The Role of Alternaria and its Major Allergen, Alt a 1, in the Pathogenesis of Allergic Airway Disorders

Amanda Cronin Rumore

Dissertation submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biological Sciences

Christopher B. Lawrence, Chair
Daniel G. Capelluto
Joseph O. Falkinham, III
Liwu Li

March 21, 2012
Blacksburg, VA

Keywords: Alternaria, Alt a 1, allergy, asthma, innate immunity, IRAK, RXLR-motifs, lipid binding, cellular entry

The Role of Alternaria and its Major Allergen, Alt a 1, in the Pathogenesis of Allergic Airway Disorders

Amanda Cronin Rumore

ABSTRACT

Chronic exposure to the ubiqutious airborne fungus, *Alternaria alternata*, has long been implicated in the development and exacerbation of human allergy and asthma. Alt a 1 was identified previously by several groups as the major allergen secreted by *A. alternata*, due to its IgE-specific reactivity with sera from atopic patients. Despite the well-documented clinical importance of Alternaria and its major allergen, little knowledge exists regarding their role and interaction with the innate immune system. Here for the first time we characterize the innate immune response to *A.alternata* and verify the significance of Alt a 1 in contributing to this response in human airway cells and murine models. Our studies establish a baseline response for both a chronic and single-challenge murine inhalation model with Alternaria spores. Both models demonstrate live conidia induce a robust response, arguably more pathologically relevant compared to studies employing Alternaria extracts. We also elucidate the overall importance of Alt a 1 by utilizing recombinant Alt a 1 protein, *A. alternata* (*Δalt a 1*) deletion mutants, and an *A.alternata* (*Alt a 1+)* overexpression mutant. Both Alt a 1 protein and *A. alternata* conidia stimulated production of pro-inflammatory cytokines/chemokines in mice after a single intranasal challenge. Infiltration of effector cells (macrophages, neutrophils, eosinophils, and

lymphocytes) into the lungs along with other hallmarks of airway inflammation was observed. In addition, Alt a 1 protein and conidia evoked secretion of pro-inflammatory cytokines in treated human airway epithelial cells while the *Alt a 1*+overxpression mutant induced a significantly higher response. In contrast, spores of *Δalt a 1* caused an attenuated response in both human cells and murine lungs suggesting that this single protein may play a major role in inducing the innate immune response in airway epithelium at the organismal level. Finally, we identified key biochemical properties of the Alt a 1 protein including a single histidine required for esterase activity and a unique RXLR-like motif which controls Alt a 1's ability to bind external lipids and enter human airway cells. Overall, these results improve our understanding of how Alternaria induces innate immunity and identifies possible therapeutic targets within allergenic proteins.

Dedicated to big dreams and those who make them a reality…

Acknowledgements

"It takes a village….."

The following work would not have been possible without the help of many teachers, advisors, administrators, lab mates, family and friends. Although I cannot thank each and every person who has had a hand in education to this point, I would like to highlight a select few who have dedicated part of their life to improving mine.

I have been very fortunate to have been able to work under the guidance of my advisor, Dr. Christopher Lawrence. I would like to thank him for giving me my first taste of research as an undergraduate and the confidence to pursue a PhD. I am eternally grateful for his inspiration and support towards not only my academics, but also my teaching, service, and personal endeavors. Our deep discussions, both scientific and philosophical, have prepared me well for the future ahead. Though words cannot express my gratitude for his role in my graduate success, I thank him from the bottom of my heart and I am proud to call him my advisor, mentor, and friend.

I also extend many thanks to the rest of my committee, Drs., Liwu Li, Joseph Falkinham, and Daniel Capelluto for their continuous assistance and valuable suggestions throughout my graduate career. All three have given me exceptional perspectives to my studies while building my confidence as a scientist. I could not have asked for a more supportive and dynamic committee.

The research presented hereafter would have never been possible without the assistance of previous and current Lawrence Lab members. I thank Sang-Wook Park for resurrecting my

love of protein biochemistry through our thoughtful conversations and his assistance in teaching me the devious wonders of protein purification. I thank Mihaela Babiceanu for our candid talks and commiseration and for teaching me a little bit of Romanian. I thank Prathyusha Kolconda, Tristan Hayes, Reena Patel, and Saleena Khanna for always being a helping hand in the lab when I needed one. Additionally, I thank our lab's previous post-docs, Kwang-Hyung Kim and Yangrae Cho, for building my fungal molecular biology skills and teaching me the importance of detail and Ha Dang for offering me technical support with our genome browsers. I thank Brad Howard for his skillful help over the past few months and I wish him the best of luck with continuing this work and bringing it to the next level.

I also owe thanks to members of the Tyler, Li, and Bassaganya Labs for their varied assistance and technical support and for always seeming to have that one chemical, buffer, instrument, etc. that I needed at the last minute. I especially thank Shiv Kale for his assistance with much of my protein work and teaching me more about lipid biochemistry than I ever wanted to know. In addition, I thank the VBI Facilities personnel, especially Linda Correll, Susan Huckle, and Sheryl Locascio, for making sure I always had the best equipment and space required to complete my research.

Outside of research, my numerous service and teaching activities have given me a profound appreciation for the "behind-the-scene" work of the Graduate School faculty and staff. I thank Dean Karen DePauw for her distinguished support of my own endeavors and those of all graduate students. Her hard work and compassion to improve graduate education has truly inspired me to be a better educator and citizen scholar. I thank both Monika Gibson and Lauren Surface for their assistance with programming and Robert Mayorga for his always helpful and positive attitude and encouragement. It was also an honor to build my leadership and teamwork

skills with my fellow members of the Graduate Student Assembly. Michelle, Jonathan, Ganesh, Shree, Courtney, and many others – your help, kind words, and moments of laughter have made my hard work so much more rewarding and graduate school far more enjoyable.

I owe an incredible amount of my knowledge and success to all of the amazing teachers I have been fortunate to have throughout the years. From Mr. Moore, my 6th-grade science teacher, to my most challenging college professors, you have all inspired me to be a lifelong learner and the desire to follow in your footsteps. Without these passionate and engaged educators I would have never embarked on my professorial path and I can only hope their energy and pedagogical talent is something I can emulate in my own classroom.

My time at Virginia Tech is sadly coming to a close, but, without question, my nine years here in Blacksburg have brought me strong friendships, immense personal growth, and my happiest memories that I will always be grateful for. From my first days in Lee Hall as a freshman, to being surrounded by 66,000 Hokie friends in Lane Stadium, to the classes, clubs, workgroups, socials, and of course dinners in West End, Virginia Tech has always been a place I feel home. My adoration and devotion was only reinforced by the outpouring of support and evident resilience of my Hokie Nation following the tragedy of April 16th. I will neVerforgeT the 32 Hokies lost on that day and I will continue to preach *Ut Prosim* for them and for every Hokie who shares my love and compassion for this special place.

I offer my sincerest gratitude to my mother and father, Dennis and Deborah Cronin, who have spent the last twenty-seven years providing me with every opportunity to succeed. They have taught me the value of hard work, how to endure through the seemingly impossible, and gave me just enough tough love when I needed it. My achievements have and will continue to

be a product of their parenting and I will never be able to thank them enough for everything they have given me. I only hope they are as proud of me as I am of them for getting me here.

I am also grateful for the lessons from my grandparents whose own perseverance has inspired me throughout my life. They have always advised me on the value of an education and I cannot begin to express the positive role they have played in making me the person I am today.

And finally, but most importantly, I owe my greatest love and gratitude to my husband Michael. For nearly ten years he has been my biggest champion, supporting me unconditionally regardless of how outrageous each and every plan of mine has been. He has been my shoulder to cry on, the one I have shared my proudest moments with, and the one to keep me grounded throughout this journey. His love and encouragement are why I am here today. I cannot wait to spend our lives together, build our own family, and continue to support each other each and every day. I love you with the biggest heart and smallest amount of doubt that you are my soulmate now and forever.

And, of course….

GO HOKIES!!! ☺

Attribution

I declare that with the exception of the statements below, all work reported herein was performed by myself.

Chapter 3: IRAK-$1^{-/-}$ and IRAK-$M^{-/-}$ mice were kindly provided by Dr. Liwu Li, Department of Biological Sciences, Virginia Tech.

Chapter 4: generation of fungal mutants (*Δalta1*, *Δalta1-rec*, Alta1+) and Southern blot hybridization were performed by Mihaela Babiceanu. Superarray and qRT-PCR were performed by Brad Howard.

Chapter 5: Significant technical assistance for experiments performed was provided by Sang-Wook Park and Shiv Kale.

Table of Contents

Chapter Page

List of Figures

Figure **Page**

Chapter IV:

Chapter V:

Chapter VI:

List of Tables

Chapter **Page**

Chapter I:

Introduction and Overview of Research

Introduction:

Alternaria alternata is one of the most clinically relevant airborne fungi associated with allergic airway disorders including asthma and allergic rhinitis. Moreover, allergy to Alternaria and its major allergen, Alt a 1, pose a significant public health threat for which few therapies exist. Although research involving commercially available Alternaria extract has led to some understanding of the fungi's ability to cause a shift to a Th2 immune response, these crude fungal preparations do not adequately mimic natural exposure to Alternaria spores. Furthermore, despite empirical evidence showing the importance of Alt a 1 in Alternaria-associated allergy, the immunogenic potential and deeper understanding of the biochemical properties of Alt a 1 have not been scientifically established. Thus, we have chosen to thoroughly explore the mammalian immune response to *A.alternata* conidia (spores) and the Alt a 1 protein in both murine models and human lung cell lines. In addition, we intend to elucidate the underlying role of the major allergen Alt a 1, and identify key biochemical properties of this protein in order to provide potential therapeutic targets against Alternaria and potentially other fungal allergens.

Alternaria species.

Human airways are constantly exposed to aeroallergens produced by fungi, often at higher levels and for longer duration than other allergens including pollens[1]. *Alternaria alternata* has been one fungal species consistently associated with human airway disorders[3,4]. *Alternaria*

spores are easily dispersed by windy weather conditions in both arid and humid environments, making them among the most common potent airborne allergens[2]. This species typically consists of single conidiophores that are relatively short, usually simple, or 1–3 branched[5]. The conidia range ~20-60µm in length x ~9-20µm in width, sizably larger than other pathogenic/allergenic fungi such as *Aspergillus* spp. which can be less than 10µm. Despite its clinical significance, it is often difficult to distinguish *A.alternata* from other species primarily due to a wide range of variation in spore sizes and morphologies within a single species, which may hinder epidemiological and diagnostic research (Figure 1). For example, *A. tenuissima* has similar branching patterns as *A. alternata* but exhibits minor differences in the degree of branching and conidia are often morphologically longer and more narrow[6,7]. Another species, *A. brassicicola*, a notorious plant pathogen, shares nearly identical growth characteristics, nutrient source utilization, and *melanization* with *A.alternata* , but is not commonly associated with airway disorders. Importantly, *A. brassicicola* although morphologically similar to *A. alternata* in some respects, does not grow as well at higher temperatures. In summary, although fairly analogous to other Alternaria species in growth and morphological characteristics, *A.alternata* and its major allergen, Alt a 1, have been most frequently associated with human respiratory disorders. Thus, only those highly skilled and experienced in the art of morphological fungal taxonomy are capable of differentiating Alternaria at the species level.

Immune response to inhaled fungi

Chronic exposure to *Alternaria alternata*, has been associated with an increased risk of developing atopic or allergic asthma, allergic rhinitis, chronic rhinosinusitis (CRS), or exacerbation of a preexisting asthmatic condition. Along with classical allergen sensitization, a

sustained innate immune response to a constantly inhaled allergen may promote and prolong overall airway inflammation (Figure 2). Inhaled spores will easily activate the airway epithelium, which results in both neutrophilic and antigen presenting cell (APC) recruitment[8]. The innate cells contribute the localized inflammatory response while the increase of APCs increases the likeliness for sensitization to the fungal allergen. Although this initial response to inhaled fungal spores is typically short-lived, frequent exposure over a period of time will result in the classical signs of airway hyperresponsiveness, including eosinophilic inflammation, increased levels of IgE, tissue fibrosis, and an infiltration of Th2 cells and related cytokines such as IL-4, IL-5 and IL-13 (reference). The cross-talk between the innate and adaptive immune response, especially at the epithelial interface, has recently gained increased attention [9]. It is now well accepted that both immune reactions should be investigated in order to build a clearer understanding of how a pathogen interacts with its mammalian host especially in the context of allergic diseases.

Mouse models of fungal airway disorders

In order to study the effect of Alternaria conidia on the mammalian immune response, well defined mouse models for innate and chronic exposure must be established. Although a few published mouse models have lead to some insight into Alternaria's interaction in vivo[10,11], they have mainly utilized potent fungal extracts or unnatural sensitization and challenge schemas. We believe the most pathologically relevant murine model will be one which employs Alternaria conidia and more natural routes of exposure. This includes analyzing the effect of low and high doses of inhaled conidia and the response within a few hours compared to a few days after exposure. A standardized mouse model with defined baseline characterisitcs in wild-type mice is

essential for testing the importance of both immune system and fungal components such as using transgenic mice or fungal mutants. The approach for this research is defined in **Aim 1**.

Innate immune signaling

The mammalian innate immune response is composed of multifaceted and well-controlled signaling cascades. Toll-like receptor (TLR) signaling pathways contain many molecules which interact to regulate transcription factors ultimately leading to the induction or suppression of genes that coordinate the inflammatory response[12]. IRAK-1 and IRAK-M are well defined kinases of classic and alternative TLR signaling respectively[13]. Genotypic abnormalities in IRAK-1 and IRAK-M have been linked to allergic and/or inflammatory disorders[14]. However, no research has established if IRAK-1 and IRAK-M are involved in innate immune signaling in response to Alternaria. Germline knock-out mice for IRAK-1 and IRAK-M exist and can be used to better understand the role of each kinase following an Alternaria challenge. Changes in pro-inflammatory cytokine production, effector cell recruitment, and the antifungal response of macrophage can be assessed in regard to Alternaria challenge. Full mouse models and cellular studies using bone-marrow derived macrophage to study the function of IRAK-1 and IRAK-M in the innate immune response to Alternaria are described in **Aim 2**

Molecular approaches to studying Alternaria major allergen Alt a 1

Alt a 1 is the major allergen secreted by *A. alternata*, due to its IgE-specific reactivity with sera from atopic Alternaria patients yet no known innate immunological activity has been assigned to this clinically relevant protein[15–19]. Since our lab previously found that Alt a 1 has been evolutionarily conserved across Alternaria species[20], its functional role at the organismal

level must be examined. Unfortunately, the mechanisms involved in the inflammatory response evoked by Alternaria or its major allergen are poorly understood. Recent data suggests that the innate function of the airway epithelium plays an important role in the response to inhaled antigens[21–24]. Additional research on other allergens such as dust mite and Aspergillus indicate the respitory epithelium readily responds and assists in promoting the pro-inflammatory immune response [25,26]. Since the airway epithelium is at the interface of the initial immune response, it is an ideal candidate for developing specific therapeutics to prevent activation. Our preliminary data confirms *A.alternata* spores readily germinate in the presence of airway epithelial cells at physiologically relevant temperatures (Figure 4).

Molecular biology techniques allow us to fully delete, mutate, or overexpress fungal proteins in the native species and then investigate phenotypic changes in the organism. Additionally, we can use these mutant isolates in mammalian cell culture experiments and mouse models to assess changes in the host immune response. These types of mutants are an extremely valuable tool when studying a single protein and its effect on an immunological response. If airway epithelial cells show a decreased response to an *A.alternata* Alt a 1 deletion mutant and a non-functional Alt a 1 protein compared to the native forms, we can infer that this single protein may play a major role in inducing the innate immune response in airway epithelium either through direct action of the protein itself, or in overall changes to the organism itself (i.e. cell wall architecture, etc.). Experimental deletion and overexpression of Alt a 1 in Alternaria and subsequent effects on the response of human airway epithelial cells is described in **Aim 3**.

The Alt a 1 protein has been previously described to have esterase enzyme activity and specific IgE binding to the sera of Alternaria-sensitized patients, but comprehensive biochemical analysis of the protein has not been performed[16,27]. Characterizing both the enzymatic activity

and mechanism of cell entry for the Alt a 1 protein may help better establish targeted therapies against the allergen. The ability of fungal effector proteins to bind and enter plant and animal cells has been linked to a unique sequence of residues which form lipid binding motifs[28,29]. This motif, termed "RXLR" binds phosphatidylinositol 3-phosphate (PI3P) on the membrane surface triggers endocytosis[30] (Figure 5). Bioinformatic analysis of the Alt a 1 amino acid sequences reveals it contains a single RXLR-like lipid binding motif, KWYS, at position 85 – 88 (Figure 6). Additionally, Alt a 1's amino acid sequence contains a single histidine at position 84 which would be critical in forming the catalytic triad in the active site of many types of enzymes, including esterase. Ironically, the histidine is positioned directly before the RXLR-like motif, making mutations in one and conserving the other difficult to obtain. Careful considerations will need to be made to find mutations that will allow one to test the role of the histidine and RXLR-like residues in the function of Alt a 1.

A small number of studies has shown lipid-mediated endocytosis of allergens or other effector molecules to be essential to their mechanism of cellular entry[31–33]. Moreover, the ability for an allergen to cross the epithelial barrier may be an important characteristic trait in order for it to come in contact with antigen presenting cells (such as mature dendritic cells), mast cells, and T cells. We intend to test Alt a 1's capacity to bind and enter human airway cells and determine if the predicted RXLR-like motif mediates this mechanism. In order to elucidate the overall importance of the RXLR-like motif or enzyme activity in Alt a 1's ability to bind lipids and enter cells, experiments can utilize fluorescently-labeled Alt a 1 proteins in human airway epithelial cell studies. Confocal laser microscopy as well as kinetic measurements of protein uptake over short and long incubation times of the epithelial cells with the wild-type and mutant protein, along with subsequent cytokine analysis, will convey the importance of lipid binding

and entry or enzyme activity on the innate immune response. Overall, the production and characterization of wild-type and mutant Alt a 1 proteins will help identify a possible mechanism of immune exposure. **Aim 4** illustrates the biochemical studies of Alt a 1 and further explains challenges and quandaries of recombinant Alt a 1 production.

A clear understanding of the biochemical role of the Alt a 1 protein in addition to its function at the organismal level is vital for development of potential therapeutic targets for Alternaria allergies. Furthermore, comprehensive knowledge of the the mammalian immune response to whole Alternaria conidia is essential for future studies involving this clinically relevant organism. The results from the studies outlined below will not only give insight into the host response to Alternaria, but have the capability to be explored across other known fungal allergens to discover similar targets for patients with multiple fungal allergies.

Overview of Research

Aim 1: Develop a mouse model to study the innate immune response to *Alternaria alternata*

Hypothesis: Intranasal administration of Alternaria alternata conidia can induce a powerful innate inflammatory response in the murine lung following a single challenge or chronic exposure

Objective: In order to study the immune response to live Alternaria conidia and the Alternaria major allergen, Alt a 1, we aimed to develop a new and unique in-vivo mouse model of fungal-induced allergic airway inflammation. Our goal was to create two mouse models; one, to examine the innate or "early-phase" response and another model to study the chronic response. Both models would only employ intranasal exposure to the fungus without the traditional intraperitoneal sensitization schema in order to create the most natural response. The purpose of creating these simple yet distinctive models is to provide substantial preliminary data which shows time and dose dependent results to ensure the models are well suited for future experimental use with mutant fungal strains or allergens or transgenic mouse lines. It is important to establish a baseline response for identifying new key pro-inflammatory mediators induced in the lung during Alternaria stimulation. It is also imperative for testing common or previously described mediators or allergy/asthma which may or may not be produced in response to Alternaria. Most importantly, both models will employ fresh, whole, and living, Alternaria conidia or pure allergen protein rather than fungal culture filtrates, extracts, or fragments. This will induce the most natural response possible in an in-vivo model.

When establishing our model we will use ordinary measurements of airway inflammation such as changes in weight, total number of cells in the bronchoalveolar lavage fluid, increase in serum IgE levels, histopathological evidence of effector cell infiltration in the lung, and measure

changes in cytokine/chemokines expressed in the lung by ELISA and real-time PCR. The results of **Aim 1** will ensure the success of our future experimentation using mouse models of allergic airway inflammation.

Aim 2: Determine contribution of IRAK-1 and IRAK-M in innate immune signaling in response to Alternaria

Hypothesis: IRAK-1 and IRAK-M are involved in regulating the innate immune response to Alternaria.

Objective: The goal of **Aim 2** is to investigate the role of IRAK-1 and IRAK-M, two components of Toll-like receptor (TLR) signaling pathways, in response to Alternaria. The work will supplement the mouse model work completed in **Aim 1** by employing IRAK-1 and IRAK-M deficient mice and murine macrophages to study the role of these two genes in both mouse and cell culture models. We will use the baseline dose and time-dependent results of **Aim 1** to help complete this experimentation. We are specifically interested in finding changes in pro-inflammatory gene expression when IRAK-1 or IRAK-M is deleted from the signaling pathway. We hypothesize removal of either gene will cause an abnormal response of pro-inflammatory cytokines to the fungus which will be compared the responses found in **Aim 1**. We will examine the expression of various pro-inflammatory cytokines in both the macrophage cell culture and murine lung using cytokine antibody arrays and real-time PCR analysis. We will also complete histopathological examination of the lungs of IRAK-1 and IRAK-M deficient mice treated with Alternaria antigens to observe any increase or decrease in common signs of allergic airway inflammation such as effector cell recruitment, mucous production, and structural changes. Finally, we will test the antifungal effectiveness of IRAK-1 and IRAK-M deficient macrophages in response to Alternaria conidia. The results of this work will shed new light on the role of IRAK-1 and IRAK-M and may suggest new therapies involving their signaling pathways.

Aim 3: Establish the innate immune response to Alternaria major allergen Alt a 1 in human airway epithelial cells

Hypothesis: Production of the allergen Alt a 1 is required for induction of the innate immunity in response to A.alternata conidia in human airway cells.

Objective: In order to describe the biological role of the Alt a 1 major allergen in *A. alternata*, we will test the immunogenicity of the Alt a 1 protein and mutant Alternaria strains lacking or overexpressing Alt a 1. We will employ fungal molecular biology techniques to produce three classes of mutants: a deletion mutant *(*Δ*alta1*) where the Alta1 gene has been completely replaced with a selectable marker gene, the corresponding complementation mutant (Δ*alta1-rec*) with Alta1 restored, and an Alt a 1 overexpression strain (*Alta1*+) with a constitutive ToxA promoter upstream of the Alta1 gene . We will verify mutations by PCR and Southern analysis and will further confirm Alta1 expression levels by real-time PCR and secreted Alta1 levels by Western Blot and ELISA using an Alt a 1 specific antibody. The phenotypes of each mutant will be thoroughly analyzed to verify any changes which may cause a difference in the mammalian response to the fungus. This will include microscopic evaluation of conidia, calculation of germination rates, and assessing nutrient utilization on various carbon sources. Following this confirmation, we will treat both primary human airway epithelial cells and immortalized cell lines with the Alt a 1 protein or conidia from the wild-type and mutant *A.alternata* strains to assess differences in immunogenicity. We will measure the levels of cytokines IL-6 and IL-8 in the cell culture supernatant following stimulation to evaluate if the absence of or overexpression of Alt a 1 changes the innate immune response of the epithelial cells. Furthermore, we will complete a global gene expression analysis of multiple targets by use of QRT-PCR array harboring primers corresponding to a set of fungal innate immunity target genes to find unique

downstream mediators of Alt a 1. These results will be confirmed by real-time PCR analysis. Overall, the goal of **Aim 3** is to further establish Alt a 1 as a major component of *A.alternata* causing immune stimulation and to provide the tools in the form of mutant *A.alternata* strains for future in vivo characterization in mice.

Aim 4: Characterize key biochemical properties of Alt a 1 and their role in the response of human airway epithelial cells

Hypothesis: *The Alt a 1 protein possesses lipid binding capabilities and esterase activity which contribute to its ability to enter the epithelium and induce innate immunity.*

Objective: The fourth aim of the project will focus on in-depth analysis of the Alt a 1 protein including biochemical properties which may affect its immunogenicity. Specifically, we will investigate the role of the single RXLR-like motif within Alt a 1 by expressing wild-type and mutant forms with modified motifs in *E.coli* and then employ recognized experimental procedures to discern differences in their lipid-binding capacity. We will also explore the potential enzymatic activity of the protein by mutating the single histidine residue to a non-functional amino acid (alanine). These proteins will also be tagged with a fluorescent marker and incubated with human bronchalveolar epithelial cells to assess their ability to bind and enter the cells. The goal of these experiments will be to distinguish the importance of lipid binding and cellular entry, or enzymatic activity, or both in the response of human airway cells to Alt a 1. The results of fully defining Alt a 1's specific mechanism of entry and/or action will not only give a valuable target for allergy therapies, but will open up research on these properties for other known fungal allergens.

Since recombinant production of the Alt a 1 allergen is difficult due to various aspects of the protein's structure and biochemical nature a portion of **Aim 4** will discuss strategies which were used in the experiments leading up to completion of **Aim 4** including vector construction, culture conditions, and purification processes. We will hypothesize on why certain strategies were unable to produce purified Alt a 1 and discuss additional approaches which should be tested in the future to further improve recombinant production of Alt a 1.

REFERENCES:

1. Horner, W. E., Helbling, A., Salvaggio, J. E. & Lehrer, S. B. Fungal allergens. *Clin Microbiol Rev* **8**, 161–179 (1995).
2. Wilken-Jensen, K. & Gravesen, S. *Atlas of Molds in Europe Causing Respiratory Allergy*. (ASK Publishing: 1984).
3. Bartra, J., Belmonte, J., Torres-Rodriguez, J. M. & Cistero-Bahima, A. Sensitization to Alternaria in patients with respiratory allergy. *Front. Biosci.* **14**, 3372–3379 (2009).
4. Pulimood, T. B., Corden, J. M., Bryden, C., Sharples, L. & Nasser, S. M. Epidemic asthma and the role of the fungal mold Alternaria alternata. *J. Allergy Clin. Immunol.* **120**, 610–617 (2007).
5. Taralova, E. H., Schlecht, J., Barnard, K. & Pryor, B. M. Modelling and visualizing morphology in the fungus Alternaria. *Fungal Biol* **115**, 1163–1173 (2011).
6. Pryor, B. M. & Michailides, T. J. Morphological, pathogenic, and molecular characterization of alternaria isolates associated with alternaria late blight of pistachio. *Phytopathology* **92**, 406–416 (2002).
7. Simmons, E. *Alternaria, an Identification Manual*. (American Phytopathological Society: 2007).
8. Schuh, J. M., Blease, K., Kunkel, S. L. & Hogaboam, C. M. Chemokines and cytokines: axis and allies in asthma and allergy. *Cytokine Growth Factor Rev.* **14**, 503–510 (2003).
9. Kato, A. & Schleimer, R. P. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr. Opin. Immunol.* **19**, 711–720 (2007).
10. Denis, O. *et al.* Chronic intranasal administration of mould spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodelling. *Immunology* **122**, 268–278 (2007).
11. Havaux, X., Zeine, A., Dits, A. & Denis, O. A new mouse model of lung allergy induced by the spores of Alternaria alternata and Cladosporium herbarum molds. *Clin. Exp. Immunol.* **139**, 179–188 (2005).
12. Akira, S. Toll-like receptor signaling. *J. Biol. Chem.* **278**, 38105–38108 (2003).
13. Gan, L. & Li, L. Regulations and roles of the interleukin-1 receptor associated kinases (IRAKs) in innate and adaptive immunity. *Immunol. Res.* **35**, 295–302 (2006).
14. Arcaroli, J. *et al.* Variant IRAK-1 haplotype is associated with increased nuclear factor-kappaB activation and worse outcomes in sepsis. *Am. J. Respir. Crit. Care Med.* **173**, 1335–1341 (2006).
15. Aden, E. *et al.* Standardization of Alternaria alternata: extraction and quantification of alt a 1 by using an mAb-based 2-site binding assay. *J. Allergy Clin. Immunol.* **104**, 128–135 (1999).
16. Asturias, J. A. *et al.* A sensitive two-site enzyme-linked immunosorbent assay for measurement of the major Alternaria alternata allergen Alt a 1. *Ann. Allergy Asthma Immunol.* **90**, 529–535 (2003).
17. De Vouge, M. W. *et al.* Molecular cloning of IgE-binding fragments of Alternaria alternata allergens. *Int. Arch. Allergy Immunol.* **116**, 261–268 (1998).
18. Kleine-Tebbe, J. *et al.* Predominance of the major allergen (Alt a I) in Alternaria sensitized patients. *Clin. Exp. Allergy* **23**, 211–218 (1993).
19. Kurup, V. P., Vijay, H. M., Kumar, V., Castillo, L. & Elms, N. IgE binding synthetic peptides of Alt a 1, a major allergen of Alternaria alternata. *Peptides* **24**, 179–185 (2003).

20. Hong, S. G., Cramer, R. A., Lawrence, C. B. & Pryor, B. M. Alt a 1 allergen homologs from Alternaria and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genet. Biol.* **42**, 119–129 (2005).
21. Gershwin, L. J. Effects of allergenic extracts on airway epithelium. *Curr Allergy Asthma Rep* **7**, 357–362 (2007).
22. Andersson, M., Downs, S., Mitakakis, T., Leuppi, J. & Marks, G. Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitized children. *Pediatr Allergy Immunol* **14**, 100–105 (2003).
23. Boitano, S. *et al.* Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. *Am. J. Physiol. Lung Cell Mol. Physiol.* **300**, L605–614 (2011).
24. Fahy, J. V. Remodeling of the airway epithelium in asthma. *Am. J. Respir. Crit. Care Med.* **164**, S46–51 (2001).
25. Kauffman, H. F., Tamm, M., Timmerman, J. A. B. & Borger, P. House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. *Clin Mol Allergy* **4**, 5 (2006).
26. Borger, P. *et al.* Proteases from Aspevgillus fumigatus Induce Interleukin (IL)-6 and IL-8 Production in Airway Epithelial Cell Lines by Transcriptional Mechanisms. *Journal of Infectious Diseases* **180**, 1267–1274 (1999).
27. Sáenz-de-Santamaría, M., Guisantes, J. A. & Martínez, J. Enzymatic activities of Alternaria alternata allergenic extracts and its major allergen (Alt a 1). *Mycoses* **49**, 288–292 (2006).
28. Dou, D. *et al.* RXLR-mediated entry of Phytophthora sojae effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* **20**, 1930–1947 (2008).
29. Kale, S. D. & Tyler, B. M. Identification of lipid-binding effectors. *Methods Mol. Biol.* **835**, 393–414 (2012).
30. Kale, S. D. *et al.* External Lipid PI3P Mediates Entry of Eukaryotic Pathogen Effectors into Plant and Animal Host Cells. *Cell* **142**, 284–295 (2010).
31. Joenväärä, S. *et al.* Caveolar transport through nasal epithelium of birch pollen allergen Bet v 1 in allergic patients. *J. Allergy Clin. Immunol.* **124**, 135–142.e1–21 (2009).
32. van der Meer-Janssen, Y. P. M., van Galen, J., Batenburg, J. J. & Helms, J. B. Lipids in host-pathogen interactions: pathogens exploit the complexity of the host cell lipidome. *Prog. Lipid Res.* **49**, 1–26 (2010).
33. Lemmon, M. A. Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* **9**, 99–111 (2008).

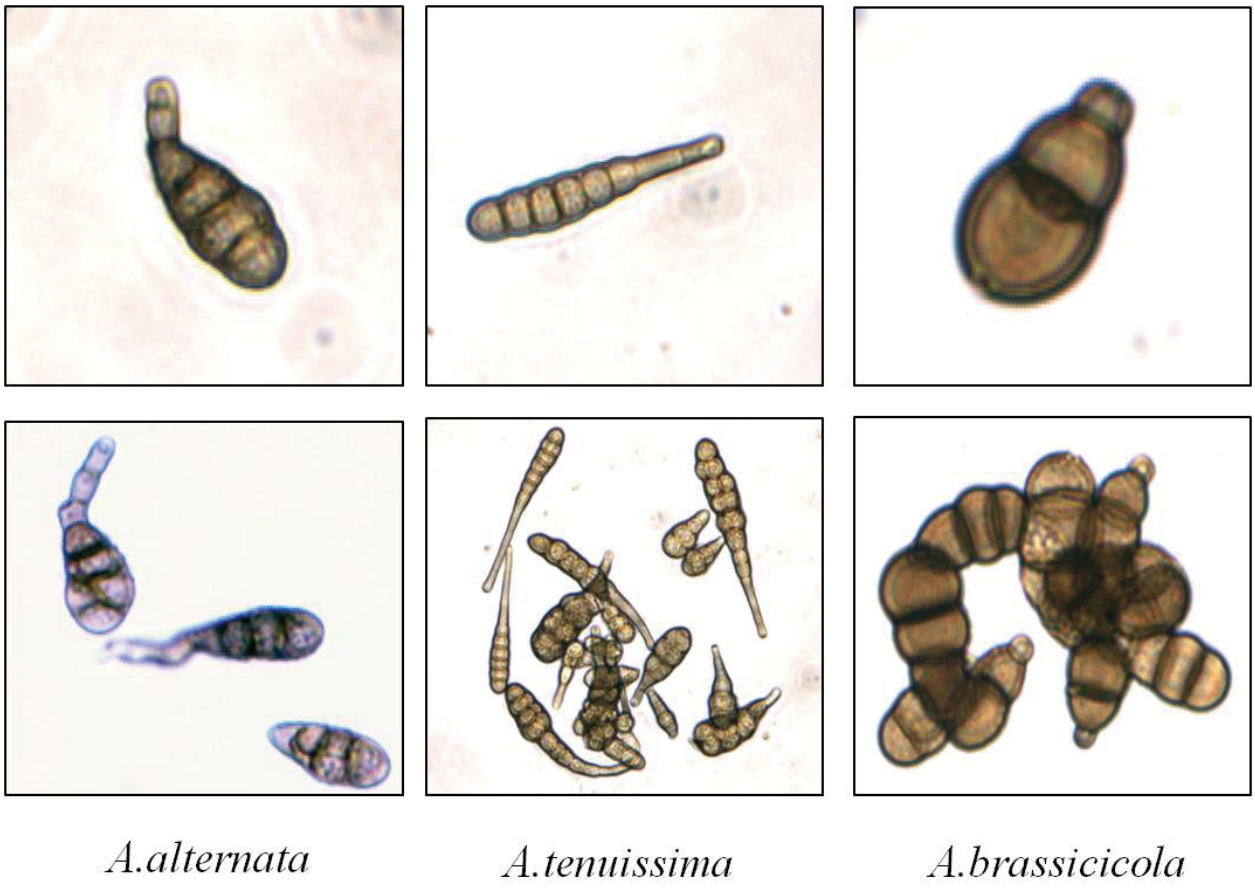


Figure 1.1: Morphological variation among Alternaria species. Conidia from *A.alternata* (ATCC: 66981), *A.tenuissima* (ATCC: 11680), and *A.brassicicola* (ATCC: 96836) were collected in water from a 7-day old PDA plate and imaged under bright field microscopy (Magnification: 40x).

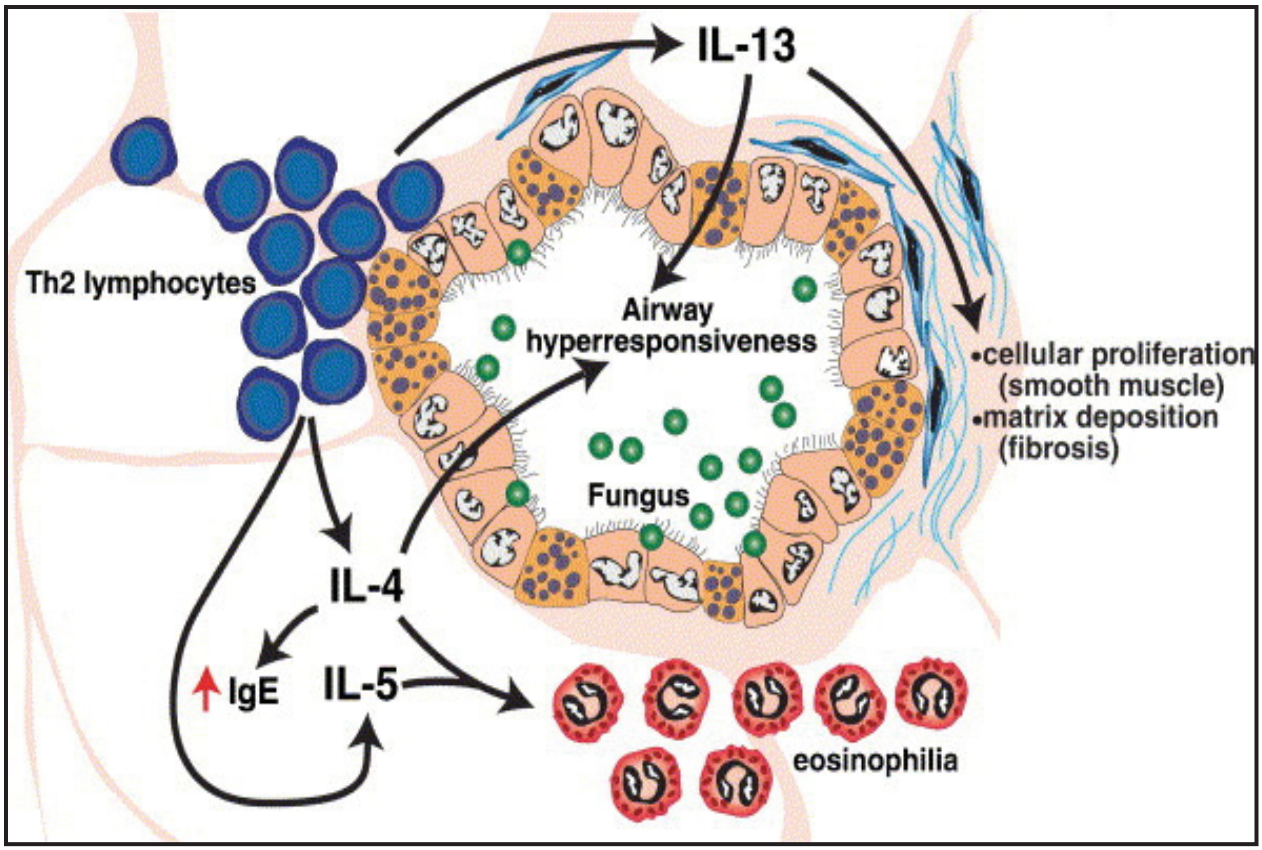


Figure 1.2: Innate immune response to fungus (Schuh et al, 2003).

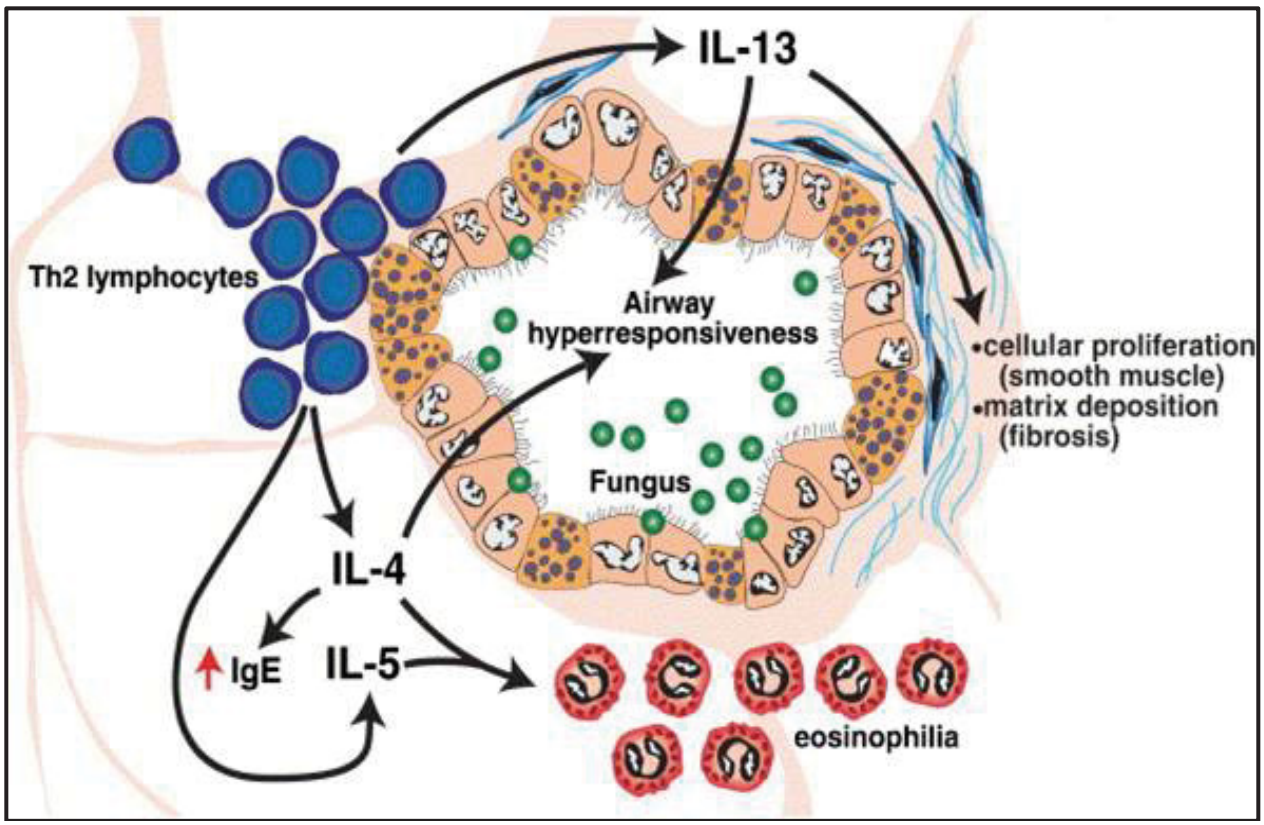


Figure 1.3: Propagation of the Th2-driven chronic allergic airway response (Schuh et al, 2003).

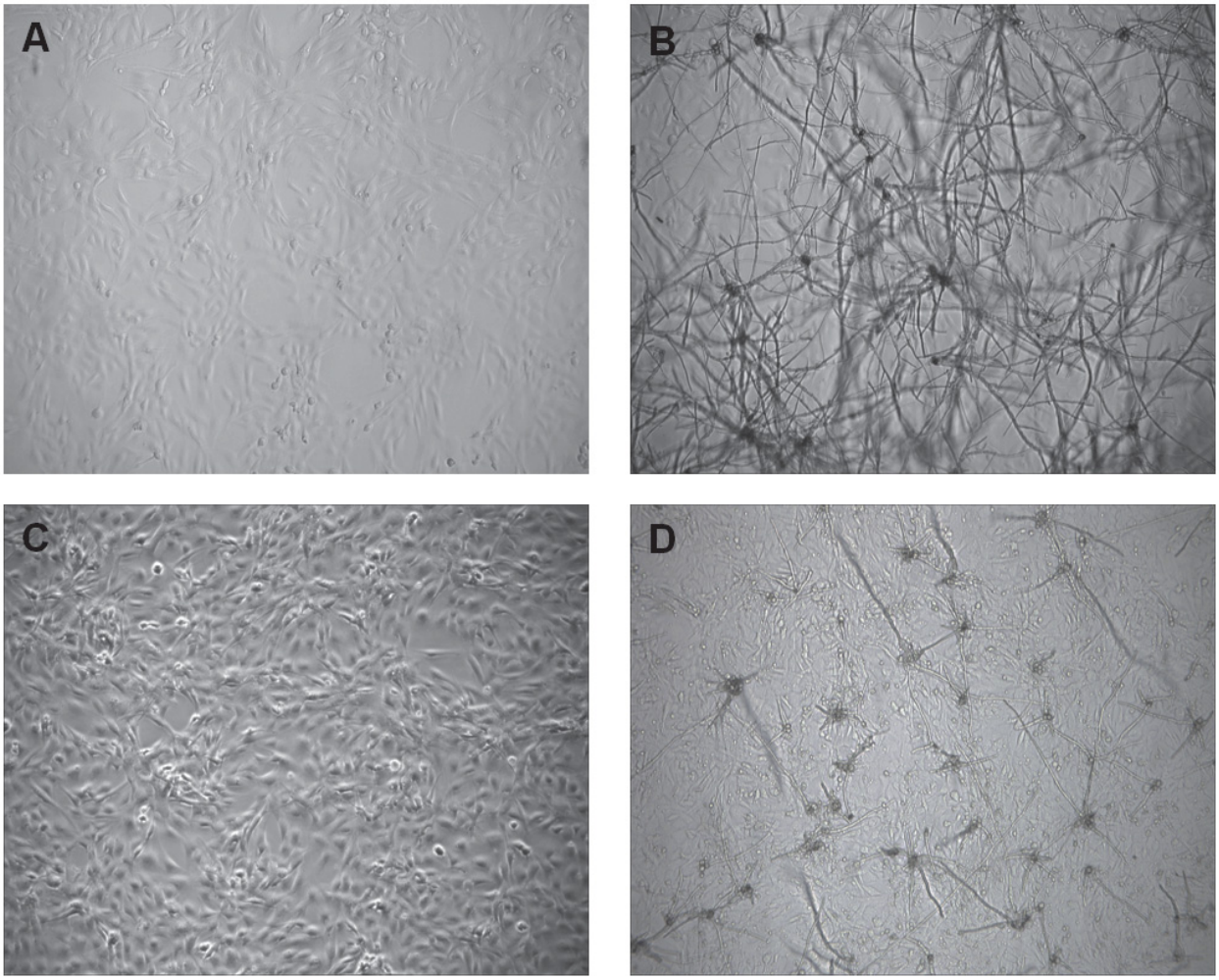


Figure 1.4: Germinating *A.alternata* in the presence of human airway epithelial cells. BEAS-2B cells were incubated with live *A.alternata* spores for 24hrs under normal conditions at either (B) 34°C or (D) 37°C and 5% CO_2. Control cells were left untreated (A and C). Cultures were imaged using an inverted phase-contrast microscope. Magnification: 40X. Bars = 20μm.

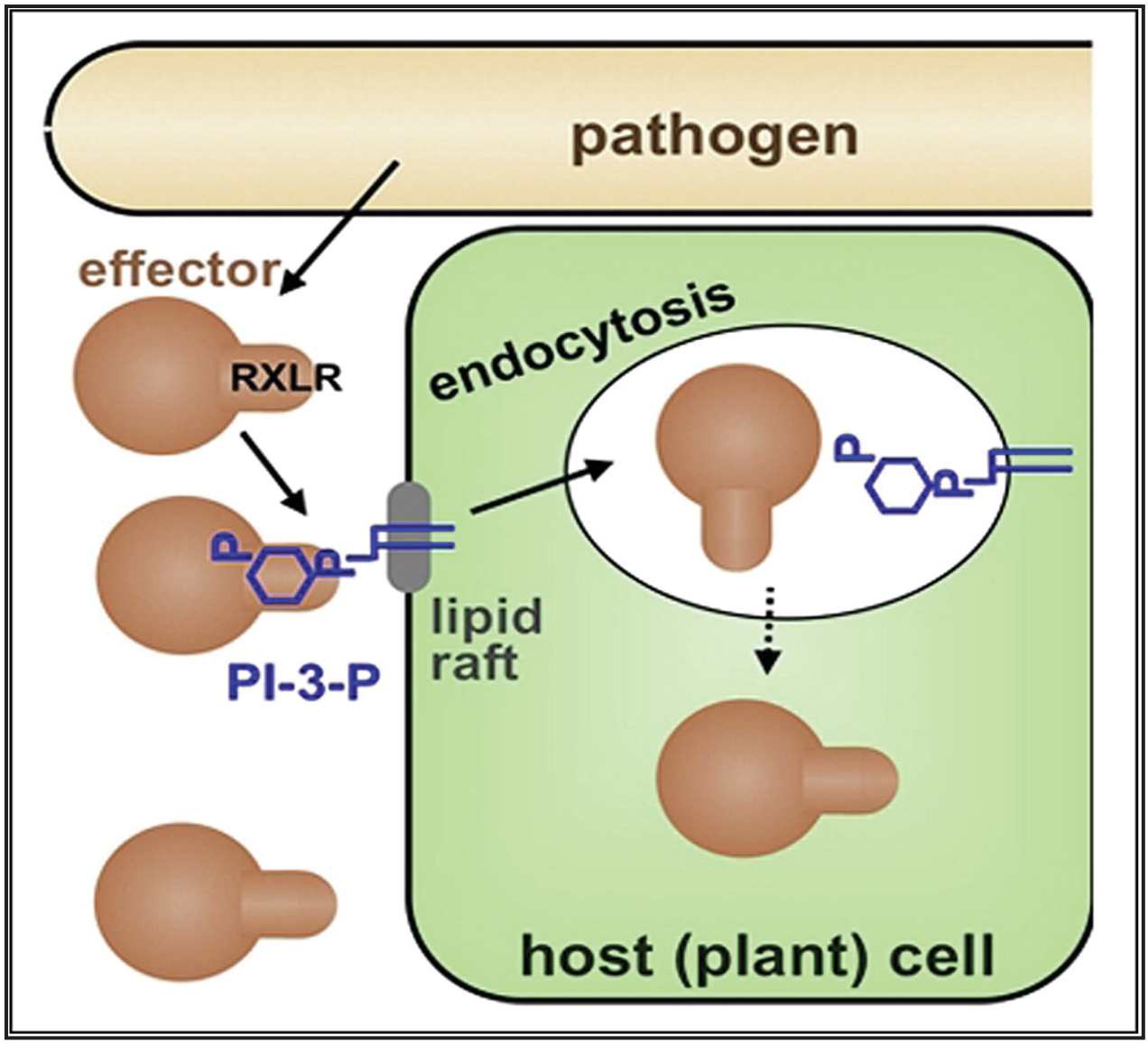


Figure 1.5: Model for effector entry. Binding of effectors via their RXLR domains to PI-3-P, possibly located in lipid rafts, leads to entry by endocytosis. The mechanism of escape from endosomes is currently unknown. The moderate affinity of the effectors for PI-3-P facilitates binding on the outer surface but dissociation from PI-3-P inside the cell. (Kale et al, 2010).

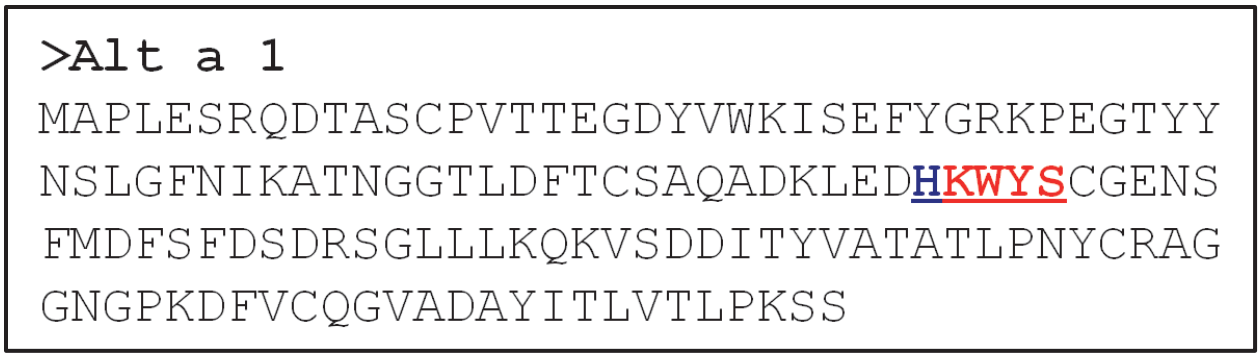


Figure 1.6. Amino acid sequence of the Alternaria allergen Alt a 1. The protein sequence indicates a single histidine at position 84 highlighted in BLUE and the putative RXLR, 85KWYS88 highlighted in RED.

Chapter II

Establishing murine models of airway inflammation to study effects of chronic Alternaria exposure and innate immunity

ABSTRACT:

The pathogenesis of allergy and asthma often initiates with an innate immune response. *Alternata alternata* is a well-documented source of airborne allergens with the compelling ability to induce an immune response *in vivo*. However few murine models have been developed to define specific parameters such as inoculation or sample collection for analyzing the innate immune response to *Alternaria* and typically utilize complex, potent fungal extracts to enhance the airway response. Furthermore, no studies have distinctly characterized this response using whole fungal spores. In this study we illustrate two novel mouse models of airway inflammation that utilize the spores of *A. alternata* to study the *in vivo* innate immune response without sensitization. Our data reveal that *A. alternata* spores are immunogenic in an inhalation mouse model after both chronic exposure, and more significantly, a single intranasal challenge as defined by cell infiltration into the lung, pro-inflammatory cytokine production, and histopathological markers of airway disease. In the chronic model, mice were either intranasally challenged with *A.alternata* spores or *Alternaria* extract. Resulting bronchoalveolar lavages (BALs) collected following these treatments contained an increase in total cells including numerous macrophages, neutrophils, eosinophils and lymphocytes while the extract caused a markedly greater response compared to the spores. Histopathology also revealed laminar

thickening of the bronchioles in both treatments with the extract again giving a greater, but less pathologically relevant response. However, secretion of Th2 cytokines including IL-4, IL-6, and IL-13 were found in the BAL following treatment with either spores or extract with IL-4 being more significantly enhanced in response to spores than extract. Total serum IgE was also more elevated in the spore treated mice than those treated with extract suggesting that spore treatment was more powerful at driving Th2 immunity. For the innate model, we used a single intranasal challenge and found this technique was sufficient for accurately studying the initial immune response to inhaled fungal spores. Total cell recruitment into the lung was evident from six to thirty-six hours after the challenge which specifically correlated with signs of cachexia. Both general inflammatory and Th2 coordinating cytokines were detectable although their expression changed over the 36-hour window suggesting sample collection times must be precise for assessing the levels of particular cytokines. Intriguingly, we were also able to test the effectiveness of our model by comparing the response to a single-challenge with wild-type or *Δalta1* spores which have had the major *Alternaria* allergen gene, Alta1, deleted. The single challenge model proved to be suitable for detecting measurable differences between the wild-type and mutant strain. Collectively, both of these models will serve as vital tools in elucidating the innate and chronic immune response to *Alternaria*.

Introduction:

Fungi are known to play a vital role in the pathogenesis of multiple human disorders including asthma, allergic rhinitis, bronchopulmonary mycoses, and airway hypersensitivity (1). Many of these fungi are common components of the atmospheric aerospora and their counts vary widely in indoor and outdoor environments with season, geographic regions, and climatic conditions (2, 3). This ubiquitous distribution makes it nearly impossible to avoid contact with the fungus and potential sensitization, an important factor given exposure has been defined as the most important risk factor for the development and exacerbation of fungal allergies and asthma (4). Furthermore, fungal organisms can have a greater impact on the host airway and immune system than other allergenic sources, such as pollen and dust, due to their active production of toxins, proteases, enzymes, and potentially volatile organic compounds (5–7)

Alternaria alternata is saprophytic fungus which is considered one of the most important aeroallergens in the United States (8, 9). Sensitization to *A.alternata* has been linked to particularly severe and persistent cases of asthma, especially in children (10, 11). Furthermore, in one study, patients with chronic rhinosinusitis (CRS) exhibit a hypersensitive immune response to *Alternaria* more than other aeroallergens (12). Multiple studies have shown a correlation between a large proportion of atopic patients having skin reactivity to *Alternaria* (13–15). The major allergen of *Alternaria* has been identified as Alt a 1 due to its IgE reactivity to serum of sensitized individual, is constitutively expressed and is rapidly secreted by germinating fungal spores (16–20). We have successfully produced a deletion mutant *A.alternata* strain with a full replacement of the Alt a 1 allergen gene to utilize in models of allergic airway disorders (21). By deletion of the allergen gene we can test the importance of its release in regulating the immune response.

Since allergy and asthma pose a sizeable health threat to the human population, animal models have been designed to investigate their pathogenesis, understand underlying processes and pathways, and discover potential therapeutic targets (22). Most of these models, designed to study effects of acute or chronic challenges, examine the clinical manifestations of an adaptive immune response following sensitization. Frequently mice are intraperitoneally injected with chicken egg ovalbumin (OVA) to create an "asthmatic" or sensitized phenotype. Once the adaptive immune response has had enough time to develop against the agent, typically 21 days after the initial injection, the mice are then intranasally challenged with OVA over a few days (acute) or many weeks (chronic) to induce robust allergic pulmonary inflammation in the airway. Although this is a routine model for testing new compounds and their ability to improve symptoms of airway inflammation, it does not offer much to assessing the natural pathogenesis of the disorder since OVA is not a common human allergen and the sensitization scheme is relatively artificial. Other studies have utilized similar models to test the ability of specific allergens to induce the immune response, but they also employed either an adjuvant or carrier protein to condition the response (23, 24).

Mouse models of *Alternaria*-induced airway inflammation have well established the fungus's ability to trigger and sustain hallmarks of allergy and hypersensitivity, but have focused on chronic exposure and/or used prepared fungal extracts instead of the actual organism (25–27). Fungal extracts pose a particular challenge since they are highly variable lot to lot especially in their allergen content and inflammatory potential (28, 29). One published model examined the acute response to *Alternaria* spores without sensitization, however the mice were challenged with an abnormally large, pathologically irrelevant quantity of spores (one million spores/dose) over three consecutive dosing days (30). Another model looked at the response following a

single intranasal challenge, but this study used *Alternaria* extract as the treatment (31). Regrettably the authors did not give any indication the extract was tested for potentially immune stimulating compounds such as endotoxin or organic compounds and thus their results may be erroneous. Alternatively, another model set out to establish the chronic response to *Alternaria* spores but was unable to complete the treatments due to induced cachexia and instead extract was used for examining the chronic response to *Alternaria* (32).

Polarization of the inflammatory response after fungal exposure is due to increased expression of Th2-promoting cytokines (33). In chronic allergic pulmonary disorders, IL-4, IL-6, and IL-13 are important in the maintenance and severity of airway response. IL-6 is released by epithelial cells, macrophages, and T cell subsets in response to various stimuli, including specific allergens (34). It can influence effector cell functions and appears to be essential for goblet cell metaplasia and mucus hypersecretion in an allergic model of asthma (35). There is also an apparent correlation between increased levels of IL-6 in the bronchoalveolar lavage (BAL) and serum and atopic patients. IL-13 is a significant cytokine inducing the pathophysiological features of asthma (36). It is readily secreted by airway epithelial cells and Th2 cells following stimulus (37, 38). IL-13 is multifactorial in organizing the immune response including supporting survival and degranulation of eosinophils, promoting activation of macrophage, and mucous hypersecretion (39–41). Its existence at high levels in the airways of allergic and asthmatic patients is also well documented (42–44). Furthermore, it has the ability to direct human B-cells to synthesize IgE, leading to a traditional allergic reaction – allergen specific mast cell degranulation (45). IL-4 is also important for IgE isotype switching, mucus secretion, and differentiation of T helper type 2 lymphocytes leading to cytokine release (46, 47). Polymorphisms in the IL-4 gene locus have been implicated in persistent asthma (48) .

Moreover, abnormally high levels of IL-4 are frequently found in the airways of asthmatic patients including children (49–52).

The innate immune response to airborne fungi features another set of cytokine markers. The potent neutrophil chemoattractant KC(CXCL1), is likely the murine structural homolog of the human innate cytokine Interleukin-8 (53). KC(CXCL1) has been shown to be important in the response to inflammatory stimuli and restoring mucosal integrity in mice (54). In humans, IL-8 is released by epithelial cells and resident macrophage and its primary function is in neutrophil and antigen presenting cell (APC) recruitment. It is well documented in the airway epithelial cell response to fungi and allergens (55–57). In mice, resident cell expression of KC(CXCL1) has been shown to be important in inflammatory cell recruitment into the lung tissue (58, 59). Additionally, its functionality in neutrophil recruitment and balancing of an overt response appears to be essential for the innate immune reaction to fungi in the lung (60, 61). A more recently discovered cytokine of innate immunity is thymic stromal lymphopoietin *(TSLP), which is primarily produced by non-hematopoietic cells such as epithelial cells and fibroblasts and* initiates the release of T-cell promoting chemokines from monocytes, enhances the maturation of dendritic cells, and induces Th2 cytokines (62). TSLP is secreted by epithelial cells in response to both proteolytic enzymes and fungi and may play a role in the crosstalk between epithelial cells and dendritic cells, leading to their activation (63, 64). Overall, KC(CXCL1) and TSLP production are markers of acute airway inflammation and their presence can indicate the effectiveness of our proposed innate mouse model of airway disease.

Despite the increased prevalence of allergy and asthma in developed countries, few clinical therapies have been uncovered for their treatment (65, 66). In order for therapies to be tested and for results to be understood, precise murine models must be developed. The goal of

this study was to describe the development of two distinct mouse models of *Alternaria*- induced inflammation. The chronic model examines how repeated exposure to *Alternaria* spores can quickly induce hallmark signs of airway inflammation in a non-sensitized host and proves a significant distinct response to *Alternaria* spores compared to the more commonly employed extract. The innate model investigates the immediate response of the airway following a single challenge of *Alternaria* spores. Substantial data will be presented regarding the importance of optimal intranasal inoculums and sample collection times as well as this model's ability to characterize differences between the wild-type *Alternaria* spores and a *Δalta1* deletion mutant. Both models can be readily employed to studying the innate immune response to *Alternaria*.

Materials and Methods:

Fungal cultures and extracts:

*Alternaria alternata (*ATCC #66981) was used as wild-type *Alternaria* in all experiments. The *A.alternata Δalta1* deletion mutant was constructed on the background of ATCC 66981 and features a complete replacement of the allergen Alta1 gene as previously described(21). Fungi were propagated on potato-dextrose -agar (PDA) (0.4% potato starch, 2% dextrose, 1.5% agar) and were grown at 25°C in the dark. For intranasal challenges, spores were harvested into sterile water from a 7-day old PDA plate. Suspensions were standardized to equal concentrations to 1 x 10^6 spores/mL after counting on a hemacytometer and then used immediately in intranasal challenges. Preparations were made on each treatment day from the same culture plate.

Culture filtrate *Alternaria* extract (called *Alternaria* extract subsequently) was obtained commercially from Greer Laboratories (Lenoir, NC). Lyophilized extract was resuspended in PBS to a concentration of 1mg/mL. Aliquots of this preparation were stored frozen at -20°C until use in murine experiments.

Animals and immunizations

Female BALB/cJ (BALB/c) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were 8 - 12 weeks old at time of experiments. The procedures and handling of the mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Polytechnic Institute and State University (IACUC approval number 09-178-VBI). Mice were housed in isolation chambers with individual air and water supply and provided food and water *ad libitum*.

For chronic and innate challenge experiments, mice were lightly anaesthetized with isofluran (Abbott Laboratories) in individual chambers of a rodent anesthesia machine with precision vaporizer (Impac6, VetEquip) at a concentration of 2 - 3% and oxygen at 2 L/min. When the mice were unresponsive but breathing comfortably, 50 µl of spore or extract solution was directly applied on the nostrils. Control mice were treated with 50 µl of DPBS. The animals were allowed to slowly inhale the liquid and then to recover in a supine position under a heat lamp. For the chronic model, 4 - 5 mice were in each treatment group in two separate experiments. In the single-challenge model, samples were collected from 2 – 3 mice per treatment at 6, 12, 24, and 36 hours following the challenge. The control group contained 4 mice which were sacrificed at the 24 hour time point.

Changes in total body weight of each animal were assessed by weighing immediately before initial challenge and then again directly before euthanasia. In chronic experiments, mice were weighed before each challenge and observed for signs of cachexia each day throughout duration of experiment.

Serum collection and total IgE determination

Mice were placed under general anesthesia by intraperitoneal injection of ketemine (100 mg/kg)/ xylazine (10 mg/mL) solution. Blood was collected from the retro-orbital plexus by gentle rotation of a non-heparinized micro-hematocrit capillary tube against the medial canthus. Blood was collected into a sterile 1.5mL tube and allowed to clot for 30 min. Serum was separated via centrifugation at 1000 *x g* for 10 min in a tabletop centrifuge. Serum was removed to a fresh microcentrifuge tube at stored at -80°C until analysis.

IgE levels in sera were determined using a sandwich ELISA following the manufacturer's instructions (Biolegend. Plates were developed by the addition of 100 µl of TMB and the reaction was stopped with 50 µl of 2N H_2SO_4 and O.D. was read at 450 nm with an automatic plate reader (Modulus Microplate, Turner Biosystems). Serum titer was determined by comparison with standard curves of purified mouse IgE.

Bronchoalveolar lavage

Mice were euthanized by an overdose of pentobarbital (FatalPlus) 24 hours after the last challenge. The trachea was exposed and incised. An 18 gauge blunt-end needle was inserted into the trachea and BAL fluid was harvested by washing the lungs twice with 1 ml of DPBS. BAL was centrifuged at 500 *x g*, red-blood cells were lysed by addition of ACK lysing buffer, cells were again pelleted by centrifugation, and the cell pellet was resuspended in RPMI media. The cell-free supernatant was stored at -80°C prior to ELISA analysis. An aliquot of cells were diluted into a trypan blue/DPBS solution and counted on a hemacytometer. Differential cell counts were obtained by counting at least 100 cells on cytospin slides stained with Diff-Quick (Jorvet).

Histopathological analysis

Tissues for histopathological examination were collected 24 hours after the last intranasal instillation. Following collection of BAL fluid, the lungs were inflated with 10% buffered formalin, latter embedded in paraffin, and then sectioned (5 µm) and stained with hematoxylin, eosin, and saffron (HES) for examination of pulmonary architecture.

Cytokine ELISA

Cytokines were measured from the cell-free bronchoalveolar lavage fluid (BALs). The levels of IL-4, IL-6, IL-13, KC (CXCL1), and TSLP were quantified by ELISA using commercial kits according to the instructions provided by the manufacturers (Biolegend). Briefly, plates were coated with primary antibody for the selected cytokines and incubated overnight. Plates were washed, blocked with assay diluent, and 100µl of BAL sample or cytokine standard were added per well. Plates washed and then incubated with secondary antibody. Following another wash, streptavidin-HRP was added to each well, plates were again washed, and then developed by addition of TMB reagent. Once desired level of development was reached, the reaction was stopped by addition of 2N H_2SO_4. The absorbance was read at 450nm on a standard plate reader (Modulus Microplate, Turner Biosystems).

Results:

Chronic administration of *Alternaria* spores or extract causes inflammatory cell infiltration into the lung

The overall goal for the first model was determining a viable challenge schematic for chronic exposure to live spores similar to fungal extract. Since previous studies have shown chronic administration of *Alternaria* mold spores results in severe cachexia (32) we closely monitored mice over the 14-day experiment for clinical signs of distress such as loss of lean body mass and fat mass, weakness, fatigue, and inflammation surrounding the nasal and ocular passages. Weight was measured prior to each challenge and total change in body weight over the course of the experiment was calculated. While the control group of mice gained an average of 1.8g between initial and final challenge, the spore challenged mice only gained an average of 0.2125g while the extract treated mice lost an average of 0.22g in total body weight (Figure 1a). The spore and extract treated mice did not exhibit visible signs of lethargy; however, the nostrils of the extract treated mice showed more redness and irritation compared to the PBS or spore treated mice, likely due to the high protease and potential toxin and or allergen content of the extract (6, 25, 28, 67) These medical observations suggest the potency of the extract may be greater than the actual organism.

To characterize pulmonary inflammation in the chronic mouse model, BALB/c mice intranasally challenged with 5 x 10^5 *Alternaria* spores or 50μg of *Alternaria* extract (in 100ul total volume) every other day over 14 days (total of eight challenges). Control mice were treated with an equal volume of DPBS.Twenty-four hours after the final challenge, BAL fluid samples were analyzed for total cell counts and the number of individual macrophages, neutrophils, eosinophils and lymphocytes. Figure 2b shows that the total number of cells obtained in BAL significantly increased with chronic challenge of spores or extract; however, the extract

treatment resulted in a 50% greater increase compared to the *Alternaria* spores. Examination of the difference among the total cell population collected in the BAL fluid showed intranasal challenges of both spores and extract leads to the accumulation of a high number of common inflammatory cells including neutrophils, eosinophils, and macrophage and to a lesser extent, lymphocytes (Figure 2c). All of these cell types play a significant role in orchestrating the innate and adaptive immune response in the lung.

Histopathological evidence of airway remodeling in a non-sensitized chronic model

Histological examinations of lung sections from mice challenged with *Alternaria* spores or extract were compared to lung sections of the DPBS control. Results of BAL fluid analysis were confirmed by significant observation of cell recruitment into the lung tissue of from both spore and extract challenges. While neutrophils were the largest observed subpopulation of cells, there was also a marked accumulation of eosinophils around the bronchioles (Figure 3). A severe thickening of the airway epithelium, especially surrounding the bronchioles, was also evident resulting in considerable constriction or complete occlusion of airway passages. As with the total BALF cell counts, the histopathologic observations showed the *Alternaria* extract induced a more severe response in the lung tissue, but, more importantly, the *Alternaria* spores stimulated a potent response as well.

Presence of Th2 cytokines in BALF

Production of Th2 cytokines are necessary for induction of allergic airway inflammation (reference). IL-4 and IL-13 are required for maintenance of Th2 cell lineage and of critical importance to stimulate B-cell immunoglobulin (Ig) class switch to IgE (45, 68). IL-6 has been

shown to be required for mucous production following inhalation of fungal allergens (35). The presence of IL-4, IL-6 and IL-13 was analyzed by ELISA in the BALFs (Figure 4). Interestingly, the spores induced IL-4 a much greater levels while the extract only slightly stimulated IL-4, suggesting *Alternaria* spores interact differentially with the immune cells of the lung compared to the *Alternaria e*xtract. Both treatments induced production of IL-6 and IL-13 at similarly high levels compared to the DPBS control.

A single challenge of *Alternaria* spores results in increased total IgE levels

In order to deduce the necessity for prior intraperitoneal (i.p) sensitization for production of IgE, we testing total serum IgE levels following the final intranasal challenge. We found the mice treated with *Alternaria* spores had a significant increase in total IgE levels, while the extract did not induce IgE to levels considerably greater than the DPBS control (Figure 5). This confirms that i.p. sensitization is not necessary for sensitization to *Alternaria* spores and that use of a more natural inhaled sensitization approach is possible for models using live spores. It may also suggest that spores compared to extract possess greater capacity via a greater induction of IL-4 to induce IgE class switching.

A single-challenge of *Alternaria* spores causes early signs of cachexia and cell recruitment into the lung

We developed a single-challenge mouse model in order to study the early and late phase of the initial innate immune response to inhaled *Alternaria*. Because mice have a very high rate of metabolic activity, we closely examined changes in total body weight in the hours following the single challenge. Within the first six hours post-challenge, a decrease in total body was

evident and this weight loss trend continued for 24 hours after the challenge before recovering to positive change in weight after 36hrs (Figure 6a). Since the control mice were collected at 24 hrs after their challenge of PBS, we can directly compare this group to the 24 hr spore challenged group. We see the control group gained an average of 0.5g while the treatment group lost an average 0.5g yielding a net difference of 1.0g in total body weight. This was initial evidence that a single-challenge of *Alternaria* spores was enough to cause a clinical response in a murine model.

Analysis of the BAL fluid revealed a significant increase in the total cell population within the lungs (Figure 6b). At 6hr following the challenge, an increase was seen but not statistically different from the control. However at 12, 24, and 36hr post challenge the total cell count is substantially increased compared to the PBS control. Total cell infiltration appears to peak at 12 – 24hr following the challenge before a considerable decrease after 36hr though still elevated at this time. Most interesting is that the total cell count is proportional to weight loss, suggesting that as cell numbers increase, metabolic activity decreases in the mice. This would agree with the common clinical response to an inhaled immune stimulus and strengthens our understanding regarding the effectiveness of this single-challenge model.

Histopathology reveals cell infiltration into the lung is primarily neutrophilic after a single intranasal challenge of *Alternaria* spores:

Lung tissue was collected and analyzed from the control and spore treated mice at the 24 hr timepoint. The spore challenged mice displayed primarily neutrophilic infiltration into their airway with a slight increase in eosinophils presents compared to the untreated control. There

was also minor but noteworthy thickening of the luminal wall, like to do goblet cell activation (69).

Both innate and Th2 cytokines are found following a single challenge of *Alternaria* spores

Since the single-challenge model is aimed at examining the innate immune response and we saw evidence neutrophil recruitment, we chose to measure the level of the neutrophil chemoattractant KC(CXCL1). We found increased levels of this cytokine were detectable as early as six hours following the intranasal challenge (Figure 8a). The levels of KC show a modest drop after this preliminary spike, but remain elevated through the 36 hr measurement period. This would support the results of the total cell counts; since KC(CXCL1) levels are highest at 6 hr it is reasonable to assume at time points immediately following we would see the highest cell counts (especially neutrophils- Figure 6). As a contrast to this primarily innate cytokine, we also measured IL-13, an important cytokine in shaping the adaptive immune response. Changes in the level of IL-13 found in the BAL remained insignificant until after 12 hrs post challenge, when levels quickly increased and remained high at the 36hr timepoint.

Single-challenge model is effective for analyzing differences in total cell infiltration between wild-type and mutant *Alternaria* strains

We chose to design a single-challenge model in order to test the innate immune response against various mutant fungal strains in the future. We previously manipulated an *A.alternata Δalta1* deletion mutant which has a full replacement of the allergen gene Alta1. We utilized this strain to see if we could detect a difference in the murine airway immune response after a single challenge of the mutant or the wild-type strain. Previous *in vitro* analysis of human airway

epithelial cells revealed the *Δalta1* strain has a decreased ability to induce major innate immune cytokines (Rumore et al, unpublished results). Based on our previous data in cell line experiments, we hypothesized the mutant would have a reduced capacity to recruit cells into the airway. Results of our BAL analysis following a single challenge with wild-type or *Δalta1* indicate that although the mutant was able to increase recruitment of cells into the lungs, it was at a significantly lower amount (Figure 9).

Evaluating optimal *Alternaria* spore inoculum for single-challenge model

Previous studies using intranasal challenges of fungal spores have featured sizable diversity in the inoculums utilized. Due to *Alternaria*'s large spore size, it is accurate to use fewer total spores than other fungi, such as *Aspergillus*. However, for consistency in our future experiments, we chose to test a range of spore concentrations to determine which inoculum size was optimal for evaluating differences between two *Alternaria* strains. We again challenged mice one time with either wild-type *Alternaria* or the *Δalta1* mutant but at amounts ranging from 1×10^4 to 1×10^5. We measured the level of the chemokine KC(CXCL1) in the BAL fluid by ELISA for each inoculum amount (Figure 10). We found we could detect the most significant difference between the wild-type and mutant at the lowest (1×10^4) and highest (1×10^5) amount tested. This would suggest it is difficult to distinguish what role the mutation is playing in the immune response at concentrations in between very low and very high doses of spores.

Differences in early and late phase immune response following a single challenge of *Alternaria* spores

Our single-challenge data would suggest the innate immune response is rapidly activated within the first few hours following the challenge and additionally changes dramatically over the first two days. Thus the window of examining various cytokines is important in the context of sample collection time. TSLP has been found to be an important innate cytokine involved in conditioning antigen-presenting cells, driving Th2 immunity, and its expression is enhanced in mouse model of asthma-like airway disorders (63, 70). We previously test BAL fluids from previous experiments for TSLP and found variability among the results especially in samples at or after 24hrs following challenge (data not shown). We utilized the BALF from the timecourse experiment comparing the wild-type and *Δalta1* mutant to see if differences in TSLP production could be detected. We found that the greatest amount of TSLP was found 6 hr after challenge and that levels were reduced 48hr after the challenge (Figure 11). This shows that TSLP is swiftly produced or released in the airway but its presence is not sustained unless the stimulus is consistently inhaled. We also confirmed that we will need to collect samples at both early (less than 6 hr) and late (more than 36 hr) timepoints following a single challenge in order to get an accurate view of the murine airway's innate immune response to inhaled fungi.

Discussion:

Alternaria exposure is well characterized in the development of allergic disease, especially asthma (9, 10, 25, 71), however few mouse models have thoroughly examined the innate immune response to the living fungus. In this study we developed a new mouse model using the spores of *A. alternata to directly measure the innate immune response and response caused by chronic, but not sensitized, intranasal exposure.* We also set to describe the advantage of using whole spores versus the potent fungal extract used in most previous studies. The spores can mimic a more natural contact with *Alternaria* in the environment compared to only exposing the host to the proteins, allergens, and cell wall components they would normally encounter following inhalation instead of the numerous intracellular elements, including toxins, found in *Alternaria* culture extract preparations. Unlike *Aspergillus fumigatus*, *Alternaria* spores are too large to be engulfed and do not colonize the airway to a large extent. They are rapidly cleared by beating cilia and thus normal exposure would likely be sporadic and in intervals rather than unlike previous models of heavy and consistent exposure over a short time period. Our results demonstrate that *A. alternata* spores have the ability to induce a measurable immune response in both a chronic exposure model and a single-challenge model in an unsensitized host. That being said, we were able to detect some spores even at 36 hours following a single challenge in murine lungs by examination of tissue sections with a microscope.

The goal of this study was to establish a new murine model using whole *Alternaria* spores to study the innate immune response and encourage use of spores over the culture extract as the response to each is detectable but sometimes distinctive to each. We also aimed to determine optimal inoculums and sample collection times to catch various responses of the innate immune system following a single intranasal challenge of *Alternaria* spores. Although a previous model involving three consecutive intranasal challenges of *Alternaria* highlighted some

of the innate immune response (30), more pathologically relevant results may have been masked by the large inoculums size and multiple treatments. As our innate model shows, several markers of airway inflammation are evident after a single challenge, thus three challenges may have only exacerbated the original response. The collected information is essential for future testing of various fungal mutants and their effect on the innate immune response in a murine system. The primarily neutrophilic infiltration in the innate model agrees with previous reports showing the importance of these cells in resolving acute fungal sinusitis (72). It was essential that our model was able to stimulate their recruitment and the histopathology revels neutrophils may be the most important cell in studying the initial innate immune response immediately following challenge with *Alternaria*. Accordingly, our innate model also showed significant increase in the neutrophil chemoattractant KC(CXCL1), highlighting the functionality of the immune response. The source of KC(CXCL1) may be resident tissue macrophage in the lung tissue combined with the orchestration of a distinct TLR signaling pathway (73). Indeed it has been shown that IL-8 in nasal lavage is a marker for CRS in humans (reference). We also found a nearly immediate release of TSLP which can function as an initiator of Th2 response (74, 75). This finding is complimentary to previous studies which have found TSLP up-regulated in mouse models of allergy (70). It is also notable that release/production of TSLP was most elevated at the first timepoint reviewed (6hr) and had reduced by nearly half 48 h after the single challenge. Chronic models likely sustain this production, assisting Th2 polarization. The fact that our Alt a 1 deletion mutant was unable to induce TSLP at any timepoint is reflective that major allergen production is required for epithelial activation and subsequent TSLP release. Our use of an *Alternaria* deletion mutant strengthens the significance of our model and confirms our ability to test mutant strains *in vivo*. Since mouse models have more frequently employed recombinant

fungal proteins to study an allergen's specific ability to induce immune response, such as work done on *A.fumigatus* allergen Asp f 1 (76), we aimed to instead utilize a live organism with a deletion of its major allergen gene without sensitization to study the effect of the allergen on the innate immune response. The results of our innate model proves that a fairly active immune response is triggered quickly after a single challenge of a fungal allergen as described by cell recruitment and cytokine production.

Our chronic model highlights that intraperitoneal sensitization is not necessary for induction of a pronounced Th2 response. Although this model ends before a secondary, or full adaptive, immune response is possibly reached, we still see evidence of Th2 promoting cytokines and a spike in serum IgE. This suggests our chronic model would likely be enhanced by examining a secondary response at closer to 21-days following the initial challenge. Besides presenting this intermediate phase of the immune response, our chronic model also demonstrates differences in the immune response to *Alternaria* spores versus extract. We eliminated batch-to-batch variation by utilizing extract from the same lot number in all intranasal challenge and confirmed that the extract gives an artificially greater response compared to the more ordinary response we found with the spores. Our chronic model also improves upon a previous chronic model with *Alternaria* spores which had to be discontinued due to cachexia in the mice due to the use of very large spore concentrations (32). We hypothesize that this preceding model would have worked with a lower intranasal spore dosage as we have shown.

The use of *Alternaria* extract in in vitro and *in vivo* experimentation can artificially mimic *Alternaria* but is not as pathologically relevant compared to using live *Alternaria* spores. For instance, studies have shown fungal proteases to be important in immune activation (77). But many of the cytokines found elevated in response to protease activities found in the extract,

including GM-CSF, Eotaxin, and RANTES(25, 63, 78, 79).), were not found to be induced in our model using whole spores. Consequently, preliminary data generated by our lab using *Alternaria* extract in epithelial cell and macrophage culture revealed a significant difference in the transcriptional profile after stimulation with extract or spores. The observed differences between spore and extract treatment could be attributed to the potentially high concentration of mycotoxins produced by *Alternaria* (80) since the extract preparations feature condensed amounts of fungal matter, including intracellular components. Thus treatment with extract likely exposes the host to a greater quantity of all fungal components which may not mimic dose encountered in nature. Additionally, a comprehensive review of murine allergic airway diseases (MAAD) concludes murine strains, allergens used, and experimental setup greatly influences the induction of particular cytokines (81). We conclude that although the *Alternaria* extract has given great insight into *Alternaria*'s ability to activate a Th2-type immune response, future studies must employ whole fungal spores to accurately interpret the immune response *in vivo*.

In summary, we have shown *Alternaria* spores can induce a measurable innate immune response in a murine model of fungal exposure. Mice who were chronically challenged with mold spores or extract developed severe signs of airway inflammation including cell infiltration of primarily pro-inflammatory cells including eosinophils, increased levels of Th2 cytokines, elevated serum IgE, and histopathological markers such as epithelial thickening. The results also confirmed the potent *Alternaria* extract can advance this response beyond that of the spores, suggesting its use is not logical in such sensitive experiments.

Both of these models establish essential parameters for inoculum size and sample collection time as well as expected levels of distinct cytokines. Furthermore, the result of our single-challenge model comparing the wild-type and *Δalta1* mutant proves it to be an important

tool for future testing of fungal mutants and their ability to stimulate the innate immune response. The data presented here will be invaluable in the further elucidation of the *in vivo* innate immune response to allergenic fungi by means of inhalation mouse models using whole fungal spores.

References:

1. Bush, R. K., J. M. Portnoy, A. Saxon, A. I. Terr, and R. A. Wood. 2006. The medical effects of mold exposure. *J. Allergy Clin. Immunol.* 117: 326–333.
2. Green, B. J., J. K. Sercombe, and E. R. Tovey. 2005. Fungal fragments and undocumented conidia function as new aeroallergen sources. *J. Allergy Clin. Immunol.* 115: 1043–1048.
3. Nasser, S. M., and T. B. Pulimood. 2009. Allergens and thunderstorm asthma. *Curr Allergy Asthma Rep* 9: 384–390.
4. Clearing the Air: Asthma and Indoor Air Exposures. .
5. Qin, J.-C., Y.-M. Zhang, L. Hu, Y.-T. Ma, and J.-M. Gao. 2009. Cytotoxic metabolites produced by Alternaria no.28, an endophytic fungus isolated from Ginkgo biloba. *Nat Prod Commun* 4: 1473–1476.
6. Kauffman, H. F., J. F. Tomee, M. A. van de Riet, A. J. Timmerman, and P. Borger. 2000. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J. Allergy Clin. Immunol.* 105: 1185–1193.
7. Simon-Nobbe, B., U. Denk, V. Pöll, R. Rid, and M. Breitenbach. 2008. The spectrum of fungal allergy. *Int. Arch. Allergy Immunol.* 145: 58–86.
8. Sanchez, H., and R. K. Bush. 2001. A review of Alternaria alternata sensitivity. *Rev Iberoam Micol* 18: 56–59.
9. Salo, P. M., S. J. Arbes Jr, M. Sever, R. Jaramillo, R. D. Cohn, S. J. London, and D. C. Zeldin. 2006. Exposure to Alternaria alternata in US homes is associated with asthma symptoms. *J. Allergy Clin. Immunol.* 118: 892–898.
10. Bush, R. K., and J. J. Prochnau. 2004. Alternaria-induced asthma. *J. Allergy Clin. Immunol.* 113: 227–234.
11. 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. *Eur. Respir. J.* 27: 615–626.
12. Shin, S.-H., J. U. Ponikau, D. A. Sherris, D. Congdon, E. Frigas, H. A. Homburger, M. C. Swanson, G. J. Gleich, and H. Kita. 2004. Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi. *J. Allergy Clin. Immunol.* 114: 1369–1375.
13. Pulimood, T. B., J. M. Corden, C. Bryden, L. Sharples, and S. M. Nasser. 2007. Epidemic asthma and the role of the fungal mold Alternaria alternata. *J. Allergy Clin. Immunol.* 120: 610–617.
14. Lopez, M., and J. E. Salvaggio. 1985. Mold-sensitive asthma. *Clin Rev Allergy* 3: 183–196.
15. Randriamanantany, Z. A., I. Annesi-Maesano, D. Moreau, C. Raherison, D. Charpin, C. Kopferschmitt, F. Lavaud, A. Taytard, F. De Blay, and D. Caillaud. 2010. Alternaria sensitization and allergic rhinitis with or without asthma in the French Six Cities study. *Allergy* 65: 368–375.
16. Achatz, G., H. Oberkofler, E. Lechenauer, B. Simon, A. Unger, D. Kandler, C. Ebner, H. Prillinger, D. Kraft, and M. Breitenbach. 1995. Molecular cloning of major and minor allergens of Alternaria alternata and Cladosporium herbarum. *Mol. Immunol.* 32: 213–227.
17. Asturias, J. A., M. C. Arilla, I. Ibarrola, E. Eraso, R. González-Rioja, and A. Martínez. 2003. A sensitive two-site enzyme-linked immunosorbent assay for measurement of the major Alternaria alternata allergen Alt a 1. *Ann. Allergy Asthma Immunol.* 90: 529–535.
18. Kurup, V. P., H. M. Vijay, V. Kumar, L. Castillo, and N. Elms. 2003. IgE binding synthetic peptides of Alt a 1, a major allergen of Alternaria alternata. *Peptides* 24: 179–185.

19. Twaroch, T. E., E. Arcalís, K. Sterflinger, E. Stöger, I. Swoboda, and R. Valenta. 2011. Predominant localization of the major Alternaria allergen Alt a 1 in the cell wall of airborne spores. *Journal of Allergy and Clinical Immunology* .
20. Sercombe, J. K., W. Eduard, T. C. Romeo, B. J. Green, and E. R. Tovey. 2006. Detection of allergens from Alternaria alternata by gold-conjugated anti-human IgE and field emission scanning electron microscopy. *J. Immunol. Methods* 316: 167–170.
21. Babiceanu, M. 2011. Analysis of the allergenic potential of the ubiquitous airborne fungus Alternaria using bioinformatics. .
22. Nials, A. T., and S. Uddin. 2008. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 1: 213–220.
23. 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.
24. Tournoy, K. G., J. C. Kips, C. Schou, and R. A. Pauwels. 2000. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin. Exp. Allergy* 30: 79–85.
25. Kobayashi, T., K. Iijima, S. Radhakrishnan, V. Mehta, R. Vassallo, C. B. Lawrence, J.-C. Cyong, L. R. Pease, K. Oguchi, and H. Kita. 2009. Asthma-related environmental fungus, Alternaria, activates dendritic cells and produces potent Th2 adjuvant activity. *J. Immunol.* 182: 2502–2510.
26. Ahn, B.-H., Y.-H. Park, and S.-H. Shin. 2009. Mouse model of Aspergillus and Alternaria induced rhinosinusitis. *Auris Nasus Larynx* 36: 422–426.
27. van den Brûle, S., J. Heymans, X. Havaux, J.-C. Renauld, D. Lison, F. Huaux, and O. Denis. 2007. Profibrotic effect of IL-9 overexpression in a model of airway remodeling. *Am. J. Respir. Cell Mol. Biol.* 37: 202–209.
28. Agarwal, M. K., R. T. Jones, and J. W. Yunginger. 1982. Immunochemical and physicochemical characterization of commercial Alternaria extracts: a model for standardization of mold allergen extracts. *J. Allergy Clin. Immunol.* 70: 432–436.
29. Aden, E., B. Weber, J. Bossert, M. Teppke, E. Frank, R. Wahl, H. Fiebig, and O. Cromwell. 1999. Standardization of Alternaria alternata: extraction and quantification of alt a 1 by using an mAb-based 2-site binding assay. *J. Allergy Clin. Immunol.* 104: 128–135.
30. Havaux, X., A. Zeine, A. Dits, and O. Denis. 2005. A new mouse model of lung allergy induced by the spores of Alternaria alternata and Cladosporium herbarum molds. *Clin. Exp. Immunol.* 139: 179–188.
31. Doherty, T. A., N. Khorram, K. Sugimoto, D. Sheppard, P. Rosenthal, J. Y. Cho, A. Pham, M. Miller, M. Croft, and D. H. Broide. 2012. Alternaria Induces STAT6-Dependent Acute Airway Eosinophilia and Epithelial FIZZ1 Expression That Promotes Airway Fibrosis and Epithelial Thickness. *Journal of Immunology (Baltimore, Md.: 1950)* .
32. Denis, O., S. van den Brûle, J. Heymans, X. Havaux, C. Rochard, F. Huaux, and K. Huygen. 2007. Chronic intranasal administration of mould spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodelling. *Immunology* 122: 268–278.
33. 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.
34. Kishimoto, T. 2010. IL-6: from its discovery to clinical applications. *International Immunology* 22: 347–352.

35. Neveu, W. A., J. B. Allard, O. Dienz, M. J. Wargo, G. Ciliberto, L. A. Whittaker, and M. Rincon. 2009. IL-6 is required for airway mucus production induced by inhaled fungal allergens. *J. Immunol.* 183: 1732–1738.
36. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
37. Allahverdian, S., N. Harada, G. K. Singhera, D. A. Knight, and D. R. Dorscheid. 2008. Secretion of IL-13 by airway epithelial cells enhances epithelial repair via HB-EGF. *Am. J. Respir. Cell Mol. Biol.* 38: 153–160.
38. Li, L., Y. Xia, A. Nguyen, Y. H. Lai, L. Feng, T. R. Mosmann, and D. Lo. 1999. Effects of Th2 Cytokines on Chemokine Expression in the Lung: IL-13 Potently Induces Eotaxin Expression by Airway Epithelial Cells. *The Journal of Immunology* 162: 2477–2487.
39. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 103: 779–788.
40. Tomkinson, A., C. Duez, G. Cieslewicz, J. C. Pratt, A. Joetham, M. C. Shanafelt, R. Gundel, and E. W. Gelfand. 2001. A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. *J. Immunol.* 166: 5792–5800.
41. Cihakova, D., J. G. Barin, M. Afanasyeva, M. Kimura, D. Fairweather, M. Berg, M. V. Talor, G. C. Baldeviano, S. Frisancho, K. Gabrielson, D. Bedja, and N. R. Rose. 2008. Interleukin-13 Protects Against Experimental Autoimmune Myocarditis by Regulating Macrophage Differentiation. *Am J Pathol* 172: 1195–1208.
42. Kroegel, C., P. Julius, H. Matthys, J. C. Virchow Jr, and W. Luttmann. 1996. Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. *Eur. Respir. J.* 9: 899–904.
43. Naseer, T., E. M. Minshall, D. Y. Leung, S. Laberge, P. Ernst, R. J. Martin, and Q. Hamid. 1997. Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am. J. Respir. Crit. Care Med.* 155: 845–851.
44. Humbert, M., S. R. Durham, P. Kimmitt, N. Powell, B. Assoufi, R. Pfister, G. Menz, A. B. Kay, and C. J. Corrigan. 1997. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J. Allergy Clin. Immunol.* 99: 657–665.
45. Punnonen, J., G. Aversa, B. G. Cocks, A. N. McKenzie, S. Menon, G. Zurawski, R. de Waal Malefyt, and J. E. de Vries. 1993. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acad. Sci. U.S.A.* 90: 3730–3734.
46. Swain, S. L., A. D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145: 3796–3806.
47. Steinke, J. W., and L. Borish. 2001. Th2 cytokines and asthma — Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respiratory Research* 2: 66.
48. Isidoro-García, M., I. Dávila, E. Laffond, E. Moreno, F. Lorente, and R. González-Sarmiento. 2005. Interleukin-4 (IL4) and Interleukin-4 receptor (IL4RA) polymorphisms in asthma: a case control study. *Clinical and Molecular Allergy* 3: 15.
49. Walker, C., W. Bauer, R. K. Braun, G. Menz, P. Braun, F. Schwarz, T. T. Hansel, and B. Villiger. 1994. Activated T cells and cytokines in bronchoalveolar lavages from patients with

various lung diseases associated with eosinophilia. *Am. J. Respir. Crit. Care Med.* 150: 1038–1048.
50. Parronchi, P., M. De Carli, R. Manetti, C. Simonelli, M. P. Piccinni, D. Macchia, E. Maggi, G. Del Prete, M. Ricci, and S. Romagnani. 1992. Aberrant interleukin (IL)-4 and IL-5 production in vitro by CD4+ helper T cells from atopic subjects. *Eur. J. Immunol.* 22: 1615–1620.
51. Moser, R., J. Fehr, and P. L. Bruijnzeel. 1992. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J. Immunol.* 149: 1432–1438.
52. Daher, S., L. M. Santos, D. Solé, M. G. De Lima, C. K. Naspitz, and C. C. Musatti. 1995. Interleukin-4 and soluble CD23 serum levels in asthmatic atopic children. *J Investig Allergol Clin Immunol* 5: 251–254.
53. Kobayashi, Y. 2006. Neutrophil infiltration and chemokines. *Crit. Rev. Immunol.* 26: 307–316.
54. Shea-Donohue, T., K. Thomas, M. J. Cody, Aiping Zhao, L. J. Detolla, K. M. Kopydlowski, M. Fukata, S. A. Lira, and S. N. Vogel. 2008. Mice deficient in the CXCR2 ligand, CXCL1 (KC/GRO-alpha), exhibit increased susceptibility to dextran sodium sulfate (DSS)-induced colitis. *Innate Immun* 14: 117–124.
55. Tomee, J. F., R. van Weissenbruch, J. G. de Monchy, and H. F. Kauffman. 1998. Interactions between inhalant allergen extracts and airway epithelial cells: effect on cytokine production and cell detachment. *J. Allergy Clin. Immunol.* 102: 75–85.
56. Borger, P., G. H. Koëter, J. a. B. Timmerman, E. Vellenga, J. F. C. Tomee, and H. F. Kauffman. 1999. Proteases from Aspevgillus fumigatus Induce Interleukin (IL)-6 and IL-8 Production in Airway Epithelial Cell Lines by Transcriptional Mechanisms. *Journal of Infectious Diseases* 180: 1267–1274.
57. King, C., S. Brennan, P. J. Thompson, and G. A. Stewart. 1998. Dust Mite Proteolytic Allergens Induce Cytokine Release from Cultured Airway Epithelium. *The Journal of Immunology* 161: 3645–3651.
58. Mohsenin, A., M. D. Burdick, J. G. Molina, M. P. Keane, and M. R. Blackburn. 2007. Enhanced CXCL1 production and angiogenesis in adenosine-mediated lung disease. *FASEB J.* 21: 1026–1036.
59. García-Ramallo, E., T. Marques, N. Prats, J. Beleta, S. L. Kunkel, and N. Godessart. 2002. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. *J. Immunol.* 169: 6467–6473.
60. Tani, M., M. E. Fuentes, J. W. Peterson, B. D. Trapp, S. K. Durham, J. K. Loy, R. Bravo, R. M. Ransohoff, and S. A. Lira. 1996. Neutrophil infiltration, glial reaction, and neurological disease in transgenic mice expressing the chemokine N51/KC in oligodendrocytes. *J. Clin. Invest.* 98: 529–539.
61. Mehrad, B., M. Wiekowski, B. E. Morrison, S.-C. Chen, E. C. Coronel, D. J. Manfra, and S. A. Lira. 2002. Transient lung-specific expression of the chemokine KC improves outcome in invasive aspergillosis. *Am. J. Respir. Crit. Care Med.* 166: 1263–1268.
62. Reche, P. A., V. Soumelis, D. M. Gorman, T. Clifford, Liu Mr, M. Travis, S. M. Zurawski, J. Johnston, Y. J. Liu, H. Spits, R. de Waal Malefyt, R. A. Kastelein, and J. F. Bazan. 2001. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J. Immunol.* 167: 336–343.
63. Kouzaki, H., S. M. O'Grady, C. B. Lawrence, and H. Kita. 2009. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J. Immunol.* 183: 1427–1434.

64. Fontenot, D., H. He, S. Hanabuchi, P. N. Nehete, M. Zhang, M. Chang, B. Nehete, Y.-H. Wang, Y.-H. Wang, Z.-M. Ma, H.-C. Lee, S. F. Ziegler, A. N. Courtney, C. J. Miller, S.-C. Sun, Y.-J. Liu, and K. J. Sastry. 2009. TSLP production by epithelial cells exposed to immunodeficiency virus triggers DC-mediated mucosal infection of CD4+ T cells. *Proc. Natl. Acad. Sci. U.S.A.* 106: 16776–16781.
65. Holt, P. G., D. H. Strickland, M. E. Wikström, and F. L. Jahnsen. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* 8: 142–152.
66. Holt, P. G., P. D. Sly, F. D. Martinez, S. T. Weiss, B. Björkstén, E. von Mutius, and U. Wahn. 2004. Drug development strategies for asthma: in search of a new paradigm. *Nat. Immunol.* 5: 695–698.
67. Vailes, L., S. Sridhara, O. Cromwell, B. Weber, M. Breitenbach, and M. Chapman. 2001. Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. *Journal of Allergy and Clinical Immunology* 107: 641–646.
68. Kuhn, R., K. Rajewsky, and W. Muller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science* 254: 707–710.
69. Southam, D. S., R. Ellis, J. Wattie, W. Glass, and M. D. Inman. 2008. Goblet Cell Rebound and Airway Dysfunction with Corticosteroid Withdrawal in a Mouse Model of Asthma. *American Journal of Respiratory and Critical Care Medicine* 178: 1115–1122.
70. Bird, L. 2005. TSLP: key role in allergic responses confirmed. *Nat Rev Immunol* 5: 747.
71. Boitano, S., A. N. Flynn, C. L. Sherwood, S. M. Schulz, J. Hoffman, I. Gruzinova, and M. O. Daines. 2011. Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. *Am. J. Physiol. Lung Cell Mol. Physiol.* 300: L605–614.
72. Rodriguez, T. E., N. R. Falkowski, J. R. Harkema, and G. B. Huffnagle. 2007. Role of neutrophils in preventing and resolving acute fungal sinusitis. *Infect. Immun.* 75: 5663–5668.
73. De Filippo, K., R. B. Henderson, M. Laschinger, and N. Hogg. 2008. Neutrophil Chemokines KC and Macrophage-Inflammatory Protein-2 Are Newly Synthesized by Tissue Macrophages Using Distinct TLR Signaling Pathways. *The Journal of Immunology* 180: 4308 –4315.
74. Omori-Miyake, M., and S. F. Ziegler. 2012. Mouse Models of Allergic Diseases: TSLP and Its Functional Roles. *Allergology International: Official Journal of the Japanese Society of Allergology* 0.
75. Saenz, S. A., M. Noti, and D. Artis. 2010. Innate immune cell populations function as initiators and effectors in Th2 cytokine responses. *Trends Immunol.* 31: 407–413.
76. Alvarez-García, E., E. Batanero, R. García-Fernández, M. Villalba, J. G. Gavilanes, and A. Martínez-del-Pozo. 2010. A deletion variant of the Aspergillus fumigatus ribotoxin Asp f 1 induces an attenuated airway inflammatory response in a mouse model of sensitization. *J Investig Allergol Clin Immunol* 20: 69–75.
77. Shin, S.-H., Y.-H. Lee, and C.-H. Jeon. 2006. Protease-dependent activation of nasal polyp epithelial cells by airborne fungi leads to migration of eosinophils and neutrophils. *Acta Otolaryngol.* 126: 1286–1294.
78. Matsuwaki, Y., K. Wada, H. Moriyama, and H. Kita. 2011. Human eosinophil innate response to Alternaria fungus through protease-activated receptor-2. *Int. Arch. Allergy Immunol.* 155 Suppl 1: 123–128.
79. Matsuwaki, Y., K. Wada, T. A. White, L. M. Benson, M. C. Charlesworth, J. L. Checkel, Y. Inoue, K. Hotta, J. U. Ponikau, C. B. Lawrence, and H. Kita. 2009. Recognition of fungal protease activities induces cellular activation and eosinophil-derived neurotoxin release in human eosinophils. *J. Immunol.* 183: 6708–6716.

80. Yekeler, H., K. Bitmiş, N. Ozçelik, M. Z. Doymaz, and M. Calta. 2001. Analysis of toxic effects of Alternaria toxins on esophagus of mice by light and electron microscopy. *Toxicol Pathol* 29: 492–497.
81. Finkelman, F. D., S. P. Hogan, G. K. K. Hershey, M. E. Rothenberg, and M. Wills-Karp. 2010. Importance of cytokines in murine allergic airway disease and human asthma. *J. Immunol.* 184: 1663–1674.

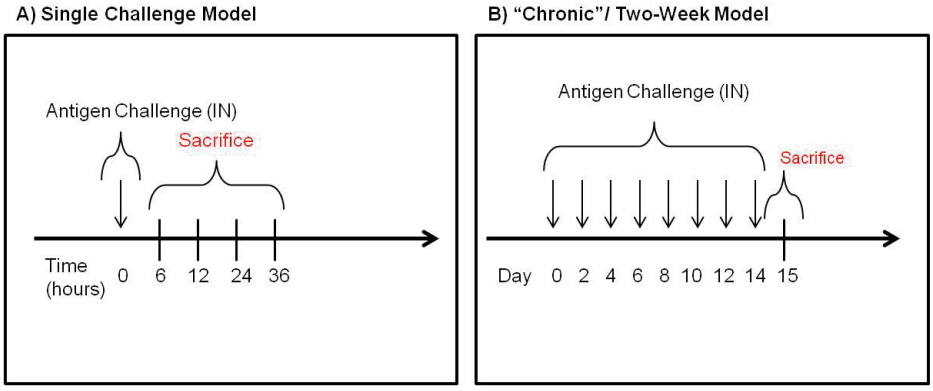


Figure 2.1: Experimental schedule for intranasal challenges in innate and chronic mouse models. For the innate model, female Balb/c mice (8 – 12 weeks old) were intranasally administered *Alternaria* wild-type spores (5 x 10^4) or a *A.alternata Δalta1* deletion mutant. In the chronic model, mice were challenge every other day over two-weeks with *Alternaria* wild-type spores (5 x 10^4) or culture extract (50µg). PBS served as the control treatment in all experiments. Arrows indicate challenges and sample collection are signified by straight bars.

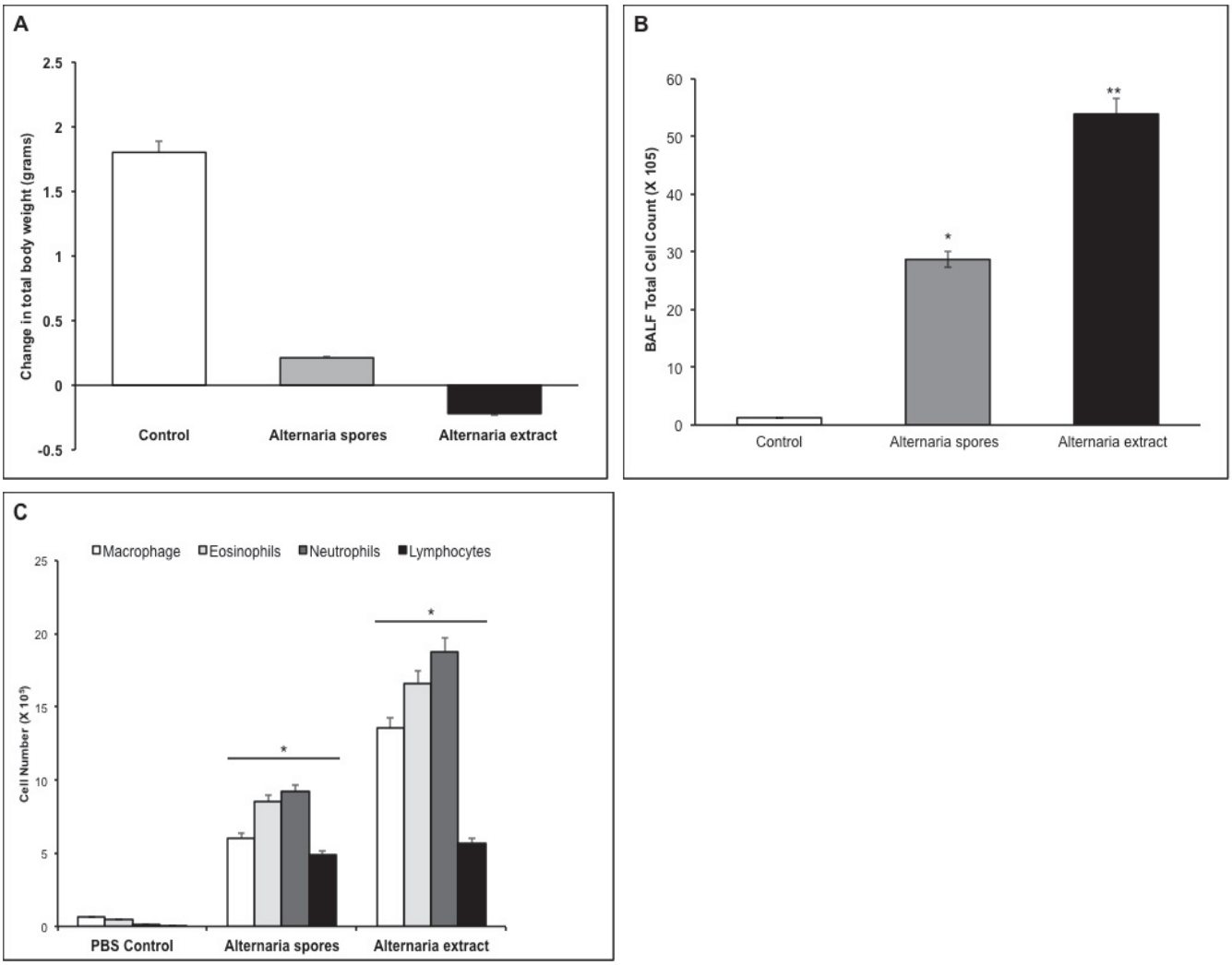


Figure 2.2: Chronic administration of *Alternaria* spores or culture extract induces changes in body weight and increased inflammatory cell recruitment in the lungs. Female BALB/c mice were intranasally challenged with PBS (control), 5×10^4 *A. alternata* wild-type spores in PBS, or *Alternaria* culture extract (50μg) in PBS every other day for 14 days. (A) Comparison of change in total body weight; mice were weighed before the initial challenge and 24 h after the final challenge. (B) Total cell recruitment in the lungs of challenged mice. BALs were collected 24 h later after the last challenge and the total cell number in each BAL was determined by counting on hemocytometer. (C) Differential cell count of the inflammatory subpopulation in the BALs 24 h after the last challenge. Data are expressed as mean ± SD (n = 4). *$P < 0.05$.

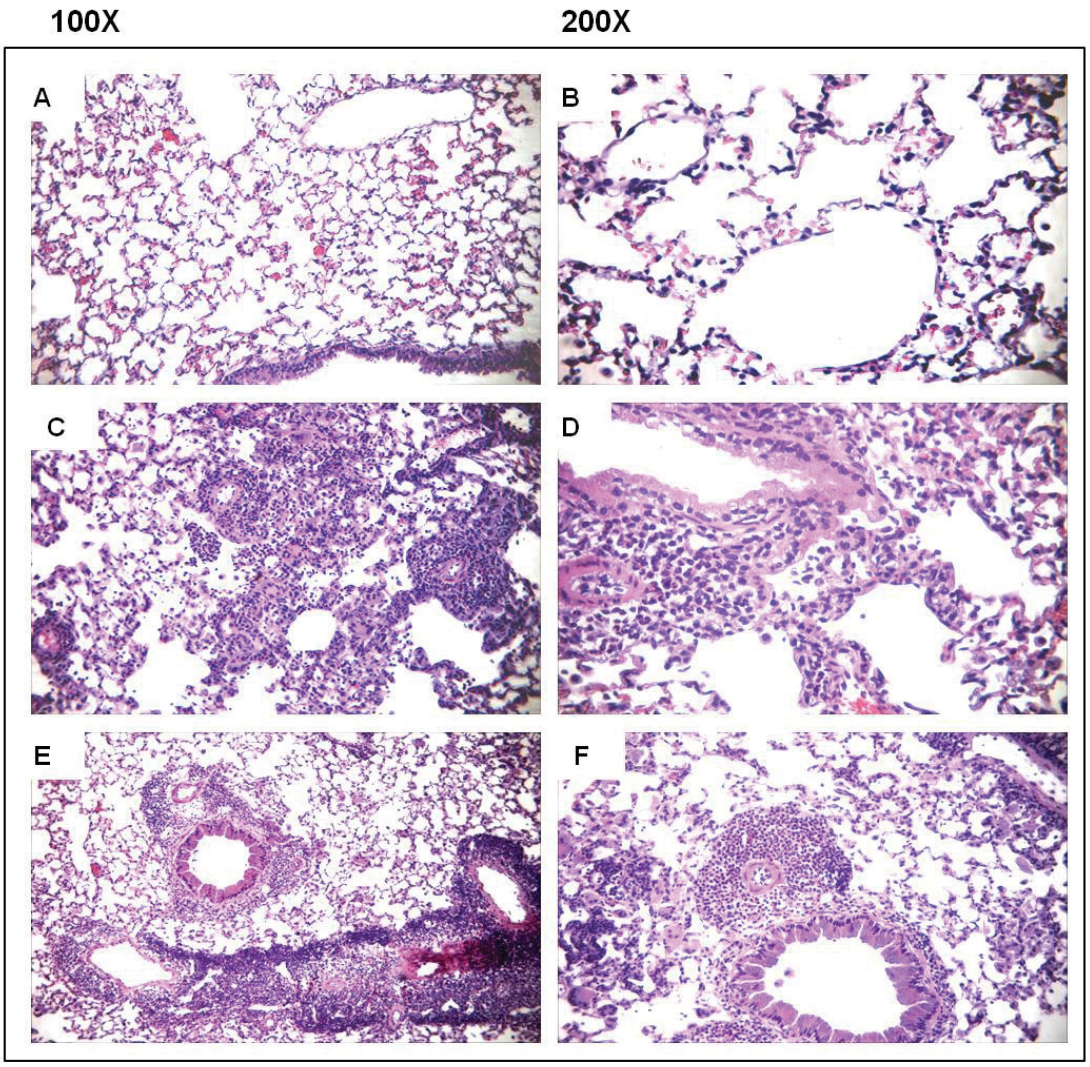


Figure 2.3: Representative histopathological changes in lungs of naïve mice challenge with *Alternaria* spores or extract. Representative histopathological changes of female BALB/c mice (8-12 weeks old) challenged intranasally with PBS (A-B), 5 X 10^5 *Alternaria* wild-type spores (C-D) or 50µg *A. alternata* extract (E-F) every other day over 14 days. Lungs were removed 24 hours after the last challenge, inflated and fixed in 10% formalin, and stained with hematoxylin and eosin (H&E).

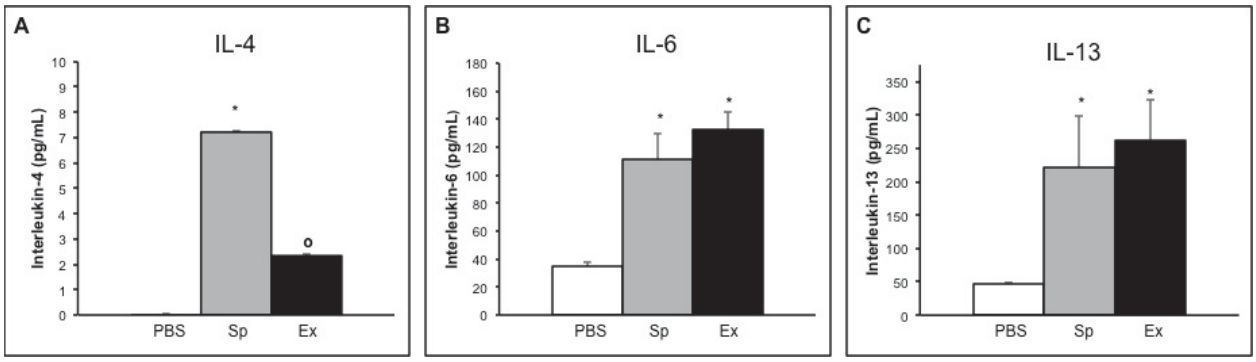


Figure 2.4: *Alternaria* spores and *Alternaria* culture extract induce Th2 cytokines in a chronic mouse model of allergic airway inflammation. Female Balb/c mice (8-12 weeks old) were intranasally challenged every other day over 14 days with equal volumes of PBS (control), or *Alternaria* wild-type spores (5 X 10^4), or *Alternaria* antigens (50µg) PBS. Mice were euthanized 24 hours after the last challenge and bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with 2mL of DPBS. The BALF was centrifuged at 500*xg* for 5 minutes and the cell-free supernatant was used for analyzing levels of (A) IL-4, (B) IL-6, or (C) IL-13 by standard ELISA. n = 4 per treatment group. Asterisk (*) denotes statistically significant compared to untreated control; circle (o) indicates significance compared to spore treatment. $P<0.05$.

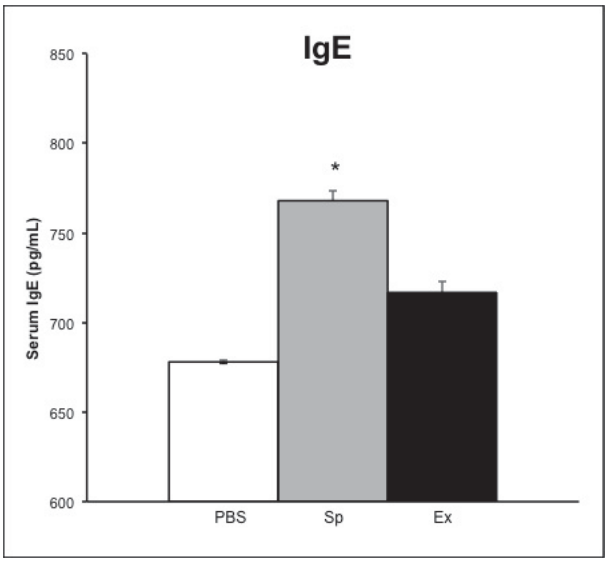


Figure 2.5: *Alternaria* spores but not extract induce increase in serum IgE levels during chronic challenge. Female Balb/c mice (8-12 weeks old) were intranasally challenged every other day over 14 days with equal volumes of PBS (control), or Alternaria wild-type spores (5 X 10^4), or Alternaria antigens (50µg) PBS. Blood was collected from the retro-orbital plexus 24 hrs after final challenge. Serum was separated by centrifugation and total IgE was measured by enzyme-linked immunosorbent asay (ELISA) following the manufacturer's instructions (Biolegend). Asterisk (*) denotes statistically significant compared to untreated control. n = 4 per treatment. $P<0.05$

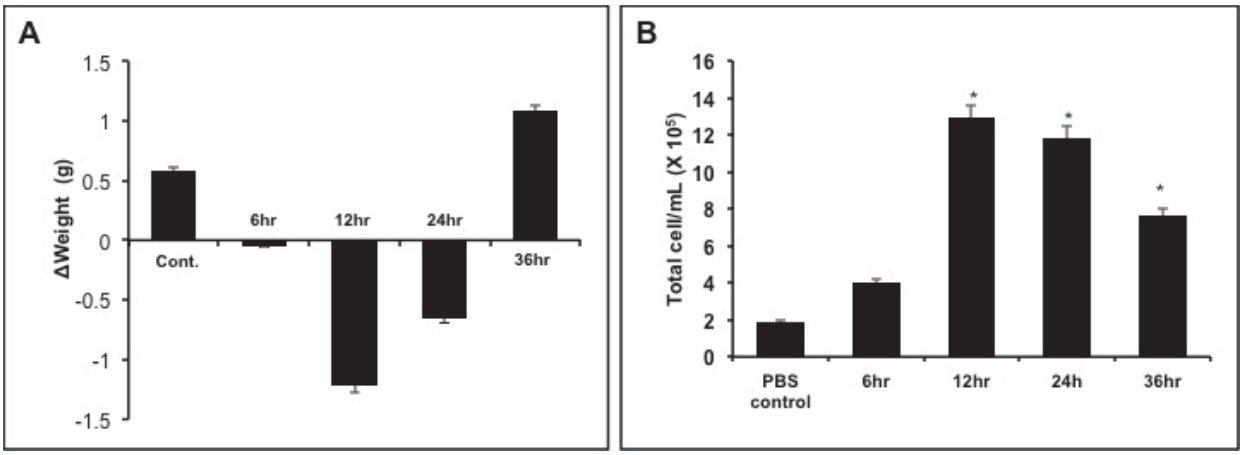


Figure 2.6: A single intranasal challenge of *Alternaria* spores induces early signs of cachexia and stimulates effector cell recruitment. Female Balb/c mice (8-12 weeks old) were intranasally challenged with equal volumes of PBS (control) or 5 x10^5 *A.alternata* wild-type spores. A) The mice were weighed prior to initial challenge and immediately before euthanasia at indicated timepoints. B) Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with DPBS. Total cell counts were assessed by staining with trypan blue and counting on hemacytometer. n = 4/treatment group. $P<0.05$.

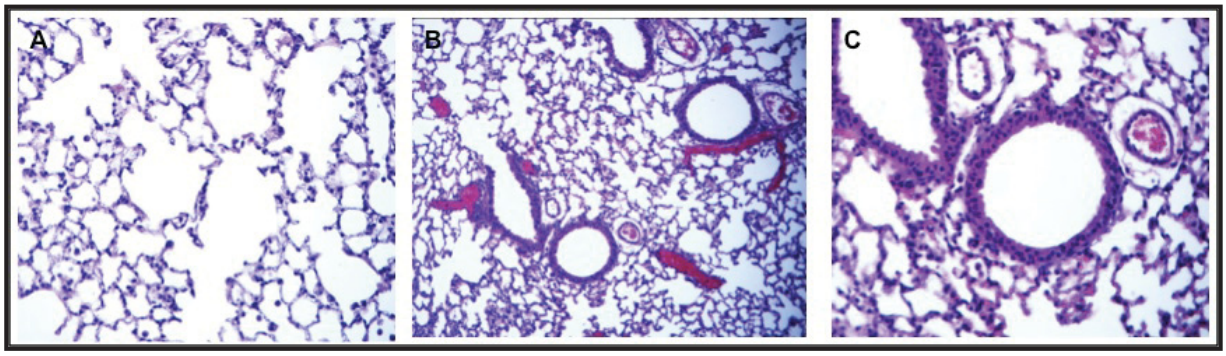


Figure 2.7: Representative histopathological changes in lungs of naïve mice after a single challenge with *Alternaria* spores. Representative histopathological changes of female BALB/c mice (8-12 weeks old) challenged intranasally one time with PBS (A) or 5 X 10^5 *Alternaria* wild-type spores (B - C). Lungs were removed 24 hours after the last challenge, inflated and fixed in 10% formalin, and stained with hematoxylin and eosin (H&E).

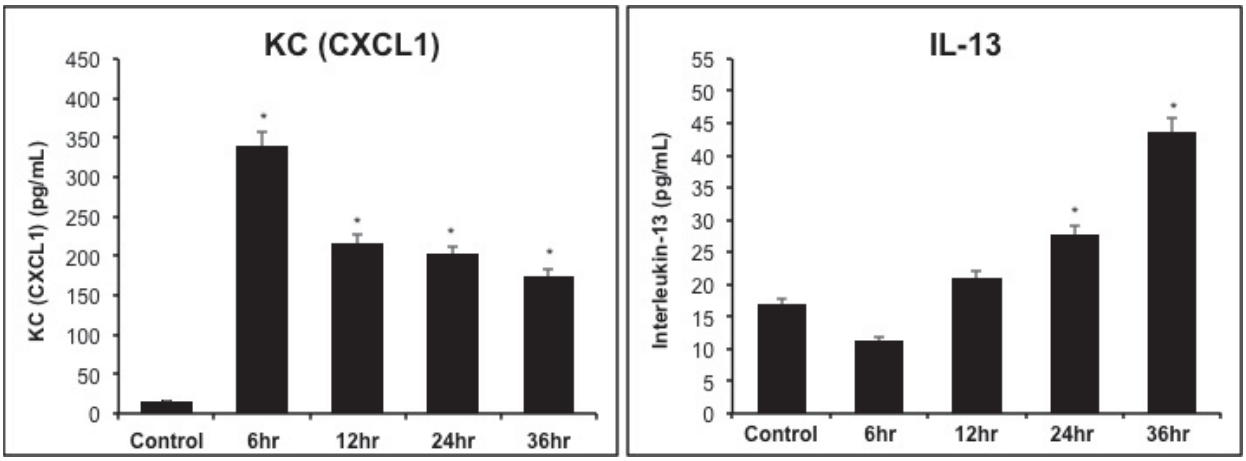


Figure 2.8: A single intranasal challenge of *Alternaria* spores stimulates inflammatory cytokines production in lungs. Female Balb/c mice (8-12 weeks old) were intranasally challenged with equal volumes of PBS (control), or 5 x10^5 *A.alternata* wild-type spores in PBS. Mice were euthanized at indicated times after the challenge and bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with DPBS. The BALF was centrifuged at 500xg for 5 minutes and the cell-free supernatant was used for analyzing levels of KC (CXCL1) and IL-13 by standard ELISA following the manufacturer's instructions (Biolegend). N = 4/treatment group. $P<0.05$.

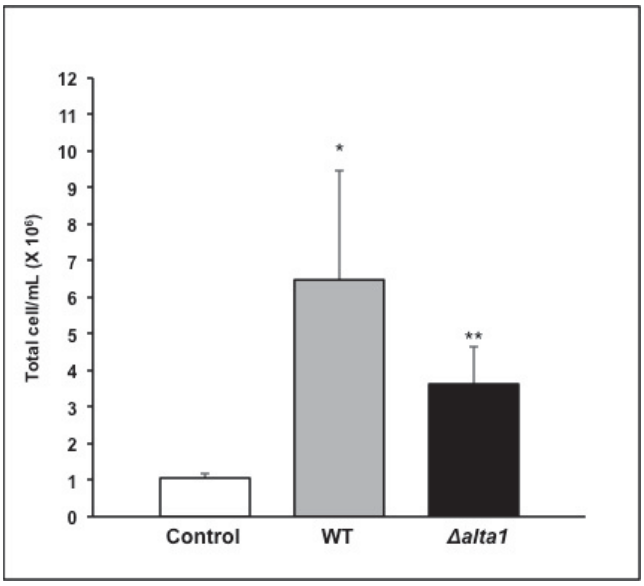


Figure 2.9: Use of a *Δalta1 Alternaria* mutant gives a reduced cytokine response after a single intranasal challenge, confirming effectiveness of model. Female Balb/c mice (8-12 weeks old) were intranasally challenged with equal volumes of PBS (control), or 5 x 10^5 *A.alternata* wild-type or *Δalta1* spores in PBS. Mice were euthanized 24 hours after the single challenge and bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with DPBS. Total cell counts were determined by staining with trypan blue and counting on a hemacytometer. N = 4/treatment group. $P<0.05$.

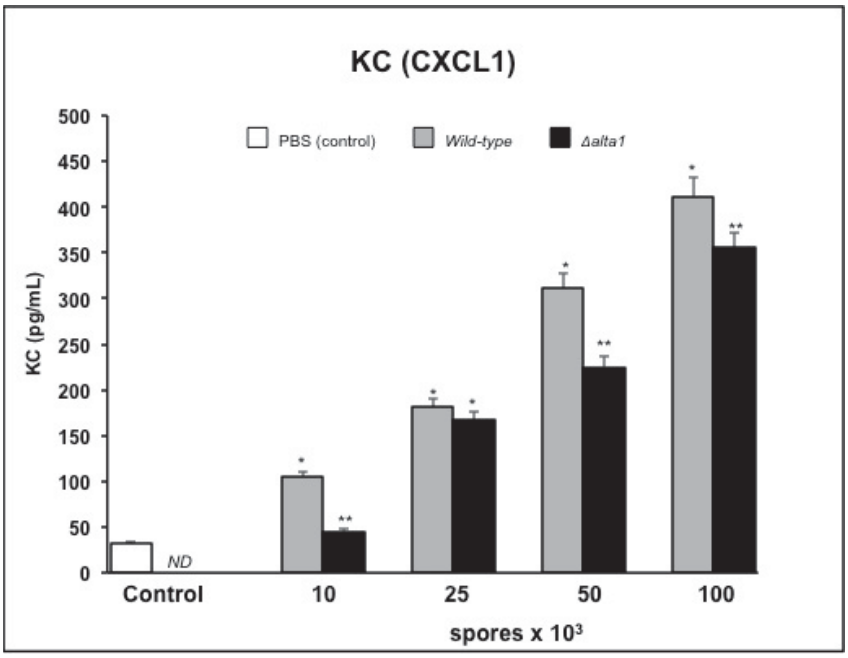


Figure 2.10: Determination of optimal spore challenge. Female Balb/c mice (8-12 weeks old) were intranasally challenged with equal volumes of PBS (control), or indicated amount of *A.alternata* wild-type or *Δalta1* spores in PBS. Mice were euthanized 24 hours after the challenge and bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with DPBS. The BALF was centrifuged at 500xg for 5 minutes and the cell-free supernatant was used for analyzing levels of KC (CXCL1) by standard ELISA. Asterisk (*) indicates significance against control; double asterisk (**) indicates significant difference from both control and wild-type treatment. N = 4/treatment group. $P<0.05$.

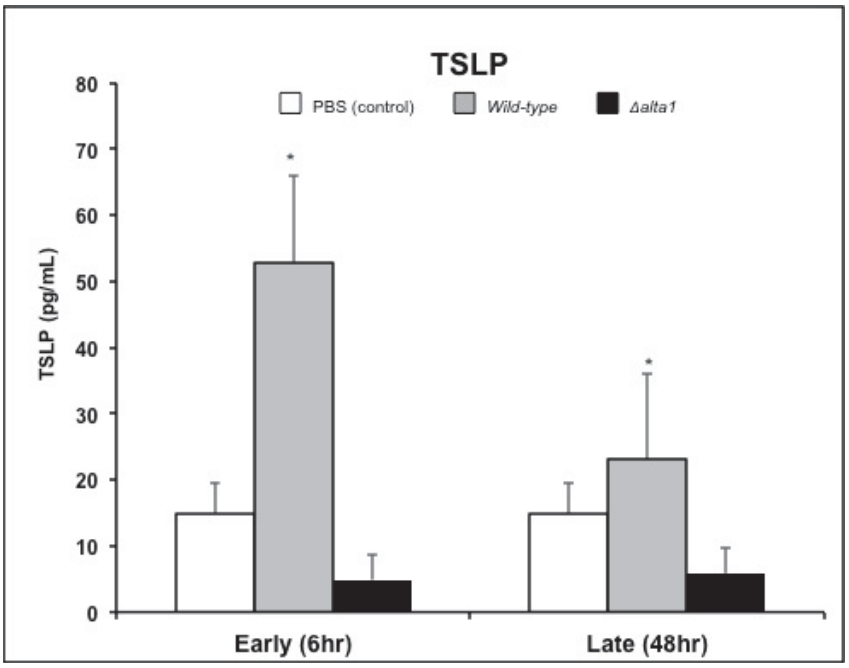


Figure 2.11: An *A.alternata Δalta1* fails to induce the cytokine TSLP after a single intranasal challenge of spores at both early and late phase response. BALB/c mice (n = 2) were lightly anaesthetized by isoflurane and then intranasally challenged with a single dose of 5 x 10^4 *Alternaria alternata or A.alternataΔalta1* spores in PBS or PBS alone (control). At the indicated timepoints, mice were euthanized and lungs were immediately washed with PBS. Cell-free BALF supernatant was used for ELISA quantification of TSLP following manufacturer's instructions (Biolegend). $P<0.05$.

Chapter III

IRAK-1 attenuates the allergic immune response to *Alternaria* in cell and mouse models

Abstract:

Fungal allergies and asthma pose a significant public health problem but the etiology of these diseases remains poorly understood and little is known about the role of innate immune signaling in the pathogenesis of these disorders. Significant research has shown that the ubiquitous airborne fungus, *Alternaria alternata*, is clinically associated with acute and chronic human airway disorders. However, our knowledge regarding the interaction between Alternaria and innate immune response signaling is poorly understood. The signaling kinase IRAK-1 and its negative regulator, IRAK-M, have been associated with controlling the innate signaling of pro-inflammatory cytokines. In this study we show changes in IRAK-1 and IRAK-M expression levels in murine macrophage stimulated with Alternaria. We identify differences in the response of key inflammatory molecules by comparing normal and IRAK-1-deficient (IRAK-$1^{-/-}$) and IRAK-M-deficient (IRAK-$M^{-/-}$) macrophage cells upon *A.alternata* spore stimulation. Finally we have developed a mouse model for the examination of allergic lung inflammation in IRAK-1-deficient mice upon *Alternaria* challenge to investigate the role of this innate signaling gene in the airway inflammatory response. We found that both IRAK-1 and IRAK-M function in innate immune signaling to Alternaria and that deletion of IRAK-1 causes an aberrant and hyper inflammatory response in an in vivo mouse model of Alternaria airway inflammation. Although

our data shows IRAK-M expression decreases during Alternaria stimulation in normal macrophage, we did not find that deletion of IRAK-M had any effect on the immune response in either macrophage or mice. Our findings suggest that Alternaria is capable of stimulating a significant innate immune response in both the airway and macrophage and that functional IRAK-1 is required for perpetuating an inflammatory response to Alternaria.

Introduction

Chronic airway inflammatory diseases, such as asthma and chronic rhinosinusitus (CRS), are complex and multi-factorial disorders which represent a significant public health problem as their incidence have significantly increased over the past 40 years (1, 2). It is now important to investigate why a strong immune response is elicited by various ubiquitous fungi and pinpoint where dysregulation may lead to chronic inflammation in response to these fungi. Since there is increasing evidence for a strong link between the innate and adaptive immune systems, it is imperative to investigate innate immune signaling in response to fungal stimuli(3–5).

Inflammatory airway disorders are thought to result from a maladaptive inflammatory response to ubiquitous environmental proteins in genetically susceptible persons (6). These disorders, including atopic asthma, allergic rhinitis, chronic rhinosinusitis (CRS), and airway-hyperreactivity disorder (AHR), have long been associated with exposure to environmental fungi (7). Despite the clinical importance and worldwide abundance of fungi, relatively few investigations have focused on the pathophysiology of fungal asthma (8).

Alternaria alternata is one fungal species which has been well characterized as a trigger of allergic rhinosinusitus, severe asthma, and airway hyperreactivity (9). Even though the fungal kingdom contains a number of species which produce a wide range of well defined protein allergens, comparative genomics has found the allergen ortholog of *A.alternata's* allergenic protein, Alt a 1, to be present in only a limited number of species (10); these findings suggest *A.alternata* may be a uniquely more powerful elucidator of airway inflammation than other environmental fungi.

Even though *A.Alternaria* exposure has been epidemiologically described to have a powerful association with airway disorders, few components of the induced molecular signaling

cascades in the host have been investigated. The detailed etiology of fungal-induced airway inflammation and hyper responsiveness is yet to be elucidated, but many reports support the notion that specific inflammatory mediators, such as the production of cytokines, chemokines, and transcription factors, directly affect the dysregulated innate immune response leading to inflammation (11).

In a recent study using a mouse model of Alternaria induced asthma, a strong inflammatory response was observed within the airway tissue (12). In this study, the allergic response to Alternaria was characterized by increased serum IgE levels, increases in inflammation-associated chemokines and cytokines, and a dramatic infiltration of effector cells such as neutrophils, eosinophils, and macrophage into the airway epithelium. It is well characterized that these effector cells produce cytokines and chemokines which further recruit more cells to the site of inflammation. A chronic infiltration of these inflammatory cells leads to a thickening of the mucosal lining and airway epithelial tissue remodeling (12). Eosinophils are of particular interest in allergic airway disorders like asthma and CRS because of their strong degranulation response to Alternaria (6). Two recent studies have shown Alternaria conidia induce a strong EDN (eosinophil-derived neurotoxin) response in normal human eosinophils and a stronger EDN release by eosinophils of asthmatic patients (7). The second study found the eosinophils were activated and EDN production was stimulated by the fungal cell wall component β-glucan, not chitin; furthermore, β-glucan induced the production of the inflammatory cytokines IL-8, MIP-1a, and MCP-1 (6). These findings encourage a deeper examination of the fungal-effector cell interaction at the molecular level.

Toll-like receptors (TLRs) are a class of membrane-spanning proteins which enable the host to recognize a large number of structurally conserved pathogen-associated molecular

patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), viral RNA, CpG-containing DNA and flagellin (13). They have also been shown to play a pivotal role in both the innate and adaptive immune response by initiating a specific host defense response (13). TLR-dependent signaling pathways result in the expression of effector molecules, such as cytokines and chemokines, which contribute to the activation of the immediate innate immune response (14, 15).

Ten distinct TLRs have been described in humans, expressed in various combinations in cells of the immune system as well as other cell types (16).The mRNA of all ten TLRs has been described in human nasal airway tissue and 2005 study by Fransson *et al*. demonstrated an up-regulation of TLR2, TLR3 and TLR4 in the nasal mucosa of patients with symptomatic allergic rhinitis (17, 18)

The role of TLRs as a primary part of the host's microbe defense system has been shown in several studies, but their possible function as mediators in allergy and asthma remains to be established. Several authors have recently suggested the significance of TLRs in the pathophysiology of allergic rhinitis and asthma, thus indicating the importance to study their role during allergic airway inflammation (3, 19). In several airway model systems, stimulation of TLRs results in changes in the production of stimulatory molecules, such as cytokines and chemokines, thereby affecting and further upgrading the airway inflammation (20).

There is accumulating evidence that fungi elicit the innate immune response through TLR2/MYD88 dependent pathway (21). Interestingly, dectin-1, a major receptor for fungal β-glucan, is highly expressed on the surface of alveolar macrophages and neutrophils and collaborates with TLR-2 in the production of inflammatory mediatiors (22). It was also found that zymosan (a crude mixture of glucans,mannan, proteins, chitin, and glycolipids extracted

from the cell membrane of fungi) induces signaling through TLR2/6 and the receptor is also recruited to the surface of zymosan-stimulated macrophages (23, 24). Further analysis showed zymosan can induce NFκB activation through TLR2/6 signaling pathways (25). It is by this knowledge we choose to investigate a key component of the signaling cascade from TLR2/6: interleukin-1-receptor-associated-kinase-1.

The discovery of Toll-like-receptors (TLRs), with their role as initiators of the innate immune response and associated inflammation, suggests they are likely involved in the recognition of fungal allergens. Recently, interleukin-1-receptor-associated kinase-1 (IRAK-1) has been shown to play a critical role in the regulation of the signaling cascade evolving from Toll-like-receptors (TLRs). This kinase network ultimately produces transcription factors for pro-inflammatory cytokines and chemokines. It is believed a disruption in a component of this signaling cascade may lead to unregulated production of molecules which illicit a strong inflammatory response. Furthermore, continued stimulation may cause the inflammatory response to become chronic.

IRAKs are intracellular kinases that can be recruited to the TLR complex and mediate diverse downstream signaling (26). Currently, the IRAK family consists of IRAK-1, 2, M, and 4. The expression patterns of these members differ, but IRAK-1 and IRAK-4 have been found to be expressed in all tissue types (27). It has been established that activation and/or disengagement of the IRAK function leads to diverse signaling events which can alter the host inflammatory response. This is supported by research which links genetic variations in human IRAK genes to various inflammatory diseases (26).Thus, IRAKs play a critical role in the direction, persistence, and regulation of the inflammatory response; further characterization of its role is essential for understanding chronic inflammatory disorders such as asthma. While each member of the IRAK

family has a distinct function, our study focuses on the role of IRAK-1 due to its strong association with the regulation of pro-inflammatory transcription factors.

IRAK-1 was first described as a signal transducer for the pro-inflammatory cytokine interleukin-1 (IL-1) and was later implicated in signal transduction of other members of the Toll-like receptor (TLR)/IL-1 receptor (IL-1R) family (28). Functioning through an intracellular kinase cascade, IRAK-1 is one molecule which mediates the downstream production of nuclear-factor-kappa-B (NF-κB), a well characterized nuclear regulator of cytokine, chemokine, cell adhesion molecule, growth factor, and immunoreceptor expression (29). However, it has been shown that IRAK-1 phosphorylation is not strictly necessary for NF-κB activation, as IRAK-1 knockout mice still produce NF-κB (30). Despite this knowledge, it is well known that incorrect regulation of NF-κB production has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development (31). Furthermore, even though IRAK-1 does not exclusively regulate NF-kB production, abnormal IRAK-1 function has a strong association with chronic inflammatory disease.

It had been previous described that those with genetic variations in the human IRAK-1 gene exhibit an increased host inflammatory response (32). A 2007 study by Liu *et al* showed that an IRAK-1 variant (Leu → Ser alteration at aa 532), exhibited increased NF-κB activity (30). The study also showed autophosphorylation of the IRAK-1 variant was greater than that found with wild-type IRAK-1, suggesting genetic alterations in the IRAK-1 gene may cause altered downstream function. Additionally, the variant IRAK-1 had greater interaction with TNFR-associated factor 6 (TRAF6) than the wild-type IRAK-1. These results demonstrate that variant IRAK-1 is associated with alterations in multiple intracellular events that are likely to contribute to altered NF-κB activation and resultant inflammatory responses.

Population based studies have shown humans with variations in the IRAK-1 gene have an increased severity of atherosclerosis and are at a higher risk for diabetes and hypertension (33). Mutations have also been implicated in an increased rate of mortality in sepsis patients (34). It has also been described that an IRAK-1 gene deletion in mice leads to an increased overall susceptibility to infection (35). The genetic link between IRAK-1 gene variations and increased severity and susceptibility to infection makes it an interesting target for further characterization of airway inflammatory disease.

Signaling through TLRs 1, 2, and 5-9 are mediated by receptor-associated Myeloid differentiation factor 88 (MyD88). MyD88-deficient mice have been generated and found to be completely defective in their responses to IL-1 and the IL-1-related cytokine, IL-18 (36). The response to LPS was also shown to be abolished (37). Furthermore, MyD88-deficient macrophages were shown to be completely unresponsive to other immunostimulatory components including peptidoglycan, lipoproteins, CpG DNA, flagellin, and imidazoquinolines, demonstrating the essential role of MyD88 in the response to all TLR responses (38–42).

The MyD88-dependent pathway recruits IRAK-1 and IRAK-4 to the TLR/MyD88 complex. Both IRAK1/4 interact with MyD88 through their death domains. IRAK-4 phosphorylates IRAK-1 in its kinase activation loop, triggering IRAK-1 kinase activity. Once activated, IRAK-1 likely autophosphorylates residues on its N-terminus. TRAF6 is also recruited to the receptor complex via interaction with IRAK-1. Three TRAF6 binding motifs (Pro-*X*-Glu-*X*-*X*-aromatic/acidic residue) are found in IRAK-1, as well as one in IRAK-M and two in IRAK-2 (43). TRAF6 then activates transforming growth factor β-activated kinase 1 (TAK1), which then activates the inhibitor kappa B kinases (IKK), Jun N-terminal kinases (JNK), and p38 (27). The common result is the activation of NFκB and the transcription of co-stimulatory molecules.

Toll-interacting protein (Tollip) is also another regulatory molecule in the TLR/IRAK-1 signaling cascade. In resting cells, Tollip forms a complex with members of the IRAK family, thereby preventing NF-κB activation (24). Upon activation Tollip/IRAK-1 complexes are recruited to the cognate receptor, resulting in the rapid autophosphorylation of IRAK-1 and its dissociation from the receptor. At the same time, IRAK phosphorylates Tollip, which may lead to the dissociation of Tollip from IRAK-1 and its rapid ubiquitination and degradation. Tollip is believed to function primarily to maintain immune cells in a quiescent state and to facilitate the termination of IL-1R/TLR-induced cell signaling during inflammation and infection (44).

Although the kinase activity of IRAK-1 increases following IL-1 stimulation, IRAK-1 kinase activity is not required for its signaling function, because overexpression of a kinase-defective mutant of IRAK-1 is observed to strongly induce NF-κB activation in cells otherwise deficient for IRAK-1 (27).Other research has shown IRAK-1-deficient mice and cell lines showed diminished cytokine production in response to IL-1 and LPS; nevertheless some response remained, suggesting that IRAK-2 or IRAK-M might compensate to some extent for the lack of IRAK-1 (45, 46). Even though IRAK-1 activity is not exclusively responsible for the production of transcription factors, its regulation is clearly important for correct function of the signaling cascade.

The components of the TLR/IRAK signaling pathway are unique to controlling production of downstream pro-inflammatory mediators. By advancing our understanding of how this signaling cascade responds to specific allergenic fungi such as *A.alternata*, we may be able to identify potential novel therapeutic approaches for fungal induced airway disorders.

Materials and Methods:

Fungal Materials

A.alternata (ATCC strain #66981) was cultured at 25°C on potato dextrose agar (Difco). Conidia were gently harvested from 7-day-old plates with sterile inoculating loop and diluted into sterile water, counted with hemocytometer, and concentration adjusted to 1 x 10^6 conidia/mL. Lyophilized culture filtrate extracts of *A. alternata* were obtained from Greer Laboratories and resuspended in PBS at a concentration of 1mg/mL.

Isolation and culture of murine bone marrow-derived macrophages

Wild-type (C57BL/6) and IRAK-$1^{-/-}$, and IRAK-$M^{-/-}$ mice with C57BL/6 background were kindly provided by Dr. Liwu Li at Virginia Polytechnic Institute and State University. Animals were bred and maintained under specific pathogen-free conditions; all animal experiments were done with the approval of and in accordance with regulatory guidelines and standards set by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University. Mice (8-weeks-old) were euthanized by cervical dislocation after brief anesthetization by isoflurane. Bone marrow from tibia and femur was obtained by flushing with DMEM. Bone marrow cells were cultured in a 125×50mm non-tissue-culture-treated dish with 50ml DMEM medium containing 30% L929 cell supernatant, 1mM Sodium Pyruvate, 50μM 2-Mercaptoethanol and 2 mM glutamine. Samples were kept at 37°C, 5% CO_2. On the third day of culture, the cells were fed with additional 20ml fresh medium and cultured for additional three days. Cells were harvested and washed with PBS, resuspended in DMEM supplemented with 10% FBS, plated at a density of 1 x 10^6 cells/well in six-well plates and allowed to rest overnight before treatment.

Macrophage Stimulation

Macrophages were treated with fresh Alternaria conidia at a 4:1 macrophage:conidia ratio or 50μg of Alternaria culture filtrate. Control macrophage samples were not inoculated with any stimuli. Samples were kept at 37°C, 5% CO_2 for 24hrs. Cell culture supernatant was collected, and centrifuged for 15min. at 13,000 rpm. Cell-free supernatant was transferred to sterile microcentrifuge tube and stored at -80°C until cytokine analysis. Cells were then washed twice with DPBS and lysed in TRIzol reagent (Invitrogen) and also stored at -80°C.

Morphological Analysis of Macrophage:

Macrophage culture samples were examined under light microscopy using the Olympus CKX41 inverted light microscope at the indicated time points post-inoculation. Images were taken with the QImaging MicroPublisher 3.3 (Nikon) digital imager.

Mouse Model of Allergic Airway Inflammation:

Wild-type (C57BL/6) and IRAK-$1^{-/-}$, and IRAK-$M^{-/-}$ mice (8 – 12 weeks old) were lightly anesthetized with isoflurane and intranasally administered with 50 μl PBS or 50 μg *Alternaria* culture filtrate every three days for six days (three challenges total). Mice were euthanized by cervical dislocation 24hrs after the last challenge. After cannulating the trachea, the lungs were lavaged with DPBS (1.0 ml). The supernatants of bronchoalveolar lavage (BAL) fluids were collected and stored at −80°C for cytokine assays. Whole lungs were homogenized in 1.0 ml TRIzol reagent (Invitrogen) and stored at -80°C.

Histopathology

Lungs were excised, fixed in 10% buffered formalin, paraffin embedded, sectioned to 5*u*m, and stained with H&E. Slides were examined under light microscope for cell infiltration and changes in alveolar structure.

Membrane Based Cytokine Assay:

The membrane based cytokine antibody array (RayBiotech Inflammatory Mouse Cytokines Array 1) was completed to the manufacturer's instructions for both macrophage and bronchalveolar lavage fluid (BALF) samples. Membranes were blocked at room temperature and then incubated with pooled samples (cell-free supernatant) overnight, shaking at 4°C. Membranes were washed and incubated with biotin-conjugated-anti-cytokines. Following another wash, membranes were then incubated with HRP-conjugated streptavidin antibodies. The membranes were exposed for 30 seconds to BioMax Light x-ray film (Kodak). Film was developed on SRX-101A (Konica) film developer. All steps, except overnight incubation, were performed at room temperature. Film was visually examined for differences between experimental and control samples.

mRNA Isolation and RT-PCR

Total mRNA was isolated from macrophage and lung homogenates using the TRIzol reagent (Invitrogen) and following the manufacturer's instructions. For cDNA synthesis, 1µg of total RNA was reverse-transcribed using the Tetro cDNA Synthesis Kit (Bioline). For RT and real-time PCR reactions, 1µl of cDNA was used per reaction using the primers described in Table 1 and either the GoTaq Green PCR MasterMix (Promega) or iQ SYBR Green Supermix (Bio-Rad). Results were normalized against the positive control gene GAPDH.

Results:

IRAK-1 and IRAK-M expression in murine macrophage following stimulation with Alternaria antigens

In order to investigate if IRAK-1 and IRAK-M may be involved in signaling in response to Alternaria, we tested the expression level of each mediator in murine macrophages. Naïve macrophages were harvested from the bone-marrow of wild-type (C57BL/6) and then stimulated with Alternaria antigens or left unstimulated for 24hrs. The mRNA levels of IRAK-1 and IRAK-M in the stimulated and unstimulated macrophages were then measuring by RT-PCR. As seen in Figure 1, IRAK-M was reduced to a non-detectable level in the treated samples while IRAK-1 expression increased nearly three-fold.

Alternaria antigens stimulate pro-inflammatory cytokines in macrophages

Although changes in expression of both IRAK-1 and IRAK-M were detectable, we also needed to establish that the experiment successfully induced changes in secreted cytokine levels in the macrophage. This was essential to establishing the baseline of our experiments and for later confirming the correlation between IRAK genes and cytokine production. By using a cytokine antibody array, we were able to measure 84 cytokines from a sample of pooled cell supernatant. This global analysis identified the chemokine CXCL1 and the cytokines MCP-1 and M-CSF were induced by Alternaria. Thus we labeled these three as markers for our experiments involving macrophage and Alternaria stimulation.

CXCL1 is produced by macrophage in a time-dependent manner in response to Alternaria

We more deeply examined the production of chemokine CXCL1 in macrophage stimulated with Alternaria antigens since it showed the greatest change in our global cytokine antibody array. We measured the mRNA levels of CXCL1 in macrophage at 0, 2, 4, 6, 8, 12, and 24 hours following Alternaria antigen stimulation. Figure 3 shows agarose gel analysis of RT-PCR for CXCL1. Nearly absent before stimulation, CXCL1 is progressively more highly expressed over time. The RT-PCR for the housekeeping gene GAPDH is shown as a standard positive control.

IRAK-1$^{-/-}$ mice show increased expression of IL-13 in a mouse model of Alternaria induced allergic airway inflammation

The ability to study the role of IRAK-1 and IRAK-M in vivo was possible by developing a mouse model of allergic airway inflammation using IRAK-1 and IRAK-M deficient mice. Wild-type mice of the C57BL/6 background were used since this is the background of the IRAK-1$^{-/-}$ and IRAK-M$^{-/-}$ mice. The mice of each genotype were intranasally challenged with Alternaria antigens every 3 days for a total of 3 treatments. Control mice were challenged with an equal volume of DPBS. Lungs were excised 24hrs after the last challenge and mRNA was extracted from homogenized lung tissue. Figure 4a shows the purity and integrity of the extracted mRNA from each genotype and are representative of the 4 samples collected for each genotype group.

Since IL-13 is regarded as one of the most significant Th2 cytokine involved in allergic airway inflammation (47) we examined the success of inducing an allergic airway response in our mouse model by first measuring IL-13 expression in the murine lungs. Following cDNA synthesis of equimolar amounts of mRNA, we measured IL-13 by RT-PCR as shown in Figure 4b. IL-13 mRNA was detected in the IRAK-1 deficient lung samples while both the WT and

IRAK-M deficient mice showed negligible expression of IL-13 in response to Alternaria antigen challenge. We also quantitatively measure IL-13 expression in the mRNA samples using real-time PCR shown in Figure 4C. IRAK-$1^{-/-}$ mice had a 35-fold change in IL-13 expression while the WT and IRAK-$M^{-/-}$ had non-significant fold-changes of IL-13.

Deletion of IRAK-1 causes hyper-response of pro-inflammatory cytokines in lungs of mice challenged with Alternaria

We also globally analyzed other cytokines known to have a role in allergic airway inflammation including CXCL1, IFN-γ, IL-4, IL-5, and MIP-2α by real-time PCR. We did not measure the levels in the IRAK-$M^{-/-}$ lungs samples since our macrophage and IL-13 results did not indicate any differences between IRAK-$M^{-/-}$ and WT samples. The mRNA analysis of various other cytokines indicates that IRAK-1 deficient mice show a "hyper" response to Alternaria by expressing a greater number of cytokines. The greatest fold differences were observed for IL-5 and MIP-2α transcripts. Although the other cytokines measured trended towards higher levels in the IRAK-1, they were not found to be statistically significant.

Histopathological changes are evident in lungs of both WT and IRAK-$1^{-/-}$ mice treated with Alternaria antigens

Following the same mouse model for allergic airway inflammation used for expression analysis, lungs were also histopathologically examined for cell infiltration and changes in alveolar structure. Figure 6 shows H&E stained sections of lung. The control samples, 6a and 6b, show clear alveoli with smooth outer lining. There was minimal effector cell infiltration and only a few resident macrophage and eosinophils present. However, the lungs of both WT and IRAK-

1-/- mice in Fig. 6c and 6d show severe changes including narrowing of alveoli and excessive eosinophil and macrophage infiltration. The lungs of IRAK-1 deficient mice also exhibited structural changes in the alveoli such as columnar breakdown and mucosal thickening. Furthermore, total effector cell count per lung area was greater for the IRAK-$1^{-/-}$ samples (data not shown). Histopathological grading would result in both genotypes being severely inflamed but the IRAK-1 deficient mice would score higher in overall severity.

IRAK-1-/- macrophage cannot effectively suppress Alternaria germination

An important quality of macrophage is their ability to limit fungal germination and thus preventing a fungal infection. We examined the ability for Alternaria spores to germinate in the presence of both WT and IRAK-1 deficient marcrophage. We visually imaged the cell culture at 1hr, 6hr, and 24hr following initial inoculation. Figure 7 indicates abnormal germination by Alternaria in the presence of WT macrophage. This is noted by the lack of germ tubes visible after 1hr in 7a and then abnormal germination signified by "bulbuous" growth in 6b. By 24hrs the conidia were unable to germinate normally and multiple small germ tubes are visible on a single spore in 6c instead of the 1-3 tubes that were more often observed . Nearly all of the spores incubated with the IRAK-1 deficient macrophage began to germinate after 1hr (6d) and continued to show normal growth at 6hr (6e) although some bulbousgrowth was evident. Interestingly, by 24hr the spores showed the same abnormal conidiagenesis as the WT treated samples (6f).

Discussion:

Our results investigating the response of both wild-type (WT), IRAK-1-deficient (IRAK-$1^{-/-}$), and IRAK-M-deficient (IRAK-$M^{-/-}$) macrophage to Alternaria challenge showed that deletion of IRAK-1 caused a hyper-response to the fungus. An in vivo mouse model of allergic airway inflammation also confirmed these results. Interestingly, deletion of IRAK-M appears to have no different effect compared to the WT in both cell culture and mouse models but is clearly down-regulated in WT macrophage in response to Alternaria, suggesting it may play a role in signaling not detected in our studies. A recent study showed that IRAK-M did not regulate the TLR-2-induced classical NFκB pathway as p65/RelA phosphorylation and nuclear translocation were unchanged in wild-type and IRAK-$M^{-/-}$ cells and demonstrated that the observed inhibitory effect of IRAK-M was primarily limited to the TLR2 ligand, instead of TLR4 (48). Furthmore, there exists much stronger evidence for the involvement of TLR2 rather than TLR4 in fungal allergy and asthma (49). Thus it seems that the role for IRAK-M may be more vital in the alternative NFκB pathway rather than in the classical NFκB pathway as originally thought (50).

Identifying key downstream response mediators to Alternaria was an important goal of our work. In order to effectively study the role of various components of signaling cascades, reliable and strong markers need to be known. Our cytokine arrays were able to detect the key mediators CXCL1, MCP-1, and MCSF produced from WT macrophage in response to Alternaria. The strongest induction corresponded to CXCL1, a neutrophil chemoattractant which has been implicated in allergic and inflamed airways (51–53). We also confirmed CXCL1 expression over time in response to Alternaria which showed a detectable level as early as two hours post-exposure and continued through 24hrs. This type of prolonged and sustained CXCL1 expression would be likely with constant exposure to Alternaria.

Numerous studies of fungal airway exposure have employed different animal exposure and sensitization techniques, each with inherent advantages and disadvantages (54). Here we have used a unique model which effectively measures the innate immune response after only three intranasal exposures. We were able to measure IL-13, one of the most important mediator of Th2 driven allergic inflammatory diseases (55), at surprisingly high levels in IRAK-1 deficient mice but not in WT or IRAK-M deficient lung tissue. This is likely due to the tight control of innate signaling in normal mice which prevents this overt expression and the alternative pathway for IRAK-M as described above. Thus the deletion of IRAK-1 causes an unregulated, inducible IL-13 expression not normally found during such a short exposure to fungi like Alternaria. Furthermore, histopathological evidence confirmed that deletion of IRAK-1 caused greater effector cell infiltration into the lung tissue, another significant hallmark of asthma (56). This increase could be explained by the increase in chemoattractant cytokines being produced due to the IRAK-1 deletion; it has been hypothesized that dysregulation in TLR-related pathways correlates with allergy and asthma development (57).

Macrophages constitute an important and primary line of defense against any infection (58) and function as modulators of the immune response (59). Normally, macrophage are easily able to engulf fungal spores such as *Aspergillus fumigatus* (60); however, Alternaria spores are too large to be fully enveloped. Thus the macrophage must first break down or somehow inhibit germination of the fungus in order to prevent infection (61). We found that IRAK-1 deficient macrophage were slower and less effective at inhibiting Alternaria germination in vitro. Since the macrophage of both WT and IRAK-1 deficient backgrounds were able to prevent germination after 24hrs, we would conclude that IRAK-1 may play a role in preventing germination in the early phase of exposure.

In summary, our data strongly indicates that IRAK-1, but not IRAK-M, helps regulate the innate immune response to Alternaria. The deletion, or dysregulation, of TLR-IRAK-1 signaling may lead to a hyper response to the fungus and thus greater or more frequent allergic and/or asthmatic symptoms. By better understanding this signaling cascade we can provide a novel mechanism for future therapeutic strategies in asthmatic patients.

Future Studies

Investigating the role of IRAK-1 in the downstream production of pro-inflammatory mediators following *A. alternata* spore challenge will be immensely useful in further characterization of the possible TLR pathway leading to or contributing to airway inflammation; furthermore, the construction of a mouse model will aid in future functional studies of the interaction between IRAK-1 and chronic airway inflammatory disorders.

Characterize the downstream production of inflammatory cytokines in both WT and IRAK $^{-/-}$ cell lines in response to *A.alternata* challenge:

Our preliminary data shows Alternaria conidia do elicit a response from WT and IRAK$^{-/-}$ macrophages, but these did not determine the full range of cytokines which are being produced in response to Alternaria. Additionally, recent research has shown that eosinophils may play the largest role in the airway inflammatory response. Increased levels of both neutrophils and dendritic cells have also been found in the airways of asthmatic patients. In order to form a better understanding of each cell type's role during Alternaria stimulation as well as the function of IRAK-1, it would be beneficial to harvest naïve eosinophils, dendritic cells, and neutrophils from the bone-marrow of the WT, IRAK-1-/-, and IRAK-M-/- mice to study the response of each

individually. Both time and dose dependent studies would give additional information regarding the role of IRAK-1 and IRAK-M in the innate immune response. A more thorough global cytokine analysis, such as a microarray with a greater number of targets, should be performed to identify more response targets to be measure. ELISA or other quantitative analysis of response proteins should be utilized to gauge differing levels between genotypes. Most importantly, experiments should utilize both the Alternaria antigen preparation and whole spores. By stimulating with both Alternaria whole conidia and extract, we can determine differences between each approach. A previous study successfully elucidated an allergic inflammatory response in normal female BALB/c mice; however, researchers used a spore extract, which likely exasperated the response due to the high concentration of fungal proteins (8). The group's follow-up study did try to establish a model using whole conidia, but intranasal administration of [2 $X10^5$] conidia induced cachexia after five weeks and treatment was discontinued (12). These in vitro studies will provide valuable data to be applied to the future mouse models of allergic airway inflammation.

Following preliminary characterization through in vitro studies, a new mouse model can be developed to elucidate the role of IRAK-1 in vivo during Alternaria mold challenge. This would include improving the current model to show both an early innate response and a chronic (sensitized response). By examining all aspects of allergic airway inflammation in the WT and IRAK-$1^{-/-}$ and IRAK-$M^{-/-}$ mice including bronchoalveolar lavage fluid for cell types and cytokines, histopathological analysis of lung tissue, cytokine expression in the airway, and serum IgE levels, a comprehensive understanding of the role IRAK-1 and IRAK-M in Alternaria-induced airway inflammation can be assessed.

References

1. Phipps, S., C. E. Lam, P. S. Foster, and K. I. Matthaei. 2007. The contribution of TLRs to the pathogenesis of asthma. *Immunol. Cell Biol.* 85: 463–470.
2. Shin, S.-H., J. U. Ponikau, D. A. Sherris, D. Congdon, E. Frigas, H. A. Homburger, M. C. Swanson, G. J. Gleich, and H. Kita. 2004. Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi. *J. Allergy Clin. Immunol.* 114: 1369–1375.
3. Heine, H., and E. Lien. 2003. TLRs and their function in innate and adaptive immunity. *Int. Arch. Allergy Immunol.* 130: 180–192.
4. Barrett, N. A., and K. F. Austen. 2009. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* 31: 425–437.
5. Saenz, S. A., M. Noti, and D. Artis. 2010. Innate immune cell populations function as initiators and effectors in Th2 cytokine responses. *Trends Immunol.* 31: 407–413.
6. Yoon, J., J. U. Ponikau, C. B. Lawrence, and H. Kita. 2008. Innate antifungal immunity of human eosinophils mediated by a beta 2 integrin, CD11b. *J. Immunol.* 181: 2907–2915.
7. 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.
8. Havaux, X., A. Zeine, A. Dits, and O. Denis. 2005. A new mouse model of lung allergy induced by the spores of Alternaria alternata and Cladosporium herbarum molds. *Clin. Exp. Immunol.* 139: 179–188.
9. Black, P. N., A. A. Udy, and S. M. Brodie. 2000. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. *Allergy* 55: 501–504.
10. Bowyer, P., M. Fraczek, and D. W. Denning. 2006. Comparative genomics of fungal allergens and epitopes shows widespread distribution of closely related allergen and epitope orthologues. *BMC Genomics* 7: 251.
11. Amrani, Y., H. Chen, and R. A. Panettieri Jr. 2000. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? *Respir. Res.* 1: 49–53.
12. Denis, O., S. van den Brûle, J. Heymans, X. Havaux, C. Rochard, F. Huaux, and K. Huygen. 2007. Chronic intranasal administration of mould spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodelling. *Immunology* 122: 268–278.
13. Bauer, S., D. Hangel, and P. Yu. 2007. Immunobiology of TLRs in allergic disease. *Immunobiology* 212: 521–533.
14. Sabroe, I., R. C. Read, M. K. B. Whyte, D. H. Dockrell, S. N. Vogel, and S. K. Dower. 2003. TLRs in health and disease: complex questions remain. *J. Immunol.* 171: 1630–1635.
15. Sabroe, I., L. C. Parker, A. G. Wilson, M. K. B. Whyte, and S. K. Dower. 2002. TLRs: their role in allergy and non-allergic inflammatory disease. *Clin. Exp. Allergy* 32: 984–989.
16. Cario, E., and D. K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect. Immun.* 68: 7010–7017.
17. Vandermeer, J., Q. Sha, A. P. Lane, and R. P. Schleimer. 2004. Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and TLRs. *Arch. Otolaryngol. Head Neck Surg.* 130: 1374–1380.
18. Fransson, M., M. Adner, J. Erjefält, L. Jansson, R. Uddman, and L.-O. Cardell. 2005. Up-regulation of TLRs 2, 3 and 4 in allergic rhinitis. *Respir. Res.* 6: 100.

19. O'Neill, L. A. J., C. E. Bryant, and S. L. Doyle. 2009. Therapeutic targeting of TLRs for infectious and inflammatory diseases and cancer. *Pharmacol. Rev.* 61: 177–197.
20. Sha, Q., A. Q. Truong-Tran, J. R. Plitt, L. A. Beck, and R. P. Schleimer. 2004. Activation of airway epithelial cells by toll-like receptor agonists. *Am. J. Respir. Cell Mol. Biol.* 31: 358–364.
21. Viriyakosol, S., J. Fierer, G. D. Brown, and T. N. Kirkland. 2005. Innate immunity to the pathogenic fungus Coccidioides posadasii is dependent on Toll-like receptor 2 and Dectin-1. *Infect. Immun.* 73: 1553–1560.
22. Taylor, P. R., G. D. Brown, D. M. Reid, J. A. Willment, L. Martinez-Pomares, S. Gordon, and S. Y. C. Wong. 2002. The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J. Immunol.* 169: 3876–3882.
23. Kataoka, K., T. Muta, S. Yamazaki, and K. Takeshige. 2002. Activation of macrophages by linear (1right-arrow3)-beta-D-glucans. Impliations for the recognition of fungi by innate immunity. *J. Biol. Chem.* 277: 36825–36831.
24. Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 401: 811–815.
25. Sato, M., H. Sano, D. Iwaki, K. Kudo, M. Konishi, H. Takahashi, T. Takahashi, H. Imaizumi, Y. Asai, and Y. Kuroki. 2003. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. *J. Immunol.* 171: 417–425.
26. Ringwood, L., and L. Li. 2008. The involvement of the interleukin-1 receptor-associated kinases (IRAKs) in cellular signaling networks controlling inflammation. *Cytokine* 42: 1–7.
27. Akira, S. 2003. Toll-like receptor signaling. *J. Biol. Chem.* 278: 38105–38108.
28. Janssens, S., and R. Beyaert. 2003. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol. Cell* 11: 293–302.
29. Lee, J. I., and G. J. Burckart. 1998. Nuclear factor kappa B: important transcription factor and therapeutic target. *J Clin Pharmacol* 38: 981–993.
30. Liu, G., Y. Tsuruta, Z. Gao, Y.-J. Park, and E. Abraham. 2007. Variant IL-1 receptor-associated kinase-1 mediates increased NF-kappa B activity. *J. Immunol.* 179: 4125–4134.
31. Albensi, B. C., and M. P. Mattson. 2000. Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse* 35: 151–159.
32. Gan, L., and L. Li. 2006. Regulations and roles of the interleukin-1 receptor associated kinases (IRAKs) in innate and adaptive immunity. *Immunol. Res.* 35: 295–302.
33. Lakoski, S. G., L. Li, C. D. Langefeld, Y. Liu, T. D. Howard, K. B. Brosnihan, J. Xu, D. W. Bowden, and D. M. Herrington. 2007. The association between innate immunity gene (IRAK1) and C-reactive protein in the Diabetes Heart Study. *Exp. Mol. Pathol.* 82: 280–283.
34. Arcaroli, J., E. Silva, J. P. Maloney, Q. He, D. Svetkauskaite, J. R. Murphy, and E. Abraham. 2006. Variant IRAK-1 haplotype is associated with increased nuclear factor-kappaB activation and worse outcomes in sepsis. *Am. J. Respir. Crit. Care Med.* 173: 1335–1341.
35. Verdrengh, M., J. A. Thomas, and O. H. Hultgren. 2004. IL-1 receptor-associated kinase 1 mediates protection against Staphylococcus aureus infection. *Microbes Infect.* 6: 1268–1272.
36. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9: 143–150.

37. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.
38. Takeuchi, O., A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P. F. Mühlradt, and S. Akira. 2000. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* 164: 554–557.
39. Takeuchi, O., K. Takeda, K. Hoshino, O. Adachi, T. Ogawa, and S. Akira. 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int. Immunol.* 12: 113–117.
40. Häcker, H., R. M. Vabulas, O. Takeuchi, K. Hoshino, S. Akira, and H. Wagner. 2000. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J. Exp. Med.* 192: 595–600.
41. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410: 1099–1103.
42. Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3: 196–200.
43. Ye, H., J. R. Arron, B. Lamothe, M. Cirilli, T. Kobayashi, N. K. Shevde, D. Segal, O. K. Dzivenu, M. Vologodskaia, M. Yim, K. Du, S. Singh, J. W. Pike, B. G. Darnay, Y. Choi, and H. Wu. 2002. Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* 418: 443–447.
44. Zhang, G., and S. Ghosh. 2002. Negative regulation of toll-like receptor-mediated signaling by Tollip. *J. Biol. Chem.* 277: 7059–7065.
45. Kanakaraj, P., P. H. Schafer, D. E. Cavender, Y. Wu, K. Ngo, P. F. Grealish, S. A. Wadsworth, P. A. Peterson, J. J. Siekierka, C. A. Harris, and W. P. Fung-Leung. 1998. Interleukin (IL)-1 receptor-associated kinase (IRAK) requirement for optimal induction of multiple IL-1 signaling pathways and IL-6 production. *J. Exp. Med.* 187: 2073–2079.
46. Thomas, J. A., J. L. Allen, M. Tsen, T. Dubnicoff, J. Danao, X. C. Liao, Z. Cao, and S. A. Wasserman. 1999. Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase. *J. Immunol.* 163: 978–984.
47. Mitchell, J., V. Dimov, and R. G. Townley. 2010. IL-13 and the IL-13 receptor as therapeutic targets for asthma and allergic disease. *Curr Opin Investig Drugs* 11: 527–534.
48. Su, J., T. Zhang, J. Tyson, and L. Li. 2009. The Interleukin-1 Receptor-Associated Kinase M Selectively Inhibits the Alternative, Instead of the Classical NFκB Pathway. *Journal of Innate Immunity* 1: 164–174.
49. Chiou, Y.-L., and C.-Y. Lin. 2009. Der p2 activates airway smooth muscle cells in a TLR2/MyD88-dependent manner to induce an inflammatory response. *J. Cell. Physiol.* 220: 311–318.
50. Flannery, S., and A. G. Bowie. 2010. The interleukin-1 receptor-associated kinases: Critical regulators of innate immune signalling. *Biochemical Pharmacology* 80: 1981–1991.
51. Cao, J., G. Ren, Y. Gong, S. Dong, Y. Yin, and L. Zhang. 2011. Bronchial epithelial cells release IL-6, CXCL1 and CXCL8 upon mast cell interaction. *Cytokine* .
52. Fitzpatrick, A. M., M. Higgins, F. Holguin, L. A. S. Brown, and W. G. Teague. 2010. The molecular phenotype of severe asthma in children. *J. Allergy Clin. Immunol.* 125: 851–857.e18.

53. Jones, C. P., S. C. Pitchford, C. M. Lloyd, and S. M. Rankin. 2009. CXCR2 mediates the recruitment of endothelial progenitor cells during allergic airways remodeling. *Stem Cells* 27: 3074–3081.
54. Templeton, S. P., A. D. Buskirk, B. J. Green, D. H. Beezhold, and D. Schmechel. 2010. Murine models of airway fungal exposure and allergic sensitization. *Med. Mycol.* 48: 217–228.
55. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 103: 779–788.
56. Mazzarella, G., A. Bianco, E. Catena, R. De Palma, and G. F. Abbate. 2000. Th1/Th2 lymphocyte polarization in asthma. *Allergy* 55 Suppl 61: 6–9.
57. Reijmerink, N. E., R. W. B. Bottema, M. Kerkhof, J. Gerritsen, F. F. Stelma, C. Thijs, C. P. van Schayck, H. A. Smit, B. Brunekreef, G. H. Koppelman, and D. S. Postma. 2010. TLR-related pathway analysis: novel gene-gene interactions in the development of asthma and atopy. *Allergy* 65: 199–207.
58. Bhatia, S., M. Fei, M. Yarlagadda, Z. Qi, S. Akira, S. Saijo, Y. Iwakura, N. van Rooijen, G. A. Gibson, C. M. St. Croix, A. Ray, and P. Ray. 2011. Rapid Host Defense against Aspergillus fumigatus Involves Alveolar Macrophages with a Predominance of Alternatively Activated Phenotype. *PLoS One* 6.
59. Ma, J., T. Chen, J. Mandelin, A. Ceponis, N. E. Miller, M. Hukkanen, G. F. Ma, and Y. T. Konttinen. 2003. Regulation of macrophage activation. *Cell. Mol. Life Sci.* 60: 2334–2346.
60. Luther, K., M. Rohde, K. Sturm, A. Kotz, J. Heesemann, and F. Ebel. 2008. Characterisation of the phagocytic uptake of Aspergillus fumigatus conidia by macrophages. *Microbes and Infection* 10: 175–184.
61. Bonnett, C. R., E. J. Cornish, A. G. Harmsen, and J. B. Burritt. 2006. Early neutrophil recruitment and aggregation in the murine lung inhibit germination of Aspergillus fumigatus Conidia. *Infect. Immun.* 74: 6528–6539.

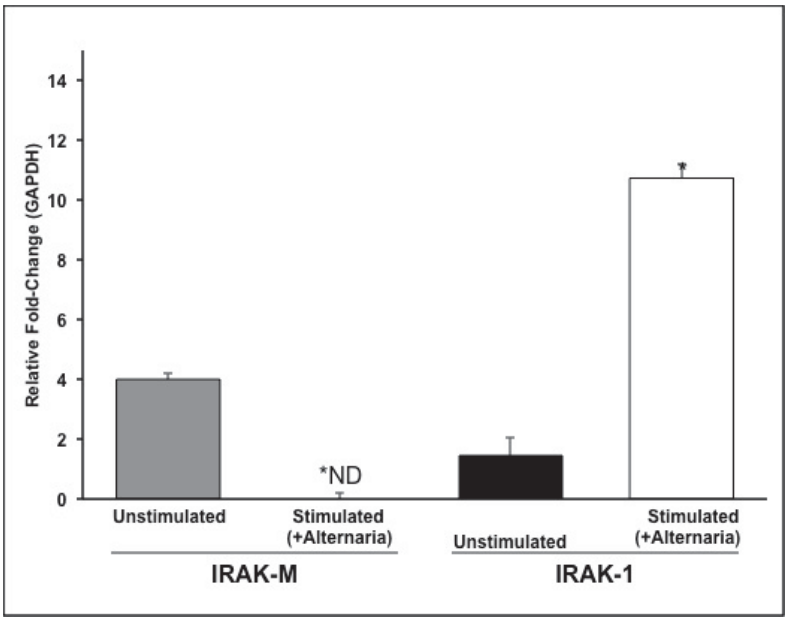


Figure 3.1: Alternaria antigens induce expression of IRAK-1 and down-regulation of IRAK-M. Bone marrow derived macrophages were harvested from the femurs and tibias of C57BL/6 mice. BMDMs were plated at a concentration of 1 $X10^6$ cell per well, serum starved for 24hrs, and then incubated with either PBS (control) or 50μg Alternaria antigens for 24hrs. Quantification of IRAK-1 and IRAK-M mRNA normalized to GAPDH levels. ND, not detected. Asterisk (*), statistically significant compared to untreated control. (n = 6).

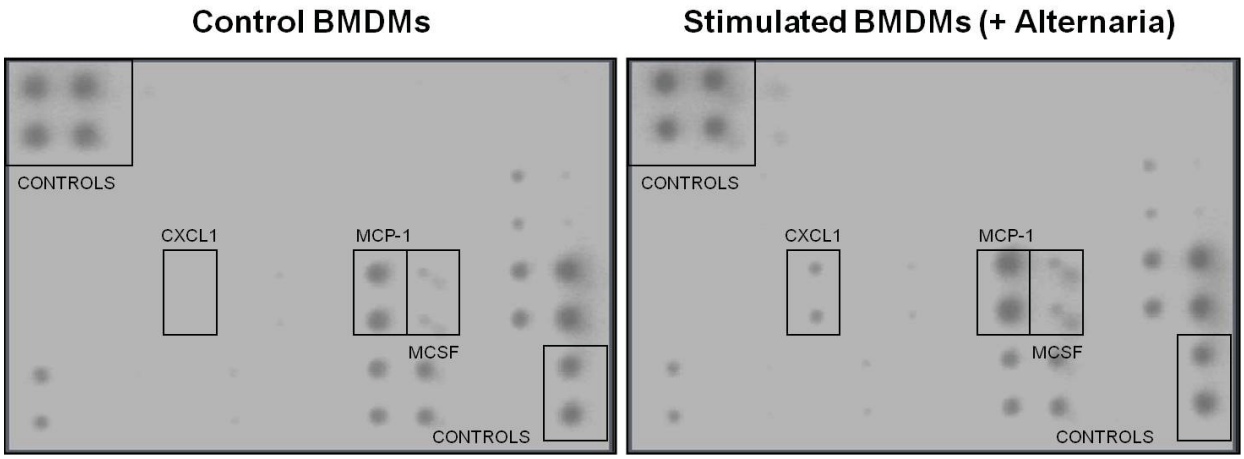


Figure 3.2. Alternaria antigens stimulated production of pro-inflammatory cytokines in murine BMDMs. Macrophage were harvested from the tibias of 8-week old C57BL/6 mice, cultured, and plated at 1 x10^6 cells/well in 6-well plates. Samples were treated with 50µg of Alternaria antigens for 24hrs. Cell-free supernatants were pooled from six samples and applied to Inflammatory Mouse Cytokines Array 1 (RayBio) and changes in cytokine levels were visually assessed.

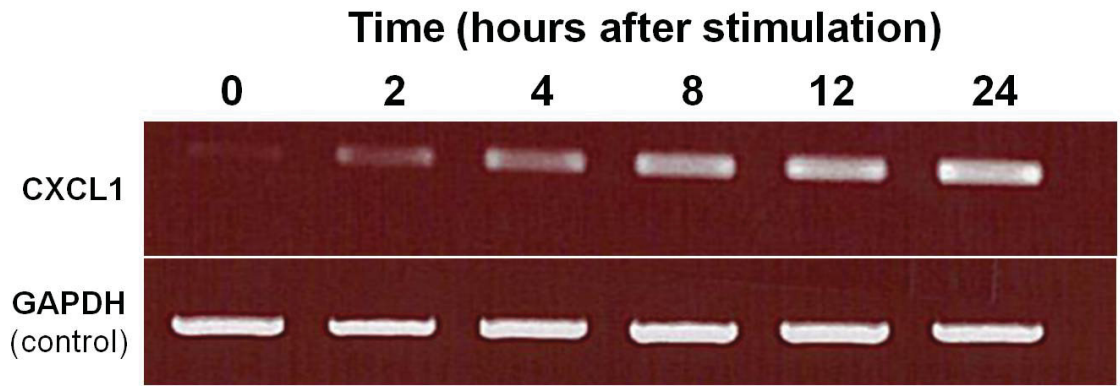


Figure 3.3. Expression of CXCL1 in BMDMs increases over time following Alternaria stimulation. BMDMs were seeded at a density of 1 x 10^6 cells/well in 6-well plates. Following a 24hr serum starvation, cells were treated with 50μg of Alternaria antigens. Cells were collected in TRIzol at the times indicated following initial treatment. Following mRNA extraction, cDNA was synthesized, and RT-PCR was performed with CXCL1 specific primers. Reactions were separated on a 1% agarose gel and visualized by ethidium bromide.

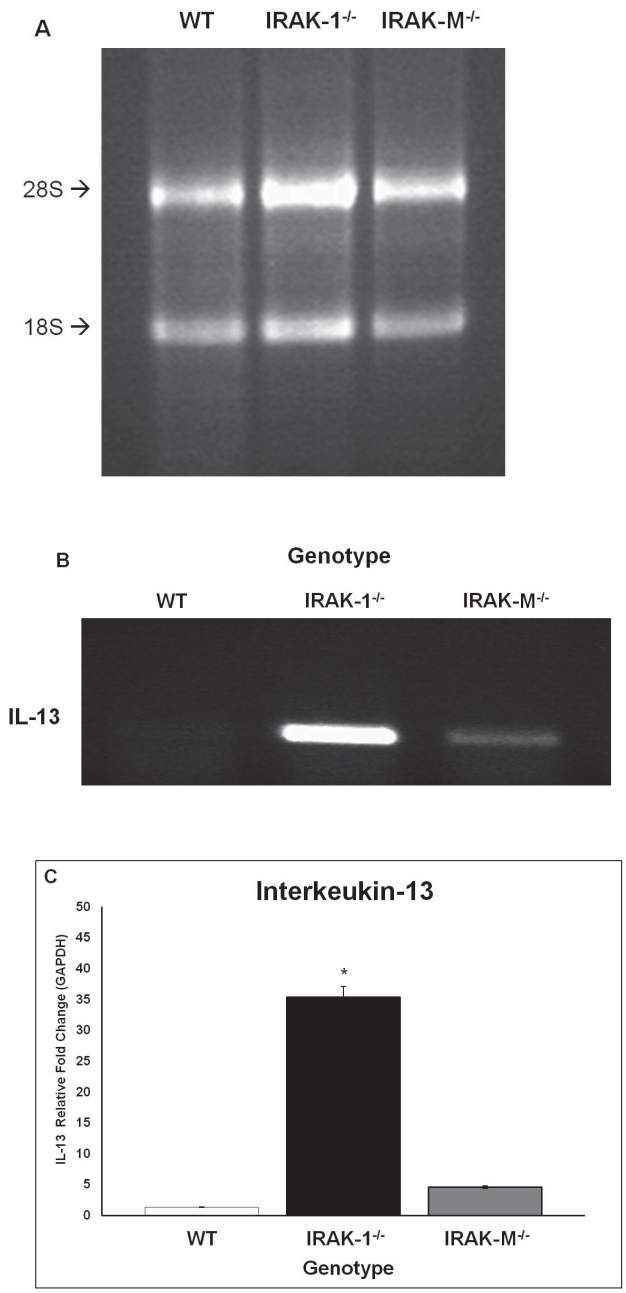


Figure 3.4: IRAK$^{-/-}$ mice have increased expression of IL-13 in response to Alternaria. Wild-type (C57BL/6), IRAK-1$^{-/-}$, and IRAK-M$^{-/-}$ mice were intranasally challenged with 50ug Alternaria antigens every 3 days for 6 days. Control mice were challenged with an equal volume of sterile DPBS. Lungs were excised 24hrs after last challenge and homogenized in TRIzol. Purity of mRNA (A) and RT-PCR of cDNA for IL-13 (B) was examined on 1% agarose gel stained with ethidium bromide. (C) Real-time PCR analysis of IL-13 was normalized to GAPDH (n = 4).

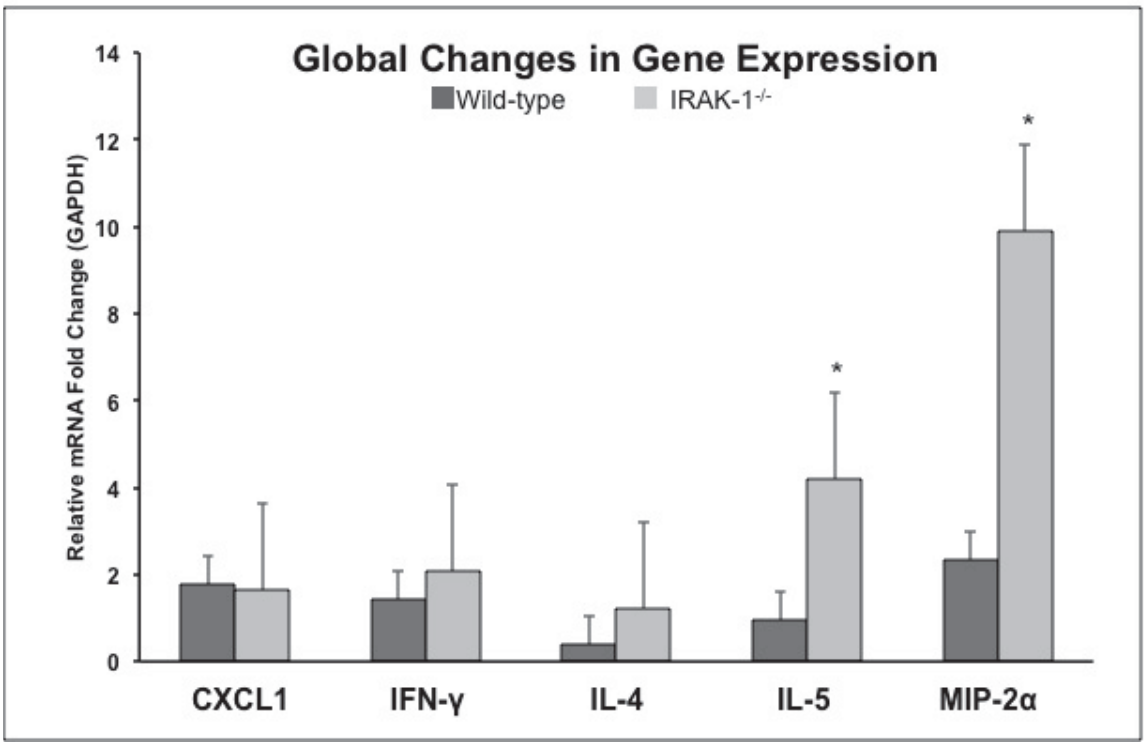


Figure 3.5: IRAK$^{-/-}$ mice exhibit higher levels of pro-inflammatory mediators in response to Alternaria. Wild-type (C57BL/6) and IRAK-1$^{-/-}$mice were intranasally challenged with 50ug Alternaria antigens every 3 days for 6 days. Control mice were challenged with an equal volume of sterile DPBS. Lungs were excised 24hrs after last challenge and homogenized in TRIzol. RNA was extracted and reverse-transcribed. The resulting cDNA was used for real-time PCR for the indicated cytokines with quantification normalized to GAPDH (n = 4).

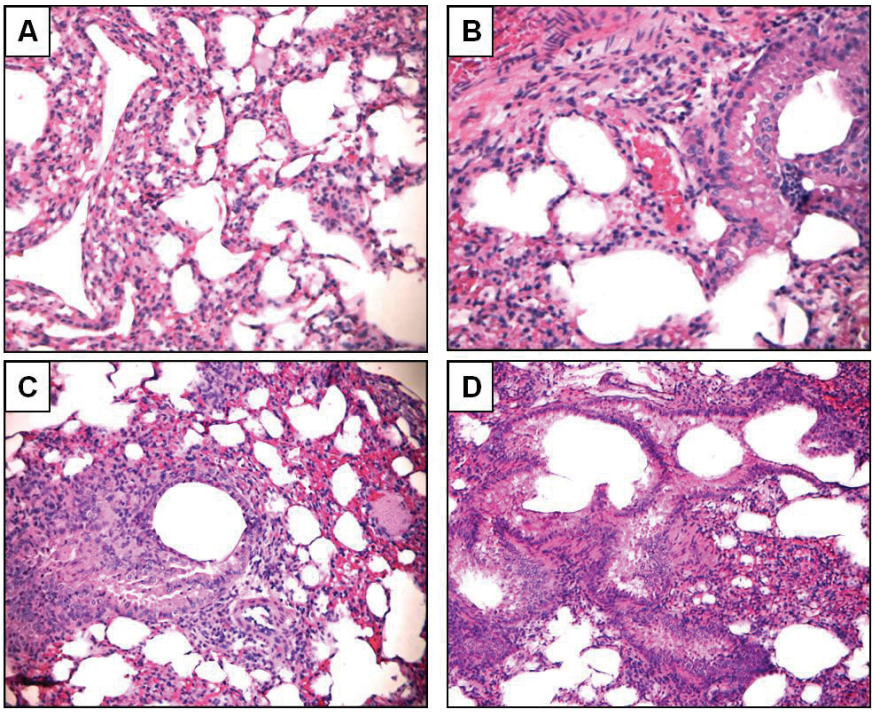


Figure 3.6: Histopathological analysis indicates greater effector cell infiltration into lungs of IRAK$^{-/-}$ mice after Alternaria challenge. Wild-type (C57BL/6) and IRAK-1$^{-/-}$ mice were intranasally challenged with 50ug Alternaria antigens every 3 days for 6 days. Control mice were challenged with an equal volume of sterile DPBS. Twenty-four hours after the last intranasal challenge, the lungs were excised, fixed in 10% buffered formalin, paraffin embedded, sectioned to 5*u*m, and stained with H&E. Panels A and B are control samples and C and D are treated samples, WT and IRAK-1$^{-/-}$ respectively. Images are representative of group (n=4/group).

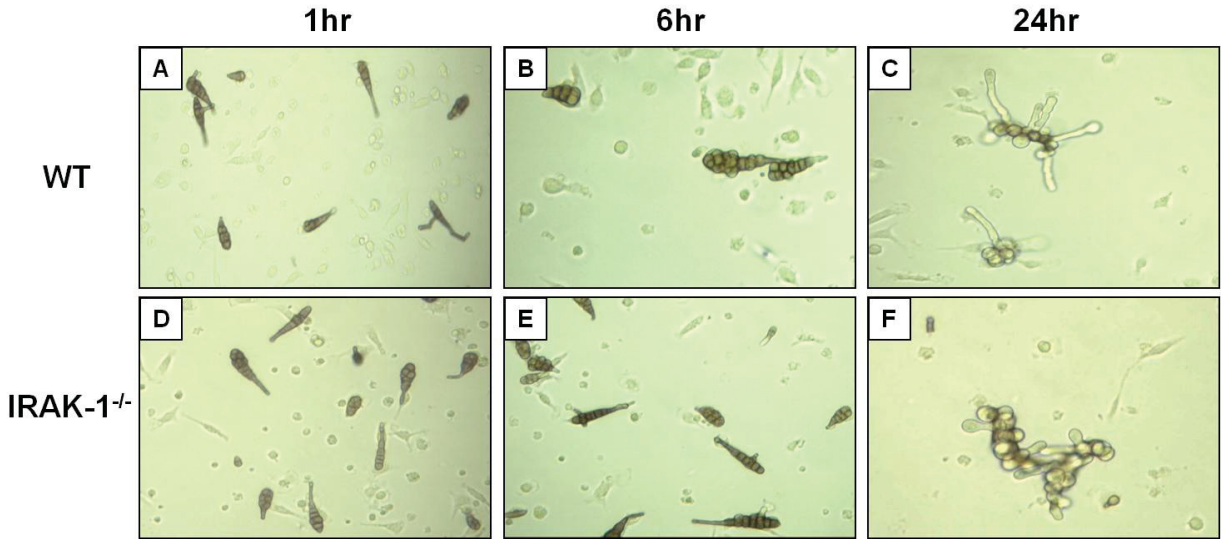


Figure 3.7. IRAK-1-deficient macrophage show less anti-fungal activity. Macrophage were harvested from the tibias of 8-week old wild-type (C57BL/6) and IRAK-$1^{-/-}$ mice, cultured, and plated at 1 x10^6 cells/well in 6-well plates. Cells were treated with 5 x 104 *A.alternata* conidia. Cultures were images using an inverted microscope at the indicated time points.

Chapter IV

Major allergen Alt a 1 modulates the innate immune response to the ubiquitous airborne fungus, *Alternaria alternata* in human airway epithelial cells

ABSTRACT

Exposure to the ubiqutious airborne fungus, *Alternaria alternata*, has long been associated with an increased risk of developing atopic or allergic asthma, chronic rhinosinusitis (CRS), or exacerbation of a preexisting asthmatic condition. Alt a 1 is the major allergen secreted by *A. alternata* due to its IgE-specific reactivity with sera from atopic Alternaria patients yet no known immunological activity has been assigned to this clinically relevant protein. In order to elucidate the overall importance of Alt a 1 in immune response to Alternaria, we utilized an *A. alternata* (*Δalta1*) deletion mutant and an *A. alternata* (Alta1+) overexpression mutant in human airway epithelial cell studies. Although the mutants have analogous phenotypes regarding growth rates, and conidial and hyphal morphology compared to the wild-type, the *Δalta1* mutant exhibits a significantly reduced ability to stimulate the pro-inflammatory cytokines IL-6, IL-8, and MCP-1 in the human bronchoalveolar epithelial cell line BEAS-2b. In addition, cells exposed to the Alta1+ overexpression mutant dramatically increased cytokine production compared to the wild-type, suggesting that this single protein may play a major role in inducing the innate immune response in the airway epithelium at the organismal level. Importantly, the effect of deletion or overexpression of Alt a 1 in cell cultures was confirmed using normal and diseased primary human bronchial cells. These findings have important implications regarding the importance of

Alt a 1 in the innate immune response to Alternaria and further confirm Alt a 1 as a potential therapeutic target for Alternaria allergy.

Introduction

Chronic airway inflammatory diseases, such as asthma and chronic rhinosinusitis (CRS), are complex and multi-factorial disorders which represent a significant public health problem as their incidence have significantly increased over the past 40 years (1, 2). Considerable research has shown that the ubiquitous airborne fungus, *Alternaria alternata*, is clinically associated with acute and chronic human airway disorders including epidemiological studies indicating that up to 70% of mold-allergic patients have skin test reactivity to Alternaria (3, 4). Sensitivity to Alternaria has been shown to not only be a risk factor for asthma, but can also directly lead to the development of severe and potentially fatal asthma often more than any other fungus (5). In a recent survey by the National Institute of Environmental Health & Safety (NIEHS) of 831 homes containing 2456 individuals, the prevalence of current symptomatic asthma increased with increasing *Alternaria* concentrations and higher levels of *Alternaria* antigens in the environment significantly raised odds of having had asthma symptoms during the preceding year more so than other examined antigens (6). Additionally, Alternaria sensitization has been identified as one of the most important factors in the onset of childhood asthma in the southwest deserts of the U.S. and other arid regions (7, 8). Despite this evidence showing the global correlation between Alternaria and airway disorders, few studies have examined the interaction between Alternaria spores and the human airway.

Our knowledge regarding the communication between Alternaria spores and host cells in regards to the innate immune response has been limited by the fact that most studies have employed potent fungal extracts in their experimentation rather than the organism itself (9–12). These fungal preparations do not adequately mimic natural host-fungal interactions and are highly variable resulting in markedly different biochemical properties such as protease activity and diverse chitin and glucan content (13). Furthermore, these protease-heavy extracts may be

pathologically irrelevant in some regards and cause additional damage to the epithelium (14) thereby enhancing the response phenotype in both in vitro and in vivo models. Most importantly, the concentration of specific allergens across different commercially-available extracts varies widely (15, 16).

The discovery of Alternaria allergens to date has only identified three major and five minor allergenic proteins (17, 18) Most of these allergens are fairly conserved proteins with known function such as enolase, ribosomal proteins, nuclear transport factor, and aldehyde dehydrogenase (19–21). However the biological role of the Alternaria major allergen, Alt a 1, has not been established. Alt a 1 is defined as the major Alternaria allergen due to its specific binding to IgE antibodies in sera from patients who are sensitive to Alternaria as demonstrated by skin prick or RAST testing (22, 23); Alt a 1 is a relatively small protein (157 amino acids) and forms a dimer of 28 kd, which dissociates into 14.5-kd and 16-kd subunits under reducing conditions yet has no known function in fungal metabolism or ecology (22, 24). The Alt a 1 protein is secreted and highly abundant in dormant spores and hyphae and predominantly localized in the cell wall (25, 26). In addition, it is released during spore germination and hyphael growth (27, 28). Native and recombinant forms of Alt a 1 are reported to have esterase enzymatic activity (29). Although Alt a 1's basic biochemical properties have been characterized, no work has elucidated its ability to stimulate the innate immune system.

The airway epithelium acts not only as a physical barrier against fungal allergens, but also plays a crucial role in initiating and augmenting the airway host defense mechanism (30). When an allergen comes in contact with the epithelium it can trigger multiple events including disruption of tight junctions, activation of mucous metaplasia, and stimulate production of pro-inflammatory cytokines and chemokines (31). Specific innate immunity cytokines and

chemokines which have been detected from the epithelium after stimulation with allergenic fungi include IL-6, IL-8, MCP-1, and RANTES (32–34). This allergen activation of the airway epithelium can provide polarizing conditions for an aggressive Th2 response (35). Additionally, damage to the epithelium leads directly to disruption of tight junctions (36) and eventual airway remodeling, both hallmarks of chronic airway disorders including allergen induced asthma (37–41).

In this present study we advance our understanding of the biological role of the major allergen, Alt a 1, in stimulating the mammalian innate immune response. By construction and utilization of an Alt a 1 deletion mutant (Δ*alta1*) and an Alt a 1 overexpression mutant (Alta1+) we show for the first time that the production and release of Alt a 1 influences the innate immune response to *A.alternata* spores in human airway epithelial cells.

MATERIALS AND METHODS

Fungal strains and growth conditions:

Alternaria alternata strain ATCC 66981 was used for all molecular manipulations. Except where indicated, strains were propagated on potato-dextrose -agar (PDA) (0.4% potato starch, 2% dextrose, 1.5% agar) or in glucose-yeast extract broth (GYEB) (1% glucose, 0.5% yeast extract). Both solid and liquid cultures were growth at 25°C and in the dark.

For cell stimulation experiments, conidia were harvested into sterile water from a 7-day old PDA plate supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone). Conidial suspensions were standardized to equal concentrations after counting on a hemocytometer and then used immediately in assays.

Tissue culture:

The human bronchial epithelial cell line BEAS-2B, derived from human bronchial epithelium transformed by an adenovirus 12-SV40 virus (81) was cultured in RPMI: 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone) at 37°C and 5% (v/v) CO_2 in 75cm^2 tissue culture flasks. Cells were routinely subculture by trypsinization at 80% confluence.

The primary normal bronchial epithelial cells (NHBE) were purchased from Lonza. The primary diseased human bronchial epithelial cells (DHBE) from an asthmatic donor were a gift from Dr. Hirohito Kita, Department of Immunology, Mayo Clinic, Rochester, MN. NHBE and DHBE cells were grown in 75 cm2-plastic flasks in bronchial epithelial basel medium (BEBM, Lonza)) supplemented with bovine pituitary extract (52 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (0.5 ng/ml), epinephrine (0.5 μg/ml), transferrin (10

μg/ml), insulin (5 μg/ml), retinoic acid (0.1 μg/ml), triiodothyronine (6.5 μg/ml), gentamicin (50 μg/ml) and amphotericin B (50 μg/ml). The medium was changed every other day until cells were 60-80% confluent.

Recombinant Alt a 1:

Purified recombinant Alt a 1 (rAlt a 1) was purchased from Indoor Biotechnologies (Charlottesville, VA). For use in immunological experiments, endotoxin was removed by filtering through Detoxi-Gel Endotoxin Removal Columns (Pierce) following the manufacturer's instructions. The protein was then concentrated using a 3kDa Amicon Ultra-4 Centrifugal Filter (Millipore) and resuspended in phosphate buffered saline (pH 7.4) at a concentration of 1mg/mL.

Construction of *alta1* deletion and overexpression constructs, fungal transformation, and complementation in *A. alternata*:

The Alt a 1 deletion mutant (*Δalta1)* was generated by cloning the flanking region of *Alt a 1* (1000 bp upstream and downstream of *Alt a 1* gene locus) into the plasmid pCB1636 containing the antibiotic resistance gene HygB for selection as previously described (42, 65). Using the wild-type *A.alternata* genomic DNA sequence, primers with a restriction enzyme site at each end were designed in order to amplify the 1kb flanking regions of *Alt a 1* gene locus. The primers used for the 1kb region upstream and downstream of the Alt a 1 gene are described in Supplementary Table 1. The PCR products were digested and sequentially cloned into the fungal transformation vector pCB1636 (82). The plasmid construct was transformed in *E. coli* DH5α competent cells and sequence verified for the insert and HygB cassette. The plasmid was used as the template for PCR amplification using M13 forward and M13 reverse primers. The PCR

product was purified with PCR Cleanup kit (Qiagen) and further concentrated to 1 mg/ml under vacuum before fungal transformation. The fungal transformation of protoplasts was performed as we have described previously (65). Individual transformants were selected and transferred to a fresh PDA + hygromycinB (30µg/mL) plate between 7 – 14 days after transformation. The transformants were further isolated by transferring a single spore to a fresh PDA + HygB plate.

For creation of the complementation mutant (*Δalta1-rec*), coding regions of the *Alt a 1* wild-type gene were reintroduced into the Δ*Alt a 1* mutant background along with approx 1kb long native promoter sequence and a nourseothricine resistance cassette (NAT). Genomic DNA from *A. alternata* was used as a template to amplify a 1.4 kb fragment using the primers described in Supplementary Table 1. Again M13 reverse and M13 forward set of primers were used and the fragment obtained was transformed into the Δ*Alt a 1* mutant following the same protocol (65). Transformants were selected on PDA + nourseothricine (50µg/mL) and further isolated as described above.

For the overexpression mutant (*Alta1+*), the strong constitutive promoter from *Pyrenophora tritici-repentis,* ToxA, was fused to wild type *Alt a 1* gene (123). The ToxA promoter was cloned at the 5' of the *Alt a 1* coding sequence in the pCB1636 vector. Primers used for addition of the ToxA promoter are described in Supplementary Table 1. The final construct was transformed into wild-type *A.alternata* as described (65).

Transformants of the *Δalta1* mutant was confirmed by Southern blot analysis and the Δ*alta1-rec* and *Alta1+* transformants were confirmed by PCR of genomic DNA using the primers listed in Supplementary Table 2.

Preparation of *A. alternata* nucleic acids:

Total DNA and RNA were isolated from each strain as previously described (63, 64). Briefly, mycelium grown for 7 days in GYEB medium was ground under liquid nitrogen and the resulting powder was used for DNA and RNA extraction. DNA was extracted using the DNeasy Plant Kit (Qiagen) following the manufacturer's fungal DNA extraction protocol. RNA was extracted using the RNeasy Plant Kit (Qiagen) plus on-column DNase digestion. Concentration and purity was measured by absorbance at 280nm using a Nanodrop spectrophotometer (ND-1000).

Southern Blotting

A total of 3µg genomic DNA was digested from each fungal strain with restriction enzymes BamH1 and EcoRV (New England Biolabs) and separated on a 0.8% agarose gel. The DNA was transferred to a Hybond N^+ nylon membrane (Amersham) and then U.V. cross-linked at 120 mJ (Spectronics corporation) to the membrane. The DNA probes for *Alta1* and *HygB* were synthesized using a PCR DIG Probe Synthesis Kit following the manufacturer's protocols (Roche). The hybridization of membrane was performed at 50ºC using the Block and Wash Buffer Kit (Roche). The blot was first hybridized with the *Alta1* specific probe. The same blot was subsequently stripped and hybridized with *HygB* probe. The membrane was washed in a final solution of 0.1× SSC (0.15M NaCl, 0.015M sodium citrate) and 0.1% sodium dodecylsulfate (SDS) at 68ºC. Visualization of the blot was performed using the DIG Luminescent Detection Kit for Nucleic Acids (Roche).

Determination of fungal growth rates:

Hyphal growth rates were compared by measuring radial growth on various carbon sources including potato dextrose agar (PDA), glucose minimal media (GluMM) (83), or galactose minimal medium (GalMM) which was identical to GluMM except for substitution of glucose with an equimolar amount of galactose. For each strain, a 1cm^3 plug was aseptically obtained from the outer boundary of a 7-day old PDA plate and placed mycelium side up in the center of the indicated solid media. Plates were incubated under normal conditions (25°C, in the dark) and the colony diameters were measured once a day over 7 days. Values are average radial growth from three plates per experiment and the experiment was repeated twice.

Conidial morphology:

Conidia were collected in sterile water from 7-day old PDA plates, placed on glass slides, and examined and imaged on a Zeiss Axioskop 2 microscope with the Axiovision 3.0 software microscope at 10X to 40X magnification.

Preparation of Fungal Culture Filtrate:

Conidia from each strain were harvested from a 7-day old PDA plate by addition of 5mL phosphate buffered saline (PBS) to the plate and gentle agitation with the back of a cell lifter. Conidial concentration in the preparations was quantified by counting on a hemocytomer and 5 x 10^6 conidia were inoculated into 25mL GYEB. Cultures were grown for 7 days at 150rpm, 25°C, and in the dark. Mycelia and conidia were removed by successive vacuum filtration across 0.8µm, 0.45µm and 0.2µm nitrocellulose filters. The collected filtrate was reduced 4x to a using a 3 kDa Amicon Ultra-4 Centrifugal Filter (Millipore). Total protein concentration was assayed by the BCA Assay (Pierce) following the manufacturer's instructions.

Detection of Alt a 1 in Culture Filtrate:

A total of 50µg of total protein from the fungal culture filtrates were separated on a 12% SDS-PAGE gel as previously described by Laemmli (84). The samples were electrophoretically transferred to a nitrocellulose membrane (Immobilon) and probed with anti-Alt a 1 antibody (Indoor Biotechnologies). Bound antibodies were detected with HRP-conjugated anti-mouse IgG (Promega) and membranes were developed using the ECL Detection Reagent (GE Healthcare).

The amount of Alt a 1 in the culture filtrates was further quantified using a commercially available Alt a 1 ELISA Kit (Indoor Biotechnologies) following the manufacturer's instructions.

Epithelial Cell Stimulation:

Epithelial cells (BEAS-2b) were seeded (1×10^6 cells/well) in six-well tissue culture plates (BD) and allowed to adhere overnight. Monolayers were washed once using Dulbecco's Phosphate Buffered Saline (Ca^{++}/Mg^{++} free) ($DPBS^{-/-}$; Hyclone) and serum-starved for two hours in RPMI: 1640 medium without supplements. Cells were washed twice more with $DPBS^{-/-}$ and cultured in a final volume of 1.5mL RPMI: 1640 without supplements.

The normal human bronchial epithelial (NHBE) primary cells were dissociated with trypsin/EDTA and cultured in BEBM media (Lonza) containing the same supplements as above but with the exception of the anti-fungal amphotericin B. The cells were seeded at a density of (1×10^5 cells/well) in six-well dishes and allowed to grow until 80% confluency. Cells were washed with Hank's Balanced Salt Solution (HBSS) and cultured in 1.5mL of BEBM media with normal supplements except amphotericin B.

Both BEAS-2b and the primary cells were inoculated with the indicated number and appropriate strain of *A.alternata* wild-type or mutant spores, rAlt a 1, or left untreated (control)

and incubated at 37°C and 5% CO_2 for 24 hours. Following treatment, the supernatants were collected and debris was removed by centrifugation (2500x*g*, 5min, 4°C) before storing at -80°C. The cells were then washed twice with $DPBS^{-/-}$ before RNA was extracted. All cell stimulation experiments were done in triplicate and repeated twice.

RNA Extraction and qRT- PCR:

Total RNA was purified from the treated and untreated BEAS-2b cells by addition of TRIzol (Invitrogen) directly to the cells and then following the manufacturer's protocol. RNA was further purified and genomic DNA eliminated by using the RNeasy Cleanup Kit (Qiagen) with on-column DNase digestion. First-strand synthesis was performed from 1µg total RNA with the Tetro cDNA Synthesis Kit (Bioline) using random primers. Real-time PCR using an iCycler following the manufacturer's instructions (Biorad) was then completed using the SYBR Green Supermix (Bio-Rad) and primers listed in Supplementary Table 3. Reactions were performed in duplicate and gene expression was normalized to the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase using the formula $2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = [(Ct_{\text{target gene}})\text{sample}\ (Ct_{GAPDH})\text{sample}]/[(Ct_{\text{target gene}})\text{reference}-(Ct_{GAPDH})\text{reference}]$.

Human Antifungal Response PCR Array:

The RT^2 Profiler PCR Array (SABiosciences) was performed with total RNA from treated and untreated BEAS-2b cells as described above. The first-strand cDNA was generated using the RT^2 First-strand Kit (SABiosciences) and 84 genes related to the human antifungal response markers were measured following the manufacturer's instructions.

Cytotoxicity Measurement:

The primary normal (NHBE) and diseased (asthmatic) (DHBE) human airway epithelial cells were plated and stimulated as described above. At the indicated timepoints, 50 µl of cell culture supernatant was aseptically removed the level of lactate dehydrogenase (LDH) was determined using the CytoTox 96 Non-Radioactive Assay Kit (Promega). Positive and negative control cells were treated with either 100 µl of 10% Triton X-100 (1% v/v final concentration) or PBS respectively. Samples were incubated with LDH substrate for 5 minutes and then the absorbance was read on a microplate reader at 490 nm.

ELISA:

The release of IL-6, IL-8, and MCP-1 in the cell culture supernatants was quantified by ELISA according to the instructions provided by the manufacturer (eBiosciences).

RESULTS:

Generation of Alt a 1 deletion and overexpression mutant strains

For these studies we utilitzed previously created mutants in our lab (Babiceanu 2010). However, their construction will be described again for ease of reading. In order to investigate the importance of Alt a 1 in fungal development and pathogenesis we created three classes of *A. alternata* mutants, including a deletion mutant (Δ*alta1)*, a complemented mutant (*Δalta1-rec*), and an Alt a 1 overexpression mutant (Alta1+) using the gene targeting strategy described in Figure 1. The deletion of Alt a 1 was completed by fully replacing the Alt a 1 gene with the Hygromycin B phosphotransferase (HygB) gene using a linear replacement construct (Figure 1A) through a process that relies on two homologous recombination events described in our previously published methods (42). In order to verify the observed phenotypes were in fact due to deletion of Alt a1, the *Δalta1* mutant was complemented by reintroduction of the wild-type Alt a 1 gene into the *Δalta1* background (Figure 1B). The overexpression mutant was created by fusing the strong constitutive promoter, ToxA, from *Pyrenophora tritici-repentis,* to the wild type *Alt a 1* gene (Figure 1C) (43) . Deletion of Alt a 1 was confirmed by Southern blot analysis. As shown in Figure 2A, the probe for the *alta1* detected the gene in the wild-type (~650bp size) but not in any of the five *Δalta1* mutants. Instead, the probe for the hygromycin B, HygB, gene (~550bp size) detected the presence of the resistance gene in the deletion mutants but not in the wild-type. Re-integration and overexpression of Alta1 was confirmed by PCR using Alta1 and HygB specific primers using extracted genomic DNA of each strain as a template (Figure 2B). The amplification of the Alternaria actin gene was used as a positive control for the PCR reactions.

Alt a 1 production in Alta1-deficient and overexpression strains:

Next, the mRNA expression and secreted Alt a 1 protein levels in the wild-type and mutant strains were evaluated. All strains were cultivated in GYEB under normal conditions for 7 days before mRNA and protein levels were measured. As shown in Figure 3A, mRNA transcripts were detected by semi-quantitative RT-PCR. Expression of Alt a 1 was not found in the *Δalta1* mutant while transcript levels in the *Δalta1-rec* mutant were similar to the wild-type. Additionally, the Alta+ mutant had more copies of the Alt a 1 transcript compared to the wild-type and complement. We confirmed Alt a 1 secretion from germinating conidia by Western blot. Figure 3B shows the detection of Alt a 1 in the GYEB culture supernatant by ant-Alta1 antibody. As expected from the mRNA data, we found complete absence of Alt a 1 protein in culture filtrates of a deletion mutant and higher levels of secreted Alt a 1 in the overexpression mutant while the complement showed comparable levels to wild type. We also utilized an Alt a 1 specific ELISA to quantify the total amount of Alt a 1 in the culture filtrates (Figure 3C). The results of the ELISA directly supported and confirmed the results of the Western blot.

Effects of deletion or overexpression of Alt a 1 on growth rate and conidial morphology

To exclude the implication that deletion of Alt a 1 alters the normal germination and growth of *A.alternata*, we utilized growth experiments and visually examined fungal morphology. Analysis of growth characteristics and conidial morphology of the *Δalta1*, *Δalta1-rec*, and Alta*1*+mutants indicated that they were indistinguishable from the wild-type. We calculated average radial growth rates by measuring the colony diameter of each strain on various carbon sources (Figure 4A). The rate of growth for each strain was determined by averaging growth on three separate plates for each strain during three separate experiments. A paired *t-test* was performed for each mutant in comparison with the wild type *A. alternata*. The probability of t-test was also

calculated with $p \leq 0.05$ considered significant. On normal solid media (PDA) there was no difference in growth rate measured between the wild-type and mutants. To assess if deletion or overexpression gave the fungus an increased or decreased ability to grow under nutrient stress, we measured growth on glucose minimal media (GluMM) and galactose minimal media (GalMM). Again, no variation was seen in the growth rates of the mutant strains compared to the wild-type. Moreover, we observed no changes in colony morphology, pigmentation, or elevation on different solid media (Figure 4B). Conidial morphology and hyphal germination were visually examined by light microscopy at 10x and 40x magnification (Figure 4C). Hyphal fragmentation and septal branching patterns appeared normal in all mutants. Conidial structure including cell wall profile, segmentation, and chain formation of all mutants were also all observed as representative when compared to the wild-type. Overall, there was no indication of gross morphological or growth related differences between the wild-type and mutants that may contribute to phenotypic changes in host response during infection.

Both *A.alternata* spores and Alt a 1 protein alone can induce pro-inflammatory cytokines

The ability of *A.alternata* spores and Alt a 1 protein itself to induce common pro-inflammatory cytokines in human airway cells was examined. BEAS-2b bronchoalveolar cells were treated with either 50μg/mL rAlta1 or 5×10^5 wild-type *A.alternata* spores or left untreated (control) for 24hrs. The levels of IL-6 and IL-8 in the cell culture supernatant were measured by ELISA (Figure 5). The wild-type *A.alternata* spores were able to induce a significant increase in both cytokines. Interestingly, the rAlt a 1 protein was also able to stimulate IL-6 and IL-8 to levels greater than 50% of the spores alone. This is surprising since multiple components of the fungal cell wall such as chitin and beta-glucan as well as proteases have been implicated in Alternaria's

ability to stimulate the immune response (44–46). Additionally, we removed any residual endotoxin present in the rAlta1 preparation to ensure the response was a result of the Alt a 1 protein and not contaminants from production in *E.coil.* The cell culture supernatant was also tested for the presence of cytokine markers IL-33, TSLP, TNF-a, and GM-CSF; however, none of these were found to be induced in airway epithelial cells by Alternaria conidia or rAlt a 1 (data not shown).The results indicate that secretion of Alt a 1 by *A.alternata* play a significant role in the response of the epithelium to the conidia.

Alt a 1 mediates stimulation of human airway epithelial cells to Alternaria conidia

Since pulmonary epithelial cells are conceptually the first cell type to release cytokines and chemokines in response to an allergen (14, 30, 47–49) we treated human airway epithelial cells with the various strains of *A. alternata* to explore the role of Alt a 1 in stimulating IL-6, IL-8 and MCP-1. BEAS-2b cells were treated with an equal number of spores of the same age from the wild-type, or *Δalta1*, *Δalta1-rec*, and Alta1+ mutants. As described in Figure 6, stimulation of the cells with the *Δalta1* strain resulted in markedly reduced induction of IL-6, IL-8 and MCP-1 as compared to the wild-type and complemented strains while the Alt a 1 overexpression strain caused a notable increase in these three inflammatory markers compared to the wild-type.

Effects of deletion and overexpression of Alt a 1 is confirmed in primary human airway epithelial cells

In order to explore the implications of deletion or overexpression of Alt a 1 in a human model, we utilized primary human epithelial cells from a normal (non-diseased) donor to more closely assess the potential in-vivo response of epithelial cells to Alternaria. The experiment was

conducted in triplicate, with each sample receiving 5 x 10^5 conidia or left as an untreated control. The release of IL-6, IL-8 and CXCL10 into the cell culture supernatant was measured by ELISA (Figure 7). For cells treated with the wild-type or complemented mutant, we found an increase of 67%, 85%, and 65% for IL-6, IL-6, and CXCL10 respectively. In cells treated with the Alt a 1 deletion mutant, we saw a decrease of 32%, 25%, and 50% for IL-6, IL-8, and CXCL10 compared to the wild-type and complement treated samples. The overexpression mutant induced IL-6, IL-8, and CXCL10 at levels 35%, 15%, and 59% greater than the wild-type and complement treated samples. All increases or decreases in the levels of these three cytokines were statistically significant $p<0.05$ using a student's t-test. We also calculated cytotoxicity of the treatments by measuring LDH release to confirm the differences in immune response was not being caused by cell death. The LDH assay was conducted as described in the Material and Methods. The results, shown in Figure 7B, illustrate that cell viability was reduced by less than 2% in all cell samples treated with conidia. Although this is higher than the untreated control, there was no statistical difference in the percentage of cell death between treated samples. We also include images in Figure 7C depicting the epithelial cell culture treated with conidia for 24 hours. The conidia have all germinated at visibly equal amounts and the epithelial cells do not appear to be impacted by treatment as demonstrated by normal morphology (pseudopodial) and no evidence of chromatin condensation or cytoplasmic blebbing compared to the untreated control. Collectively, the results of our primary airway epithelial cell model suggest that the secretion of Alt a 1 significantly influences the response of the human airway to *A.alternata* and that cell death is not a factor in the observed response.

Alt a 1 is involved in activation of multiple innate immune pathways

We also investigated the role of Alt a1 in the activation of antifungal markers in human airway cells. Total RNA was prepared from BEAS-2b cells treated with wild-type or *Δalta1* spores as well as untreated (control) cells and applied to the RT^2 Profiler PCR Array (SABiosciences) which contained markers for 84 antifungal genes. Full results of the array can be found in Supplementary Table 3. In summary, the array detected the same response for IL-6, IL-8, and MCP-1 (CCL2), thus confirming the previously conducted ELISA analysis from cell culture supernatants. Additionally, the array detected a host of other genes including CXCL1, CXCL10, CASP1, IL-1b, IL-23a, PTPN6, CD14, MAP kinases, PTX3, TLR3, TLR4, and TIRAP in the wild-type treated samples but were induced at much lower levels in the *Δalta1* treated samples. Interestingly, transcripts for common markers associated with airway disorders, including RANTES (CCL2), CCR1, and CXCL2 were not detected following either of the treatments. Following normalization against the untreated control, 10 targets which were found to be most significantly different between the wild-type and *Δalta1* treated samples were chosen for further analysis. The genes selected were IL-6, IL-8, MCP-1, CXCL1, CXCL10, CXCL11, CASP1, STAT1, MyD88, MAPK14, CD14, TLR3, and TLR4. Primers were designed from the cDNA coding sequence of each gene and are described in Supplementary Table 3. For comparison, qRT-PCR was conducted on triplicate RNA samples to confirm array results of the wild-type and *Δalta1* treated samples as well as samples from *Δalta1-rec* and *Atla1+* treated cells. The results for fold-change differences of each gene when normalized to GAPDH are shown in Figure 8. Again, the Alt a 1 deletion mutant had markedly less influence on the response of the human airway cells for multiple gene targets involved in innate immune pathways and cytokine production including IL-6, IL-8, MCP-1, CXCL10, CXCL11, STAT1, MyD88, and CASP1 while the overexpression induced a greater response. The targeted qRT-PCR did not find marked

differences between the deletion or overexpression mutants and wild-type for MAPK14, CXCL1, CD14, or TLR4. There was a slightly reduced response in TLR3 expression in the *Δalta1* samples and a slightly higher response in the Alta1+ samples. The differences between the Superarray and follow-up qRT-PCR can be attributed to the nature of the Superarray results only comparing a single wild-type and *Δalta1* treated RNA sample while the qRT-PCR contained three biological replications and two technical replications. The results of the RT^2 PCR Array and subsequent confirmation by qRT-PCR not only confirm previous ELISA results for IL-6, IL-8, MCP-1, and CXCL10 but also reveal the involvement or lack thereof for many other molecules involved in innate immune signaling.

DISCUSSION:

The significance of Alternaria sensitization in correlation with airway disorders has been well established (50–53). Additionally, the major allergen from *A.alternata* has been identified as Alt a 1 for its substantial reactivity with sera from sensitized individuals and a documented IgE binding domain (54, 55); however, no known function or role in pathogenesis has been found for this pertinent protein. Despite evidence based on assays involving potent Alternaria extracts (56–59), there is limited data showing the interaction of actual Alternaria spores with the human immune system.

As the first physical barrier to inhaled organisms and allergens, the epithelium is now recognized to play a central role in the regulation of airway inflammatory status, structure, and function in normal and diseased airways (48). More specifically epithelial cell activation can result in the release of proinflammatory cytokines and chemokines that attract inflammatory effector cells like neutrophils, macrophages, eosinophils, and lymphocytes. The production and release of cytokines and chemokines have been of particular interest in allergic conditions such as asthma and allergic rhinitis (60). In this manuscript we present convincing evidence for the role of Alt a 1 in the response of human airway epithelial cells to *Alternaria alternata* conidia.

We first established an *in-vitro* bronchoalveolar cell model for investigating the interaction between Alternaria conidia and the airway epithelium and compared these to similar results using the traditional Alternaria extract (data not shown). We also utilized rAlt a 1 to show the response of epithelial cells to the protein was similar to the wild-type conidia. Both the conidia and Alt a 1 protein induced IL-6, IL-8, and MCP-1 three well classified cytokines linked Alternaria and other fungal allergens (61, 62)

Once we determined the ideal conditions for induction of a response by *A.alternata*, we set out to explore if secreted Alt a 1 from germinating conidia can modulate the epithelial response to whole spores. To do this, we constructed an *A.alternata* Δ*alta1* deletion mutant, a Δ*alta1*-rec complemented mutant (reintroduction of the wild type *Alt a 1* gene in the Δ*alta1* deletion mutant background), and an *Alta1*+ (Alt a 1 overexpression) mutant. The deletion and complementation transformations were conducted using well established methods from our lab (42, 63–65) which uses homologous recombination of linearized DNA to completely replace the target gene with a resistance gene. The overexpression strain was created by fusing the constitutive promoter ToxA to the 5'-end of Alt a 1. The mutations were successfully confirmed by Southern blot and PCR and subsequently Alt a 1 production was measured and quantified from the culture filtrates of each strain by Western blot and ELISA using an anti-Alta1 antibody. Alt a 1 was not detected in the deletion mutant at either the mRNA and protein level while the overexpression had an excessive abundance of Alt a 1 transcripts and protein secretion compared to the wild-type. We used these three mutants along with wild-type *A. alternata* to assess the role of Alt a 1 in inducing hallmarks of innate immunity in human airway epithelial cells due to the presence or absence of Alt a 1.

We took measures to establish that the mutants had similar growth and condial morphology compared to the wild-type. We found no indication that deletion or overproduction of Alt a 1 altered the normal phenotype or the conidia or hyphae. Growth rates on normal solid media (PDA) and nutrient-depleted media (GluMM and GalMM) were similar for all mutants compared to the wild-type. We also visually examined the conidia and hyphae of each strain and found no difference in cell wall formation, hyphael fragmentation, or septal branching.

Despite the markedly similar phenotype of the *Δalta1* and *Alta1+* mutants, their ability to stimulate host epithelial cells was dramatically unlike the response elicited from wild-type spores. The Δ*alta1* mutant had impaired ability to induce the pro-inflammatory markers IL-6, IL-8, and MCP-1 in human airway cells. Whereas the *Alta1+* overexpression mutant significantly increased levels of IL-6, IL-8, and MCP-1 compared to the wild-type. The secretion and effect of pro-inflammatory cytokines and chemokines in promoting a significant inflammatory response are well defined (47, 66-67). The presence of all three molecules has been linked to patients diagnosed with fungal allergen sensitivities (68–70). We also confirmed the results of the epithelial cell line used in initial experiments by also treating primary human airway epithelial cells with wild-type and mutant conidia. We show a nearly identical response in the primary cells compared to the transformed cell line for differences between the Alt a 1 deletion and overexpression mutants in their ability to induce production of IL-6, IL-8, and MCP-1. This data confirms the epithelial cell response in the immortalized cell line was similar to what would likely be seen the in-vivo response. Furthermore, by quantitatively measuring the release of lactose dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, we showed that there is no difference in cytotoxicity between the wild-type, *Δalta1, Δalta1-rec*, and Alta1+ strains. This confirms our immunological data is a result of the difference in the conidial genotype and not that one strain is more or less cytotoxic. Since Alternaria is ubiquitous in nature and at presumably high levels in some homes and climates (71–74) it can be suggested that constant inhalation of Alternaria spores could contribute to a persistently activated epithelium. Furthermore, we can conclude by our data that Alt a 1 may be the major factor from the inhaled fungal spores which is influencing the epithelium's response.

Deletion of Alt a 1 also attenuated the induction of key innate signaling molecules in a global transcriptional analysis of multiple pathways. The profiling experiments highlight the participation of key innate immune signaling pathways in response to Alternaria. Our Superarray analysis found over twenty targets involved in the anti-fungal immune response which were not induced by the Alt a 1 deletion mutant including the small inducible cytokine CXCL1, the chemoattractant chemokine CXCL10, the inflammatory precursor CASP1 and its substrate IL-1b, the pro-inflammatory and T-cell differentiating cytokine IL-23a, the protein tyrosine phosphatase PTPN6, the pattern recognition receptor CD14, the protein kinase cluster of MAPK genes, the inflammatory response pentraxin-related protein PTX3, and the receptors TLR3 and TLR4 their adaptor molecule TIRAP. The subsequent qRT-PCR showed that IL-6, IL-8, MCP-1, CXCL10, CXCL11, CASP1, and TLR3 were most closely controlled by the presence of Alt a 1. CXCL10 is another chemokine involved in airway hyperreactiviy and inflammation (75) while CASP1 has been implicated in both the inflammatory response to mite allergen and cigarette smoke in the airway (76, 77). The small IFN-γ, inducible chemokine, CXCL11 defined as a CCR3 antagonist and may play anti-inflammatory role or at least assist in balancing the Th2-skewed response (82). Intriguingly, certain genes, which have been previously shown to be part of the inflammatory airway response, were not detected including RANTES, CCR1, TLR4, MAP14K, CD14, and CXCL1 (83–85). Additionally, STAT1 and MyD88, components of two separate innate immune signaling cascades, were both found to be somewhat affected by the presence or absence of Alt a 1. Although these expression results are only from one cell type, the findings give multiple targets for further exploration as to their involvement with interacting with *A.alternata* spores and the allergen Alt a 1. Future experiments could examine other cell types and their response to conidia and/or allergen protein as opposed to potent fungal extracts which

contain intracellular proteins, carbohydrates, and toxins which are not normally secreted by the fungus after inhalation and germination and thus would differentially induce other target genes.

In conclusion, our results provide substantial evidence that Alt a1 is responsible for a significant portion of the innate inflammatory response to *A.alternata* spores in human airway cells. Most notably we show for the first time in-vitro experimentation utilizing airway epithelial cells and the actual the fungal organism as opposed to prepared extract or a single fungal component. Furthermore, we, to the best of our knowledge, are the first to show an attenuated response to an entire organism by deletion of its major allergen including work done with the other major allergenic fungi, Aspergillus, Penicillum, and Cladosporium. It is likely that the secretion of Alt a 1 by inhaled conidia induces the initial pro-inflammatory cytokines and chemokines as well as disruption of the epithelial barrier. Over time, this stimulation could lead to more distinguishing symptoms of allergy and asthma such as mucous production, effector cell infiltration, and airway remodeling. It is now essential to further characterize the biochemical properties of the Alt a 1 protein and its interaction with the epithelium as an avenue to potential therapeutic targets.

REFERENCES:

1. Grant EN, Wagner R, Weiss KB (1999) Observations on emerging patterns of asthma in our society. *J Allergy Clin Immunol* 104:S1–9.
2. Mudarri D, Fisk WJ (2007) Public health and economic impact of dampness and mold. *Indoor Air* 17:226–235.
3. Andersson M, Downs S, Mitakakis T, Leuppi J, Marks G (2003) Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitized children. *Pediatr Allergy Immunol* 14:100–105.
4. O'Hollaren MT et al. (1991) Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. *N Engl J Med* 324:359–363.
5. Gergen PJ, Weiss KB (1992) The increasing problem of asthma in the United States. *Am Rev Respir Dis* 146:823–824.
6. Gergen PJ, Turkeltaub PC (1992) The association of individual allergen reactivity with respiratory disease in a national sample: data from the second National Health and Nutrition Examination Survey, 1976-80 (NHANES II). *J Allergy Clin Immunol* 90:579–588.
7. Bartra J, Belmonte J, Torres-Rodriguez JM, Cistero-Bahima A (2009) Sensitization to Alternaria in patients with respiratory allergy. *Front Biosci* 14:3372–3379.
8. Halonen M, Stern D, Wright A, Taussig L, Martinez F (1997) Alternaria as a major allergen for asthma in children raised in a desert environment. *Am J Respir Crit Care Med* 155:1356–1361.
9. Boitano S et al. (2011) Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. *Am J Physiol Lung Cell Mol Physiol* 300:L605–614.
10. Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P (2000) Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J Allergy Clin Immunol* 105:1185–1193.
11. Kouzaki H, O'Grady SM, Lawrence CB, Kita H (2009) Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 183:1427–1434.
12. Melkamu T, Squillace D, Kita H, O'Grady SM (2009) Regulation of TLR2 expression and function in human airway epithelial cells. *J Membr Biol* 229:101–113.
13. Gregory LG, Lloyd CM (2011) Orchestrating house dust mite-associated allergy in the lung. *Trends in Immunology* 32:402–411.
14. Kauffman HF (2003) Interaction of environmental allergens with airway epithelium as a key component of asthma. *Curr Allergy Asthma Rep* 3:101–108.
15. Vailes L et al. (2001) Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. *Journal of Allergy and Clinical Immunology* 107:641–646.
16. Aden E et al. (1999) Standardization of Alternaria alternata: extraction and quantification of alt a 1 by using an mAb-based 2-site binding assay. *J Allergy Clin Immunol* 104:128–135.
17. Sanchez H, Bush RK (2001) A review of Alternaria alternata sensitivity. *Rev Iberoam Micol* 18:56–59.
18. Breitenbach M, Simon-Nobbe B (2002) The allergens of Cladosporium herbarum and Alternaria alternata. *Chem Immunol* 81:48–72.
19. Achatz G et al. (1995) Molecular cloning of major and minor allergens of Alternaria alternata and Cladosporium herbarum. *Mol Immunol* 32:213–227.
20. Simon-Nobbe B et al. (2000) IgE-binding epitopes of enolases, a class of highly conserved fungal allergens. *J Allergy Clin Immunol* 106:887–895.

21. Weichel M et al. (2003) Nuclear transport factor 2 represents a novel cross-reactive fungal allergen. *Allergy* 58:198–206.
22. De Vouge MW et al. (1996) Isolation and expression of a cDNA clone encoding an Alternaria alternata Alt a 1 subunit. *Int Arch Allergy Immunol* 111:385–395.
23. Kleine-Tebbe J et al. (1993) Predominance of the major allergen (Alt a I) in Alternaria sensitized patients. *Clin Exp Allergy* 23:211–218.
24. Barnes CS et al. (1996) Production of a recombinant protein from Alternaria containing the reported N-terminal of the Alt a1 allergen. *Adv Exp Med Biol* 409:197–203.
25. Twaroch TE et al. (2011) Predominant localization of the major Alternaria allergen Alt a 1 in the cell wall of airborne spores. *Journal of Allergy and Clinical Immunology*. Available at: http://www.jacionline.org/article/S0091-6749(11)01576-4/fulltext [Accessed November 15, 2011].
26. Sercombe JK, Eduard W, Romeo TC, Green BJ, Tovey ER (2006) Detection of allergens from Alternaria alternata by gold-conjugated anti-human IgE and field emission scanning electron microscopy. *J Immunol Methods* 316:167–170.
27. Ibarrola I et al. (2004) Production profile of the major allergen Alt a 1 in Alternaria alternata cultures. *Ann Allergy Asthma Immunol* 93:589–593.
28. Mitakakis T (2001) Spore germination increases allergen release from Alternaria. *Journal of Allergy and Clinical Immunology* 107:388–390.
29. Sáenz-de-Santamaría M, Guisantes JA, Martínez J (2006) Enzymatic activities of Alternaria alternata allergenic extracts and its major allergen (Alt a 1). *Mycoses* 49:288–292.
30. Kato A, Schleimer RP (2007) Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr Opin Immunol* 19:711–720.
31. Gershwin LJ (2007) Effects of allergenic extracts on airway epithelium. *Curr Allergy Asthma Rep* 7:357–362.
32. Neveu WA et al. (2009) IL-6 is required for airway mucus production induced by inhaled fungal allergens. *J Immunol* 183:1732–1738.
33. Kauffman HF (2006) Innate immune responses to environmental allergens. *Clin Rev Allergy Immunol* 30:129–140.
34. Shin S-H, Lee Y-H (2010) Airborne fungi induce nasal polyp epithelial cell activation and Toll-like receptor expression. *Int Arch Allergy Immunol* 153:46–52.
35. Schuh JM, Blease K, Kunkel SL, Hogaboam CM (2003) Chemokines and cytokines: axis and allies in asthma and allergy. *Cytokine Growth Factor Rev* 14:503–510.
36. Hassim Z, Maronese SE, Kumar RK (1998) Injury to murine airway epithelial cells by pollen enzymes. *Thorax* 53:368 –371.
37. Chetta A et al. (1997) Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 111:852–857.
38. Fahy JV (2001) Remodeling of the airway epithelium in asthma. *Am J Respir Crit Care Med* 164:S46–51.
39. Venkatesan N, Siddiqui S, Jo T, Martin JG, Ludwig MS (2011) Allergen-induced airway remodeling in Brown Norway rats: structural and metabolic changes in glycosaminoglycans. *American Journal of Respiratory Cell and Molecular Biology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21852687 [Accessed November 9, 2011].
40. Vignola AM, Chanez P, Bonsignore G, Godard P, Bousquet J (2000) Structural consequences of airway inflammation in asthma. *J Allergy Clin Immunol* 105:S514–517.

41. Vignola AM et al. (2001) Airway remodeling in the pathogenesis of asthma. *Curr Allergy Asthma Rep* 1:108–115.
42. Ke Y et al. (1988) Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. *Differentiation* 38:60–66.
43. Cho Y et al. (2009) Identification of novel virulence factors associated with signal transduction pathways in Alternaria brassicicola. *Mol Microbiol* 72:1316–1333.
44. Cho Y et al. (2006) A high throughput targeted gene disruption method for Alternaria brassicicola functional genomics using linear minimal element (LME) constructs. *Mol Plant Microbe Interact* 19:7–15.
45. Sweigard J, Chumley F, Carroll A, Farrall L, Valent B (1997) Vectors for fungal transformation. *Fungal Genetics Newsletter* 44:52–53.
46. Cho Y et al. (2007) The Fus3/Kss1 MAP kinase homolog Amk1 regulates the expression of genes encoding hydrolytic enzymes in Alternaria brassicicola. *Fungal Genet Biol* 44:543–553.
47. Kim K-H, Cho Y, LA Rota M, Cramer RA Jr, Lawrence CB (2007) Functional analysis of the Alternaria brassicicola non-ribosomal peptide synthetase gene AbNPS2 reveals a role in conidial cell wall construction. *Mol Plant Pathol* 8:23–39.
48. Shimizu K, Keller NP (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. *Genetics* 157:591–600.
49. LAEMMLI UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 227:680–685.
50. L M Ciuffetti RPT A single gene encodes a selective toxin causal to the development of tan spot of wheat. *The Plant Cell* 9:135.
51. Van Dyken SJ et al. (2011) Fungal chitin from asthma-associated home environments induces eosinophilic lung infiltration. *J Immunol* 187:2261–2267.
52. Yoon J, Ponikau JU, Lawrence CB, Kita H (2008) Innate antifungal immunity of human eosinophils mediated by a beta 2 integrin, CD11b. *J Immunol* 181:2907–2915.
53. Shin S-H, Lee Y-H, Jeon C-H (2006) Protease-dependent activation of nasal polyp epithelial cells by airborne fungi leads to migration of eosinophils and neutrophils. *Acta Otolaryngol* 126:1286–1294.
54. Holgate ST (2002) Airway inflammation and remodeling in asthma: current concepts. *Mol Biotechnol* 22:179–189.
55. Proud D, Leigh R (2011) Epithelial cells and airway diseases. *Immunol Rev* 242:186–204.
56. Ryu J-H, Kim C-H, Yoon J-H (2010) Innate immune responses of the airway epithelium. *Mol Cells* 30:173–183.
57. Fernández C et al. (2011) Asthma related to Alternaria sensitization: an analysis of skin-test and serum-specific IgE efficiency based on the bronchial provocation test. *Clin Exp Allergy* 41:649–656.
58. Bousquet P-J et al. (2010) Assessment of allergen sensitization in a general population-based survey (European Community Respiratory Health Survey I). *Ann Epidemiol* 20:797–803.
59. Katotomichelakis M et al. (2011) Clinical significance of Alternaria alternata sensitization in patients with allergic rhinitis. *American Journal of Otolaryngology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21907454 [Accessed November 14, 2011].
60. Mokhtari Amirmajdi M et al. (2011) Alternaria in patients with allergic rhinitis. *Iran J Allergy Asthma Immunol* 10:221–226.

61. De Vouge MW et al. (1998) Molecular cloning of IgE-binding fragments of Alternaria alternata allergens. *Int Arch Allergy Immunol* 116:261–268.
62. Zhang L et al. (1995) N-terminus of a major allergen, Alt a I, of Alternaria alternata defined to be an epitope. *Int Arch Allergy Immunol* 108:254–259.
63. Inoue Y, Matsuwaki Y, Shin S-H, Ponikau JU, Kita H (2005) Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J Immunol* 175:5439–5447.
64. Okano M et al. (2011) Role of fungal antigens in eosinophilia-associated cellular responses in nasal polyps: a comparison with enterotoxin. *Clin Exp Allergy* 41:171–178.
65. Kobayashi T et al. (2009) Asthma-related environmental fungus, Alternaria, activates dendritic cells and produces potent Th2 adjuvant activity. *J Immunol* 182:2502–2510.
66. van den Brûle S et al. (2007) Profibrotic effect of IL-9 overexpression in a model of airway remodeling. *Am J Respir Cell Mol Biol* 37:202–209.
67. Mills PR, Davies RJ, Devalia JL (1999) Airway epithelial cells, cytokines, and pollutants. *Am J Respir Crit Care Med* 160:S38–43.
68. Barnes PJ (2008) The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 118:3546–3556.
69. Shin S-H, Ye M-K (2011) The effect of nano-silver on the activation of nasal polyp epithelial cells by Alternaria, Der P1 and staphylococcal enterotoxin B. *Int Immunopharmacol* 11:1691–1696.
70. Chung KF (2001) Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 34:50s–59s.
71. Nickel R, Beck LA, Stellato C, Schleimer RP (1999) Chemokines and allergic disease. *J Allergy Clin Immunol* 104:723–742.
72. Cao J et al. (2011) Bronchial epithelial cells release IL-6, CXCL1 and CXCL8 upon mast cell interaction. *Cytokine*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22030312 [Accessed November 14, 2011].
73. Perić A et al. (2010) Proinflammatory cytokine levels in nasal fluid as indicators of severity of nasal polyposis. *Acta Clin Croat* 49:395–403.
74. Walker C et al. (1994) Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am J Respir Crit Care Med* 150:1038–1048.
75. Abe K (2011) Assessment of Home Environments with a Fungal Index Using Hydrophilic and Xerophilic Fungi as Biological Sensors. *Indoor Air*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21995759 [Accessed November 14, 2011].
76. Oliveira M, Delgado L, Ribeiro H, Abreu I (2010) Fungal spores from Pleosporales in the atmosphere of urban and rural locations in Portugal. *J Environ Monit* 12:1187–1194.
77. Salo PM et al. (2005) Dustborne Alternaria alternata antigens in US homes: results from the National Survey of Lead and Allergens in Housing. *J Allergy Clin Immunol* 116:623–629.
78. Stern DA et al. (2004) Dynamic changes in sensitization to specific aeroallergens in children raised in a desert environment. *Clin Exp Allergy* 34:1563–1669.
79. Medoff BD et al. (2002) IFN-γ-Inducible Protein 10 (CXCL10) Contributes to Airway Hyperreactivity and Airway Inflammation in a Mouse Model of Asthma. *The Journal of Immunology* 168:5278 –5286.
80. Dai X et al. (2011) Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes. *J Allergy Clin Immunol* 127:806–814.e1–4.

81. Eltom S et al. (2011) P2X7 receptor and caspase 1 activation are central to airway inflammation observed after exposure to tobacco smoke. *PLoS ONE* 6:e24097.
82. Su Y-C, Rolph MS, Hansbro NG, Mackay CR, Sewell WA (2008) Granulocyte-Macrophage Colony-Stimulating Factor Is Required for Bronchial Eosinophilia in a Murine Model of Allergic Airway Inflammation. *J Immunol* 180:2600–2607.
83. Schuh JM, Blease K, Hogaboam CM (2002) The role of CC chemokine receptor 5 (CCR5) and RANTES/CCL5 during chronic fungal asthma in mice. *FASEB J* 16:228–230.
84. Carpenter KJ et al. (2005) Therapeutic targeting of CCR1 attenuates established chronic fungal asthma in mice. *Br J Pharmacol* 145:1160–1172.
85. Shahan TA, Sorenson WG, Paulauskis JD, Morey R, Lewis DM (1998) Concentration- and time-dependent upregulation and release of the cytokines MIP-2, KC, TNF, and MIP-1alpha in rat alveolar macrophages by fungal spores implicated in airway inflammation. *Am J Respir Cell Mol Biol* 18:435–440.

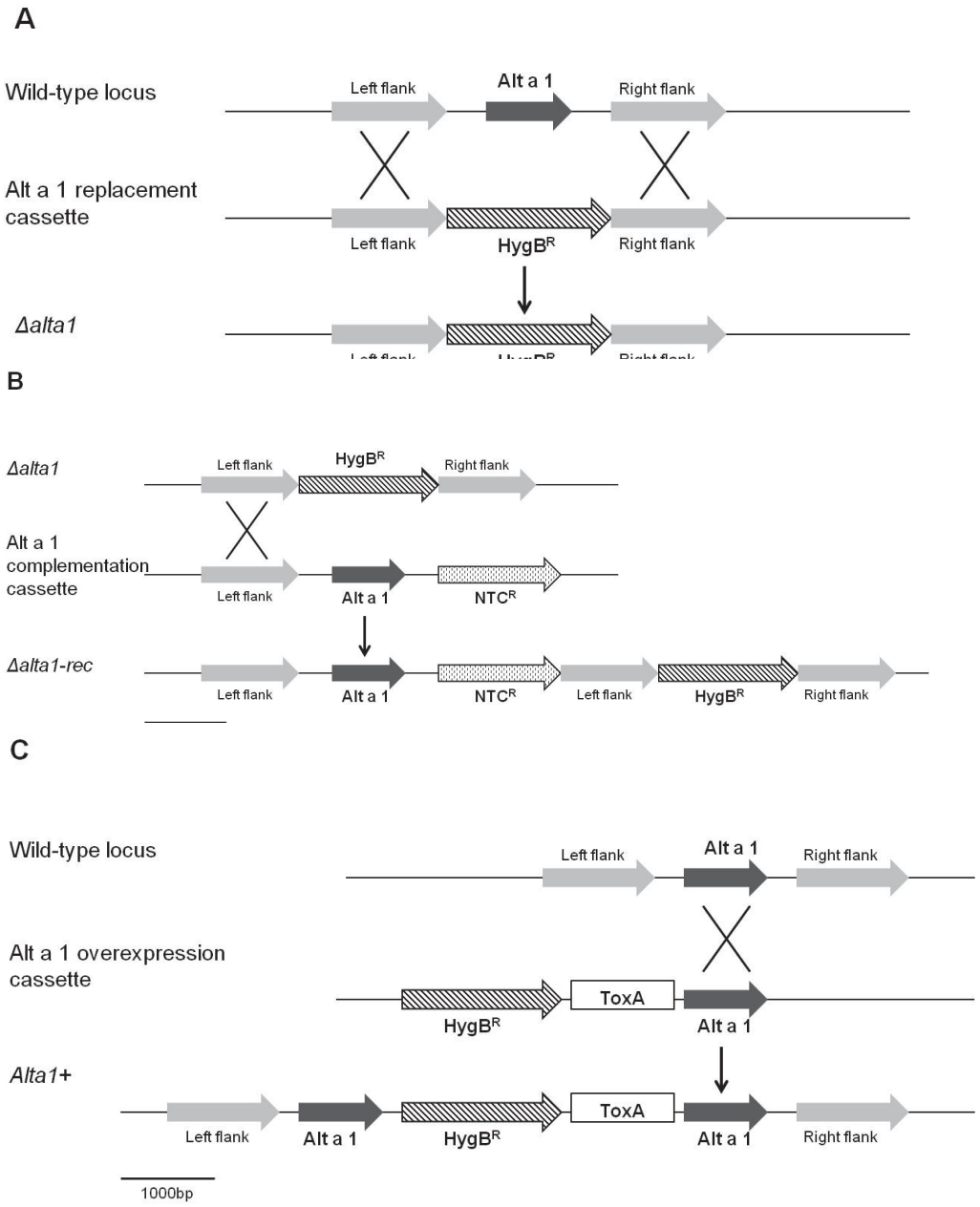


Figure 4.1: Deletion, complementation, and overexpression of Alt a 1 in *A.alternata*. Schematic representation of the *Alta1* wild-type gene locus and replacement insert used for replacement (A), complementation (B) and overexpression (C) of Alta1 in *A.alternata*. The light gray arrows represent the 1kb flanking regions of the *Alt a 1* gene. HygB, hygromycin B resistance gene; NTC, nourseothricin resistance gene; ToxA, constitutive promoter gene from *Pyrenophora tritici-repentis*. Homologous recombination events are shown with a large "X" between target genomic locus.

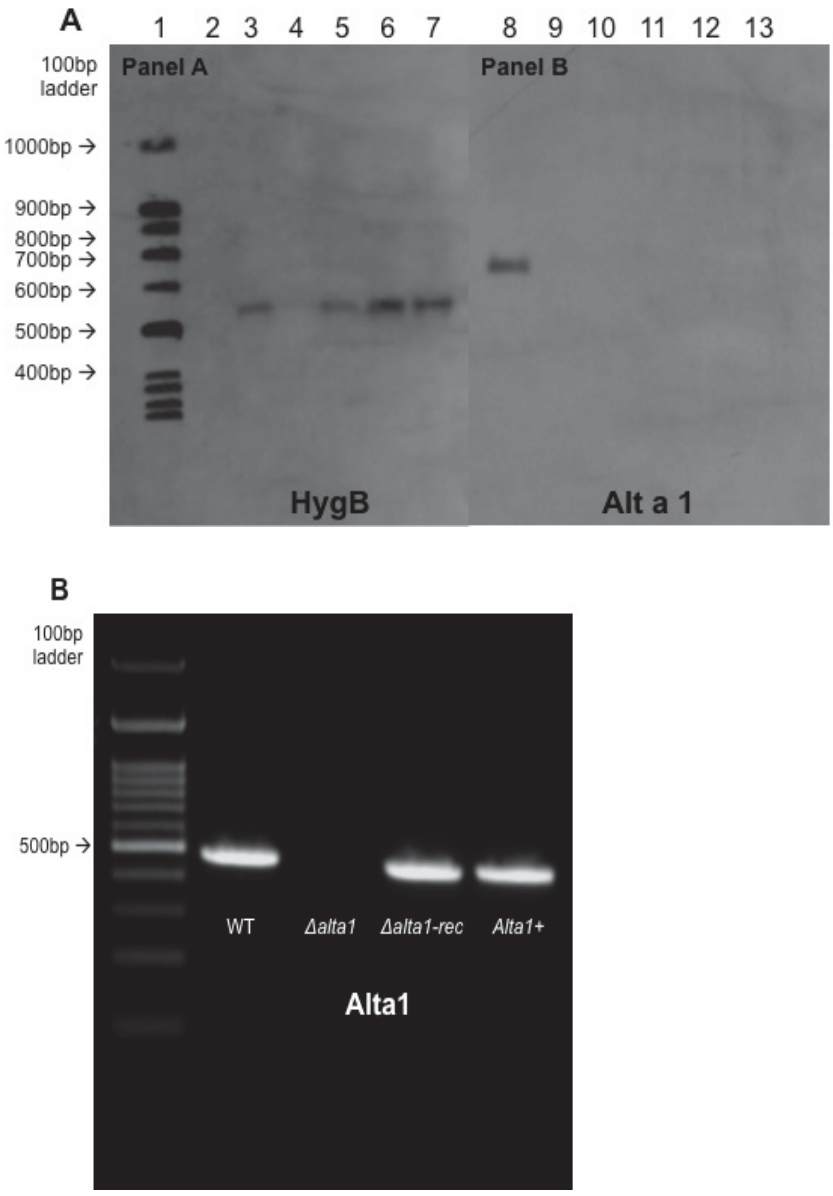


Figure 4.2: Southern blot and PCR verification of *Δalta1* mutant. (A) For the Southern blot analysis, genomic DNA (3 µg per well) was digested from the wild-type and five individual transformants with BamH1 and EcoRV, blotted, and hybridized with a DNA probe for HygB (Panel A) and Alta1 (Panel B). Lane 1: 100bp ladder, Lane 2: *A.alternata* wild-type, Lane 3 – 7: *Δalta1* transformants, Lane 8: *A. alternata* wild-type, Lane 9 – 13: *Δalta1* transformants. (B) Presence of Alt a 1 following transformation was confirmed in the *Δalta1-rec* and Alta1+ strains by PCR on genomic DNA using Alt a 1 specific primers as described in Supplementary Table 2.

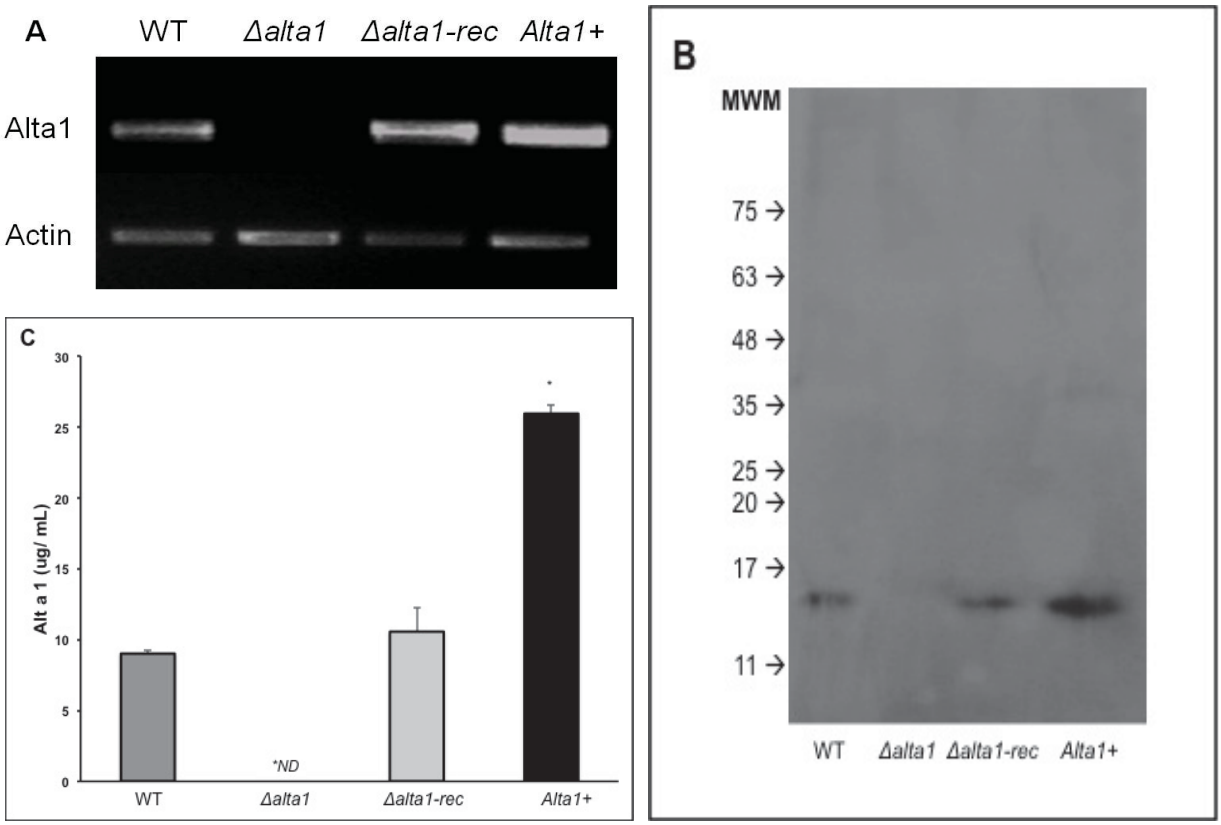
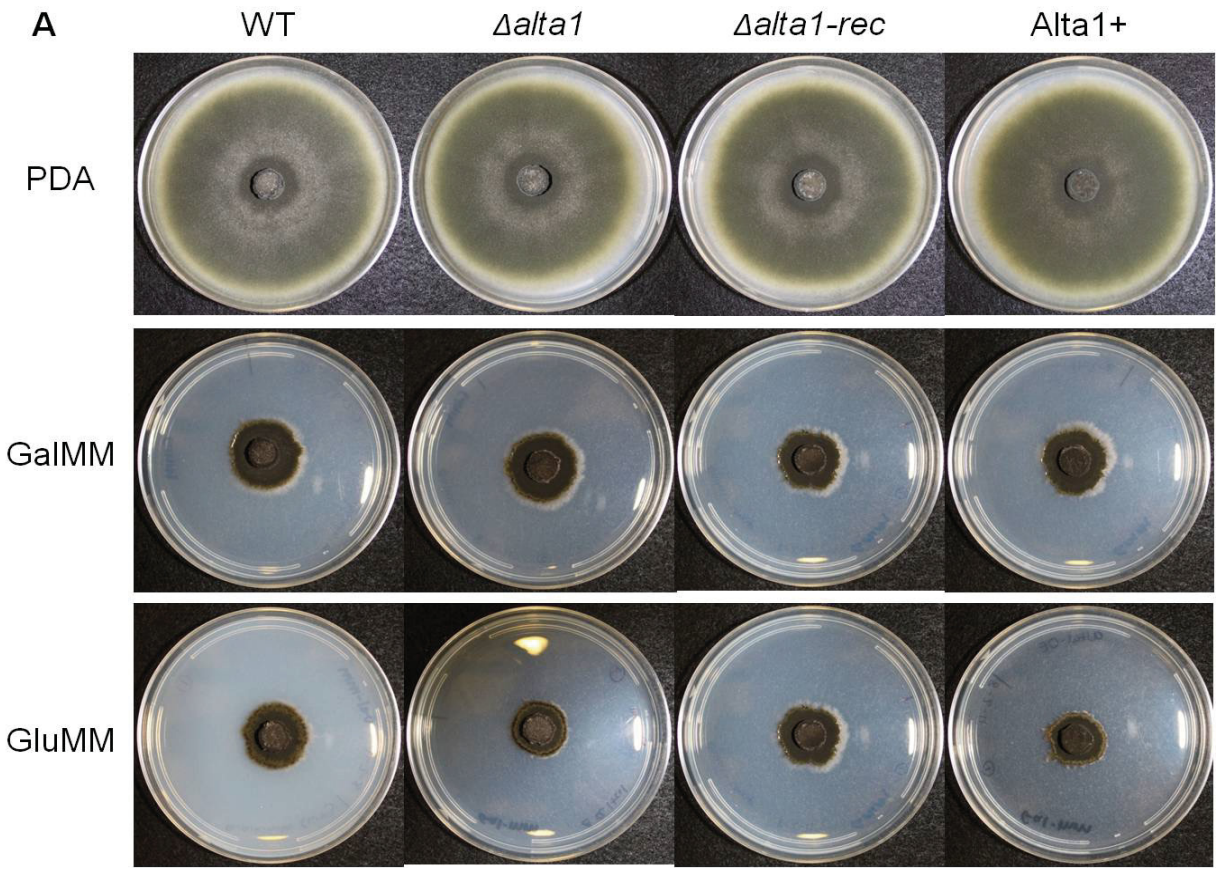
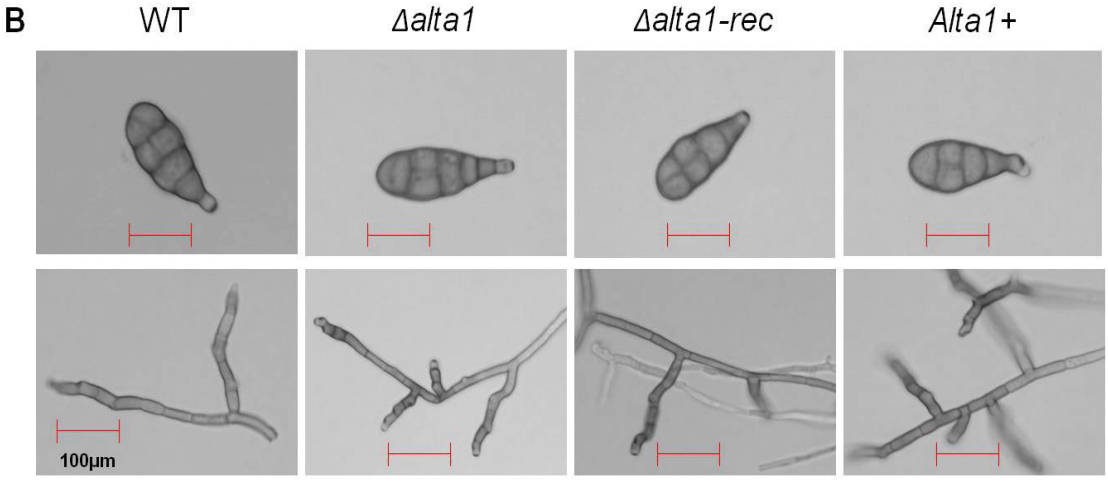
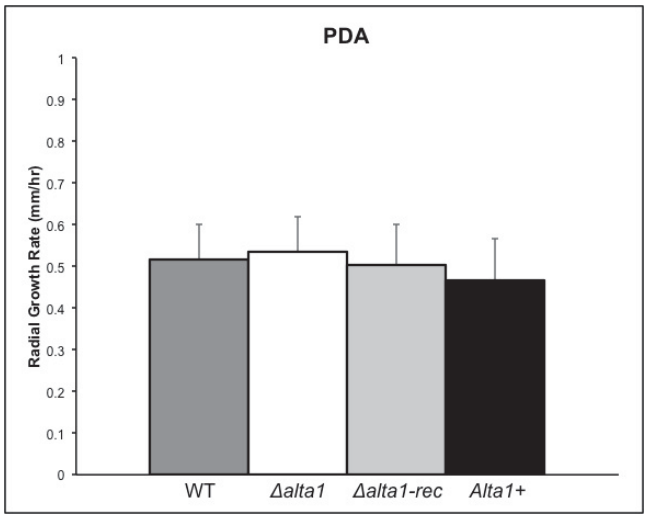
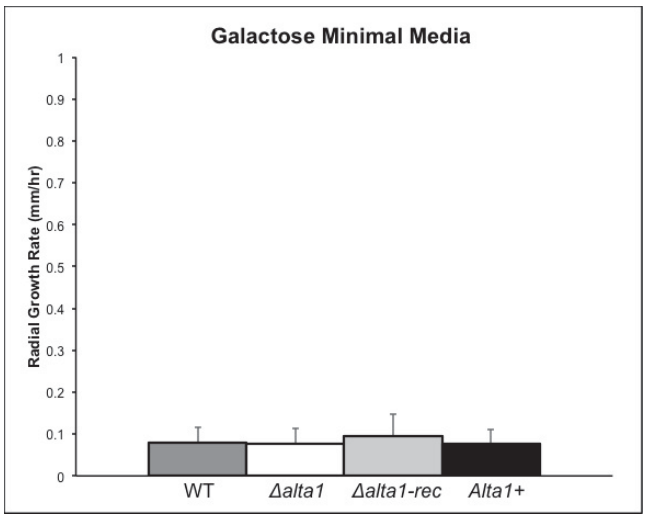


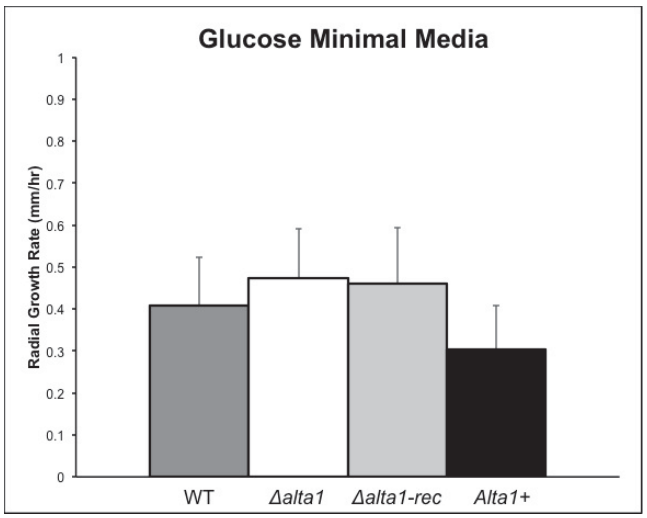
Figure 4. 3: Detection of Alt a 1 production levels in wild-type and Alt a 1 mutant strains. The wild-type and mutant strains were grown for 7-days in GYEB. Mycelium was collected, dried, and ground in liquid nitrogen. The culture filtrate (25mL) was filtered, concentrated in a 3kDA ultrafiltration unit, and total protein concentration was assessed by the BCA Assay. (A) The mRNA expression levels of Alt a 1 in the mycelium were measured by quantitative real-time (qRT-PCR) after synthesis of cDNA from RNA of each strain. Lane 1: 100kB ladder, Lane 2: wild-type *A.alternata,* Lane 3: *Δalta1*, Lane 4: *Δalta1-rec*, Lane 5: *Alta1+*. **(B)** Secretion of Alt a 1 into the GYEB culture supernatant was visualized by Western blot using Alt a 1 specific antibody. **Lane 1:** wild-type *A.alternata,* **Lane 2:***Δalta1*, **Lane 3:** *Δalta1-rec*, **Lane 4:** Alta1+. (C) Quantitative measurement of Alt a 1 in the culture filtrates was assessed by Alt a 1 specific ELISA (ug/mL). Filtrates were prepared as described in (B). ND: not detected. Asterisks (*) indicates statistically significant from wild-type. $p<0.05$.


A
WT
Δalta1
Δalta1-rec
Alta1+
PDA
GalMM
GluMM


B
WT
Δalta1
Δalta1-rec
Alta1+
100μm


PDA
Radial Growth Rate (mm/hr)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
Δalta1
Δalta1-rec
Alta1+


Galactose Minimal Media
Radial Growth Rate (mm/hr)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
Δalta1
Δalta1-rec
Alta1+


Glucose Minimal Media
Radial Growth Rate (mm/hr)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
Δalta1
Δalta1-rec
Alta1+

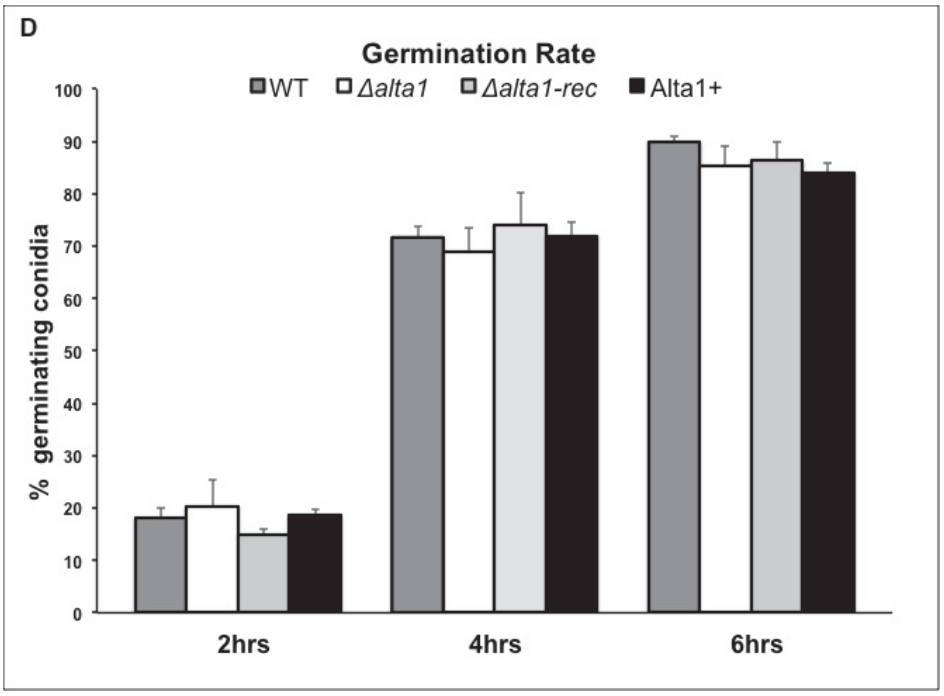


Figure 4.4: Analysis of growth and conidial morphology of Alternaria mutants. (A) Pigmentation and radial growth of mutant and wild-type strains were similar on various carbon sources. A 1cm^3 plug of each strain was placed on the indicated media and incubated at 25°C for 7 days. PDA: potato dextrose agar, GalMM: galactose minimal media, GluMM: glucose minimal media. (B) Light micrographs of 7-day-old conidia and hyphae of *A. alternata* wild-type and *Δalta1*, *Δalta1-rec*, and *Alta1+* mutants taken at 40x magnification. Bars = 100 µm. No difference in conidiation or hyphal fragmentation was observed. (C) Average growth rates of the *Δalta1*, *Δalta1-rec*, and Alta1+ strains were compared to the wild-type (WT) *A.alternata*. Strains were incubated on various carbon sources including dextrose (PDA), glucose (GluMM), and galactose (GalMM) at 25°C and radial growth was measured once a day for 7 days. No significant difference in growth rate between the mutants and the wild-type were detected. The experiment was conducted in triplicate and repeated twice. (D) Germination of fresh conidia from the wild-type, *Δalta1*, *Δalta1-rec*, and *Alta1+* strains was assessed on PDA. For each time point, 100 conidia were counted. Data presented is the mean +/- standard deviation of two separate experiments conducted in triplicate.

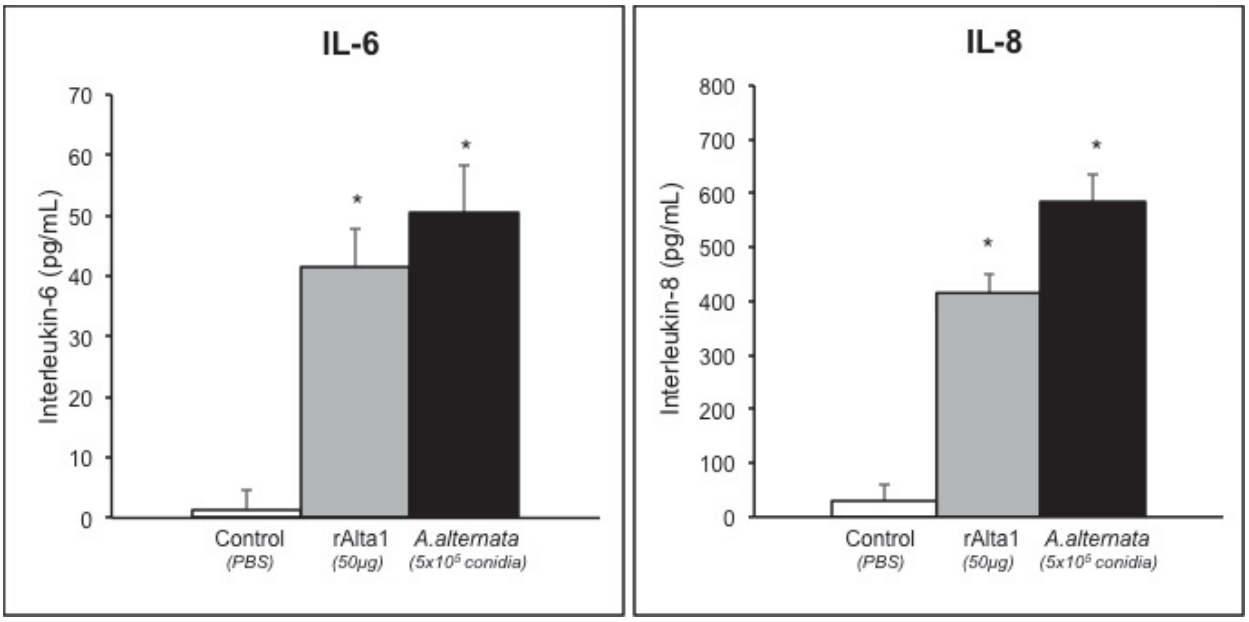


Figure 4.5: The Alt a 1 allergen protein can induce pro-inflammatory cytokines. Release of IL-6 and IL-8 into culture supernatant was measured by ELISA after a 24hr incubation of BEAS-2b cells treated with 5 x 10^5 wild-type *A.alternata* spore or 50µg of endotoxin-free rAlta1, or left untreated (control). Results are from three independent experiments done in triplicate, $p < 0.05$.

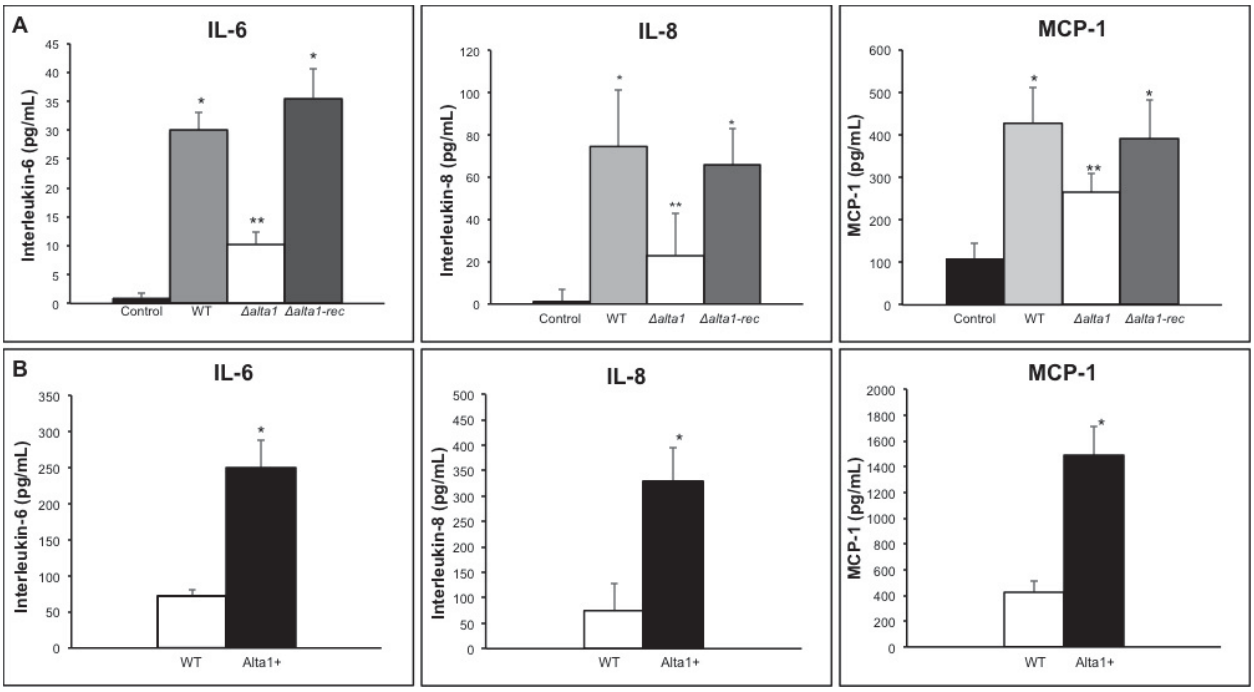
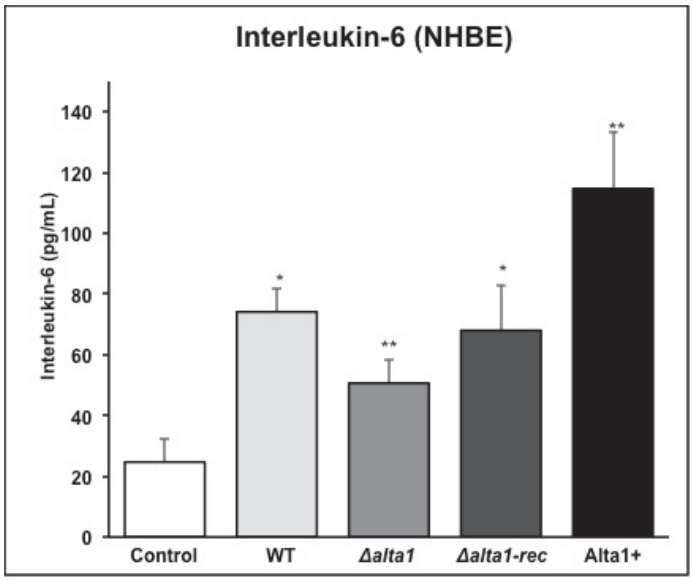
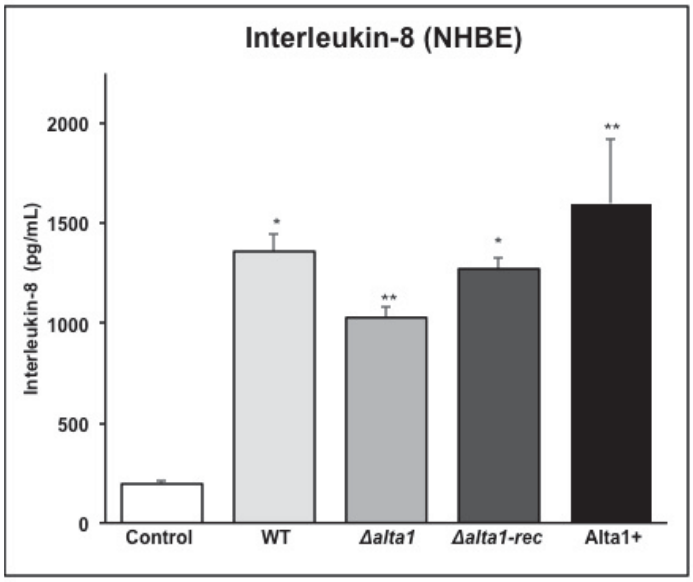
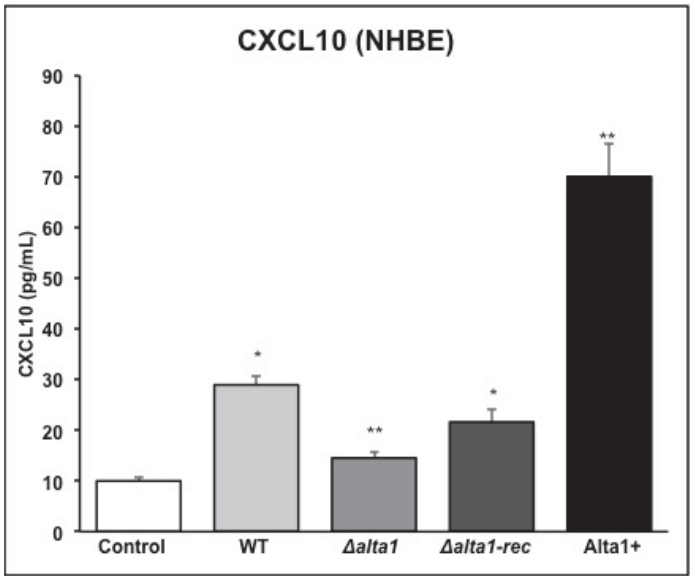


Figure 4.6: Alt a 1 is required for release of IL-6, IL-6, and MCP from human airway epithelial cells stimulated with *A.alternata* conidia. The levels of IL-6, IL-8, and MCP-1 were quantified by ELISA from the culture supernatants of BEAS-2b epithelial cells treated with the wild-type and mutant fungal strains. For each cytokine, (A) comparison of cytokine levels between control, wild-type, *Δalta1*, and *Δalta1-rec*. (B) comparison of conidia overexpressing Alt a 1 (Alta1+) to wild-type conidia. All results indicate the mean ± standard error. All experiments were performed in triplicate on three separate occasions. For all panels, single asterisk indicates significantly reduced or enhanced epithelial cell response compared to the control; a double asterisks signifies difference from *A.alternata* wild-type and *Δalta1-rec* strains, $p < 0.05$.


Interleukin-6 (NHBE)
Interleukin-6 (pg/mL)
140
120
100
80
60
40
20
0
Control
WT
Δalta1
Δalta1-rec
Alta1+


Interleukin-8 (NHBE)
Interleukin-8 (pg/mL)
2000
1500
1000
500
0
Control
WT
Δalta1
Δalta1-rec
Alta1+


CXCL10 (NHBE)
CXCL10 (pg/mL)
90
80
70
60
50
40
30
20
10
0
Control
WT
Δalta1
Δalta1-rec
Alta1+

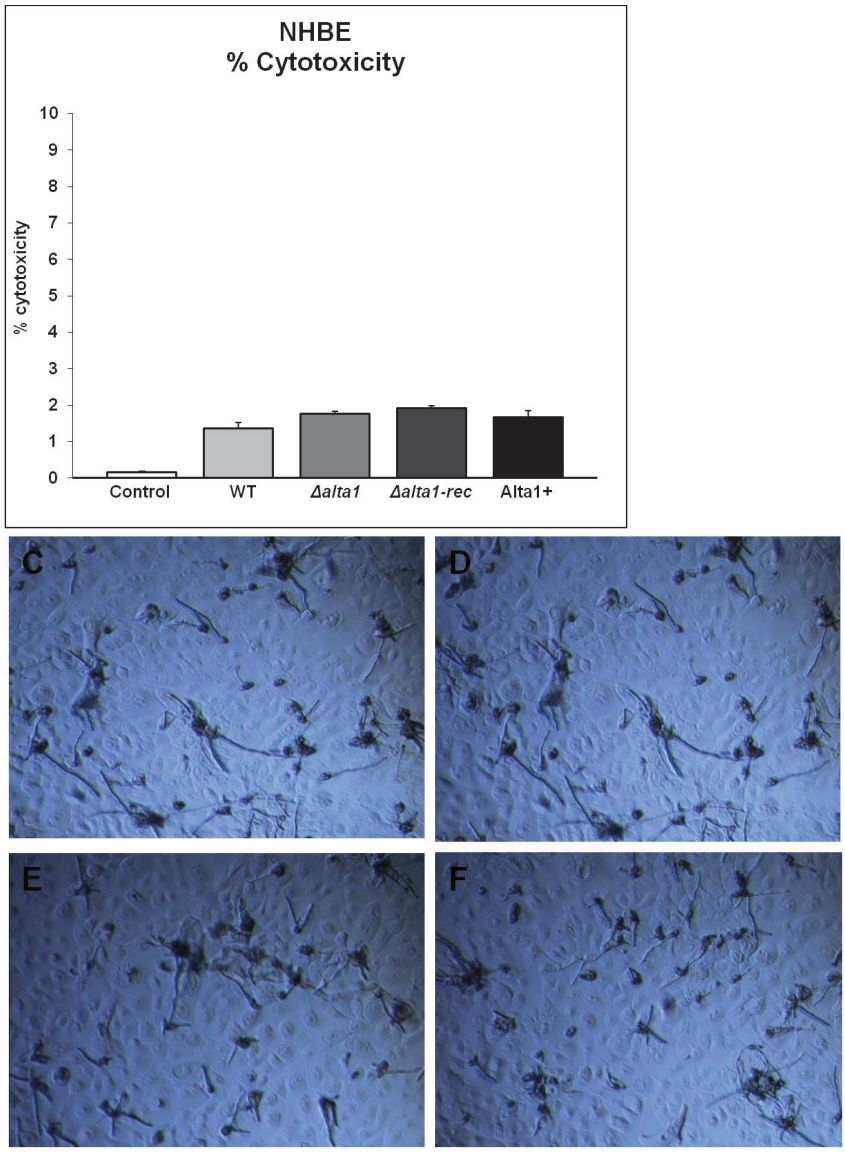
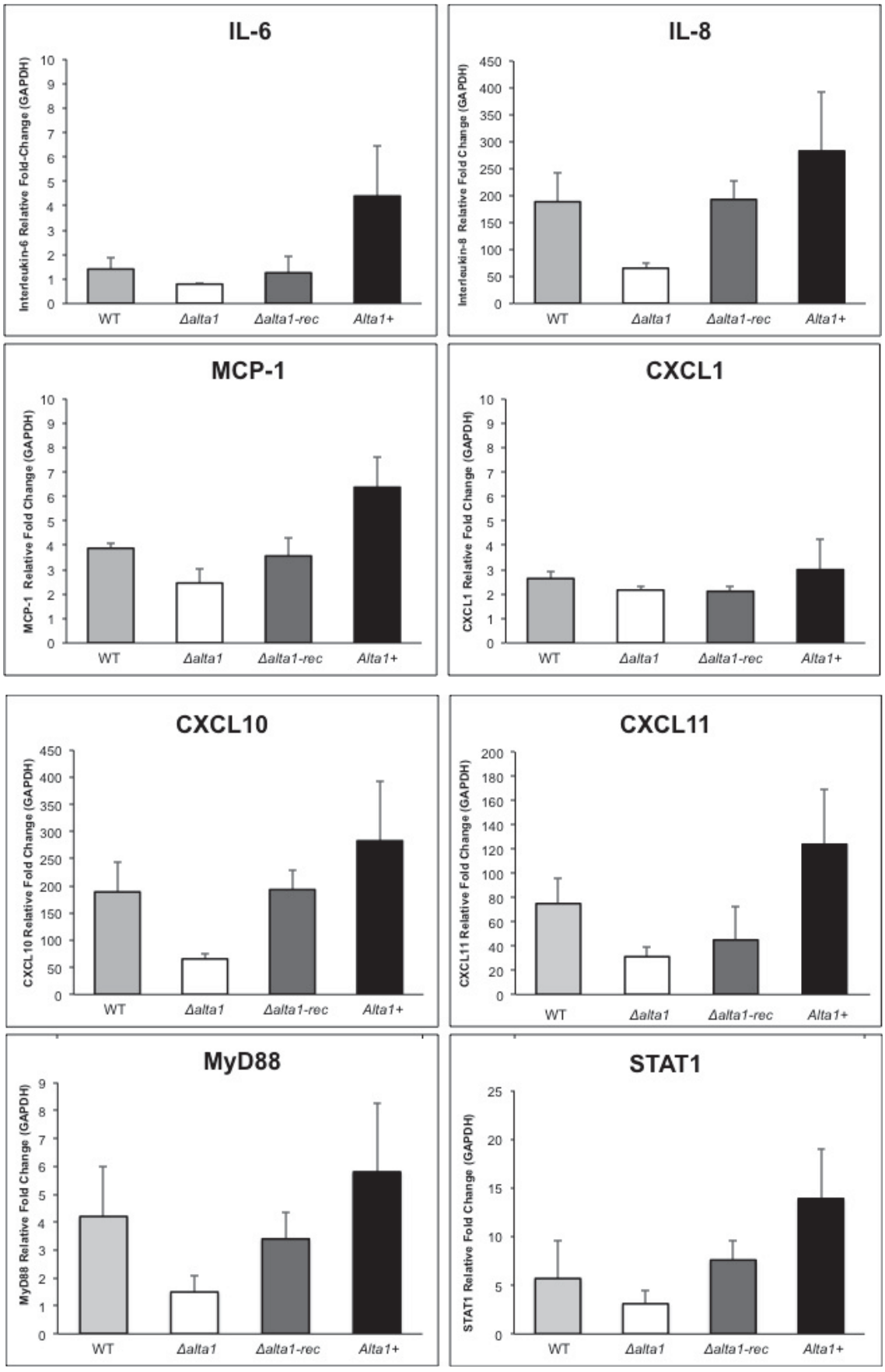


Figure 4.7: Primary human bronchial epithelial cells respond to deletion or overexpression of Alt a 1. Normal (non-diseased) human primary epithelial cells (NHBEs) were treated with wild-type and *Δalta1*, *Δalta1-rec*, and Alta1+ conidia for 24hrs. (A) The levels of IL-6, IL-8 and MCP-1 were measured from the cell culture supernatant following treatment. (*) = statistically significant compared to untreated control; (**) = statistically significant against wild-type and *Δalta1-rec* at 95% confidence. (B) The cell culture supernatant was assayed for LDH to determine cytotoxicity. Positive control cells were lysed with 1% Triton X-100 prior to sampling and negative control cells were treated with PBS. $p<0.05$. *(*C-F) Germinating conidia from *A.alternata* wild-type, *Δalta1*, *Δalta1-rec*, and Alta1+ mutants respectively in presence of human airway epithelial cells. Normal human bronchial epithelial cells were incubated with live conidia for 24 hours under normal conditions. Cultures were imaged using a inverted phase-contrast microscope. Magnification: 40X.


IL-6
Interleukin-6 Relative Fold-Change (GAPDH)
0 1 2 3 4 5 6 7 8 9 10
WT Δalta1 Δalta1-rec Alta1+
IL-8
Interleukin-8 Relative Fold Change (GAPDH)
0 50 100 150 200 250 300 350 400 450
WT Δalta1 Δalta1-rec Alta1+
MCP-1
MCP-1 Relative Fold Change (GAPDH)
0 1 2 3 4 5 6 7 8 9 10
WT Δalta1 Δalta1-rec Alta1+
CXCL1
CXCL1 Relative Fold Change (GAPDH)
0 1 2 3 4 5 6 7 8 9 10
WT Δalta1 Δalta1-rec Alta1+
CXCL10
CXCL10 Relative Fold Change (GAPDH)
0 50 100 150 200 250 300 350 400 450
WT Δalta1 Δalta1-rec Alta1+
CXCL11
CXCL11 Relative Fold Change (GAPDH)
0 20 40 60 80 100 120 140 160 180 200
WT Δalta1 Δalta1-rec Alta1+
MyD88
MyD88 Relative Fold Change (GAPDH)
0 1 2 3 4 5 6 7 8 9
WT Δalta1 Δalta1-rec Alta1+
STAT1
STAT1 Relative Fold Change (GAPDH)
0 5 10 15 20 25
WT Δalta1 Δalta1-rec Alta1+

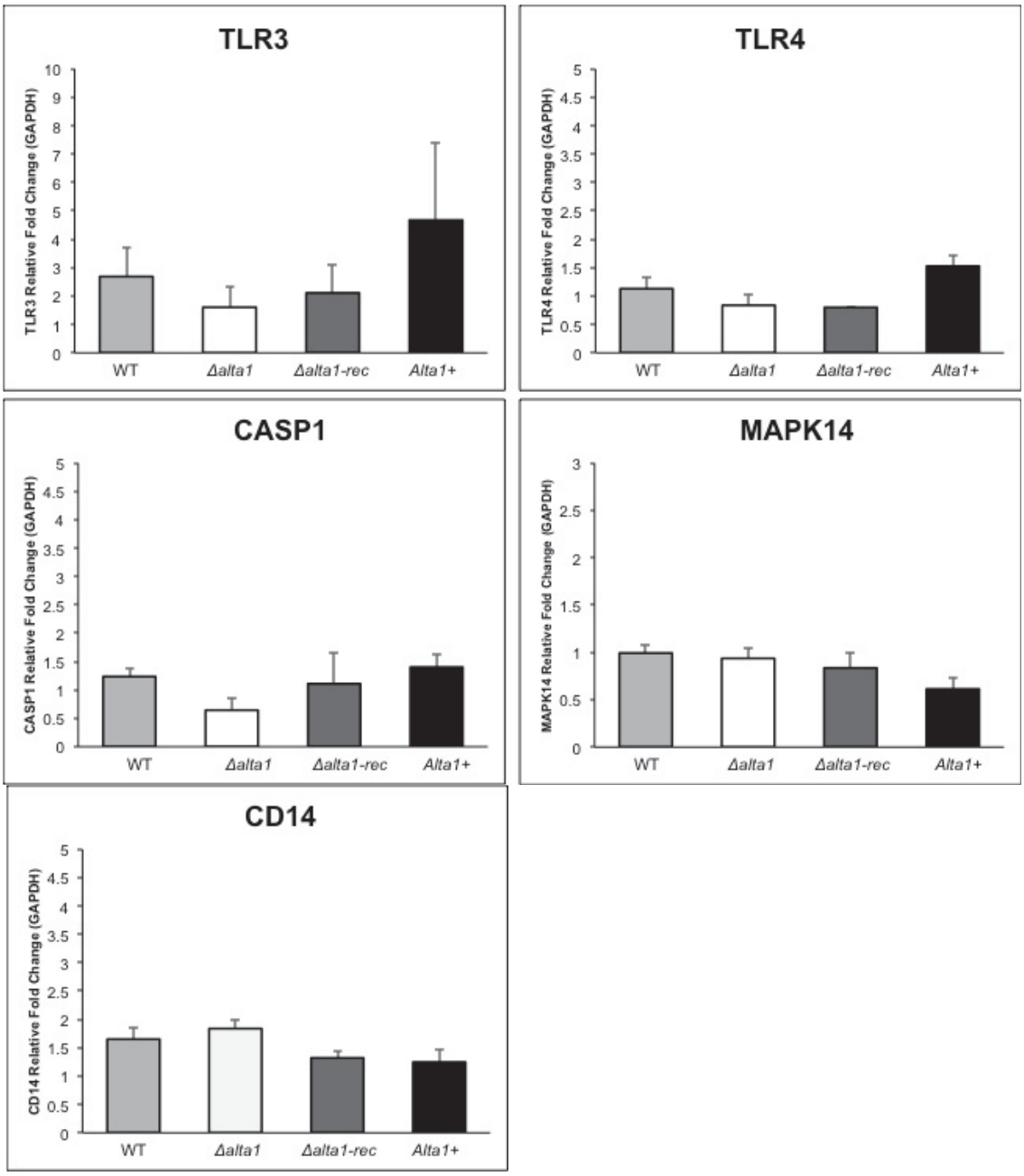


Figure 4.8: Expression of innate antifungal immunity genes in response to *A.alternata* conidia is controlled by present of Alt a 1. Transcript levels of antifungal genes from BEAS-2b cells treated with conidia from *A. alternata* wild-type*, Δalta1, Δalta1-rec,* and *alta1+* mutants. Fold differences in the expression of each gene of the treated samples were normalized against the untreated control (set to transcript level 1). Data are mean ± SD of two independent experiments completed in triplicate.

Table 4.1: Primers used for construction of *Δalta1*, *Δalta1-rec*, and Alta1+ mutant strains
Primer name Sequence (5' → 3')

Δalta1	
5'Kpn_before_alta1	AGGGTACCGCAATGCACTCACGGCTCAAAGTTCCATCATCT
3' Xho1_before_alta1	AGCTCGAGGAGGTTGAAGAATAGAGTTTTGGTAGTTGAGTAGTTG
5'HindIII_after_alta1	AGAAGCTTGCGATAGTTGGTCGAGCATATAAGAAGCATCAACTTC
3' BamH1_after_alta1	AGGGATCCGTACTCACCCTAGCCTTCCTGTAACATTGGAAAG
Δalta1-rec	
5'Apa1_alta1(500)_pNat	GGGCCCATCATAATGGACGTCACATCTCGTATA
3'Sal1_alta1(500)_pNat	GTCGACGTGATGGTGATGGTGATGAGAGCT
Alta1+	
5' ToxA_HindIII	AGAAGCTTGATCGAGACTACTATAGGGCG
3'ToxA_ EcoRV	AGGATATCGGCCTATAT TCATTCATTGT
5'EcoR1_Alta1	GAATTCATGCAGTTCACCACCATCGCCTCTCTCTTC
3'Pst_Alta1	AACTGGAGTTAAGAGCTCTTGGGGAGAGTGACGAGGGTGAT

Table 4.1: Oligonucleotides used for generation of replacement, complementation and overexpression of Alt a 1 in *A.alternata*. Primers were generated from genomic DNA sequence of *Alternaria alternata* [http://alternaria.vbi.vt.edu/lannot/index.shtml].

Primer name:	Sequence (5’ → 3’)
5’Alt_a_1	ATGCAGTTCACCACCATCGCCTCTCTCTTC
3’ Alt_a_1	TTAAGAGCTCTTGGGGAGAGTGACGAGGGT
5’ HygB_forward	CGTTAACTGATATTGAAGGAGC
3’ HygB_reverse	AAAGGAATAGAGTAGATGCCGA
5’ Actin_forward	ATGACACAGATTGTTTTCGAGACCTT
3’ Actin_reverse	GCTCTGCAGGATTGTAGCATGTAACA

Table 4.2: PCR Primers used for construction of Southern hybridization probes and PCR

Table 4.3: Primers used for qRT-PCR of selected antifungal makers.

Gene:	Forward Primer:	Reverse Primer:
MCP-1	TGTCCCAAAGAAGCTGTGATC	ATTCTTGGGTTGTGGAGTGAG
CD14	CAGAGGTTCGGAAGACTTATCG	TTCGGAGAAGTTGCAGACG
CASP1	TTTCTGCTCTTCCACACCAG	AATGAAAATCGAACCTTGCGG
CXCL1	AACCGAAGTCATAGCCACAC	CCTCCCTTCTGGTCAGTTG
CXCL10	CCTTATCTTTCTGACTCTAAGTGGC	ACGTGGACAAAATTGGCTTG
TLR4	TGCGTGAGACCAGAAAGC	TTAAAGCTCAGGTCCAGGTTC
MAPK14	TCAGTCCTTTGAAAGCAGGG	ACAGTGAAGTGGGATCAACAG
TLR3	TCAACTTTCTGATAAAACCTTTGCC	AGATGACAAGCCATTATGAGACA
IL-8	TCCTGATTTCTGCAGCTCTG	GTCCACTCTCAATCACTCTCAG
IL-6	GACAGCCACTCACCTCTT	TGTTTTCTGCCAGTGCC
GAPDH	CTCTGACTTCAACAGCGAC	TGGTCCAGGGGTCTTACT

Table 4.3: Primers used for qRT-PCR of selected antifungal makers. All primers were generated from the RefSeq sequence for each gene.

Table 4.4: Profile of transcriptional differences in human antifungal genes from human airway epithelial cells in response to wild-type and *Δalta1 A.alternata*

Gene	Wild-type	*Δalta1*	Gene	Wild-type	*Δalta1*	Gene	Wild-type	*Δalta1*
BCL10	2.2038	-1.0425	**FCGR3A**	-1.3755	-2.2346	**NLRP3**	7.1602	4.4076
C3	14.3204	5.063	**FCN1**	2.5315	1.2658	**NPTX1**	-1.3755	-2.2346
C5AR1	6.2333	2.7132	**FOS**	65.7993	32.8996	**PLCG2**	1.6702	1.0281
CARD9	5.8159	2.0562	**IFNG**	-1.3755	-2.2346	**PTGS2**	-1.3755	-1.2834
CASP1	526.3943	186.1085	**IKBKB**	1.5583	-1.3755	**PTPN6**	122.7858	32.8996
CASP8	61.3929	23.2636	**IL10**	-1.3755	-2.2346	**PTX3**	398.9323	114.5632
CCL2	1209.3365	372.217	**IL12A**	2.2038	1.181	**PYCARD**	13.3614	5.4264
CCL20	16.4498	5.4264	**IL12B**	-1.3755	-2.2346	**RAF1**	-202.2506	-867.0672
CCL5	-18305.632	-51776.147	**IL18**	4.724	2.362	**SCARF1**	1.3566	-1.815
CCR1	4.4076	2.7132	**IL1A**	20.2521	12.4666	**SFTPD**	-8.3397	2.7132
CD14	186.1085	49.8665	**IL1B**	122.7858	70.5219	**SOCS3**	1.5583	1.5583
CD207	-1.3755	-2.2346	**IL1R1**	3.3404	1.3566	**ST3GAL5**	1.1019	-1.1975
CD209	-1.3755	-2.2346	**IL2**	-1.3755	-2.2346	**STAT1**	20.2521	10.8528
CD36	1.1019	-2.0849	**IL23A**	122.7858	24.9333	**SYK**	-1.3755	-2.2346
CD40	5.4264	1.6702	**IL6**	61.3929	40.5042	**TIRAP**	61.3929	43.4113
CD5	-1.3755	-2.2346	**IL8**	1488.8679	372.217	**TLR2**	7.6741	2.362
CD83	43.4113	18.8959	**IRAK1**	1.7901	1.0281	**TLR4**	65.7993	14.3204
CHIA	-1.3755	-2.2346	**IRAK4**	-1.0425	-1.4743	**TLR9**	3.5801	-2.2346
CLEC6A	-1.3755	-2.2346	**ITGAM**	-1.3755	-2.2346	**TNF**	4.4076	1.0281
CLEC7A	-1.3755	-2.2346	**ITGB2**	-1.3755	-2.2346	**TRAF6**	-704.2774	-3983.9947
COLEC12	-1.3755	-2.2346	**JUN**	7.6741	3.8371	**B2M**	1.454	-1.0425
CSF2	9.4479	4.1125	**LYN**	4.1125	2.2038	**HPRT1**	-2.7511	-1.2834
CSF3	-1.3755	-2.2346	**MALT1**	5.063	2.9079	**RPL13A**	1.2658	-1.1975
CXCL1	564.1754	75.5835	**MAP2K4**	491.1432	173.6454	**GAPDH**	1.7901	1.454
CXCL10	199.4661	86.8227	**MAP3K7**	564.1754	229.1264	**ACTB**	-1.1975	1.1019
CXCL11	32.8996	20.2521	**MAPK14**	1209.3365	491.1432	**HGDC**	-1.3755	-2.2346
CXCL2	8.8152	4.4076	**MAPK8**	199.4661	106.8913	**RTC**	1.9185	2.5315
CXCL9	4.724	2.2038	**MBL2**	-1.3755	-2.2346	**RTC**	916.5057	694.5814
F2RL1	18.8959	8.8152	**MRC1**	-1.3755	-2.2346	**RTC**	-404.5012	-613.1091
F3	213.7825	86.8227	**MYD88**	4.1125	2.9079	**PPC**	-62.2499	-133.4356
FCGR1A	2.0562	-2.2346	**NFKB1**	1.7901	1.0281	**PPC**	-176.0694	-404.5012
FCGR2A	17.6305	5.8159	**NFKBIA**	3.1167	2.362	**PPC**	-572.051	-1067.4849

Table 4.4: Profile of transcriptional differences in human antifungal genes from human airway epithelial cells in response to wild-type and *Δalta1 A.alternata*. Transcriptional levels of 84 antifungal- related genes were assessed by qRT-PCR using the RT^2 Profiler Superarray (SABiocsiences). BEAS-2b epithelial cells were left untreated (control) or treated with *A.alternata* wild-type or *Δalta1* conidia for 24hrs. Relative fold change in the expression of each gene between the wild-type and *Δalta1* samples were normalized against the untreated control. Red indicates a significant difference between wild-type and *Δalta1* treatment. Results are from two independent experiments where RNA was pooled from three separate treatments on each occasion.

Chapter V

Purification and assesment of key biochemical properties of Alternaria major allergen Alt a 1

ABSTRACT

Allergic airway hyperreactivity disorders pose a significant health risk for which few reliable therapies exist. Alt a 1 is the major allergen secreted by the fungus *A. alternata*, due to its IgE-specific reactivity with sera from atopic Alternaria patients yet no known innate immunological activity has been assigned to this clinically relevant protein. Alt a 1 is predicted to have esterase activity in addition to a unique lipid-binding motif (RXLR) that facilitates entry of many pathogenic proteins including those from fungi. In this present study we have utlized recombinant protein production statgegies to study the lipid binding capacity and esterase acitivity of Alt a 1 and how these properties influence the mammalian innate and adaptive immune responses. We established that Alt a 1 itself is immunogenic in both airway epithelial cells and an in vivo mouse model by its ability to induce production of pro-inflammatory cytokines and effector cell recruitment. In order to study the biochemical properties of Alt a 1, mutations to the single histidine and predicted RXLR motif were introduce and fusion proteins with or without fluourscent tags were purified. We confirm Alt a 1 possesses esterase activity which requires a functional histidine. Notably, we found that the putative RXLR-motif is required for preferential binding to the phospholipid, phosphatidylinositol-3-phosphate PI3P and entry into human airway cells. However, Alt a 1 enzymatic activity is required for disruption of epithelial cell membrane integrity and IL-13 production in a sensitized host. These noteworthy

results provoked our intensive design of a production and purification strategy for Alt a 1 fusion proteins which resulted in ultra-pure and endotoxin free protein for future immunological studies.

Introduction:

Human airways are constantly exposed to aeroallergens produced by fungi, often at higher levels and for longer duration than other allergens including pollens[1,2]. Unfortunetly, the past few decades have resulted in few clinically viable therapies to treat airway diseases[3]. Chronic exposure to the ubiqutious fungus, *Alternaria alternata*, has been associated with an increased risk of developing atopic or allergic asthma, allergic rhinitis, chronic rhinosinusitis (CRS), or exacerbation of a preexisting asthmatic conditions[4–6]. The mechanisms involved in the inflammatory response evoked by Alternaria are poorly understood, but recent data indicates that the innate immune functions of the airway epithelium play an important role[7,8].

Diagnosis of *A. alternata* sensitization is often hampered by the variability and complexity of fungal extracts, and thus simplification of the diagnostic procedures with purified allergens has been investigated[9]. The majority of the known Alternaria allergens are intracellular proteins with predicted activities of enolase, ribosomal proteins, nuclear transport factor, and aldehyde dehydrogenase[10–12]. Numerous clinical studies suggest that Alt a 1 is the major *Alternaria* allergen since it elicits the most intense and consistent IgE-mediated reaction in *Alternaria*-sensitized individuals[13,14] Alt a 1 is stored in the cell well of the fungus and readily secreted during germination[15–18]. Alt a 1 is a relatively small protein (157 amino acids) and forms a dimer of 28 kd, which dissociates into 14.5-kd and 16-kd subunits under reducing conditions[19–21]. Additionally, the Alt a 1 protein has been shown to possess esterase enzymatic activity[22]. Inspection of the Alt a 1 amino acid sequence shows only one histidine (at position 84) which would be required for formation of the catalytic triad of an esterase. Pure Alt a 1 protein produces a similar reaction as total allergen extracts in the majority (80-90%) of subjects by skin prick tests (SPT)[23,24]. Thus, Alt a 1, either in its natural or recombinant form, is likely to be sufficient for a reliable diagnosis of *A. alternata* sensitization and to induce skin prick reactivity

comparable with that produced by commercially available *A. alternata* extract. However, apart from its reactivity to serum IgE, no other immunological activity has been assigned to Alt a 1. Since allergens themselves do not contain any known structural characteristics defining them as allergens[25], we currently cannot predict how Alt a 1 acts as a stimulant. Thus, there is clearly a need to elucidate exactly by what mechanism Alt a 1 interacts with the immune system leading to the advancement of allergic diseases.

Many pathogens secrete effector proteins which assist in their ability to avoid, suppress or modulate an immune response towards an antagonistic pathway. There is overwhelming evidence that many of these effectors manipulate membrane lipids to gain entry into various host cell types[26]. Specific amino acid sequence motifs have been implicated as necessary for this lipidome exploitation. One of the best characterized lipid binding motifs in fungi is the RXLR motif known to be important in the entry mechanism of plant pathogen effectors[27,28]. Evidence suggests that the RXLR motif binds phosphoinositides present on the host cell surface to trigger lipid raft-mediated endocytosis and uptake[29]. The preceding study also proved that the lipid phosphatidylinositol-3-phosphate (PI3P) is nearly ubiquitous on the surface of human airway cells[29]. This exposes a potential mechanism by which other proteins containing RXLR-motifs can bind and enter. There is an established set of rules for what substitutions are possible in the motif that still result in a functional RXLR. The arginine at position 1 can be substituted by another positively charged residue (K or H) and leucine at position 3 can be substituted with a large hydrophobic residue (I, M, F, Y, or W), while the fourth position can accept many substitutions[30]. Examination of the Alt a 1 amino acid sequence reveals a unique RXLR-like motif of KWYS at position 85 – 88. However there is no current information available regarding Alt a 1's ability to enter airway cells or if this motif has any function in vivo.

The airway epithelium is a critical component of the innate immune response in addition to assisting in directing the adaptive immune response[31,32]. Once disregarded as important in guiding the Th2-driven allergic response, there is now increasing evidence that the epithelium can generate a full repertoire of cytokines and lipid mediators, and recruit effector cells [33]. Alternaria has been shown to have the powerful ability to activate the airway epithelium and direct production of pro-inflammatory cytokines such as IL-6 and IL-8[34–37]. Although these studies did not examine if the Alt a 1 allergen plays a role in the epithelial cell response, reports of other individual allergen protein molecules activating the epithelium are readily available[38–41].

It has been reported that allergens of many types are readily transported across the airway epithelium[42,43]. Common non-proteolytic allergenic proteins, such as birch tree allergen Bet v 1 and grass pollen Phl p 1, can bind and permeate the epithelial barrier[44–46]. Allergic patients also have an increased ability in allergen capture and processing by antigen presenting cells (APC)[47]. This disease characteristic is potentially due to underlying damage to the epithelial layer leading to a polarized airway. Under normal circumstances tight junctions prevent inhaled particles and allergens from accessing the subepithelial mucosa[48,49]. In the case of allergens, transepithelial delivery or disruption would likely facilitate the passage of allergens and increase their detection by dendritic cells, T cells, mast cells, and other effector cells contributing to hallmarks of asthma[49,50]. There are current studies suggesting this transport is mediated by interaction with lipids on the cell surface[51]. Unfortunately, a molecular mechanism by which the allergen can bind surface lipid and enter epithelial cells has not yet been discovered.

We show here that Alt a 1 is itself an immunogenic protein with the ability to induce pro-inflammatory cytokines and effector cell recruitment in epithelial cells and murine model. We have also identified a unique-RXLR-like motif which mediates Alt a 1's capacity to bind

phospholipid PI3P and enter human airway cells. We confirmed Alt a 1 is indeed enzymaticallly active possessing esterase activity. Moreover, the protein's single histidine residue is required for its enzymatic activity. Both of these characteristics appear to play a role in the immune response to the allergen. Finally, we describe in detail a production and purification strategy for recombinant Alt a 1 with selected mutations to further study Alt a 1's structural characeristics.

Materials and Methods:

Recombinant Alt a 1:

Purified recombinant Alt a 1 (rAlt a 1) was purchased from Indoor Biotechnologies (Charlottesville, VA). For use in immunological experiments, endotoxin was removed by filtering through Detoxi-Gel Endotoxin Removal Columns (Pierce) following the manufacturer's instructions. The protein was then concentrated using a 3kDa Amicon Ultra-4 Centrifugal Filter (Millipore) and resuspended in phosphate buffered saline (pH 7.4) at a concentration of 1mg/mL.

Epithelial Cell Stimulation:

Human airway cells (A549) were seeded (1×10^6 cells/well) in six-well tissue culture plates (BD) and allowed to adhere overnight. Monolayers were washed once using Dulbecco's Phosphate Buffered Saline (Ca^{++}/Mg^{++} free) (DPBS; Hyclone) and serum-starved for two hours in RPMI: 1640 medium without supplements. Cells were washed twice more with DPBS and cultured in a final volume of 1.5mL RPMI: 1640 without supplements. Cells were treated with 50μg of rAlt a 1 or the Alt a 1 fusion proteins prepared in house as indicated in figure legends. For spore stimulation experiments, *Alternaria alternata* strain ATCC 66981 was used. Spores were harvested into sterile water from a 7-day old PDA plate supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone). Spore suspensions were standardized to 1 x 10^3 spores/μl after counting on a hemocytometer and then used immediately in assays. In all assays, control cells were treated with DPBS. Cells were incubated at 37°C and 5% CO_2 for 24 hours before the supernatants were collected and debris was removed by centrifugation (2500x*g*, 5min, 4°C) and stored at -80°C. The cells were then washed twice with DPBS before RNA was extracted.

RNA Extraction and qRT- PCR:

Total RNA was purified cells by addition of TRIzol (Invitrogen) directly to the cells and then following the manufacturer's protocol. RNA was further purified and genomic DNA eliminated by using the RNeasy Cleanup Kit (Qiagen) with on-column DNase digestion. First-strand synthesis was performed from 1μg total RNA with the Tetro cDNA Synthesis Kit (Bioline) using random primers. Quantitative real-time PCR for IL-6 was completed using the SYBR Green Supermix (Bio-Rad) and gene specific primers for IL-6, (Forward: 5'-GACAGCCACTCACCTCTT -3', Reverse: 5'- TGTTTTCTGCCAGTGCC -3'). Reactions were performed in duplicate and gene expression was normalized to the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase using the formula $2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = [(Ct_{target\ gene})sample\ (Ct_{GAPDH})sample]/[(Ct_{target\ gene})reference\ (Ct_{GAPDH})reference]$.

Membrane-based Cytokine Profile Assays:

Cell-free supernatant was applied to the membrane based cytokine antibody array (RayBiotech Inflammatory Human Cytokines Array 1) and assay was completed to the manufacturer's instructions. Membranes were blocked at room temperature and then incubated with samples (cell-free supernatant) overnight, shaking at 4°C. Membranes were washed and incubated with biotin-conjugated-anti-cytokines. Following another wash, membranes were then incubated with HRP-conjugated streptavidin antibodies. The membranes were exposed for 30 seconds to BioMax Light x-ray film (Kodak). Film was developed on SRX-101A (Konica) film developer. All steps, except overnight incubation, were performed at room temperature. Film was visually examined for differences between experimental and control samples.

Murine Models:

Female BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were 8 - 12 weeks old at time of experiments. The procedures and handling of the mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Polytechnic Institute and State University (IACUC approval number 08-211BIOL). Mice were housed in filter-top cages and provided food and water *ad libitum*.

For intranasal challenges, mice were lightly anaesthetized with isofluran (Abbott Laboratories) in individual chambers of a rodent anesthesia unit with precision vaporizer (Impac6, VetEquip Inc.) at a concentration of 2 - 3% and oxygen at 2 L/min. When the mice were unresponsive but breathing comfortably, the protein or spore solution was applied to the nostrils. Control mice were treated with 50µl of DPBS. The animals were allowed to slowly inhale the liquid and then to recover in a supine position under a heat lamp.

In the chronic model, intranasal exposure was repeated every other day over 14 days for a total of 8 challenges. Changes in total body weight of each animal were assessed by weighing immediately before initial challenge and then again directly before euthanasia. In the sensitized model, mice were sensitized by intraperitoneal injections of native Alt a 1+ aluminum hydroxide adjuvant (Imject, Pierce) on Days 1 and 14. Intranasal challenges were conducted every day for three days on Days 21 -23. In both models, samples were collected 24 h following the last challenge.

Serum IgE and Bronchoalveolar Lavage:

Terminal blood was collection was completed by cardiac puncture. Briefly, a 22 gauge needle attached to a 1mL syringe was inserted into the heart at a 30 degree angle towards the

chin. The plunger was slightly withdrawn to create a vacuum and the maximum amount of blood was retrieved. Mice were then immediately euthanized by cervical dislocation. Serum was separated via centrifugation at 1000 *x g* for 10 min in a tabletop centrifuge. Serum was removed to a fresh microcentrifuge tube at stored at -80°C until analysis.

Bronchoalveolar lavage

The trachea was exposed and incised. An 18 gauge blunt-end needle was inserted into the trachea and BAL fluid was harvested by washing the lungs twice with 1 ml of DPBS. BAL was centrifuged at 500*xg*, red-blood cells were lysed by addition of ACK lysing buffer, cells were again pelleted by centrifugation, and the cell pellet was resuspended in RPMI media. The cell-free supernatant was stored at -80°C prior to ELISA analysis. For total cell counts, an aliquot of cells were diluted into a trypan blue/DPBS solution and counted on a hemacytometer.

Histopathological analysis

Following collection of BAL fluid, the lungs were inflated with 10% buffered formalin, latter embedded in paraffin, and then sectioned (5 μm) and stained with hematoxylin and eosin (H&E) for examination of pulmonary architecture.

ELISA:

Cytokine levels in the cell-free BAL or culture supernatant were quantified by standard ELISA per manufacturer's instructions.

Synthetic DNA:

Sequences for native Alt a 1 and the mutants Alt a 1_His^{84-} (nKWYS), Alt a 1_$^{85}RXLR^{88}$- (HqvvS), (HqWYS), (HKvvS), and (HKWvS) were designed and synthesized by GenScript Corporation, with codon usage optimized for *E.coli* expression. The amino acid sequences are summarized in Figure 5B.

Expression Vectors

The Gateway expression vectors used to generate the Alt a 1 fusion proteins are summarized in Figure5C and were constructed following the manufacturer's instructions (Invitrogen). Briefly, the BP clonase reaction was used to introduce the synthetic coding DNA containing flanking attB recognition sties into the pDONR vector. Following genetic sequencing confirmation, the LR clonase reaction was used to transfer DNA fragments from entry clones into destination vectors summarized in Figure 5C. The product of recombination reactions was used to transform competent *E. coli* B21(DE3) using heat shock.

Protein Expression in *E.coli*:

BL21(DE3) *E. coli* cells containing plasmids encoding each fusion protein were grown were grown in 500 mL of Luria-Bertani media containing 100 µg/mL ampicillin in a 1 liter flask shaken at 250 rpm at 37ºC to an OD600 of 0.6. Protein expression was induced with 1mM IPTG and cell growth was continued at 16ºC for 14 hours. Cells were harvested by centrifugation at 8,000 *x g* for 20 min at 4°C and stored at -20ºC.

Purification of Alt a 1 Fusion Proteins:

Cells were lysed by sonication in chilled 0.01M phosphate buffered saline (PBS, pH 7.4) plus 1 mg/mL lysozyme per 4 mL/g wet cell weight. Lysed cells were centrifuged at 10,000 *x g* for 20 min at 4°C and then fusion proteins were collected from the supernatant using containing glutathione-sepharose 4B beads (GE Healthcare). The beads were washed in a column with at least 5 volumes of PBS then eluted in 1 mL fractions with GST elution buffer (PBS, 10 mM reduced glutathione, pH 6.8). Fractions were pooled and concentrated to 1mL using a 3 kDa concentrator device (Amicon).

The GST fusion was removed from the Alt a 1_pSDK3 proteins by digesting 1mg of protein with 1 unit of thrombin (Novagen, restriction grade) at 20°C overnight. The reaction was then applied to His-Bind Resin Column (Novagen). The column was then washed with at least 5 volumes PBS, pH 7.2 and eluted in 1 mL fractions with elution buffer (PBS, 250 mM imidazole). Fractions were pooled then concentrated to 1mL in PBS using a 3 kDa concentrator device (Amicon). Protein concentration was quantified spectrophometrically on Nanodrop (Thermo) by reading absorbance at 280nm. The purity was assessed by gel electrophoresis (Figure 6).

Lipid Filter-Binding Assay:

Lipid filter-binding assays were performed as previously described (Kale, et al.). Lipids (from Cayman Chemical, Ann Arbor, Michigan or Avanti Polar Lipids) were dissolved in DMSO. Lipid filters were prepared by spotting 1 µl of each lipid at appropriate concentrations onto Hybond-C-extra membranes (GE Healthcare). Membranes were incubated in 20 mL of blocking buffer (10mM Tris-HCl, pH 7.2, 150mM NaCl, 0.1% Tween-20, 3% fatty-acid free bovine serum albumin, Sigma # A-7030) for 1 hr at room temperature. The Alt a 1 protein (20 µg) was added to the blocking buffer and incubated overnight at 4ºC. After washing, bound

proteins were detected with goat anti-GST (GE Healthcare). Unbound antibody was removed by washing and then probed with HRP conjugate (donkey-anti-goat IgG-HRP; Santa Cruz Biotechnology). Detection was carried out using ECL reagent (Thermo Scientific).

Human Airway Epithelial Cell Uptake assays:

For uptake assay, human lung adenocarcinoma cells A549 (ATCC CCL-185) were seeded at a concentration of 5.0 x 10^4 per well in a 96-well tissue culture plate. Cells were washed with Dulbecco's Phosphate Buffer Saline (Ca2+/Mg2+ free) (DPBS; Gibco) and cultured in 250 µL RPMI, 10% FBS, 1% penicillin/streptomycin. Following adherence, cells were serum starved for two hours by a media exchange with 250ul RPMI alone. Cells were washed twice with DPBS and then 100ul of RPMI + fusion protein was added to each well (100 µg/mL, final concentration) and incubated in the same conditions at 37ºC for 15 minutes or 24 hours as indicated. No protein controls were 100ul of RPMI alone. Cells were then washed twice with DPBS, trypisinized and immediately examined under confocal microscopy (Zeiss LSM 510).

In order to assay inhibition of entry, Alt a 1(WT)- mCherry (0.2mg/mL) was pre-incubated with *inositol 1,3-disphosphate* (1,3-IP2) (1mM) in RPMI (500uM final concentration IP2, 0.1mg/mL protein) for 15mins at room temperature and then added to cells as indicated above.

Esterase Activity Assay:

The hydrolysis of *p*-nitrophenyl acetate by Alt a 1 fusion proteins was carried out at 25°C for 10 min in 150 µL of 50 mM phosphate buffer, pH 6.5. Substrate solutions were prepared

from 100mM p-nitrophenyl stock using reaction buffer (RB, 75mM phosphate buffer, pH 6.0). The substrate solution was plated in 96-well test plates (BD) and either RB (control) or protein (~1µg) was added to each well. The reaction was incubated at room temperature in the dark for 5-30mins until a positive (yellow) color was visualized. The absorbance of each sample was measured at 400nm and the steady-state kinetic parameter of each protein, K_m , was quantified using the Michaelis-Menten plot as previously described[52].

Production and purification of Alt a 1_pET21dc proteins for immunological studies:

Construction of Alt a 1 proteins for use in future immunological experiments is summarized in Table 3. Alt a 1 was PCR amplified from genomic *A.alternata* DNA with addition of NcoI/NotI restriction sites. The PCR product was cloned into pET21dc vector and correct insertion was confirmed by genetic sequencing. This plasmid was used as the backbone for QuickChange Mutagenesis (Agilent) for the desired mutations. Briefly, reactions were set-up containing 5uL of reaction buffer, 2ul of Alt a 1_pET21dc plasmid DNA, 1umol of forward and reverse primer containing the mutation, 10umol dNTPs, brought to a total volume of 50ul with water. The PCR reaction included an initial denaturation of 95°C(30sec) and no final extension. The cycling conditions were as follows for a total of 18 cycles: 95°C(30s) → 55°C(1min)→ 68°C(5.5min). The Dpn restriction enzyme was then added to the reaction to degrade the methylated parental strand. The mutated plasmid was then transformed into XL-Blue supercompetent cells and plated on LB-amp plates containing 80ug/mL X-gal and 20mM IPTG. Positive (blue) colonies were confirmed for the correct mutation by genetic sequencing.

A total of 2 litres of BL21(DE3) *E. coli* cells containing Alt a 1_pET21dc plasmids encoding each fusion protein were grown in Luria-Bertani media containing 100 µg/mL

ampicillin at 250 rpm at 37ºC to an OD600 of 0.6. Protein expression was induced with 1mM IPTG and cell growth was continued at 16ºC for 14 hours. Cells were harvested by centrifugation at 8,500 *x g* for 10 min at 4°C. Cells were lysed by sonication in chilled 0.01M phosphate buffered saline (PBS, pH 7.4) plus 1 mg/mL lysozyme per 4 mL/g wet cell weight. Lysed cells were centrifuged at 10,000 *x g* for 20 min at 4°C. The supernatant was applied to Ni-NTA agarose and allowed to gently mix end-over-end for 1 hr. The solution was washed in a column with 10 volumes of Wash Buffer (PBS, 30mM imidizole) and eluted by addition of 4 volumes of Elution Buffer (PBS, 250mM imidazole). Proteins were separated from contaminants using a *ÄKTAprime Plus HPLC System* (GE Healthcare). The sample (~2 – 3mg) was applied to HiPrep Sephacryl, 26 × 600 mm column (GE Healthcare) and separated at a flow rate of 1 mL/min using a linear gradient (PBS, pH 7.4). The column was washed and equilibrated at initial conditions for at least 10 min and sample fraction collection was initiated approximately 40 – 50 min following sample injection. Fractions were selected based on chromatographic analysis and purity of each fraction was assessed on SDS-PAGE with Silver Staining (Pierce). Pure fractions were pooled and concentrated to 1mL using a 3 kDa concentrator device (Amicon). Final purity analysis was completed on a 14% Native-PAGE gel and stained with Coomassie Brilliant Blue.

Endotoxin Removal and Testing of Alt a 1_pETdc proteins:

Residual endotoxin was removed from purified Alt a 1_pET21dc protein preparations by decontamination on endotoxin removal columns (Detoxi-Gel endotoxin removing columns, Pierce). Briefly, resin was equilibrated in 1% sodium deoxycholate followed by 5 volume washes of PBS. The protein (1mL) was applied to the column and incubated for 1 hr. Protein was eluted by addition of PBS and endotoxin levels were measured by ToxinSensor^{TM} Chromogenic

LAL Endotoxin Assay Kit (Genscript) with a threshold of 1U/mL. All solutions were prepared in pyrogen-free water or PBS.

Results:

Alt a 1 induces immune response in human airway cells and murine lungs

The first aim of our study was to establish Alt a 1's ability to induce an immune response in both human airway epithelial cells and a murine model. Previous studies from our laboratory had shown the *A.alternata* spores are potently immunogenic and were thus used as a positive control in these experiments. By using both *A.alternata* spores and rAlt a 1 as treatments, we could generally assess the degree of the immune response induced by interaction with Alt a 1 alone compared to other fungal components. In order to determine which cytokines would serve as the best markers for our experiments, we utilized a membrane based cytokine assayed which tested for 42 common human pro-inflammatory cytokines in the cell culture supernatant (Figure 1). Both IL-6 and IL-8 were predominantly found from airway cells treated with spores or Alt a 1. Human CXCL1/GROα, which has homologous neutrophil chemoattractant function to IL-8, was also detected as was the antigen-presenting cell recruiter, CCL2/MCP-1. Since IL-8, CXCL1/GROα, and CCL2/MCP-1 have similar function, we chose IL-8 and IL-6 as markers for testing of immunogenicity of our purified Alt a 1 proteins.

The results of the cytokine array were confirmed both transcriptionally by qRT-PCR (IL-6) and translationally by ELISA (IL-8) (Figure 2). In the case of IL-6, the Alt a 1 protein was able to enhance the transcription of this cytokine more than the fungal organism. However, levels of IL-8 were fairly similar for both treatments. Potentially, transcription was lagging in the case of the cells treated with Alt a 1 and ELISA would have reflected this difference as well.

Since the allergic airway response is a carefully orchestrated occurrence involving more than epithelial cells, we also examined if Alt a 1 could induce a similar pro-inflammatory immune response in vivo. We intranasally challenged mice every other day over 14 days with

either Alt a 1 protein or *A.alternata* spores. We again found the protein was nearly as immunogenic as the live organism. Both treatment groups demonstrated reduce weight gain compared to the control mice, a sign of cachexia frequently associated with allergic models (Figure3A). The spores and Alt a 1 protein were also able to significantly enhance the recruitment of effector cells into the lung as described to total cell counts in the bronchoalveolar lavage fluid (Figure 3B). Although differential cell counts were not assessed due to sampling integrity issues, histopathological examination of the lung tissue was completed (Figure 3C). The lung pathology was assessed for cell infiltration, bronchiole lumen thickening, and increase in mucous production. The Alt a 1 protein appeared to enhance neutrophil recruitment more than the spores; however the spores stimulated more eosinophilia and a greater thickening of the bronchioles. This primarily eosinophilic response elicited by the spores was confirmed by the detection the IL-13, the predominant cytokine involved in allergic airway inflammation, in the BAL fluid. Notably, the Alt a 1 protein itself was also able to induce production of IL-13. There may be the requirement of other fungal components in addition to the allergen in order to fully coordinate the entire allergic response. The results of our in vivo and in vitro testing confirm Alt a 1's immunogenic competence.

Identification and mutation of Alt a 1 motifs

The amino acid sequence of Alt a 1 predicts a protein of ~18kDa in size as well as a single histidine at position 84. Interestingly, we identified a unique RXLR-motif (KWYS) directly following the histidine residue at positions 85 – 88 (Figure 5A); the motif was predicting using the rules established for other pathogenic proteins as previously described[30,53]. RXLR motifs have been proven to be necessary for fungal effector entry into both plant and animal

cells[29]. In order to establish if Alt a 1's predicted RXLR motif or single histidine were essential for its biochemical properties, we introduced selected mutations into the motifs to alter their functionality as summarized in Figure 5B. The coding DNA for the native and mutated Alt a 1 sequences was synthetically synthesized and codon optimized for *E.coli* (Genscript) with the addition of attB1 sites for use in the Gateway cloning system (Invitrogen). We utilized three expression vectors (Figure 5C). The first vector (pSDK3) contained a N-terminal glutathione-S-transferase (GST) tag and C-terminal 6x-histidine (6x-his) tag while the other two were identical except for the addition of either an green fluorescent protein tag (GFP, pSDK2), or red fluorescent protein tag (mCherry, pSDK3) fused to the C-terminus of Alt a 1 before the his-tag.

Before testing all of the various RXLR mutations, we chose the constructs for the mutation of the single histidine (nKWYS) and the most severe RXLR mutation (HqvvS) for initial analysis. The purification of both the Alt a 1_pSDK3 and pSDK2 for the native (HKWYS), nKWYS, and HqvvS constructs are shown in Figure6AB. These three preparations were used for the subsequent characterization experiments.

Alt a 1 putative RXLR-motif is required for lipid binding

Following confirmation of Alt a 1's immunologic activities, we sought to further characterize its unique properties. We were first interested in determining if Alt a 1 can bind common lipid molecules found almost ubiquitously on mammalian cell membranes. This knowledge would gauge if Alt a 1 may be able to enter or cross the epithelial cell barrier in the airway. We used a lipid filter binding assay for testing of lipid binding capacity as previously described[53]. First we tested the native Alt a 1 against thirteen principle lipids and found it preferentially binds phosphatidylinositol-3-phosphate (PI3P) (Figure 7A). Binding to any other

phospholipids was not detected including the close variants PI's such as PI5P. With this information we utilized a gradient lipid filter assay to determine capacity to bind PI3P and confirm inability to bind PI4P and PI5P. We found native Alt a 1 was able to bind as little as 25pmol of PI3P and PI4P at high concentrations (200pmol). Most interestingly, when we tested the Alt a 1 his^{84} and RXLR (HqvvS) mutants, we found that the removal of histidine did not affect Alt a 1's binding capacity to PI3P while the HqvvS mutation abolished the protein's ability to bind phospholipid (Figure 7B). This data suggests that the predicted RXLR, but not enzymatic activity involving the single histidine, is required for Alt a 1's lipid binding capability.

Catalytic activity of Alt a 1

Alt a 1 was previously shown to be a potential esterase and phosphatase[22]. If Alt a 1 is an esterase, the histidine would be required for the coordination of the catalytic triad. We examined the ability of Alt a 1 and the His^{84}- and RXLR mutant to hydrolyze the simple substrate *p*-nitrophenyl acetate (C_2). We found the native Alt a 1 to have a steady-state kinetic, K_m, of 2.5 mM ± 0.34 (Figure 8). Predictably, the Alt a 1 with asparagine substituted for the histidine residue did not have any detected esterase activity suggesting that Alt a 1 is indeed an esterase. Although the histidine is still intact in the Alt a 1 (HqvvS) protein, it did not exhibit detectable esterase activity. This is likely due to such a large replacement directly following the histidine residue causing structural changes which are preventing the formation of the active site.

Alt a 1 enters human airway cells by binding surface lipid PI3P

Once we established that Alt a 1's ability to bind lipid PI3P was controlled by its predicted RXLR, we decided determine if the motif also controls entry into human airway cells.

In the first assay we incubated human bronchoalveolar cells with 25ug of native Alt a 1 fused to mCherry for 15min and confocal laser microscopy revealed the protein was endocytosed and accumulated in yet to be determined, unknown cytoplasmic vesicles (Figure 9). There was no accumulation of protein on the cell membrane since cells were treated with a protease (trypsin) and washed with DPBS prior to imaging in order to remove membrane bound protein. The control cells are shown to validate autofluorescense was not the cause of the imaged structures. The next experiment shows that, like lipid binding, the predicted Alt a 1 RXLR motif mediates entry into airway cells (Figure 10C). This Alt a 1 (HqvvS)-mCherry fusion was unable to enter airway cells in any instance. Moreover, the histidine mutant, with a fully functional RXLR and PI3P binding capability, is still able to enter the cells (Figure 10B). Since both mutations abolish esterase activity, we used a competitive inhibitor of 1,3IP2 in conjunction with native Alt a 1-mCherry to determine if enzymatic activity was playing a role in the entry. We found that use of a PI3P inhibitor prevented entry of the enzymatically active native protein (Figure 10E) suggesting its activity is not required for entry. Finally, we show that entry of Alt a 1 is consistent across all cells in the suspension (Figure 10D).

In order to further asses the importance of enzymatic activity in Alt a 1's capacity to enter airway cells, we incubated the cells with native Alt a 1 fused to GFP at 37°C and 4°C for 24 hrs. Interestingly, we observed significant membrane degradation as evident by "ruffling" in addition to equivalent entry compared to the Alt a 1-mCherry fusion protein (Figure 11A). This was not seen with the shorter incubation time of 15 min. These images would suggest that not only can Alt a 1 enter airway epithelial cells, but it can also exit the cell (transcytosis) and fragment the membrane, a type of injury which, in large amounts over a period of time, could lead to tissue injury. The membrane integrity was still clear of any degradation in the cells incubated with Alt

a 1-GFP at 4°C and the control cells (Figure 11BC), but the protein was unable to enter and was found strongly bound to the extracellular membrane at refrigerated temperatures (Figure 11B). Collectively, this would suggest that the putative RXLR of Alt a 1 is required for binding to the membrane but that some class of activity is required by the cells for endocytosis.

Pro-inflammatory response of human airway cells to Alt a 1 is potentially controlled by putative RXLR-motif

Since epithelial cells are likely the first cell type activated in the airway's response to an allergen, and we have previously determined Alt a 1 can induce the innate immune cytokine, IL-8, we measured the role of activity and entry in the stimulation of IL-8. We found a slight increase in IL-8 if the protein could still enter but lacked enzyme activity but this level did not approach levels exhibited by treatment with the the native protein (Table 2). We did not detect any amount of IL-8 if Alt a 1 was unable to enter cells and had no esterase activity. The native Alt a 1 induced a substantial amount of IL-8 in the airway cells, further confirming our previous results.

Role of Alt a 1 in the allergic response in-vivo

Given that avoidance of sensitization to a ubiquitous allergen is nearly impractical, we tested if inhibiting entry of an allergen in a sensitized host would be a potential therapeutic target. We sensitized mice to Alt a 1 by two intraperitoneal injections of the protein + adjuvant two weeks apart and then intranasally challenged them with either the native or mutant Alt a 1 proteins or PBS control (Figure 12A). We found that entry was essential for the stimulation of IgE release (Figure 12B) but not stimulation of IL-13 (Figure 12C). Those these are very

preliminary results with a small sample size (n =3/group) we consider them sufficient for further exploration.

Purification strategies of Alt a 1 for immunological studies

Our airway epithelial cell culture and sensitized mouse model data implore expanded testing of Alt a 1. In order to elicit the most natural response, we needed an established protocol for purification of Alt a 1 protein with minimal tags or additional amino acids not found in the native sequence. We cloned the genomic DNA sequence of Alt a 1 (Table 3A) into the vector pET21dc at restriction sites Nco1/Not1 to avoid as few extra residues as possible. The translated product has no additional C-terminal amino acids and the N-terminus features a 3x-his tag and five mostly small residues before the 6x-his tag (Table 3B). This Alt a 1_pET21dc construct was then used as the backbone for mutagenesis to generate the desired mutations. The primers used for QuickChange mutatgenesis (Agilent) are listed in Table 3C. All constructs were confirmed by sequencing and transformed into BL21(DE3) *E.coli* cells for expression.

Unfortunately, expression and solubility of Alt a 1 is less than those constructs with extensive fusions. Thus, two liters of total culture is necessary for enough starting material for our extensive purification strategy. The initial culture results in a few milligrams of total Alt a 1_pET21dc protein however it is highly contaminated with additional soluble proteins from *E.coli* following purification on Ni-NTA agarose (Figure 13A). We found that HPLC results in the best separation of Alt a 1 from these proteins (Figure 13B). The chromatogram suggests large (>70kDa) contaminating proteins elute first, followed by the mid-sized (20 – 40 kDA) proteins and finally Alt a 1 (~14 – 18 kDa) (Figure 13C). The HPLC fractions were analyzed by ultra-sensitive Silver Staining (Pierce) of an SDS-PAGE gel. The cleanest fractions were then selected

and concentrated to 1mL in PBS which contains ~1mg total protein. The purity was confirmed by both regular and native PAGE analysis which confirms Alt a 1 forms a dimer of ~36kD from two monomers of ~18kDA (Figure 13 DE). The final purification approach is endotoxin removal and verification by chromogenic LAL testing at a threshold of 10U/mL. This purification strategy now allows us to produce high-quality Alt a 1 protein for future immunological experiments.

Discussion:

In order to elucidate the overall importance of Alt a 1 in immune responses to Alternaria, we utilized a recombinant Alt a 1 protein, including Alt a 1 mutant proteins deficient in enzymatic activity and/ or RXLR-like motifs. Both the Alt a 1 protein and *A. alternata* (wild type spores) stimulated the production of the Th2-skewing cytokine, IL-13, in mice following chronic intranasal challenges of spores protein. Infiltration of effector cells (macrophages, neutrophils, eosinophils, and lymphocytes) into the airways along with other hallmarks of airway inflammation was also observed. Alt a 1 protein and wildtype Alternaria spores also evoked the secretion of pro-inflammatory cytokines (IL-6 and IL-8) in treated airway epithelial cells. In contrast, Alt a 1 with a non-functional histidine or RXLR, were unable to induce cytokine production compared to native protein suggesting that this single protein may play a major role in inducing innate immune responses in airway epithelium at the organismal level. We also confirmed Alt a 1 entry in to airway epithelial cell is mediated by its binding to membrane lipid PI3P and esterase activity is controlled through a single histidine. These findings could have important implications regarding importance of Alt a 1 in Alternaria related allergies.

The fact that Alt a 1 can activate the epithelium and possibly cross it potentiates its ability to further develop the adaptive immune response by interaction with APCs. Resident dendritic cells are located directly beneath the epithelial basement membrane and any disruption to the epithelial barrier may increase their ability to have direct contact with antigens bound or transcytosing the epithelium[54]. Our data would suggest that Alt a 1's ability to bind and enter airway cells may be one of the reasons it is the major Alternaria allergen. Furthermore, by blocking this mechanism, we may be able to prevent mast cell degranulation in Alt a 1 allergic patients, dendritic cell activation and subsequent Th2 cascade.

Since the presumably endosomal vesicles we found the in the airway cells containing Alt a 1 are nearly identical to those found for other fungal effector proteins, we conclude that Alt a 1 can enter airway cells via lipid-raft mediated endocytosis. This function may be closely related to the hypothesized caveolae-dependent mechanism of Bet v 1[51]. Further investigations are necessary to determine if Alt a 1 escapes the endosomal compartment and is able to trancytose the epithelium similar to the system identified for allergen Phl p 1[46]. Transcytotic function may be crucial for an allergen to be more likely to bind allergen specific IgE on mast cells and be processed by APCs over other innocuous proteins secreted by the fungus. Our sensitized mouse model may have proved that Alt a 1 must cross epithelial cell barrier to be able to cause mast cell degranulation and increase B-cell production of specific IgE, however this model must be rigorously repeated with a larger sample size and more thorough investigation.

Pro-inflammatory cytokines, such as IL-13 are rapidly produced by resident effector cells once activated by signals from the epithelium. Alt a 1 was able to induce IL-13 in both a chronic and sensitized mouse model. We hypothesize this response is more likely mediated by decreased membrane integrity caused by pro-longed exposure to the Alt a 1's as described previously for injury.[55] Although it is also possible that repeated disturbance to the membrane phosphoinositides could provide an innate signal for IL-13 production[56,57]. It is also important in the future to assess whether or not PI-3-P itself is a substrate for Alt a 1's enzymatic activities especially in light of the fact that it has demonstrable esterase and phosphatase activities. If we find this to be true, it will also be important to test if PI-3-P break down products are indeed the source of innate immune response activated by Alt a 1.

We have also identified potential RXLR-like motifs in other allergens including dust mite Tyr p 2, (KVLK), timothy grass Phl p 6 (KLIE, KTFE, and RIIA), and bee venom, PLA2

(RIGD, RIIY, and KFYD). Hypothetical motifs are also found in Der p 2 (dust mite, *D.pteronyssinus*) Pen c 1 (Penicillium), and Cla h 8 (Cladosporium); however the multitude of motifs within these allergens will require considerable mutational analysis. If these motifs or those of other allergen proteins are identified to be essential for cellular entry and subsequent immune response, the value of the RXLR motif in predicting potential allergenicity will be tremendous. Furthermore, manipulation of this motif may then be able to be used to develop medical treatments for atopic patients. There are currently many clinical trials utilizing recombinant allergens for immunotherapy instead of the classical fungal extract preparation[58,59]. These products allow new formulations of immunotherapy tailored to the patient. Recombinant-based approaches are mostly focused on genetic modification of allergens to produce molecules with reduced allergenic activity and conserved antigenicity[60]. Our described process for obtaining highly pure Alt a 1 is clearly an important tool to have on hand for testing new immunotherapeutic approaches. Additionally, introducing mutations to the RXLR motif rendering the protein non-functional may be a potential approach to producing safer allergens for treatment.

Since Alt a 1 is predicted to be fairly hydrophobic due to a multitude of large charged residues and a predicted overall negative charge (-6) at pH 7.0, a large amount of culture is required to obtain enough protein in the soluble fraction. Although retrieval from the insoluble fraction is possible, the additional time and reagents combined with the necessity for refolding makes it less feasible for production of proteins for immunotherapy. Although the method outlined here is effective in production of pure protein, we may need to construct additional fusions to increase solubility for increased yield. It has been shown that addition of fluorescent protein tags improve solubility[61]. Mammalian cell culture systems offer a more natural

expression system, including post -translational modifications, but are nearly hundred fold more expensive than microbial systems[62]. If a more suitable expression condition or system can be found, the purification schematic described here can easily be employed to produce pure Alt a 1 protein for use in immunologic studies.

Collectively, our results give great insight on Alt a 1's immunogenicity and mechanism of entry into airway eptihelial cells. The combined enzymatic activity and lipid binding capability prove to be important in the mammalian innate and adaptive immune response to the allergen. In addition, we provide tools for future endeavors in identification of potential therapies against inhaled fungal allergens.

REFERENCES:

1. O'Hollaren, M. T. *et al.* Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. *N. Engl. J. Med.* **324**, 359–363 (1991).
2. Nasser, S. M. & Pulimood, T. B. Allergens and thunderstorm asthma. *Curr Allergy Asthma Rep* **9**, 384–390 (2009).
3. Holt, P. G. *et al.* Drug development strategies for asthma: in search of a new paradigm. *Nat. Immunol.* **5**, 695–698 (2004).
4. Andersson, M., Downs, S., Mitakakis, T., Leuppi, J. & Marks, G. Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitized children. *Pediatr Allergy Immunol* **14**, 100–105 (2003).
5. Salo, P. M. *et al.* Exposure to Alternaria alternata in US homes is associated with asthma symptoms. *J. Allergy Clin. Immunol.* **118**, 892–898 (2006).
6. Bush, R. K. & Prochnau, J. J. Alternaria-induced asthma. *J. Allergy Clin. Immunol.* **113**, 227–234 (2004).
7. Fahy, J. V. Remodeling of the airway epithelium in asthma. *Am. J. Respir. Crit. Care Med.* **164**, S46–51 (2001).
8. Kato, A. & Schleimer, R. P. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr. Opin. Immunol.* **19**, 711–720 (2007).
9. Vailes, L. *et al.* Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. *Journal of Allergy and Clinical Immunology* **107**, 641–646 (2001).
10. Weichel, M. *et al.* Nuclear transport factor 2 represents a novel cross-reactive fungal allergen. *Allergy* **58**, 198–206 (2003).
11. Bowyer, P., Fraczek, M. & Denning, D. W. Comparative genomics of fungal allergens and epitopes shows widespread distribution of closely related allergen and epitope orthologues. *BMC Genomics* **7**, 251 (2006).
12. Huby, R. D., Dearman, R. J. & Kimber, I. Why are some proteins allergens? *Toxicol. Sci.* **55**, 235–246 (2000).
13. Achatz, G. *et al.* Molecular cloning of major and minor allergens of Alternaria alternata and Cladosporium herbarum. *Mol. Immunol.* **32**, 213–227 (1995).
14. Kurup, V. P., Vijay, H. M., Kumar, V., Castillo, L. & Elms, N. IgE binding synthetic peptides of Alt a 1, a major allergen of Alternaria alternata. *Peptides* **24**, 179–185 (2003).
15. Ibarrola, I. *et al.* Production profile of the major allergen Alt a 1 in Alternaria alternata cultures. *Ann. Allergy Asthma Immunol.* **93**, 589–593 (2004).
16. Twaroch, T. E. *et al.* Predominant localization of the major Alternaria allergen Alt a 1 in the cell wall of airborne spores. *Journal of Allergy and Clinical Immunology* (2011).doi:10.1016/j.jaci.2011.10.008
17. Sercombe, J. K., Eduard, W., Romeo, T. C., Green, B. J. & Tovey, E. R. Detection of allergens from Alternaria alternata by gold-conjugated anti-human IgE and field emission scanning electron microscopy. *J. Immunol. Methods* **316**, 167–170 (2006).
18. Green, B. J., Mitakakis, T. Z. & Tovey, E. R. Allergen detection from 11 fungal species before and after germination. *J. Allergy Clin. Immunol.* **111**, 285–289 (2003).
19. Breitenbach, M. & Simon-Nobbe, B. The allergens of Cladosporium herbarum and Alternaria alternata. *Chem. Immunol.* **81**, 48–72 (2002).
20. Deards, M. J. & Montague, A. E. Purification and characterisation of a major allergen of Alternaria alternata. *Mol. Immunol.* **28**, 409–415 (1991).

21. Barnes, C. S. *et al.* Production of a recombinant protein from Alternaria containing the reported N-terminal of the Alt a1 allergen. *Adv. Exp. Med. Biol.* **409**, 197–203 (1996).
22. Sáenz-de-Santamaría, M., Guisantes, J. A. & Martínez, J. Enzymatic activities of Alternaria alternata allergenic extracts and its major allergen (Alt a 1). *Mycoses* **49**, 288–292 (2006).
23. De Vouge, M. W. *et al.* Isolation and expression of a cDNA clone encoding an Alternaria alternata Alt a 1 subunit. *Int. Arch. Allergy Immunol.* **111**, 385–395 (1996).
24. Kleine-Tebbe, J. *et al.* Predominance of the major allergen (Alt a I) in Alternaria sensitized patients. *Clin. Exp. Allergy* **23**, 211–218 (1993).
25. Aalberse, R. C. Structural biology of allergens. *J. Allergy Clin. Immunol.* **106**, 228–238 (2000).
26. van der Meer-Janssen, Y. P. M., van Galen, J., Batenburg, J. J. & Helms, J. B. Lipids in host-pathogen interactions: pathogens exploit the complexity of the host cell lipidome. *Prog. Lipid Res.* **49**, 1–26 (2010).
27. Dou, D. *et al.* RXLR-mediated entry of Phytophthora sojae effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* **20**, 1930–1947 (2008).
28. Gu, B. *et al.* Rust secreted protein Ps87 is conserved in diverse fungal pathogens and contains a RXLR-like motif sufficient for translocation into plant cells. *PLoS ONE* **6**, e27217 (2011).
29. Kale, S. D. *et al.* External Lipid PI3P Mediates Entry of Eukaryotic Pathogen Effectors into Plant and Animal Host Cells. *Cell* **142**, 284–295 (2010).
30. Tyler, B. M. Entry of Oomycete and Fungal Effectors into Host Cells. 243–275doi:10.1002/9781119949138.ch10
31. Holt, P. G., Strickland, D. H., Wikström, M. E. & Jahnsen, F. L. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* **8**, 142–152 (2008).
32. Hammad, H. & Lambrecht, B. N. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* **8**, 193–204 (2008).
33. Barrett, N. A. & Austen, K. F. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* **31**, 425–437 (2009).
34. Shin, S.-H. & Lee, Y.-H. Airborne fungi induce nasal polyp epithelial cell activation and Toll-like receptor expression. *Int. Arch. Allergy Immunol.* **153**, 46–52 (2010).
35. Doherty, T. A. *et al.* Alternaria Induces STAT6-Dependent Acute Airway Eosinophilia and Epithelial FIZZ1 Expression That Promotes Airway Fibrosis and Epithelial Thickness. *Journal of Immunology (Baltimore, Md.: 1950)* (2012).doi:10.4049/jimmunol.1101632
36. Kobayashi, T. *et al.* Asthma-related environmental fungus, Alternaria, activates dendritic cells and produces potent Th2 adjuvant activity. *J. Immunol.* **182**, 2502–2510 (2009).
37. Kauffman, H. F., Tomee, J. F., van de Riet, M. A., Timmerman, A. J. & Borger, P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J. Allergy Clin. Immunol.* **105**, 1185–1193 (2000).
38. Kauffman, H. F., Tamm, M., Timmerman, J. A. B. & Borger, P. House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. *Clin Mol Allergy* **4**, 5 (2006).
39. Wong, C. K. *et al.* House dust mite allergen Der p 1 elevates the release of inflammatory cytokines and expression of adhesion molecules in co-culture of human eosinophils and bronchial epithelial cells. *Int. Immunol.* **18**, 1327–1335 (2006).
40. Tai, H.-Y. *et al.* Pen ch 13 allergen induces secretion of mediators and degradation of occludin protein of human lung epithelial cells. *Allergy* **61**, 382–388 (2006).

41. Röschmann, K. *et al.* Timothy grass pollen major allergen Phl p 1 activates respiratory epithelial cells by a non-protease mechanism. *Clin. Exp. Allergy* **39**, 1358–1369 (2009).
42. Toppila-Salmi, S., Renkonen, J., Joenväärä, S., Mattila, P. & Renkonen, R. Allergen interactions with epithelium. *Curr Opin Allergy Clin Immunol* **11**, 29–32 (2011).
43. Renkonen, R. *et al.* Allergens are transported through the respiratory epithelium. *Expert Rev Clin Immunol* **6**, 55–59 (2010).
44. Mogensen, J. E. *et al.* The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. *Biochemistry* **46**, 3356–3365 (2007).
45. Renkonen, J. *et al.* Birch pollen allergen Bet v 1 binds to and is transported through conjunctival epithelium in allergic patients. *Allergy* **64**, 868–875 (2009).
46. Blume, C. *et al.* Human epithelial cells of the respiratory tract and the skin differentially internalize grass pollen allergens. *J. Invest. Dermatol.* **129**, 1935–1944 (2009).
47. von Garnier, C. *et al.* Allergic airways disease develops after an increase in allergen capture and processing in the airway mucosa. *J. Immunol.* **179**, 5748–5759 (2007).
48. Holgate, S. T. Airway inflammation and remodeling in asthma: current concepts. *Mol. Biotechnol.* **22**, 179–189 (2002).
49. Xiao, C. *et al.* Defective epithelial barrier function in asthma. *J. Allergy Clin. Immunol.* **128**, 549–556.e1–12 (2011).
50. Proud, D. & Leigh, R. Epithelial cells and airway diseases. *Immunol. Rev.* **242**, 186–204 (2011).
51. Joenväärä, S. *et al.* Caveolar transport through nasal epithelium of birch pollen allergen Bet v 1 in allergic patients. *J. Allergy Clin. Immunol.* **124**, 135–142.e1–21 (2009).
52. Hernández, A. & Ruiz, M. T. An EXCEL template for calculation of enzyme kinetic parameters by non-linear regression. *Bioinformatics* **14**, 227–228 (1998).
53. Kale, S. D. & Tyler, B. M. Identification of lipid-binding effectors. *Methods Mol. Biol.* **835**, 393–414 (2012).
54. van Rijt, L. S. & Lambrecht, B. N. Dendritic cells in asthma: a function beyond sensitization. *Clin. Exp. Allergy* **35**, 1125–1134 (2005).
55. Allahverdian, S., Harada, N., Singhera, G. K., Knight, D. A. & Dorscheid, D. R. Secretion of IL-13 by airway epithelial cells enhances epithelial repair via HB-EGF. *Am. J. Respir. Cell Mol. Biol.* **38**, 153–160 (2008).
56. Wong, K.-W. & Isberg, R. R. Arf6 and phosphoinositol-4-phosphate-5-kinase activities permit bypass of the Rac1 requirement for beta1 integrin-mediated bacterial uptake. *J. Exp. Med.* **198**, 603–614 (2003).
57. Lemmon, M. A. Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* **9**, 99–111 (2008).
58. Valenta, R. *et al.* From allergen genes to allergy vaccines. *Annu. Rev. Immunol.* **28**, 211–241 (2010).
59. Bordas-Le Floch, V. *et al.* Expression and Characterization of Natural-Like Recombinant Der p 2 for Sublingual Immunotherapy. *International Archives of Allergy and Immunology* **158**, 157–167 (2012).
60. Ferreira, F. *et al.* Modified recombinant allergens for safer immunotherapy. *Inflamm Allergy Drug Targets* **5**, 5–14 (2006).
61. Pédelacq, J.-D., Cabantous, S., Tran, T., Terwilliger, T. C. & Waldo, G. S. Engineering and characterization of a superfolder green fluorescent protein. *Nat. Biotechnol.* **24**, 79–88 (2006).

62. Singh, M. B. & Bhalla, P. L. Recombinant expression systems for allergen vaccines. *Inflamm Allergy Drug Targets* **5**, 53–59 (2006).

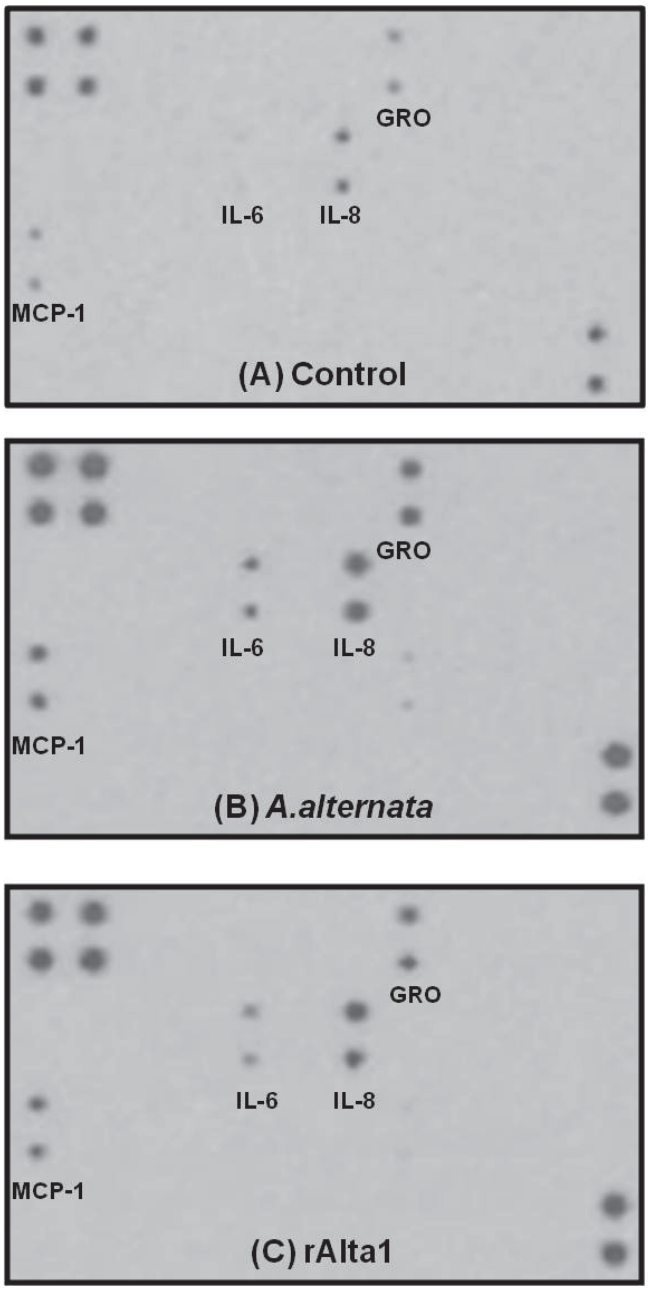


Figure 5.1: Alternaria major allergen Alt a 1 induces pro-inflammatory cytokines in human bronchoalveolar epithelial cells. BEAS-2b cells were seeded at a density of 1 X 10^6 cells per well in a six-well plate at 37°C, 5% CO_2. Following a two hour serum starvation, cells were incubated with (A) PBS (control), (B) 5 x 10^4 Alternaria spores, or (C) 50ug rAlt a 1 for 24hrs. The supernatant from three biological replicates were pooled and the cell-free supernatant was applied to the RayBio Inflammatory Human Cytokines Array 1 and developed per the manufacturer's instructions.

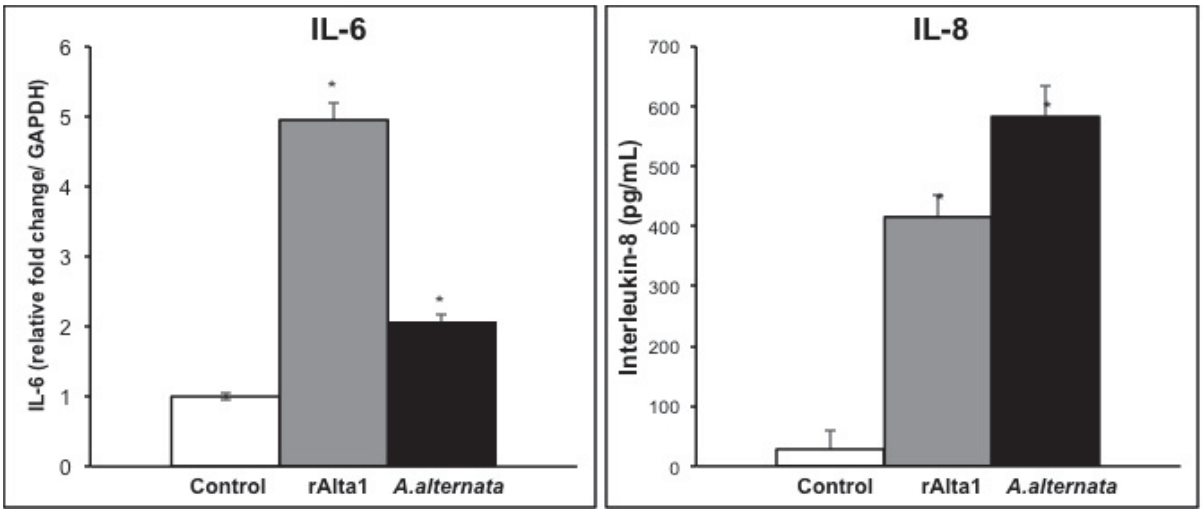


Figure 5.2: Confirmation of allergen Alt a 1's ability to induce of IL-6 and IL-8 in human airway epithelial cells. The results of the cytokine array were confirmed by measuring levels of IL-8 and IL-8 in the cell culture supernatant by either quantitative real-time PCR (IL-6) or ELISA (IL-8) was conducted per the manufacturer's instructions. $P < 0.05$.

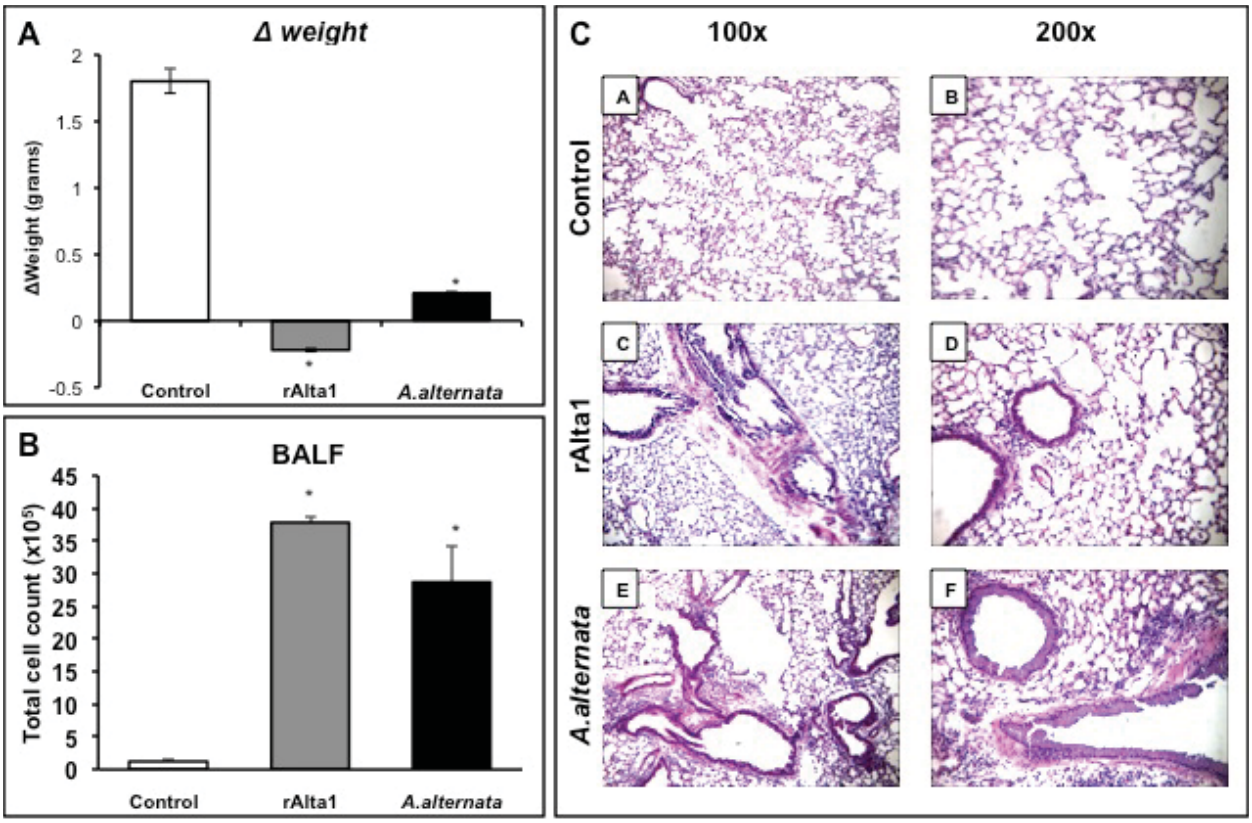


Figure 5.3: Alt a 1 can stimulate markers of allergic airway hypersensitivity. Female BALB/c mice (8-12 weeks old) were challenged every two days over fourteen days with PBS (control), rAlt a 1 (50µg), or *A.alternata* spores (1 X 10^5). (A) Change in total body weight was calculated by difference between start weight before initial challenge and weight immediately prior to euthanasia. (B) Total bronchoalveolar cell counts. Lungs were washed twice with DPBS and cells were counted on a haemocytometer. (C) Representative histopathological changes in the lung tissue. Lungs were removed 24 hours after the last challenge, inflated and fixed in 10% formalin, and stained with hematoxylin and eosin (H&E). Data are shown as group mean ± SEM (n = 4/group).

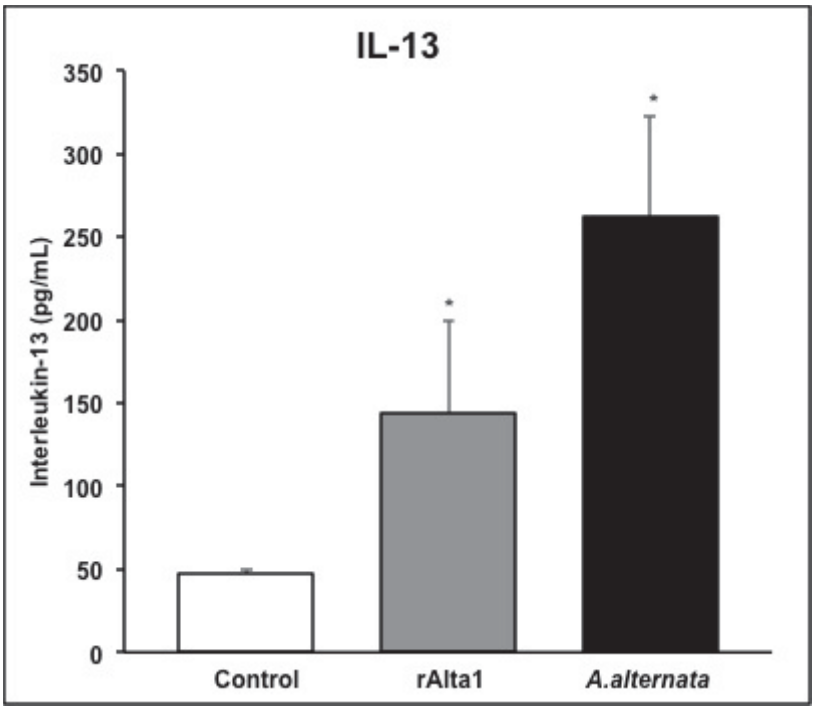


Figure 5.4. Alternaria allergen Alt a 1 promotes Th2 cytokine production in murine airway. The level of IL-13 in the cell-free bronchoalveolar lavage was measured by ELISA following the manufacturer's instructions. Data are shown as group mean ± SEM (n = 4/group); $p<0.05$.

A) Alt a 1 Native protein Sequences	MAPLESRQDTASCPVTTEGDYVWKISEFYGRKPEGTYYNSLGFNIKATNGGTLD FTCSAQADKLEDHKWYSCGENSFMDFSFDSDRSGLLLKQKVSDDITYVATATLP NYCRAGGNGPKDFVCQGVADAYITLVTLPKSS

B) Mutations of the Alta1 RXLR-like motif	Alta1……^{70}LDFTCSAQADKLEDHKWYSCGENSFMDFSFD^{100}……
	Alta1 (nKWYS) ----------NKWYS----------
	Alta1 (HqvvS) ----------HQVVS----------
	Alta1 (HqWYS) ----------HQWYS----------
	Alta1 (HKWvS) ----------HKWVS----------
	Alta1 (HKvvS) ----------HKVVS----------

C) pSDK3-Alta1

pSDK2-Alta1-mCherry

pSDK1-Alta1-GFP

Figure 5.5: Schematic diagram of Alt a 1 native protein sequence, selected mutations of putative RXLR motif, and expression vectors. (A) Native Alt a 1 protein sequence with single histidine at position 84 highlighted in green and the putative RXLR, 85KWYS88 highlighted in red. (B) Mutations of the Alt a 1 histidine or RXLR predicted RXLR motif. Dashes indicated identical residues. (C) Constructs for the expression of native and mutated Alt a 1 proteins. All vectors contain N-terminal GST tag (aqua), thrombin cleavage site (yellow), attB cloning recognition sites (black), and enterokinase site (magenta). Fluorescently tagged proteins contain an additional mCherry (red) or GFP (green) tag followed by a poly-histidine tag (purple).

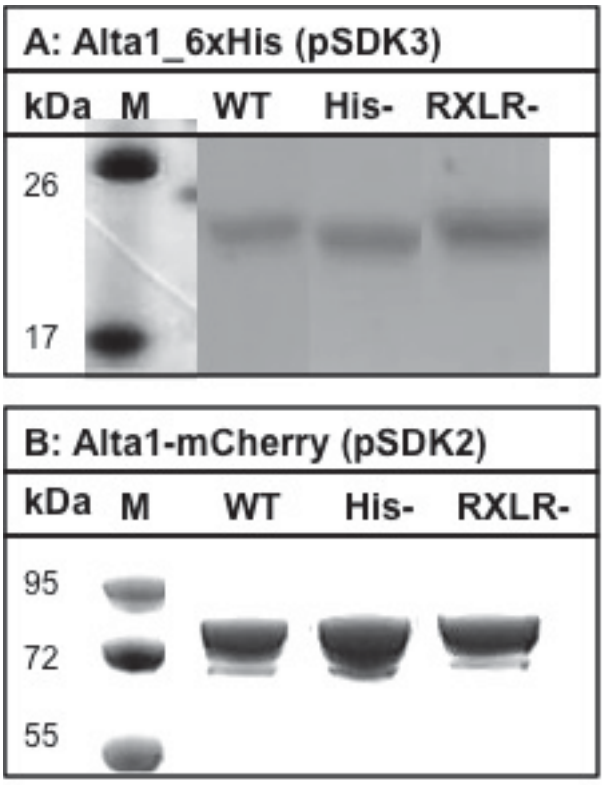


Figure 5.6: SDS-PAGE Analysis of Alt a 1 fusion proteins. Synthetic DNA sequences were codon-optimized for *E.coli* and synthesized by Genscript to include the desired Alt a 1 native or mutated sequence flanked by attB cloning sites. DNA was Gateway cloned into pDONR for introduction by homologous recombination into the desired destination vectors *E.coli* BL21 cells containing plasmids encoding each fusion protein were grown at 37°C to OD600 = 0.6. Protein expression was induced with 1mM IPTG and cell growth was continued for 12 hours at 16°C. Proteins were then collected from the cell-free supernatant using Ni-NTA agarose (Qiagen). The GST tag was removed by overnight digestion with thrombin. The protein was separated from the cleaved GST by running the protein through glutathione sepharose 4B (GE Healthcare). The flow-through containing the fusion protein was collected and concentrated using a 3kDa cut-off concentrator (Amicon). Purified Alt a 1 fusion proteins were electrophoresed on SDS-polyacrylamide gels, then stained with Coomassie Brilliant Blue. (A): Alt a 1 with GST tag removed (~18kDa). (B):GST-Alt a 1-mCherry (~75kDa). M = molecular weight markers.

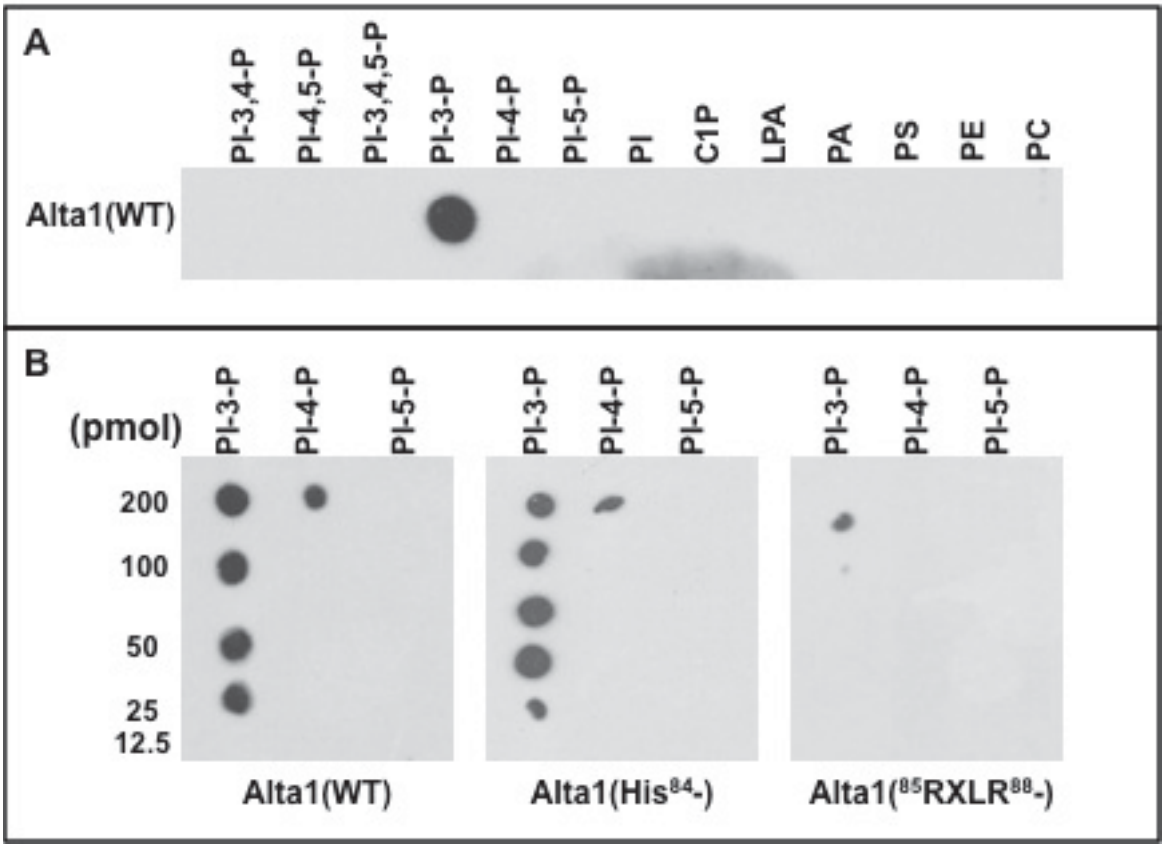


Figure 5.7: Alt a 1 putative RXLR is required for preferential binding to phosphatidylinositol-3-phosphate. Lipids were dissolved in DMSO then spotted at appropriate concentrations onto membranes. Membranes were incubated in blocking buffer for 1 hr, then the probed with 20ug of protein and incubated overnight at 4ºC. After washing, bound proteins were detected with goat anti-GST antibody followed by an HRP-secondary antibody conjugate. Detection was carried out using ECL reagent. (A) binding capacity of Alt a 1(WT) to thirteen principle lipids. Exposure = 1 minute. (B) gradient binding capacity of Alt a 1(WT) and the mutants Alt a 1(His^{84}-) and Alt a 1(^{85}RXLR^{88}-) to PI-3-P, PI-4-P, and PI-5-P (Exposure = 15min).

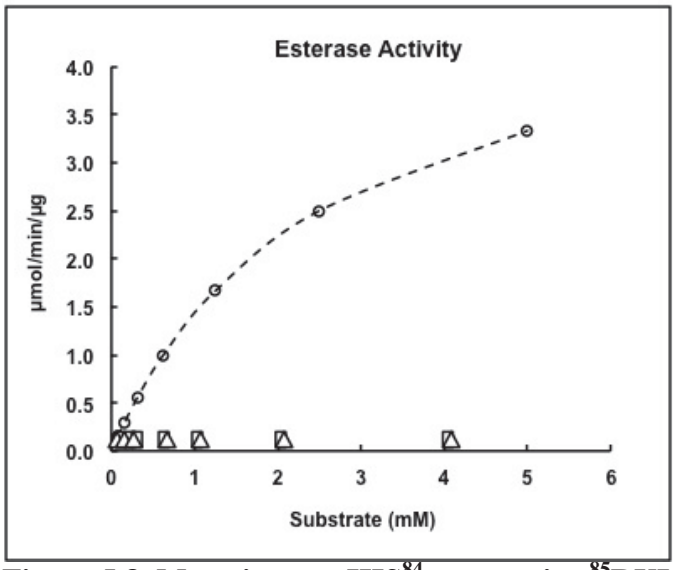


Figure 5.8. Mutations on HIS^{84} or putative ^{85}RXLR^{88} motif of Alt a 1 abolished esterase activity. The hydrolysis of *p*-nitrophenyl acetate (C_2) with Alt a 1 (circles), Alt a 1(His^{84}-) (squares) and Alt al (^{85}RXLR^{88}-)(triangles) was carried out at 25°C for 10 min in 150 µL of 50 mM phosphate buffer, pH 6.5. Lines represent the global fit of all data (n=3) to the equation for Michaelis-Menten plot using *Anemona* (Hernández and Ruiz, 1998 Bioinformatics 14:227). The steady-state kinetic parameter, K_m, of Alt a1 was determined to be 2.5 mM ± 0.34.

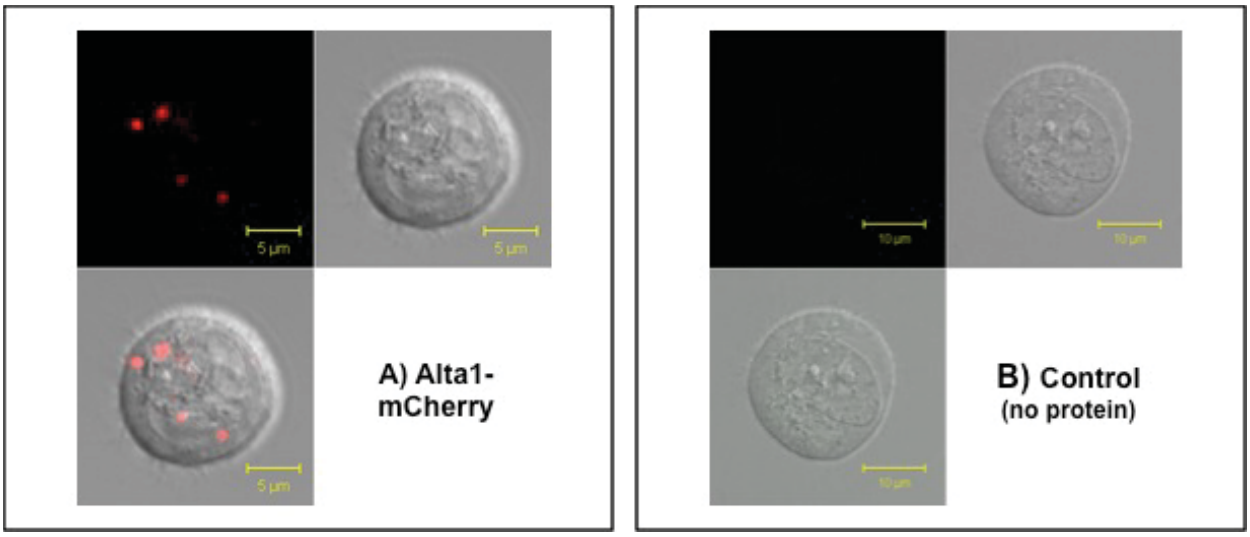


Figure 5.9: Alt a 1 enters human airway epithelial cells. Human bronchoalveolar epithelial cells (A549) were incubated for 15min with 25ug/mL purified Alt a 1-mCherry (A) or no protein (B). Lower left panels are light micrograph image (upper right panels) overlaid with fluorescent image (upper left panel).

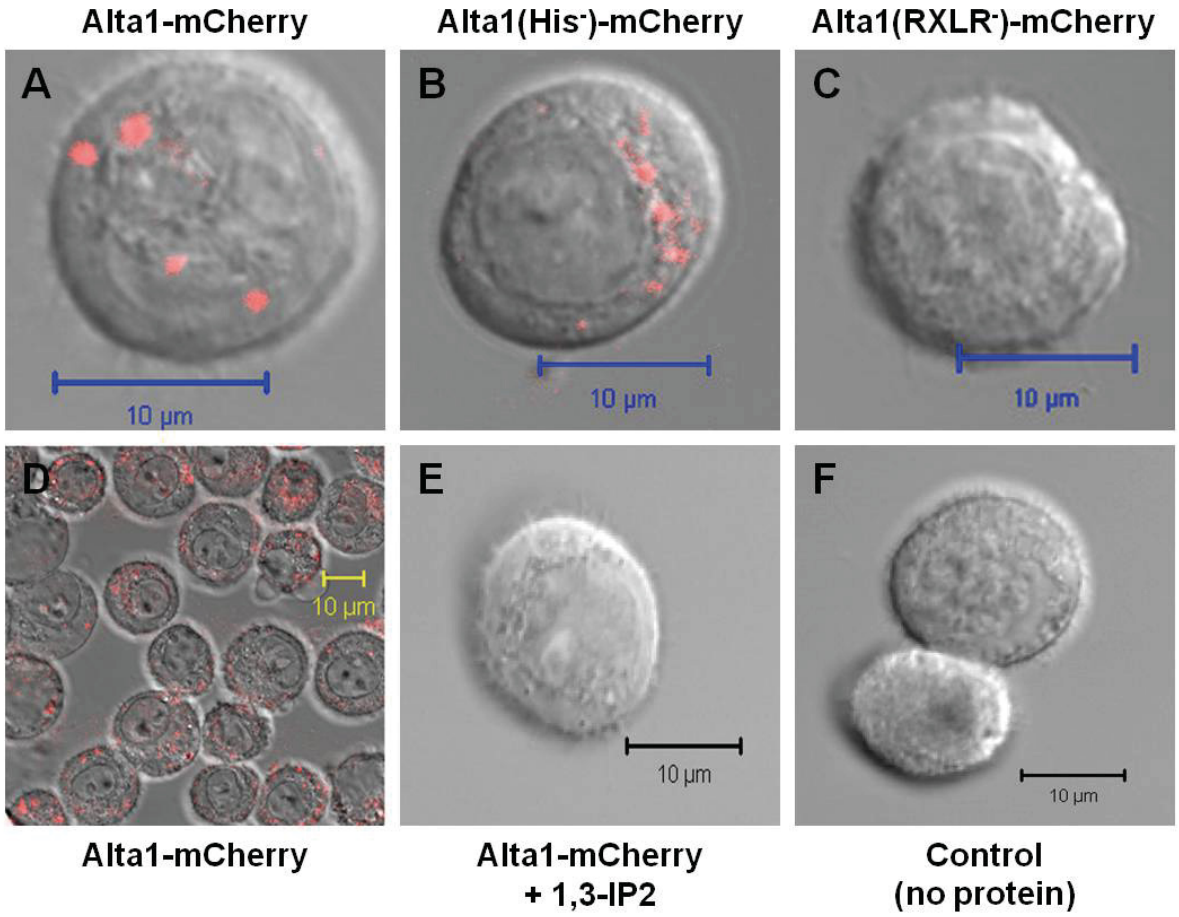


Figure 5.10: Putative RXLR of Alt a 1 is required for entry into human bronchoalveolar epithelial cells (A549). Cells were incubated with purified Alt a 1-mCherry fusion protein for 15min at 37°C. For all treatments, cells were washed twice with DPBS, trypsinized, and examined using confocal microscopy. (A) Alt a 1-mCherry, (B) Alt a 1-His^{84}-mCherry, (C) Alt a 1-^{85}RXLR^{88}-mCherry. (D) Representative level of entry for Alt a 1-mCherry into multiple cells. (E) To assay inhibition of entry, Alt a 1-mCherry was pre-incubated for 15 minutes with the competitive inhibitor, inositol-1,3-diphosphate (1,3-IP2). Cells were then exposed to the Alt a 1-mCherry protein for 15 minutes at 37°C. (F): Control cells were incubated with serum-free medium without addition of any protein. (Bar = 10*um*).

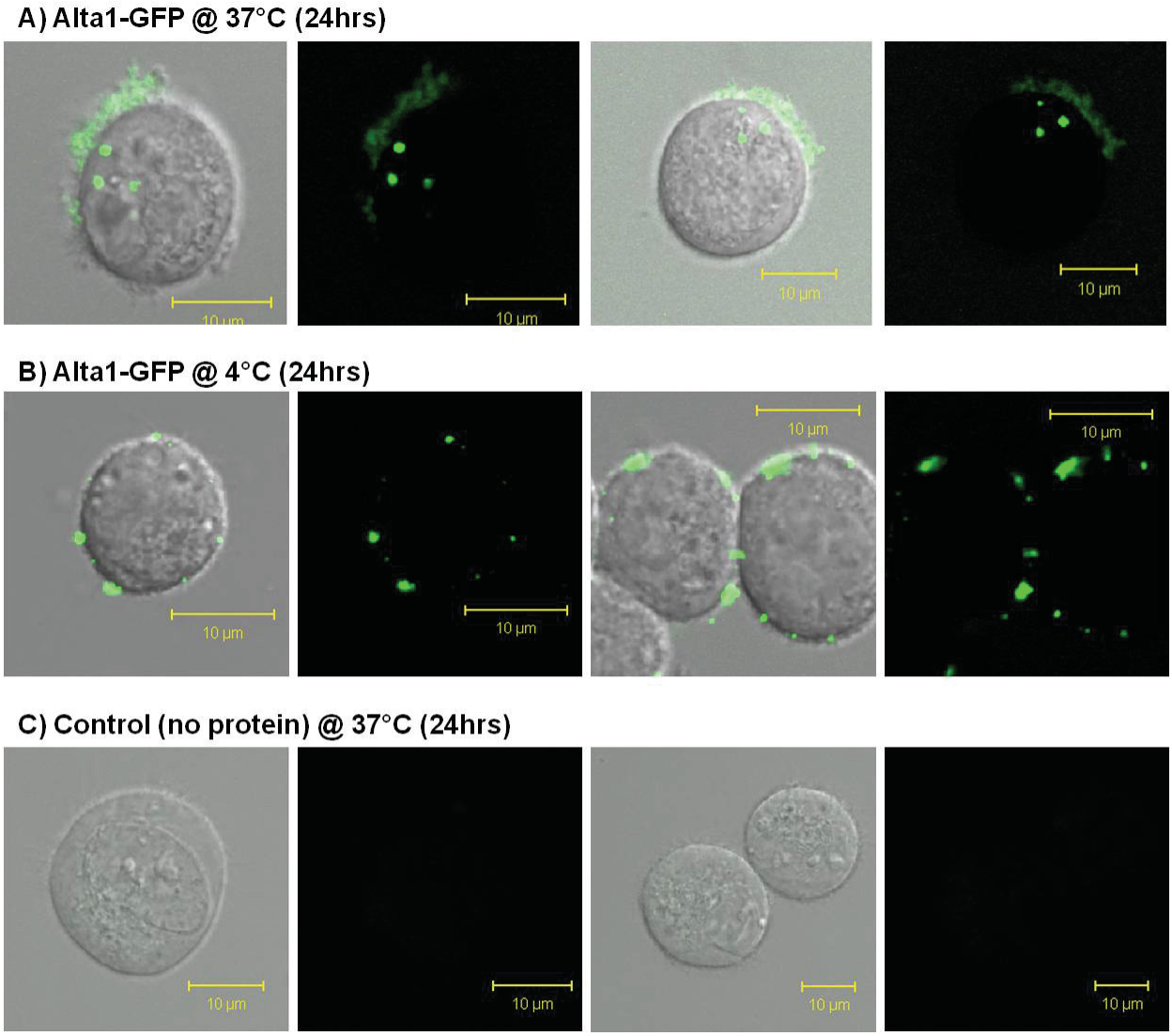


Figure 5.11. Prolonged exposure to Alt a 1 affects cell membrane integrity and endocytosis of Alt a 1 is inhibited at 4°C. Human airway epithelial cells (A549) were incubated with Alt a 1-GFP for 24 hours at either (A) 37°C or (B) 4°C. (C) Control cells were not exposed to protein. Cell membrane ruffling as shown is representative of all cells . (Bar = 10*um*).

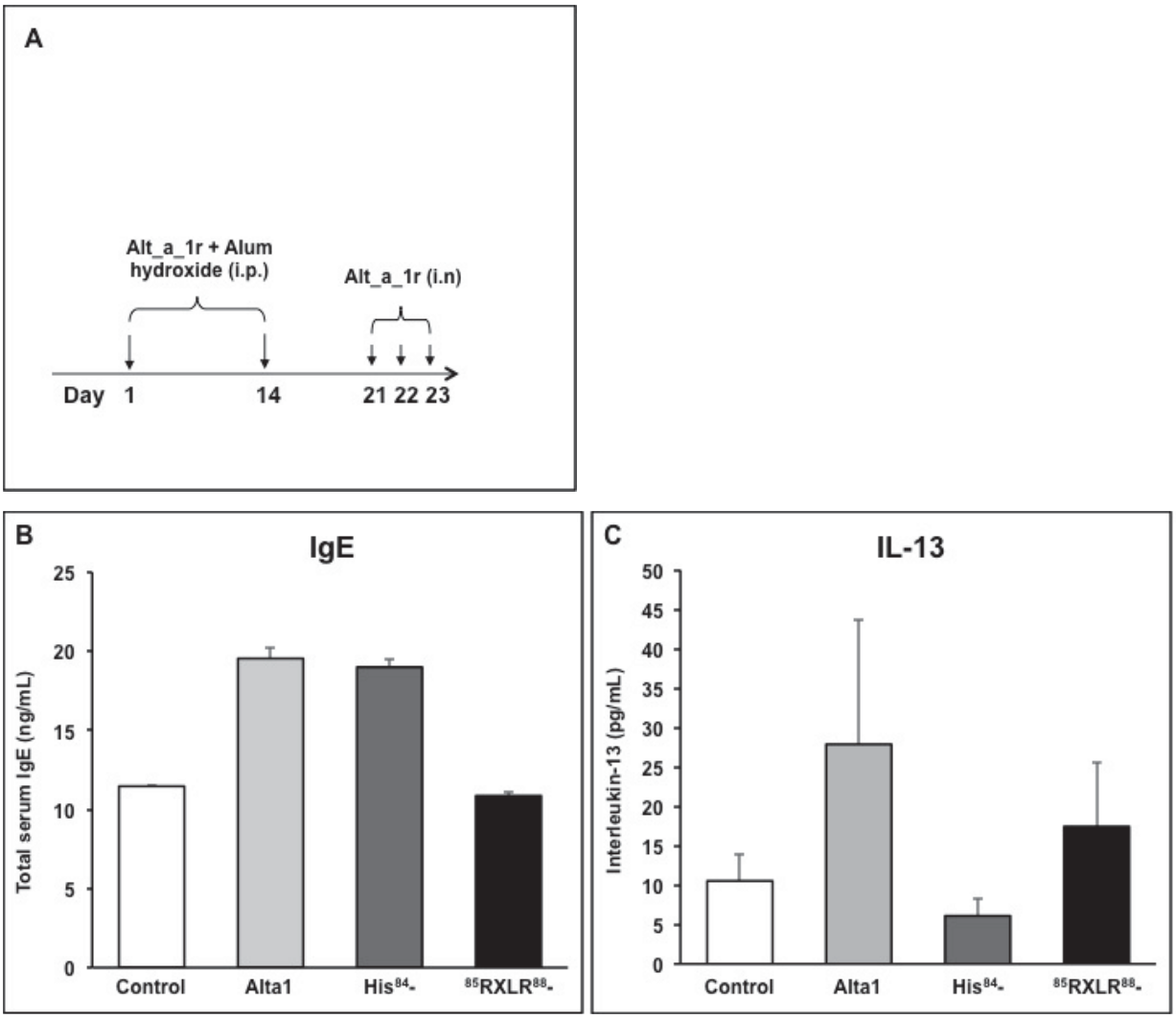


Figure 5.12. Functional RXLR-motif may be required for allergic response in-vivo. (A) Female BALB/c mice (aged 6 – 8 weeks) were sensitized by intraperitoneal injections of native Alt a 1+adjuvant on Days 1 and 14. Mice were then intranasally challenged with either Alt a 1, Alt a 1(His^{84}-), or Alt a 1(^{85}RXLR^{88}) on Day 21, 22, and 23. Control mice were challenged with PBS. Serum and BAL fluid were collected 24 h following the last challenge. (B) Total serum IgE and (C) IL-13 from the cell-free BAL were measured by ELISA following the manufacturer's instructions (Biolegend). The data are expressed as the mean ± S.E., n = 3/group

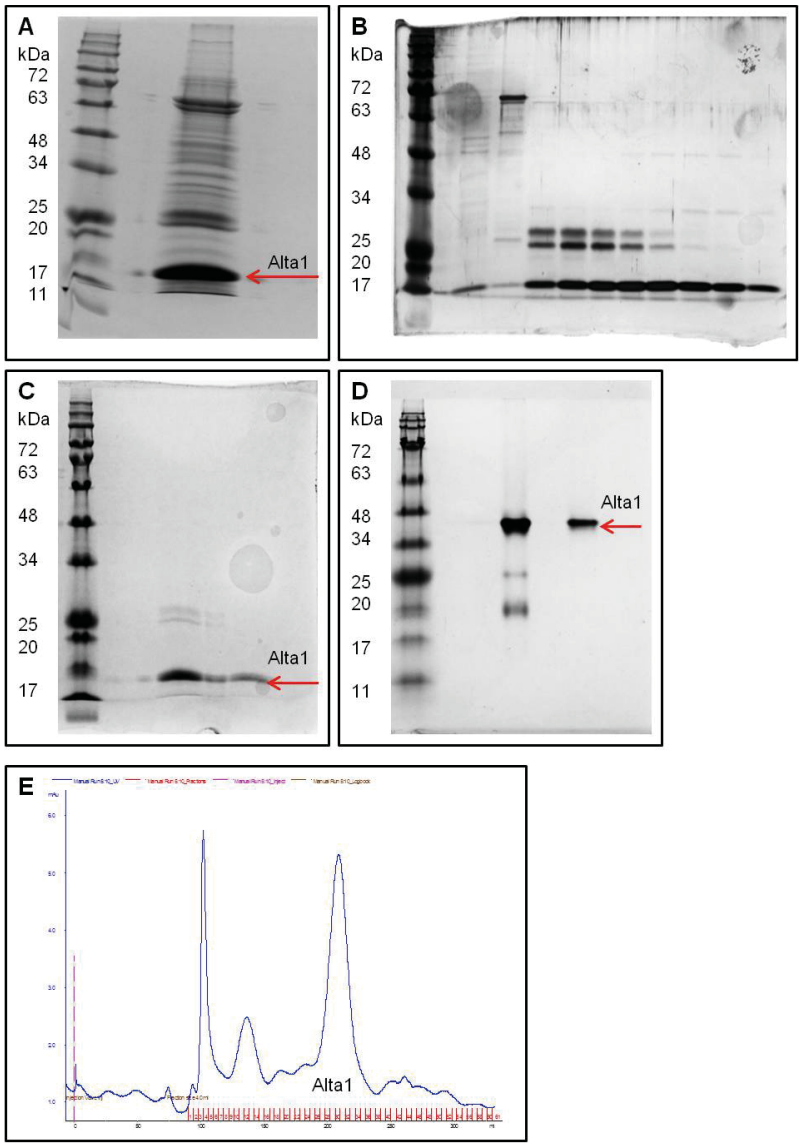


Figure 5.13: Production and purification of Alt a 1_pET21 fusion proteins for immunological studies. *E.coli* BL21 cells containing plasmids encoding each fusion protein were grown at 37°C to OD600 = 0.6. Protein expression was induced with 1mM IPTG and cell growth was continued for 12 hours at 16°C. Proteins were then purified from the cell-free supernatant using Ni-NTA agarose (Qiagen). The protein was further separated by high performance liquid chromatography and fractions identified by HPLC detector were analyzed on SDS-PAGE gel and Silver Stain (Pierce) following the manufacturer's instructions. Fractions containing pure Alt a 1 were concentrated using a 3kDa cut-off concentrator (Amicon) and electrophoresed on SDS-PAGE and Native-PAGE gels. Proteins were detected by staining with Coomassie Brilliant Blue. (A): SDS-PAGE analysis of elution of Alt a 1_pET from Ni-NTA agarose. B) SDS-PAGE analysis of selected fractions after HPLC. C) SDS-PAGE analysis of protein from fractions 6 - 8 following concentration to 1mL in PBS D) Native-PAGE analysis of final protein product. E) Representative chromatogram from HPLC of Alt a 1 proteins. M = molecular weight markers.

Table 1: Lipids used for lipid-filter binding assays

PI-3,4-P	phosphatidylinositol-3,4-bisphosphate
PI-4,5-P	phosphatidylinositol-4,5-bisphosphate
PI-3,4,5-P	phosphatidylinositol-3,4,5-triphosphate
PI-3-P	phosphatidylinositol-3-phosphate
PI-4-P	phosphatidylinositol-4-phosphate
PI-5-P	phosphatidylinositol-5-phosphate
PI	phosphatidylinositol
C1P	ceramide-1-phosphate
LPA	lysophosphatidic acid
PA	phosphatidic acid
PS	phosphatidylserine
PE	phosphatidylethanolamine
PC	phosphatidylcholine

Table 5.1. Lipid compounds used in lipid filter binding assays. All lipids were purchased from Cayman Chemical or Avanti Polar Lipids and dissolved in DMSO.

Treatment	IL-8 (pg/mL)
PBS	ND
Alta1	415.38 ± 35.3
Alta1(His^{84}-)	41.03 ± 20.52
Alta1 (^{85}RXLR^{88})-	ND

ND = not detected, p<0.05

Table 5.2: IL-8 production from human airway cells is mediated by activity and entry of Alt a 1. Human airway epithelial cells were exposed to indicated protein or control for 24 hrs. The amount of IL-8 in the cell-free cell culture supernatant was assessed by ELISA following the manufacturer's instructions (Biolegend). Values shown as mean ± SD of 4 biological replications. ND = not detected, P<0.05.

A) >ALta1_NS (coding)

ATGGGCTCCTCTCGAGTCTCGCCAGGACACCGCATCCTGCCCTGTTACCACTGAGGGTGACT
ACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCGGAAGGAACCTACTACAACAGCCTC
GGCTTCAACATCAAGGCCACCAACGGAGGAACCCTCGACTTCACCTGCTCTGCTCAGGCCGA
TAAGCTTGAGGAC**CACAAGTGGTACTCC**TGTGGCGAGAACAGCTTCATGGACTTCTCTTTCG
ACAGCGACCGCAGCGGTCTGCTCCTGAAGCAGAAGGTTAGCGACGACATCACCTATGTCGCT
ACCGCCACTCTTCCCAACTACTGCCGCGCTGGCGGTAACGGCCCTAAGGACTTTGTCTGCCA
GGGTGTTGCCGACGCCTACATCACCCTCGTCACTCTCCCCAAGAGCTCT

B) >Alta1_NS_pET21d (amino acid)

Met A P L E S R Q D T A S C P V T T E G D Y V W K I S E F Y G
R K P E G T Y Y N S L G F N I K A T N G G T L D F T C S A Q A
D K L E D **H K W Y S** C G E N S F M D F S F D S D R S G L L L K
Q K V S D D I T Y V A T A T L P N Y C R A G G N G P K D F V C
Q G V A D A Y I T L V T L P K S S H H H A A A L E H H H H H H
Stop

C) Primer:	Sequence:
F'-Alta1_(HqvvS)	5'- gccgataagcttgaggaccaccaggtggtctcctgtggcgagaacagctt-3'
R'-Alta1_(HqvvS)	5'- aagctgttctcgccacaggagaccacctggtggtcctcaagcttatcggc-3'
F'-Alta1-(HqWYS)	5'-taagcttgaggaccaccagtggtactcctgtgg-3'
R'-Alta1-(HqWYS)	5'-ccacaggagtaccactggtggtcctcaagctta-3'
F'-Alta1-(HKvvS)	5'-gataagcttgaggaccacaaggtggtctcctgtggcgagaacagctt-3'
R'-Alta1-(HKvvS)	5'-aagctgttctcgccacaggagaccaccttgtggtcctcaagcttatc-3'
F'-Alta1-(HKWvS)	5'-tgaggaccacaagtgggtctcctgtggcgagaac-3'
R'-Alta1-(HKWvS)	5'-gttctcgccacaggagacccacttgtggtcctca-3'

Table 5.3: Construction of Alt a 1_pET21dc. The coding sequence for Alt a 1 was amplified from genomic *A.alternata* DNA and flanking NcoI/NotI restrictions sites were introduced by PCR. The PCR product was cloned into vector pET21dc and insertion was confirmed by sequencing of the purified plasmid. Mutations were introduced by Quick-Change Mutagenesis on the Alt a 1_pET21dc backbone using the primers listed in Table 3 and confirmed by sequencing of the purified plasmids. A) Native coding Alt a 1 sequence. B) Amino acid sequence of Alt a 1_pET21dc. C) Primers used for quick change mutagenesis of Alt a 1_pET21dc.

Chapter VI:

Conclusions and Future Directions

Alternaria alternata is a common aeroallergen increasingly implicated in the development and exacerbation in a variety of human airway disorders. This environmentally ubiquitous fungus has been shown through epidemiologic studies in the United States and Europe to be strongly associated with the development, persistence, and severity of allergic asthma and CRS (1). Skin reactions to the antigens of *Alternaria alternata* are associated with a high risk of allergic respiratory conditions in both children and young adults including episodes of life-threatening asthmatic attacks (2, 3). These inflammatory airway disorders have emerged as a significant public health problem and impose a major burden on health care resources for which few therapies exist (4). Alt a 1 has been identified as the major Alternaria allergen due to its specific reactivity with atopic patient sera however insufficient information exists regarding the structure and function of this clinically relevant protein. Until the studies presented here, very few studies have examined the mechanism by which Alternaria spores and its major allergen, Alt a 1, interact with the immune response to promote the pathogenesis of allergic disease. Furthermore, the role of the innate immune response, such as the response of the airway epithelium, is now regarded as an integral component to the establishment and promotion of the inflammatory response to allergens (5–7).

My work here has focused on establishing baseline models for measuring the innate immune response to Alternaria spores and Alt a 1 in both epithelial cell culture and murine systems. Additionally I have examined the biochemical properties of Alt a 1 to better understand the mechanism that leads to its recognition as the major allergen. Many of the techniques used in

my research were well established but were not designed to study the innate immune response. The most important overall aspect of the methods I utilized in all of my work is the use of live fungal spores as opposed to the commonly employed "extract." As discussed in Chapter II and Chapter IV, these prepared fungal extracts induce an artificial response due to the highly concentrated levels of intracellular enzymes, toxins, and cell debris they contain (8, 9). Although they are far easier to apply in cell and mouse models, I have made a conscientious effort to establish methods that employ the actual live organism or pure allergen protein so the most natural and likely response is measured.

The first system I employed was the use of a basic mouse model to examine both the innate and chronic immune response to Alternaria spores in-vivo. This research is described in Chapter II. Previous mouse models have employed large doses of Alternaria extract and/or the use of an adjuvant to promote a more robust immune response (10–12). One model attempted to measure the response to intranasal spore challenges but found the mice suffering from cachexia due to the high dose and discontinued the treatment (13). The previously mentioned study did find that three consecutive intranasal challenges induced a response, however our data would suggest that the large dose and frequency are not the most ideal choice for examining either type of immune response. My models have addressed these issues by not only employing fungal spores but also carefully assessing proper standards using time and dose dependent studies. I found that a single challenge with an approximate intranasal dose of 5×10^4 conidia achieves optimal results in the airway without heavily compromising other aspects of the murine system. I also discovered that detection of specific cytokines is dependent on the sampling time post challenge. This is highly important knowledge for future testing of newly discovered cytokines and their potential involvement in Alternaria signaling.

Since it is now widely recognized that chronic airway diseases are usually a result of continuous or intermittent allergen exposure via inhalation, there has been a recent focus on developing chronic allergen exposure models without arbitrary sensitization schemas (14). The chronic model I developed adopts this theory and features a continuous inhalation model in which the mice exhibit signs of chronic Alternaria exposure without prior sensitization with an adjuvant. The results of our chronic model also compare the murine lung response to Alternaria spores and extract. I showed the extract to be far more immunostimulatory in terms of inflammatory cell recruitment, however the spores were similar if not more potent than the extract in terms of cytokine production and levels of total serum IgE. This suggests the extract's robust components may have a more damaging effect on the tissue pathology and greater ability to recruit a repertoire of inflammatory cells, but the spores are more relevant at the molecular level regarding cytokine production and specific cell recruitment. The innate model was a newly designed system that has not before been used for studying the response to a single challenge of Alternaria spores. I first employed the model to establish baseline readings for the airway response to a single intranasal challenge of spores. This included measuring weight change, cytokine production, and cell infiltration. I then tested the effectiveness of the model for distinguishing changes in the innate immune response when challenged with the wild-type fungus or an Alt_a_1 deletion mutant (*Δalta1*). Previous results in epithelial cells (see Chapter IV) suggested the deletion mutant would be less immunogenic, but measuring differences in a single cell line is much less complicated than an entire mammalian system. Intriguingly our model was effective for testing differences and I found a lower total cell count in the bronchoalveolar lavage and decreased levels of the cytokines CXCL1 and TSLP in the mice treated with the *Δalta1* compared to the wild type. It is important to also note that detecting the

differences in cytokine levels between the wild-type and *Δalta1* was contingent on the intranasal dose and time post-inoculation. These attributes must be taken into serious consideration in the future use of this single-challenge model. Overall, the results of Chapter II establish an easy-to-use murine model, which will be an incredibly useful tool for studying the innate immune response of the airway to fungal spores.

In Chapter III, I delve more deeply into the potential innate signaling cascade Alternaria is activating. The interaction of fungal spores, and possibly allergens themselves, with the Toll-like receptors (TLRs) on the surface of innate immune cells such as alveolar macrophage and epithelial cells, and subsequent activation of their downstream signaling cascades determines the effectiveness of eliminating fungal material (15). While the epithelium is the largest tissue surface area of the lung, the alveolar macrophage is the most abundance resident immune cell and serves a critical role in shaping the adaptive immune response (16). Thus I chose to examine the role of two important kinases involved in TLR signaling, IRAK-1 and IRAK-M in both macrophage cells and a chronic mouse model. Epidemiological evidence suggests polymorphisms in the IRAK-1 gene may lead to an increase in inflammatory disorders (17). Alternatively, IRAK-M is generally considered a negative activator of innate immune signaling in which overexpression may lead to deactivation of the innate immunity signaling process and an ineffective response to invading pathogens (18). Interestingly, it has been observed that IRAK-M primarily affects the alternative activation pathway in macrophages which is more frequently connected with chronic airway disorders (19, 20). Many studies have already illustrated the effects of a dysregulated signaling pathway and have spurred interest in the exploration of novel therapeutic targets that include suppression and neutralization of inflammasome activation through these kinase signaling cascades (21).

Thus in order to contemplate a vigorous in-depth analysis of signaling, I first established if expression of either IRAK-1 or IRAK-M was changed in macrophage cells in response to stimulation with Alternaria antigen. Using real-time PCR analysis, I found that IRAK-M expression was downregulated while IRAK-1 was upregulated as would be predicted in a normal and functional innate immune response. Before pursuing avenues using IRAK-1 and IRAK-M deficient mice to better investigate the role of each kinase, I performed a comprehensive test of the cell culture media to find which cytokines were being produced in normal cells to use as my baseline for the knock-out cell lines. I found CXCL1, MCP-1, and MCSF to be produced at the highest level in response to Alternaria antigens. I then performed a time-course and examined the transcriptional level of CXCL1 and confirmed it increases consistently over a 24hr period. With this baseline established, I utilized my chronic inhalation mouse model with IRAK-$1^{-/-}$ and IRAK-$M^{-/-}$ mice and found that the deletion of IRAK-1 caused the mice to overly express IL-13 in response to Alternaria antigens. Further analysis of the IRAK-$1^{-/-}$ samples showed evidence that global changes in pro-inflammatory gene expression was much greater in IRAK-$1^{-/-}$ lung tissue than in wild-type mice. Histopathological evidence confirmed these results with IRAK-$1^{-/-}$ tissue visually confirming greater cell infiltration. The IRAK-$M^{-/-}$ mice did not reveal an aberrant response compared to the wild-type samples. For future studies, I would highly suggest utilizing the innate mouse model with the IRAK-$1^{-/-}$ mice and challenging with live spores or Alt a 1. This will confirm the spores or direct allergen are also capable of inducing the same response as the extract and a more comprehensive transcriptional analysis may give insight into specific cytokines controlled through IRAK-1 signaling. Also, the role of IRAK-M should be more deeply analyzed to verify its involvement in signaling. Overall, Chapter III provides preliminary

data for detailed examination of the role of IRAK-1 and IRAK-M in innate immune signaling to Alternaria.

Since Alt a 1 has been described as the major allergen of Alternaria and I have a declared interest in establishing that Alternaria spores induce a profoundly more natural innate immune response, Chapter IV examines the role of Alt a 1 at the organismal level. I chose to use an Alt a 1 deletion mutant (*Δalta1)* as well as an Alt a 1 overexpression mutant (Alta1+) to examine how each differentially stimulated the innate immune response compared to the wild type. The construction of the fungal mutants with deletion and overexpression of Alt a 1 were previously described (22). However, a substantial examination of their phenotype was not yet completed. I confirmed the desired mutants were indeed expressionless or overexpressing Alt a 1 by qRT-PCR analysis, Western blot, and Alt a 1 specific ELISA. Before any immunological testing was completed, I performed a comprehensive analysis of the mutants to confirm there were no outstanding morphological or physiological attributes that could influence the immune response. This included visually examining the fungal spores, calculating germination rate, and analyzing of growth on various carbon sources. I found no difference in the measured characteristics between the mutants and the wild type, suggesting Alt a 1 does not affect the phenotype of Alternaria species. I then tested the response of human airway epithelial cells to the wild type and mutant spores by measuring the release of pro-inflammatory cytokines into the cell culture supernatant. I found the *Δalta1* mutant was unable to induce cytokines near levels of the wild type while the Alta1+ overexpression mutant was heavily more immunogenic than the wild type. I also tested the response in primary human airway cells from a normal donor. Here I not only confirmed the previous results but also show that neither the wild type nor mutant strains are cytotoxic towards human airway cells. Further analysis of innate antifungal signaling markers

revealed multiple signaling targets which may be involved in Alt a 1's recognition by epithelial cells.

The data presented in Chapter IV provides a solid account for the importance of Alt a 1 in innate immune signaling. There are two experiments I would recommend as the next avenue of exploration related to this project. First, the results of our morphological analysis suggests Alt a 1 is not necessary for normal germination or growth of the organism and thus these results do not shed light on Alt a 1's potential function within Alternaria species such as nutrient acquisition or degradation. Recent studies have shown the apparently abundant localization of Alt a 1 within the cell wall of the spore (23). A proteomics based experiment could examine changes during germination of the mutants compared to the wild type and provide a high throughput representation of specific pathways within Alternaria which are affected by Alt a 1. Additionally, an immunohistochemical approach using an Alt a 1 specific antibody could be used to track the localization and release of Alt a 1 during the germination process. The second project that is now necessary is a full mouse model using the single-challenge method I have developed in Chapter II. This will provide the necessary in-vivo data to confirm the importance of Alt a 1 in a spore model and strengthen Alt a 1 as a potential therapeutic target.

Continuing with characterization of the Alt a 1 allergen, Chapter V studies the biochemical properties of the protein that may be linked to its ability to enter and stimulate airway cells. Some work has predicted Alt a 1 to have enzymatic activity and overall protease activity of Alternaria extract has been implicated in activation of the epithelium (24–27). Furthermore, enzymatic activities of other allergens has been linked to cytokine production in epithelial cells (28–30). Additional studies have shown various allergens have the ability to enter airway cells, which may be mediated by a lipid dependent mechanism (31–33). Interestingly,

collaborative work previously completed in our lab helped identify a unique lipid binding motif in fungal effector proteins which helps the proteins bind and enter host cells (34). This motif, termed “RXLR”, mediates protein binding to lipid PI3P, abundantly found on the cell surface of epithelial cells, and triggers endocytosis.

The premise of Chapter V was to identify the enzymatic activity as well as potential lipid-binding motif of Alt a 1 and utilize molecular tools and recombinant protein production to test the importance of these characteristics in the epithelial cells response to Alt a 1. The first part of Chapter V establishes that rAlt a 1 alone can induce an immune response similar to that of the wild type Alternaria spores. This was confirmed in both epithelial cells and a chronic intranasal mouse model by measuring pro-inflammatory cytokine production and inflammatory cell recruitment. This was an essential task given that the immune potential of Alt a 1 had never been explored despite its known reactivity to atopic patient sera. Once I verified the immunogenicity of Alt a 1, I examined the protein for esterase activity and lipid-binding capabilities. An esterase activity test confirmed Alt a 1 is indeed an esterase. A lipid blot for testing of thirteen ubiquitous lipids found Alt a 1 preferentially binds PI3P. With this knowledge, I created recombinant Alt a 1 proteins with selected mutations to modify enzymatic activity and lipid-binding capacity. Changing the single histidine residue to an asparagine rendered the esterase catalytic triad non-functioning, which was confirmed by an esterase assay. Unfortunately, the histidine is directly before the predicted RXLR-like motif of KWYS. Mutation of this motif also abolished esterase activity, but this was likely due to introduction of such a severe mutation within the protein. Fortunately, I was still able to confirm that the histidine mutant, with an intact predicted RXLR motif, could still bind PI3P while the RXLR mutant (KWYS → qvvS) could not.

Using fluorescently tagged versions of the native, histidine, and RXLR mutant proteins, I tested their capability to enter human airway cells to determine if this was controlled by esterase activity or lipid-binding. I found that wild type and histidine mutants were able to enter airway cells and collect within unknown endosomal vesicles while the RXLR mutant could not. Further analysis also showed prolonged exposure to Alt a 1 lead to membrane degradation which was abolished by reducing enzymatic activity by incubation at 4°C. In all, this data would suggest that the predicated lipid binding motif of Alt a 1 is required for entry into human airway cells but damage to epithelial tissue is a results of continued exposure to Alt a 1's esterase activity. This theory is confirmed in a sensitized mouse model. The ability for Alt a 1 to cross the epithelium and lead to subsequent mast cell degranulation is dependent on its predicted RXLR-like motif.

The final portion of Chapter V discusses the development of a detailed production plan for large amounts of highly pure recombinant protein for use in immunological studies. The method addresses issues regarding Alt a 1's solubility as well as the importance of obtaining ultra pure protein through use of HPLC and endotoxin removal. The development of this method was absolutely necessary for the production of acceptable proteins for use in cell culture and mouse model and will be a another valuable tool in the future of this work.

Chapter V provides a substantial amount of information regarding Alt a 1's biochemical properties and describes an important mechanism for allergen entry. Most importantly, the work described in Chapter V sets the foundation for similar exploration in all known fungal allergens. As outlined in Table 6.1, allergens from insects, plants, and other fungi all contain predicted RXLR-like motifs within their amino acid sequence. The methods and techniques used in Chapter V could easily be employed to test importance of lipid binding and cell entry for these other well-known allergens, leading to a comprehensive understanding of allergen entry.

Additionally, an easily reproducible mouse model of *A.fumigatus* Asp f 1 sensitization has already established its ability to test mutant proteins as potential candidates for immunotherapy and could be used as a model for testing Alt a 1 and other allergens in vivo (35). Most importantly, since many allergic individuals are sensitized to more than one organism, discovering a mechanism that blocks entry, and hopefully symptoms, of multiple allergens at once would be a breakthrough for therapeutic drug development.

Understanding the complexity of the innate immune response to common airborne fungi may ultimately lead to efficient therapies against aeroallergens. As the prevalence of allergic asthma has increased over the past few decades, the medical options have focused on desensitization treatments such as sublingual (SLIT) or subcutaneous immunotherapy (SCIT). There is acceptable evidence that shows that children with allergies to grass pollen, Alternaria, and house dust mite can be benefit from SCIT therapy (36). However, very few controlled studies have examined the efficacy and safety of immunotherapy since there is a lack of standardized extracts for the diagnosis and actual treatment (37). These methods have shown great promise, but due to the high cost and multiple treatments in order to complete the therapy, they may not be justifiable to all patients. Moreover, these therapeutic approaches have failed to shed light on the basic immunological mechanisms the allergen is activating. The desensitization process is simply dampening the disease, or "hiding" it from the immune system without much known on how they are actually functioning. This does not help advance medical treatments nor does it help researchers better understand the complex mechanisms involved in the allergic response.

It is my hope that the work I have presented here is the beginning of a thorough and enlightening era of allergen research that focuses on a better understanding of the innate immune

response and how the mechanisms of this response can be targeted for the development of safer and more economical alternatives to the current therapies. It is increasingly clear that the prevalence of allergies and asthma will continue to rise and thus broadening our understanding of the mammalian innate immune response to common aeroallergens will help us more quickly and effectively find a desirable cure for allergic airway disorders.

References:

1. Knutsen, A. P., R. K. Bush, J. G. Demain, D. W. Denning, A. Dixit, A. Fairs, P. A. Greenberger, B. Kariuki, H. Kita, V. P. Kurup, R. B. Moss, R. M. Niven, C. H. Pashley, R. G. Slavin, H. M. Vijay, and A. J. Wardlaw. 2012. Fungi and allergic lower respiratory tract diseases. *J. Allergy Clin. Immunol.* 129: 280–291.
2. Andersson, M., S. Downs, T. Mitakakis, J. Leuppi, and G. Marks. 2003. Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitized children. *Pediatr Allergy Immunol* 14: 100–105.
3. Downs, S. H., T. Z. Mitakakis, G. B. Marks, N. G. Car, E. G. Belousova, J. D. Leüppi, W. Xuan, S. R. Downie, A. Tobias, and J. K. Peat. 2001. Clinical importance of Alternaria exposure in children. *Am. J. Respir. Crit. Care Med.* 164: 455–459.
4. Jackson, D. J., A. Sykes, P. Mallia, and S. L. Johnston. 2011. Asthma exacerbations: origin, effect, and prevention. *J. Allergy Clin. Immunol.* 128: 1165–1174.
5. Barrett, N. A., and K. F. Austen. 2009. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* 31: 425–437.
6. Hammad, H., and B. N. Lambrecht. 2008. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 8: 193–204.
7. Kauffman, H. F. 2003. Interaction of environmental allergens with airway epithelium as a key component of asthma. *Curr Allergy Asthma Rep* 3: 101–108.
8. Breitenbach, M., and B. Simon-Nobbe. 2002. The allergens of Cladosporium herbarum and Alternaria alternata. *Chem. Immunol.* 81: 48–72.
9. Vailes, L., S. Sridhara, O. Cromwell, B. Weber, M. Breitenbach, and M. Chapman. 2001. Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. *Journal of Allergy and Clinical Immunology* 107: 641–646.
10. Ahn, B.-H., Y.-H. Park, and S.-H. Shin. 2009. Mouse model of Aspergillus and Alternaria induced rhinosinusitis. *Auris Nasus Larynx* 36: 422–426.
11. Havaux, X., A. Zeine, A. Dits, and O. Denis. 2005. A new mouse model of lung allergy induced by the spores of Alternaria alternata and Cladosporium herbarum molds. *Clin. Exp. Immunol.* 139: 179–188.
12. Doherty, T. A., N. Khorram, K. Sugimoto, D. Sheppard, P. Rosenthal, J. Y. Cho, A. Pham, M. Miller, M. Croft, and D. H. Broide. 2012. Alternaria Induces STAT6-Dependent Acute Airway Eosinophilia and Epithelial FIZZ1 Expression That Promotes Airway Fibrosis and Epithelial Thickness. *Journal of Immunology (Baltimore, Md.: 1950)* .
13. Denis, O., S. van den Brûle, J. Heymans, X. Havaux, C. Rochard, F. Huaux, and K. Huygen. 2007. Chronic intranasal administration of mould spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodelling. *Immunology* 122: 268–278.
14. Nials, A. T., and S. Uddin. 2008. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 1: 213–220.
15. Akira, S. 2003. Toll-like receptor signaling. *J. Biol. Chem.* 278: 38105–38108.
16. Byers, D. E., and M. J. Holtzman. 2011. Alternatively activated macrophages and airway disease. *Chest* 140: 768–774.
17. Arcaroli, J., E. Silva, J. P. Maloney, Q. He, D. Svetkauskaite, J. R. Murphy, and E. Abraham. 2006. Variant IRAK-1 haplotype is associated with increased nuclear factor-kappaB activation and worse outcomes in sepsis. *Am. J. Respir. Crit. Care Med.* 173: 1335–1341.

18. Ringwood, L., and L. Li. 2008. The involvement of the interleukin-1 receptor-associated kinases (IRAKs) in cellular signaling networks controlling inflammation. *Cytokine* 42: 1–7.
19. Li, L., J. Su, and Q. Xie. 2007. Differential regulation of key signaling molecules in innate immunity and human diseases. *Adv. Exp. Med. Biol.* 598: 49–61.
20. Kurowska-Stolarska, M., B. Stolarski, P. Kewin, G. Murphy, C. J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I. B. McInnes, D. Xu, and F. Y. Liew. 2009. IL-33 Amplifies the Polarization of Alternatively Activated Macrophages That Contribute to Airway Inflammation. *J Immunol* 183: 6469–6477.
21. Henderson, C., and R. Goldbach-Mansky. 2010. Monogenic IL-1 Mediated Autoinflammatory and Immunodeficiency Syndromes: Finding the Right Balance in Response to Danger Signals. *Clin Immunol* 135: 210–222.
22. Babiceanu, M. 2011. Analysis of the allergenic potential of the ubiquitous airborne fungus Alternaria using bioinformatics. .
23. Twaroch, T. E., E. Arcalís, K. Sterflinger, E. Stöger, I. Swoboda, and R. Valenta. 2011. Predominant localization of the major Alternaria allergen Alt a 1 in the cell wall of airborne spores. *Journal of Allergy and Clinical Immunology* .
24. Sáenz-de-Santamaría, M., J. A. Guisantes, and J. Martínez. 2006. Enzymatic activities of Alternaria alternata allergenic extracts and its major allergen (Alt a 1). *Mycoses* 49: 288–292.
25. Boitano, S., A. N. Flynn, C. L. Sherwood, S. M. Schulz, J. Hoffman, I. Gruzinova, and M. O. Daines. 2011. Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. *Am. J. Physiol. Lung Cell Mol. Physiol.* 300: L605–614.
26. Shin, S.-H., Y.-H. Lee, and C.-H. Jeon. 2006. Protease-dependent activation of nasal polyp epithelial cells by airborne fungi leads to migration of eosinophils and neutrophils. *Acta Otolaryngol.* 126: 1286–1294.
27. Kouzaki, H., S. M. O'Grady, C. B. Lawrence, and H. Kita. 2009. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J. Immunol.* 183: 1427–1434.
28. Borger, P., G. H. Koëter, J. a. B. Timmerman, E. Vellenga, J. F. C. Tomee, and H. F. Kauffman. 1999. Proteases from Aspevgillus fumigatus Induce Interleukin (IL)-6 and IL-8 Production in Airway Epithelial Cell Lines by Transcriptional Mechanisms. *Journal of Infectious Diseases* 180: 1267–1274.
29. Chen, J.-C., J.-G. Chuang, Y.-Y. Su, B.-L. Chiang, Y.-S. Lin, and L.-P. Chow. 2011. The Protease Allergen Pen c 13 Induces Allergic Airway Inflammation and Changes in Epithelial Barrier Integrity and Function in a Murine Model. *J. Biol. Chem.* 286: 26667–26679.
30. Kauffman, H. F., M. Tamm, J. A. B. Timmerman, and P. Borger. 2006. House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. *Clin Mol Allergy* 4: 5.
31. Joenväärä, S., P. Mattila, J. Renkonen, A. Mäkitie, S. Toppila-Salmi, M. Lehtonen, P. Salmi, S. Lehti, J. Mäkinen, R. Sormunen, T. Paavonen, and R. Renkonen. 2009. Caveolar transport through nasal epithelium of birch pollen allergen Bet v 1 in allergic patients. *J. Allergy Clin. Immunol.* 124: 135–142.e1–21.
32. Renkonen, J., P. Mattila, S. Lehti, J. Mäkinen, R. Sormunen, T. Tervo, T. Paavonen, and R. Renkonen. 2009. Birch pollen allergen Bet v 1 binds to and is transported through conjunctival epithelium in allergic patients. *Allergy* 64: 868–875.

33. Mogensen, J. E., M. Ferreras, R. Wimmer, S. V. Petersen, J. J. Enghild, and D. E. Otzen. 2007. The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. *Biochemistry* 46: 3356–3365.
34. Kale, S. D., B. Gu, D. G. S. Capelluto, D. Dou, E. Feldman, A. Rumore, F. D. Arredondo, R. Hanlon, I. Fudal, T. Rouxel, C. B. Lawrence, W. Shan, and B. M. Tyler. 2010. External Lipid PI3P Mediates Entry of Eukaryotic Pathogen Effectors into Plant and Animal Host Cells. *Cell* 142: 284–295.
35. Alvarez-García, E., E. Batanero, R. García-Fernández, M. Villalba, J. G. Gavilanes, and A. Martínez-del-Pozo. 2010. A deletion variant of the Aspergillus fumigatus ribotoxin Asp f 1 induces an attenuated airway inflammatory response in a mouse model of sensitization. *J Investig Allergol Clin Immunol* 20: 69–75.
36. Larenas-Linnemann, D. E. S., D. R. Pietropaolo-Cienfuegos, and M. A. Calderón. 2011. Evidence of effect of subcutaneous immunotherapy in children: complete and updated review from 2006 onward. *Ann. Allergy Asthma Immunol.* 107: 407–416.e11.
37. Martínez-Cañavate Burgos, A., A. Valenzuela-Soria, and A. Rojo-Hernández. 2007. Immunotherapy with Alternaria alternata: present and future. *Allergol Immunopathol (Madr)* 35: 259–263.

Allergen	Amino acid sequence
PLA 2 (bee venom)	MQVVLGSLFLLLLSTSHGWQIRDRIGDNELEERIIYPGTLWCGHGNKSSGPN ELGRFKHTDACCRTHDMCPDVMSAGESKHGLTNTASHTRLSCDCDDKFYD CLKNSADTISSYFVGKMYFNLIDTKCYKLEHPVTGCGERTEGRCLHYTVDK SKPKVYQWFDLRKY
Phl p 6 (timothy grass pollen)	MVAMFLAVAVVLGLATSPTAEGGKATTEEQKLIEDVNASFRAAMATTANV PPADKYKTFEAAFTVSSKRNLADAVSKAPQLVPKLDEVYNAAYNAADHAA PEDKYEAFVLHFSEALRIIAGTPEVHAVKPGA
Try p 2 (*T.putrescentiae*, dust mite allergen)	MKFLILFALVAVAAAGQVKFTDCGKKEIASVAVDGCEGDLCVIHKSKPVHV IAEFTANQDTCKIEVKVTGQLNGLEVPIPGIETDGCKVLKCPLKKGTKYTMN YSVNVPSVVPNIKTVVKLLATGEHGVLACGAVNTDVKP
Der p 2 (*D.pteronyssinus*, dust mite allergen)	MMYKILCLSLLVAAVARDQVDVKDCANHEIKKVLVPGCGSEPCIIHRGKPF QLEAVFEANQNTKTAKIEIKASIDGLEVDVPGIDPNACHYMKCPLVKGQQY DIKYTWNVPKIAPKSENVVVTVKVMGDDGVLACAIATHAKIRD
Pen c 1 (*P.citrinum*, major allergen)	MGFLKVLATSLATLAVVDAGTLLTASNTDAVIPSSYIVVMNDDVSTAEFNT HREWATNVHARLSRRKNGETGPGKHFEINGLKGYTASFDESTAKDIANDPA VKYIEPDMIVNATANVVQSNVPSWGLARISSKRTGTTSYTYDSTAGEGVVF YGVDTGIDISHSDFGGRAKWGTNVVDNDNTDGNGHGTHTASTAAGSKYGV AKKATLVAVKVLGADGSGTNSGVISGMDWAVKDAKSRGANGKYVMNMS LGGEFSKAVNDAAANVVKSGIFLSVAAGNEAENASNSSPASAAEVCTIAAST STDGSASFTNFGSVVDLYAPGQSITAAYPGGGSKTLSGTSMAAPHVAGVAA YLMALEGVSAGNACARIVQLATSSISRAPSGTTSKLLYNGINV
Cla h 8 (*C.herbarum*, major allergen)	MPGQQATKHESLLDQLSLKGKVVVVTGASGPKGMGIEAARGCAEMGAAV AITYASRAQGAEENVKELEKTYGIKAKAYKCQVDSYESCEKLVKDVVADFG QIDAFIANAGATADSGILDGSVEAWNHVVQVDLNGTFHCAKAVGHHFKER GTGSLVITASMSGHIANFPQEQTSYNVAKAGCIHMARSLANEWRDFARVNSI SPGYIDTGLSDFVPKETQQLWHSMIPMGRDGLAKELKGAYVYFASDASTYT TGADLLIDGGYTTR

Figure 6.1. Predicted RXLR-like motifs in other known allergens. Amino acid sequences of common allergens were examined for RXLR-like motifs following previously described rules. Residues highlighted in yellow are considered to be the most likely motifs within each sequence.