ANALYSIS OF THE ALLERGENIC POTENTIAL OF THE UBIQUITOUS AIRBORNE FUNGUS ALTERNARIA USING BIOINFORMATICS

Mihaela Corina Babiceanu

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
Genetics, Bioinformatics and Computational Biology

Christopher B. Lawrence, Chair
David R. Bevan
Liwu Li
Joao C. Setubal

June 15th, 2011
Blacksburg, Virginia

Key words: Alternaria, allergen, Alt a 1

ABSTRACT

Analysis of the allergenic potential of the ubiquitous airborne fungus Alternaria using bioinformatics

Mihaela Corina Babiceanu

Among the environmental airborne fungi one of the most common is *Alternaria alternata.* From a clinical perspective Alternaria has long been associated with IgE-mediated, histamine-dependent mold allergy, allergic rhinitis, chronic rhinosinusitis (CRS), and asthma. Recently it has been shown that an abnormal immunological response to Alternaria most likely contributes to the pathogenesis of upper respiratory airway disorders. In this body of work, we present for the first time results of several sets of experiments including, 1) the analysis of *A. alternata* spore germination expressed sequence tags (ESTs), 2) the survey of global allergen homologues in fungal genomes, and 3) the first microarray experiment investigating airway epithelial cell responses to spores of this fungus.

In the first project, the analysis of the EST dataset offered a first look into the gene content of A. alternata and represents the beginning of future research of this ubiquitous fungus. Annotation and classification of ESTs revealed a number of genes that could be involved in the immunomodulation process of the human immune response toward fungi. We also discovered that the majority of known allergens are expressed during the spore germination phase of *A. alternata*.

For investigating the allergenic potential of fungi in the second project we developed a whole genome approach by querying fungal genome sequences (*A. alternata*, *A. brassicicola*, and *Aspergillus fumigatus*) with a database of all known allergenic proteins from a taxonomically diverse group of organisms. Interestingly, we identified homologues of diverse types of allergens in these fungal genomes and also many homologues of allergens from other organisms including those from pollen, insects, and venoms.

Finally, in the third project we investigated global gene expression changes of human airway cells in response to *A. alternata* and an Δ*alt a 1* deletion mutant. We found that wild type

Alternaria spores induced significant changes in gene expression patterns in human airway epithelial cells, especially known immune response genes. Furthermore, results of these analyses revealed that Alt a 1 is a major factor in inducing epithelial inflammatory responses.

ACKNOWLEDGEMENTS

Apart from my work, the success of this project depends largely on the encouragement and guidelines of many others. I take this opportunity to express my gratitude to the people who gave me the possibility to complete this thesis.

I owe my deepest gratitude to my mentor Christopher B. Lawrence whose help, stimulating suggestions, guidance and encouragement helped me in all the steps that I have to take in research and in writing this thesis. Our talks enabled me to develop an understanding of a particular subject and realize how a true scientist thinks. I'll always be grateful for the freedom that he offered me in research, for our talks about the shared interests outside science and for the friendship that we developed in all these years.

I would like to show my greatest appreciation to my doctoral committee: David R. Bevan, Liwu Li and Joao Setubal. I can't thank them enough for their tremendous support and help. I felt motivated and encouraged every time I spoke with them. Without their guidance this project would not have materialized.

I want to thank Lawrence Lab colleagues who became my friends Amanda Rumore, Prathyusha Kolconda, Sang-Wook Park and Ha X. Dang for supporting me in my research work Also, I want to special recognize the help and support that I received from Nalvo Almeida from Setubal Lab.

I wish to express my gratitude to my family for their understanding and love, through the duration of my PhD studies: to my smart and beautiful daughter Laura, who, one day, I hope will understand that sometimes I was not available for her as much as she and I would have wanted, and to my husband whose success and determination inspired me.

I want to specially thank my friend Diego Cortes, with whom I have had the pleasure of sharing classes and working with and whose words and actions over the years, has taught me a lot about myself and the incomprehensible life's paths. Also, I want to thank to my friend Juan Ruiz from whom I hope to have learned about perseverance, resilience and a little bit of Spanish. I had the chance here, in Blacksburg, to have around me a very eclectic, cosmopolite and amazing group of ladies. These girls are smart, beautiful, sarcastic, deep and funny and they were

the daily support that anybody can wish for. Susan Bland, the word's magician, not only helped me learn about and understand better a new world: English, but also she made me discovered "the" new world: America. Shelana Ryan with her sweetness and innocence made me reevaluate my cynical views of life. Isabel Osorio da Fonseca thought me about lack of barriers between countries, languages and universality of human sources of happiness. Anne-Collins Albimino showed me what effortless grace and refinement is. Ella Kromin who introduced me to a world that mixes very successfully practicality with sophistication. Becky Fritz showed me that a pragmatic, efficient person can be expressive. All these people and so many others have touched my life in one way or the other and are very important to me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABPA	Allergic Bronchopulmonary Aspergillosis
CD	Cluster of Differentiation
CEBPD	CCAAT/enhancer-binding protein delta
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
CRS	Chronic Rhinosinusitis
CXCL1	Chemokine ligand 1
CXCL10	Chemokine ligand 10
CXCL11	Chemokine ligand 11
CXCL6	Chemokine ligand 6
EST	Expressed Sequence Tag
GalMM	Galactose Minimal media
GluMM	Glucose Minimal media
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL13	Interleukin 13
IL32	Interleukin 32
IL4	Interleukin 4
IL5	Interleukin 5
IL7R	Interleukin 7 receptor
IL8	Interleukin 8
MBP	Major basic protein
NFKB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PBMC	Polymorphic Blood Mononuclear Cells
PDA	Potato Dextrose Agar
RT-PCR	Real Time- Polymerase Chain Reaction
STAT2	Signal transducer and activator of transcription 2
Th2 cell	T helper cell type 2
TRL3	Toll-Like receptor 3

CHAPTER 1

SPECIFIC AIMS

The main purpose of this project is to expand our knowledge about the complex relationship between humans and the ubiquitous airborne fungus, *Altenaria alternata*.

Based on what is known at this point this interaction is multifaceted and not entirely understood. Substantial clinical and scientific evidence suggests that *A. alternata* plays a significant role in the onset and development of human airway disorders such as allergic rhinitis, atopic asthma, and chronic rhinosinusitis (CRS). The primary hypothesis we are investigating is that immunomodulatory enzymes secreted by the clinically important fungus, Alternaria, are a major trigger that results in the induction of strong Th2 adaptive immune responses in the airways. This research consists of three specific aims described in more details below.

Aim 1. Analysis of *Alternaria alternata* spore germination Expressed Sequence Tags (ESTs). The primary objective of this specific aim is to further our knowledge of the molecular events occurring during spore germination of *A. alternata* in an *in vitro* condition of pathological relevance. Spores will be germinated in the presence of mucin, a common component of the epithelial lining of airways. Ultimately this aim will result in the development of an expanded Alternaria proteome database by combining existing Alternaria genomic resources with these additional expressed sequence tags (ESTs) derived from Alternaria grown in the presence of mucin.

One other goal of the *A. alternata* EST project is to characterize both putative secreted and membrane-bound proteins that may be involved in the interaction between the fungus and the human epithelial cells. The multitude of data sets that are going to be derived from these analyses, based on different type of selections and using different methods of characterization, will provide important information about the genes that we intend to follow up with laboratory techniques like RT-PCR on the selected fungal genes. Bioinformatics will be performed to assign putative functions to ESTs using a combination of Blast and analyses of predicted functional domains of translated proteins. Specific attention will be given to comparing conceptually

translated ESTs to known allergens and other known immunostimulatory proteins from fungi as well as other organisms. We are also interested in identifying and characterizing *A. alternata* sequences that are unique compared to the genome sequence of the plant pathogen, *A. brassicicola* using Blast algorithms.

Aim 2. Investigating the allergenic potential of *Alternaria alternata in silico*. The primary objective of this aim is to identify and characterize the repertoire of allergen homologues of *A. alternata* using bioinformatics. These results will then be compared to repertoires of allergen homologues identified in *A. brassicicola* and *A. fumigatus*. *A. brassicicola* will be used because of the close resemblance of the two Alternaria genomes and because there are no known reports of this plant pathogenic species being allergenic to humans although an Alt a 1 homolog has been previously identified (1). *A. fumigatus* will be used as a reference potent allergenic fungus with a genome sequence available.

The first analysis that will be performed is to characterize allergen homologues found in the predicted *A. brassicicola* proteome and the hypothetical proteome encoded by the *A. alternata* ESTs using a database of over 1400 allergens from a variety of organisms including fungi that are publicly available from the Allergen Online database (http://www.allergenonline.org/) as query. Specific analyses using known fungal allergen proteins, including several previously described *A. alternata* allergens, will be used as query sequences against the predicted *A. brassicicola* proteome and against the *A. alternata* unisequence EST set. The hits and their level of identity are going to be transformed into a heat map depicting the level of homology of *A. alternata* and *A. brassicicola* genes with known fungal allergens. One additional result from these studies will be the identification of novel allergen homologues in the *A. fumigatus* proteome that have yet to be discovered.

Aim 3. The role of Alt a 1 in Alternaria biology. In order to characterize the biological role of the Alt a 1 major allergen in *A. alternata*, we will perform *in silico* analysis using protein modeling software and more in depth informatic analyses.

We will also produce and characterize different types of fungal mutants.. Using fungal molecular biology techniques we intend to produce three classes of mutants: a Δ*alt a 1* deletion mutant and corresponding, restored, complementation mutant and a mutant overexpressing the

Alt a 1 protein. Various phenotypic analyses will be performed using this collection of strains such as growth and germination rates, general morphology, and substrate utilization. Furthermore the human respiratory epithelial cell line (BEAS-2B) will be treated with the wild type *A. alternata* spores and the Δa*lt a 1* deletion mutant spores. Subsequently, RNA will be isolated from the human cells and used in microarray analyses in order to determine if any human genes are differentially expressed in lung cells due to the presence of the wild type fungus itself or the absence of a*lt a 1* in the fungus.

With these approaches we will attempt to answer or shed light on several questions:

1. What *A. alternata* proteins may play a significant role in the immune response developed following Alternaria exposure?

2. Which of these proteins are important in triggering the immune reactions in airway epithelial cells and other cell types exposed to Alternaria? Furthermore, which of these proteins are responsible for eosinophilic Th2-like airway inflammation?

3. Are *A. alternata* proteins playing a role in the modulation of allergic immune response to different triggers like fungi allergens or to other type of allergens?

4. Is *A. alternata* playing a role in allergenicity by itself, independently or at least is playing an adjuvant role in the allergenicity and allergenic response to other triggers?

5. Is the Alt a 1 major allergen important for basic biological and/or developmental aspects of the fungus?

6. Is Alt a 1 playing another immunological role in addition to the known IgE-binding function of this protein?

Our attempt to further our knowledge of these processes and mechanisms will provide a better understanding of the pathophysiology of airway disorders. Identification of immunostimulatory/immunomodulatory fungal proteins and elucidating the mechanisms of persistent airway inflammation in the majority of airway disorders due to recognition and response to these proteins will help to provide specific and effective therapies and novel diagnostic approaches useful at the point of care.

CHAPTER 2

Background and Significance

Introduction

Human airways are continuously exposed to ubiquitous environmental fungi (molds) and very often occur at a higher level and for a longer duration than that of pollen or other known airborne allergens. One of the most common of these environmental fungi is *Alternaria alternata.* From a clinical perspective, Alternaria has long been associated with IgE-mediated, histamine-dependent mold allergy, allergic rhinitis, and asthma. Increasing clinical, immunological, molecular, and biochemical evidence strongly supports the role of Alternaria in the etiology and pathology of another major respiratory disorder, chronic rhinosinusitis (CRS) (2-4).

Fungal spores and hyphae are ubiquitous in the environment, and respiratory exposure to these structures is almost constant throughout the year. The most common airborne fungi include species found within the genera Cladosporium, Alternaria, Penicillium, and Aspergillus, although a multitude of other species are commonly found in any type of environment and climate (5-7). Other fungi such as Candida and Trichophyton are natural skin or gut inhabitants. House dust mites, cat dander or grass pollen are also common environmental sources of allergenic proteins, but fungi alone have the ability to actively germinate and secrete additional molecules in the respiratory tract. Fungi may also infect the host skin or at least attempt to colonize the respiratory tract. This is the greatest difference and probably the most important factor in the pathologic potential and capabilities of fungi in regards to allergic inflammation. Thus, it is possible that fungi have a much greater impact on an individual in terms of triggering host immune responses against these potential pathogens from an allergenic perspective while concomitantly producing non-allergenic toxins, enzymes, and other pro-inflammatory factors that may play an accessory role in triggering and exacerbating chronic inflammatory disorders by stimulating innate immune responses and influencing the development of adaptive Th2 immunity.

Alternaria alternata

Alternaria is a cosmopolitan dematiaceous (phaeoid) fungus commonly isolated from plants, soil, food, and indoor air environments. The production of the dark pigment melanin is one of its major characteristics (Figure 1.1).

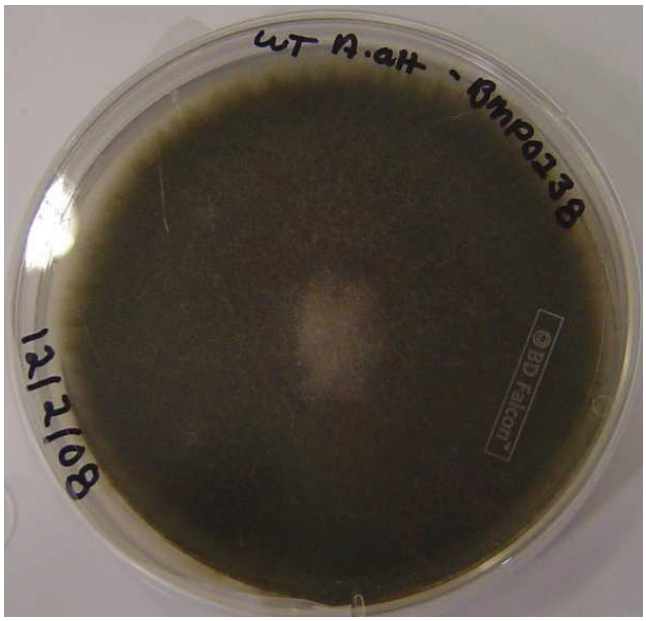

Figure 1.1 Alternaria alternata grown on PDA (potato dextrose agar) for 6 days.

The genus Alternaria currently contains approximately 100 to several hundred species, although specific data are difficult to assess due to the increase of nomenclatural synonyms of questionable taxonomic validity. However, among these species, *A*. *alternata* is by far the most common in the environment and isolated from invasive human infections and sinuses (8). Some authorities suggest that *A. alternata* is a representative species complex rather than a single entity and consists of several heterogeneous members and morphovariants. While *A. chartarum, A. dianthicola, A. geophilia, A. infectoria, A. stemphyloides,* and *A. teunissima* are among the other species isolated from infections, some Alternaria strains reported as causative agents remain unspecified (9).

Alternaria spores have emerged as opportunistic pathogens particularly in patients with immunosuppression, such as bone marrow transplant patients (8, 9). They are also one of the

causative agents of phaeohyphomycosis. Cases of onychomycosis, sinusitis, ulcerated cutaneous infections, and keratitis, as well as visceral infections and osteomyelitis due to Alternaria have been reported (10). In immunocompetent patients, Alternaria colonizes the paranasal sinuses, which may lead to chronic hypertrophic sinusitis in certain individuals. In immunocompromised patients colonization may result in the development of invasive disease (11). It is known to be one of the multitudes of agents that is producing otitis media in agricultural field workers (12). Since Alternaria species are ubiquitous in nature, they are also common laboratory contaminants. Thus, their isolation in culture requires cautious evaluation (13).

The size of the Alternaria spores is on average 13x37 µm and, because of its larger spore size compared to most fungi, the spores are inhalable but not respirable, therefore unlikely to reach deep into the lung. *Alternaria* spp. grow rapidly and the colony size reaches a diameter of 3 to 9 cm following incubation at 25°C for 7 days on potato dextrose agar (PDA). The colony is flat, and is typically covered initially by grayish, short, aerial hyphae. The surface is grayish to white initially that later darkens and becomes greenish black or olive brown with a light border. The reverse side is typically brown to black due to pigment production (14).

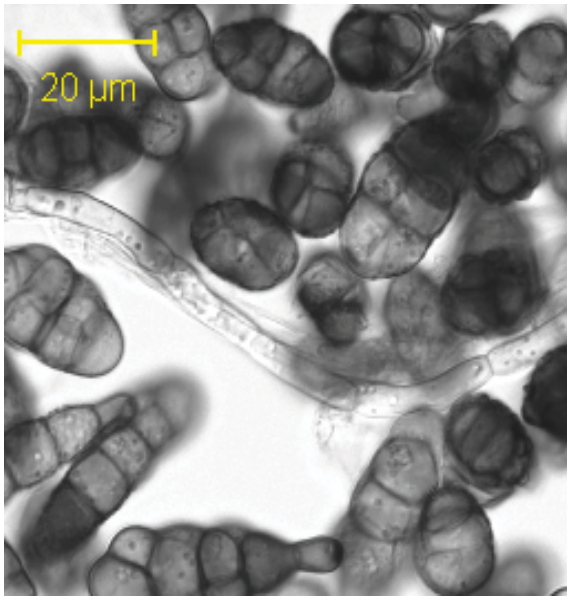

Figure 1.2 *A. alternata* spores.

Alternaria spores have septate, brown highly melanized hyphae (Figure 1.2). Conidiophores are also septate and brown in color, occasionally producing a zigzag appearance.

They bear simple or branched large conidia (7-10 x 23-34 μm) which have both transverse and longitudinal septations. These conidia may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid, darkly pigmented, muriform, smooth or roughened. The end of the conidium nearest the conidiophore is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia (15, 16). Dark colored filamentous hyphae are observed in the sections of infected tissue stained with hematoxilin and eosin. Fontana-Masson silver stain, which is specific to melanin, may be applied (14) if the pigment formation is not obvious.

Airway Disorders and Alternaria-Overview

As mentioned previously, human airways are continuously exposed to ubiquitous environmental fungi and it has recently been demonstrated that an abnormal immunologic response to these organisms most likely contributes to the pathogenesis of inflammatory airway disorders, especially the superior part of the respiratory tract. The exposure to the fungi is more intense and for a longer time than the exposure to pollen and other known allergens. In the air there are many particles and among them many fungi, but, one of the most common fungi present in the air in any part of the world is *Alternaria alternata*.

A. alternata is one of the most frequently found molds as a causative agent of type I, IgE-mediated, hypersensitivity allergies in indoor and outdoor environments especially in regions with a warm and/or arid climate. It has been proven for a long time that sensitivity to *A. alternata* is a common cause not only of atopic or allergic asthma (17), but also of allergic rhinitis and upper respiratory tract symptoms can develop or worsen when the level of spores in the environment is very high (18). Sensitization to the fungus *A. alternata* has been recognized and reported in many studies.

An epidemiological study in a large sample of patients (from Italy and Austria), with known respiratory tract symptoms suggestive of allergic disease, showed that 19% of patients were sensitized to fungi and 66% of these were positive for *A. alternata* (19). Many factors have been reported to contribute to sensitization to allergens such as exposure conditions, geographic and climatic areas, host genetics/population, but also to different characteristics of diagnostic extracts (20).

Alternaria species are considered some of the most important fungi responsible for allergenic reactions in humans (21). Despite the large spore size, spore dispersal may occur for hundreds of miles from the source. In temperate climates, airborne Alternaria spores are detectable from May to November, with peaks in late summer and autumn. Counts of Alternaria on dry, windy days can be in the range of 500 to 1,000 spores per cubic meter in grass- or grain-growing areas. In the United States alone it has been found that 3.6% of the population is sensitized to mold and in particular Alternaria (22). Moreover, diagnosis of *A. alternata* sensitization is hampered by the variability and complexity of fungal extracts, and thus simplification of the diagnostic procedures with purified allergens should be and are being pursued. Alt a 1, classified as a major secreted allergen from Alternaria, either in its natural or recombinant form, is sufficient for a reliable diagnosis of *A. alternata* sensitization and induces skin prick reactivity comparable with that produced by *A. alternata* extract (23).

Alternaria and Asthma

Asthma is common in the developed world and increasing in frequency, despite better living conditions. In 2002 for example, there were 15,960,496 adults with self-reported asthma in the USA (24). There were 17 million visits to the doctor concerning asthma and asthma ranked 8th in terms of visits to the doctor in the USA. Asthma is a major cause of chronic illness, affecting as many as 25% of schoolchildren in regions of high prevalence (25). Considerable research is currently being directed towards understanding the role of genetic susceptibility to allergy and asthma (26).

Asthma being one of the most important and most prevalent disorders in human pathology suggests a serious multifaceted disease. Asthma is defined as a condition with clinical symptoms of cough, wheeze and shortness of breath characterized by variable airflow limitation (obstruction), increased responsiveness of the airways to various external stimuli (hyperreactivity, twitchiness), inflammation, with mucous and cells infiltration (primarily eosinophils but often includes neutrophils, macrophages, and lymphocytes) in the airways and airway wall modifications known as remodeling. Inflammation is the primary cause of asthma and if persistent, leads to permanent changes in the airways, often permanent, making them even more hyperreactive.

Our current understanding of asthma suggests that the inflammation in the airways is primarily eosinophilic and neutrophilic in nature. Chronic airway inflammation in asthma is characterized by the preponderance of eosinophils in the airway walls and lumens. Virtually the only mechanism for creating eosinophilic asthma in children is allergy. The allergens that are the most important for creating the eosinophilic inflammation in the airways of children with asthma are the indoor perennial allergens, mites, dander, molds (including Alternaria, Aspergillus, Penicillium and Cladosporium) and cockroaches.

In asthmatic patients, the penetration of allergens into the lungs leads to airway inflammation characterized by a peribronchial infiltration of CD4+ T cells, macrophages, eosinophils and neutrophils. Asthmatic patients also present with a goblet cell metaplasia/ hyperplasia and characteristic modifications of the airway wall including epithelial hyperplasia, thickening of the basement membrane, subepithelial fibrosis and increased airway smooth muscle mass (27, 28). It is believed that these modifications finally lead to airway hyperactivity (AHR) to specific and non specific stimuli although the exact mechanisms of AHR pathogenesis are still unclear. Thus far, the role of fungi as a primary exogenous driver of asthma has been incompletely explored, possibly because exposure is universal but highly variable in time and intensity and hard to measure.

The apical epithelial surfaces of mammalian respiratory, gastrointestinal, and reproductive tracts are coated by mucus (a mixture of water, ions, glycoproteins, proteins, and lipids). Mucosal components are secreted apically by goblet cells in polarized epithelium and by secretory cells in the submucosal glands. Mucus provides a protective barrier against pathogens and toxins and contributes to the innate defensive system in mucosal immunology. Mucin glycoproteins are the major macromolecular constituents of epithelial mucus and have been implicated in health and disease for a very long time. Airway mucins are major components of the soluble layer and/or viscoelastic gel that comprise lung mucus in healthy airways and contribute to the mucociliary defense system that protects the lungs against pathogens and environmental toxins. Mucus and mucins are overproduced in the airways of patients with chronic airway diseases, which greatly contribute to airway obstruction in patients with asthma, chronic obstructive pulmonary diseases (COPD), or cystic fibrosis (CF).

Sensitivity to the fungus *A. alternata* is a common cause of asthma. Epidemiological studies indicate that Alternaria sensitivity is closely linked with the development of asthma (22,

29, 30). In addition, up to 70 % of mold-allergic patients have skin test reactivity to Alternaria and the sensitivity to Alternaria has been shown to be a risk factor for asthma (22, 29-31). Indeed as early as 1941 Harris (32) exposed patients to 1 g of Alternaria powder dispersed in a 700 cubic feet room and he proved he can provoke asthma and rhinitis symptoms in 10 out of 12 patients with positive skin tests to Alternaria who had a history compatible with sensitivity to the fungus. It has also been demonstrated that inhalation of either Alternaria or Penicillium spores in quantities comparable with those encountered by natural exposure can induce both immediate and late phase asthma in sensitive individuals (33).

In the United States, 35 million people have sinus problems, and 20 million people have asthma (34). For a long time, even without scientific evidence, physicians knew that a sinus infection can substantially contribute to the frequency and severity of asthma attacks. Asthma and sinusitis are both recognized as intricate diseases in the literature for a long time. Over time there have been different approaches for the treatment of these diseases, depending on the possibilities that the current science was offering at that point in time or depending on the previous knowledge of the physician.

In order to help asthmatic people sinus surgery was considered one of the treatment options between 1940 and 1950. The sinusal mucosa was removed together with the opening of the nasal airway and, in this way, results were quite efficacious for some patients. In the 1960s, improvements after sinus surgery were thought to be related more to the stress reaction than to the surgical technique; therefore, sinus surgery became less popular as a principle of asthma management. With the introduction of CT scanning in the 1970s, accurately pinpointing the location and extent of the sinus pathology became possible. A return to corrective surgery for individuals with sinusitis and for individuals with asthma has occurred, showing the benefits of clearing sinus pathology. In the 1980s, functional endoscopic sinus surgery and the ability to physiologically improve sinus function became available. In the 1990s, as CT scanning enhanced visualization of the sinus and as endoscopic surgery, especially with the computer-assisted techniques, enhanced the ability to improve sinus function, physicians are returning to sinus treatment as an aid to asthma management. Further aids to treatment have included newer antibiotics and emphasis on function of the cilia. Newer medications, such as the corticosteroids sprays, have given new options for treatment by reducing inflammation. Indeed, many allergists now emphasize the role of these drugs in treating asthma and sinusitis.

Alternaria associated Allergy

Allergy is the term used for a set of diseases mediated by immunologic mechanisms. Allergic disorders include allergic rhinitis, conjunctivitis, asthma, urticaria, angioedema, food allergy, drug allergy, and anaphylaxis. Allergic disorders share the common pathology of inflammation of affected tissues. Allergy requires sensitization to an allergen and specific immunologic response upon re-exposure to that same allergen. Pathogenesis involves production and release of cytokines, chemokines, and lipid mediators which cause tissue damage and recruitment of inflammatory effector cells.

Diagnosis often involves documenting responses to allergen such as in skin testing, radioallergosorbant allergen testing (RAST), or bronchial provocation testing. Current therapies include allergen avoidance, antihistamines, leukotriene modifiers, corticosteroids, phosphodiesterase inhibitors, humanized monoclonal anti-IgE, and immunotherapy.

The prevalence of allergies has been increasing but to date allergy, as a systemic or site-specific disease, affects approximately 20-30% of the general population, and is considered a major cause of morbidity, disability, decreased quality of life, and major loss of productivity throughout the world. In fact the WHO now lists allergy as one of the most prevalent chronic diseases worldwide. Genetic as well as environmental factors contribute to the onset and development of allergic diseases. The evolution and characterization of the disease is dictated by the site of exposure to allergen, but different diseases have a very similar pathophysiological process (35).

Indoor allergens typically consist of those derived from dust mites, cockroaches and animal dander while plant pollens are typical outdoor allergens (25). Fungal spores are universal atmospheric components (indoor and outdoor) and are also recognized as important causes of respiratory allergies not only because of the proteins that are classified as allergens but also because of the capacity of fungi to actively germinate and attempt to infect the host skin or attempt to colonize the respiratory tract (36). More than 80 genera of fungi have been associated with symptoms of respiratory allergy and the prevalence of this type of allergy is estimated at 20–30% among atopic individuals and up to 6% in the general population (37, 38). Importantly,

many well known allergenic proteins have been described in Aspergillus, Alternaria and other fungi (Supplementary Table 1) (39).

There are currently over 1,400 clinically relevant, verified allergens (http://www.allergenonline.org/). This database of allergens contains proteins from a variety of sources like fungi, pollen, insects, venoms, grass, and food. The list includes many biochemical categories but proteases, by far, are the most represented group across taxonomic groups. Many studies have been conducted regarding the role of proteases in the pathogenesis of asthma, allergies and airway disorders. Furthermore, many aeroallergens associated with asthma, like house dust mite allergens and various fungal allergens, are proteases. One of the most important classes of proteolytic allergens is tryptase (a type of serine protease). In *in vitro* studies tryptase promotes human mast cell and eosinophil degranulation, induces migration of eosinophils and neutrophils, amplifies the bronchoconstrictor effects of histamine on lung tissue, and stimulates the growth of airway fibroblasts, smooth muscle cells, and epithelial cells in the process called airway remodeling (40, 41).

Alt a 1, the major allergen from *A. alternata*

Alternaria species are considered some of the most important fungi that are responsible for allergenic reactions in humans and additionally in horses causing what is commonly called wheezing disease (21). Most of the existing Alternaria allergens are fairly conserved proteins with known function such as enolase, ribosomal proteins, nuclear transport factor, and aldehyde dehydrogenase, (42-44). The biological functions of many other allergenic proteins are unknown (42, 45). The most notable example is Alt a 1, the major allergen secreted by *A. alternata* and a protein with no known function in fungal metabolism or ecology described as of yet (45, 46).

Diagnosis of *A. alternata* sensitization is hampered by the variability and complexity of fungal extracts, and thus simplification of the diagnostic procedures with purified allergens has been investigated. Currently, in many of the allergy medical clinics in the U.S.A., for the sensitization test to *A. alternata,* pure Alt a 1 protein is used in lieu of total fungal extract because it was proven to produce the same reaction in the human subjects as the total extract of proteins from *A. alternata* (23). Alt a 1, either in its natural or recombinant form, is sufficient for a reliable diagnosis of *A. alternata* sensitization and induces skin prick reactivity comparable

with that produced by total proteins *A. alternata* extract in the vast majority of Alternaria sensitized patients (23).

Alt a 1 is the *A. alternata* protein that elicits the most intense allergic reaction in humans found thus far and yet no known biological function has been assigned to this protein. Even though Alt a 1 has been extensively produced by recombinant technology, the fact that the biological activity remains unclear has made it difficult to fully characterize it from immunological perspectives, beyond identification of IgE binding sites (47).

Interestingly, in a study published in 2006 it was reported that enzymatic activity corresponding to phosphatase and esterase was associated with native and *E. coli* produced recombinant Alt a 1 (48). These findings suggest that the possible structural differences between the recombinant and the native proteins do not affect the enzymatic activity in a significant manner. In addition, this study reported that the same enzyme activities were found in cellular extracts and culture filtrates from all strains examined.

In studies published in 2003 and 2004 by the Lawrence Laboratory an ortholog of the *alt a 1* gene from *A. alternata*, called *alt b 1* from *A. brassicicola* was found to be highly up-regulated during the infection process of this species on the model flowering plant *Arabidopsis thaliana* suggesting that the gene/gene product may be involved in plant pathogenicity (1, 49). Although, the level of similarity between the two homologous genes is very high (92%), there are many other known proteins that are homologues between the two types of Alternaria that have higher levels of similarity (98%, 99%) (49, 50). In another study by Hong et al., 2004 (51), it was shown that approximately 52 species of Alternaria and very closely related taxa possess a highly conserved *alt a 1* gene like *A. brassiciola alt b 1* suggesting that all species of Alternaria are potentially allergenic.

In 2001 it was reported that germinating spores of Alternaria release significantly higher amounts of allergen than non-germinating spores using immunostaining techniques (52). The authors hypothesized that Alt a 1 is secreted or released from growing germ tubes, based on the previously known fact that germinating spores of Aspergillus were reported to secrete more allergens than non-germinating spores (53). In a study in 2003 (49), results of virtual Northern analysis seemed to confirm these Alternaria allergen release results to a degree. The study states that “if one assumes that transcript abundance corresponds to allergen release, then clearly more allergen is released from *A. brassicicola* spores germinating in nutrient-rich environments than

non-germinating spores". Identification of the specific environmental or pathophysiological local conditions that trigger Alt a 1 production and its release represents a very important step in the elucidation of the role of Alt a 1 in the human pathology.

Chronic Rhinosinusitis (CRS).

Like atopic asthma, CRS is one of the most prevalent diseases in the general population, estimated to affect approximately 14.1% (29.2 million) of the adults in the US (34, 54). More specifically, the National Center for Health Statistics reported that 14.1% of the US adults recalled a health professional's diagnosis of sinusitis at least once in their lifetime (34). Chronic sinusitis is a very common illness with a substantial health care impact; in the US the direct cost was estimated at $5.6 billion in 1996 and the indirect costs were estimated to include more than 70 million lost activity days per year (55). In 1997 in the US there were 786,000 emergency department visits for rhinosinusitis and out of these 545,000 were for CRS specifically (56).

CRS is commonly diagnosed on the basis of symptoms alone, even though it is commonly known that this method is often unreliable. The lack of a clear definition of CRS therapies has limited the ability to provide effective clinical care (medicine or other treatment methods) or develop new drug therapies that can provide a better outcome of the treatment meaning to treat the original reason of the entire disease. CRS is a disease characterized by chronic inflammation of the nasal and paranasal sinus mucosa that lasts more than 12 weeks and is associated with mucosal alterations ranging from mild thickening of the sinusal mucosa to its final stage that is inflammatory mucosal thickening and, in some patients, polypoid changes often abbreviated CRSwnp (CRS with nasal polyps) (34). This definition is now commonly accepted but the struggle with the proper definition made the Sinus and Allergy Health Partnership (SAHP) set up a multidisciplinary team to develop a definition that will allow clinicians and researchers to more accurately diagnose and treat this disease.

Historically speaking, the absence of a widely accepted definition for CRS has resulted in a lack of research aimed at the understanding the pathophysiology of this disease and to, eventually, improve the CRS treatment. Although etiological factors contributing to chronic rhinosinusitis, like ostial blockage, delayed recovery of mucociliary function, mucus recirculation, and microbial factors in sinusitis have been defined, there is still a lack of

knowledge of the natural history and of inflammatory factors in chronic sinusitis (57). Over time there have been a number of debates about the proper diagnostic and effective treatment (medical or surgery) that are the most effective for CRS. Patients with CRS present with long term nasal congestion, lost sense of smell and most of the time irreversible, difficult to manage headaches and, the most important are recurrent acute exacerbations that appear as a result of secondary bacterial infections (58). It is already known that the quality of life is more affected for a patient with CRS than that one with congestive heart failure (59). These patients have almost always additional associated comorbidity conditions such as asthma, eczema and otitis media. Approximately 20% of patients with chronic sinusitis also have nasal polyposis and a subset of these patients suffer from the aspirin-sensitivity syndrome often associated with asthma and rhinitis. There is conflict in the criteria for diagnosis of acute or chronic sinusitis. Patients who have been symptomatic for 3 weeks or less are considered to have acute disease while those having prolonged symptoms lasting longer than 12 weeks or more are referred to as having chronic disease. Thus, in order to have effective therapeutic means it is essential to have a better understanding of the pathogenesis and etiology of the CRS and related disorders.

In acute rhinosinusitis patients present a variety of signs and symptoms. The purulent discharge (in anterior or in posterior) is believed to be one of the most significant findings in the diagnosis of rhinosinusitis. For the clinical evaluation there are major criteria (purulent drainage, headache, facial pain or pressure, nasal congestion/blockage, decreased smelling sensation and fever) and minor criteria (halitosis, fever – non acute rhinosinusitis, weakness, dental pain, ear fullness -clicking noises and pain, cough and in children, irritability) (57, 60, 61). In the case of chronic sinusitis, this disease is considered “very likely” if two or more of the above mentioned major criteria or one major and two minor criteria are found in the history and exam over a 6-12 week time interval. There are patients that may present with re-activation of the disease which may include acute rhinosinusitis occurring at least 3-4 times/year and lasting for a minimum of 10 days. It has been proposed that computerized tomography be used to assess the evolution of the disease and the effectiveness of the treatment. If the modifications persist on computerized tomography of the paranasal sinuses are still present after 4 weeks following therapy, the condition can be considered chronic (60).

Inflammation is thought to play a key role in the development and onset of the pathogenesis of CRS. Two major types of inflammatory processes are observed to be associated

with CRS. The first one is the infectious inflammation, which is associated with acute sinusitis with either bacterial or viral etiology. The second CRS-associated inflammatory process is noninfectious inflammation which is described to have pathognomonic signs, the presence of eosinophils and mixed mononuclear cells, and the relative absence of the neutrophils in the mucus (57). Specific to CRS is the presence of sinus mucosal abnormalities, represented by the persistence of eosinophilic inflammation and an increased number of T cells (62). The T cells are responsible for the production of cytokines, among them the most important being IL5. IL5 is a major regulator of eosinophil accumulation in tissues, and can modulate eosinophil behavior at every stage from maturation to survival. The presence of the eosinophils and IL5 secreting T cells strongly suggests a possible T helper 2 or Th2-like immune response as has been described for helminthes defense. Most likely, the pathological signs seen in CRS are a result of the overlapping of the two major inflammatory processes. Although there is still debate over a comprehensive explanation of the whole pathology for CRS there have been several significant new developments described thereafter.

Upper and lower airway mucins are major components of the soluble layer and/or viscoelastic gel that comprise lung mucus in healthy airways and contribute to the mucociliary defense system that protects the lungs against pathogens and environmental toxins. The most significant difference between the CRS patients and healthy individuals is the very small number of neutrophils in mucosa (63, 64). Although in patients with CRS there is evidence for the IL8 secretion in the mucosa it is still unknown whether or not cytokines or chemokines are responsible for the relatively low number of neutrophils in sinuses (65). In subjects classified as “non-allergic” the highest levels of IL8 and the highest percentages of lavage neutrophilia were observed and this is in contrast with patients with CRS and associated allergic rhinitis that had a modest increase in the number of neutrophils and IL8 levels in lavage fluid. In another study (66) there were shown increased level of IL8 by performing Quantitative PCR in sinus mucosa obtained from sinus surgery and they found that there is a correlation between the radiographic extent of disease on preoperative sinus CT scans and the level of IL8. The reason for discrepancy in these two studies regarding IL8 levels in the non-allergic patients and CRS patients is still a mystery.

Chronic Rhinosinusitis Distinctive Features and Association with Alternaria

Although the pathogenesis and etiology are far from being completely understood, there are several distinctive features associated with CRS. The most important marker of CRS is represented by the presence of nasal polyps. Another important feature is the inflammatory thickening and polypoid changes of the sinusal mucosa, this process being present even in the situation that the polyps are absent. The recurrence of polyps after "successful" polypectomy surgery is a very common occurrence and clearly illustrates that CRS has many features of a chronic and persistent inflammation. The pathologic process of airway remodeling found in asthma is also noted in the sino-nasal samples from patients with CRS (4).

The histologic hallmark of CRS is seen in almost all CRS patients with or without polyps, and is represented by a chronic inflammatory infiltrate of lymphocytes, plasma cells, and eosinophils, (67, 68) that has been shown to be significantly similar to the bronchial mucosa from patients with asthma. In fact CRS is often termed "asthma of the upper airways". In several studies it was shown that more than 40% of the CRS patients are clinically non-atopic and do not present specific IgE antibody (necessary to prove the atopy) (69).The association of eosinophilia with the extent of disease is clearly independent of atopy although some theories suggest atopy may influence the overall severity of the disease, (69, 70). Another notable finding in the case of CRS patients, in their sinus mucosa, (71-73), is the increased numbers of CD4+ T lymphocytes positive for Th1 and Th2 cytokines, such as IL5, IL13, and IFN-γ. These findings are very suggestive for a rather complex immunologic mechanism.

In a recent study in 2004 (3), the possibility that the common airborne fungi are the main trigger in the onset and the pathogenesis of persistent sinus inflammation that appear in CRS was investigated. In this study it was clearly shown that lymphocytes from CRS patients had a pro-inflammatory response (production of IL5, IL13, IFN-γ) to antigen preparations from ubiquitous airborne fungi including Alternaria, Aspergillus, Cladosporium and Penicillum. Although there are measurable responses due to exposure to antigens from Aspergillus and Cladosporium, the most statistically significant and pronounced response was to antigen preparations derived from Alternaria. This data strongly suggests that CRS patients (and possibly a large number of asthmatic patients) react to specific secreted molecules from Alternaria via antigen processing and T-cell recognition. PBMCs (polymorphic blood mononuclear cells: lymphocytes and

monocytes) from all of the patients with CRS produced high levels of cytokines (IL5, IL13, and IFN-γ) but PBMCs from the normal individuals did not.

Eosinophils and Airway Disorders

The eosinophils are granular bi-lobed leukocytes that can be stained by eosin (eosinophil = eosin + philein, to love in Greek) and represent approximately 2 to 5% of granulocytes in a non-allergic person. In the last few decades there has been an increased interest regarding the role of the eosinophil in several physiologic and pathologic processes. Eosinophil progenitors are released from the bone marrow into the circulation and different chemotactic factors are responsible for their presence to the site of action. Development and maturation of eosinophils can also occur in situ in peripheral sites of inflammation containing pre-existing increased tissue eosinophil numbers.

Activated eosinophils play a role in allergy, asthma, defense against parasitic diseases, granulomatous disorders, fibrotic conditions and in development of several types of malignant tumors. During various allergic and non-allergic inflammatory reactions, the eosinophils are recruited from the blood stream to the sites of inflammation. The recruitment is accomplished by specific soluble mediators released by immune cells, and some other, known and unknown stimuli, trigger the degranulation (the release of eosinophil granule proteins) (74). Indeed in a recent Alternaria-related study by Yoon et al., it was shown that Alternaria hyphae and beta-glucan, a common component of fungal cell walls, can cause massive degranulation (75). Moreover, it was also shown in this study that the physical interaction of eosinophils with live Alternaria spores and hyphae and not necessarily secreted fungal products induced degranulation. These eosinophil granules contain a number of preformed enzymes and other proteins, including cationic proteins such as major basic protein (MBP), eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase. Other granule enzymes include lysophospholipase, collagenase, β-glucuronidase, and arylsulfatase B.

Eosinophil granules contain several cytotoxic proteins (76) including, the most important, the MBP, which is directly toxic to extracellular microorganisms as well as host tissue. Major basic protein and eosinophil peroxidase are known to be toxic to the respiratory epithelial tissues, lung tissue and tracheal epithelium *in vitro* (77). CRS specimens show that the epithelial damage

is colocalized with MBP deposition (4, 68, 78) *in vitro*, MBP directly damages respiratory and sinus epithelium and these actions are done in a time- and dose-dependent manner (79, 80). These granules contain a number of preformed enzymes and other proteins, including cationic proteins such as MBP, eosinophil cationic protein, eosinophil derived neurotoxin, and eosinophil peroxidase. Other granule enzymes include lypophospholipase, collagenase, beta-glucuronidase, and arylsulfatase B (81).

The principal granule protein in eosinophils is MBP, a highly positively charged, 117-amino acid polypeptide with a molecular weight of 14 kDa. In an experiment with cultured human nasal epithelial cells it was shown that physiologic concentrations of MBP upregulate expression of intercellular adhesion (82). Moreover, in this manner MBP can induce eosinophil degranulation and the production of IL8 by these cells (2). In this manner, MBP can act as an autocrine factor to potentiate further eosinophil recruitment and activation in the airways. The highly cationic MBP reacts relatively easy with acidic lipids, disordering lipid membranes and resulting in fusion and lysis (83). MBP is a potent bactericidal and helminthotoxic agent that is also toxic to tumor cells and other mammalian cells, including the airway epithelium. MBP has diverse cytotoxic and non-cytotoxic effects on many different cell types due to interactions with cell-surface anion charges. The importance of the cationic charge is supported by the observation that synthetic polycations of similar size and charge, such as poly-L-arginine and poly-L-lysine, mimic many of the effects of MBP. There are different studies that suggest that MBP plays a key role in the alterations of airway function that accompany eosinophilic airway inflammation. Initial *in vivo* (84) and *in vitro* studies (85, 86) suggested that the effects of MBP on airway smooth-muscle contractility occurred indirectly, by the effects on the airway epithelium. It has been shown that the exposure to MBP damages the airway epithelial barrier (79, 87) and increases epithelial permeability to hydrophilic tracers (88), and, in this way, is exposing the underlying airway smooth muscle to higher concentrations of bronchoactive substances. In addition to actions on the airway epithelium, MBP exert neuromodulatory influences on airway reactivity. An important observation has been made in that eosinophils cluster around airway nerves in patients with asthma.

CHAPTER 3

Analysis of expressed sequence tags (ESTs) during *Alternaria alternata* spore germination reveals high immunogenic potential

Introduction

Human airways are continuously exposed to ubiquitous environmental fungi (molds) and very often occur at a higher level and for a longer duration than that of pollen or other known airborne allergens. One of the most common of these environmental fungi is *Alternaria alternata.* From a clinical perspective, Alternaria has long been associated with IgE-mediated, histamine-dependent mold allergy, allergic rhinitis, and asthma. Increasing clinical, immunological, molecular, and biochemical evidence strongly supports the role of Alternaria in the etiology and pathology of another major respiratory disorder, chronic rhinosinusitis (CRS) (2-4).

Fungal spores and hyphae are ubiquitous in the environment, and respiratory exposure to these structures is almost constant throughout the year. The most common airborne fungi include species found within the genera *Cladosporium, Alternaria, Penicillium,* and *Aspergillus*, although a multitude of other species are commonly found in any type of environment and climate (20). House dust mites, cat dander and grass pollen are also common environmental sources of allergenic proteins, but fungi alone have the ability to actively germinate and secrete additional molecules in the respiratory tract. Fungi may also infect the host skin or at least attempt to colonize the respiratory tract. This is the greatest difference and probably the most important factor in the pathologic potential and capabilities of fungi in regards to allergic inflammation. Thus, it is possible that fungi have a much greater impact on an individual in terms of triggering host immune responses against these potential pathogens from an allergenic perspective while concomitantly producing non-allergenic toxins, enzymes, and other pro-inflammatory factors that may play an accessory role in triggering and exacerbating chronic

inflammatory disorders by stimulating innate immune responses and influencing the development of adaptive Th2 immunity.

Alternaria species are considered some of the most important fungi that are responsible for allergenic reactions in humans and additionally in horses causing what is commonly called wheezing disease (21). Most of the existing Alternaria allergens are fairly conserved proteins with known function such as enolase, ribosomal proteins, nuclear transport factor, and aldehyde dehydrogenase (42-44). The biological functions of other allergenic proteins are unknown (42, 45). The most notable example is Alt a 1, the major allergen secreted by *A. alternata* and a protein with no known function in fungal metabolism or ecology described as of yet (45, 46).

In addition to known IgE-binding allergens from Alternaria it has been hypothesized that other secreted fungal proteins may contribute to airway inflammation by stimulating innate immune responses. One example is enzymes with proteolytic activity that function through protease activated receptors such as PAR-2 and subsequently induce the production of proinflammatory cytokines (89). Serine and aspartic protease activities found in Alternaria antigen preparations have been shown to function through PAR-2 in these recent studies (90).

The primary objective of this study was to further our knowledge of the molecular events occurring during spore germination of *A. alternata* in an *in vitro* condition of pathological relevance using an EST sequencing approach. The use of ESTs to identify genes expressed by an organism in a specific environment or condition is well documented. In our study, spores were germinated in the presence of mucin, a common component of the epithelial lining of airways, RNA was harvested from fungal cells, and ESTs were produced and analyzed. Besides investigating the expression of known allergens during spore germination, one other goal of the *A. alternata* EST project was to characterize both putative secreted and membrane-bound proteins, especially those with predicted enzymatic activities like proteases, which may be involved in the interaction between the fungus and the human epithelial cells. Results of these analyses will be discussed.

Results

EST library construction and analysis

In order to produce a catalog of the genes transcribed by fungus *A. alternata* during spore germination in the presence of mucin, a cDNA library was created from RNA extracted from sporelings grown at 30^{o}C for 24 hours. The total RNA was sent to Washington University Genome Center, a library was created, and approximately 27,000 paired end reads were obtained using Sanger sequencing methodology. The high quality reads were later trimmed, clustered and assembled using PCAP software into 1,443 contigs and 6,605 singletons as a unisequence set (100). The EST sequences were annotated through similarity comparison with the NCBI non-redundant protein database (NR) using BlastX (91) and protein family databases using the suite of Interpro tools (92). We found 4,603 sequences (57%) to have homologous sequences in the NR database (e-value $\leq 10^{-5}$) and 2,943 unique proteins were hit including hypothetical proteins. In addition, using the same e-value cutoff as above, we performed Blastn against a recently sequenced *A. alternata* genome harboring 11,968 predicted gene models (CDS). Our results show that the EST assembly represented 3,620/11,968 predicted genes/proteins or approximately 30% of the gene space. Thus, we feel this assembly represents a substantial portion of the gene repertoire in *A. alternata*. The higher number of hits (unique proteins) to the *A. alternata* genome-derived CDS database compared to NR is most likely due to Alternaria-specific genes/proteins not yet annotated or available in NR.

Table 3.1 EST read statistics (A, B and C)

A Total Reads = 26,880

PREFIX	COUNT	DIR	PASS	FAIL	XAVLEN	SUCC_PCT
AKAB	**13440**	**forward**	9796	3644	538.25	72.89
AKAB	**13440**	**reverse**	9486	3954	467.07	70.58

B

Total input reads	19282
Total input bases	14659712 bp
Total Q20 bases	9726189 bp
Average Q20 bases per read	504 bp
Average read length	760 bp
Placed reads	18170
(reads in scaffold	12501)
(reads in singleton	5669)
Unplaced reads	1112
Chaff rate	5.77%
Q20 base redundancy	7.7X
Total prefin reads input	0
Total prefin reads unused	0
***** Contiguity**	Contig ***
Total contig number	1369
Total contig bases	1250014 bp
Total Q20 bases	1176893 bp
Q20 bases %	94.10%
Average contig length	913 bp
Maximum contig length	3613 bp
N50 contig length	1006 bp
N50 contig number	480
Major contig (> 2000 bp) number	15
Major_contig bases	35886 bp
Major_contig Q20 bases	34025 bp
Major_contig Q20 base percent	94.80%
***** Genome Contents *****	
Total GC count	627694, (50.2%)
Total AT count	622320, (49.8%)
Total NX count	0, (0%)
Total	1250014

C

	Top tier (up to 1031000 bp):	Middle tier (1031000 bp -- 1224000 bp):	Bottom tier (1224000 bp -- end):
Supercontig number	964	326	79
Average length	1070 bp	593 bp	322 bp
Longest length	3613 bp	715 bp	419 bp
Contig bases in this tier	1031305 bp	193307 bp	25402 bp
Q20 bases in this tier	972115 bp	181314 bp	23464 bp
Q20 base percentage	94.20%	93.70%	92.30%
N50 supercontig length	1081 bp	611 bp	363 bp
N50 supercontig number	375	145	32

Prediction and classification of putative function of *A. alternata* ESTs using Interpro and assignment of gene ontology (GO) terms

Following assembly of the ESTs we performed a hypothetical translation (6-frames) into protein sequences and subsequently used the Interpro suite of bioinformatics tools (99, http://www.ebi.ac.uk/interpro/). Using the Interpro suite of analysis tools, 2,583 sequences (32%) were found to contain at least one putative functional protein domain (e-value $\leq 10^{-5}$).

In addition, we identified 2,791 unique domains within the translated ESTs. Further characterizations of the predicted proteins were obtained by assigning gene ontology (GO) terms using Interpro2Go. We were able to assign 1,777 unique GO terms to our ESTs. A sequential classification was then performed on the GO terms assigned, using the GO Slim resources (93), for determining the highest hierarchical parent of all the GO terms that we identified in the data set. We intended to pinpoint the activities and the processes that these proteins are associated with during spore germination. The results are summarized in Table 3.2 and visualized in Figure 3.1. We found Metabolic Process as the GO term most highly represented in this analysis (3,249 ESTs) while Transport Activity was the least represented (620 ESTs).

Each of these categories of proteins were characterized one step further by separating them into more specific classes of activities giving a more thorough and meaningful snapshot of the spore germination *A. alternata* trasncriptome as shown in Figure 3.2 (for proteins categorized to be involved in metabolic processes), Figure 3.3 (for Cell Component), Figure 3.4 (Catalytic Activity), Figure 3.5 (Binding) and Figure 3.6 (Transport Activity). This further classification

shows that many genes involved in translation (ribosome, nucleotide binding), hydrolytic and proteolytic activity are represented, as well as transporter activity.

Table 3.2 Distribution of the GO Slim customized results for the ESTs from A. alternata spores germinating in the presence of mucin.

Biological Process	Number of ESTs
Binding	1,706
Catalytic Activity	2,014
Cell Component	2,091
Metabolic Processes	3,249
Transport Activity	620

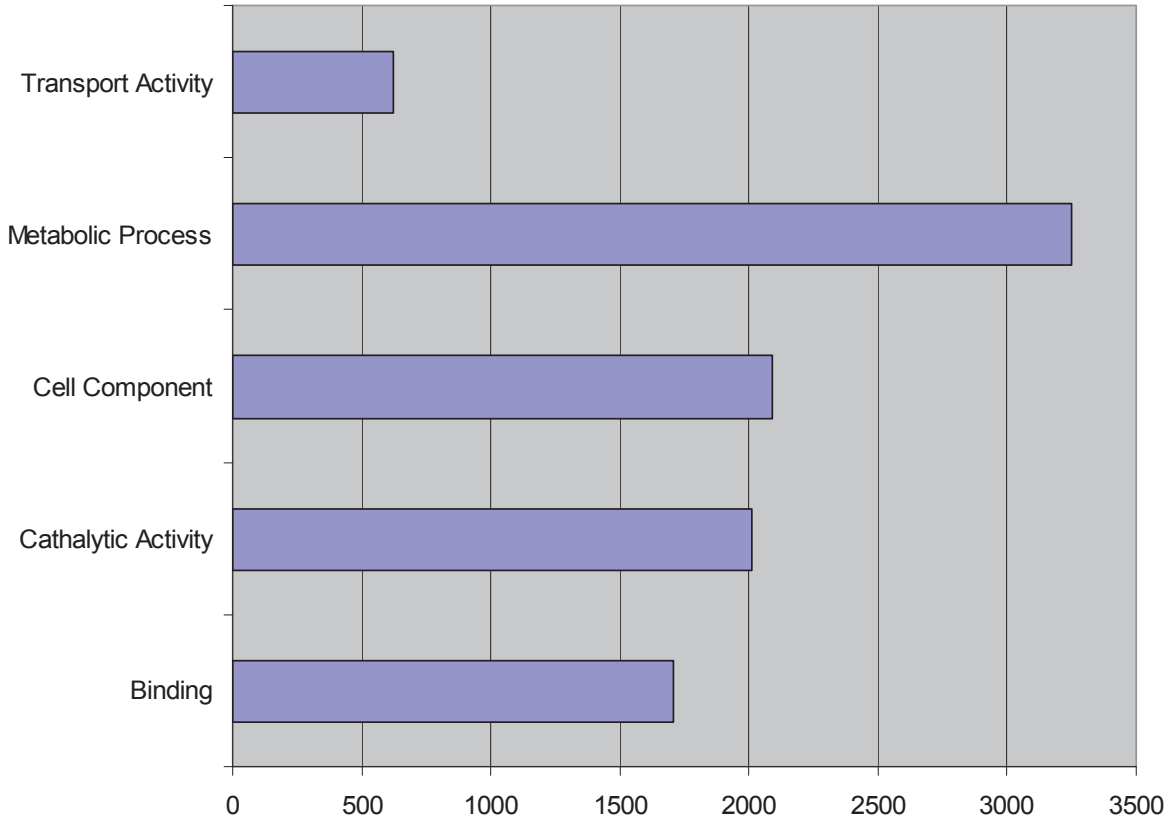

Figure 3.1 Classification of the Alternaria ESTs data based on the highest level of hierarchy (1,177 GO Slim customized terms assigned).

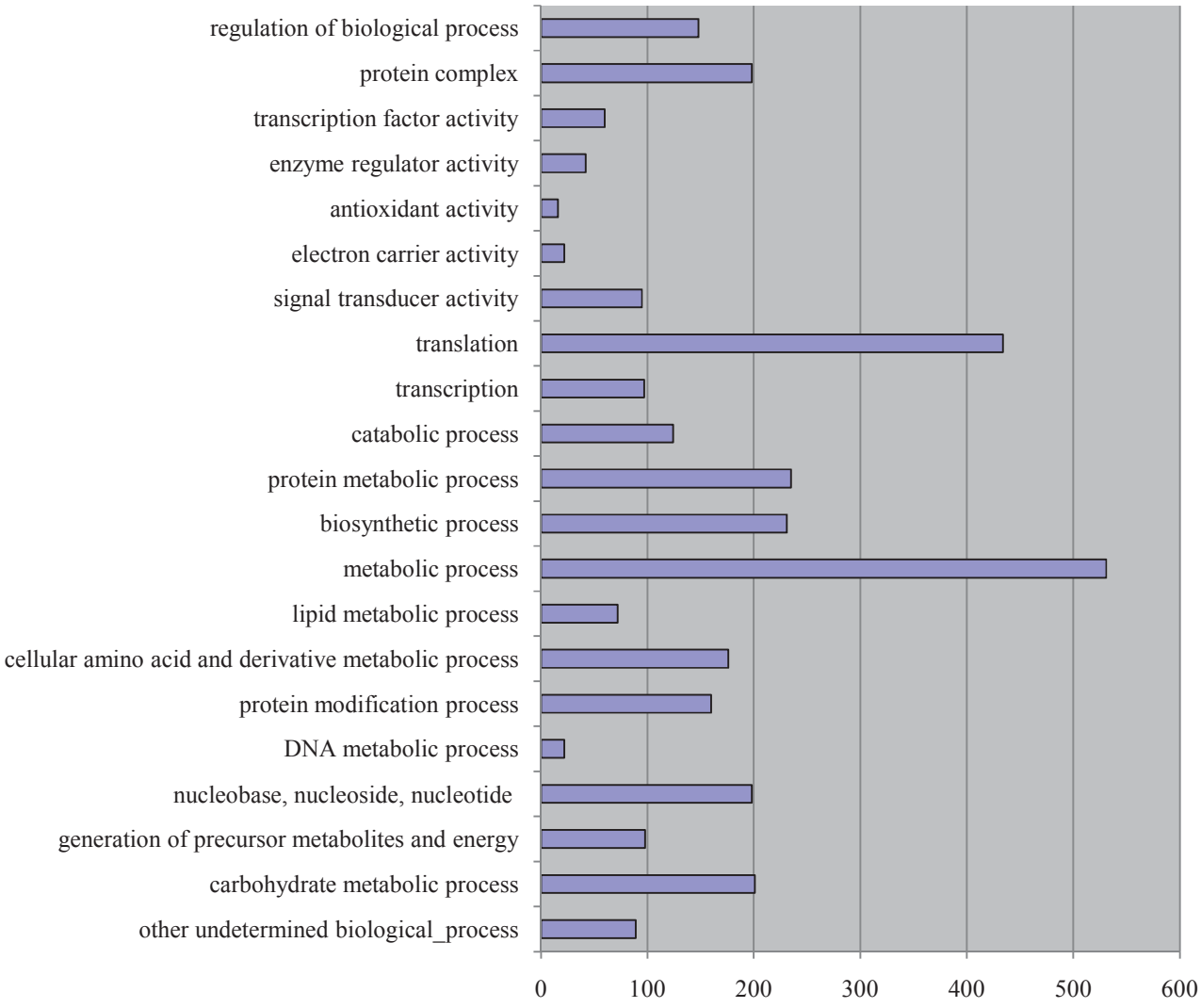

Figure 3.2 Distribution of proteins involved in metabolic processes based on GO Slim second highest hierarchy in this case.

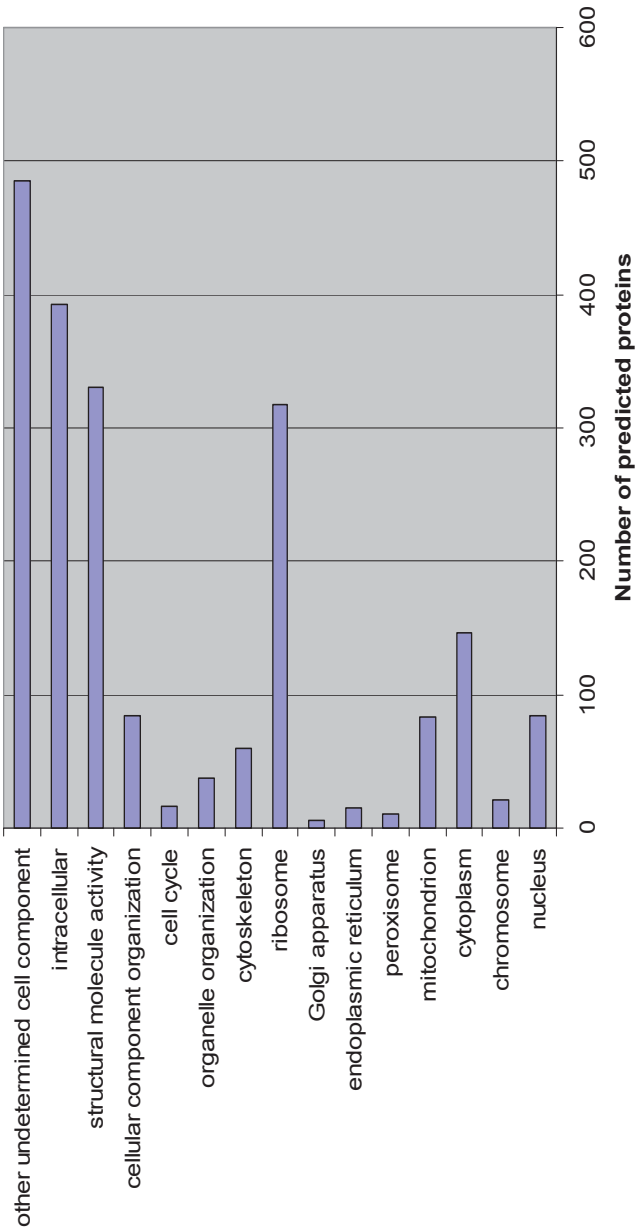

Figure 3.3 Distribution of the proteins corresponding to cell components using GO Slim analysis.

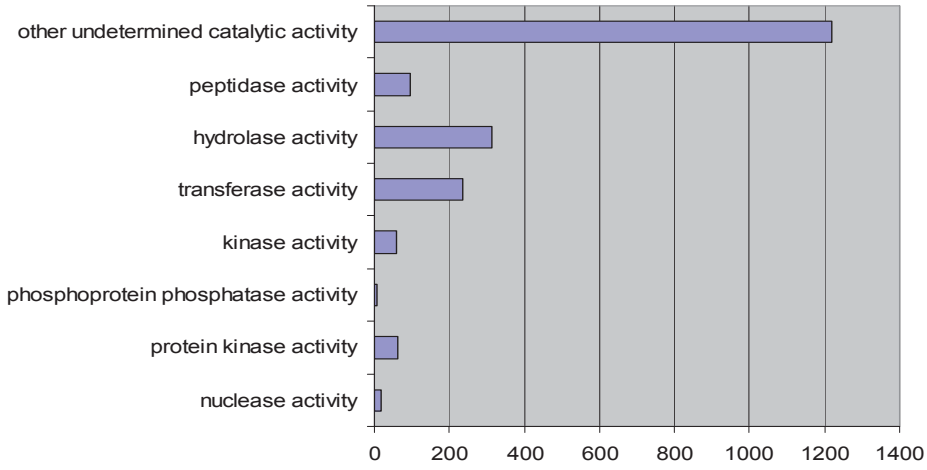

Figure 3.4 Distribution of the proteins that have a putative function of catalytic activity using GO Slim.

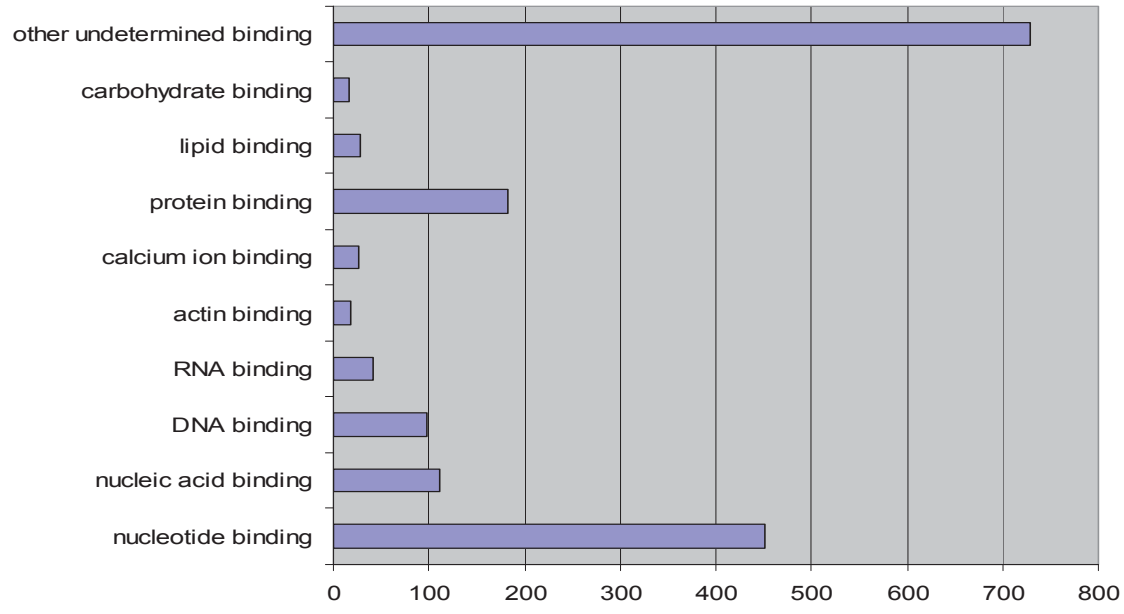

Figure 3.5 Distribution of the binding activity proteins data obtained with GO Slim.

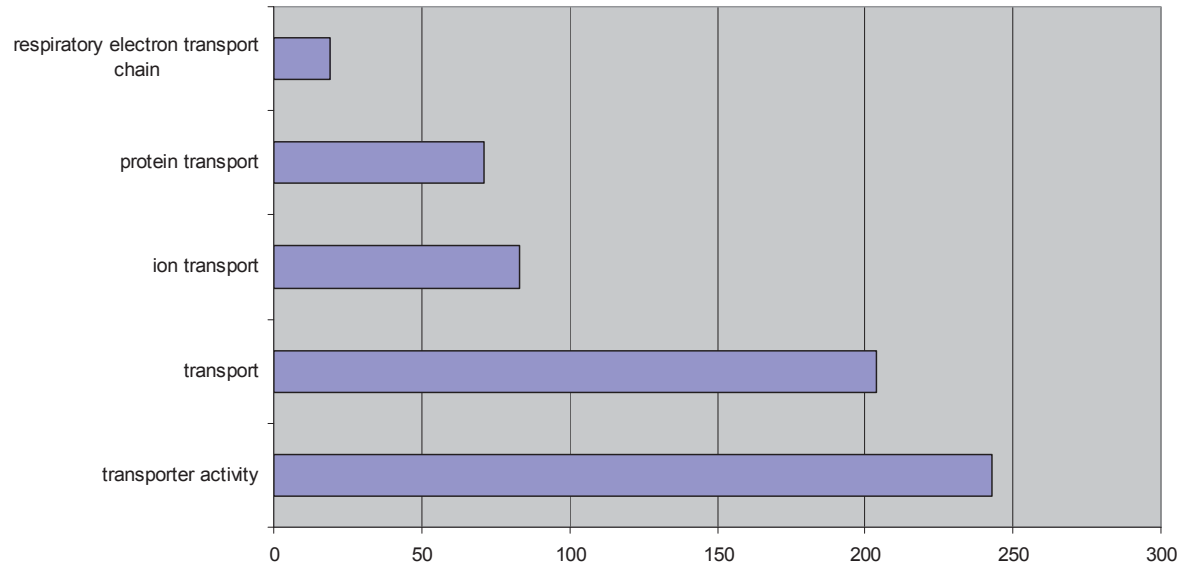

Figure 3.6 Putative transport activity proteins distribution chart, data obtained with GO Slim.

Classification of ESTs representing putative proteases

Since many known allergens or immunostimulatory proteins from various organisms have been reported to possess protease activity and to be involved in the onset and development of upper respiratory disease, we were also interested in classifying the ESTs most likely to encode proteolytic enzymes (31, 90, 94-96). For this analysis we used the MEROPS database (http://merops.sanger.ac.uk/). The MEROPS database is a comprehensive information resource for peptidases (also termed proteases, proteinases and proteolytic enzymes) and the proteins that inhibit them and allows one to perform blast analyses. The MEROPS database uses a hierarchical, structure-based classification of the peptidases. In this database system, each peptidase is assigned to a Family on the basis of statistically significant similarities in amino acid sequence, and families that are thought to be homologous are grouped together in a Clan. The classification of putative proteases found in our EST assembly was obtained using the web-based Batch Blast interface at the MEROPS website.

The data generated from this in silico experiment are found in Table 3.3 with 60 unique putative proteases identified within the ESTs. The most common family of proteases in this set was the metalloprotease family (30/60) followed by serine protease family (23/60), cysteine protease family (5/60) and finally aspartic protease family (2/60) and depicted in Figure 3.7. Furthermore, the metalloproteases (Figure 3.8) and serine proteases (Figure 3.9) were further subdivided into clans. For example, the most represented clan of the predicted serine type proteases dataset is S10, a carboxypeptidase (Figure 3.8).

Table 3.3 Distribution of the proteases in EST library based on the prediction of protease function obtained with MEROPS.

Type of protease	The total # of sequences/protease class in the data set	The # of proteases predicted to be secreted by Signal P 3.0 within a class
Aspartic Protease	2	1
Cysteine Protease	5	5
Serine Protease	23	9
Metalloprotease	30	13

Next we determined the number of putative proteases that are predicted to be secreted using Signal P3.0 analysis server (http://www.cbs.dtu.dk/services/SignalP/) because these may be considered more pathologically relevant than intracellular enzymes. Results are depicted in Table 3.3. The SignalP 3.0 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms: Gram-positive prokaryotes, Gram-negative prokaryotes, and eukaryotes. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models (97). The percentages of metalloproteases and serine proteases that were predicted to be secreted are 39.1 % and 43.3 % respectively. One aspartic protease (50%) and all five of the putative cysteine proteases (100%) were predicted to be secreted.

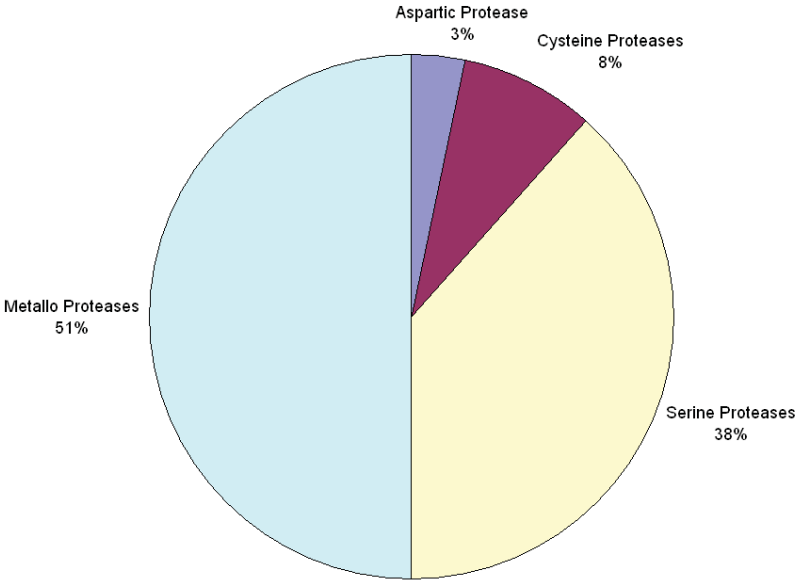

Figure 3.7 The distribution of the proteases in the EST library based on the prediction of proteases function, data generated with MEROPS.

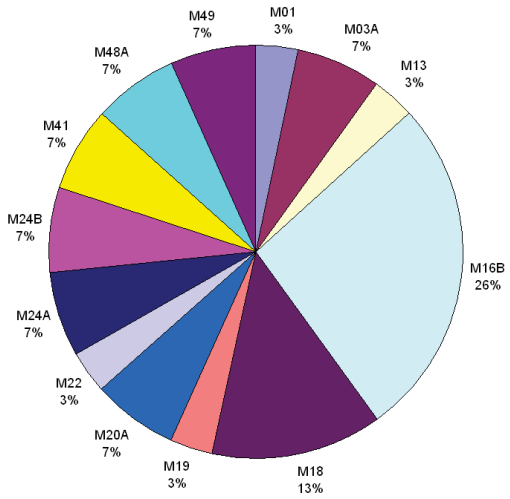

Figure 3.8 The distribution of the metalloproteases based on their function subdivision predicted by MEROPS. We have detected metalloproteses like methionyl aminopeptidase 1(M24A), aminopeptidase P (M24B), aminopeptidase I (M18), glutamate carboxypeptidase (M20A), thimet oligopeptidase (M03A) or FtsH peptidase (M41) and Ste24 peptidase (M48A)

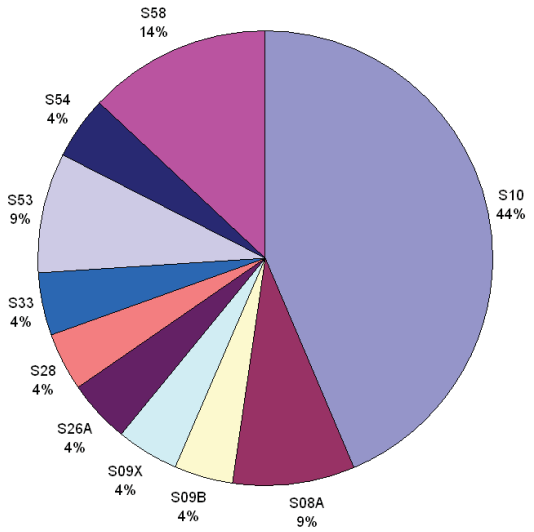

Figure 3.9 MEROPS generated serine protease subdivision based on their biochemical function predicted by MEROPS. We were able to identify carboxypeptidase Y (S10), subtilisin Carlsberg (S08A), DmpA aminopeptidase (S58) or sedolisin (S53).

Verification of gene expression using RT-PCR

In order to further check the quality of our EST dataset we used cDNAs prepared from RNA harvested from fungal spores germinating in the presence or absence of mucin, a glycoprotein that is commonly found in epithelial lining of airway. We not only chose to investigate all the known Alternaria allergens but also other genes of interest including a predicted serine protease, aspartic protease and S-adenosyl-homocysteine hydrolase. The results of the amplification are shown in (Figure 3.10.). Results of this experiment revealed that all of the genes were expressed in both conditions. Moreover, none of the target genes selected appeared to be regulated in a significant manner due to the presence of mucin. It is important to mention that we were able to amplify the major Alternaria allergen Alt a 1 although it was not discovered to be in the EST dataset.

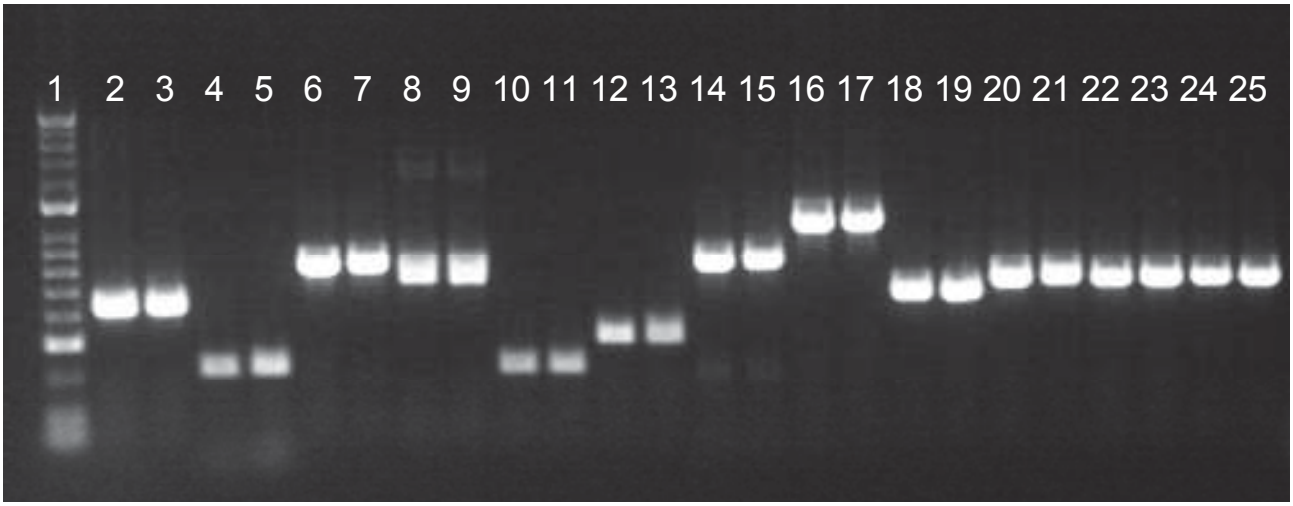

Figure 3.10 Selected genes amplified from cDNA from *A. alternata* grown in GYEB and GYEB supplemented with 1% mucin. Lane 1, DNA marker HyperLadder II, from Bioline (Portland, OR). The remaining lanes with even numbers depict amplification products using cDNA from *A. alternata* grown in GYEB as template and the odd numbered lanes depict amplification products using cDNA from *A. alternata* grown in GYEB with 1% mucin as template. Lane 1 is molecular weight marker; Lanes 2 and 3 are Alt a 1; Lanes 4 and 5, Alta2; Lanes 6 and 7, Alta3; Lanes 8 and 9, Alta4; Lanes 10 and 11, Alta5; Lanes 12 and 13, Alta6; Lanes 14 and 15, Alta7; Lanes 16 and 17, Alt a 10; Lanes 18 and 19, Alt a 12; Lanes 20 and 21, S-adenosyl-homocysteine hydrolase; Lanes 22 and 23, Aspartic protease and Lanes 24 and 25, Serine protease.

Table 3.4 Primers sequences used for selected gene amplification.

Primer Name	Sequence
5' Alt a 1	ATGCAGTTCACCACCATCGCCTC
3' Alt a 1	TTAAGAGCTCTTGGGGAGAGT
5' Alt a 2	GGACTGGGCACAACTCCTAA
3' Alt a 2	AAGTCTGGCCATGGAAATTG
5' Alt a 3	GGAGTCGGAGAGAGTGTTGC
3' Alt a 3	AGCCCAACAAGTCCATCAAC
5'Alt a 4	CATTCCACCACAGCAAAATG
3'Alt a 4	GCACCGAAGAGGAAGTTGTC
5'Alt a 5	TTGGTATCGAGGCTGACTCC
3'Alt a 5	CCTCCTTCTCCTCCTCCTTG
5' Alt a 6	TTGGTATCGAGGCTGACTCC

Primer Name	Sequence
3' Alt a 6	GAGAGATTGGCCGGATCATA
5' Alt a 7	AGCCAATTCGTTCAAGATGG
3' Alt a 7	GGCAACAGCCTCGTAGAAAG
5' Alt a 10	ACCAGGTGTCATCAACGTCA
3' Alt a 10	GACACGGTCTTGGTCTGGAT
5' Alt a 12	CGTATGCAAGTCGTGTCACC
3' Alt a12	ACCGTCGCAATGTCTACCTC
5' SAH	CGCCGTTATTGTTCATTCCT
3' SAH	CTTGCCCAGATCATGCTGTA
5' Aspartic Protease	AACCATGACGTTCCTGAAGC
3' Aspartic Protease	CATCGACGAGAGCCACTACA
5' Serine Protease	CGGGCAGTGTAAAAGGTCAT
3' Serine Protease	CTTCTACTCGGCCATTCTCG

Identification of *Alternaria alternata* specific genes

Next we aimed to identify ESTs that represented *A. alternata* specific genes as compared to the plant pathogenic species, *A. brassicicola*. Our hypothesis was that it is possible *A. alternata* may possess unique proinflammatory proteins since there has not been a report of *A. brassicioola* being associated with allergic airway disorders. Different types of bioinformatics methods were used to analyze the unisequence set to investigate this hypothesis.

The first exercise performed with the EST unisequence set was to determine how many of the ESTs represented unique genes in *A. alternata* as compared to *A. brassicicola*. For this initial analysis, the *A. alternata* unisequence EST dataset was compared to the *A. brassicicola* genome to identify not only the similarities but also the differences that are found between the two strains of Alternaria using PROmer (98). The PROmer program can generate alignments based upon the six-frame translations of both input sequences and is a component of the MUMmer software package (http://mummer.sourceforge.net/) (37).

Using PROmer, we determined 1,017 *A. alternata* regions (from contigs and singletons) at least 500 bp in length with no similarity with *A. brassicicola* regions. Using an e-value cutoff of 10^{-30} to avoid false positives, these 1,017 specific regions were then blasted against the NR

database and resulted in a target set of 1,126 proteins. Using tBlastn (10^{-5} e-value cutoff) these 1,126 proteins were reciprocally blasted back against the *A. brassicicola* genome to insure that the proteins were truly specific to *A. alternata*. The resulting high quality set consisted of 226 proteins. These 226 protein regions were then clustered using OrthoMCL (99). OrthoMCL was used to characterize and cluster the proteins based on their similarity. This set of regions represents a good group of candidates of unique genes of *A. alternata* comparative with *A. brassicicola*. Collectively, based upon results of PROmer, blast analyses and OrthoMCL there were 47 ESTs predicted to be solely unique to *A. alternata* related to *A. brassicicola*. Most of these unique genes have hypothetical or unknown function but it was possible to annotate several of them and putative biochemical functions were assigned. Some of these included proteins predicted to have chloramphenicol acetyl transferase, monoamine oxidase, ornithine decarboxylase, alternative oxidase, NADH/ubiquinone oxidoreductase and NADH dehydrogenase activities. The immunological/pathological relevance of these unique proteins remains to be elucidated.

In addition, using BlastX (e value cutoff of 10^{-20}) we were able to identify 733 *A. alternata* sequences, (9.11% of the total unisequence set) corresponding to predicted secreted *A. brassicicola* proteins. It is important to mention that approximately 6% of the *A. brassicicola* proteome is predicted to be secreted based on SignalP 3.0 analysis (http://www.cbs.dtu.dk/services/SignalP/) performed earlier this year (Lawrence et al, unpublished data). The higher percentage of *A. alternata* genes predicted to encode secreted proteins could be due to larger number of genes expressed during spore germination that correspond to these types of secreted proteins important for the breakdown of complex carbohydrates and proteins as nutrient sources.

Discussion

This study represents the first larger-scale investigation into the transcriptome of *Alternaria alternata*. We chose to sequence and characterize spore germination ESTs as an initial step in identifying genes and corresponding proteins that could play a role in the development and exacerbation of allergic disease caused by Alternaria.

Information obtained by classifying ESTs and assigning GO and GOslim terms were informative regarding the level of activity and the type of processes that are taking place during the spore germination process of *A. alternata* in the presence of mucin. The fact that the metabolic and catalytic processes are very well represented is encouraging and somewhat expected. Hydrolases and proteases are the most represented terms in the group of catalytic proteins and this fact is suggestive that mucin may be eliciting at least a subset of processes that are known to be involved in the allergic and immunomodulatory reactions.

The characteristic destruction of tissue in inflammatory diseases is to a large extent mediated by an excess of serine proteases and matrix metalloproteases (100-102). It was previously shown that proteases in extracts of the allergenic fungus *Aspergillus fumigatus* cause epithelial cell desquamation and release of proinflammatory cytokines (103). Alkaline serine proteases such as Asp f 13, Asp fl 13, and Asp o 13 are known allergens from *A. fumigatus*, *A. flavus*, and *A. oryzae*, respectively (104). Asp fl 13 (from *A. flavus*) was shown to have 100 % identity at the amino acid level with Asp o 13 (105). Similar serine proteases, Pen b 13, Pen c 13, and Pen n 13 with sequence homology to the *Aspergillus* spp. f13-type allergen protease have also been identified from various species of Penicillium (106). In another study, the same group of researchers (107) showed that the alkaline and/or vacuolar serine proteinases are major allergens in Aspergillus and Penicillium. Although no known Alternaria allergens have been classified as proteases, in our present study we identified 60 putative proteases from Alternaria including many from the serine class. In a recent study (108) the impact of serine proteases from *A. alternata* on lung inflammation in vivo and on cleaving protease-activated receptor-2 (PAR-2) was examined *in vitro*. *A. alternata* culture filtrate applied to the airway in nonsensitized Balb/c mice induced a protease-dependent lung inflammation. Moreover, *A. alternata* filtrates applied to human bronchial epithelial cells (16HBE14) induced changes in intracellular Ca^{2+} concentration consistent with PAR-2 activation. It is important to note that these studies examined serine protease activity using specific inhibitors and did not identify the individual proteases in filtrates responsible for this type of immunological activity. Thus, our EST data may provide candidate serine protease sequences to investigate in future studies.

We also identified two aspartic proteases in our EST dataset with one being classified as being secreted. It has previously been shown that aspartic type protease activity from commercially available *A. alternata* antigen extracts was important for inducing PAR-2

dependent immunological responses including cytokine production (IL6, IL8, and TSLP) in airway epithelial cells (89). Moreover, in a separate study aspartic protease activity in these same antigen preparations was also found to be responsible for inducing eosinophil activation and degranulation (95). Thus, our EST data may provide aspartic protease candidates for future immunological experimentation.

RT-PCR-based amplification of candidate genes confirmed their presence (Figure 3.1) in our cDNA library. However, we cannot infer that any of these specific genes are upregulated or downregulated because of the presence of mucin. Investigation into additional mucin-inducible candidate genes by RT-PCR is currently underway. A large-scale comparative approach (Alternaria whole genome microarray or RNA-seq) to identify mucin-inducible genes may be possible in the future now that the *A. alternata* genome sequence has become available. On a different note, we included the major *A. alternata* allergen, Alt a 1 as a candidate in our RT-PCR studies as the only gene in this particular set not found in our library. Alt a 1 was easily amplified using RT-PCR although this gene was not found in our assembled data set. The fact that this sequence was not present in our EST set was initially concerning since Alt a 1 is considered a highly abundant secreted protein from Alternaria. It is possible that our library does not represent the entire transcriptome during spore germination or that Alt a 1 specific sequences were eliminated during the quality control step of library processing prior to assembly. With over 6,000 singletons in our assembly out of 19,000 high quality sequences (Table 3.1) we might estimate a redundancy rate of about 66%.

In summary, the EST dataset obtained in this project offers a first look into the gene content of *A. alternata* and represents the beginning of future research regarding identifying immunologically relevant proteins from this ubiquitous allergic airway disorder-associated fungus.

Methods

Culturing Fungi, RNA extraction, and cDNA synthesis and library construction

A. alternata (ATCC11680) was cultured on potato dextrose agar (PDA) at 24 °C for seven days. Spores were harvested by washing plates with glucose yeast extract broth (GYEB)

with or without 1% mucin (bovine sinus maxillary gland mucin, Sigma Aldrich, St. Louis, MO). Spore concentration for each sample was adjusted to 50,000 spores/ml using a hemacytometer. Spores were allowed to germinate for 24 hours at 30°C. Germlings were harvested by centrifuguation (5 minutes at 5,000 x g and immediately frozen in liquid nitrogen.

Total RNA from the *A. alternata* germlings was isolated using the phenol-guanidine isothiocyanate based reagent Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was used to synthesize cDNA using the Cloneminer cDNA library construction kit and cloned into the pDONR vector following the manufacturer protocols (Invitrogen, Carlsbad, CA). Inserts were sequenced bidirectionally using primers -21UPpOT as the 5' primer and -28RPpOT as the 3' primer: -21UPpOT - TGTAAAACGACGGCCAGT-28RPpOT - CAGGAAACAGCTATGACC.

DNA sequencing in 384-well plates was performed on ABI prism using Sanger method. Quality assessment and assembly of reads was performed using PCAP software package (109).

Annotation of ESTs

The assembled ESTs (contigs and singletons) were then annotated using a variety of software tools. In addition to basic blast analyses (98) against genbank nr database we used the the Interpro suite of tools (99). The Interpro suite of tools (http://www.ebi.ac.uk/interpro/) integrates predictive models or 'signatures' representing protein domains, families and functional sites from multiple, diverse source databases: Gene3D, PANTHER, Pfam, PIRSF, PRINTS, ProDom, PROSITE, SMART, SUPERFAMILY and TIGRFAMs. In InterPro (92), identifiable features found in known proteins, can be applied to new protein sequences. It is important to note that Interpro also includes SignalP 3.0 analysis as one tool for prediction of signal peptides (extracellular secretion signal). InterPro analysis also assigns gene ontology (GO) terms as part of the output. We also performed Signal P3.0 analysis separately utilizing the resources at CBS (http://www.cbs.dtu.dk/services/SignalP/).

Using the GO categorization, genes from different species can be compared based on their GO annotations. GO Slim, GO Slim generic (93) and AMIGO (110) were used for the analysis of EST dataset. Using GO Slim all the GO terms assigned to the EST dataset were placed at the highest parent that is found in the hierarchy of a specific gene.

The MEROPS database (http://merops.sanger.ac.uk/) was used to annotate putative proteases in our EST dataset. MEROPS is a comprehensive information resource for peptidases (also termed proteases, proteinases and proteolytic enzymes) and the proteins that inhibit them and allows one to perform blast analyses. The MEROPS database uses a hierarchical, structure-based classification of the peptidases. In this database system, each peptidase is assigned to a Family on the basis of statistically significant similarities in amino acid sequence, and families that are thought to be homologous are grouped together in a Clan. The classification of putative proteases found in our EST assembly was obtained using the web-based Batch Blast interface at the MEROPS website.

Identification of *A. alternata* specific genes

The annotated *A. brassicicola* (ATCC96836) genome used in these studies is publicly available (http://genome.jgi-psf.org/Altbr1/Altbr1.info.html). The first step consisted of running PROmer (37). PROmer generates alignments based upon the six-frame translations of both input sequences. The input files included the *A. alternata* EST contigs and singletons and the *A. brassicicola* whole genome assembly. As output, PROmer returned all approximate matching regions between each pair of contigs and/or singletons. The resulting output file represented the regions (contigs or singletons) from *A. alternata* that are at least 500 bp and that don't have any significant similarity with *A. brassicicola*. Using *A. alternata* specific regions as query sequences we ran Blastx (91) against nr database in order to find predicted proteins in these regions. The resulting set of hits was used in reciprocal blast analysis against the *A. brassicicola* genome. The set of unique proteins following reciprocal blast was then subject to analysis by OrthoMCL (99). OrthoMCL was used to characterize and cluster the proteins based on their similarity.

RT-PCR

For the gene specific PCR amplifications we used the GoTaq Green Master Mix (Promega Corporation, Madison, WI) which is a premixed, ready-to-use solution containing GoTaq DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. PCR was performed using the manufacturer's protocol. Primers used for these amplifications are shown in Table 3.4.

CHAPTER 4

The Alternaria allergome.

Introduction

More than 80 genera of fungi have been associated with symptoms of respiratory allergy and the prevalence of this kind of allergy is estimated at 20–30% among atopic individuals and up to 6% in the general population (37, 38). Human airways are continuously exposed to ubiquitous environmental fungi and one of the most common is Alternaria. Fungal allergy including allergic rhinitis, conjunctivitis, bronchial asthma, and allergic bronchopulmonary mycoses results from exposure to spores or hyphal fragments. From a clinical perspective, Alternaria has long been associated with IgE-mediated, histamine-dependent mold allergy, allergic rhinitis, and asthma. For example, in one study, nineteen percent of patients with known respiratory tract symptoms suggestive of allergic disease were found to be sensitized to fungi and 66% of them were positive to *A. alternata* (111).

Over the years there have been a multitude of studies describing fungal allergens, their biochemical activities, and their structures (112). Several themes have emerged from these studies. One of these themes is that several types of allergens have been found to correspond to highly conserved proteins across the fungal kingdom and are cross-reactive in fungal sensitized patients. A few examples of these include superoxide dismutase, ribosomal proteins, and enolase (112, 113). The other theme that has become more apparent is that in addition to the conserved allergens, there are fungal genera specific allergens as well. For example, the secreted ribotoxin Asp f 1 is found only in *Aspergillus fumigatus* and closely related species (114), Alt a 1, the major secreted allergen from Alternaria is also specific to this fungal genus and closely related taxa (47, 115). Thus, one could hypothesize IgE reactivity to the conserved allergens serves as a diagnostic for fungal sensitivity in general and the species specific allergens useful as a tool to identify sensitivity to specific fungal genera or species.

Recently a few studies have reported using bioinformatics to survey the distribution of mold allergen homologues at the whole genome level in sequenced fungal genomes (5). For example, using a non-redundant set of clinically validated mold allergens (~99) from several different fungal species as a query for blast analyses against the *A. fumigatus* genome sequence, over 50 homologues were found that exceeded the minimum criteria of e $\leq 10^{-5}$ and at least 100 aminoacids alignment length.. The authors reported that the results of this study suggest that *A. fumigatus* may produce many more allergen candidates than have been clinically validated and warrants further investigation.

In our lab we are studying the allergenic potential of fungi in general and Alternaria spp. more specifically. There have been thirteen allergens described in *A. alternata* fungus to date (Table 4.1).

Table 4.1 The known biological functions of A .alternata major and minor allergens (http://www.allergon.com)

Allergen	Function	Obsolete Name	References
Alt a 1 Major allergen	Esterase, a heat stable protein		(42, 112, 116)
Alt a 2 Major allergen	Aldehyde dehydrogenase		(42, 112)
Alt a 3	Heat shock protein 70		(42, 112, 117)
Alt a 4	Disulfide isomerase		(42, 112)
Alt a 5	Ribosomal protein P2	Alt a 6	(42, 112, 117)
Alt a 6	Enolase	Alt a 5, 11	(42, 112, 116)
Alt a 7	YCP4 protein		(42, 112, 116)
Alt a 8	Mannitol dehydrogenase		(40)
Alt a 10	Aldehyde dehydrogenase		(42, 112, 116, 117)
Alt a 12	Acid ribosomal protein P1		(112, 117)
Alt a 13	Glutathione-S-transferase		(112)
Alt a 70kDa	Unknown		(116)
Alt a NTF2	Nuclear transport Factor 2		(44)

Alt a 1 is a secreted allergen causing sensitization in asthmatics, 93% of asthma patients were reported to have IgE to Alt a 1 in one study (118). Sixty-one percent of individuals with asthma have IgE to Alt a 2, another allergen (117). Alt a 3 is recognized by 5% of allergic subjects (117). For the enolase Alt a 5, the reported range of allergenicity varies from 20% (119)

to 50% (120). Alt a 6 and Alt a 7 bound IgE in approximately 7% of Alternaria sensitive individuals (42). Only 2% of Alternaria sensitive individuals have IgE to bound Alt a 10. Thus, only Alt a 1 and Alt a 2 may be considered major allergens and the rest classified as minor allergens based on several studies mentioned above (112, 115). Two additional allergens in *A. alternata,* Alt a 70kDa, a 70 kDa protein recognized by 87% of patients in one study (116) and Alt a NTF2, a nuclear transport factor 2 protein recognized by only 3% of patients in another study (44) have also been reported. However, blastx analysis using the Alt a 70kDa cDNA sequence available in genbank nr has revealed that it is a chimera between Alt a 1 and a portion of the vector used for cloning (Lawrence, unpublished data). Thus, it is not considered a new allergen in our opinion and explains the high levels (87%) of IgE reactivity in Alternaria-sensitized patients (107).

The mechanisms underlying the strong clinical association between allergic respiratory disorders and fungal sensitivity are not entirely clear (111). In fact, it was recently reported that there appears to be a strong correlation of fungal allergy and people with severe asthma (121). In this clinical study, over 70% of individuals having severe asthma (2 or more trips to the emergency room due to asthma attack over the last year) also displayed fungal sensitivity and this was not true for sensitivity to other allergen sources (cat, pollen, dustmite, etc.). Over 30% of mild asthmatics also displayed fungal sensitivity but were often pan-allergic, meaning that they had allergies to multiple organisms and or sources.

In this project we have used bioinformatics approaches to survey, evaluate and characterize the predicted allergen homolog set (allergome) in several fungal genomes, including *A. alternata, A.brassicicola* and *A. fumigatus.* In contrast to the previous studies surveying mold allergen homologues within *A. fumigatus*, the novelty of our study lies in the fact that we surveyed fungal genomes for the presence of homologues corresponding to all known allergens regardless or origin. We discovered that hundreds of allergen homologues from diverse sources are present in fungal genomes. Our results strongly suggest that fungi contain tremendous allergenic potential and fungal exposure could be responsible for eliciting sensitivity to allergens from other organisms.

Results

In a first *in silico* experiment, we sought to define the fungal-specific allergome in the *A. brassicicola* genome and *A. alternata* EST datasets in a similar manner as previous studies with *A. fumigatus* (5). A set of 99 non-redundant known fungal allergens was used as query and Blastp and tBlastn algorithms were applied. Levels of identity were transformed into a heat map depicting the level of homology of *A. alternata* ESTs and *A. brassicicola* genes with the known fungal allergens (Figure 4.1). Raw output data from the blast analyses is also presented in Supplementary Table 1). In the *A. brassicicola* genome we found homologues to the vast majority of known fungal allergens (76/99). We found lower but significantly high (72/99) numbers of homologues in the *A. alternata* ESTs but that is to be expected since this is based on mRNA sequences and these represent only 30% of the predicted gene space in the *A. alternata* genome (see Chapter 3). We also present data regarding the distribution of known Alternaria allergens amongst the set of 99 clinically validated fungal allergens (Figure 4.2). As expected the majority of Alternaria allergens are homologous with other known fungal allergens with Alt a 1 being specific to *A. alternata* in the context of this analysis.

Next, in order to define the allergome (the suite of putative allergenic proteins) in the predicted *A. fumigatus, A. brassicicola* and *A. alternata* proteomes and a hypothetical proteome encoded by a set of *A. alternata* spore germination ESTs we used a set of 1,407 known allergens (pan-allergome) from diverse organisms (fungi, pollen, insects, venoms, grass, food, etc.) as a query for blast analysis. Local fungal genome databases (predicted *A. brassicicola, A. alternata* and *A. fumigatus* proteomes, and the hypothetical proteome encoded by a set of approximately 19,000 high quality reads *A. alternata* spore germination ESTs was investigated using Blast (Blastp and tBlastn respectively) algorithms. Using $e \leq 10^{-10}$ and minimum of 100 amino acids alignment length as a cutoff, the comparison of *A. fumigatus A. alternata* and *A. brassicicola* with 1,407 known allergens revealed that all three genomes harbored high numbers of allergen homologues in diverse classes (>220 hits/genome) (Figure 4. 3 and Table 4.2).

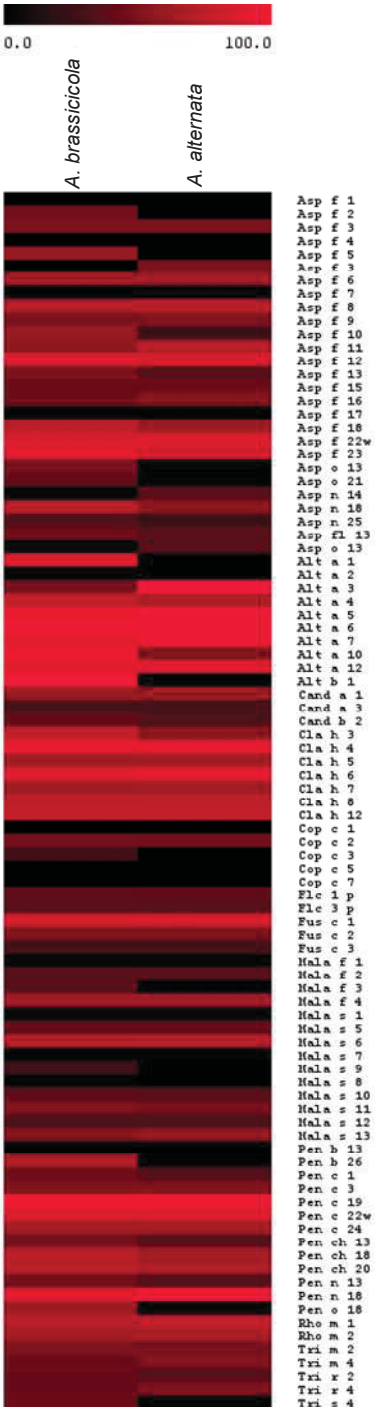

Figure 4.1 A heat map showing the homologies of known fungal allergens with predicted proteins of *A. brassicicola* and *A. alternata*. The Blastp and tblastn hits and their level of identity were transformed into a heat map depicting the level of homology of *A. alternata* and *A. brassicicola* genes with known fungal allergens. The heat map was generated using the MultiExperiment Viewer (MeV 4-3-01).

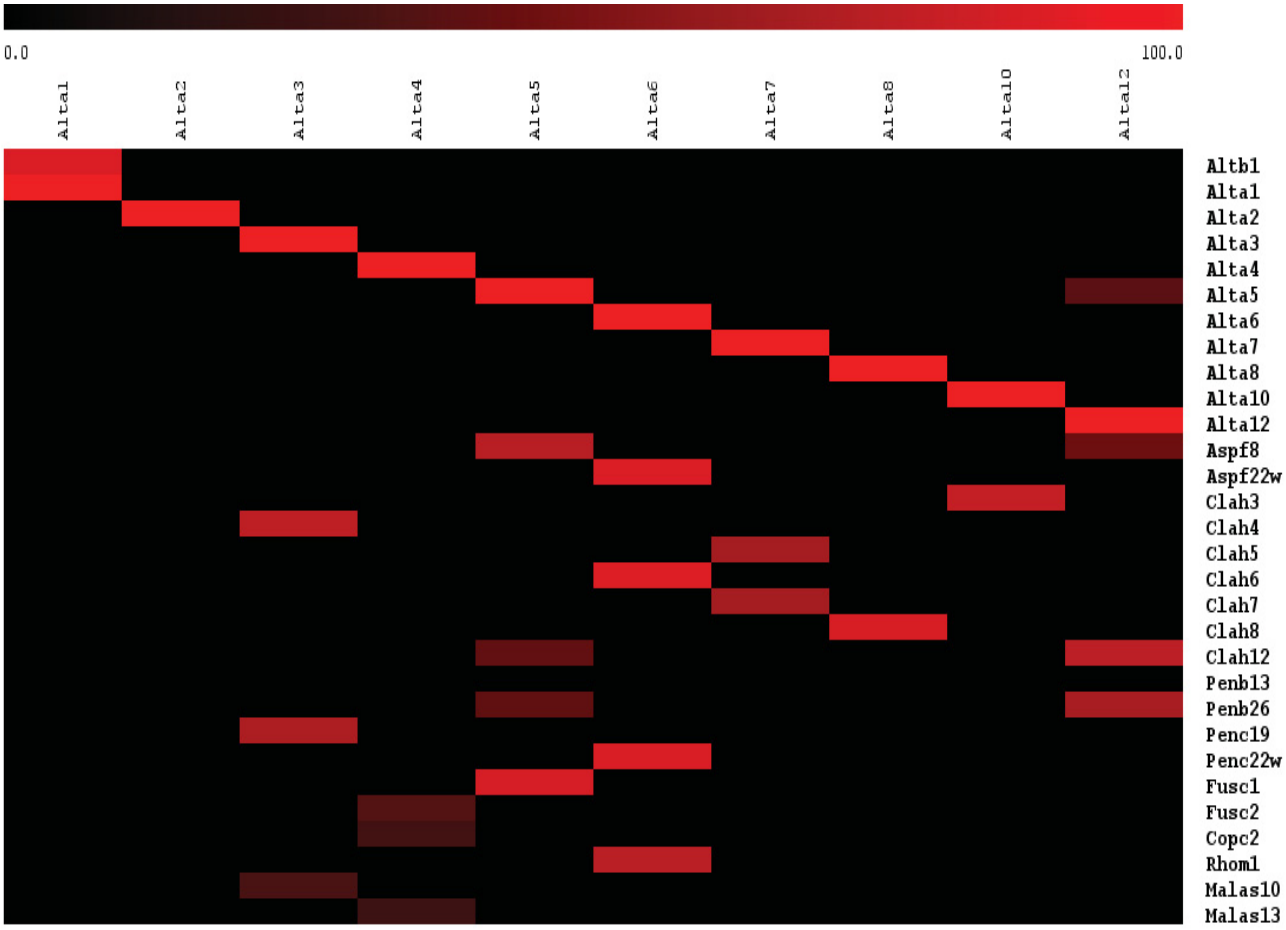

Figure 4.2 Comparison between the majors allergens from *A. alternata* and known fungal allergens. The Blastp hits and their level of identity were transformed into a heat map depicting the level of homology of *A. alternata* genes with known fungal allergens. The MeV 4-3-01 was used to generate the heat map.

Interestingly, using these parameters we also found 72 allergen homologues in the *A. alternata* ESTs (Table 4.2, Figure 4.4). Among these data sets investigated, the *A. alternata*

genome contained the highest number (274) of allergen homologues. Interestingly, *A. alternata* had almost double the number of potential allergens of the "contact" class compared to *A. brassicicola* and *A. fumigatus*. We also found that the *A .alternata* genome alone harbored bacterial allergen homologues. As expected, homologs to known fungal/mold allergens were the most abundant class in all of these datasets.

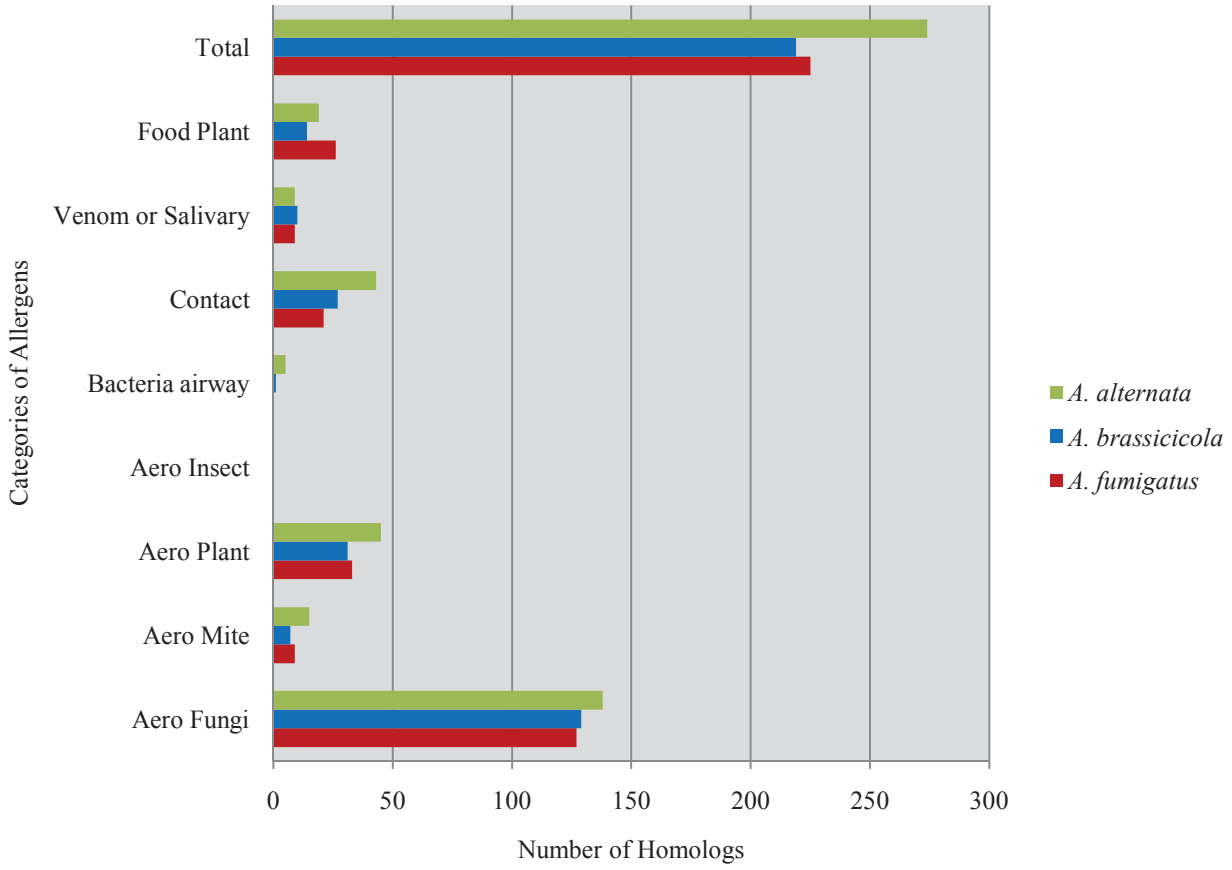

Figure 4.3 Potential allergenic homologs in A. fumigatus, A. alternata and A. brassicicola genomes

Table 4.2. Distribution of allergen homologs in fungi. Table depicts various classes of allergens found in genomes (Aspergillus fumigatus, Alternaria brassicicola, Alternaria alternata) and A. alternata spore ESTs (cDNA library derived from mRNA extracted from spores germinating in the presence of sinus mucin).

	Alternaria. alternata	*Aspergillus fumigatus*	*Alternaria brassicicola*	*Alternaria alternata* ESTs
Aero Fungi	138	127	129	50
Aero Mite	15	9	7	1
Aero Plant	45	33	31	7
Aero Insect	0	0	0	1
Bacteria airway	5	0	1	0
Contact	43	21	27	6
Venom or Salivary	9	9	10	2
Food Plant	19	26	14	5
Total	274	225	219	72

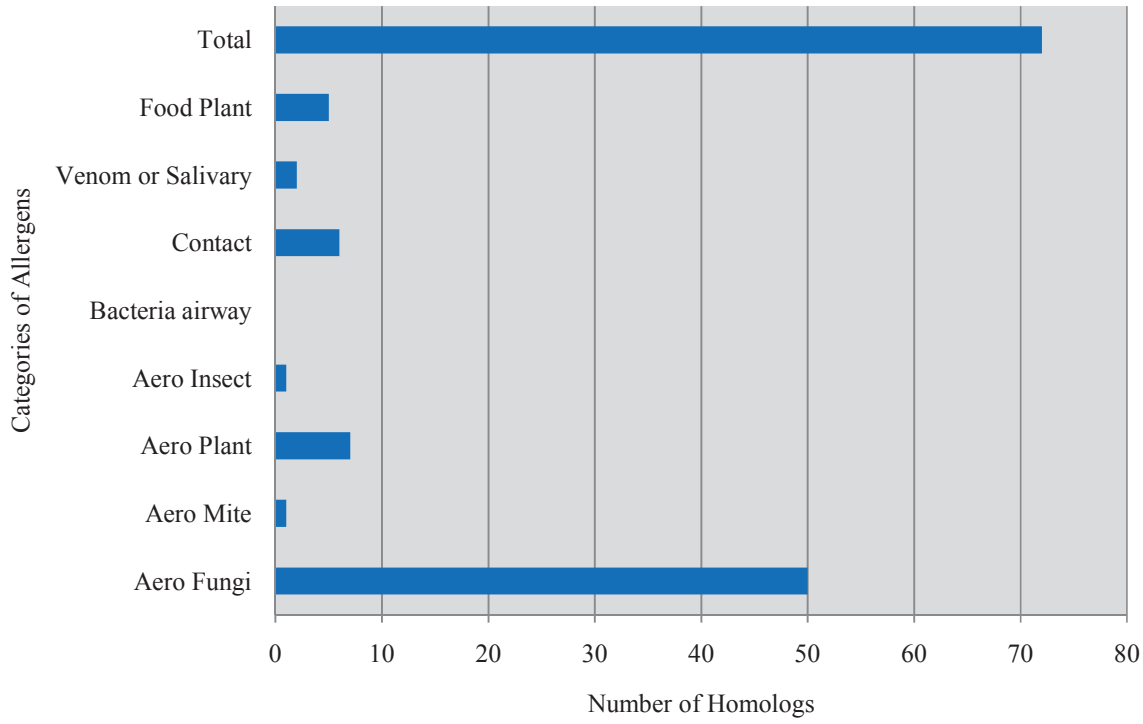

Figure 4.4 Potential allergen homologs in *A. alternata* ESTs dataset

Discussion

We have investigated the allergenic potential of two known allergenic fungi, Alternaria and Aspergillus using a bioinformatics approach to discover and characterize allergen homologs derived from diverse organisms. Interestingly our data strongly suggest that fungi possess the capacity to produce many and diverse types of potential allergens originally found in organisms and bio-sources such as fungi, plants and pollen, dustmite and cockroach, and venoms for example.

As stated previously, homologs of 76 out of 99 known fungal allergens were found in the predicted *A. brassicicola* proteome and 72/99 in a relatively modest set of *A. alternata* ESTs (mRNA derived from spores germinating in the presence of sinus mucin). In addition, a larger

scale analysis of allergens in the fungi investigated revealed that the *A. alternata* genome contained the highest total number of homologues of allergens of diverse classes. This suggests that the genus Alternaria in a general sense has high allergenic potential especially during the spore germination process. It is our hypothesis that long term exposure to high levels of fungal spores, especially Alternaria in light of our findings, may result in sensitization of humans to allergens from other organisms or sources such as pollens, insects, or dustmite for example. Moreover, the fact that at least 45 proteins in *A. brassicicola* genome and 43 in *A. alternata* ESTs that maintain at least 50% identity with known fungal allergens could have pathological and clinical significance.

Except for Alt a 1 (the major *A. alternata* allergen) and Alt a 2, the remaining known Alternaria allergens have been previously described as having potentially cross-reactive homologues in other fungi. Queries using the Alt a 6 enolase protein resulted in the most hits in the allergome fungal dataset with hits to Asp f 22 (identity 88%), Cla h 6 (89%), Pen c 22w (87%) and Pho m 1 (74%). These high levels of identity are most likely due to the highly conserved nature of enolases across taxa. In a previous study it was reported 19 allergens from other species have at least 50 % identity with *A. fumigatus* proteins suggesting that proteins from *A. fumigatus* can or might cross-react with serum from patients sensitized to these fungi and that these proteins are possibly "cryptic" allergens (112). Interestingly, in one part of our study we have shown that 45 proteins from *A. brassicicola* and at least 43 from *A. alternata* have homology with known fungal allergens. The criteria of selection of these proteins was the same as was used in an *A fumigatus* study (e-value $\leq 10^{-10}$ and identity at the amino acid level of at least 50%) thus suggesting that Alternaria has double the level of "cryptic" allergen homologues compared to Aspergillus and could be clinically relevant.

The presence of genes/proteins homologs with known allergens does not, by itself, prove that these proteins identified in either *A. brassicicola* or *A. alternata* are allergenic or even cross-reactive. Many other criteria have to be implemented for such an affirmation such tests with sera from known Alternaria sensitive patients whereby IgE cross-reactivity to that protein would be measured.

Methods

Data and Bioinformatics

The data used in this project have different sources. For the *A. fumigatus* genome we used the data publicly available from Af293 strain (122), for *A. brassicicola* (ATCC96836) we used the genome that is available in the Lawrence lab (unpublished data) and for *A.alternata* (ATCC11680*)* we used both a genome sequence and a cDNA library obtained from RNA isolated during spore germination in the presence of mucin (see Chapter 3 methods).

A set of 1407 known allergens and another set of 99 known fungal allergen proteins, including several previously described *A. alternata* allergens, were used as query sequences for Blastp (e value cutoff of 10^{-20}) analysis against the predicted *A. brassicicola, A. alternata,* and *A. fumigatus* proteomes. TBlastn (e value 10^{-20}) was performed using the *A. alternata* unisequence EST set (31). The data obtained regarding the hits in all three genomes and the EST data set was manually curated to eliminate redundancy. As criteria of selection for the final data sets were the length of the alignment at the amino acids level (being longer than 100 amino acids), the level of identity (the highest level was chosen against a smaller one) and the p value (the smaller p-value).

The Blastp and tblastn hits and their level of identity were transformed into a heat map depicting the level of homology of *A. alternata* and A. brassicicola genes with known fungal allergens. For the heat map generation, the MultiExperimentViewer (MeV) software, a component of the TM4 suite, was used. MeV is an open source Java application and is hosted at SourceForge. In a subsequent *in silico* experiment, the allergens that have previously been found and described in *A. alternata* were compared with all known fungal allergens to date that were compiled based on the approach presented by Bowyer and Denning (5).

CHAPTER 5

Investigations of Alt a 1 function.

Introduction

Alternaria species are considered some of the most important fungi that are responsible for allergenic reactions in humans and additionally in horses causing what is commonly called wheezing disease (21). Most of the existing Alternaria allergens are fairly conserved proteins with known function such as enolase, ribosomal proteins, nuclear transport factor, and aldehyde dehydrogenase (42-44). The biological functions of some allergenic proteins from various organisms are unknown (42, 45). The most notable example is Alt a 1, the major allergen secreted by *A. alternata* and a protein with no known function in fungal metabolism or ecology described as of yet (45, 46).

Diagnosis of *A. alternata* sensitization is hampered by the variability and complexity of fungal extracts, and thus simplification of the diagnostic procedures with purified allergens has been investigated. Currently, in many of the allergy medical clinics in the U.S.A., for the sensitization test to *A. alternata,* pure Alt a 1 protein is used in lieu of total fungal extract because it was proven to produce the same reaction in the human subjects as the total extract of proteins from *A. alternata* (23). Alt a 1, either in its natural or recombinant form, is sufficient for a reliable diagnosis of *A. alternata* sensitization and induces skin prick reactivity comparable with that produced by total proteins *A. alternata* extract in the vast majority of Alternaria sensitized patients (23).

Alt a 1 is the *A. alternata* protein that elicits the most intense allergic reaction in humans found thus far and yet no known biological function has been assigned to this protein. Even though Alt a 1 has been extensively produced by recombinant technology, the fact that the biological activity remains unclear has made it difficult to fully characterize it from immunological perspectives, beyond identification of IgE binding sites (47).

Interestingly, in a study published in 2006 it was reported that enzymatic activity corresponding to phosphatase and esterase was associated with native and *E. coli* produced

recombinant Alt a 1 (48). These findings suggest that the possible structural differences between the recombinant and the native proteins do not affect the enzymatic activity in a significant manner. In addition, this study reported that the same enzyme activities were found in cellular extracts and culture filtrates from all strains examined.

In the present study we utilized a combination of approaches to further our understanding of the biological role of Alt a 1 in the context of both fungal biology as well as allergic inflammation and innate immune responses. We used a bioinformatics approach to produce a hypothetical structural model of the Alt a 1 protein, we produced and purified recombinant Alt a 1 and utilized it in several growth inhibition experiments using bacteria and fungi. Moreover, the effect of rAlt a 1 on the growth of wild type *A. alternata* was evaluated not only on solid media but also in liquid media and also by evaluating spore germination in the presence of Alt a 1.

We produced three classes of *A. alternata* mutants, an Δ*alt a 1* deletion mutant (KO), a complemented mutant (reintroduction of the wild type *Alt a 1* gene in the Δ*Alt a 1* deletion mutant background) and an Alt a 1 overexpression mutant using a strong constitutive promoter, from *Pyrenophora tritici-repentis* called the ToxA promoter fused to the wild type *Alt a 1* gene (123). We used these three mutants along with wild type *A. alternata* to assess the role of Alt a 1 in fungal biology. A subset of these mutants was used to investigate the changes in gene expression in human lung epithelial cells following exposure to spores especially due to the presence or absence of Alt a 1.

Results

In our attempt to initially characterize structural aspects of the Alt a 1 protein, Using the Alt a 1 amino acid sequence and Protein Predict software, we found that the Alt a1 protein can be classified as: H (alpha-helix) 14.01%, E (beta-strand) 36.94% and L (neither helix, nor strand) is 49.04 %. The alpha-helix is at the N-terminus and there are nine beta-strand motifs alternating with turn motifs (Figure 5.1) (http://www.predictprotein.org).

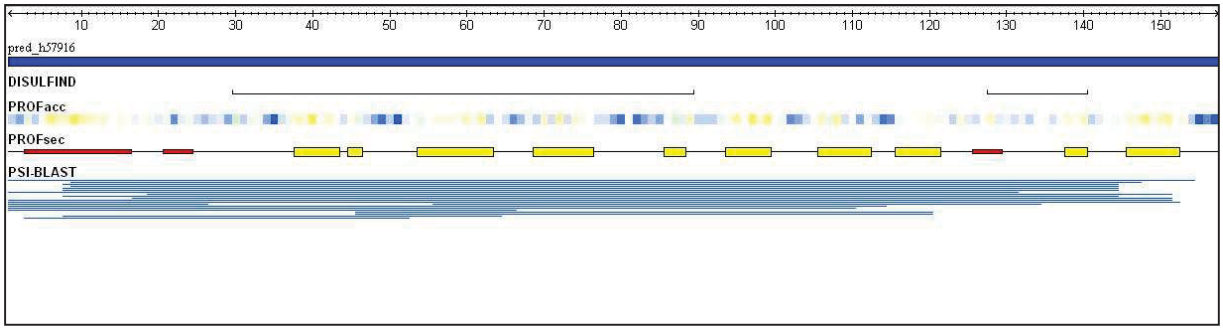

Figure 5.1 Alt a1 alpha-helix and beta-strands obtained using Protein Predict (http://www.predictprotein.org)

In addition, a 3D model was generated (Figure 5.2) using WebMod, the online version of Modeler (124). The generated model was considered a weak model due to the lack of suitable templates available. However, one template: 1JRH_H showed acceptable homology (35% Sequence identity of the alignment) and was useful for 3D structure prediction. The 1JRH_H is a 219 AA length protein that is an anti-human interferon gamma receptor monoclonal antibody (116). It binds the receptor and inhibits binding by IFN-gamma (125). We are currently testing if Alt a 1 can bind this receptor or other cytokine receptors in vitro using pull down approaches.

Because the generated model was useful but probably not the most accurate depiction of three dimensional structure we produced recombinant Alt a 1 (rAlt a 1) in an *E. coli* expression system to be used for obtaining the crystal structure in the future and also additional lab experiments described later.

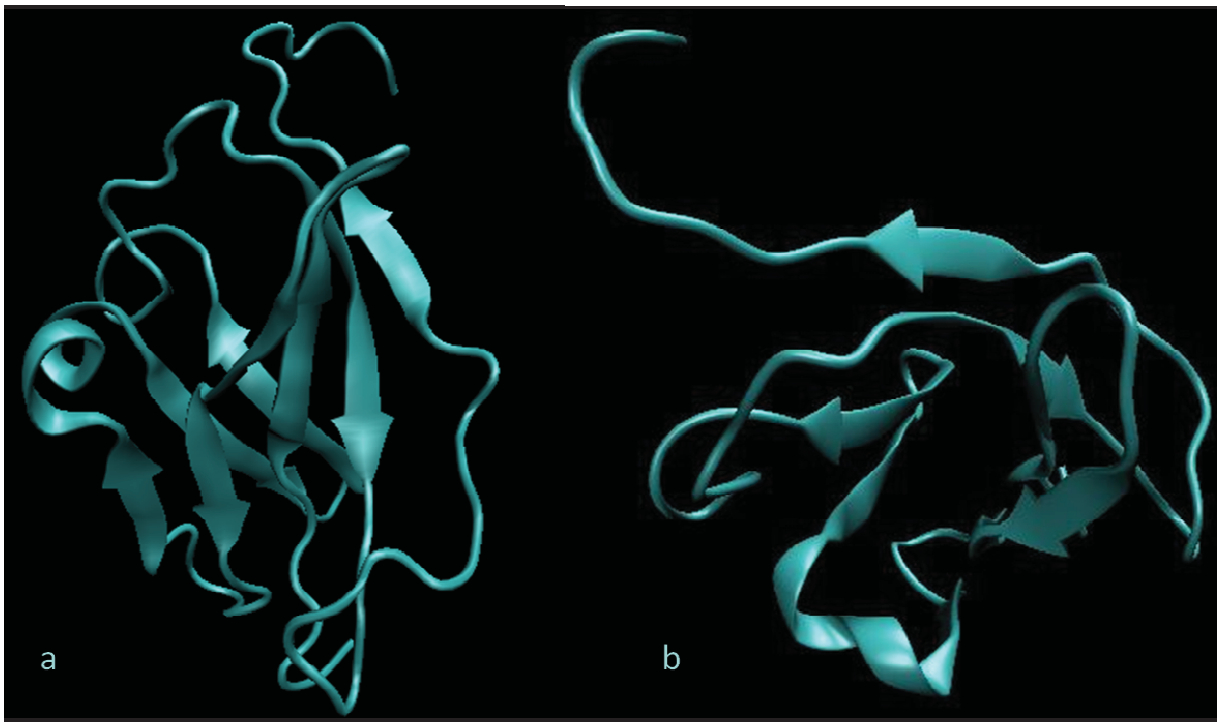

Figure 5.2 Cartoon representation of the model Alt a 1 protein from *A. alternata* (homology modeling) shown in two perspectives. The pictures were generated and rendered in VMD (126).

The recombinant Alt a 1 (rAlt a 1) purification process is shown in Figure 5.3. Furthermore, elution fractions 5 and 6 were subsequently concentrated and the original buffer was exchanged to 0.2M Sodium Phosphate pH 7.5. The pure rAlt a 1 protein was assessed for its enzymatic activity using the Apizym system (bioMerieux Vitek, Inc., Hazelwood, MO) and our results confirm the enzymatic activity reported previously for Alt a 1 (phosphatase and esterase-data not shown). In addition we uncovered two other possible enzymatic activities for Alt a 1, esterase lipase and naphthol-AS-Bi-phosphohydrolase. Experiments utilizing pure rAlt a1 in regards to growth inhibition-type assays are described later.

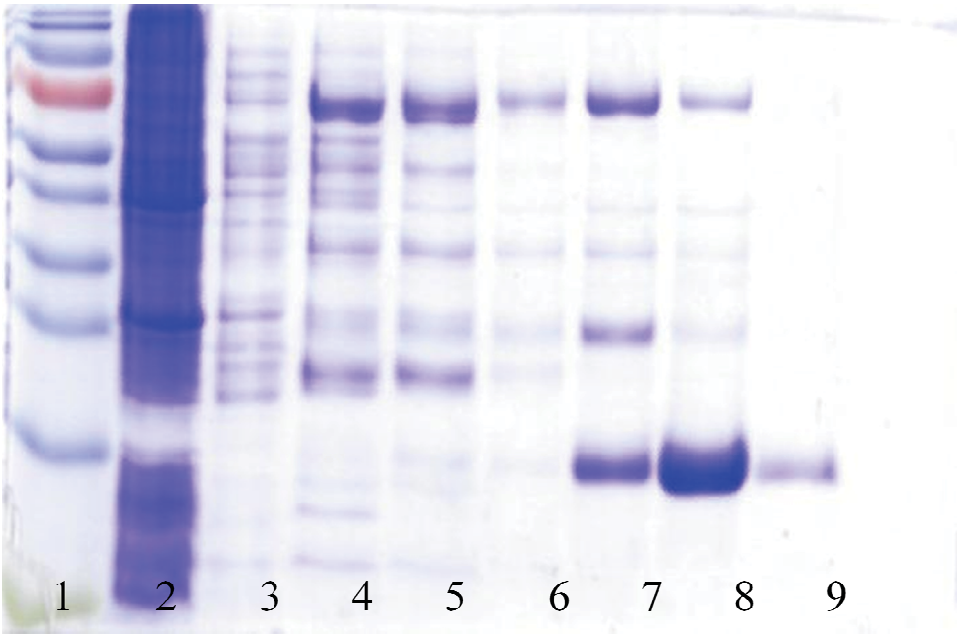

Figure 5.3 Recombinant Alt a 1 protein purification. Fractions were run on a 12% SDS polyacrylamide gel. Lane 1, protein marker (EZ-Run Pre-stained Rec Protein Ladder, Fisher Bioreagents, Pittsburg, PA); Lane 2, the flow through column fraction; lane 3 is the first washing fraction; lane 4-9 are elution fractions with sodium phosphate and ammonium sulfate as base and increasing concentration (50, 70, 85, 100, 250 and 500mM) of imidazole for each elution. Gel was stained with Coomassie blue.

Production of Alternaria Alt a 1 mutant strains

We produced three classes of *A. alternata* mutants, an Δ*alt a 1* deletion mutant (KO), a complemented mutant (reintroduction of the wild type *Alt a 1* gene in the Δ*Alt a 1* deletion mutant background) and an Alt a 1 overexpression mutant using a strong constitutive promoter, from *Pyrenophora tritici-repentis* called the ToxA promoter fused to the wild type *Alt a 1* gene (123). We used these three mutants along with wild type *A. alternata* to assess to role of Alt a 1 in fungal biology. A subset of these mutants was used to investigate the changes in gene expression in human lung epithelial cells following exposure to spores especially due to the presence or absence of Alt a 1. For the Δ*alt a 1* deletion mutant, the Alt a 1 gene was replaced by Hygromycine B phosphotransferase (HygB) gene using a linear replacement construct through a process that relies on two homologous recombination events using our previously published methods (127).

After the transformation of *A. alternata* protoplasts, the resulting Δ*alt a 1* deletion mutants were confirmed using Southern hybridization for five individual transformants. All five transformants had the *Alt a 1* gene deleted (Figure 5.4).

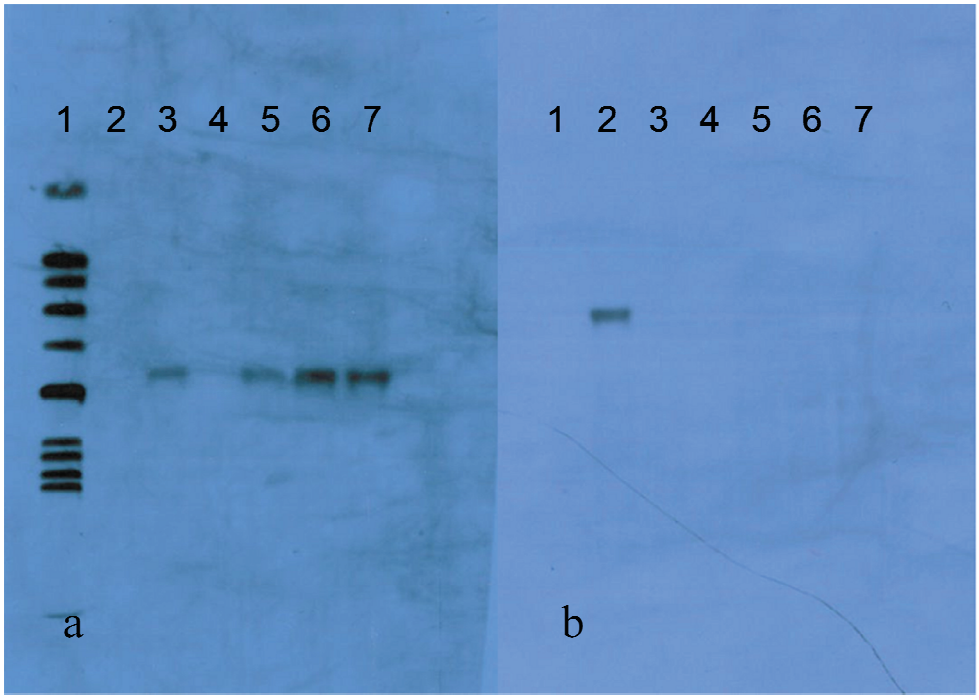

Figure 5.4 Southern hybridization of Alternaria transformants. Genomic DNA was extracted from 5 individual transformants, digested with Hind III and electrophoresed on agarose gels and transferred to Hybond N membranes (Amersham). Hybridization results of the blot probed with HygB, (Panel a) and Alt a 1 (Panel b) are shown. Lane 1, 100 bp DNA ladder (Promega, Madison, WI); Lane 2, *A. alternata* wild type and lanes 3 -7 *A. alternata* transformant genomic DNA. Note HygB present only in transformants (not wild type, untransformed fungus, panel a) and the absence (deletion) of the Alt a 1 gene in transformants 3-7 (panel b).

In addition to gene deletion, we also created complementation strains and overexpression strains. Western blot using a commercially available Alt a 1 specific antibody was used as a validation method for all three types of mutants obtained in relation with Alt a 1 gene: Δ*alt a 1* deletion mutant, the complemented deletion mutant and the overexpression mutant in comparison with the wild type *A. alternata* (Figure 5.5). As expected 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 compared to levels exhibited by wild type.

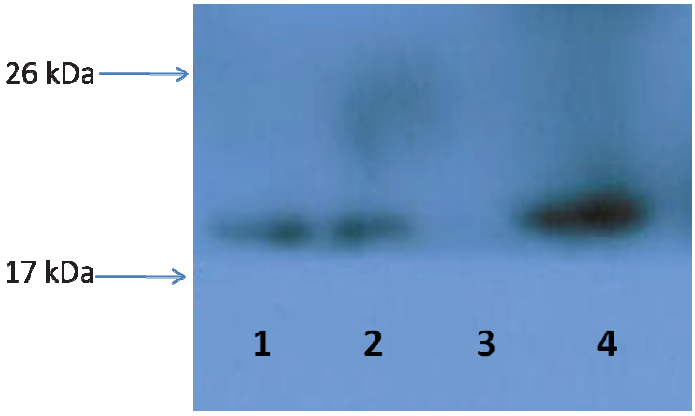

Figure 5.5 Western Blot analysis for Alt a 1 protein production in mutant strains. Lane 1. Ladder (Fisher Bioreagents, Pittsburgh, PA); lane 2 the *A. alternata* wild type; lane 3 complement mutant; lane 4 the Δ*Alt a 1* deletion mutant and lane 5 overexpression mutant.

In order to evaluate and characterize the three mutants, a growth experiment was set up using different types of solid and liquid media. The growth rate of wild type *A. alternata* was compared with that of the three mutants. The solid media that were selected were PDA (potato dextrose agar), GluMM (glucose minimal media) and GalMM (galactose minimal media). The measure of the diameters of growth over seven days were followed and registered and can be found in the supplementary Table 2. In Table 5.1 measurements are reported for each day as mean plus/minus standard deviation. 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.

Interestingly, on both PDA (potato dextrose agar) and GluMM (glucose minimal media) we observed the trend that the growth rate of the Δ*alt a 1* deletion mutant was faster than the wild type, complement, and overexpression strains. The overexpression strain had the slowest growth rate of all strains tested. These growth patterns were observed on both PDA and GluMM, with higher differences for GluMM. From a statistical point of view the rate of growth for the Δ*alt a 1* mutant was higher than *A. alternata* wild type with a range between 116 % (day 1) to

146% (day 2). The rate of growth of the overexpression mutant in comparison with *A. alternata* wild type varies from 70% (day 6) to 83% (day 2). A probability of a paired t-test was calculated for all three mutants in comparison with the wild type for each day and most of p-values are situated below the classical threshold of ≤ 0.05. The p-values are shown in the last column of Table 5.1 (Figure 5.7). Figure 5.8 shows day 7 data for growth experiments with the trend for enhanced or slightly more rapid growth in the deletion mutant compared to the wild-type and slower growth due to Alt a 1 overexpression.

Table 5.1 Growth rate of Alternaria mutants. Growth rates of Alt a 1 KO (deletion), complementation, and overexpression mutants on solid media were compared with the wild type over seven days. Mean and standard deviation of measured growth for each day is shown along with the statistical significance (p-value) compared to wild type at that particular day. The probability of significance using a paired t-test is reported data marked with "*" exhibited $p \leq 0.05$.

		GluMM	GalMM	PDA	p-value
WT	day 1	6.33+0.57	6+0.00	7+0.00	
	day 2	13.33+1.15	8+1.00	21+1.00	
	day 3	23+2.00	11+1.00	35+1.00	
	day 4	33.33+3.05	13.33+1.52	48+1.52	
	day 5	48.33+2.88	14.33+0.57	61+1.73	
	day 6	57+2.64	15.67+1.15	70+1.15	
	day 7	65+2.64	17.33+1.52	81+1.52	
Alt a 1 KO	day 1	7.33+0.57	7.33+0.57	8.3+0.57	0.1012
	day 2	19.33+0.57	8.66+0.57	21+1.00	0.0013*
	day 3	30.33+0.57	10+0.00	35+1.00	0.0037*
	day 4	41.67+1.52	12.33+0.57	50+0.57	0.0134*
	day 5	58+2.00	15+1.00	64+1.52	0.0089*
	day 6	67.67+3.51	17+1.00	73+0.57	0.0137*
	day 7	75.67+4.04	18.33+1.52	85+0.57	0.0158*
Alt a 1 Comp	day 1	6.33+0.57	6+0.00	7+0.00	1
	day 2	15+2.00	7+0.00	21+0.57	0.1296
	day 3	28+1.00	9+1.00	32+1.52	0.0377*
	day 4	39.33+1.52	12.67+0.57	47+1.15	0.0591
	day 5	54.33+2.08	14+1.00	60+1.00	0.1515
	day 6	63+2.64	17.33+0.57	72+1.52	0.0442*
	day 7	72.67+0.57	19.67+0.57	80+2.3	0.0171*

		GluMM	GalMM	PDA	p-value
Alt a 1 OE	day 1	6+0.00	6+0.00	7+0.00	0.4226
	day 2	10+1.00	7+0.00	15+0.00	0.0634
	day 3	20+1.00	9.66+0.57	28+1.15	0.0955
	day 4	27.67+0.57	11.67+0.57	41+1.15	0.0926
	day 5	35.67+0.57	12.67+0.57	54+1.52	0.0208*
	day 6	40.33+0.57	14.67+1.15	65+0.57	0.0122*
	day 7	50+1.00	16.67+0.57	74+1.15	0.0052*

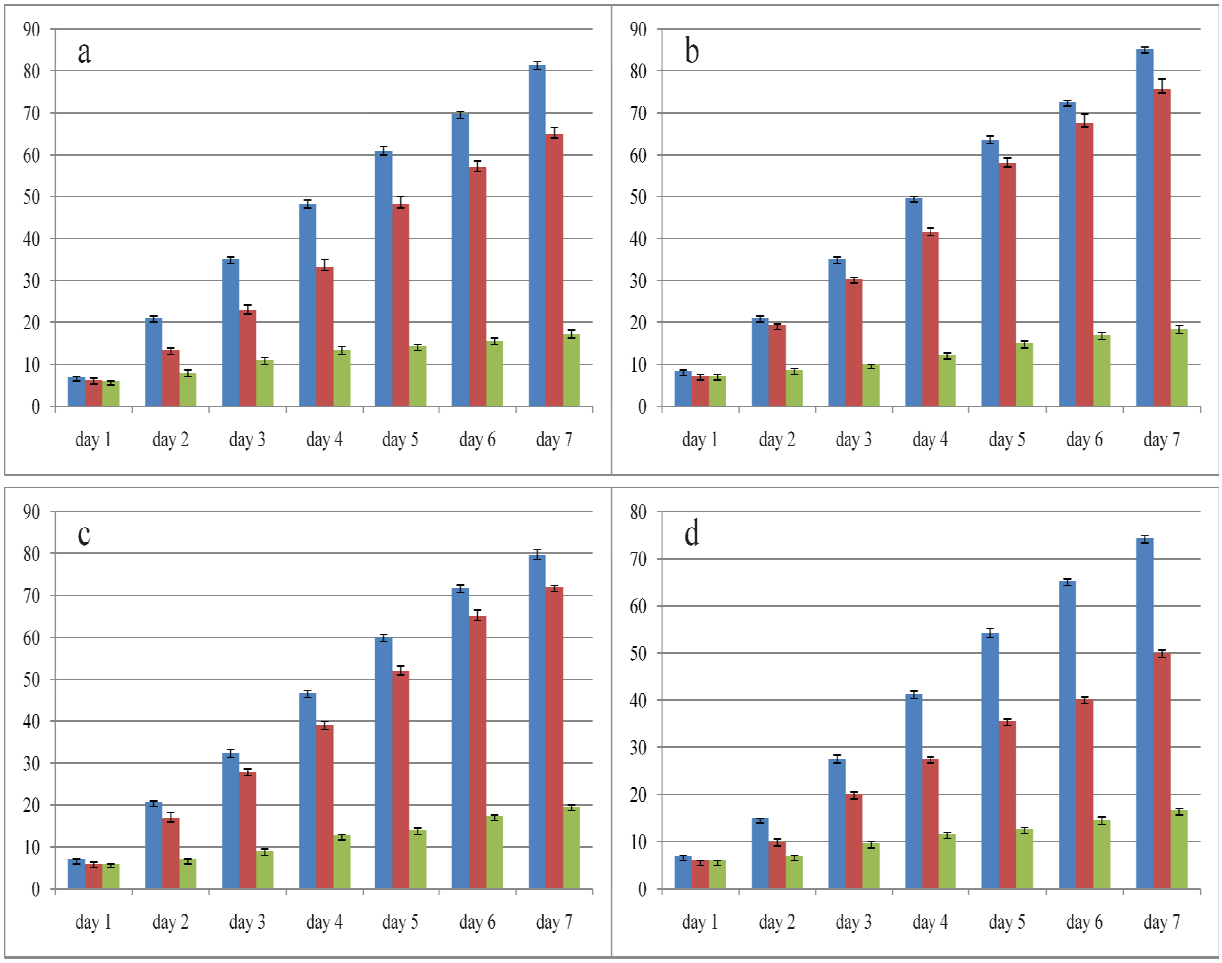

Figure 5.6. The growth chart of *A. alternata* and its three mutants. Panel a wild type A. alternata, panel b *Δalt a 1* deletion mutant, panel c is the complement Alt a 1 gene mutant and panel d is the overexpressed Alt a 1 mutant. With blue is represented the PDA (potato dextrose agar) growth, in red is the GluMM (glucose minimal media) growth and GalMM (galactose minimal media) is in green. The columnar representation contains also the standard error of mean (SEM) associated with each type of growth and each mutant.

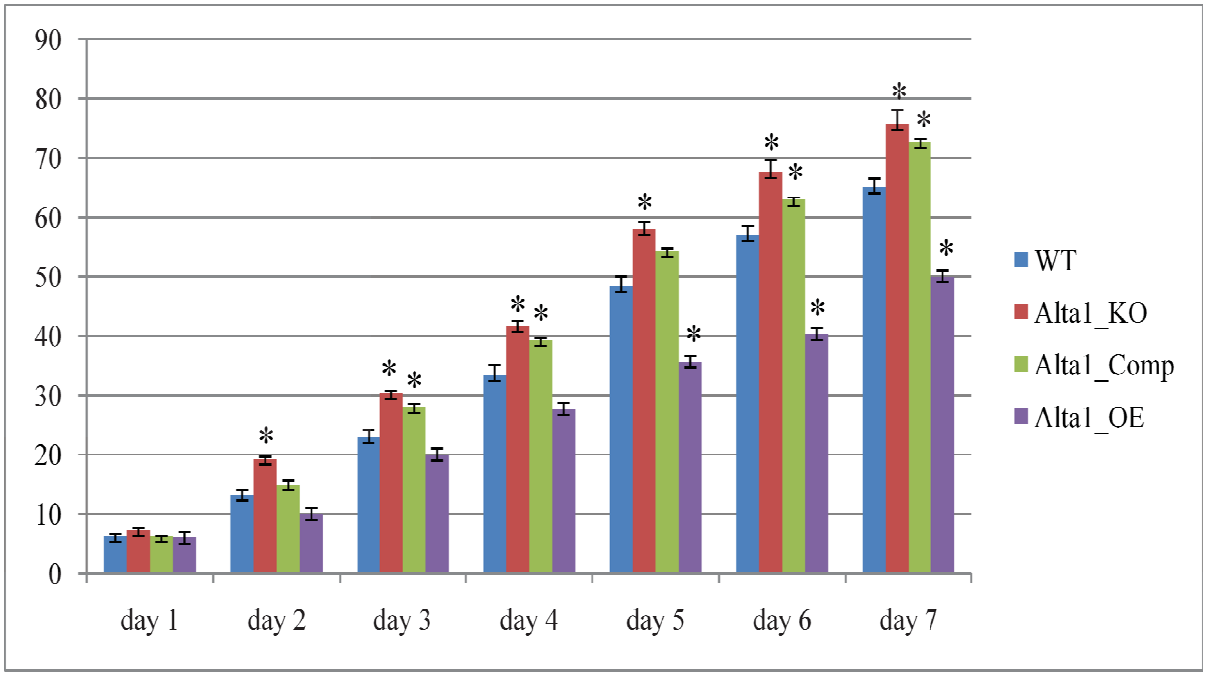

Figure 5.7 The growth chart representing wild type *A. alternata* (represented in this chart with blue) and its three mutants Δ*alt a 1* deletion mutant (red), the complementation Alt a 1 mutant (green) and the overexpression Alt a 1 mutant (purple) grown in GluMm (glucose minimal media). With * are the data that were proven to have statistical significance by the p≤ 0.05.

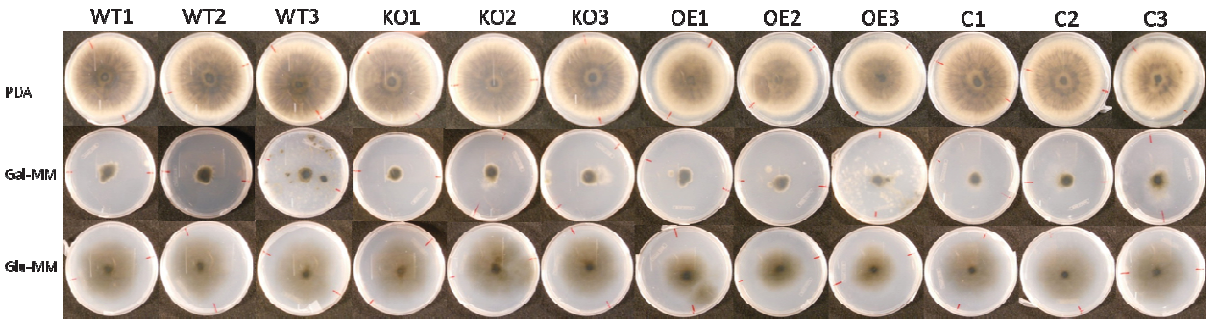

Figure 5.8 Day seven of the inhibition experiment for the wild type *A. alternata* and the three mutants grown on potato dextrose agar (PDA), galactose minimal media (GalMM) and glucose minimal media (GalMM). The wild type is noted with WT and the three replicates noted with WT, the Δ*Alt a 1* deletion mutant named KO with its three replicates, the Alt a 1 overexpression (OE) and the complement Alt a 1 mutant (C).

Next we tested the effects of pure recombinant Alt a 1 on germination of *A. alternata* spores after treatment with 10ug and 30ug of rAlt a 1. The spores were analyzed microscopically and the germinated and non-germinating spores were counted. In the Table 5.2 are shown the results of counting 100 spores and classifying them as germinated and non-germinated spores after 6 hours, at 24 °C in GYEB liquid media. No marked effect was observed with 10 ug sample so only 30 ug data is presented in Table 5.2.

Table 5.2 Count of *A. alternata* germinating and non-germinating spores after 6 hours of growth at 24 degree Celsius with and without rAlt a 1.

	WT 6 hours		**WT+Alt a1 6 hours**	
Experiment	Non-germinating spores	Germinating spores	Non-germinating spores	Germinating spores
1	20	80	48	52
2	19	81	50	50
3	17	83	49	51

We noticed a difference not only in the number of the germinating spores but also length of germ tubes emerging from spores that did germinate. We noticed a decreased length and number of germ tubes in *A. alternata* in the presence of rAlt a 1 compared to untreated spores. Figure 5.9 depicts our typical observations.

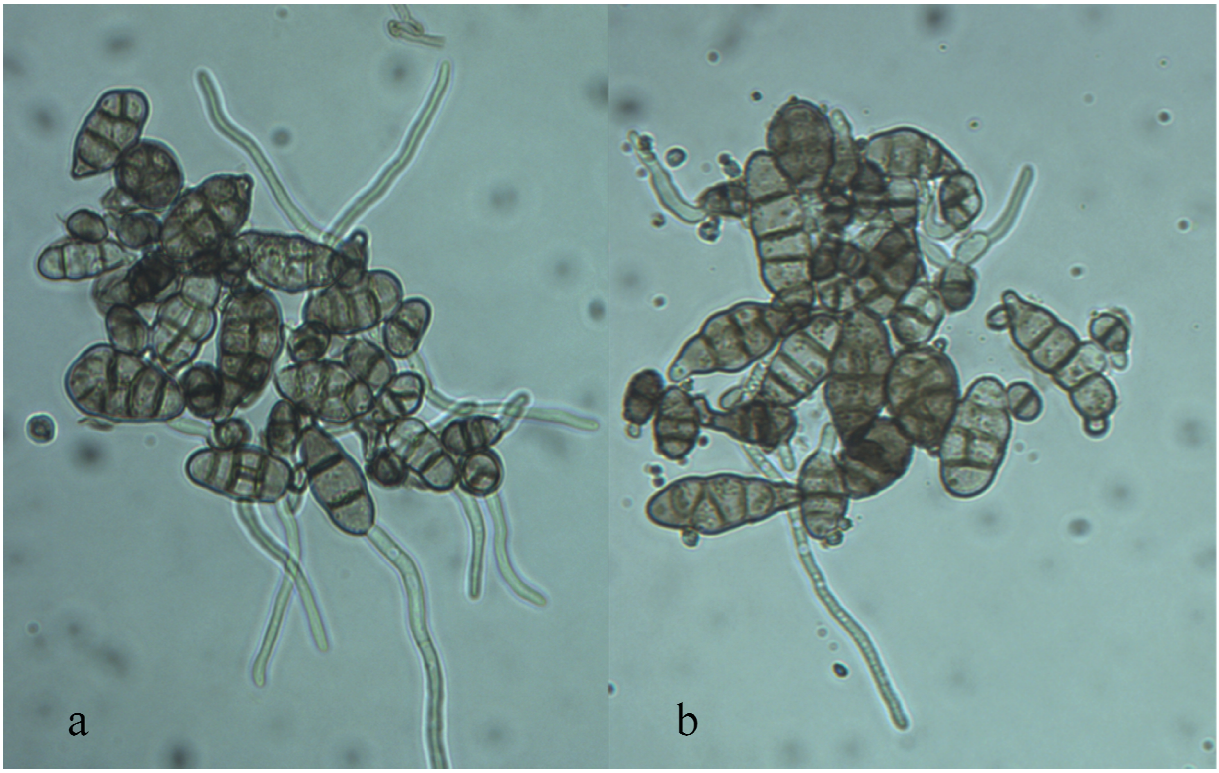

Figure 5.9 Spore germination of *A. alternata* in the presence and absence of Alt a 1. Panel a *A. alternata* spores germinating in GYEB for six hours and panel b, *A. alternata* spores germinating in the presence of 30ug rAlt a 1. Experiment was performed at 24 °C.

Microarray analysis of differential gene expression in human respiratory epithelial cells exposed to Alternaria

In order to investigate global gene expression changes in airway epithelial cells in response to Alternaria we performed microarray analysis with Affymetrix gene chips. More specifically, BEAS-2B cells were treated with PBS (negative control), 50,000 spores of Alternaria wild type or Δ*alt a 1*deletion strain for 24 hours. RNA was then extracted from the human cells and used in Affymetrix experiments (Human Genome U133 Plus 2.0 Array).

After applying various statistical analyses (GCRMA) (128) using the R software package version 2.12 (http://www.r-project.org/) a significant set of genes that were differentially

expressed, either up-regulated or down regulated, was determined for each set of contrasts. For the BEAS-2B cells in the presence of wild type *A. alternata* spores versus BEAS-2B cells alone the numbers of significantly differentially expressed genes were 613 genes. In this set out of 613 genes, 79 genes were down-regulated and 534 are up-regulated. For the other contrast the BEAS-2B cells in the presence of Δ*alt a 1* deletion mutant spores versus BEAS-2B cell alone the resulting set of significant genes contains 757 genes, out of which 172 are down-regulated and 585 are up-regulated.

The data that was obtained from the statistical analysis was further analyzed using a combination of two important pathway and network analysis systems: KEGG and Ingenuity Pathway System (IPA, Ingenuity Systems, http://www.ingenuity.com/index.html).

An excel file containing the gene names and the fold change was uploaded to IPA, where genes were analyzed with the Ingenuity Knowledge Base that contains information from scientific publications regarding direct and indirect relationship between genes and proteins. Interestingly, the biological processes corresponding to our gene sets were shown to be involved to a large extent in immunological processes, cell growth, or cell death. A subset of genes of interest including gene expression changes obtained and their fold change in the two experimental treatments are shown in Table 5.3 and are divided into functional groups. The clusters of biological processes corresponding to wild type and Δ*alt a 1* deletion mutant spores are visualized in Figure 5.10. Most of the biological processes of interest are higher following exposure to wild type spores compared to Δ*alt a 1* deletion mutant spores with only three notable exceptions: cellular function and maintenance, death receptor signaling and growth hormone signaling. What is notable from our results depicted in the table and the chart is that most of the genes that are differentially expressed in the two treatments are similar with similar values of the fold change. We are not only interested in cellular response to wild type spores but we are also interested in gene expression levels that appear to be related to presence or absence of Alt a 1. The genes that appear to be differentially expressed in the human epithelial cells exposed to *A. alternata* wild type set and not in the Δ*alt a 1* set are the ones that could provide important information about Alt a 1, and its immunostimulatory function(s).

Table 5.3 Genes modulated in response to *A. alternata* wild type and Δ *Alt a 1*. With ND are genes that are not detected in the data sets.

Gene Symbol	Gene Title	Log_2 Fold Change *A. alternata*	Log_2 Fold Change Δ*Alt a 1*
Antigen presentation			
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	0.65097457	1.5568065
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	1.1096765	2.5595385
Apoptosis			
ADAR	adenosine deaminase, RNA-specific	1.14590536	ND
CCNE1	cyclin E1	ND	1.1666131
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	0.56551629	ND
IFI27	interferon, alpha-inducible protein 27	4.65603791	7.702955
IFI35	interferon-induced protein 35	1.76001073	2.9860577
IFI44	Interferon-induced protein 44	4.15711781	7.6107134
IFI44L	interferon-induced protein 44-like	6.00263872	ND
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	4.03623614	4.4998068
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	3.76972291	3.6912687
IFITM1	interferon induced transmembrane protein 1 (9-27)	2.96243495	3.9491757
IFNB1	interferon, beta 1, fibroblast	ND	2.8149255
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	0.51758507	ND
JAG1	jagged 1 (Alagille syndrome)	-0.8081865	ND
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	ND	-1.03905
RAI3	retinoic acid induced 3	ND	1.8075446
RARB	retinoic acid receptor, beta	0.97818259	ND
TBK1	TANK-binding kinase 1	ND	-0.9966
TFRC	transferrin receptor (p90, CD71)	-0.8397467	1.3023675
TRAF3	TNF receptor-associated factor 3	ND	1.6268193
Cell death			
ADAR	adenosine deaminase, RNA-specific	1.14590536	ND
ATF3	activating transcription factor 3	1.10797694	1.7346913
BCL2L13	BCL2-like 13 (apoptosis facilitator)	0.52296967	-5.059352
BIRC3	baculoviral IAP repeat-containing 3	0.98305348	ND
C1S	complement component 1, s subcomponent	0.74615409	3.7390417
CALR	calreticulin	ND	-3.559982
CASP4	caspase 4, apoptosis-related cysteine protease	0.574165	1.7303177
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	3.59621156	1.0815056
IL15RA	interleukin 15	ND	1.0437345
PRKCE	protein kinase C, epsilon	ND	1.5369216
SHC1	SHC (Src homology 2 domain containing) transforming protein 1	ND	-3.343258
SOD2	superoxide dismutase 2, mitochondrial	0.61288258	ND
STAT1	signal transducer and activator of transcription 1, 91kDa	3.29214003	4.2463173
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	-0.6963861	ND
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	3.56038916	3.0996202
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	0.94264779	ND
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	0.63944737	ND

Gene Symbol	Gene Title	Log_2 Fold Change *A. alternata*	Log_2 Fold Change Δ*Alt a 1*
Cell signaling			
IRF1	interferon regulatory factor 1	1.11069812	2.0961462
IRF9	interferon regulatory factor 9	2.1248919	2.7170165
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.53471933	ND
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	ND	-1.693109
Cell-to-cell signaling and interaction.			
ICAM	intercellular adhesion molecule 1	0.67685677	ND
NRP1	neuropilin 1	0.5255687	ND
RARRES1	retinoic acid receptor responder (tazarotene induced) 1	1.89662228	ND
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	1.02763707	2.1047223
Cellular growth and proliferation			
BCL2L1	BCL2-like 1	ND	-5.059352
CALM1	calmodulin 1 (phosphorylase kinase, delta)	ND	0.8541916
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	1.71915157	ND
ERG1	early growth response 1	ND	5.3129164
GNG11	guanine nucleotide binding protein (G protein), gamma 11	ND	2.2381173
IFIH1	interferon induced with helicase C domain 1	4.26764898	ND
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	3.77371266	3.887956
IFIT5	interferon-induced protein with tetratricopeptide repeats 5	2.17720153	ND
IL7R	interleukin 7 receptor	-0.5464045	ND
MUC1	Mucine 1	2.36452031	ND
NOLC1	nucleolar and coiled-body phosphoprotein 1	ND	0.7088789
PPP1R12B	protein phosphatase 1, regulatory (inhibitor) subunit 12B	0.68524102	ND
ZBP1	Z-DNA binding protein 1	0.8271084	ND
STAT2	signal transducer and activator of transcription 2, 113kDa	0.97188712	-2.06544
Immune cell trafficking			
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	0.84423737	2.0174811
CCL2	chemokine (C-C motif) ligand 2	0.89221729	ND
CCL26	chemokine (C-C motif) ligand 26	0.53350918	1.9361974
CCL5	chemokine (C-C motif) ligand 5	0.69771732	1.8074532
CCND1	cyclin D1	0.71764829	ND
CD14	CD14 molecule	1.00134271	ND
CD47	CD47 molecule	0.45005715	ND
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	1.0298698	ND
CXCL10	chemokine (C-X-C motif) ligand 10	0.0001458	2.0865849
CXCL11	chemokine (C-X-C motif) ligand 11	2.34670889	ND
CXCL2	chemokine (C-X-C motif) ligand 2	0.55870594	ND
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	0.83314456	ND
IL1A	interleukin 1, alpha	0.64956412	ND
IL32	interleukin 32	0.62725969	ND
IL8	interleukin 8	1.70951448	2.649068
MYD88	myeloid differentiation primary response gene (88)	1.4430827	2.2681156
TLR3	toll-like receptor 3	1.57533289	ND

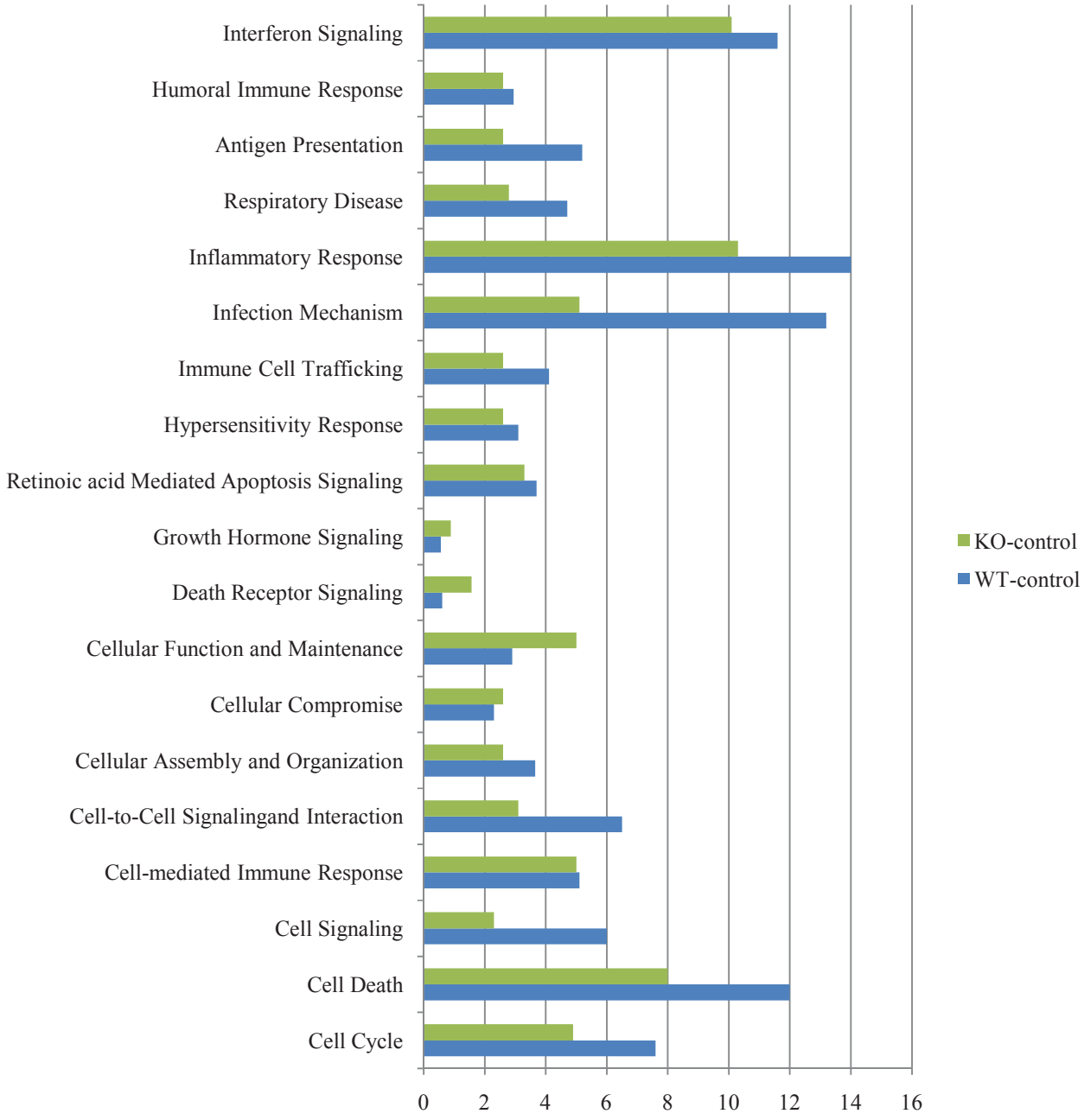

Figure 5.10 Biological functions and processes in human airway cells exposed to wild type *A. alternata* spores and to Δ*alt a 1* deletion mutant spores. The x axis depicts the biological processes and on the y axis is the –log (p-value) as produced by IPA analysis.

Next, using the KEGG Pathway Analysis System we were able to visualize some of the networks that are created based on our set of differentially expressed genes pertaining to each of the two experiments. Using the KEGG pathway analysis, the input being the two datasets, not only are genes involved in one specific pathway highlighted but also the nature of regulation (down-regulated or up-regulated). Figures 5.11 and 5.12 show the Cytokine-Cytokine interactions pathway for the genes that were either down-regulated or up-regulated for the human respiratory epithelial cells in the presence of *A. alternata* and Δa*lt a 1* mutant spores. Chemokine signaling pathway is shown in Figure 5.13 for the reaction of the human cells to wild type *A. alternata* and Figure 5.14 for the response to Δa*lt a 1* mutant spores.

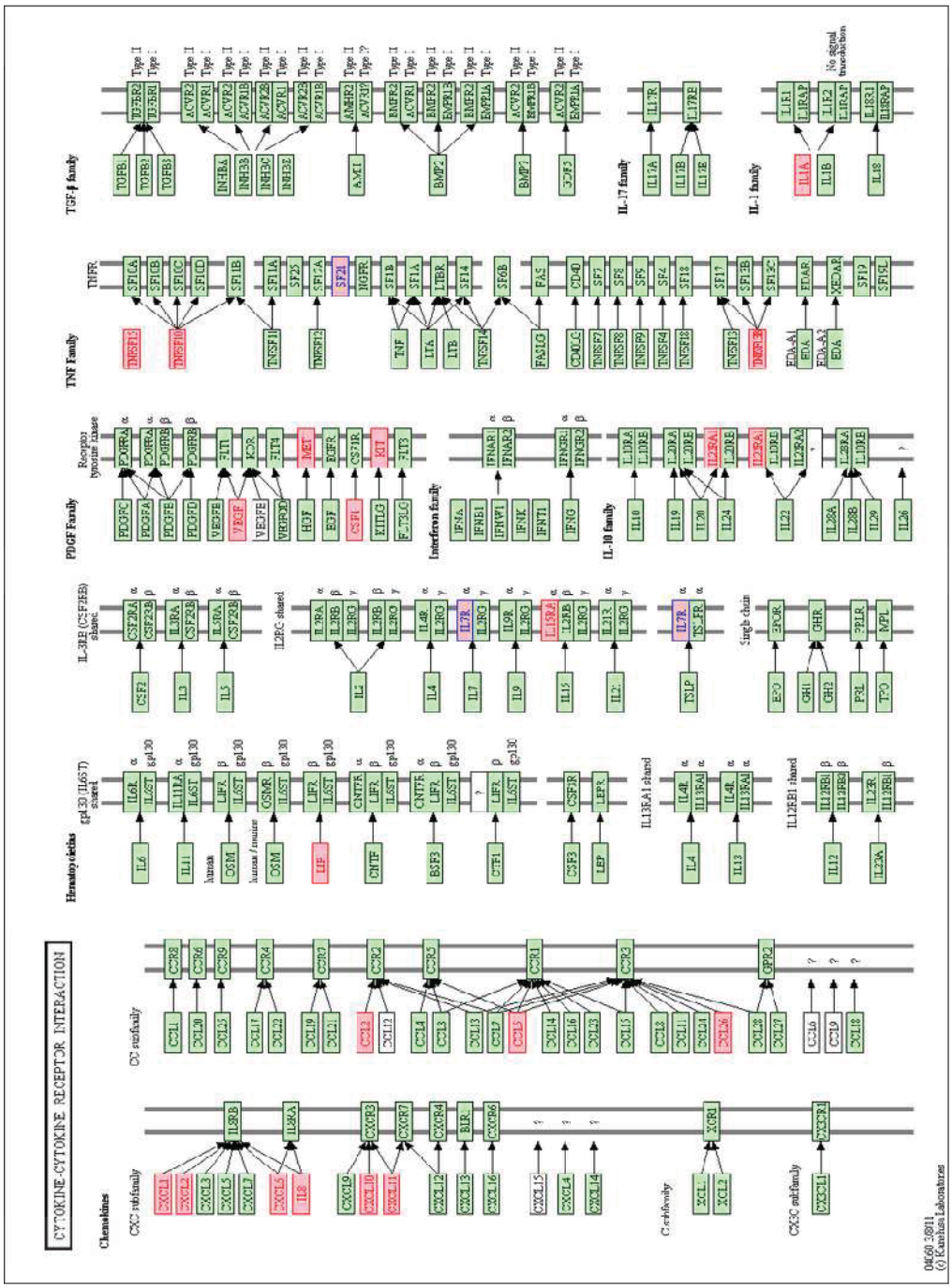

Figure 5.11 KEGG analysis of Cytokine-Cytokine receptor interaction with the genes differentially expressed by human respiratory epithelial cells in the presence of spores from wild type *A. alternata* fungus. Genes that are up-regulated are shown in red and down-regulated in blue.

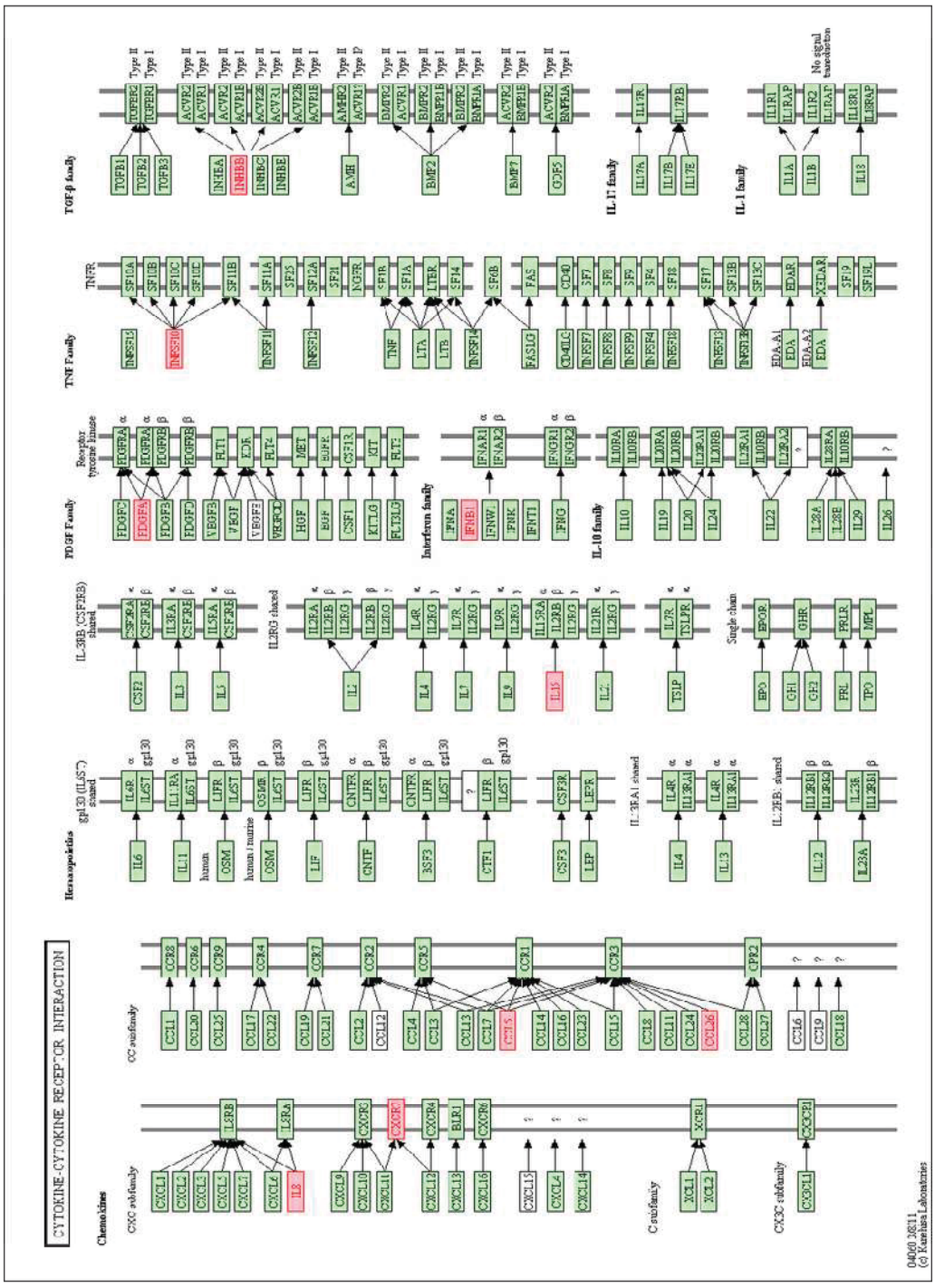

Figure 5.12 KEGG analysis of Cytokine-Cytokine receptor interaction with the genes differentially expressed by human respiratory epithelial cells in the presence of spores from Δ*alt a 1* deletion mutant. Genes that are up-regulated are shown in red and down-regulated in blue.

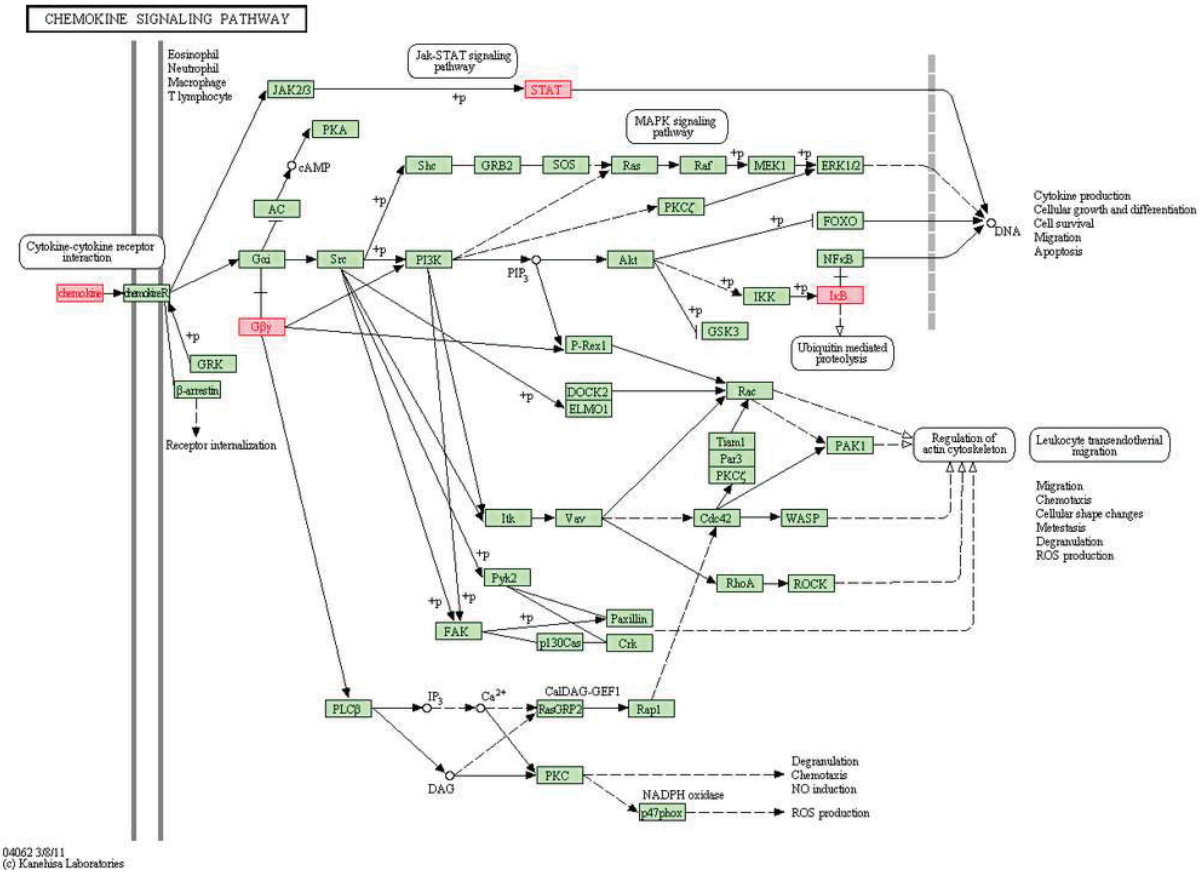

Figure 5.13 KEGG analysis of Chemokine signaling pathway with the genes differentially expressed by human respiratory epithelial cells in the presence of spores from wild type *A. alternata* fungus. Genes that are up-regulated are shown in red and down-regulated in blue.

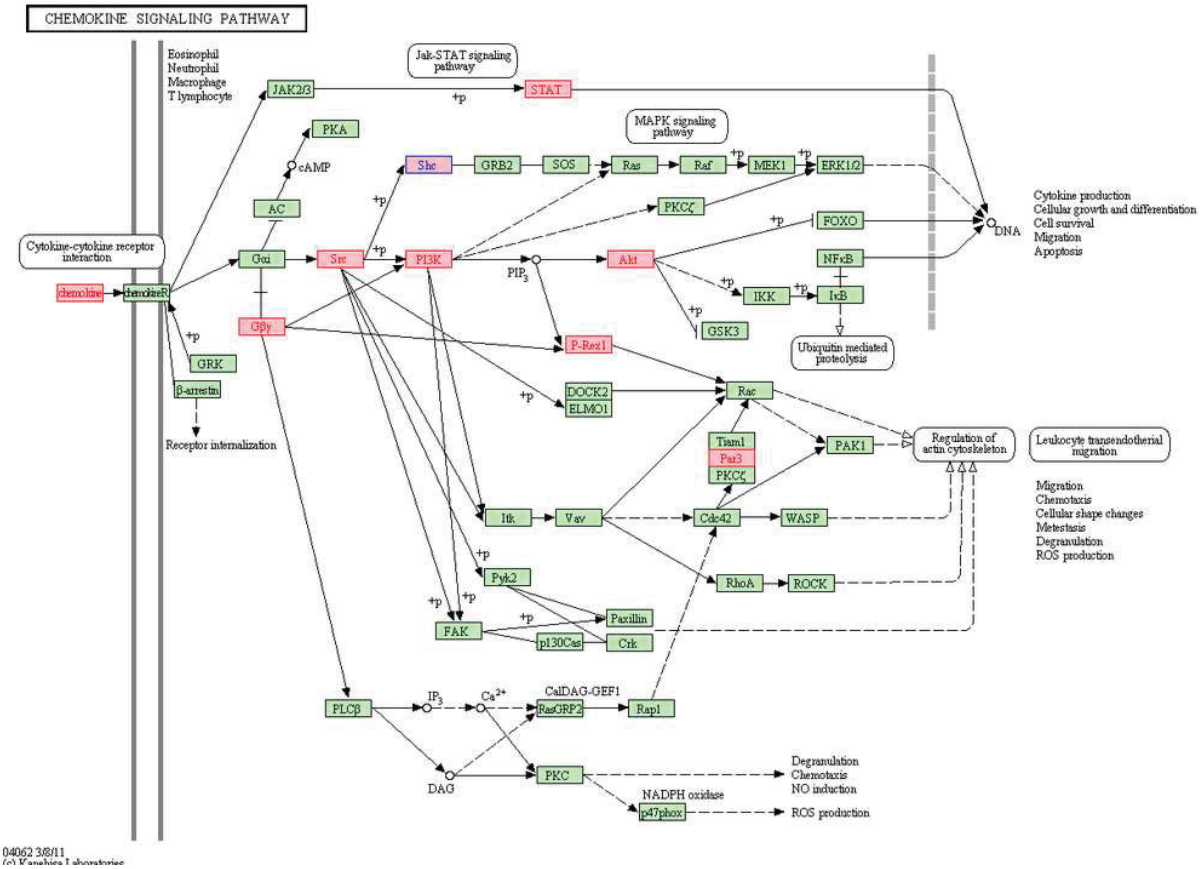

Figure 5.14 KEGG analysis of Chemokine signaling pathway with the genes differentially expressed by human respiratory epithelial cells in the presence of spores from Δ*alt a 1* deletion mutant. Genes that are up-regulated are shown in red and down-regulated in blue.

Other pathways that were significant in the immunologic processes are Toll-like receptor signaling pathway (Figure 5.15), B-cell receptor signaling pathway (Figure 5.16) and antigen processing and presentation (Figure 5.17).

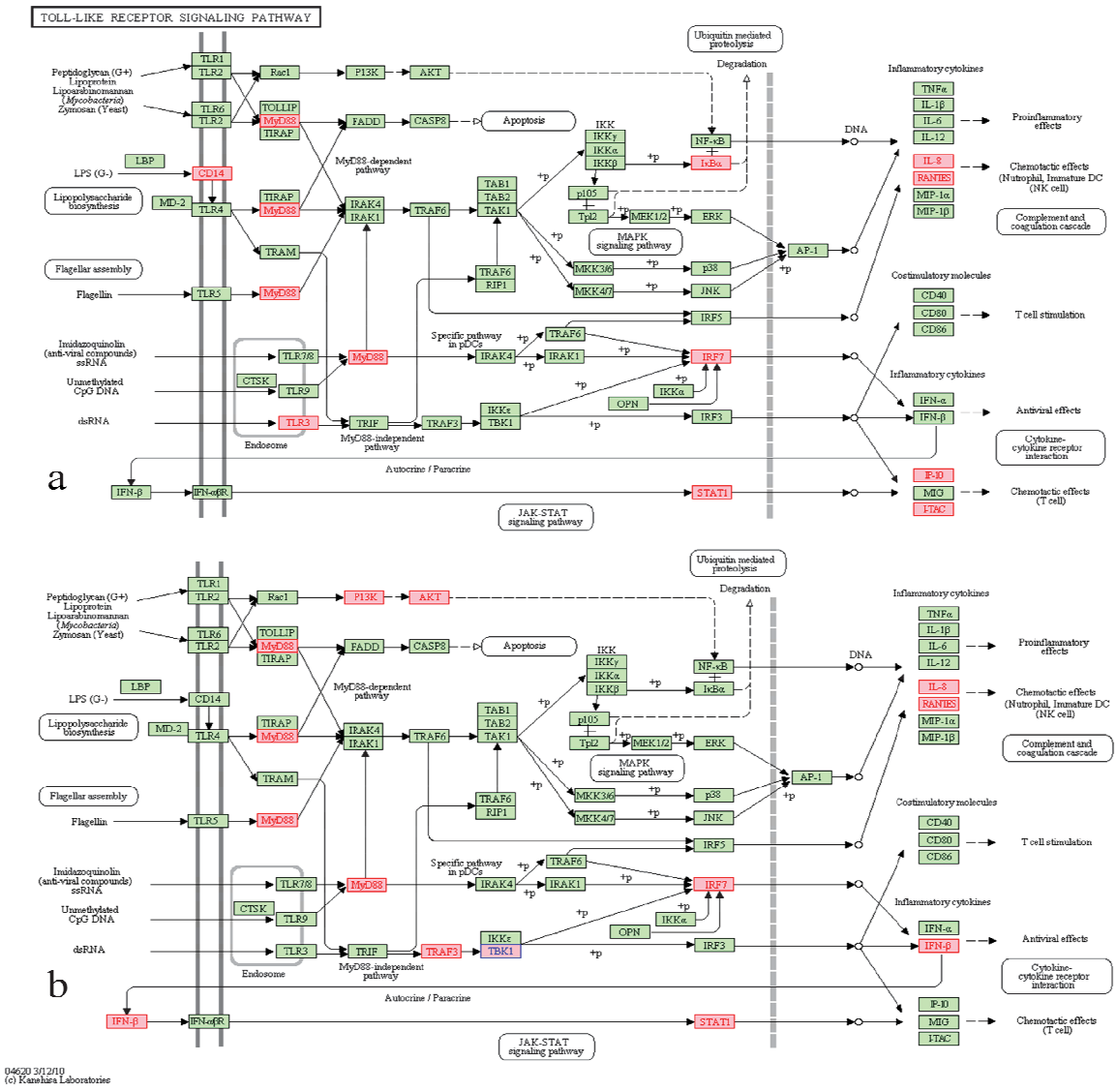

Figure 5.15 KEGG analysis of Toll-like receptor signaling pathway with genes that are differentially expressed by human respiratory epithelial cells in the presence of spores from wild type *A. alternata* (panel a) and *Δalt a 1* deletion mutant (panel b). Genes that are up-regulated are shown in red and down-regulated in blue.

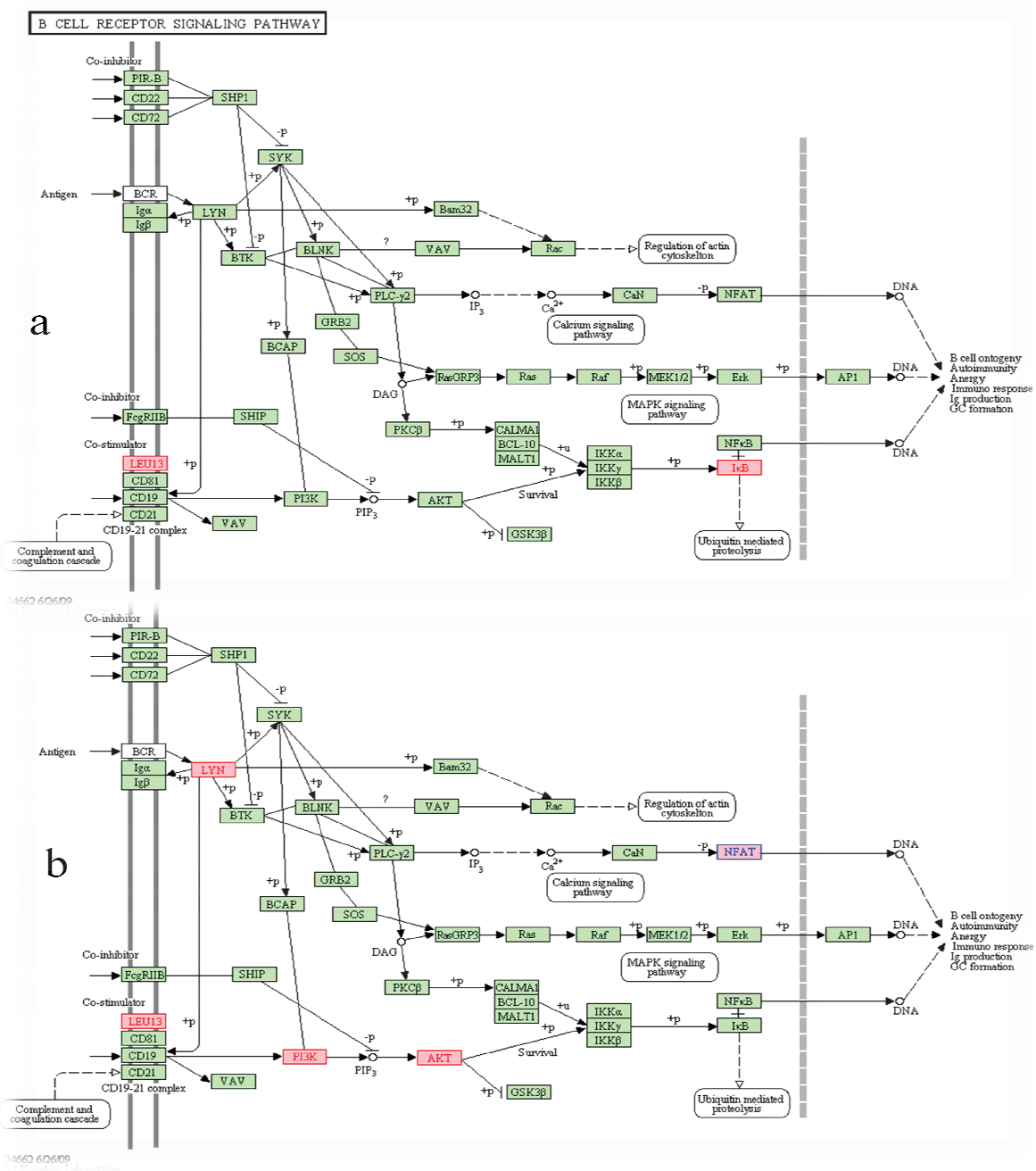

Figure 5.16 KEGG analysis of B-cell receptor signaling pathway with genes that are differentially expressed by human respiratory epithelial cells in the presence of spores from wild type *A. alternata* (panel a) and *Δalt a 1* deletion mutant (panel b). Genes that are up-regulated are shown in red and down-regulated in blue.

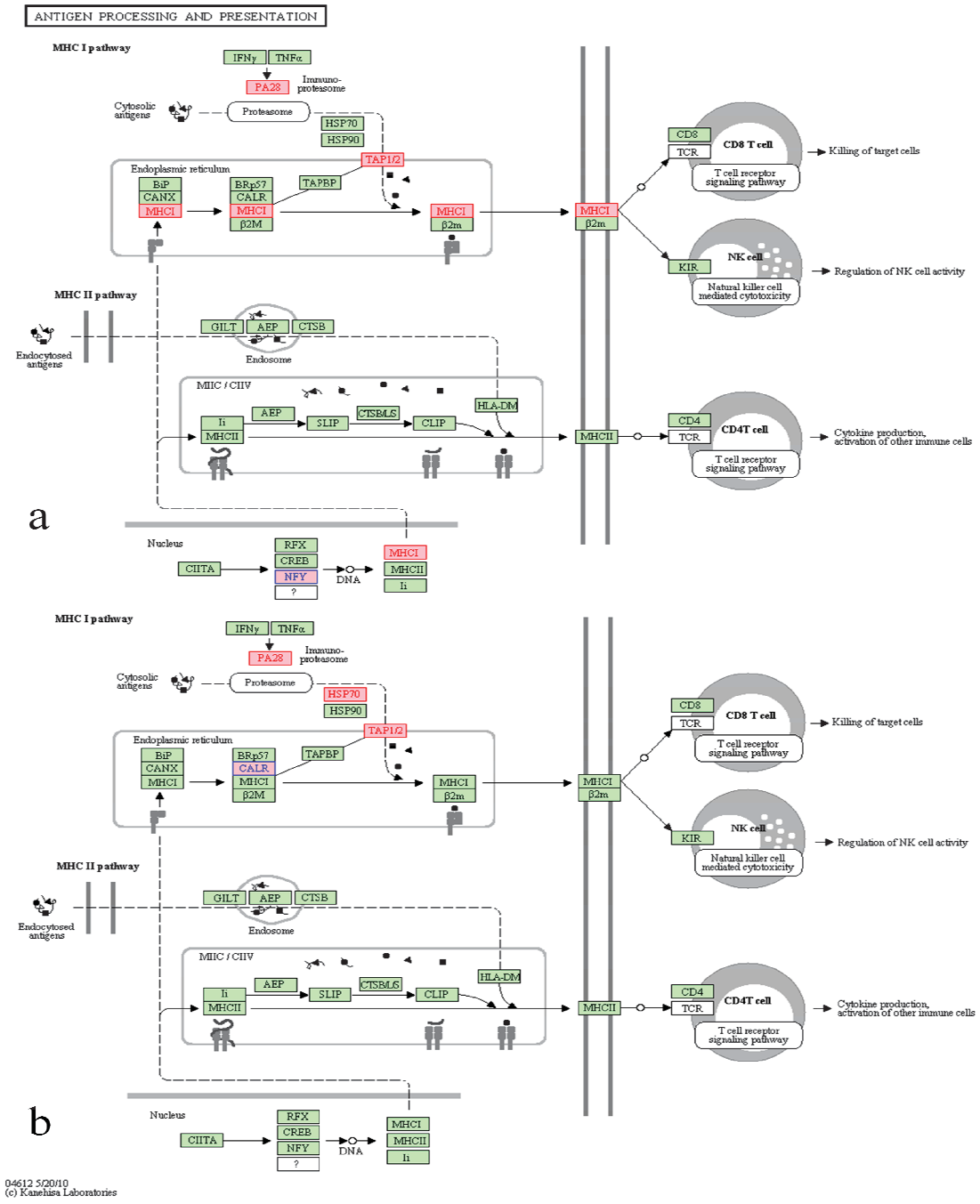

Figure 5.17 KEGG analysis of antigen processing and presentation mechanisms with genes that are differentially expressed by human respiratory epithelial cells in the presence of spores from wild type *A. alternata* (panel a) and *Δalt a 1* deletion mutant (panel b). Genes that are up-regulated are shown in red and down-regulated in blue.

The same set of genes were further on analyzed with IPA (Ingenuity Pathway System) having the goal to pinpoint the genes and related pathways that are specifically differentially expressed in the human respiratory epithelial cells in the presence of wild type spores versus cells exposed to Δ*alt a 1* deletion mutant spores. We were able to select genes that we can conclude with high confidence that were uniquely differentially expressed by the BEAS-2B cells in the presence of *A. alternata* spores as opposed to the genes that BEAS-2B cell expressed in the presence of Δ*alt a 1* deletion mutant. In this set we grouped genes by clusters. The first cluster is the immunologic group where we found genes that pertain to C-X-C motif like: CXCL1 (Chemokine ligand 1), CXCL6, (Chemokine ligand 6), CXCL10 (Chemokine ligand 10), CXCL11 (Chemokine ligand 11), gene that is also a chemokine but from the C-C motif group CCL2 (chemokine ligand 2). In the same group activity we also unveiled the receptors like the CD (cluster of differentiation) group containing proteins like TLR3 (Toll-like receptor 3 also known as CD283), ICAM (Inter-Cellular Adhesion Molecule 1 also known as CD54), CD14 (Cluster of differentiation 14), CD47 (Cluster of Differentiation 47), JAG1 (also designated as CD339**),** IL7R (interleukin 7 receptor), or MYD88 (Myeloid differentiation primary response gene 88). Under the same immunological umbrella, another group was identified and contains interleukins like IL1A (Interleukin 1 alpha), IL8 (Interleukin 8), IL32 (Interleukin 32). An interesting group contains CEBPD (CCAAT/enhancer-gcells inhibitor, alpha), TNFRSF21 (Tumor necrosis factor receptor superfamily, member 21), TNFSF15 (tumor necrosis factor superfamily 15), TNFSF13B (Tumor necrosis factor superfamily 13B), STAT2 (Signal transducer and activator of transcription 2) and MUC1 (Mucin 1, cell surface associated**).**

The rest of the genes that are unique to the reaction of human epithelial cells to wild type spores *A. alternata* are: CYR61 (Cysteine-rich 61), BIRC3 (Baculoviral IAP repeat-containing protein 3), DHX58 (DEXH Asp-Glu-X-His box polypeptide 58), SOD2 (Superoxide dismutase 2), CCND1 (G1/S-specific cyclin-D1), EIF2AK2 (eukaryotic translation initiation factor 2-alpha kinase 2), PPP1R12B (Protein phosphatase 1 regulatory subunit 12B), ADAR (Double-stranded RNA-specific adenosine deaminase) and ZBP1 (Z-DNA-binding protein 1).

As for the genes that appear to be differentially expressed in the human respiratory epithelial cells in the presence of Δ*alt a 1* deletion mutant spores we were able to select genes

like: TBK1 (TANK binding kinase1), TRAF3 (TNF receptor-associated factor 3), PRKCE (Protein kinase) IFNB1 (interferon, beta 1, fibroblast), TBK1 C epsilon type), PIK3C2A (Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha polypeptide), IL15RA (Interleukin 15 receptor, alpha subunit), CALM1 (calmodulin 1), GNG11 (Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11), ERG1 (the human *Ether-à-go-go* Related Gene), NOLC1 (Nucleolar phosphoprotein p130), ERBB3 (Receptor tyrosine-protein kinase erbB-3), CALR (calreticulin), BCL2L1 (Bcl-2-like protein 1), IL8 (Interleukin 8), NFAT5 (Nuclear factor of activated T-cells 5), CCL26 (Chemokine ligand 26), CCNE1 (G1/S-specific cyclin-E1) and SHC1 (SHC-transforming protein 1).

From both groups, the genes that appear to be differentially expressed by human epithelial cells in the presence of *A. alternata* wild type spores and in the presence of Δ*alt a 1* spores, we selected several genes to be validated by amplifying them with real-time PCR (RT-PCR) from the mRNA that was originally used for the microarray experiment. The genes were selected based on their known function, especially in regards to immunological relevance. These genes were: CXCL10, CXCL11, CEBPD, TRL3, IL7R, IL32 and STAT2 and the primers that were used for the selected genes are found in Table 5.4. The results of RT-PCR are shown in Figure 5.18. We noticed that CXCL10 is highly expressed in the human respiratory cells exposed to the Δ*alt a 1* spores compared to levels observed in cells treated with *A. alternata* wild type. We observed similar trends for IL32. In the case of TLR3 the cells exposed to wild type *A. alternata* showed higher expression than the ones exposed to Δ*alt a 1*. An interesting result was also obtained for STAT2 which appears to be down-regulated in the cells exposed to Δ*alt a 1* spores in comparison with control or with the exposure to wild type *A. alternata*.

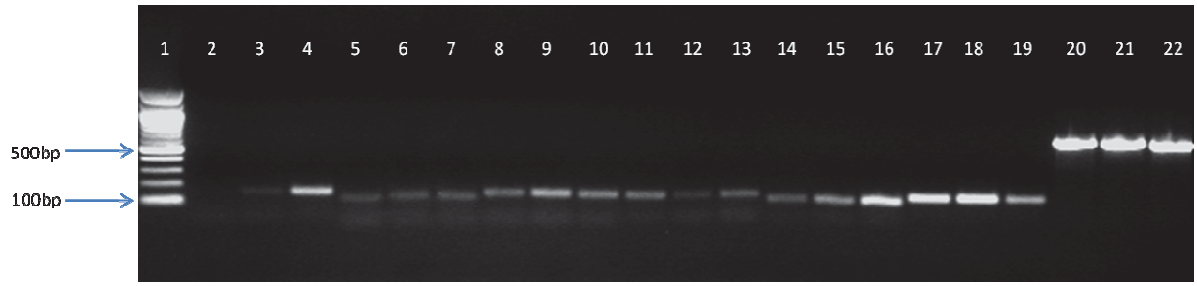

Figure 5.18 Selective gene RT-PCR amplification for respiratory human epithelial cells exposed to *A. alternata* wild type spores and Δ*alt a 1* deletion mutant spores. Lane 1 100 kB ladder; CXCL10 with lane 2 control cells, lane 3 wild type exposure, lane 4 Δ*alt a 1*: CEBPD lane 5 control, lane 6 wild type, lane7 Δ*alt a 1*; TLR3 with lane 8 control, lane 9 wild type and lane10 Δ*alt a 1*; IL7R lane 11 control, lane 12 wild type, lane 13 Δ*alt a 1*; IL32 lane 14 control, lane 15 wild type, lane 16 Δ*alt a 1*; STAT2 lane 17 control, lane 18 wild type and lane 19 Δ*alt a 1*; actin as a positive control lane 20 control, lane 21 wild type and lane 22 Δ*alt a 1*.

Table 5.4 The primers that were used for RT-PCR , validation of microarray results.

Name Primer	Sequence
5'CXCL10	GCATCAGCATTAGTAATCAACCTG
3'CXCL10	TGGCCTTCGATTCTGGATTC
5'CEBPD	CATCGACTTCAGCGCCTAC
3'CEBPD	GCCTTGTGATTGCTGTTGAAG
5'TLR3	AAGGAAAGGCTAGCAGTCATC
3'TLR3	GCAACTTCATGGCTAACAGTG
5'IL7R	CGCCAGGAAAAGGATGAAAAC
3'IL7R	GCCTTTAAAATAGTGATCAGGGATG
5'Il32	TGCACCAGGCCATAGAAAG
3'Il32	GGTAGCCCTCTTTGAAGTCG
5'STAT2	ACCCTAATCAGAGCCCAAATG
3'STAT2	TCAATCCAGACAGCCAAGTAC
5'Actin	ACGTTGCTATCCAGGCTGTGCTAT
3'Actin	ACTCCTGCTTGCTGATCCACATCT

Discussion

Alt a1 is the *A. alternata* protein that elicits the most intense allergic reaction in humans found thus far and yet no known biological function has been assigned to this protein and no mechanism or pathway of action is known to date. By producing three mutants related to the *Alt a 1* gene we have generated important tools that can be used to assess the role that Alt a 1 plays in fungal biology and interactions with humans and/or mouse.

Our growth experiments with these mutants suggest that Alt a 1 may partially inhibit the growth of the fungus. This has some possible explanation: either the activity of the protein itself may lead to growth inhibition or a specific amino acid sequence and conformation of the protein may be involved in phenotype growth inhibition. It has been speculated before the existence of "the possibility that there may be universal fungal metabolites, which may account for hyphal directional growth of all filamentous fungi" and also "a species-specific component may drive hyphal morphogenesis for diverse distantly related fungal species" (129) . Also another possible explanation regarding fungal growth inhibition is regarding the glucose uptake (130, 131) and the pH influence (132) on the hyphal growth or spores germination. Because we didn't notice the same trend in growth for the fungi growth on galactose minimal media (GalMM) we can speculate that glucose and its uptake is the main factor affecting the growth of mutants and for the wild type. However, more experimentation in the future will be required to support this hypothesis. Also, the fact that the germination of spores was somewhat influenced by the presence of Alt a 1 in the media is interesting. We can speculate that Alt a 1 has influenced the germination and growth of the fungus *A. alternata* through the regulation on the glucose uptake mechanisms. The self-inhibition role that Alt a 1 is playing in the radial growth of *A. alternata* can be further investigated. In addition, we found in pilot experiments that Alt a 1 had a mild inhibitory effect against *E. coli* growth further supporting our hypothesis.

Activation of epithelial cells can result in immediate host defense responses. 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. Activation of epithelium and production of cytokines and chemokines have been of particular interest in allergic conditions such as asthma and allergic rhinitis (133).

Our microarray experiments have provided for the first time a profile of the genes differentially expressed in airway epithelial cells due to Alternaria exposure. Based upon our results, we found substantial evidence for the induction of many genes previously found to be associated with the innate immune response. Moreover, our approach with our deletion mutant has allowed us to some degree to identify candidate genes that may be regulated by Alt a 1. For example, we found many chemokines in the set of genes that are specific to the cells exposed to *A. alternata* wild type, and do not appear to be up-regulated by the Δ*alt a 1* deletion mutant. Among these chemokines, interferon γ-inducible protein CXCL10 is important in the recruitment of Th1 cells involved in host immune defense against intracellular pathogens. The fact that it was previously proven to be secreted by respiratory epithelial cells (134) and appears in our data set is important especially because it does not appear in the set of genes differentially expressed by the Δ*alt a 1* spores. We can infer that just Alt a 1 alone may be the trigger that promotes up-regulation and release of CXCL10. A similar situation was observed for CXCL11 a gene with its activity strongly induced by IFN-γ and IFN-β, and weakly induced by IFN-α. Other chemokines that appear to be induced by wild type Alternaria specifically are CXCL1, a potent neutrophil chemoattractant, found in the early phase of experimental and clinical asthma and in the inflamed airway mucosa in COPD (135, 136), and CXCL6, also an inflammation-associated chemoattractant.

One of the most exciting observations we made was the up-regulation of Toll-like Receptor 3 (TLR3) only in cells treated with wild type spores and not the deletion mutant. Previously it was shown that (TLR3)-mediated challenge of airway epithelium elicited a strong chemokine response including CCL2 and CXCL10, underscoring their shared role in the response to microbial stimuli (137-140). It was previously reported that Rhinoviruses, a major agent responsible for the common cold, could increase the expression of TLR3 on the human bronchial epithelial cells, and trigger exacerbation of the pulmonary allergic reaction through TLR3/TRIF-dependent or TLR3/IRF3-dependent pathways implying that TLR3 may play an important role in asthma (141-143). A recent study reported that application of specific TLR agonists showed a protective effect for asthma (144). However, long-term activation of TLR3 induced inflammation and impaired lung function in mice (145). Down-regulation of TLR3 expression using siRNAs in rats caused a decrease of serum IgE levels and a reduction of IL4 mRNA expression in immune organs in an allergic asthma model (146). The same study

suggested that TLR3 systemically modulated disease development in asthma model rats by reducing serum IgE release via IL4 down-regulation, which may provide a vital clue for further research in the asthma pathogenesis and suggest a new target for asthma treatment (146). It is important to note that in this study (146), mice were sensitized to and subsequently challenged with OVA (ovalbumin) and thus the role of TLR3 in regards to fungal induced allergic asthma has never been studied. Our microarray results suggest that it may be worth investigating the role of TLR3 in fungal induced inflammation in the future. Also in the same study(146), the expression of CXCL10 paralleled TLR3 expression, suggesting that TLR3 is essential for CXCL10 release in respiratory epithelial cells. In our data sets not only did we observe TLR3 up-regulated in the BEAS-2B cells exposed to *A. alternata* but we also found CXCL10 slightly upregulated in the human epithelial cells exposed to *A. alternata* wild type and even more upregulated in the cells exposed to Δ*Alt a 1* deletion mutant. Our RT-PCR results validate our microarray experiment. The activity of TLR3 is also regulated through the Myd88 adaptor, which is highly upregulated in our dataset from human epithelial cells in response to *A. alternata* wild type. MyD88 signaling ultimately results in activation of the NFKB transcription factor (6, 147). Based upon our data, we might assume that the presence of Alt a 1 is triggering NFKB in human respiratory epithelial cells and subsequently a subset of chemokines, cytokines, and immunoglobulin production. However, we did notice that NFKBIA was upregulated by the wild type and not the deletion mutant. NFKBIA inhibits the activity of NFKB proteins by masking its nuclear localization signal (NLS) and keeping them in an inactive state in the cytoplasm (148). NFKB plays a key role in regulating the immune response to infection. Misregulation of NFKB has been linked to cancer, inflammatory and autoimmune diseases and septic shock. We can hypothesize based on our results that because cells exposed to wild type *A. alternata* induced this inhibitor but not the deletion mutant, the Alt a 1 protein may be suppressing certain arms of the innate immune response and possibly shifting the balance from Th1 to more of a Th2 type inflammatory response.

In summary, our studies indicate that TLR3 may contribute to the exacerbation of inflammatory reaction in response of respiratory epithelial cells to *A. alternata*. We speculate that the TLR3 signaling pathway may be an attractive therapeutic target for asthmatic and/or allergic reactions and warrants further investigation in the context of our experimental system.

We also found ICAM1 in the set of genes upregulated by the presence of *A. alternata* and not by the Δ*alt a 1* mutant. The expression of ICAM1 is upregulated by proinflammatory cytokines, most notably (TNF-α) and IFNγ (149). ICAM1 has previously been implicated in the inflammatory component of several lung diseases, including tuberculosis and asthma (150).

Interleukin-8 (IL8), a C-X-C chemokine, is a chemoattractant and activator for neutrophils and T cells (151, 152) and more recently has been shown to have a role in monocyte recruitment (153). Although it appears in both our data set it was induced to higher levels in the human respiratory epithelial cells exposed to wild type *A. alternata*.

TNFRSF 21 may be involved in inflammation and immune regulation but is mostly known as death receptor (154). In our data sets this receptor appears down-regulated which is in opposition with what was expected. TNFSF15 and TNFSF13B are genes that play an important role in immunity by T helper cells activation (154), are known as death receptor ligands and appear to be upregulated in our dataset. This is considered to be expected when cells are exposed to cell death causing agents like necrotrophic fungi such as Alternaria.

MUC1 mucin is secreted by respiratory epithelial cells (155) and has functions ranging from lubrication to cell signaling to chemical barrier formation. It is very interesting that the human cells in our experiment have responded to wild type *A. alternata* spores by increasing the expression of the MUC1 gene but not in the case of exposure to Δa*lt a 1* deletion mutant spores. We can infer that the protein Alt a 1 may represent a trigger for the secretion of MUC 1, a trigger that is missing in the Δ*alt a 1* mutant.

CEBPD protein is another important transcription factor for the regulation of genes involved in immune and inflammatory responses and may be involved in the regulation of genes associated with activation and/or differentiation of macrophages (156). It was previously reported that CEBPD gene is associated with severe acute respiratory syndrome (157). It is regulated by IL17 production and it is known that specific IL17 isoforms are commonly associated with allergic responses. We found CEBPD is also upregulated in the wild type *A. alternata* that contains major allergen Alt a 1.

In conclusion, the ability of *A. alternata* and Δ*alt a 1* deletion mutant to induce selective activation of different pathways, immunological or the other type, could lead to the coordinate regulation of multiple, functionally related chemokines and signaling pathways in airway epithelium. It is likely that Alt a 1 acts as a major inducer of epithelial inflammatory responses,

by differentially expressing genes that independently or in combination play an important role in local inflammation and ultimately systemic responses to *A. alternata*. Unmasking the molecular mechanisms of Alt a 1 function may likely uncover novel therapeutic strategies for blockade of proinflammatory pathways associated with asthma, COPD, and other lung inflammatory diseases.

Methods

Fungal cultures

Alternaria alternata, isolate ATCC11680, was routinely cultured on PDA media (Difco, Kansas City, MO, U.S.A.). For the growth experiment we additionally used two media types Glucose minimal media (GluMM) as previously described (158) and Galactose minimal media (GalMM) where we replace glucose from GluMM with galactose in the same molar concentration.

DNA isolation

Fungi were grown for 2 to 3 days in 50 ml of GYEB media. Approximately 0.2 g of mycelia was harvested and filtered with Miracloth (Calbiochem, Darmstadt, Germany), semi-dried with paper towels, and ground into fine powder with a mortar and pestle in the presence of liquid nitrogen. The powder was resuspended in cell lysate buffer from DNeasy Plant mini Kit from Qiagen (Palo Alto, CA, USA) and the DNA was extracted according to the manufacturer's protocol.

Bacterial genomic DNA was extracted from the transformants after growing them overnight in LB media with Plasmid Maxi Kit from Qiagen (Palo Alto, CA, USA) following the manufacturer instructions.

Fungal Transformation

In order to obtain the KO mutant the flanking region of *Alt a 1* (1000 bp upstream and downstream of *Alt a 1* gene locus) pieces were cloned in pCB1636 plasmid. The pCB1636 plasmid already contains the antibiotic resistance HygB gene used as marker for selection as previously described (159), (127).

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 of the Alt a 1 gene were 5' AGGGTACCGCAATGCACTCACGGCTCAAAGTTCCATCATCT which has a KpnI restriction site included and 3' AGCTCGAGGAGGTTGAAGAATAGAGTTTTGGTAGTTGAGTAGTTG that has an XhoI restriction site for the region before Alt a 1 gene, and 5' AGAAGCTTGCGATAGTTGGTCGAGCATATAAGAAGCATCAACTTC having a HindIII and 3' AGGGATCCGTACTCACCCTAGCCTTCCTGTAACATTGGAAAG with a BamHI site for the 1kb region positioned after Alt a 1 gene. The PCR products were digested and subsequently sequentially cloned into the fungal transformation vector pCB1636 (160). The plasmid construct was transform in *E. coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA). The plasmid construct was sequenced to verify the vector. The resulting plasmid was then used as template DNA to amplify between M13 forward and M13 reverse priming site that contained the Hyg B phosphotransferase gene under control of trpC fungal promoter gene and the cloned sequences of interest. The PCR products were purified with PCR Clean up kit (Qiagen, Palo Alto, CA, USA) and further increased their concentration to 1ug/ul in a Speedvac (Eppendorf, Barkhauseweg, Germany) and used in fungal transformation.

The fungal transformation of protoplasts was performed as we have described previously (159). Following transformation, spores from transormants were further purified by single spore isolation.

For creation of the complementation mutant, coding regions of the Alt a 1 wild type gene were reintroduced in the Δ*Alt a 1* mutant along with approx 1kb long native promoter sequence and a Nourseothricine resistance cassette. Genomic DNA from *A. alternata* was used as a template to amplify a 1.4 kb fragment using 5' AGGGTACCGTTCATGAACGGACCGACAGGAACTC and 3' primer AACTGCAGTTAAGAGCTCTTGGGGAGAGTGACGAGGGTGAT. Again M13 reverse and M13 forward set of primers were used and the fragment obtained was transformed in the Δ*Alt a 1* mutant following the same protocol (159).

For the overexpression mutant, the ToxA promoter was cloned 5' of the *Alt a 1* coding sequence in the pCB1636 vector. Primers used for cloning were 5' AGAAGCTTGATCGAGACTACTATAGGGCG with a HindIII site and 3'

AGGATATCGGCCTATATTCATTCATTGT having a EcoRV site for the ToxA promoter and the pairs 5' GAATTCATGCAGTTCACCACCATCGCCTCTCTCTTC having an EcoRI site and 3' AACTGGAGTTAAGAGCTCTTGGGGAGAGTGACGAGGGTGAT with a PstI restriction site for the *Alt a 1* coding sequence.

Southern Blotting

Fungal genomic DNA (3ug) was digested with selected enzymes (BamH1 and EcoRV) (New England BioLab, Beverly, MA, U.S.A.) than was size separated on a 0.8% agarose gel, followed by transfer to Hybond N+ nylon membrane (Amersham, Piscataway, NJ, U.S.A.). The DNA probes for *Alt a 1* and *HygB* were synthesized using a PCR digoxigenin (DIG) Probe Synthesis Kit following the manufacturer's protocols (Roche Diagnostics, Indianapolis, IN). Hybridization of membrane was performed at 50ºC using the Block and Wash Buffer set according to the manufacturer's manual (Roche Diagnostics, Indianapolis, IN). The blot containing DNA from the wild type and from the five mutants was first hybridized with *Alt a 1* specific probe. The same blot was subsequently stripped and hybridized with *HygB* probe according to the manufacturer's specifications (Roche Diagnostics, Indianapolis, IN). Membrane was washed in a final solution of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecylsulfate at 68ºC. Visualization of the blot was performed using DIG Luminescent Detection Kit for Nucleic Acids (Roche Diagnostics, Indianapolis, IN).

Western Blotting

The fungi were grown in GYEB media for seven days and the media from these cultures were loaded on a 12% SDS-polyacrylamide gel and separated with a NuPAGE (Invitrogen) system. After separation, the samples were transferred to a nitrocellulose membrane with an Invitrogen iBlot system. Primary anti-Alt a 1 antibodies (Indoor Biotechnologies, Charlottesville, VA) were used according to manufacturer's protocols and HRP-conjugated antibodies (Promega Corporation, Madison, WI) were used as secondary antibody. Chemiluminescent detection was used to develop the blot with CDP-Star using the ECL Plus Blotting Detection System kit according to the manufacturer's instructions (Amersham, Piscataway, NJ, U.S.A).

Alt a 1 protein production and purification

Alt a 1 gene engineered with a C-terminal 6xHis tag was PCR amplified from Alternaria cDNA using following set of primers 5' CCATGGCGATGCAGTTCACCACCATC that has a NcoI site and 3' GCGGCCGCGTGGTGGTGAGAGCTCTTGGGGAGAG with a NotI site and subsequently cloned into the pET21dc vector After sequence verification, the resulting plasmid was transformed into *E. coli* DH5α. A single colony was used to prepare a starter culture and was used to inoculate 2 liters of LB media. After inducing with IPTG (final concentration 0.3mM) cells were collected, lysed using sonication, centrifuged, and supernatant containing proteins was resuspended in the binding buffer following the manufacturer's manual for Ni-NTA His-Binding Resin from Novagen (San Diego, CA, USA). Six elution fractions were obtained with 50mM sodium phosphate and 150 mM ammonium sulfate and increasing concentrations (50, 70, 85, 100, 250 and 500mM) of imidazole for each elution. After purification with Ni-NTA column the initial elution buffer of the pure rAlt a 1 was exchanged to 0.2M Sodium Phosphate pH 7.5 buffer. SDS-PAGE and Western Blot was performed to confirm presence of 6x-His tag on purified Alt a 1 protein. The anti-His tag antibodies and the secondary anti-mouse antibodies that were used were from Promega (Promega Corporation, Madison, WI).

The enzymatic activity was assessed with API-ZYM kit (bioMerieux Vitek, Inc., Hazelwood, MO) following the manufacturer's protocol.

Cell culture and treatment

The human bronchial epithelial cell line BEAS-2B, derived from human bronchial epithelium transformed by an adenovirus 12-SV40 Adeno 12 hybrid virus, was cultured in 75cm^2 tissue-culture flasks in RPMI:1640 medium (Hyclone) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. Once the cells reached 80% confluence they were seeded in six-well tissue culture dishes, at a concentration of 1 X 10^6 cells per well and allowed to adhere overnight. Cells were then 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 then washed twice more with DPBS and cultured in a final volume of 1.5mL RPMI:1640 without supplements. Cells were then treated with 5 X 10^5 *Alternaria alternata* or *Δalt a 1* spores or an equal volume of DPBS (control). Cells were incubated at 37°C and 5% CO_2 for 24 hours. After

incubation, the cell culture supernatants were collected, debris removed by centrifugation at 1500xg for 5minutes, and stored at -80°C. Cells were washed twice with DPBS before RNA was extracted.

RNA extraction

Total RNA was purified via a hybrid protocol using TRIzol (Invitrogen, Carlsbad, CA) and the RNeasy Cleanup Kit (Qiagen, Valencia, CA). One mL of TRIzol was added per well and cells were gently collected and lysed using a cell lifter. RNA extraction was carried out per manufacturer's instructions until addition of ethanol. Following this step, the preparation was added to the RNeasy Mini Spin Column and purified per manufacturer instructions with on-column DNase digestion. RNA was eluted in 100µl of RNase-free water and purity and concentration was assessed with microanalysis (Agilent Bioanalyzer 2100). RNA samples were stored at -20°C until microarray analysis.

Microarray analysis and data processing

Affymetrix® GeneChip Human Genome U133 Plus 2.0 Array (HG-U 133 Plus 2.0) containing 47,000 genes and variants which represents approximately 39,000 characterized human genes was used for gene expression analysis. Preparation of *in vitro* RNA, oligonucleotide array hybridization and scanning were performed according to Affimetrix (Santa Clara, CA) protocols in the VBI Core Laboratory Facility. A probe set-based gene expression data file was generated from quantified image files with the GeneChip Multi-Array Average (GCRMA) method (128) together with packages from the BioConductor tool suite (http://www.bioconductor.org/), using R version 2.12 (http://www.r-project.org/) and annotated with Unigene annotation from the February 2009 mapping version of the human genome. The comparison of each of the treatments with control or between treatments, by the analysis of all 9 CEL files simultaneously, revealed a data matrix of probe sets in which each value indicated the calculated log abundance of each gene probe under the two treatments and the control conditions. Using GCRMA, background subtraction, quantile normalization and gene data summarization was performed. Differential expression analysis was performed using the linear modeling features of the limma package (161). In order to correct the p-values the Holm (162) multiple-testing adjustment was applied. All genes in a comparison of interest with an adjusted

p-value ≤ 0.05 were considered as statistically significant regardless of the fold difference in expression level.

Pathways analysis and network building

Pathway analysis and network were generated with of IPA (Ingenuity Systems, http://www.ingenuity.com/index.html). Also KEGG Pathway Analysis was used (http://www.genome.jp/kegg/pathway.html) for the pathways finder and pathway graphics.

RT-PCR

RNA was extracted as described above and converted into cDNA templates using Tetro cDNA synthesis kit (Bioline, Tauton, MA). For the gene specific PCR amplifications we used the GoTaq Green Master Mix (Promega Corporation, Madison, WI) which is a premixed, ready-to-use solution containing GoTaq DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. PCR was performed using the manufacturer‘s protocol. Primers used for these amplifications are shown in Table 5.3.

CHAPTER 6

Conclusions

The vast majority of the species of airborne fungi are saprophytic and not pathogenic to plants, animals or humans. Fungi such as Fusarium, Alternaria or Trichosporon, had been thought to represent contamination or harmless colonization when isolated from humans. More recently, the role of these and other newly recognized fungi as serious pathogens has been clearly established. Among such fungi are members of the Aspergillus, Fusarium, Alternaria, Mucor genera, comprising an emerging fungal pathogen group in humans. In this body of work, we present for the first time results of several sets of experiments including, 1) the analysis of *A. alternata* spore germination expressed sequence tags (ESTs), 2) the survey of global allergen homologues in fungal genomes, and 3) the first microarray experiment investigating airway epithelial cell responses to this fungus.

With this study we have generated new information about the relatively poorly studied fungus *Alternaria alternata* in regards to its immunological/allergenic potentials. Our data might also suggest that *A. alternata* plays an important role in onset, development and maintenance of allergies to other fungi or for other upper respiratory diseases in the same way that was suggested that Asp f 1, a ribotoxin, may prime the atopic host for allergic reaction by damaging epithelial tissue.

The EST dataset obtained in this project offers a first look into the gene content of *A. alternata* and represents the beginning of future research of this ubiquitous but still not completely understood fungus. Annotation and classification of ESTs revealed a number of genes that could be involved in the immunomodulation process of the human immune response toward fungi and other known allergens. Detailed analysis of this dataset has provided several important features of the *A. alternata* transcriptome such as conserved genes as well as possible *A. alternata* specific genes, assignment of genes to GO categories, identification of genes expressed in the presence of mucin. In summary, it is anticipated that this study is a significant contribution to enhance genomic resources that will eventually impact the knowledge about *A. alternata*.

In our EST study we have shown that proteins from *A. brassicicola* and from *A. alternata* have homology with known fungal allergens. The criteria used were the same that proved that allergens from other species have similarity with *A. fumigatus* proteins suggesting that proteins from *A. fumigatus* (or other fungi) might cross react with serum from patients sensitized to these fungi and that these proteins are possible cryptic allergens. In addition, we investigated the presence and numbers of homologs of known *A. alternata* allergens in the set of fungal allergens. In our study we have identified numerous potential allergenic proteins through homology to existing allergen sequences across taxa, a useful, though imperfect, tool for prediction of whether an unknown protein may either be allergenic or cross reactive at either T cell, B cell or mast cell level.

The survey of allergen homologues at a genomic level among the three fungal genomes and one EST data set represents a new approach to discover, evaluate and assess the allergenic potential of fungi. Interestingly our data strongly suggests that fungi possess the capacity to produce many and diverse types of potential allergens originally found in organisms and bio-sources such as fungi, plants and pollen, dustmite and cockroach, and venoms for example. In order to test this hypothesis in the future one might envision sensitizing mice to fungal extracts and then testing animals to see if they possess IgE that can cross react with allergens from other sources like dustmite or pollen. Although the plethora of data that we obtained is very informative, we have to interpret it with caution because there is considerable evidence supporting the hypothesis that all fungi may contain allergen orthologs that may cross-react. Still, based on the genomic approach that we have implemented in this study, it can be said, at this point, that without laboratory experiment data to confirm, the theoretical allergenic potential of *A. alternata* is unexpectedly high, higher than *A. fumigatus*, known to have the greatest number of proven fungal allergenic proteins.

Among the proven and hypothetical allergens, our studies with Alt a 1, the major allergen from *A. alternata,* represent an important source of complex information. Based upon results of our work, the immunological properties of Alt a 1 could extend beyond what is known to date about this unique gene. Based on our results the Alt a 1 protein plays an important role in generating inflammation that appears in asthma and other upper respiratory diseases by acting as trigger in several important pathways, for example, the TLR3 pathway and subsequent activation of NFKB via MyD88 dependent or independent mechanisms. The ability of *A. alternata* and Δ*alt*

a 1 deletion mutant to induce selective activation of different pathways could lead to the coordinate regulation of multiple, functionally related chemokines and signaling pathways in airway epithelium. It is likely that Alt a 1 acts as a controller of epithelial inflammatory responses, by differentially inducing genes that independently or in combination play an important role in promoting local inflammation and systemic response to *A. alternata* in particular. Unmasking the molecular mechanisms of Alt a 1 function may likely uncover novel therapeutic strategies for blockade of proinflammatory pathways pathogenic for asthma, allergies, and other lung inflammatory diseases.

REFERENCES

1. Cramer RA, Lawrence CB. Cloning of a gene encoding an Alt a 1 isoallergen differentially expressed by the necrotrophic fungus Alternaria brassicicola during Arabidopsis infection. Appl Environ Microbiol. 2003;69(4):2361-4. PMCID: 154767.

2. Kita H, Abu-Ghazaleh RI, Sur S, Gleich GJ. Eosinophil major basic protein induces degranulation and IL-8 production by human eosinophils. J Immunol. 1995;154(9):4749-58.

3. Shin SH, Ponikau JU, Sherris DA, Congdon D, Frigas E, Homburger HA, et al. Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi. J Allergy Clin Immunol. 2004;114(6):1369-75.

4. Ponikau JU, Sherris DA, Kephart GM, Kern EB, Gaffey TA, Tarara JE, et al. Features of airway remodeling and eosinophilic inflammation in chronic rhinosinusitis: is the histopathology similar to asthma? J Allergy Clin Immunol. 2003;112(5):877-82.

5. Bowyer P, Denning DW. Genomic analysis of allergen genes in Aspergillus spp: the relevance of genomics to everyday research. Med Mycol. 2007;45(1):17-26.

6. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335-76.

7. Larsen LS. A three-year-survey of microfungi in the air of Copenhagen 1977-79. Allergy. 1981;36(1):15-22.

8. Morrison VA, R. J. Haake, and D. J. Weisdorf. . The spectrum of non-Candida fungal infections following bone marrow transplantation. . Medicine (Baltimore) 1993;72:78-89.

9. Vartivarian SE, E. J. Anaissie, and G. P. Bodey. . Emerging fungal pathogens in immunocompromised patients: classification, diagnosis, and management. . Clinical Infectious Diseases. 1993(17):S487-S91.

10. Anaissie EJ, G. P. Bodey, and M. G. Rinaldi. Emerging fungal pathogens. European Journal of Clinical Microbiology & Infectious Diseases 1989;8:323-30.

11. Vennewald I, Henker, M., Klemm, E., Seebacher, C. Fungal colonization of the paranasal sinuses. Mycoses. 1999(42):33-6.

12. Wadhwani K, Srivastava, A.K. Fungi from otitis media of agricultural field workers. Mycopathologia 1984(88):155-9.

13. Pritchard RC, Muir, D.B. Black fungi: a survey of dematiaceous hyphomycetes from clinical specimens identified over a five year period in a reference laboratory 1987.

14. Collier L, Balows, A., Sussman, M. 9 ed. Arnold, London, Sydney, Auckland, New York.: Topley & Wilson's; 1998.

15. Larone DH. Medically Important Fungi A Guide to Identification. 3 ed. Washington, D.C.: ASM Press, ; 1995.

16. St-Germain G, and R. Summerbell. . Identifying Filamentous Fungi A Clinical Laboratory Handbook. 1 ed. Belmont, California: Star Publishing Company; 1996.

17. Halomen M, Stern, D.A., Wright, A.L., Taussig, L.M., Martinez, F.D. . Alternaria as a major allergen for asthma in children raised in a desert enviroment. American Journal of Respiratory Critical Care. 1997;155:1356-61.

18. Downs S, Mitakakis, T., Leuppi, J., Marks, G. Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitised children. . Pediatric Allergy Immunology. 2003(4):100-5.

19. Mari A, Schneider, P., Wally, V., Breitenbach, M., Simon-Nobbe, B., . Sensitization to fungi: epidemiology, comparative skin tests, and IgE reactivity of fungal extracts. Clinical Experimental Allergy 2003;33:1429-38.

20. D'Amato G, Spieksma FT. Aerobiologic and clinical aspects of mould allergy in Europe. Allergy. 1995;50(11):870-7.

21. Al-Doory Y, Domson, J.F. Muold Alleregy. Philadelphia: Lea & Febiger; 1984.

22. Gergen PJ, Turkeltaub PC. 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. 1992;90(4 Pt 1):579-88.

23. Asturias JA, Ibarrola I, Ferrer A, Andreu C, Lopez-Pascual E, Quiralte J, et al. Diagnosis of Alternaria alternata sensitization with natural and recombinant Alt a 1 allergens. J Allergy Clin Immunol. 2005;115(6):1210-7.

24. CDC. Adult self-reported current asthma prevalence rate and prevalence by state or territory. Centers for Disease Control and Prevention; 2002 [updated 2002; cited]; Available from: www.cdc.gov/asthma/brfss/02/current/tableC1.htm. .

25. Kay AB. Allergy and allergic diseases. First of two parts. N Engl J Med. 2001;344(1):30-7.

26. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat Rev Immunol. 2001;1(1):69-75.

27. Arm JP, , Lee, T.H. The pathobiology of bronchial asthma. Advanced Immunology. 1992(51):323-82.

28. Busse WW, Lemanske RF, Jr. Asthma. N Engl J Med. 2001;344(5):350-62.

29. Halonen M, Stern DA, Wright AL, Taussig LM, Martinez FD. Alternaria as a major allergen for asthma in children raised in a desert environment. Am J Respir Crit Care Med. 1997;155(4):1356-61.

30. Peat JK, Tovey E, Mellis CM, Leeder SR, Woolcock AJ. Importance of house dust mite and Alternaria allergens in childhood asthma: an epidemiological study in two climatic regions of Australia. Clin Exp Allergy. 1993;23(10):812-20.

31. Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM. The link between fungi and severe asthma: a summary of the evidence. Eur Respir J. 2006;27(3):615-26.

32. Harris LH. Experimental reproduction of respiratory mold allergy. Journal of Allergy and Clinical Immunology. 1941(12):279-89.

33. Licorish K, Novey HS, Kozak P, Fairshter RD, Wilson AF. Role of Alternaria and Penicillium spores in the pathogenesis of asthma. J Allergy Clin Immunol. 1985;76(6):819-25.

34. Lethbridge-Cejku M, Schiller JS, Bernadel L. Summary health statistics for U.S. adults: National Health Interview Survey, 2002. Vital Health Stat 10. 2004(222):1-151.

35. Lichtenstein LM. Allergy and the immune system. Sci Am. 1993;269(3):116-24.

36. Kurup VP, Shen, H., Banerjee, B. . Respiratory fungal allergy. Microbes Infect. 2000(2):1101-10.

37. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. Genome Biol. 2004;5(2):R12.

38. Zureik M, Neukirch C, Leynaert B, Liard R, Bousquet J, Neukirch F. Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey. BMJ. 2002;325(7361):411-4.

39. Kurup VP, Crameri, R., . www.aspergillus.man.ac.uk/secure/articles. . 2010 [updated 2010 October 2010; cited October 22 2010]; Available from.

40. Fish JE, Peters SP. Airway remodeling and persistent airway obstruction in asthma. J Allergy Clin Immunol. 1999;104(3 Pt 1):509-16.

41. Kips JC, Pauwels RA. Airway wall remodelling: does it occur and what does it mean? Clin Exp Allergy. 1999;29(11):1457-66.

42. Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, et al. Molecular cloning of major and minor allergens of Alternaria alternata and Cladosporium herbarum. Mol Immunol. 1995;32(3):213-27.

43. Simon-Nobbe B, Probst G, Kajava AV, Oberkofler H, Susani M, Crameri R, et al. IgE-binding epitopes of enolases, a class of highly conserved fungal allergens. J Allergy Clin Immunol. 2000;106(5):887-95.

44. Weichel M, Schmid-Grendelmeier P, Fluckiger S, Breitenbach M, Blaser K, Crameri R. Nuclear transport factor 2 represents a novel cross-reactive fungal allergen. Allergy. 2003;58(3):198-206.

45. De Vouge MW, Thaker AJ, Curran IH, Zhang L, Muradia G, Rode H, et al. Isolation and expression of a cDNA clone encoding an Alternaria alternata Alt a 1 subunit. Int Arch Allergy Immunol. 1996;111(4):385-95.

46. Barnes CS, Pacheco F, Landuyt J, Rosenthal D, Hu F, Portnoy J. Production of a recombinant protein from Alternaria containing the reported N-terminal of the Alt a1 allergen. Adv Exp Med Biol. 1996;409:197-203.

47. Kurup VP, Vijay HM, Kumar V, Castillo L, Elms N. IgE binding synthetic peptides of Alt a 1, a major allergen of Alternaria alternata. Peptides. 2003;24(2):179-85.

48. Saenz-de-Santamaria M, Guisantes JA, Martinez J. Enzymatic activities of Alternaria alternata allergenic extracts and its major allergen (Alt a 1). Mycoses. 2006;49(4):288-92.

49. Cramer RA, Lawrence CB. Identification of Alternaria brassicicola genes expressed in planta during pathogenesis of Arabidopsis thaliana. Fungal Genet Biol. 2004;41(2):115-28.

50. Pryor BM, Bigelow DM. Molecular characterization of Embellisia and Nimbya species and their relationship to Alternaria, Ulocladium and Stemphylium. Mycologia. 2003;95(6):1141-54.

51. Hong SG, Cramer RA, Lawrence CB, Pryor BM. Alt a 1 allergen homologs from Alternaria and related taxa: analysis of phylogenetic content and secondary structure. Fungal Genet Biol. 2005;42(2):119-29.

52. Mitakakis TZ, Barnes C, Tovey ER. Spore germination increases allergen release from Alternaria. J Allergy Clin Immunol. 2001;107(2):388-90.

53. Sporik RB, Arruda LK, Woodfolk J, Chapman MD, Platts-Mills TA. Environmental exposure to Aspergillus fumigatus allergen (Asp f I). Clin Exp Allergy. 1993;23(4):326-31.

54. Benninger MS, Ferguson BJ, Hadley JA, Hamilos DL, Jacobs M, Kennedy DW, et al. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngol Head Neck Surg. 2003;129(3 Suppl):S1-32.

55. Ray NF, Baraniuk JN, Thamer M, Rinehart CS, Gergen PJ, Kaliner M, et al. Healthcare expenditures for sinusitis in 1996: contributions of asthma, rhinitis, and other airway disorders. J Allergy Clin Immunol. 1999;103(3 Pt 1):408-14.

56. Statistics NCfH. Ambulatory care visits to physicians offices, hospital, outpatient departments, and emergency departments: United States, 1997. Hyattsville, MD: National Center for Health Statistics.; 1999.

57. Hamilos DL. Chronic sinusitis. J Allergy Clin Immunol. 2000;106(2):213-27.

58. Bishai WR. Issues in the management of bacterial sinusitis. Otolaryngol Head Neck Surg. 2002;127(6 Suppl):S3-9.

59. Gliklich RE, Metson R. The health impact of chronic sinusitis in patients seeking otolaryngologic care. Otolaryngol Head Neck Surg. 1995;113(1):104-9.

60. Delafuente JC, Davis TA, Davis JA. Pharmacotherapy of allergic rhinitis. Clin Pharm. 1989;8(7):474-85.

61. Gwaltney JM, Jr., Hendley JO, Phillips CD, Bass CR, Mygind N, Winther B. Nose blowing propels nasal fluid into the paranasal sinuses. Clin Infect Dis. 2000;30(2):387-91.

62. Ponikau JU, Sherris DA, Kephart GM, Kern EB, Congdon DJ, Adolphson CR, et al. Striking deposition of toxic eosinophil major basic protein in mucus: implications for chronic rhinosinusitis. J Allergy Clin Immunol. 2005;116(2):362-9.

63. Demoly P, Crampette L, Mondain M, Campbell AM, Lequeux N, Enander I, et al. Assessment of inflammation in noninfectious chronic maxillary sinusitis. J Allergy Clin Immunol. 1994;94(1):95-108.

64. Hamilos DL, Leung DY, Wood R, Meyers A, Stephens JK, Barkans J, et al. Chronic hyperplastic sinusitis: association of tissue eosinophilia with mRNA expression of granulocyte-

macrophage colony-stimulating factor and interleukin-3. J Allergy Clin Immunol. 1993;92(1 Pt 1):39-48.

65. Demoly P, Crampette L, Mondain M, Enander I, Jones I, Bousquet J. Myeloperoxidase and interleukin-8 levels in chronic sinusitis. Clin Exp Allergy. 1997;27(6):672-5.

66. Rhyoo C, Sanders SP, Leopold DA, Proud D. Sinus mucosal IL-8 gene expression in chronic rhinosinusitis. J Allergy Clin Immunol. 1999;103(3 Pt 1):395-400.

67. Stoop AE, van der Heijden HA, Biewenga J, van der Baan S. Eosinophils in nasal polyps and nasal mucosa: an immunohistochemical study. J Allergy Clin Immunol. 1993;91(2):616-22.

68. Harlin SL, Ansel DG, Lane SR, Myers J, Kephart GM, Gleich GJ. A clinical and pathologic study of chronic sinusitis: the role of the eosinophil. J Allergy Clin Immunol. 1988;81(5 Pt 1):867-75.

69. Newman LJ, Platts-Mills TA, Phillips CD, Hazen KC, Gross CW. Chronic sinusitis. Relationship of computed tomographic findings to allergy, asthma, and eosinophilia. JAMA. 1994;271(5):363-7.

70. Hoover GE, Newman LJ, Platts-Mills TA, Phillips CD, Gross CW, Wheatley LM. Chronic sinusitis: risk factors for extensive disease. J Allergy Clin Immunol. 1997;100(2):185-91.

71. Hamilos DL, Leung, D.Y., Wood, R., Cunningham, L., Bean, D.K., Yasruel, Z., Schotman, E., Hamid, Q. Evidence for distinct cytokine expression in allergic versus nonallergic chronic sinusitis. Journal of Allergy and Clinical Immunology. 1994;96:537-44.

72. Sanchez-Segura A, Brieva JA, Rodriguez C. T lymphocytes that infiltrate nasal polyps have a specialized phenotype and produce a mixed TH1/TH2 pattern of cytokines. J Allergy Clin Immunol. 1998;102(6 Pt 1):953-60.

73. Hamilos DL, Leung DY, Wood R, Bean DK, Song YL, Schotman E, et al. Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF-alpha. Am J Respir Cell Mol Biol. 1996;15(4):443-50.

74. Kita H, Adolphson, C.R., Gleich, G.J. Biology of eosinophils. 6 ed. St. Louis: N. F. Adkinson, Jr., B. S. Bochner, J. W. Yunginger, S. T. Holgate, W. W. Busse, F. E. R. Simons; 2003.

75. Yoon J, Ponikau JU, Lawrence CB, Kita H. Innate antifungal immunity of human eosinophils mediated by a beta 2 integrin, CD11b. J Immunol. 2008;181(4):2907-15. PMCID: 2614918.

76. Abu-Ghazaleh RI, Dunnette SL, Loegering DA, Checkel JL, Kita H, Thomas LL, et al. Eosinophil granule proteins in peripheral blood granulocytes. J Leukoc Biol. 1992;52(6):611-8.

77. Gleich GJ, Adolphson CR, Leiferman KM. The biology of the eosinophilic leukocyte. Annu Rev Med. 1993;44:85-101.

78. Fujisawa T, Kephart GM, Gray BH, Gleich GJ. The neutrophil and chronic allergic inflammation. Immunochemical localization of neutrophil elastase. Am Rev Respir Dis. 1990;141(3):689-97.

79. Frigas E, Loegering DA, Gleich GJ. Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. Lab Invest. 1980;42(1):35-43.

80. Hisamatsu K, Ganbo T, Nakazawa T, Murakami Y, Gleich GJ, Makiyama K, et al. Cytotoxicity of human eosinophil granule major basic protein to human nasal sinus mucosa in vitro. J Allergy Clin Immunol. 1990;86(1):52-63.

81. Gleich GJ, Adolphson CR. The eosinophilic leukocyte: structure and function. Adv Immunol. 1986;39:177-253.

82. Altman LC, Ayars GH, Baker C, Luchtel DL. Cytokines and eosinophil-derived cationic proteins upregulate intercellular adhesion molecule-1 on human nasal epithelial cells. J Allergy Clin Immunol. 1993;92(4):527-36.

83. Popken-Harris P, Thomas L, Oxvig C, Sottrup-Jensen L, Kubo H, Klein JS, et al. Biochemical properties, activities, and presence in biologic fluids of eosinophil granule major basic protein. J Allergy Clin Immunol. 1994;94(6 Pt 2):1282-9.

84. Uchida DA, Ackerman SJ, Coyle AJ, Larsen GL, Weller PF, Freed J, et al. The effect of human eosinophil granule major basic protein on airway responsiveness in the rat in vivo. A comparison with polycations. Am Rev Respir Dis. 1993;147(4):982-8.

85. Coyle AJ, Mitzner W, Irvin CG. Cationic proteins alter smooth muscle function by an epithelium-dependent mechanism. J Appl Physiol. 1993;74(4):1761-8.

86. Brofman JD, White SR, Blake JS, Munoz NM, Gleich GJ, Leff AR. Epithelial augmentation of trachealis contraction caused by major basic protein of eosinophils. J Appl Physiol. 1989;66(4):1867-73.

87. Motojima S, Frigas E, Loegering DA, Gleich GJ. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. Am Rev Respir Dis. 1989;139(3):801-5.

88. Hulsmann AR, Raatgeep HR, den Hollander JC, Bakker WH, Saxena PR, de Jongste JC. Permeability of human isolated airways increases after hydrogen peroxide and poly-L-arginine. Am J Respir Crit Care Med. 1996;153(2):841-6.

89. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol. 2009;183(2):1427-34. PMCID: 2706924.

90. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. J Allergy Clin Immunol. 2004;114(5):997-1008; quiz 9.

91. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25(17):3389-402.

92. Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, et al. InterPro: the integrative protein signature database. Nucleic Acids Res. 2009;37(Database issue):D211-5.

93. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25-9. PMCID: 3037419.

94. Janoff A. Proteases and lung injury. A state-of-the-art minireview. Chest. 1983;83(5 Suppl):54S-8S.

95. Matsuwaki Y, Wada K, White TA, Benson LM, Charlesworth MC, Checkel JL, et al. Recognition of fungal protease activities induces cellular activation and eosinophil-derived neurotoxin release in human eosinophils. J Immunol. 2009;183(10):6708-16.

96. Shin MH, Chung YB, Kita H. Degranulation of human eosinophils induced by Paragonimus westermani-secreted protease. Korean J Parasitol. 2005;43(1):33-7.

97. Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc. 2007;2(4):953-71.

98. Delcher AL, Phillippy A, Carlton J, Salzberg SL. Fast algorithms for large-scale genome alignment and comparison. Nucleic Acids Res. 2002;30(11):2478-83. PMCID: 117189.

99. Li L, Stoeckert CJ, Jr., Roos DS. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 2003;13(9):2178-89.

100. Janoff A. Elastase in tissue injury. Annu Rev Med. 1985;36:207-16.

101. Ohbayashi H. Matrix metalloproteinases in lung diseases. Curr Protein Pept Sci. 2002;3(4):409-21.

102. Tetley TD. Macrophages and the pathogenesis of COPD. Chest. 2002;121(5 Suppl):156S-9S.

103. Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. J Allergy Clin Immunol. 2000;105(6 Pt 1):1185-93.

104. Shen HD, Lin WL, Tam MF, Wang SR, Tsai JJ, Chou H, et al. Alkaline serine proteinase: a major allergen of Aspergillus oryzae and its cross-reactivity with Penicillium citrinum. Int Arch Allergy Immunol. 1998;116(1):29-35.

105. Yu CJ, Chiou SH, Lai WY, Chiang BL, Chow LP. Characterization of a novel allergen, a major IgE-binding protein from Aspergillus flavus, as an alkaline serine protease. Biochem Biophys Res Commun. 1999;261(3):669-75.

106. Shen HD, Lin WL, Liaw SF, Tam MF, Han SH. Characterization of the 33-kilodalton major allergen of Penicillium citrinum by using MoAbs and N-terminal amino acid sequencing. Clin Exp Allergy. 1997;27(1):79-86.

107. Shen HD, Tam MF, Chou H, Han SH. The importance of serine proteinases as aeroallergens associated with asthma. Int Arch Allergy Immunol. 1999;119(4):259-64.

108. Boitano S, Flynn AN, Sherwood CL, Schulz SM, Hoffman J, Gruzinova I, et al. Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. Am J Physiol Lung Cell Mol Physiol. 2011;300(4):L605-14. PMCID: 3075100.

109. Huang X, Wang J, Aluru S, Yang SP, Hillier L. PCAP: a whole-genome assembly program. Genome Res. 2003;13(9):2164-70. PMCID: 403719.

110. Carbon S, Ireland A, Mungall C, Shu S, Marshall B, Lewis S, et al. AmiGO: online access to ontology and annotation data. Bioinformatics. 2008.

111. Mari A, Schneider P, Wally V, Breitenbach M, Simon-Nobbe B. Sensitization to fungi: epidemiology, comparative skin tests, and IgE reactivity of fungal extracts. Clin Exp Allergy. 2003;33(10):1429-38.

112. Vijay HM, Kurup VP. Fungal allergens. Clin Allergy Immunol. 2008;21:141-60.

113. Horst M, Hejjaoui A, Horst V, Michel FB, Bousquet J. Double-blind, placebo-controlled rush immunotherapy with a standardized Alternaria extract. J Allergy Clin Immunol. 1990;85(2):460-72.

114. Arruda LK, Mann BJ, Chapman MD. Selective expression of a major allergen and cytotoxin, Asp f I, in Aspergillus fumigatus. Implications for the immunopathogenesis of Aspergillus-related diseases. J Immunol. 1992;149(10):3354-9.

115. Saenz-de-Santamaria M, Postigo I, Gutierrez-Rodriguez A, Cardona G, Guisantes JA, Asturias J, et al. The major allergen of Alternaria alternata (Alt a 1) is expressed in other members of the Pleosporaceae family. Mycoses. 2006;49(2):91-5.

116. Portnoy J, Pacheco F, Upadrashta B, Barnes C. A double monoclonal antibody assay for the Alternaria allergen GP70. Ann Allergy. 1993;71(4):401-7.

117. Bush RK, Sanchez H, Geisler D. Molecular cloning of a major Alternaria alternata allergen, rAlt a 2. J Allergy Clin Immunol. 1999;104(3 Pt 1):665-71.

118. Curran IH, Young NM, Burton M, Vijay HM. Purification and characterization of Alt a-29 from Alternaria alternata. Int Arch Allergy Immunol. 1993;102(3):267-75.

119. Breitenbach M, Simon-Nobbe B. The allergens of Cladosporium herbarum and Alternaria alternata. Chem Immunol. 2002;81:48-72.

120. Unger A, Stoger P, Simon-Nobbe B, Susani M, Crameri R, Ebner C, et al. Clinical testing of recombinant allergens of the mold Alternaria alternata. Int Arch Allergy Immunol. 1999;118(2-4):220-1.

121. O'Driscoll BR, Hopkinson LC, Denning DW. Mold sensitization is common amongst patients with severe asthma requiring multiple hospital admissions. BMC Pulm Med. 2005;5:4. PMCID: 550663.

122. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature. 2005;438(7071):1151-6.

123. Ciuffetti LM, Tuori RP, Gaventa JM. A single gene encodes a selective toxin causal to the development of tan spot of wheat. Plant Cell. 1997;9(2):135-44. PMCID: 156906.

124. Fiser A, Sali A. Modeller: generation and refinement of homology-based protein structure models. Methods Enzymol. 2003;374:461-91.

125. Sogabe S, Stuart F, Henke C, Bridges A, Williams G, Birch A, et al. Neutralizing epitopes on the extracellular interferon gamma receptor (IFNgammaR) alpha-chain characterized by homolog scanning mutagenesis and X-ray crystal structure of the A6 fab-IFNgammaR1-108 complex. J Mol Biol. 1997;273(4):882-97.

126. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. J Mol Graph. 1996;14(1):33-8, 27-8.

127. Cho Y, Kim KH, La Rota M, Scott D, Santopietro G, Callihan M, et al. Identification of novel virulence factors associated with signal transduction pathways in Alternaria brassicicola. Mol Microbiol. 2009;72(6):1316-33.

128. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4(2):249-64.

129. Bottone EJ, Nagarsheth N, Chiu K. Evidence of self-inhibition by filamentous fungi accounts for unidirectional hyphal growth in colonies. Can J Microbiol. 1998;44(4):390-3.

130. Brandao RL, Castro IM, Passos JB, Nicoli JR, Thevelein JM. Glucose-induced activation of the plasma membrane H(+)-ATPase in Fusarium oxysporum. J Gen Microbiol. 1992;138 Pt 8:1579-86.

131. Eraso P, Portillo F. Molecular mechanism of regulation of yeast plasma membrane H(+)-ATPase by glucose. Interaction between domains and identification of new regulatory sites. J Biol Chem. 1994;269(14):10393-9.

132. Viegas CA, Rosa MF, Sa-Correia I, Novais JM. Inhibition of Yeast Growth by Octanoic and Decanoic Acids Produced during Ethanolic Fermentation. Appl Environ Microbiol. 1989;55(1):21-8. PMCID: 184048.

133. Mills PR, Davies RJ, Devalia JL. Airway epithelial cells, cytokines, and pollutants. Am J Respir Crit Care Med. 1999;160(5 Pt 2):S38-43.

134. Spurrell JC, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. Am J Physiol Lung Cell Mol Physiol. 2005;289(1):L85-95.

135. Anderson P. Pin1: a proline isomerase that makes you wheeze? Nat Immunol. 2005;6(12):1211-2.

136. Anderson P. Post-transcriptional control of cytokine production. Nat Immunol. 2008;9(4):353-9.

137. Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004;16(1):3-9.

138. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, et al. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem. 2005;280(7):5571-80.

139. Matsukura S, Kokubu F, Kurokawa M, Kawaguchi M, Ieki K, Kuga H, et al. Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-kappaB and/or IRF-3 in airway epithelial cells. Clin Exp Allergy. 2006;36(8):1049-62.

140. Ritter M, Mennerich D, Weith A, Seither P. Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. J Inflamm (Lond). 2005;2:16. PMCID: 1315317.

141. Torres D, Dieudonne A, Ryffel B, Vilain E, Si-Tahar M, Pichavant M, et al. Double-stranded RNA exacerbates pulmonary allergic reaction through TLR3: implication of airway epithelium and dendritic cells. J Immunol. 2010;185(1):451-9.

142. Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, Johnston SL. Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. J Virol. 2005;79(19):12273-9. PMCID: 1211516.

143. Wang Q, Nagarkar DR, Bowman ER, Schneider D, Gosangi B, Lei J, et al. Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. J Immunol. 2009;183(11):6989-97. PMCID: 2920602.

144. Aumeunier A, Grela F, Ramadan A, Pham Van L, Bardel E, Gomez Alcala A, et al. Systemic Toll-like receptor stimulation suppresses experimental allergic asthma and autoimmune diabetes in NOD mice. PLoS One. 2010;5(7):e11484. PMCID: 2900205.

145. Stowell NC, Seideman J, Raymond HA, Smalley KA, Lamb RJ, Egenolf DD, et al. Long-term activation of TLR3 by poly(I:C) induces inflammation and impairs lung function in mice. Respir Res. 2009;10:43. PMCID: 2694181.

146. Meng L, He X, Zhu W, Yang X, Jiang C, Sun Q, et al. TLR3 and TLR7 modulate IgE production in antigen induced pulmonary inflammation via influencing IL-4 expression in immune organs. PLoS One. 2011;6(2):e17252. PMCID: 3045401.

147. Toubi E, Shoenfeld Y. Toll-like receptors and their role in the development of autoimmune diseases. Autoimmunity. 2004;37(3):183-8.

148. May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today. 1998;19(2):80-8.

149. Beck-Schimmer B, Madjdpour C, Kneller S, Ziegler U, Pasch T, Wuthrich RP, et al. Role of alveolar epithelial ICAM-1 in lipopolysaccharide-induced lung inflammation. Eur Respir J. 2002;19(6):1142-50.

150. Wegner CD, Gundel RH, Reilly P, Haynes N, Letts LG, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. Science. 1990;247(4941):456-9.

151. Thomas LH, Wickremasinghe MI, Sharland M, Friedland JS. Synergistic upregulation of interleukin-8 secretion from pulmonary epithelial cells by direct and monocyte-dependent effects of respiratory syncytial virus infection. J Virol. 2000;74(18):8425-33. PMCID: 116353.

152. Baggiolini M, Walz A, Kunkel SL. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest. 1989;84(4):1045-9. PMCID: 329758.

153. Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Jr., et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature. 1999;398(6729):718-23.

154. Eder J. Tumour necrosis factor alpha and interleukin 1 signalling: do MAPKK kinases connect it all? Trends Pharmacol Sci. 1997;18(9):319-22.

155. Gendler SJ. MUC1, the renaissance molecule. J Mammary Gland Biol Neoplasia. 2001;6(3):339-53.

156. Cassel TN, Nord M. C/EBP transcription factors in the lung epithelium. Am J Physiol Lung Cell Mol Physiol. 2003;285(4):L773-81.

157. Reghunathan R, Jayapal M, Hsu LY, Chng HH, Tai D, Leung BP, et al. Expression profile of immune response genes in patients with Severe Acute Respiratory Syndrome. BMC Immunol. 2005;6:2. PMCID: 546205.

158. Shimizu K, Keller NP. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. Genetics. 2001;157(2):591-600. PMCID: 1461531.

159. Cho Y, Davis JW, Kim KH, Wang J, Sun QH, Cramer RA, Jr., et al. A high throughput targeted gene disruption method for Alternaria brassicicola functional genomics using linear minimal element (LME) constructs. Mol Plant Microbe Interact. 2006;19(1):7-15.

160. Sweigard JA, Carroll AM, Kang S, Farrall L, Chumley FG, Valent B. Identification, cloning, and characterization of PWL2, a gene for host species specificity in the rice blast fungus. Plant Cell. 1995;7(8):1221-33. PMCID: 160946.

161. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.

162. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A. 2003;100(16):9440-5. PMCID: 170937.

APPENDIX

Supplementary Tables

Supplementary Table 1 The homology level between known fungal allergens and A. brassicicola predicted proteins (whole genome) and A. alternata proteome form spore germination EST collection.

Described Allergen	A. brassicicola Homologs	A. alternata Homologs
Asp f 2	2 (46, 40)	No Significant Hits
Asp f 3	2 (49, 39)	1 (49)
Asp f 5	1 (59)	No Significant Hits
Asp f 3	No Significant Hits	1 (49)
Asp f 6	2 (64, 55)	3 (64, 55, 51)
Asp f 7	No Significant Hits	No Significant Hits
Asp f 8	2 (72, 48)	2 (73, 48)
Asp f 9	5 (48, 49, 43, 40, 30)	5 (52, 44, 39, 44, 32)
Asp f 10	7 (60, 44, 48, 25, 26, 25, 35)	1 (26)
Asp f 11	11 (55, 57, 55, 55, 51, 56, 55, 49, 45, 44)	5 (64, 56, 56, 44, 72)
Asp f 12	1 (88) 0.0	2 (86, 65)
Asp f 13	5 (46, 43, 39, 49, 39)	1 (37)
Asp f 15	1 (43)	1 (44)
Asp f 16	5 (35, 45, 35, 29, 38)	5 (53, 46, 39, 44)
Asp f 18	5 (75, 43, 34, 39, 42)	2 (63, 51)
Asp f 22w	1 (87)	2 (88, 87)
Asp f 23	2 (85, 91)	1 (85) 0.0
Asp o 13	5 (45, 40, 35, 48, 36)	No Significant Hits
Asp o 21	1 (41)	No Significant Hits
Asp n 14	No Significant Hits	1 (38)
Asp n 18	5 (74, 43, 34, 38, 42)	1 (56)
Asp n 25	6 (27, 27, 27, 26, 28, 34)	1 (27)
Asp fl 13	5 (45, 40, 35, 45, 36)	1 (37)
Asp o 13	No Significant Hits	1 (37)
Alt a 1	1 (88)	No Significant Hits
Alt a 3	1 (48)	2 (99, 47)
Alt a 4	2 (78, 31)	4 (69, 60, 68, 68)
Alt a 5	2 (94, 43)	2 (98, 43)
Alt a 6	1 (99)	1 (98)
Alt a 7	2 (91, 82)	1 (98)
Alt a 8	23 (98, 58, 39, 40, 37, 42, 32, 30, 27, 45, 30, 26, 30, 42, 26, 33, 22, 40, 25, 42, 35, 27, 48)	13 (99, 82, 35, 34, 45, 26, 25, 36, 29, 27, 31, 25, 33)

Alt a 10	21 (94, 93, 54, 43, 45, 39, 34, 36, 37, 35)	10 (53, 48, 35, 35, 35, 30, 40, 35, 35, 34)
Alt a 12	3 (90, 40, 39)	1 (90)
Alt b 1	1 (100)	1 (98)
Cand a 1	32 (60, 42, 61, 40, 41, 31, 30, 30, 34, 31)	11 (61, 42, 30, 29, 42, 41, 24, 31, 26, 29, 57)
Cand a 3	1 (30)	1 (30)
Cand b 2	2 (34, 41)	1 (34)
Cla h 3	21 (78, 76, 52, 45, 41, 39, 35, 35, 35, 32)	10 (55, 45, 37, 36, 41, 31, 42, 37, 40, 39)
Cla h 4	6 (62, 57, 47, 92, 30, 24)	7 (83, 56, 93, 43, 55, 36, 32)
Cla h 5	2 (64, 58)	1 (65)
Cla h 6	1 (89)	2 (88, 92)
Cla h 7	2 (64, 58)	1 (65)
Cla h 8	39 (75, 42, 36, 33, 34, 32, 31, 32, 32, 28)	10 (75, 34, 42, 29, 60, 43, 28, 28, 26, 25)
Cla h 12	3 (78, 45, 39)	2 (77, 41)
Cop c 2	4 (47, 42, 33, 33)	4 (46, 42, 41, 33)
Cop c 3	1 (28)	No Significant Hits
Flc 1 p	3 (39, 23, 19)	1 (40)
Flc 3 p	3 (37, 22, 19)	1 (37)
Fus c 1	3 (84, 45, 60)	2 (85, 44)
Fus c 2	5 (50, 47, 38, 36, 35, 28)	4 (50, 49, 44, 37)
Fus c 3	1 (30)	1 (30)
Mala f 2	2 (37, 36)	1 (37)
Mala f 3	2 (38, 37)	No Significant Hits
Mala f 4	2 (64, 55)	4 (64, 55, 43, 44)
Mala s 5	2 (42, 38)	1 (42)
Mala s 6	11 (68, 67, 62, 63, 61, 70, 51, 50, 44, 42)	6 (68, 71, 66, 52, 64, 63)
Mala s 9	1 (30)	No Significant Hits
Mala s 10	6 (28, 27, 25, 24, 27, 39)	3 (37, 22, 24)
Mala s 11	2 (51, 46)	3 (47, 46, 45)
Mala s 12	22 (33, 33, 30, 31, 30, 28, 28, 29, 28, 32)	2 (26, 32)
Mala s 13	8 (53, 42, 44, 35, 34, 34, 29, 30)	6 (48, 42, 41, 37, 36, 58)
Pen b 26	3 (69, 39, 42)	No Significant Hits
Pen c 1	5 (43, 39, 44, 36, 36)	1 (36)
Pen c 3	2 (51, 40)	1 (51)
Pen c 19	6 (60, 57, 47, 32, 97, 26)	6 (82, 55, 97, 32, 28, 45)
Pen c 22w	1 (87)	2 (87, 88)
Pen c 24	2 (65, 63)	1 (65)
Pen ch 13	5 (47, 38, 43, 45, 36)	1 (36)
Pen ch 18	5 (73, 39, 36, 40, 35)	2 (65, 50)
Pen ch 20	2 (73, 37)	2 (69, 37)
Pen n 13	5 (47, 38, 43, 45, 36)	1 (36)
Pen n 18	4 (90, 46, 38, 40)	4 (84, 98, 68, 36)
Pen o 18	5 (63, 41, 35, 38, 42)	No Significant Hits
Rho m 1	1 (74)	2 (72, 77)

Rho m 2	5 (43, 65, 39, 40, 41)	3 (68, 49, 41)
Tri m 2	5 (50, 50, 43, 45, 43)	2 (48, 45)
Tri m 4	3 (43, 40, 38)	1 (52)
Tri r 2	5 (41, 42, 38, 41, 36)	1 (36)
Tri r 4	3 (42, 40, 37)	1 (51)
Tri s 4	3 (43, 40, 38)	No Significant Hits

Supplementary Table 2. Growth study of the wild type A. alternata and three mutants in a seven day time interval. The mutants used are Δ*alt a 1* deletion gene (KO), the complementation mutant of this gene (Comp Alt a 1), and the Alt a 1 overexpression mutant (OE Alt a 1).

	GluMM			GalMM			PDA			
day 1	6	6	6	6	6	6	7	7	7	WT
day 2	12	14	14	9	8	7	20	21	22	
day 3	21	23	25	11	12	10	34	35	36	
day 4	30	34	36	13	15	12	48	47	50	
day 5	45	50	50	14	15	14	60	60	63	
day 6	54	58	59	15	17	15	69	69	71	
day 7	62	66	67	17	19	16	80	81	83	
day 1	7	7	7	7	8	7	9	8	8	KO_Alt a 1
day 2	19	20	19	9	9	8	22	21	20	
day 3	30	31	30	10	10	10	36	35	34	
day 4	40	43	42	12	12	13	50	50	49	
day 5	56	60	58	15	16	14	64	65	62	
day 6	64	71	68	18	17	16	72	73	73	
day 7	72	80	75	20	18	17	85	85	86	
day 1	7	6	6	6	6	6	7	7	7	Comp_Alt a 1
day 2	13	15	17	7	7	7	20	21	21	
day 3	27	29	28	10	9	8	32	34	31	
day 4	38	41	39	13	12	13	46	48	46	
day 5	56	55	52	15	13	14	60	61	59	
day 6	60	64	65	17	18	17	72	73	70	
day 7	73	73	72	20	20	19	81	81	77	
day 1	6	6	6	6	6	6	7	7	7	OE_Alt a 1
day 2	10	11	9	7	7	7	15	15	15	
day 3	20	19	21	10	10	9	27	27	29	
day 4	28	27	28	11	12	12	42	40	42	
day 5	36	35	36	12	13	13	54	53	56	
day 6	41	40	40	14	14	16	65	65	66	
day 7	51	50	49	17	16	17	73	75	75	