CHARACTERIZING HUMIDITY, SEX, AND B-CELL GENE REGULATION IN FUNGAL ALLERGIC

ASTHMA

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Emma Claire Kusick

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Microbiological Sciences

April 2020

Fargo, North Dakota

North Dakota State University
Graduate School

Title

CHARACTERIZING HUMIDITY, SEX, AND B-CELL GENE REGULATION IN
FUNGAL ALLERGIC ASTHMA

By

EMMA KUSICK

The Supervisory Committee certifies that this ***disquisition*** complies with North

Dakota State University's regulations and meets the accepted standards for the

degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Jane Schuh

Chair

Dr. Teresa Bergholz

Dr. Penelope Gibbs

Dr. Sumit Ghosh

Approved:

5/4/2020	Dr. John McEvoy
Date	Department Chair

ABSTRACT

Asthma is a debilitating lung disease that affects nearly 300 million people worldwide. Environments with high humidity and subsequent mold exposure often trigger allergic asthma. Sex differences have been reported in the incidence, prevalence, and severity of asthma. B-lymphocytes are recruited in high numbers to the allergic lung in response to the inhalation of *Aspergillus fumigatus* mold spores (conidia). In this work, we used a mouse model of allergic fungal asthma to assess environmental humidity, sex, and B-lymphocytes in an inhalational model of allergic fungal asthma. Our results showed that animals sensitized in low humidity conditions had no airway hyperresponsiveness (AHR), inflammation, but an increase in IgG3 antibody production. Males weighed more than females, female mice had more fibrosis and produced more IgG3 Ab, but sex showed no impact on low humidity. C19$^+$ B-lymphocytes differentially downregulated multiple genes related to allergic asthma returning the body to homeostasis.

ACKNOWLEDGEMENTS

Earning my M.S. has been one of the most challenging, but exciting years of my life. The two additional years spent at NDSU were well worth the growing pains, and I gained more friendships and learned so much about myself to be successful in life.

First, I would like to thank Dr. Jane Schuh for being my adviser during this experience. She was always there to provide guidance, direction, and support throughout the process. I have learned so much about how to become a good scientist and solve problems in different ways. You are truly a strong female role model of mine, and I appreciate that you were willing to take me under your wing. Thank you for refusing to let me give up when times were rough and showed me that this degree is for myself.

Second, I would like to thank Scott Hoselton for being the mentor, teacher, listener, advice giver, and so on. You answered question after question, and without you this would not have been possible. You are the person holding the Schuh laboratory steady, and you have helped me become a better-trained researcher.

Third, I would like to thank my committee members, Dr. Teresa Bergholz, Dr. Penelope Gibbs, and Dr. Sumit Ghosh for their support and helpful suggestions. Although our time was brief together, I enjoyed the committee meetings and you made a learning and encouraging environment. Dr. Bergholz, I appreciated that your door was always open, and I enjoyed working with you during my time as the Graduate Student Association President. Dr. Gibbs, I could always find a smile from you and enjoyed hearing all your stories about life. Dr. Ghosh, without you and your previous research, my research would not exist. You were amazing to learn from as both an undergraduate and graduate student.

To the members of the Schuh lab over the years, you have been a source of friendship, encouragement, and advice in the classroom, laboratory, and life throughout these years. I could not have made it through all this without you- Thank you!

Friends and Family:

I have had the pleasure of gaining many friends throughout my undergraduate and graduate career at NDSU. Although many of them left after undergrad, they were still there to support during this journey. I was able to meet new graduate students from other departments that soon became friends and

fellow fighters through all this. Thank you to everyone that told me I could do this, and I hope to return the support and laughter someday.

I could not have achieved this without my parents, Bo and Cheryl Kusick, and my brother Owen Kusick. You have shown me that dedication, hard work, and encouragement will allow me to follow my dreams and that you will always be there to help pick me up if I falter. The four of us can get through anything, and I owe you three everything.

Finally, I wish to thank my husband, Kyle Mason, for everything he has done for me and supporting me while I finished up my degree. Having you there to listen about frustrating days when everything had gone wrong even though you did not understand exactly what I was doing helped me immensely. I am finally finished and thanks for waiting for me so we can begin our next adventure.

DEDICATION

I dedicate this to all the strong women in my life who taught me to be fearless. Family, friends, mentors,

and colleagues who showed me that a girl can do anything she sets her mind to.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab ....Antibody

AERD..Aspirin Exacerbated Respiratory Disease

AHR ....Airway hyperresponsiveness

Ag ....Antigen

APC ....Antigen Presenting Cells

ATCC...American Type Culture Collection

BAL...Bronchoalveolar lavage

BCR ....B-cell Receptor

CD...Cluster of differentiation

DC...Dendritic cells

EACACC ....European Collection of Cell Cultures

ECM...Extracellular matrix

GERD...Gastroesophageal Reflux Disease

GC ....Goblet cell

HA..Hyaluronan

H&E ....Hematoxylin and Eosin

HMM ....High Molecular Mass

HPF..High powered field

IFN...Interferon

Ig..Immunoglobulin

IH..Inhalation

IN..Intranasal

IL...Interleukin

IP ....Intraperitoneal

JCRB ....Japanese Collection of Research Bioresources

LPS...Lipopolysaccharide

LMM..Low Molecular Mass

1. LITERATURE REVIEW

1.1. Asthma

Asthma is a chronic inflammatory disease of the lung, characterized by recurrent episodes of reversible airway obstruction and wheezing. An acute asthmatic attack occurs after the immediate inhalation of an injurious agent. Prolonged and persistent inflammation is then triggered when the host's tissues respond insufficiently to overcome the effects of the injurious agent[1]. These 'injurious agents' can be pollutants and irritants (intrinsic asthma), or allergens (extrinsic asthma). They stimulate the lung epithelium and elicit the immune response to release inflammatory cells and mediators[2]. These mediators, such as histamine, leukotrienes, and various cytokines release free radicals and nitric oxide that damage the epithelial cells lining the airways resulting in a subsequent increase in mucus production that clogs the airways and tissue remodeling that narrows the airways[3]. Once damaged, the epithelial cells become more sensitive and overreact to stimuli[2]. Asthma affects 8-10% of the U.S. (25 million) with a large number of those being children[4]. The annual cost of living with the asthma has increased to $56 billion or roughly $3,259 per person in terms of health care dollars, time away from work and school, and premature death[4]. Due to widespread education, access to appropriate medical care, and increasingly better treatment options, fatalities from asthma are rare in the U.S., but over 3,300 deaths still occur every year[4]. Worldwide, there are still nearly 250,000 deaths attributable to asthma exacerbations, with most of these being avoidable if access to suitable medical care was available[4].

Asthma occurs in two phenotypes: intrinsic or extrinsic. Symptoms are similar between the two forms range from wheezing, shortness of breath, and chest tightness to life-threatening anaphylaxis, but the triggers for each are different. Intrinsic asthma is not mediated by IgE; thus, it is a 'non-allergic' form of asthma. Intrinsic asthma typically has a later onset and occurs in 10-30% of those suffering from asthma[5]. This type can be caused by a wide variety of chemical pollutants and irritants such as cigarette smoke, scents, or hairspray. Other triggers include anxiety, atmospheric conditions, or exercise. There is also an association of intrinsic asthma and nasal polyps, rhinosinusitis, gastroesophageal reflux disease (GERD), and/or aspirin-exacerbated respiratory disease (AERD)[6].

Extrinsic asthma is mediated by inhaled allergens crosslinking IgE on the surface of effector cells, resulting in the degranulation of mast cells and eosinophils[7]. Allergic asthmatics have elevated IgE

levels and eosinophil numbers circulating in their blood and an increase in both mast cells and eosinophils present in their lungs. Most asthma sufferers have an associated allergic sensitization that occurs in 80% of asthmatic children and 60% asthmatic adults[8]. This type may be caused by allergens such as dust mite, pet dander, pollen, and mold or a combination of multiple allergen triggers[9].

There are several treatment options currently available to those who suffer from this disease. Most people require both short-term and long-term drug treatments. These treatments can vary on the amount time it takes and the mechanism the drug uses to help provide relief. Short-term treatments are used as rapid relievers whereas long-term treatments provide systematic sustained relief. Short-acting beta2-agonist (SABA) immediately relax tight muscles around the airways that cut down air flow during an asthma attack. Long-term treatments have various mechanisms of action; some open the airways, others reduce inflammation, and some prevent the immune response from acting against allergic triggers. Long-term treatment options include inhaled corticosteroids and long-acting beta2-agonists, nebulized Cromolyn, anti-IgE Omalizumab, leukotriene modifiers, or Theophylline[10]. Inhaled corticosteroids suppress airway inflammation by inhibiting phospholipase A_2 that reduces arachidonic acid which is essential for building inflammatory mediators[11]. Corticosteroids reduce vasoconstriction by reversing mucosal edema and secretion[11]. Beta2-agonists release constricted airways by activating adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP) to cause smooth muscle relaxation[12]. Corticosteroids help beta2-agonists open the airways by increasing the expression of the receptor that binds to beta2-agonists[13]. Nebulized Cromolyn inhibits mast cell degranulation and prevents the release of histamine[14]. Cromolyn therapy can block airway hyperreactivity in chronic allergen exposure[15]. Omalizumab is a recombinant monoclonal anti-antibody that inhibits IgE functions by blocking free serum IgE and prevents the binding of IgE to cellular receptors[16]. Leukotriene modifiers are antagonists that block the transcription of leukotrienes, which cause bronchoconstriction and infiltration of inflammatory cells[17]. Theophylline increases cAMP to activate protein kinase A, thereby inhibiting TNFα and leukotriene synthesis to reduce inflammation and bronchoconstriction[18].

Treatments may be optimally individualized for the patient based on the type, trigger, and severity of asthma. Asthma is classified as controlled or uncontrolled based on of the number of daytime symptoms, nighttime symptoms, short-acting beta agonist (SABA) use, activity limitations, and oral steroid

bursts[19]. In controlled intermittent asthma, a person has daytime symptoms fewer than two days a week and nighttime symptoms fewer than two times a month[20]. There is no activity limitation and the use of SABA is less than two days a week with oral steroids being used no more than once a year[20]. In controlled persistent (mild to severe) asthma, a person has daytime symptoms more often than two days a week and nighttime symptoms multiple times per month[20]. There are activity limitations and SABA use is more than two days a week. When these measures do not suffice, oral steroids are required multiple times per year[20]. Uncontrolled asthma occurs when the treatment options are no longer effective to control asthma symptoms and dosages must be adjusted or new medications implemented. For those suffering from asthma, it is important to be aware asthma triggers and to avoid those triggers by creating an Asthma Action Plan[21]. The Asthma Action Plan outlines three zones: Green (every day control and maintenance medications), Yellow (Green Zone medication and quick-relief medicines), and Red (Emergency situations requiring hospitalization)[21, 22]. While there is no cure for asthma, finding the right balance of treatment options allows those who suffer from asthma to live normal lives. There is a genetic component to allergic asthma. Children of asthmatic parents are more likely to develop asthma[23]. Genes involved with cytokine production, MHC Class II receptors, regulation of immune cell development, airway remodeling, smooth muscle reactivity, and inflammatory responses have all been shown to have an association with allergy and asthma[24]. The genetic inheritance of asthma is influenced by economic, cultural, and environmental factors[25].

The environment has significant impacts on the development and continuing of allergic asthma by exposure to allergens, infections organisms, and other factors that irritate the mucosal linings of the airways. Respiratory viral and bacterial infections are a contributing factor in the development of asthma as studies have shown that 80% of asthma attacks occurring were preceded by respiratory viral infection in children[24]. The environment contains both nonallergic and allergic triggers. Nonallergic triggers include smoke, odors, cold air and weather, chemicals, medications, exercise, and hormonal changes[26]. Allergic triggers include seasonal pollen, mold spores, dust mites, animal dander, and food[26]. Specially, molds can be found in both indoor and outdoor environments, but prefer warm, moist environments[27]. Studies have shown an association between mold sensitivity and increased bronchial reactivity in children and asthma severity with increased intensive care admissions in adults[28, 29].

Studies have also shown that nonallergic fungal cell wall components are involved in mold-related respiratory symptoms[30, 31]. Interventions that reduce moisture and humidity, such as dehumidification, air conditioning, and increased ventilation, have been shown to reduce exposure to molds[20]. The humidity of the environment has been shown to both alleviate and exasperate asthma symptoms. Low humidity conditions have been recommended as a potential treatment option to those suffering from asthma if the humidity levels stay between 30-50%[32]. Extreme low humidity, below 15%, is another trigger for those suffering from asthma as dry air causes the airways to become irritated and swollen worsening the symptoms [21]. High humidity has been shown to increase mold and mildew which allows for more sensitization to occur as well as the increased prevalence of allergic triggers in the environment[33]. Understanding the environment and how the body reacts to changes in the environment are important aspects to control for in allergic asthma modeling.

1.2. Model

Our lab focuses on the inflammatory processes and outcomes of fungal allergic asthma. Allergic asthma causes an IgE-mediated response that occurs when the immune system is triggered by an environmental allergen[24]. Roughly 70% of those suffering from asthma have symptoms that are associated with IgE-mediated reactions[24]. When an individual inhales the allergen, it is taken up and processed by antigen presenting cells (APC), typically a dendritic cell, the APC then travels to the draining lymph node to present the antigen to naïve T-cells and B-cells[24]. If the lymphocyte is specific for the antigen that is presented, the naïve T- or B- cell becomes activated. T-cells produce IL-4, which in turn triggers B-cells to produce IgE, which is the hallmark antibody of an allergic response[24]. In allergic hosts, circulating IgE binds to high affinity FcεRI receptors that allows mast cells and eosinophils to respond very quickly to a subsequent encounter with the antigen by cross-linking these receptors on the cell surface[24]. After repeated exposure to the allergen, the sensitized mast cells will degranulate, releasing inflammatory mediators, vasoactive amines, and lipid mediators[24]. These cause an immediate hypersensitivity that is characterized by vasodilation, congestion, and edema at the site of inflammation[24]. Inflammatory cytokines will cause a delayed late phase reaction that is characterized by the accumulation of inflammatory cells: neutrophils, eosinophils, basophils, and helper T-cells[24]. In

4

addition, repeated exposure to the allergen may cause epithelial damage and increases mucus and subepithelial smooth muscle hypertrophy, leading to increased airway obstruction[34].

A diverse array of allergens have been used to study allergic asthma: ovalbumin, house dust mite, cockroach antigen, and *Aspergillus fumigatus* to name a few[35]. For the past several decades, the most common sensitizing agent used in the study of allergic asthma was ovalbumin[36]. Egg white proteins do elicit an allergic response in common murine strains, and the allergen is both cheap and easy to use. As a consequence of its nearly universal use in the field of allergic research, the chemical reagents for ovalbumin as well as the cell and animal models that were developed for use by the research community were substantial are the compounding reasons that the ovalbumin model was maintained for such a long time as the primary tool for studying allergic asthma[37]. However, egg white is not a clinically relevant respiratory allergen and slowly other models have been introduced to compare and contrast immune responses against the standard of ovalbumin and have become more widely accepted as not only an acceptable research tool, but a preferred method. Our research laboratory uses the fungal species *Aspergillus fumigatus* to sensitize and induce allergic asthma[38, 39]. The *Aspergillus* genus contains over 182 species including *A. fumigatus* which is commonly found in the soil and produces spores that are highly allergenic[40]. Each day, people inhale roughly 200-300 conidia. Most people can remove the conidia by innate mechanisms. However, in those suffering from allergic asthma, conidia continually prime the allergic immune response to react in those suffering from allergic asthma[41]. *A. fumigatus* and other fungi can affect the lungs by acting as aeroallergens or as a pathogen causing infection[42]. Those who are sensitized to *A. fumigatus* have more impaired lung function and higher rates of bronchiectasis that causes more tissue damage[43].

Currently, there are a variety of murine models used to study allergic asthma. Mouse models have been used to replicate asthma in published articles since 1994 and have become the most widely used species because they are easy to use, maintain, and handle[44]. There is also a wide variety of specific reagents, multiple murine and genetically engineered transgenic murine strains for modeling airway diseases [45]. Since mice do not develop asthma naturally, models must include a sensitization and a challenge phase [46]. Adjuvants are used to increase allergen immunogenicity in mice to increase sensitization whereas humans are sensitized via mucosa exposure to allergen which is then followed by a

Th2 immune response[47]. Acute mice models have been able to reproduce, IgE, airway inflammation, epithelial hypertrophy, and airway hyperresponsiveness. However, the pattern and distribution of pulmonary inflammation found in mice is different than in humans[48]. Murine models can also lack chronicity of the response to allergens and a tolerance after repeated allergen exposure can build up[46]. Chronic mice models have been able to more closely reproduce characteristics seen in asthmatic humans. Th2- dependent allergic inflammation and remolding of airways has been seen in both[49]. While animal models have been beneficial to our understanding of the mechanisms of asthma and have allowed for the development of treatments, it is important to remember that no animal model can replicate all features of the human disease and models should be thoughtfully planned to recapitulate as closely as possible the features of human disease.

Various sensitization protocols and mouse strains may be used to research both acute and chronic allergic asthma. Most murine models follow a format of first sensitizing the animal to a foreign protein via an intraperitoneal injection, with or without an adjuvant, to elicit global sensitization to the antigen and break tolerance[50]. The animal then receives further antigen exposure by several routes. In models using fungal conidia (spores) of the common environmental respiratory allergen *A. fumigatus*, secondary exposure may be carried out by involuntary aspiration, intranasal instillation, intratracheal injection, nebulization, or dry inhalation of airborne conidia. Typically, an animal is sensitized with two to three intraperitoneal, intranasal, and/or subcutaneous injections spaced a week to two weeks apart, then the animal is challenged on secondary exposure[50-52]. This secondary exposure elicits a local allergic reaction in the lungs characterized by an increase in the number of eosinophils and other immune cells present, epithelial thickening, and airway hyperresponsiveness and changes in mucus production[50, 53].

The inhalational (IH) model fungal murine model of asthma follows an 8-week protocol in which mice are sensitized to fungal extracts delivered in an adjuvant via intraperitoneal (IP) and subcutaneous (SC) routes followed by intranasal (IN) sensitization of the airways with *A. fumigatus* extracts in saline over three weeks, and finally 2 allergen challenges with inhaled *A. fumigatus* conidia 2 weeks apart. This leads to IgE production, as well as recruitment and activation of masts cells and eosinophils to the lungs. Mucus production, airway hyperresponsiveness (AHR), lymphocytic pulmonary inflammation, and

peribronchial fibrosis is observed[38, 39]. Samples are collected at specific timepoints after the second

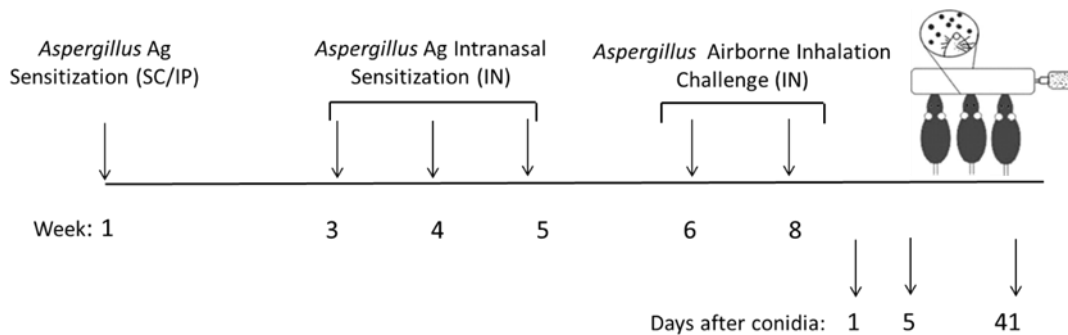allergy challenge as shown in **Figure 1**[38, 39].

Figure 1. *Aspergillus fumigatus* **sensitization and inhalation model**. Naïve C57Bl/6 mice were sensitized with subcutaneous (SC) and intraperitoneal (IP) injections of soluble *A. fumigatus* antigen in alum followed by three intranasal (IN) inoculations with soluble antigen in PBS. In an inhalation (IH) challenge, sensitized mice were exposed to airborne *A. fumigatus* conidia for 10 min.

Previous research has shown that eosinophils peak on Day 3 after the conidia challenge and B-

lymphocytes peak on Day 5. Chronic fibrosis has been tracked through at least day 42 with no reduction

in the deposition of collagen noted[54].

1.3. Immune Response to Fungus

Normally when contemplating the response to an allergen, we are looking at an abnormally

aggressive response to an innocuous agent. This is not the case with the response to fungi if an infection

is initiated. Fungal infections can be rapidly fatal[24]. When a fungal spore enters the lung, the host

response starts with an innate immune response. The mucus membranes of the respiratory tract form a

physical barrier with epithelial cells creating tight junctions, which block the passage of the allergen, and

mucus, which binds the conidia in a viscous secretion containing glycoproteins (mucins). Ciliary action in

the bronchial tree moves the allergen up and out of the airways[24]. If the physical barrier is breached,

macrophages and neutrophils are present to defend against the allergen. Macrophages bind and

phagocytose components of the fungal cell wall using pattern recognition receptors (PRR's) such as Toll-

like Receptor(TLR)-2 and -4[55]. Neutrophils will phagocytose the conidia using TLR-2 and -4[56].

Neutrophils destroy the conidia using oxidative mechanisms and reactive nitrogen intermediates; all

damage conidia membranes and DNA[57-59]. Afterwards, a release of destroyed particles, cytokines,

and activated antigen presenting cells will initiate the adaptive immune response[59].

During an adaptive immune response, conidia entering the lung are engulfed by an antigen presenting cell (APC), typically a dendritic cell. Macrophages, B-lymphocytes, and follicular dendritic cells are capable of presenting antigens[60]. The dendritic cells present the antigen to a naïve CD4+ T-cell that differentiates into an activated Th-cell causing a priming of the Th1, Th17, or Th2 immune response[61]. The various immune responses are elicited based on 1) activation of particular cytokine genes, 2) commitment to a chosen response pathway (Th17 or Th2), 3) suppression of the opposing cytokine gene, and 4) stabilization and continuation of the chosen response pathway[62]. The Th17 type of immune response produces the cytokines Interleukin 17A(IL-17A), Interleukin 17F (IL-17F), and Interleukin 22 (IL-22) that act on neutrophils, macrophages, and B-cells to protect the body from fungi and are involved in chronic inflammatory diseases[63]. Some asthmatic patients have shown high levels of Interferon gamma (IFN-γ), IL-17, and neutrophils in the lungs indicating that there is a Th17 response. However, studies have found that Interleukin 13 (IL-13) and Interleukin (IL-4), Th2 mediated cytokines, have the ability to repress Th17 genes thus activating a Th2 response[64, 65].

When a Th2 immune response is initiated, activated Th-cells produce IL-4 that activates B-lymphocytes to differentiate into plasma cells. These cells begin to produce Immunoglobulin E (IgE), the hallmark antibody of allergic asthma, which bind to the Fc-receptors on granulocytes such as mast cells, basophils, and eosinophils. Plasma cells also produce Interleukin 5 (IL-5), which activates eosinophils, another hallmark cell of the allergic response. The granulocytes are now primed for a Th2 immune response. On re-exposure to *A. fumigatus* conidia, antigen crosslinks surface IgE on mast cells, basophils, or eosinophils causing them to degranulate, releasing inflammatory mediators such as histamines and leukotrienes. Histamine is an organic nitrogenous compound that causes blood vessel dilation and nitric oxide release. The inflammatory mediators cause vasodilation, mucus secretion, nerve stimulation, smooth muscle contraction, and an influx of inflammatory cells to the lung. The symptoms include a reactive airway that makes breathing difficult and, in severe cases, anaphylaxis can occur. Anaphylaxis is a serious allergic response involving chest tightness, difficulty breathing (which can be life threating if the airways are affected), swelling, lowered blood pressure, and sending a patient into shock that can be fatal is not treated immediately[9].

1.4. Sex Differences

Sex differences have been noted in many different types of diseases including asthma, cardiovascular disease, autoimmune disease, neurological and psychiatric disorders, birth defects, and many types of cancers. Sex differences are observed in the severity of the diseases, in the onset of the disease, and in presence in one sex and complete absence in the other sex[66]. The National Institute of Health developed the Office of Research on Women's Health (ORWH) in September 1990. ORWH is designed to promote women's health research because studies have shown that over half of the people participating in clinical research studies are women, but a majority of pre-clinical research has been performed in male animals and male cell lines[67]. In an attempt to fully represent both sexes, the NIH has developed policies to assure that grant applicants account for the biological differences of sex in research and adding funding supplements to use for the opposite sex used in the original grants[67].

Sex related differences have been investigated on different facets of the immune system such as cell proliferation, selective recruitment of immune cells to the site of infection, general inflammation, immune activation, and hormone involvement. Females fight infections with a more robust innate and adaptive immune response that can allow for quick clearing of pathogens when compared to males. But in other cases, for example H1N1 influenza virus, the immune response is so robust that it actually is responsible for causing more fatalities in women than in men[68]. A study performed in rats investigating neutrophil stimulation and recruitment in skin injury showed that male rats had more and prolonged neutrophil accumulation and tissue injury during a two-hour reperfusion than female rats. This was attributed to the fact that male mice had increased surface expression of CXCL5/CXCL6 on adhesion molecules than females[69]. In Peyer's patches and spleen cells, in BALB/c and C57BL/6 mice, that have colitis, males have a lower percentage of T-cells and natural killer cells in both Peyer's patches and spleen but higher percentages of Th1 cells and dendritic when compared to females[70]. Numerous studies have shown that the role of hormones play an important role in dictating sex differences. Estrogen has been found to be pro-inflammatory by increasing the activity of NF-kappa B in human T-cells and survival of those T-cells, whereas testosterone exerts anti-inflammatory effects by inhibiting the expression and release of inflammatory chemokines and cytokines [71-73]. In respiratory diseases, males have higher mortality rates in acute diseases, whereas women are more negatively impacted by chronic

9

diseases[74]. For asthma, specifically, boys tend to have higher prevalence and more severe cases than girls. However following puberty, the phenotype switches to women having higher and more severe cases of asthma than men[1]. Once menopause is reached in women, the prevalence of asthma in women drops and becomes equal to that of men[75, 76]. By looking at sex differences, scientists can have a better understanding of diseases and develop evidence-based treatment options for each sex.

1.5. B-lymphocytes

B-lymphocytes are continuously produced throughout the life of humans and mice. B-lymphocytes are initially produced from the fetal liver, then from hematopoietic stem cells in the bone marrow. B-lymphocytes can be grouped into four categories: Marginal zone B-cells, B1 cells, B2 cells, and B-regulatory cells. Marginal zone B-cells are derived from the fetal liver, are found in the marginal zone of the spleen and other lymphoid tissues, and are short lived[24]. Marginal zone B-cells are responsible for T-cell independent responses and Immunoglobulin M (IgM) antibody production[77]. B1-cells are derived from the fetal liver, make up 66% of the B-cell population, and are found in tissues[24]. B1-cells are responsible for T-cell independent responses and IgM and Immunoglobulin A (IgA) antibody production[24]. B2 (follicular or activated B-cells) are derived from the bone marrow, but mature and are activated in the lymph organs. These cells are part of the adaptive immune response requiring collaboration with helper T-cells to fight infection, producing antibodies, performing antigen presentation, producing regulatory cytokines, and in some cases developing into memory cells[24]. B-regulatory cells are a small subset, 5% of the B-cell population, that are responsible for producing anti-inflammatory cytokines such as interleukin 10 (IL-10) and Transforming growth factor-beta (TGFβ) that suppress the immune system and return the body to homeostasis[78].

B-lymphocytes have three main functions in the body: antigen presentation, antibody production, and cytokine production. In antigen presentation, B-cells are constantly moving from one lymphoid organ to the next through blood vessels (T-cells zone) into follicles (B-cell zone) by using the CXCR5 chemokine receptor that binds to the chemokine CXCL13 which is secreted by dendritic cells[24]. The process of antigen presentation occurs in two steps: 1) uptake of antigen by the B-cell and 2) movement and antigen presentation to an activated helper T-cell. The uptake of antigen uses the B-cell receptor (BCR)-mediated mechanism[79]. The BCR is a membrane-bound immunoglobulin found on the outer membrane

10

that has an affinity for a given antigen[80]. The receptor internalizes the bound antigen into endosomal vesicles and processes the antigen into peptides that are presented on the B-cell surface for recognition by helper T-cells[24]. B-cells process and present specific and nonspecific antigens differently. Specific antigens allow the B-cell receptor to function with high efficiency and induce B-cell activation, which then causes the activation of T-cells. Nonspecific antigens create the opposite effect, decreasing B-cell activation and inactivating T-cells[81]. B-cells can receive antigen from antigen presenting cells such as macrophages or dendritic cells or can take up the antigen themselves via the BCR, although this alone is inadequate to stimulate a productive B-cell response[82]. The BCR will cross-link when binding the antigen and increases expression of proteins promoting survival and cell cycle, antigen presentation molecules, cytokine receptors, and the CCR7 chemokine receptor. Activated B-cell survival is increased and cells proliferate, interact with Th-cells, respond to circulating cytokines, and migrate to T-cell areas for recognition by Th-cells[24].

After the activated B-cell has endocytosed the antigen, processed it, and presented the antigen on its surface via Class II MHC-peptide molecules, it will begin to move to the edge of the B-cell zone. Helper T-cells are activated by antigen presentation in the T-cell zone and once activated, will down regulate CCR7 and upregulate CXCR5 to move to the edge on the T-cell zone in the spleen to interact with activated B-cells. The activated helper T-cell also expresses CD40 ligand (CD40L) that binds to the CD40 receptor on antigen stimulated B-cells and induces B-cell proliferation and differentiation into plasma cells[24].

In antibody production, the activated Th-cell will express CD40L and secrete cytokines that help guide the proliferating B cells, which will be activated by cytokines and CD40 interaction from the Th-cell to proliferate and initiate differentiation into plasma cells that produce antibodies. Antibodies, or immunoglobulins, are glycosylated proteins found on the surface of B-cells and are also secreted by B-cells as effector molecules. They are comprised of four protein chains: two heavy (VH and CH) and two light (VL and CL) chains that are linked via a disulfide bond in a Y-shaped structure[83]. There are five isotypes of antibodies (IgM, IgD, IgG, IgA, and IgE). The effector mechanism is determined by isotype switching of the heavy-chain region of the antibody[83]. On a naïve B cell, the antibodies present on the surface of a B-cell are IgM and Immunoglobulin D (IgD)[84]. During an immune response, specific

11

cytokines will be released from activated CD4+ T-cells to induce isotype switching[84]. These cytokines act as specific signals that will make changes in the switch region of the c gene in naive B-cells by use of AID, a cytidine deaminase that converts cytosine to uracil[24]. The uracil is removed, and the DNA is repaired leaving a segment of DNA with only one isotype gene that is permanently expressed on the now activated B-cell[84]. Immunoglobulin G (IgG) is the most common isotype found in the human body and contains four subclasses with different effector functions: IgG1, IgG2, IgG3, and IgG4. IgG1 and IgG3 antibodies are produced in response to protein antigens whereas IgG2 and IgG4 are produced in response to polysaccharide antigens[85]. In mice, the IgG isotype contains four subclasses: IgG1, IgG2a, IgG2b, and IgG3 with the hierarchy of IgG3>> IgG2b > IgG2a>> IgG1[86]. The IgG antibodies also help to neutralize toxins and viruses. IgA is the second most common isotype found in the body and contains two subclasses: IgA1 and IgA2. IgA is responsible for protecting mucosal surfaces from toxins, viruses, and bacteria[87]. IgM is the third most common isotype found in the body and is associated with primary immune response and is the first immunoglobulin expressed in B-cell development[88]. This type of antibody would be considered the first line of defense in the body as well as helps regulate the immune response. IgD is the fourth most common isotype and is only found bound as BCR bound to the cell. The functions of IgD are poorly understood, but it may be protective against respiratory pathogens[89]. IgE is the least common antibody and is associated with hypersensitivity and allergic reactions. IgE is the hallmark cytokine of allergic asthma and bind to a high affinity receptor allowing more IgE to bind to mast cells, basophils, and eosinophils in preparation for antigen binding[90]. Omalizumab is a humanized, monoclonal antibody that has been shown to treat moderate to severe allergic asthma[91]. Omalizumab binds to the constant region of circulating IgE which prevents non-bound IgE from interacting with high- and low- affinity IgE receptors on mast cells, basophils, dendritic cells, and B-lymphocytes[92]. This reduces the volume of mediators released in the allergic response leading to a decreased Th2 immune response. Anti-IgE therapy has shown to decrease the number of serious asthmas exacerbations and asthma-related emergency room visits and hospitalization[93].

In cytokine production, activated B-cells will produce cytokines to help coordinate an effective immune response. Cytokines help regulate inflammatory cells, cell differentiation, and migration for both the innate and adaptive immune response[94]. The effects of cytokines are dependent on their high-

affinity binding to a receptor on a specific target cell. Allergic asthma produces high levels of pro-inflammatory cytokines (IL-4, IL-5, IL-8, IL-12, IL-13, IL-17A, IL-17F, IL-18, TNFα, TGFβ) and chemokines (CCL11/Eotaxin)[95]. B-cells specifically produce IL-4, IL-10, and TNFα[96]. Tumor necrosis factor alpha (TNFα) plays a role in the regulation of B-cell proliferation and differentiation by upregulating the transcription of NF-κB, which is part of the canonical cytokine pathway[97]. IL-4 is typically produced by B-cells when the cells are also secreting IgE antibodies. IL-4 has been shown to facilitate IgE-dependent mast cell activation and increase expression of Eotaxin. Eotaxin is a chemokine for eosinophils produced by the epithelial cells, smooth muscle cells, and fibroblasts[98]. Eotaxin prevents eosinophil apoptosis, promotes eosinophil activation, and other inflammatory cytokines that increase inflammation and lung remodeling in allergic asthma[99]. IL-5 is produced by CD8^{+} T-cells to regulate eosinophil function such as development, activation, recruitment to the lungs using Eotaxin, and survival[100, 101]. IL-5 is responsible for increased AHR in asthmatic patients[101]. Interleukin (IL-13) promotes class switching of IgM to IgE antibody production by B-cells, goblet cell differentiation, activation of fibroblasts, and airway hyperresponsiveness[93]. IL-13 also increases eosinophil function in the absence of IL-5 and Eotaxin[100]. TGFβ secreted mostly by eosinophils and fibroblasts as well as B-cells and is responsible for extensive airway remodeling and activating multiple intracellular pathways[102]. TGFβ effects airway remodeling by increasing the survival of inflammatory cells stimulating the proliferation of fibroblasts, and increasing mucus production by goblet cells[102].

Anti-inflammatory cytokines are produced to return the body to homeostasis. IFNγ is typically produced by Th1-cells and upregulates pathogen recognition, antigen processing and presentation in macrophages, inhibition of cellular proliferation, and viral infections of cells[103]. IFNγ plays a suppressive role for Th2 immune response and counteracts the effects of cytokines IL-4 and IL-5[104]. IL-10 is produced by CD4 T-helper cells in Th1 response to assist a return to homeostasis. B-regulatory cells prevent inflammatory responses by basophils, mast cells, and eosinophils by inhibiting antigen-presentation and expression of MHC class II and co-stimulatory molecules[105, 106].

1.6. Summary

In summary, asthma affects many aspects of life from health to economics. There is a close correlation between asthma exacerbations and hospital admissions and season, temperature, humidity,

and exposure to air pollution or allergens. This disease has different triggers and treatments that show differences between males and females, but there is relatively little known about specific mechanisms of action in fungal allergic asthma in regard to sex. Allergic asthma caused by *A. fumigatus* conidia elicits a Th2 type of response with pro-inflammatory cells, cytokines, and mediators. B-lymphocytes help to prime and maintain the immune response by performing antigen presentation and producing antibodies and cytokines. My thesis aims to characterize three areas of fungal allergic asthma by examining: 1) the *in vivo* model in low (<20%) relative humidity 2) sex the *in vivo* model in low humidity and 3) B-lymphocyte gene expression.

2. CHARACTERIZATION OF FUNGAL ALLERGIC ASTHMA IN DRY CONDITIONS

2.1. Introduction

The murine model of fungal allergic asthma that was developed in the Schuh laboratory produces airway hyperresponsiveness (AHR), eosinophilic inflammation, increased IgE and cytokine production, as well as fibrosis, smooth muscle hyperplasia, mucus hypersecretion, and airway wall remodeling[39]. Working with a murine model requires that the health of the animals be assessed daily to comply with approved protocols that are in line with the Office of Laboratory Animal Welfare and the NDSU Institutional Animal Care and Use Committee. This visual assessment includes monitoring for deviations from normal behavior, such as (changes in grooming, eating, drinking, and activity,) in addition to physical changes, such as (loss of fur, weight, wounds, or physical deficits). Serological testing as well as using sentinel animal protocol is used to ensure specific pathogen-free conditions and common murine pathogens are tested for every six months, providing that the animals are free from secondary infections that would confound immunological data for allergy and inflammation studies. Housing conditions of the animals are temperature controlled with 12-hr lighting cycles and 10+ air exchanges[107].

Both the abundance of allergens and the display of asthma symptoms can vary depending on climate conditions. For example, there is a close correlation between asthma exacerbations and hospital admissions and season, temperature, humidity, and exposure to air pollution or allergens[108, 109]. The association has been quantified: for every 10% increase in indoor humidity, there is a 2.7% increase in asthma prevalence[110]. In the study of fungal allergic asthma, high humidity levels allows for the growth of fungus and the subsequent release of more allergenic spores into the environment[21, 111]. Weather conditions in North Dakota can be extreme. Although the humidity in parts of the summer months may be considered 'muggy' with average outdoor humidity levels reaching as high as 75% in July, the average outdoor humidity for the winter months from November to April is 0%[112]. The NDSU Institutional Animal Care and Use Committee, relaying the recommendations of the Guide for the Care and Use of Laboratory Animals, recommends facility humidity within the range of 30-60%[107]. Since comfortable, suitable room humidity and temperature ranges are similar for humans and mice, these parameters had been considered acceptable in the murine housing facility located in room 106 Van Es Hall with concern only at times when heating or cooling for the building was generally unacceptable to humans (the colony is

housed in a room that is interior to the building and is connected to building heating and ventilation). It was only when unexplained changes in the murine colony became an issue that the humidity in the room was investigated. This was an extended process that weighed many other parameters of the model—antigen source and makeup, new operators' technique, animal vendor, infection, etc.—before concluding that humidity was playing a large part in the physical characteristics (reduced fecundity, small litter size, runting) and immunological response (reduced inflammation and poor development of architectural changes in the asthmatic lung) that we were experiencing.

After installing a humidity detector and tracking the average humidity in the colony through several seasons, we found that there was a vast fluctuation in humidity with a good share of the fall and winter having undetectable levels of indoor humidity. To mediate this problem, we had a Honeywell True Ease Flow Through Humidifier installed in the ductwork coming into the room. This was insufficient to increase the in-room humidity significantly and a Lasko Model 1128 Recirculating Console portable humidifier was added. Even with both units engaged, we were able to reach only 15%-20% relative humidity consistently. However, this appears to have significantly alleviated the problem.

While this situation was frustrating because it slowed down our normal research, it provided an opportunity to work through a vital animal colony management problem systematically. We examined our animal husbandry, care, and maintenance, as well as our model and techniques from various angles to come to the result. It also allowed the characterization of the immune response to *A. fumigatus* in an unusual situation of lower humidity, giving insight into the pathology of the fungus.

2.2. Materials and Methods

Animals. C57Bl/6 mice were purchased from Jackson Labs (Bar Harbor, ME, USA) and were bred and maintained in a specific pathogen-free facility for the duration of this study. Animals (4-6 months old) were fed LabDiet 5k67™ Rat and Mouse Auto-Ovals (PMI Nutrition International, LLC, St. Louis, MO, USA) and given water throughout the study and housed on Alpha-dri™ bedding (Shepherd Specialty Papers, Watertown, TN, USA) in micro filter topped cages (Ancare, Bellmore, NY, USA). Prior approval for these studies was obtained from the Institutional Animal Care and Use Committee of NDSU. After samples had been collected, it was noted that in-room humidity was extremely low and was likely low

during the entire lives of the animals. Relative humidity ranges are classified as: low humidity (<20%) and normal humidity (40-60%).

Antigen preparation and conidia culture. A. fumigatus antigen was purchased from Greer Laboratories (Lenoir, NC, USA). Lyophilized A. fumigatus culture was reconstituted in 5mL of phosphate buffered saline (PBS) per ATCC recommendations, and 60µL aliquots of the suspension were stored at 4°C until use. A single aliquot of A. fumigatus was grown on 10ml of Sabouraud dextrose agar (SDA) in a 25-cm^2 cell culture flask for eight days at 37°C. The use of A. fumigatus was approved by the NDSU Institutional Biosafety Committee.

Allergen sensitization and conidia inhalation challenge. To create an allergic phenotype, mice were injected subcutaneously (SC) and intraperitoneally (IP) with a 100 ul of soluble A. fumigatus antigen (Greer Laboratories, Lenoir, NC, USA) Antigen was prepared by suspending 2ug in 0.1mL PBS and 0.1mL Imject® Alum (Pierce, Rockford, IL, USA). Two weeks later, mice were started on a series of three, weekly 4µg intranasal (IN) inoculations of soluble A. fumigatus extract (Greer Laboratories, Lenoir, NC, USA) in 20µL of PBS. One week after the final IN inoculation, mice were exposed via nose only inhalation to eight day old A. fumigatus conidia, as previously described[39]. This inhalational challenge was repeated 14 days later. Prior to the inhalational challenge, mice were anesthetized using a solution of ketamine (75mg/kg) and xylazine (25mg/kg), and their noses were placed in the inoculation chamber to inhale mature A. fumigatus conidia for ten minutes as shown in **Figure 1** in the literature review. On Days 1, 5, and 41 post allergen challenge, animals were anesthetized with pentobarbital (150mg/kg) and a trachea tube was inserted for sample collection. This was the termination point of the study.

Airway hyperresponsiveness measurement. Mice were anesthetized using pentobarbital (150mg/kg) (Midwest Vet Supply), intubated, and ventilated using a Harvard Mini Vent, type 845 (Harvard Apparatus). Buxco whole body plethysmograph (Buxco Research Co., Wilmington, NC) was used to measure lung resistance and compliance. The system was calibrated to a breath volume of 225µL and breaths/minute of 150. The baseline value for airway resistance was measured for each animal before being administered a nebulized dose of methacholine (0, 4, 8, and 16 mg/mL) to determine AHR each time point.

Collection of serum, bronchoalveolar lavage (BAL) contents, and lung tissue. Blood was collected and centrifuged at 13,000xg for 10 minutes at 4ºC, to obtain serum which was stored at -80°C until use. 1mL of sterile PBS was inserted into the lungs via a trachea tube for bronchoalveolar lavage. Cells from the BAL fluid were pelleted via Microfuge 18 Centrifuge at 2,000xg for 10 minutes at 4ºC   (Beckman Coulter, Brea, CA), resuspended in 200µl of PBS, then Cytospun (Thermo Shandon, Waltham, MA) at 200xg for 5 minutes at room temperature onto slides and stained with Quik-Dip (Mercedes Medical, Sarasota, FL) stain. Cells from five, random high-powered fields (HPF, 1000X) were counted to determine the mean number of cells per HPF in the airway lumen of each mouse. Right lungs were harvested and used for RNA and protein extraction. Left lungs were fixed with 10% neutral buffered formalin, processed, and embedded in paraffin for histological sectioning. 5µm tissue sections of the left lung were stained with Hematoxylin and Eosin (H&E) (Thermo-Scientific, Kalamazoo, MI) to examine inflammation, Periodic Acid Schiff's stain (PAS) (Thermo-Scientific, Kalamazoo, MI) to examine mucus-producing goblet cells, and Gomori's Trichrome stain (Thermo-Scientific, Kalamazoo, MI) to examine collagen deposition in naïve and allergic mice[38]. For each Periodic Acid Schiff's stained lung section, a 100µm interval along the largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch) was examined for PAS-stained cells. For each Gomori's Trichrome stained lung section, 40-50 points were measured at 50µm intervals along lateral bronchiolar branches. A perpendicular line was drawn from the basement membrane through the full thickness of the collagen immediately below. The mean collagen thickness was reported for each sample, and the mean of the means was reported for each sex for histological analysis.

Quantification of serum for IgE, IgA, IgG1, IgG2a, IgG3. Total IgE, IgA, IgG1, IgG2a and IgG3(eBioscience, Vienna, Austria) were quantified via specific ELISA in serum according to the manufacturer's protocol. Briefly, plates were coated with anti-IgE, or anti-IgA, or anti-IgG1, or anti-IgG2a, or anti-IgG3 capture antibodies and incubated overnight at 4°C. Washed plates were blocked for 2 hours at room temperature in PBS with 1% Tween™ and 10% Bovine Serum Albumin. Washed plates were incubated in serum samples (1:100 for IgE, 1:2000 for IgA, 1:500 for IgG1, 1:5000 for IgG2a, 1: 100,000 for IgG3) at room temperature for 1 hour. Dilutions for each sample were predetermined by previous dilution series per antibody. Plates were washed, then incubated in detection antibody, enzyme

conjugate, and developed in substrate. A Stop Solution (eBioscience™ Stop Solution Cat. No.

BMS409.0100) was added and plates were read at 450nm. The minimum detection limits for the kits were

5ng/mL for IgE, 0.391ng/mL for IgA, 3.12ng/mL for IgG1, 3.91ng/mL for IgG2a, and 1.95ng/mL for IgG3.

Statistical analysis. Results from sensitized and challenged groups were compared to a non-

sensitized, unchallenged, control group (Day 0). An unpaired Student two-tailed *t* test with a Welch

correction was used to determine statistical significance with Prism GraphPad software (San Diego, CA,

USA). Group sizes were as follows: at day 0 (naïve) $n = 6$ mice, at day 1 $n = 10$ mice, at day 5 $n = 10$

mice, and at day 41 $n = 4$ mice. For each allergic timepoint compared with the naïve control, statistical

significance is indicated as follows: *$p = 0.01$-0.05, **$p = 0.001$-0.01, ***$p < 0.001$, ****$p < 0.0001$. All results

are expressed as the means $\pm$ SEM.

2.3. Results

Low humidity conditions do not affect weight in mice born, raised, and sensitized in low humidity.

To determine the effects of dry conditions, adult mice were weighed at predetermined timepoints **(Figure

2)**. There was no statistical difference between groups. Mice born and raised in low humidity appeared

smaller and had rough coats compared to mice born and raised in normal humidity conditions (data not
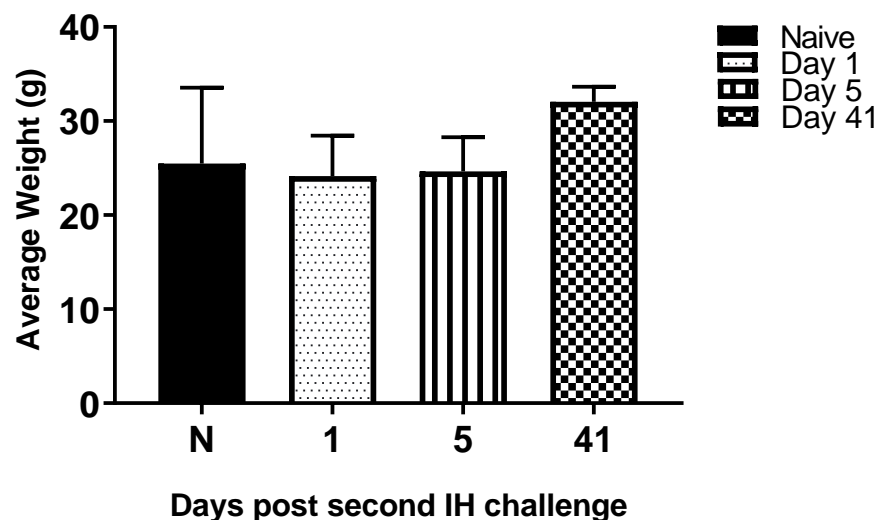
shown).

Figure 2. The weight of mice in low humidity. Mice were exposed to *A. fumigatus* according to the schedule shown in **Figure 2**. Mice were weighed days 1, 5, and 41 after final allergen challenge. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (n =4-10 mice per group).

Mice raised in low humidity fail to develop airway hyperresponsiveness after inhalation to A. fumigatus *conidia.* Airway response measurements from all study animals were used to determine the baseline mean for airway hyperresponsiveness before nebulized methacholine. Inhalation of *A. fumigatus* did not significantly increase airway hyperresponsiveness (AHR) at day 1, 5, or 41 as compared to naïve animals **(Figure 3)**.
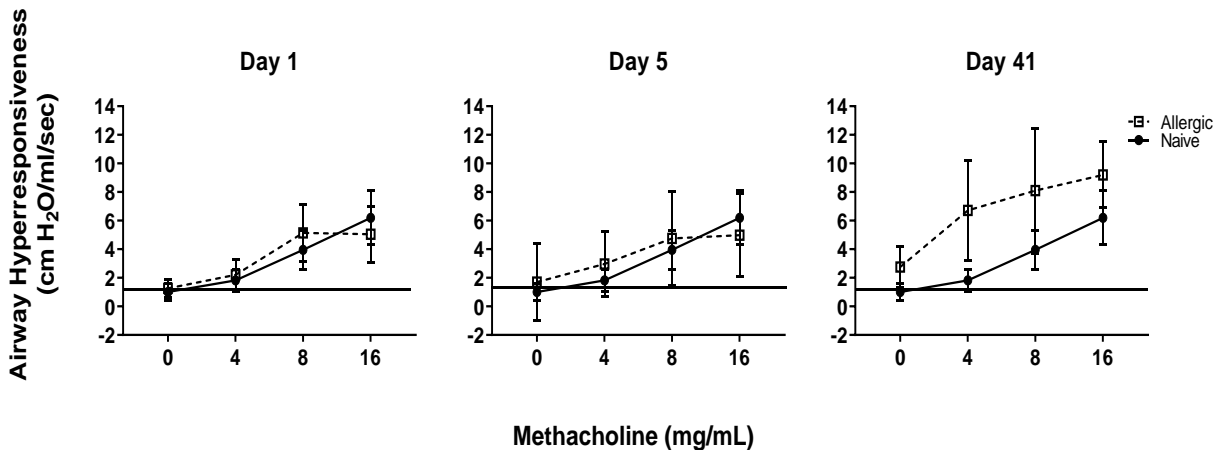

Figure 3. Inhalation of *A. fumigatus* conidia in low humidity does not increase airway hyperresponsiveness. Baseline airway response was obtained (0.87 ± 0.15 cmH$_2$O/mL/sec is shown as solid line) before methacholine challenge. Airway responses from increasing doses of nebulized methacholine (0, 4, 8, and 16 mg/mL) were recorded. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (*n* =4-10 mice per group).

Fewer inflammatory cells are recruited to the allergic airways after fungal conidia challenge in low humidity. Using morphometric analysis of macrophages, neutrophils, eosinophils, and lymphocytes in the lumen of the lungs, we assessed inflammatory cell recruitment to the lungs of allergen-sensitized animals that had inhaled conidia **(Figure 4)**. In naïve animals, alveolar macrophages averaged 5 cells/HPF while no other cell types were present **(Figure 4A, B, C, D)**. The most prominent cell types in the BAL 1 day after the second conidia challenge showed macrophages (average of 5 cells/HPF) and neutrophils (average 8 cells/HPF) **(Figure 4A, B).** Macrophages (average of 12 cells/HPF), neutrophils (average of 25 cells/HPF) and eosinophils (average of 15 cells/HPF) were prominent cell types identified in the BAL 5 days after the second conidia challenge **(Figure 4A, B, &C)**. Eosinophils averaged 15 cells/HPF **(Figure 4C)** in the BAL 5 days after multiple inhalations of conidia indicated an allergic response. Macrophages

(average of 10 cells/HPF) and lymphocytes (average of 2cells/HPF) were prominent cell types identified

in the BAL 41 days after the second conidia challenge **(Figure 4A, D)**.

Days post second IH challenge
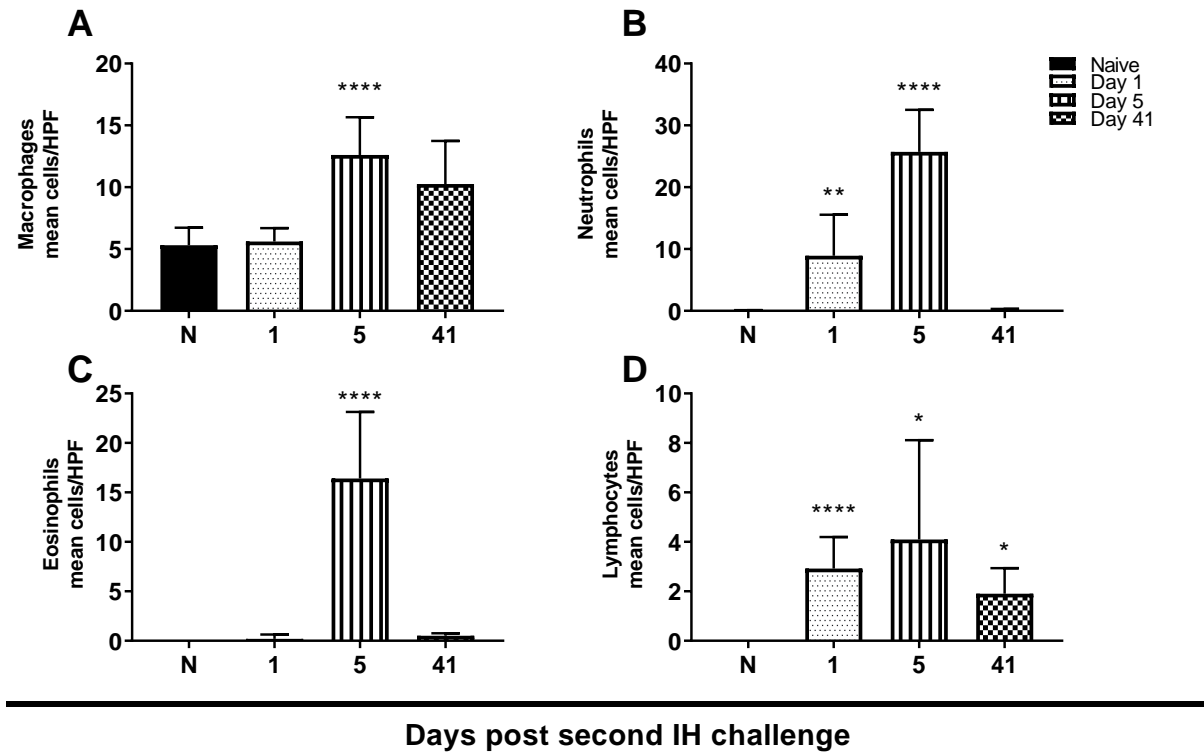
Figure 4. Effect of *A. fumigatus* conidia inhalation on inflammatory cells in the allergic lung in low humidity. Cells were cytospun onto coded microscope slides and Quik-Dip stained. Five randomly selected (HPF, 1000X) were counted per sample. Airway inflammation was marked by the presence of **(Figure 4A)** macrophages, **(Figure 4B)** neutrophils, **(Figure 4C)** eosinophils, and **(Figure 4D)** lymphocytes in naive and allergic C57Bl/6 mice. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (*n* =4-10 mice per group). *p<0.05, **p<0.01, ****p<0.0001 is significant when compared to naive control.

Mice sensitized and challenged under low humidity conditions failed to exhibit tissue

inflammation. Total lung inflammation was assessed by examining H&E stained lung sections **(Figure 5).**

There was little peribronchial inflammation evident at any of the three timepoints examined for this study

(Figure 5B-D). Although some smooth muscle hyperplasia and peribronchial fibrosis could be seen in the

histological sections, overall inflammation was similar to the amount of inflammation seen in naïve mice.

No statistical significance between naïve and any of the three timepoints total H&E score.

Naïve	Day 1	Day 5	Day 41
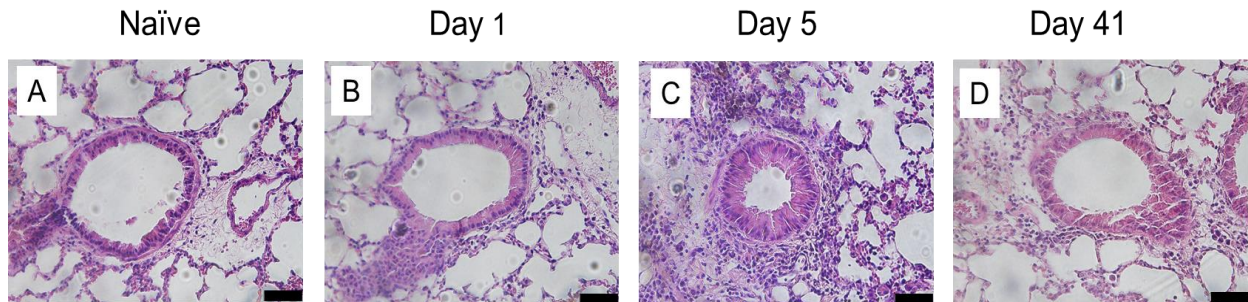
Figure 5. Inhalation of *A. fumigatus* conidia does not increase pulmonary inflammation in low humidity. Representative pictures (200x) of H&E stained lung sections of allergen-challenged day 1,5, and 41 C57Bl/6 mice **(Figure 5A-D).** Bar represents 50µm. There was little inflammation seen at the three timepoints compared to naïve.

Inhalation of Aspergillus fumigatus *increases goblet cell production mice at day 1, 5 and 41 in low humidity.* Goblet cells (mucus-producing cells) were assessed by counting PAS-stained cells and representing them as a percentage of total epithelial cells lining the lateral airways of each lung section **(Figure 6)**. For each sample, a 100µm interval along the largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch) was examined for PAS-stained cells **(Figure 6A)**. There was a significant increase in the number of goblet cells (~35% of total) at day 1 **(Figure 6B)**. There is a significant number of goblet cells (~50% of total) lining the airways at day 5 **(Figure 6B)**. The total number of goblet cells (only ~15% of total) decreases by day 41 after the second conidia challenge compared to the day 5 time point. **(Figure 6D).**
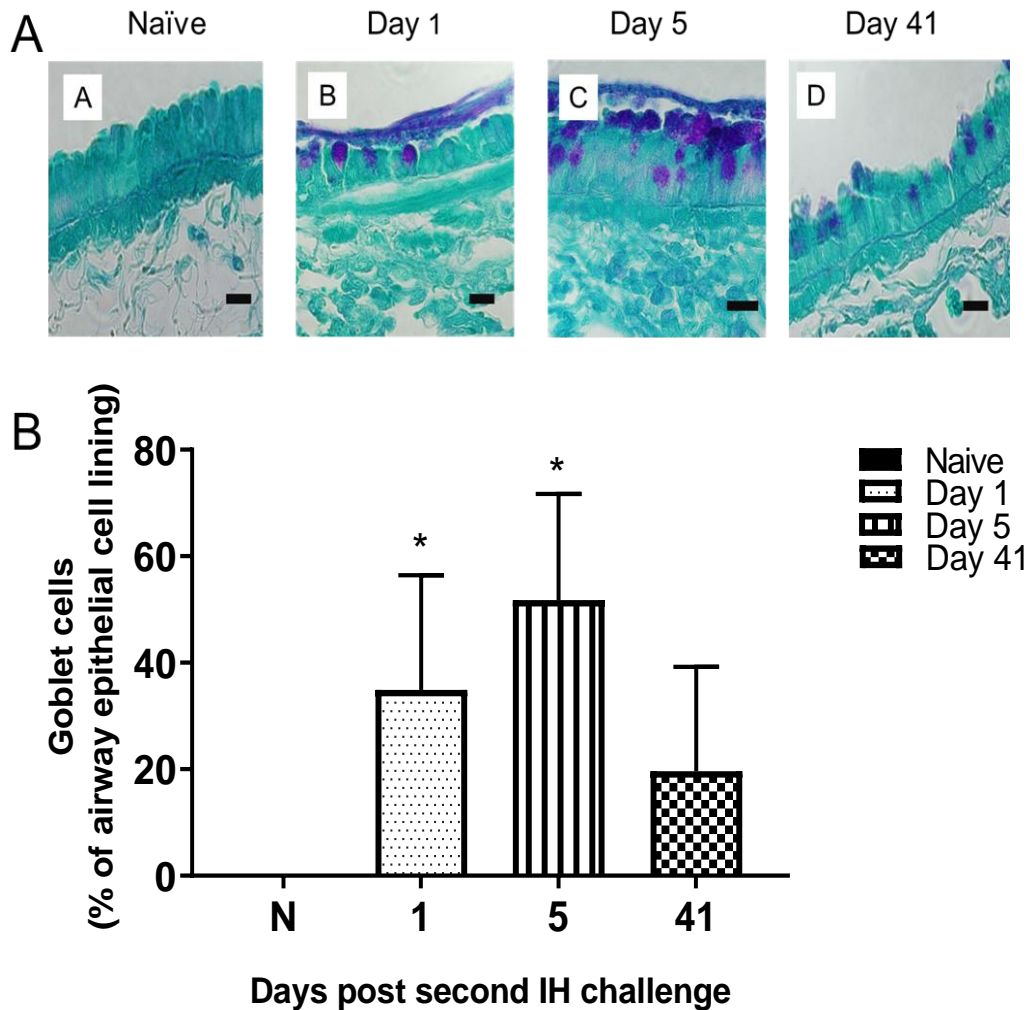
Figure 6. Goblet cell metaplasia is increased after the inhalation of *A. fumigatus* conidia in low humidity. Day 1, 5, and 41 C57BL/6 mice show that goblet cells and mucus production were present in the airways. Representative image of goblet cell production **(Figure 6A)**. Bar represents 10µm. Goblet cell numbers were reported as the percent of total epithelial cells along 100µm segments lining the lateral branches of the lung **(Figure 6B)**. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (n=4-10 mice per group). *$p<0.05$ is significance when compared to naïve control.

Inhalation of Aspergillus fumigatus *significantly increases collage deposition at day 1, 5, and 41 in low humidity.* Collagen production was assessed by measuring the thickness of Gomorri's trichome stained peribronchial collagen from the basement membrane to the lumen of the lung **(Figure 7)**. For each sample, at least 50 discrete points were measured at 50µm intervals along the largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch). A perpendicular line was drawn from the point on the basement membrane through the full thickness of the collagen immediately below **(Figure 7A)**. The mean collagen thickness was reported for each sample, and the

23

mean of the means was reported for each group. There is a significant amount of collagen produced at

day 1 (average of 15µm thickness), day 5 (average of 20µm thickness), and day 41 (average of 30µm

thickness) **(Figure 7B)**. Collagen accumulation did not diminish over the time course of this study. This

phenomenon has been seen and extended in other studies by our laboratory in both BALB/c and

C57BL/6 mice[38, 51, 113].
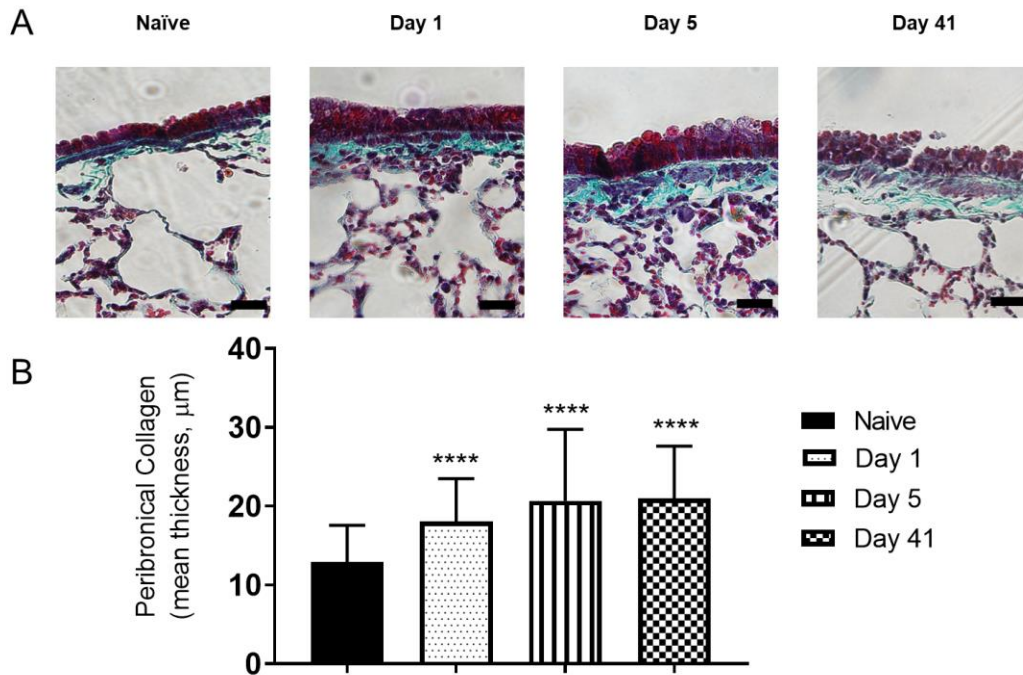
Figure 7. Effect of *A. fumigatus* conidia on collagen thickness in low humidity. Gomorri's trichrome
stain was used to visualize collagen deposition in whole lung sections **(Figure 7A)**. Bar represents 50µm.
Approximately 40-50 points were measured at 50µm intervals along the L2 or L3 lateral branches of the
airway. A perpendicular line was drawn from the point on the basement membrane through the full
thickness of the collagen deposition below. The mean collagen thickness was reported for each timepoint
(Figure 7B). Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All
values are expressed as mean $\pm$ SEM (*n* =4-10 mice per group). ****p<0.0001 is significant when
compared to naïve control.

Fungal inhalation in low humidity conditions resulted in significant increased serum IgE and IgG3

levels in allergic mice at day 1, 5, and 41. IgE is significantly increased in at day 5 mice over naïve

indicating an allergic response **(Figure 8A)**. IgG3 is significantly increased in mice at day 1 and day 5

compared to naïve **(Figure 8B)**. IgA is not significantly changed in allergic mice at all three-time points

compared to naïve **(Figure 8C)**. IgG1 is not significantly changed in mice at all three-time points

compared to naïve **(Figure 8D)**. IgG2a is not significantly changed in mice at day 1 and day 5 compared

to naïve **(Figure 8E)**.

Days post second IH challenge
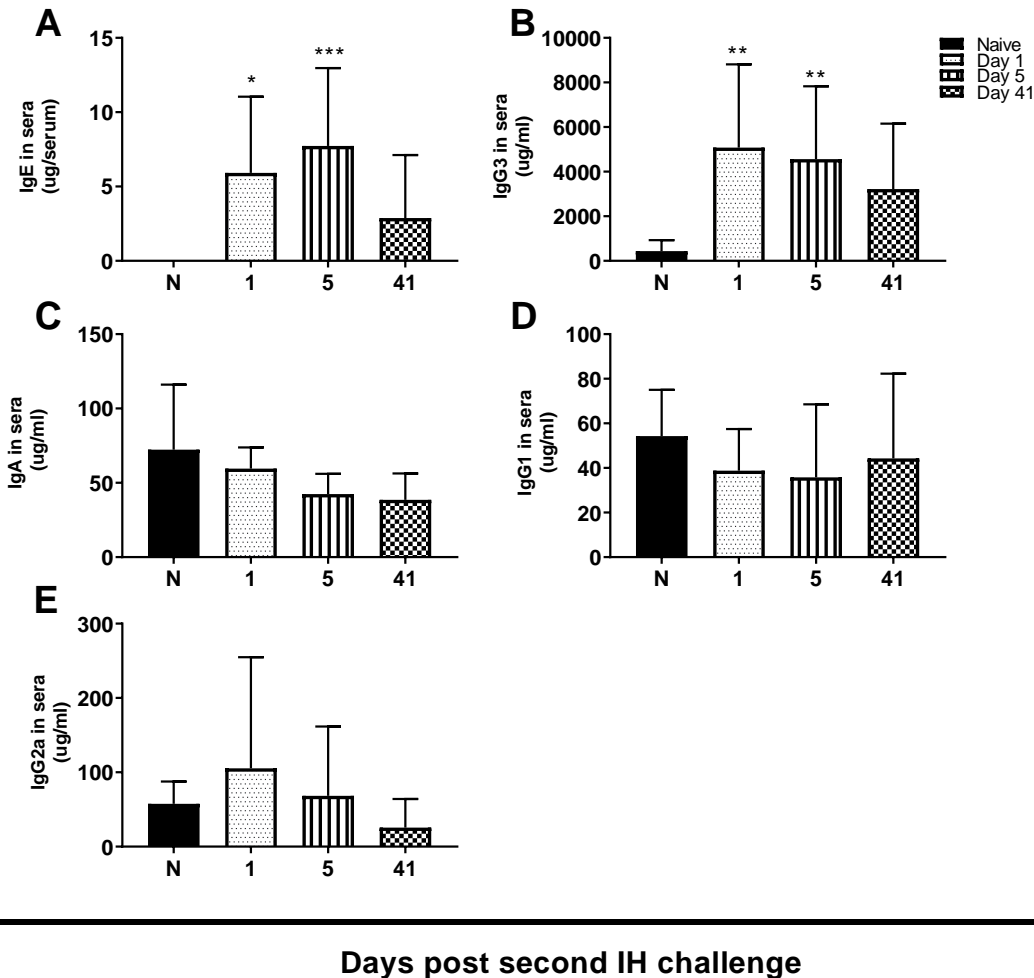
Figure 8. Effect of inhaled *A. fumigatus* on the production of IgE, IgA, IgG1, IgG2a, IgG3 in low humidity. Mice were sensitized and exposed to *A. fumigatus* per **Figure 2**. Ab isotypes were quantified by specific ELISA in serum at days 1, 5, and 41. IgE **(Figure 8A)** IgG3 **(Figure 8B)** IgA **(Figure 8C)** IgG1 **(Figure 8D)** IgG2a **(Figure 8E)**. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (*n* =4-10 mice per group). *p<0.05, **p<0.01, **p<0.001 is significant when compared to naïve control.

2.4. Discussion

Low humidity conditions have been recommended as a potential treatment option to those suffering from asthma if the humidity levels stay between 30-50%[32]. We would have expected that lower humidity in the mouse room, below 15%, would have contributed to the allergic asthma phenotype by increasing airway hyperresponsiveness, inflammatory cells present in the lungs, and overall inflammation because lower humidity has been shown to cause the airways to become more irritated, which will become more sensitive to the conidia. Humidity extremes can result in physiological and morphologic changes that can negatively affect the animal and outcome of research protocols[107]. To

characterize humidity in our model, we examined the following readouts: weight, airway hyperresponsiveness, inflammation via BAL and histological staining, and antibody production.

Overall, low humidity had no impact on the weight of model animals compared to naïve that were raised in similar conditions. Mice born, raised, and used for studies in low humidity conditions appeared smaller and had rougher looking coats compared to historical data of the colony in normal humid conditions. The mice weighed 25-35 grams which is in the normal range for adult mice (males 20-35g and females 18-25g)[114]. However, the low humidity affected the mice coats and the overall breeding productivity of the colony.

Airway hyperresponsiveness was not significantly increased in allergic mice over naïve after inhalation of *A. fumigatus* conidia in low humidity conditions. In normal humid conditions, our model has shown significant increases airway hyperresponsiveness after multiple inhalational challenges of *A. fumigatus* conidia[51]. A study performed on rats examining exercise-induced bronchoconstriction showed that humid conditions were more likely to increase airway hyperresponsiveness[115]. Another study examining outdoor temperature and humidity influencing methacholine tests showed that more humid conditions increase the prevalence of airway hyperresponsiveness[116].

There was little lung inflammation indicated by the slight increase of inflammatory cells in the allergic mice at the different timepoints compared to naïve. The low humidity conditions showed an influx of 15 macrophages/HPF, 30 neutrophils/HPF, 25 eosinophils/HPF, and 10 lymphocytes/HPF. Normal humid conditions have shown 40 macrophages/HPF, 40 neutrophils/HPF, 200 eosinophils/HPF, and 100 lymphocytes/HPF[51]. H&E stained sections showed no difference in inflammation between naïve and all three timepoints in low humidity conditions. In normal humid conditions, there is severe inflammation after multiple inhalations of conidia. In the low humidity conditions, eosinophils were present in the lungs at day 5 and still produced IgE antibodies at all three timepoints indicating an allergic phenotype. In normal humid conditions, there are more eosinophils present, but the levels of serum IgE is similar to the levels of IgE in the less humid conditions[51]. Low humidity conditions do not prevent the production of IgE, but do not allow for proper growth of allergens and minimizes the number of encounters of allergen to elicit an allergic response[117].

The low humidity conditions produced goblet cells at all three timepoints compared to naïve. Day 5 significantly had more goblet cells produced compared to naïve. In normal humid conditions, goblet cells are produced and the percentage of goblet producing cells are similar to the percentage of goblet producing cells in lower humidity conditions[51, 113]. Low humidity conditions are known to cause an increase in mucus production in the disease Vasomotor Rhinitis (VMR)[118, 119]. VMR is characterized by the excess amount of mucous produced by the mucous membranes and can be caused by dry conditions. The low humidity conditions dry out the nasal passage and the mucous membranes over produce mucus leading to excess mucus in the nasal cavities[120].

The low humidity conditions produced significant peribronchial collagen deposition at all three timepoints compared to naïve controls. In humid conditions, peribronchial collagen deposition is increased compared to naïve controls and the thickness of collagen is similar to the thickness of collagen in humid conditions[51, 113]. There is little research on the effects of humidity on collagen deposition.

The low humidity conditions produced no significant differences in serum levels of IgA, IgG1, and IgG2a in allergic mice at day 1, 5, and 41 compared to naïve. In normal humid conditions, no significant difference of IgA and IgG2a production is observed in allergic mice when compared to naïve controls[51, 113]. In normal humid conditions, there is a significant increase in IgG1 production compared to naïve controls[113]. IgG1 antibodies are produced in response to protein antigens[85]. A study examining cystic fibrosis association with allergic bronchopulmonary aspergillosis (ABPA) showed increased IgG1 antibody titers after colonization of *A. fumigatus[121].* There is little research on the effects of humidity on IgG1 antibody production.

The low humidity conditions produced a significant amount of IgG3 antibodies in allergic mice compared to naïve. We have not studied the amount of IgG3 production in normal humid conditions. IgG3 produces antibodies against protein antigens[85]. The IgG3 antibody response was significantly increased in allergic mice than any of the other antibody responses. We hypothesize that IgG3 is acting as a neutralizing antibody and providing protection in the airways from *A. fumigatus* conidia. IgG3 has been shown to act as a neutralizing antibody in a lupus flare murine model and in several viral diseases such as Dengue fever and HIV[122-124].

We observed no increase in airway hyperresponsiveness, inflammatory cells present, and total inflammation in low humidity conditions. The low humidity conditions prevented the allergic asthmatic phenotypes of airway hyperresponsiveness and overall inflammation aside from reducing IgE Ab levels.

3. CHARACTERIZATION OF SEX IN FUNGAL ALLERGIC ASTHMA IN DRY CONDITIONS

3.1. Introduction

The murine model of fungal allergic asthma that was developed in the Schuh laboratory produces airway hyperresponsiveness (AHR), eosinophilic inflammation, increased IgE and cytokine production, as well as fibrosis, smooth muscle hyperplasia, and mucus hypersecretion that account for airway wall remodeling[39]. This model has been used to examine the effects in multiple mouse strains (C57BL/6, JH, VPACK 2K/O, BALB/c, µMT) at acute and chronic timepoints. However, the model has never been used to examine the characterization of the immune response to *A. fumigatus* in sex.

Studies have shown an increase in asthma in boys before puberty and after puberty, an increase in asthma in women[125]. These asthmatic women have a poorer quality of life that requires more frequent and greater use of oral corticosteroids and short acting-beta-agonists[125]. Asthmatic women also have an increase in healthcare visits and costs and a higher mortality rate than men suffering from asthma[125]. There are several different asthmatic mouse models looking at sex differences. In those using ovalbumin, researchers have found an increase in inflammatory cells, Th2 cytokines, immunoglobulins IgE and IgA, and airway remodeling in female mice compared to male mice[126, 127]. In those using a lipopolysaccharide (LPS) antigen, researchers have found an increase in airway hyperresponsiveness in male mice compared to female mice[127]. These animal studies and epidemiology studies have shown that sex differences do exist in asthma; therefore, it is important to examine sex for the characterization of our model in all types of environmental conditions and to observe if sex is linked to low humidity conditions.

3.2. Materials and Methods

Animals. C57Bl/6 mice were purchased from Jackson Labs (Bar Harbor, ME, USA) and were bred and maintained in a specific pathogen-free facility for the duration of this study. Animals (4-6 months old) were fed LabDiet 5k67™ Rat and Mouse Auto-Ovals (PMI Nutrition International, LLC, St. Louis, MO, USA) and given water throughout the study and housed on Alpha-dri™ bedding (Shepherd Specialty Papers, Watertown, TN, USA) in micro filter topped cages (Ancare, Bellmore, NY, USA). Prior approval for these studies was obtained from the Institutional Animal Care and Use Committee of NDSU. Relative humidity ranges are classified as: low humidity (<20%) and normal humidity (40-60%).

Antigen preparation and conidia culture. A. fumigatus antigen was purchased from Greer Laboratories (Lenoir, NC, USA). Lyophilized *A. fumigatus* culture was reconstituted in 5mL of phosphate buffered saline (PBS) per ATCC recommendations, and 60μL aliquots of the suspension were stored at 4°C until use. A single aliquot of *A. fumigatus* was grown on 10mL of Sabouraud dextrose agar (SDA) in a 25-cm^2 cell culture flask for eight days at 37°C. The use of *A. fumigatus* was approved by the NDSU Institutional Biosafety Committee.

Allergen sensitization and conidia inhalation challenge. To create an allergic phenotype, mice were injected subcutaneously (SC) and intraperitoneally (IP) with a 100 ul of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) Antigen was prepared by suspending 2ug in 0.1mL PBS and 0.1mL Imject® Alum (Pierce, Rockford, IL, USA). Two weeks later, mice were started on a series of three, weekly 4μg intranasal (IN) inoculations of soluble *A. fumigatus* extract (Greer Laboratories, Lenoir, NC, USA) in 20μL of PBS. One week after the final IN inoculation, mice were exposed via nose only inhalation to eight day old *A. fumigatus* conidia, as previously described[39]. This inhalational challenge was repeated 14 days later. Prior to the inhalational challenge, mice were anesthetized using a solution of ketamine (75mg/kg) and xylazine (25mg/kg), and their noses were placed in the inoculation chamber to inhale mature *A. fumigatus* conidia for ten minutes as shown in **Figure 1** in the literature review. On Days 1, 5, and 41 post allergen challenge, animals were anesthetized with pentobarbital (150mg/kg) and a trachea tube was inserted for sample collection. This was the termination point of the study.

Airway hyperresponsiveness measurement. Mice were anesthetized using pentobarbital (150mg/kg) (Midwest Vet Supply), intubated, and ventilated using a Harvard Mini Vent, type 845 (Harvard Apparatus). Buxco whole body plethysmograph (Buxco Research Co., Wilmington, NC) was used to measure lung resistance and compliance. The system was calibrated to a breath volume of 225μL and breaths/minute of 150. The baseline value for airway resistance was measured for each animal before being administered a nebulized dose of methacholine (0, 4, 8, and 16 mg/mL) to determine AHR at each time point.

Collection of serum, bronchoalveolar lavage (BAL) contents, and lung tissue. Blood was collected and centrifuged at 13,000xg for 10 minutes at 4°C, to obtain serum which was stored at -80°C until use. 1mL of sterile PBS was inserted into the lungs via a trachea tube for bronchoalveolar lavage. Cells from

the BAL fluid were pelleted via Microfuge 18 Centrifuge at 2,000xg for 10 minutes at 4ºC   (Beckman

Coulter, Brea, CA), resuspended in 200µl of PBS, then Cytospun (Thermo Shandon, Waltham, MA) at

200xg for 5 minutes at room temperature onto slides and stained with Quik-Dip (Mercedes Medical,

Sarasota, FL) stain. Cells from five, random high-powered fields (HPF, 1000X) were counted to determine

the mean number of cells per HPF in the airway lumen of each mouse. Right lungs were harvested and

used for RNA and protein extraction. Left lungs were fixed with 10% neutral buffered formalin, processed,

and embedded in paraffin for histological sectioning. 5µm tissue sections of the left lung were stained with

Hematoxylin and Eosin (H&E) (Thermo-Scientific, Kalamazoo, MI) to examine inflammation, Periodic Acid

Schiff's stain (PAS) (Thermo-Scientific, Kalamazoo, MI) to examine mucus-producing goblet cells, and

Gomori's Trichrome stain (Thermo-Scientific, Kalamazoo, MI) to examine collagen deposition in naïve

and allergic mice[38]. For each Periodic Acid Schiff's stained lung section, a 100µm interval along the

largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch)

was examined for PAS-stained cells. For each Gomori's Trichrome stained lung section, 40-50 points

were measured at 50µm intervals along lateral bronchiolar branches. A perpendicular line was drawn

from the basement membrane through the full thickness of the collagen immediately below. The mean

collagen thickness was reported for each sample, and the mean of the means was reported for each sex

for histological analysis.

Quantification of serum for IgE, IgA, IgG1, IgG2a, IgG3. Total IgE, IgA, IgG1, IgG2a and

IgG3(eBioscience, Vienna, Austria) were quantified via specific ELISA in serum according to the

manufacturer's protocol. Briefly, plates were coated with anti-IgE, or anti-IgA, or anti-IgG1, or anti-IgG2a,

or anti-IgG3 capture antibodies and incubated overnight at 4°C. Washed plates were blocked for 2 hours

at room temperature in PBS with 1% Tween™ and 10% Bovine Serum Albumin. Washed plates were

incubated in serum samples (1:100 for IgE, 1:2000 for IgA, 1:500 for IgG1, 1:5000 for IgG2a, 1: 100,000

for IgG3) at room temperature for 1 hour. Dilutions for each sample were predetermined by previous

dilution series per antibody. Plates were washed, then incubated in detection antibody, enzyme

conjugate, and developed in substrate. A Stop Solution (eBioscience™ Stop Solution Cat. No.

BMS409.0100) was added and plates were read at 450nm. The minimum detection limits for the kits were

5ng/mL for IgE, 0.391ng/mL for IgA, 3.12ng/mL for IgG1, 3.91ng/mL for IgG2a, and 1.95ng/mL for IgG3.

Statistical analysis. Allergic C57BL/6 WT animals were compared to each sex and to their respective naïve controls at each timepoint. An unpaired Student two-tailed *t* test with a Welch correction was used to determine statistical significance with Prism GraphPad software (San Diego, CA, USA). All results are expressed as the means $\pm$ SEM. Group sizes were as follows: at day 0 (naïve) $n = 3$ mice/sex, at day 1 $n = 5$ mice/sex, at day 5 $n = 5$ mice/sex, and at day 41 $n = 1$ female mouse/4 male mice. No statistical analysis for sex differences was performed Day 41 samples as only one female C57BL/6 mouse was viable through the end of the study. For each allergic timepoint compared with the naïve control, statistical significance is indicated as follows: *$p = 0.01$-0.05, **$p = 0.001$-0.01, ***$p < 0.001$. Where appropriate, ^ indicates statistical difference between the female and male C57Bl/6 WT mice (^$p = 0.05$).

3.3. Results

Adult male mice weigh significantly more than female mice. To determine the weight of mice in the model, we weighed the mice at predetermined time points **(Figure 9)**. All male mice weighed significantly more than female mice except for day 41 post second inhalation conidia challenge **(Figure 9)**.
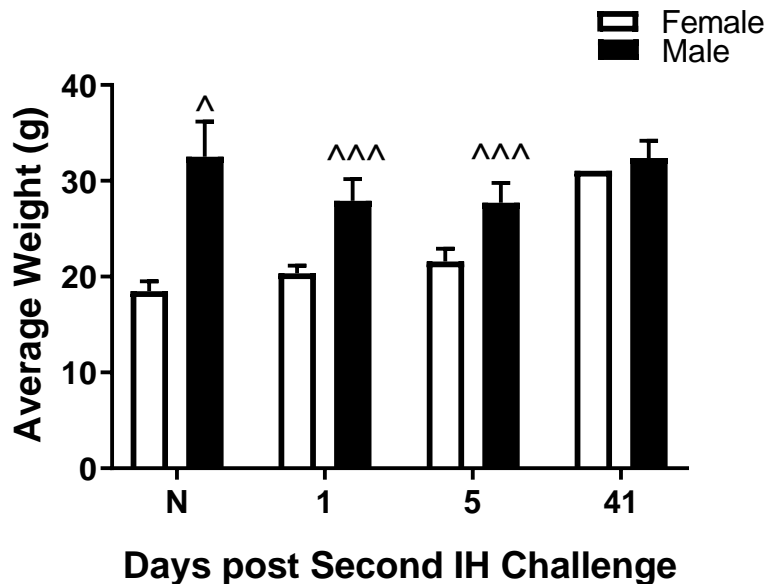
Figure 9. Weight of sex-matched mice in low humidity. Mice were exposed to *A. fumigatus* according to the schedule shown in **Figure 2**. At days 1, 5, and 41 mice were weighed before sample collection. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (n =3-5 mice per group). ^$p < 0.5$, ^^^$p < 0.001$ is significant when compared between males and females at each respective timepoint.

Airborne fungal challenge results in little airway hyperresponsiveness in male and female mice

after sensitization to A. fumigatus *in low humidity*. Airway response measurements from all study animals were used to determine the baseline mean for airway hyperresponsiveness prior to nebulized methacholine. Inhalation of *A. fumigatus* does not significantly increase airway hyperresponsiveness (AHR) in either male mice or female mice at day 1, 5 and 41 (**Figure 10**). No differences were seen between male and female mice in AHR at any of the respective timepoints.
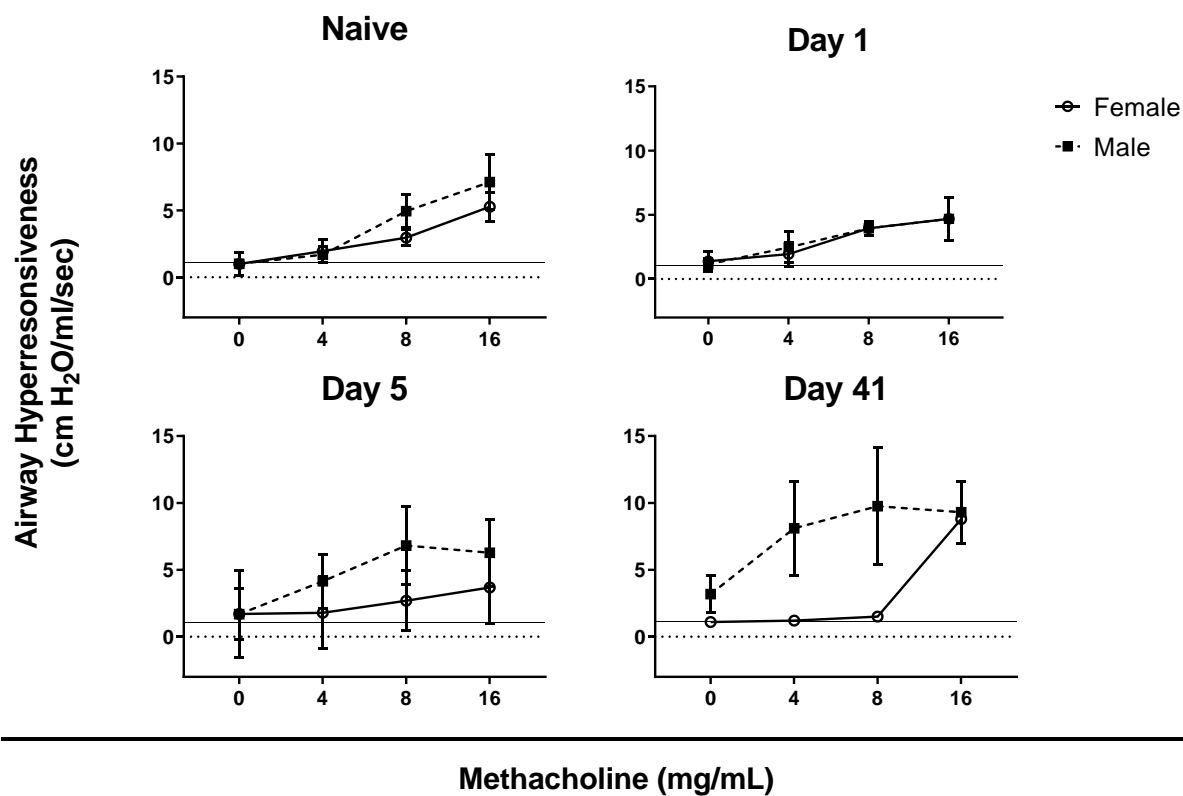
Figure 10. Inhalation of *A. fumigatus* conidia shows no differences in airway hyperresponsiveness between male and female mice in low humidity. Baseline response was obtained (0.87±0.15 cmH$_2$O/mL/sec is shown as solid line) prior to methacholine challenge. Nebulized methacholine (0,4,8, and 16 mg/mL) challenges were recorded**.** Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (*n* =3-5 mice per group). There was no statistical significance between males and females.

Fewer inflammatory cells are recruited to the allergic airways after fungal conidia challenge in

both females and males in low humidity. Inflammatory cell recruitment to the lungs of allergen-sensitized animals that had inhaled conidia was evaluated using morphometric analysis of macrophages, neutrophils, eosinophils, and lymphocytes in the lumen of the lungs **(Figure 11)**. In naïve animals, the

dominant cell type seen were alveolar macrophages (average of 5 cell/HPF) for males and females **(Figure 11)**. Macrophages (average of ~5 cells/HPF for female and male), neutrophils (average of ~8 cells/HPF for female and male), and lymphocytes (average of ~4cells/HPF for female, average of ~2 cells for male) were identified in the BAL on day 1 **(Figure 11)**. Macrophages (average of ~13 cells/HPF for female, average of ~11 cells/HPF for male), neutrophils (average of ~25 cells/HPF for female, average of ~22 cells/HPF for male), and eosinophils (average of ~12 cells/HPF for female, average of ~18 cells/HPF for male) were prominent cell types identified in the BAL 5 days after the second conidia challenge **(Figure 11)**. On day 41 post second conidia challenge, the dominant cell type seen was macrophages (average of ~10 cells/HPF) **(Figure 11).** Eosinophils present **(Figure 11)** in the BAL of both male and female mice indicate an allergic response after multiple inhalations of conidia. There was no statistical difference in any cell type between male and female mice at all timepoints.

Days post second IH challenge

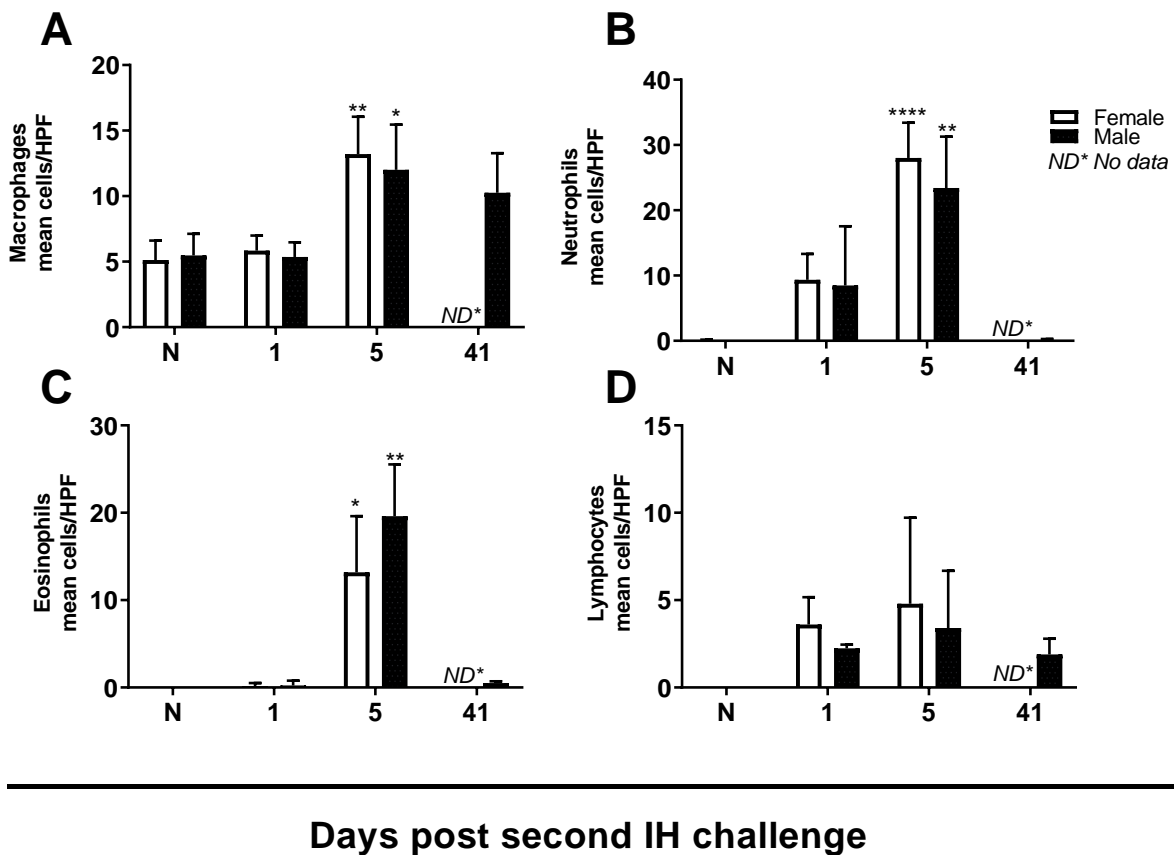**Figure 11. Effect of *A. fumigatus* conidia inhalation on inflammatory cells in the allergic lung of male and female mice in low humidity.** Cells were cytospun onto coded microscope slides and Quik-Dip stained. Five randomly selected (HPF, 1000X) were counted per sample. Airway inflammation was marked by the presence of **(Figure 11A)** macrophages, **(Figure 11B)** neutrophils, **(Figure 11C)**

eosinophils, and **(Figure 11D)** lymphocytes in naive and allergic C57Bl/6. There was more inflammation present at D5 **(Figure 11C)**. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (*n* =3-5 mice per group). *p<0.05, **p<0.01, ****p<0.0001 is significant when compared to the respective naive controls. There was no statistical significance between males and females.

Inhalation of A. fumigatus *does not cause significant differences in overall lung inflammation between male and female mice in low humidity.* Overall lung inflammation was evaluated using H&E stained lung sections **(Figure 12)**. Representative images of female mice **(Figure 12A-D)** and male mice **(Fig 12E-H).** No inflammation was observed at day 1 **(Figure 12B, F)** for both males and females. No significant changes in inflammation were observed at day 5 **(Figure 12C, G)** and at day 41 **(Figure 12D, H)** between males and females. No statistical significance of the total H&E scores between respective naïve controls and any of the sexes at all three timepoints was observed.
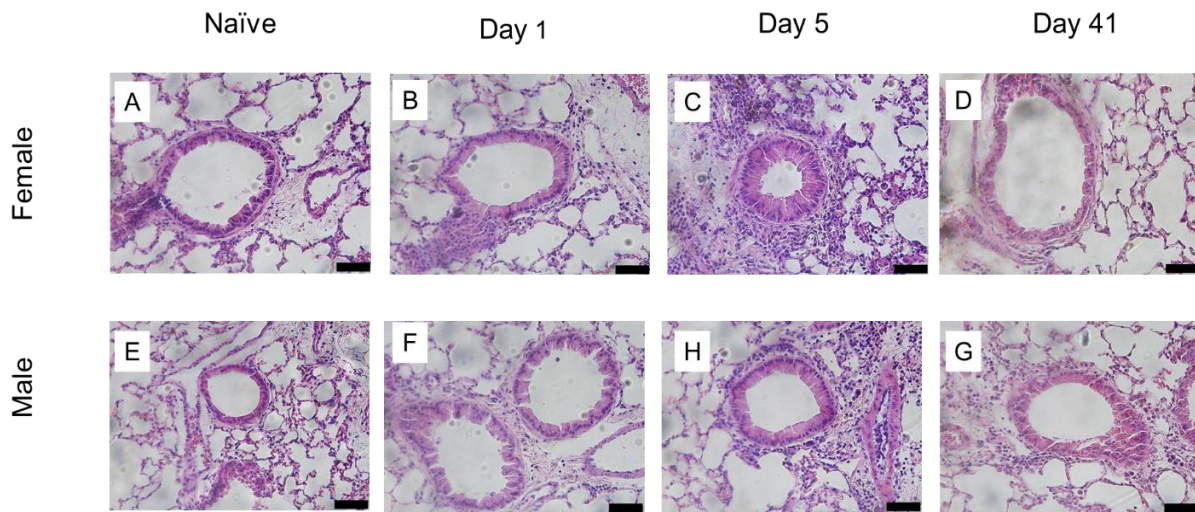
Figure 12. Inhalation of *A. fumigatus* conidia does not cause differences in pulmonary inflammation between male and female mice in low humidity. Representative pictures of H&E stained lung sections of allergen challenged C57Bl/6 female mice **(Figure 12A-D)** and male mice **(Figure 12E-H)**. No inflammation was observed at Day 1 **(Figure 12B, F)** for both males and females. No significant changes in inflammation were observed at Day 5 **(Figure 12C, G)** and at Day 41 **(Figure 12D, H)** for both males and females. Bar represents 50µm.

Inhalation of Aspergillus fumigatus *significantly increases goblet cell production in male mice at day 1 and in both male and female mice at day 5 in low humidity.* Goblet cells (mucous producing cells) were assessed by counting PAS-stained cells and representing them as a percentage of total epithelial cells lining the lateral airways of each lung section **(Figure 13)**. A representative image is shown **(Figure 13A)**. For each sample, a 100µm interval along the largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch) was examined for PAS-stained cells. At day 1,

male (~45% of total) mice have a significant increase in goblet cells, but not significantly more than

female mice at day 1 **(Figure 13B).** At day 5, both male (~60% of total) and female (~38% of total) mice

have significantly more goblet cells over naïve animals **(Figure 13B)**. At day 41, no significant changes in

the percentage of goblet cells in both male and female (~20% of total) were observed compared to naïve.

There is no statistical difference in the percentage of goblet cells between male and female mice at days

1, 5, and 41 **(Figure 13B)**.
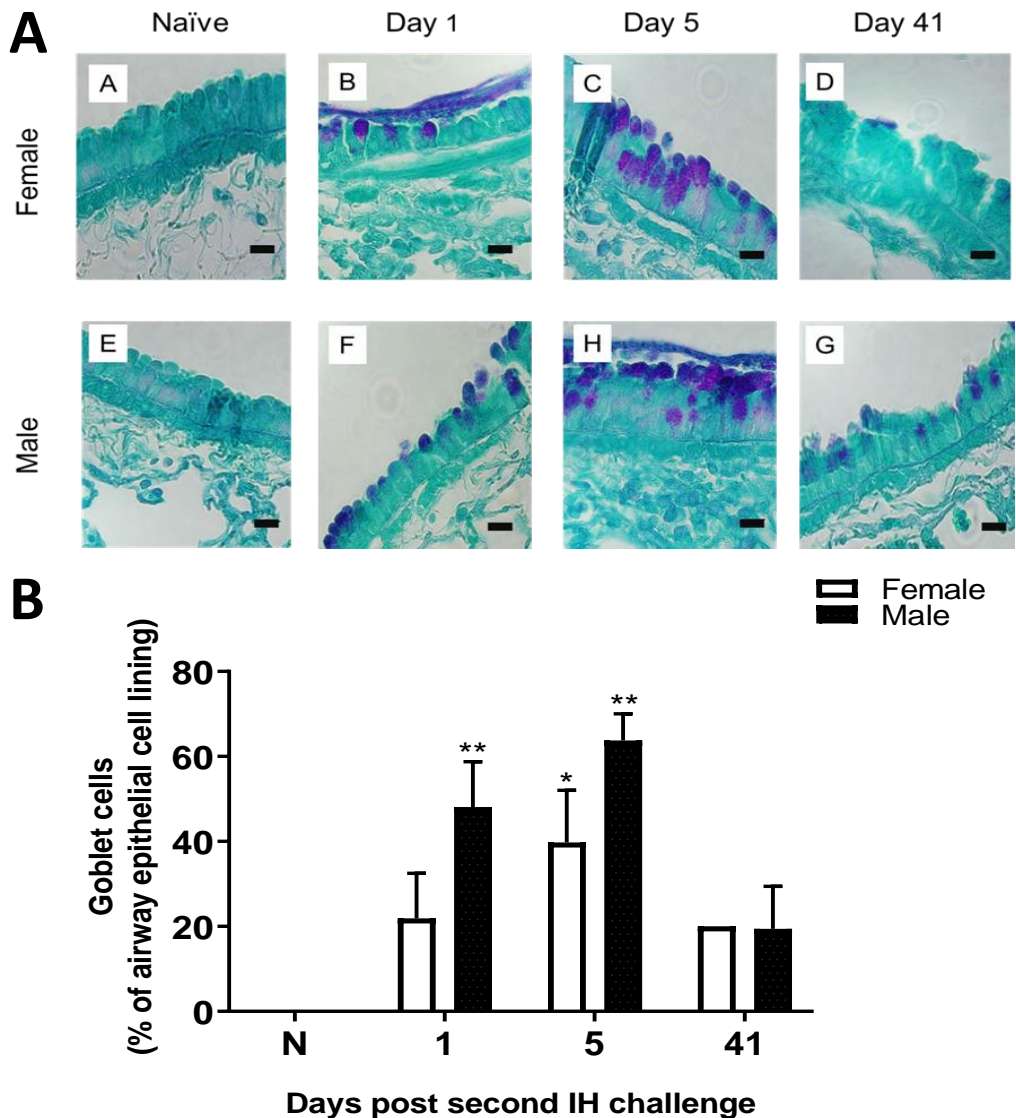
Figure 13. Inhalation of *A. fumigatus* **conidia increases goblet cell metaplasia in male and female mice in low humidity.** Both male and female C57BL/6 mice show that goblet cells and mucus production were present in the airways **(Figure 13A)**. Bar represents 10µm. Goblet cell numbers were reported as the percent of total epithelial cells along 100µm segments lining the lateral branches of the lung **(Figure 13B)**. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values

are expressed as mean $\pm$ SEM (n =3-5 mice per group). *p<0.5, **p<0.01 is significant when compared to respective naïve controls. There was no statistical significance between males and females.

Inhalation of Aspergillus fumigatus *significantly increases collage deposition in female mice at day 5 in low humidity.* Collagen production was assessed by measuring the thickness of Gomorri's trichome stained peribronchial collagen from the basement membrane to the lumen of the lung **(Figure 14)**. A representative image is shown **(Figure 14A)**. For each sample, at least 50 discrete points were measured at 50μm intervals along the largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch). A perpendicular line was drawn from the point on the basement membrane through the full thickness of the collagen immediately below **(Figure 14B)**. The mean collagen thickness was reported for each sample, and the mean of the means was reported for each group. At day 1, there is no significant collagen produced by either sex (average of ~15μm thickness) compared to naïve controls **(Figure 14B)**. At day 5, female mice produce significantly more collagen (average of ~25μm thickness) than the respective naïve control and male mice (average of ~15μm thickness) **(Figure 14B)**. At day 41, no significant amounts of collagen produced were observed in female mice (average of ~23μm thickness) or male mice (average of ~20μm) **(Figure 14B).**
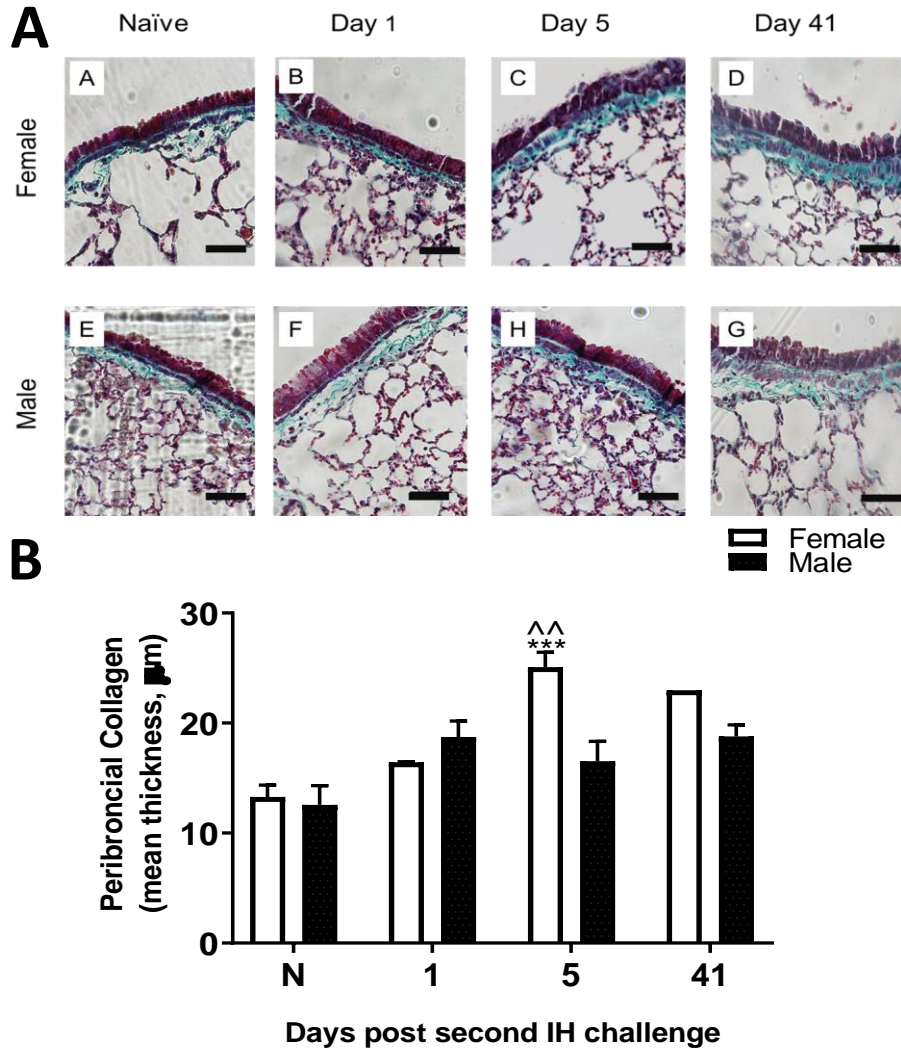
Figure 14. Effect of *A. fumigatus* conidia on collagen thickness in male and female mice in low humidity conditions. Gomorri's trichrome stain was used to visualize collagen deposition in whole lung sections. Representative pictures of male and female trichrome stained sections **(Figure 14A)**. Bar represents 50μm. Approximately 40-50 points were measured at 50μm intervals along lateral branches. A perpendicular line was drawn from the point on the basement membrane through the full thickness of the collagen deposition below. The mean collagen thickness was reported for each sex **(Figure 14B)**. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (*n* =3-5 mice per group). ***p<0.01 is significant when compared to respective naïve controls. ^^*p*<0.1 is significant when compared between males and females at respective timepoints.

Fungal inhalation of A. fumigatus *in low humidity conditions resulted in significantly increased serum IgE in females, increased IgE in male, and significantly increased IgG3 in females at day 1 and day 5 compared to males.* IgE is significantly increased in female mice over naïve females at day 5 indicating an allergic response **(Figure 15A)**. IgG3 is significantly increased in female mice at both day 1 and day 5 compared to respective naïve control and is significantly increased in female mice compared to

male mice at both day 1 and day 5 **(Figure 15B).** IgA is not significantly changed between males and

females at any timepoint **(Figure 15C)**. IgG1 is not significantly changed between males and females at

any timepoint **(Figure 15D)**. IgG2a is not significantly changed between males and females at any

timepoint **(Figure 15E)**.

Days post second IH challenge
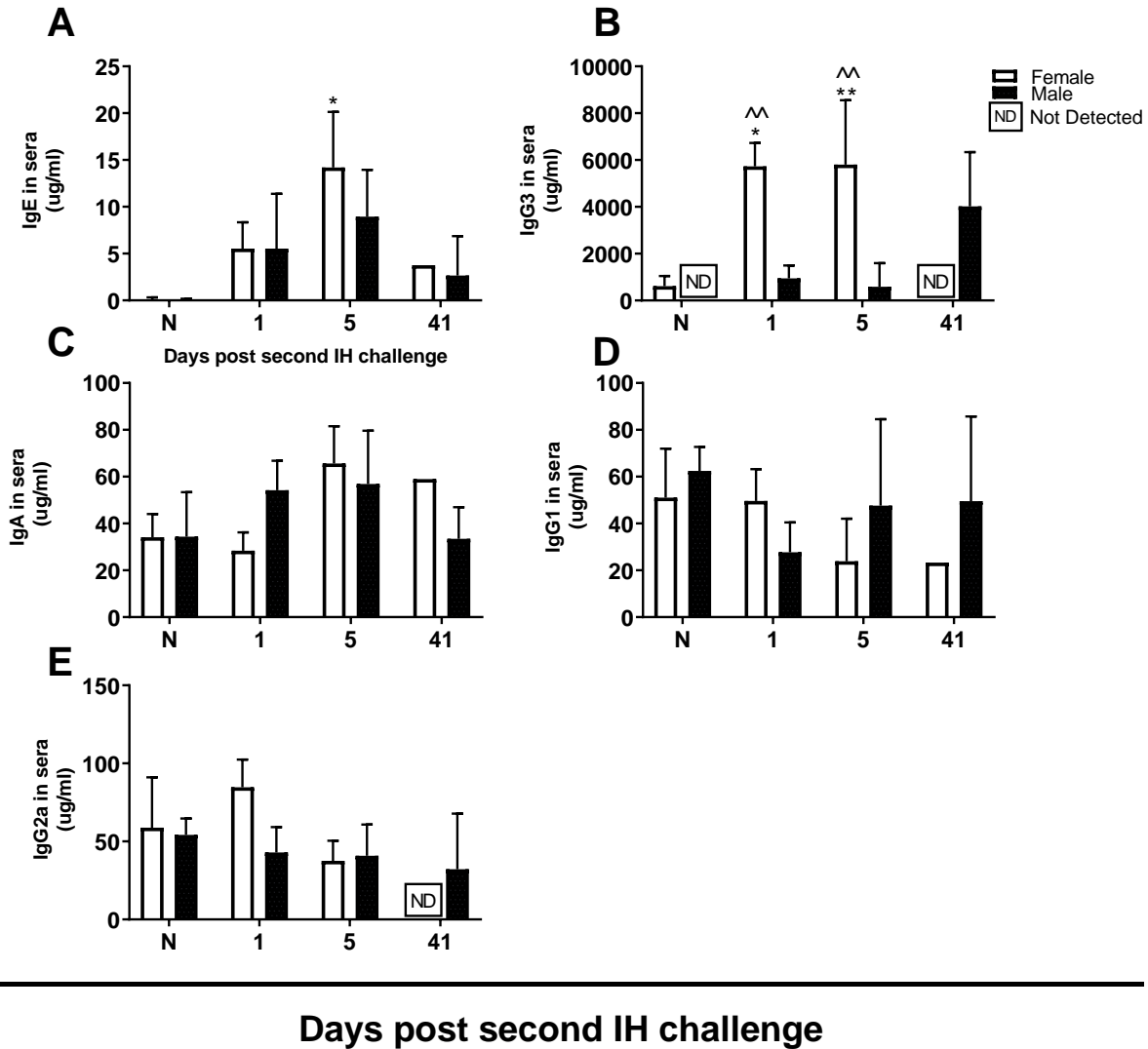
Figure 15. Effect of inhaled *A. fumigatus* in low humidity on the production of IgE, IgA, IgG1, IgG2a, IgG3 in male and female mice. Mice were sensitized and exposed to A. fumigatus per **Figure 2**. Ab isotypes were quantified by specific ELISA in serum at days 1 and 5. IgE **(Figure 15A)** IgG3 **(Figure 15B)** IgA **(Figure 15C)** IgG1 **(Figure 15D)** IgG2a **(Figure 15E)**. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM (n =3-5 mice per group). *$p<0.05$, **$p<0.01$ is significant when compared to respective naïve control. ^^$p<0.05$ is significant when compared between males and females at respective timepoints.

3.4. Discussion

Sex has been a factor in the severity, onset, and treatment of fungal allergic asthma[66]. To characterize sex in our *A. fumigatus* model in low humidity conditions, we examined the following readouts: weight, airway hyperresponsiveness, inflammation via BAL and histological staining, and antibody production at different timepoints of and the summary of results is shown in **Tables 1 - 3**.

Table 1. Summary of Sex Characteristics at Day 1

Sex	Weight	AHR	BAL	H&E	PAS	TRI	IgE	IgA	IgG1	IgG2a	IgG3
Male	++	++	++	+	++	++	++	++	++	++	+
Female	+	++	++	+	++	++	++	++	++	++	++

At day 1, male mice weigh significantly more than female mice. Female mice have significantly more Ig3 production.

Table 2. Summary of Sex Characteristics at Day 5

Sex	Weight	AHR	BAL	H&E	PAS	TRI	IgE	IgA	IgG1	IgG2a	IgG3
Male	++	++	++	+	++	+	++	++	++	++	+
Female	+	++	++	+	++	++	++	++	++	++	++

At day 5, male mice weigh significantly more than female mice. Female mice have significantly more collagen deposition and IgG3 antibody production.

Table 3. Summary of Sex Characteristics

Sex	Weight	AHR	BAL	H&E	PAS	TRI	IgE	IgA	IgG1	IgG2a	IgG3
Male	++	++	++	+	++	+	++	++	++	++	+
Female	+	++	++	+	++	++	++	++	++	++	++

Our model showed that male mice weigh significantly more than female mice. While our male mice were in the normal weight range, epidemiological data and mouse models of asthma have shown that there is a relationship between obesity and asthma where there is a strong correlation between female obesity and the adult onset of asthma [128, 129]·[130].

Male and female mice have no significant difference in airway hyperresponsiveness after inhalation of *A. fumigatus* conidia in low humidity. The literature shows inconsistencies with AHR between sexes. For example, a model using intratracheal lipopolysaccharides administration to sensitize the airway with a nebulized methacholine challenge showed that male mice had more significant airway

hyperresponsiveness than female mice[131]. A model using ovalbumin in BALB/c mice (that are more responsive than C57Bl/6 mice[132]) showed no difference in AHR between male and female mice[133]. However, male C57Bl/6 mice have shown to be hyper-reactive toward methacholine without allergic sensitization. Effects of sex on AHR must be evaluated by the model being used. The trend is opposite in humans as epidemiological studies have shown that women have a higher bronchial hyperresponsiveness then men[134]. While mouse models are useful for studying asthma, they do not always accurately represent what is seen in humans.

Overall inflammation showed no significant differences between male and female mice in low humidity. The number of inflammatory cells in the BAL wash fluid was similar between male and female mice. However, other mouse models have shown that female mice had an increase in the total number of cells as well as specific increases in eosinophils, lymphocytes, and myeloid dendritic cells[126, 127]. The H&E stained lung sections showed no difference in inflammation between sex after *A. fumigatus* inhalation in low humidity. This finding is opposite to a study using LPS to illicit inflammation that found LPS-treated male mice had more severe inflammation by having a higher histopathological score over LPS-treated female mice[131].

Female mice had more significant collagen deposition than male mice indicating more airway remodeling and more fibrosis in female mice at day 5. A study looking at Bleomycin-induced pulmonary fibrosis in rats showed that female rats had more severe fibrosis by having higher levels of lung collagen deposition and fibrotic cytokine expression than male rats[135]. Fibrosis has been linked to hormones. Estradiol was shown to up-regulate pro-inflammatory cytokines IL-1β and TNF-α while downregulating anti-inflammatory cytokines such as IL-10[136]. When Estradiol was given to male mice, there was a promotion of a Th2 type of response noted by the increase of IL-4[137]. Progesterone was shown to up-regulate anti-inflammatory cytokines and decrease the amount of IL-4 present[136].

Antibody production was increased in both male and female mice after inhalation of *A. fumigatus* conidia in low humidity. There was no significant difference in serum levels of IgE, IgA, IgG1, and IgG2a between male and female mice. However, an ovalbumin study showed that female mice produce more IgE and IgA than male mice[127]. Another study examining sex in allergic rhinitis using *Schistosoma mansoni* egg antigen showed that female mice were able to produce more IgE and that sex played no

role in IgG1 production[138]. There is little research on female mice producing more IgG2a than males in a Th2 type of immune response. However, a study on immune responses and viral shedding after a Seoul virus infection in rats showed that male rats had higher levels of IgG2a antibodies with a Th1 type of immune response[139].

Female mice produced significantly more IgG3 antibodies than male mice in low humidity. IgG3 produces antibodies against protein antigens[85]. Allergen-specific IgG can contribute to a Th2 mediated allergic inflammation due to Fc-gamma receptor (FcγR) signaling on antigen presenting cells that will induce airway inflammation[140]. A study examining serum antibody responses in mice infected with the parasite *Giardia muris* showed that females had higher IgG3 production than males[141]. In other sex-linked chronic inflammatory diseases, such as systemic lupus, the reduction of IgG3 via tamoxifen showed beneficial therapeutic effects[142].

Overall, no sex differences were observed in our model other than weight and IgG3 antibody production. Therefore, sex is not linked to the asthmatic response produced to inhalation of *A. fumigatus* in low humidity.

4. FUNGAL ALLERGIC B-LYMPHOCYTE GENE REGULATION

4.1. Introduction

B-cells have been shown to play an important role in the immune response. They are primarily responsible for antigen presentation, antibody production, and cytokine production. In allergic asthma, B-cells are responsible for priming the immune response with IgE. In the Th2 immune response, activated CD4+T-cells produce IL-4 that activates B-lymphocytes to differentiate into plasma cells. These cells begin to produce IgE, the hallmark antibody of allergic asthma, which bind to the Fc-receptors on mast cells, basophils, and eosinophils. Plasma cells also produce IL-5 which activates eosinophils, the hallmark granulocyte of allergic asthma. On re-exposure to *A. fumigatus* conidia, the antigen is crossed linked with surface IgE on mast cells, basophils, or eosinophils which will cause them to degranulate; releasing inflammatory mediators such as histamines and leukotrienes that start the cascade of signals initiating the allergic response. B-cells are critical in developing a Th2 immune response and should be further examined as possible therapeutic targets[143, 144]. Our model has shown that B-cells are migrating to the lungs, where inflammation is occurring, as well as producing large amounts of IgE to illicit the allergic immune response[54]. We wanted to determine what role the B-cells play regarding several different aspects: antibody production, apoptosis, activation, adhesion, cytokine production, response to antigen, and signaling factors by examining the gene expression of allergic B-cells. To examine the role of the B-cell, we isolated and purified allergic CD19+ B-cells from the spleens of challenged male and female mice using magnetic cell sorting. Then, cDNA from the purified CD19+ B-cells was made from RNA to be used for qPCR expression on custom gene expression plates with 96 different B-cell genes.

4.2. Materials and Methods

Animals. C57Bl/6 mice were purchased from Jackson Labs (Bar Harbor, ME, USA) and were bred and maintained in a specific pathogen-free facility for the duration of this study. Animals (4-6 months old) were fed LabDiet 5k67™ Rat and Mouse Auto-Ovals (PMI Nutrition International, LLC, St. Louis, MO, USA) and given water throughout the study and housed on Alpha-dri™ bedding (Shepherd Specialty Papers, Watertown, TN, USA) in micro filter topped cages (Ancare, Bellmore, NY, USA). Prior approval for these studies was obtained from the Institutional Animal Care and Use Committee of NDSU. After samples had been collected, it was noted that in-room humidity was extremely low and was likely low

during the entire lives of the animals. Relative humidity ranges are classified as: low humidity (<20%) and normal humidity (40-60%).

Antigen preparation and conidia culture. A. fumigatus antigen was purchased from Greer Laboratories (Lenoir, NC, USA). Lyophilized *A. fumigatus* culture was reconstituted in 5mL of phosphate buffered saline (PBS) per ATCC recommendations, and 60µL aliquots of the suspension were stored at 4°C until use. A single aliquot of *A. fumigatus* was grown on 10ml of Sabouraud dextrose agar (SDA) in a 25-cm^2 cell culture flask for eight days at 37°C. The use of *A. fumigatus* was approved by the NDSU Institutional Biosafety Committee.

Allergen sensitization and conidia inhalation challenge. To create an allergic phenotype, mice were injected subcutaneously (SC) and intraperitoneally (IP) with a 100 ul of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) Antigen was prepared by suspending 2ug in 0.1mL PBS and 0.1mL Imject® Alum (Pierce, Rockford, IL, USA). Two weeks later, mice were started on a series of three, weekly 4µg intranasal (IN) inoculations of soluble *A. fumigatus* extract (Greer Laboratories, Lenoir, NC, USA) in 20µL of PBS. One week after the final IN inoculation, mice were exposed via nose only inhalation to eight day old *A. fumigatus* conidia, as previously described[39]. This inhalational challenge was repeated 14 days later. Prior to the inhalational challenge, mice were anesthetized using a solution of ketamine (75mg/kg) and xylazine (25mg/kg), and their noses were placed in the inoculation chamber to inhale mature *A. fumigatus* conidia for ten minutes as shown in **Figure 1** in the literature review. On Days 5 and 41 post allergen challenge, animals were anesthetized with pentobarbital (150mg/kg) and a trachea tube was inserted for sample collection. This was the termination point of the study. Day 21 IMIT instillation was performed as described by Lawrenz, et al.[145] with a slight modification of culture and anesthesia used. Briefly, mice were anesthetized using a cocktail of ketamine (75mg/kg) and xylazine (25mg/kg) in PBS. Once sedated, a 2% lidocaine solution was placed on the back of the throat and at least 5 minutes elapsed before intubation was attempted, for the lidocaine to take full effect. The mice were then placed supine on an angled platform, the tongue retracted, and catheter placed in the trachea, using an otoscope fitted with an intubation speculum for help with visualization. Once intubation was confirmed, the blunt needle of a syringe containing 50µL of a 4 x 10^7 conidia/mL suspension, as

described above, plus 150µL air gap was dispensed through the catheter directly to the lungs. This procedure was performed once per week for four consecutive weeks.

Sample collection of serum. Blood was collected and centrifuged at 13,000xg for 10 minutes to obtain serum which was stored at -80°C until use.

Quantification of serum IgE. Total IgE (eBioscience, Vienna, Austria) was quantified via specific ELISA in serum according to the manufacturer's protocol. Briefly, plates were coated with anti-IgE capture antibodies and incubated overnight at 4°C. Washed plates were blocked for 2 hours at room temperature in PBS with 1% Tween™ and 10% Bovine Serum Albumin. Washed plates were incubated in serum samples in a 1:100 dilution for IgE at room temperature for 1 hour. Plates were washed, then incubated in detection antibody, enzyme conjugate, and developed in substrate. A Stop Solution (eBioscience™ Stop Solution Cat. No. BMS409.0100) was added and plates were read at 450nm. The minimum detection limits for the kit was 5ng/mL.

B-cell isolation and purification. A splenectomy was performed on each mouse post second inhalation challenge of *A. fumigatus*. A single cell suspension was made by mechanical dissociation of the tissue by injecting DMEM/High Glucose (GE Life Sciences, Pittsburgh, PA) through a 10ml syringe into the spleen to release splenocytes. Cells were pelleted via centrifugation (AllegraX-15R, Beckman Coulter, Brea, CA) at 600xg for 5 minutes at 4°C. Then RBC's were lysed with Ammonium Chloride Lysis Buffer (Fargo, ND), passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA) and resuspended in 40mL of DMEM/High Glucose (GE Life Sciences, Pittsburgh, PA). B-lymphocytes were isolated using an Anti-CD 19+ Magnetic Bead purification kit (Miltenyi Biotech Inc., Auburn CA). Briefly, spleen cells were incubated with 10 µl of anti-FITC Microbeads per 10^7 cells (Miltenyi Biotec Inc., Auburn, CA) and CD19-positive B lymphocyte were positively selected using the Quadro MACS system with LS columns (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's guidelines. Following purification, total concentration of CD19+ B-cells per sample was determined using a hemocytometer. CD19+ B-cells were pelleted at 300xg for 5 minutes at 4°C, supernatant removed, and stored at -80°C in aliquots of $6X10^6$cells/mL.

Flow Cytometry. $6X10^6$ isolated spleen cells, unbound cells from B-cell purification, and CD19+ magnetic bead labeled B-cells were suspended in PBS with 5% FBS (Thermo-Scientific, Logan, UT) to a

final concentration of 1 X 10^7 cells/ml. Fc receptors were blocked with anti-mouse CD16/CD32, 0.5mg/mL (eBioscience, San Diego, CA) for 10 minutes at 4°C. Cells were then labeled with the following Abs: extracellularly 1µg of anti-mouse CD19 PerCP-Cy5.5 (eBioscience, San Diego, CA) and 1µg of C74 FITC (BD Biosciences, San Jose, CA) or intracellularly 1µg of CD74 FITC (BD Biosciences, San Jose, CA) using a BD Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD Biosciences, San Jose, CA). Samples were preincubated with respective labeled Abs for 30 minutes in the dark at 4°C and then washed with PBS 5%FBS before the samples were analyzed using a FACSCalibur (Flow cytometer, BD Biosciences). A minimum of 25,000 events were acquired and the data was analyzed using Flowjo™ software (Tree Star, Inc., Ashland, OR).

   Gene Expression via qPCR. $6x10^6$ purified B-cells were subjected to RNA purification using the Sure-prep True Total RNA Purification Kit (Fisher Scientific, Pittsburgh, PA). Total RNA was analyzed using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). The A260/280 ratios were between 1.90 and 2.1 for all samples. 1µg of RNA per sample was then DNase treated using 1 unit of RQ1 RNase-Free DNase (Promega, Madison, WI) and 1µL of RQ1 RNase-Free DNase Reaction Buffer (Promega, Madison, WI) for 30 minutes at 37°C. 1µL of RQ1 DNase Stop Solution (Promega, Madison, WI) was then added and incubated for 10 minutes at 65°C to inactivate the DNase. Total concentration of DNase treated RNA was determined using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). 1µg of DNase treated RNA was then reverse transcribed via iScript Reverse Transcription Supermix for qRT-PCR from BioRad according to the manufacturer's instructions (BioRad, Hercules, CA). The reaction protocol ran with the following parameters: priming for 5 min at 25°C, reverse transcription for 20 min at 46°C, and RT inactivation for 1 min at 95°C. cDNA was stored at -80°C for later use.

   Custom 96-well PCR plates were created from BioRad (Hercules, CA). Briefly, genes were selected based on function: antibody production, apoptosis, activation, adhesion, cytokine production, response to antigen, and signaling factors. BioRad allergy, immune response, and B-cell plates were used as references to create custom PCR plates. The real time reactions contained a 5µL of cDNA/sample (1:20 dilution) was combined with 10µL of 2X PrimePCR™ SYBR® Green Assay (BioRad, Hercules, CA) and 4µL Nuclease Free H_2O (Millipore, Billerica, MA) per well, with each well containing

1μL of a lyophilized primer, of a 96-well custom plate. Plates were then run on CFX96 Real-Time Thermal

Cycler (BioRad, Hercules, CA). The qRT-PCR reaction was run with the following parameters: 2 min at

95°C to denature the cDNA and to activate Taq polymerase, followed by 40 cycles of 5 s at 95°C and 30 s

at 60°C. Analysis was performed using Bio-Rad CFX Manger 3.0 expression software (Hercules, CA).

GeNorm was used to determine the two most stable expressed control genes between naïve samples

and day 1, 5, and 41 for each sex shown below in **Table 4**[146]. Samples were then normalized to the

GeNorm determined reference genes per respective set of qRT-PCR data. The relative fold difference

was calculated using the $2^{\Delta\Delta C_T}$ method.

Table 4. GeNorm Reference Genes per Timepoint

Time Point	Gene
D5 Female	*Hmgb1*
	Nfkbia
D21 Female	*Irak1*
	Lgals1
D41 Female	*Pak2*
	Il2rg
D5 Male	*Blnk*
	Hprt
D21 Male	*Irak2*
	Tnfrsf1b
D41 Male	*Cd38*
	Lgals1

Statistical analysis. Allergic C57BL/6 WT animals were compared to each sex and to their

respective naïve controls at each timepoint. An unpaired Student two-tailed *t* test with a Welch correction

was used to determine statistical significance with Prism GraphPad software (San Diego, CA, USA). All results are expressed as the means $\pm$ SEM. Group sizes were as follows: at day 0 (naïve) $n = 3$ mice/sex, at day 1 $n = 5$ mice/sex, at day 5 $n = 5$ mice/sex, and at day 41 $n = 1$ female mouse/4 male mice. No statistical analysis for sex differences was performed Day 41 samples as only one female C57BL/6 mouse was viable through the end of the study. For each allergic timepoint compared with the naïve control, statistical significance is indicated as follows: *$p = 0.01$-0.05

4.3. Results

Inhalation of Aspergillus fumigatus *elicits an allergic response by increasing IgE production.* IgE antibodies were detected in the serum of both male and female mice indicating an allergic response after multiple inhalations of conidia **(Figure 16)**. Since IgE is elevated, fungal allergic B-cells are present in the body.
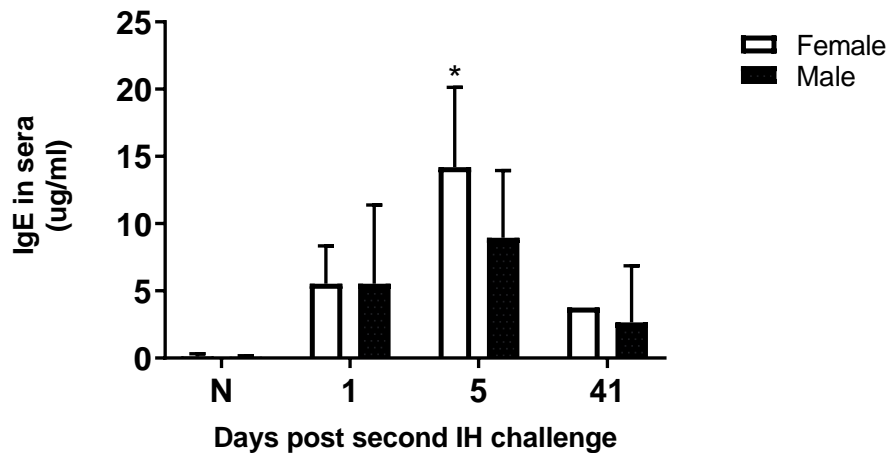
Figure 16. Effect of inhaled *A. fumigatus* on the production of IgE. Mice were sensitized and exposed to A. fumigatus according to **Figure 2**. IgE was quantified by ELISA in serum in naïve animals and days 1, 5, and 41. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as mean $\pm$ SEM ($n = 3$-5 mice per group). *$p < 0.05$ is significant when compared to respective naïve control.

CD19+ B-cells are present in the spleen of allergen challenged mice and can be isolated using magnetic cell sorting. To characterize potential B-cell genes activated in the allergic model, we isolated spleen lymphocytes from naïve and allergic mice using magnetic cell sorting and analyzed the B lymphocyte populations with a flow cytometer for purity **(Figure 17)**. CD19+ B-cells are present in the spleens of allergic mice B **(Figure 17A, D)**. Few CD19+ B-cells were washed out during magnetic cell sorting **(Figure 17B, E)**. Pure CD19+ B-cells were isolated from the spleen **(Figure 17C, F)**.
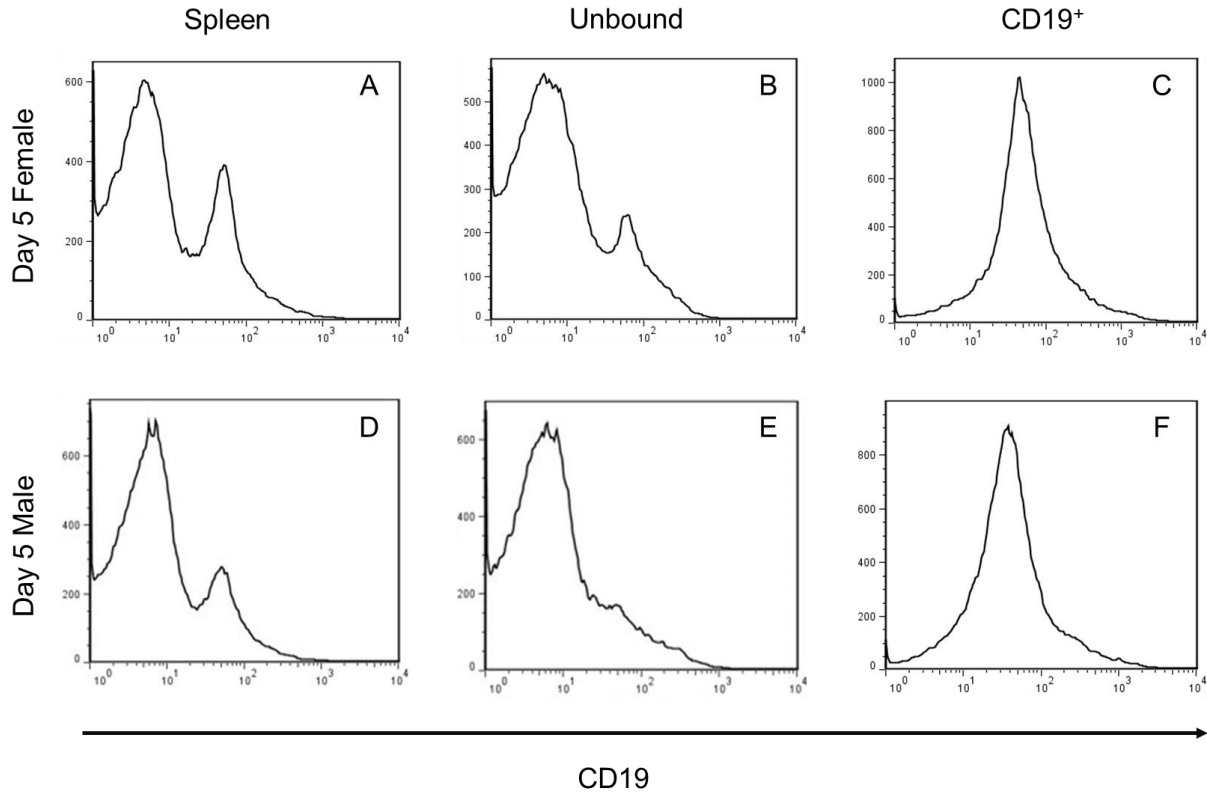
Figure 17. Purification of CD19+ B cells from the allergic spleen. Spleen cells from naïve and allergic animals were analyzed using flow cytometry to show CD19+ B cells present before purification **(Figure 17A, D)**. The unbound cells passing through the magnetic column were analyzed to show that few CD19+ B cells were lost **(Figure 17B, E)**. The sample of purified CD19+ B cells once removed from the magnetic column **(Figure 17C, F)**.

Overview of CD19+ B-cell gene expression. To examine what functions and roles B-cells are performing in fungal allergic asthma, custom gene expression plates with 92 different genes associated with: antibody production, apoptosis, activation, adhesion, cytokine production, response to antigen, and signaling factors were created and analyzed. CD19+ B-cells were isolated and purified, then mRNA was used to perform qPCR. Genes examined are shown in **Table 5.** Both male and female CD19+B-cells downregulate *Cd74* at day 5, 21, and 41 post inhalation of *A. fumigatus* conidia examined via qPCR using BioRad custom gene expression plates. The expression of CD74 was examined both extracellularly and intracellularly using flow cytometry. Expression of CD74 failed to show differences between naïve and Day 5 CD19+ B-cells. No differences in levels of expression were observed between males and females. Data not shown.

Table 5. 96-well Custom PCR Plate Genes

Function	Gene
Antibody Production & Regulation	Blimp1
	IL-4ra
	Apoe
	Ccr6
	IL-10
	Pax5
Apoptosis	Cflar
	Bax
	Birc5
	Tgm2
	IgF1r
	Gnaq
Activation, Maturation, & Cytokine Production	Ccr7
	Cdc42
	Cdc42ep4
	Doc8
	Hmgb1
	Ighm
	IL6
	IL6ra
	IL6st
	IL7r
	Irf8
	Malt1
	Nedd4
	Socs1
	Socs3
	Stat1
	Spp1
	Trem1
	Tnf
	Tgfb1
	IL10ra
	Cd81
Adhesion & Motility	Alcam
	Capn2
	Cd151
	Cd38
	Vcam1

Table 5. 96-well Custom PCR Plate Genes (continued)

Function	Gene
Interaction with Cells & Extracellular Matrix	Abcc5
	Cd44
	Fermt3
	Lgals1
	Mmp14
	Rhamm
	Sdc1
	Sparc
	Vcan
	Tsg6
	Gjb2
Signaling, Metabolism, & Transcription Factors	Acly
	Cd81
	Fkbp8
	Fmr1
	Foxo1
	Foxo4
	Foxp3
	Gna13
	Gng5
	IL2rg
	Mapk1
	Ripk2
	Sigirr
Proliferation	Blnk
	Btk
	Caprin1
	Mki67
Response to Antigen	Cd14
	Cd36
	Cd74
	Irak3
	Irf7
	Tlr2
	Tlr4
	Gpr116

Table 5. 96-well Custom PCR Plate Genes (continued)

Function	Gene
NFkB	Cd27
	Cc2d1a
	Irak1
	Irak2
	Nfkb1
	Nfkbia
	Nfkbiz
	Nod2
	Rela
	S100a8
	S100a9
	Tnfrsf1a
	Tnfrsf1b

Allergic CD19⁺ B-cells upregulate and downregulate specific genes varies by sex at day 5. Day 5 genes with fold change expression greater than 5-fold change compared to respective naïve controls are shown in **Table 6**. There are a wide variety of functions being regulated in the CD19⁺ allergic B-cells. Female CD19⁺ B-cells downregulate 8 genes (*Gna13, Nfkb1, Cd74, Ccr7, Cd81, Cd38, Bax, Irf8*) and upregulate 6 genes (*Hprt, Gng5, S100a8, Fmr1, Fkbp8, Mki67*). Male CD19⁺ B-cells downregulate 15 genes (*Fermt3, Irak1, Tnfrsf1b, Nedd4, Btk, Tlr2, S100a9, Pax5, Lgals1, Cd74, Ccr7, Cd81, Cd38, Bax, Irf8*) and upregulate 4 genes (*Cdc42ep4, Nod2, Scd1, Tnfaip6*). Both female and male CD19⁺ B-cells downregulate 6 genes (*Cd74, Ccr7, Cd81, Cd38, Bax, Irf8*).

Table 6. Day 5 CD19⁺ B-cell Gene Expression

Day 5 Female					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Cd74	-61.26	Response to antigen/ Proliferation	*Hprt*	33.06	Purine Synthesis
Gna13	-22.45	Signaling/ Metabolism/ Transcription	*Gng5*	9.74	Signaling/ Metabolism/ Transcription
Ccr7	-20.91	Activation	*S100a8*	8.6	Proinflammatory
Cd81	-13.35	Signaling	*Fmr1*	8.31315	Signaling/ Metabolism/ Transcription

Table 6. Day 5 CD19^{+} B-cell Gene Expression (continued)

Day 5 Female					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Bax	-10.42	Apoptosis	*Fkbp8*	6.73	Signaling
Cd38	-8.23	Adhesion and motility	*Mki67*	5.02	Proliferation
Irf8	-6.71	Th17			
Nfkb1	-6	Proinflammatory			
Day 5 Male					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Fermt3	-31.69	Extracellular Matrix	*Tnfaip6*	13.76	Activation/ Maturation
Irak1	-21.99	Proinflammatory	*Cdc42ep4*	5.2	Activation/ Maturation
Cd81	-21.06	Signaling	*Nod2*	5.67	Proinflammatory
Tnfrsf1b	-16.2	Proinflammatory	*Sdc1*	4.56	Extracellular Matrix
Bax	-15.27	Apoptosis			
Irf8	-10.91	Th17			
Ccr7	-9.83	Activation			
Cd74	-9.39	Response to antigen/ Proliferation			
Nedd4	-9.36	Activation/ Maturation			
Btk	-9.27	Proliferation			
Tlr2	-8.98	Response to antigen			
Cd38	-6.17	Adhesion and motility			
S100a9	-5.41	Proinflammatory			
Pax5	-5.05	Ab production and regulation			
Lgals	-5.02	Extracellular Matrix			

Allergic CD19^{+} B-cells upregulate and downregulate specific genes varies by sex at day 21. Day 21 genes with greater than 4-fold expression compared to respective naïve controls are shown in **Table 7**. There are a wide variety of functions being regulated in the allergic CD19^{+} B-cells. Female CD19^{+} B-cells downregulate 8 genes (*NFκb1, Stat1, Irf8, Cd36, Cd40, Cd74, Foxo1, Pax5*) and upregulate 3 genes (*Cd27, Fkbp8,* Fmr1). Male CD19^{+} B-cells downregulate 5 genes (*Fermt3, Dock8, Cd74, Foxo1, Pax5*) and upregulate 9 genes (*Gng5, Hprt, Sdc1, Irak3, Vcam1, IL10, Cd27, Fkbp8, Fmr1*). Both female and

male CD19$^+$ B-cells downregulate 3 genes (*Cd74, Foxo1, Pax5*) and upregulate 3 genes (*Cd27, Fkbp8, Fmr1*).

Table 7. Day 21 CD19$^+$ B-cell Gene Expression

Day 21 Female					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Cd74	-324.67	Response to antigen /Proliferation	*Cd27*	4.55	Proinflammatory
Nfkb1	-35.14	Proinflammatory	*Fkbp8*	4.95	Signaling/ Metabolism/ Transcription Factors
Stat1	-13.47	Activation/ Maturation	*Fmr1*	4.5	Signaling/ Metabolism/ Transcription Factors
Foxo1	-13.39	Signaling/ Metabolism/ Transcription Factors			
Irf8	-9.8	Th17			
Pax5	-7.07	Ab production and maturation			
Cd36	-5.63	Response to antigen			
Cd40	-5.33	Signaling			
Day 21 Male					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Cd74	-384.22	Response to antigen/ Proliferation	*Fkbp8*	12.45	Signaling/ Metabolism/ Transcription Factors
Foxo1	-10.35	Signaling/ Metabolism/ Transcription Factors	*Gng5*	12.34	Signaling/ Metabolism/ Transcription Factors
Fermt3	-9.58	Extracellular Matrix	*Hprt*	7.85	Purine Synthesis
Pax5	-5.46	Ab production and regulation	*Fmr1*	6.82	Signaling/ Metabolism/ Transcription Factors
Dock8	-4.39	Activation/ Maturation	*Cd27*	6.56	Proinflammatory
			Sdc1	5.58	Extracellular Matrix
			Irak3	4.7	Response to antigen
			Vcam1	4.11	Adhesion/Motility
			IL10	4.1	Cytokine production/ Anti-inflammatory

Allergic CD19+ B-cells upregulate and downregulate specific genes varies by sex at day 41. Day 41 genes with greater than 3-fold change in expression compared to respective naïve control are shown in **Table 8**. There are a wide variety of functions being regulated in the allergic CD19+ B-cells. Female CD19+ B-cells downregulate 12 genes (*Fermt3, Ccr7, Cd81, Lgals, Irf8, Foxo1, Nfkb1, IL4ra, Cd36, Gapdh, Irak2, Cd74*) and upregulate 1 gene (*Gng5*). Male CD19+ B-cells upregulate 10 genes (*Trem1, Sdc1, Tlr4, Hprt, IL10, Cd44, NFκbia, Fmr1, S100a8, Gng5*) and downregulate 1 gene (*Cd74).* Both female and male CD19+ B-cells downregulate 1 gene (*Cd74*) and up-regulate 1 gene (*Gng5*).

Table 8. Day 41 CD19+ B-cell Gene Expression

Day 41 Female					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Fermt3	-71.68	Extracellular Matrix	*Gng5*	15.88	Signaling/ Metabolism/ Transcription Factors
Ccr7	-65.64	Activation			
Cd81	-41.55	Signaling			
Lgals	-24.27	Extracellular Matrix			
Irf8	-11.33	Th17			
Cd74	-10.05	Response to antigen/ Proliferation			
Foxo1	-8.63	Signaling/ Metabolism/ Transcription Factors			
Nfkb1	-8.61	Proinflammatory			
Il4ra	-6.13	Ab production (IgE)			
Cd36	-4.8	Response to antigen			
Gapdh	-4.38	Metabolism			
Irak2	-4.2	Proinflammatory			

Table 8. Day 41 CD19+ B-cell Gene Expression (continued)

Day 41 Male					
Gene	**Down Regulation**	**Function**	**Gene**	**Up Regulation**	**Function**
Cd74	-3.39	Response to antigen/ Proliferation	*Trem1*	42.16	Activation/ Maturation/ Cytokine Production
			Gng5	32.42	Signaling/ Metabolism/ Transcription Factors
			Sdc1	23.24	Extracellular Matrix
			Tlr4	21.53	Response to antigen
			Hprt	15.72	Purine Synthesis
			IL10	6.85	Cytokine production/ Anti-inflammatory
			Cd44	4.96	Extracellular Matrix
			Nfkbia	4.68	Proinflammatory
			Fmr1	4.59	Signaling/ Metabolism/ Transcription Factors
			S100a8	4.38	Proinflammatory

4.4. Discussion

We wanted to determine what role the B-cells play regarding several different aspects: antibody production, apoptosis, activation, adhesion, cytokine production, response to antigen, and signaling factors. CD19+ B-cells were successfully isolated from the spleens of allergic male and female mice. The gene expression profile of 92 different B-cell genes was created to have a better understanding of how B-cells are affected by *A. fumigatus* and by biological sex.

Normalization to a reference gene allows for accurate gene expression analysis[147, 148]. Available bioinformatics software, GeNorm, was used to determine a reference gene[146]. GeNorm identifies the optimal reference gene by calculating the geometric mean of all the logarithmically transformed expression ratios of the potential reference genes[146]. Then a pairwise variation is used to identify the most stable expression reference gene by eliminating the worst-scoring housekeeping gene, recalculating and removing worst-scoring genes until the optimal two references genes exist[146]. Using this method, the most stable reference genes were calculated for each timepoint per sex rather than using known house-keeping genes.

Two known house-keeping genes were reflected changes in regulation in multiple time points compared to respect naïve controls. In CD19^{+} B-cells: females up-regulated *Hprt*, day 21 males up-regulate *Hprt*, day 41 females downregulate *Gapdh*, and day 41 males up-regulate *Hprt*. *Hprt*, Hypoxanthine Phosphoribosyltransferase 1, encodes for an enzyme that is used to make purine nucleotides via the purine salvage pathway[149]. A study examining the preimplantation developmental stages of X-linked genes in bovine embryos found that *Hprt* mRNA expression was significantly higher in female embryos than in male embryos[150]. *Gapdh*, Glyceraldehyde-3 Phosphate Dehydrogenase, encodes for a protein that catalyzes the sixth step of glycolysis (Glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate yielding one nicotinamide adenine dinucleotide (NADH) molecule)[149]. Expression of *GAPDH* is upregulated with proliferation, activation, and differentiation[151, 152]. A study examining the levels of *GAPDH* in BAL fluid cells and endobronchial tissues in asthmatics by competitive reverse transcription-polymerase chain reaction (RT-PCR) showed 10 times lower levels of expression in asthmatics compared to naïve controls[147]. Expression of *GAPDH* has also shown to be differentially expressed in females and males. A studying examining glutamic acid decarboxylase (GAD$_{65}$) in rodent hypothalamus found that *GAPDH* expression was significantly increased in either females or males compared to the opposite sex depending on which areas of the hypothalamus were studied[153] Another study examining stem cell therapy showed that female bone marrow-derived mesenchymal cells have higher levels of *GAPDH* expression and affected the cellular behaviors[154]. Therefore, using the most stable normalization genes chosen mathematically rather than picking the most commonly used normalization genes gives a more accurate representation of genes being regulated.

Of the 92 different genes examined, both day 5 female and male CD19^{+} B-cells downregulate 6 genes: *Cd74, Ccr7, Cd81, Cd38, Bax,* and *Irf8*. *Cd74*, MHC II invariant chain, encodes for a type 2 transmembrane protein that plays multiple roles regarding antigen presentation, cell proliferation, and cell survival[149]. *Ccr7*, Chemokine receptor type 7, encodes for a G-protein coupled receptor found on the plasma membrane and is responsible to activating B-cells in chronic inflammation[149, 155, 156]. *Cd81* encodes for a cell surface glycoprotein that is responsible signal transduction in cell that regulates development, activation, growth, and motility[149]. *Cd38* encodes for a cell surface glycoprotein that is responsible for cell-adhesion and single transduction[149]. Both *Cd81* and *Cd38* have been shown to be

57

factors in airway hyperresponsiveness[157, 158]. *Irf8*, Interferon regulatory factor 8, is a protein coding gene that has roles in the development of B-cells and regulating the Th1, Th2, Th17 mediated responses[149]. *Irf8* has been shown to silence Th17 cell differentiation and plays a role in basophil and mast cell development[159, 160]. *Bax*, BCL2 Associated X, encodes for a protein that acts as an apoptosis regulator[149]. *Bax* has been shown to play a role in mucous hyperplasia[161]. We classify these 6 genes as genes that are impacted by our model and can examine in future studies. We expected these genes to be upregulated as we see increases in AHR, mucus production, cells becoming activated and proliferation, etc. We hypothesize that the B-cells in the spleen at day 5 no longer needs these genes to be upregulated and are starting to return the body to homeostasis.

We can continue this idea of returning to homeostasis by looking specifically at the day 5 female CD19$^+$ B-cells. Day 5 female CD19$^+$ B-cells were shown to downregulate 8 genes (*Cd74, Ccr7, Cd81, Cd38, Bax, Irf8, Gna13, & Nfkb1*) and upregulate 6 genes (*Hprt, Gng5, S100a8, Fmr1, Fkbp8, Mki67*) compared to naïve female CD19$^+$ B-cells **(Figure 18)**.

Bax, Ccr7, Cd38, Cd81, Gna13, Trem1
Downregulated
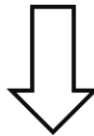
Nfκb1
Pro-inflammatory Chemokines, Cytokines, Adhesion Molecules, Proliferation, Survival, Ig Class Switching

Figure 18. Hypothetical Day 5 Female CD19$^+$ B-cell Pathway. At day 5, 8 genes were found to be downregulated in allergic female CD19$^+$ B-cells. These genes can be linked to the Nfkb1 pathway, which is responsible for pro-inflammatory chemokines, cytokines, adhesion molecules, proliferation, survival, and Ig Class switching. These genes are being downregulated hence turning "off" the pro-inflammatory response.

Ccr7, Cd81, Cd38, and *Gna13* are all part of the MAPK (mitogen-activated protein kinases) pathway which acts as a signal to transcribe or inhibit the transcription of *NFκB*, Nuclear Factor kappa B, which is responsible for producing pro-inflammatory chemokines and cytokines and adhesion molecules[149, 156]. These four genes are all down-regulated which act to turn off the transcription of

Nfκb1 and stop the production of pro-inflammatory mediators[149]. Products from the pathway, such as

Bax, were down-regulated indicating the stopping of transcription[149].

While the day 5 male CD19⁺ B-cells down-regulate 9 genes (*Fermt3, Irak1, Tnfrsf1b, Nedd4, Btk,*

Tlr2, S100a9, Pax5, Lgals1) and up-regulate 4 genes (*Cdc42ep4, Nod2, Scd1, Tnfaip6*), the genes do not

form a complete picture to support the hypothesis of homeostasis. The downregulated genes *Tnfrsf1b*,

Tlr2, Lgals1, Btk, Irak1, and *Pax 5* all play roles in the MAPK pathway[149, 162] **(Figure 19)**.

Btk, Lgals, Irak1, Pax5, Tlr2, Tnfrsf1b
Downregulated

Nfκb1
Returning to homeostasis

Figure 19. Hypothetical Day 5 Male CD19⁺ B-cell Pathway. At day 5, 6 genes were found to be downregulated in allergic male CD19⁺ B-cells. These genes can be linked to the MAPK pathway which is a chain of proteins in a cell that communicates a signal from the cell surface to the DNA in the nucleus. The downregulation of these genes causes the body to return to homeostasis.

These should be supporting the downregulation of *NFκB;* however, there is no change in

regulation of *NFκB* at day 5 compared to naïve. Males are known to have less B-cells and poorer

functioning B-cells compared to females[139].

Of the 92 different genes examined, both day 21 female and male CD19⁺ B-cells downregulate 3

genes (*Cd74, Foxo1,* and *Pax5*) and up-regulate 3 genes (*Cd27, Fkbp8, Fmr1*). *Cd74*, MHC II invariant

chain, encodes for a type 2 transmembrane protein that plays multiple roles regarding antigen

presentation, cell proliferation, and cell survival[149]. *Foxo1,* Forkhead box 1, encodes for transcription

factors for early B-cell development, B-cell IgG1 class switching, and CXCXR-mediated signaling

events[105, 149, 163]. *Pax5*, paired box protein 5, encodes for a b-cell specific activator that is express at

early stages of b-cell differentiation[149]. *Cd27*, is responsible for regulating B-cell activation and

immunoglobulin synthesis[149, 164]. *Fkbp8*, FK506 Binding Protein 8, is an immunophilin protein

responsible for immunoregulation, protein folding/tracking, and Ig class switching[149, 165]. *Fmr1*, Fragile

X Mental Retardation 1, is responsible for binding RNA, is involved in RNA trafficking from the nucleus to

the cytoplasm, and increased levels are associated with increased levels of IL-10[149, 166]. We classify these 6 genes as genes that are impacted by our model and can examine in future studies. We expected these downregulated genes (*Cd74, Foxo1,* and *Pax5*) to be upregulated as we see B-cells becoming activated and proliferating. It is possible that these up-regulated genes (*Cd27, Fkbp8,* Fmr1) are being upregulating as the body is making memory B-cells and there is the production of anti-inflammatory IL-10. We hypothesize that the CD19^{+} B-cells in the spleen at day 21 are starting to return the body to homeostasis.

We can continue this idea of returning to homeostasis by looking specifically at the day 21 female CD19^{+} B-cells. Day 21 female CD19^{+} B-cells were shown to downregulate 8 genes (*Cd74, Foxo1, Pax5, Stat1, Nfkb1, Cd36, Cd40, Irf8*) and upregulate 3 genes (*Cd27, Fkbp8, Fmr1*) compared to naïve female B-cells **(Figure 20)**.

Irf8, Pax5, Stat1
Downregulated

Nfκb1
Returning to homeostasis

Cd36, Cd40, Foxo1, Stat1
Downregulated

Antigen Presentation to T-cells

Cd27, Fkbp8
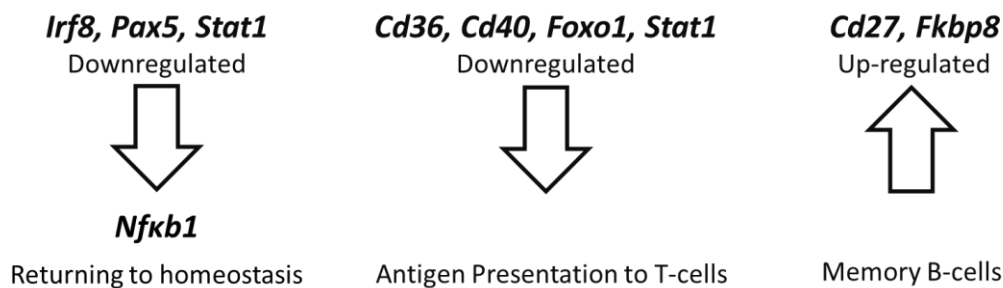Up-regulated

Memory B-cells

Figure 20. Hypothetical Day 21 Female CD19^{+} B-cell Pathways. At day 21, 8 genes were found to be downregulated and 3 genes were found to be down regulated in allergic female CD19^{+}B-cells. 3 genes can be linked to the NfκB1 pathway. 3 genes can be linked to processes involving antigen presentation to T-cells. 2 genes can be linked to processes involving memory B-cells.

Cd36, Cd40, Foxo1, and *Stat1* are all involved in the process of antigen presentation by B-cells[24, 167, 168]. These genes no longer need to be upregulated as the B-cells have stopped antigen processing and presenting to allow the body to return to a normal state. *Pax5, Irf8,* and *Stat1* are all involved in the signal transduction of *NFκB*, Nuclear Factor Kappa B, which is responsible for producing pro-inflammatory chemokines and cytokines and adhesion molecules[149, 169]. These three genes are all downregulated which act to turn off the transcription of *NFkb1* and stop the production of pro-inflammatory mediators. The upregulation of *Cd27* and *Fkbp8* are involved of Ig isotype switching to make memory B-cells for the next interaction of *A. fumigatus[165, 170]*.

Homeostasis can also be seen by examining the day 21 male CD19+ B-cells as they downregulate 5 genes (*Cd74, Foxo1, Pax5, Fermt3, Dock8*) and upregulate 9 genes (*Cd27, Fkbp8, Fmr1, IL-10, Irak3, Gng5, Hprt, Sdc1, Vcam1*) compared to naïve male CD19+ B-cells **(Figure 21)**.

IL-10, Irak3
Up-regulated

Cd74, Fermt3, Dock8, Foxo1
Downregulated

Cd27, Fkbp8, Fmr1, Vcam1
Up-regulated

Anti-inflammatory
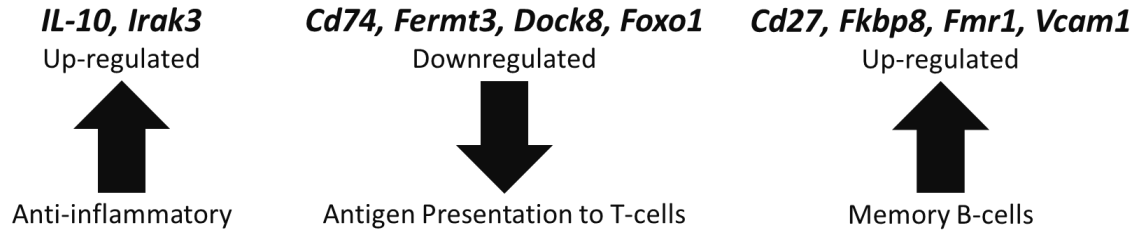
Antigen Presentation to T-cells

Memory B-cells

Figure 21. Hypothetical Day 21 CD19+ Male B-cell Pathways. At day 21, 5 genes were found to be downregulated and 9 genes were found to be upregulated in allergic male CD19+ B-cells. 2 genes can be linked to anti-inflammatory response. 4 genes can be linked antigen presentation to T-cells. 4 genes can be linked to processes involving memory B-cells.

Cd74, Foxo1, Fermt3, and *Dock8* are all involved in the process of cell-cell signaling and antigen presentation by B-cells[168, 171]. These genes no longer need to be upregulated at the B-cells have stopped antigen presentation. The upregulation of *Cd27, Fkbp8, Fmr1,* and *Vcam1* are involved of Ig isotype switching to make memory B-cells for the next interaction of *A. fumigatus[165, 170]*. There is the upregulation of *IL-10* and *Irak3* to produce an anti-inflammatory signal to return to homeostasis[172, 173].

Of the 92 different genes examined, both day 41 female and male CD19+ B-cells downregulate 1 gene (*Cd74*) and up-regulate 1 gene (*Gng5*). *Cd74*, MHC II invariant chain, encodes for a type 2 transmembrane protein that plays multiple roles regarding antigen presentation, cell proliferation, and cell survival[149]. *Gng5*, G Protein Subunit Gamma 5, is a trimeric membrane associated point responsible for signal transduction and is a common target in therapy for allergic asthma[149, 174, 175].

Examining the idea of homeostasis continues with day 41 female CD19+ B-cells as they were shown to downregulate 12 genes (*Cd74, Fermt, Ccr7, Cd81, Lgals, Irf8, Foxo1, Nfkb1, IL4ra, Cd36, Gapdh, Irak2*) and upregulate 1 gene (*Gng5*) compared to naïve female CD19+ B-cells **(Figure 22)**.

Cd81, Lgals, Irf8, Irak2
Downregulated

Ccr7, Cd36, Fermt3, Foxo1, IL4ra
Downregulated

⬇ ⬇

Nfκb1

Returning to homeostasis

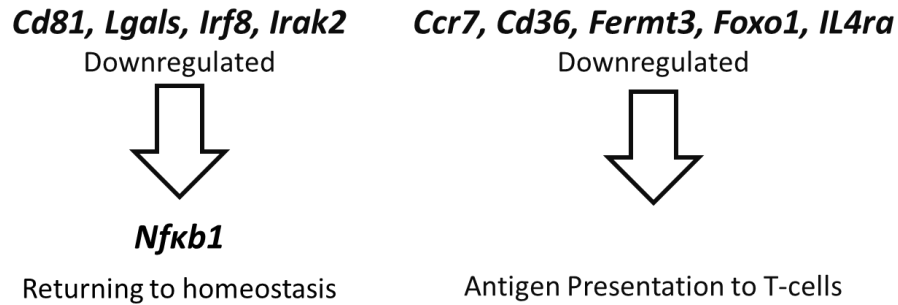Antigen Presentation to T-cells

Figure 22. Hypothetical Day 41 Female CD19⁺ B-cell Pathways. At day 41, 12 genes were found to be downregulated and 1 gene was found to be upregulated in allergic female B-cells. 4 genes can be linked to the Nfkb1 pathway returning the body to homeostasis. 5 genes can be linked to antigen presentation to T-cells.

Ccr7, Cd36, Fermt3, Foxo1, and *Il4ra* are involved with cell-cell signaling for B-cells to act as antigen presenting cells[149, 156, 176, 177]. By day 41, there is no need for antigen presentation as the immune response to *A. fumigatus* has been cleared. *Cd81, Irf8, Irak2, Lgals* all involved in the signal transduction of *NFκB*, Nuclear Factor Kappa B, which is responsible for producing pro-inflammatory chemokines and cytokines and adhesion molecules[149, 162, 177]. These four genes are all downregulated which act to turn off the transcription of *Nfκb1* and stop the production of pro-inflammatory mediators.

Day 41 male CD19⁺ B-cells were shown to downregulate 1 gene (*Cd74*) and up-regulate 10 genes (*Gng5, Trem1, Sdc1, Tlr4, Hprt, IL-10, Cd44, Nfκbia, Fmr1, S100a8*) compared to naïve male CD19⁺ B-cells **(Figure 23)**.

Cd44, Il10ra, Nfkbia, Tlr4
Up-regulated

⬆
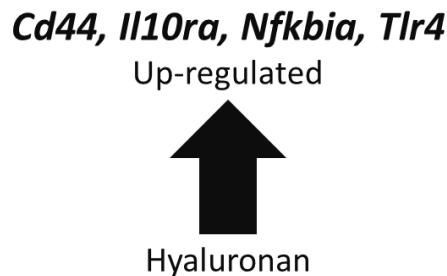
Hyaluronan

Figure 23. Hypothetical Day 41 Male CD19⁺ B-cell Pathway. At day 41, 1 gene was found to be downregulated and 10 genes were found to be upregulated in allergic male B-cells. 4 genes are involved in the signaling or processing of hyaluronan.

Fmr1 and *IL-10* are responsible for returning the body to homeostasis[105, 166]. *Cd44, IL-10, Nfκbia,* and *Tlr4* are all involved in the signaling or processing of hyaluronan[178-181]. Hyaluronan (HA) is a non-sulfated glycosaminoglycan polymer component of the ECM[182]. HA exists as high molecular

mass (HMM) ($>10^7$DA) in the native state in the lung and under inflammatory conditions, HMM Ha is broken down into low molecular mass (LMM HA) (35-400kDA) and is pro-inflammatory[183-185]. Our lab has shown that hyaluronan stimulates B-cell chemotaxis and cytokine production[186].

Both male and female CD19$^+$B-cells downregulate *Cd74* at day 5, 21, and 41 post inhalation of *A. fumigatus* conidia examined via qPCR using BioRad custom gene expression plates. The expression of CD74 was examined both extracellularly and intracellularly using flow cytometry. Expression of CD74 failed to show differences between naïve and Day 5 CD19$^+$ B-cells. Protein expression is has traditionally examined using protein assays, western blots, or immunoblots[187, 188]. Novel techniques such as protein quantitation rationing (PQR) are being developed to examine the protein expression with less time consuming experiments, less destruction of cells, and overall better accuracy of the results[189]. In order to understand the expression of CD74, other assays need to be performed. CD74, also known as MHC II invariant chain, encodes for a type 2 transmembrane protein that plays multiple functions both extracellularly and intracellularly in B-lymphocytes. There are over 4×10^6 receptors found on the surface, and each receptor has a half-life of 10 minutes[190]. Functions include antigen presentation, regulating mature B-cell survival, and promoting cell survival. In antigen presentation, CD74 forms complexes with MHC II molecules to help shield the binding groove against premature peptide binding and directs the processing of antigen presentation[191]. In asthma, CD74 has been show to induce pulmonary inflammation[192]. In allergic asthma, CD74 complexes with CD44 to bind macrophage Migration Inhibitory Factor (MIF) to promote B-cell proliferation. MIF is present in many cells types and is pro-inflammatory. An OVA model of asthma study showed that MIF deficient mice were unable to develop airway hyperresponsiveness, eosinophilia, and mucus metaplasia[193]. This study showed the importance of CD74 in creating and maintaining the allergic Th2 response. Activated CD74 also promotes cell survival inducing a signaling pathway that leads to the activation of NF-κB, allowing the B-cell to elicit a pro-inflammatory response. CD74 activated NF-κB will also upregulate transcription of Bcl-X$_L$ that rescues B-cells from apoptosis[194]. More research is needed to further understand the role of CD74 and its function in allergic B-cells and impact in fungal allergic asthma.

5. GENERAL DISCUSSION

Asthma is a chronic lung disease that inflames the airways. This disease is dependent on the environment to elicit different phenotypes and affects males and females disproportionately. There are different mouse models of allergic asthma that use the following antigens: ovalbumin, lipopolysaccharides, house dust mite, and *Aspergillus fumigatus* to illicit the allergic response[38, 126, 127, 131, 195], these antigens create a Th2 immune response with pro-inflammatory cells, cytokines, and mediators. Our model specifically uses *Aspergillus fumigatus* to sensitize and challenge mice and increases in IgE production, recruitment of eosinophils, mucous production, airway hyperresponsiveness (AHR), leukocytic pulmonary inflammation, and peribronchial fibrosis are observed[38, 39].

To characterize low humidity in our model, we examined the following readouts: weight, airway hyperresponsiveness, inflammation via BAL and histological staining, and antibody production. Overall, low humidity had no impact on the weight of model animals compared to naïve that were raised in similar conditions. Mice born, raised, and used for studies in low humidity conditions appeared smaller and had rougher looking coats compared to historical data of the colony in normal humid conditions. Airway hyperresponsiveness was not significantly increased over naïve in the allergic model under low humidity conditions. In normal humid conditions, our model has shown significant increases airway hyperresponsiveness after multiple inhalational challenges of *A. fumigatus* conidia[51]. There was little lung inflammation indicated by the slight increase of inflammatory cells. Eosinophils were present on day 5, and the allergic model still produced IgE antibodies indicating an allergic phenotype. In humid conditions, there is more eosinophils present, but the levels of serum IgE is similar to the lower humidity conditions[51]. Lower humidity conditions do not prevent the production of IgE, but do not allow for proper growth of allergens and minimize the number of encounters of allergen to elicit an allergic response[117]. The low humidity conditions did produce goblet cells and collagen deposition. In normal humid conditions, goblet cells are produced and the percentage of goblet producing cells are similar to the percentage of goblet producing cells in dry conditions[51, 113]. The lower humidity conditions produced a significant amount of IgG3 antibodies.

We observed no increase in airway hyperresponsiveness, inflammatory cells present, and total inflammation in low humidity conditions. The low humidity conditions prevented the allergic asthmatic

64

phenotype aside from reducing IgE Ab levels. The lack of inflammation leading to a lack of airway hyperresponsiveness is important to note due to the fact that the allergic "asthma attack" is not being observed. The amount and frequency of asthma attacks are the reason people seek out treatment[20]. Rather than taking drugs, lowering the humidity could become an easier and safer potential treatment option, but further studies are required to examine this concept. It important to know how humidity affects the immune response in fungal allergic asthma so accurate models can be studied. The development of a model that is humidity tolerant allows for year-round productive research to be conducted. The lower humidity also allowed for multiple areas of the model to be examined including antigen source and makeup, new operators' technique, animal vendor, and possible infections. Understanding each component of the model allows for better research training of graduate students.

To examine if the allergic phenotype in low humidity varied by sex, we examined the following readouts: weight, airway hyperresponsiveness, inflammation via BAL and histological staining, and antibody production. Our model showed that male mice weigh significantly more than female mice. Female mice have more collagen deposition and IgG3 antibody production. Overall, sex has little impact on airway hyperresponsiveness, inflammatory cells, and antibody production in lower humidity conditions in our model. Sex has no impact on the overall role of B-lymphocytes at multiple timepoints throughout our model as there were few phenotypic differences observed in the allergic model. By examining sex and finding little difference in the immune response to *A. fumigatus* in low humidity, we can conclude that sex is not linked to the allergic asthmatic phenotype. It is important that we studied sex and noted no differences observed in C57Bl/6 allergic mice because we can now use either sex or a mix without skewing the results towards a certain sex, aside from IgG3 antibody production, in studying fungal allergic asthma in low humidity conditions.

We wanted to determine what role the B-cells play regarding several different aspects: antibody production, apoptosis, activation, adhesion, cytokine production, response to antigen, and signaling factors. CD19+ B-cells were successfully isolated from the spleens of allergic male and female mice. The gene expression profile of 92 different CD19+ B-cell genes was created to have a better understanding of how B-cells are affected by *A. fumigatus* and by biological sex. The reference genes for the BioRad plates were determined using GeneNorm software. The gene expression plates showed that *Hprt*

expression was increased at day 21 and 41 in males and *Gapdh* expression was decreased in females at day 41 compared to respective naïve controls. Using a mathematical calculation to determine stable reference genes prevented skewing the data based off of traditional information for normalization of qPCR. Reviewing the literature to determine the "best" control genes based off of the disease and tissues being studied should be step one of designing a qPCR experiment. It is important to note that while qPCR has the advantages of rapid detection of nucleic acids from a wide range of samples, there can be technical deficiencies that are limiting to the process. Poor nucleic acid quality, inadequately designed primer sequences, and inappropriate analysis normalizing to inaccurate reference genes can lead to incorrect interpretation of gene expression[196].

CD19+ B-lymphocytes have 6 genes (*Cd74, Ccr7, CD81, CD38, Bax,* and *Irf8*) in males and females at day 5, 6 genes (*Cd74, Foxo1, Pax5, Cd27, Fkbp8,* and *Fmr1*) in males and females at day 21, and 2 genes (*Cd74* and *Gng5*) in males and females at day 41 that have potential roles in our allergic asthma model and require further studies. We showed that sex has little impact on the overall role of B-lymphocytes in our model as few phenotypic differences were observed.

There are a variety of skills and techniques that I have learned as a graduate student that I now apply to my current position as a Cytogenetic Technologist at Integrated Genetics. My work involves the culturing and analysis of prenatal cells to determine if genetic abnormalities are present. After samples are delivered and are set-up *as in vivo* cultures, daily monitoring and maintenance must be performed. Once cells are actively dividing, cultures are harvested for analysis and results are sent to patients.

The tissue culture techniques I learned in completing my masters are applicable to my current position. Aseptic technique is one of the most important skills learned in a laboratory. This technique minimizes contamination of the cultures from bacteria, viruses, and fungi as well as prevent cross-contamination from other cultures. This technique has been stressed in all my undergraduate classes, graduate classes, and now my work. Tissue culture requires the monitoring the cell growth of different culture types. Due to classes involving tissue culture, I now have familiarity with adherent and suspension cultures. It is vital to understand the different medias, reagents, and equipment used in the growth of cultures. Each component and the concentration of the component in media is optimized to ensure the health and growth of the cells. Understanding what and why we are using what we are using allows for

troubleshooting issues with poor cellular growth. The familiarity with biological safety cabinets, inverted-phase microscopes, incubators, and sterilization techniques have allowed me to successfully master tissue culturing techniques in my career.

My thesis work required the use of fine motor dexterity to insert endotracheal tubes, injections of drugs, and dissection of tissues for cell collection. The work also required the identification of different tissue types such as the lungs, spleen, lymph nodes, and nasal passages. This fine motor movement and understanding of tissue types has allowed me to excel in the area of identifying and isolating chorionic villi from maternal material efficiently and effectively to yield dividing cells for further studies.

Although my analysis in my thesis involved data points and graphs and not analyzing chromosomes, the process of analyzing and interpreting the results has carried over into my current work. Concepts such as use and knowledge of microscopy, analysis, and accuracy have been used to report the data for my thesis and the results for the patient. For example, I needed eosinophils to be present to determine that I had allergic model. I stained BAL wash fluid, from *A. fumigatus* challenged mice, with Quik-Dip stain to be able to identify eosinophils under a microscope. I could then count the number of eosinophils and compare the numbers to non-allergic mice to determine if I had an allergic model. In my work, I need a karyotype to visualize if a fetus is abnormal or normal. I stain fixed prenatal cells with Wright Giemsa stain to be able to identify chromosomes under a microscope. I could then count the number of chromosomes, analyze the banding pattern, and determine if the fetus is normal. Both processes use different staining techniques; however, the processes are similar in that I must interpret what the microscope slides are showing and report the answer.

There are other aspects of working in a laboratory that cross from being a student into an employee. Scientists follow protocols to produce results. As a graduate student, I followed established model protocols and amended or developed my own protocols to produce results. As a quality assurance person at LabCorp, I am responsible for editing protocols, submitting to the correct departments for approval, and implementing the changes. I used my knowledge of working with protocols to perform my duties successfully as well as be able to make suggestions to improve the outcomes of the protocols. Scientists also follow strict guidelines for safety and quality control. As a graduate researcher, I had a responsibility to follow IUCAC policies, laboratory policies, and NDSU's safety guidelines to ensure that I

was conducting my experiments correctly. I was required to understand how IUCAC protects animal rights and how I could best perform my experiments without causing harm or distress to the mice. Scientists must document equipment, protocols, and results to be able to complete an experiment successfully. Learning how to have good documentation practices regarding my laboratory notebooks for protocols and results in graduate school has carried over into my being responsible for multiple electronic logs throughout the different departments at Integrated Genetics.

While my thesis taught me a variety of technical skills for the workplace, there are other skills that I believe have made me even more of a successful scientist. I learned how to work with others in a research laboratory. I had to share resources: mice, equipment, reagents, and even advisors to complete my thesis. I learned how to best interact with multiple personalities and to ask for help when needed. I learned how to use preparation and time management to study for classes and balance a school and research environment. I also learned how to perform multiple experiments in a day. As an employee, I am responsible for performing multiple tasks at once to complete an experiment and can do so successfully because of the effort my research my thesis required. I can perform my duties as productively as possible to be able to move onto the next task. I learned how to troubleshoot experiments and can think critically through issues that arise.

6. CONCLUSIONS

In characterizing low humidity in our model, we showed that lower humidity produces no airway hyperresponsiveness, inflammation, and increases antibody production (IgG1 and IgG3) in response to inhalation of *A. fumigatus*. Low humidity has no effect on the goblet cell production, fibrosis, and antibody production (IgE, IgA, and IgG2a). Future experiments would include developing a model that displays allergic phenotypes regardless of humidity and fixing our humidity conditions to have a working model of fungal allergic asthma year-round.

While characterizing sex in our low humidity model, we showed that sex plays a role in some measurements, such as weight and antibody production (IgG3), but overall sex plays little to no role in the allergic asthma response to *A. fumigatus*. Other models of asthma have shown that sex does play a part in eliciting the allergic response. More sex-specific research needs to be performed to have a better understanding of why asthma has phenotypic differences based on sex. Future experiments would include characterizing sex in other environmental conditions and exploring the effect of sex in other aspects of the model. There is also a need to examine sex in the other murine strains such as JH, VPACK 2K/O, BALB/c, and μMT that have been used in the laboratory.

B-lymphocytes have 6 genes (*Cd74, Ccr7, CD81, CD38, Bax,* and *Irf8*) in males and females at day 5, 6 genes (*Cd74, Foxo1, Pax5, Cd27, Fkbp8,* and *Fmr1*) in males and females at day 21, and 2 genes (*Cd74* and *Gng5*) in males and females at day 41 that have potential roles in our allergic asthma model. CD74 was then further examined using flow cytometry and showed no difference in protein expression between naïve and allergic C19⁺ B-cells. Future experiments would include further elucidating the role of *Cd74* regarding its interaction in hyaluronan deposition, airway remodeling, and allergic asthma. The downregulated genes show that the immune system was returning to homeostasis. Little phenotypic evidence of inflammatory cells and inflammation also helps to prove the return to homeostasis. We showed that sex has no impact on the overall role of B-lymphocytes at multiple timepoints throughout our model as there were few phenotypic differences observed in the allergic model. Future experiments would include further elucidating the role of potential model genes and use these techniques to examine other cell populations and cell locations in the model.

Beyond the allergic asthma research, this work shows the need for examining all aspects a mouse model undergoes to accurately report findings.

REFERENCES

1. Casimir, G.J., et al., *Sex and inflammation in respiratory diseases: a clinical viewpoint.* Biology of Sex Differences, 2013. **4**: p. 16-16.

2. Grainge, C.L. and D.E. Davies, *Epithelial injury and repair in airways diseases.* Chest, 2013. **144**(6): p. 1906-12.

3. *Inflammation 101: Inflammation and Asthma*. Inflammation: the Host Response to Injury 2011 [cited 2017.

4. VitalSigns, C. *Asthma in the US*. 2011 May 3 2011 [cited 2015; Available from: www.cdc.gov/vitalsigns/asthma.

5. Peters, S.P., *Asthma phenotypes: nonallergic (intrinsic) asthma.* J Allergy Clin Immunol Pract, 2014. **2**(6): p. 650-2.

6. Romanet-Manent, S., et al., *Allergic vs nonallergic asthma: what makes the difference?* Allergy, 2002. **57**(7): p. 607-13.

7. Ostergaard, P.A., *Non-IgE-mediated asthma in children.* Acta Paediatr Scand, 1985. **74**(5): p. 713-9.

8. Johansson, S.G. and J. Lundahl, *Asthma, atopy, and IgE: what is the link?* Curr Allergy Asthma Rep, 2001. **1**(2): p. 89-90.

9. *Asthma Basics*. Asthma and Allergy Foundation of America.

10. *Treatments*. What is Asthma 2014 [cited 2016 December 9th]; Available from: https://www.nhlbi.nih.gov/health/health-topics/topics/asthma/treatment.

11. Townley, R.G. and F. Suliaman, *The mechanism of corticosteroids in treating asthma.* Ann Allergy, 1987. **58**(1): p. 1-6.

12. Sears, M.R., et al., *Regular inhaled beta-agonist treatment in bronchial asthma.* The Lancet, 1990. **336**(8728): p. 1391-1396.

13. Johnson, M., *Interactions between corticosteroids and beta2-agonists in asthma and chronic obstructive pulmonary disease.* Proc Am Thorac Soc, 2004. **1**(3): p. 200-6.

14. *Cromolyn Sodium - Drug Summary*. Prescribers' Digital Reference.

15. Murphy, S. and H.W. Kelly, *Cromolyn sodium: a review of mechanisms and clinical use in asthma.* Drug Intell Clin Pharm, 1987. **21**(1 Pt 1): p. 22-35.

16. D'Amato, G., et al., *A review of anti-IgE monoclonal antibody (omalizumab) as add on therapy for severe allergic (IgE-mediated) asthma.* Therapeutics and Clinical Risk Management, 2007. **3**(4): p. 613-619.

17. Montuschi, P., *Role of Leukotrienes and Leukotriene Modifiers in Asthma.* Pharmaceuticals, 2010. **3**(6): p. 1792-1811.

18. Hansel, T.T., et al., *Theophylline: mechanism of action and use in asthma and chronic obstructive pulmonary disease.* Drugs Today (Barc), 2004. **40**(1): p. 55-69.

19. Taussig, L. and L. Caverly *Uncontrolled asthma: When to step up thearpy.* 2011.

20. *Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007.* J Allergy Clin Immunol, 2007. **120**(5 Suppl): p. S94-138.

21. *Asthma Treatment & Managment.* Asthma 2017 [cited 2017.]

22. *Asthma Action Plan.* 2017; Available from: http://m.kidshealth.org/en/parents/action-plan-sheet.html.

23. Mukherjee, A.B. and Z. Zhang, *Allergic asthma: influence of genetic and environmental factors.* J Biol Chem, 2011. **286**(38): p. 32883-9.

24. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Cellular and Molecular Immunology.* Vol. 8th Edition. 2015, Philadelphia, PA: Elsevier Saunders.

25. Scirica, C.V. and J.C. Celedón, *Genetics of asthma: potential implications for reducing asthma disparities.* CHEST Journal, 2007. **132**(5_suppl): p. 770S-781S.

26. Kelly, W.F.I., *Allergic and Environmental Asthma Overview of Asthma*, B.M. Cohee, Editor. April 13, 2017, Medscape: Online.

27. Diette, G.B., et al., *Environmental Issues in Managing Asthma.* Respir Care, 2008. **53**(5): p. 602-17.

28. Halonen, M., et al., *Alternaria as a major allergen for asthma in children raised in a desert environment.* Am J Respir Crit Care Med, 1997. **155**(4): p. 1356-61.

29. Zureik, M., et al., *Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey.* Bmj, 2002. **325**(7361): p. 411-4.

30. Douwes, J., et al., *(1-->3)-beta-D-glucan and endotoxin in house dust and peak flow variability in children.* Am J Respir Crit Care Med, 2000. **162**(4 Pt 1): p. 1348-54.

31. Douwes, J. and N. Pearce, *Invited Commentary: Is Indoor Mold Exposure a Risk Factor for Asthma?* American Journal of Epidemiology, 2003. **158**(3): p. 203-206.

32. Weller, S., et al., *IgM(+)IgD(+)CD27(+) B cells are markedly reduced in IRAK-4–, MyD88-, and TIRAP- but not UNC-93B–deficient patients.* Blood, 2012. **120**(25): p. 4992-5001.

33. Chavarria, J.F., *Short report: Asthma admissions and weather conditions in Costa Rica.* Arch Dis Child, 2001. **84**(6): p. 514-5.

34. Cohn, L., J.A. Elias, and G.L. Chupp, *Asthma: mechanisms of disease persistence and progression.* Annu Rev Immunol, 2004. **22**: p. 789-815.

35. Balenga, N.A., et al., *A fungal protease allergen provokes airway hyper-responsiveness in asthma.* Nat Commun, 2015. **6**: p. 6763.

36. Kumar, R.K., C. Herbert, and P.S. Foster, *The "classical" ovalbumin challenge model of asthma in mice.* Curr Drug Targets, 2008. **9**(6): p. 485-94.

37. Nials, A.T. and S. Uddin, *Mouse models of allergic asthma: acute and chronic allergen challenge*, in *Dis Model Mech*. 2008. p. 213-20.

38.   Hoselton, S.A., et al., *An inhalation model of airway allergic response to inhalation of environmental Aspergillus fumigatus conidia in sensitized BALB/c mice.* Med Mycol, 2010. **48**(8): p. 1056-65.

39.   Schuh, J.M. and S.A. Hoselton, *An inhalation model of allergic fungal asthma: Aspergillus fumigatus-induced inflammation and remodeling in allergic airway disease.* Methods Mol Biol, 2013. **1032**: p. 173-84.

40.   Wang, K., D.W. Ussery, and S. Brunak, *Analysis and prediction of gene splice sites in four Aspergillus genomes.* Fungal Genetics and Biology, 2009. **46**(1, Supplement): p. S14-S18.

41.   Latgé, J.-P., *Aspergillus fumigatus and Aspergillosis.* Clinical Microbiology Reviews, 1999. **12**(2): p. 310-350.

42.   Denning, D.W., et al., *Fungal allergy in asthma–state of the art and research needs.* Clinical and Translational Allergy, 2014. **4**: p. 14-14.

43.   Fairs, A., et al., *IgE sensitization to Aspergillus fumigatus is associated with reduced lung function in asthma.* Am J Respir Crit Care Med, 2010. **182**(11): p. 1362-8.

44.   Lukacs, N.W., et al., *Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma.* Am J Respir Cell Mol Biol, 1994. **10**(5): p. 526-32.

45.   Bonamichi-Santos, R., et al., *Microbiome and Asthma: What Have Experimental Models Already Taught Us?* J Immunol Res, 2015. **2015**: p. 614758.

46.   Aun, M.V., et al., *Animal models of asthma: utility and limitations*, in *J Asthma Allergy*. 2017. p. 293-301.

47.   Ishida, W., et al., *Adjuvants determine the contribution of basophils to antigen sensitization in vivo.* Immunol Lett, 2011. **136**(1): p. 49-54.

48.   McMillan, S.J. and C.M. Lloyd, *Prolonged allergen challenge in mice leads to persistent airway remodelling.* Clin Exp Allergy, 2004. **34**(3): p. 497-507.

49.   Kumar, R.K., et al., *Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma.* Clin Exp Allergy, 2002. **32**(7): p. 1104-11.

50.   Bates, J.H.T., M. Rincon, and C.G. Irvin, *Animal models of asthma*, in *Am J Physiol Lung Cell Mol Physiol*. 2009. p. L401-10.

51.   Samarasinghe, A.E., S.A. Hoselton, and J.M. Schuh, *A comparison between intratracheal and inhalation delivery of Aspergillus fumigatus conidia in the development of fungal allergic asthma in C57BL/6 mice.* Fungal biology, 2011. **115**(1): p. 21-29.

52.   Wagers, S., et al., *The allergic mouse model of asthma: normal smooth muscle in an abnormal lung?* J Appl Physiol (1985), 2004. **96**(6): p. 2019-27.

53.   Zhang, Y., et al., *Influence of the route of allergen administration and genetic background on the murine allergic pulmonary response.* Am J Respir Crit Care Med, 1997. **155**(2): p. 661-9.

54.   Ghosh, S., et al., *B lymphocytes regulate airway granulocytic inflammation and cytokine production in a murine model of fungal allergic asthma.* Cell Mol Immunol, 2015. **12**(2): p. 202-12.

55.   Meier, A., et al., *Toll-like receptor (TLR) 2 and TLR4 are essential for Aspergillus-induced activation of murine macrophages.* Cell Microbiol, 2003. **5**(8): p. 561-70.

56.   Braedel, S., et al., *Aspergillus fumigatus antigens activate innate immune cells via toll-like receptors 2 and 4.* Br J Haematol, 2004. **125**(3): p. 392-9.

57.   Song, J.-S., et al., *Neutrophil elastase causes MUC5AC mucin synthesis via EGF receptor, ERK and NF-kB pathways in A549 cells.* The Korean journal of internal medicine, 2005. **20**(4): p. 275-283.

58.   Boyle, K.B., L.R. Stephens, and P.T. Hawkins, *Activation of the neutrophil NADPH oxidase by Aspergillus fumigatus.* Ann N Y Acad Sci, 2012. **1273**: p. 68-73.

59.   Heinekamp, T., et al., *Interference of Aspergillus fumigatus with the immune response.* Seminars in Immunopathology, 2015. **37**(2): p. 141-152.

60.   Hughes, C.E., et al., *Antigen-Presenting Cells and Antigen Presentation in Tertiary Lymphoid Organs.* Frontiers in Immunology, 2016. **7**(481).

61.   Maggi, E., *The TH1/TH2 paradigm in allergy.* Immunotechnology, 1998. **3**(4): p. 233-244.

62.   Kaiko, G.E., et al., *Immunological decision-making: how does the immune system decide to mount a helper T-cell response?* Immunology, 2008. **123**(3): p. 326-338.

63.   Cosmi, L., et al., *Th17 cells: new players in asthma pathogenesis.* Allergy, 2011. **66**(8): p. 989-998.

64.   Choy, D.F., et al., *TH2 and TH17 inflammatory pathways are reciprocally regulated in asthma.* Sci Transl Med, 2015. **7**(301): p. 301ra129.

65.   McKinley, L., et al., *TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice.* J Immunol, 2008. **181**(6): p. 4089-97.

66.   Ober, C., D.A. Loisel, and Y. Gilad, *Sex-Specific Genetic Architecture of Human Disease.* Nature reviews. Genetics, 2008. **9**(12): p. 911-922.

67.   Clayton, J.A. and F.S. Collins, *Policy: NIH to balance sex in cell and animal studies.* May 2014, Nature News: Nature Publishing Group.

68.   Sankaran-Walters, S., et al., *Sex differences matter in the gut: effect on mucosal immune activation and inflammation.* Biol Sex Differ, 2013. **4**(1): p. 10.

69.   Madalli, S., et al., *Sex-specific regulation of chemokine Cxcl5/6 controls neutrophil recruitment and tissue injury in acute inflammatory states.* Biol Sex Differ, 2015. **6**: p. 27.

70.   Elderman, M., et al., *Sex impacts Th1 cells, Tregs, and DCs in both intestinal and systemic immunity in a mouse strain and location-dependent manner.* Biology of Sex Differences, 2016. **7**: p. 21.

71.   Hirano, S., D. Furutama, and T. Hanafusa, *Physiologically high concentrations of 17beta-estradiol enhance NF-kappaB activity in human T cells.* Am J Physiol Regul Integr Comp Physiol, 2007. **292**(4): p. R1465-71.

72.   Zhang, Y., et al., *Endogenous sex hormones and C-reactive protein in healthy Chinese men.* Clin Endocrinol (Oxf), 2013. **78**(1): p. 60-6.

73. Maggio, M., et al., *The relationship between testosterone and molecular markers of inflammation in older men.* J Endocrinol Invest, 2005. **28**(11 Suppl Proceedings): p. 116-9.

74. Falagas, M.E., E.G. Mourtzoukou, and K.Z. Vardakas.

75. Becklake, M.R. and F. Kauffmann, *Gender differences in airway behaviour over the human life span.* Thorax, 1999. **54**(12): p. 1119-38.

76. Troisi, R.J., et al., *Menopause, postmenopausal estrogen preparations, and the risk of adult-onset asthma. A prospective cohort study.* Am J Respir Crit Care Med, 1995. **152**(4 Pt 1): p. 1183-8.

77. Garraud, O., et al., *Revisiting the B-cell compartment in mouse and humans: more than one B-cell subset exists in the marginal zone and beyond.* BMC Immunol, 2012. **13**: p. 63.

78. Rosser, E.C. and C. Mauri, *Regulatory B cells: origin, phenotype, and function.* Immunity, 2015. **42**(4): p. 607-12.

79. Harwood, N.E. and F.D. Batista, *Antigen presentation to B cells.* F1000 Biology Reports, 2010. **2**: p. 87.

80. Rodriguez-Pinto, D., *B cells as antigen presenting cells.* Cell Immunol, 2005. **238**(2): p. 67-75.

81. Chen, X. and P.E. Jensen, *The role of B lymphocytes as antigen-presenting cells.* Arch Immunol Ther Exp (Warsz), 2008. **56**(2): p. 77-83.

82. Batista, F.D. and N.E. Harwood, *The who, how and where of antigen presentation to B cells.* Nat Rev Immunol, 2009. **9**(1): p. 15-27.

83. Hoffman, W., F.G. Lakkis, and G. Chalasani, *B Cells, Antibodies, and More.* Clinical Journal of the American Society of Nephrology : CJASN, 2016. **11**(1): p. 137-154.

84. Stavnezer, J., J.E.J. Guikema, and C.E. Schrader, *Mechanism and Regulation of Class Switch Recombination.* Annual review of immunology, 2008. **26**: p. 261-292.

85. Parlowsky, T., et al., *Neonatal pemphigus vulgaris: IgG4 autoantibodies to desmoglein 3 induce skin blisters in newborns.* J Am Acad Dermatol, 2003. **48**(4): p. 623-5.

86. Michaelsen, T.E., et al., *The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of Neisseria meningitidis.* Scand J Immunol, 2004. **59**(1): p. 34-9.

87. Woof, J.M. and J. Mestecky, *Mucosal immunoglobulins.* Immunol Rev, 2005. **206**: p. 64-82.

88. Boes, M., *Role of natural and immune IgM antibodies in immune responses.* Mol Immunol, 2000. **37**(18): p. 1141-9.

89. Chen, K. and A. Cerutti, *The Function and Regulation of Immunoglobulin D.* Current opinion in immunology, 2011. **23**(3): p. 345-352.

90. Chang, T.W., et al., *Anti-IgE antibodies for the treatment of IgE-mediated allergic diseases.* Adv Immunol, 2007. **93**: p. 63-119.

91. Fahy, J.V. and H.A. Boushey, *Targeting IgE with monoclonal antibodies: the future is now.* Clin Exp Allergy, 1998. **28**(6): p. 664-7.

92.   Kopp, M.V., *Omalizumab: Anti-IgE therapy in allergy.* Curr Allergy Asthma Rep, 2011. **11**(2): p. 101-6.

93.   Corren, J., *Role of interleukin-13 in asthma.* Curr Allergy Asthma Rep, 2013. **13**(5): p. 415-20.

94.   Testar, J., *Cytokines: Introduction.* British Society for Immunology.

95.   *Asthma/Airway Inflammation & Cytokines.* Affymetrix: eBioscience: Cytokine Atlas.

96.   Opata, M.M., et al., *B Cell Production of Tumor Necrosis Factor in Response to Pneumocystis murina Infection in Mice.* Infection and Immunity, 2013. **81**(11): p. 4252-4260.

97.   Rieckmann, P., J.M. Tuscano, and J.H. Kehrl, *Tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) in B-lymphocyte function.* Methods, 1997. **11**(1): p. 128-32.

98.   Conroy, D.M. and T.J. Williams, *Eotaxin and the attraction of eosinophils to the asthmatic lung.* Respiratory Research, 2001. **2**(3): p. 150.

99.   Steinke, J.W. and L. Borish, *Th2 cytokines and asthma — Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists.* Respiratory Research, 2001. **2**(2): p. 66-70.

100.   Mattes, J., et al., *Intrinsic Defect in T Cell Production of Interleukin (IL)-13 in the Absence of Both IL-5 and Eotaxin Precludes the Development of Eosinophilia and Airways Hyperreactivity in Experimental Asthma.* The Journal of Experimental Medicine, 2002. **195**(11): p. 1433.

101.   Hamelmann, E. and E.W. Gelfand, *IL-5-induced airway eosinophilia – the key to asthma?* Immunological Reviews, 2001. **179**(1): p. 182-191.

102.   Makinde, T., R.F. Murphy, and D.K. Agrawal, *The regulatory role of TGF-beta in airway remodeling in asthma.* Immunol Cell Biol, 2007. **85**(5): p. 348-56.

103.   Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.

104.   Teixeira, L.K., et al., *The role of interferon-g on immune and allergic responses.* Memórias do Instituto Oswaldo Cruz, 2005. **100**: p. 137-144.

105.   Chung, F., *Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma.* Mediators of Inflammation, 2001. **10**(2): p. 51-59.

106.   Hawrylowicz, C.M. and A. O'Garra, *Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma.* Nat Rev Immunol, 2005. **5**(4): p. 271-283.

107.   Council, N.R., *Guide for the Care and Use of Laboratory Animals.* Vol. 8th Edition. Washington, DC: National Academies Press.

108.   Chen, C.-H., S. Xirasagar, and H.-C. Lin, *Seasonality in Adult Asthma Admissions, Air Pollutant Levels, and Climate: A Population-based Study.* Journal of Asthma, 2006. **43**(4): p. 287-292.

109.   Hashimoto, M., et al., *Influence of climate factors on emergency visits for childhood asthma attack.* Pediatrics international, 2004. **46**(1): p. 48-52.

110.   Weiland, S., et al., *Climate and the prevalence of symptoms of asthma, allergic rhinitis, and atopic eczema in children.* Occupational and Environmental Medicine, 2004. **61**(7): p. 609-615.

111. D'Amato, G. and L. Cecchi, *Effects of climate change on environmental factors in respiratory allergic diseases.* Clin Exp Allergy, 2008. **38**(8): p. 1264-74.

112. *Average Weather in Fargo North Dakota, United States.* [cited 2017.

113. Ghosh, S., S.A. Hoselton, and J.M. Schuh, *mu-chain-deficient mice possess B-1 cells and produce IgG and IgE, but not IgA, following systemic sensitization and inhalational challenge in a fungal asthma model.* J Immunol, 2012. **189**(3): p. 1322-9.

114. *Body Weight Information for C57Bl/6.* [cited 2017.

115. Arantes-Costa, F.M., et al., *Effects of ventilation, humidity and temperature on airway responsiveness to methacholine in rats.* Eur Respir J, 2002. **19**(6): p. 1008-14.

116. Sposato, B., et al., *Seasons can influence the results of the methacholine challenge test.* Annals of Thoracic Medicine, 2012. **7**(2): p. 61-68.

117. Nielsen, G.D., et al., *IgE-mediated asthma and rhinitis I: a role of allergen exposure?* Pharmacol Toxicol, 2002. **90**(5): p. 231-42.

118. Thornton, D.J., K. Rousseau, and M.A. McGuckin, *Structure and function of the polymeric mucins in airways mucus.* Annu Rev Physiol, 2008. **70**: p. 459-86.

119. Crump, V. *Vasomotor Rhinitis (VMR) or idiopathic non-allergic rhinitis.* 2003.

120. Naclerio, R.M., C. Bachert, and J.N. Baraniuk, *Pathophysiology of nasal congestion.* International Journal of General Medicine, 2010. **3**: p. 47-57.

121. Skov, M., et al., *Immunoglobulin allotypes and IgG subclass antibody response to Aspergillus fumigatus in cystic fibrosis patients.* J Cyst Fibros, 2004. **3**(3): p. 173-8.

122. Rodrigo, W.W.S.I., et al., *Dengue virus neutralization is modulated by IgG antibody subclass and Fcγ receptor subtype.* Virology, 2009. **394**(2): p. 175-182.

123. Cavacini, L.A., et al., *Binding and neutralization activity of human IgG1 and IgG3 from serum of HIV-infected individuals.* AIDS Res Hum Retroviruses, 2003. **19**(9): p. 785-92.

124. Zagury, D., et al., *IFNalpha kinoid vaccine-induced neutralizing antibodies prevent clinical manifestations in a lupus flare murine model.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5294-9.

125. Kynyk, J.A., J.G. Mastronarde, and J.W. McCallister, *Asthma, the sex difference.* Curr Opin Pulm Med, 2011. **17**(1): p. 6-11.

126. Melgert, B.N., et al., *Macrophages: regulators of sex differences in asthma?* American journal of respiratory cell and molecular biology, 2010. **42**(5): p. 595-603.

127. Takeda, M., et al., *Gender difference in allergic airway remodelling and immunoglobulin production in mouse model of asthma.* Respirology, 2013. **18**(5): p. 797-806.

128. Shore, S.A., *Obesity and asthma: lessons from animal models.* Journal of Applied Physiology, 2007. **102**(2): p. 516-528.

129. Delgado, J., P. Barranco, and S. Quirce, *Obesity and asthma.* J Investig Allergol Clin Immunol, 2008. **18**(6): p. 420-425.

130. Weiss, S.T., *Obesity: insight into the origins of asthma.* Nat Immunol, 2005. **6**(6): p. 537-9.

131. Card, J.W., et al., *Gender Differences in Murine Airway Responsiveness and Lipopolysaccharide-Induced Inflammation.* Journal of immunology (Baltimore, Md. : 1950), 2006. **177**(1): p. 621-630.

132. Gueders, M.M., et al., *Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production.* Inflamm Res, 2009. **58**(12): p. 845-54.

133. Corteling, R. and A. Trifilieff, *Gender comparison in a murine model of allergen-driven airway inflammation and the response to budesonide treatment.* BMC pharmacology, 2004. **4**(1): p. 1-9.

134. Bakke, P.S., V. Baste, and A. Gulsvik, *Bronchial responsiveness in a Norwegian community.* Am Rev Respir Dis, 1991. **143**(2): p. 317-22.

135. Gharaee-Kermani, M., et al., *Gender-Based Differences in Bleomycin-Induced Pulmonary Fibrosis.* The American Journal of Pathology, 2005. **166**(6): p. 1593-1606.

136. Tam, A., et al., *The role of female hormones on lung function in chronic lung diseases.* BMC Womens Health, 2011. **11**: p. 24.

137. Huber, S.A. and B. Pfaeffle, *Differential Th1 and Th2 cell responses in male and female BALB/c mice infected with coxsackievirus group B type 3.* J Virol, 1994. **68**(8): p. 5126-32.

138. Yamatomo, T., et al., *Sex-related differences in the initiation of allergic rhinitis in mice.* Allergy, 2001. **56**(6): p. 525-31.

139. Klein, S.L., B.H. Bird, and G.E. Glass, *Sex differences in immune responses and viral shedding following Seoul virus infection in Norway rats.* The American journal of tropical medicine and hygiene, 2001. **65**(1): p. 57-63.

140. Williams, J.W., M.Y. Tjota, and A.I. Sperling, *The contribution of allergen-specific IgG to the development of th2-mediated airway inflammation.* J Allergy (Cairo), 2012. **2012**: p. 236075.

141. Daniels, C.W. and M. Belosevic, *Serum antibody responses by male and female C57Bl/6 mice infected with Giardia muris.* Clin Exp Immunol, 1994. **97**(3): p. 424-9.

142. Sthoeger, Z., H. Zinger, and E. Mozes, *Beneficial effects of the anti-oestrogen tamoxifen on systemic lupus erythematosus of (NZBxNZW)F1 female mice are associated with specific reduction of IgG3 autoantibodies.* Annals of the Rheumatic Diseases, 2003. **62**(4): p. 341-346.

143. Drake, L.Y., et al., *B cells play key roles in th2-type airway immune responses in mice exposed to natural airborne allergens.* PLoS One, 2015. **10**(3): p. e0121660.

144. Wypych, T.P., et al., *Role of B cells in TH cell responses in a mouse model of asthma.* J Allergy Clin Immunol, 2018. **141**(4): p. 1395-1410.

145. Lawrenz, M.B., et al., *Intubation-mediated Intratracheal (IMIT) Instillation: A Noninvasive, Lung-specific Delivery System.* Journal of Visualized Experiments : JoVE, 2014(93): p. 52261.

146. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. Research0034.

147. Glare, E.M., et al., *beta-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels.* Thorax, 2002. **57**(9): p. 765-70.

148. Albershardt, T.C., B.M. Iritani, and A. Ruddell, *Evaluation of reference genes for quantitative PCR analysis of mouse lymphocytes.* Journal of immunological methods, 2012. **384**(1-2): p. 196-199.

149. *Genecards.*

150. Gutierrez-Adan, A., et al., *Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage.* Mol Reprod Dev, 2000. **55**(2): p. 146-51.

151. Tricarico, C., et al., *Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies.* Anal Biochem, 2002. **309**(2): p. 293-300.

152. Sugden, K., et al., *Housekeeping gene expression is affected by antidepressant treatment in a mouse fibroblast cell line.* J Psychopharmacol, 2010. **24**(8): p. 1253-9.

153. Perrot-Sinal, T.S., A.M. Davis, and M.M. McCarthy, *Developmental sex differences in glutamic acid decarboxylase (GAD(65)) and the housekeeping gene, GAPDH.* Brain Res, 2001. **922**(2): p. 201-8.

154. Tan, R., et al., *GAPDH is critical for superior efficacy of female bone marrow-derived mesenchymal stem cells on pulmonary hypertension.* Cardiovasc Res, 2013. **100**(1): p. 19-27.

155. Kawakami, M., et al., *The role of CCR7 in allergic airway inflammation induced by house dust mite exposure.* Cell Immunol, 2012. **275**(1-2): p. 24-32.

156. Pereira, J.P., L.M. Kelly, and J.G. Cyster, *Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses.* International Immunology, 2010. **22**(6): p. 413-419.

157. Deng, J., et al., *Allergen-induced airway hyperreactivity is diminished in CD81-deficient mice.* The Journal of Immunology, 2000. **165**(9): p. 5054-5061.

158. Guedes, A.G.P., et al., *CD38 and Airway hyperresponsiveness: Studies on human airway smooth muscle cells and mouse models.* Canadian journal of physiology and pharmacology, 2015. **93**(2): p. 145-153.

159. Ouyang, X., et al., *Transcription factor IRF8 directs a silencing programme for TH17 cell differentiation.* Nat Commun, 2011. **2**: p. 314.

160. Sasaki, H., et al., *Transcription factor IRF8 plays a critical role in the development of murine basophils and mast cells.* Blood, 2015. **125**(2): p. 358-69.

161. Stout, B.A., et al., *STAT1 activation causes translocation of Bax to the endoplasmic reticulum during the resolution of airway mucous cell hyperplasia by IFN-gamma.* J Immunol, 2007. **178**(12): p. 8107-16.

162. Anginot, A., et al., *Galectin 1 modulates plasma cell homeostasis and regulates the humoral immune response.* Journal of immunology (Baltimore, Md. : 1950), 2013. **190**(11): p. 5526-5533.

163. Dengler, H.S., et al., *Distinct roles for Foxo1 at multiple stages of B cell differentiation.* Nature immunology, 2008. **9**(12): p. 1388-1398.

164. Agematsu, K., *Memory B cells and CD27.* Histol Histopathol, 2000. **15**(2): p. 573-6.

165. Crabtree, G.R. and E.N. Olson, *NFAT signaling: choreographing the social lives of cells.* Cell, 2002. **109 Suppl**: p. S67-79.

166. Marek, D., et al., *Carriers of the fragile X mental retardation 1 (FMR1) premutation allele present with increased levels of cytokine IL-10.* J Neuroinflammation, 2012. **9**: p. 238.

167. Won, W.J., M.F. Bachmann, and J.F. Kearney, *CD36 is differentially expressed on B cell subsets during development and in responses to antigen.* J Immunol, 2008. **180**(1): p. 230-7.

168. Chung, S., et al., *FoxO1 regulates allergic asthmatic inflammation through regulating polarization of the macrophage inflammatory phenotype.* Oncotarget, 2016. **7**(14): p. 17532-46.

169. Fulkerson, P.C., et al., *Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma.* J Immunol, 2004. **173**(12): p. 7565-74.

170. Hamzaoui, A., et al., *Elevation of serum soluble E-selectin and VCAM-1 in severe asthma.* Mediators of Inflammation, 2001. **10**(6): p. 339-342.

171. Jabara, H.H., et al., *DOCK8 functions as an adaptor that links TLR-MyD88 signaling to B cell activation.* Nat Immunol, 2012. **13**(6): p. 612-20.

172. Wang, X., et al., *Activated Syndecan-1 shedding contributes to mice colitis induced by dextran sulfate sodium.* Dig Dis Sci, 2011. **56**(4): p. 1047-56.

173. del Fresno, C., et al., *Inflammatory responses associated with acute coronary syndrome up-regulate IRAK-M and induce endotoxin tolerance in circulating monocytes.* J Endotoxin Res, 2007. **13**(1): p. 39-52.

174. Postma, D.S., *Gender differences in asthma development and progression.* Gend Med, 2007. **4 Suppl B**: p. S133-46.

175. Smolonska, J., et al., *Common genes underlying asthma and COPD? Genome-wide analysis on the Dutch hypothesis.* The European respiratory journal, 2014. **44**(4): p. 860-872.

176. Wojciechowski, W., et al., *Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus.* Immunity, 2009. **30**(3): p. 421-33.

177. Croston, G.E., Z. Cao, and D.V. Goeddel, *NF-kappa B activation by interleukin-1 (IL-1) requires an IL-1 receptor-associated protein kinase activity.* J Biol Chem, 1995. **270**(28): p. 16514-7.

178. Hua, Z. and B. Hou, *TLR signaling in B-cell development and activation.* Cell Mol Immunol, 2013. **10**(2): p. 103-6.

179. Rothenberg, M.E., *CD44 — a sticky target for asthma.* Journal of Clinical Investigation, 2003. **111**(10): p. 1460-1462.

180. Ali, S., et al., *Functional genetic variation in NFKBIA and susceptibility to childhood asthma, bronchiolitis, and bronchopulmonary dysplasia.* J Immunol, 2013. **190**(8): p. 3949-58.

181. Gomez-Aristizabal, A., K.P. Kim, and S. Viswanathan, *A Systematic Study of the Effect of Different Molecular Weights of Hyaluronic Acid on Mesenchymal Stromal Cell-Mediated Immunomodulation.* PLoS One, 2016. **11**(1): p. e0147868.

182.   Meyer, K. and J. Palmer, *The Polysaccharide of the Vitreous Humor.* Journal of Biological Chemistry, 1934. **107**: p. 629.

183.   Ghosh, S., et al., *Hyaluronan fragments as mediators of inflammation in allergic pulmonary disease.* Immunobiology, 2015. **220**(5): p. 575-88.

184.   Noble, P.W., *Hyaluronan and its catabolic products in tissue injury and repair.* Matrix Biol, 2002. **21**(1): p. 25-9.

185.   Noble, P.W. and D. Jiang, *Matrix regulation of lung injury, inflammation, and repair: the role of innate immunity.* Proc Am Thorac Soc, 2006. **3**(5): p. 401-4.

186.   Ghosh, S., et al., *Hyaluronan stimulates ex vivo B lymphocyte chemotaxis and cytokine production in a murine model of fungal allergic asthma.* Immunobiology, 2015. **220**(7): p. 899-909.

187.   Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.

188.   Renart, J., J. Reiser, and G.R. Stark, *Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure.* Proc Natl Acad Sci U S A, 1979. **76**(7): p. 3116-20.

189.   Lo, C.A., et al., *Quantification of Protein Levels in Single Living Cells.* Cell Rep, 2015. **13**(11): p. 2634-2644.

190.   Roche, P.A., et al., *Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(18): p. 8581-8585.

191.   Villadangos, J.A., et al., *Proteases involved in MHC class II antigen presentation.* Immunol Rev, 1999. **172**: p. 109-20.

192.   Takahashi, K., et al., *Macrophage CD74 contributes to MIF-induced pulmonary inflammation.* Respir Res, 2009. **10**: p. 33.

193.   Magalhaes, E.S., et al., *Macrophage migration inhibitory factor is essential for allergic asthma but not for Th2 differentiation.* Eur J Immunol, 2007. **37**(4): p. 1097-106.

194.   Starlets, D., et al., *Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival.* Blood, 2006. **107**(12): p. 4807-16.

195.   Hammad, H., et al., *House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells.* Nature medicine, 2009. **15**(4): p. 410-416.

196.   Bustin, S.A., et al., *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.