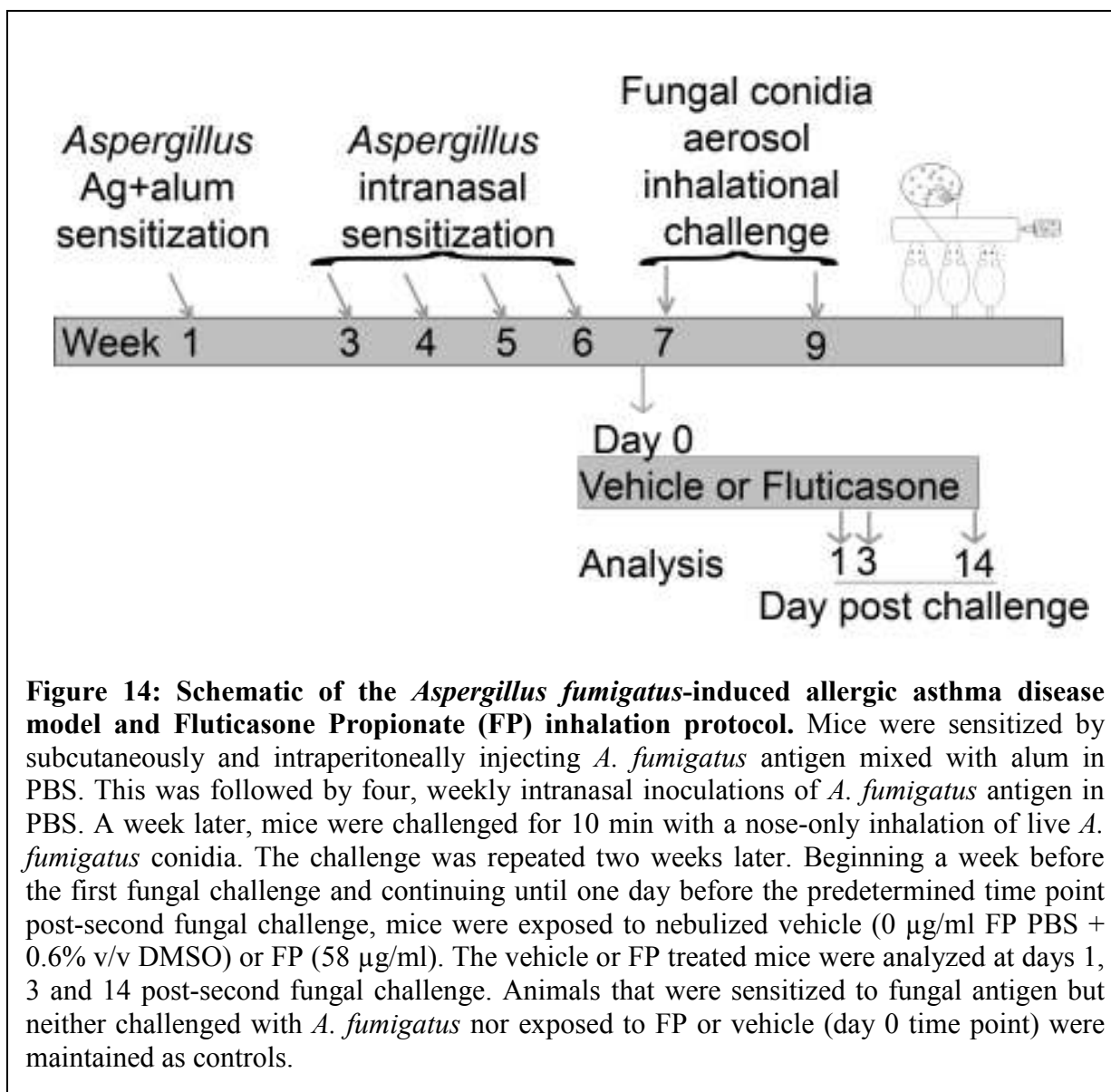


Figure 14: Schematic of the *Aspergillus fumigatus*-induced allergic asthma disease model and Fluticasone Propionate (FP) inhalation protocol. Mice were sensitized by subcutaneously and intraperitoneally injecting *A. fumigatus* antigen mixed with alum in PBS. This was followed by four, weekly intranasal inoculations of *A. fumigatus* antigen in PBS. A week later, mice were challenged for 10 min with a nose-only inhalation of live *A. fumigatus* conidia. The challenge was repeated two weeks later. Beginning a week before the first fungal challenge and continuing until one day before the predetermined time point post-second fungal challenge, mice were exposed to nebulized vehicle (0 µg/ml FP PBS + 0.6% v/v DMSO) or FP (58 µg/ml). The vehicle or FP treated mice were analyzed at days 1, 3 and 14 post-second fungal challenge. Animals that were sensitized to fungal antigen but neither challenged with *A. fumigatus* nor exposed to FP or vehicle (day 0 time point) were maintained as controls.

Fluticasone Propionate inhalation

FP is recommended in doses of 400-1000 µg/day for a 70-Kg adult human [41,51]. The 176-µg/kg/day intended mouse dose for inhalation was estimated as an equivalent of 1000 µg/day human dose using the body surface area normalization method, which takes several biology parameters into consideration, including oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function [86]. The inhaled dose (ID) can be calculated using the equation provided by Alexander D. J. *et. al.* [3]: $ID (mg/kg) = C_{chamber} (mg/L)/BW (kg) \times RMV (L/min) \times D (min)$, where C is the concentration of the substance in the exposure chamber, BW is the mouse body weight (0.035 kg), RMV is the respiratory minute volume for mice ($0.608 \times BW (kg)^{0.852}$) and D is the duration of exposure (10 min in this study). Using this equation and the intended mouse dose for inhalation (176 µg/kg), the concentration of FP in the chamber ($C_{chamber}$) was estimated. With the pump flow rate of 1200 ml/min and nebulizer rate of 0.4 ml/min, the concentration of FP desired in the nebulizer ($C_{nebulizer}$) to deliver $C_{chamber}$ dose was estimated using the formula: $C_{chamber} \times \text{pump flow rate} = C_{nebulizer} \times \text{nebulizer rate}$. $C_{nebulizer}$ for FP was estimated to be 58 µg/ml. A dose of 6 µg/ml used in our initial study did not significantly reduce the inflammatory cell count at day 3 post-second fungal challenge.

FP was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and stored at room temperature until use. Immediately before inhalation exposures, FP was diluted to 58 µg/ml in PBS and added to the nebulizer. For nose-only exposure of mice to nebulized FP or vehicle (PBS + 0.6% v/v DMSO), a bench top *inExpose* (Scireq, Montreal, QC, Canada) inhalation exposure system fitted with an Aeroneb Lab Nebulizer Unit (Aerogen Limited, Galway, Ireland) with a pore size of 4-6 µm,

was used inside a fume hood. Mice were restrained with soft wired restraints and were allowed to acclimatize for 5 min before exposure to vehicle or FP. A sealed chamber was maintained with the nares of the mice exposed to the nebulized FP in the chamber.

The mice sensitized to fungal extract were randomly separated into two groups and treated once daily with FP or vehicle. As depicted in **Figure 14**, the treatment began a week before the first fungal challenge and continued until one day before the pre-determined time point after the second fungal challenge. Previous studies using our allergic asthma model suggested that waves of inflammatory cells are recruited in the bronchoalveolar space of the lung post-second challenge with live *A. fumigatus* conidia. Peak leukocyte recruitment time points were selected so that the effect of inhaled FP on the recruitment of different cell types, including neutrophils (peaking at day 1 post-second challenge), eosinophils (peaking at day 3), and lymphocytes (peaking at day 28) could be evaluated. The FP treatment was started a week prior to the first fungal challenge in order to establish a prophylactic dosing regimen which was continued through the time when a leukocyte recruitment peak was expected. At these time points, the airway hyperresponsiveness (AHR) was measured and thereafter, the serum, bronchoalveolar lavage fluid (BALF), and lung tissue samples were collected.

Airway hyperresponsiveness

Mice were anesthetized with 0.001 mg/kg sodium pentobarbital (Butler, Columbus, OH), and the trachea was cannulated and connected to a Harvard pump ventilator (Harvard apparatus, Reno, NV). At days 1, 3, and 14 post-second fungal challenge, baseline airway responses were recorded, and peak AHR after intravenous methacholine (420 µg/kg) provocation was assessed using a Buxco whole-body plethysmograph (Buxco, Troy, NY), as previously described [49].

Morphometric analysis of leukocyte accumulation

After AHR measurement, 1 ml of sterile PBS was used to lavage the bronchoalveolar space of the mouse. The cell-free BALF was obtained by centrifugation at $7500 \times g$ for 10 min. While the BALF was stored at -20°C , the cellular fraction was resuspended in sterile PBS for morphometric analysis. The cells were cytopun (Shandon Scientific, Runcorn, UK) onto glass microscope slides, stained with Quik-Dip differential stain (Mercedes Medical; Sarasota, FL), and quantified based on morphology. Mean numbers of macrophages/monocytes, neutrophils, eosinophils, and lymphocytes were determined from five random high power fields (1000X) per sample.

Serum and BALF antibody analysis

Approximately 500 μl of blood was collected from each mouse by ocular bleed and centrifuged at $13000 \times g$ for 10 min to yield serum, which was stored at -20°C until use. The antibody levels were determined in the serum and BALF, as an indicator of systemic and lung mucosal (local) antibody levels, respectively. Total IgE and IgG₁ was determined using mouse specific ELISA kits, as per manufacturer's directions. The IgE ELISA kit was purchased from BD Biosciences (San Jose, CA), and the kit for IgG₁ ELISA was purchased from Bethyl Laboratories Inc. (Montgomery, TX). Diluted serum samples were used for IgE (1:250) and IgG₁ (1:5000) quantification. BALF samples were diluted 1:2 and used for IgE and IgG₁ quantification.

Histological analysis

The whole left lungs were harvested and fixed in 10% neutral buffered formalin. The lungs were paraffin-embedded and sectioned to 5- μm thickness. These sections were affixed to microscope slides and stained with hematoxylin and eosin stain (Dako North America Inc, Carpinteria, CA) to assess pulmonary inflammation and Periodic acid Schiff (PAS) stain

(Richard Allan Scientific Inc., Kalamazoo, MI) to visualize goblet cell metaplasia in the lung. Sections were coverslipped using xylene based mounting media (Richard Allan Scientific Inc., Kalamazoo, MI, USA) and visualized through bright field microscopy. Representative photomicrographs were obtained using a Zeiss Z1 AxioObserver inverted microscope (Carl Zeiss Microscopy LLC, Thornwood, NY). A photometric analysis package called Olympus MicroSuite™ (Olympus America Inc., Center Valley, PA) was used to obtain the percentage of magenta-colored, mucus-producing goblet cells to total columnar epithelial cells from 5 randomly selected 200- μ m segments of basement membrane in the lateral bronchial branches or small airways of mice lungs. Mean measurements for each lung section were calculated, and the percentage of goblet cells for a each group was obtained by taking the mean of the mean measurements from each mouse in its respective group.

Statistical analysis

All results are expressed as the mean \pm the standard error of mean. GraphPad Prism5 (GraphPad Inc., LaJolla, CA) was used to calculate statistics; differences between groups were tested with a two-tailed unpaired student's *t*-test with Welch's correction. In all cases, p-value < 0.05 was considered statistically significant.

Results

Fluticasone Propionate inhalation did not lead to fungal outgrowth in the lung

Several case studies in humans have reported the development of invasive *Aspergillosis* (IA) infection with inhaled fluticasone propionate (FP) [10,16,37,60,78]. However, in the current study, we did not observe any fungal hyphae growth on the hematoxylin and eosin (H&E) stained lung sections obtained from mice treated with inhaled FP for 3-5 weeks. Furthermore,

none of the mice treated with FP showed symptoms of an invasive disease or died during the study.

Fluticasone Propionate inhalation did not significantly reduce the inflammatory cell count in the lung

Leukocyte recruitment in the lung is a defining pathophysiological characteristic of asthma exacerbation. To investigate the anti-inflammatory effect of FP on *Aspergillus fumigatus* induced allergic asthma, mice were given a 1 mg/day human equivalent dose of nebulized FP via nose-only inhalation. The lungs of control mice (Day 0 controls, indicated by a dashed line in **Fig. 15**) that were sensitized to fungal extract but were neither challenged with live *A. fumigatus* conidia nor exposed to vehicle or FP treatment, had alveolar macrophages (AMs) as the predominant cell population and a negligible percentage of neutrophils, eosinophils and lymphocytes. Waves of leukocytes infiltrated the bronchoalveolar space of murine lung in response to live *A. fumigatus* conidia inhalation. While AMs remained a steady population in the lung (**Fig. 15A**), neutrophils peaked at day 1 (**Fig. 15B**), eosinophils peaked at day 3 (**Fig. 15C**) and lymphocytes peaked at day 14 (**Fig. 15D**) post-second fungal challenge. At days 1, and 14, the recruitment of AMs (**Fig. 15E**), neutrophils (**Fig. 15F**), eosinophils (**Fig. 15G**) and lymphocytes (**Fig. 15H**) was largely unaffected by the inhaled FP.

To further assess the effect of inhaled FP on inflammatory cell numbers, we analyzed the H&E stained lung sections from vehicle and FP treated allergic mice (**Fig. 16**). At days 1, 3 and 14, dense inflammatory pockets were observed around the bronchioles and blood vessels of mice from both the groups. At day 3 post-second challenge, a reduction in inflammation was observed around the small airways, however, the extent of the response varied from animal-to-animal in that group. Increase in vascular smooth muscle thickness following a double challenge of live *A.*

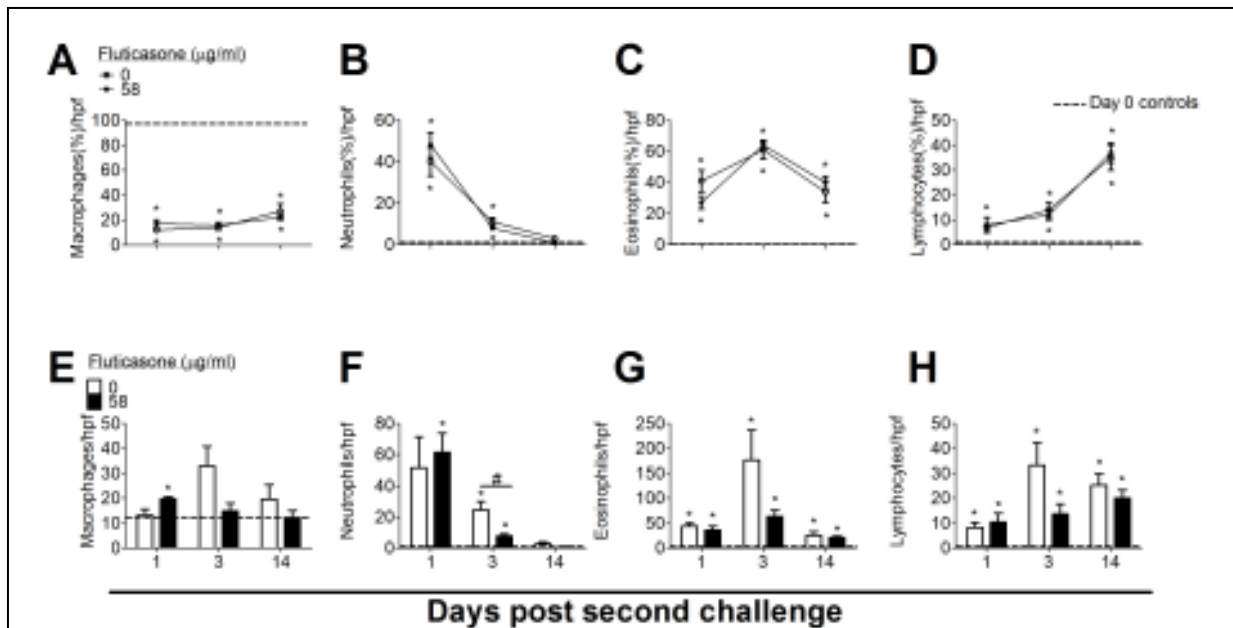


Figure 15: Effect of FP inhalation on differential inflammation in the bronchoalveolar lavage fluid (BALF). Mice were sensitized to fungal extracts, challenged with live *A. fumigatus* conidia and treated with nebulized vehicle or FP. Percentage (A-D) and counts (E-H) of macrophages (A, E), neutrophils (B, F), eosinophils (C, G) and lymphocytes (D, H) obtained in the BALF of allergic mice treated with vehicle (0 µg/ml FP; open circles or white bars) or FP (58 µg/ml; closed circles or black bars) were identified based on morphology and differential staining. Day 0 control mice values are represented by a dashed line. The results are represented as % of total cells per high power field (hpf; 1000X) (A-D) or mean cell number per hpf (E-H). Bars represent mean±SEM, n=4-6 mice/group. *, #, p value<0.05, as compared with day 0 control mice or mice exposed to vehicle, respectively.

fumigatus conidia, is a key feature of our allergic asthma model [90]. As observed on H&E stained lung sections, the increase in vascular smooth muscle thickness was unaffected by FP inhalation.

Fluticasone Propionate inhalation led to a transient increase in airway hyperresponsiveness without any effect on goblet cell metaplasia

Airway hyperresponsiveness (AHR) is a key symptom of asthma, and can be affected by inflammatory and structural changes in the lung [18]. Compared to vehicle treated or day 0 control mice, the inhalation of 1 mg/day human equivalent dose of FP resulted in a transient

increase in AHR (**Fig. 17A**), at day 3 post-second fungal challenge. The AHR was similar in both the groups at days 1 and 14 post-second fungal challenge.

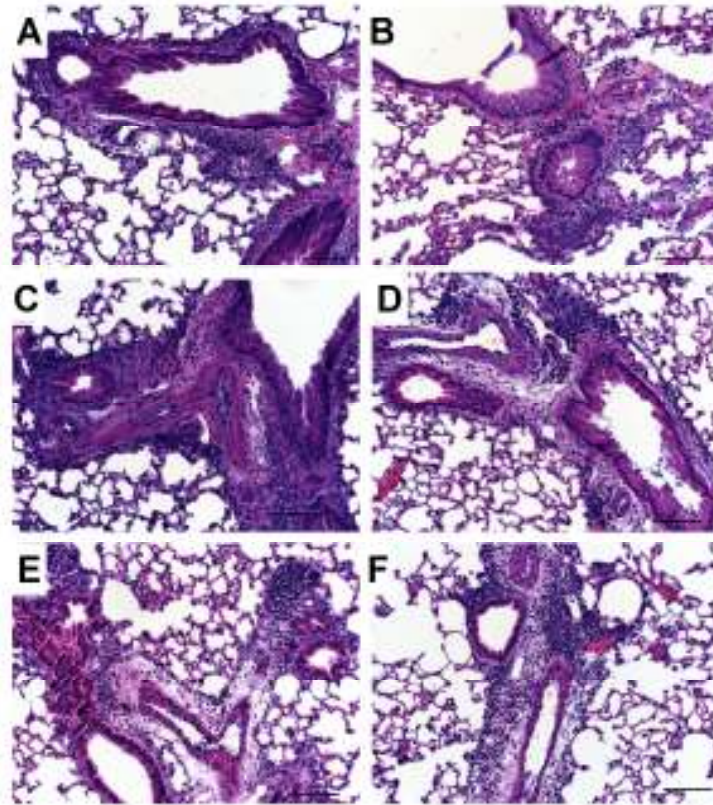
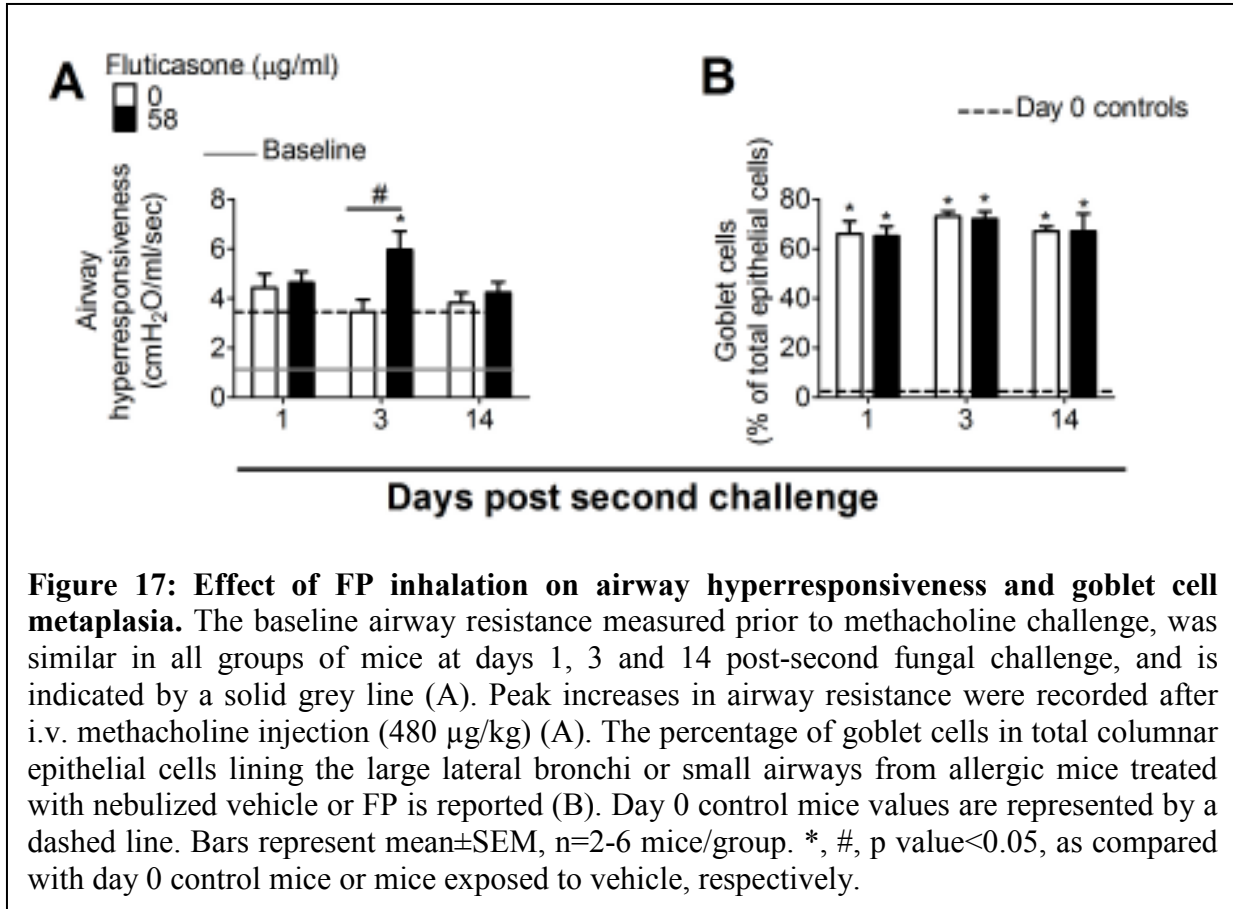


Figure 16: Effect of FP inhalation on the inflammatory cell recruitment in the peribronchovascular region of the lung. Representative photomicrographs of H&E stained lung sections of allergen challenged mice treated with vehicle (left panel) or FP (right panel) at days 1 (A and B), 3 (C and D), and 14 (E and F) post-second fungal challenge. The staining indicated insignificant reduction in peribronchovascular inflammation or perivascular smooth muscle hypertrophy in the lungs of FP treated mice. Day 0 control mice did not show any inflammation or smooth muscle hypertrophy (data not shown). Scale bars = 100 μ m.

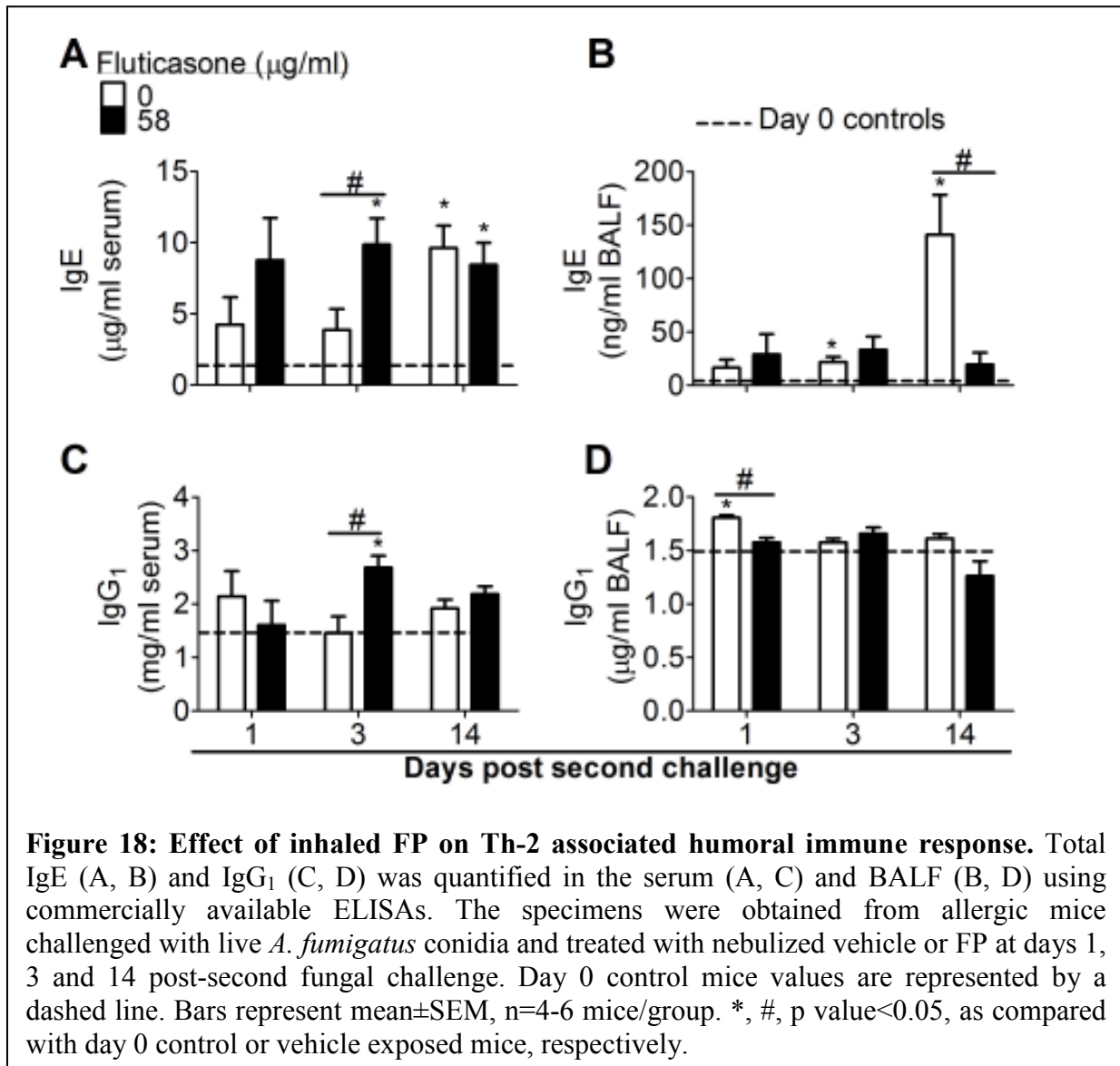
Excessive mucus production can lead to the physical obstruction of smaller caliber airways [88]. Therefore, we analyzed the Periodic acid-schiff stained lung sections for goblet cell metaplasia (**Fig. 17B**). Both vehicle and FP treated mice had significantly elevated goblet cell percentage interspersed in the columnar epithelial cell lining of the airways, at all the tested time points. FP inhalation did not have any effect on the goblet cell metaplasia in the murine lung.

Moreover, as previously described, an increase in vascular smooth muscle thickness was unaffected by FP inhalation. Taken together, these data suggest that the increase in AHR in FP treated mice did not correlate with enhanced structural changes in the lung as quantified by histological staining and measurements.



Fluticasone Propionate inhalation elicited dynamic changes in Th-2 associated antibody levels

Elevated serum IgE and IgG₁ are attributed to increased Th-2 cytokine levels [29,40] and are considered as the hallmarks of allergic asthma. Compared to the day 0 control mice (dashed line in **Fig. 18A and B**), no differences were observed in the antibody levels at day 1 post-second challenge. However, dynamic changes were observed with FP inhalation at day 3 and day 14 post-second fungal challenge. At day 3, there was a significant increase in serum IgE (**Fig. 18A**) and serum IgG₁ (**Fig. 18C**) levels in the FP treated mice compared to the vehicle treated



and day 0 control mice. While the serum IgG₁ levels receded back to the sensitization levels (day 0 control mice) by day 14 post-second fungal challenge (Fig. 18C), the serum IgE levels were significantly elevated in vehicle and FP treated mice, over day 0 controls (Fig. 18A).

While effects on serum antibody levels are likely to indicate the systemic effects of FP inhalation, the mucosal antibody levels, determined in the washings of bronchoalveolar space (BALF), are likely to suggest a local effect of inhaled FP and/or *A. fumigatus*. Compared to FP treated mice and day 0 control mice, vehicle treated mice showed an uninhibited increase in

BALF IgE levels, which started at day 3 and was prominent (~7 fold difference) by day 14 post-second fungal challenge (**Fig. 18B**). However, the FP treated mice did not show an increase in mucosal IgE levels at any timepoint, compared to the day 0 controls (**Fig. 18B**). The differences in BALF IgG₁ were not as dramatic as BALF IgE levels (**Fig. 18D**), but indicated a similar trend. Compared to the day-0 control mice, the BALF IgG₁ levels were significantly elevated in vehicle treated mice at day 1 post-second fungal challenge, but not in FP treated mice (**Fig. 18D**).

Discussion

Although the anti-inflammatory effects of fluticasone propionate (FP), a commonly prescribed ICS, are well documented, there is no experimental report to show its effects on fungus induced allergic inflammation. The present study shows that at the tested dosage, FP inhalation did not significantly reduce the inflammatory cell number in the peribronchovascular region or lumen of the lung in a murine model of live *Aspergillus fumigatus* induced allergic asthma. Similarly, a reduction in goblet cell metaplasia and perivascular smooth muscle hypertrophy was not observed in the histological staining. However, at day 3 post-second fungal challenge, there was a significant increase in airway hyperresponsiveness (AHR), total serum IgE and IgG₁. But, an increase in the bronchoalveolar lavage fluid (BALF) IgE and IgG₁ was inhibited in the FP treated mice.

Corticosteroids (CS) are the most potent and effective anti-inflammatory drugs currently available for asthma management [69]. They target multiple inflammatory pathways simultaneously, and exert their anti-inflammatory effects on structural as well as inflammatory cells in the airways. While their broad-spectrum immunosuppressive properties are beneficial for their efficacy in asthma symptom management, the risk for opportunistic infections is increased

with their usage. Prolonged use of ICS can potentially lead to infections with oral or respiratory pathogens. Oral candidiasis is the most commonly reported adverse effect with the use of ICS [69]. Additionally, reports of reactivation of tuberculosis [52,95], increased risk of severe pneumonia [39,97] and decreased clearance of *Klebsiella pneumoniae* [76] associated with the use of ICS, have also been published. Of particular interest to our lab, were the case studies documenting the development of invasive *Aspergillosis* (IA) infection with inhaled FP [10,16,37,60,78].

Systemic administration of corticosteroids is routinely employed for inducing a state of immunosuppression to develop murine experimental models of IA [6,98,99]. However, the effect of ICS on *A. fumigatus* induced lung infection and/or allergic inflammation in a murine model has never been documented. In the present study, hematoxylin and eosin (H&E) stained lung sections and slides prepared with BAL cells from FP treated mice did not show an outgrowth of *A. fumigatus* hyphae, and none of the mice treated with FP demonstrated any signs of illness during the treatment protocol. This protection against an invasive disease by *A. fumigatus*, despite FP inhalation, is in accordance with the fact that the inflammatory cell numbers in the peribronchovascular region and the lumen of the lung was not significantly reduced, compared to the vehicle treated mice, with the tested dosage. The lack of the effect of FP treatment on inflammatory cell counts is in accordance with the *Klebsiella Pneumoniae* infection study in mice. However, unlike our study, they reported increased mice mortality due to impaired clearance of the bacteria and reduced expression of infection induced cytokines [76]. Other studies in murine models have shown qualitative and quantitative differences in the effects of FP treatment on inflammatory cell counts, with reduction in all or just one cell type in the BALF [26,61,87,96,101,103].

Although the neutrophil count in the BAL sample was significantly reduced in the FP treated mice at day 3 post-second challenge, this finding is of nominal significance given the minor contribution of neutrophils to the inflammatory profile at this time point. Additionally, informed by the results of a publication by Dr. Borna Meharad's group, the role of neutrophils in *A. fumigatus* clearance in an allergic murine host is questionable. They showed that in contrast to naïve (non-allergic) mice depleted of neutrophils, the mice sensitized to *A. fumigatus* extract and then challenged with live *A. fumigatus* conidia (allergic mice) did not succumb to an invasive disease, and effectively cleared the fungi by day 7 post-inoculation [63,75]. These results suggest that in addition to neutrophils, allergy specific cells and mediators might play an important role in *A. fumigatus* clearance in the murine host. In this regard, evidence has been provided for the role of pro-allergy Th-2 cells [82] and eosinophils [81,109] in fungal clearance, and further investigation is warranted.

An interesting finding was a transient increase in AHR in FP treated mice compared to the vehicle treated mice, at day 3 post-second challenge. Contradictory results have been obtained for AHR measurements from other murine models of allergic asthma, where the variations exist in the type of inflammatory/infectious trigger, the type of measurements used to assess AHR, and whether FP was used alone or in combination with other drugs [87,96,103]. However, unlike the present study, none of them reported an increase in AHR with FP inhalation, suggesting a unique role of *A. fumigatus* induced inflammation and concurrent treatment with FP in increased AHR. The differences in pathophysiological changes observed with histological staining, including peribronchovascular inflammation, smooth muscle hypertrophy and goblet cell metaplasia; or inflammatory cell counts in the BALF, were insignificant to explain the significant increase in AHR in FP treated mice at day 3. At the least,

a conclusion can be made that FP inhalation was ineffective at preventing an asthma exacerbation in fungus associated asthma and may in fact result in transient worsening of the symptoms in response to fungus inhalation.

While multiple independent and additive pathways can contribute to an increase in AHR [47,105,107], we hypothesize that a direct interaction of inhaled FP, alone or in combination with viable *A. fumigatus*, with the structural and/or immune cells, may lead to increased immune system activation and/or damage thereby leading to increased AHR. Although overt fungal growth was not detected as previously described, the possibility of having increased fungal/antigen load due to uncleared fungus, in the presence of the anti-inflammatory effects of FP, at this time point, cannot be ruled out. In addition to immune activation, damage to the structural cells, particularly to the epithelial cells, and subsequent remodeling events may also lead to increased AHR in the lung [18,50]. The effects of FP on apoptosis of inflammatory cells is well documented [8]. Additionally, *in vitro* [33,104] and mice experiments [34] have provided evidence for epithelial cell apoptosis with the use of ICS, although these findings have been challenged in the context of the human population [20,44]. In contrast, *A. fumigatus* has been shown to inhibit the apoptosis of epithelial cells [13,38] and macrophages [102]. Given these findings, the overall effect of inhaled FP and *A. fumigatus* on apoptosis in the lung could not be predicted. In the present study, apoptosis was investigated by analyzing the levels of caspase 3 and cleaved caspase 3 expressions in the lung homogenates via western blot (data not shown). Although caspase 3 levels were detected in the lung homogenates from mice treated with vehicle or FP, we did not detect any cleaved caspase 3 in the samples from either group, suggesting an absence of detectable apoptosis via this method.

While ICS have revolutionized the management of airway inflammation and AHR in asthma patients, a controversy surrounding their use relates to their paradoxical effects [27,83,89]. CS induced anti-inflammatory effects are likely to shift the Th-1/Th-2 balance in the favor of a pro-allergic Th-2 type by suppressing IFN- γ , (inhibitor of IL-4 mediated Th-2 differentiation), or IL-12 (promoter of Th-1 cytokine expression) production and IL-12R function [7,36,84,106]. Additionally, CS can promote the synthesis of pro-allergic IgE antibody isotype, by increasing the expression of CD40L (second signal required for isotype switching) expression in the presence of IL-4 [7,83,89,106]. The first reports for CS mediated transient increases in serum IgE in allergy/asthma patients were published in 1978 [94,110]. Since then, *in vitro*, *ex vivo* and *in vivo* studies have demonstrated the CS mediated increases in IgE, and the effect varied from 2-1000 fold [2,48,59,106,108]. In contrast, the use of an ICS (FP) in nasal allergy patients resulted in the inhibition of mucosal IgE synthesis [19,32,35] and serum IgE [73]. The findings in humans have been replicated in certain murine models, where ICS use resulted in increased serum IgE and/or Th-2 cytokine levels [58,106]. Timing of CS introduction [106], route of administration [58] and timing of sampling [94], has been proposed to account for differences in the studies. While the controversy exists, in the present study we observed a transient elevation in Th-2 associated, serum IgE and IgG₁ in the allergic mice treated with FP inhalation at day 3 post-second fungal challenge. At day 14 post-second challenge, the serum IgE was elevated and similar in vehicle and FP treated group, but the BALF IgE was significantly decreased in FP treated mice compared to vehicle treated mice. The BALF IgG₁ showed a transient decrease at day 1 post-second challenge. Although the significance of dysregulated IgE with ICS use remains unresolved for clinical management of asthma, the observations in the present study do reproduce the published findings in humans, in an experimental murine model

of fungus induced allergic asthma. Lastly, it is interesting to speculate that a transiently enhanced shift of Th-1/Th-2 balance in favor of Th-2 response, at day 3 post-second fungal challenge, resulted in increased Th-2 associated processes, namely AHR, serum IgE and IgG₁ and decreased Th-1 associated neutrophil counts.

The observations of the present study must be viewed in the light of certain limitations, which are typical of inhalation studies. Significant differences occur in the anatomy and physiology of mice and humans, which may impact the pharmacological outcomes [15,67,85]. Most importantly, unlike humans, mice are obligate nose breathers, which may result in deposition and clearance of the inhaled dose in the nasopharyngeal region, besides in the trachea, bronchi and the lungs. Additionally, the inhaled dose measurements are difficult, compared to other routes of administration. The inhaled dose is a function of several parameters related to the delivery apparatus such as; flow rate, the type of nebulizer, nebulization rate and particle size [25]. Moreover, large inter-subject variability may result from differences in the breathing pattern and lung geometry of individual mice [77]. Even in the clinical settings, the lung exposure via aerosol delivery is between 5 and 50% with the remaining being deposited in the oropharynx and subsequently swallowed [57,70]. Indeed, the inhaler technique can significantly impact the symptom control in asthma patients [65,74].

Although our dose selection for this study was based on rational grounds to mimic the maximum prescribed dose in humans, there is no way to accurately calculate the local versus systemic bioavailability or high versus low dose of inhaled FP for mice. In clinical settings, ICS dose is titrated to result in a reduction in inflammatory numbers. In the present study, a significant reduction in inflammatory cell numbers was not observed with the tested dosage,

which is in alignment with the equivocally established clinical efficacy of ICS in fungus associated asthma [2,46,55,56,93].

Albeit dose-dependent, differences in antibody levels observed with FP inhalation reproduce the published findings in humans. Nonetheless, in order to understand the dynamic host-fungus interaction in the changing physiology of an allergic lung treated with an anti-inflammatory ICS, animal models which closely mimic the normal human exposure are absolutely critical. Indeed, the fungus induced allergic asthma model used in this study mimics the human exposure allowing repeated inhalation of *A. fumigatus* spores and ICS. Moreover, it successfully reproduces the acute and chronic hallmarks of a fungus induced lung disease, and permit the examination of temporal and spatial interactions of cells and their mediators in the changing physiology of an allergic lung.

Overall, our results point towards a critical limitation in current asthma symptom management. While airway hyperresponsiveness identifies the asthmatic patients, the underlying inflammatory trigger (infectious or non-infectious) or the extent and nature of inflammatory processes are rarely measured in clinical settings. For instance, the diagnosis of fungus associated asthma and the extent of colonization in the lung is not only technically challenging, but also leads to varying extents of Th-2 predominant inflammation. Although ICS are considered as the gold standards for asthma symptom management, the symptoms are likely to return with subsequent allergen exposure in the absence of adequate prophylactic measures. The response to ICS can significantly vary with the type of underlying inflammatory processes. Therefore, identification of the primary etiologies and asthma phenotype is absolutely necessary in designing better therapeutics for asthma.

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GENERAL DISCUSSION

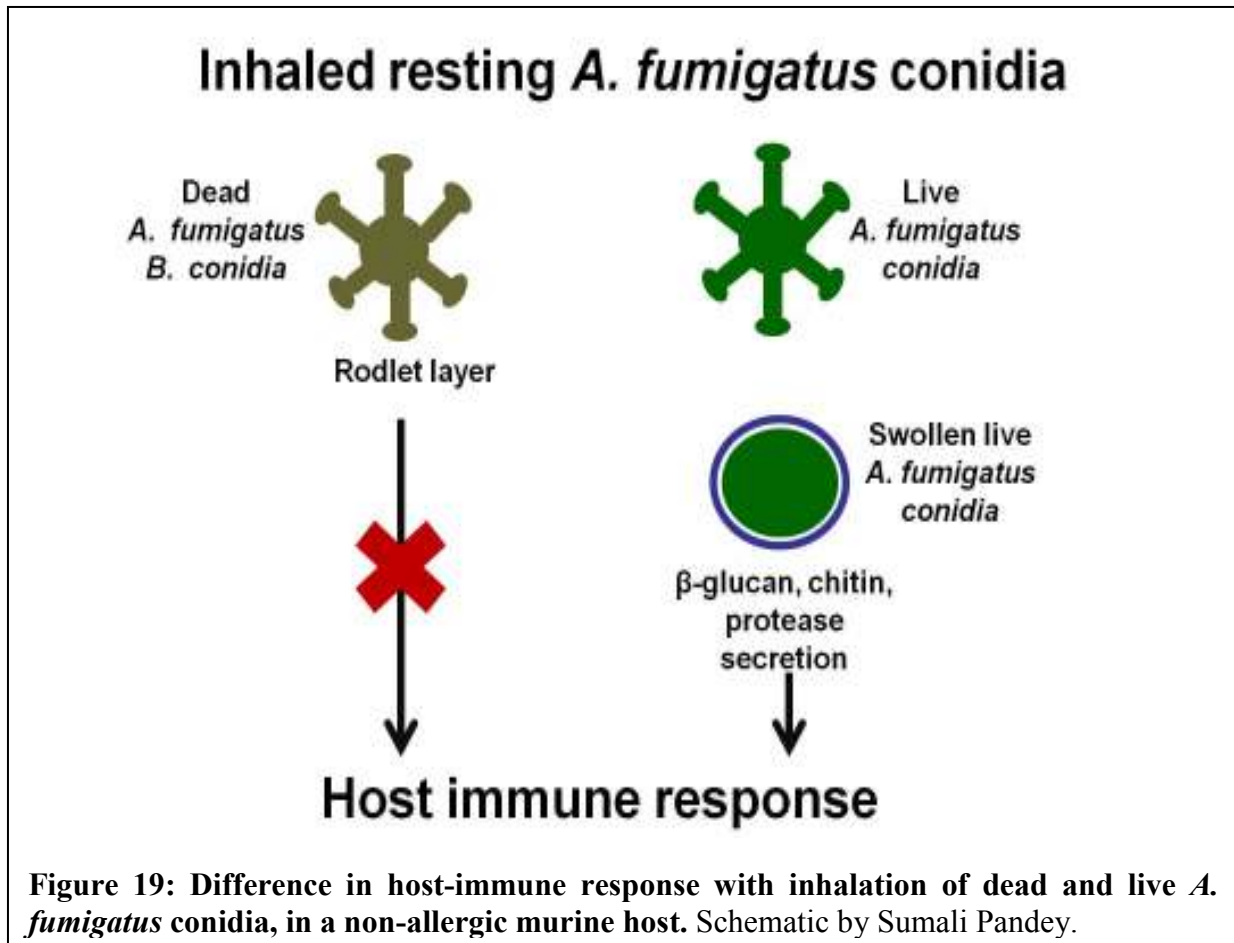
Although previous studies have addressed the immune related effects of *A. fumigatus*, deoxynivalenol (DON), and fluticasone propionate (FP), this research project was unique in utilizing inhalation as the route of delivery of these agents, and/or in investigating the effects of these immunomodulatory agents in an *A. fumigatus* induced allergic asthma disease model.

Our first objective in this project was to assess the ability of inhaled irradiation-killed and live *A. fumigatus* conidia to induce an allergic asthma disease. In the environment, exposure to both live and dead *A. fumigatus* spores occurs. However, previous studies comparing host-immune responses to live and dead (typically killed by autoclaving) *A. fumigatus* conidia are not only limited by an unnatural route of human exposure, but also exhibit variable levels of inflammation and Th-2 (pro-allergic) responses [6,108,160,323,339]. Moreover, reports comparing the pulmonary histopathological changes associated with live or dead *A. fumigatus* conidia are lacking. Our results demonstrated that in a naive murine host (without prior exposure to fungal antigens) live, but not dead *A. fumigatus* conidia, have a greater tendency to elicit acute and chronic symptoms of allergic asthma.

The differences observed with inhalation of dead and live *A. fumigatus* conidia in non-allergic mice, are pointing towards the emerging conclusion from several different studies that conidial swelling, the first step of germination, is essential in display of immunogenic surface moieties; such as chitin and/or β -glucan for the recruitment of eosinophils and neutrophils [6,160,333,415] (**Fig. 19**).

Since we administered dry, resting conidia, the swelling of live *A. fumigatus* conidia most likely occurred inside the murine lungs and triggered the recruitment of granulocytes. Dead *A. fumigatus* conidia, due to their incapability to swell and get rid of the immunologically inert

surface rodlet layer, probably provide insufficient antigens and/or PAMPs to trigger the immune response (**Fig. 19**). This phenomenon demonstrates that a non-allergic host focuses the pulmonary inflammatory response towards spores that carry an invasive potential. However, the dormant conidia, which are of no threat to the host, are cleared by alveolar macrophages, without launching a massive inflammatory event.



An acute response to live *A. fumigatus* conidia was characterized by neutrophils, eosinophils, macrophages, and antibodies such as IgA, IgG₁ and IgG_{2a} (**Fig. 20**). Although the role of antibodies and B lymphocytes in defense against *A. fumigatus* remains inconclusive [62,124,259,323,410], and is an active area of research in our lab [124], the importance of phagocytes (neutrophils and macrophages) in preventing an invasive disease is well

Acute

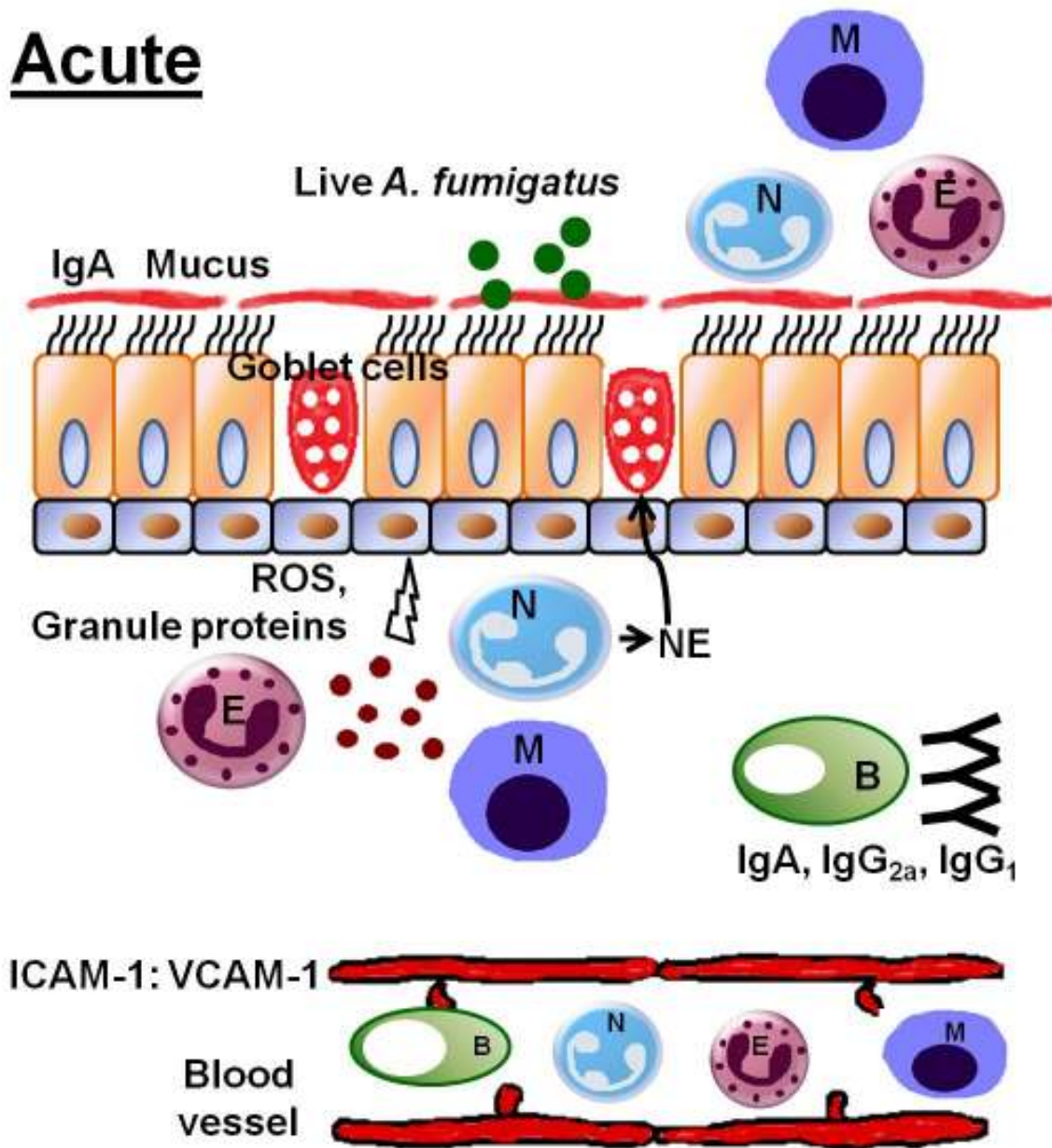
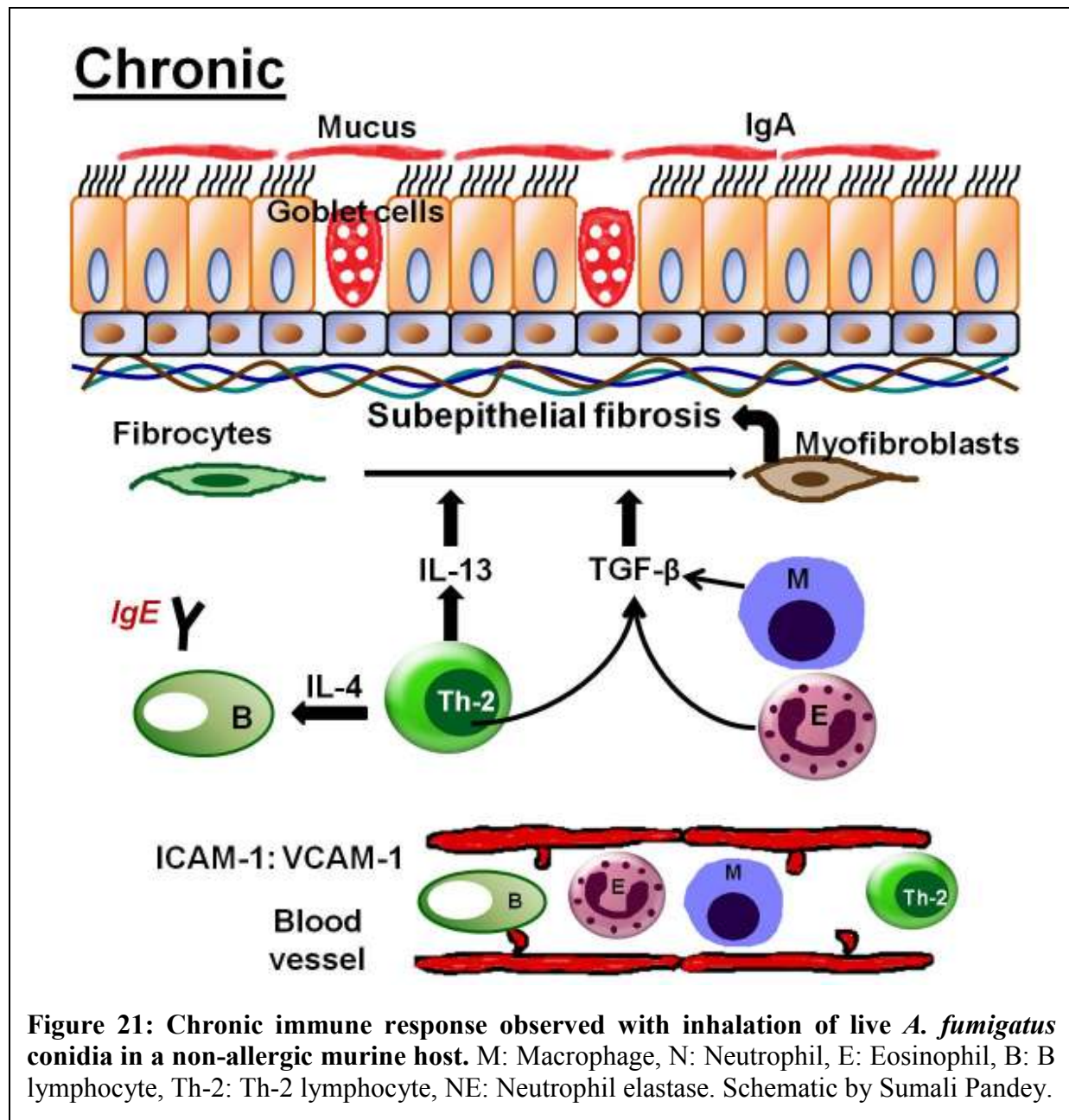


Figure 20: Acute immune response observed with inhalation of live *A. fumigatus* conidia in a non-allergic murine host. M: Macrophage, N: Neutrophil, E: Eosinophil, B: B lymphocyte, NE: Neutrophil elastase. Schematic by Sumali Pandey.

demonstrated. Research has shown, that in the normal lung, inhaled conidia are phagocytosed and subsequently killed by the resident alveolar macrophages (AMs) through oxidative burst mechanisms [175,315]. Neutrophils are recruited to the lungs quickly after and kill the fungi by release of reactive oxygen, proteases, or granule proteins or through the formation of

extracellular traps [50,245,365]. Neutrophils are absolutely essential in preventing an invasive disease by *A. fumigatus* [121,254,319,356]. Additionally, eosinophils have also been shown to play an essential role in fungal clearance [322,450]. However, research pertaining to the role of eosinophils in *A. fumigatus* infection, specifically, is lacking. Although the granulocytes and macrophages are critical in preventing *A. fumigatus* invasion, their recruitment in the peribronchovascular region can cause substantial lung tissue damage due to the release of reactive oxygen species and toxic granule proteins (**Fig. 20**).

In the present study, irrespective of the underlying allergic immune status, challenge with live, but not the dead conidia, led to increased collagen deposition. This correlated with increased granulocyte recruitment in response to live conidia challenge. While the role of neutrophils in collagen deposition is possibly limited to causing tissue injury, eosinophils, macrophages and Th-2 lymphocytes are likely to play an important role in pro-fibrotic repair, by regulating the recruitment, proliferation or activation of fibroblasts [444], as shown in **Figure 21**. Thus, it is possible that waves of leukocytes, with varying functions are recruited in the lung to orchestrate various phases of wound and pro-fibrotic repair after live *A. fumigatus* conidia challenge. In this regard, an interesting observation was that *ccl17* mRNA was up-regulated at day 7 post-fungal challenge with live, but not the dead, *A. fumigatus* conidia in non-allergic mice. CCL17 is an epithelium derived chemokine specific for CCR4⁺ cells, which is expressed on several cell types including Th-2 cells [295,348], and macrophages [33]. Additionally, fungal proteases have been implicated in up-regulating CCL17 mediated Th-2 response [203]. The up-regulated *ccl17* gene expression in live but not the dead *A. fumigatus* conidia challenged mice, correlated with increased fibrosis in these mice (**Fig. 21**). A reduction of bleomycin induced pulmonary fibrosis was observed with neutralization of CCL17 [33]. Given these findings, it is



tempting to hypothesize that up-regulation of *ccl17* at day 7 post live *A. fumigatus* challenge, marks the shift in host-immune response from a tissue damaging, acute inflammatory response to a pro-fibrotic repair response (Fig. 22), and a time-dependent neutralization of *ccl17* may result in reduced pulmonary fibrosis in this model.

The airway epithelial cells play an important role in removing the inhaled particles, by trapping them in the mucus and subsequently sweeping them away with the mucociliary

escalator. While largely considered as an innate immune response in healthy people, the Th-2 predominant immune response in asthma patients results in chronic mucus hypersecretion [77,434]. Indeed, metaplasia of airway epithelial cells into mucus producing GC is a common pathophysiological feature in asthma patients, and results in airway obstruction. In the present study, a non-allergic murine host challenged with live, but not the dead conidia *A. fumigatus* conidia showed significantly increased mucus producing GC. The finding suggested that mucus production was not only an innate immune response to particulate matter, but can be significantly affected with the viability status of the inhaled substance. This raises a question that which fungal associated factors might contribute to GC metaplasia in *in vivo* settings? Indeed, research has shown that proteases produced by *A. fumigatus* can induce a Th-2 dominant response [199,215], which may result in increased GC metaplasia. Furthermore, Oguma T. *et al.*'s *in vitro* study demonstrated that a potent serine protease activity in *A. fumigatus* was essential to induce the expression of mucin producing *Muc5ac* gene and mucin secretion in the bronchial epithelial cells [286]. While these studies have established a critical role for proteases in *A. fumigatus* induced mucus secretion, they have either utilized purified protease extracts and/or are *in vitro*. Therefore, present study demonstrates the importance of factors associated with viable, dry, aerosolized conidia (mimicking natural human exposure), and potentially an *in situ* production of proteases, in directly or indirectly promoting GC metaplasia.

The next question we asked was: does an allergic murine host, with a predominant Th-2 type immunity in place, respond differently to dead and live *A. fumigatus* conidia inhalation? In order to mimic an allergic state in the murine host, we exposed mice to fungal extracts via systemic and intranasal routes, before challenging them with live or dead *A. fumigatus* conidia. While fungal sensitization induced humoral response, these mice did not exhibit pulmonary

Acute to chronic

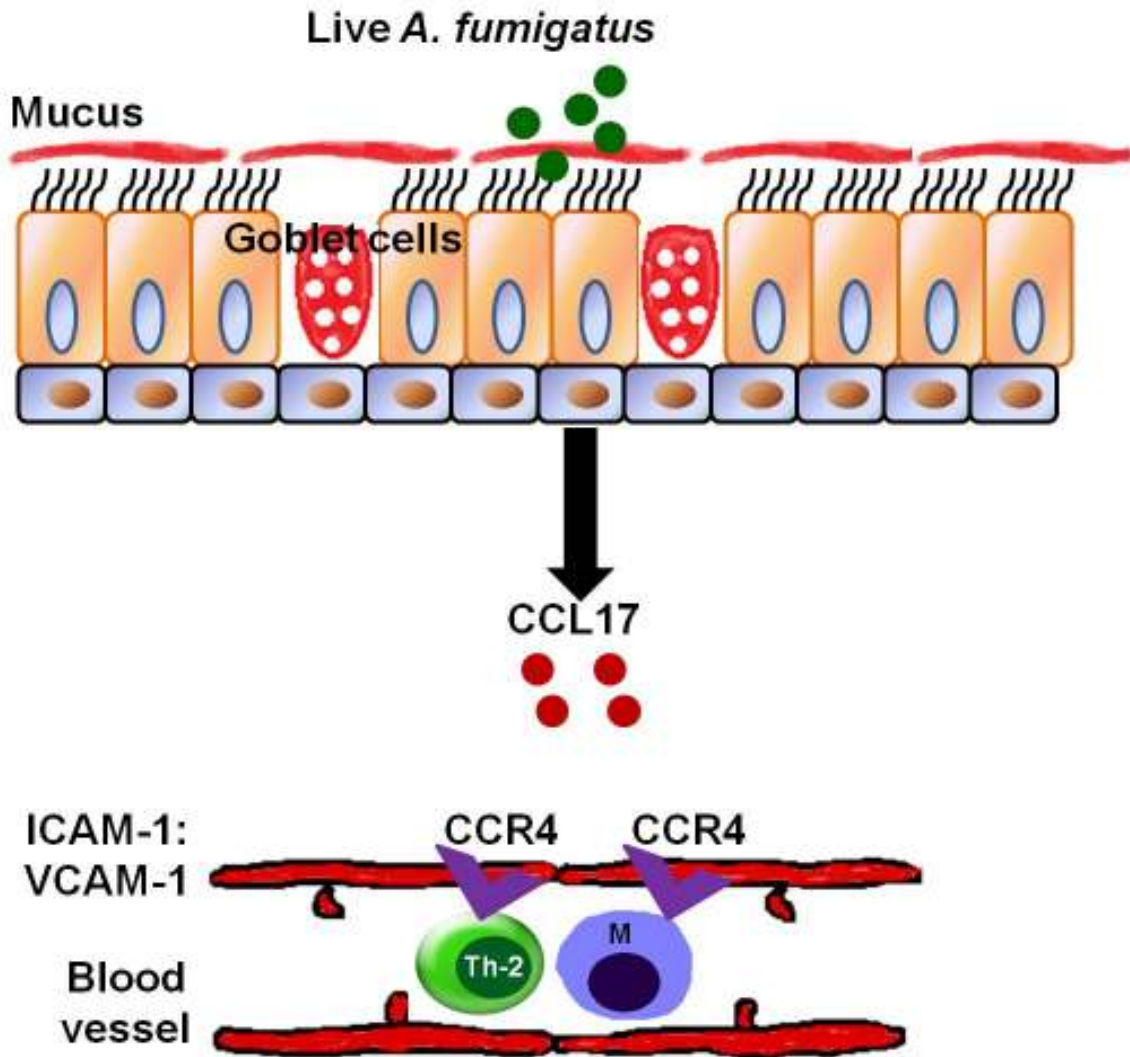
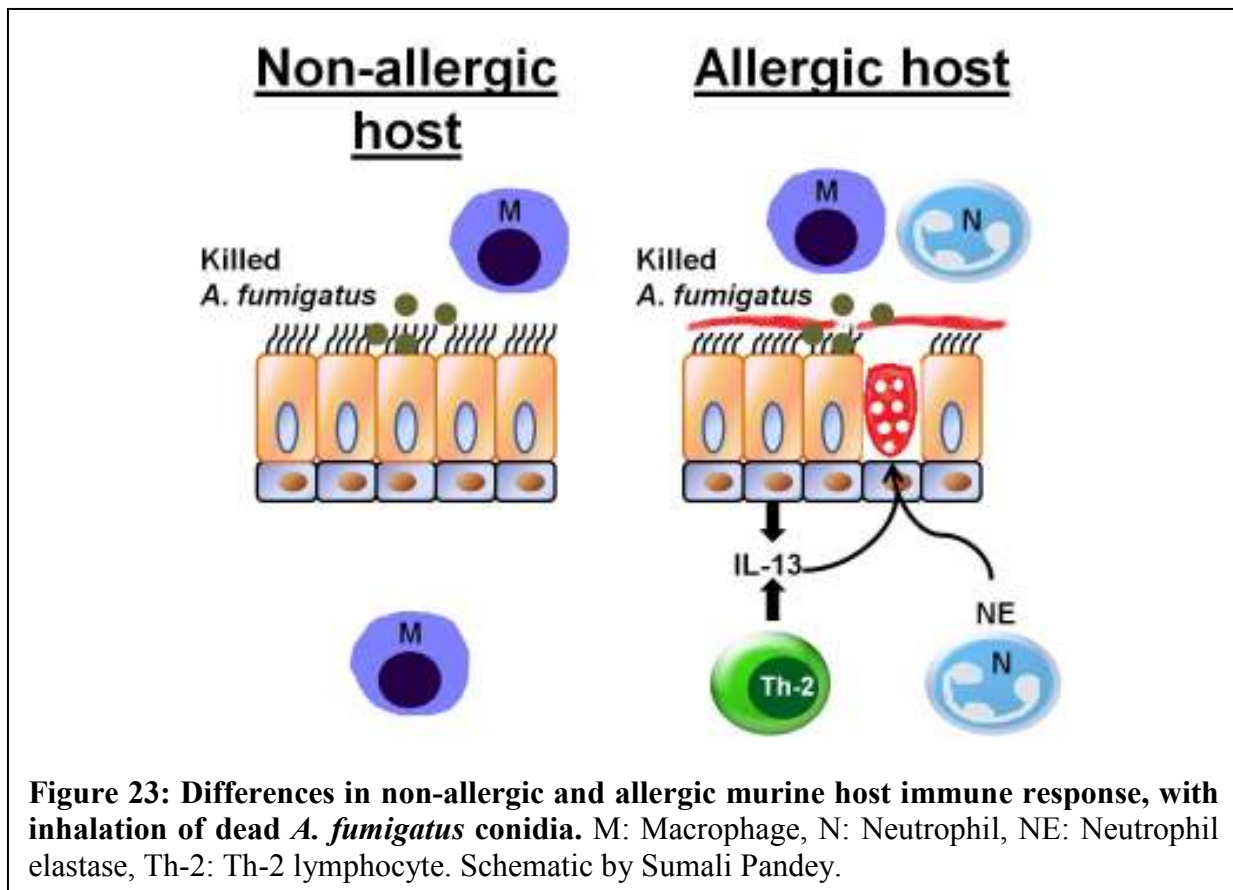


Figure 22: Proposed role for *ccl17*, in marking a shift from tissue-damaging acute response to pro-fibrotic chronic immune response, with inhalation of live *A. fumigatus* conidia in a non-allergic murine host. M: Macrophage, Th-2: Th-2 lymphocyte. Schematic by Sumali Pandey.

inflammation and airway remodeling changes, unless challenged with live or dead *A. fumigatus* conidia. This suggests that pulmonary inflammation and airway remodeling changes in the lung require a trigger with an inhaled substance, than just an elevated humoral antibody response in

place. A key difference between allergic and non-allergic host was observed with regard to inhalation of dead conidia (**Fig. 23**). Compared to non-allergic host, inhalation of dead conidia in an allergic host led to neutrophilic response similar to live conidia. Furthermore, a significant increase in GC metaplasia was observed in these mice, compared to their non-allergic counterparts (**Fig. 23**). Since infiltration of neutrophils, and eosinophils [101,279,424] or mucus plugging [59] of the airways may lead to asthma exacerbation [426], these findings indicate that an allergic host is substantially sensitive to develop asthma symptoms even with the inhalation of innocuous non-viable fungal spores, compared to their non-allergic counterparts.



Although the reason for neutrophil recruitment in response to dead *A. fumigatus* conidia, in an allergic host is not immediately apparent, it is important to note that sensitization alone did not recruit any neutrophils to the murine lung. Therefore, requirement for a conidial ligand

(Pathogen Associated Molecular Pattern; PAMP) seems necessary. Since both live and dead conidia recruited neutrophils in an allergic host, our observations suggest that the PAMP is independent of conidial swelling or metabolic state of the host in these mice. Our findings also suggest that β -glucan (displayed on swollen conidia)/dectin-1 independent signaling pathways might play an important role in neutrophil recruitment, in response to *A. fumigatus* conidia.

Another key observation was that, in an allergic murine host, GC metaplasia was significantly enhanced even with the inhalation of dead conidia. This suggested that, at least in our experimental settings, the pre-existing Th-2 predominant immunity in allergic mice was sufficient to partially compensate for the reduced GC metaplasia response observed with dead conidia challenge in non-allergic mice. Alternatively, neutrophil elastase has been shown to induce *Muc5ac* gene expression and mucus production [103,373,421]. Since elevated neutrophil recruitment and GC metaplasia levels correlated in allergic mice challenged with dead or live conidia, neutrophil elastase production might have contributed to increased GC% in these mice. Nonetheless, dead conidia inhalation served as a trigger for the induction of GC metaplasia, since no GC was observed in sensitized mice in the absence of conidia challenge.

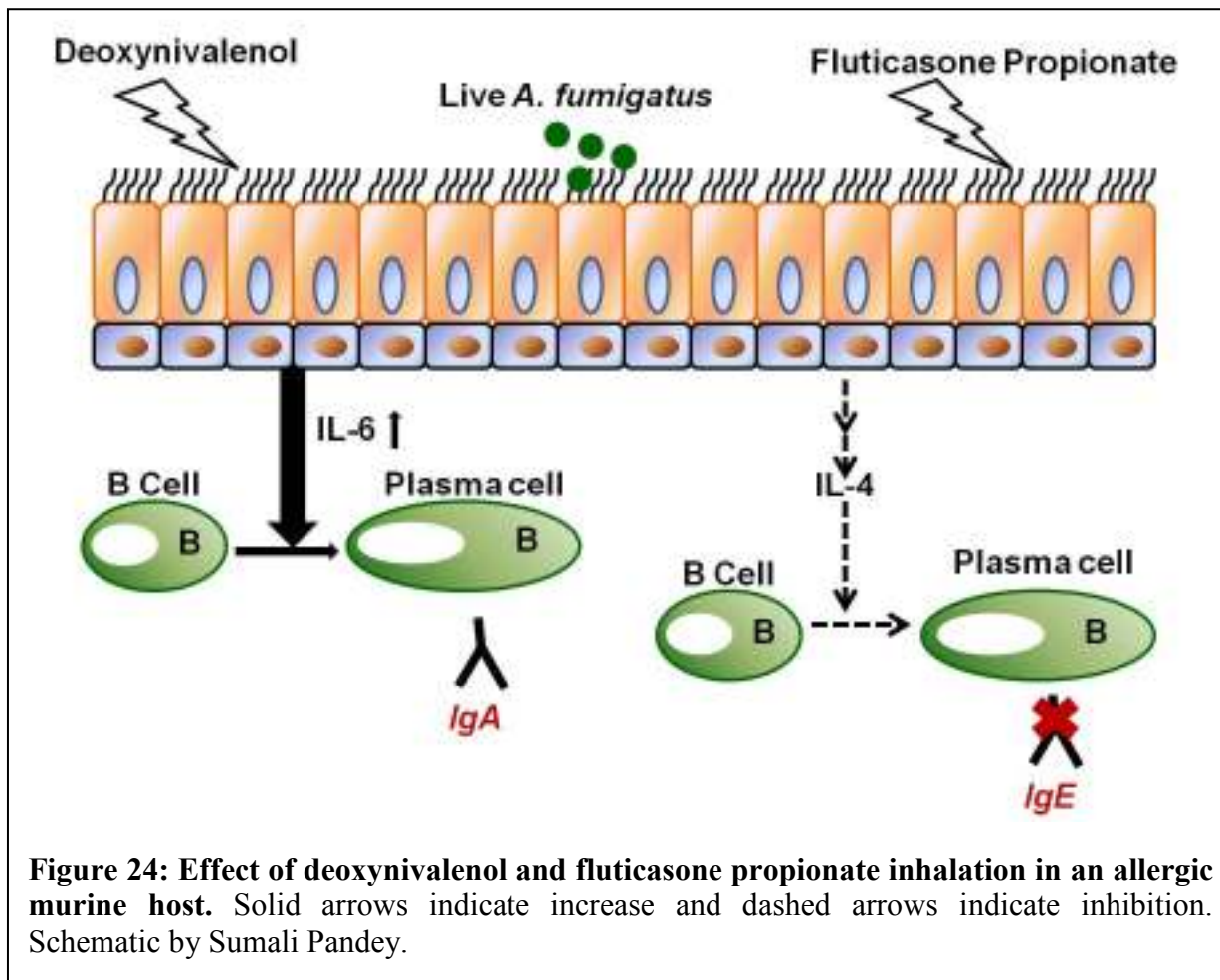
A. fumigatus is a source of 23 listed allergens [389], and substantial host-pathogen interaction underlies the pathophysiology of *A. fumigatus* induced infection, as noted in the observations listed above. A meta-analysis study showed 15-48% prevalence rate for *Aspergillus* sensitization in bronchial asthma [3]. In addition to moldy buildings, repeated inhalation of high doses of *A. fumigatus*, over an extended period of time may occur in agricultural environments [141,146]. Therefore, we next hypothesized that in a person allergic to mold such as *A. fumigatus*, repeated inhalation of an immunomodulatory agent in an agricultural setting may substantially change or damage the lung, thereby influencing the outcome of allergic asthma.

Occupational exposure to grain dust may be an underlying cause or an exacerbating agent in allergic diseases that are prevalent in farmers [25,26], who represent 30% of adults disabled by respiratory illness [392]. Our literature search revealed that deoxynivalenol, a trichothecene mycotoxin produced by *Fusarium* species, is one of the most prevalent mycotoxins found in barley, corn, rye, safflower seeds, wheat, and mixed feeds [318,411]. Additionally, DON is a potent immunomodulatory agent [179,261,307] [34,207,308,309] [19,70,86,294,305,307,310,311,427,456,457], but its effects on allergic asthma have been investigated in only one study, and was limited to serum IgE as a readout [179].

While repeated inhalation of DON in an allergic farm worker is highly likely, the research in this domain is lacking. DON is routinely detected in grain dust released during grain production, transport, and storage, making inhalation exposure in these environments exceedingly possible [140,210,211,218,278]. However, most of the immune related effects of DON have been established using human or mouse cell culture models, or in animal models involving oral ingestion of DON. To our knowledge there is only one study, that has investigated the effects of DON delivered intranasally, and found that DON was more toxic when delivered via nasal route compare to oral route [14], which makes it all the more pertinent to test DON's effects when delivered via inhalation. Using our previously established murine model of *A. fumigatus* induced allergic asthma, we tested the effect of inhaled DON, administered once a day, for 6 consecutive days. Our study shows that inhalation of DON in an experimental allergic asthma model elicits a dose- and time- dependent increase in local (BALF) and systemic (serum) IgA levels. However, at the tested dosages, inhaled DON did not have any adjuvant effect on allergic asthma mediators (e.g. serum IgE or Th-2 cytokines) or symptom (e.g. GC metaplasia).

While the elevation in serum IgA levels observed with DON inhalation was short lived, the increased BALF IgA levels persisted until three weeks after withdrawal of DON treatment (at day 28 post-allergen challenge). This suggests that lung associated mucosal tissue is the predominant site of inhaled DON mediated IgA production. Previous studies with dietary DON have shown an indispensable role for IL-6 since dietary DON induced serum or mesangial IgA increase was not observed in IL-6 deficient mice [306]. In the lung, epithelial cells are likely to be a significant source of IL-6 production [292,400]. Additionally, airway epithelial cells can secrete other cytokines involved in IgA production, namely IL-2, -4, -5, -10, and TGF- β [350]. Furthermore, epithelial cells produce a glycoprotein called secretory component, which confers stability against proteases to secretory IgA and allows polymeric IgA to be translocated from the basolateral side of epithelial cells into the bronchoalveolar space [350]. In the absence of inflammation in the lung, as on day 28-post fungal challenge, epithelial cells are likely to be the key players in regulating IgA production in the lung. Therefore, we hypothesize that airway epithelial cells are principal regulators in DON mediated IgA increase, and the effects are mediated through increased IL-6 production by airway epithelial cells (**Fig. 24**). Further studies in non-allergic and allergic mice, are needed to validate this hypothesis.

Lastly, we wanted to investigate the extent to which the inhaled corticosteroid (ICS), fluticasone propionate, can alter the host-pathogen interaction, and consequent asthma pathology in a murine model of live *A. fumigatus* induced allergic asthma. Corticosteroids are the most potent and effective anti-inflammatory drugs currently available for asthma management [99]. They target multiple inflammatory pathways simultaneously, and exert their anti-inflammatory effects on structural as well as inflammatory cells in the airways. While their broad-spectrum immunosuppressive properties are beneficial for their efficacy in asthma symptom management,



the risk for opportunistic infections is increased with their usage. Prolonged use of CS can potentially lead to infections with oral or respiratory pathogens. Of particular interest to our lab, were the case studies documenting the development of invasive *Aspergillosis* (IA) infection with inhaled FP [29,48,102,220,312]. At the tested dosage, the FP treatment did not significantly reduce the number of inflammatory cells or goblet cell metaplasia. However, our model allowed us to investigate the temporal and spatial changes associated with ICS treatment in a murine model of fungus-induced allergic asthma. Only the lung mucosal effects are mentioned here, and the readers are referred to chapter 3, for detailed account on changes in serum antibody titers. Compared to the vehicle treated mice, there was a transient increase in AHR at day 3 post-second fungal challenge. However at day 14 post-second challenge, an increase in

bronchoalveolar lavage fluid IgE was inhibited in FP treated mice. IL-4 is required for isotype class switching of B cells to IgE secreting plasma cells. We hypothesize that down-stream effects of a direct interaction of airway epithelium with fluticasone propionate prevents the up-regulation of IL-4 in *A. fumigatus* challenged mice, thereby preventing an increase in BALF IgE in fluticasone propionate treated mice (**Fig. 24**).

Airway epithelium forms a critical interface between inhaled environmental substances and structural or inflammatory cells in the lung parenchyma. Besides being a physical barrier, epithelial cells can recognize microbes through pattern recognition receptors (PRRs), secrete mucus, pro and anti-inflammatory mediators to recruit other cells, as well as anti-microbial substances. Additionally, Dr. Stephen Holgate's research described the epithelial mesenchymal trophic unit, which maintains tissue homeostasis, but is dysregulated in asthma. This formed the key driving notion, behind this project. According to Dr. Holgate, repeated damage to the epithelium, due to inhaled biologically active substances, proteases, irritants etc. result in production of signals that act on underlying mesenchyme to propagate and amplify inflammatory and airway remodeling responses in the airways. We tested this hypothesis in this project by exposing the murine lung to repeated inhalational insults with dead or live *A. fumigatus* conidia, and immunomodulatory agents such as deoxynivalenol and fluticasone propionate. Of the four substances tested, we found that *in situ* recognition of dry, inhaled live *A. fumigatus* conidia elicits a full spectrum of allergic asthma, while dead spores fail to do so. These results are likely to be important while considering fungal-exposure assessment methods, and for making informed therapeutic decisions for mold-associated diseases. Moreover, DON and FP do not significantly influence the allergic asthma phenotype, except for influencing the antibody titers.

Research has provided evidence for interaction of *A. fumigatus* conidia [293], deoxynivalenol [179] and fluticasone propionate [326,399] with the airway epithelium. In the experimental studies, mechanism based insights are gathered from *in vitro* studies. However, their major shortcoming is that they do not accurately represent an *in vivo* scenario. The epithelium rarely works as an isolated unit. Several other cell types such as the underlying smooth muscle cells, endothelial cells, alveolar macrophages, and recruited inflammatory cells, cooperate with the epithelium in orchestrating the host-immune response. Therefore, *in vivo* studies using inhalation as the route of delivery have been the best way to investigate epithelium's response to inhaled substances, and to establish cause- and effect-relationships. With the advent of laser capture microdissection techniques, it is now possible to capture the airway epithelium from lung sections of naïve mice or mice challenged with inhaled substances. Therefore, an option to explore the proteome and transcriptome of airway epithelium in health and disease, without relying on *in vitro* settings, isn't a distant future.

The observations of the present project must be viewed in the light of certain limitations, which are typical of inhalation studies. Significant differences occur in the anatomy and physiology of mice and humans, which may impact the pharmacological outcomes [44,266,332]. Most importantly, unlike humans, mice are obligate nose breathers, which may result in deposition and clearance of the inhaled dose in the nasopharyngeal region, besides in the trachea, bronchi and the lungs. Additionally, the inhaled dose measurements are difficult, compared to other routes of administration. The inhaled dose is a function of several parameters related to the delivery apparatus such as; flow rate, the type of nebulizer, nebulization rate and particle size [68]. Moreover, large intersubject variability may result from differences in the breathing pattern and lung geometry of the individual mice [299]. Even in the clinical settings the lung exposure

via aerosol delivery is between 5 and 50% with the remaining being deposited in the oropharynx and subsequently swallowed [197,270]. Indeed, the inhaler technique can significantly impact the symptom control in asthma patients [258,296]. Lastly, dose, frequency of exposure and mouse strain-dependent differences may lead to variations in the extent of response.

CONCLUSIONS

Asthma is characterized by an increased sensitivity to inhaled substances. Our objective in this study was to investigate the ability of inhaled immunomodulatory agents, encountered either in environmental or clinical settings, to elicit or affect the symptoms of fungus-induced allergic asthma. We chose inhalation as the route of delivery since it best mimics the natural route of human exposure, and ensures direct interaction of the inhaled substance with airway epithelium. We showed that repeated inhalation of live *A. fumigatus* spores is more likely to induce symptoms of allergic asthma disease. However, in individuals with established allergy to fungal antigens, asthma symptoms might be exacerbated by the inhalation of live or dead spores. Additionally, in allergic mice repeated inhalation of DON has the potential to influence the disease progression by dysregulating IgA levels, and FP treatment did not prevent the development of allergic asthma, but introduced dynamic changes in the lung physiology and antibody levels. Further mechanism-centered studies in non-allergic and allergic murine host would shed light on the ability of DON and FP to influence the antibody production.

Our study showed that out of the substances tested, repeated interaction of live *A. fumigatus* conidia can potentially alter the lung mucosa in order to establish a full spectrum of allergic asthma disease. By analyzing potential mechanisms, a critical interaction of airway epithelium with live *A. fumigatus* or its products in asthma pathogenesis is highlighted, which would inform mechanism-based future studies. In conclusion, this project is likely to aid in establishing cause and effect relationships for airway epithelium's interaction with the environmental substances. Insights gathered would help in forming evidence based standards for fungus-related exposures, and in making informed therapeutic decisions for fungus-associated diseases.

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