IMMUNOMODULATORY ROLE OF B LYMPHOCYTES AND HYALURONIC ACID IN A MURINE MODEL OF ALLERGIC ASTHMA

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

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program: Cellular and Molecular Biology
Major Department: Veterinary and Microbiological Sciences

July 2012

Fargo, North Dakota

North Dakota State University

Graduate School

Title

IMMUNOMODULATORY ROLE OF B LYMPHOCYTES AND HYALURONIC ACID IN A MURINE MODEL OF ALLERGIC ASTHMA

By

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ABSTRACT

In the world today, asthma affects more than 235 million people. The widespread prescription of inhaled corticosteroids—the current gold standard of asthma control medication—allows many asthmatics to live symptom-free and has significantly reduced the number of deaths due to asthma. However, when the disease is poorly controlled, for example due to ubiquitous exposure to airborne fungal conidia, this chronic inflammatory disease often results in lung dysfunction caused by airway architectural changes.

The role of B lymphocytes in allergic asthma has been relegated to the production of IgE with relatively little being known about the trafficking of these cells in the tissues or their role(s) in the affected tissue. As a first step in ascertaining their function, the initial aim of this project was to characterize the recruitment and localization of B cells in the murine lung in response to *Aspergillus fumigatus* inhalation. We found that $CD19^{+}CD23^{+}$ B2 lymphocytes were recruited to the lungs after fungal inhalation and that IgA-, IgE-, IgG-producing cells localized around the large airways. The second aim of the project was to begin defining the impact that these B lymphocytes have on the allergic lung. By using mice that were deficient of conventional B cells, we were able to demonstrate that the allergic phenotype was retained, although the impact of tissue B1 B cells cannot yet be ruled out. We then investigated the ability of hyaluronic acid (HA), a major component of the extracellular matrix (ECM) generated at sites of chronic inflammation, to recruit and modulate B lymphocyte functions in allergic fungal disease. We found that B lymphocytes undergo chemotaxis in response to LMM HA, while HMM HA had little to no effect on B cell chemotaxis. Furthermore, HA-mediated B lymphocyte chemotaxis was significantly inhibited by blocking the CD44 HA receptor. We also demonstrated that LMM HA fragments elicit the production of the pro-fibrotic cytokines IL-10 and TGF-β1 by B

lymphocytes. These observations suggest a previously unrecognized role for B lymphocytes and HA in the context of allergy and represent novel pathways by which B lymphocytes may contribute to airway inflammation and airway remodeling.

ACKNOWLEDGEMENTS

Completing my PhD is probably the most challenging activity of the first 28 years of my life. The best and worst moments of my doctoral journey have been shared with a number of people. It has been a great privilege to spend 5 years in the Department of Veterinary and Microbiological Sciences at North Dakota State University, and its members will always remain dear to me.

My first debt of gratitude goes to my adviser and mentor, Dr. Jane Marie Schuh, for all the opportunities given and for believing in me and my work. She patiently provided the vision, encouragement, and advice necessary for me to succeed through the doctoral program and complete my dissertation. I have had the privilege to work on a number of projects and had the opportunity to present my work at national meetings which allowed me to grow and think as a scientist. The joy and enthusiasm she has for her research was contagious and motivational for me, even during tough times in the PhD pursuit. I appreciate the freedom you gave me to follow my dreams in your laboratory and showed me a way to achieve them and earn this degree.

I would like to express my heartfelt gratitude to Scott Hoselton for being an excellent teacher and a patient listener. You have been like a mentor to me and without you this would have not been possible. When I reflect back and think what I was when I came to NDSU and what I am today, I understand how big role you and Dr. Schuh have played in training me to become a skillful researcher. Thank you!

Special thanks to my committee members, Dr. Mark Sheridan, Dr. John McEvoy, Dr. Birgit Pruess, and Dr. Glenn Dorsam, for their support, guidance, and helpful suggestions. I would like to mention that I really enjoyed all the committee meetings and exams during the

course of my program as you people created a very comfortable and encouraging environment. Dr. Sheridan, I appreciate all the times you have left your office door open for discussing my plan of study and helping me in various aspects in the Cellular and Molecular Biology program. Dr. McEvoy, I really enjoyed taking your class and had a wonderful time working with you on the search committee. You are one of the nicest people I know, and I thank you for all your support and help. Dr. Dorsam, I would like to mention that your class in Biochemistry I enjoyed the most at NDSU. Hats off to your teaching style!!! And I really thank you for all the scientific advice and encouragement over the years. Dr. Pruess, thank you for all the support during my stay at NDSU. It was really a pleasure working with you on the search committee and the various poster sessions of your class. I thank you for always encouraging the graduate students.

The members of the Schuh lab over the years have contributed immensely to my personal and professional time at North Dakota State University. They have been source of friendship as well as good advice. Thank you!

I would also like to acknowledge our department chair, Dr. Charlene Wolf-Hall, for giving me an opportunity to serve on the faculty search committee. This was one of the best experiences I could get such early in my career. I wish to thank Dr. Neil Dyer and Dr. Eugene Berry for giving me wonderful recommendation letters which helped me secure the North Dakota Experimental Program to Stimulate Competitive Research (ND EPSCoR) doctoral dissertation fellowship. I would like to thank Center of Biomedical Research Excellence (COBRE) and ND EPSCoR for the fellowship which made it possible for me to fulfill my degree requirements without the added burden of employment.

Friends & Family:

My friends in US, India, and other parts of the world were sources of constant laughter, joy, and support. Special thanks to all of them for all the wonderful time and moments you people have provided me over the years. You all mean a lot to me. I appreciate everything!

This dissertation is dedicated to my grandparents, Sukumar and Meena Sarkar, and my mother Chanda Ghosh. My grandparents and my mother taught me the value of higher education and always encouraged me to follow my dreams. Their love and support was my driving force and I owe them everything.

I would also like to thank my Aunt's Sandipa and Shubha Sarkar, and my Uncle Sanjay Sarkar for all their support and help over the years. Special thanks to my brother Amit Ghosh and sister-in-law Kokilla Ghosh who have been a role model to me over the years and for helping and supporting me in every aspect of my life over the years. Without you all it would not have been possible for me to stay and study in USA.

Finally, I wish to thank my fiancée, Neha Khanna, for everything she has done for me over the years and for tolerating my anger and frustration throughout the years I spend in grad school. She had complete faith in me and my intellect even when I felt like digging hole and crawling into one because I didn't have faith in myself. She already has my heart so I will just give her a heartfelt "thanks". You are a ROCKSTAR!

FRONTISPIECE

"The important thing is not to stop questioning; curiosity has its own reason for existing. One cannot help but be in awe when contemplating the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of the mystery every day. The important thing is not to stop questioning; never lose a holy curiosity."

-Albert Einstein

Quotation cited fromwww.asl-associates.com/einsteinquotes.htm, June, 2012

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LIST OF ABBREVIATIONS

Ab…………………………………Antibody

ABPA……………………………..Allergic Bronchopulmonary Aspergillosis

Ag…………………………………Antigen

AHR………………………………Airway hyperresponsiveness

AMs……………………………….Alveolar Macrophages

ASM………………………………Airway Smooth Muscle

BAL……………………………….Bronchoalveolar lavage

BCR……………………………….B cell receptor

CD…………………………………Cluster of differentiation

DC…………………………………Dendritic cell

EAR………………………………..Early-phase allergic reaction

ECM……………………………….Extracellular matrix

EGF………………………………..Epidermal growth factor

GM-CSF…………………………..Granulocyte macrophage colony stimulating factor

HA………………………………...Hyaluronic acid

HDM……………………………....House dust mite

HSCs……………………………....Hematopoietic stem cells

ICAM-1……………………………Intercellular adhesion molecule

GC…………………………………Goblet cell

IFN…………………………………Interferon

Ig…………………………………...Immunoglobulin

IL…………………………………...Interleukin

IMs…………………………………Interstitial Macrophages

LAR………………………………...Late-phase allergic reaction

LFA-1………………………………Lymphocyte function-associated antigen

MCs…………………………………..Mast cells

MM…………………………………..Molecular mass

RHAMM………………………….…Receptor for hyaluronic acid mediated motility

SCF……………………………….…Stem cell factor

TGF…………………………………Transforming growth factor

T_H …………………………………...T helper

TNF…………………………………Tumor necrosis factor

TLR…………………………………Toll like receptors

VCAM-1……………………………Vascular cell adhesion molecule

VEGF………………………………Vascular endothelial growth factor

VLA-4………………………………Very Late Antigen-4

GENERAL INTRODUCTION

Significance of the research

Asthma is a chronic disorder of the airways that affects more than 235 million people worldwide (1-6). Inflammation, mucus hypersecretion, and structural remodeling of the allergic airway wall results in wheezing, coughing, and shortness of breath that are commonly associated with asthma symptoms. While deaths due to asthma exacerbations are fewer than 5,000 per year in the United States (4), the direct and indirect economic cost of this disease is nearly 56 billion dollars a year (8). Of this, individuals who are sensitized to fungal allergens bear a disproportionately large financial burden due to increased medical provider visits to control their asthma and emergency center treatment and hospitalization when their symptoms are not controlled (9). Fungal spores, such as those produced by the saprophytic fungus *Aspergillus fumigatus*, are ubiquitous both indoors and outdoors and in many different environments making avoidance strategies largely ineffective. *A. fumigatus* is a common mold that produces several human allergens. While the fungus is relatively harmless to those who are non-atopic, it may generally exacerbate respiratory symptoms in asthmatics or cause severe asthma with fungal sensitization in those who are allergic to it. In instances where the immune system is compromised due to illness or chemotherapy, *A. fumigatus* can grow. If the fungal growth is held in check, an Aspergilloma will form. This may cause local inflammation and bleeding, but usually is not immediately life threatening. However, if the host cannot limit fungal growth, the fungus can disseminate through the circulatory system, causing a critically dangerous disease called Invasive Aspergillosis. Mortality rates for this disease are between 40-90% even with appropriate anti-fungal therapies. Our research focuses on the host's response to *A. fumigatus* in the setting of the allergic lung, but also has practical applications in other disease states.

Cells from both the innate and adaptive branches of the immune system (10), structural cells of the lung (10, 11), and ECM components (12) have been linked to asthma pathogenesis, but many aspects of the disease's pathophysiology remain unclear. For example although IgE antibodies (Abs) have long been recognized as important mediators of the allergic immune response, there is relatively little known about the other roles of B cells, which produce them, in the context of allergic asthma. Recent studies have shown that B lymphocytes are present in the sputum of asthmatic patients (13), and our laboratory's investigators have observed B cells in and around the airways of allergic animals after exposure to airborne allergens. The aim of this doctoral work was to characterize the role that B cells may play in allergic fungal asthma.

Experimental models are important tools to identify cellular mechanisms that initiate and maintain disease. In addition, they help to identify new targets for drug treatment. Most of the models of allergic asthma employ ovalbumin, which has little clinical relevance as an inhalational allergen. On the other hand, models that utilize allergens that are naturally inhaled such as molds, cockroach antigens, pollens, and house dust mite antigens are becoming popular among researchers who recognize the importance of using clinically relevant allergens in model systems that accurately mimic human disease. Most models that employ *A. fumigatus* either nebulize lyophilized extract (14) or inject suspended conidia directly into the trachea (15). Although these models provide a means to study allergy, they do not employ the natural route of *A. fumigatus* exposure and disease in humans. Our lab has developed an inhalational model system of fungal allergic asthma. Using this model system we can study the different cellular and molecular aspects associated with the host's response to viable, inhaled fungal conidia (spores).

Specific aims of the dissertation project

The objective of this research was to determine the effect of B cells in the context of the allergic lung that has been sensitized and exposed to inhaled fungus. In this dissertation, we elucidate the spatial and temporal pattern of B lymphocyte recruitment to the lung after allergen challenge and explore the impact of the microenvironment of the lung on the recruited cells. In particular, we have begun to study the effects of pulmonary hyaluronic acid (HA) on recruited B cells as the HA macromolecule form changes at sites of chronic inflammation/tissue injury. The central hypothesis for this work is that B cells have a local role in the response to inhaled fungal spores. Based on our laboratory's previous findings, research results in the course of the doctoral program, and literature in the field; we developed our working hypotheses for the three studies that are presented in this dissertation. They are as follows: 1) B cells are recruited to the allergic lung in response to fungal conidia inhalation, 2) B cells contribute directly to allergic airway remodeling, and 3) structural high molecular mass HA is cleaved by inflammatory cell egress to the airway at sites of chronic inflammation generating low molecular mass HA that will impact B lymphocyte functions. The results from these studies have been included in this dissertation as manuscripts that have been accepted or submitted for publication. The rationale for this research was to identify the role of B lymphocytes in the microenvironment of the allergic lung in response to fungus, in order to understand the process of pulmonary fungal responses and to develop new therapeutic targets.

Specific Aim 1. Characterize the recruitment and localization of B lymphocytes in allergic lungs after exposure to inhaled fungal spores. Our working hypothesis is that B lymphocytes will be recruited to the allergic airways in response to fungal challenge with *A. fumigatus* and will perform effector functions at the site of inflammation.

Specific Aim 2. Determine the extent to which B lymphocytes exacerbate allergic airway wall remodeling. Our working hypothesis is that B lymphocytes produce Abs and cytokines that support the T_H2-mediated transformation of the allergic airway.

Specific Aim 3. Determine the extent to which the pulmonary extracellular matrix impacts B lymphocyte recruitment and function. The working hypothesis is that the degradation of high molecular mass hyaluronic acid at sites of chronic inflammation increases B cell recruitment to the allergic airways and promotes the production of pro-remodeling cytokines.

Organization of the dissertation

The dissertation has been organized to provide the reader with thorough background information of the current literature on allergic asthma that has guided this research. The literature review that follows is intended to provide a focused account of asthma pathology, highlighting the cellular components of the immune system that play a key role in the immune response in fungal asthma. Animal models of allergic asthma are presented and information relating to B lymphocytes and hyaluronic acid, which has been a central research focus for this work, is also included in the literature review section.

The subsequent chapters provide an account of the research carried out and the discoveries of each of the individual research projects in the format of journal manuscripts that have either been reviewed and accepted for publication through the academic peer review process or are currently under review. Each manuscript is a stand-alone document providing a detailed introduction, methods, results, discussion, and references. There is a general discussion section that combines the findings of all the research projects at the end of the dissertation. An

additional list of references from the literature review and general discussion has been included at the end of the dissertation.

LITERATURE REVIEW

Asthma

According to the clinical definition as recommended by the National Asthma Education Program Expert Panel Report, "Asthma is a lung disease with the following characteristics: (i) airway obstruction that is reversible (but not completely so in some patients) either spontaneously or with treatment, (ii) airway inflammation, and (iii) increased airway responsiveness to a variety of stimuli" (7, 16, 17). Asthma is a chronic inflammatory disease of the airways that affects around 235 million people in the world (6). In the U.S. alone, asthma affects approximately 20 million people (17, 18) and the annual cost associated with asthma treatments is $56 billion (8, 18, 19). Over the past three decades, the incidence of asthma in the western world has increased and is now one of the most common chronic diseases in the world (20). In most cases, patients with asthma have a genetic predisposition to atopy. The physiologic basis for atopy is currently believed to be a dysregulated cellular and humoral response to an allergen. In addition to genetic factors, environmental factors such as lifestyle, infections, and pollution also influence cellular processes of the immune system in a complex and interrelated manner. In addition to the atopic T_H2-type immune response that characterizes allergic asthma, additional cellular factors such as those derived from the structural cells of the airways also play an important role in regulating immune responses in the lung and the development of chronic, self-perpetuating airway inflammation that leads to functional changes and ultimately to irreversible airway remodeling (21).

Asthma is categorized as either intrinsic and extrinsic asthma (22). Intrinsic asthma is known as non-allergic asthma and is triggered by other factors such as anxiety, stress, exercise, cold air, dry air, hyperventilation, viruses or other irritants (22, 23). Extrinsic asthma on the other

hand, is also known as allergic asthma and is triggered by the inhalation of allergens such as house dust mite or cockroach antigens, pollen, animal dander, or molds (22, 24). Most of the symptoms associated with allergic and non-allergic asthma are similar and may include wheezing, coughing, shortness of breath, and chest pain which may occur several times a day or week. Allergic asthma is the most common form of asthma, affecting over 50% of the 20 million asthma sufferers (25). More than 2.5 million children under age 18 suffer from allergic asthma (18).

The response to inhaled allergens in allergic asthma is comprised of two phases. The early phase, which occurs immediately after allergen exposure, and the late phase which starts six to nine hours following allergen provocation (26). The early-phase allergic reaction (EAR) is mediated by allergen which activates the IgE immunoglobulin bound to mast cells and other granulocytes (27). IgE binds to these cells via the high affinity receptor FcεR1, which causes degranulation and the release of a number of mediators such as histamine, prostaglandins, leukotrienes, and reactive oxygen species, which facilitate smooth muscle contraction, mucus hypersecretion, and vasodilation (28). As blood vessels dilate and become more permeable as a result of the inflammatory process, plasma proteins bypass the tight junctions of the epithelium and are released into the airway lumen, interfering with mucociliary clearance (29). The late-phase allergic reaction (LAR) involves the recruitment of inflammatory cells and their subsequent production of cytokines that direct the late response. Monocytes; neutrophils; T_H2 lymphocytes; and eosinophils, whose production of high affinity FcεR1 and accumulation in the lungs is a hallmark of allergic asthma are classic examples of inflammatory cells recruited in response to an inhaled allergen (30). The LAR is considered to be a model system for studying the mechanisms of chronic inflammation (31, 32).

Airway hyperresponsiveness (AHR) is one of the most prominent clinical complaints of asthma exacerbation and a component of the LAR. AHR is defined as an exaggerated obstructive response of the airways to a variety of pharmacological, chemical, or physical stimuli including histamine, methacholine, sulphur dioxide, fog, and cold air (33, 34). Although, a number of studies in humans and animals have shown the relationship between variable AHR and inflammation of the airways, it is uncertain whether airway inflammation directly influences AHR, or if both characteristics develop simultaneously in response to triggers. Despite intensive research, the mechanisms underlying acute and chronic AHR are poorly understood and animal models are indispensable to the unraveling of the mechanisms underlying AHR at the cellular and molecular level.

Airway inflammation has been widely demonstrated in all forms of asthma. Many studies have shown an association between the extent of inflammation and the clinical severity of asthma (35). Animal models and human patients with asthma have demonstrated the influx of several key leukocytes in the lung. The main cell type that is implicated in allergic asthma is the eosinophil. In addition, both allergic and non-allergic asthmatic patients have the accumulation of neutrophils, macrophages, mast cells, and lymphocytes in the lung. In addition, structural cells play a critical role in reguklating the immune response in the microenvironment of the lung. The following section provides more insight into important cell types associated with allergic asthma.

Cells involved in asthma

Mast cells. Mast cells (MCs) play an important role in the pathogenesis of asthma (36). They are thought to be the main link between IgE and AHR (37). Mast cells are found in the bronchial airway connective tissue and in the peripheral intra-alveolar spaces in the lungs with

different profiles of chemical release, and their numbers increase after allergen exposure (38). In asthmatic patients, mast cells are localized within the bronchial smooth muscle bundles and bronchial epithelium, and infiltrate into the airway mucosal glands. Furthermore, in asthmatics, the number of degranulated mast cells is increased (38). Re-exposure with the sensitizing allergen leads to cross-linking of the FcεRI:IgE receptor complex on mast cells (39). This cross-linking triggers the immediate activation of signaling cascades which result in mast cell degranulation, releasing a number of inflammatory mediators such as histamines, prostaglandins, leukotrienes, and TNF-α. The impact of mast cell degranulation is felt immediately in the respiratory tract which constricts and spasms due to nerve ending perturbation, smooth muscle constriction, edema, and mucus secretion (40).

Basophils. Basophilic granulocytes share many common features with mast cells. They also express the high affinity IgE Fc receptor, produce T_H2-type cytokines, and release histamine as one of their granulocytic mediators. Basophils are produced from a lineage separate from that of mast cells. They develop from $CD34^+$ pluripotent stem cells, differentiate, and develop in the bone marrow. While mast cells are tissue-associated, basophils are blood granulocytes. Upon re-exposure, FcεRI-bound IgE binds to the sensitizing allergen, and basophils are recruited to the activated, bronchial mucosa, which leads to granule exocytosis and mediator release (41).

Eosinophils. Blood and tissue eosinophilia are characteristic features of allergic inflammation and asthma (42). The presence of these granulocytes has been documented in various asthma studies, although their exact role in the disease process still remains unclear (35, 43, 44). While the accumulation and presence of eosinophils are considered to be hallmarks of asthma, previous reports on allergic and non-allergic asthma have found a profound difference in the number of eosinophils (45). It appears that not all asthma is characterized by eosinophil

accumulation in the airways. The role of eosinophils in the immune response or immunopathology of asthma remains speculative. A number of studies suggest that eosinophils may contribute to airway remodeling through their actions which injure the epithelium and thicken the basement membrane or cause smooth muscle hypertrophy (42, 46-55).

Neutrophils. Neutrophils play an important role in innate immunity by protecting individuals against infectious agents and can cause significant damage when they accumulate at the site of inflammation, particularly in the airways. Once neutrophils get activated in the peripheral blood, they undergo intravascular migration, adhesion to the endothelium, and migration to the site of inflammation. Neutrophils outnumber eosinophils in sputum of patients with acute exacerbations of asthma (56). In acute severe asthma, neutrophil-induced pathophysiology is induced by the release of neutrophil elastase (57), which mediates mucus hypersecretion, and by the release of other neutrophil mediators that increase the vascular permeability and promote exudation of plasma (58).

Macrophages. Among the different cell types present in the allergic airway, macrophages have emerged as an important participant in disease pathogenesis because of their excessive and prolonged activation during both the inflammatory and repair phases (59). Alveolar macrophages (AMs) and interstitial macrophages (IMs) represent the 2 major populations of macrophages present in the lung (59). Whether these cells are functionally distinct is not known, but both serve as important sentinels in the lung.

Alveolar macrophages play an important role in the activation of dendritic cells (DCs) since DCs rely on the cytokines secreted by macrophages to determine whether or not Ags are innocuous or present potentially dangerous pathogenic infection. Under inflammatory

conditions, recruited monocytes can replenish the AM population. These macrophages also exhibit a unique activation pattern upon exposure to prototypical T_H1- or T_H2-type cytokines. Those macrophages that mature in the presence of IFN-γ are categorized as "classically activated macrophages" denoted as M1, while the macrophages which develop in the presence of IL-4 and IL-13 are categorized as "alternatively activated macrophages" denoted by M2. Studies have shown that alveolar macrophages are capable of both enhancing or suppressing inflammatory responses in allergic asthma and must be programmed to implement the effector responses appropriate to the needs of the moment (26, 59, 60).

Dendritic cells. The main function of dendritic cells (DCs) is antigen presentation. In addition, DCs also play a role in the chemotaxis of T cells in ongoing inflammation. Depending upon the stimulus, human DCs are capable of producing CCL2, CCL3, CCL4, CCL17, CCL2, and CXCL8 (61). Production of CCR4 ligands (CCL17 and CCL22) by myeloid DCs suggest that these cells can recruit T_H2 cells and/or $CD4^+CD25^+$ T regulatory cells at sites of inflammation during the late-phase allergic reaction (62). The total number of lung-resident DCs is increased in asthmatics, which could be an important factor in the persistence of the chronic T cell-mediated allergic inflammation that contributes to remodeling and AHR in chronic asthma (63).

Structural cells. Many structural cells including epithelial cells, endothelial cells, airway smooth muscle (ASM) cells, and fibroblasts have been shown to have pro-inflammatory roles (64). Injury to the airway epithelium can affect AHR in multiple ways, as the epithelium performs a number of different functions (60). First, the epithelium acts as a physiological barrier to diffusion (26). After epithelial injury, inhaled allergens, dust, irritants, agonists or gases might reach the underlying smooth muscle more easily (26). Second, epithelial layers protect intra-

epithelial nerves from being stimulated by inhaled products. If this layer is damaged, the sensory nerves involved in the release of neuropeptides are exposed and bronchoconstriction is induced. Third, the epithelial cell has a metabolic function, and any dysfunction results in an increase in the concentration of several contractile agents. And last, the epithelial cells synthesize mucus, cytokines, and chemokines, which are secreted to participate in inflammatory reactions and releases epithelium-derived relaxin factors such as prostaglandin E2 (PGE2) and nitric oxide (NO). These factors may protect the airways from excessive bronchoconstriction. When the epithelium is damaged, the decrease in the release of these relaxing substances results in excessive bronchoconstriction (65).

Airway endothelial cells play a role in the recruitment and activation of basophils, eosinophils, and lymphocytes into the airways because they express adhesion molecules and produce chemoattractants on their luminal side (60).

Human ASM cells are involved in the pathogenesis of asthma, as both hyperplasia and hypertrophy of ASM cells contribute to AHR and airway obstruction (66). In addition, these cells have inflammatory surface receptors such as CD44, ICAM-1 (Intercellular adhesion molecule-1), VCAM-1 (Vascular cell adhesion molecule-1) and can be activated to release inflammatory mediators which contribute to bronchial inflammation and airway dysfunction (66).

Fibroblasts play a role in maintaining tissue integrity and are also involved in the repair process in response to inflammation. In addition, they have been shown to contribute to airway remodeling. Myofibroblasts are the key source of collagen in asthma, and their numbers are increased in asthma, correlating with the extent of collagen deposition (67). Sub-epithelial fibrosis at the level of the reticular lamina is thought to be an early and fundamental change

within the airways that can be observed even in newly diagnosed asthma (68). This fibrosis is characterized by an increased deposition of collagens I, II, and V, glycoproteins (fibronectin and tenascin), and proteoglycans (lumican, versican, and biglycan) (68-70). This increase in the deposition of macromolecules may also form a compartment where adhesion molecules, cytokines, and other inflammatory mediators are stored, perpetuating the inflammation (71).

T lymphocytes. There are various subsets of $CD4^+$ T cells: T_H1, T_H2, T_H9, T_H17, T_{regs}, and T_{FH}. Polarization of each T_H subset is dictated largely by the cytokines produced by the activating DC. The DC cytokine profile is, in turn, dictated by the type of antigen and the microenvironment in which the DC was activated. $CD8^+$ T cells are cytotoxic and target cells that present their cognate Ag on MHC I for apoptosis.

The dependence of allergic immune responses on thymus-derived lymphocytes has been known since the late 1960s, shortly after the description of IgE (72). In the late 1980s, the phenotype of asthma was suggested to be due to chronic activation of helper T cells sustained by allergen exposure which perpetuated an inflammatory response in and around the bronchi through the release of lymphokines (73). The T cell hypothesis of asthma developed around observations of a T_H2 type T cell cytokine profile in acute severe asthma and LAR that seemed to initiate and maintain the disease (74). Clinical and experimental data have provided considerable evidence to show that T_H2 cells impact atopic, non-atopic, and occupational asthma (75); however, if and how T cells instigate the immunopathology associated with the clinical features of airway hyperresponsiveness, airway obstruction, and airway wall remodeling are not yet fully understood. T helper cells' interaction with either other immune cells or with structural cells in the lung may influence the development of clinical symptoms, and some studies have shown that the number of activated T lymphocytes correlates with the number of blood

eosinophils in allergic disease (74). In addition, it is well known that allergen-specific IgE synthesis by B cells is T cell-dependent through cognate activation of B lymphocytes and the T_H2 cell-derived cytokines, IL-4 and IL-13 (76).

There is much current interest in the regulatory T cells. Regulatory T cells (Treg) play an important role in regulating T_H2 responses to allergens and maintaining functional tolerance (77). These cells have been described in both mice and humans, including the naturally occurring $CD4^+CD25^+$ T cells, IL-10-producing T cells, and TR1 cells (78). Whether, such cells can be induced therapeutically in asthma remains to be established.

B lymphocytes. Human B lymphocyte development begins in the fetal liver during mid to late gestation, then continues after birth and throughout the lifetime of the individual in the bone marrow, albeit at a reduced rate as the person ages (79). B lymphocytes are unique in that they are the only cells in the immune system that produce Abs. Five isotypes of Ab (IgM, IgD, IgG, IgE, and IgA) may be produced after activation of a B lymphocyte. With the exception of IgD, which is not secreted, all Abs may either be surface-bound to effect cell functions on the B cell or secreted as effector molecules for the humoral immune response. The functions of the different isotypes are largely dependent upon their ability to bind antigen via their variable region. They may do nothing more than neutralize the effect of a toxin by binding to it or they may activate the classical complement pathway or be bound by Fc receptors specific for an individual isotype on effector granulocytes or phagocytes to elicit tissue responses. In addition, some isotypes have particular jobs in unique developmental stages (IgG in fetal and neonatal protection) or anatomical sites (IgA at mucosal surfaces).

There are two main populations of B lymphocytes present in the body, referred as B-1 and B-2 B cells. B-1 B cells are considered part of the innate immune system, while B-2 B cells

function in adaptive responses. Beyond functional differences, the development of B-1 and B-2 cells is also distinct (80).

During postnatal development, B-2 cells are derived from hematopoietic stem cells (HSCs) in the bone marrow (81-84). This process results in the development of cells that express IgM on their surfaces. These cells then migrate to the spleen where they undergo maturation into either follicular or marginal-zone B cells (85). The majority of the B lymphocytes present in the secondary lymphoid organs are comprised of B-2 lymphocytes (80, 86, 87). Following exposure to antigen and signals from helper T cells, follicular B cells can undergo immunoglobulin class switching, somatic hypermutation, and differentiation into plasma and memory B cells (88).

B-1 B cell development occurs primarily during the perinatal period during late gestation and during the first month after birth (89). These cells persist throughout the life of the individual by self-renewal. Approximately half of the B cells present in the pleural and peritoneal cavities are B-1 B cells (90, 91). They are innate-like lymphocytes that respond rapidly to infection. However, in contrast to B-2 cells, B-1 B cells respond to a limited range of T-independent antigens. The B-1 and B-2 cell populations both express the pan B cell marker CD19. However, they can be identified based on the differential expression of additional cell-surface determinants that include CD23, CD9, CD21, CD5 (87, 89, 92, 93). B-1 B cells can be further subdivided based on the differential expression of CD5, as B-1a B cells are CD5+ and B-1b B cells do not express this surface marker.

With the abundant IL-4 cytokine production that accompanies allergic diseases, B cells produce and secrete IgE upon activation. This IgE plays an important effector function in both the EAR and LAR phases, as it mediates the degranulation and activation of mast cells (94).

However, the cellular role of B cells in the development and maintenance of allergic asthma may exceed the production of IgE, and this was a main focal point of the work that will be discussed in detail in the later sections of this dissertation.

The cytokine network in asthma

Cytokines play a key role in orchestrating the chronic inflammation and structural changes of the respiratory tract in asthma by recruiting, activating, and promoting the survival of various inflammatory cells in the respiratory tract (7). To date, about 50 different cytokines have been identified in asthma, but their role in the pathophysiology of this complex disease syndrome remains unclear.

The different cytokines released are classified as lymphokines (cytokines that are released by T cells and regulate immune responses), pro-inflammatory cytokines (cytokines that amplify the inflammatory process), growth factors (cytokines that promote cell survival and result in structural changes in the airways), chemokines (cytokines that are chemotactic to inflammatory cells), and anti-inflammatory cytokines (cytokines that negatively modulate the inflammatory response). Some of the important cytokine classes are discussed in detail in the following section.

Lymphokines. In asthma, lymphokines are produced by T_H2 cells and are more commonly referred to as T_H2 cytokines. In patients with asthma, there is an increase in the number of $CD4^+$ T_H cells in the airways, which are predominantly of the T_H2 subtype (16), as there is an increase in the $GATA3^+$ transcription factor that regulates the differentiation of naïve T cells into T_H2 cells (95). These T_H2 cells are characterized by the secretion of cytokines IL-4, IL-5, IL-9, and IL-13. IL-4 supports the differentiation of T_H0 cells to a T_H2 phenotype and may also be

important in the initial sensitization to allergens. In addition, it is important for isotype switching of B cells from IgG-producers to IgE producing cells [**Fig 1** (7) {Figure modified from reference}]. IL-4 and IL-13 may share receptor component, and some of their functions overlap. For instance, IL-13 mimics IL-4 in inducing IgE secretion and causing structural changes in the airways, but it does not play a role in the differentiation of T_H0 cells. IL-13 has attracted particular attention in asthma as a therapeutic target as it not only induces airway hyperresponsiveness but also exacerbates the development of the structural changes seen in chronic asthma, including goblet cell hyperplasia, airway smooth muscle proliferation, and sub-epithelial fibrosis (96).

IL-5 is critically involved in the differentiation of eosinophils from bone marrow precursor cells and it also prolongs eosinophil survival (7). Systemic and local administration of IL-5 to asthmatic patients results in an increase in $CD34^+$ eosinophil precursors (97). In experimental models, blockade of IL-5 reduces eosinophil numbers in the lungs and inhibits allergic responses (98, 99). In humans, blocking IL-5 reduces circulating and sputum eosinophils, but has no effect on either allergic responses or AHR (100).

IL-9 overexpression has been associated with eosinophilia, mucus hypersecretion, mastocytosis, AHR, and increased expression of T_H2 cytokines and IgE (101). IL-9 blockade inhibits pulmonary eosinophilia, mucus hypersecretion, and AHR after allergen challenge in sensitized mice (101). Many of the effects of IL-9 in mice (eosinophilic inflammation and mucus hypersecretion) are mediated via the increased release of IL-13 (102), whereas its effects on mast cell expansion and B cells seem to be direct (101). IL-9 plays an important role in differentiation and proliferation of mast cells and works synergistically with stem cell factor (SCF) (103).

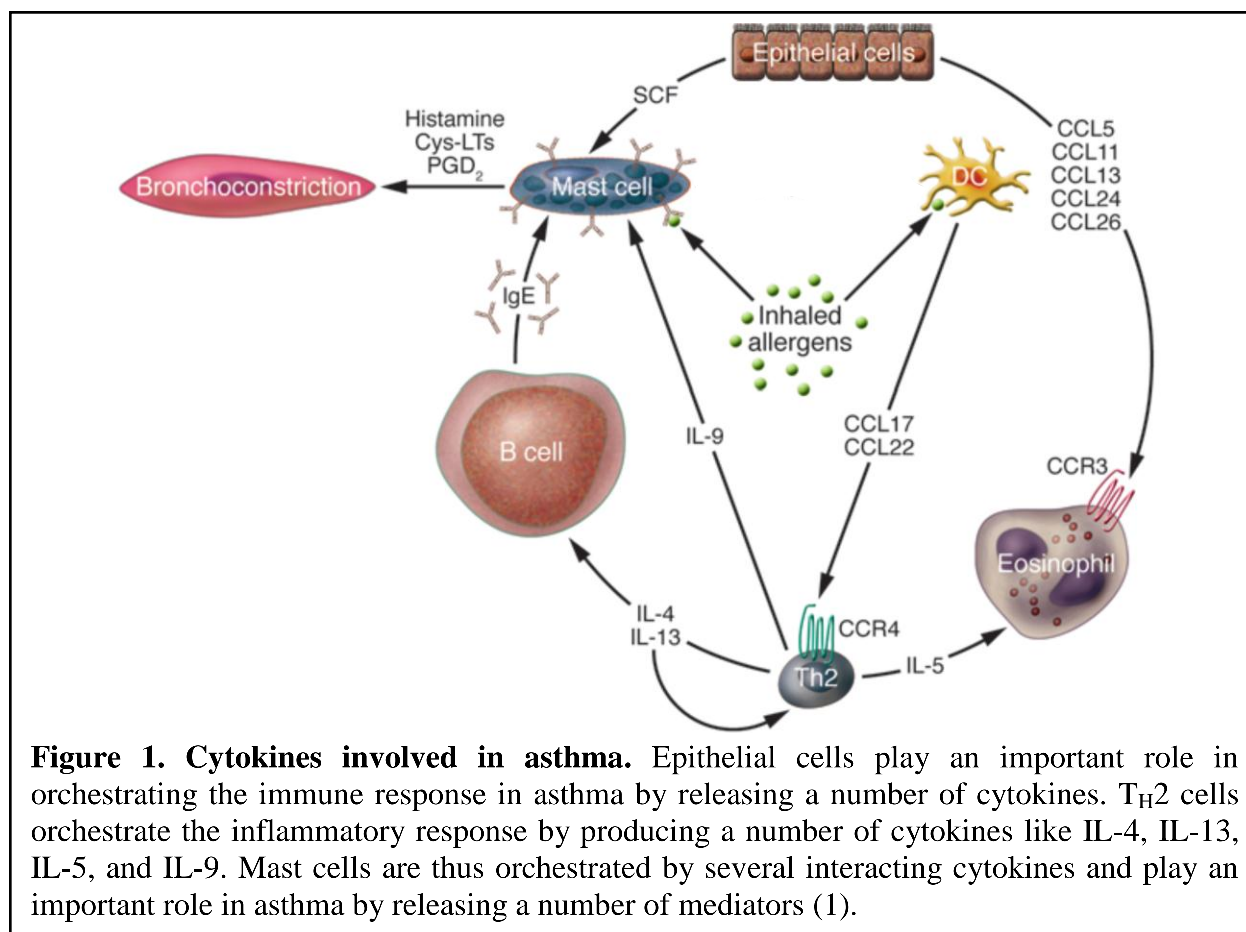

Figure 1. Cytokines involved in asthma. Epithelial cells play an important role in orchestrating the immune response in asthma by releasing a number of cytokines. T_H2 cells orchestrate the inflammatory response by producing a number of cytokines like IL-4, IL-13, IL-5, and IL-9. Mast cells are thus orchestrated by several interacting cytokines and play an important role in asthma by releasing a number of mediators (1).

Over the past few years, IL-17's role in allergic asthma has been studied widely. Research using an intratracheal model of *Aspergillus fumigatus*-induced disease demonstrated a central role for IL-17 in driving eosinophilia in T_H2-mediated airway inflammation (104, 105). In other work, IL-17 was shown to induce eotaxin-1 expression in human airway smooth muscle cells (106).

Pro-inflammatory cytokines. Increased NF-κB leads to the increased expression of multiple inflammatory genes in asthma, and pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are found in increased concentrations in the sputum and BAL fluid of asthmatic patients (7). Blocking these cytokines has been of clinical benefit in other chronic inflammatory diseases, therefore there has been considerable interest in determining whether the same approach might also be useful in asthma.

Many cells have the capacity to produce TNF-α, including mast cells, T cells, epithelial cells, and airway smooth muscle cells. Inhaled TNF-α induces AHR and airway inflammation mediated by neutrophils in asthmatic subjects (107). TNF-α directly acts on human airway smooth muscles to increase the contractile response to spasmogens and thus may play a role in AHR. Blocking TNF-α with etanercept reduced AHR and improved lung function in a small study which included asthmatic patients (108). Larger studies are now underway in patients with severe asthma.

Another inflammatory cytokine, IL-1β, is elevated in asthmatic airways and has been shown to activate a number of inflammatory genes. IL-1 receptor antagonists reduce AHR induced by allergens in mice, but human recombinant IL-1Ra is not effective in the treatment of asthma (109).

IL-6 often works in concert with other cytokines and it provides a link between the innate and the adaptive immune system. IL-6 is present in higher amounts in patients with asthma and may play a role in the expansion of T_H2 and T_H17 cells.

Thymic stromal lymphoprotein (TSLP) is increased in asthmatic patients (110). TSLP is released by airway epithelial cells and its synergistic interaction with IL-1β and TNF-α results in the release of T_H2 cytokines from mast cells independent of T cells (111). It also plays a key role in programming DCs and in recruiting T_H2 cells in the airways (112). Blocking Abs to this receptor have showed considerable promise as it inhibits TSLP mediated T_H2 cell activation in mice and non-human primates (113).

Growth factors. There are several cytokines involved in airway inflammation that promote the differentiation and survival of inflammatory cells or activation of structural cells,

contributing to airway remodeling. They are known as growth factors. Some of the growth factors include granulocyte macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β1 (TGF-β1).

GM-CSF plays a role in the differentiation and survival of eosinophils, neutrophils, and macrophages. Airways of asthmatic patients strongly express GM-CSF. SCF is the ligand of the c-Kit tyrosine kinase receptor, which is expressed by several structural and inflammatory cells (114). SCF is produced by epithelial cells, airway smooth muscle cells, endothelial cells, fibroblasts, mast cells, and eosinophils. It is critical for the growth of mast cells and generation of CD34+ progenitors.

Another set of growth factors EGF and VEGF both are upregulated in asthmatic patients. EGF activates EGFR tyrosine kinases that are also activated by TGF-α and may play a critical role in regulating mucus production in asthmatic patients while VEGF plays an important role in regulating the growth of new vessels and vascular leak into the asthmatic airways (115).

Transforming growth factor (TGF)-β1 is a member of a superfamily of pleiotropic cytokines which participates in the phenotype of asthma. The cellular origin of TGF-β1 is not clear, but it is known that TGF-β1 is widely expressed throughout the body. Most of the resident structural and immune cells of the lung as well as those immune cells that are recruited to the lungs are capable of expressing and secreting TGF-β1 (116). The general understanding among asthma researchers is that TGF-β1 is upregulated in asthma but the overall theme of its function is not clear from the published literature and the cellular origin in the asthmatic lung is still in debate. TGF-β1, like IL-10, has immunoregulatory effects that are largely mediated by the

induction of FoxP3, resulting in the suppression of T_H1 and T_H2 cells, so it is also called an anti-inflammatory cytokine.

Anti-inflammatory cytokines. Although most cytokines increase or orchestrate the inflammatory response in asthma, some cytokines have inhibitory or anti-inflammatory effects. IL-12, through the release of IFN-γ, can suppress T_H2 cytokine release. TGF-β1 has potential immunoregulatory effects through inhibition of $CD4^+$ T cells. And IL-10 has anti-inflammatory effects that inhibit the synthesis of a number of inflammatory proteins (TNF-α, GM-CSF, IL-5) and chemokines. IL-10 transcription is reduced in asthmatic patients (117). However, the role of IL-10 in *Aspergillus* induced asthma is controversial, as it plays different roles at different stages of disease: an enhancing effect when conidia dominate and a suppressive effect during the late stages of the disease (118). Furthermore, increased IL-10 production has been associated with an increase in levels of total and specific IgE, IgG1, and IgA reflecting to a role of IL-10 in promoting a T_H2 response to *A. fumigatus* Ags (119-122).

Mouse models of allergic asthma

Our ability to manipulate and interrogate the genetic makeup of the mouse provides a very powerful means to perform controlled experiments in mammals (123). Mouse model systems may be altered to be either acute or chronic. In acute mouse models, animals are sensitized against the Ag mixed with an adjuvant, and challenged once or multiple times with the Ag alone prior to preselected time points for analysis (124). On the other hand, in chronic mouse models, animals are first sensitized with Ag mixed in alum and then challenged multiple times before samples are harvested for analysis (125). Therefore, the terms acute and chronic do not refer to the duration of sensitization or challenges, rather it refers to the outcome that needs to be

measured. The difference in the two modeling systems lies in the ability to mimic airway remodeling events such as airway fibrosis, smooth muscle cell hyperplasia, and angiogenesis. Chronic allergen exposure in mice appears to be now the model of choice for studying the role of specific cell types.

There are a number of murine model systems that are used to study human asthma. Although the allergen choice of each of the model system may be different, the basic sensitization and challenge procedures are similar. Most models use an adjuvant along with the Ag for the sensitization process, as adjuvants help to enhance the immune response against weekly immunogenic Ags. A defined amount of Ag is required to activate the immune response in laboratory animals, referred as " window of immunogenicity" (126). A slight variation in the amount of Ag administered can lead to the development of immune tolerance. For this reason, adjuvants are used and minute amounts of Ag are co-administered along with the adjuvants to elicit stronger immune responses as adjuvant-free protocols require a greater number of allergen exposures to attain effective sensitizations (127). In all allergic asthma model systems, the type, load, and route of allergen delivery determines the strength of response. The most commonly used allergic asthma model system uses the OVA allergen (128). This OVA allergen has minimum human implications and new model systems like house dust mite (HDM), cockroach Ags, and fungi are now being used to replace OVA in the laboratory (53, 129, 130).

Aspergillus fumigatus *induced model systems of allergic asthma.* Although other fungal genera including *Bipolaris, Curvularia, Cladosporium, Penicillium,* and *Alternaria* are also suspected culprits in the growing epidemic of allergy and asthma (131-133), the airborne fungal pathogens that have received the greatest research attention are found in the genera *Aspergillus* (134). Based on the worldwide sampling of indoor and outdoor air, members of genus

Aspergillus are among the most prevalent of airborne fungal spores. The genus *Aspergillus* consists of more than 180 species of which *Aspergillus fumigatus* is the most common human pathogen and allergen (135). *A. fumigatus* poses a significant problem in home and hospital environments where it readily infects patients with various forms of immunodeficiency (134). This filamentous fungi has rapid growth rates and can produce millions of tiny spores known as conidia (135). These spores can remain airborne for long durations and humans are estimated to breathe in hundreds of spores each day (118, 136, 137). The size (2µm - 3µm) and shape of the spores allow their inhalation all the way to the alveoli (136). *A. fumigatus* also affects individuals with fully functioning immune systems. For example, the intrapulmonary growth and persistence of *A. fumigatus* elicits chronic hypersensitivity reaction in the lung that is commonly referred to as allergic bronchopulmonary aspergillosis (ABPA). Several features of ABPA are similar to asthma. Although ABPA is clinically diagnosed with confidence in only 1-2% of chronic asthmatics, this may be a finding related to the fact that colonization of the lung by *Aspergillus* is rarely reported in immunocompetent individuals. However, the lack of *Aspergillus* colonization in the asthmatic lung does not rule out a major exacerbating role for this fungus in the vast majority of other asthmatics that do not present with the clinically defined ABPA.

Animal models that use *Aspergillus fumigatus* have evolved over the past 22 years, initially from the utilization of extracts from *A. fumigatus* cultures to induce allergic airway disease (121, 138). As conidia are responsible for human disease, most of the models scrape conidia from fungal cultures grown on Sabaouraud Dextrose Agar using PBS containing tween-80 (139). The usual model of spore-induced airway disease is to administer a specific number of spores in suspension by intratracheal inoculation ito animals previously sensitized with fungal Ags (140, 141). Although these models provide a closer representation of human airway disease,

they fail to take full advantage of the dispersal strategies that allow conidia to be inhaled deep into the lung as the suspension certainly inhibits their movement into the small airways. Additionally, tween-80 that is needed to prevent clumping of the spores may cause disruption of the spore coat, so the inoculum is even more removed from the normal inhalation exposure.

Our laboratory has developed an *A. fumigatus* murine model to study human fungal asthma that exploits the nature of *A. fumigatus* spores by exposing sensitized mice to airborne spores (52). In this model system, exposure to fungal spores yields the hallmark symptoms that are associated with allergic asthma including elevated IgE levels in serum and BAL, eosinophillic inflammation, AHR, and airway remodeling. This fungal murine model system recapitulates the acute as well as chronic features associated with allergic asthma and was employed for all the projects tested as part of this dissertation project.

B lymphocytes in the development and maintenance of allergic asthma

B cells are unique in that they are the only cells in the immune system that can produce Ab. As in the blood, the majority of pulmonary B cells express CD19 and CD20 and bear surface immunoglobulins indicating a mature phenotype (142). Of the Ab isotypes produced by B lymphocytes—IgM, IgD, IgG, IgE, and IgA—only IgD is not secreted. It performs the function of a B cell receptor (BCR) only. Each of the other Abs have specific effector functions largely dependent upon the availability of receptors on cells of the innate immune system.

Resident plasma cells have been observed in the lungs of both human asthma sufferers and mice under experimental allergic airways protocols (143). A number of immunoglobulin isotypes are believed to play an important role in the pulmonary response to fungi. IgA is the predominant isotype present in the respiratory tract and is actively pumped across the epithelium

to provide innate mucosal protection (144-146). Selective IgA deficiency in clinic patients is associated with an increased prevalence of atopy (147, 148). The IgG subtype, IgG1, which is a T_H2-elicited antibody, is cytophillic to mast cells (149); and IgG2a, which is produced by T_H1-activated B cells, plays a role in host protection against fungal growth (150); while IgE enables mast cell degranulation (151). IgE has long been recognized as a perpetrator of asthma exacerbations, and anti-IgE therapies have been used successfully for treatment (152-154). During asthma exacerbations, B cells in all stages of activation and differentiation are found in increased numbers in the blood of asthmatic patients (155). B cells are also present in the bronchial mucosa of asthmatics. In the setting of invasive disease, Abs to *Aspergillus* proteins have been noted in patients with aspergillomas and invasive disease (156, 157), although the characterization and role of these Abs is not well documented (158). While allergen-specific Abs are recognized as contributing factors in the immunopathology of an aberrant response against an innocuous allergen such as pollen or animal dander (159), they have also been suggested to be part of the successful clearance of fungus from the airways (160, 161).

While a strong phagocytic defense is essential for effective clearance of the inhaled spores and opsonization by Abs may assist this process, it is becoming clear that B cells may play other roles in the target tissues. In addition to Ab production, the B lymphocytes can present Ag to T cells in the tissue and can produce cytokines at the point of infection and/or may contribute to the development of immunopathology (162). Over the last 30 years, there has been growing evidence supporting B cell regulatory functions like $Foxp3^+$ regulatory T cells (Treg), which suppress Ag (Ag)- specific immune effector cells, making negative immune regulation a focus of work to characterize the pathogenesis of allergic inflammatory diseases (163-167). The absence or loss of these regulatory B cells exacerbates disease symptoms in allergic (including

contact hypersensitivity and anaphylaxis) and autoimmune diseases (168-171). Moreover, B cells have been shown to regulate immunological or allergic inflammation and T cell-mediated autoimmunity through the production of IL-10 (168, 169) as well as other regulatory B cell subsets, including TGF-β producing Br3 (172).

Currently, little is known about the spatial and temporal orchestration of B cells in the allergic lung in response to fungal allergens/pathogens. In this dissertation study, we employed a murine model of *A. fumigatus*-induced allergic lung disease that uses the inhalation of unmanipulated, dry spores in order to determine the recruitment of B cells into the pulmonary space, their localization, and their production of major Ab isotypes in the context of the fungal allergen-sensitized lung. This is a critical step in determining the specific function of B cells, Abs, and B cell-produced cytokines in the response to environmental fungal exposures in the asthmatic patient. In addition, we used a knockout model system to deduce the role of B lymphocytes in allergic asthma.

The role of hyaluronic acid in the development and maintenance of allergic asthma

Recent studies have shown that ECM components play an important role in the attachment of cells, tissue growth and repair (173), proliferation and differentiation (174), cell migration and activation (175), cell survival/delay of apoptosis (176), and chemotaxis (177). Clearly, they may have direct and significant impact of the development and persistence of inflammation in many disease states, including asthma. Moreover, studies have demonstrated that, under certain circumstances, ECM components can function in cellular signaling (178) and can deliver signals leading to or regulating cellular proliferation (179). ECM components have been reported to play an important role in regulating host response to lung injury. Accumulation

of ECM components can be seen in tissue injury following a variety of insults such as those that occur in the adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), and chronic persistent asthma (180). Among the different ECM components studied, the glycosaminoglycan (GAG) hyaluronan (HA) undergoes dynamic regulation during inflammation (181). HA, which is mainly synthesized by fibroblasts (182, 183), exists as a high molecular mass (HMM) polymer usually in excess of 10^6 D in its native form (184). In the lungs, the HA content is 15-150mg/g dry weight which is mainly localized in the peri-bronchial and inter- and peri-alveolar tissue. Under disease conditions this HMM HA is hydrolysed into low molecular mass HA (LMM HA) (185). Recently, several studies have shown that LMM HA and HMM HA have opposite functions (181). Importantly, LMM HA has pro-inflammatory effects (186), while HMM HA can block the pro-inflammatory effects of LMM HA and helps to support the tissue integrity (187).

HA binding proteins play an important role in cellular signal transduction and two of the most widely studied receptors of HA are CD44 and RHAMM (Receptor for hyaluronic acid-mediated motility) (188, 189). To date, CD44 is the best characterized transmembrane hyaluronan receptor and is present on most cell types (190, 191). Recent studies have shown, CD44-expression on eosinophils and its up-regulation by IL-5 or GM-CSF (192). In addition, it has been reported that there is an increase in the expression of CD44 on eosinophils from late-phase bronchoalveolar lavage fluid (BALF) of patients with asthma (193). Furthermore, CD44 deficient mice exhibit minor abnormalities in hematopoiesis and lymphocyte circulation (194, 195) and that CD44 undergoes dynamic regulation on eosinophils and macrophages. Whether CD44 impacts B lymphocyte recruitment and function in allergic diseases is still not clear.

However, there is growing evidence to suggest that interaction between HA and CD44 may play an important role in the regulation of functions of lymphoid and myeloid cells (196).

In the studies included in this dissertation, we also examined the role of high and low molecular mass HA on B lymphocyte recruitment and function. The purpose of this study was to explore the extent to which LMM HA and HMM HA promote the recruitment of B lymphocytes to the lungs, to describe the role of the CD44 receptor in the migration of B lymphocytes, and to determine the role of LMM HA and HMM HA in the activation of B lymphocytes.

The therapeutic potential of B lymphocytes and hyaluronic acid in allergic asthma

B cell numbers and Ab production is altered in various diseases allowing it to be used as therapeutic models of many diseases. Asthma and other allergic diseases are caused by aberrant immune responses. Soluble IgE molecules, produced by immune cells known as B cells, are key immune mediators of these diseases. Therapeutic targeting of IgE in the blood can neutralize its effects and is an effective treatment for moderate-to-severe allergic asthma. However, this approach does not halt IgE production and patients need to be treated repeatedly. Recently, a team of researchers at Genentech Inc., South San Francisco, have developed a way to specifically eliminate IgE-producing B cells, providing a potential new long-lasting therapeutic approach for treating asthma and other allergic diseases (197). IgE-producing B cells express surface IgE that is slightly different than the IgE molecules that they secrete. When mice expressing human IgE were treated with a monoclonal antibody that blocks human IgE, the IgE levels in the blood decreased substantially as did the number of IgE-producing B cells (197). In addition to blocking the IgE receptor, other methods of exploiting the B cell therapeutic potential should be explored as these B cells have multiple functions. One way would be to

enhance/decrease the cytokine production functions of B lymphocytes in allergic diseases. On the other hand, HA homeostasis is deranged in asthma and there is an increase in the accumulation of LMM HA in the asthmatic lung. Targeting the pro-inflammatory cycle mediated by LMM HA and exploring the therapeutic potential of HMM HA (HMM HA has been shown to inhibit acute lung injury in a sepsis model) in asthma may present novel therapeutic strategies in the treatment of asthma.

Despite an enormous increase in the understanding of the immune mechanisms underlying allergic asthma, it is disappointing to see that only a fraction of this has been translated into new treatments. One possible reason is the lack of understanding of the disease chronicity and the environmental factors that contribute to the allergic disease in addition to the allergen. What is now needed is a clearer understanding of the origins of allergy and the factors responsible for increasing the incidence of allergic asthma with a view of developing preventive as well as therapeutic strategies.

PAPER 1. $CD19^+CD23^+$ B2 LYMPHOCYTES ARE RECRUITED TO THE ALLERGIC AIRWAYS OF BALB/C MICE IN RESPONSE TO THE INHALATION OF *ASPERGILLUS FUMIGATUS* CONIDIA

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Sumit Ghosh, Scott A. Hoselton, and Jane M. Schuh

In Review: The Open Immunology Journal

Abstract

Fungal sensitization in patients with asthma often indicates an unusual disease course in which traditional asthma treatments have little effect and in which morbidity is particularly severe. Airway hyperresponsiveness, inflammatory infiltrates, smooth muscle increases, and irreversible fibrotic remodeling of the bronchial architecture are features of allergic fungal asthma. The systemic production of IgE has long been associated with the immunopathogenesis of allergic asthma; however, the role of B lymphocytes and their products in the response to fungal allergens remains unclear. In the present study, we hypothesize that B lymphocytes are recruited to the allergic lung to impact the allergic response. Using a murine fungal aeroallergen model to mimic the human syndrome, we tracked the recruitment of B cells into the lung after fungal challenge and found that $CD19^{+}CD23^{+}$ B2 lymphocytes are recruited to the allergic lung in a dynamic process. IgA, IgG2a, and IgE were prominent in the serum and bronchoalveolar lavage fluid of allergic animals. It was evident that a tissue-centric production of these Abs was possible, and IgA-, IgG-, and IgE-producing cells from the allergic lung were identified by flow cytometry. This study shows for the first time that $CD19^{+}CD23^{+}$ B2 lymphocytes are recruited to the lungs after inhalation of fungal conidia and their recruitment has a significant impact on the Ab production in the pulmonary compartment in the context of fungal allergy.

Introduction

Aspergillus fumigatus is a saprophytic mold with an important environmental function in carbon and nitrogen cycling (1). Its hydrophobic spores are readily dispersed in the environment and, when inhaled, are small enough to navigate the airways of the lung far beyond the barriers of the ciliated epithelium (2). Cellular innate (neutrophil- and macrophage-mediated) and adaptive (T_H1-mediated) immune responses protect against infection by *Aspergillus* in a normal lung (3-6), but *A. fumigatus* can induce or exacerbate allergies of the upper and lower airways, and its ubiquitous dissemination in indoor and outdoor environments limits the effectiveness of avoidance strategies. Sensitization to *Aspergillus* is common in atopic individuals and *Aspergillus fumigatus* is responsible for about 16-38% of *Aspergillus* related illness in humans (7, 8). In asthmatic individuals *Aspergillus* sensitization can herald a particularly difficult to treat disease termed Severe Asthma with Fungal Sensitization (9). In immunocompromised patients or those with previous lung damage, *A. fumigatus* may germinate and its growth may invade local blood vessels causing disseminated fungal disease with mortality rates ranging from 40-90% (1, 10).

A number of immunoglobulin isotypes are believed to be important in pulmonary response to fungi. At the earliest interface with the lung, IgA from resident B cells is actively pumped across the epithelium to provide innate mucosal protection (11). We have previously shown that IgA production is upregulated in the allergic murine bronchoalveolar lavage (BAL) fluid after exposure to inhaled spores (12). In the immunocompetent, non-atopic host, IgG2a from follicular B2 cells is associated with a T_H1 response and has been shown to arrest fungal development, preventing germination of the fungus (13). In allergic responses, IgE functions in the activation/degranulation of granulocytes. While mast cell degranulation is typically

associated with allergic immunopathology, recent work indicates that the degranulation of eosinophils in the lumen may provide protection in response to fungus (14). In the setting of invasive disease, antibodies to *Aspergillus* proteins have been noted in patients with aspergillomas and invasive disease (15, 16), although the characterization and role of these antibodies is yet not well documented (17).

While a strong phagocytic defense is essential for effective clearance of the inhaled spores and opsonization by Abs may assist this process, it is becoming clear that B cells may play other roles in target tissues where their ability to supply antibody or cytokines at the point of infection or to present antigen to T cells in the tissue may support the development of a productive immune response and/or may contribute to the development of immunopathology (18).

Currently, little is known about the spatial and temporal orchestration of B cells in the allergic lung in response to fungal allergens/pathogens. In this study, we employed a murine model of *A. fumigatus*-induced allergic lung disease that uses the inhalation of unmanipulated, dry spores in order to determine the recruitment of B2 cells into the pulmonary space, the localization of B cells in the allergic lung, and their production of three major Ab isotypes in the context of the fungal allergen-sensitized lung. This is a critical step in determining the specific function of B cells, Abs, and B cell-produced cytokines in the response to environmental fungal exposures in the asthmatic patient.

Materials and Methods

Animals. BALB/c 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 (5-9 weeks old) were fed and given water *ad libitum* throughout the study and housed on Alpha-dri™ paper 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 North Dakota State University.

Antigen preparation and conidia culture. Soluble *A. fumigatus* extract was purchased from Greer Laboratories (Lenoir, NC, USA) and fungal culture stock (strain NIH 5233) was purchased from American Type Culture Collection (Manassas, VA, USA). A lyophilized *A. fumigatus* was reconstituted in 5ml phosphate buffered saline (PBS) and 60-µl aliquots of the suspension were stored at 4˚C until use. A single aliquot of *A. fumigatus* was grown on Sabouraud dextrose agar (SDA) in a 25-cm^2 cell culture flask for 8 days at 37°C. The use of *A. fumigatus* was approved by the NDSU Institutional Biosafety Committee.

Allergen sensitization and conidia inhalation challenge. To elicit allergen sensitization, mice were injected subcutaneously (SC) and intraperitoneally (IP) with a total of 10 µg of soluble *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) dissolved in 0.1 ml PBS and 0.1 ml Imject® Alum (Pierce, Rockford, IL, USA) (12, 19, 20). Two weeks later, mice were started on a series of five, weekly 20-µg intranasal (IN) inoculations consisting of soluble *A. fumigatus* extract (Greer Laboratories, Lenoir, NC, USA) dissolved in 20 µl of normal saline. One week after the final IN inoculation, mice were exposed to *A. fumigatus* by inhalation of mature, airborne conidia, as previously described (19). Briefly, mice were anesthetized using a cocktail of ketamine (75 mg/kg) and xylazine (25 mg/kg), and their noses were placed in the

inoculation chamber where they inhaled mature *A. fumigatus* conidia for 10 min. The sensitization and challenge model is detailed in **Figure 2**. At specified time points after allergen challenge, animals were anesthetized with pentobarbital (150 mg/kg) and tracheostomized for sample collection.

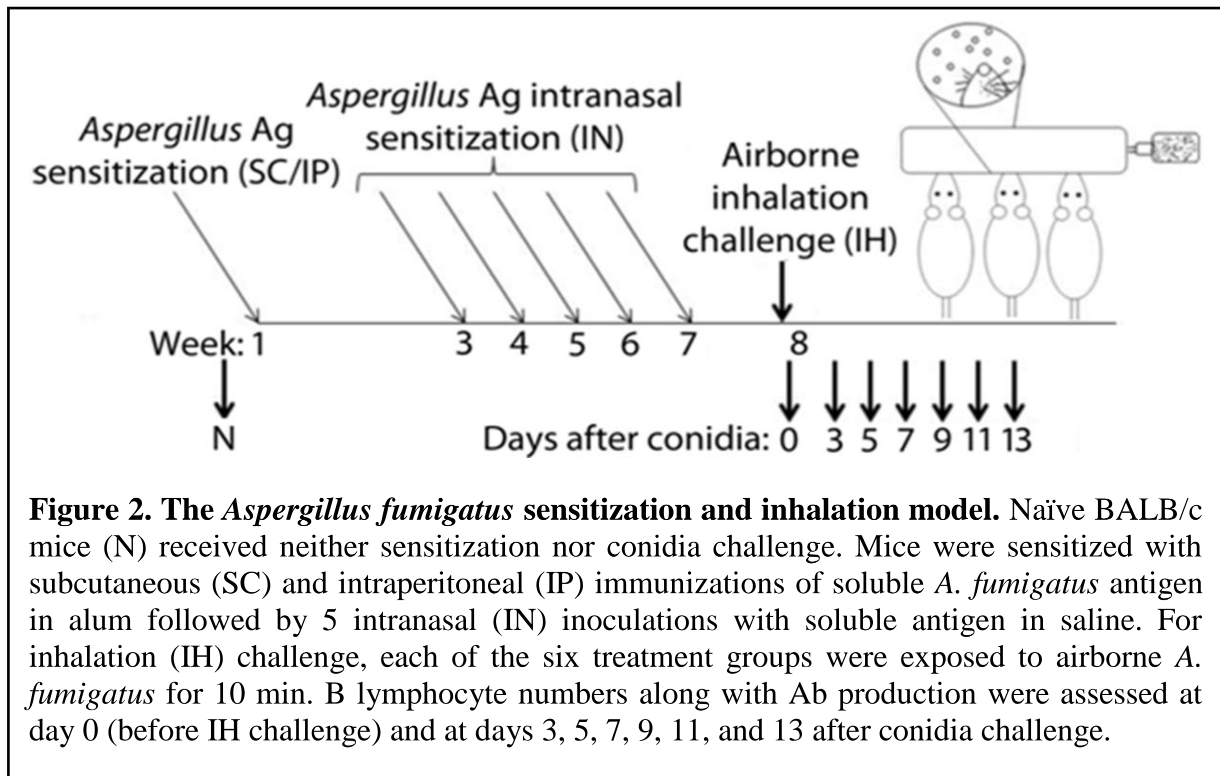

Figure 2. The *Aspergillus fumigatus* sensitization and inhalation model. Naïve BALB/c mice (N) received neither sensitization nor conidia challenge. Mice were sensitized with subcutaneous (SC) and intraperitoneal (IP) immunizations of soluble *A. fumigatus* antigen in alum followed by 5 intranasal (IN) inoculations with soluble antigen in saline. For inhalation (IH) challenge, each of the six treatment groups were exposed to airborne *A. fumigatus* for 10 min. B lymphocyte numbers along with Ab production were assessed at day 0 (before IH challenge) and at days 3, 5, 7, 9, 11, and 13 after conidia challenge.

Sample collection for serum, bronchoalveolar lavage (BAL), and lung tissue. Animals were exsanguinated under anesthesia. The blood was centrifuged at 13,000 x*g* for 10 min to obtain serum, which was stored at -20°C until use. One milliliter of sterile PBS was used to lavage the bronchoalveolar space. Cells were separated from BAL fluid by centrifugation and added to the single cell suspensions obtained from the lung tissue for use in flow cytometry. BAL fluid was stored at -20°C for later analysis. Right lungs were harvested and used for flow cytometry. Left lungs were fixed in an inflated state with 10% neutral buffered formalin and

embedded in paraffin for histological sectioning. Longitudinal 5-µm tissue sections across the coronal plane of the left lung were used for histological analysis.

Quantification of serum and BAL fluid for IgE, IgG2a, and IgA. Total IgE, IgG2a (BD OptEIA, San Diego, CA, USA), and IgA (Bethyl Laboratories, TX, USA) were quantified via specific ELISA in serum and BAL according to manufacturer's guidelines. Serum samples were diluted in PBS (1:100 for IgE, 1:5000 for IgG2a and 1:500 for IgA). The detection limits for the kits were 1.6 ng/ml for IgE, 3.1 ng/ml for IgG2a, and 15.625 ng/ml for IgA.

Flow cytometry. Minced right lungs from animals at days 0 (sensitized, but not challenged), 3, 5, 7, 9, 11, and 13 were subjected to collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) digestion and red blood cell lysis. For collagenase digestion minced lung sections were treated with 0.04% collagenase IV in DMEM and were incubated at 37^{0}C for 1 hour. For antibody staining, the cells were suspended in PBS with 1% BSA (Sigma Aldrich, St. Louis, MO, USA) to a final cell concentration of 1 x 10^{7} cells/ml. Fc receptors were blocked with anti-mouse CD16/CD32 (1 µg /1 x 10^{6} cells) for 10 min on ice. Samples were surface stained with a cocktail containing 1 µg of anti-mouse CD19 FITC and 1 µg of anti-mouse CD23 PE (eBiosciences, San Diego, CA, USA) for 30 min in the dark at 4°C. For intracellular staining, 1 x 10^{6} cells were fixed and permeabilized using BD Cytofix/Cytoperm™kit (BD Biosciences, San Jose, CA, USA). These cells were then stained for intracellular and extracellular immunoglobulins with FITC labeled antibodies for mouse IgE, IgA (eBiosciences, San Diego, USA), and IgG (Invitrogen, Camarillo, CA, USA). Data acquisition was performed using the FACSCaliber flow cytometer (BD Biosciences, San Jose, USA) and analyzed using BD CellQuest Pro software (BD Biosciences, San Jose, USA).

Histological analysis of inflammation and IgA-, IgG-, and IgE-producing cells in the lung. H&E staining and immunohistochemistry (IHC) was carried out on serial sections of allergic lungs 5 days post conidia inhalation. For IHC, sections affixed to glass slides were submerged in 10-mM citric acid at pH 6.0 and microwaved in a pressure cooker for 10 min for antigen retrieval. Staining was performed using the HRP-AEC Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's recommended protocol. Samples were incubated with 5 µg/ml goat anti-mouse IgA, IgG, or IgE (Southern Biotech, Birmingham, AL, USA) overnight at 4°C in a humidified chamber. Incubation with secondary antibodies was carried out at room temperature for 1h. Incubation with horseradish peroxidase followed by 3-amino-9-ethylcabazole yielded a red precipitate in areas of positive staining. The sections were counterstained, and corresponding areas of H&E-stained and IHC-stained were photographed using a Zeiss Axio Observer Z1 micrscope (Carl Zeiss MicroImaging, Gottingen, Germany).

Statistical analysis. Results from sensitized and challenged groups were compared to the sensitized, but unchallenged, control group (day 0). Prism GraphPad software (San Diego, CA, USA) was used to analyze the data using an unpaired Student's two-tailed *t* test with Welch's correction, n=4-5 lungs per time point. * $p < 0.05$ was reported as statistically significant for these experiments.

Results

Inhalation of fungal conidia results in a temporal increase in Ab production in mice sensitized with A. fumigatus *extract.* To determine the temporal production of Ab isotypes that are known mediators of fungus-induced innate, allergic, and adaptive immune responses, we used specific ELISAs to analyze serum levels of IgA, IgG2a, and IgE at predetermined time points after *A. fumigatus* conidia inhalation in sensitized mice. In addition, we assayed levels of these same Abs in pooled BAL fluid samples as an indication of their relative availability in the lung tissue.

We show in this study that IgA is increased in serum (**Fig 3A**) and, even more noticeably, in BAL fluid (**Fig 3B**) as early as 3 days after inhalation. Production of IgG2a, an isotype prominent in the T_H1-mediated cellular response, was not significantly increased in the serum of sensitized mice that had been challenged with conidia as compared to animals that had undergone sensitization only (**Fig 3C**). Although statistical significance could not be determined due to the pooling of BAL fluid samples, the levels of IgG2a in the lung increased from an undetectable level in the unchallenged, sensitized animals to nearly 5-µg/ml at day 11 after inhalation, suggesting a local production accompanying the inhalation of *A. fumigatus* conidia and/or significant vascular leakage at this time point (**Fig 3D**). IgE, the humoral effector of the allergic response, was significantly increased in the serum by day 3 after conidia inhalation (**Fig 3E**). Serum IgE levels continued to rise throughout the study, with sensitized and challenged animals measuring a 900-fold increase above control animals at day 13 after conidia inhalation. A similar trend of increased IgE availability was noted in the BAL fluid, with peak IgE detection noted at day 11 after conidia inhalation as compared with levels in day-0 control animals (**Fig 3F**).

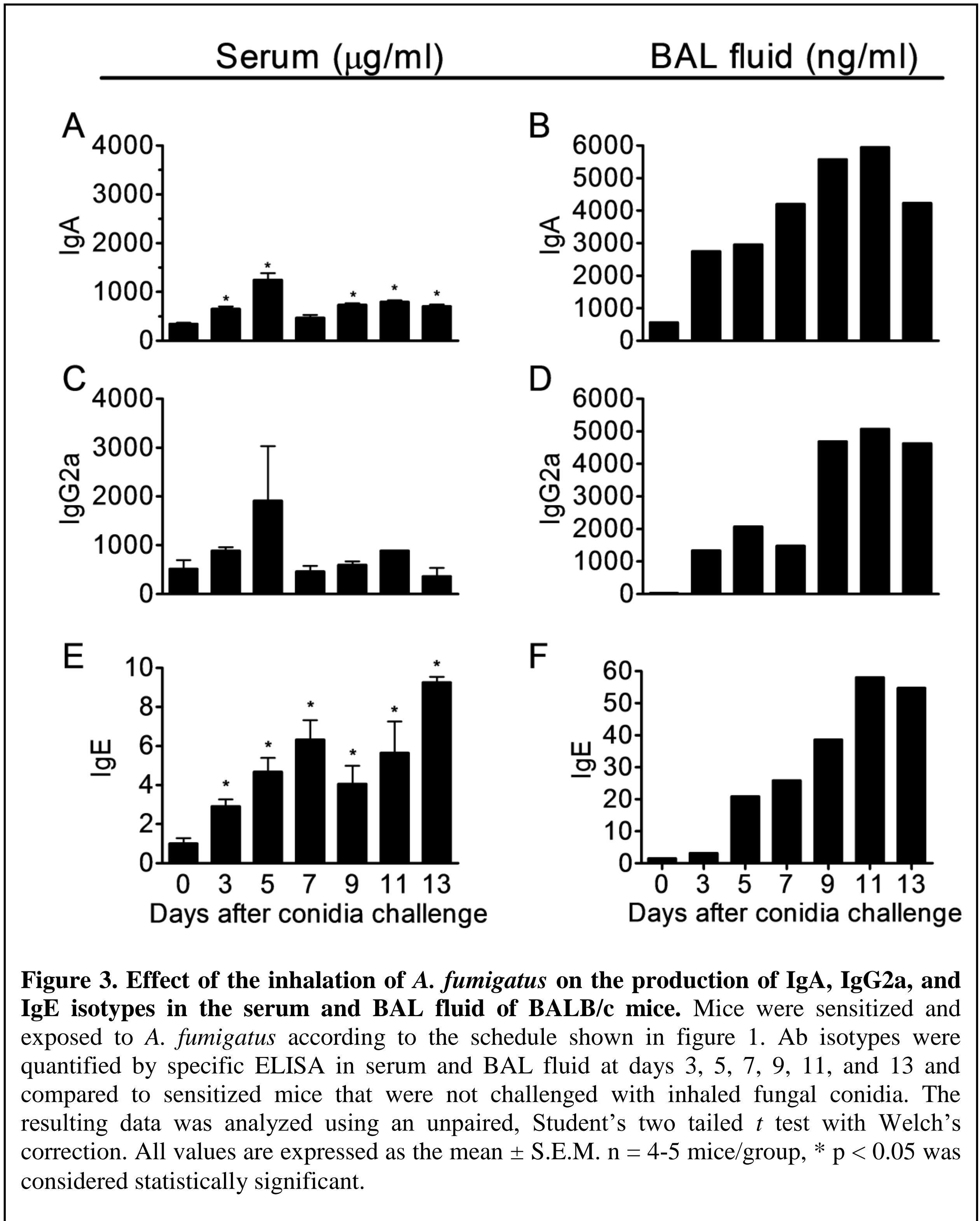

Figure 3. Effect of the inhalation of *A. fumigatus* on the production of IgA, IgG2a, and IgE isotypes in the serum and BAL fluid of BALB/c mice. Mice were sensitized and exposed to *A. fumigatus* according to the schedule shown in figure 1. Ab isotypes were quantified by specific ELISA in serum and BAL fluid at days 3, 5, 7, 9, 11, and 13 and compared to sensitized mice that were not challenged with inhaled fungal conidia. The resulting data was analyzed using an unpaired, Student's two tailed *t* test with Welch's correction. All values are expressed as the mean ± S.E.M. n = 4-5 mice/group, * $p < 0.05$ was considered statistically significant.

B lymphocytes are recruited to the sensitized lungs of BALB/c mice after conidia inhalation. Flow cytometry was used to track the recruitment of ($CD19^+CD23^+$) B lymphocytes (conventional B2 cells) (21-23) into the allergic lung as a result of the inhalation of *A. fumigatus* spores to determine if B2 cells were a potential source of isotype-switched Abs in response to inhaled fungus. Single cell suspensions of pulmonary cells were pooled at each time point and stained with anti-CD19 and anti-CD23 for the detection of B2 cells. The $CD19^+CD23^+$ B2 cells were actively recruited to the pulmonary tissues after allergen inhalation (**Fig 4**). B2 cell recruitment increased rapidly and peaked at day 5 after conidia inhalation with an average of 1.7 million B2 cells per lung counted in the sensitized and challenged animals.

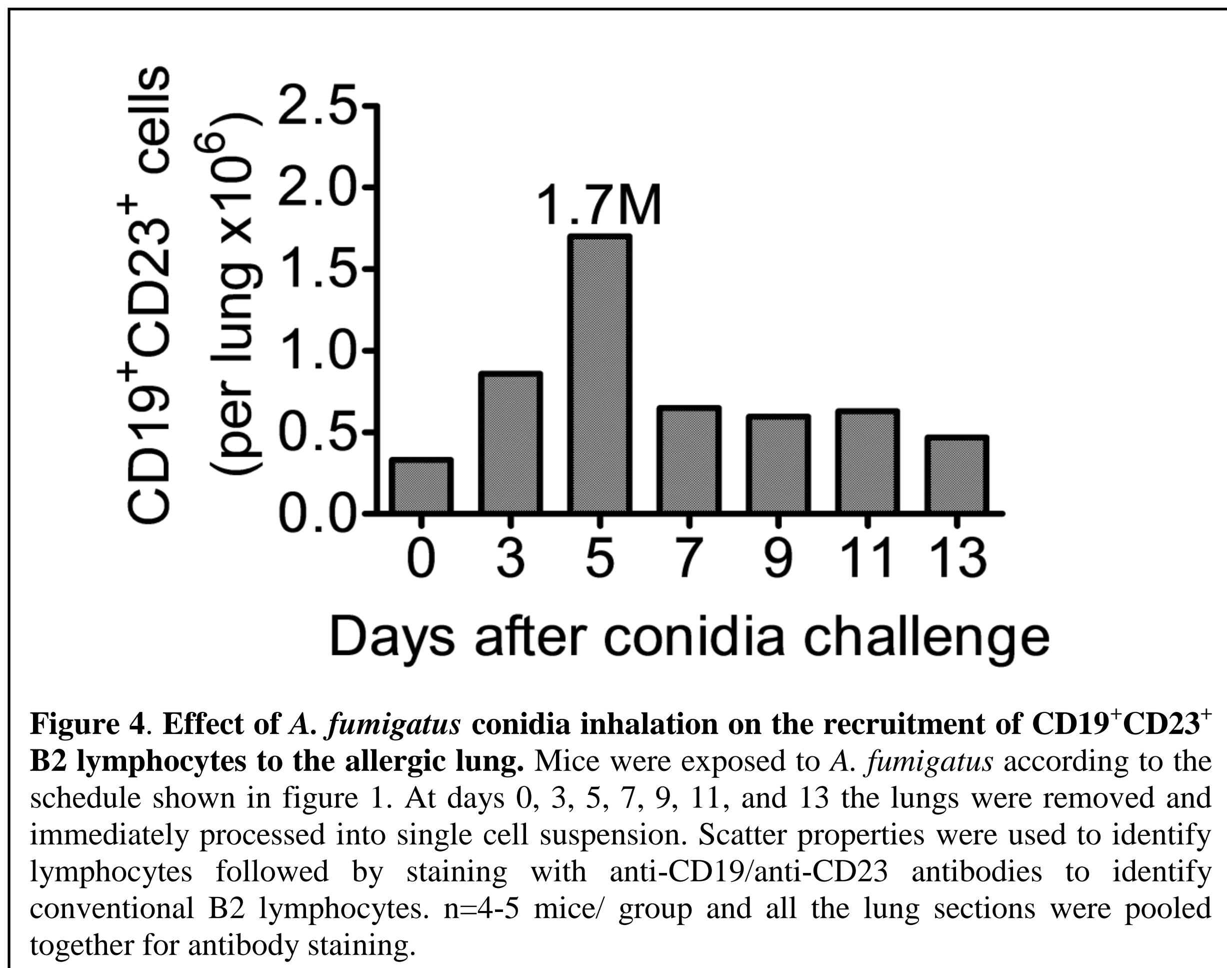

Figure 4. **Effect of *A. fumigatus* conidia inhalation on the recruitment of $CD19^+CD23^+$ B2 lymphocytes to the allergic lung.** Mice were exposed to *A. fumigatus* according to the schedule shown in figure 1. At days 0, 3, 5, 7, 9, 11, and 13 the lungs were removed and immediately processed into single cell suspension. Scatter properties were used to identify lymphocytes followed by staining with anti-CD19/anti-CD23 antibodies to identify conventional B2 lymphocytes. n=4-5 mice/ group and all the lung sections were pooled together for antibody staining.

IgA, IgG, and IgE are produced in the lungs of allergic mice after conidia inhalation. Cells expressing intracellular and extracellular IgA, IgG, and IgE were assessed by flow cytometry on pooled single cell suspensions of lung cells. IgA^+ cells constituted a relatively

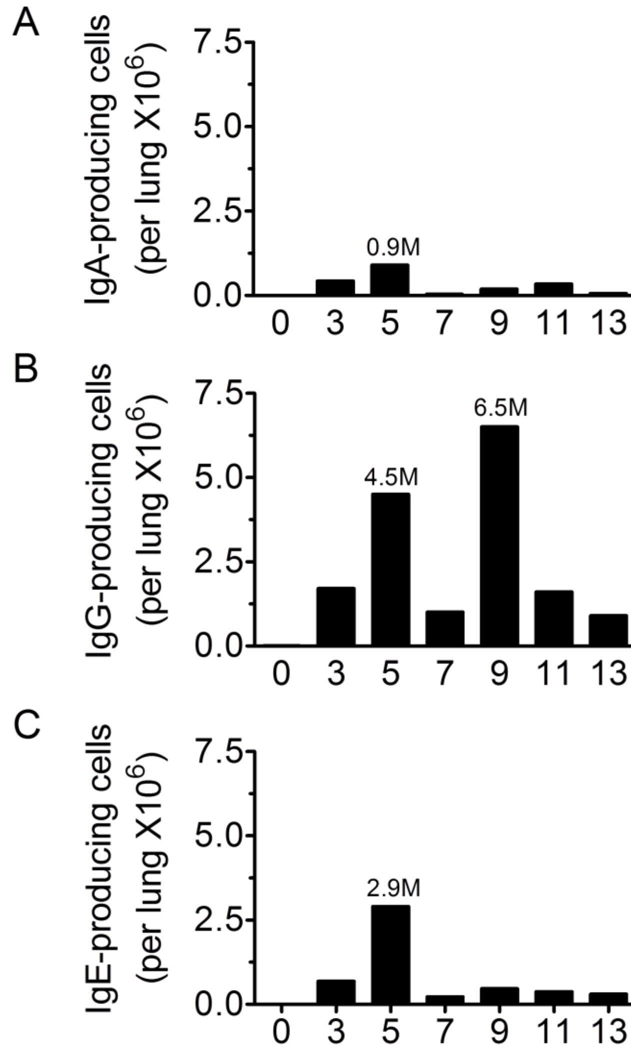

Figure 5. Effect of *A. fumigatus* conidia inhalation on Ab-producing cells in the allergic murine lung. Mice were exposed *to A. fumigatus* according to the schedule shown in figure 1. At days 0, 3, 5, 7, 9, 11, and 13 the lungs were harvested and immediately processed into a single cell suspension. Fluorescent anti-IgE, IgG and IgA antibodies were used to identify antibody producing cells. n=4-5 mice/ group and all the lung sections were pooled together for antibody staining.

minor population in the allergic lung. The largest population of IgA^+ cells was noted at day 5 after allergen challenge, but these accounted for less than 11% of the Ab-producing cells of the lung (**Fig 5A**). Resident IgA^+ cells were clearly evident around the large airways when visualized on tissue sections immunohistochemically stained with anti-IgA antibodies **(Fig 6A inset)**. IgG^+ cells made up the largest population of the three isotypes analyzed, with separate peaks at days 5 and 9 after inhalation of conidia (**Fig 5B**).

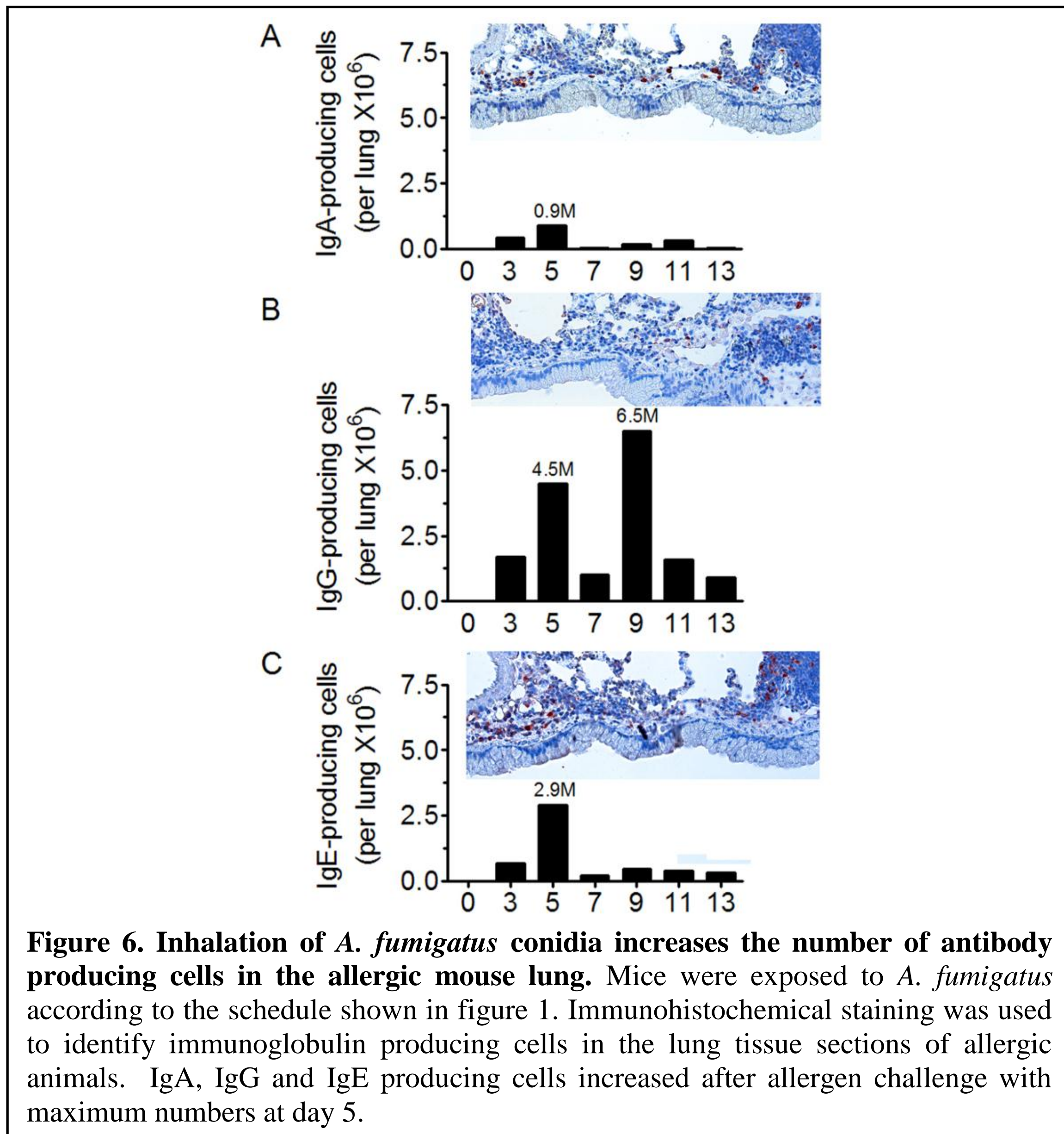

Figure 6. Inhalation of *A. fumigatus* conidia increases the number of antibody producing cells in the allergic mouse lung. Mice were exposed to *A. fumigatus* according to the schedule shown in figure 1. Immunohistochemical staining was used to identify immunoglobulin producing cells in the lung tissue sections of allergic animals. IgA, IgG and IgE producing cells increased after allergen challenge with maximum numbers at day 5.

Even though the IgG-producing cells outnumbered IgA- or IgE-producing cells, IgG^+ cells were rarely noted in the IHC-stained tissue sections at day 5 after conidia challenge (**Fig 6B inset**). IgE^+ cells were most notable in the lung 5 days after allergen challenge (**Fig 5C**) and were clearly evident in the tissues around blood vessels and large airways (**Fig 6C inset**).

Discussion

Aspergillus species are among the most well recognized and best studied fungal pathogens of humans and animals. Exposure to this species can result in a range of disease states from allergic rhinitis to invasive aspergillosis. The innate immune system functions to bar fungal infection and, when that fails, both humoral and cell-mediated adaptive responses function to eliminate fungal pathogens (24). The contribution of humoral immune responses against fungal pathogens and allergens has been the topic of debate. While it is evident that cell-mediated responses play an important role in fungal clearance (25), the relative importance of serum and BAL antibodies to fungal components has been more difficult to establish (26, 27).

B lymphocytes are the tuners of humoral immunity and they develop a specific immune response to *A. fumigatus* as they have been documented to produce *A. fumigatus* specific IgA, IgG, and IgE antibodies (28). They perform three important functions. In addition to producing antibodies, B cells present antigen to T cells to indirectly support the allergic phenotype. They can also produce cytokines to directly impact fibrosis and other airway changes (29) suggesting that these cells can play an important role in the development and maintenance of allergic disease.

In this study, we show the temporal recruitment of ($CD19^{+}CD23^{+}$) B2 lymphocytes to the allergic lung after exposure to conidia, placing these cells in a role to produce antibody isotypes that directly affect the immune response in the lung. Although not the focus of the current study, these cells could have other roles in the development and maintenance of the phenotype that is seen in the allergic lung.

The mucociliary elevator removes the majority of inhaled microbes from the surface of the pulmonary epithelium. B cells support the mucosal immune response by pumping IgA dimers of limited antigenic repertoire across the epithelium onto the luminal surface of the lung (30). In our study, IgA-producing cells were found to be localized around the large airways. This corresponds with their role as an innate defense immunoglobulin pumped across the columnar epithelium in an attempt to block fungal infection. IgA has been found to be critically important to fungal elimination and a strong immune defense against mold spores (31). The production of IgA after exposure to fungal conidia is important because it shows the dynamic activation of this cell type in response to a potential pathogen/particulate.

When the host inhales conidia in large doses or at continuous low levels, fungal sensitization resulting in an aggressive pulmonary allergic response to *Aspergillus* antigens may arise. The allergic response overlaps the innate cellular component in the sensitized individual resulting in elevated IgE, airway hyperresponsiveness, and peribronchial inflammation. As an example of this collaboration of innate and adaptive immune responses recruitment of both innate response cells and increased IgA production coincident with lymphocyte recruitment to the lung suggests a coordinated strategy for fungal elimination in the lung. Persistent T_H2-mediated responses give rise to increased peribronchial smooth muscle and collagen deposition which characterizes the airway wall remodeling that often accompanies allergic lung disease (32). In this study, increased IgA levels corresponded with increased IgE in serum and BAL. In addition, IgE-producing cells were localized near the large airways of allergic animals. This IgE production is the hallmark of allergic asthma, as it has the ability to crosslink antigen to elicit mast cell degranulation and the activation of T_H2 immune cascade. Even IgG2a, which is produced by T_H-1-associated B cells (33), was elevated throughout the time points and IgG

producing cells were present near the large airways indicating its importance in host protection against fungal growth. Nonetheless, the enhanced recruitment of $CD19^+CD23^+$ B2 cells along with increased availability of IgA, IgG2a, and IgE in the serum and BAL fluid advocates for the investigation of humoral defenses against *A. fumigatus*.

Allergic responses have been suggested as an aberrant immune response mounted against particulates that in many cases are not associated with pathogenic organisms (e.g., pollen, animal dander, and various chemicals). However, two prominent pathogens that have evolved in conjunction with their human hosts—helminthic worms and fungi—both of which are luminal pathogens that are perhaps the driving force behind a luminal immune response that has evolved as the allergic response. Eosinophilia is a hallmark of the allergic airway, and we have previously shown these cells are aptly recruited to the lungs of allergic mice after challenge with inhaled conidia (19). Contemporary research has revealed that B cells traffic to the lung in disease states (29) and that isotype switching can occur within the lung (34).

The lower airways of the normal lung are typically considered to be sterile of bacterial microbes; however, this is not the case for the compact extremely hydrophobic spores of *A. fumigatus*. These tiny spores can travel well past the ciliated epithelium into small airways of the lung where their density is ideal for deposition. Once lodged in the alveoli, their thermotolerance and permissive substrate use provides the necessary characteristics for an opportunistic pathogen. We have shown previously that inhaled *A. fumigatus* spores are capable of navigating the airways to lodge in the very small peripheral airways of the mouse (19). Many studies previously have demonstrated that T lymphocytes and T_H2 cytokines play an important role in the development of allergic lung disease and the downstream events, including inflammation, eosinophilia, mast cell accumulation/activation, and airway remodeling (35-38). However, very

little is known about the B cells which are the tuners of humoral immunity. Previous studies concerning B lymphocytes in allergic disease have concluded that B cells play no role in the development of allergic disease as characterized by eosinophilia and AHR (39, 40). Other studies have suggested that the contribution of B cells is primary via IgE (41). However, in our study we see that recruitment of $CD19^{+}CD23^{+}$ (conventional B2) cells impacts the antibody phenotype of allergic animals. It would be interesting to find the signals which might have evoked the $CD19^{+}CD23^{+}$ B2 lymphocytes to get recruited to the lungs.

B lymphocytes are active players in host defense as shown by many *in vitro* studies. While the exact mechanism by which B lymphocytes modulate the allergic response is not known yet, it is possible that these cells may act in synergy with the T lymphocytes and affect the cytokine milieu of the lungs in addition to antibody production. This is in part supported by the fact that antibody production against inhaled antigens is altered by pulmonary inflammation resulting from diverse pathogenesis (27). However, further studies are needed to determine the exact role of B lymphocytes in the induction of T_H2 cytokines, MHC II expression, and T cell activation

In conclusion, the current report shows for the first time that *A. fumigatus* sensitization and conidia challenge can recruit ($CD19^{+}CD23^{+}$) B2 lymphocytes to the allergic lung. In addition to this, we show a role of $CD19^{+}CD23^{+}$ B2 lymphocytes in immunoglobulin secretion in the context of fungal allergy. Taken as a whole this research supports the increasing body of knowledge that $CD19^{+}CD23^{+}$ B2 lymphocytes impact antibody responses in allergic asthma. Further studies analyzing the various functions of B cells using a knockout model system will shed light on the intricate cellular functions of these cells in allergic asthma and may lead to better treatment for this devastating disease.

Acknowledgements

The authors wish to thank Dr. Pawel Borowicz (North Dakota State University) and the Advanced Imaging and Microscopy Laboratory at North Dakota State University for imaging support using the Zeiss Z1 AxioObserver inverted microscope.

Contribution

The work reported in this paper has been conducted by Mr. Sumit Ghosh. Analysis of the data and manuscript preparation has been done with the help of the co-authors.

References

1. Dagenais, T. R., and N. P. Keller. 2009. Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. *Clin Microbiol Rev* 22: 447-465.
2. Latge, J. P. 1999. Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev* 12: 310-350.
3. Beck, O., M. S. Topp, U. Koehl, E. Roilides, M. Simitsopoulou, M. Hanisch, J. Sarfati, J. P. Latge, T. Klingebiel, H. Einsele, and T. Lehrnbecher. 2006. Generation of highly purified and functionally active human TH1 cells against Aspergillus fumigatus. *Blood* 107: 2562-2569.
4. Grazziutti, M. L., J. H. Rex, R. E. Cowart, E. J. Anaissie, A. Ford, and C. A. Savary. 1997. Aspergillus fumigatus conidia induce a Th1-type cytokine response. *J Infect Dis* 176: 1579-1583.
5. Traynor, T. R., and G. B. Huffnagle. 2001. Role of chemokines in fungal infections. *Med Mycol* 39: 41-50.
6. Murdock, B. J., A. B. Shreiner, R. A. McDonald, J. J. Osterholzer, E. S. White, G. B. Toews, and G. B. Huffnagle. 2011. Coevolution of TH1, TH2, and TH17 responses during repeated pulmonary exposure to Aspergillus fumigatus conidia. *Infect Immun* 79: 125-135.
7. Maurya, V., H. C. Gugnani, P. U. Sarma, T. Madan, and A. Shah. 2005. Sensitization to Aspergillus antigens and occurrence of allergic bronchopulmonary aspergillosis in patients with asthma. *Chest* 127: 1252-1259.
8. Schwartz, H. J., K. M. Citron, E. H. Chester, J. Kaimal, P. B. Barlow, G. L. Baum, and M. R. Schuyler. 1978. A comparison of the prevalence of sensitization to Aspergillus antigens among asthmatics in Cleveland and London. *J Allergy Clin Immunol* 62: 9-14.
9. Agarwal, R. 2009. Allergic bronchopulmonary aspergillosis. *Chest* 135: 805-826.
10. Lin, S. J., J. Schranz, and S. M. Teutsch. 2001. Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis* 32: 358-366.
11. Lorenz, R. G., V. J. McCracken, and C. O. Elson. 2005. Animal models of intestinal inflammation: ineffective communication between coalition members. *Springer Semin Immunopathol* 27: 233-247.
12. Samarasinghe, A. E., S. A. Hoselton, and J. M. Schuh. 2011. The absence of VPAC2 leads to aberrant antibody production in Aspergillus fumigatus sensitized and challenged mice. *Peptides* 32: 131-137.
13. Yuan, R., R. Clynes, J. Oh, J. V. Ravetch, and M. D. Scharff. 1998. Antibody-mediated modulation of Cryptococcus neoformans infection is dependent on distinct Fc receptor functions and IgG subclasses. *J Exp Med* 187: 641-648.
14. Inoue, Y., Y. Matsuwaki, S. H. Shin, J. U. Ponikau, and H. Kita. 2005. Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J Immunol* 175: 5439-5447.
15. el-Dahr, J. M., R. Fink, R. Selden, L. K. Arruda, T. A. Platts-Mills, and P. W. Heymann. 1994. Development of immune responses to Aspergillus at an early age in children with cystic fibrosis. *American journal of respiratory and critical care medicine* 150: 1513-1518.

16. Krasnick, J., P. A. Greenberger, M. Roberts, and R. Patterson. 1995. Allergic bronchopulmonary aspergillosis: serologic update for 1995. *J Clin Lab Immunol* 46: 137-142.
17. Hohl, T. M., H. L. Van Epps, A. Rivera, L. A. Morgan, P. L. Chen, M. Feldmesser, and E. G. Pamer. 2005. Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog* 1: e30.
18. Lund, F. E., M. Hollifield, K. Schuer, J. L. Lines, T. D. Randall, and B. A. Garvy. 2006. B cells are required for generation of protective effector and memory CD4 cells in response to Pneumocystis lung infection. *J Immunol* 176: 6147-6154.
19. Hoselton, S. A., A. E. Samarasinghe, J. M. Seydel, and J. M. Schuh. 2010. An inhalation model of airway allergic response to inhalation of environmental Aspergillus fumigatus conidia in sensitized BALB/c mice. *Medical mycology : official publication of the International Society for Human and Animal Mycology* 48: 1056-1065.
20. Samarasinghe, A. E., S. A. Hoselton, and J. M. Schuh. 2011. A comparison between intratracheal and inhalation delivery of Aspergillus fumigatus conidia in the development of fungal allergic asthma in C57BL/6 mice. *Fungal biology* 115: 21-29.
21. Oliveira, F. L., A. M. Aguiar, R. Borojevic, and M. C. El-Cheikh. 2005. IgE expression on the surface of B1 and B2 lymphocytes in experimental murine schistosomiasis. *Braz J Med Biol Res* 38: 1033-1042.
22. Wang, H., and S. H. Clarke. 2003. Evidence for a ligand-mediated positive selection signal in differentiation to a mature B cell. *J Immunol* 171: 6381-6388.
23. Martin, F., and J. F. Kearney. 2001. B1 cells: similarities and differences with other B cell subsets. *Current opinion in immunology* 13: 195-201.
24. Ibrahim-Granet, O., B. Philippe, H. Boleti, E. Boisvieux-Ulrich, D. Grenet, M. Stern, and J. P. Latge. 2003. Phagocytosis and intracellular fate of Aspergillus fumigatus conidia in alveolar macrophages. *Infection and immunity* 71: 891-903.
25. Cenci, E., A. Mencacci, C. Fe d'Ostiani, G. Del Sero, P. Mosci, C. Montagnoli, A. Bacci, and L. Romani. 1998. Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. *The Journal of infectious diseases* 178: 1750-1760.
26. Polonelli, L., A. Casadevall, Y. Han, F. Bernardis, T. N. Kirkland, R. C. Matthews, D. Adriani, M. Boccanera, J. P. Burnie, A. Cassone, S. Conti, J. E. Cutler, R. Frazzi, C. Gregory, S. Hodgetts, C. Illidge, W. Magliani, G. Rigg, and G. Santoni. 2000. The efficacy of acquired humoral and cellular immunity in the prevention and therapy of experimental fungal infections. *Medical mycology : official publication of the International Society for Human and Animal Mycology* 38 Suppl 1: 281-292.
27. Yoshizawa, Y., Y. Ohtani, T. Inoue, S. Miyake, A. Ikeda, M. Tanba, and V. P. Kurup. 1995. Immune responsiveness to inhaled antigens: local antibody production in the respiratory tract in health and lung diseases. *Clin Exp Immunol* 100: 395-400.
28. Walker, C. A., P. Fitzharris, J. L. Longbottom, and A. J. Taylor. 1989. Lymphocyte sensitization to Aspergillus fumigatus in allergic bronchopulmonary aspergillosis. *Clin Exp Immunol* 76: 34-40.
29. Lindell, D. M., A. A. Berlin, M. A. Schaller, and N. W. Lukacs. 2008. B cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. *PLoS One* 3: e3129.

30. Macpherson, A. J., K. D. McCoy, F. E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. *Mucosal Immunol* 1: 11-22.
31. Blanco, J. L., and M. E. Garcia. 2008. Immune response to fungal infections. *Veterinary immunology and immunopathology* 125: 47-70.
32. Rapaka, R. R., and J. K. Kolls. 2009. Pathogenesis of allergic bronchopulmonary aspergillosis in cystic fibrosis: current understanding and future directions. *Med Mycol* 47 Suppl 1: S331-337.
33. Mukherjee, S., S. C. Lee, and A. Casadevall. 1995. Antibodies to Cryptococcus neoformans glucuronoxylomannan enhance antifungal activity of murine macrophages. *Infection and immunity* 63: 573-579.
34. Snow, R. E., R. Djukanovic, and F. K. Stevenson. 1999. Analysis of immunoglobulin E VH transcripts in a bronchial biopsy of an asthmatic patient confirms bias towards VH5, and indicates local clonal expansion, somatic mutation and isotype switch events. *Immunology* 98: 646-651.
35. Blease, K., C. Jakubzick, J. Westwick, N. Lukacs, S. L. Kunkel, and C. M. Hogaboam. 2001. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J Immunol* 166: 5219-5224.
36. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263.
37. Kuperman, D. A., X. Huang, L. L. Koth, G. H. Chang, G. M. Dolganov, Z. Zhu, J. A. Elias, D. Sheppard, and D. J. Erle. 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8: 885-889.
38. Larche, M., D. S. Robinson, and A. B. Kay. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 111: 450-463; quiz 464.
39. Korsgren, M., J. S. Erjefalt, O. Korsgren, F. Sundler, and C. G. Persson. 1997. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 185: 885-892.
40. Hamelmann, E., K. Takeda, J. Schwarze, A. T. Vella, C. G. Irvin, and E. W. Gelfand. 1999. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am J Respir Cell Mol Biol* 21: 480-489.
41. Oshiba, A., E. Hamelmann, K. Takeda, K. L. Bradley, J. E. Loader, G. L. Larsen, and E. W. Gelfand. 1996. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J Clin Invest* 97: 1398-1408.

PAPER 2. μ-CHAIN-DEFICIENT MICE POSSESS B1 CELLS AND PRODUCE IgG AND IgE, BUT NOT IgA, FOLLOWING SYSTEMIC SENSITIZATION AND INHALATIONAL CHALLENGE IN A FUNGAL ASTHMA MODEL

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Published: August 1st, 2012, doi:10.4049/jimmunol.1200138

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Abstract

Allergic Bronchopulmonary Aspergillosis (ABPA) is often difficult to treat and results in morbidity associated with chronic airway changes. This study assessed the requirement for B cells and their products in the allergic pulmonary phenotype in a murine model of fungal allergic asthma that mimics ABPA. C57BL/6 and μMT mice (assumed to lack peripheral B cells), were sensitized with *Aspergillus fumigatus* extract and challenged with two inhalation exposures of live conidia to induce airway disease. Airway hyperresponsiveness after methacholine challenge, peribronchovascular inflammation, goblet cell metaplasia, and fibrotic remodeling of the airways was similar between μMT mice and their wild type counterparts (C57BL/6). Surprisingly, even in the absence of the μ-chain, these μMT mice produced IgE and IgG antibodies, although the antibodies induced did not have specificity for *A. fumigatus* antigens. In contrast, IgA was not detected in either the lavage fluid or serum of μMT mice that had been exposed to *A. fumigatus*. Our findings also reveal the existence of $CD19^{+}CD9^{+}IgD^{+}$ B-1 cells in the lungs of the μMT animals. These data show the μMT mice to have a developmental pathway independent of the canonical μ-chain route that allows for their survival upon antigenic challenge with *A. fumigatus* conidia, although this pathway does not seem to allow for the normal development of antigen-specific repertoires. Additionally, the study shows that IgA is required for neither clearance nor containment of *A. fumigatus* in the murine lung, since fungal outgrowth was not observed in the μMT animals after multiple inhalation exposures to live conidia.

Introduction

Allergic asthma is characterized by reversible airway obstruction due to the recruitment of leukocytes to the lung in response to an inhaled allergen (1). Increased mucus production in the airways, smooth muscle mass around the large airways, and peribronchial collagen deposition further narrows the airway lumen and restricts normal airway compliance contributing to airway obstruction (2-6). Sensitization to fungi with production of IgE and/or colonization by fungal species often signals a disease course that is particularly difficult to treat and results in chronic architecture changes in the lung causing long-term morbidity (7).

Aspergillus fumigatus has a number of characteristics that make it an ideal aeroallergen and opportunistic pathogen of humans. Its small conidia are ubiquitous in indoor and outdoor environments and can remain airborne for long periods of time (8). The size and shape of the conidia are such that they may be inhaled deep into the lung tissue, past the mucociliary elevator that clears many particulates from the airways (9). Holding an environmental niche as a carbon and nitrogen recycler in compost piles, it can take advantage of a wide range of substrates and can grow at the high internal body temperature that discourages most fungal species (10).

Resident plasma cells have been observed in the lungs of both human asthma sufferers and mice under experimental allergic airways protocols (11). Secretory IgA is recognized as an integral part of the innate mucosal response that protects the upper respiratory tract (12-14), and selective IgA deficiency in clinic patients is associated with an increased prevalence of atopy (15, 16). The IgG subtype IgG1, which is a T_H2-elicited antibody, is cytophillic to mast cells (17); and IgG2a, which is produced by T_H1-activated B cells, plays a role in host protection against fungal growth (18). As instigators of humoral immunity, B lymphocytes provide specificity to allergens in the production of IgE Abs that enable mast cell degranulation (19). IgE

has long been recognized as a perpetrator of asthma exacerbations, and anti-IgE therapies have been used successfully for treatment (20-22). During asthma exacerbations, B cells in all stages of activation and differentiation are found in increased numbers in the blood of asthmatic patients (23). B cells are also present in the bronchial mucosa of asthmatics (24). While allergen-specific antibodies (Abs) are recognized as contributing factors in the immunopathology of an aberrant response against an innocuous allergen such as pollen or animal dander (25), they have also been suggested to be part of the successful clearance of fungus from the airways (26, 27).

The aim of the current study was to determine the extent to which B cells impact the development and maintenance of the phenotype of the allergic lung. We used mice that, due to a homozygous targeted disruption of the membrane exon of the Ig μ-chain, are deficient of peripheral B cells, known as μMT (28). Using an *Aspergillus fumigatus* murine inhalation model developed in our laboratory to mimic human fungal asthma (29), we compared the effects of repeated *A. fumigatus* inhalation in C57BL/6 wild type controls and μMT animals. We found that the absence of the μ-chain did not alter the pulmonary pathology that results from inhalation of *A. fumigatus* in allergic animals: AHR, peribronchial inflammation, epithelial changes, and collagen deposition were equivalent to wild type controls. Surprisingly, we found that repeated *A. fumigatus* conidia exposure resulted in elevated IgE, IgG1 (in bronchoalveolar lavage, BAL), and IgG2a production in sensitized μMT mice, although IgA was undetectable in the μMT animals. This has implications both for the role of the B cell in the allergic lung and for IgA in the response to fungal allergens. We report for the first time that, even in the absence of the immunoglobulin μ-chain, IgG1 (only in BAL), IgG2a, and IgE isotypes were produced in animals after exposure to fungal antigens, but IgA was not made. The Abs produced after fungal exposure showed no antigen specificity for *A. fumigatus*. Our findings also reveal the previously

unreported presence of B-1 cells ($CD19^{+}CD9^{+}IgD^{+}$) in the lungs of μMT mice, even in the complete absence of B-2 cells. Taken together, we show for the first time that μMT mice have B-1 cells in the lungs and that these animals produce selected isotypes through a μ-independent pathway in the context of the fungal allergen-exposed lung.

Materials and Methods

Experimental animals. C57BL/6 and μMT mice (5-9 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed on Alpha-dri^{TM} paper bedding (Shepherd Specialty Papers, Watertown, TN, USA) in micro filter-topped cages (Ancare, Bellmore, NY, USA) in a specific pathogen-free facility with *ad libitum* access to food and water. The study described was performed in accordance with the Office of Laboratory Animal Welfare guidelines and was approved by the North Dakota State University Institutional Animal Care and Use Committee.

Antigen preparation and A. fumigatus *culture.* Soluble *A. fumigatus* antigen extract was purchased from Greer Laboratories (Lenoir, NC, USA) and fungal culture stock (strain NIH 5233) was purchased from American Type Culture Collection (Manassas, VA, USA). The *A. fumigatus* culture was reconstituted in 5ml PBS, and 60-μl aliquots were stored at 4°C until use. All experiments that utilized *A. fumigatus* were conducted with prior approval of the Institutional Biological Safety Committee of North Dakota State University.

Allergen sensitization and challenge by airborne delivery system. Animals were sensitized per Hogaboam's published protocol (30), with the exception that alum was used as the adjuvant. Mice were sensitized globally with 10 μg of *A. fumigatus* antigen (Greer Laboratories) in 0.1 ml normal saline (NS) mixed with 0.1 ml of Imject Alum (Pierce, Rockford, IL, USA) which was injected subcutaneously (0.1 ml) and intraperitoneally (0.1ml). After two weeks, mice were given a series of three intranasal, weekly 20-μg doses of *A. fumigatus* antigen in 20 μl of NS. Animals were challenged as previously described with a 10-min nose-only aerosol exposure to live *A. fumigatus* conidia (29). Each anesthetized mouse was placed supine with its nose in an inoculation port inhaling the live fungal conidia for 10 min. Two weeks after the first allergen

challenge, mice were subjected to a second 10-min aerosol fungal challenge. Naïve animals from both the groups were neither sensitized nor challenged. After the second allergen exposure, the mice were separated into groups of five for analysis at day 0 (sensitized, but not challenged) or days 7 or 28 after the second aerosol challenge. Day 7 after challenge had been previously determined to be the peak of B cell recruitment into the allergic lungs, and leukocyte inflammation was assessed at this time point. Airway wall remodeling can be seen as early as 7 days after the second aerosol challenge in this model, and the changes to the lung architecture continue to accrue through at least day 28 after the second inhalation of fungal conidia. The day-28 time point was chosen to assess epithelial changes, as well as peribronchial fibrosis. The experimental protocol is depicted in Figure 7.

Airway hyperresponsiveness measurement. Mice were anesthetized using sodium pentobarbital (Butler, Columbus OH; 0.1mg/0.01kg of mouse body weight), intubated, and ventilated with a Harvard pump ventilator (Harvard Apparatus, Reno, NV, USA) to assess allergic airway responses. Restrained plethysmography (Buxco, Troy, NY, USA) was used to assess airway hyperresponsiveness. Before performing readings, the system was first calibrated and the stroke volume set at 225 with the stroke/min set at 150. The value for baseline airway resistance was measured for each animal before an intravenous injection of acetyl-β-methacholine (420μg/kg) was administered to determine AHR at each time point.

Sample collection. Approximately 500 μl of blood was removed from each mouse via ocular bleed and centrifuged at 13,000 x*g* for 10 min to yield serum. Serum was stored at -20°C until use. Bronchoalveolar lavage (BAL) was performed on each mouse with 1.0 ml sterile normal saline (NS). The BAL contents were centrifuged at 2000 x*g* for 10 min to separate cells from fluid. The BAL fluid was stored at -20°C until use, and cells were used immediately for

morphometric analysis. Left lungs were harvested and fixed in 10% neutral buffered formalin for histological analysis.

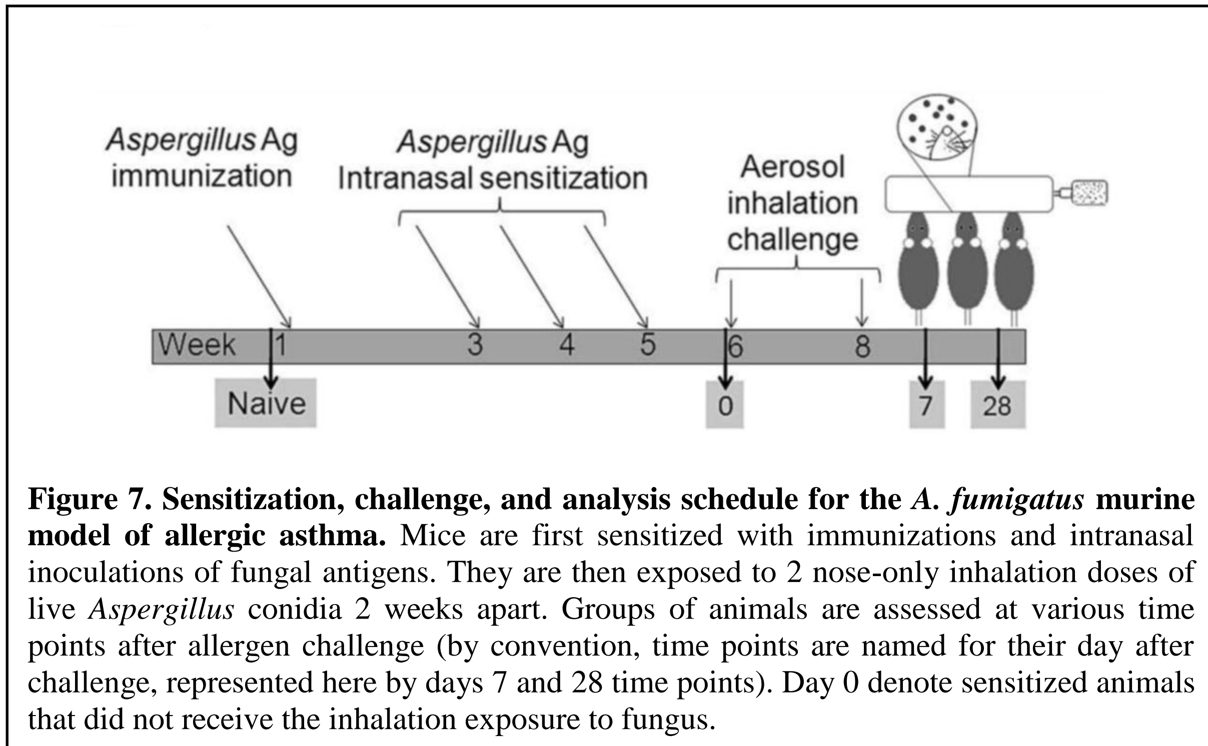

Figure 7. Sensitization, challenge, and analysis schedule for the *A. fumigatus* murine model of allergic asthma. Mice are first sensitized with immunizations and intranasal inoculations of fungal antigens. They are then exposed to 2 nose-only inhalation doses of live *Aspergillus* conidia 2 weeks apart. Groups of animals are assessed at various time points after allergen challenge (by convention, time points are named for their day after challenge, represented here by days 7 and 28 time points). Day 0 denote sensitized animals that did not receive the inhalation exposure to fungus.

Morphometric and histological analysis. BAL cells were cytospun (Shandon Scientific, Runcorn, UK) onto microscope slides and differentially stained (Quick-Dip stain, Mercedes Medical, Sarasota, FL, USA). Cells from five, random high-powered fields (HPFs) were counted to determine the mean number of each cell type per HPF in the airway lumen of each mouse.

Formalin-fixed, paraffin-embedded lungs were cut longitudinally across the coronal plane in 5-µm sections and stained with hematoxylin and eosin (H&E) to assess inflammation and with periodic acid Schiff's (PAS) stain (Richard-Allan Scientific, Kalamazoo, MI, USA) for the analysis of goblet cells.

Evaluation of collagen thickness. Gomori's trichrome (Richard-Allan Scientific, Kalamazoo, MI, USA) was used to stain histological sections to assess collagen deposition in naïve and allergic mice as described previously by Hoselton *et al.* (29). For each sample, at least

50 discrete points were measured at 50-μm 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. The mean collagen thickness was reported for each sample, and the mean of the means was reported for each group.

Quantification of serum and BAL IgE, IgG1, IgG2a, and IgA. The total IgE (BD OptEIA, San Diego, CA, USA), IgG1 (Immunology Consultants Laboratory, Portland, OR, USA), IgG2a (BD OptEIA, San Diego, CA, USA), and IgA (Bethyl laboratories, TX, USA) in serum and BAL were quantified via specific ELISA according to manufacturer's guidelines. Serum samples were diluted in PBS 1:100 for IgE, 1:500 and 1:5000 for IgG1 and IgG2a, and 1:500 for IgA. BAL samples were pooled and diluted 1:5 for IgE and IgG2a, 1:2 for IgG1, or undiluted for IgA. The detection limits for the kits were1.6 ng/ml for IgE, 6.25 ng/ml for IgG1, 3.1 ng/ml for IgG2a, and 15.625 ng/ml for IgA.

A. fumigatus-*specific antibody detection.* ELISA plates were coated with 100 μl/well of a 20-μg/ml sample of *A. fumigatus* antigen (Greer Laboratories) diluted in coating buffer (pH 9.6, 15 mM Na_2CO_3, and 35 mM $NaHCO_3$) and incubated overnight at 4°C. The next day, the plates were washed three times with PBS containing 0.05% tween-20, and 200 μl of blocker (3% BSA in coating buffer) was added to each well. Plates were incubated in the dark for 2 h at room temperature and washed 3 times with PBS-tween. After blocking, 100 μl/well of serially diluted serum or BAL from C57BL/6 and μMT mice diluted in PBS-tween/1% BSA (10^{-1} to 10^{-8} for serum and 1:2 to 1:64 for BAL fluid) was added to each well and incubated for 1 h. Plates were washed 5 times with PBS-tween, and 100 μl/well of diluted goat anti-mouse Ig-HRP (Southern Biotech, Birmingham, AL, USA) secondary antibody was added. Following a 1-h incubation, the

plates were again washed 5 times, and 100 µl per well of TMB substrate (BD Biosciences, San Jose, CA, USA) was added. The absorbance was read at 650 nm using a Synergy HT microplate reader (BioTek, Winooski, VT, USA). In addition, serum and BAL samples were tested to check the specificity of individual subclasses of antibody (IgG1 and IgE) for *A. fumigatus*. For this, rat anti-mouse IgG1-AKP (1:5000 dilution, BD Pharmingen, San Jose, CA, USA) and goat anti-mouse IgE-HRP (1:500 dilution, Thermo Scientific, Rochester, NY, USA) secondary Abs were used in place of Ig-HRP. The absorbance was read at 650 nm when TMB was used as a substrate and at 405 nm when p-Nitrophenyl phosphate disodium salt hexahydrate was used as a substrate (Sigma-Aldrich Corp., St. Louis, MO, USA) using a Synergy HT microplate reader.

Flow cytometry. Minced lungs from naïve animals and at days 0 (sensitized, but not challenged) and 7 were subjected to collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) digestion and red blood cell lysis. For collagenase digestion, minced lung sections were treated with 0.04% collagenase IV in DMEM and were incubated at 37°C for 1 h with gentle agitation. For flow cytometry analysis, the cells were suspended in PBS with 1% BSA (Sigma Aldrich, St. Louis, MO, USA) to a final concentration of 1×10^7 cells/ml. Fc receptors were blocked with anti-mouse CD16/CD32 (1 µg /1×10^6 cells) for 10 min on ice. The following Abs were used for phenotypic characterization of B lymphocytes using flow cytometry: PerCP-Cy5.5-Anti-CD19, PE-anti-CD23, Alexa flour 647-Anti-CD9, FITC-anti-IgD, FITC-anti-IgM, FITC-anti-IgG, FITC-anti-IgE, FITC-anti-IgA, (all Abs were purchased from eBiosciences, San Diego, CA, USA). The samples were pre-incubated with combinations of directly labeled Abs for 30 min in the dark and then washed with PBS 1% BSA twice before the samples were analyzed using an Accuri® C6 Flow Cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA) or a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). A minimum of 50,000 events were

acquired and the data was analyzed using Flowjo™ software (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis. Allergic C57BL/6 wild type and μMT animals were compared to each other and to their respective naïve controls at each time point. An unpaired, Student's two tailed *t* test with Welch's correction was used to determine statistical significance with Prism Graph Pad software (San Diego, CA, USA). p=0.01-0.05 (*), p = 0.001-0.01(**), and p <0.001 (***) indicates statistical difference when each of the mouse strains were compared to their naïve controls. Where appropriate, # indicates statistical difference between the C57BL/6 and μMT mice. $p < 0.05$. All results are expressed as the mean ± SEM.

Results

Airborne fungal challenge results in airway hyperresponsiveness in µMT mice after sensitization to A. fumigatus. In the present study, airway physiology of both murine groups (i.e. C57BL/6 and µMT animals) was monitored before allergen challenge at day 0 and at days 7 and 28 post second conidia inhalation **(Fig 8)**. Airway response measurements from all study animals were used to determine the baseline mean for airway hyperresponsiveness prior to methacholine

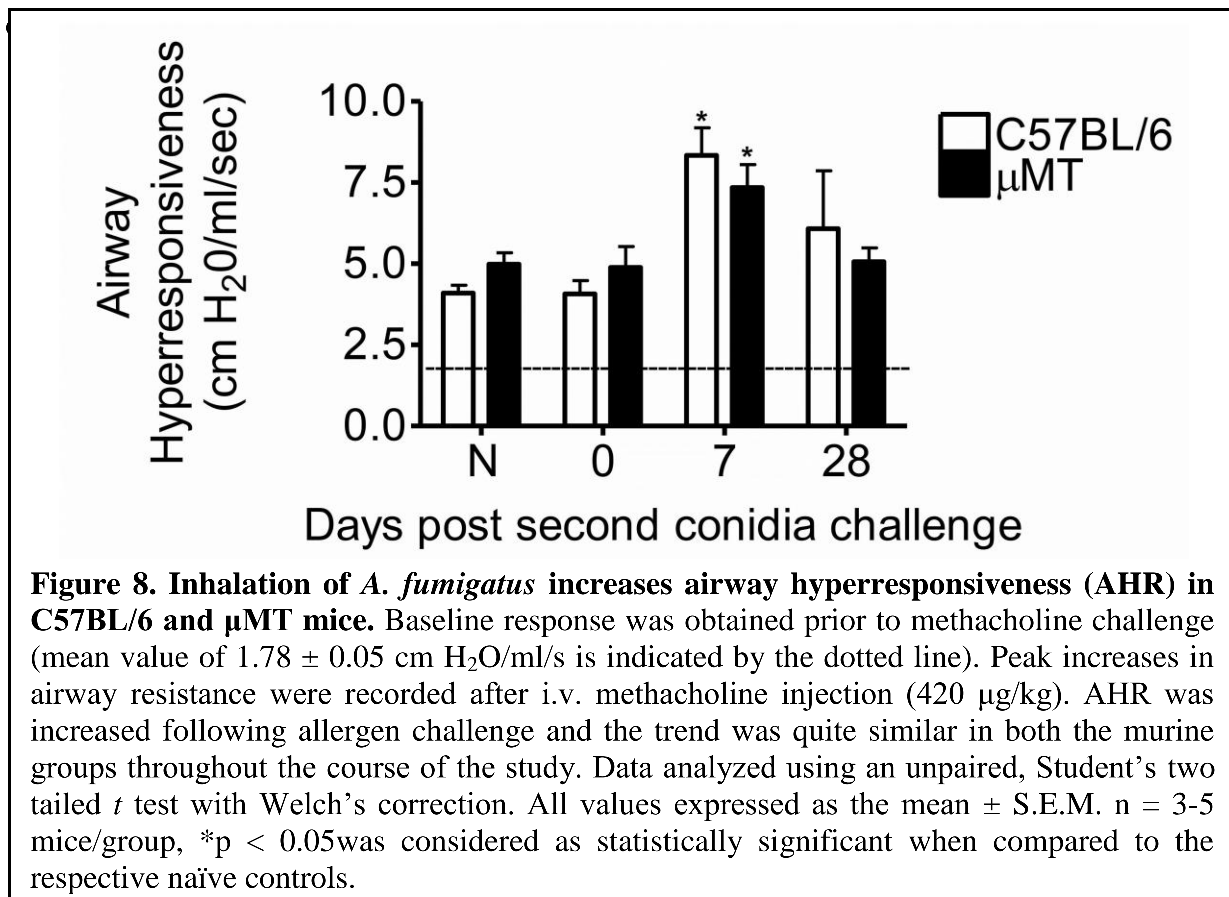

Figure 8. Inhalation of *A. fumigatus* increases airway hyperresponsiveness (AHR) in C57BL/6 and µMT mice. Baseline response was obtained prior to methacholine challenge (mean value of 1.78 ± 0.05 cm H_2O/ml/s is indicated by the dotted line). Peak increases in airway resistance were recorded after i.v. methacholine injection (420 µg/kg). AHR was increased following allergen challenge and the trend was quite similar in both the murine groups throughout the course of the study. Data analyzed using an unpaired, Student's two tailed *t* test with Welch's correction. All values expressed as the mean ± S.E.M. n = 3-5 mice/group, *p < 0.05was considered as statistically significant when compared to the respective naïve controls.

Sensitized animals from wild type and µMT groups that were not challenged with spore inhalation showed no difference in airway resistance values when compared to naïve animals in their respective groups. At day 7 after 2 conidia challenges, airway hyperresponsiveness was significantly increased in both murine groups as compared to naive controls **(Fig 8)**; however, there was no difference in the AHR values of µMT animals as compared to the C57BL/6 wild

type animals. By day 28 after the second conidia challenge, AHR values for both murine groups returned to baseline, irrespective of the presence or absence of the μ-gene.

Leukocytes are recruited to the allergic airways after fungal conidia challenge in μMT mice. Leukocyte recruitment to the lungs of allergen-sensitized animals that had inhaled conidia was evaluated using H&E-stained lung sections and morphometric analysis of BAL cells. Naïve animals from both groups exhibited no pulmonary inflammation **(Fig 9A & B)**. Similarly, sensitized C57BL/6 and μMT animals that did not inhale spores (day 0) showed no evidence of inflammation **(Fig 9C & D, respectively)**. However, upon allergen challenge, both C57BL/6 and μMT animals actively recruited inflammatory cells to the lungs. Allergic animals exhibited prominent perivascular and peribronchial leukocyte inflammation 7 days after the second spore challenge **(Fig 9E & F)**. The pattern of perivascular and peribronchial inflammation was similar in the C57BL/6 and μMT animals at day 7, and inflammation was largely resolved in both strains by day 28 post challenge **(Fig 9G & H)**.

Morphometric analysis of monocyte/macrophage lineage cells, neutrophils, eosinophils, and lymphocytes was performed to estimate the relative makeup of the cellular inflammation and to monitor leukocyte egress into the airway lumen **(Fig 10)**. In naïve and sensitized animals that were not challenged (day 0), alveolar macrophages were the dominant cell type **(Fig 10A)**. Neutrophils, lymphocytes, and particularly eosinophils were prominent cell types identified in the BAL 7 days after the second conidia challenge **(Fig 10B, C, & D)**. Eosinophils were the most numerous cell type counted **(Fig 10C)** in the BAL of both C57BL/6 and μMT mice at day 7 after the second conidia exposure, emphasizing the polarization of the immune response in favor of allergy after multiple inhalations of conidia.

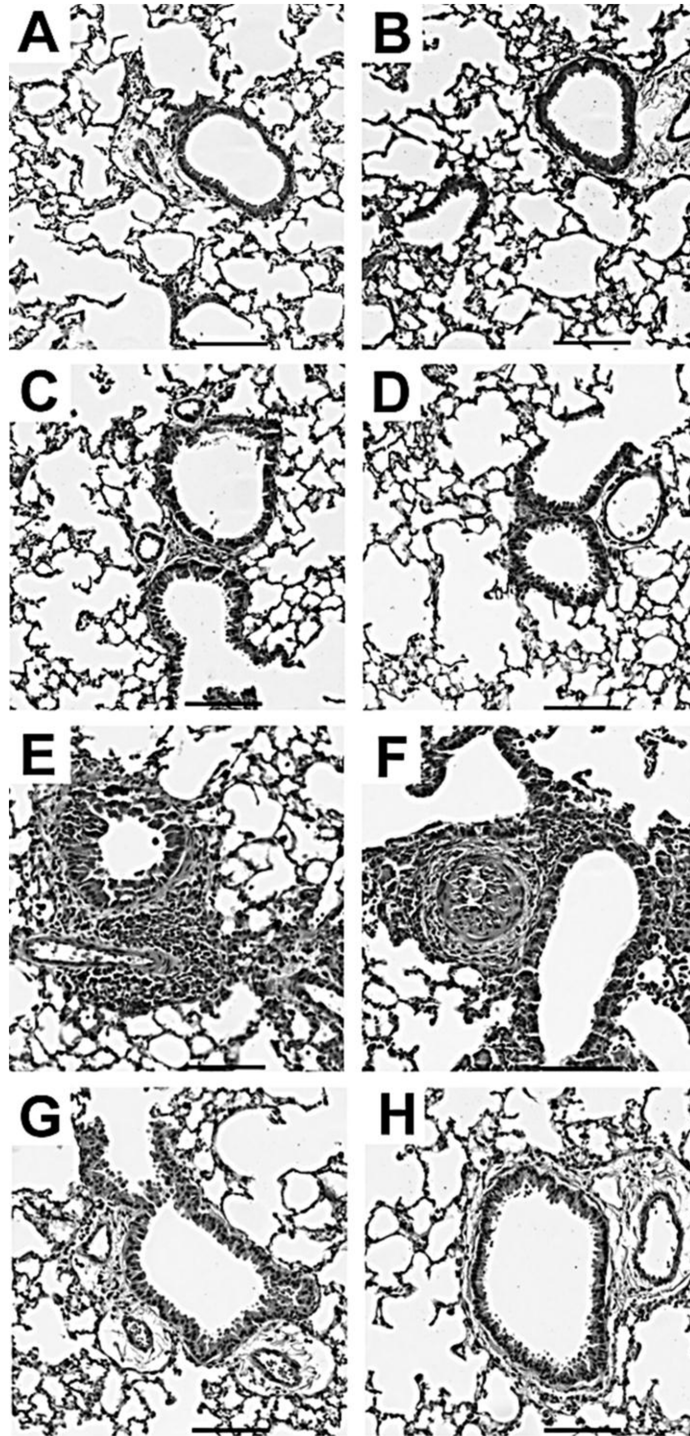

Figure 9. **Inhalation of *A. fumigatus* conidia increases pulmonary inflammation in C57BL/6 and µMT mice.** Representative photomicrographs of H&E stained lung sections of allergen-challenged C57BL/6 (left column) and µMT (right column) mice. Naïve and day 0 mice in both the groups did not show inflammation (Fig. 3A-D). Peribronchovascular inflammation was prominent at day 7 post second conidia challenge in both groups (Fig. 3E&F) and subsided well into day 28 (Fig.3G&H). Scale Bar = 100µm.

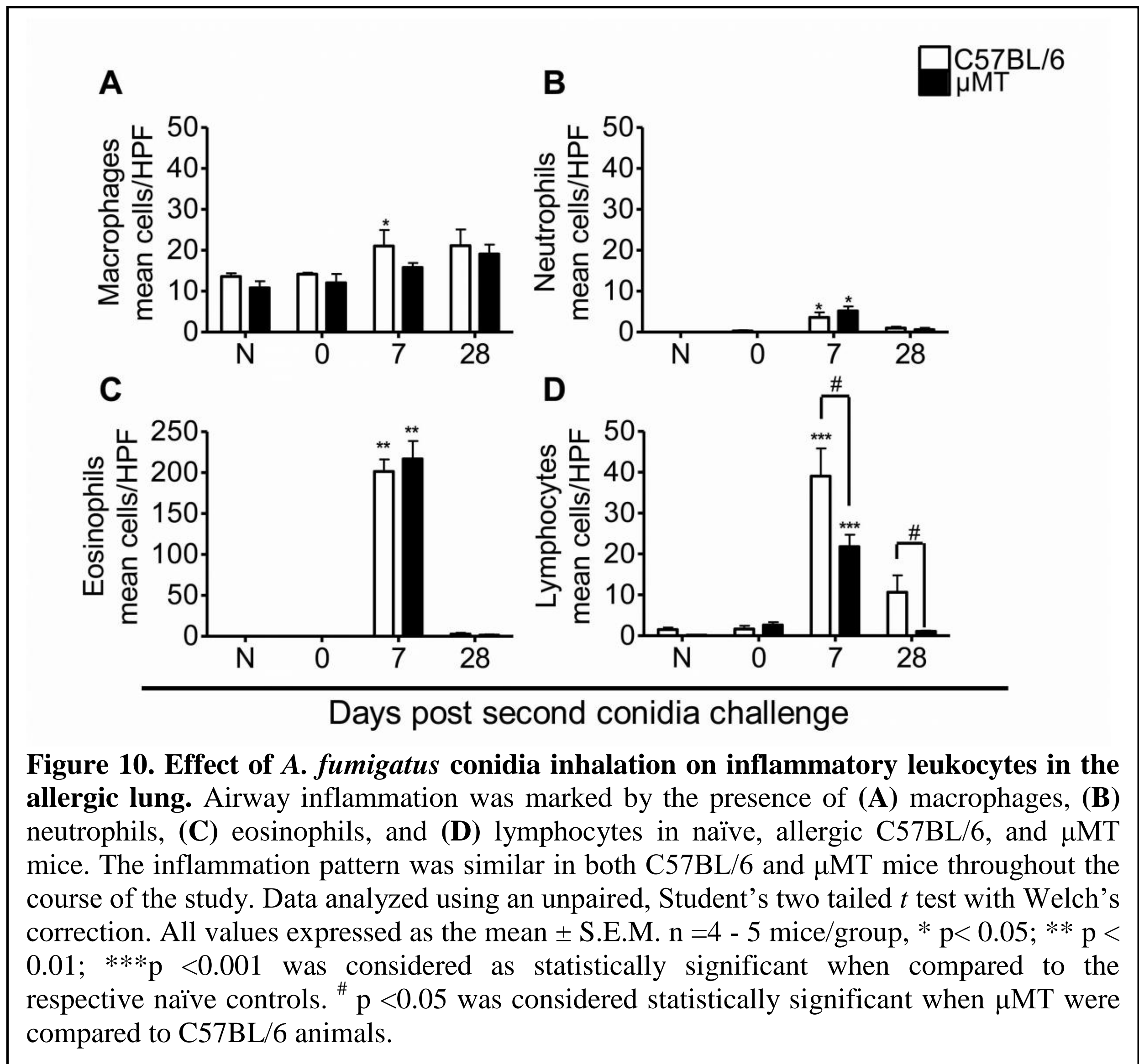

Figure 10. Effect of *A. fumigatus* conidia inhalation on inflammatory leukocytes in the allergic lung. Airway inflammation was marked by the presence of **(A)** macrophages, **(B)** neutrophils, **(C)** eosinophils, and **(D)** lymphocytes in naïve, allergic C57BL/6, and µMT mice. The inflammation pattern was similar in both C57BL/6 and µMT mice throughout the course of the study. Data analyzed using an unpaired, Student's two tailed *t* test with Welch's correction. All values expressed as the mean ± S.E.M. n =4 - 5 mice/group, * $p< 0.05$; ** $p < 0.01$; ***$p <0.001$ was considered as statistically significant when compared to the respective naïve controls. $^{\#}$ $p <0.05$ was considered statistically significant when µMT were compared to C57BL/6 animals.

At day 28 post challenge, macrophages were again the major cellular component of the BAL compartment with very few neutrophils **(Fig 10A & B)**.The inflammation pattern was similar between C57BL/6 and µMT animals, with eosinophils dominating at day 7 in both murine groups when they were compared to their naïve controls.

Inhalation of fungal conidia changes the airway architecture in allergic C57BL/6 mice and allergic µMT mice. In the present study, goblet cells were assessed by counting PAS-stained cells and representing them as a percentage of total epithelial cells lining the second or third

lateral airways in each histological section. Goblet cell metaplasia was not observed in the day 0 (sensitized, but not challenged) animals of either the C57BL/6 or μMT groups **(Fig 11C)**. Challenge with *A. fumigatus* conidia resulted in a marked increase in the percentage of goblet cells lining the airways **(Fig 11C)**. As compared to sensitized animals that did not receive inhaled conidia, the number of goblet cells was increased dramatically (~65% of total) but equally in both groups 7 days post challenge **(Fig 11A, B, & C)**.

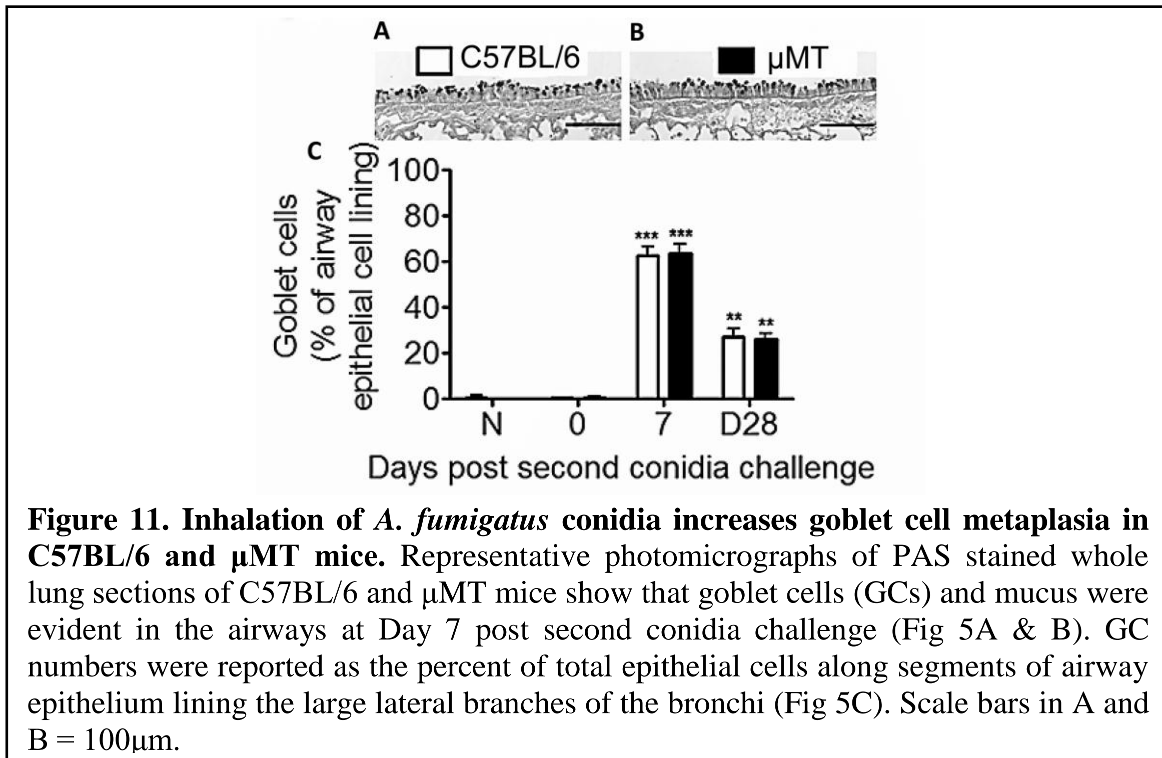

Figure 11. Inhalation of *A. fumigatus* conidia increases goblet cell metaplasia in C57BL/6 and μMT mice. Representative photomicrographs of PAS stained whole lung sections of C57BL/6 and μMT mice show that goblet cells (GCs) and mucus were evident in the airways at Day 7 post second conidia challenge (Fig 5A & B). GC numbers were reported as the percent of total epithelial cells along segments of airway epithelium lining the large lateral branches of the bronchi (Fig 5C). Scale bars in A and B = 100μm.

By day 28 after the second conidia challenge, fewer goblet cells were noted in the allergic lungs of both the C57BL/6 and the μMT group as compared to the day-7 time point, although there was no difference in the number of goblet cells between the wild type and the μMT groups (**Fig 11C,** ~27% of the total epithelial cells for each). Collagen accumulation in the peribronchial space of allergic C57BL/6 or μMT animals was significantly increased at both day 7 and day 28 after the second conidia inhalation as compared to sensitized animals that had not been exposed to inhaled conidia **(Fig 12)**. In contrast to the pattern seen in goblet cell metaplasia, collagen

accumulation did not diminish over the time course of this study. This phenomena has been seen and extended in other studies by our laboratory in both BALB/c and C57BL/6 mice (29, 31).

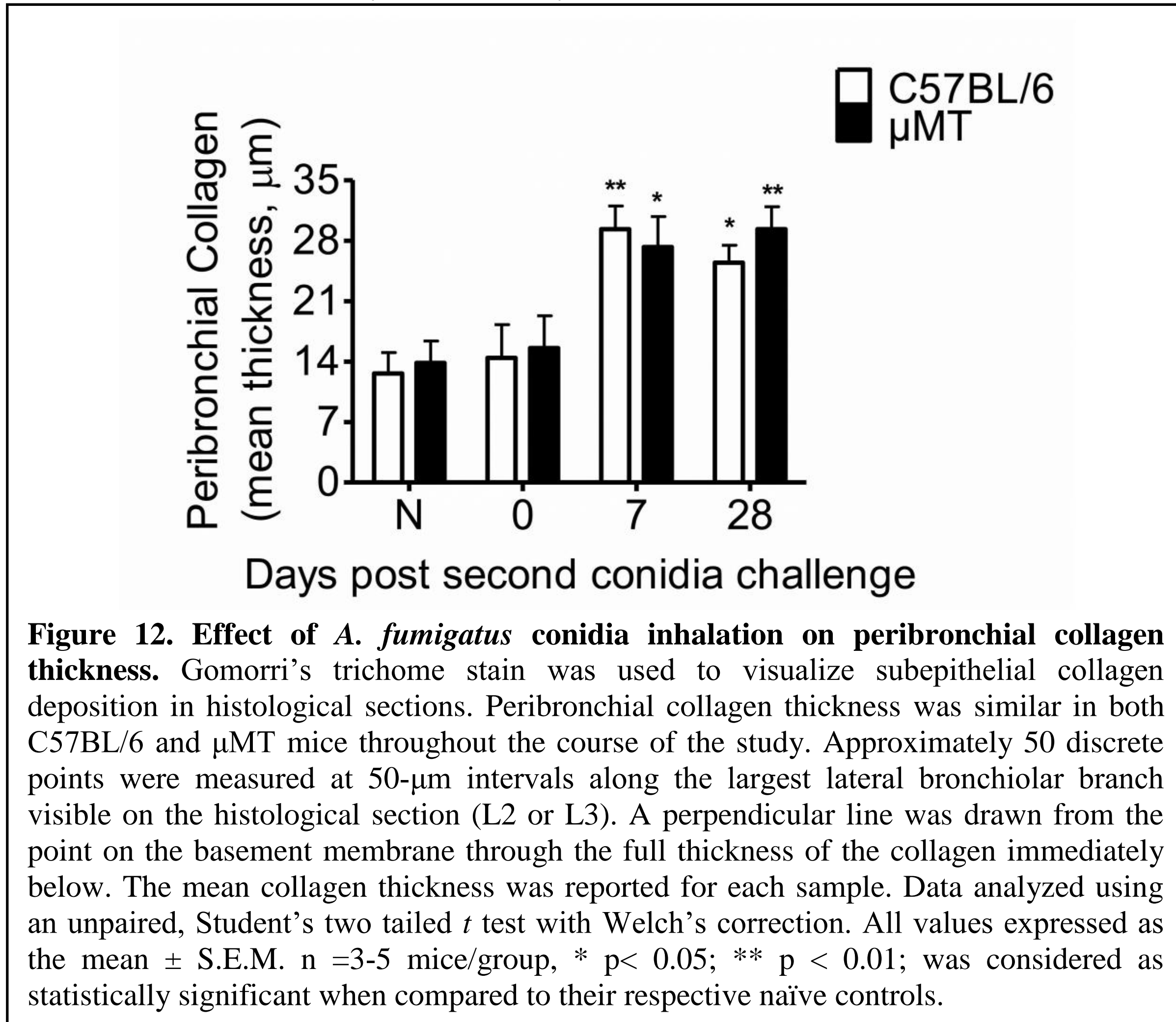

Figure 12. Effect of *A. fumigatus* conidia inhalation on peribronchial collagen thickness. Gomorri's trichome stain was used to visualize subepithelial collagen deposition in histological sections. Peribronchial collagen thickness was similar in both C57BL/6 and μMT mice throughout the course of the study. Approximately 50 discrete points were measured at 50-μm intervals along the largest lateral bronchiolar branch visible on the histological section (L2 or L3). A perpendicular line was drawn from the point on the basement membrane through the full thickness of the collagen immediately below. The mean collagen thickness was reported for each sample. Data analyzed using an unpaired, Student's two tailed *t* test with Welch's correction. All values expressed as the mean ± S.E.M. n =3-5 mice/group, * $p < 0.05$; ** $p < 0.01$; was considered as statistically significant when compared to their respective naïve controls.

Fungal inhalation resulted in increased serum IgA, IgG1, IgG2a, and IgE levels in allergic C57BL/6 mice, while μMT mice exhibited elevated IgG1 in BAL, IgG2a and IgE in serum. In the present study, inhalation of *A. fumigatus* conidia resulted in an increase in the BAL IgA from C57BL/6 mice at day 7 after 2 conidia inhalations. IgA Abs were not detected in either serum or the BAL fluid of μMT mice (**Fig 13A & B**). IgG1 was detected in the BAL fluid of allergic μMT mice 7 days after 2 exposures to conidia, but was not found in the serum **(Fig 13C & D).** Although serum IgG2a levels in naïve μMT animals were significantly lower than wild type, sensitization with fungal antigens stimulated its production to levels equivalent to those of

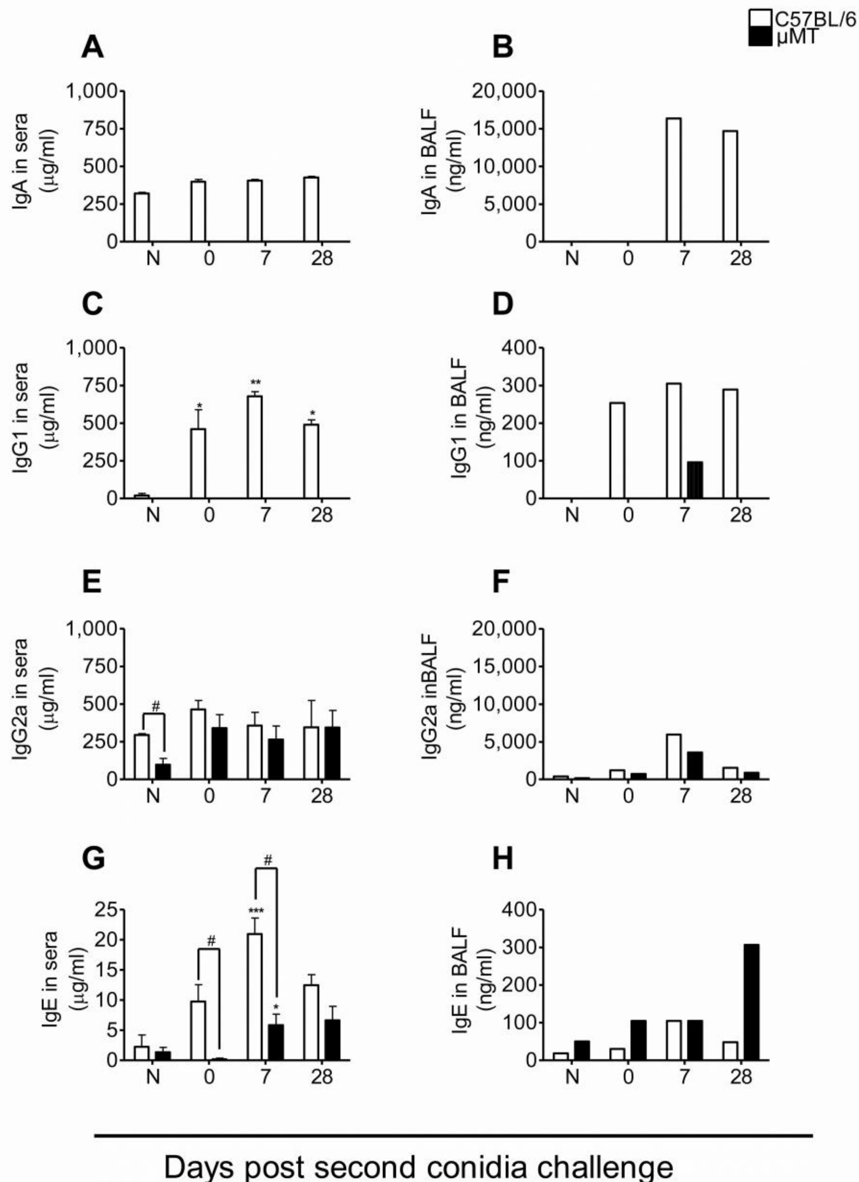

Figure 13. Inhalation of *A. fumigatus* conidia induces µMT mice to produce IgG1 (only in BAL), IgE and IgG2a in serum and bronchoalveolar lavage (BAL) fluid. The Ab levels of C57BL/6 and µMT mice were compared to naïve animals and to each other at each time point. ELISA's indicated that the µMT mice produced antibodies in response to *A. fumigatus* allergen challenge. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ± S.E.M. n = 4-5 mice/group, * p< 0.05; *** p <0.001 was considered significant as compared to naïve controls. # p <0.05 was considered statistically significant when µMT were compared to C57BL/6 animals. No statistics are shown for Ig ELISAs for BAL fluid as the samples from each time point were pooled and run as a single sample.

wild type, and the μ-deficient animals matched IgG2a levels throughout the rest of the study (**Fig 13E**). IgG2a was also detected in the BAL fluid of the μMT mice (**Fig 13F**). IgE was elevated in *A. fumigatus*-sensitized and challenged C57BL/6 and μMT animals (**Fig 13G & H**). Even though there was a significant difference in the IgE levels of C57BL/6 and μMT mice, the production of IgE was significantly higher at day 7 post second conidia challenge in both the murine groups when they were compared to their respective naïve controls, suggesting that isotype switching to an allergic phenotype was possible even in the μMT mice. However, IgE production in C57BL/6 mice was 4X higher than μMT levels at day 7 after the second inhalation.

To investigate the extent to which the antibodies produced as a result of fungal sensitization and inhalation exposure were specific to *A. fumigatus,* serial dilutions of serum and BAL samples from C57BL/6 and μMT mice were collected at day 7 after the second conidia exposure and analyzed against the sensitizing antigen. The specificity of antibodies to *A. fumigatus* at day 7 post second conidia challenge (when the antibody levels are higher in serum and BAL) of both C57BL/6 and μMT mice are shown in figure 8. The serum and BAL antibodies produced in the C57BL/6 mice were specific to *A. fumigatus* (filled square), while the ones produced in μMT mice (filled circle) appeared to be non-specific and the values were comparable to those of naïve control animals **(Fig 14A & B)**. When the specificity of individual subclasses of Abs (IgE and IgG1) for *A. fumigatus* was tested, we observed similar results (data not shown).

$CD19^+CD9^+IgD^+$ B-1 cells are present in the lungs of μMT mice despite a lack of the Ig μ chain. It has previously been shown that μMT mice on the BALB/c, but not C57BL/6 background, display an incomplete block in B cell development and harbor mature B cells in secondary lymphoid organs (32, 33).

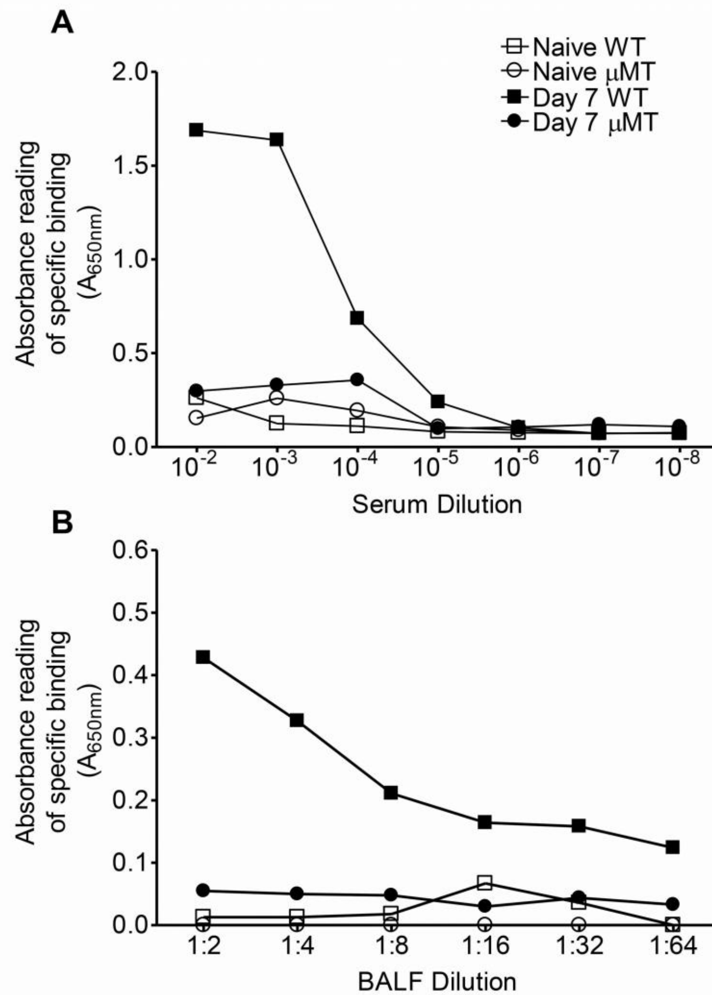

Figure 14. Inhalation of *A. fumigatus* conidia induces specific serum and BAL antibody production in C57BL/6 mice, but not in μMT mice. Serum and BAL samples from the C57BL/6 and μMT mice were pooled and serial dilution was used to evaluate antibody titers. **(A)** The specificity of total serum antibody from C57BL/6 (Day 7 WT) and μMT (day 7 μMT) for *A. fumigatus* was evaluated against *A. fumigatus* Ag. **(B)** The specificity of total BAL antibody from C57BL/6 (Day 7 WT) and μMT (day 7 μMT) for *A. fumigatus* Ag. C57BL/6 mice produced specific antibodies for *A. fumigatus*, while none were detected in μMT mice.

Although all μMT animals used here were on the C57BL/6 background, we considered the possibility that *A. fumigatus* exposure might overcome the B cell developmental block (28, 33). In the present study, after inhalation with *A. fumigatus* conidia, $CD19^+$ B cells were detected in the lungs of μMT mice at day 7 after the second conidia inhalation and their numbers were

fewer than the WT controls **(Fig 15C, D, E & F)**. When evaluated, $CD19^+$ B cells were also detected in the lungs of naïve μMT mice (data not shown).

Given the fact that Abs produced in the μMT mice were not specific for *A. fumigatus,* we looked for the presence of B-1 lymphocytes in the lungs as these cells are known to produce Abs in a non-specific manner and they predominate in the pleural and peritoneal cavities (34). In addition to B-1 cells, we also looked for B-2 cells in the lungs of μMT mice as these conventional B-2 cells form a major population of lymphocytes which is present in the body (35, 36). The $CD19^+$ B cell population in the lungs of C57BL/6 WT mice expressed either CD9 (as B-1 cells are $CD9^+$) (34, 37) or CD23 (as B-2 cells are $CD23^+$ and have low to no expression of CD9) (36, 38), showing the presence of both B-1 and B-2 B cells **(Fig 15C & E)**. On the contrary, $CD19^+$ B cells that were present in the lungs of μMT mice did not express CD23 indicating the absence of B-2 lymphocytes **(Fig 15F)**. Similar to the B-1 population in C57BL/6 WT mice, the μMT $CD19^+$ B cells expressed CD9, illustrating the presence of B-1 lymphocytes (34) **(Fig 15D)**.

It has been shown that IgD can substitute for IgM if it is expressed early in the B cell development process (39). As such, we looked for the expression of IgD on the $CD19^+CD9^+$ cells that were present in the lungs of C57BL/6 WT and μMT mice using flow cytometry. IgD was expressed on the $CD19^+CD9^+$ cells present in the lungs of C57BL/6 and μMT mice at day 7 post second conidia challenge **(Fig 15G & H)**. As expected, IgM positive cells were not detected in either naïve or *A. fumigatus* challenged μMT mice (data not shown). These data demonstrate that in the μMT mice, IgD can substitute for IgM early in B-1 cell development.

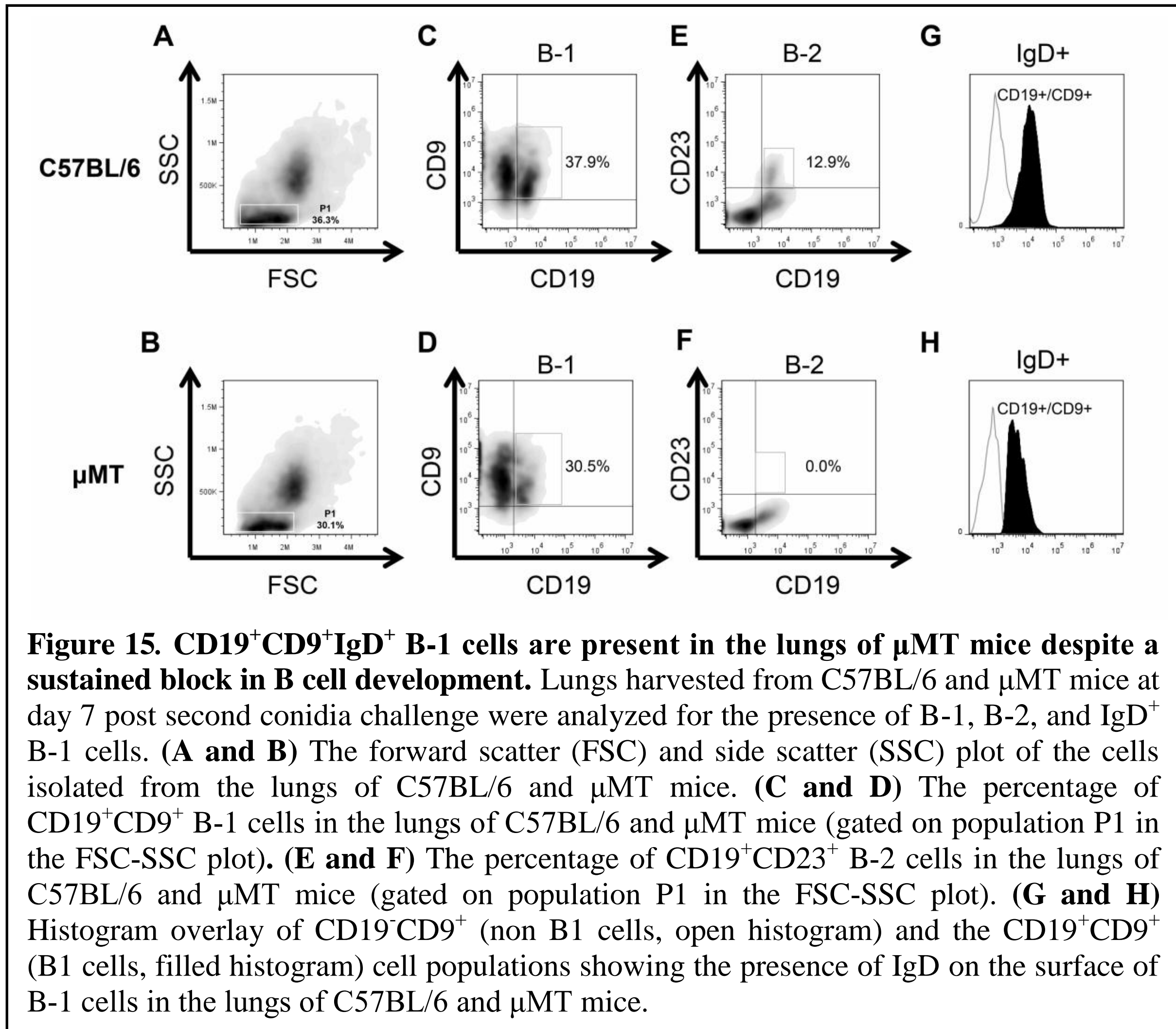

Figure 15. $CD19^+CD9^+IgD^+$ B-1 cells are present in the lungs of μMT mice despite a sustained block in B cell development. Lungs harvested from C57BL/6 and μMT mice at day 7 post second conidia challenge were analyzed for the presence of B-1, B-2, and IgD^+ B-1 cells. **(A and B)** The forward scatter (FSC) and side scatter (SSC) plot of the cells isolated from the lungs of C57BL/6 and μMT mice. **(C and D)** The percentage of $CD19^+CD9^+$ B-1 cells in the lungs of C57BL/6 and μMT mice (gated on population P1 in the FSC-SSC plot)**. (E and F)** The percentage of $CD19^+CD23^+$ B-2 cells in the lungs of C57BL/6 and μMT mice (gated on population P1 in the FSC-SSC plot). **(G and H)** Histogram overlay of $CD19^-CD9^+$ (non B1 cells, open histogram) and the $CD19^+CD9^+$ (B1 cells, filled histogram) cell populations showing the presence of IgD on the surface of B-1 cells in the lungs of C57BL/6 and μMT mice.

Discussion

In the current study, we show that the localized production of IgG1, IgG2a, and IgE is elicited in μMT mice in response to systemic fungal sensitization and inhalational challenge in an experimental allergic asthma model. In addition to the localized production, our work demonstrates that μ-deficient mice produced systemic IgG2a and IgE Abs after exposure to *A. fumigatus* extract antigens followed by inhalation of *A. fumigatus* conidia. However, when tested in binding assays with the *Aspergillus* antigens that were used to sensitize the animals, the Ab isotypes from the μMT animals proved to be non-specific, while the antibodies produced by the μ-sufficient controls were specific.

In the present work using a fungal trigger to elicit allergic airways disease, the characteristic signs and symptoms of allergic airway disease were present. AHR, pulmonary inflammation, excessive mucus production, and serum IgE in μMT mice were comparable to C57BL/6 controls. In contrast, studies using a repeated aerosol exposure to OVA showed reduced lung inflammation and mucus hypersecretion in μMT mice as compared to controls (40, 41). In addition, OVA-challenged mice failed to develop AHR, suggesting a possible role of B cells in the development of AHR in response to OVA (40, 41). So, in addition to the type of Ab that can be elicited in μMT animals, the type of immune response is also different with different antigenic stimuli, dissecting further the role of B cell activation in response to fungal pathogens/allergens.

IgM and IgA immunoglobulins share a number of similarities. IgA is related more closely to IgM than other isotypes, with the μ and α chains sharing a characteristic long secretory segment (42). In addition, both IgA and IgM can form multimers in conjunction with the J chain

and both can be secreted at the mucosal surfaces coupled to the polymeric Ig receptor (43). Some investigators have speculated that expression of IgA may act as a surrogate for membrane IgM in B cell development in some instances (43). Secretory IgA has been recognized as an integral part of the innate mucosal response that protects the upper respiratory tract (12, 13), and selective IgA deficiency in clinic patients has been associated with an increased prevalence of atopy (15, 16). In experimental conditions, μMT mice infected with *Salmonella* did produce IgA (43), and we expected that, if any isotype was made in the μMT mice in response to fungal stimulation, IgA would be that isotype. However, we found no IgA in the serum or the BAL of μMT mice. This suggests that: 1) other Ab isotypes can substitute for IgM in B cell development, 2) that the type of antigenic stimulus dictates isotype development in the μMT mouse, even when the context of the exposure (mucosal delivery) is similar (although not identical), and 3) that IgA is not necessary for fungal containment in this model.

In previous work using μMT mice on a C57BL/6 background, a very sensitive method to detect low levels of FcεRI-bound IgE on basophils showed that IgE was made in μMT mice after prolonged exposure to *Heligmosomoides polygyrus*, *Tricuris muris*, or *Schistosoma mansoni* gut parasites (44). In fungal sensitization and challenge, our results show a similar capacity for an IgE response in μMT mice after treatment with *Aspergillus* Ags. In the current study, a robust IgE response was readily quantified by ELISA, and elevated IgE levels were sustained throughout the study. Our findings show for the first time that a conserved mucosal humoral response, which is not mediated through IgM, may significantly impact the response to, not only gut parasites, but inhaled fungal pathogens as well. Interestingly, in studies in which ovalbumin (OVA) was used as a surrogate for clinically relevant inhaled allergens, no IgE was produced in μMT mice (3, 40).

In addition to IgE, we report for the first time IgG1 production in the BAL and IgG2a production in the serum and BAL of μMT mice on a C57BL/6 background. IgG1, which is associated with T_H2-type responses elicited by IL-4, was elevated only in the BAL and only after fungal challenge, suggesting a localized production of this Ab isotype. IgG2a, which plays an important role in fungal opsonization and clearance (18), was elevated throughout the study in both C57BL/6 and μMT mice. This further supports the fact that antigenic stimulus dictates isotype development in the μMT mice and that without the benefit of the μ gene a small percentage of pre-B cells can escape elimination, switch to downstream immunoglobulin heavy chains, and respond to antigens (33, 45).

The canonical pathway of B cell ontogeny requires surface expression of the μ Ig chain at an early pre-B cell stage (46). Indeed, until recently only B cells which express IgM were believed to migrate from the bone marrow to the peripheral lymphoid organs (33), and membrane-bound IgM expression was thought to be essential for B cell maturation and differentiation to Ab-producing cells. However, recent research using μMT mice has shown that the expression of the μ Ig chain is not an absolute requirement for B cell survival (33, 43, 44, 47). These genetically altered animals have been useful tools in understanding the complex biological processes associated with different diseases.

Although Ab-binding ELISAs were not attempted in the helminth infection study, the IgE was functional in that it elicited IL-4 production by basophils (44). In their study, the Ab was produced at a low concentration and Ab-producing B cells could not be detected in the central or peripheral lymph organs. In the current study, while μMT mice were able to produce Abs after sensitization to and challenge with fungal Ags, our results show that the Abs produced by the μ-deficient animals had no affinity for *A. fumigatus* Ags as compared to wild type. Together, these

results suggest a tissue-centric Ab production, which mandated the assessment of B cell populations in the lung.

In investigating the potential source of Abs in μMT mice, no other study has examined the presence of $CD19^{+}IgD^{+}$ cells in the lungs. Since we did not find $CD19^{+}IgD^{+}$ or $CD19^{+}IgM^{+}$ cells in the bone marrow or spleens of naïve mice (data not shown), we hypothesized that tissue-resident B-1 cells act as the source of Abs in μMT mice. We were able to detect IgD-expressing $CD19^{+}CD9^{+}$ B-1 cells in the lungs of μMT mice using flow cytometry, supporting a tissue resident B-1 cell as a source for localized Ab production. These observations are consistent with the notion that B cells can receive switching signals in peripheral sites (43, 44, 48, 49), a process that may occur in the allergic lung.

In summary, we provide conclusive evidence that B-1 cells can impact asthma pathophysiology in the absence of conventional B-2 lymphocytes. From these studies, we report two significant conclusions. First, the route of the pathogenic/allergenic challenge as well as the type of antigen has a significant impact on the generation of Ab responses in μMT mice lacking the normal pathway for B-2 cell maturation, as different types of antigen yield very different outcomes. The second major finding is that as a B-2 cell KO mouse, μMT animals may be very useful to determine the role of B-1 cells in response to various pulmonary insults. Future studies may include elucidating the mechanism for B-1 isotype switching in the lung and B-1 cells' contribution to protective responses, which would have important implications for experimental analysis and for understanding normal B-1 and B-2 cell activation in health and disease.

Acknowledgements

The authors wish to thank Dr. Pawel Borowicz (North Dakota State University) and the Advanced Imaging and Microscopy Laboratory at North Dakota State University for imaging support using the Zeiss Z1 AxioObserver inverted microscope. We also thank Dr. Jodie Haring from the Core Biology Facility at NDSU for technical assistance with flow cytometry.

Contribution

The work reported in this paper has been conducted by Mr. Sumit Ghosh. The data analysis and manuscript preparation has been done with the help of the co-authors.

References

1. Boulet, L. P. 2009. Irreversible airway obstruction in asthma. *Current allergy and asthma reports* 9: 168-173.
2. Bradley, B. L., M. Azzawi, M. Jacobson, B. Assoufi, J. V. Collins, A. M. A. Irani, L. B. Schwartz, S. R. Durham, P. K. Jeffery, and A. B. Kay. 1991. Eosinophils, T-Lymphocytes, Mast-Cells, Neutrophils, and Macrophages in Bronchial Biopsy Specimens from Atopic Subjects with Asthma - Comparison with Biopsy Specimens from Atopic Subjects without Asthma and Normal Control Subjects and Relationship to Bronchial Hyperresponsiveness. *J Allergy Clin Immun* 88: 661-674.
3. Korsgren, M., J. S. Erjefalt, O. Korsgren, F. Sundler, and C. G. Persson. 1997. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 185: 885-892.
4. Frigas, E., S. Motojima, and G. J. Gleich. 1991. The eosinophilic injury to the mucosa of the airways in the pathogenesis of bronchial asthma. *The European respiratory journal. Supplement* 13: 123s-135s.
5. Seminario, M. C., and G. J. Gleich. 1994. The role of eosinophils in the pathogenesis of asthma. *Curr Opin Immunol* 6: 860-864.
6. Kumar, R. K., and P. S. Foster. 2001. Murine model of chronic human asthma. *Immunology and cell biology* 79: 141-144.
7. Denning, D. W., B. R. O'Driscoll, C. M. Hogaboam, P. Bowyer, and R. M. Niven. 2006. The link between fungi and severe asthma: a summary of the evidence. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 27: 615-626.
8. Tsuji, S., and K. Ogawa. 2011. [Chronic pulmonary aspergillosis]. *Nihon rinsho. Japanese journal of clinical medicine* 69: 1462-1467.
9. Cramer, R. A., A. Rivera, and T. M. Hohl. 2011. Immune responses against Aspergillus fumigatus: what have we learned? *Current opinion in infectious diseases* 24: 315-322.
10. Knutsen, A. P., and R. G. Slavin. 2011. Allergic bronchopulmonary aspergillosis in asthma and cystic fibrosis. *Clin Dev Immunol* 2011: 843763.
11. Bice, D. E., R. H. Gray, M. J. Evans, and B. A. Muggenburg. 1987. Identification of plasma cells in lung alveoli and interstitial tissues after localized lung immunization. *Journal of leukocyte biology* 41: 1-7.
12. Suzuki, K., M. Maruya, S. Kawamoto, and S. Fagarasan. 2010. Roles of B-1 and B-2 cells in innate and acquired IgA-mediated immunity. *Immunological reviews* 237: 180-190.
13. Mogi, G. 1975. Secretory immunoglobulin A in oral and respiratory passages in man. *Ann Otol Rhinol Laryngol* 84 Suppl 20: 1-23.
14. Mantis, N. J., N. Rol, and B. Corthesy. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology* 4: 603-611.
15. van Asperen, P. P., M. Gleeson, A. S. Kemp, A. W. Cripps, S. B. Geraghty, C. M. Mellis, and R. L. Clancy. 1985. The relationship between atopy and salivary IgA deficiency in infancy. *Clin Exp Immunol* 62: 753-757.
16. Ostergaard, P. A. 1980. Clinical and immunological features of transient IgA deficiency in children. *Clin Exp Immunol* 40: 561-565.

17. Kaplan, C., J. C. Valdez, R. Chandrasekaran, H. Eibel, K. Mikecz, T. T. Glant, and A. Finnegan. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. *Arthritis Res* 4: 54-58.
18. Mukherjee, S., S. C. Lee, and A. Casadevall. 1995. Antibodies to Cryptococcus neoformans glucuronoxylomannan enhance antifungal activity of murine macrophages. *Infect Immun* 63: 573-579.
19. Zeimpekoglou, K**.** 2008. The Role of B lymphocytes in lung disease. *PNEUMON* 21: 196-199.
20. Laitinen, L. A., A. Laitinen, and T. Haahtela. 1993. Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am Rev Respir Dis* 147: 697-704.
21. Sears, M. R., B. Burrows, E. M. Flannery, G. P. Herbison, C. J. Hewitt, and M. D. Holdaway. 1991. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 325: 1067-1071.
22. Rolinck-Werninghaus, C., U. Wahn, and E. Hamelmann. 2005. Anti-IgE therapy in allergic asthma. *Curr Drug Targets Inflamm Allergy* 4: 551-564.
23. Poryadin, G. V., N. E. Zhuravleva, J. M. Salmasi, A. N. Kazimirsky, L. Y. Semenova, S. A. Polner, and T. A. Chervinskaya. 2002. Immunological mechanisms of recovery from an acute stage in patients with atopic bronchial asthma. *Russ J Immunol* 7: 259-264.
24. Zhu, J., Y. S. Qiu, D. J. Figueroa, V. Bandi, H. Galczenski, K. Hamada, K. K. Guntupalli, J. F. Evans, and P. K. Jeffery. 2005. Localization and upregulation of cysteinyl leukotriene-1 receptor in asthmatic bronchial mucosa. *American journal of respiratory cell and molecular biology* 33: 531-540.
25. Knutsen, A. P. 2011. Immunopathology and immunogenetics of allergic bronchopulmonary aspergillosis. *Journal of allergy* 2011: 785983.
26. Rapaka, R. R., D. M. Ricks, J. F. Alcorn, K. Chen, S. A. Khader, M. Zheng, S. Plevy, E. Bengten, and J. K. Kolls. 2010. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina. *J Exp Med* 207: 2907-2919.
27. Flicker, S., E. Gadermaier, C. Madritsch, and R. Valenta. 2011. Passive immunization with allergen-specific antibodies. *Current topics in microbiology and immunology* 352: 141-159.
28. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350: 423-426.
29. Hoselton, S. A., A. E. Samarasinghe, J. M. Seydel, and J. M. Schuh. 2010. An inhalation model of airway allergic response to inhalation of environmental Aspergillus fumigatus conidia in sensitized BALB/c mice. *Medical mycology : official publication of the International Society for Human and Animal Mycology* 48: 1056-1065.
30. Hogaboam, C. M., K. Blease, B. Mehrad, M. L. Steinhauser, T. J. Standiford, S. L. Kunkel, and N. W. Lukacs. 2000. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. *The American journal of pathology* 156: 723-732.
31. Samarasinghe, A. E., S. A. Hoselton, and J. M. Schuh. 2011. A comparison between intratracheal and inhalation delivery of Aspergillus fumigatus conidia in the development of fungal allergic asthma in C57BL/6 mice. *Fungal biology* 115: 21-29.

32. Hasan, M., B. Polic, M. Bralic, S. Jonjic, and K. Rajewsky. 2002. Incomplete block of B cell development and immunoglobulin production in mice carrying the muMT mutation on the BALB/c background. *European journal of immunology* 32: 3463-3471.
33. Orinska, Z., A. Osiak, J. Lohler, E. Bulanova, V. Budagian, I. Horak, and S. Bulfone-Paus. 2002. Novel B cell population producing functional IgG in the absence of membrane IgM expression. *European journal of immunology* 32: 3472-3480.
34. Won, W. J., and J. F. Kearney. 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J Immunol* 168: 5605-5611.
35. Natasha Kushnir, N. A. B., Adrian W. Zuercher, Susan E. Coffin, Charlotte A. Moser, Paul A. Offit, and John J. Cebra. 2001. B2 but Not B1 Cells Can Contribute to CD4+ T-Cell-Mediated Clearance of Rotavirus in SCID Mice *Journal of Virology*: 5482-5490.
36. Oliveira, F. L., A. M. Aguiar, R. Borojevic, and M. C. El-Cheikh. 2005. IgE expression on the surface of B1 and B2 lymphocytes in experimental murine schistosomiasis. *Braz J Med Biol Res* 38: 1033-1042.
37. Martin, F., and J. F. Kearney. 2001. B1 cells: similarities and differences with other B cell subsets. *Curr Opin Immunol* 13: 195-201.
38. Wang, H., and S. H. Clarke. 2003. Evidence for a ligand-mediated positive selection signal in differentiation to a mature B cell. *J Immunol* 171: 6381-6388.
39. Lutz, C., B. Ledermann, M. H. Kosco-Vilbois, A. F. Ochsenbein, R. M. Zinkernagel, G. Kohler, and F. Brombacher. 1998. IgD can largely substitute for loss of IgM function in B cells. *Nature* 393: 797-801.
40. Jungsuwadee, P., M. Benkovszky, G. Dekan, G. Stingl, and M. M. Epstein. 2004. Repeated aerosol allergen exposure suppresses inflammation in B-cell-deficient mice with established allergic asthma. *Int Arch Allergy Immunol* 133: 40-48.
41. Hamelmann, E., A. T. Vella, A. Oshiba, J. W. Kappler, P. Marrack, and E. W. Gelfand. 1997. Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 94: 1350-1355.
42. Warr, G. W., K. E. Magor, and D. A. Higgins. 1995. IgY: clues to the origins of modern antibodies. *Immunol Today* 16: 392-398.
43. Macpherson, A. J., A. Lamarre, K. McCoy, G. R. Harriman, B. Odermatt, G. Dougan, H. Hengartner, and R. M. Zinkernagel. 2001. IgA production without mu or delta chain expression in developing B cells. *Nat Immunol* 2: 625-631.
44. Perona-Wright, G., K. Mohrs, J. Taylor, C. Zaph, D. Artis, E. J. Pearce, and M. Mohrs. 2008. Cutting edge: Helminth infection induces IgE in the absence of mu- or delta-chain expression. *J Immunol* 181: 6697-6701.
45. Waisman, A., A. L. Croxford, and F. Demircik. 2008. New tools to study the role of B cells in cytomegalovirus infections. *Med Microbiol Immunol* 197: 145-149.
46. Melchers, F., E. ten Boekel, T. Seidl, X. C. Kong, T. Yamagami, K. Onishi, T. Shimizu, A. G. Rolink, and J. Andersson. 2000. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. *Immunological reviews* 175: 33-46.
47. Melamed, D., E. Miri, N. Leider, and D. Nemazee. 2000. Unexpected autoantibody production in membrane Ig-mu-deficient/lpr mice. *J Immunol* 165: 4353-4358.
48. KleinJan, A., J. G. Vinke, L. W. Severijnen, and W. J. Fokkens. 2000. Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis

patients. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 15: 491-497.

49. Samitas, K., J. Lotvall, and A. Bossios. 2010. B cells: from early development to regulating allergic diseases. *Archivum immunologiae et therapiae experimentalis* 58: 209-225.

PAPER 3. THE IMPACT OF HYALURONIC ACID ON B LYMPHOCYTE RECRUITMENT AND FUNCTION IN A MURINE FUNGAL ALLERGIC ASTHMA MODEL

Sumit Ghosh, Scott A. Hoselton, and Jane M. Schuh

Abstract

Allergic Bronchopulmonary Aspergillosis (ABPA) is a particularly severe form of allergic asthma that is difficult to treat and results in morbidity associated with chronic airway changes. B lymphocytes are well recognized in the development of IgE responses which exacerbate asthma symptoms, but may also be important in a protective response against inhaled fungi. Further, these lymphocytes may play a direct role in the maintenance of airway wall fibrosis as they can produce cytokines. Recent studies have shown that extracellular matrix components participate in cell attachment, chemotaxis, cellular activation and differentiation, tissue growth and repair, and cell survival indicating that they may play an important role in the development and persistence of both inflammation and fibrosis in ABPA. In the present study, we investigated the ability of hyaluronic acid, a major component of the extracellular matrix (ECM) generated at sites of inflammation, to recruit and modulate B lymphocyte functions in allergic fungal disease. Using an *Aspergillus fumigatus* murine inhalation model to mimic human asthma, we compared the effects of low and high molecular mass HA (LMM HA and HMM HA) on B lymphocyte chemotaxis and function. We found that B lymphocytes undergo chemotaxis in response to LMM HA while HMM HA had little to no effect. LMM HA-mediated B lymphocyte chemotaxis was significantly inhibited by blocking the CD44 receptor. Furthermore, we demonstrated that LMM HA fragments elicit the production of pro-fibrotic/pro-remodeling cytokines IL-10 and TGF-β1 by B lymphocytes. These observations suggest previously unforeseen interactions between B lymphocytes and LMM HA in the context of allergy and response to fungi. They represent novel pathways by which B lymphocytes may contribute to airway remodeling.

Introduction

Asthma is a debilitating disease of the airways that affects over 235 million people globally (World Health Organization, 2011). Asthmatic airways sensitized to a particular allergen respond violently to subsequent exposures, resulting in asthma attacks which can be fatal (1). Some of the hallmarks of chronic airway inflammation of patients with severe or persistent asthma include the accumulation of activated eosinophils (2-4), neutrophils, lymphocytes (5), and extracellular matrix (ECM) components in the airways (1).

Many studies have shown that T lymphocytes and T_H2 cytokines play an important role in the development of allergic lung disease and the downstream events, including inflammation, eosinophilia, mast cell accumulation/activation, and airway remodeling (6-9). However, much less is known about the mechanisms that govern the development and maintenance of B lymphocytes, and their role in allergic asthma remains unresolved. Previous studies have shown that B cells play no role in the development of allergic disease (10, 11); while other studies have shown the primary contribution of B cells is via IgE production (12, 13). On the contrary, some studies have shown that a significant number of B cells are recruited during chronic allergic lung disease (14) suggesting that they may have a role to play in allergic asthma, although their exact role in asthma still remains unclear.

Recent studies have shown that ECM components play a role in the attachment of cells, tissue growth and repair (15, 16), proliferation and differentiation (17), cell migration and activation (18), cell survival/delay of apoptosis (19), and chemotaxis (20), indicating that the ECM may play an important role in the development and persistence of inflammation. Moreover, studies have demonstrated that ECM components can function in cellular signaling under certain circumstances (21) and can deliver signals leading to or regulating cellular proliferation (22).

The ECM components have been reported to play an important role in regulating host response to lung injury. Accumulation of ECM components can be seen in tissue injury following a variety of insults such as those that occur in the adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, and chronic persistent asthma (23). Among the different ECM components studied, the glycosaminoglycan (GAG) hyaluronan (HA), which is a non-sulfated GAG polymer made of repeating disaccharide units and a major component of ECM, undergoes dynamic regulation during inflammation (24). HA is mainly synthesized by fibroblasts (25, 26) and it exists as a HMM HA polymer usually in excess of 10^6 D in its native form (27). In the lungs, the HA content is 15-150mg.$^{g-1}$ dry weight which is mainly localized in the peri-bronchial and inter-alveolar/peri-alveolar tissue and under diseased conditions this HMM HA is broken down into LMM HA (15, 28, 29). More recently, several studies have shown that LMM HA, but not HMM HA, exhibits pronounced biologic effects on cells and in tissues (24). Most importantly, LMM HA has multiple pro-inflammatory effects not observed for HMM HA (30). In fact, HMM HA can block the pro-inflammatory effects of LMM HA and helps support tissue integrity (31).

HA fragments of low MM ($<5 \times 10^5$ D), but not high MM ($> 5 \times 10^6$ D), have been shown to stimulate the murine alveolar macrophage cell line MH-S and macrophages recruited to the sites of inflammation to produce chemokines and cytokines macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α and IL-12 (32, 33). In addition, LMM HA and has been shown to induce nitric oxide synthase through a nuclear factor- κB (NF-κB) dependent mechanism, (34) suggesting LMM HA may be an important regulator of inflammatory cell activation at sites of chronic inflammation. Apart from macrophages, LMM

HA has been shown to stimulate gene expression in eosinophils, endothelial cells, and some epithelial cells (32).

HA binding proteins play an important role in cellular signal transduction and two of the most widely studied receptors of HA are CD44 and RHAMM (Receptor for Hyaluronan - mediated motility) (35, 36). Of these two receptors, CD44 is a structurally variable and multifunctional glycoprotein of the cell surface which is the most widely studied receptor for HA. To date, CD44 is the best characterized transmembrane hyaluronan receptor and is present on most cell types (37, 38). Recent studies have shown, CD44-expression on eosinophils and its up-regulation by IL-5 or GM-CSF (39). In addition, it has been reported that there is an increase in the expression of CD44 on eosinophils from late-phase bronchoalveolar lavage fluid (BALF) of patients with asthma (40). Additionally, CD44 deficient mice develop and exhibit minor abnormalities in hematopoiesis and lymphocyte circulation (41, 42). Although, it is known that CD44 undergoes dynamic regulation on eosinophils and macrophages very little is known about CD44 expression on B lymphocytes and its role in B lymphocyte interaction with hyaluronic acid, cell recruitment, and activation. However, there is growing evidence to suggest that interaction between HA and CD44 may play an important role in the regulation of functions of lymphoid and myeloid cells (43).

The aim of this study was to determine the extent to which hyaluronic acid fragments generated at sites of inflammation/tissue injury impact B lymphocyte recruitment and function. The purpose of this study was (a) To identify whether LMM HA and HMM HA play a role in the recruitment of B lymphocytes to the lungs; (b) to identify the role of the CD44 receptor in the migration of B lymphocytes; (c) and to determine the role of LMM HA and HMM HA in the activation of B lymphocytes. We found that LMM HA had a pronounced effect on B lymphocyte

recruitment, and activation. We also report that the hyaluronan binding protein CD44 is responsible for B lymphocyte migration in response to LMM HA. In addition, we have found that LMM HA impacts production of pro-fibrotic/pro-remodeling cytokines TGF-β1 and IL-10 from B cells. These observations suggest a previously unforeseen role of B lymphocytes and LMM HA, and thus novel pathways by which B lymphocytes may contribute to the regulation of chronic airway inflammation and airway remodeling in asthma.

Materials and Methods

Experimental animals. C57BL/6 male and female mice (5-9 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed on Alpha-dri™ paper bedding (Shepherd Speciality Papers, Watertown, TN, USA) in micro filter-topped cages (Ancare, Bellmore, NY, USA) in a specific pathogen-free facility with ad libitum access to food and water. The study described was performed in accordance with the Office of Laboratory Animal Welfare guidelines and was approved by the North Dakota State University Institutional Animal Care and Use Committee.

Antigen preparation and A. fumigatus *culture.* Soluble *A. fumigatus* antigen extract was purchased from Greer Laboratories (Lenoir, NC, USA) and fungal culture stock (strain NIH 5233) was purchased from American Type Culture Collection (Manassas, VA, USA). The *A. fumigatus* culture was reconstituted in 5 ml PBS, and 60-µl aliquots were stored at 4°C until use. All experiments that utilized *A. fumigatus* were conducted with prior approval of the Institutional Biological Safety Committee of North Dakota State University.

Allergen sensitization and challenge by nose only inhalational model. Animals were sensitized as per Hogaboam's published protocol (44), with the exception that alum was used as the adjuvant. Mice were sensitized globally with 10 µg of *A. fumigatus* antigen (Greer Laboratories) in 0.1 ml normal saline (NS) mixed with 0.1 ml of Imject Alum (Pierce, Rockford, IL, USA) which was injected subcutaneously (0.1 ml) and intraperitoneally (0.1 ml). After two weeks, mice were given a series of three, weekly 20-µg doses of *A. fumigatus* antigen in 20 µl of NS. Animals were challenged as previously described with a 10-min nose-only aerosol exposure to live *A. fumigatus* conidia (45). Each anesthetized mouse was placed supine with its nose in an inoculation port inhaling the live fungal conidia for 10 min. Two weeks after the first allergen

challenge, mice were subjected to a second 10-min aerosol fungal challenge. Naïve animals from both the groups were neither sensitized nor challenged. After the second allergen exposure, the mice were separated into groups of 15-18 for analysis at days 5 and 28 after the second aerosol challenge. Day 5 after challenge had previously been determined to be the peak of B cell recruitment into the allergic lungs, and this time point was chosen to assess leukocyte inflammation and hyaluronic acid levels. The day-28 time point was chosen to represent chronic stages of asthma.

Sample collection. Approximately 500 µl of blood was removed from each mouse via ocular bleed and centrifuged at 13,000 X*g* for 10 min to yield serum. Serum was stored at -20°C until use. Bronchoalveolar lavage (BAL) was performed on five mice/group with 1.0 ml sterile normal saline (NS). The BAL contents were centrifuged at 2000 X*g* for 5 min to separate cells from fluid. The BAL fluid was stored at -20°C until use, and cells were used immediately for morphometric analysis. Left lungs were harvested and fixed in 10% neutral buffered formalin for histological analysis.

Quantification of hyaluronic acid in serum and BAL. The total hyaluronic acid levels in undiluted serum and BAL were quantified via specific competitive ELISA according to the manufacturer's guidelines (Echelon Biosciences, Salt Lake City, Utah, USA). The detection limit of the kit was 50 ng/ml.

Tissue harvest and staining. Left lungs were harvested following a bronchoalveolar lavage with 1 ml of saline. Lungs were inflated and fixed in 10% neutral buffered formalin and paraffin-embedded. Hematoxylin and Eosin (Richard-Allan Scientific, Kalamazoo, MI, USA) staining was performed on 5-µm sections across the coronal planes of the lungs to determine

inflammation. Immunostaining for HA was performed as previously published (46). Briefly, sections were incubated overnight at 4°C in 3 μg/ml of HA-binding protein (Seikagaku Corporation, Japan) diluted in phosphate buffered saline with 1% bovine serum albumin. Washed sections were then incubated for one hour in avidin conjugated horseradish peroxidase (Vector Labs, Irvine, CA, USA) and developed in 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) for 10 min and counterstained in hematoxylin. Controls that were not incubated in HA-binding protein did not yield brown precipitate. The same locations within the lung and staining from serial sections were photographed.

Characterization and purification of B lymphocytes. Mice were first anesthetized using sodium pentobarbital (Butler, Columbus OH; 0.1 mg/0.01 kg of mouse body weight), and spleens and lungs were removed aseptically. To prepare the single cell suspension lungs and spleen were pooled together. Lungs from six different animals and spleens from three different animals were pooled together to prepare the single cell suspension. Minced lungs from naïve animals and animals at days 5 and 28 post second aerosol challenge were subjected to collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) digestion in complete medium (DMEM containing penicillin/streptomycin/fungizone [PSF] and 10% fetal bovine serum [FBS]) at 37°C for 1 hr with mild agitation. The cells were then dispersed through a 40-μm cell strainer (BD Biosciences, San Jose, CA, USA) using a 10-ml syringe. The cells were then washed with sterile-PBS twice before they were treated with ammonium chloride cell lysis buffer to remove the red blood cells. Syringe perfusion with DMEM and a sterile 22-gauge needle was used to prepare a single cell suspension of spleen. These spleen cells were first washed with sterile PBS and then treated with ammonium chloride lysis buffer to lyse red blood cells. Before antibody staining, the lung and spleen cells were first counted using a hemocytometer and re-suspended in

PBS with 1% BSA (Sigma Aldrich, St. Louis, MO, USA) to get a final cell concentration of 1 x 10^7 cells/ml. Fc receptors were blocked with 1 µg anti-mouse CD16/CD32 per 10^6 cells (eBiosciences, San Diego, CA, USA) for 10 min on ice. Before the spleen and lung cells were sorted for B lymphocytes using magnetic activated cell sorting (MACS), we aliquoted 10 million cells from each tube for the extracellular flow cytometry analysis. The following Abs were used for phenotypic characterization of B lymphocytes using flow cytometry: FITC-anti-CD19, PE-anti-CD44, PE-Anti-CD49d (All Abs were purchased from eBioscience, San Diego, CA, USA) and PE-Cy™ 7 Anti-CD11a (BD Biosciences, San Jose, CA, USA). The samples were pre-incubated with combinations of directly labeled antibodies (1 µg/million cells) for 30 min in dark at 4°C and then washed with PBS 1% BSA twice before the samples were analyzed using BD FACSCalibur (BD Biosciences, San Jose, USA). A minimum of 50,000 events were acquired and the data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

For the purification of B lymphocytes, spleen and lung cells were first incubated with 0.0625 µg of FITC-anti-CD19 antibody per million cells for 30 minutes in dark at 4°C (eBioscience, San Diego, CA, USA) (47). The cells were then washed with sterile-PBS to remove unbound antibodies before sorting using magnetic beads. The cells were then incubated with 10 µl of anti-FITC Microbeads per 10^7 cells (Miltenyi Biotec, Auburn, CA, USA), and CD19-positive B lymphocytes were positively selected using the quadro MACS system with LS columns (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer instructions. After sorting, the B lymphocytes were re-suspended in complete medium (DMEM with 10% FBS, PSF, Glutamine, 2-ME) to get a final concentration of 2 x 10^7 cells/ml. The purity of sorted B cells was between 90-95% when analyzed by flow cytometry, and these cells were further used for the chemotaxis assay and cell culture experiments. All the samples were analyzed with a BD

FACSCalibur (BD Biosciences, San Jose, USA) and data analyzed using FlowJo software as mentioned above.

Chemotactic activity of low and high molecular mass hyaluronic acid and ex vivo culture of B lymphocytes. To assess chemotactic activity of LMM HA (40 kDa) and HMM HA (500 kDa) (Lifecore Biomedical LLC, Chaska, MN, USA), isolated B lymphocytes from spleen and lungs were subjected to an *in vitro* chemotaxis assay in a 96-well modified Boyden chamber appropriate for the evaluation of lymphocyte chemotaxis (ECM515, Millipore Corporation, Billerica, MA, CA, USA). Sorted B lymphocytes were re-suspended at 2×10^7 cells/ml in complete medium (Dulbecco's modified Eagle's medium with 10% FBS, PSF, Glutamine, 2-ME) and each well was seeded with one million B cells. Blocking of CD44 was done by pre-incubating B lymphocytes with 50 μg/ml anti-CD44 neutralizing Ab (48, 49) (clone IM7; BD Biosciences Pharmingen, San Diego, CA, USA) for 2 h. *In vitro* assays were then performed in a 96-well polycarbonate filter plate appropriate for leukocyte/lymphocyte chemotaxis (ECM515, Millipore Corporation, Billerica, MA, CA, USA). Purified B cells (1×10^6 cells) were added to each well in the top filter plate portion of the assembly, and 150 μl of 400 μg/ml solution of LMM HA and HMM HA (50, 51) was added to the respective bottom feeder wells. The whole setup was kept in a 37^0C incubator (5% CO_2, constant humidity) for 16 h. The migrated cells were then detached, lysed, and labeled with a fluorescent dye that exhibited strong fluorescence when bound to cellular nucleic acids. Sample fluorescence was measured with a Synergy HT fluorescence microplate reader (BioTek, Winooski, VT, USA) using 480/520nm filter set.

For B lymphocyte *ex vivo* culture experiments, sorted B lymphocytes (1 million cells/ 50 μl) were seeded in a 96 well cell culture cluster (Corning, New York, USA). Blocking of CD44 was done as mentioned above with 50 μg/ml anti-CD44 (48). LMM HA and HMM HA (0.5

mg/ml) (43) in complete medium were then added to the respective wells containing B lymphocytes so that the total volume in each well was 200 µl. The whole setup was kept in a 37°C incubator for 48 hrs. IL-10 and TGF-β1 were then quantified in the supernatant via specific ELISA's according to the manufacturer's guidelines (eBiosciences, San Diego, CA, USA).

Quantification of TGF-β1, IL-10 in serum, BAL and cell culture supernatants. The total TGF-β1 and IL-10 levels in serum, BAL, and cell culture supernatants were quantified via specific ELISA's according to manufacturer's guidelines (eBiosciences, San Diego, CA, USA). The serum samples were diluted 1:7 for the TGF-β1 ELISA, while undiluted serum samples were used for IL-10 ELISA. Cell culture supernatants and BAL fluid were diluted 1:1.4 for TGF-β1 ELISA, while undiluted samples were used for IL-10 ELISA. The detection limits of the kit were 7.8 ng/ml for TGF-β1 and 31.2 ng/ml for IL-10.

Statistical analysis. Allergic C57BL/6 wild type animals were compared to their respective naïve controls at each time point and B cells cultured in the presence of LMM HA were compared to control B cells (media alone). Results are expressed as mean ± SEM. Data were evaluated using an unpaired, student's two tailed *t* test with Welch's correction to determine statistical significance with GraphPad Prism software (San Diego, CA, USA). $*p < 0.05$ indicates statistical difference when allergic animals were compared to their respective naïve controls and when B cells cultured in the presence of LMM HA were compared to control B cells (media alone). # represents the statistical difference when B cells cultured in the presence of blocking antibody (CD44) and LMM HA were compared to B cells that were cultured in the presence of LMM HA alone.

Results

Inhalational fungal challenge with A. fumigatus results in airway hyperresponsiveness, increased IgE production, and inflammation in allergen challenged mice. In the present study, airway physiology of C57BL/6 mice was monitored before allergen challenge in naïve animals and at days 5 and 28 post second conidia inhalation **(Fig 16A)**. Airway response measurements from all study animals were used to determine the baseline mean for airway hyperresponsiveness prior to methacholine challenge **(Fig 16A, dotted line, 1.89 ± 0.09 cm H_20/ml/s)**. At day 5 after 2 conidia challenges, airway hyperresponsiveness was significantly increased in C57BL/6 mice as compared to naive controls **(Fig 16A).** By day 28 after the second conidia challenge, AHR values returned to the baseline level. In addition, IgE which is the hallmark of allergic asthma, was elevated in BAL and serum at days 5 and 28 post second conidia inhalation **(Fig 16B & 16C)**.

We also examined the temporal and spatial recruitment of leukocytes in tissue sections of naïve and allergic animals at days 5 and 28 after allergen challenge. Morphometric identification of monocytes/macrophages, neutrophils, eosinophils, and lymphocytes was performed to estimate the relative makeup of the cellular inflammation and to monitor leukocyte egress into the airway lumen **(Fig 16D-G).** Lymphocytes, and particularly eosinophils, were prominent cell types identified in the BAL 5 days after the second conidia challenge with very few neutrophils (**Fig 16E, 16F & 16G)** emphasizing that multiple inhalations of conidia polarize the immune response in favor of allergy and that lymphocytes form a major percentage of cells along with eosinophils in the allergic lung. By day 28 post challenge, macrophages were only the major cellular component of the BAL compartment with very few lymphocytes **(Fig 16D-G)**.

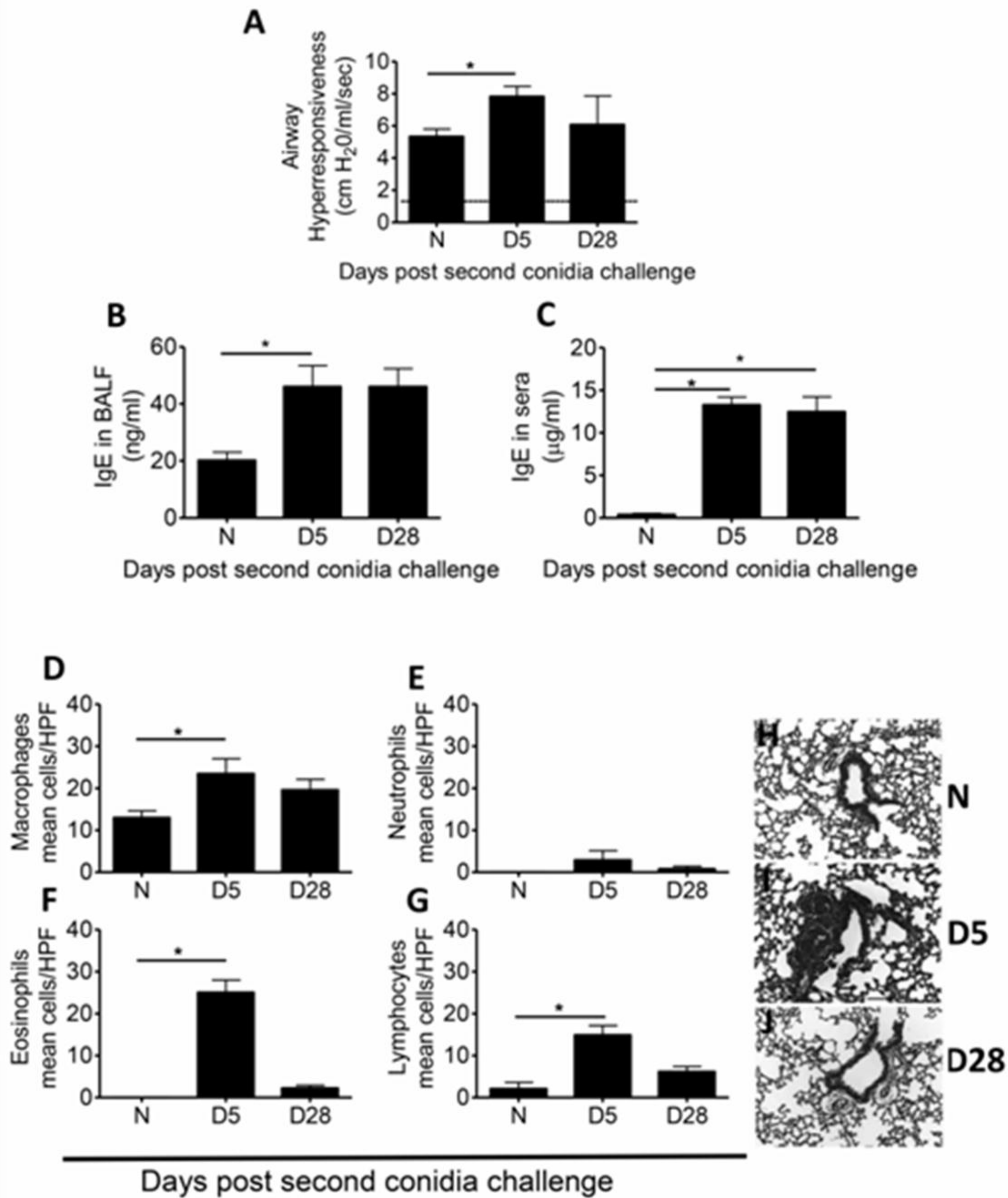

Figure 16. Effect of *A. fumigatus* conidia inhalation on AHR, IgE production, and inflammation in allergic C57BL/6 mice. (A) Peak AHR after 420 µg/kg methacholine provocation (baseline 1.89 ± 0.09 cm H_2O/ml/s indicated by the line). **(B & C)** IgE Ab levels in the BAL and serum of naïve (N) and allergic animals after conidia inhalation. Airway inflammation marked by the presence of macrophages **(D)**, neutrophils **(E)**, eosinophils **(F)**, and lymphocytes **(G)** in naïve and allergic mice. Representative photomicrographs of H&E stained lung sections of naïve (H) and allergen-challenged **(I & J)** mice. Data analyzed using an unpaired, Student's two tailed *t* test with Welch's correction. All values expressed as the mean ± S.E.M. n = 3-5 mice/group, *$p < 0.05$ as compared to naïve. Scale bars=100 µm

Similarly, perivascular and peribronchial tissue inflammation was increased in allergic animals at day 5 post second conidia challenge **(Fig 16H-J)**.

HA levels were elevated in the BAL, serum, and lungs of allergen challenged mice. HA in asthma and COPD has a lower molecular mass because under diseased conditions, HMM HA is generally broken down into LMM HA (28, 29, 52) and often lung injuries are associated with increased concentrations of hyaluronic acid (HA) in the BALF (53, 54). In the present study, inhalation of *A. fumigatus* conidia resulted in an increase in the concentration of HA in both BAL and serum of C57BL/6 mice at day 5 post the second conidia challenge (**Fig 17A & 17B**). By day 28 after the second aerosol challenge, the HA levels go back to naïve levels in both serum and BALF **(Fig 17A & 17B)** which also coincides with the resolution of inflammation in our murine fungal allergy model system **(Fig 17D-J).**

We also examined HA deposition in perivascular and peribronchial spaces in naïve and allergic animals. Naïve lungs had minimal evidence of HA around airways and blood vessels **(Fig 17C & 17F)**. HA deposition increased after second conidia challenge and localized to the perivascular and peribronchial areas where inflammatory cells are present **(Fig 17D & 17G)**. After conidia inhalation, intense HA deposition was observed around the distal airways and blood vessels of conidia-challenged allergic lungs **(Fig 17D)**. While HA localization was reduced by day 28, intense staining was still clearly evident surrounding the blood vessels and in the peribronchial areas **(Fig 2D & 2G)**. There was a significant correlation between the severity of asthma as measured by airway hyperresponsiveness, IgE Ab levels, and eosinophilia (**Fig 16)** and the HA levels in BAL, serum, and lung tissue sections **(Fig 17A-H)** indicating significant pulmonary damage in response to fungal challenge.

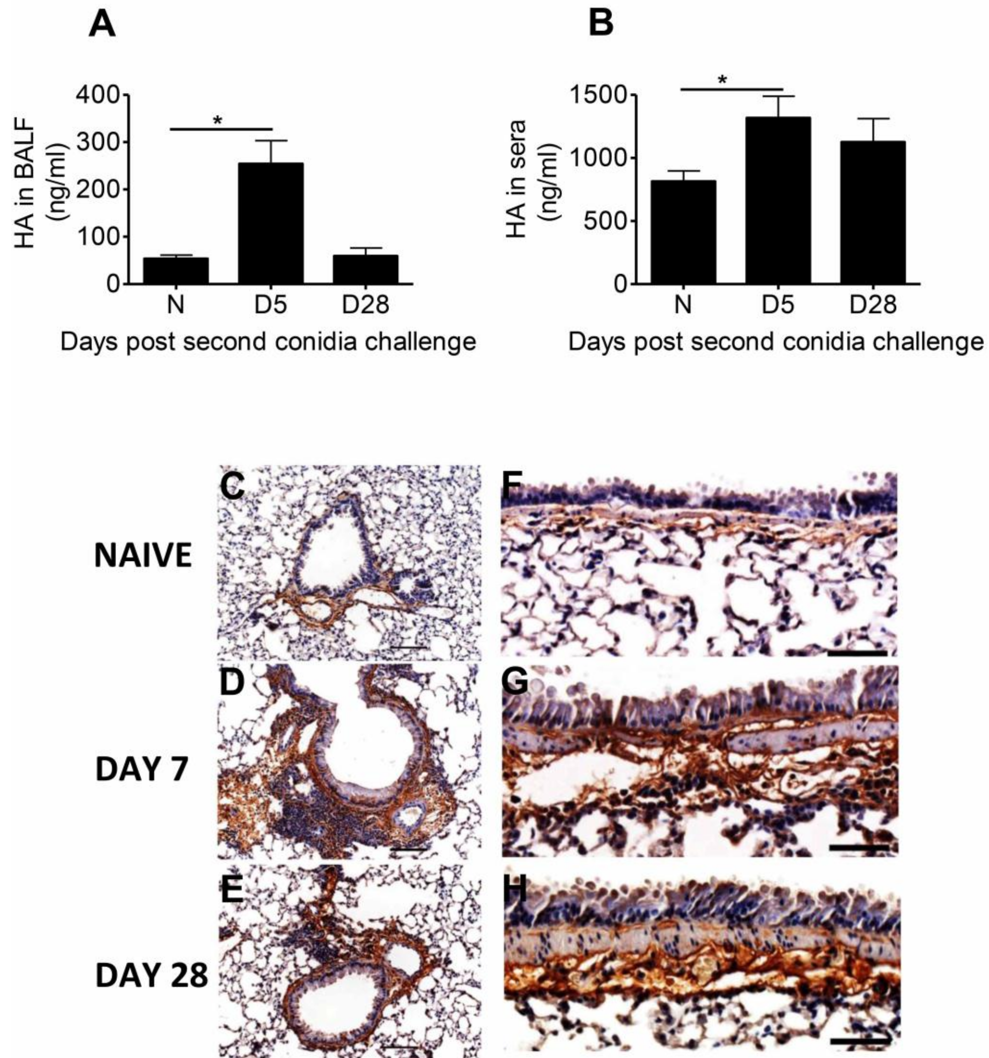

Figure 17. Effect of inhalation of *A. fumigatus* conidia on the levels of hyaluronic acid (HA) in bronchoalveolar lavage (BAL) fluid, serum, and lung tissue sections. ELISA's were carried out to measure HA levels in C57BL/6 mice after fungal challenge. (A) HA levels in BAL fluid of naïve and allergic animals. (B) HA levels in serum of naïve and allergic animals. (C-H) Immunostaining of HA in lung sections of naïve and allergic mice. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ±S.E.M. n = 3-5 mice/group, * $p < 0.05$; was considered statistically significant when compared to the respective naïve controls. Scale bars =100 μm (C,D,E) and 50μm (F,G,H)

CD19$^+$ B cell numbers are increased in the allergic lung and spleen. We next examined whether B lymphocytes were a significant component of the inflammation associated with allergic disease when HA levels are elevated in the BAL, serum, and lungs of allergic animals. To investigate this, mice were sensitized and challenged with *A. fumigatus* and at days 5 and 28 following the second conidia challenge the numbers of CD19$^+$ B cells, in the lung and spleen were determined by flow cytometry and were compared to the respective naïve controls. The percentage of lung CD19$^+$ B cells (in the lymph+ gate) increased at day 5 after second conidia challenge. **(Fig 18E & 18G)** at day 5 post second conidia challenge. A similar phenomenon was observed in the spleen of allergic animals. The percentage of CD19$^+$ B cells (in the lymph+ gate) increased in the spleen of allergic animals from (34.02% ± 2.48) to (46.05% ± 1.93) at day 5 after second conidia challenge **(Fig 18L & 18N)**. By day 28, the number of B lymphocytes decreased in the lung **(Fig 18F & 18G)** suggesting a resolution of inflammation which also coincides with a decrease in the HA levels in BAL of allergic animals. However, the number of B cells in the spleen did not decrease by day 28 **(Fig 18M & 18N)**. This data demonstrates that B cells are a major component of the pulmonary and splenic environment associated with allergic asthma when the HA levels are higher in the BAL and serum of allergic animals.

CD19$^+$CD44$^+$ B cells are elevated in lung and spleen of allergen challenged mice. We characterized the role of CD44 in the initiation, progression, and cellular inflammation in a murine model of allergic asthma at different time points after two fungal challenges as CD44 is the most widely studied receptor for HA. To determine changes in the CD44 numbers in the lung and spleen lymphocyte population after *A. fumigatus* conidia challenge, we first isolated the lung and spleen cells from naïve and allergic mice and analyzed the B lymphocyte populations by a flow cytometer for CD44 expression.

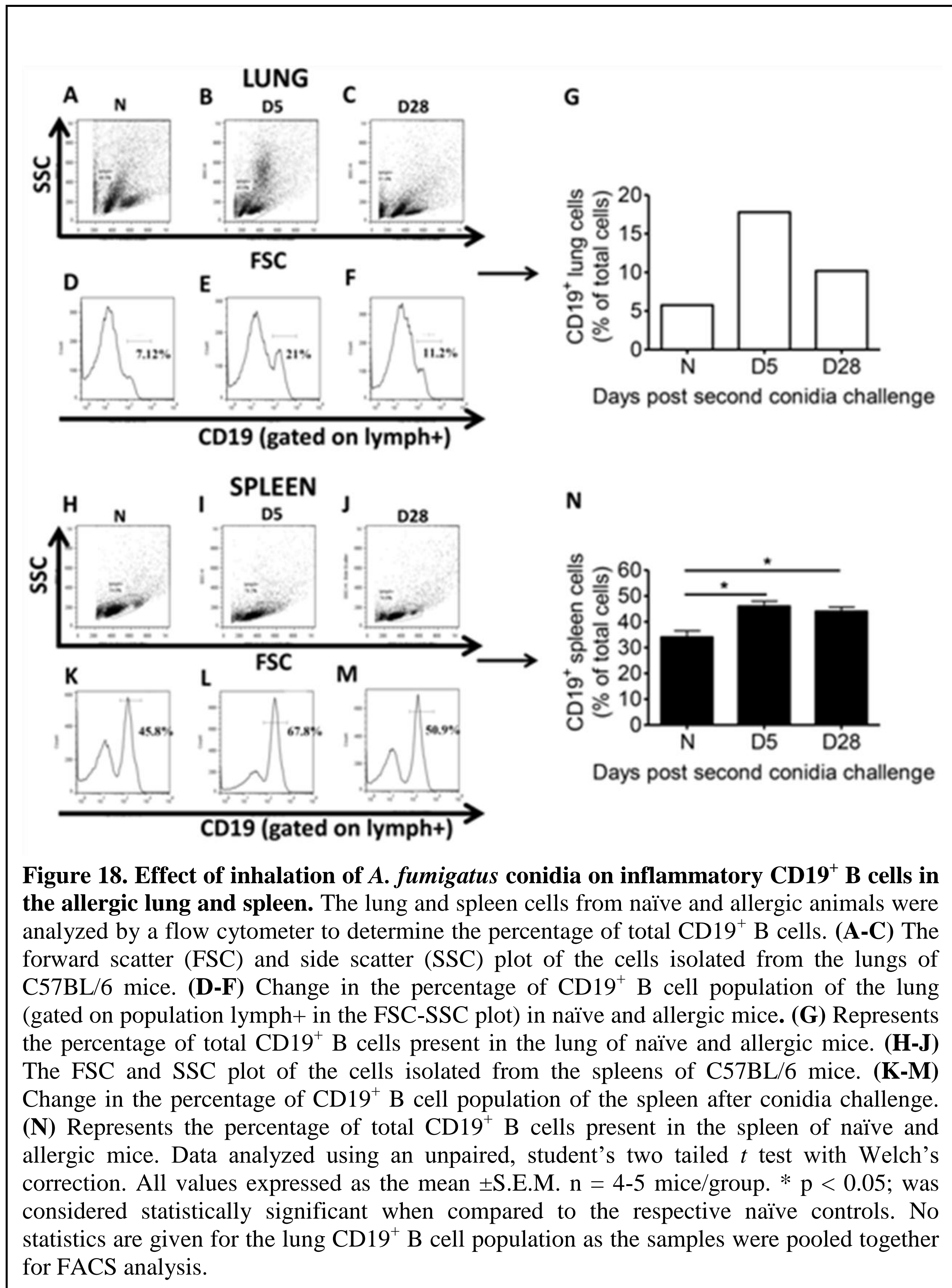

Figure 18. Effect of inhalation of *A. fumigatus* conidia on inflammatory CD19$^+$ B cells in the allergic lung and spleen. The lung and spleen cells from naïve and allergic animals were analyzed by a flow cytometer to determine the percentage of total CD19$^+$ B cells. **(A-C)** The forward scatter (FSC) and side scatter (SSC) plot of the cells isolated from the lungs of C57BL/6 mice. **(D-F)** Change in the percentage of CD19$^+$ B cell population of the lung (gated on population lymph+ in the FSC-SSC plot) in naïve and allergic mice**.** **(G)** Represents the percentage of total CD19$^+$ B cells present in the lung of naïve and allergic mice. **(H-J)** The FSC and SSC plot of the cells isolated from the spleens of C57BL/6 mice. **(K-M)** Change in the percentage of CD19$^+$ B cell population of the spleen after conidia challenge. **(N)** Represents the percentage of total CD19$^+$ B cells present in the spleen of naïve and allergic mice. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ±S.E.M. n = 4-5 mice/group. * $p < 0.05$; was considered statistically significant when compared to the respective naïve controls. No statistics are given for the lung CD19$^+$ B cell population as the samples were pooled together for FACS analysis.

We found that CD44 expressing $CD19^+$ lung B cells were increased at day 5 after two conidia challenges **(Fig 19E & 19G)** when they were compared to their respective naïve controls. However, by day 28 post second conidia challenge CD44 expressing $CD19^+$ decreased when compared to naïve controls and this timepoint coincides with the resolution of inflammation and low HA levels in BAL (**Fig 19F & 19G**). CD44 expressing $CD19^+$ B cells were also increased in the spleen of allergic animals **(Fig 19L & 19N)**. This data suggests that B cells present in allergic lungs and spleen have the receptor for HA and they may interact with HA via CD44.

$CD19^+CD49d^+$ and $CD19^+CD11a^+$ numbers are increased in the lung and spleen of A. fumigatus *challenged mice.* The up-regulation/expression of activation markers CD49d and CD11a is considered one of the mechanisms by which B lymphocytes mediate immune responses. These molecules play an important role in accumulating infiltrating cells at allergic, inflammatory sites as they can interact with ligands on endothelial cells and impact cell migration. In our study, we determined whether B cells in the lung and spleen up-regulate or express the integrin molecules CD49d and CD11a as these molecules are required for interaction with its ligands on the endothelial surface and extravasation to the site of infection/tissue injury. To study this, B cell populations of naïve and allergic animals were analyzed using flow cytometry. After allergen challenge, at day 5 post second conidia challenge, the number of CD49d and CD11a expressing B cells was increased in the lung (**Fig 20E, 20H, 20J & 20K**). Similarly, there was an increase in the number of $CD19^+CD49d^+$ and $CD19^+CD11a^+$ B cells in the spleen **(Fig 20P, 20S, 20U & 20V)**. These results suggests that after allergen challenge the number of B cells expressing the activation markers CD49d and CD11a are increased in the allergic lung suggesting that these integrin molecules may be playing a role in B cell migration to the site of tissue injury.

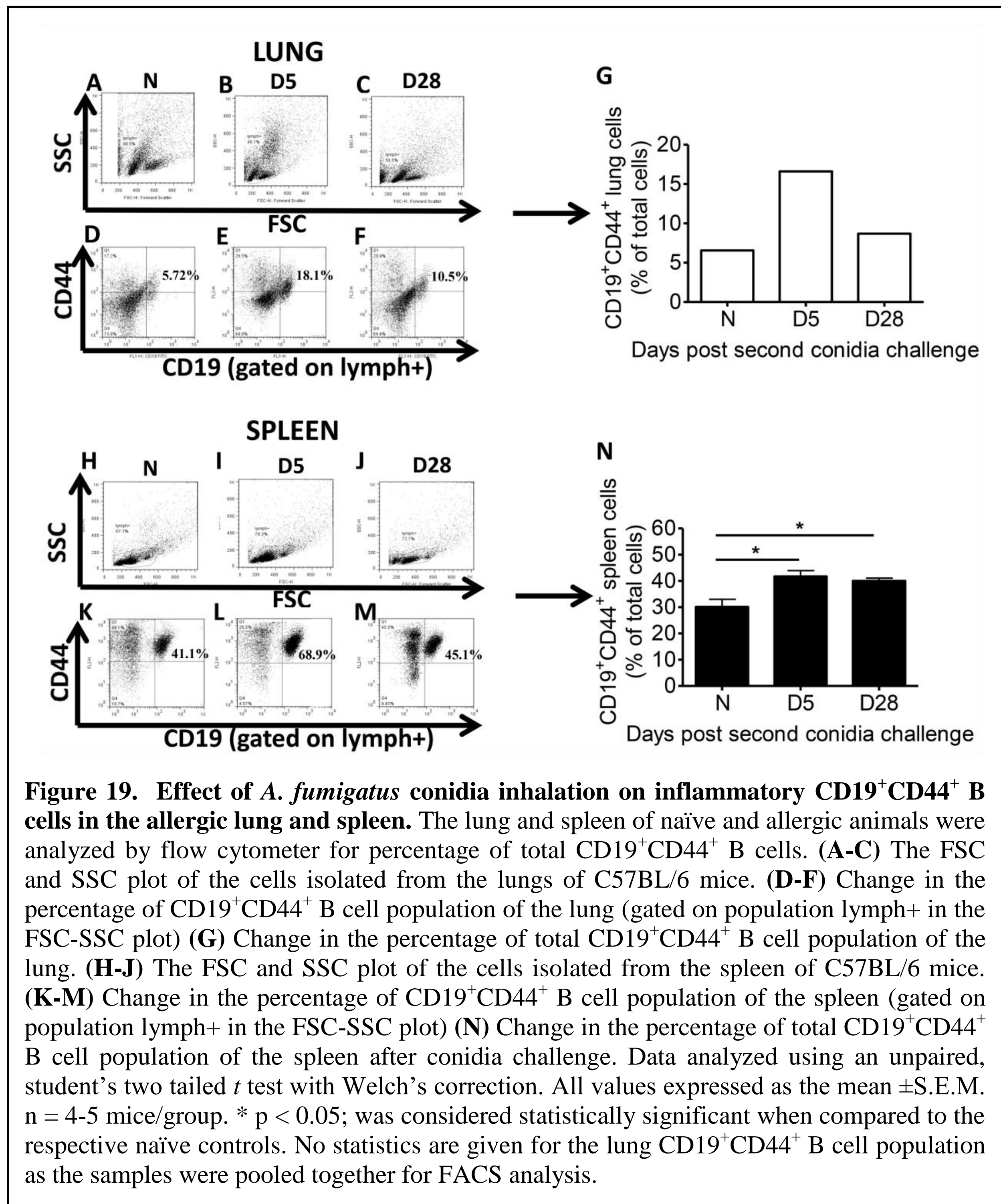

Figure 19. Effect of *A. fumigatus* conidia inhalation on inflammatory CD19$^+$CD44$^+$ B cells in the allergic lung and spleen. The lung and spleen of naïve and allergic animals were analyzed by flow cytometer for percentage of total CD19$^+$CD44$^+$ B cells. **(A-C)** The FSC and SSC plot of the cells isolated from the lungs of C57BL/6 mice. **(D-F)** Change in the percentage of CD19$^+$CD44$^+$ B cell population of the lung (gated on population lymph+ in the FSC-SSC plot) **(G)** Change in the percentage of total CD19$^+$CD44$^+$ B cell population of the lung. **(H-J)** The FSC and SSC plot of the cells isolated from the spleen of C57BL/6 mice. **(K-M)** Change in the percentage of CD19$^+$CD44$^+$ B cell population of the spleen (gated on population lymph+ in the FSC-SSC plot) **(N)** Change in the percentage of total CD19$^+$CD44$^+$ B cell population of the spleen after conidia challenge. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ±S.E.M. n = 4-5 mice/group. * $p < 0.05$; was considered statistically significant when compared to the respective naïve controls. No statistics are given for the lung CD19$^+$CD44$^+$ B cell population as the samples were pooled together for FACS analysis.

CD44 is necessary for low molecular mass HA-mediated chemotaxis of B lymphocytes.

To investigate the role of HA and CD44 in HA-mediated motility of B lymphocytes, spleen B lymphocytes were isolated and a modified Boyden chamber assay was performed using LMM

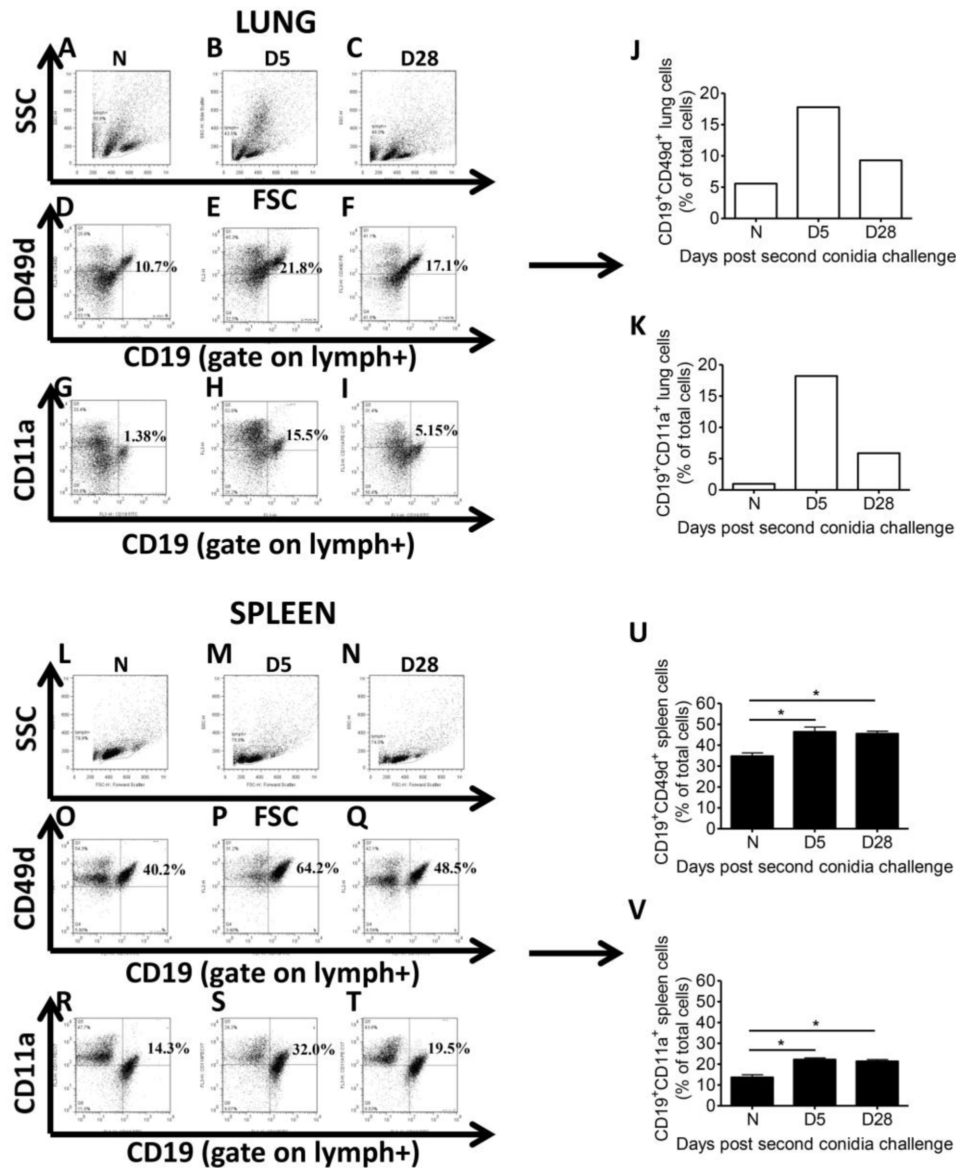

Figure 20. Effect of *A. fumigatus* conidia inhalation on trafficking of inflammatory $CD19^+$ B cells in the allergic lung and spleen. Lung and spleen derived $CD19^+$ B cells were evaluated for the integrin molecules CD49d and CD11a which play an important role in leukocyte transmigration. **(A-C)** The FSC and SSC plot of the cells isolated from the lungs of C57BL/6 mice. **(D-I)** Change in the percentage of $CD19^+CD49d^+$ and $CD19^+CD11a^+$ cells of the lung (gated on population lymph+ in the FSC-SSC plot). **(J&H)** Percentage of total $CD19^+CD49^+$ and $CD19^+CD11a^+$ B cells in the lung of naïve and allergic mice. **(L-N)** The FSC and side SSC plot of the cells isolated from the spleen of C57BL/6 mice. **(O-T)** Change in the percentage of $CD19^+CD49d^+$ and $CD19^+CD11a^+$ cells of the spleen (gated on population lymph+ in the FSC-SSC plot). **(U&V)** Percentage of total $CD19^+CD49^+$ and $CD19^+CD11a^+$ B cells in the spleen of naïve and allergic mice. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ±S.E.M. n = 4-5 mice/group. * $p < 0.05$; was considered statistically significant when compared to the respective naïve controls. No statistics are given for the lung $CD19^+$ $CD49d^+$ and $CD19^+CD11a^+$ cell populations as the samples were pooled together for FACS analysis.

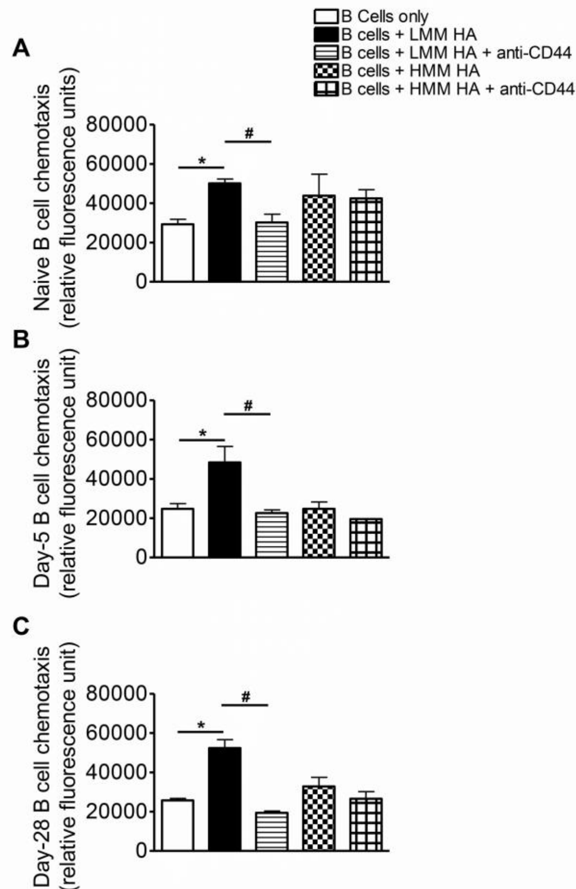

Figure 21. *Ex vivo* CD44-dependent CD19$^+$ B lymphocyte migration. Using a modified Boyden chamber chemotactic activity of isolated spleen CD19$^+$ B lymphocytes towards LMM HA and HMM HA was measured. **(A)** Chemotactic activity of CD19$^+$ B lymphocytes isolated from naïve animal towards LMM HA and HMM HA. **(B)** Chemotactic activity of CD19$^+$ B lymphocytes isolated from an allergic animal at day 5 post second conidia challenge towards LMM HA and HMM HA. **(C)** Chemotactic activity of CD19$^+$ B cells isolated from a day 28 mice post second conidia challenge towards LMM HA and HMM HA. Alternatively, HA's action was blocked by CD44-neutralizing Ab. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ±S.E.M. n = 3, * $p < 0.05$; was considered statistically significant when B cells + LMM HA were compared to B cells only controls. # <0.05 was considered statistically significant when B cells + LMM HA + anti-CD44 were compared to B cells + LMM HA.

HA (40kDa) and HMM HA (500kDa) as the chemoattractant. LMM HA significantly increased the chemotactic activity of isolated B lymphocytes from naïve and allergic animals at day 5 and day 28 post second conidia challenge **(Fig 21A, 21B & 21C)**. On the contrary, high molecular mass HA had no effect on the chemotactic activity of B lymphocytes **(Fig 21A, 21B & 21C)**.

CD44 is considered the best characterized receptor for HA which plays an important role in cell-cell interaction, cell adhesion, and migration. To determine whether CD44 has an impact on B lymphocyte chemotaxis in response to LMM HA we blocked the CD44 receptor using an anti-CD44 neutralizing Ab. Blocking of CD44 decreased the chemotaxis of spleen B lymphocytes isolated from naïve mice towards LMM HA **(Fig 21A)**. Furthermore, chemotaxis of B lymphocytes isolated from allergic mice was decreased when CD44 receptor was blocked (**Fig 21B & 21C)**. These results demonstrate that that LMM HA provides a signal for B lymphocyte chemotaxis that may be acting via CD44.

LMM HA mediated IL-10 and TGF-β1 production by B lymphocytes. B cells have been shown to produce IL-10 which promotes a T_H2 /pro-remodeling phenotype (55). In our murine model system, IL-10 was elevated at day 5 post second conidia challenge in the BAL **(Fig 22A)** of allergic animals and this time point coincides with increased $CD19^+$B lymphocyte numbers in the lung and the spleen **(Fig 18G & 18N)** and increased HA concentrations in BAL and serum **(Fig 17A & 17B)**. To determine whether HA impacted IL-10 cytokine production by B cells we isolated B cells and cultured them with LMM HA and HMM HA and IL-10 production was examined using ELISA. As shown in **Figure 22C**, LMM HA stimulated naïve spleen B cells to produce IL-10 after 48 hours of HA incubation. However, blocking CD44 returns IL-10 production to that of cells alone (statistically) **(Fig 22C)**. This data indicates that LMM HA and B lymphocyte interaction may be involved in IL-10 production by B lymphocytes at the site of chronic inflammation which is independent of CD44 receptor. Another cytokine, which promotes a Th2 and pro-fibrotic phenotype like IL-10, is the pleiotropic growth regulatory factor TGF-β1 (56). Like IL-10, the levels of TGF-β1 were elevated at day 5 post second conidia challenge, when HA levels are elevated in the BAL and lung tissue sections of allergic animals.

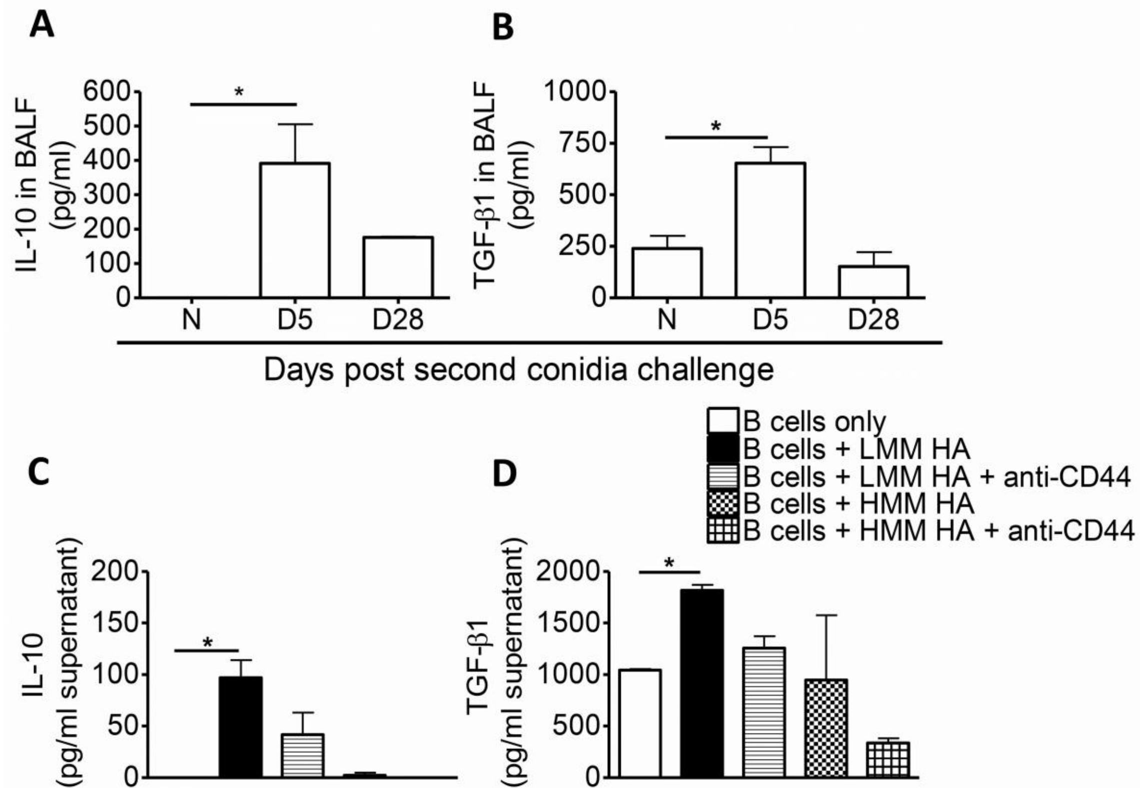

Figure 22. Effect of *A. fumigatus* conidia challenge and hyaluronic acid fragments on IL-10 and TGF-β1 production. (A) Change in IL-10 levels in the BALF of naïve and allergic animals **(B)** Change in TGF-β1 levels in the BALF of naïve and allergic animals. Spleen B lymphocytes were harvested from wild-type C57BL/6 mice. Equal concentrations of isolated B lymphocytes were treated with LMM HA and HMM HA. Supernatant was harvested and analyzed for levels of IL-10 and TGF-β1. **(C)** Represents the IL-10 production by B lymphocytes in response to LMM HA and HMM HA. **(D)** Represents the TGF-β1 production by B lymphocytes in response to LMM HA and HMM HA. Alternatively, HA's action was blocked by CD44-neutralizing Ab. Data analyzed using an unpaired, student's two tailed *t* test with Welch's correction. All values expressed as the mean ±S.E.M. n = 2-5, * $p < 0.05$; was considered statistically significant when compared to the respective naïve controls and when B cells + LMM HA were compared to B cells only controls.

Discussion

In the current study, we show that LMM HA, but not HMM HA, has a pronounced effect on B lymphocyte recruitment in a murine model of allergic asthma. In addition, we show that LMM HA can stimulate B lymphocytes to produce the pro-fibrotic/pro-remodeling cytokines IL-10 and TGF-β1. These results are in agreement with previous data in which LMM HA but not HMM HA has been shown to activate the murine alveolar macrophage cell line MH-S or macrophages recruited to the sites of tissue injury to produce a panel of cytokines (24, 32, 33), and eosinophils to produce TGF-β1 (24). Although, the mechanisms responsible for these different functions of HA based on the different molecular sizes are still unclear. A possible explanation is that that LMM HA may bind firmly to induce receptor crosslinking than does the HMM HA, although, this possibility needs further investigation (24).

HA has been shown to exert direct effects on cells and on the extracellular matrix components suggesting a role of HA in wound repair. The pro-inflammatory cytokines, TNF-α and IL-1β, have been shown to induce cell surface expression of HA (57). Furthermore, HA has been shown to stimulate the migration and proliferation of a number of cell types including smooth muscle cells (58), fibroblasts (59), immune cells (60), and endothelial cells (61). HA also activates monocytes to macrophages (62) and increases cytokine gene expression by macrophages (32) and fibroblasts (63). Increased accumulation of HA has been associated with inflammation associated with acute injury to several organ systems including the lungs. Recent studies have suggested that LMM HA and HMM HA have opposite functions and that LMM HA promotes inflammation and that interaction of HA and its receptors preceded and promotes the inflammatory response to injury (50). Even in our murine fungal model of allergic asthma we report similar findings of increased HA concentrations in serum and BALF of allergic animals

indicating a diseased state. In addition, we show that HA fragments not only impact the functions of macrophages, eosinophils, and fibroblasts it can also affect the function of B lymphocytes suggesting that LMM HA can impact the functions of different cell types in the context of mucosal immunity.

CD44, one of the well characterized HA receptors which has different functions on different cell populations, has been shown to play a vital role in lymphocyte homing, lymphopoiesis, T cell activation, and metastasis (64-70). Studies using CD44-deficient mice challenged with non-infectious lung injury have shown sustained infiltration of inflammatory cells within the alveolar interstitium, increased mortality, and LMM HA accumulation at 14 days, as well as impaired clearance of neutrophils in association with decreased TGF-β1 activation (50) suggesting a role of CD44 in cytokine production. However, very less is known about the functional regulation or expression of CD44 on B cells (43, 71, 72)**.** Previous studies have shown that normal B cells bind to HA to a lower degree than activated B cells, particularly those stimulated by IL-5 (64, 73), and that interferon-γ (IFN-γ) inhibits CD44-Hyaluronan interactions in normal human B lymphocytes (74). In fungal sensitization and challenge, our results show a similar capacity for a CD44-HA interaction by B cells after treatment with *Aspergillus fumigatus* conidia. A possible explanation can be that elevated IL-5 and IL-4 levels in allergic animals which inhibit the production of IFN-γ may promote B lymphocyte – HA interaction via CD44. However, in our model, LMM HA stimulated naïve B cells to undergo chemotaxis indicating that it may directly activate B cells by binding to CD44 **(Fig 23)**. In our fungal model system, production of IL-10 and TGF-β1 cytokines by B lymphocytes was not dependent on CD44 suggesting that other receptors may be involved in regulating this process **(Fig 23)**. It is probable that toll like-receptors 2 and 4 (TLR-2 and TLR-4) present on B cells (75)

may provide the signals for cytokine production as TLR-2 and TLR-4 have been shown to interact with fragmented hyaluronic acid which are increased in the setting of severe trauma and acute lung injury (76), but this remains to be determined.

Studies using repeated aerosol exposure to OVA showed reduced AHR, lung inflammation, and mucus production in muMT mice (77, 78). Alternatively, it has been demonstrated that B lymphocytes mainly contribute in allergic asthma via IgE (79, 80). While allergen-specific antibodies are recognized as contributing factors in the immunopathology of an aberrant response against an innocuous allergen such as pollen or animal dander (81), they have also been suggested to be part of the successful clearance of fungus in the airways (82). However, whether B lymphocytes exacerbate pulmonary inflammation or contribute to lung fibrosis remains unknown. In our murine model of allergic asthma, *A. fumigatus* challenge resulted in an increase in the concentration of HA in the BAL and serum when B cell numbers are elevated in lung and spleen. LMM HA appeared to play a role in the migration of B lymphocytes to the site of tissue injury which was dependent on the CD44 receptor and the activation markers CD49d and CD11b, indicating a possible activation mechanism in B cells through ECM-cell modes at sites of chronic inflammation, including asthma, as it has been suggested for other cell leukocytes. In addition, the LMM HA stimulated B cells to produce the pro-fibrotic/pro-remodeling cytokines IL-10 and TGF-β1. These results suggest that: 1) CD44 on B cells can interact with LMM HA and mediate downstream signaling pathways. 2) B cells may switch to a pro-fibrotic profile when they interact with LMM HA at the site of tissue injury. Collectively, these results support a novel mechanism of localized immune modulation mediated by B lymphocytes when they interact with LMM HA.

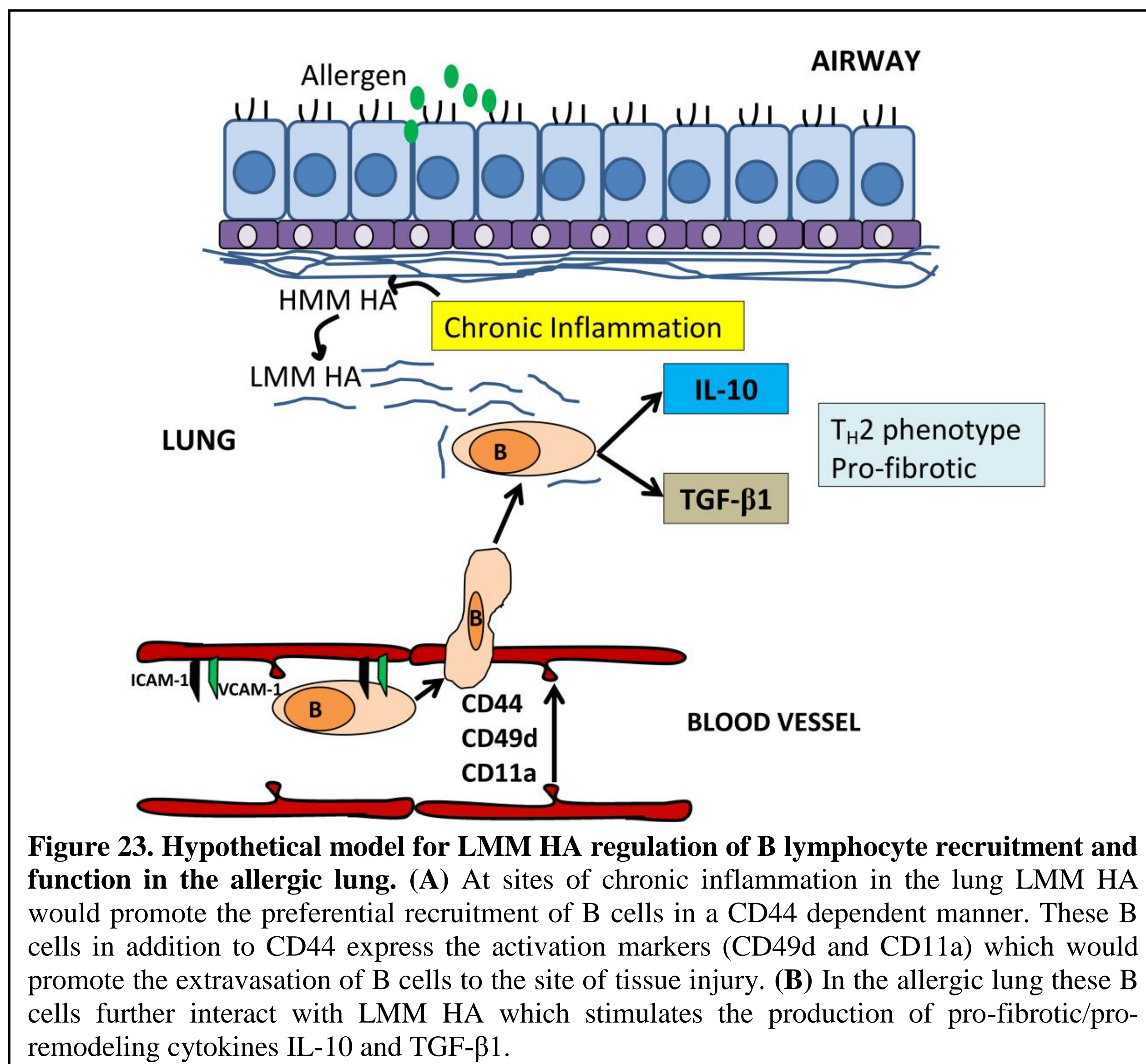

Figure 23. Hypothetical model for LMM HA regulation of B lymphocyte recruitment and function in the allergic lung. (A) At sites of chronic inflammation in the lung LMM HA would promote the preferential recruitment of B cells in a CD44 dependent manner. These B cells in addition to CD44 express the activation markers (CD49d and CD11a) which would promote the extravasation of B cells to the site of tissue injury. **(B)** In the allergic lung these B cells further interact with LMM HA which stimulates the production of pro-fibrotic/pro-remodeling cytokines IL-10 and TGF-β1.

An important outcome of this study is the increased release of TGF-β1 and IL-10 by B lymphocytes, which is crucial in the maintenance of T_H2 responses and crucial in the development of airway remodeling and fibrosis in allergic asthma in response to LMM HA. TGF-β1 has long been established to play a role in airway remodeling (83-85). It has been shown to be associated with epithelial changes, subepithelial fibrosis, airway smooth muscle remodeling, and mucus upregulation (85). While the role of IL-10 in *Aspergillus* induced asthma is controversial, it plays different roles at different stages of disease: an enhancing effect when

conidia dominate and a suppressive effect during the late stages of the disease (86). Furthermore, increased IL-10 production has been associated with an increase in levels of total and specific IgE, IgG1, and IgA reflecting to a role of IL-10 in promoting a T_H2 response to *A. fumigatus* antigens (87-90).

In conclusion, we identified that LMM HA generated at sites of inflammation in the lung in response to fungal challenge may serve to recruit B lymphocytes to the lungs and that this migration is partly dependent on CD44 **(Fig 23)**. In addition, we identified that LMM HA can function as an important signaling molecule for B lymphocytes and can impact the cytokine function of B lymphocytes by inducing the production of IL-10 and TGF-β1 which are critical to the maintenance of allergic inflammatory responses and the development of tissue fibrosis/ airway remodeling **(Fig 23)**. Further characterization of the lineage and the impact of LMM HA on B lymphocyte antibody production and Ag presentation may yield important insights into the function of B lymphocytes and LMM HA in mediating allergic airway responses.

Acknowledgements

The authors wish to thank Aaron Mertens, Akshat Sharma, Breanne Steffan, and Sumali Pandey for assistance with sample collection; Dr. Amali Samarasinghe for providing samples for HA staining; Dr. McCarthy Lab, University of Minnesota for performing the hyaluronic acid staining; and Dr. Pawel Borowicz (North Dakota State University) and the Advanced Imaging and Microscopy Laboratory at North Dakota State University for imaging support using the Zeiss Z1 AxioObserver inverted microscope.

Contribution

The work reported in this paper was conducted by Mr. Sumit Ghosh. The data analysis and manuscript preparation has been done with the help of the co-authors.

References

1. Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay. 1989. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 140: 1745-1753.
2. Kay, A. B. 2005. The role of eosinophils in the pathogenesis of asthma. *Trends Mol Med* 11: 148-152.
3. Busse, W. W., and J. B. Sedgwick. 1992. Eosinophils in asthma. *Ann Allergy* 68: 286-290.
4. Calhoun, W. J., J. Sedgwick, and W. W. Busse. 1991. The role of eosinophils in the pathophysiology of asthma. *Ann N Y Acad Sci* 629: 62-72.
5. Lee, S. Y., S. J. Kim, S. S. Kwon, Y. K. Kim, K. H. Kim, H. S. Moon, J. S. Song, and S. H. Park. 2001. Distribution and cytokine production of CD4 and CD8 T-lymphocyte subsets in patients with acute asthma attacks. *Ann Allergy Asthma Immunol* 86: 659-664.
6. Blease, K., C. Jakubzick, J. Westwick, N. Lukacs, S. L. Kunkel, and C. M. Hogaboam. 2001. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J Immunol* 166: 5219-5224.
7. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263.
8. Kuperman, D. A., X. Huang, L. L. Koth, G. H. Chang, G. M. Dolganov, Z. Zhu, J. A. Elias, D. Sheppard, and D. J. Erle. 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8: 885-889.
9. Tournoy, K. G., J. C. Kips, and R. A. Pauwels. 2001. The allergen-induced airway hyperresponsiveness in a human-mouse chimera model of asthma is T cell and IL-4 and IL-5 dependent. *J Immunol* 166: 6982-6991.
10. Korsgren, M., J. S. Erjefalt, O. Korsgren, F. Sundler, and C. G. Persson. 1997. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *The Journal of experimental medicine* 185: 885-892.
11. Hamelmann, E., K. Takeda, J. Schwarze, A. T. Vella, C. G. Irvin, and E. W. Gelfand. 1999. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *American journal of respiratory cell and molecular biology* 21: 480-489.
12. Lack, G., A. Oshiba, K. L. Bradley, J. E. Loader, D. Amran, G. L. Larsen, and E. W. Gelfand. 1995. Transfer of immediate hypersensitivity and airway hyperresponsiveness by IgE-positive B cells. *Am J Respir Crit Care Med* 152: 1765-1773.
13. Oshiba, A., E. Hamelmann, K. Takeda, K. L. Bradley, J. E. Loader, G. L. Larsen, and E. W. Gelfand. 1996. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *The Journal of clinical investigation* 97: 1398-1408.
14. Lindell, D. M., A. A. Berlin, M. A. Schaller, and N. W. Lukacs. 2008. B cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. *PLoS One* 3: e3129.

15. Liang, J., D. Jiang, Y. Jung, T. Xie, J. Ingram, T. Church, S. Degan, M. Leonard, M. Kraft, and P. W. Noble. Role of hyaluronan and hyaluronan-binding proteins in human asthma. *J Allergy Clin Immunol* 128: 403-411 e403.
16. Raghow, R. 1994. The role of extracellular matrix in postinflammatory wound healing and fibrosis. *FASEB J* 8: 823-831.
17. Erle, D. J., and R. Pytela. 1992. How do integrins integrate? The role of cell adhesion receptors in differentiation and development. *American journal of respiratory cell and molecular biology* 6: 459-460.
18. Akiyama, S. K., S. S. Yamada, W. T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J Cell Biol* 109: 863-875.
19. Frisch, S. M., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124: 619-626.
20. Norris, D. A., R. A. Clark, L. M. Swigart, J. C. Huff, W. L. Weston, and S. E. Howell. 1982. Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. *J Immunol* 129: 1612-1618.
21. Bourguignon, L. Y., V. B. Lokeshwar, X. Chen, and W. G. Kerrick. 1993. Hyaluronic acid-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. *J Immunol* 151: 6634-6644.
22. Hall, C. L., C. Wang, L. A. Lange, and E. A. Turley. 1994. Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. *J Cell Biol* 126: 575-588.
23. Jiang, D., J. Liang, and P. W. Noble. 2007. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol* 23: 435-461.
24. Ohkawara, Y., G. Tamura, T. Iwasaki, A. Tanaka, T. Kikuchi, and K. Shirato. 2000. Activation and transforming growth factor-beta production in eosinophils by hyaluronan. *American journal of respiratory cell and molecular biology* 23: 444-451.
25. Spicer, A. P., and J. A. McDonald. 1998. Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J Biol Chem* 273: 1923-1932.
26. Castor, C. W., T. D. Fremuth, A. M. Furlong, and G. W. Jourdian. 1983. Hyaluronic acid and proteoglycan synthesis by lung fibroblasts in basal and activated states. *In vitro* 19: 462-470.
27. Laurent, T. C., and J. R. Fraser. 1992. Hyaluronan. *FASEB J* 6: 2397-2404.
28. Klagas, I., S. Goulet, G. Karakiulakis, J. Zhong, M. Baraket, J. L. Black, E. Papakonstantinou, and M. Roth. 2009. Decreased hyaluronan in airway smooth muscle cells from patients with asthma and COPD. *Eur Respir J* 34: 616-628.
29. Noble, P. W. 2002. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol* 21: 25-29.
30. Pure, E., and R. K. Assoian. 2009. Rheostatic signaling by CD44 and hyaluronan. *Cell Signal* 21: 651-655.
31. Hodge-Dufour, J., M. W. Marino, M. R. Horton, A. Jungbluth, M. D. Burdick, R. M. Strieter, P. W. Noble, C. A. Hunter, and E. Pure. 1998. Inhibition of interferon gamma induced interleukin 12 production: a potential mechanism for the anti-inflammatory activities of tumor necrosis factor. *Proc Natl Acad Sci U S A* 95: 13806-13811.
32. McKee, C. M., M. B. Penno, M. Cowman, M. D. Burdick, R. M. Strieter, C. Bao, and P. W. Noble. 1996. Hyaluronan (HA) fragments induce chemokine gene expression in

alveolar macrophages. The role of HA size and CD44. *The Journal of clinical investigation* 98: 2403-2413.
33. Hodge-Dufour, J., P. W. Noble, M. R. Horton, C. Bao, M. Wysoka, M. D. Burdick, R. M. Strieter, G. Trinchieri, and E. Pure. 1997. Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. *J Immunol* 159: 2492-2500.
34. McKee, C. M., C. J. Lowenstein, M. R. Horton, J. Wu, C. Bao, B. Y. Chin, A. M. Choi, and P. W. Noble. 1997. Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. *J Biol Chem* 272: 8013-8018.
35. Toole, B. P. 2001. Hyaluronan in morphogenesis. *Semin Cell Dev Biol* 12: 79-87.
36. Toole, B. P., T. N. Wight, and M. I. Tammi. 2002. Hyaluronan-cell interactions in cancer and vascular disease. *J Biol Chem* 277: 4593-4596.
37. Karjalainen, J. M., R. H. Tammi, M. I. Tammi, M. J. Eskelinen, U. M. Agren, J. J. Parkkinen, E. M. Alhava, and V. M. Kosma. 2000. Reduced level of CD44 and hyaluronan associated with unfavorable prognosis in clinical stage I cutaneous melanoma. *Am J Pathol* 157: 957-965.
38. Tzircotis, G., R. F. Thorne, and C. M. Isacke. 2005. Chemotaxis towards hyaluronan is dependent on CD44 expression and modulated by cell type variation in CD44-hyaluronan binding. *J Cell Sci* 118: 5119-5128.
39. Sano, K., K. Yamauchi, H. Hoshi, M. Honma, G. Tamura, and K. Shirato. 1997. CD44 expression on blood eosinophils is a novel marker of bronchial asthma. *Int Arch Allergy Immunol* 114 Suppl 1: 67-71.
40. Matsumoto, K., J. Appiah-Pippim, R. P. Schleimer, C. A. Bickel, L. A. Beck, and B. S. Bochner. 1998. CD44 and CD69 represent different types of cell-surface activation markers for human eosinophils. *American journal of respiratory cell and molecular biology* 18: 860-866.
41. Protin, U., T. Schweighoffer, W. Jochum, and F. Hilberg. 1999. CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. *J Immunol* 163: 4917-4923.
42. Schmits, R., J. Filmus, N. Gerwin, G. Senaldi, F. Kiefer, T. Kundig, A. Wakeham, A. Shahinian, C. Catzavelos, J. Rak, C. Furlonger, A. Zakarian, J. J. Simard, P. S. Ohashi, C. J. Paige, J. C. Gutierrez-Ramos, and T. W. Mak. 1997. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* 90: 2217-2233.
43. Rafi, A., M. Nagarkatti, and P. S. Nagarkatti. 1997. Hyaluronate-CD44 interactions can induce murine B-cell activation. *Blood* 89: 2901-2908.
44. Hogaboam, C. M., K. Blease, B. Mehrad, M. L. Steinhauser, T. J. Standiford, S. L. Kunkel, and N. W. Lukacs. 2000. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. *Am J Pathol* 156: 723-732.
45. Hoselton, S. A., A. E. Samarasinghe, J. M. Seydel, and J. M. Schuh. An inhalation model of airway allergic response to inhalation of environmental Aspergillus fumigatus conidia in sensitized BALB/c mice. *Med Mycol* 48: 1056-1065.
46. Simpson, M. A., C. M. Wilson, and J. B. McCarthy. 2002. Inhibition of prostate tumor cell hyaluronan synthesis impairs subcutaneous growth and vascularization in immunocompromised mice. *Am J Pathol* 161: 849-857.

47. Mikus, L. D., L. A. Rosenthal, R. L. Sorkness, and R. F. Lemanske, Jr. 2001. Reduced interferon-gamma secretion by natural killer cells from rats susceptible to postviral chronic airway dysfunction. *American journal of respiratory cell and molecular biology* 24: 74-82.
48. Vachon, E., R. Martin, J. Plumb, V. Kwok, R. W. Vandivier, M. Glogauer, A. Kapus, X. Wang, C. W. Chow, S. Grinstein, and G. P. Downey. 2006. CD44 is a phagocytic receptor. *Blood* 107: 4149-4158.
49. Yamawaki, H., S. Hirohata, T. Miyoshi, K. Takahashi, H. Ogawa, R. Shinohata, K. Demircan, S. Kusachi, K. Yamamoto, and Y. Ninomiya. 2009. Hyaluronan receptors involved in cytokine induction in monocytes. *Glycobiology* 19: 83-92.
50. Zaman, A., Z. Cui, J. P. Foley, H. Zhao, P. C. Grimm, H. M. Delisser, and R. C. Savani. 2005. Expression and role of the hyaluronan receptor RHAMM in inflammation after bleomycin injury. *American journal of respiratory cell and molecular biology* 33: 447-454.
51. Tamoto, K., H. Nochi, M. Tada, S. Shimada, Y. Mori, S. Kataoka, Y. Suzuki, and T. Nakamura. 1994. High-molecular-weight hyaluronic acids inhibit chemotaxis and phagocytosis but not lysosomal enzyme release induced by receptor-mediated stimulations in guinea pig phagocytes. *Microbiology and immunology* 38: 73-80.
52. Noble, P. W., and D. Jiang. 2006. Matrix regulation of lung injury, inflammation, and repair: the role of innate immunity. *Proceedings of the American Thoracic Society* 3: 401-404.
53. Sahu, S., and W. S. Lynn. 1978. Hyaluronic acid in the pulmonary secretions of patients with asthma. *Biochem J* 173: 565-568.
54. Sahu, S. C. 1980. Hyaluronic acid. An indicator of pathological conditions of human lungs? *Inflammation* 4: 107-112.
55. Watanabe, R., N. Ishiura, H. Nakashima, Y. Kuwano, H. Okochi, K. Tamaki, S. Sato, T. F. Tedder, and M. Fujimoto. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *J Immunol* 184: 4801-4809.
56. Commodaro, A. G., J. P. Peron, J. Genre, C. Arslanian, L. Sanches, C. Muccioli, L. V. Rizzo, and R. Belfort, Jr. IL-10 and TGF-beta immunoregulatory cytokines rather than natural regulatory T cells are associated with the resolution phase of Vogt-Koyanagi-Harada (VKH) syndrome. *Scand J Immunol* 72: 31-37.
57. Mohamadzadeh, M., H. DeGrendele, H. Arizpe, P. Estess, and M. Siegelman. 1998. Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *The Journal of clinical investigation* 101: 97-108.
58. Savani, R. C., C. Wang, B. Yang, S. Zhang, M. G. Kinsella, T. N. Wight, R. Stern, D. M. Nance, and E. A. Turley. 1995. Migration of bovine aortic smooth muscle cells after wounding injury. The role of hyaluronan and RHAMM. *The Journal of clinical investigation* 95: 1158-1168.
59. Hardwick, C., K. Hoare, R. Owens, H. P. Hohn, M. Hook, D. Moore, V. Cripps, L. Austen, D. M. Nance, and E. A. Turley. 1992. Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. *J Cell Biol* 117: 1343-1350.
60. Savani, R. C., N. Khalil, and E. A. Turley. 1995. Hyaluronan receptor antagonists alter skin inflammation and fibrosis following injury. *Proc West Pharmacol Soc* 38: 131-136.

61. Trochon, V., C. Mabilat, P. Bertrand, Y. Legrand, F. Smadja-Joffe, C. Soria, B. Delpech, and H. Lu. 1996. Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro. *Int J Cancer* 66: 664-668.
62. Noble, P. W., F. R. Lake, P. M. Henson, and D. W. Riches. 1993. Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor-alpha-dependent mechanism in murine macrophages. *The Journal of clinical investigation* 91: 2368-2377.
63. Kobayashi, H., and T. Terao. 1997. Hyaluronic acid-specific regulation of cytokines by human uterine fibroblasts. *Am J Physiol* 273: C1151-1159.
64. Lesley, J., R. Hyman, and P. W. Kincade. 1993. CD44 and its interaction with extracellular matrix. *Adv Immunol* 54: 271-335.
65. Herrlich, P., M. Zoller, S. T. Pals, and H. Ponta. 1993. CD44 splice variants: metastases meet lymphocytes. *Immunol Today* 14: 395-399.
66. Naor, D., R. V. Sionov, and D. Ish-Shalom. 1997. CD44: structure, function, and association with the malignant process. *Adv Cancer Res* 71: 241-319.
67. Lesley, J., N. Howes, A. Perschl, and R. Hyman. 1994. Hyaluronan binding function of CD44 is transiently activated on T cells during an in vivo immune response. *The Journal of experimental medicine* 180: 383-387.
68. Shimizu, Y., G. A. Van Seventer, R. Siraganian, L. Wahl, and S. Shaw. 1989. Dual role of the CD44 molecule in T cell adhesion and activation. *J Immunol* 143: 2457-2463.
69. DeGrendele, H. C., P. Estess, L. J. Picker, and M. H. Siegelman. 1996. CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. *The Journal of experimental medicine* 183: 1119-1130.
70. DeGrendele, H. C., P. Estess, and M. H. Siegelman. 1997. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 278: 672-675.
71. Murakami, S., K. Miyake, C. H. June, P. W. Kincade, and R. J. Hodes. 1990. IL-5 induces a Pgp-1 (CD44) bright B cell subpopulation that is highly enriched in proliferative and Ig secretory activity and binds to hyaluronate. *J Immunol* 145: 3618-3627.
72. Kryworuckho, M., F. Diaz-Mitoma, and A. Kumar. 1995. CD44 isoforms containing exons V6 and V7 are differentially expressed on mitogenically stimulated normal and Epstein-Barr virus-transformed human B cells. *Immunology* 86: 41-48.
73. Hathcock, K. S., H. Hirano, S. Murakami, and R. J. Hodes. 1993. CD44 expression on activated B cells. Differential capacity for CD44-dependent binding to hyaluronic acid. *J Immunol* 151: 6712-6722.
74. Marko Kryworuchko, F. D.-M. a. A. K. 1999. Interferon-gamma inhibits CD44-Hyaluronan interactions in Normal Human B Lymphocytes. *Experimental Cell Research*: 241-252.
75. Chassin, C., M. Picardeau, J. M. Goujon, P. Bourhy, N. Quellard, S. Darche, E. Badell, M. F. d'Andon, N. Winter, S. Lacroix-Lamande, D. Buzoni-Gatel, A. Vandewalle, and C. Werts. 2009. TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial pathogen, Leptospira interrogans. *J Immunol* 183: 2669-2677.
76. Lorne, E., H. Dupont, and E. Abraham. Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine? *Intensive Care Med* 36: 1826-1835.

77. Jungsuwadee, P., M. Benkovszky, G. Dekan, G. Stingl, and M. M. Epstein. 2004. Repeated aerosol allergen exposure suppresses inflammation in B-cell-deficient mice with established allergic asthma. *Int Arch Allergy Immunol* 133: 40-48.
78. Hamelmann, E., A. T. Vella, A. Oshiba, J. W. Kappler, P. Marrack, and E. W. Gelfand. 1997. Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proc Natl Acad Sci U S A* 94: 1350-1355.
79. Laitinen, L. A., A. Laitinen, and T. Haahtela. 1993. Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am Rev Respir Dis* 147: 697-704.
80. Sears, M. R., B. Burrows, E. M. Flannery, G. P. Herbison, C. J. Hewitt, and M. D. Holdaway. 1991. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 325: 1067-1071.
81. Knutsen, A. P. Immunopathology and immunogenetics of allergic bronchopulmonary aspergillosis. *J Allergy (Cairo)* 2011: 785983.
82. Rapaka, R. R., D. M. Ricks, J. F. Alcorn, K. Chen, S. A. Khader, M. Zheng, S. Plevy, E. Bengten, and J. K. Kolls. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina. *The Journal of experimental medicine* 207: 2907-2919.
83. Minshall, E. M., D. Y. Leung, R. J. Martin, Y. L. Song, L. Cameron, P. Ernst, and Q. Hamid. 1997. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *American journal of respiratory cell and molecular biology* 17: 326-333.
84. Doherty, T., and D. Broide. 2007. Cytokines and growth factors in airway remodeling in asthma. *Curr Opin Immunol* 19: 676-680.
85. Halwani, R., S. Al-Muhsen, H. Al-Jahdali, and Q. Hamid. Role of transforming growth factor-beta in airway remodeling in asthma. *American journal of respiratory cell and molecular biology* 44: 127-133.
86. Latge, J. P. 1999. Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev* 12: 310-350.
87. Kurup, V. P., G. Grunig, A. P. Knutsen, and P. S. Murali. 1998. Cytokines in allergic bronchopulmonary aspergillosis. *Res Immunol* 149: 466-477; discussion 515-466.
88. Kurup, V. P., V. Hari, J. Guo, P. S. Murali, A. Resnick, M. Krishnan, and J. N. Fink. 1996. Aspergillus fumigatus peptides differentially express Th1 and Th2 cytokines. *Peptides* 17: 183-190.
89. Kurup, V. P., B. W. Seymour, H. Choi, and R. L. Coffman. 1994. Particulate Aspergillus fumigatus antigens elicit a TH2 response in BALB/c mice. *J Allergy Clin Immunol* 93: 1013-1020.
90. Wang, J. M., M. Denis, M. Fournier, and M. Laviolette. 1994. Experimental allergic bronchopulmonary aspergillosis in the mouse: immunological and histological features. *Scand J Immunol* 39: 19-26.

GENERAL DISCUSSION

Of the estimated 3.5-5.1 million fungal species that are predicted from high throughput environmental screening (198), *Aspergillus* species are among the most well recognized and best studied fungal pathogens of humans and animals. Exposure to *Aspergillus* can result in a range of disease states from allergic rhinitis to invasive aspergillosis. The growth habit and physical characteristics of *A. fumigatus* make it an ideal aeroallergen and opportunistic pathogen of humans. Allergic fungal asthma is a chronic disease that is important from both a personal and public perspective. Sensitization and colonization by this fungal species often signals a disease course that is particularly difficult to treat and results in chronic architecture changes in the lung causing long-term morbidity (199, 200), reduced productivity and quality of life, as well as increased burden for medical treatment.

In hosts with competent immune systems that encounter low-level airborne *Aspergillus*, the innate immune system bars fungal infection and, when it fails to fully arrest the fungus, adaptive responses function to eliminate fungal pathogens (201). While it is evident that cell-mediated responses are critical to successful fungal clearance (202), the relative importance of Ab-mediated responses to fungal pathogens has been more difficult to establish (203, 204). Research has shown that B cells contribute to the phenotype of allergic asthma through the production of IgE Abs (205), although this seems to have little impact on the eosinophilia or AHR that accompanies allergic asthma (206, 207). Most of the research on B cells in asthma has dealt with Ab production, and little is known about the role of these cells in the allergic lung.

B lymphocytes mediate humoral immunity and can perform three important functions. In addition to producing Ab, B cells present Ag to T cells to indirectly support the allergic phenotype. They can also produce cytokines to directly and indirectly exacerbate airway wall

remodeling (94) and impact the function of other cells in the local environment, suggesting that these cells can play an important role in the development and maintenance of allergic disease. During the early and late-phase asthmatic reaction, B cells in all stages of activation and differentiation are found in increased numbers in the blood of asthmatic patients (155). B cells are also present within the alveolar spaces and pulmonary parenchyma of asthmatics (143, 208).

Secretory IgA has been shown to play a role in the innate mucosal response that protects the upper respiratory tract (144-146), and selective IgA deficiency in clinic patients is associated with an increased prevalence of atopy (147, 148). The IgG subtype, IgG1, which is a T_H2-elicited antibody, is cytophillic to mast cells (149); and IgG_{2a}, which is produced by T_H1-activated B cells, plays a role in host protection against fungal growth (150). While, IgE Abs have been shown to induce mast cell degranulation (151). IgE has long been recognized as a perpetrator of asthma exacerbations, and anti-IgE therapies have been used successfully for treatment (152-154). While allergen-specific Abs are recognized as contributing factors in the immunopathology of an aberrant response against an innocuous allergen such as pollen or animal dander (159), they have also been suggested to be part of the successful clearance of fungus from the airways (160, 161).

The results presented in this dissertation show for the first time that *A. fumigatus* sensitization and inhalation triggers the recruitment of $CD19^+CD23^+$ B2 lymphocytes to the allergic lung and that they secrete immunoglobulins, placing them in the appropriate context for participation in the development and maintenance of allergic responses **(Fig 24)**. We demonstrate that IgA- and IgE-producing cells are localized around the large airways. The secretion of IgA is triggered after allergen challenge with inhaled fungal spores, which supports

its role as a mucosal defense molecule that is pumped across the columnar epithelium in an attempt to block fungal infection.

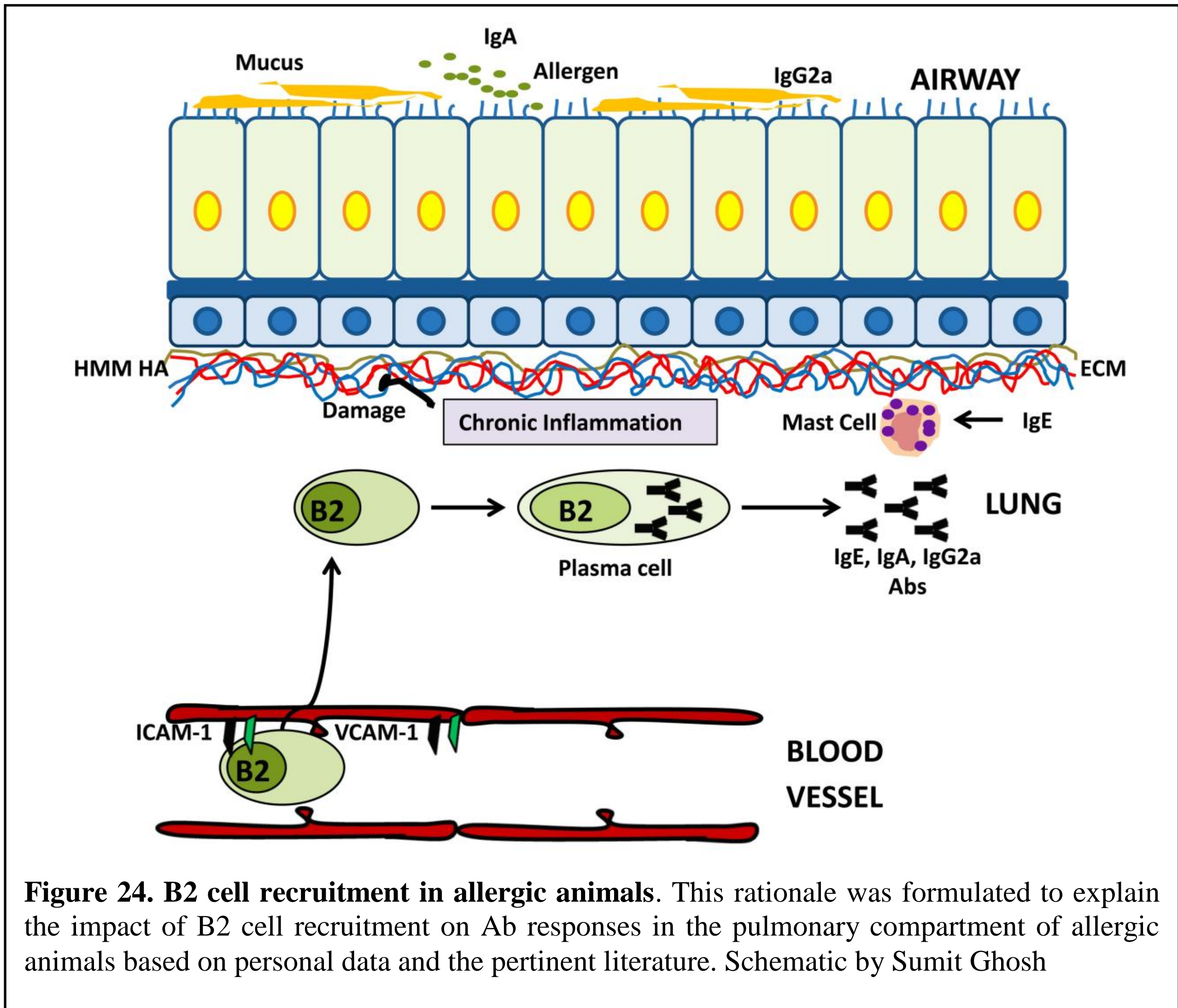

Figure 24. B2 cell recruitment in allergic animals. This rationale was formulated to explain the impact of B2 cell recruitment on Ab responses in the pulmonary compartment of allergic animals based on personal data and the pertinent literature. Schematic by Sumit Ghosh

IgE levels corresponded with increased IgA in serum and BAL in these studies. While IgE+ cells were very rare in naïve lung samples, IgE-producing cells were localized around the large airways of allergic animals. IgE is a hallmark of T_H2-mediated allergic sensitization in the adaptive immune response and has the ability to crosslink Ag to elicit granulocyte activation and degranulation. IgG_{2a}, which is produced by T_H1-associated B cells (150), was elevated throughout the studies indicating its importance in host protection against fungal growth.

This research on the determination of the recruitment of B2 cells in the lungs makes an important contribution to the literature. The spatial and temporal availability of B cells in the lung is the first step in elucidating their function in potentially interacting with T lymphocytes at the point of infection or affecting the cytokine milieu of the lungs, in addition to Ab production (204). The cellular interplay between cells in allergic asthma is sufficiently complex that it will require examining this syndrome with a number of strategies. One of these strategies would be to identifying the signals that differentiate which lymphocytes are recruited to various areas of the lungs in an intact mouse system. Other elements that will help to address the potential contributions of B cells to the overall phenotype of asthma will include an evaluation of MHC II and cytokine expression that may impact T cell clones' activation in the tissues and activation of other immune cells or structural cells in the development of immunopathologies associated with fungal asthma.

Knockout animals are a time-honored method of demonstrating the importance of a gene product or cell type in a disease process. We attempted this strategy using a μMT B cell KO system. It is important to note that a knockout system does not have all of the components of normal development. As such, the muMT system was employed to give a general idea of the requirement of B cells in the generation and maintenance of allergic fungal asthma, but there is always the variable of system artifact that is an inherent with a KO system to be considered. This was very evident when using the muMT "B cell KO" mouse. We discovered that, not only did the mouse have B-1 B cells, but these cells produced Abs in significant quantities.

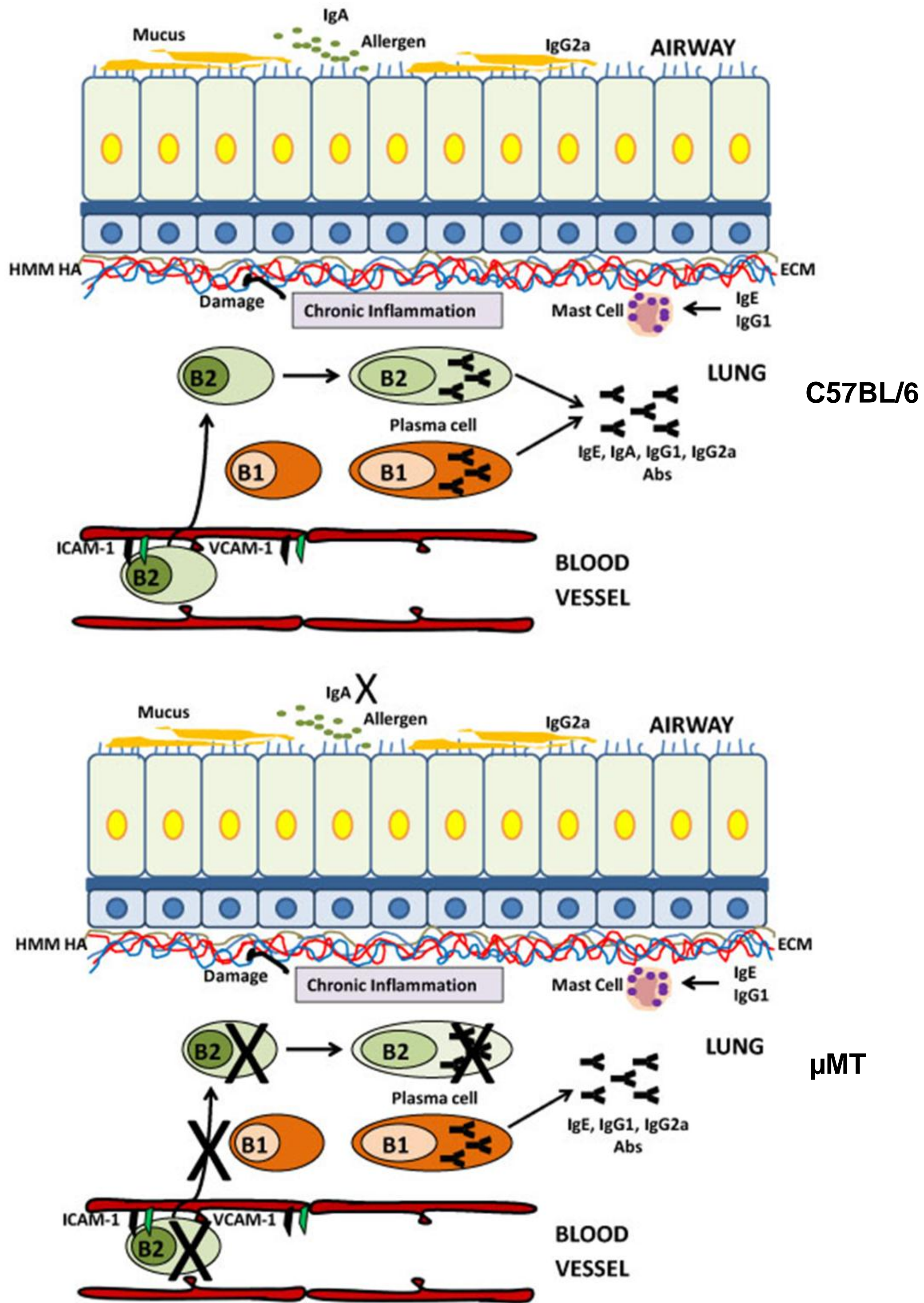

Figure 25. Immunoregulation by B1 cells in the absence of B2 cells in µMT mice. The rationale was formulated to explain the differences between the C57BL/6 and µMT animals based on personal data and the pertinent literature. Schematic by Sumit Ghosh.

The canonical pathway of B cell development requires the surface expression of the μ Ig chain at an early pre-B cell stage (209) and mice lacking this μ chain, known as μMT mice, have been useful tools in understanding the complex biological processes associated with B cell/Ab deficiency in different diseases. Using μMT mice in our fungal allergy model system, we report for the first time the existence of only $CD19^{+}CD9^{+}IgD^{+}$ B-1 cells in the lungs of the μMT animals and that even in the absence of B-2 cells the lung pathophysiology can be maintained in μMT mice **(Fig 25)**. We also report a tissue centric IgG1, IgG2a, and IgE Ab production in μMT mice after sensitization and challenge with fungal Ags in a non-specific manner. Additionally, the study shows that IgA, which plays an important role in protecting mucosal surfaces, is not required for either clearance or containment of *A. fumigatus* in the murine lung, since fungal outgrowth was not observed in the muMT animals after multiple inhalation exposures to live conidia.

Using μMT mice in our inhalation fungal asthma system has two important implications. First, the type of Ag and the route of infection have a significant impact on the generation of Ab responses in μMT mice lacking the normal pathway for B-2 cell maturation, as different types of Ag yield very different outcomes (210, 211). The second major finding of this study is that, as a B-2 cell KO mouse, μMT animals may be useful to determine the role of B-1 cells in response to pulmonary insults. Further research is necessary for elucidating the mechanism for B-1 isotype switching in the lung and their contribution to protective responses, which would have important implications for experimental analysis and for understanding normal B-1 and B-2 cell activation in health and disease.

One of the important benefits of using an experimental animal system to model a complex disease syndrome like asthma is that you can recreate a similar environment to that of the human allergic lung. This has been very important in the research we are doing with HA in the activation of B cells in the allergic lung. Over the past few years, a number of studies have shown that ECM and ECM degradation products may be associated with airway fibrosis and a decline in lung function (24, 173, 212). An accumulation of ECM components can be seen in tissues following a variety of insults such as those that occur in the adult respiratory distress syndrome (ARDS), chronic asthma, and idiopathic pulmonary fibrosis (180). Among the different ECM components studied, HA undergoes dynamic regulation resulting in accumulation of lower molecular mass species during tissue injury and inflammation (22, 24, 181, 185, 212, 213). We envision the waves of granulocytes (neutrophils and eosinophils) hydrolyzing HA as they enter the lumen of the airway as having a major impact on this process. LMM HA has been shown to exhibit pronounced biologic effects on specific cells and tissues (213, 214). The mechanisms that dictate the different functions of HA based on the different molecular size are still being determined, but a possible explanation for LMM HA's role in inflammation is that it may bind more firmly than does the HMM HA, inducing receptor crosslinking and allowing signaling (181).

CD44, the most widely studied HA receptor, has different functions on different cell populations and has been shown to play a vital role in lymphocyte homing, lymphopoiesis, T cell activation, and metastasis in certain situations (215-221). Its expression and functional regulation on B cells is less well understood (196, 222, 223). Previous studies have shown that normal B cells bind to HA to a lower degree than activated B cells, particularly those stimulated by IL-5 (215, 224), and that IFN-γ inhibits CD44-HA interactions in normal human B lymphocytes

(225). Additionally, CD44-deficient mice develop and exhibit minor abnormalities in hematopoiesis and lymphocyte circulation (194, 195). However, there is growing evidence to suggest that interaction between HA and CD44 may play an important role in the regulation of functions of lymphoid and myeloid cells (196).

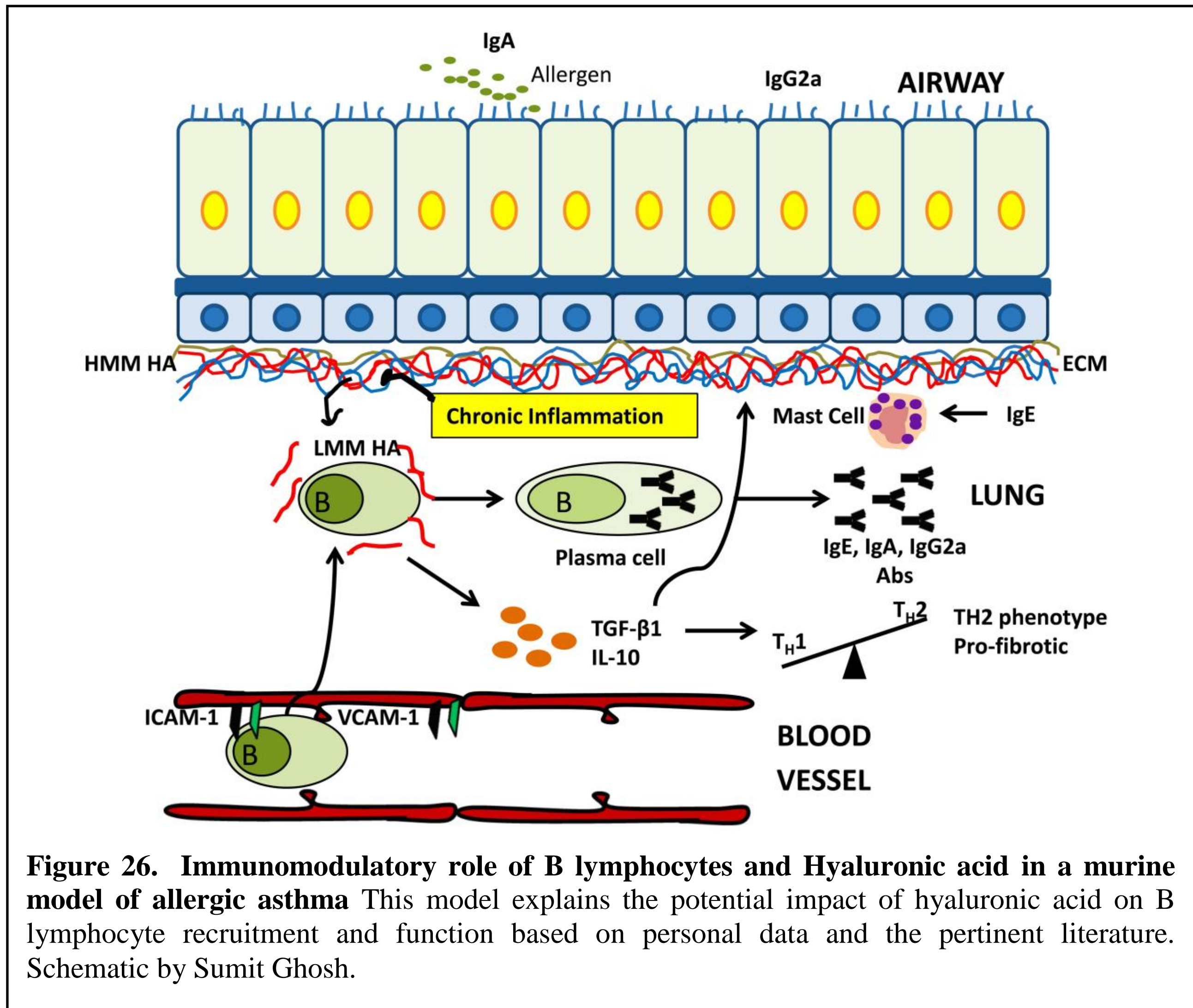

Figure 26. Immunomodulatory role of B lymphocytes and Hyaluronic acid in a murine model of allergic asthma This model explains the potential impact of hyaluronic acid on B lymphocyte recruitment and function based on personal data and the pertinent literature. Schematic by Sumit Ghosh.

While allergen-specific Abs are recognized as contributing factors in the immunopathology of an aberrant response against an innocuous allergen such as fungi, pollen or animal dander (159), they have also been suggested to be part of the successful clearance of fungus in the airways (160). Whether B lymphocytes exacerbate pulmonary inflammation or

contribute to lung fibrosis remains still unknown. The data presented in this dissertation imply that LMM HA generated at sites of chronic inflammation may serve to recruit B lymphocytes to the lungs in a CD44 dependent manner **(Fig 26)**. In addition, we report that LMM HA can function as an important signaling molecule for B lymphocytes and can impact the cytokine function of B lymphocytes by inducing the production of IL-10 and TGF-β1 which are critical to the development of tissue fibrosis/airway remodeling **(Fig 26)**. Further studies, on how LMM HA mediates cytokine production by B lymphocytes would be of great interest. Emerging evidence shows that TLR signaling plays an important role in mediating immune responses to HA (24, 226).

TGF-β1 has long been established to play a role in airway remodeling (227-229). It has been shown to be associated with epithelial changes, sub-epithelial fibrosis, airway smooth muscle remodeling, and mucus upregulation (229). The role of IL-10 in *Aspergillus*-induced asthma has not been established definitively, as it seems to play different roles at different stages of disease; an enhancing effect when conidia dominate and a suppressive effect during the late stages of the disease (118). Furthermore, increased IL-10 production has been associated with an increase in levels of total and Ag-specific IgE, IgG1, and IgA, probably reflecting its role in promoting a T_H2 response to *A. fumigatus* Ags (119-122). Further characterization of the lineage of B lymphocytes, the receptors involved in cytokine production, and the impact of LMM HA on B lymphocyte antibody function and Ag presentation may yield important insights into the function of B lymphocytes and LMM HA in mediating allergic airway responses.

CONCLUSIONS

Our aim for this dissertation was to begin to elucidate the role of B lymphocytes in the initiation and maintenance of allergic asthma. Our studies reveal that B2 cells are recruited to the lungs in response to fungal challenge and that B-1 cells are triggered to produce Ab even in the absence of conventional B-2 lymphocytes. Further, our studies reveal the role of LMM HA (a component of ECM) generated at the sites of chronic inflammation/tissue injury in facilitating B lymphocyte recruitment. Further, LMM HA induces B cell activation through the production of the pro-fibrotic/pro-remodeling cytokines IL-10 and TGF-β1, which are critical to the maintenance of the allergic inflammatory responses. Collectively, these results indicate that both different B cell subsets and LMM HA may contribute to the development and maintenance of the allergic phenotype. Further studies using a total B cell knockout mouse, characterizing the lineage of B cells, and exploring the impact of LMM HA on B lymphocyte Ab production function and Ag presentation may yield important insights into the function of B lymphocytes in mediating allergic airway responses and may help to develop new therapeutic targets for diseases of the mucosa such as asthma. Beyond allergic asthma research, this work may also provide important information about anatomical and functional aspects of general B cell development that are currently not known.

REFERENCES CITED IN THE LITERATURE REVIEW AND THE GENERAL DISCUSSION

1. Types of Asthma, http://www.webmd.com/asthma/guide/types-asthma.
2. Types of Asthma, http://asthma.about.com/od/typesofasthma/Types_of_Asthma_Asthma_Classifications_and_More.htm.
3. 2011. Asthma Fact Sheet, http://www.who.int/mediacentre/factsheets/fs307/en/index.html.
4. 2009. Center For Disease Control and Prevention. National Center For Heath Statistics. Final Vital Statistics Report. Deaths: Final Data for 2007.
5. 2011. The Asthma Foundation, http://www.asthmanz.co.nz/.
6. Asthma Facts, http://www.nhlbi.nih.gov/guidelines/asthma/.
7. Barnes, P. J. 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 118: 3546-3556.
8. Barnett, S. B., and T. A. Nurmagambetov. 2011. Costs of asthma in the United States: 2002-2007. *J Allergy Clin Immunol* 127: 145-152.
9. Porter, P. C., V. Ongeri, A. Luong, F. Kheradmand, and D. B. Corry. 2011. Seeking common pathophysiology in asthma, atopy and sinusitis. *Trends Immunol* 32: 43-49.
10. Eisenbarth, S. C., S. Cassel, and K. Bottomly. 2004. Understanding asthma pathogenesis: linking innate and adaptive immunity. *Current opinion in pediatrics* 16: 659-666.
11. Pichavant, M., A. S. Charbonnier, S. Taront, A. Brichet, B. Wallaert, J. Pestel, A. B. Tonnel, and P. Gosset. 2005. Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J Allergy Clin Immunol* 115: 771-778.
12. Royce, S. G., L. Tan, A. A. Koek, and M. L. Tang. 2009. Effect of extracellular matrix composition on airway epithelial cell and fibroblast structure: implications for airway remodeling in asthma. *Ann Allergy Asthma Immunol* 102: 238-246.
13. Kidney, J. C., A. G. Wong, A. Efthimiadis, M. M. Morris, M. R. Sears, J. Dolovich, and F. E. Hargreave. 1996. Elevated B cells in sputum of asthmatics. Close correlation with eosinophils. *American journal of respiratory and critical care medicine* 153: 540-544.
14. Vallor, A. C., W. R. Kirkpatrick, L. K. Najvar, R. Bocanegra, M. C. Kinney, A. W. Fothergill, M. L. Herrera, B. L. Wickes, J. R. Graybill, and T. F. Patterson. 2008. Assessment of Aspergillus fumigatus burden in pulmonary tissue of guinea pigs by quantitative PCR, galactomannan enzyme immunoassay, and quantitative culture. *Antimicrobial agents and chemotherapy* 52: 2593-2598.
15. Schuh, J. M., K. Blease, and C. M. Hogaboam. 2002. CXCR2 is necessary for the development and persistence of chronic fungal asthma in mice. *J Immunol* 168: 1447-1456.
16. Barnes, P. J. 2008. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 8: 183-192.
17. Saavedra-Delgado, A. M., and S. G. Cohen. 1991. Huang-Ti, the Yellow Emperor and the Nei Ching: antiquity's earliest reference to asthma. *Allergy Proc* 12: 197-198.
18. 2009. Center for Disease Control and Prevention, 2009.

19. Eder, W., M. J. Ege, and E. von Mutius. 2006. The asthma epidemic. *N Engl J Med* 355: 2226-2235.
20. Lotvall, J., L. Ekerljung, E. P. Ronmark, G. Wennergren, A. Linden, E. Ronmark, K. Toren, and B. Lundback. 2009. West Sweden Asthma Study: prevalence trends over the last 18 years argues no recent increase in asthma. *Respir Res* 10: 94.
21. Georas, S. N., J. Guo, U. De Fanis, and V. Casolaro. 2005. T-helper cell type-2 regulation in allergic disease. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 26: 1119-1137.
22. Noble, P. W., and D. Jiang. 2006. Matrix regulation of lung injury, inflammation, and repair: the role of innate immunity. *Proceedings of the American Thoracic Society* 3: 401-404.
23. Martinez, F. D., and P. J. Helms. 1998. Types of asthma and wheezing. *Eur Respir J Suppl* 27: 3s-8s.
24. Jiang, D., J. Liang, J. Fan, S. Yu, S. Chen, Y. Luo, G. D. Prestwich, M. M. Mascarenhas, H. G. Garg, D. A. Quinn, R. J. Homer, D. R. Goldstein, R. Bucala, P. J. Lee, R. Medzhitov, and P. W. Noble. 2005. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med* 11: 1173-1179.
25. 2011. World Health Organization.
26. Verstraelen, S., K. Bloemen, I. Nelissen, H. Witters, G. Schoeters, and R. Van Den Heuvel. 2008. Cell types involved in allergic asthma and their use in in vitro models to assess respiratory sensitization. *Toxicology in vitro : an international journal published in association with BIBRA* 22: 1419-1431.
27. Arshad, S. H., and S. Holgate. 2001. The role of IgE in allergen-induced inflammation and the potential for intervention with a humanized monoclonal anti-IgE antibody. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 31: 1344-1351.
28. Xiang, Z., M. Block, C. Lofman, and G. Nilsson. 2001. IgE-mediated mast cell degranulation and recovery monitored by time-lapse photography. *J Allergy Clin Immunol* 108: 116-121.
29. Greiff, L., I. Erjefalt, C. Svensson, P. Wollmer, U. Alkner, M. Andersson, and C. G. Persson. 1993. Plasma exudation and solute absorption across the airway mucosa. *Clinical physiology (Oxford, England)* 13: 219-233.
30. De Monchy, J. G., H. F. Kauffman, P. Venge, G. H. Koeter, H. M. Jansen, H. J. Sluiter, and K. De Vries. 1985. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *The American review of respiratory disease* 131: 373-376.
31. Holgate, S. T. 1993. The 1992 Cournand Lecture. Asthma: past, present and future. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 6: 1507-1520.
32. Bousquet, J., P. K. Jeffery, W. W. Busse, M. Johnson, and A. M. Vignola. 2000. Asthma. From bronchoconstriction to airways inflammation and remodeling. *American journal of respiratory and critical care medicine* 161: 1720-1745.
33. Woolcock, A. J., S. D. Anderson, J. K. Peat, J. I. Du Toit, Y. G. Zhang, C. M. Smith, and C. M. Salome. 1991. Characteristics of bronchial hyperresponsiveness in chronic obstructive pulmonary disease and in asthma. *The American review of respiratory disease* 143: 1438-1443.

34. Hargreave, F. E., P. M. O'Byrne, and E. H. Ramsdale. 1985. Mediators, airway responsiveness, and asthma. *J Allergy Clin Immunol* 76: 272-276.
35. Bousquet, J., P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, and et al. 1990. Eosinophilic inflammation in asthma. *N Engl J Med* 323: 1033-1039.
36. Venkatesha, R. T., E. Berla Thangam, A. K. Zaidi, and H. Ali. 2005. Distinct regulation of C3a-induced MCP-1/CCL2 and RANTES/CCL5 production in human mast cells by extracellular signal regulated kinase and PI3 kinase. *Mol Immunol* 42: 581-587.
37. Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J Clin Pathol* 54: 577-589.
38. Stone, K. D., C. Prussin, and D. D. Metcalfe. 2010. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 125: S73-80.
39. Chang, T. W., F. M. Davis, N. C. Sun, C. R. Sun, D. W. MacGlashan, Jr., and R. G. Hamilton. 1990. Monoclonal antibodies specific for human IgE-producing B cells: a potential therapeutic for IgE-mediated allergic diseases. *Biotechnology (N Y)* 8: 122-126.
40. Brightling, C. E., F. A. Symon, S. S. Birring, P. Bradding, I. D. Pavord, and A. J. Wardlaw. 2002. TH2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 110: 899-905.
41. Nouri-Aria, K. T., A. M. Irani, M. R. Jacobson, F. O'Brien, E. M. Varga, S. J. Till, S. R. Durham, and L. B. Schwartz. 2001. Basophil recruitment and IL-4 production during human allergen-induced late asthma. *J Allergy Clin Immunol* 108: 205-211.
42. Venge, P. 2010. The eosinophil and airway remodelling in asthma. *The clinical respiratory journal* 4 Suppl 1: 15-19.
43. Holgate, S. T. 1999. The epidemic of allergy and asthma. *Nature* 402: B2-4.
44. Trivedi, S. G., and C. M. Lloyd. 2007. Eosinophils in the pathogenesis of allergic airways disease. *Cell Mol Life Sci* 64: 1269-1289.
45. Amin, K., D. Ludviksdottir, C. Janson, O. Nettelbladt, E. Bjornsson, G. M. Roomans, G. Boman, L. Seveus, and P. Venge. 2000. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. *American journal of respiratory and critical care medicine* 162: 2295-2301.
46. Pearlman, D. S. 1999. Pathophysiology of the inflammatory response. *J Allergy Clin Immunol* 104: S132-137.
47. Reed, C. E. 1994. The importance of eosinophils in the immunology of asthma and allergic disease. *Annals of allergy* 72: 376-380.
48. Makino, S., and T. Fukuda. 1995. Eosinophils and allergy in asthma. *Allergy Proc* 16: 13-21.
49. Kariyawasam, H. H., and D. S. Robinson. 2007. The role of eosinophils in airway tissue remodelling in asthma. *Current opinion in immunology* 19: 681-686.
50. Williams, T. J. 2004. The eosinophil enigma. *J Clin Invest* 113: 507-509.
51. Clark, K., L. Simson, N. Newcombe, A. M. Koskinen, J. Mattes, N. A. Lee, J. J. Lee, L. A. Dent, K. I. Matthaei, and P. S. Foster. 2004. Eosinophil degranulation in the allergic lung of mice primarily occurs in the airway lumen. *Journal of leukocyte biology* 75: 1001-1009.
52. Hoselton, S. A., A. E. Samarasinghe, J. M. Seydel, and J. M. Schuh. 2010. An inhalation model of airway allergic response to inhalation of environmental Aspergillus fumigatus

conidia in sensitized BALB/c mice. *Medical mycology : official publication of the International Society for Human and Animal Mycology* 48: 1056-1065.
53. Samarasinghe, A. E., S. A. Hoselton, and J. M. Schuh. 2011. A comparison between intratracheal and inhalation delivery of Aspergillus fumigatus conidia in the development of fungal allergic asthma in C57BL/6 mice. *Fungal biology* 115: 21-29.
54. Samarasinghe, A. E., S. A. Hoselton, and J. M. Schuh. 2011. The absence of VPAC2 leads to aberrant antibody production in Aspergillus fumigatus sensitized and challenged mice. *Peptides* 32: 131-137.
55. Hogaboam, C. M., K. Blease, and J. M. Schuh. 2003. Cytokines and chemokines in allergic bronchopulmonary aspergillosis (ABPA) and experimental Aspergillus-induced allergic airway or asthmatic disease. *Frontiers in bioscience : a journal and virtual library* 8: e147-156.
56. Sampson, A. P. 2000. The role of eosinophils and neutrophils in inflammation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 30 Suppl 1: 22-27.
57. Sommerhoff, C. P., J. A. Nadel, C. B. Basbaum, and G. H. Caughey. 1990. Neutrophil elastase and cathepsin G stimulate secretion from cultured bovine airway gland serous cells. *J Clin Invest* 85: 682-689.
58. Liu, H., S. C. Lazarus, G. H. Caughey, and J. V. Fahy. 1999. Neutrophil elastase and elastase-rich cystic fibrosis sputum degranulate human eosinophils in vitro. *The American journal of physiology* 276: L28-34.
59. Moreira, A. P., and C. M. Hogaboam. 2011. Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 31: 485-491.
60. Smit, J. J., and N. W. Lukacs. 2006. A closer look at chemokines and their role in asthmatic responses. *Eur J Pharmacol* 533: 277-288.
61. Bloemen, K., S. Verstraelen, R. Van Den Heuvel, H. Witters, I. Nelissen, and G. Schoeters. 2007. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunology letters* 113: 6-18.
62. Hammad, H., H. H. Smits, C. Ratajczak, A. Nithiananthan, E. A. Wierenga, G. A. Stewart, A. Jacquet, A. B. Tonnel, and J. Pestel. 2003. Monocyte-derived dendritic cells exposed to Der p 1 allergen enhance the recruitment of Th2 cells: major involvement of the chemokines TARC/CCL17 and MDC/CCL22. *Eur Cytokine Netw* 14: 219-228.
63. Lipscomb, M. F., and B. J. Masten. 2002. Dendritic cells: immune regulators in health and disease. *Physiol Rev* 82: 97-130.
64. Laberge, S., and S. El Bassam. 2004. Cytokines, structural cells of the lungs and airway inflammation. *Paediatr Respir Rev* 5 Suppl A: S41-45.
65. Folkerts, G., and F. P. Nijkamp. 1998. Airway epithelium: more than just a barrier! *Trends Pharmacol Sci* 19: 334-341.
66. Johnson, S. R., and A. J. Knox. 1997. Synthetic functions of airway smooth muscle in asthma. *Trends Pharmacol Sci* 18: 288-292.
67. Brewster, C. E., P. H. Howarth, R. Djukanovic, J. Wilson, S. T. Holgate, and W. R. Roche. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 3: 507-511.

68. Roche, W. R., R. Beasley, J. H. Williams, and S. T. Holgate. 1989. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1: 520-524.
69. Wilson, J. W., and X. Li. 1997. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 27: 363-371.
70. Huang, J., R. Olivenstein, R. Taha, Q. Hamid, and M. Ludwig. 1999. Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. *American journal of respiratory and critical care medicine* 160: 725-729.
71. Bergeron, C., and L. P. Boulet. 2006. Structural changes in airway diseases: characteristics, mechanisms, consequences, and pharmacologic modulation. *Chest* 129: 1068-1087.
72. Frew, A. J. 1998. T cells in Allergy and Asthma. *Rev. Esp. Alergol Inmunol Clín* 13.
73. Kay, A. B. 2006. The role of T lymphocytes in asthma. *Chem Immunol Allergy* 91: 59-75.
74. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 326: 298-304.
75. Larche, M., D. S. Robinson, and A. B. Kay. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 111: 450-463; quiz 464.
76. Vercelli, D. 2001. Immunoglobulin E and its regulators. *Curr Opin Allergy Clin Immunol* 1: 61-65.
77. Larche, M. 2007. Regulatory T cells in allergy and asthma. *Chest* 132: 1007-1014.
78. McHugh, R. S., and E. M. Shevach. 2002. The role of suppressor T cells in regulation of immune responses. *J Allergy Clin Immunol* 110: 693-702.
79. Miosge, L. A., and C. C. Goodnow. 2005. Genes, pathways and checkpoints in lymphocyte development and homeostasis. *Immunol Cell Biol* 83: 318-335.
80. Montecino-Rodriguez, E., and K. Dorshkind. 2006. New perspectives in B-1 B cell development and function. *Trends Immunol* 27: 428-433.
81. Hardy, R. R., and K. Hayakawa. 2001. B cell development pathways. *Annu Rev Immunol* 19: 595-621.
82. Nagasawa, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* 6: 107-116.
83. Matthias, P., and A. G. Rolink. 2005. Transcriptional networks in developing and mature B cells. *Nat Rev Immunol* 5: 497-508.
84. Medina, K. L., and H. Singh. 2005. Gene regulatory networks orchestrating B cell fate specification, commitment, and differentiation. *Curr Top Microbiol Immunol* 290: 1-14.
85. Pillai, S., A. Cariappa, and S. T. Moran. 2005. Marginal zone B cells. *Annu Rev Immunol* 23: 161-196.
86. Natasha Kushnir, N. A. B., Adrian W. Zuercher, Susan E. Coffin, Charlotte A. Moser, Paul A. Offit, and John J. Cebra. 2001. B2 but Not B1 Cells Can Contribute to CD4+ T-Cell-Mediated Clearance of Rotavirus in SCID Mice *Journal of Virology*: 5482-5490.
87. Oliveira, F. L., A. M. Aguiar, R. Borojevic, and M. C. El-Cheikh. 2005. IgE expression on the surface of B1 and B2 lymphocytes in experimental murine schistosomiasis. *Braz J Med Biol Res* 38: 1033-1042.
88. McHeyzer-Williams, L. J., and M. G. McHeyzer-Williams. 2005. Antigen-specific memory B cell development. *Annu Rev Immunol* 23: 487-513.

89. Won, W. J., and J. F. Kearney. 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J Immunol* 168: 5605-5611.
90. Lam, K. P., and K. Rajewsky. 1999. B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. *J Exp Med* 190: 471-477.
91. Montecino-Rodriguez, E., and K. Dorshkind. 2012. B-1 B cell development in the fetus and adult. *Immunity* 36: 13-21.
92. Martin, F., and J. F. Kearney. 2001. B1 cells: similarities and differences with other B cell subsets. *Current opinion in immunology* 13: 195-201.
93. Wang, H., and S. H. Clarke. 2003. Evidence for a ligand-mediated positive selection signal in differentiation to a mature B cell. *J Immunol* 171: 6381-6388.
94. Lindell, D. M., A. A. Berlin, M. A. Schaller, and N. W. Lukacs. 2008. B cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. *PLoS One* 3: e3129.
95. Ho, I. C., and S. Y. Pai. 2007. GATA-3 - not just for Th2 cells anymore. *Cell Mol Immunol* 4: 15-29.
96. Wills-Karp, M., and M. Chiaramonte. 2003. Interleukin-13 in asthma. *Curr Opin Pulm Med* 9: 21-27.
97. Stirling, R. G., E. L. van Rensen, P. J. Barnes, and K. F. Chung. 2001. Interleukin-5 induces CD34(+) eosinophil progenitor mobilization and eosinophil CCR3 expression in asthma. *American journal of respiratory and critical care medicine* 164: 1403-1409.
98. Corren, J. 2012. Inhibition of interleukin-5 for the treatment of eosinophilic diseases. *Discovery medicine* 13: 305-312.
99. Kouro, T., and K. Takatsu. 2009. IL-5- and eosinophil-mediated inflammation: from discovery to therapy. *International immunology* 21: 1303-1309.
100. Flood-Page, P., C. Swenson, I. Faiferman, J. Matthews, M. Williams, L. Brannick, D. Robinson, S. Wenzel, W. Busse, T. T. Hansel, N. C. Barnes, and G. International Mepolizumab Study. 2007. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *American journal of respiratory and critical care medicine* 176: 1062-1071.
101. McNamara, P. S., and R. L. Smyth. 2005. Interleukin-9 as a possible therapeutic target in both asthma and chronic obstructive airways disease. *Drug News Perspect* 18: 615-621.
102. Temann, U. A., Y. Laouar, E. E. Eynon, R. Homer, and R. A. Flavell. 2007. IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. *International immunology* 19: 1-10.
103. Sonoda, Y., H. Sakabe, Y. Ohmisono, S. Tanimukai, S. Yokota, S. Nakagawa, S. C. Clark, and T. Abe. 1994. Synergistic actions of stem cell factor and other burst-promoting activities on proliferation of CD34+ highly purified blood progenitors expressing HLA-DR or different levels of c-kit protein. *Blood* 84: 4099-4106.
104. Murdock, B. J., N. R. Falkowski, A. B. Shreiner, A. A. Akha, R. A. McDonald, E. S. White, G. B. Toews, and G. B. Huffnagle. 2012. Interleukin-17 drives pulmonary eosinophilia following repeated exposure to Aspergillus fumigatus conidia. *Infection and immunity* 80: 1424-1436.
105. Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, V. Quesniaux, F. Fossiez, B. Ryffel, and B. Schnyder. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 203: 2715-2725.

106. Rahman, M. S., A. Yamasaki, J. Yang, L. Shan, A. J. Halayko, and A. S. Gounni. 2006. IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J Immunol* 177: 4064-4071.
107. Thomas, P. S., D. H. Yates, and P. J. Barnes. 1995. Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. *American journal of respiratory and critical care medicine* 152: 76-80.
108. Kips, J. C., J. H. Tavernier, G. F. Joos, R. A. Peleman, and R. A. Pauwels. 1993. The potential role of tumour necrosis factor alpha in asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 23: 247-250.
109. Rosenwasser, L. J. 1998. Biologic activities of IL-1 and its role in human disease. *J Allergy Clin Immunol* 102: 344-350.
110. Shikotra, A., D. F. Choy, C. M. Ohri, E. Doran, C. Butler, B. Hargadon, M. Shelley, A. R. Abbas, C. D. Austin, J. Jackman, L. C. Wu, L. G. Heaney, J. R. Arron, and P. Bradding. 2012. Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma. *J Allergy Clin Immunol* 129: 104-111 e101-109.
111. Semlali, A., E. Jacques, L. Koussih, A. S. Gounni, and J. Chakir. 2010. Thymic stromal lymphopoietin-induced human asthmatic airway epithelial cell proliferation through an IL-13-dependent pathway. *J Allergy Clin Immunol* 125: 844-850.
112. Liu, Y. J., V. Soumelis, N. Watanabe, T. Ito, Y. H. Wang, W. Malefyt Rde, M. Omori, B. Zhou, and S. F. Ziegler. 2007. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* 25: 193-219.
113. Seshasayee, D., W. P. Lee, M. Zhou, J. Shu, E. Suto, J. Zhang, L. Diehl, C. D. Austin, Y. G. Meng, M. Tan, S. L. Bullens, S. Seeber, M. E. Fuentes, A. F. Labrijn, Y. M. Graus, L. A. Miller, E. S. Schelegle, D. M. Hyde, L. C. Wu, S. G. Hymowitz, and F. Martin. 2007. In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J Clin Invest* 117: 3868-3878.
114. Ashman, L. K. 1999. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol* 31: 1037-1051.
115. Feltis, B. N., D. Wignarajah, L. Zheng, C. Ward, D. Reid, R. Harding, and E. H. Walters. 2006. Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma. *American journal of respiratory and critical care medicine* 173: 1201-1207.
116. Bosse, Y., and M. Rola-Pleszczynski. 2007. Controversy surrounding the increased expression of TGF beta 1 in asthma. *Respir Res* 8: 66.
117. Pretolani, M., and M. Goldman. 1997. IL-10: a potential therapy for allergic inflammation? *Immunol Today* 18: 277-280.
118. Latge, J. P. 1999. Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev* 12: 310-350.
119. Kurup, V. P., G. Grunig, A. P. Knutsen, and P. S. Murali. 1998. Cytokines in allergic bronchopulmonary aspergillosis. *Res Immunol* 149: 466-477; discussion 515-466.
120. Kurup, V. P., V. Hari, J. Guo, P. S. Murali, A. Resnick, M. Krishnan, and J. N. Fink. 1996. Aspergillus fumigatus peptides differentially express Th1 and Th2 cytokines. *Peptides* 17: 183-190.

121. Kurup, V. P., B. W. Seymour, H. Choi, and R. L. Coffman. 1994. Particulate Aspergillus fumigatus antigens elicit a TH2 response in BALB/c mice. *J Allergy Clin Immunol* 93: 1013-1020.
122. Wang, J. M., M. Denis, M. Fournier, and M. Laviolette. 1994. Experimental allergic bronchopulmonary aspergillosis in the mouse: immunological and histological features. *Scand J Immunol* 39: 19-26.
123. Zosky, G. R., and P. D. Sly. 2007. Animal models of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 37: 973-988.
124. Sarpong, S. B., L. Y. Zhang, and S. R. Kleeberger. 2003. A novel mouse model of experimental asthma. *Int Arch Allergy Immunol* 132: 346-354.
125. Nials, A. T., and S. Uddin. 2008. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 1: 213-220.
126. Hunter, R. L. 2002. Overview of vaccine adjuvants: present and future. *Vaccine* 20 Suppl 3: S7-12.
127. Blyth, D. I., M. S. Pedrick, T. J. Savage, E. M. Hessel, and D. Fattah. 1996. Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol* 14: 425-438.
128. Fuchs, B., and A. Braun. 2008. Improved mouse models of allergy and allergic asthma--chances beyond ovalbumin. *Curr Drug Targets* 9: 495-502.
129. Birrell, M. A., A. J. Van Oosterhout, and M. G. Belvisi. 2010. Do the current house dust mite-driven models really mimic allergic asthma? *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 36: 1220-1221.
130. Kang, B., D. Vellody, H. Homburger, and J. W. Yunginger. 1979. Cockroach cause of allergic asthma. Its specificity and immunologic profile. *J Allergy Clin Immunol* 63: 80-86.
131. Lugauskas, A., A. Krikstaponis, and L. Sveistyte. 2004. Airborne fungi in industrial environments--potential agents of respiratory diseases. *Ann Agric Environ Med* 11: 19-25.
132. Green, B. J., T. Z. Mitakakis, and E. R. Tovey. 2003. Allergen detection from 11 fungal species before and after germination. *J Allergy Clin Immunol* 111: 285-289.
133. Kauffman, H. F., and S. van der Heide. 2003. Exposure, sensitization, and mechanisms of fungus-induced asthma. *Curr Allergy Asthma Rep* 3: 430-437.
134. Hogaboam, C. M., K. J. Carpenter, J. M. Schuh, and K. F. Buckland. 2005. Aspergillus and asthma--any link? *Medical mycology : official publication of the International Society for Human and Animal Mycology* 43 Suppl 1: S197-202.
135. Wang, K., D. W. Ussery, and S. Brunak. 2009. Analysis and prediction of gene splice sites in four Aspergillus genomes. *Fungal genetics and biology : FG & B* 46 Suppl 1: S14-18.
136. Latge, J. P. 2001. The pathobiology of Aspergillus fumigatus. *Trends Microbiol* 9: 382-389.
137. Hospenthal, D. R., K. J. Kwon-Chung, and J. E. Bennett. 1998. Concentrations of airborne Aspergillus compared to the incidence of invasive aspergillosis: lack of correlation. *Medical mycology : official publication of the International Society for Human and Animal Mycology* 36: 165-168.

138. Kurup, V. P., H. Choi, P. S. Murali, and R. L. Coffman. 1994. IgE and eosinophil regulation in a murine model of allergic aspergillosis. *Journal of leukocyte biology* 56: 593-598.
139. Chalupova, V. 1994. Morphological and colour mutants of Aspergillus fumigatus. *Acta Univ Palacki Olomuc Fac Med* 137: 11-14.
140. Land, C. J., B. Sostaric, R. Fuchs, H. Lundstrom, and K. Hult. 1989. Intratracheal exposure of rats to Aspergillus fumigatus spores isolated from sawmills in Sweden. *Appl Environ Microbiol* 55: 2856-2860.
141. Hogaboam, C. M., K. Blease, B. Mehrad, M. L. Steinhauser, T. J. Standiford, S. L. Kunkel, and N. W. Lukacs. 2000. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by Aspergillus fumigatus. *Am J Pathol* 156: 723-732.
142. Baraldo, S., K. Lokar Oliani, G. Turato, R. Zuin, and M. Saetta. 2007. The Role of Lymphocytes in the Pathogenesis of Asthma and COPD. *Current medicinal chemistry* 14: 2250-2256.
143. Bice, D. E., R. H. Gray, M. J. Evans, and B. A. Muggenburg. 1987. Identification of plasma cells in lung alveoli and interstitial tissues after localized lung immunization. *Journal of leukocyte biology* 41: 1-7.
144. Mantis, N. J., N. Rol, and B. Corthesy. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology* 4: 603-611.
145. Suzuki, K., M. Maruya, S. Kawamoto, and S. Fagarasan. 2010. Roles of B-1 and B-2 cells in innate and acquired IgA-mediated immunity. *Immunological reviews* 237: 180-190.
146. Mogi, G. 1975. Secretory immunoglobulin A in oral and respiratory passages in man. *Ann Otol Rhinol Laryngol* 84 Suppl 20: 1-23.
147. van Asperen, P. P., M. Gleeson, A. S. Kemp, A. W. Cripps, S. B. Geraghty, C. M. Mellis, and R. L. Clancy. 1985. The relationship between atopy and salivary IgA deficiency in infancy. *Clin Exp Immunol* 62: 753-757.
148. Ostergaard, P. A. 1980. Clinical and immunological features of transient IgA deficiency in children. *Clin Exp Immunol* 40: 561-565.
149. Kaplan, C., J. C. Valdez, R. Chandrasekaran, H. Eibel, K. Mikecz, T. T. Glant, and A. Finnegan. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. *Arthritis Res* 4: 54-58.
150. Mukherjee, S., S. C. Lee, and A. Casadevall. 1995. Antibodies to Cryptococcus neoformans glucuronoxylomannan enhance antifungal activity of murine macrophages. *Infection and immunity* 63: 573-579.
151. Zeimpekoglou, K**.** 2008. The Role of B lymphocytes in lung disease. *PNEUMON* 21: 196-199.
152. Laitinen, L. A., A. Laitinen, and T. Haahtela. 1993. Airway mucosal inflammation even in patients with newly diagnosed asthma. *The American review of respiratory disease* 147: 697-704.
153. Sears, M. R., B. Burrows, E. M. Flannery, G. P. Herbison, C. J. Hewitt, and M. D. Holdaway. 1991. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 325: 1067-1071.
154. Rolinck-Werninghaus, C., U. Wahn, and E. Hamelmann. 2005. Anti-IgE therapy in allergic asthma. *Curr Drug Targets Inflamm Allergy* 4: 551-564.

155. Poryadin, G. V., N. E. Zhuravleva, J. M. Salmasi, A. N. Kazimirsky, L. Y. Semenova, S. A. Polner, and T. A. Chervinskaya. 2002. Immunological mechanisms of recovery from an acute stage in patients with atopic bronchial asthma. *Russ J Immunol* 7: 259-264.
156. el-Dahr, J. M., R. Fink, R. Selden, L. K. Arruda, T. A. Platts-Mills, and P. W. Heymann. 1994. Development of immune responses to Aspergillus at an early age in children with cystic fibrosis. *American journal of respiratory and critical care medicine* 150: 1513-1518.
157. Krasnick, J., P. A. Greenberger, M. Roberts, and R. Patterson. 1995. Allergic bronchopulmonary aspergillosis: serologic update for 1995. *J Clin Lab Immunol* 46: 137-142.
158. Hohl, T. M., H. L. Van Epps, A. Rivera, L. A. Morgan, P. L. Chen, M. Feldmesser, and E. G. Pamer. 2005. Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog* 1: e30.
159. Knutsen, A. P. 2011. Immunopathology and immunogenetics of allergic bronchopulmonary aspergillosis. *Journal of allergy* 2011: 785983.
160. Rapaka, R. R., D. M. Ricks, J. F. Alcorn, K. Chen, S. A. Khader, M. Zheng, S. Plevy, E. Bengten, and J. K. Kolls. 2010. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina. *J Exp Med* 207: 2907-2919.
161. Flicker, S., E. Gadermaier, C. Madritsch, and R. Valenta. 2011. Passive immunization with allergen-specific antibodies. *Curr Top Microbiol Immunol* 352: 141-159.
162. Lund, F. E., M. Hollifield, K. Schuer, J. L. Lines, T. D. Randall, and B. A. Garvy. 2006. B cells are required for generation of protective effector and memory CD4 cells in response to Pneumocystis lung infection. *J Immunol* 176: 6147-6154.
163. Fillatreau, S., D. Gray, and S. M. Anderton. 2008. Not always the bad guys: B cells as regulators of autoimmune pathology. *Nat Rev Immunol* 8: 391-397.
164. Rieger, A., and A. Bar-Or. 2008. B-cell-derived interleukin-10 in autoimmune disease: regulating the regulators. *Nat Rev Immunol* 8: 486-487.
165. Mauri, C., and M. R. Ehrenstein. 2008. The 'short' history of regulatory B cells. *Trends Immunol* 29: 34-40.
166. DiLillo, D. J., T. Matsushita, and T. F. Tedder. 2010. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Annals of the New York Academy of Sciences* 1183: 38-57.
167. Lund, F. E., and T. D. Randall. 2010. Effector and regulatory B cells: modulators of CD4(+) T cell immunity. *Nat Rev Immunol* 10: 236-247.
168. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3: 944-950.
169. Mauri, C., D. Gray, N. Mushtaq, and M. Londei. 2003. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197: 489-501.
170. Haas, K. M., R. Watanabe, T. Matsushita, H. Nakashima, N. Ishiura, H. Okochi, M. Fujimoto, and T. F. Tedder. 2010. Protective and pathogenic roles for B cells during systemic autoimmunity in NZB/W F1 mice. *J Immunol* 184: 4789-4800.
171. Amu, S., S. P. Saunders, M. Kronenberg, N. E. Mangan, A. Atzberger, and P. G. Fallon. 2010. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol* 125: 1114-1124 e1118.

172. Hussaarts, L., L. E. van der Vlugt, M. Yazdanbakhsh, and H. H. Smits. 2011. Regulatory B-cell induction by helminths: implications for allergic disease. *J Allergy Clin Immunol* 128: 733-739.
173. Raghow, R. 1994. The role of extracellular matrix in postinflammatory wound healing and fibrosis. *FASEB J* 8: 823-831.
174. Erle, D. J., and R. Pytela. 1992. How do integrins integrate? The role of cell adhesion receptors in differentiation and development. *Am J Respir Cell Mol Biol* 6: 459-460.
175. Akiyama, S. K., S. S. Yamada, W. T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J Cell Biol* 109: 863-875.
176. Frisch, S. M., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124: 619-626.
177. Norris, D. A., R. A. Clark, L. M. Swigart, J. C. Huff, W. L. Weston, and S. E. Howell. 1982. Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. *J Immunol* 129: 1612-1618.
178. Bourguignon, L. Y., V. B. Lokeshwar, X. Chen, and W. G. Kerrick. 1993. Hyaluronic acid-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. *J Immunol* 151: 6634-6644.
179. Hall, C. L., C. Wang, L. A. Lange, and E. A. Turley. 1994. Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. *J Cell Biol* 126: 575-588.
180. Jiang, D., J. Liang, and P. W. Noble. 2007. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol* 23: 435-461.
181. Ohkawara, Y., G. Tamura, T. Iwasaki, A. Tanaka, T. Kikuchi, and K. Shirato. 2000. Activation and transforming growth factor-beta production in eosinophils by hyaluronan. *Am J Respir Cell Mol Biol* 23: 444-451.
182. Wilkinson, T. S., S. Potter-Perigo, C. Tsoi, L. C. Altman, and T. N. Wight. 2004. Pro- and anti-inflammatory factors cooperate to control hyaluronan synthesis in lung fibroblasts. *Am J Respir Cell Mol Biol* 31: 92-99.
183. Spicer, A. P., and J. A. McDonald. 1998. Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J Biol Chem* 273: 1923-1932.
184. Laurent, T. C., and J. R. Fraser. 1992. Hyaluronan. *FASEB J* 6: 2397-2404.
185. Klagas, I., S. Goulet, G. Karakiulakis, J. Zhong, M. Baraket, J. L. Black, E. Papakonstantinou, and M. Roth. 2009. Decreased hyaluronan in airway smooth muscle cells from patients with asthma and COPD. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 34: 616-628.
186. Pure, E., and R. K. Assoian. 2009. Rheostatic signaling by CD44 and hyaluronan. *Cell Signal* 21: 651-655.
187. Hodge-Dufour, J., M. W. Marino, M. R. Horton, A. Jungbluth, M. D. Burdick, R. M. Strieter, P. W. Noble, C. A. Hunter, and E. Pure. 1998. Inhibition of interferon gamma induced interleukin 12 production: a potential mechanism for the anti-inflammatory activities of tumor necrosis factor. *Proc Natl Acad Sci U S A* 95: 13806-13811.
188. Toole, B. P. 2001. Hyaluronan in morphogenesis. *Semin Cell Dev Biol* 12: 79-87.
189. Toole, B. P., T. N. Wight, and M. I. Tammi. 2002. Hyaluronan-cell interactions in cancer and vascular disease. *J Biol Chem* 277: 4593-4596.

190. Karjalainen, J. M., R. H. Tammi, M. I. Tammi, M. J. Eskelinen, U. M. Agren, J. J. Parkkinen, E. M. Alhava, and V. M. Kosma. 2000. Reduced level of CD44 and hyaluronan associated with unfavorable prognosis in clinical stage I cutaneous melanoma. *Am J Pathol* 157: 957-965.
191. Tzircotis, G., R. F. Thorne, and C. M. Isacke. 2005. Chemotaxis towards hyaluronan is dependent on CD44 expression and modulated by cell type variation in CD44-hyaluronan binding. *J Cell Sci* 118: 5119-5128.
192. Sano, K., K. Yamauchi, H. Hoshi, M. Honma, G. Tamura, and K. Shirato. 1997. CD44 expression on blood eosinophils is a novel marker of bronchial asthma. *Int Arch Allergy Immunol* 114 Suppl 1: 67-71.
193. Matsumoto, K., J. Appiah-Pippim, R. P. Schleimer, C. A. Bickel, L. A. Beck, and B. S. Bochner. 1998. CD44 and CD69 represent different types of cell-surface activation markers for human eosinophils. *Am J Respir Cell Mol Biol* 18: 860-866.
194. Protin, U., T. Schweighoffer, W. Jochum, and F. Hilberg. 1999. CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. *J Immunol* 163: 4917-4923.
195. Schmits, R., J. Filmus, N. Gerwin, G. Senaldi, F. Kiefer, T. Kundig, A. Wakeham, A. Shahinian, C. Catzavelos, J. Rak, C. Furlonger, A. Zakarian, J. J. Simard, P. S. Ohashi, C. J. Paige, J. C. Gutierrez-Ramos, and T. W. Mak. 1997. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* 90: 2217-2233.
196. Rafi, A., M. Nagarkatti, and P. S. Nagarkatti. 1997. Hyaluronate-CD44 interactions can induce murine B-cell activation. *Blood* 89: 2901-2908.
197. Brightbill, H. D., S. Jeet, Z. Lin, D. Yan, M. Zhou, M. Tan, A. Nguyen, S. Yeh, D. Delarosa, S. R. Leong, T. Wong, Y. Chen, M. Ultsch, E. Luis, S. R. Ramani, J. Jackman, L. Gonzalez, M. S. Dennis, A. Chuntharapai, L. DeForge, Y. G. Meng, M. Xu, C. Eigenbrot, W. P. Lee, C. J. Refino, M. Balazs, and L. C. Wu. 2010. Antibodies specific for a segment of human membrane IgE deplete IgE-producing B cells in humanized mice. *J Clin Invest* 120: 2218-2229.
198. O'Brien, H. E., J. L. Parrent, J. A. Jackson, J. M. Moncalvo, and R. Vilgalys. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Appl Environ Microbiol* 71: 5544-5550.
199. Denning, D. W., B. R. O'Driscoll, C. M. Hogaboam, P. Bowyer, and R. M. Niven. 2006. The link between fungi and severe asthma: a summary of the evidence. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 27: 615-626.
200. Knutsen, A. P., and R. G. Slavin. 2011. Allergic bronchopulmonary aspergillosis in asthma and cystic fibrosis. *Clin Dev Immunol* 2011: 843763.
201. Ibrahim-Granet, O., B. Philippe, H. Boleti, E. Boisvieux-Ulrich, D. Grenet, M. Stern, and J. P. Latge. 2003. Phagocytosis and intracellular fate of Aspergillus fumigatus conidia in alveolar macrophages. *Infection and immunity* 71: 891-903.
202. Cenci, E., A. Mencacci, C. Fe d'Ostiani, G. Del Sero, P. Mosci, C. Montagnoli, A. Bacci, and L. Romani. 1998. Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. *The Journal of infectious diseases* 178: 1750-1760.
203. Polonelli, L., A. Casadevall, Y. Han, F. Bernardis, T. N. Kirkland, R. C. Matthews, D. Adriani, M. Boccanera, J. P. Burnie, A. Cassone, S. Conti, J. E. Cutler, R. Frazzi, C.

Gregory, S. Hodgetts, C. Illidge, W. Magliani, G. Rigg, and G. Santoni. 2000. The efficacy of acquired humoral and cellular immunity in the prevention and therapy of experimental fungal infections. *Medical mycology : official publication of the International Society for Human and Animal Mycology* 38 Suppl 1: 281-292.
204. Yoshizawa, Y., Y. Ohtani, T. Inoue, S. Miyake, A. Ikeda, M. Tanba, and V. P. Kurup. 1995. Immune responsiveness to inhaled antigens: local antibody production in the respiratory tract in health and lung diseases. *Clin Exp Immunol* 100: 395-400.
205. Oshiba, A., E. Hamelmann, K. Takeda, K. L. Bradley, J. E. Loader, G. L. Larsen, and E. W. Gelfand. 1996. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J Clin Invest* 97: 1398-1408.
206. Korsgren, M., J. S. Erjefalt, O. Korsgren, F. Sundler, and C. G. Persson. 1997. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 185: 885-892.
207. Hamelmann, E., K. Takeda, J. Schwarze, A. T. Vella, C. G. Irvin, and E. W. Gelfand. 1999. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am J Respir Cell Mol Biol* 21: 480-489.
208. Zhu, J., Y. S. Qiu, D. J. Figueroa, V. Bandi, H. Galczenski, K. Hamada, K. K. Guntupalli, J. F. Evans, and P. K. Jeffery. 2005. Localization and upregulation of cysteinyl leukotriene-1 receptor in asthmatic bronchial mucosa. *Am J Respir Cell Mol Biol* 33: 531-540.
209. Melchers, F., E. ten Boekel, T. Seidl, X. C. Kong, T. Yamagami, K. Onishi, T. Shimizu, A. G. Rolink, and J. Andersson. 2000. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. *Immunological reviews* 175: 33-46.
210. Hamelmann, E., A. T. Vella, A. Oshiba, J. W. Kappler, P. Marrack, and E. W. Gelfand. 1997. Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proc Natl Acad Sci U S A* 94: 1350-1355.
211. Jungsuwadee, P., M. Benkovszky, G. Dekan, G. Stingl, and M. M. Epstein. 2004. Repeated aerosol allergen exposure suppresses inflammation in B-cell-deficient mice with established allergic asthma. *Int Arch Allergy Immunol* 133: 40-48.
212. Liang, J., D. Jiang, Y. Jung, T. Xie, J. Ingram, T. Church, S. Degan, M. Leonard, M. Kraft, and P. W. Noble. 2011. Role of hyaluronan and hyaluronan-binding proteins in human asthma. *J Allergy Clin Immunol* 128: 403-411 e403.
213. Noble, P. W. 2002. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix biology : journal of the International Society for Matrix Biology* 21: 25-29.
214. Noble, P. W., C. M. McKee, M. Cowman, and H. S. Shin. 1996. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. *J Exp Med* 183: 2373-2378.
215. Lesley, J., R. Hyman, and P. W. Kincade. 1993. CD44 and its interaction with extracellular matrix. *Adv Immunol* 54: 271-335.
216. Herrlich, P., M. Zoller, S. T. Pals, and H. Ponta. 1993. CD44 splice variants: metastases meet lymphocytes. *Immunol Today* 14: 395-399.
217. Naor, D., R. V. Sionov, and D. Ish-Shalom. 1997. CD44: structure, function, and association with the malignant process. *Adv Cancer Res* 71: 241-319.

218. Lesley, J., N. Howes, A. Perschl, and R. Hyman. 1994. Hyaluronan binding function of CD44 is transiently activated on T cells during an in vivo immune response. *J Exp Med* 180: 383-387.
219. Shimizu, Y., G. A. Van Seventer, R. Siraganian, L. Wahl, and S. Shaw. 1989. Dual role of the CD44 molecule in T cell adhesion and activation. *J Immunol* 143: 2457-2463.
220. DeGrendele, H. C., P. Estess, L. J. Picker, and M. H. Siegelman. 1996. CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. *J Exp Med* 183: 1119-1130.
221. DeGrendele, H. C., P. Estess, and M. H. Siegelman. 1997. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 278: 672-675.
222. Murakami, S., K. Miyake, C. H. June, P. W. Kincade, and R. J. Hodes. 1990. IL-5 induces a Pgp-1 (CD44) bright B cell subpopulation that is highly enriched in proliferative and Ig secretory activity and binds to hyaluronate. *J Immunol* 145: 3618-3627.
223. Kryworuckho, M., F. Diaz-Mitoma, and A. Kumar. 1995. CD44 isoforms containing exons V6 and V7 are differentially expressed on mitogenically stimulated normal and Epstein-Barr virus-transformed human B cells. *Immunology* 86: 41-48.
224. Hathcock, K. S., H. Hirano, S. Murakami, and R. J. Hodes. 1993. CD44 expression on activated B cells. Differential capacity for CD44-dependent binding to hyaluronic acid. *J Immunol* 151: 6712-6722.
225. Marko Kryworuchko, F. D.-M. a. A. K. 1999. Interferon-gamma inhibits CD44-Hyaluronan interactions in Normal Human B Lymphocytes. *Experimental Cell Research*: 241-252.
226. Termeer, C., F. Benedix, J. Sleeman, C. Fieber, U. Voith, T. Ahrens, K. Miyake, M. Freudenberg, C. Galanos, and J. C. Simon. 2002. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* 195: 99-111.
227. Minshall, E. M., D. Y. Leung, R. J. Martin, Y. L. Song, L. Cameron, P. Ernst, and Q. Hamid. 1997. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 17: 326-333.
228. Doherty, T., and D. Broide. 2007. Cytokines and growth factors in airway remodeling in asthma. *Current opinion in immunology* 19: 676-680.
229. Halwani, R., S. Al-Muhsen, H. Al-Jahdali, and Q. Hamid. 2011. Role of transforming growth factor-beta in airway remodeling in asthma. *Am J Respir Cell Mol Biol* 44: 127-133.
230. Boulet, L. P. 2009. Irreversible airway obstruction in asthma. *Curr Allergy Asthma Rep* 9: 168-173.
231. Kay, A. B. 2005. The role of eosinophils in the pathogenesis of asthma. *Trends Mol Med* 11: 148-152.
232. Busse, W. W., and J. B. Sedgwick. 1992. Eosinophils in asthma. *Annals of allergy* 68: 286-290.
233. Calhoun, W. J., J. Sedgwick, and W. W. Busse. 1991. The role of eosinophils in the pathophysiology of asthma. *Annals of the New York Academy of Sciences* 629: 62-72.
234. Lee, S. Y., S. J. Kim, S. S. Kwon, Y. K. Kim, K. H. Kim, H. S. Moon, J. S. Song, and S. H. Park. 2001. Distribution and cytokine production of CD4 and CD8 T-lymphocyte subsets in patients with acute asthma attacks. *Ann Allergy Asthma Immunol* 86: 659-664.

235. Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay. 1989. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *The American review of respiratory disease* 140: 1745-1753.
236. Blease, K., C. Jakubzick, J. Westwick, N. Lukacs, S. L. Kunkel, and C. M. Hogaboam. 2001. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J Immunol* 166: 5219-5224.
237. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263.
238. Kuperman, D. A., X. Huang, L. L. Koth, G. H. Chang, G. M. Dolganov, Z. Zhu, J. A. Elias, D. Sheppard, and D. J. Erle. 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8: 885-889.
239. Tournoy, K. G., J. C. Kips, and R. A. Pauwels. 2001. The allergen-induced airway hyperresponsiveness in a human-mouse chimera model of asthma is T cell and IL-4 and IL-5 dependent. *J Immunol* 166: 6982-6991.
240. Lack, G., A. Oshiba, K. L. Bradley, J. E. Loader, D. Amran, G. L. Larsen, and E. W. Gelfand. 1995. Transfer of immediate hypersensitivity and airway hyperresponsiveness by IgE-positive B cells. *American journal of respiratory and critical care medicine* 152: 1765-1773.