Ironing Out the Host-Fungal Interaction in Airway Epithelial Cells

Shernita Lynnae Lee

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

Reinhard C. Laubenbacher, Chair
Christopher B. Lawrence, Co-Chair
Stefan Hoops
Suzy V. Torti

March 21, 2014
Blacksburg, Virginia

Keywords: Iron metabolism, immune response, mathematical model,
host-fungal interaction, transferrin receptor, divalent metal ion transporter 1

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ABSTRACT

Aspergillus fumigatus is a ubiquitous fungus associated with several airway complications and diseases including asthma, allergies, cystic fibrosis, and most commonly Invasive Aspergillosis. The airway epithelium, a protective barrier, is the first anatomical site to interact with *A. fumigatus*. Although this host-fungal interaction is often asymptomatic for immunocompetent individuals, for immunocompromised persons, due to a weakened competence of the immune system, they have an increased likelihood of fungal infection.

This dissertation aims to investigate the effect of *A. fumigatus* on the transcriptional response of human airway epithelial cells, focusing on the relationship between innate immunity and iron regulation from the host perspective. The trace element iron is needed by both the fungus and the host for cellular maintenance and survival, but tightly controlled iron regulation in the host is required to prevent oxidative stress and cell death. The research methods in this dissertation employ a systems biology approach, by incorporating mathematical modeling, RNA-seq analysis, and experimental biology techniques to assess the role of airway epithelial cells in the host-fungal interaction. Both the quantitative and qualitative research design allows for characterization of airway epithelial cells and the downstream changes in iron importer genes. This study addresses literature gaps through analysis of the host transcriptome using multiple time points, by performing an extensive evaluation of the effect of cytokines on iron importer genes, and conceptualization of a comprehensive mathematical model of the airway epithelial cell.

The major findings suggest the following: 1) airway epithelial cells avidly respond to *A. fumigatus* through modification of the expression of immune response related genes at different infection stages, 2) during *A. fumigatus* co-incubation with airway epithelial cells, the iron importers genes respond in strikingly different ways, and 3) cytokines have a significant effect on the increase in expression of an iron importer gene. We illuminated the role of airway epithelial cells in fungal recognition and activation of the immune response in signaling cascades that consequently modify iron importer genes and hope to use this information as a platform to discover potential therapeutic targets.

This dissertation is dedicated to my loving parents, Darryl A. and Sheryl F. Lee.

Acknowledgements:

"Trust in the LORD with all your heart and lean not on your own understanding; in all your ways acknowledge him, and he will make your paths straight." Proverbs 3: 5-6

Reflecting upon this journey, there are several people I would like to acknowledge. To my advisors, Dr. Reinhard Laubenbacher and Dr. Christopher Lawrence, I thank you both for your patience, guidance, and training. Dr. Laubenbacher, you are a wonderful advisor and although it took me four tries to get it right, I found an excellent friend and mentor in you. Dr. Lawrence, although the experimental training in your lab took me well beyond my mathematics comfort zone, I thank you for the numerous discussions and tips to make experimental magic occur and for being such a supportive advisor. To my committee members, Dr. Stefan Hoops and Dr. Suzy Torti, I am grateful for the project feedback and valuable dialogue to progress this dissertation.

I would like to thank all of the past and current research members of the Lawrence and Laubenbacher groups, especially Dr. David Murrugarra, Dr. Franziska Hinkelmann, Dr. Mihaela Babiceanu, Prathyusha Koloconda, and Shivani Grover for all of your advice. To the following research members who deserve more gratitude than words can express for showing me the experimental techniques I know and truly being excellent trainers, Dr. Amanda Cronin Rumore, Dr. Sang-Wook Park, Ha Dang, and Brad Howard, I say thank you.

During my time here, I established valuable friendships. To Dr. Brian Moseley, Mrs. Maria Laubenbacher, Mrs. Betsy Williams, Traci Roberts, Dr. Elaine Nsoesie, Dr. Nikki Lewis-Huff, Bianca Baker, Sandria Gray, Adria Carbo, Dr. Kathy O'Hara, Dr. Lachelle Waller, Dr. Tamisha Vaughan, Dr. Rajat Singhania, Dr. Frank Criscone and Mrs. Joyce Randall, I thank each and every one of you.

One special person who I often attribute to my pursuit of this degree is Dr. Kristy Collins. We met at an unexpected conference in 2008, but I am confident it was destined for our paths to cross and for you to tell me about Virginia Tech and the GBCB program. You convinced me that Virginia Tech was the place to be and your excitement for the university was contagious. Thank you for all of the laughs and for being Malaysia's mother.

Dennie Munson and Dr. David Bevan, thank you for assisting me in the GBCB program and making everything seem effortless. I had countless conversations with each of you along the way, but I can attest to the fact, once I left your offices I felt relief. To Dr. Ed Smith and the initiative to maximize student development (IMSD) family, I thank you for the financial support and helping me transition to the university. IMSD catalyzed many of my friendships and it played a significant role in my professional development. To the graduate school, I thank you for being my home away from home, especially Dean Karen DePauw, Mrs. Dannette Gomez-Beane, Mrs. Ennis McCrery, Mrs. Lauren Surface, and Ms. Monika Gibson.

To my Alabama State University family, you set the stage for my success in graduate school and encouraged me to aim for the stars. To Dr. Ana Tameru, my mentor and friend, you ignited my passion for research. Mrs. Yvonne Williams, Dr. Shree Singh, and the NSF STEM family thank you for providing opportunities for students like me to gain research experience.

Next, I want to acknowledge my friends who motivated me to continue on this journey. Dr. Gena Chandler-Smith, Nyala Smith, Ayden Huff, Jasmine Heard, Tiffany Clark Jones, Arika Barnett, Keith Harrell, Lynette Wilcox, Jennifer Hartfield, and Dr. Rachelle Brunn-Bevel, I am grateful for our friendships. I also thank my sorority members of the Beta Pi and Tau Mu Omega chapters of Alpha Kappa Alpha Sorority, Inc.

Lastly, I would like to thank my supportive family. To my parents, Darryl and Sheryl Lee, I love you beyond words. Thank you for sacrificing so much over the years to instill the value of hard work and higher education. To my beautiful sisters, LaDara Lee and Darnita Perkins, thank you both for all of the calls, emails, text messages, and for just being your amazing selves. Your constant motivation inspired me even through my toughest times.

"And when I wake up, everything I went through will be beautiful"- Jill Scott

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List of Abbreviations

%	Percent
℃	Degrees Celsius
β –glucan	β-1, 3-glucan
β-defensin 2	Beta defensin 2
µg	microgram
µL	micro liter
µm	micron
A	Adenine
A549	Adenocarcinomic human alveolar basal epithelial cells
ABPA	Allergic bronchopulmonary aspergilliosis
Active IRPs	Active iron regulatory proteins
AEC	Airway epithelial cell
Af293	*Aspergillus fumigatus* Af293 strain
ATCC	American type culture collection
ATF3	Activating transcription factor 3
BAL	Bronchoalveolar lavage
BEA2B	SV-40 transformed human bronchial epithelial cells
BEGM	bronchial epithelial growth media
bp	base pairs
BPE	bovine pituitary extract
BSA-FAF	bovine serum albumin – fatty acid free
C	Cytosine
Ca	Calcium
cDNA	Complimentary deoxyribonucleic acid
CF	Cystic Fibrosis
Cm^2	Centimeter squared
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disorder
COX2	Cyclooxygenase-2
CSF2	Colony stimulating factor 2
DEX	Dexamethasone
DMT1	Divalent metal ion transporter 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco's phosphate buffered saline
FC	Fold change
Fe	Iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fpn	Ferroportin
Ft	Ferritin
FTL	Ferritin light
FXN	Frataxin
G	Guanine

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
GMM	Glucose minimal media
GO	Gene ontology
GTPase	Guanosine triphosphatase
HBEC	Human bronchial epithelial cell
heGF	Human epidermal growth factor
Hep	Hepcidin
HIF1A	Hypoxia inducible factor 1 alpha
HO-1	Heme oxygenase 1
Hrs.	Hours
IA	Invasive Aspergillosis
IPA	Invasive Pulmonary Aspergillosis
ICAM1	Intercellular adhesion molecule 1
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IGV	Integrative genomics viewer
Ikβ	Ikappa B kinase
IL	Interleukin
IL10	Interleukin 10
IL1β	Interleukin 1 beta
IL36G	Interleukin 36 gamma
IL6	Interleukin 6
IL8	Interleukin 8
IPA	Ingenuity Pathway Analysis
IRE	Iron responsive element
KEGG	Kyoto encyclopedia of genes and genomes
LCN2	Lipocalin 2
LIP	Labile iron pool
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mg	Magnesium
mL	milliliter
mRNA	Messenger ribonucleic acid
MTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
Mθs	Macrophages
N/A	Not applicable
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NHBE	Normal human bronchial epithelial
nM	Nano molar
NRAMP2	Natural resistance-associated macrophage protein 2
NRF2	Nuclear factor-like 2
OVOL1	Ovo-like zinc finger 1

PAMPs	Pathogen-associated molecular patterns
PDS	Polynomial dynamical systems
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
P-value	Probability value
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RNA-seq	RNA sequencing
RNS	Reactive nitrogen species
NO	Nitric oxide
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
siRNA	Small interfering RNA
SP-A	Surfactant protein A
SP-D	Surfactant protein D
STEAP4	Six-transmembrane epithelial antigen of prostate 4
SV-40	Simian virus 40
T	Thymine
Tf	Transferrin
TF	Transcription factor
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TFRC	Transferrin receptor
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TNF-α	Tumor necrosis factor alpha
TREM1	Triggering receptor expressed on myeloid cells 1
TSLP	Thymic stromal lymphopoietin
U	Units
U.S.	United States
UTR	Untranslated region

Preface/Attribution

Chapter 2:

The authors acknowledge Ha Dang for his assistance with RNA-Seq analysis protocol. We are also grateful to project contributors at the Virginia Bioinformatics and Reinhard Laubenbacher for their kind contribution to support the RNA-Seq data processing. Amanda Cronin-Rumore assisted with the experimental design, sample collection and preparation for RNA processing, and methodology description. Stephanie Dodson and Kiersten Utsey helped with the Tuxedo protocol analysis and examination of the differential gene expression. Christopher Lawrence assisted with validation of RNA-seq data.

Chapter 3:

Sang-wook Park assisted with the project development providing valuable insight about the data and qRT-PCR techniques. Christopher Lawrence provided the BEAS2B cells, Af293, and cell culture/sample extraction tools for this study.

Chapter 4:

Reinhard Laubenbacher supported in the creation of Cyclone, and the authors thank Paul Vines for his programming efforts to ensure optimal Cyclone usage. Brad Howard helped with primers specification and cDNA processing. All experimental supplies provided by Christopher Lawrence. Jake Weissman, John Gowins, and Tyler George, aided in model development and analysis.

Chapter 1: Background

1.1 General introduction

In recent years, increasing numbers of individuals suffer from invasive airways diseases caused by fungi [1]. Fungi possess advanced mechanisms to enable them to thrive in human hosts and cause infections, diseases, and respiratory complications. However, Brown et al. [2] reported that only 2% of major research agencies' funding in both the United States (U.S.) and the United Kingdom support fungal research. Most likely, every person will have direct interaction with fungal pathogens during his or her lifetime [3]. Fungi have adapted over time to increase their heat tolerance, modification of spore dispersion techniques, and augment their survivability techniques [4-7]. For persons with weakened immune systems, fungal exposure can result in localized infection in the lung, brain, or other organs, particularly if phagocytic cells fail to halt the fungal pathogenesis process. Although all of these locations have features that promote fungal infection, the lung is an ideal site for fungal colonization due to its moist environment, connectivity to blood sources and other organs, and nutrient availability.

Airway epithelial cells (AEC) have recently emerged as primary components in the immune response due to their active recognition of fungi, secretion of inflammatory cytokines, and activation of defense mechanisms [8, 9]. Immunocompromised persons often preserve some level of functionality in AEC, but they experience significant decrease in response time to pathogens, cellular counts, and recruitment and performance of neutrophils and alveolar macrophages [10, 11] . The adjective "immunocompromised" is a loosely defined term used to describe a person with an immune system deficit. This category of persons includes organ transplant donors and recipients, HIV/AIDS patients, asthmatics, corticosteroid users, and other immunosuppressed individuals [12]. The unstated problem is often the unknown and abrupt transition from being immunocompetent to being immunocompromised, due to adverse medicinal side effects, genetic abnormalities, or underlying medical conditions. According to the U.S. Department of Health and Human Services, approximately 28,051 persons received an organ transplant in the United States last year alone [13]. The medications to reduce the risks of organ rejection, unfortunately, increase the risk of airway diseases secondary to fungal infection. As the number of immunocompromised individuals increases, improved methods of understanding the physiology of fungal infection are necessary.

One fungus commonly associated with airway infection, *Aspergillus fumigatus*, is a ubiquitous fungus found in diverse environments and settings such as dust, soil, ground water, hospitals, and even black pepper [14-16]. *A. fumigatus* is the leading global cause of airborne fungal infections [17], estimated to infect over 200,000 persons yearly [3]. The primary function of the fungus, as in the case of most pathogens, is not to infect and threaten the health of susceptible human hosts, but rather to aid in carbon recycling and decomposition of soil and decaying matter. *A. fumigatus* spores, approximately 3μm in size, allow for easy inhalation and distribution in the airway environment, and for avoidance of mucosal clearance mechanisms in the lung [18]. *A. fumigatus* is linked to several respiratory diseases and conditions such as Invasive Aspergillosis (IA), Chronic Obstructive Pulmonary Disorder (COPD), asthma, and Cystic Fibrosis (CF) [19-21]. Its unmatched virulence, genetic variation, and rapid adaptability, makes it a critical fungus to investigate. Persons with CF also exhibit mixed colonization of fungi, but *A. fumigatus* is

commonly found in the sputum [22, 23]. For IA, a deadly disease, there are minimal healthcare options to provide fungal burden relief to its patients. Although healthcare has advanced in the treatment of IA and other *Aspergillus* induced healthcare issues, the morbidity and mortality rates are still increasing. In most cases, infection by *A. fumigatus* is not easily diagnosed until it is actively searched for or after a prolonged infection period.

Investigation of host-fungal interactions in AEC may lead to potential therapeutic targets since these cells are the first cell type to come in contact with fungal spores and, result in a direct interaction with *A. fumigatus* [8, 24]. Several limitations in current diagnosis and treatment of *Aspergillus* infections exist, especially for those with weakened immune systems. Tierney et al. [25] suggested a systems biology approach as the only way to learn valuable information about host-fungal interactions. Others agree with this notion and believe more efforts are required to understand the response of the host to fungal infection, rather than focusing solely on the behavior of the fungus [26-33].

Another aspect of the interaction between AEC and *A. fumigatus* is the battle for the essential element iron. Iron is needed by every mammalian pathogen, with the exception of *Borrelia burgodorferi* which has a manganese requirement [34]. Host cells, such as airway epithelial cells, need iron for DNA synthesis, cellular respiration, maintenance of oxygen levels, and other processes, while *A. fumigatus* needs iron for virulence and survival. Our proposed research implements a systems biology approach to extract knowledge about the response of the host to *A. fumigatus* exposure and what potential immune response and iron related mechanisms the host employs to affect the fungus.

The innate immune response of the host to *A. fumigatus* determines the efficiency of the host to expel the threat [9, 11, 12, 35, 36]. Many immune cells respond to *A. fumigatus,* but the primary responders are epithelial cells, the barrier between the external environment and the internal environment of the host [37]. Past researchers directed emphasis towards macrophages, dendritic cells, and neutrophils due to their phagocytic property and established role in immunity [38]. Yet, epithelial cells are gaining attention because of newly detected properties in response to *A. fumigatus*. One study showed the ability of epithelial cells to internalize *A. fumigatus,* and this mechanism is yet to be interpreted as either a host defense mechanism or a fungal tactic to prevent detection by other cells [39]. Another study revealed that treatment of epithelial cells with proteases from *A. fumigatus* promoted secretion of cytokines such as interleukin (IL) 6 and IL8 (neutrophil chemoattractant) which determines successful cell recruitment [40, 41].

1.2 Review of literature

1.2.1 Morphology properties of Aspergillus

The *Aspergillus* genus has over 300 species but only roughly 40 are known to affect humans, notably *A. fumigatus*, labeled the most pathogenic [42-44]. *A. fumigatus* is a saprophytic fungus associated with decaying organic matter such as hay or compost [45]. The small conidia size, roughly 2-3 μm, echinolate structure, hydrophobic property, and easy dispersion in air samples allows for unavoidable interaction with the *Aspergillus* species [14]. *Aspergillus* is capable of both sexual and asexual reproduction [14], influencing its four morphologies: dormant conidia, swollen conidia, germlings, and hyphae [46]. *A. fumigatus* has evolutionarily adapted to environmental changes, and one of those adaptations is thermotolerance. The conidia can survive at temperatures as high as 75°C, surpassing the average human body temperature [5, 47]. The optimal temperatures for *A. fumigatus* airborne conidia occur in temperate and humid locations [48]. It is estimated that since humans inhale thousands of liters of air daily, fungal spore inhalation is at least in the hundreds [48]. Its versatility contributes to its success at being one of the most prevalent pathogens distributed worldwide [5].

1.2.2 Characteristics of Aspergillus infection

Aspergillus has evolutionarily adapted to survive in humans and is related to multiple respiratory infections and diseases including asthma, allergies, IA, COPD, allergic bronchopulmonary aspergilliosis (ABPA), aspergilloma, invasive pulmonary aspergillosis (IPA), and others [11, 49, 50]. Studies suggest that at least 30% of fungal infections of cancer patients are characteristic of aspergillosis [51]. One of the most progressive *A. fumigatus* related diseases IA has a mortality rate as high as 100%, depending on the type of transplantation, host immune status, and drug usage amount and duration [52]. A five-year hospital study investigated the infection rate in persons with compromised immune systems to *A. fumigatus*, and discovered that 30% of the cases had confirmed, probable, or possible IA [53].

1.2.3 Aspergillus and asthma?

The relationship between asthma and *A. fumigatus* is a newly emerging area due to the adaptability of the fungus and its allergens [21].To quantify the amount of airborne *Aspergillus* conidia, Vermani et al. [54] measured the atmospheric concentration in Delhi and determined, in an average year, *A. fumigatus* aerial allergen concentration was 18-380.4 ng. A comparison of the spore binding differences of *Aspergillus* in normal versus asthmatic lung conditions showed that asthmatic lungs had an increased adherence of *Aspergillus* spores [55]. Of the 936 urban children participating in an intervention study, 27% were found to have sensitization to the *Aspergillus* allergen [56]. Examination of the homes of asthmatic and non-asthmatic children revealed *Aspergillus/ Penicillium* in at least 20% of homes [57]. The anti-fungal drug, voriconazole, was shown to reduce the number of exacerbations in asthmatics versus asthmatics in the placebo group, displaying promising results [58].

1.2.4 Drug treatments for A. fumigatus related complications

1.2.4.1 Epithelial cells

Many immunocompromised persons generally have multiple infections over time, and, due to corticosteroids and other immune suppressants, develop a decreased immune response [46]. Both the germinated and ungerminated form of *A. fumigatus* are susceptible to the antifungal and anti-

inflammatory drugs like amphotericin B, itraconazole, voriconazole, SCH56592, and prophylaxis, the drugs most commonly used to treat *Aspergillus* infection [59, 60]. Some current treatments, dexamethosone (DEX), for example, were shown to increase the invasiveness of *A. fumigatus* by promoting its growth through fibronectin expression, in comparison to no DEX treatment [61]. Antifungal drugs are effective for most individuals, but fungal resistance is a rising complication [62-64]. To address this, the following must be done: discover new drug targets for *A. fumigatus*, determine optimal available drug combinations, or find innovative drugs to target the varied immune response of the host.

1.2.4.2 Macrophages

Current antifungal drug treatments, such as DEX, have a direct effect on the response of other cell types such as macrophages (Mθs) to *A. fumigatus*. One hypothesis is that DEX inhibits NF-κB during *A. fumigatus* expression [65], and granulocyte macrophage colony stimulating factor (GM-CSF) mediates the minimization in cytokine production [66] which has importance in *Aspergillus* infection [67].

1.2.5 The host response to A. fumigatus

Recent research emphasizes the need to understand the response of epithelial cells to *A. fumigatus* because of their role in the recruitment of other effector cells to the infection site [37, 68, 69]. The epithelial cell layer is not just a barrier separating two disjointed anatomical regions but rather a strategic player in inflammation, innate immunity, and adaptive immunity. This section covers the themes shown in Figure 1.1.

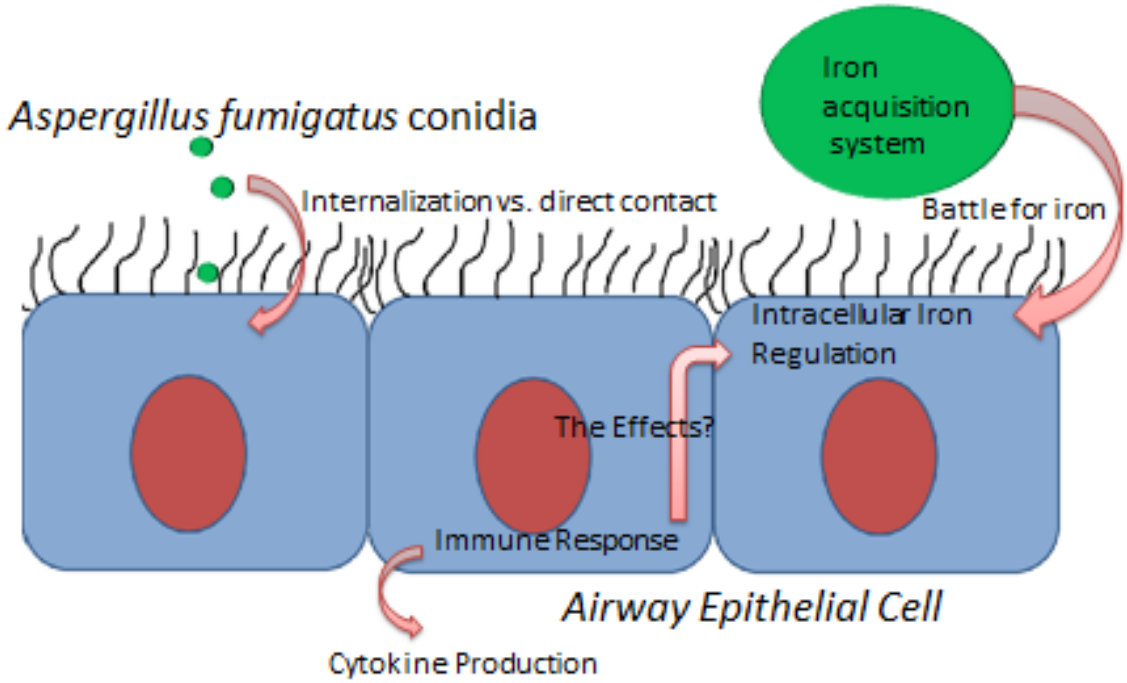

Figure 1.1. Schematic of the host-fungal interaction. *A. fumigatus* conidia interact with the airway epithelial cell and induce an immune response.

1.2.5.1 Initial fungal recognition

In order to start the signaling cascade, first *A. fumigatus* displays its pathogen-associated molecular patterns (PAMPs) and/or secreted fungal byproducts. *A. fumigatus* produces chemicals that slow down the movement of mucociliary clearance in epithelial cells. Asp f5, an enzyme from *A. fumigatus*, increases permeability of airway epithelial cells [70] and, suggest the importance of cellular integrity and recognition of various fungal forms. The most common PAMP is β-1, 3-glucan (β-glucan), a cell wall component displayed at various stages of inflammation [71, 72]. PAMPs are pathogen specific and recognized by pathogen recognition receptors (PRRs) of the host [73]. Toll-like receptors(TLRs) are a member of the PRR family and directly recognize and physically interact with PAMPs [74]. Effectiveness of TLRs determines the recognition of *A. fumigatus* in the lung and progression of disease in the host [75]. To date, there are three PRRs known to recognize *A. fumigatus*: dectin-1, toll-like receptor-2 (TLR2), and toll-like receptor -4 (TLR4). There is a debate over which TLRs recognize *A. fumigatus*, but the common agreement is that at least TLR2 and TLR4 are two TLRs involved [42, 76].The toll-like receptor-3 (TLR3) is possibly involved as well in fungal recognition [77]. The C-type lectin dectin-1, the detector of β-glucan, has been shown to be up-regulated in response to *A. fumigatus* and suggested to strongly influence a heightened immune response [78]. The expression of β-glucan depends on the form of *A. fumigatus*; it is not found on hyphae, only swollen conidia, although it makes up 50% of the cell wall [79].

1.2.5.2 Mechanisms of Aspergillus internalization by airway epithelial cells

The first study to examine the ability of epithelial cells to internalize *A. fumigatus* spores was initiated by Paris et al. [80] in 1997; They suggested the epithelial cell may serve as a reservoir for the fungus, and adherence to the plasma membrane begins as early as 2 hours of incubation. Conidia fuse with late endosomes/lysosomes, and once internalized in A549 cells, the fungus can still germinate for up to 24 hours [81]. In one study examining the percentage of cells with internalized viable and heat killed conidia in A549 cells, researchers found 30% were internalized. This percentage was preserved for both conidia states, and it appeared that germination no longer occurred in internalized conidia for at least up to 6 hours [39]. A study by Han et al. [82] supported this observation by showing the internalization of *A. fumigatus* conidia is contingent on dectin-1 expression. Significant internalization occurs as early as 6 hours, and the maximum average internalization index of approximately 25% occurs at 8 hours. Studies show that resting (non-germinating) and germinating conidia are able to induce a response in AEC [83]. The larger the number of spores, the greater the binding of *Aspergillus* to A549 cells [55].

1.2.5.3 Changes in cytokine expression

Cytokine production in the lung is essential for protection against microbial invaders relevant to both adaptive and innate immunity, reviewed in Strieter et al. [84]. Cytokines are defined as soluble mediators secreted by cells that promote cell-to-cell communication [85, 86]. Chemokines, a superfamily of cytokines, have as their main functionality the signaling to other cell types to assist epithelial cells with expulsion of the fungus. Through the MyD88- nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, TLRs eventually produce intracellular tumor necrosis factor alpha (TNF-α), using the dual activation of a TLR with the PAMPs from the fungus, as well as extracellular TNF-α binding to TNF-α receptor [76, 87]. Research has shown proteases from *A. fumigatus* induce an immune response in AEC via NF-κB which controls signaling of cytokines and other inflammatory molecules [88]. TNF-α is a

cytokine with two forms: the soluble form and the membrane form. The form produced through the previously mentioned pathway is the soluble form which activates other cytokines-interferon gamma (IFN-γ), IL-8,and IL-6 [9, 84]. Epithelial cells induced with *A. fumigatus* conidia have an increase in TNF-α production [78]. The germinating form of the fungus induces an increase in IL-8 expression in AEC [89]. The resting conidia form, however, can activate interferon beta (IFN-β) dependent pathways [83].

1.2.6 The iron of the matter

Iron is at the intersection of an optimal host immune response and minimal fungal virulence [90, 91]. Iron is needed for processes including DNA synthesis, cell respiration, and oxygen transport [92]. When the system is poorly controlled, the cell attempts to rebalance iron levels. Dietary iron is tightly controlled and it is estimated that the average person loses 1-2 mg of iron daily [93].

Defects in iron regulation contribute to multiple diseases and disorders and a recent review suggests microRNAs may inhibit key iron regulatory functions including iron import and storage [94]. In an extensive review, Kell et al. [95] examine the effects of dysregulated iron levels and the link with diseases including hemacromatosis, diabetes, Alzheimer's, atherosclerosis, and Parkinson's disease. Another review examined the connection between iron levels and cancers (breast, pancreatic, bladder, prostate) and the potential usage of iron as a therapeutic target or in cancer prevention [96]. In CF patients, there were significantly higher amounts of iron in the lung lavage [44]. Additionally, a decrease in iron availability revealed a decrease in Malaria susceptibility [97]. Iron chelators are implicated in the management of oxidative stress targeting macrophages and neutrophils [98]. A new iron-related cell death, ferroptosis, is a rising phenomenon triggered by oxidative stress [99], and perhaps may be a critical phenotype in the host-fungal interaction.

1.2.6.1 Epithelial cells and iron metabolism

Cells have distinct mechanisms for the proper iron regulation. Transferrin (Tf) bound iron is imported through the plasma membrane protein transferrin receptor 1 (TfR1) after the reduction of iron from its ferric (Fe^{3+}) to its ferrous (Fe^{2+}) form [100]. The other importer, divalent metal ion transporter 1 (DMT1), is a plasma membrane transporter protein, specific for certain epithelial cell types, specifically in the lung [101], in our case. DMT1 imports non-Tf bound iron and, when the cell is stressed, DMT1 is able to reduce oxidative stress by mediating an increase in intracellular storage [100]. Due to alternative splicing, there are two forms of DMT1: - iron responsive element (IRE) and +IRE, based on presence or absence of iron responsive elements in its 3'untranslated region [102]. TfR1 and DMT1 direct iron to the labile iron pool (LIP), which is a low molecular weight pool of catalytically available iron [100, 103]. The import of iron is dependent on the influence of active iron regulatory proteins (active IRPs), which post-transcriptionally regulate TfR1. Active IRPs also regulate other iron export and storage proteins.

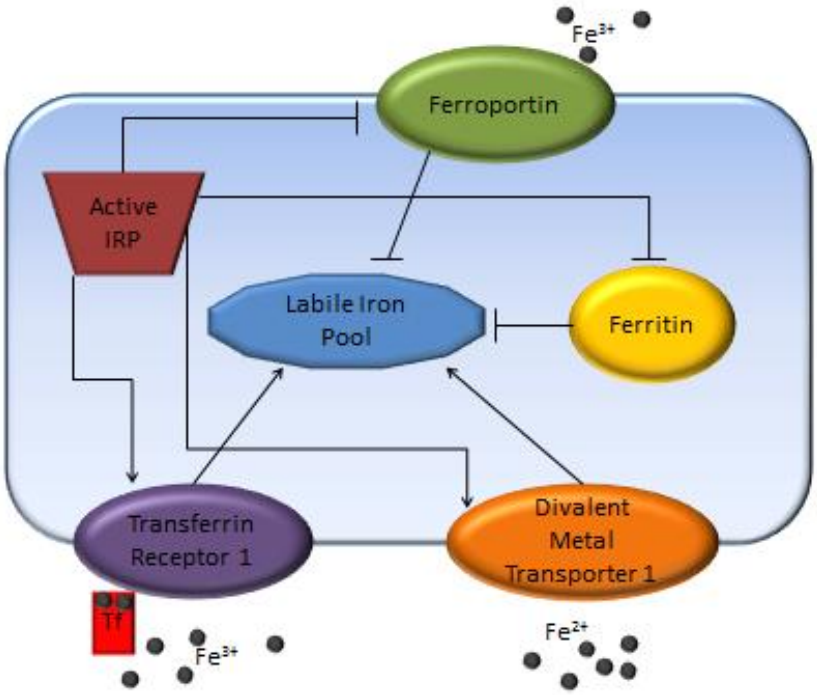

Figure 1.2: Schematic of iron regulation in the airway epithelial cell. The components are transferrin receptor 1, divalent metal transporter 1, active iron regulatory proteins (IRP), labile iron pool, ferritin, and ferroportin. Arrows indicate up-regulation/production and hammerheads indicate down-regulation/suppression.

Iron is stored in ferritin (Ft), a storage protein composed of heavy and light subunits [104], and Ft reduces the amount of free iron in the cellular environment [105]. Active IRPs are key regulators of iron homeostasis, also affecting ferroportin (Fpn), Ft, and, as previously mentioned, TfR1. Fpn is an iron exporter and is located on the plasma membrane of the cell. It removes iron no longer needed for usage in the cell and exports it to other cell types. The LIP is an indicator of available iron in the cell [106]. The core network of iron metabolism in lung epithelial cells is shown in Figure 1.1. Another essential component of iron metabolism is hepcidin (Hep). Hep is a peptide hormone produced by the liver that degrades Fpn and is essential during the infection process [107]. The role of Hep in airway epithelial cells is not fully understood, but examination of the link between Hep and IFN-γ showed that IFN-γ increases Hep expression [108].

The addition of supplemental iron causes an increase in Ft and DMT1 expression levels [109]. Reactive nitric species (RNS) like nitric oxide (NO) modify the structure of the epithelial cell, its function, and determine epithelial cell response to stimuli. Reactive oxygen species (ROS) are produced by epithelial cells and produced in response to particulates [110]. The activity of dectin-1 in fungal recognition determines ROS production in AEC [78]. A key element in oxidative stress maintenance is heme oxygenase-1 (HO-1), an iron catabolizer. When HO-1 is overexpressed in AEC, it results in decreased LIP levels, increased storage via Ft, and increased import by TfR1 [111].

1.2.6.2 Cytokines and iron regulation

Cytokine levels, previously discussed, have been shown to increase during fungal infection. Several published studies examine the independent and combined effect of cytokines on iron regulatory components. TNF-α is a master cytokine regulator and plays a role in the up-regulation of IL-1β and IFN-γ [112, 113]. IL-1β inhibits TfR1 and up-regulates Ft [104], while IFN-γ up-regulates Ft [105]. The activation of TNF-α results in an increased import of non-Tf-bound iron via DMT1 [105, 114].Evidence also supports *A. fumigatus*-induced secretion of IL6 and IL8 in lung epithelial cells, but that evidence needs to be further explored, since their effect on iron regulatory proteins has not been investigated.

1.2.6.3 Iron acquisition system of A. fumigatus

Aspergillus virulence has been linked to its capability of iron acquisition [115, 116]. *A. fumigatus* has a high affinity uptake system and a siderophore-mediated iron uptake system. It has 3-5 associated siderophores, ranging from intracellular to extracellular [117, 118]. The intracellular siderophore, ferricronin, is a storage site for iron and communicates with a collection of iron similar to the labile iron pool of the host [119]. The extracellular siderophores, fusarine C and triacetylfusarine, are tasked with the responsibility of acquiring extracellular iron [120, 121]. Triacetylfusarine has a high binding affinity for iron, and thus it can win the competition with the host for Tf-bound iron [118, 122]. Siderophores are able to use bound and unbound iron, and the fungus secretes siderophores to acquire iron, minimizing the levels of free iron in the environment. Cowen et al. [123], showed that deletion of the HAPX gene, a transcription factor, decreased *A. fumigatus* growth and sporulation in iron deplete conditions, suggesting the importance of the fungal iron requirement.

1.2.7 The appeal of proteomics and transcriptome analysis of the host-fungal interaction

Presently, there are 12 articles examining the transcriptional or proteomic response of AEC, *A. fumigatus*, or both. The sequencing of the genome of *A. fumigatus* amplified the discovery of transcriptional and protein level changes in various environmental conditions [124]. Suh et al. [125] examined multiple conidia and hyphal stages of *A. fumigatus* to assess protein similarities and differences using shotgun proteomics. Using multiplexed activity-based protein profiling approach and mass spectrometry, Wiedner et al. [126] examined activated pathways and functional changes in *A. fumigatus* in the presence and absence of serum. In 2012, high-throughput sequencing (RNA-seq) was used to find differentially expressed genes in *A. fumigatus* in two growth conditions [127]. Others investigated changes of the fungus in oxidative stress conditions [128], iron replete vs. deplete conditions [129, 130], its response to hypoxia [131], or discovery of potential fungal drug targets [132].

The first transcriptional study to investigate the airway epithelial cell and *A. fumigatus* interaction occurred in 2011 [133]. This study was pivotal to the host-fungal interaction because it simultaneously studied the changes of both involved species at one time point. This study found immune response genes are activated in the AEC and the fungus up-regulated pathways are related to iron acquisition. A functional genomics study discovered 889 differentially expressed genes comparing positive (fungal internalization) vs. negative (fungal contact) cell populations, with 376 genes up-regulated and 513 genes down-regulated related to inflammation, cytokine activity, and other categories [134]. Fekkar et al. [135] focused on the

secretome of AEC in infected vs. non infected cells and found activation of the redox system to limit cellular oxidative stress.

Therefore, the need for the transcriptional study of airway epithelial cells is crucial. The pathogen aspect has tremendously more research but more work is needed on the host side in order to progress toward potential therapeutic targets to tackle drug resistance, more work is needed on the host side. This dissertation aims to shed light on the response of AEC to *A. fumigatus*, focusing on activation of the immune response and downstream changes in iron import. Since both species require iron, we employ mathematical modeling, bioinformatics, and experimental biology to elucidate the ensuing battle over iron between the host and the fungus. In this thesis research, the work is presented as follows: Chapter 1 reviews the literature on the host-fungal interaction, innate immunity, and iron regulation. Chapter 2 examines the transcriptional response of AEC to *A. fumigatus* using bioinformatics analysis. In Chapter 3, we study the effect of IL-1β, TNF-α and IL-8 on iron importers in AEC. A mathematical model of the effect of *A. fumigatus* on AEC is presented in Chapter 4. Chapter 5 is a general discussion of the summary of findings and future studies applicable to this research project.

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Chapter 2:
Host Transcriptome Analysis of *Aspergillus fumigatus* Infection in Airway Epithelial Cells

Shernita Lee[1], Amanda Cronin-Rumore[2], Stephanie Dodson[3], Kiersten Utsey[4], Christopher Lawrence[1], Reinhard Laubenbacher[5]

[1] Virginia Bioinformatics Institute, Blacksburg, VA, USA

[2] Randolph College, Lynchburg, VA, USA

[3] University of Massachusetts Amherst, Amherst, MA, USA

[4] Carroll College, Helena, MT, USA

[5] University of Connecticut Health Center, Farmington, CT, USA

Abstract:

Aspergillus fumigatus is an opportunistic fungus which directly interacts with the airway epithelium, the first line of defense, after the respiration of spores (conidia). The epithelium is a recognized component of the innate immune response, due to its role in detecting the fungal threat and recruitment of phagocytes to the infection site. Although preceding studies have established internalization of *A. fumigatus* by airway epithelial cells, there are no transcriptional studies of the infection of airway epithelial cells at different time points of co-incubation. Hence, the goal of this study is to determine the transcriptional response of primary normal human bronchial epithelial (NHBE) cells following interaction with *A. fumigatus* conidia at 2, 6, and 12 hours. After total RNA isolation, the Illumina® HiSeq 2000 next generation sequencing system was used to characterize the transcriptional response, checking 27,024 transcripts, in duplicates, in untreated and *A. fumigatus* treated cells. RNA-Seq data analysis revealed 694, 1794, and 9006 genes displaying differential expression at 2, 6, and 12 hours, respectively. The NHBE cells had a significant number of differentially expressed genes associated with inflammation, immunity, and oxidative stress. Moreover, Gene Ontology terms up/down-regulated in the differentially expressed genes at one or more time points included regulation of response to stimulus, immune system process, and regulation of cell communication. Ingenuity Pathway Analysis revealed the top pathways triggered during fungal infection include cytokine signaling, acute phase response, and signaling by Rho family GTPases. Using qRT-PCR, candidate genes were selected for validation in airway epithelial cells treated with *A. fumigatus*. This is the first study to investigate the transcriptome of uninfected airway epithelial cells and airway epithelial cells interacting with *A. fumigatus* at multiple time points and provides important details about the response of the airway epithelium in the host-fungal interaction.

2.1 Background

Aspergillus fumigatus, a ubiquitous fungus, is responsible for a myriad of airway complications and diseases, including allergies, asthma, chronic obstructive pulmonary disorder, and invasive pulmonary aspergillosis (IPA) [1-3]. IPA results in increased fungal burden in the lung and even with treatment, has a high mortality rate primarily due to drug resistance [4]. Although the interaction with this airborne fungus is often asymptomatic, immunocompromised individuals encounter fatal consequences due to a reduction in the efficiency of the immune system. Several studies have examined the effects of *A. fumigatus* on diverse cell types, including dendritic cells, macrophages, neutrophils, and epithelial cells, all of which play a key role in fungal pathogenesis [5-22].

The small conidia size of *A. fumigatus*, 2.5-3 μm, and constant air inhalation allows for the promotion of spore inhalation, yet an effective immune system prevents spore colonization and subsequent organ damage. The average person inhales approximately one hundred spores daily, therefore interaction with fungi is unavoidable [23]. Many immune response cells interact with *A. fumigatus*, but the initial recognition is by lung epithelial cells, which serve as a barrier between inhaled particulates and the internal lung environment [24]. Airway epithelial cells, the first line of contact, sense the fungus and are avid responders through modification of host defense mechanisms.

There are two recent studies which characterize the transcriptional response of airway epithelial cells to *A. fumigatus* using 16HBE14o- cells at one time point, one focusing on direct conidial contact and the other on internalization of conidia [8, 9]. Previous studies show *A. fumigatus* conidia is capable of germination and internalization by airway epithelial cells [12, 13, 25]. We hypothesize that the response of airway epithelial cells changes based on the duration of the co-infection with *A. fumigatus*, modifying the immune response in airway epithelial cells in a time-dependent fashion. In this article, we report the results of an analysis of RNA-Seq data at three time points, 2, 6, and 12 hrs., describing highly expressed genes and activated pathways. These results serve to emphasize the role of epithelial cells in innate immunity. This is the first transcriptional analysis of this host-fungal interaction using multiple time points and advanced sequencing technology.

2.2 Materials and methods

2.2.1 Fungal strain and growth conditions

The *Aspergillus fumigatus* strain Af293 (ATCC, MYA-4609) was used with the strain propagated on Glucose Minimal Media (GMM), incubated at 37°C in the dark. Conidia were collected in Dulbecco's Phosphate Buffered Saline (Ca^{++}/Mg^{++} free) (DPBS; Hyclone) by gentle agitation and enumerated on a hemacytometer.

2.2.2 Cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD). NHBE cells were grown in bronchial epithelial growth media (BEGM) with SingleQuot (Lonza) supplements and growth factors (bovine pituitary Extract [BPE], hydrocortisone, human epidermal growth factor [hEGF], epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and bovine serum albumin – fatty acid free [BSA-FAF]) as recommended by the manufacturer.

Cells were cultured in 75 cm^2 tissue culture flasks at 37°C and 5% CO_2 until reaching 80% confluence.

2.2.3 Cell stimulation

Cells were sub-cultured into six-well tissue culture dishes at a concentration of 1 x 10^6 cells per well and allowed to adhere overnight. The cells were washed twice with DPBS and cultured in a final volume of 1.5mL BEGM media without addition of the provided gentamicin/*amphotericin-B* component to avoid potential interference from these media supplements with fungal stimulation. Nine individual samples were prepared for each treatment by addition of 0.5 x 10^6 *A. fumigatus* spores or left untreated (control). Cells were incubated at 37°C and 5% CO_2. Samples were collected at 2, 6, and 12 hrs., following initial stimulation. Culture supernatant was collected and debris was removed by centrifugation before storing at -80°C. The cells were washed twice with DPBS before RNA extraction.

2.2.4 RNA extraction and preparation:

Total RNA was collected by addition of 1mL TRIzol (Invitrogen Carlsbad, CA) directly to the cell culture plate and gentle agitation using a cell lifter. RNA extraction was carried out per manufacturer's instructions followed by clean-up using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) with on-column DNase digestion to remove residual genomic DNA. RNA was eluted in 50µl of RNase-free water and integrity (A260/A280) and concentration were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For each time point, equal amounts of RNA from three treatment replicates were pooled to one sample for a total of three samples per time point. A control replicate pool was also generated from each of the three time points (2 hrs., 6 hrs., and 12 hrs.) in the same manner. RNA samples were then stored at -80°C until further analysis.

2.2.5 Reverse transcriptase-polymerase chain reaction validation

RNA was reverse transcribed into cDNA using tetro cDNA synthesis kit (BioLine, Taunton, MA) per the manufacturer's instructions. Quantitative PCR was performed using IQ SYBR green supermix (Biorad, Hercules, CA) on BioRad iCycler thermal cycler. With Cycle 1: (1X), and Step 1 at 95.0 °C for 03:00 minutes, Cycle 2: (40X) with Step1 at 95.0 °C for 00:10 seconds and Step 2 at 55.0 °C for 00:30 seconds, and Cycle 3: (81X) at 55.0 °C-95.0 °C for 00:30 seconds.

DMT1, IL6, and IL8 mRNA levels were normalized to the housekeeping gene GAPDH. The following sequences were used, purchased from Integrated DNA Technologies (IDT):

DMT1: 5′-ATGGACTAGGTGGCGGATT-3′ and 5′ -GATAAGCCACGTGACCACA-3′;
GAPDH: 5′-ACCCACTCCTCACCTTTGA-3′ and 5′-CTGTTGCTGTACCAAATTCGT-3′.
IL8: 5′-CACTGTGTGTAAACATGACTTC-3′ and 5′ -GGAGTATGTCTTTATGCACTGAC-3′
IL6: 5′-ATTCCTTCTTCTGGTCAGAAACC-3′ and 5′-ACAAAGGATATTCAAACTGCATA-GC-3′.

Statistical analysis incorporated the two-tailed Student's *t* test, and we considered values of $p < 0.05$ statistically significant.

2.2.6 Data filtering, read mapping, and quantification of gene expression

Our samples were processed in the Iowa State University DNA facility, using the benchtop sequencer Illumina HiSeq 2000. Library preparation was performed on site and samples were multiplexed on four Illumina flow cells. Reads were single end and 50 base pairs (bp) in length.

2.2.7 Data analysis

The 50 bp reads are mapped and aligned to the human reference genome (hg19) using the alignment software, bowtie, (bowtie-bio.sourcefourge.net/index.shtml) mapping over 80% of the reads as shown in Table 2.1. Additional software used for differential expression analysis included Cufflinks and CuffDiff (cufflinnks.cbcb.umd.edu/index.html) using the raw count method to quantify changes in gene expression. For isoform detection, we employed TopHat (tophat.cbcb.umd.edu). Lastly, our visualizer was the integrative genomics viewer (IGV) (www.broadinstitute.org/igv/).

Time Point	Sample	# of reads with at least one alignment	Percentage of total reads
2 hours	Control #1	28,797,263	82.59%
	Control #2	22,869,599	83.20%
	A. fumigatus #1	38,718,785	84.58%
	A. fumigatus #2	43,342,702	84.59%
6 hours	Control #1	28,592,400	83.66%
	Control #2	29,884,703	84.50%
	A. fumigatus #1	67,420,287	84.31%
	A. fumigatus #2	55,416,122	84.41%
12 hours	Control #1	34,207,285	81.57%
	Control #2	32,352,425	84.37%
	A. fumigatus #1	20,180,954	85.25%
	A. fumigatus #2	41,832,306	84.43%

Table 2.1: Reads alignment. Column 1 is the time point (2, 6, and 12 hrs.), column 2 is each sample descriptions, column 3 lists the number of reads with at least one alignment and column 4 is the percentage of total reads mapped from each sample based on the total number of genes in the hg19 human reference genome.

2.3 Results

2.3.1 Differentially expressed genes

The reads, 50 bp in length and single-end, were mapped to the hg19 reference genome (http://hgdownload.cse.ucsc.edu/downloads.html#human) to examine read abundance. For the functional analysis of gene expression in the NHBE cells samples, the Tuxedo protocol was used to determine differential expression [26]. At 2, 6, and 12 hours, there were 694, 1794, and 9006 differentially expressed genes, respectively as shown in Figure 2.1.

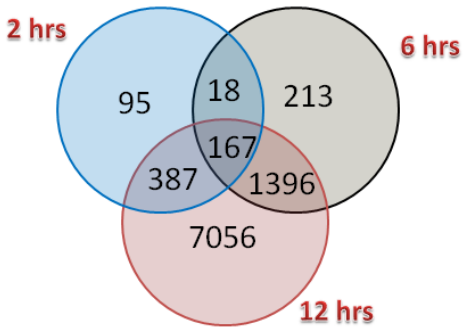

Figure 2.1: Venn diagram. Diagram of differentially expressed genes at 2 hrs., 6 hrs., and 12 hrs. in pairwise comparisons between control and *A. fumigatus* infected samples.

A high number of genes are regulated post-infection with *A. fumigatus*, primarily at later time points. Figure 2.1 shows the distribution of differentially expressed genes at 2 (blue), 6 (purple), and 12 (red) hours in *A. fumigatus* infected samples, compared to control, with a p-value < 0.05. We further analyzed these genes to categorize the basic functions at each time point, focusing on the similarities and differences in gene expression during *Aspergillus* infection.

The primary comparison included investigation of *A. fumigatus* infected samples versus control samples at the same time point; then, in order to observe the trend of genes shared across multiple comparisons, we inspected all combinations as shown in Table 2.2. Later time points, 6 and 12 hrs., maintained the highest number of shared differentially expressed genes at 1563 genes. Due to our sample size and in order to prevent exclusion of targeted genes, we considered all differentially expressed genes regardless of the fold change (FC) value. Figure 2.2 shows the total gene distribution for different FC values. As seen in Table 2.3, there are only a small number of down-regulated genes at each time point when using a common FC cut-off value of 2.

Time point comparison	2 hrs. vs. 6 hrs.	2 hrs. vs. 12 hrs.	6 hrs. vs. 12 hrs.	2 hrs. vs. 6 hrs. vs. 12 hrs.
Gene similarity	185	554	1563	167

Table 2.2: Examination of differentially expressed gene similarity. Using four comparisons: 2 hrs. fungal infection vs. 2 hrs. control with 6 hrs. fungal infection vs. 6 hrs. control, 2 hrs. fungal infection vs. 2 hrs. control with 12 hrs. fungal infection vs. 12 hrs. control, 6 hrs. fungal infection vs. 6 hrs. control with 12 hrs. fungal infection vs. 12 hrs. control, and 2 hrs. fungal infection vs. 2 hrs. control with 12 hrs. fungal infection vs. 12 hrs. control with 6 hrs. fungal infection vs. 6 hrs. control. Differentially expressed genes have p-value <0.05.

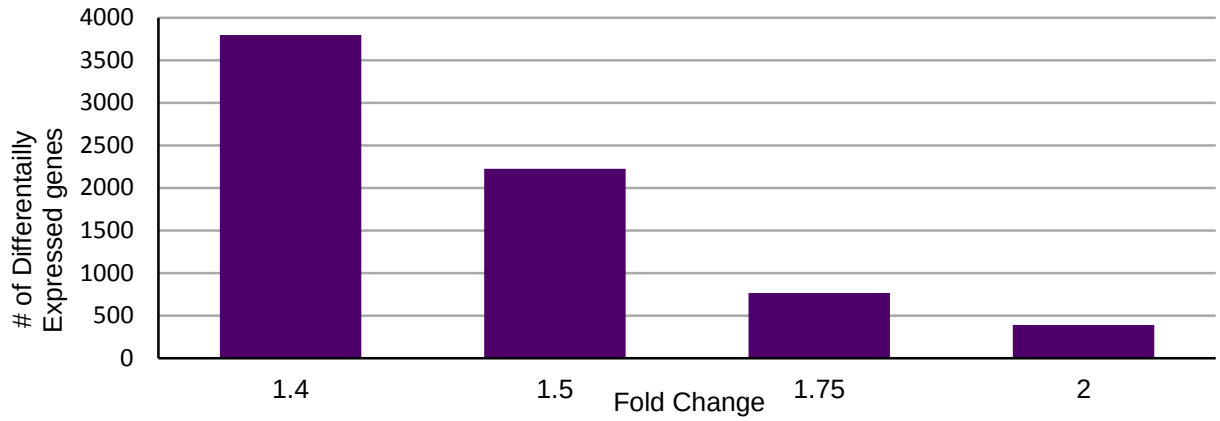

Figure 2.2: Differentially expressed genes based on fold change threshold. Distribution of differentially expressed genes over all time points for the following fold changes: ±1.4, ±1.5, ±1.75, and ±2.

Time point	**2 hrs.**	**6 hrs.**	**12 hrs.**
Total Genes	694	1794	9006
Up-regulated	9	14	333
Down Regulated	17	9	15

Table 2.3: Up/down regulated genes for ± 2 fold change. Listing of the total number of genes differentially expressed at each time point and the number of up/down regulated genes at each time point with a ± 2 fold change cut-off.

GOrilla (http://cbl-gorilla.cs.technion.ac.il/), an online tool, analyzes gene sets to determine the highly represented gene ontology (GO) functions, processes, and components, listing the number of genes in each category using false discovery rate methods [27]. Using Pantherdb (pantherdb.com), we determined the GO categories for differentially expressed genes [28]. Since the results are representative of three diverse stages of infection: early, mid, and prolonged, we analyzed the response at each time point. Table 2.4 lists five descriptive GO categories for 2, 6, and 12 hrs., comparing control versus *A. fumigatus* infected NHBE cells. The Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com) was used to determine the activated pathways at each time point, shown in Table 2.5.

2 hours	6 hours	12 hours
Response to stimulus	Regulation of cell communication	Immune system process
Actin filament organization	Negative regulation of cell cycle	Response to external stimulus
Cellular component organization	Negative regulation of signaling	Response to oxygen levels
Response to amino acid stimulus	Regulation of response to stimulus	Response to stress
Protein complex subunit organization	Negative regulation of organelle organization	Response to growth factor stimulus

Table 2.4: Gene Ontology categories. Representative GO categories for 2 hrs., 6 hrs., and 12 hrs., post *A. fumigatus* infection in differentially expressed genes using GOrilla software.

2 hrs.	6 hrs.	12 hrs.
Acute Phase Response	HIF1α Signaling	Signaling by Rho Family GTPases
HIF1α Signaling	Hypoxia Signaling in Cardiovascular System	IL-10 Signaling
IL-8 Signaling	Acute Phase Response	IL-6 Signaling
IL-6 Signaling	NRF2-Mediated Oxidative Stress Response	NF-κβ Signaling

Table 2.5: Top pathways in Ingenuity Pathway Analysis software. Listed are top IPA pathways for 2 hrs., 6 hrs., and 12 hrs. post infection with *A. fumigatus.*

Next, we focused on the following functional categories: hypoxia and oxidative stress, fungal recognition, immune response, and iron related genes, although several other pathways are induced in response to fungal interaction. These categories are selected to serve as indicators of the amount of fungal burden, the strength of the immune response, and the coordination of cellular changes in response to fungi. Using Pantherdb, we determined the functional distribution of genes at each time point, shown in Figure 2.3.

Although the number of differentially expressed genes is significantly higher at later time points, the distribution of genes is conserved for most categories. Pathway focused arrays target genes with a known association with a specific cellular function, for example, apoptosis. To assess the genes in our data set related to detailed categories, we used known associated genes on array chips from SABiosciences, a Qiagen company (www.sabiosciences.com), to check for hypoxia and oxidative stress (Figures 2.4 and 2.5) and fungal recognition (Figures 2.6 and 2.7) related genes.

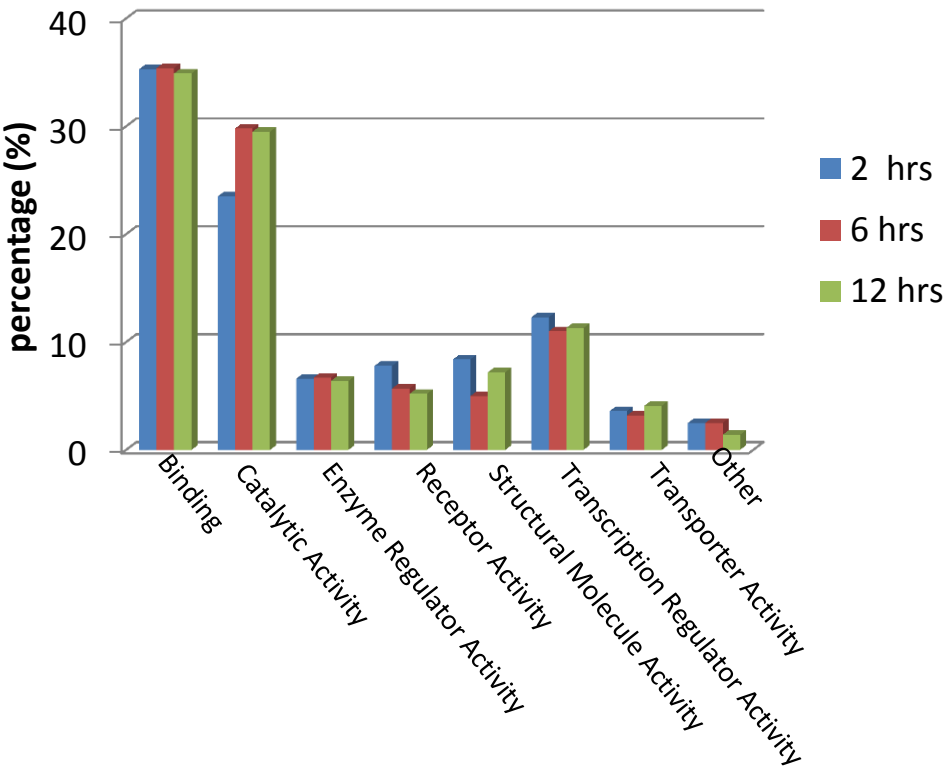

Figure 2.3: Functional categories of genes. The percentage of genes related to binding, catalytic activity, enzyme regulator activity, receptor activity, structural molecule activity, transcriptional regulatory activity, transporter activity, and other categories which include translational regulator, ion channel activity, motor activity, and antioxidant activity at 2 hrs. (blue), 6 hrs. (red), and 12 hrs. (green) are shown.

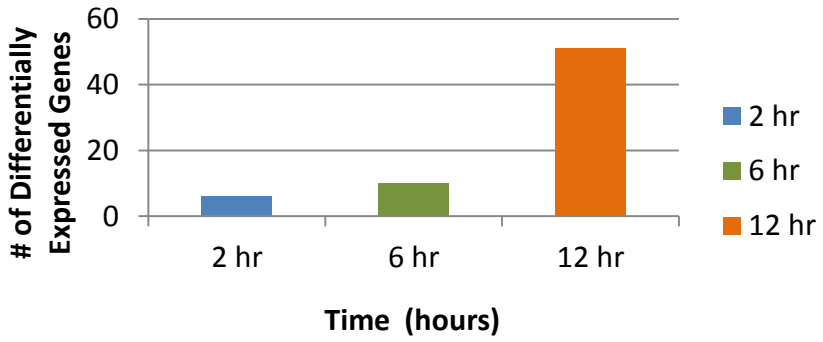

Figure 2.4: Hypoxia and oxidative stress pathway related genes. The number of differentially expressed genes found in 2 hrs. (blue), 6 hrs. (green), and 12 hrs.(orange) in NHBE cells post *A. fumigatus* infection are shown.

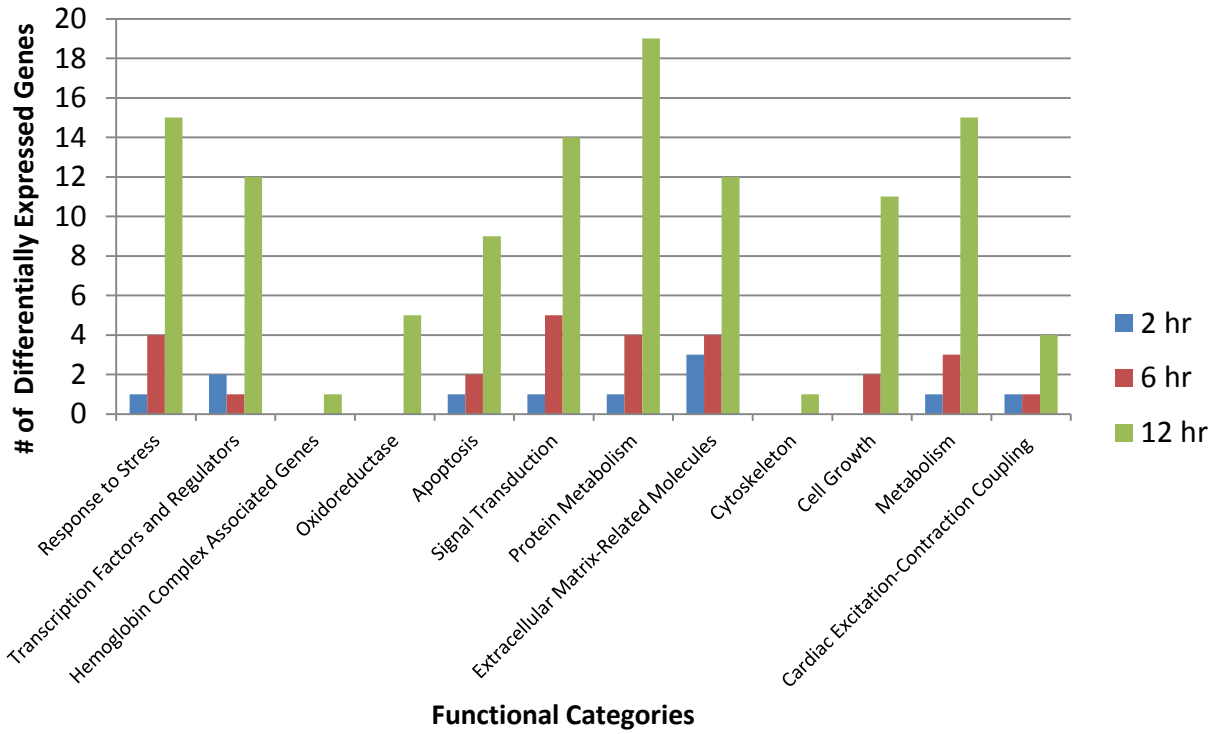

Figure 2.5: Functional categories of differentially expressed genes related to hypoxia and oxidative stress. The categories include: response to stress, transcriptional factors and regulators, hemoglobin complex associated genes, oxidoreductase, apoptosis, signal transduction, protein metabolism, extracellular matrix-related molecules, cytoskeleton, cell growth, metabolism, and cardiac excitation-contraction coupling for 2 hrs. (blue), 6 hrs. (red), and 12 hrs. (green), NHBE cells post *A. fumigatus* infection.

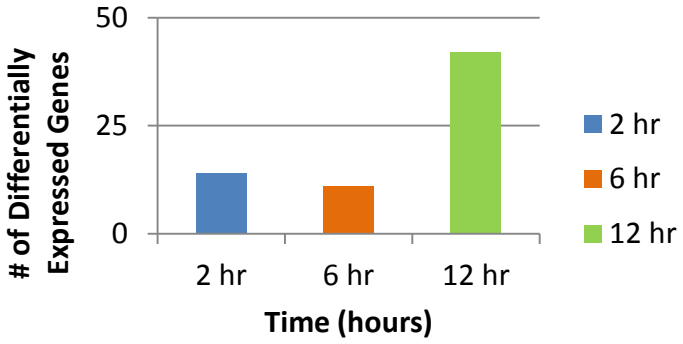

Figure 2.6: Fungal recognition pathway related genes. The number of differentially expressed genes found in 2 hrs. (blue), 6 hrs. (green), and 12 hrs.(orange), NHBE cells post *A. fumigatus* infection related to fungal recognition pathways.

Extraction of the representative top five differentially expressed genes with the highest FC revealed new information about genes actively responding to fungal stimuli (Table 2.6). For the

candidate genes, the fold change value tended to increase at both 2 and 12 hrs., but decrease at 6 hrs., except for IL6. Iron related genes are our central focus in order to determine the changes in its regulation during fungal infection. Table 2.7 describes the genes with the greatest change in response to *A. fumigatus*. The changes in iron genes were fairly subtle, which may attest to the tight regulation of iron related molecules during fungal infection. The next tier, immune response related genes, showed significant changes in expression, even as early as 2 hrs. post-infection with *A. fumigatus*, as shown in Table 2.8.

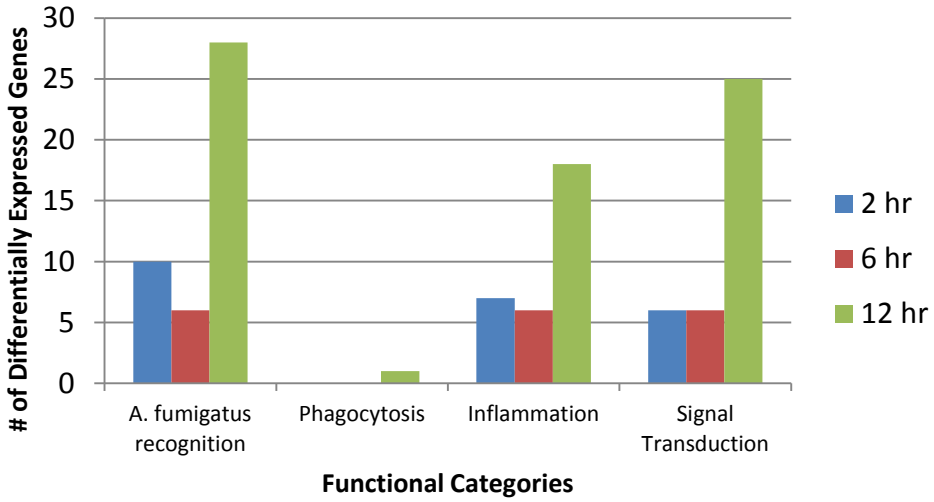

Figure 2.7: Functional categories of differentially expressed genes related to fungal recognition. The categories include: *A. fumigatus* recognition, phagocytosis, inflammation, and signal transduction for 2 hrs. (blue), 6 hrs. (red), and 12 hrs. (green), in NHBE cells post *A. fumigatus* infection.

Gene	Function	2 hrs. FC	6 hrs. FC	12 hrs. FC
IL36G	Cytokine activity	2.69	0.90	12.96
IL8	Chemokine activity	3.15	1.37	10.74
OVOL1	Transcription factor	1.96	1.40	8.87
ATF3	Transcription factor induced by cellular stress	1.99	1.28	6.62
IL6	Cytokine	1.77	2.47	5.37

Table 2.6: Top 5 differentially expressed genes. IL36G, IL8, OVOL1, ATF3, and LDN4 had the highest FC at 2 hrs., 6 hrs., and 12 hrs., post *A. fumigatus* infection.

Gene Symbol	Function	2 hrs. FC	6 hrs. FC	12 hrs. FC
LCN2	Capture siderophores	1.398	-1.102	1.988
FXN	Regulates mitochondrial iron transport	1.340	1.056	1.901
TFRC	Iron importer	-1.046	1.072	1.670
STEAP4	Iron reduction from ferric to ferrous iron	1.071	1.179	1.624
FTL	Iron storage	1.017	-1.009	1.596

Table 2.7: Top iron related genes. LCN2, FXN, TFRC, STEAP4, and FTL and the corresponding FC values at 2 hrs., 6 hrs., and 12 hrs., post infection with *A. fumigatus*.

Gene Symbol	Function	2 hrs. FC	6 hrs. FC	12 hrs. FC
IL36G	Agonist of NF-κB activation	2.689	-1.118	12.751
IL8	Inflammatory response	3.154	1.373	10.729
CSF2	Production, differentiation, function of granulocytes and macrophages	1.483	1.498	4.065
TNFAIP3	Inflammatory signaling pathways	1.210	1.1	2.86
TREM1	Stimulates neutrophils and inflammatory response	1.06	-1.47	2.85

Table 2.8: Top immune response related genes. The top genes include IL36G, IL8, CSF2, TNFAIP3, and TREM1 and the corresponding FC values at 2, 6 and 12 hrs. post-infection with *A. fumigatus*.

2.3.2 Comparison of findings with previous studies

We compared our data sets with two previous transcriptional studies using airway epithelial cells exposed to *A. fumigatus* conidia to identify similarities in gene expression. In the first study, we found 8 genes at 2 hrs., 12 genes at 6 hrs., and 58 genes at 12 hrs. in common with the 255 genes shown to be differentially expressed (FC cut-off 1.5) in the analysis of 16HBE14o- cells with a 1:1 (spore:cell) ratio at 6 hrs. only [9]. However, in another study also using 16HBE14o- cells at 6 hrs. examining the effects of internalized conidia and direct contact with the spore and cell, and a 10:1 ratio, we found 35 shared genes at 2 hrs., 70 shared genes at 6 hrs., and 244 similar genes at 12 hrs. in the 889 differentially expressed genes reported [8].

2.3.3 qRT-PCR Analysis

To confirm the changes in expression level from our RNA-Seq analysis, we chose three genes to analyze their expression using qRT-PCR. The genes, SLC11A2 (DMT1), IL6, and IL8 (Table 2.9), were selected due to their relationship in known pathways and the significant change in

expression predicted by the transcriptional analysis. Figure 2.8 shows a significant up-regulation of the chemokine IL8 at 6 and 12 hrs. post incubation with *A. fumigatus* in airway epithelial cells. IL8 expression is highly up-regulated at 12 hrs., showing an avid immune response. Examination of IL6 expression, also revealed in increase but at a later time point an increase of statistical significance (Figure 2.9). The RNA-seq analysis suggested no significant change in DMT1 expression until 12 hrs. in airway epithelial cells interacting with *A. fumigatus*, and we confirmed this in Figure 2.10.

Gene Name	2 hrs. FC	6 hrs. FC	12 hrs. FC
SLC11A2 (DMT1)	N/A	N/A	1.27
IL-6	1.77	2.47	5.37
IL-8	3.154	1.373	10.729

Table 2.9: RNA-seq fold change values for qRT-PCR validation. The genes names are listed along with the FC values, if available ($p<0.05$), otherwise not applicable (N/A).

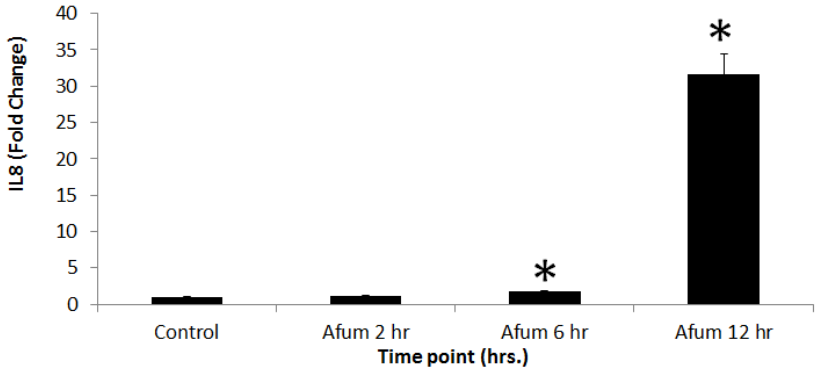

Figure 2.8: IL8 Expression in *A. fumigatus* treated airway epithelial cells. qRT-PCR results for 2, 6, and 12 hrs. post-infection.

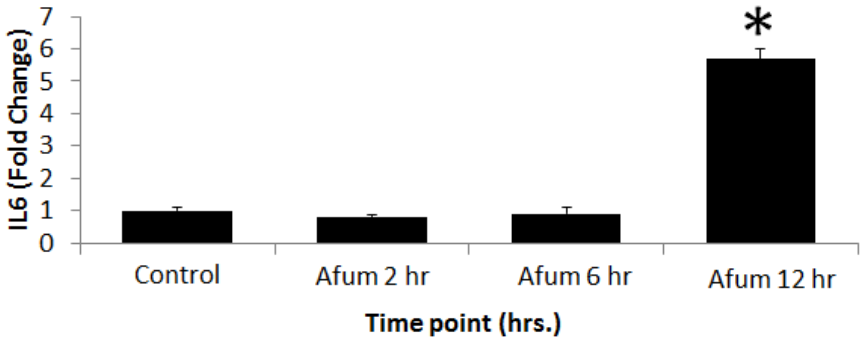

Figure 2.9: IL6 expression in *A. fumigatus* treated airway epithelial cells. qRT-PCR results for 2, 6, and 12 hrs. post-infection.

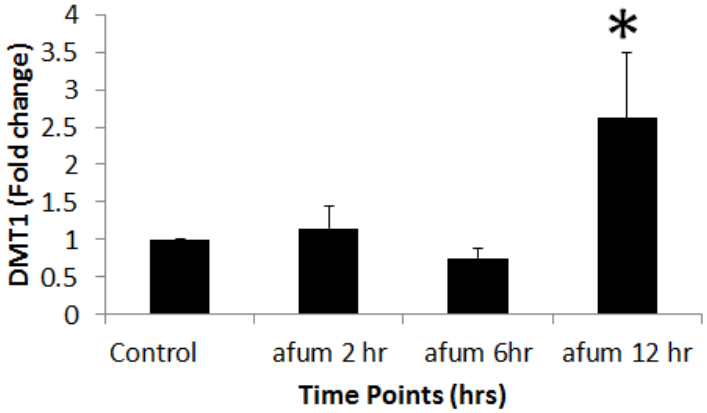

Figure 2.10: DMT1 expression in *A. fumigatus* treated airway epithelial cells. qRT-PCR results for 2, 6, and 12 hrs. post-infection.

2.4 Discussion

This is the first study to use high throughput sequencing to profile the transcriptional response of NHBE cells interacting with *A. fumigatus* conidia at three time points. We investigated the dynamics at each time point in the following categories: immune response, oxidative stress and hypoxia, and iron metabolism. We compared our results with previous studies and found similarities, although experimental conditions varied [8, 9]. We chose a minimal spore to cell ratio since we wanted to give each cell a 50% probability of interacting with conidia, versus overburdening the cells with a large number of spores for prolonged time points. While numerous studies examine the host-fungal interaction in other professional cell types, recent reviews emphasize the relevance of airway epithelial cells at the early stage of infection [24, 29].

The response of the airway epithelial cell is modified depending on the duration of the contact of the cell with conidia. Since we did not separate epithelial cells based on conidia internalization versus direct contact, our results indicate an average of the effects of the response of NHBE cells. One of the benefits of this study is that we can observe three stages of infection: resting conidia, germinated conidia, and hyphal growth, and characterize the epithelium response. To classify the biological response we used IPA, GOrilla, and Pantherdb to identify pathways, functional categories, and GO terms for the differentially expressed genes at each time point. This study is useful since it also proposes new genes and the roles they play in *Aspergillus* infection, many of which are not characterized functionally or in nomenclature. This data provides the building blocks to create a comprehensive pathway describing the response of airway epithelial cells to *A. fumigatus*.

Many of the individual genes related to the immune response had the highest fold changes. Little is known about the role of interleukin 36 gamma (IL36G) but it has agonist properties on NF-κB pathways and is associated with several diseases including neutrophillic airway inflammation [30]. IL6 and IL8 are well studied in *A. fumigatus* infection and shown by Borger et al. [31] to increase as early as 6 hrs., hence the observation of this transcriptional change confirms active fungal presence. Ovo-like zinc finger 1 (OVOL1), a transcription factor associated with cell plasticity influencing the mesenchymal to epithelial transition, but not yet linked to fungal infection [32]. However, this up-regulation of OVOL1 may be representative of a cancer-like induced trait. Activating transcription factor 3 (ATF3) is a survival mechanism induced by the cell to respond to cellular stress [33].

Although, the iron related genes did not have very high fold change values, our data showed interesting findings. Lipocalin 2 (LCN2), discovered to play an immunomodulatory role in neutrophil iron acquisition, but during inflammation, LCN2 is up-regulated in the lung epithelium [34-36]. The ability of frataxin (FXN) to promote iron-sulfur complexes is its primary function, but its role in the airway epithelium is unexplored [37]. Transferrin receptor (TFRC) during *A. fumigatus* infection was shown to have an observable decrease in expression in alveolar macrophages, and perhaps bronchial epithelial cells exhibit a similar or deviating response during prolonged infection [38]. However, six membrane epithelial antigen of prostate 4 (STEAP4), associated with inflammation, has only been shown to be up-regulated in macrophages in response to lipopolysaccharide (LPS) or Zymosan, or in obese subjects [39, 40]. In the context of the host-fungal interaction, these genes provide insight on the changes in the expression of iron related genes and should be further explored.

For the overall highest fold change in differentially expressed genes, colony stimulating factor 2 (CSF2), tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), and triggering receptor expressed on myeloid cells 1 (TREM1), are increased in late stages of infection. CSF2 is responsible for progenitor cell proliferation and lung functioning [41]. TNFAIP3, on the other hand, has a transcriptional association with NF-κB and is responsive to cytokines and stimulators, such as LPS [39, 42]. An *in vivo* study examining the effects of *A. fumigatus* on chronic asthmatic mice concluded that TREM1 levels increased in the whole lung and in the bronchoalveolar lavage (BAL) fluid, and may be a receptor in the immune response against fungi [43].

To validate changes observed in the RNA-seq analysis, we employed qRT-PCR to evaluate the expression of IL6, IL8, and DMT1. The qRT-PCR results confirm DMT1, IL6, and IL8 expression increases during co-incubation with *A. fumigatus*. Although IL6 and IL8 have previously been shown to be up-regulated in the presence of fungal conidia [9, 44], this study is the first to report the effect of *A. fumigatus* on the iron importer, DMT1 in airway epithelial cells or any airway cell type. DMT1 imports numerous metals including manganese, iron, and copper [45]. It is a hypoxia related gene and the fungus stimulates hypoxic conditions in the host which may downstream influence DMT1 regulation preventing ferrous iron uptake [46, 47]. The role of DMT1 in *A. fumigatus* infection and the potential hypoxic conditions induced by the fungus potentially has adverse effects.

Our analysis revealed NHBE cells are highly responsive to the presence of *A. fumigatus* and examination of newly discovered transcription factors and genes may allude to information about how epithelial cells recognize pathogens. The high number of differentially expressed genes, confirm the relevance of the airway epithelium in fungal infection. We showed changes in immune response genes, hypoxia and oxidative stress related genes, and transcription factors, many of which are unknown in our host-fungal interaction. This study, most importantly, generated a snapshot of the airway epithelial cells response to *A. fumigatus* at differential conidial stages, showing progression of the host response to the pathogen.

2.5 Literature citations

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Appendix A: Supplemental figures and tables

A.1 Figures

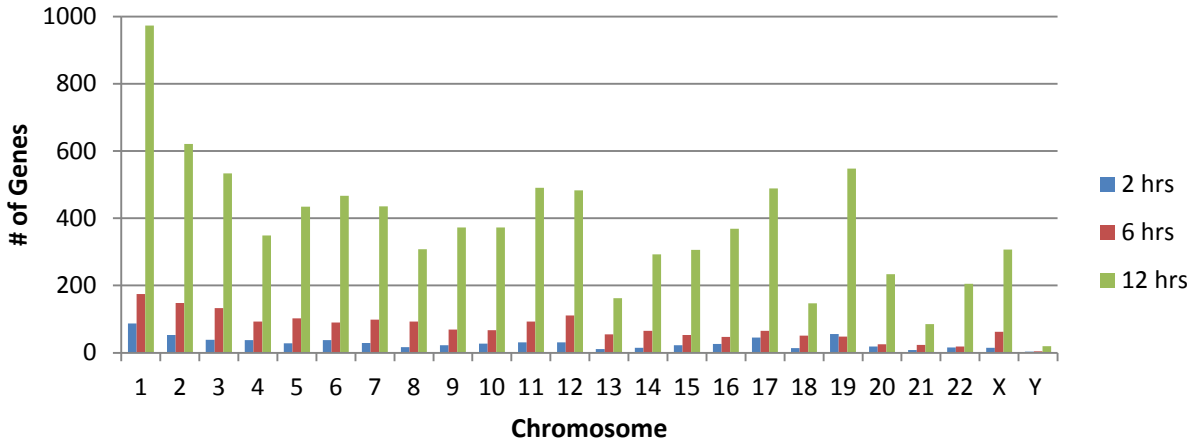

Figure A.1: Chromosome distribution for differentially expressed genes. A plot of the differentially expressed genes at 2, 6, and 12 hrs. after *A. fumigatus* infection.

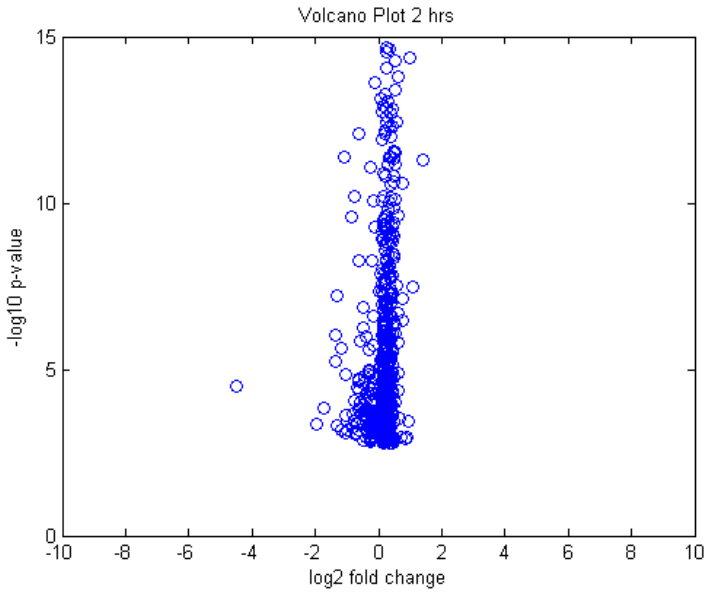

Figure A.2: Volcano plot for 2 hours. A plot for differentially expressed genes at 2 hrs. after *A. fumigatus* infection of log2foldchange vs. –log10p-value.

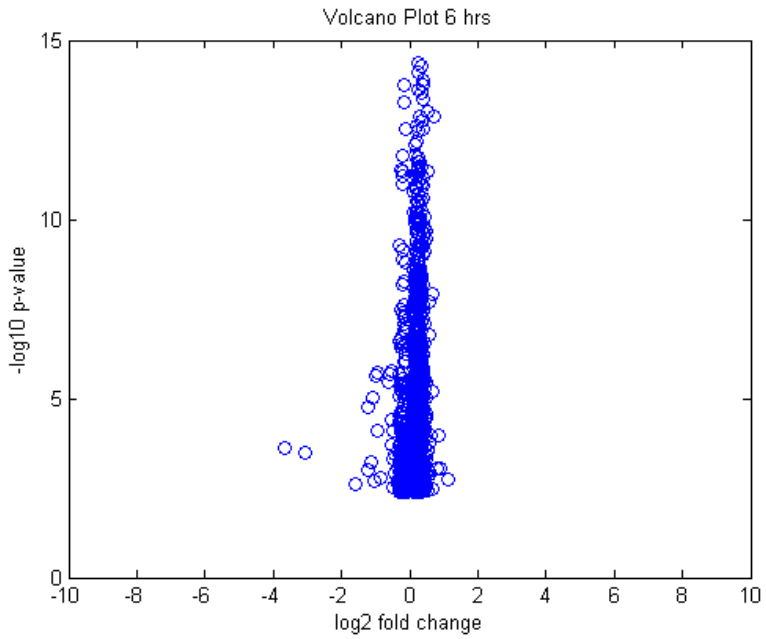

Figure A.3: Volcano plot for 6 hours. A plot for differentially expressed genes at 6 hrs. after *A. fumigatus* infection of log2foldchange vs. –log10p-value.

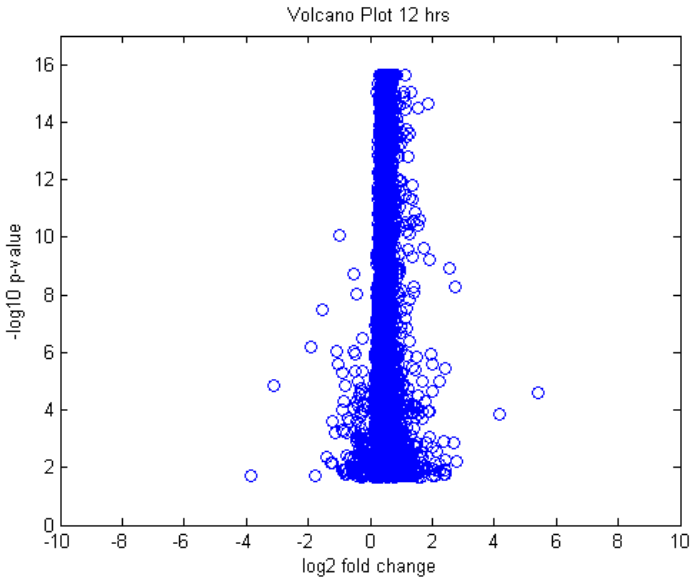

Figure A.4: Volcano plot for 12 hours. A plot for differentially expressed genes at 12 hrs. after *A. fumigatus* infection of log2foldchange vs. –log10p-value.

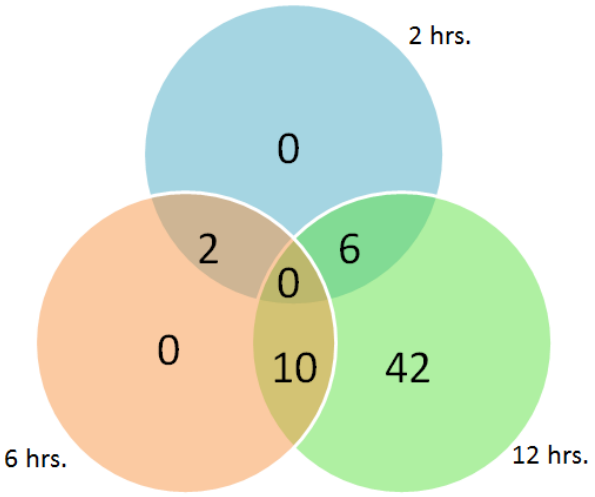

Figure A.5: Venn diagram of shared genes at each time point referencing Oosthuizen et al., 2011.

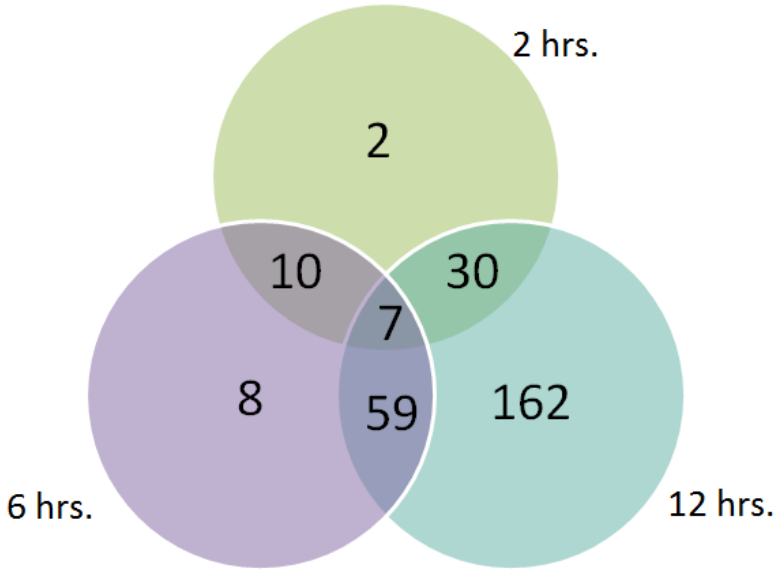

Figure A.6: Venn diagram of shared genes at each time point referencing Gomez et al., 2010.

A.2. Tables

Response to Stress:

Response to Hypoxia: ANGPTL4, ARNT2, CREBBP, EP300, HIF1A, MT3, PRKAA1.

Response to Oxidative Stress: CAT, CYGB, GPX1, IPCEF1.

Immune Response: GPI, IL1A, IL6, IL6ST, NOS2, NOTCH1, PTX3, RARA.

Other Genes Related to Stress Response: ADM, EPO, HYOU1, VEGFA.

Hemoglobin Complex Associated Genes: CYGB, EPO, HBB, HMOX1, NOS2, IPCEF1.

Oxidoreductase:

Peroxidase: CAT, CYGB, GPX1, IPCEF1.

Other Oxidoreductase-Related Genes: HIF1AN, HMOX1, MT3, NOS2, PLOD3, TH.

Transcription Factors and Regulators:

Transcription Cofactors: CREBBP, DR1, ENO1, EP300, EPAS1, KAT5, RARA.

Transcription Factors: ARNT2, BHLHE40, CREBBP, ENO1, EP300, EPAS1, HIF1A, HIF3A, KHSRP, MYBL2, PPARA, RARA.

Other Transcription Factors and Regulators: HIF1AN, NOTCH1.

Apoptosis:

Anti-apoptosis: BAX, ANGPTL4, BIRC5, IL1A, MYBL2, PEA15, PRKAA1, VEGFA.

Caspase Activity: BIRC5, CASP1.

Induction of Apoptosis: BAX, DAPK3, NUDT2.

Other Apoptosis Genes: EP300.

Signal Transduction: ADM, ARNT2, CASP1, CDC42, CREBBP, EP300, EPAS1, EPO, GNA11, HIF1A, HIF3A, HMOX1, IGFBP1, IL1A, IL6, IL6ST, IQGAP1, KIT, LEP, PLAU, RARA, VEGFA.

Protein Metabolism:

Protein Biosynthesis: EEF1A1, PDIA2 (PDIP), PRKAA1, RPL28, RPL32, RPS2, RPS7.

Protein Heterodimerization: ARNT2, HIF1A, RARA, SAE1.

Protein Homodimerization: ARNT2, RARA, VEGFA.

Protein Amino Acid Phosphorylation: DAPK3, KIT, PRKAA1.

Protein Binding: CASP1, CREBBP, ENO1, EP300, IQGAP1, NOS2, PEA15, PPP2CB, RARA.

Other Genes Related to Protein Metabolism: NAA10, CDC42, GNA11, HYOU1, MAN2B1, PLOD3, PSMB3, SUMO2, TUBA4A (TUBA1)

Extracellular Matrix (ECM)-Related Molecules:

Protease Inhibitors: BIRC5, CSTB.

Protease Molecules: AGTPBP1, CASP1, ECE1, PLAU, PSMB3.

Other Extracellular Molecules: ADM, ANGPTL4, CHGA, COL1A1, EPO, IGF2, IGFBP1, IL1A, IL6, LEP, NPY, PTX3, VEGFA.

Cytoskeleton: DCTN2, SPTBN1.

Cell Growth:

Cell Cycle: BAX, BIRC5, EP300, HK2, IGF2, IL1A, MYBL2, SSSCA1, VEGFA.

Cell Proliferation: DCTN2, IGF2, IL1A, IL6, MT3, NPY, RARA, VEGFA.

Growth Factors: GPI, IGF2, IGFBP1, IL1A, IL6, KIT, VEGFA.

Other Genes Related to Cell Growth: ENO1.

Metabolism:

Carbohydrate Metabolism: GPI, HK2, LCT, MAN2B1, PEA15, PRKAA1, SLC2A1, SLC2A4.

Lipid Metabolism: AGPAT2, ANGPTL4, PPARA, PRKAA1.

One-carbon Compound Metabolism: CA1.

Superoxide Metabolism: MT3, NOS2.

RNA Metabolism: PRPF40A (FNBP3), KHSRP, RARA, RPL28, RPS2, SNRNP70.

Other Genes Related to Metabolism: ADM, AGPAT2, MOCS3, NUDT2, TH, TST, UCP2.

Cardiac Excitation-Contraction (E-C) Coupling: ARNT2, CHGA, DAPK3, GNA11, IQGAP1, KIT, NOS2, NOTCH1, NPY, PRKAA1, SPTBN1

Table A.1: Hypoxia and oxidative stress related genes. The list of genes used to characterize the RNA-seq data.

Fungal Pattern Recognition Receptors (PRRs):
Beta-Glycan Responsive: CD36, CD5, CLEC7A (Dectin-1), ITGAM, ITGB2, SCARF1, TLR2.
Mannose/Chitin Responsive: CD207, CD209, CHIA, CLEC6A (Dectin-2), MRC1, TLR2, TLR4.
Other: COLEC12, NLRP3, NPTX1, PTX3, TLR9.

Signal Transduction:
Dectin-1 Signaling Pathway: BCL10, CARD9, CLEC7A (Dectin-1), MALT1, PLCG2, RAF1, SYK.
NFkB Signaling Pathway: BCL10, CARD9, CASP1 (ICE), CASP8 (FLICE), CD40 (TNFRSF5), IKBKB, IL10, IL1B, IRAK1, IRAK4, MALT1, MAP3K7 (TAK1), NFKB1, NFKBIA (IkBa/MAD3), STAT1, TNF.
Toll-like receptor (TLR) Signaling Pathway: CASP8 (FLICE), CD14, FOS, IRAK1, IRAK4, ITGB2, JUN, MAP2K4 (JNKK1), MAP3K7 (TAK1), MAPK14 (p38 MAPK), MAPK8 (JNK1), MYD88, NFKB1, TIRAP, TLR2, TLR4, TLR9, TRAF6.
Complement Signaling Pathway: C3, C5AR1 (GPR77), ITGAM, ITGB2, LYN, MBL2, SYK.
Nod-like Receptor (NLR) Signaling Pathway: CARD9, CASP1 (ICE), NLRP3, PYCARD (TMS1/ASC).

Inflammation: C3, CCL2 (MCP-1), CCL20 (MIP-3A), CCL5 (RANTES), CCR1, CD14, CD40 (TNFRSF5), CLEC7A (Dectin-1), CXCL1, CXCL10 (INP10), CXCL11 (I-TAC/IP-9), CXCL2, CXCL9 (MIG), F3, FOS, IL10, IL1A, IL1B, IL2, IL23A, IL6, IL8, ITGB2, LYN, MBL2, MYD88, NFKB1, PTGS2 (COX-2), TIRAP, TLR2, TLR4, TLR9, TNF.
Phagocytosis: C3, CD14, CD36, CLEC7A (Dectin-1), COLEC12, FCGR1A, FCN1, MBL2, SFTPD.
Genes Responsive to Specific Pathogenic Fungi: *Aspergillus fumigatus*: C3, CASP1 (ICE), CASP8 (FLICE), CCL2 (MCP-1), CCL20 (MIP-3A), CCL5 (RANTES), CCR1, CD14, CD40 (TNFRSF5), CD83, CLEC6A (Dectin-2), CLEC7A (Dectin-1), CSF2 (GM-CSF), CSF3 (GCSF), CXCL1, CXCL10 (INP10), CXCL11 (I-TAC/IP-9), CXCL2, CXCL9 (MIG), F2RL1, F3, FCGR1A, FCGR2A, FCN1, IFNG, IL10, IL12A, IL12B, IL18, IL1A, IL1B, IL1R1, IL6, IL8, JUN, MALT1, MAPK14 (p38 MAPK), MBL2, MYD88, NFKBIA (IkBa/MAD3), PTGS2 (COX-2), PTPN6, PTX3, SFTPD, SOCS3, ST3GAL5, STAT1, SYK, TLR2, TLR4, TLR9, TNF.

Table A.2: Fungal recognition related genes. The list of genes used to characterize the RNA-seq data.

Gene name
A2ML1
CDC42EP1
DNAJC4
DOT1L
INHBA
PHLDB2
PTPRE
SOD2

Table A.3: List of genes similar at 2hrs. to Oosthuizen et al., 2011.

Gene name
ARMC8
BCOR
DNAJC4
IRS2
LNX2
MTAP
NPEPPS
PDLIM5
PHLDB2
RAB23
RRM2
TRAF6

Table A.4: List of genes similar at 6hrs. to Ooshttuizen et al., 2011.

Gene name
A2ML1
ARMC8
ARRDC2
ARRDC3
BCOR
C11ORF61
CARD9
CDC42EP1
CMTM7
CXCL3
DIDO1
DKFZP434I0714
DOT1L
EFNA4
ETS1
FAM015B
GPR137B
HCLS1
INHBA
IRS2
IRX5
KIAA0020
KLF11
LNX2
LYPD3
MMP17
MORC2
MTAP
NF1
NKX1-2
NPEPPS
PDLIM5
PIP5K1C
PPAT
PTPRE
RAB23
RNPEPL1
RRM2
SF3A2
SLC30A1
SOD2
TAF4
TBRG1
TGM1

TGM2
THAP6
TRAF6
TSPAN14
USP43
WDR5B
WDR89
WHSC1
ZB1B5
ZNF140
ZNF426
ZNF57
ZNF595
ZNF627

Table A.5: List of genes similar at 12 hrs. to Ooshttuizen et al., 2011.

ATXN2L
CAV1
COL16A1
CXCL2
DOT1L
ECM1
GOLGA2
HMGCS1
IMP3
ITPKC
KIAA1199
KLF6
MAP4K4
MTA1
MTHFD2
NOLC1
NPAS2
PARD3
PER1
PIP5K1A

PTHLH
PYCR1
RNF41
SERPINB8
SNAI2
SOX4
STARD5
STRN4
SYNE1
TNFAIP3
TRIB3
UAP1
UPP1
VEGFC
ZNF652

Table A.6: List of genes similar at 2 hrs. to Gomez et al., 2010.

ANP32E
ARHGAP18
ATAD2
BACH1
BUB1
C1GALT1
CALD1
CCDC14
CENPE
CHMP2B
CKAP2
COL16A1
COL18A1
DDHD2
DHFR
DNAJC19

DUSP10
FKBP8
FLII
GLS
GPR126
GPR56
HIST1H2BD
HIST2H2BE
HMGCS1
HSPA6
IL4I1
ITM2B
KLF6
KNTC1
KRAS
KRT8
KYNU
L3MBTL3
LARP4
LOX
LRP8
LSM1
LSM3
LZTFL1
MEN1
MGST2
MRPL42
MTERF
NOL8
OPA1
PPM1B
PTHLH
PTPN2

PUM2
PYCR1
RNGTT
SAMD9
SELT
SGOL2
SOX4
SPAG5
STX17
TAF13
THUMPD1
TNFAIP3
TOR3A
TRIB3
TTK
UAP1
UPP1
WDFY3
ZDHHC2
ZMYM1
ZNF429

Table A.7: List of genes similar at 6 hrs. to Gomez et al., 2010.

ABLIM3
ADARB1
ANAPC1
ANKRD10
ANP32E
AOX1
ARHGAP11A
ARHGAP18
ARHGEF10L
ARMCX6

ATAD2
ATXN2L
BACH1
BAX
BLMH
BRMS1L
BUB1
CA5B
CALD1
CAMKK2
CAMTA1
CASP4
CCDC14
CDCA5
CENPE
CERK
CHMP2B
CHST11
CHUK
CKAP2
CNOT1
CPSF1
CTPS
CTSC
CUL4A
CXCL2
CXCL3
DCBLD2
DDHD2
DHFR
DHX30
DNAJC19
DOT1L

DTNBP1
DUSP10
DUSP4
ECM1
EHD4
EHMT1
ELL3
ELMO2
ENG
ETV6
FGF5
FKBP5
FKBP8
FLI1
FLII
FTH1
FTL
FUT6
GLS
GOLGA2
GPR126
GPSM2
GTF2H5
H2AFY2
HDAC4
HES1
HIST1H2BC
HIST1H2BD
HIST2H2BE
HMGB1
HMGCL
HMGCR
HMGCS1

HMOX2
HRASLS2
IDI1
IFT20
IGFBP6
IL4I1
IL6
IMP3
INPP5F
IQSEC2
ITM2B
ITPKC
KCNG1
KIAA0020
KIAA1199
KIAA1279
KIAA1704
KIF23
KLF6
KLHL12
KNTC1
KRAS
KRT18
KRT8
KYNU
L3MBTL3
LARP4
LIPE
LOX
LOXL2
LPXN
LRIG1
LRP8

LRRFIP1
LSM1
LSM3
LUC7L2
LYAR
LZTFL1
MAP4K4
MAPRE1
MCART1
MCM6
MFN1
MGST1
MGST2
MLLT11
MMAB
MMP1
MRFAP1
MRPL42
MRPS31
MT1E
MTA1
MTERF
MTERFD1
MTERFD3
MTHFD2
MUC1
MUS81
MUT
MYADM
NAV3
NCL
NDUFB11
NOL8

NOLC1
NPAS2
NUFIP1
NUP88
OASL
OLFML2A
OMA1
OPA1
ORMDL2
PARD3
PDCD2
PDCD5
PERP
PHF20
PIM2
PIP5K1A
PKP2
PLEK2
POPDC3
PPM1B
PRDX2
PTPN2
PUM2
PUS1
PYCR1
RAB13
RAD50
RANGAP1
RFWD2
RHOV
RIOK1
RNF41
RNF8

RNGTT
RPL21
RSL1D1
RUFY1
S100A16
SACM1L
SAMD9
SEC61G
SELT
SENP6
SERPINB8
SERPINE2
SGOL1
SGOL2
SH3D19
SKP2
SLC35C1
SLU7
SMARCC2
SNAI2
SNRPA1
SOX4
SRF
SSR3
ST3GAL5
STARD5
STEAP1
STRN4
STX17
SYDE1
SYNE1
SYTL2
TAF13

TAF4
TAF5L
TEAD3
TEAD4
TFCP2
THADA
THOC1
THUMPD1
TNFAIP3
TP73
TPM1
TSPAN14
TTK
UAP1
ULBP2
UPF3B
UPP1
USP4
VDR
VEGFC
WASF2
WDFY3
WDR1
WDR75
WWTR1
YWHAG
ZC3HAV1
ZDHHC2
ZMYM1
ZNF142
ZNF404
ZNF503
ZNF542

ZNF652
ZNRF1
ZRANB3

Table A.8: List of genes similar at 12 hrs. to Gomez et al., 2010.

Chapter 3:
The Effects of TNF-α, IL-1β, and IL-8 on Iron Import in Airway Epithelial Cells

Shernita Lee[1], Sang-Wook Park[1], Reinhard Laubenbacher[2], Christopher Lawrence[1]

[1] Virginia Bioinformatics Institute, Blacksburg, VA, USA

[2] University of Connecticut Health Center, Farmington, CT, USA

Abstract:

Cytokines are essential in cell to cell communication and determine avid cell recruitment, inflammation mediation, and other immunological functions. The effects of cytokines on iron import may represent protective mechanisms to control iron levels, preventing toxicity and production of reactive oxygen species. The cytokines interleukin 8 (IL-8), interleukin 1beta (IL-1β), and tumor necrosis factor alpha (TNF-α) change iron importer genes in SV-40 transformed bronchial airway epithelial cells, BEAS2B. We assessed the individual, dual, and triple cytokine combined effects on transferrin receptor 1 (TfR1) and divalent metal ion transporter 1 (DMT1) to expand current knowledge about the influence of cytokines on iron related genes. We enhanced the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor pathway, an inducer of cytokines, and examined the effects on iron importers. We show that the expression of TfR1 has little to no change in response to the presence of cytokines, while DMT1 shows increased expression in response to cytokines.

3.1 Background

The element iron is needed by organisms for biological functions including cellular maintenance, proliferation, conservation of oxygen levels, and detoxification [1, 2]. Ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron, two common oxidation states, are tightly regulated in order to prevent iron toxicity and production of oxidative stress related molecules [3]. Preservation of iron regulation is controlled by importers, exporters, and storage molecules, although many mechanisms are still poorly understood [4]. Systemic iron influences local iron levels, and iron homeostasis prevents cellular damage [5].

Free iron concentrations within the cell are controlled by two iron importers and the orchestration of iron export, storage, and intracellular iron availability. Natural resistance associated macrophage 2 (NRAMP2) or divalent metal ion transporter 1 (DMT1), is an importer of unbound ferrous iron which mediates metal absorption of minerals such as copper, zinc, nickel, and manganese [6]. Due to alternative splicing, there are two isoforms of DMT1 which differ in the 3´- untranslated region (UTR): - iron responsive element (IRE) and +IRE. Examination of the –IRE to +IRE ratio in the lung, Wang et al. [7] concluded with the addition of iron, only the –IRE isoform of DMT1 increased in expression while the +IRE isoform was non-responsive. Transferrin receptor 1 (TfR1), on the other hand, is an apical membrane protein responsible for the import of transferrin bound ferric iron. Although there is an additional form, transferrin receptor 2 (TfR2), only TfR1 is ubiquitous and post-transcriptionally regulated by the binding of iron regulatory proteins (IRPs) to the multiple IRE sites on the 3´ UTR [8, 9].

There are limited studies examining the roles of cytokines (pro- or anti-inflammatory) on airway epithelial cells, particularly the effects on iron related genes. Tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) significantly increased DMT1 expression and the expression of the iron storage protein, ferritin (Ft) [10]. TNF-α intensifies intracellular adhesion molecule 1 (ICAM-1) surface expression [11], reactive oxygen species [12], and IL-8 mRNA levels via mitogen-activated protein kinase (MAPK) pathways [13-15]. Both TNF-α and IL-1β induced human β-defensin-2 [16]. IL-1β induced IL-8 in a dose dependent manner [17]. Supplemental iron, in addition to the cytokines IL-1β or TNF-α, caused an increase in Ft expression and a decrease in TfR1 expression in A549 cells [18]. Cytokines influence multiple pathways in airway epithelial cells, depending on the inflammatory type of the cytokine and duration of the cytokine added. The goal of this study is to comprehensively examine the individual and combined effects of cytokines on the transcriptional expression of iron importers. This work elucidates the coordination of cytokines in the regulation of iron importers and whether particular cytokines have an increased or reduced response in combination with other cytokines.

Cytokine profiles indicate inflammatory regions, often characteristic for diseases, and indicate a lack of proper lung functioning. For example, in persons with bronchiectasis, chronic bronchitis, and cystic fibrosis, the concentration of IL-8 in the sputum was higher in comparison to healthy volunteers [19, 20]. Although TNF-α is generally associated with alveolar macrophages, an increase in TNF-α expression is a marker for host defenses against pathogens, for example in invasive pulmonary aspergillosis [21, 22]. TNF-α is also considered a potential target for rheumatoid arthritis due to its pro-inflammatory properties [23]. In asthmatics with fungal infections, TNF-α secretion is increased [24]. Although increased cytokine production is

beneficial at moderate levels and short periods of time, their influence on iron importers may also contribute to additional cellular damage. We aim to monitor the airway epithelium response to pro- and anti-inflammatory cytokines and determine the ability of the host to change the expression of iron importers.

3.2 Materials and methods

3.2.1 Sample preparation

SV-40 transformed human bronchial epithelial cells (BEAS2B) were cultured in 75 cm^2 tissue culture flasks at 37°C and 5% CO_2 until reaching 80% confluence. Cells were purchased from ATCC (Manassas,VA). The BEAS2B cells were grown 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.

Cells were sub-cultured into six-well tissue culture dishes at a concentration of 0.5 x 10^6 cells per well and allowed to adhere overnight. The cells were washed twice with DPBS and cultured in a final volume of 1.5 mL RPMI media without addition of the provided gentamicin/*amphotericin-B* component to avoid potential interference from these media supplements. Samples were prepared for each treatment by addition of 50 ng of TNF-α, IL-8, or IL-1β (GenScript, Piscataway Township, NJ). Cells were incubated at 37°C and 5% CO_2. Samples were collected at 0, 2, 4, and 6 hours following initial stimulation. Culture supernatant was collected and debris was removed by centrifugation before storing at -80°C. The cells were washed twice with DPBS before RNA extraction.

3.2.2 RNA extraction

Total RNA was collected by the addition of 1mL TRIzol (Invitrogen, Carlsbad, CA) directly to the cell culture plate and gentle agitation using a cell lifter. RNA extraction was carried out per manufacturer's instructions using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) with on-column DNase digestion to remove residual genomic DNA. RNA was eluted in 30µl of RNase-free water and integrity (A260/A280) and concentration were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were then stored at -80°C until further analysis.

3.2.3 Reverse transcriptase-polymerase chain reaction

RNA was reverse transcribed into cDNA using the tetro cDNA synthesis kit (BioLine, Taunton, MA) per the manufacturer's instructions. Quantitative RT-PCR was performed using IQ SYBR green supermix (Biorad, Hercules, CA) on the BioRad iCycler thermal cycler. With Cycle 1: (1X), and Step 1 at 95.0°C for 03:00 minutes, Cycle 2: (40X) with Step1 at 95.0°C for 00:10 seconds and Step 2 at 55.0°C for 00:30 seconds, and Cycle 3: (81X) at 55.0°C-95.0°C for 00:30 seconds.

TfR1 and DMT1 mRNA levels were normalized to the housekeeping gene GAPDH. The following sequences were used, purchased from Integrated DNA Technologies (IDT):

DMT1: 5′-ATGGACTAGGTGGCGGATT-3′ and 5′ -GATAAGCCACGTGACCACA-3′; TfR1: 5′-CAGGAACCGATCTCCAGTGA-3′ and 5′-CTTGATGGTGCGGTGAAGT-3′; GAPDH: 5′-ACCCACTCCTCACCTTTGA-3′ and 5′-CTGTTGCTGTACCAAATTCGT-3′.

3.2.4 Statistical analysis

Statistical analysis incorporated the two-tailed Student's *t* test, and we considered values of $p < 0.05$ statistically significant.

3.3 Results

3.3.1 Effect of individual cytokines on iron importers

The addition of 50ng of TNF-α caused an increase in DMT1 expression, but a slight decrease in TfR1 expression, although statistically insignificant (Figure 3.1). The recombinant cytokine, IL-1β, on the other hand, led to no changes in TfR1 expression, yet an increase in DMT1 expression (Figure 3.2). Lastly, IL-8 triggered a decrease in TfR1 expression and a modest decrease in DMT1 expression before returning to its basal level (Figure 3.3). Treatment with solely IL-1β, TNF-α, or IL-8 significantly modified levels of DMT1 in the airway epithelial cells.

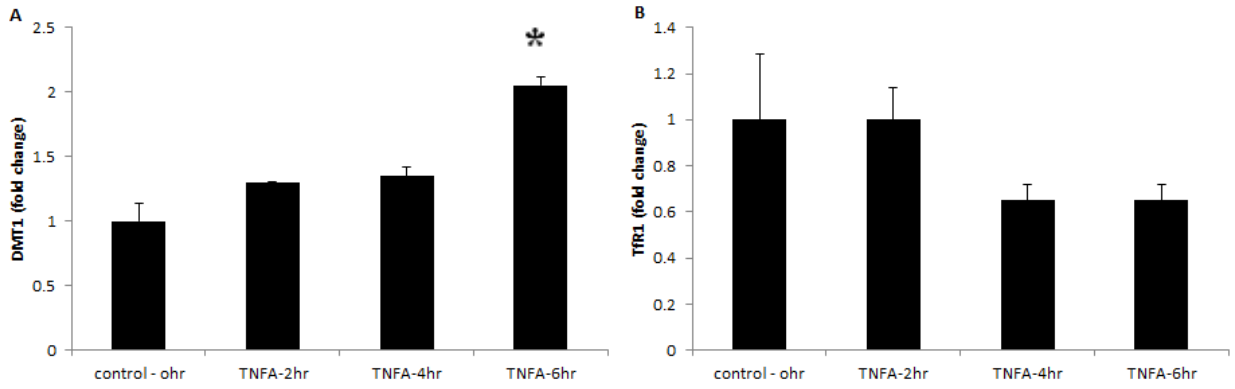

Figure 3.1: The effect of TNF-α on iron importers. A] The relative expression of DMT1 in control cells at 0 hrs. and TNF-α added at 2, 4, and 6 hrs. B] The relative expression of TfR1 at 2, 4, and 6 hrs. post TNF-α treatment.

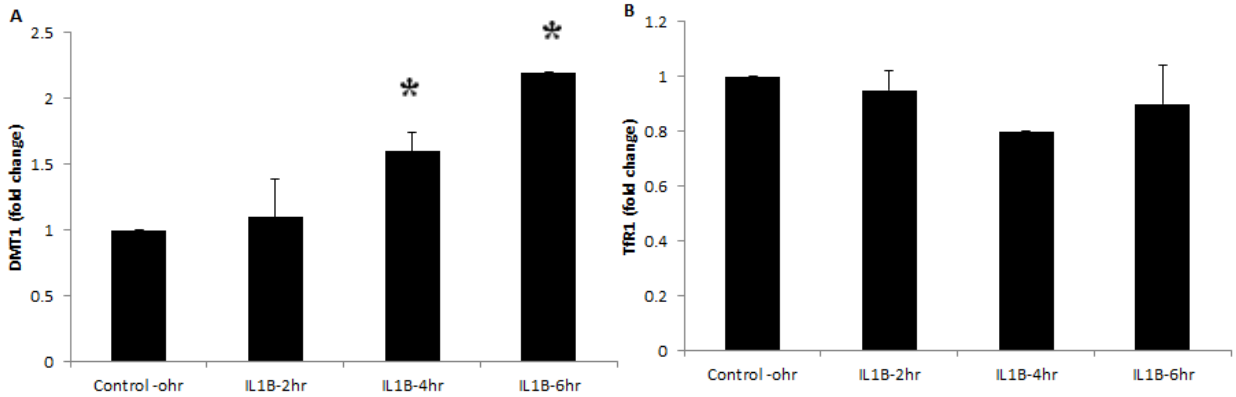

Figure 3.2: The effect of IL-1β on iron importers. A] The relative expression of DMT1 in control cells at 0 hrs. and IL-1β added at 2, 4, and 6 hrs. B] The relative expression of TfR1 at 2, 4, and 6 hrs. post IL-1β treatment.

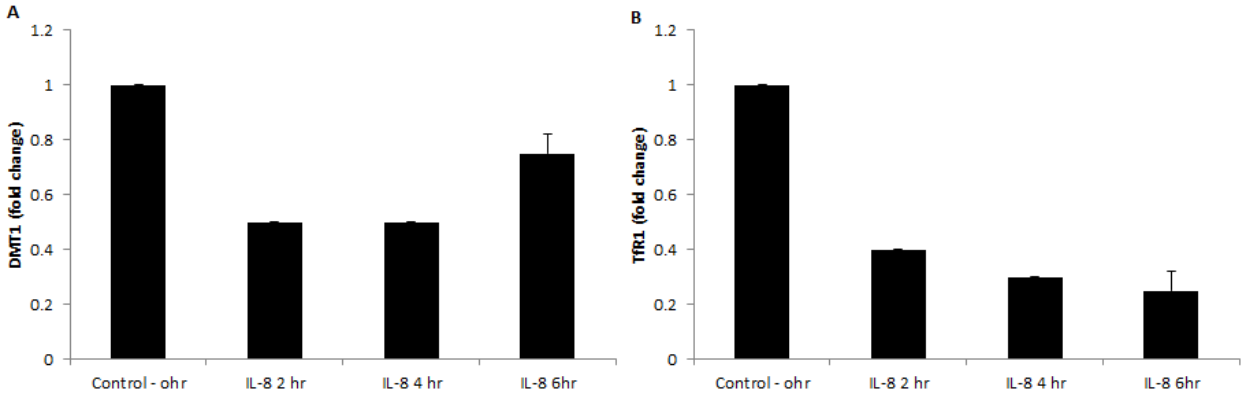

Figure 3.3: The effect of IL-8 on iron importers. A] The relative expression of DMT1 in control cells at 0 hrs. and IL-8 added at 2, 4, and 6 hrs. B] The relative expression of TfR1 at 2, 4, and 6 hrs. post IL-8 treatment.

3.3.2 Effect of dual cytokines on iron importers

Examination of the effects of two recombinant cytokines (TNF-α+IL-1β, IL-1β+IL-8, and IL-8+TNF-α) revealed the expression of TfR1 is insensitive to their presence (Figure 3.4). DMT1, on the other hand, increased for all combinations, but we observed an extremely high fold change in expression when IL-8 was involved. These observations suggest that cytokines quickly modify DMT1 expression, but do not have a substantial influence on TfR1 at early time points.

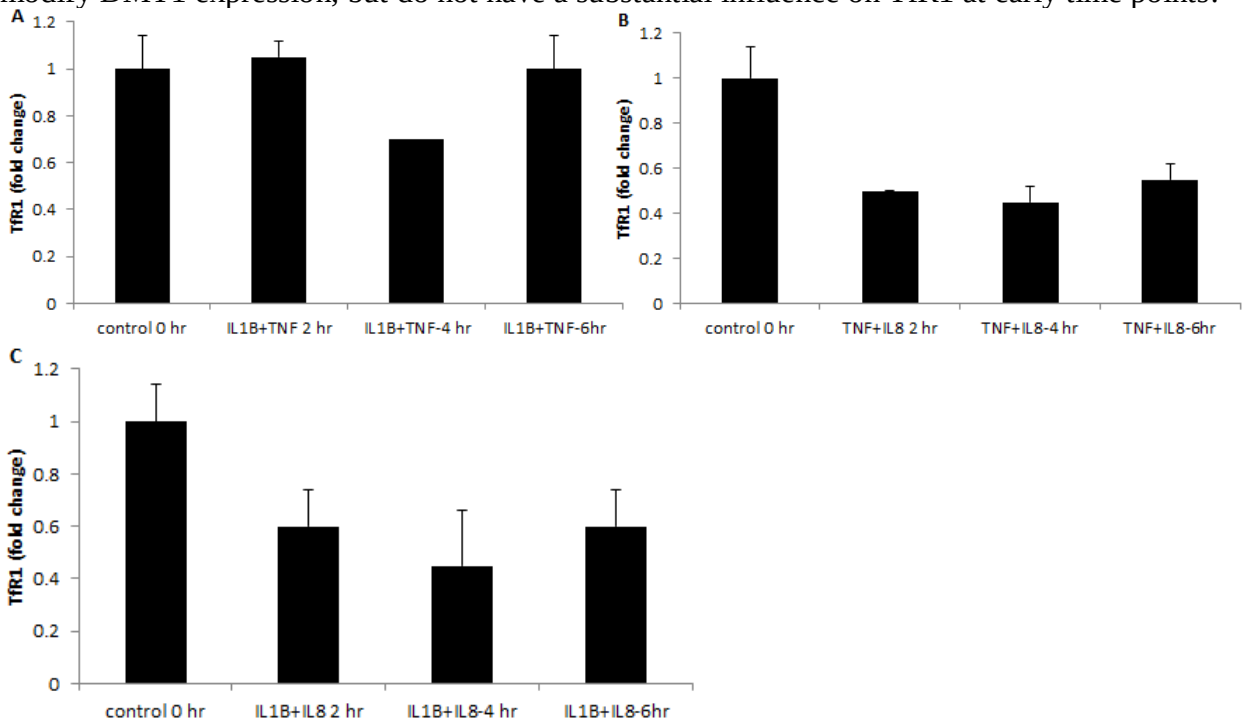

Figure 3.4: The effect of dual cytokines on TfR1. A] The relative expression of TfR1 in control cells at 0 hrs. and IL-1β and TNF-α added at 2, 4, and 6 hrs. B] The relative expression of TfR1 post-treatment with TNF-α and IL-8. C].The relative expression of TfR1 post-treatment with IL-1β and IL-8.

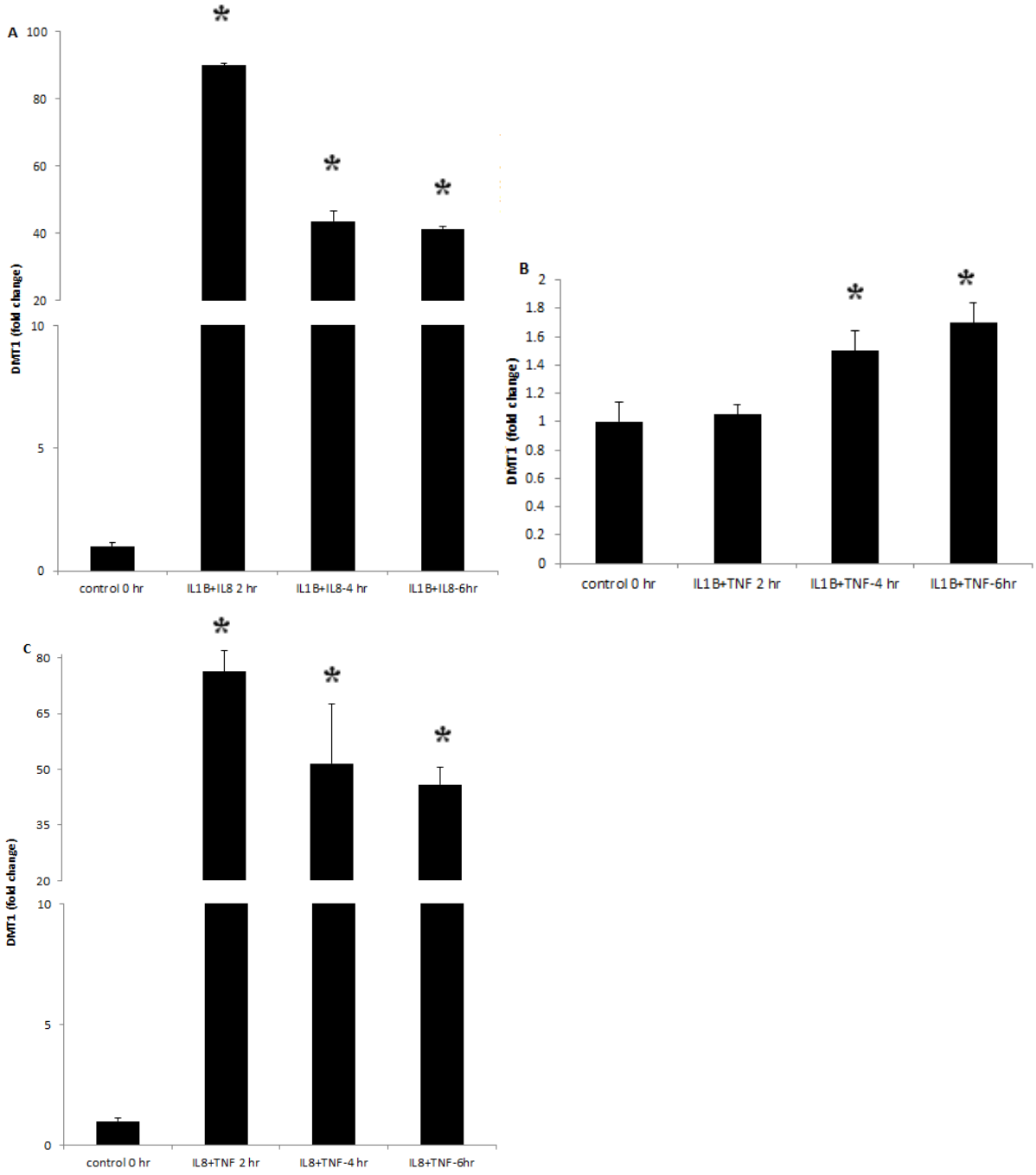

Figure 3.5: The effect of dual cytokines on DMT1. A] The relative expression of DMT1 in control cells at 0 hrs. and IL-1β and IL-8 added at 2, 4, and 6 hrs. B] The relative expression of DMT1 post-treatment with IL-1β and TNF-α. C] The relative expression of DMT1 post-treatment with TNF-α and IL-8.

3.3.3 Effect of triple cytokines on iron importers

Lastly, we investigated the changes to iron importers when all three cytokines, the cytomix of TNF-α, IL-8, and IL-1β, were added to airway epithelial cells. As anticipated, there is no change in TfR1 expression and an increase in DMT1 expression (Figure 3.6). Although DMT1

expression is not as high as the dual cytokines conditions, DMT1 maintains dependence on the presence of cytokines to influence its expression level.

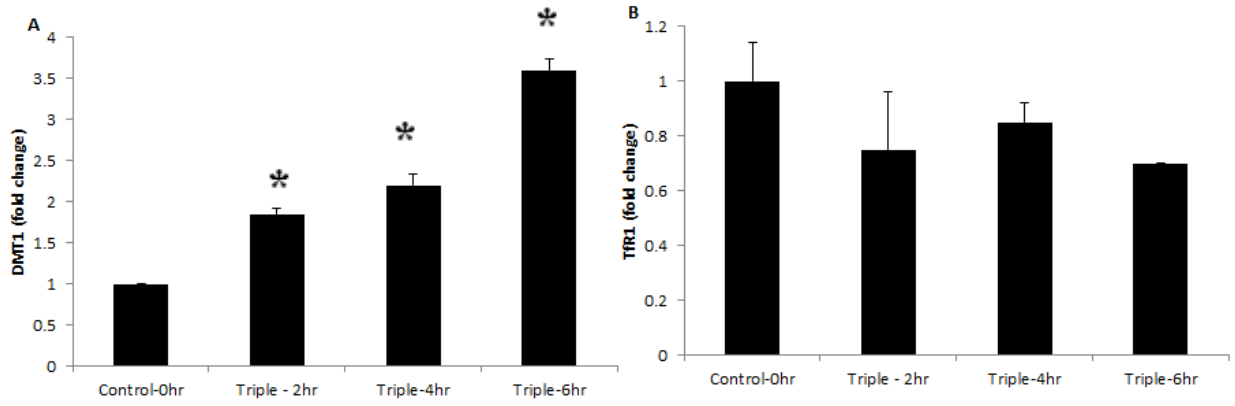

Figure 3.6: The effect of triple cytokines on iron importers. A] The relative expression of DMT1 in control cells at 0 hrs. and IL-1β, IL-8, and TNF-α added at 2, 4, and 6 hrs. B] The relative expression of TfR1 post treatment with IL-1β, IL-8, and TNF-α.

3.4 Discussion

In this study, we assessed the effects of TNF-α, IL-1β, and IL-8 on iron importer genes in BEAS2B cells. DMT1 expression was enhanced for all cytokine combinations, while TfR1 showed little change in response to cytokines. Our study is the first study to examine the influence of IL-8 on DMT1 and TfR1 in airway epithelial cells. It seems that for DMT1 expression, IL-8 plays a key role, while a modest effect on TfR1 is observed. The increase in DMT1 may be an oxidative protective mechanism by the cell to manage increased inflammation, however, this has to be further explored in the context of fungal infection [25, 26]. Presently, only two previous studies examine the effects of cytokines on iron importers in airway epithelial cells. Figure 3.7 compiles the results from this study and the observed changes in the literature, both validated and invalidated. Wang et al. [10] describes the effect of TNF-α on DMT1 results in an increase in DMT1 expression and our experimental results support this conclusion. Smirnov et al. [18], however, proposes TNF-α or IL-1β added to airway epithelial cells leads to an inhibition in TfR1 expression. Although we did not observe a comparable trend, this may be due to the use of an alveolar versus bronchial cell line and more extensive and refined time points contradict this behavior [27]. Another study in endothelial cells observed the expression of both DMT1 and TfR1 increased with TNF-α treatment, at a 1.9-2.6 fold change in DMT1 expression, consistent with our data for DMT1 [28].

TNF-α is a cytokine regulator and shown to increase NF-κB expression by inhibiting the expression of its suppressor, Ikappa B kinase (Ikβ) [29]. In our results, the single addition of TNF-α is similar to the response of IL-1β but not of IL-8. TNF-α is an inducer of IL-8 which may support the drastic increase in DMT1 expression, but even without TNF-α, IL-8 in dual combination is sufficient in its mechanistic functionality to cause such an increase in DMT1 expression. This creates a feedback mechanism (NF-κB->TNF-α-> NF-κB) on the cytokine TNF-α which is activated by NF-κB, but there may be alternative mechanisms to modify iron importers genes (Figure 3.8).

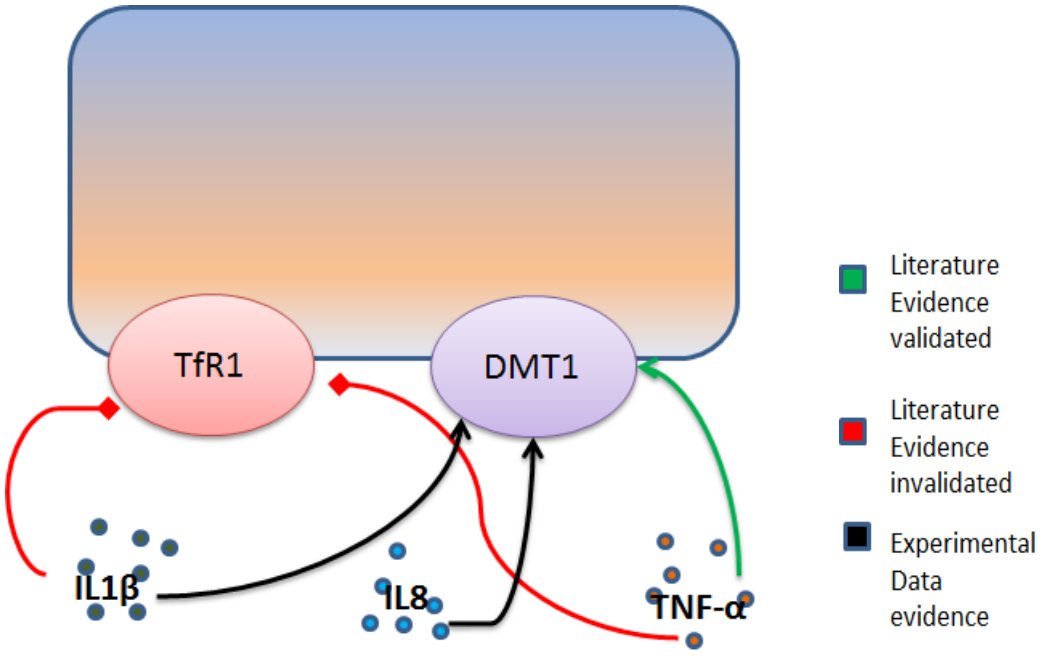

Figure 3.7: Proposed schematic of cytokine influence on iron importers. Activation (arrowhead) or inhibition (diamond) of TfR1 and DMT1 by cytokines: IL-1β, IL-8, and TNF-α. Red indicates our data disproves published results, green indicates validation of published results, and black represents new information from the experimental data.

To test whether the regulation of DMT1 is dependent of the NF-κB pathway and TfR1 is independent of the NF-κB pathway, we propose using anisomycin to inhibit NF-κB expression. In order to activate NF-κB from upstream, we will use *A. fumigatus* to activate TLR2 and TLR4 and observe the changes to both cytokine levels and the expression of DMT1 and TfR1. We demonstrated the importance of cytokines on DMT1 expression and with further analysis we aim to show a contrasting influence on the expression of TfR1, independent of the NF-κB pathway.

The innate immune response to *A. fumigatus* is profiled based on the production of cytokines including IL-8, TNF-α, and IL-6 [30-32]. Although, there is no literature evidence describing the effect of *A. fumigatus* on the expression of IL-1β, or furthermore the effect of IL-1β on TfR1 or DMT1, this preliminary data suggests IL-1β and DMT1 are important for both innate immunity of the host to fungi and emphasizes the sophisticated regulation of TfR1 in various experimental conditions. This study hints at the hypothesis that the immune response, activated by a fungal stimulus, induces the observed changes in iron regulation. Inhibition of the NF-κB pathway in future experiments will confirm if it is indeed NF-κB dependent cytokines that modify the expression of iron importer genes during fungal infection.

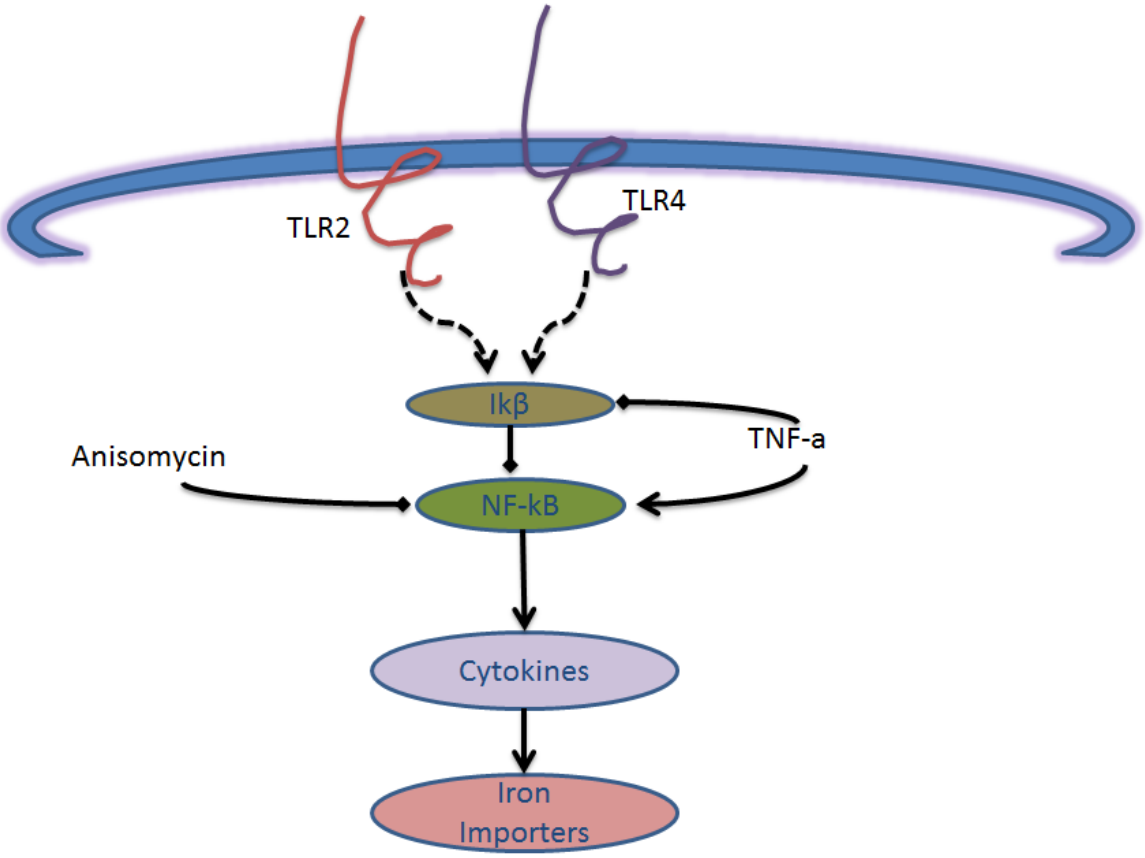

Figure 3.8: Summary of NF-κB pathway influence on iron importers. Activation of toll-like receptors (TLR2/TLR4) signals IκB which inhibits NF-κB. NF-κB regulates the expression of cytokines which downstream influences iron importers.

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Chapter 4: A Mathematical Model of the Effects of *Aspergillus fumigatus* on Iron Import in Airway Epithelial Cells

Shernita Lee[1], Tyler George[2], John Gowins[3], Jake Weissman[4], Brad Howard[1], Christopher Lawrence[1], Reinhard Laubenbacher[5]

[1] Virginia Bioinformatics Institute, Blacksburg, VA, USA

[2] Ferris State University, Big Rapids, MI, USA

[3]Western State Colorado University, Gunnison, CO, USA

[4] Bard College, Annandale-on-Hudson, NY, USA

[5] University of Connecticut Health Center, Farmington, CT, USA

Abstract:

Aspergillus fumigatus is a ubiquitous fungus that causes infections worldwide due to the continuous inhalation of *A. fumigatus* fungal conidia. Immunocompromised individuals have increased risks and compose most of the population with the aggressive disease, invasive pulmonary aspergillosis, due to a weakened competence of the immune response. The airway epithelium plays an essential role, since it is the first barrier of defense. The trace element iron is needed by both the fungus and the host for cellular maintenance and survival, but proper iron homeostasis in the host is required to prevent oxidative stress and cell death. In this paper, we present a mathematical model of an airway epithelial cell, including key immune response pathways such as nuclear factor kappa-light-chain-enhancer of activated *B* cells (NF-κB) and hypoxia inducible factor 1 alpha (HIF1A). We are interested in observing their roles in the context of fungal infection and most importantly the effect on cellular iron import via transferrin receptor (TFRC) and divalent metal ion transporter 1 (DMT1). Using the framework of polynomial dynamical systems to analyze the transcriptional network which is based on published literature and RNA-seq data, we generate several biological hypotheses to validate experimentally using qRT-PCR. Lastly, we investigate the effect of the cytokine, interleukin 1 beta (IL1B) on TFRC expression during co-incubation with *A. fumigatus*. We employ mathematics as a tool to investigate the biological role of airway epithelial cells in fungal recognition and activation of the immune response in signaling cascades that subsequently modify iron import and hope to use this information as a platform to understand the host-fungal interaction.

4.1. Background

4.1.1 *Aspergillus fumigatus* and the host defense mechanisms

There are numerous inflammatory diseases and disorders that result from exposure to fungi, most notably *A. fumigatus*, a ubiquitous species in the genus *Aspergillus*, that is commonly dispersed through airborne spores [1, 2]. In addition to infectious hyphal fragments, the conidia or spores, are 2.5-3μm in size and capable of causing a myriad of respiratory complications primarily in immunocompromised hosts [3]. *A. fumigatus* is associated with cystic fibrosis, allergic bronchopulmonary aspergillosis, chronic obstructive pulmonary disorder, and invasive aspergillosis (IA) [2, 4, 5]. The mortality rate of IA exceeds 50% and has been recorded as high as 95%, even with treatment [6-8].

As the first line of defense, airway epithelial cells determine the response of the host to fungal infection. There are several protective mechanisms such as mucus production and cilia to minimize the likelihood of direct contact of the conidia with the epithelial cell. The airborne fungus *A. fumigatus* is able to infiltrate the epithelial cell barrier in its human host using multiple tactics [6, 9]. The opportunistic fungus secretes various molecules that warn epithelial cells of its presence, triggering the innate immune response of the host. The initial timing and magnitude of the innate immune response plays a critical role in cellular defense against fungi [10]. One host defense mechanism is the expression of immune response proteins such as cytokines, small inflammatory proteins secreted by epithelial cells in response to *A. fumigatus*. They are often responsible for recruitment of other effector cells including macrophages and neutrophils [11-14].

4.1.2. Iron acquisition and homeostasis

Once inhaled by the host, *A. fumigatus* acquires iron by scavenging the extracellular environment of the host [15]. Siderophores are secreted molecules which have a high affinity for iron and are used by fungi to acquire iron and deplete extracellular iron from the host for proliferation [16-19]. The ability to acquire iron has been shown to be a key virulence factor for the fungus *A. fumigatus [20]*. The struggle between the fungus and the host for control of iron has been investigated in alveolar macrophages during *A. fumigatus* infection since macrophages are the first phagocytic cells to the infection site [17, 18]. Epithelial cells are gaining attention as well, as the first line of defense because their response determines the recruitment of other cells. Plasma membrane-bound pattern recognition receptors (PRRs) found on the human epithelial cells such as dectin-1, detect pathogen associated molecular patterns and fungal secretions (glycolipids, polysaccharides, proteases,) of *A. fumigatus* [21-24]. These secretions alert PRRs, including toll-like receptors that regulate the innate immune response through the secretion of pro-inflammatory cytokines from epithelial cells [25]. Consequently, these cytokines trigger intracellular effects which impact the iron regulatory system [26, 27].

During invasive infection, *A. fumigatus* derives its nutrition from the host environment including the redox active element iron (Fe) [28, 29]. The host needs iron for a variety of biological processes, including DNA synthesis, cellular respiration, and other mechanisms to maintain cellular integrity and functionality [30, 31]. The regulation of iron in epithelial cells is complex and governed by several feedback loops; thus, investigation of the underlying causes of changes in iron metabolism are difficult to observe and analyze [32]. Iron is a valuable nutrient tightly regulated in host cells since excess iron leads to the production of intracellular reactive oxygen

species (ROS), such as hydroxyl peroxide and superoxide, which causes toxicity and disturb iron homeostasis [33]. Ferric (Fe^{3+}) and ferrous (Fe^{2+}) are the forms of iron found in circulation. Excess iron leads to oxidative stress and contributes to cellular damage and apoptosis. Homeostasis of iron is regulated by proteins, which control the flux of iron into and out of the cell, as well as sequestration of excess iron not needed for metabolic reactions [34]. The primary control mechanisms are import, export, storage and utilization. In this paper, we focus on two iron importers. Iron is imported into the cell via the membrane proteins transferrin receptor (TFRC) and divalent metal ion transporter 1 (DMT1). TFRC imports diferric (Fe^{3+}) transferrin bound iron, while DMT1 imports ferrous iron after its reduction by a ferrireductase [35]. DMT1 is able to reduce oxidative stress by influencing an increase in intracellular iron storage [36].

This study uses a dynamic mathematical model of normal lung epithelial cells to study the effect of cytokines on the expression of iron importers, in response to *A. fumigatus* co-incubation. A mathematical model is constructed to analyze the effect of cytokines involved in the immune response on the expression of importers of iron. We validate the model, and use it as a tool to generate predictions about the host-fungal interaction, all of which are validated through *in vitro* experiments.

4.2 The modeling framework

The discrete modeling approach uses a framework where qualitative expression levels describe each model component and eliminate the need for parameter estimation which is often experimentally difficult to construct. To construct the model, we build transition tables which are representative of the biology of the network (schematic in Figure 4.3) and based on the interactions (activation/inhibition) between model components. The transition table considers all possible concentrations of the independent model component; we determine the output for each row for the dependent model components. The output is generated in the next time step column, t+1, which denotes the update of the model components. Our system updates synchronously and time is discrete. When completing the output, we build in continuity by not allowing a model component to skip a state (i.e. at time (t), x_1=0, we cannot update at time (t+1) to x_1=2). The number of model components influencing another model component determines the size of the transition table for a given species. If m= the number of transition table entries, z=the number of discrete states, and h=the number of inputs, then $m=z^h$.

Consider two nodes, A and B. When A is high, B expression is high and the expression is dependent on the expression of itself (Figure 4.1). Both nodes, A and B, take on one of two finite states, where 0 denotes low expression level and 1 denotes a high expression level. Tables 4.1 and 4.2 describe the mathematical description and the biological description of nodes A, while Tables 4.3 and 4.4 describe the mathematical description and the biological interpretation.

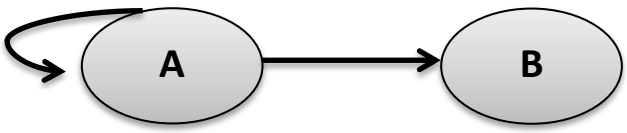

Figure 4.1: Wiring diagram for example.

A(*t*)	A(*t*+1)
0	0
1	1

Table 4.1: Transition table for node A. This table describes the state of node A and the changes in its expression at the next time step (*t*+1)

A(*t*)	A(*t*+1)
Low	Low
High	High

Table 4.2: The biological interpretation of the transition table for node A. This table describes the change in node A expression based on its starting expression level of low or high.

A(*t*)	B(*t*)	B(*t*+1)
0	0	0
0	1	0
1	0	1
1	1	1

Table 4.3: Transition table for node B. This table describes the state of node B and the changes in its expression at the next time step (*t*+1)

A(t)	B(t)	B(t+1)
Low	Low	Low
Low	High	Low
High	Low	High
High	High	High

Table 4.4: The biological interpretation of the transition table for node B. This table describes the change in node B expression level based on its starting expression level of low or high and the expression level of the input-node A.

Using our system of transition tables (Appendix B), we incorporate polynomial dynamical systems (PDS) as a tool to investigate the dynamics of the model. PDS is defined as a function $f = (f_1,\ldots,f_n) : k^n \rightarrow k^n$ with coordinate functions $f_i \epsilon k[x_1, \ldots, x_n]$ over a finite field k [37, 38]. Each x_i, is a model component (x_1-x_{13}) and its corresponding polynomial resulting from interpolation of the transition table, f_i.

Continuing with the example above, the functions from the transition tables for nodes A and B are constructed using Lagrange interpolation [39].
f(A)=A
f(B)=A
The function for node A, f(A), describes the final expression value for node A over time will be equivalent to the initial expression value of node A. On the other hand, the function for node B, f(B), describes the final expression value of node B is equal to the expression value of node A at the initial time *t*.

In order to examine the stability of our model with /without *A. fumigatus* presence and in normoxic/hypoxic conditions, we used Cyclone software (publically unavailable) which allows users to input all transition tables of the network. The program outputs the percentage of the state space at each steady state and/or limit cycle details. A steady state is reached when the cell remains fixed at the current level of concentrations achieved, whereas a cell that continuously cycles through a subset of the state space reaches a limit cycle. The relevance of each steady state is determined by the percentage of starting combinations that stabilize at that certain fixed point. The state space includes all possible expression level combinations of each of the model components (x_1, x_2,…, x_{13}). Each state describes the behavior of the lung epithelial cell for some given condition. We input 13 transition tables into Cyclone based on assumptions listed below and analyzed the results.

4.3. Model Construction Techniques

Using the transcriptional data described in Chapter 2, we characterized the differentially expressed genes to determine signaling pathways induced by *A. fumigatus* infection. Employing the search engines, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.kegg.jp/) and PathwayLinker (pathwaylinker.org), we generated a proposed connection between hypoxia inducible factor alpha (HIF-1α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways in response to *A. fumigatus*, shown in Figure 4.2. Each pathway independently contributes to the active immune response of the host through the production of cytokines and other signaling molecules.

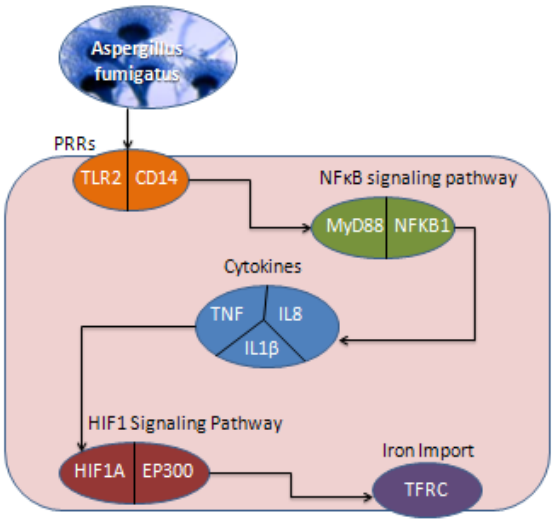

Figure 4.2: Schematic image of the pathways connecting recognition of the fungus, *A. fumigatus* to *TFRC*. Pathogen recognition receptors (PRRs), toll-like receptor 2 (TLR2) and CD14, trigger the NF-κB pathway which cause a cascade of changes by producing cytokines like tumor necrosis factor alpha (TNFA), interleukin 8 (IL8) and interleukin 1 beta (IL1B). These cytokine induce the hypoxia inducible factor 1 pathway (HIF-1α) which influences the iron importer transferrin receptor (TFRC).

We performed an extensive literature review searching for articles focusing on the following: the effect of cytokines on iron importer genes, the effect of cytokines on other cytokines, and the effect of fungi on airway epithelial cell cytokine production. We introduce the immune response connections to the iron regulatory network supported by scientific literature described in section 4.4 and the workflow is described in Figure 4.3.

Figure 4.3: Workflow for model construction. Using RNA-Seq data, KEGG pathway, PathwayLinker, and an extensive literature search, we compiled a list of candidate genes and their proposed interaction (s).

A. fumigatus activates the NF-κB pathway which promotes a signaling cascade of cytokine production based on the binding of the NF-κB transcription factor. The cytokines, through regulatory molecules such as mitogen activated protein kinases (MAPK), and phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (MTOR), influence the expression of the iron importers, DMT1and TFRC, directly or indirectly [40]. TNF-α, a master regulator, up-regulates cytokines including interleukin 1 beta (IL1B) [41, 42] and IL1B inhibits TFRC [27]. Evidence supports *A. fumigatus*-induced secretion of interleukin 8 (IL8) in lung epithelial cells, but since the effects of IL8 and other cytokines have not been investigated on iron regulatory proteins, we utilize data collected in Chapter 3 [22, 43]. The oxidative stress portion of the model is based on normoxic or hypoxic conditions. HIF1A, a transcription factor, regulates DMT1 [44]. It also influences cyclooxygenase-2 (COX2), an enzyme catalyzed during inflammation. Vascular endothelial growth factor A (VEGFA) is regulated by both COX2 and HIF1A and thought to be a protective component during inflammation through inhibition of apoptotic events [45]. There are several connections between the selected genes and genes not included in our model, but the model provides a blueprint for the basic interactions.

4.4. The Model

The model, a schematic shown in Figure 4.4, is composed of thirteen components. Eleven of the components are represented as variables, HIF1A, COX2, NFKB, IL8, IL1B, TNFA, PI3K/AKT/MTOR, MAPK8-MAPK14, VEGFA, TFRC, DMT1, and two components serve as external variables, *A. fumigatus*, and available oxygen levels. Since one portion of the model is dependent on oxygen availability and *A. fumigatus*, or another pathogen, is needed to stimulate the NF-κB pathway, we considered all combinations of the external variables. These components are relevant to iron regulation, innate immunity, and oxidative stress among other essential pathways in lung epithelial cells.

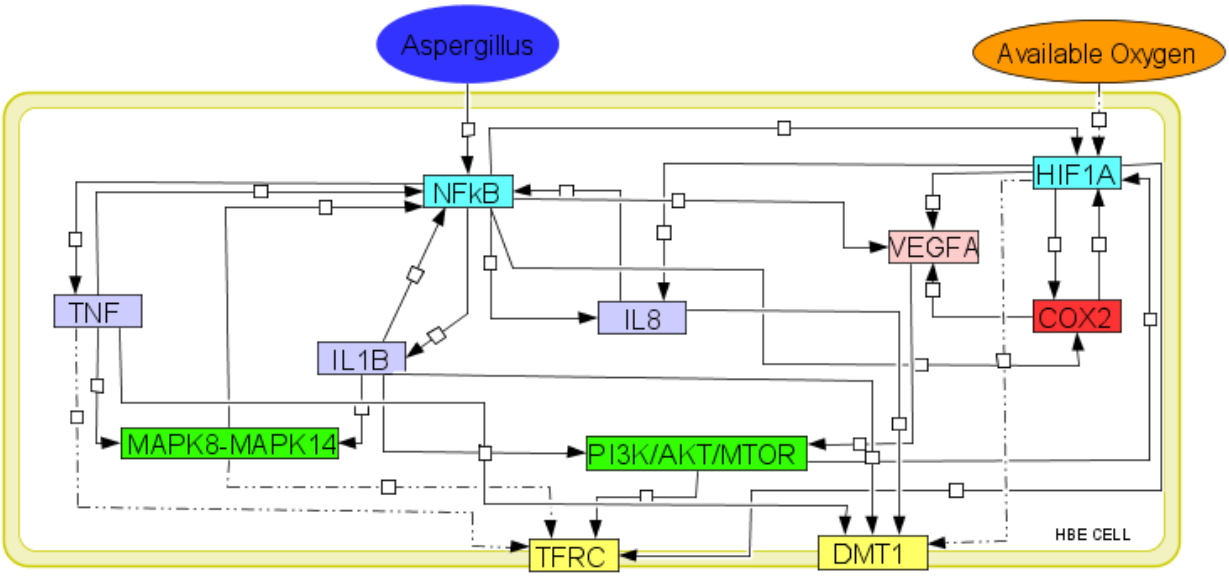

Figure 4.4: Schematic image of the model network in an airway epithelial cell. The model components include TNFA, MAPK8-MAPK14, IL1B, NFKB, TFRC, DMT1, IL8, PI3K/AKT/MTOR, VEGFA, COX2, HIF1A, and the external parameters available oxygen and *A. fumigatus*. Dashed lines represent inhibition/degradation and solid lines represent activation/up-regulation.

4.4.1 Discretization of the nodes

Descriptions of the 13 nodes, found in Table 4.5, show the number of discrete states for each node and the biological meaning. Many of the components have three state levels, such as low, medium and high; However, the meaning for nodes, even with the same number of states, may differ. Due to limitations in the literature in regards to the quantitative cut off for qualitative modeling approach, we restricted some nodes to two states and other nodes to three states, for a more precise biological description. This discretization is crucial for the construction of the transition table for each component of the model and most importantly interpretation of the steady state results of the model.

Node	Name	State Number	Description: State 0	Description: State 1	Description: State 2
1	HIF1A	3	Off (Degraded)	Medium (Moderate Degradation/ Expression)	High (No Degradation)
2	COX2	3	Off (no expression)	Medium	High
3	NFKB	3	Off (Deactivation)	Medium (Moderate Deactivation)	High (No Deactivation)
4	IL8	3	Off	Medium	High
5	IL1B	2	Off (no expression)	On	n/a
6	TNFA	2	Off (no expression)	On	n/a
7	MTOR	3	Inactive/ Low Expression	Active	Very Active
8	MAPK	3	Inactive/ Low Expression	Active	Very Active
9	VEGFA	3	Off (no expression)	Moderate Expression	High Expression
10	TFRC	3	Low	normal Levels	High
11	DMT1	3	Low	Normal levels	High
External	*Aspergillus fumigatus*	2	Absent	Present	n/a
External	Oxygen level	2	Hypoxic Conditions	Normoxic Conditions	n/a

Table 4.5: Model nodes and state descriptions. This table describes each of the 13 nodes of the schematic image and description of each state if applicable, if not, n/a is listed.

The literature contains several studies generally examining the effect(s) of one component in our model on another component in our model for different cell types. Variations in the experimental procedures and designs are the motivation for the construction of a comprehensive model to unify relevant studies. We preferred citations specific for airway epithelial cells, but due to the restricted number of studies examining all of the model components of interest, we extended our focus to other epithelial cells from other organs, endothelial cells, and other cell types. In the few interactions where this applies, we assume there is some preserved level of similarity in the biological regulation in airway epithelial cells. Table 4.6 describes each of the 28 interactions in our model and the supporting citation. The remaining 3 interactions for the DMT1 node were added based on findings in Chapter 3.

Interactions	Citations
KEY: → = upregulates/activates, ⊣ = downregulates/inhibits * = test done with epithelial cells	
Aspergillus fumigatus → NFKB	[46]
Oxygen ⊣ HIF-1A	[47]
HIF1A → COX2	[48]
NFKB → HIF1A	*[49]
HIF1A → IL8	*[50]
NFKB → VEGF	*[49]
NFKB → COX2	*[49]
COX2 → HIF1A	*[49]
HIF1A → VEGFA	[51, 52]
NFKB → IL8	*[43]
NFKB → TNFA	[53]
NFKB → IL1B	*[49]
IL1B → NFKB	*[49]
IL8 → NFKB	*[54]
MTOR → HIF1A	*[49]
MAPK ⊣ TFRC	[55]
HIF1A → TFRC	[56]
TNFA → MAPK	*[57]
TNFA → NFKB	[58]
MAPK → NFKB	*[57]
COX2 → VEGFA	*[49]
VEGF → MTOR	[59]
IL1B → MTOR	[60]
IL1B → MAPK	*[57] [61]
MTOR → TFRC	[62]
NFKB ⊣ NFKB	[63]
A. fumigatus induces hypoxia	*[64]
TNFA ⊣ TFRC	[27]
HIF1A ⊣ DMT1	[44]

Table 4.6: Model citations for each interaction. A description of each interaction in the model and the complementary literature citation.

4.4.2 Model Assumptions:

General Assumptions

Cumulative Activators/Repressors – Since activation/repression of a gene's function can occur via multiple pathways (change in transcription levels, mRNA manipulation, stabilization/degradation of final protein, etc.). We allowed the inputs of nodes to act in a cumulative manner, considering synergistic and agonistic effects if known

Equal Effects of Node Inputs – In the absence of a good method of rating the strength of the influence each node has on another, we have made it so all activators and repressors have equal strength in our model. (Exceptions include the HIF1A/Oxygen levels relationship with oxygen levels completely blocking HIF1A unless several stabilizers are present, the NFKB/*A. fumigatus* relationship where *A. fumigatus* is heavily weighted as an activator, and the combined effect of MAPK and TNFA on TFRC.

2 vs. 3 State Node Strengths –3-state nodes are capable of a more pronounced effect that 2-state nodes when highly active.

Thresholds for Activation – Generally the thresholds for activation in our model at a given level are evenly spaced according to the different possible input-activation levels. For example, if 6 levels of activation input are possible for a three-state node, we will make the following set of rules:
(A = activation input)
If A= 0 or 1, then Node = 0
If A= 2 or 3, then Node = 1
If A= 4 or 5, then Node = 2
Since we have little to no data showing what the appropriate thresholds might be, we selected thresholds to be as biologically relevant as possible.

Natural Degradation/Self Inhibition – We have constructed our rules such that the state of a node will reduce to its basal level in the absence of activators or repressors. In the absence of constant activation or stabilization, transcript levels will decrease due to the inherent instability of mRNA.

Specific Assumptions
MAPK/TNFA synergy – MAPK's effect on TFRC is mediated by IFN-γ. It has been shown that IFN-γ and TNFA have a synergistic effect which we have incorporated into our model rules [65].

NFKB/*A. fumigatus* Relationship – *A. fumigatus* infection will cause a large spike in NFKB (The effect of *A. fumigatus* is heavily weighted in model). There are also additional pathways activated by contact with fungus that are not included in our model. Since toll-like receptors, and thus NFKB, are only activated by *A. fumigatus* in immunocompromised hosts [66].

HIF1A/Oxygen Relationship – Oxygen will completely shut off HIF1A in most cases. This reflects the findings that HIF1a is degraded when oxygen is present [47].

4.5 Steady state analysis for fungus and normoxic conditions

Our model allows us to compare multiple environmental conditions, but for the purpose of this study, we are interested in the dynamics of the system when *A. fumigatus* is present and sufficient oxygen levels. The analysis results in two steady states (HIF1A, COX2, NFKB, IL8, IL1B, TNFA, MTOR, MAPK, VEGFA, TFRC, DMT1) and shows the percentage of the state space which converged to these steady states for fungus and normoxic conditions.

1222112220111 12.4943%

0121111210211 12.505%

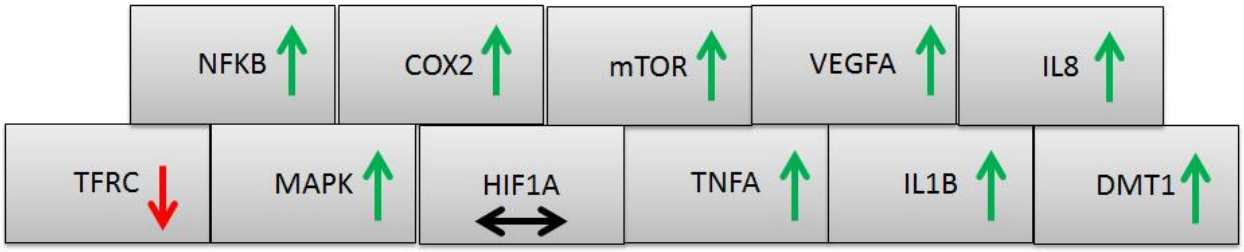

Figure 4.5: Steady state results for the presence of *A. fumigatus* and normoxic conditions in airway epithelial cells. Red arrows represent a decrease or the lowest state of expression, green arrows indicate an increase to medium or high level of expression, and the black arrow represents no change in expression.

Biologically, this fixed point represents an airway epithelial cell with activation of all immune response genes and modification of the expression of iron importer genes. The decrease in TFRC and the opposite response, increase in DMT1 expression, may be the cells way to quickly remove unbound iron. This form of iron is the easier for the fungus to acquire and generates reactive oxygen species. In order to gain a complete picture of the overall response to fungus through changes in iron regulation, this model has to be expanded to include the iron exporter and storage molecule.

4.6 Experimental materials and methods

4.6.1 Fungal strain and growth conditions:

Aspergillus fumigatus strain Af293 (ATCC, MYA-4609) was used with the strain propagated on Glucose Minimal Media (GMM) incubated at 37°C in the dark. Conidia were collected in Dulbecco's Phosphate Buffered Saline (Ca^{++}/Mg^{++} free) (DPBS; Hyclone) by gentle agitation and enumerated on a hemacytometer.

4.6.2 Cell culture

SV-40 transformed human bronchial epithelial cells (BEAS2B) were cultured in 75cm^2 tissue culture flasks at 37°C and 5% CO_2 until reaching 80% confluence. Cells were purchased from ATCC (Manassas,VA). The BEAS2B cells were grown in RPMI: 1640 medium (Hyclone) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 100U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO_2.

4.6.3 Cell stimulation:

Cells were sub-cultured into six-well tissue culture dishes at a concentration of 1 x 10^6 cells per well and allowed to adhere overnight. The cells were washed twice with DPBS and cultured in a final volume of 1.5mL RPMI media without addition of the provided gentamicin/*amphotericin-B* component to avoid potential interference from these media supplements with fungal stimulation. Nine individual samples were prepared for each treatment by addition of 0.5 x 10^6 *A. fumigatus* spores or left untreated (control). Cells were incubated at 37°C and 5% CO_2. Samples were

collected at 2, 6, and 12 hours following initial stimulation. Culture supernatant was collected and debris was removed by centrifugation before storing at -80°C. The cells were washed twice with DPBS before RNA extraction.

4.6.4 RNA Extraction and Preparation:

Total RNA was collected by addition of 1mL TRIzol (Invitrogen Carlsbad, CA) directly to the cell culture plate and gentle agitation using a cell lifter. RNA extraction was carried out per manufacturer's instructions followed by clean-up using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) with on-column DNase digestion to remove residual genomic DNA. RNA was eluted in 30µl of RNase-free water and integrity (A260/A280) and concentration were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were then stored at -80°C until further analysis.

4.6.5 Reverse transcriptase-polymerase chain reaction

RNA was reverse transcribed into cDNA using tetro cDNA synthesis kit (BioLine, Taunton, MA) per the manufacturer's instructions. Quantitative PCR was performed using IQ SYBR green supermix (Biorad, Hercules, CA) on BioRad iCycler thermal cycler. With Cycle 1: (1X), and Step 1 at 95.0 °C for 03:00 minutes, Cycle 2: (40X) with Step1 at 95.0 °C for 00:10 seconds and Step 2 at 55.0 °C for 00:30 seconds, and Cycle 3: (81X) at 55.0 °C-95.0 °C for 00:30 seconds.

TfRC, DMT1, IL1B, HIF1A, and VEGFA mRNA levels were normalized to the housekeeping gene GAPDH. The following sequences were used, purchased from Integrated DNA Technologies (IDT):
DMT1: 5′-ATGGACTAGGTGGCGGATT-3′and 5′ -GATAAGCCACGTGA-CCACA-3′;
TFRC: 5′-CAGGAACCGATCTCCAGTGA-3′ and 5′-CTTGATGGT-GCGGTGA-AGT-3′;
GAPDH: 5′-ACCCACTCCTCACCTTTGA-3′ and 5′-CTGTTGCTGTACCAAATTCGT-3′;
IL1B: 5′-AAACAGATGAAGTGCTC CTTCCAGG-3′ and 5′-TGGAGAACACCACTTGTTGCTCCA-3′; HIF1A: 5′-GAAAGCGCAAGTCTTCAAAG-3′ and 5′-TGGGTAGGAGATGGAGATGC-3′; VEGFA: 5′-AGGAGGAGGGCAGAATCATCA-3′ and 5′-CTCGATTGGAT-GGCAGTAGCT-3′.

4.6.6 IL1B knockdown using small interfering RNA (siRNA)

BEAS2B cells were seeded at a density of $0.5x10^6$ cells per well in 6-well plates. The cells were grown in DMEM containing HiPerFect Transfection reagent (Qiagen) according to the manufacturer's protocol and siRNA against IL1B (Santa Cruz Biotechnology), 5nM was added for incubation for 24 hours of incubation at 37°C and 5% CO_2. The cells were washed in DPBS, and treated for an additional 24 hours with 5 nM of IL1B siRNA at 37°C and 5% CO_2. The cells were supplemented serum free media for 2 hours prior to fungal stimulation. As a control, a non-silencing siRNA duplex nonspecific was used. We harvested cells for quantitative analysis, which showed at least a 60% reduction in IL1B expression prior to fungal treatment.

4.7 Model Validation

Using qRT-PCR, we validate the model predictions of the changes to the following genes in response to *A. fumigatus* conidia interacting with airway epithelial cells: HIF1A, IL1B, TFRC,

DMT1, and VEGFA. For HIF1A we observed no change in expression at any of the three time points (Figure 4.6).The cytokine, IL1B, increased as predicted by the model along with VEGFA (Figures 4.7 and 4.8). TFRC displayed a down-regulation trend but the decrease at 12 hrs. is statistically insignificant (Figure 4.9). Examination of DMT1 shows an increase in expression (Figure 4.10). For the cytokines, some significantly increase as early as 2 or 6 hrs. (data not shown), and others are late in changing their expression. However, we believe the changes in the iron importer genes are influenced (directly or indirectly) by cytokines and respond at later time points.

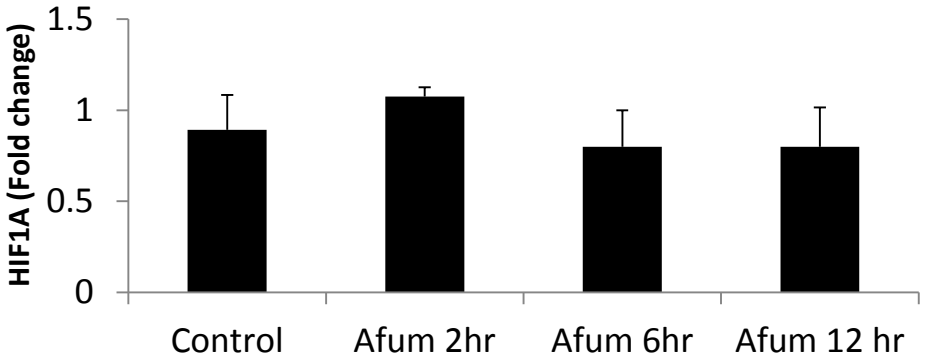

Figure 4.6: HIF1A expression after *A. fumigatus* infection. qRT-PCR results for the comparison of control at each time point with fungal infected at 2, 6, and 12 hours.

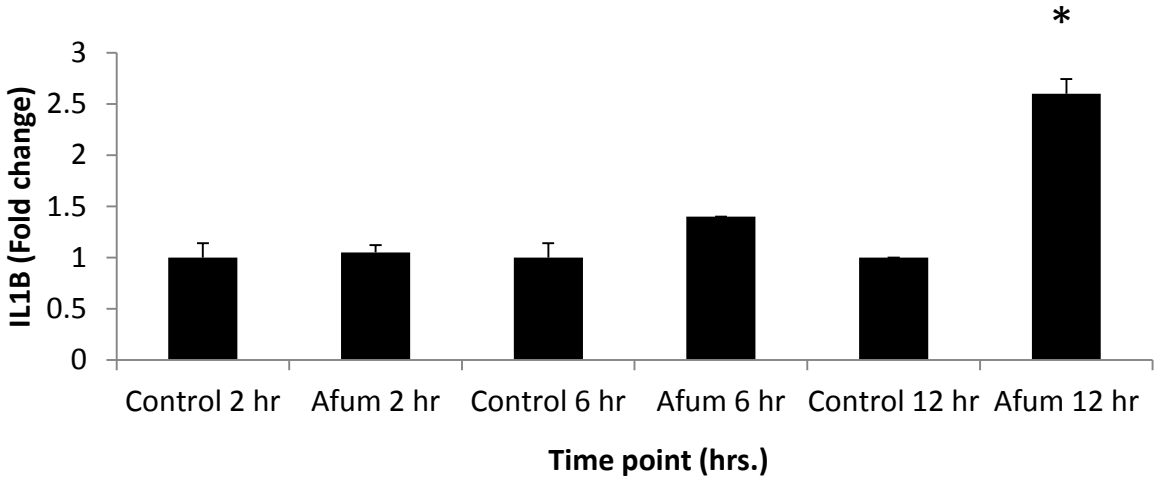

Figure 4.7: IL1B expression after *A. fumigatus* infection. qRT-PCR results for the comparison of control at each time point with fungal infected at 2, 6, and 12 hours.

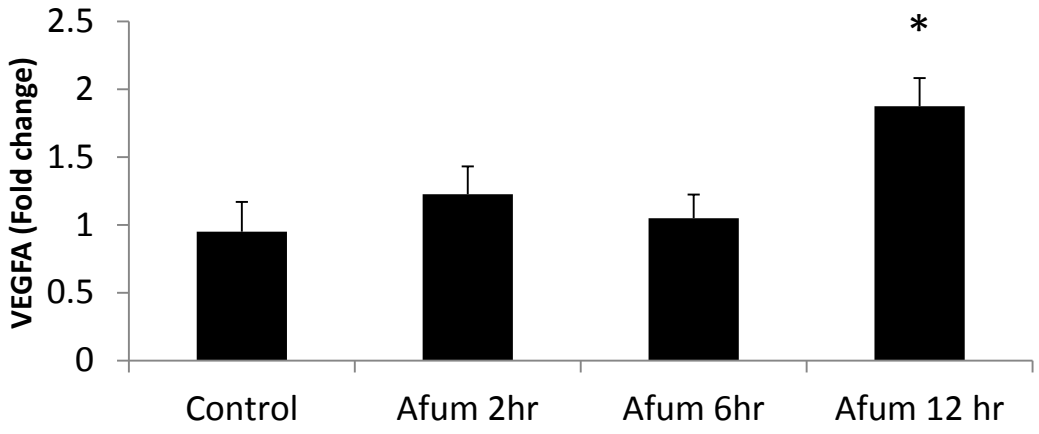

Figure 4.8: VEGFA expression after *A. fumigatus* infection. qRT-PCR results for the comparison of control at each time point with fungal infected at 2, 6, and 12 hours.

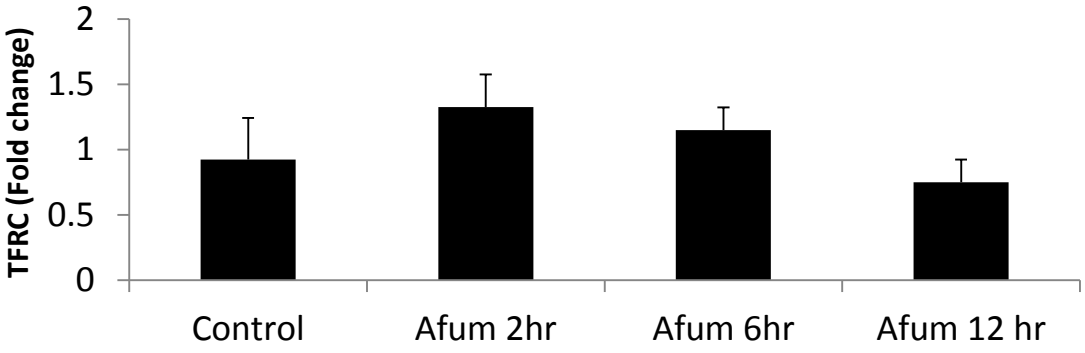

Figure 4.9: TFRC expression after *A. fumigatus* infection. qRT-PCR results for the comparison of control at each time point with fungal infected at 2, 6, and 12 hours.

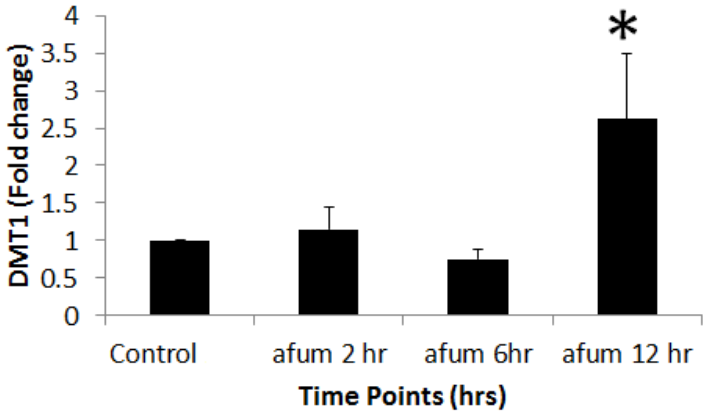

Figure 4.10: DMT1 expression after *A. fumigatus* infection. qRT-PCR results for the comparison of control at each time point with fungal infected at 2, 6, and 12 hours.

4.8 Analysis of the cytokine-iron interface in fungal infection

The down regulation in TFRC expression during fungal infection could be related to the influence of cytokines. To investigate this possibility, we used siRNA to reduce IL1B expression (Figure 4.11) and treated the airway epithelial cells with *A. fumigatus* conidia to observe the effects on TFRC. A 60% reduction in IL1B expression led to an increase in TFRC at the 12 hrs. time point (Figure 4.12). This contrast in the behavior of TFRC (Figure 4.9) suggests IL1B inhibits TFRC expression in the presence of *A. fumigatus* and indeed the cytokine is responsible for modification of TFRC change in expression.

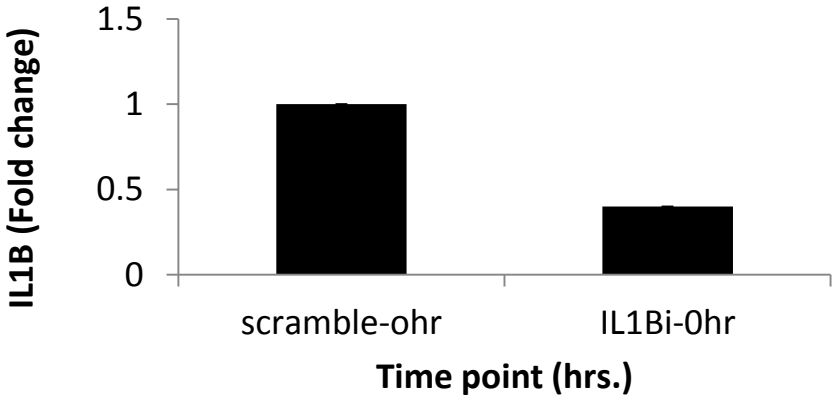

Figure 4.11: IL1B expression after knockdown. qRT-PCR results for the comparison of scramble and IL1B siRNA, both untreated at 0 hrs.

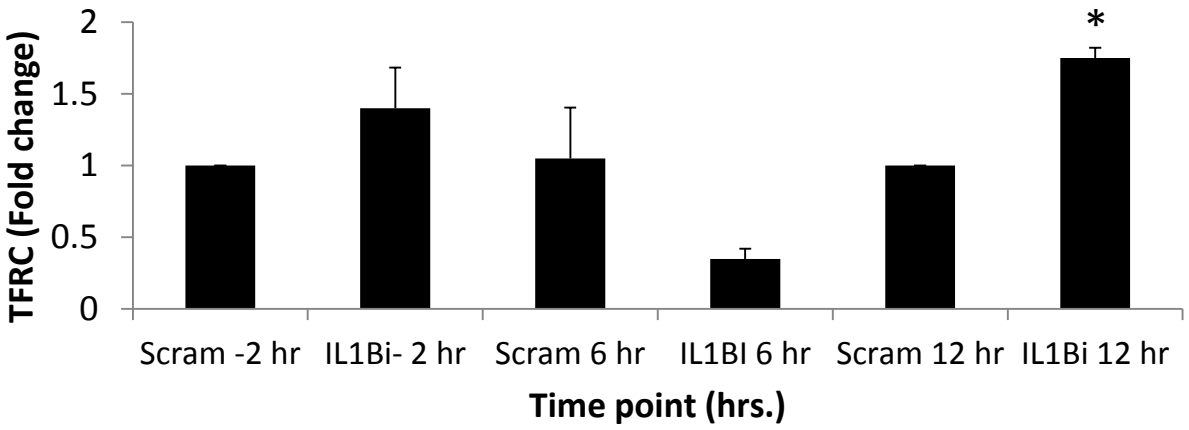

Figure 4.12: TFRC expression after *A. fumigatus* infection and IL1B knockdown. qRT-PCR results for the comparison of control at each time point with fungal infected at 2, 6, and 12 hours for IL1B knockdown and scramble control.

4.9 Discussion

This mathematical model shows the changes induced by the innate immune response, following exposure to *Aspergillus fumigatus* in human lung epithelial cells and the effects on iron importers, DMT1 and TFRC. Our model incorporates 13 components which influence the dynamics of the system. Creating a model for other cell types and the interactions between the cells is critical to understanding the immune response to *A. fumigatus*. Our model provides a localized view of the innate immune response to an opportunistic fungus and the role of the iron importers in lung epithelial cells.

The down regulation of TfR1 expression level following fungal exposure suggests the fungus prevents host cells from importing ferric iron and this trend has been observed in macrophages in response to *A. fumigatus* [18]. Meanwhile, the up-regulation of DMT1 predicted by the model was confirmed using qRT-PCR. Therefore, DMT1 plays a significant role in iron import and Although, this is the first study of the effect of *A. fumigatus* on DMT1 in any cell type, we believe it is related to fungal infection and oxidative stress [67, 68].

We also made a novel discovery about the effect of *A. fumigatus* on airway epithelial cells via VEGFA expression. VEGFA, in airway epithelial cells interacting with *Pseudomonas aeriguinosa*, was also shown to be up-regulated [69]. This provides evidence of the possible angiogenesis mechanisms in the lung and the expression of VEGFA responds to prevent cell death. This is also the first study to investigate the relationship between IL1B and *A. fumigatus*. We showed it increased in response to *A. fumigatus* and further studied the effects of IL1B on TFRC. Our data suggests IL1B inhibits TFRC when *A. fumigatus* is present and this work can be extended to see the effects on DMT1.

Our discrete modeling approach provided an efficient method to understand the effects of a stimulus, *A. fumigatus*, on lung epithelial cells, to mimic the fungal-host interaction. The novel model, allows the visualization of the dynamics of the immune response of lung epithelial cells following fungal infection and the supporting role of the iron regulatory network. Our model is novel, innovative, and fundamental in understanding the two complex systems, the host immune response to fungal infections and the role of iron, in mediating the host's immune response.

4.10 Literature citations

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Appendix A: Supplemental tables

A.1 Tables

$x13(t)$	$x7(t)$	$x2(t)$	$x3(t)$	$x1(t)$	$x1(t+1)$
0	0	0	0	0	1
0	0	0	0	1	1
0	0	0	0	2	1
0	0	0	1	0	1
0	0	0	1	1	1
0	0	0	1	2	2
0	0	0	2	0	1
0	0	0	2	1	1
0	0	0	2	2	2
0	0	1	0	0	1
0	0	1	0	1	1
0	0	1	0	2	2
0	0	1	1	0	1
0	0	1	1	1	1
0	0	1	1	2	2
0	0	1	2	0	1
0	0	1	2	1	1
0	0	1	2	2	2
0	0	2	0	0	1
0	0	2	0	1	1
0	0	2	0	2	2
0	0	2	1	0	1
0	0	2	1	1	1
0	0	2	1	2	2
0	0	2	2	0	2
0	0	2	2	1	2
0	0	2	2	2	2
0	1	0	0	0	1
0	1	0	0	1	1
0	1	0	0	2	2
0	1	0	1	0	1
0	1	0	1	1	1
0	1	0	1	2	2
0	1	0	2	0	1
0	1	0	2	1	1

0	1	0	2	2	2
0	1	1	0	0	1
0	1	1	0	1	1
0	1	1	0	2	2
0	1	1	1	0	1
0	1	1	1	1	1
0	1	1	1	2	2
0	1	1	2	0	2
0	1	1	2	1	2
0	1	1	2	2	2
0	1	2	0	0	1
0	1	2	0	1	1
0	1	2	0	2	2
0	1	2	1	0	2
0	1	2	1	1	2
0	1	2	1	2	2
0	1	2	2	0	2
0	1	2	2	1	2
0	1	2	2	2	2
0	2	0	0	0	1
0	2	0	0	1	1
0	2	0	0	2	2
0	2	0	1	0	1
0	2	0	1	1	1
0	2	0	1	2	2
0	2	0	2	0	2
0	2	0	2	1	2
0	2	0	2	2	2
0	2	1	0	0	1
0	2	1	0	1	1
0	2	1	0	2	2
0	2	1	1	0	2
0	2	1	1	1	2
0	2	1	1	2	2
0	2	1	2	0	2
0	2	1	2	1	2
0	2	1	2	2	2
0	2	2	0	0	2
0	2	2	0	1	2
0	2	2	0	2	2
0	2	2	1	0	2

0	2	2	1	1	2
0	2	2	1	2	2
0	2	2	2	0	2
0	2	2	2	1	2
0	2	2	2	2	2
1	0	0	0	0	0
1	0	0	0	1	0
1	0	0	0	2	0
1	0	0	1	0	0
1	0	0	1	1	0
1	0	0	1	2	0
1	0	0	2	0	0
1	0	0	2	1	0
1	0	0	2	2	0
1	0	1	0	0	0
1	0	1	0	1	0
1	0	1	0	2	0
1	0	1	1	0	0
1	0	1	1	1	0
1	0	1	1	2	0
1	0	1	2	0	0
1	0	1	2	1	0
1	0	1	2	2	0
1	0	2	0	0	0
1	0	2	0	1	0
1	0	2	0	2	0
1	0	2	1	0	0
1	0	2	1	1	0
1	0	2	1	2	0
1	0	2	2	0	0
1	0	2	2	1	0
1	0	2	2	2	0
1	1	0	0	0	0
1	1	0	0	1	0
1	1	0	0	2	0
1	1	0	1	0	0
1	1	0	1	1	0
1	1	0	1	2	0
1	1	0	2	0	0
1	1	0	2	1	0
1	1	0	2	2	0

1	1	1	0	0	0
1	1	1	0	1	0
1	1	1	0	2	0
1	1	1	1	0	0
1	1	1	1	1	0
1	1	1	1	2	0
1	1	1	2	0	0
1	1	1	2	1	0
1	1	1	2	2	0
1	1	2	0	0	0
1	1	2	0	1	0
1	1	2	0	2	0
1	1	2	1	0	0
1	1	2	1	1	0
1	1	2	1	2	0
1	1	2	2	0	1
1	1	2	2	1	1
1	1	2	2	2	1
1	2	0	0	0	0
1	2	0	0	1	0
1	2	0	0	2	0
1	2	0	1	0	0
1	2	0	1	1	0
1	2	0	1	2	0
1	2	0	2	0	0
1	2	0	2	1	0
1	2	0	2	2	0
1	2	1	0	0	0
1	2	1	0	1	0
1	2	1	0	2	0
1	2	1	1	0	0
1	2	1	1	1	0
1	2	1	1	2	0
1	2	1	2	0	1
1	2	1	2	1	1
1	2	1	2	2	1
1	2	2	0	0	0
1	2	2	0	1	0
1	2	2	0	2	0
1	2	2	1	0	1
1	2	2	1	1	1

1	2	2	1	2	1
1	2	2	2	0	1
1	2	2	2	1	1
1	2	2	2	2	1

Table A.1 Transition table for variable x_1

$x1(t)$	$x3(t)$	$x2(t+1)$
0	0	0
0	1	1
0	2	1
1	0	1
1	1	1
1	2	2
2	0	1
2	1	2
2	2	2

Table A.2 Transition table for variable x_2

$x12(t)$	$x5(t)$	$x8(t)$	$x4(t)$	$x3(t)$	$x6(t)$	$x3(t+1)$
0	0	0	0	0	0	0
0	0	0	0	0	1	0
0	0	0	0	1	0	2
0	0	0	0	1	1	0
0	0	0	0	2	0	2
0	0	0	0	2	1	2
0	0	0	1	0	0	0
0	0	0	1	0	1	0
0	0	0	1	1	0	0
0	0	0	1	1	1	0
0	0	0	1	2	0	2
0	0	0	1	2	1	0

0	0	0	2	0	0	0
0	0	0	2	0	1	0
0	0	0	2	1	0	0
0	0	0	2	1	1	0
0	0	0	2	2	0	0
0	0	0	2	2	1	0
0	0	1	0	0	0	0
0	0	1	0	0	1	0
0	0	1	0	1	0	0
0	0	1	0	1	1	0
0	0	1	0	2	0	2
0	0	1	0	2	1	0
0	0	1	1	0	0	0
0	0	1	1	0	1	0
0	0	1	1	1	0	0
0	0	1	1	1	1	0
0	0	1	1	2	0	0
0	0	1	1	2	1	0
0	0	1	2	0	0	0
0	0	1	2	0	1	0
0	0	1	2	1	0	0
0	0	1	2	1	1	0
0	0	1	2	2	0	0
0	0	1	2	2	1	0

0	0	2	0	0	0	0
0	0	2	0	0	1	0
0	0	2	0	1	0	0
0	0	2	0	1	1	0
0	0	2	0	2	0	0
0	0	2	0	2	1	0
0	0	2	1	0	0	0
0	0	2	1	0	1	0
0	0	2	1	1	0	0
0	0	2	1	1	1	0
0	0	2	1	2	0	0
0	0	2	1	2	1	0
0	0	2	2	0	0	0
0	0	2	2	0	1	1
0	0	2	2	1	0	0
0	0	2	2	1	1	0
0	0	2	2	2	0	0
0	0	2	2	2	1	0
0	1	0	0	0	0	0
0	1	0	0	0	1	0
0	1	0	0	1	0	0
0	1	0	0	1	1	0
0	1	0	0	2	0	2
0	1	0	0	2	1	0

0	1	0	1	0	0	0
0	1	0	1	0	1	0
0	1	0	1	1	0	0
0	1	0	1	1	1	0
0	1	0	1	2	0	0
0	1	0	1	2	1	0
0	1	0	2	0	0	0
0	1	0	2	0	1	0
0	1	0	2	1	0	0
0	1	0	2	1	1	0
0	1	0	2	2	0	0
0	1	0	2	2	1	0
0	1	1	0	0	0	0
0	1	1	0	0	1	0
0	1	1	0	1	0	0
0	1	1	0	1	1	0
0	1	1	0	2	0	0
0	1	1	0	2	1	0
0	1	1	1	0	0	0
0	1	1	1	0	1	0
0	1	1	1	1	0	0
0	1	1	1	1	1	0
0	1	1	1	2	0	0
0	1	1	1	2	1	0

0	1	1	2	0	0	0
0	1	1	2	0	1	1
0	1	1	2	1	0	0
0	1	1	2	1	1	0
0	1	1	2	2	0	0
0	1	1	2	2	1	0
0	1	2	0	0	0	0
0	1	2	0	0	1	0
0	1	2	0	1	0	0
0	1	2	0	1	1	0
0	1	2	0	2	0	0
0	1	2	0	2	1	0
0	1	2	1	0	0	0
0	1	2	1	0	1	1
0	1	2	1	1	0	0
0	1	2	1	1	1	0
0	1	2	1	2	0	0
0	1	2	1	2	1	0
0	1	2	2	0	0	1
0	1	2	2	0	1	1
0	1	2	2	1	0	0
0	1	2	2	1	1	1
0	1	2	2	2	0	0
0	1	2	2	2	1	0

1	0	0	0	0	0	2
1	0	0	0	0	1	2
1	0	0	0	1	0	1
1	0	0	0	1	1	2
1	0	0	0	2	0	1
1	0	0	0	2	1	1
1	0	0	1	0	0	2
1	0	0	1	0	1	2
1	0	0	1	1	0	2
1	0	0	1	1	1	2
1	0	0	1	2	0	1
1	0	0	1	2	1	2
1	0	0	2	0	0	2
1	0	0	2	0	1	2
1	0	0	2	1	0	2
1	0	0	2	1	1	2
1	0	0	2	2	0	2
1	0	0	2	2	1	2
1	0	1	0	0	0	2
1	0	1	0	0	1	2
1	0	1	0	1	0	2
1	0	1	0	1	1	2
1	0	1	0	2	0	1
1	0	1	0	2	1	2

1	0	1	1	0	0	2
1	0	1	1	0	1	2
1	0	1	1	1	0	2
1	0	1	1	1	1	2
1	0	1	1	2	0	2
1	0	1	1	2	1	2
1	0	1	2	0	0	2
1	0	1	2	0	1	2
1	0	1	2	1	0	2
1	0	1	2	1	1	2
1	0	1	2	2	0	2
1	0	1	2	2	1	2
1	0	2	0	0	0	2
1	0	2	0	0	1	2
1	0	2	0	1	0	2
1	0	2	0	1	1	2
1	0	2	0	2	0	2
1	0	2	0	2	1	2
1	0	2	1	0	0	2
1	0	2	1	0	1	2
1	0	2	1	1	0	2
1	0	2	1	1	1	2
1	0	2	1	2	0	2
1	0	2	1	2	1	2

1	0	2	2	0	0	2
1	0	2	2	0	1	2
1	0	2	2	1	0	2
1	0	2	2	1	1	2
1	0	2	2	2	0	2
1	0	2	2	2	1	2
1	1	0	0	0	0	2
1	1	0	0	0	1	2
1	1	0	0	1	0	2
1	1	0	0	1	1	2
1	1	0	0	2	0	1
1	1	0	0	2	1	2
1	1	0	1	0	0	2
1	1	0	1	0	1	2
1	1	0	1	1	0	2
1	1	0	1	1	1	2
1	1	0	1	2	0	2
1	1	0	1	2	1	2
1	1	0	2	0	0	2
1	1	0	2	0	1	2
1	1	0	2	1	0	2
1	1	0	2	1	1	2
1	1	0	2	2	0	2
1	1	0	2	2	1	2

1	1	1	0	0	0	2
1	1	1	0	0	1	2
1	1	1	0	1	0	2
1	1	1	0	1	1	2
1	1	1	0	2	0	2
1	1	1	0	2	1	2
1	1	1	1	0	0	2
1	1	1	1	0	1	2
1	1	1	1	1	0	2
1	1	1	1	1	1	2
1	1	1	1	2	0	2
1	1	1	1	2	1	2
1	1	1	2	0	0	2
1	1	1	2	0	1	2
1	1	1	2	1	0	2
1	1	1	2	1	1	2
1	1	1	2	2	0	2
1	1	1	2	2	1	2
1	1	2	0	0	0	2
1	1	2	0	0	1	2
1	1	2	0	1	0	2
1	1	2	0	1	1	2
1	1	2	0	2	0	2
1	1	2	0	2	1	2

1	1	2	1	0	0	2
1	1	2	1	0	1	2
1	1	2	1	1	0	2
1	1	2	1	1	1	2
1	1	2	1	2	0	2
1	1	2	1	2	1	2
1	1	2	2	0	0	2
1	1	2	2	0	1	2
1	1	2	2	1	0	2
1	1	2	2	1	1	2
1	1	2	2	2	0	2
1	1	2	2	2	1	2

Table A.3 Transition table for variable x_3

$x1(t)$	$x3(t)$	$x4(t+1)$
0	0	0
0	1	1
0	2	1
1	0	1
1	1	1
1	2	2
2	0	1
2	1	2
2	2	2

Table A.4 Transition table for variable x_4

$x3(t)$	$x5(t+1)$
0	0
1	1
2	1

Table A.5 Transition table for variable x_5

$x3(t)$	$x6(t+1)$
0	0
1	1
2	1

Table A.6 Transition table for variable x_6

$x9(t)$	$x5(t)$	$x7(t+1)$
0	0	0
0	1	1
1	0	1
1	1	1
2	0	1
2	1	2

Table A.7 Transition table for variable x_7

$x5(t)$	$x6(t)$	$x8(t+1)$
0	0	0
0	1	1
1	0	1
1	1	2

Table A.8 Transition table for variable x_8

$x1(t)$	$x2(t)$	$x3(t)$	$x9(t+1)$
0	0	0	0
0	0	1	1
0	0	2	1
0	1	0	1
0	1	1	1
0	1	2	1
0	2	0	1
0	2	1	1
0	2	2	2
1	0	0	1
1	0	1	1
1	0	2	1
1	1	0	1
1	1	1	1
1	1	2	2
1	2	0	1
1	2	1	2

1	2	2	2
2	0	0	1
2	0	1	1
2	0	2	2
2	1	0	1
2	1	1	2
2	1	2	2
2	2	0	2
2	2	1	2
2	2	2	2

Table A.9 Transition table for variable x_9

$x1(t)$	$x7(t)$	$x8(t)$	$x6(t)$	$x10(t+1)$
0	0	0	0	1
0	0	0	1	0
0	0	1	0	0
0	0	1	1	0
0	0	2	0	0
0	0	2	1	0
0	1	0	0	1
0	1	0	1	0
0	1	1	0	0
0	1	1	1	0
0	1	2	0	0
0	1	2	1	0
0	2	0	0	1
0	2	0	1	1
0	2	1	0	1
0	2	1	1	0
0	2	2	0	0
0	2	2	1	0
1	0	0	0	1
1	0	0	1	0
1	0	1	0	0
1	0	1	1	0
1	0	2	0	0
1	0	2	1	0
1	1	0	0	1
1	1	0	1	1
1	1	1	0	1
1	1	1	1	0

1	1	2	0	0
1	1	2	1	0
1	2	0	0	2
1	2	0	1	1
1	2	1	0	1
1	2	1	1	0
1	2	2	0	1
1	2	2	1	0
2	0	0	0	1
2	0	0	1	1
2	0	1	0	1
2	0	1	1	0
2	0	2	0	0
2	0	2	1	0
2	1	0	0	2
2	1	0	1	1
2	1	1	0	1
2	1	1	1	0
2	1	2	0	1
2	1	2	1	0
2	2	0	0	2
2	2	0	1	2
2	2	1	0	2
2	2	1	1	1
2	2	2	0	1
2	2	2	1	1

Table A.10 Transition table for variable x_{10}

$x1(t)$	$x4(t)$	$x5(t)$	$x6(t)$	$x11\ (t+1)$
0	0	0	0	0
0	0	0	1	0
0	0	1	0	0
0	0	1	1	1
0	1	0	0	0
0	1	0	1	1
0	1	1	0	1
0	1	1	1	1
0	2	0	0	1
0	2	0	1	2

0	2	1	0	2
0	2	1	1	2
1	0	0	1	0
1	0	1	0	0
1	0	1	1	1
1	1	0	0	0
1	1	0	1	1
1	1	1	0	1
1	1	1	1	1
1	2	0	0	1
1	2	0	1	1
1	2	1	0	1
1	2	1	1	1
2	0	0	1	0
2	0	1	0	0
2	0	1	1	0
2	1	0	0	0
2	1	0	1	0
2	1	1	0	0
2	1	1	1	0
2	2	0	0	1
2	2	0	1	1
2	2	1	0	1
2	2	1	1	1

Table A.11 Transition table for variable x_{11}

$x12(t)$	$x12(t+1)$
0	0
1	1

Table A.12 Transition table for external variable x_{12}

$x13(t)$	$x13(t+1)$
0	0
1	1

Table A.13 Transition table for external variable x_{13}

Chapter 5

Concluding remarks

This chapter summarizes key findings and provides insight of future research directions in the field of host-fungal interactions. It also emphasizes the benefits of this interdisciplinary work, mathematical modeling, and the novelty of our approach to study the effects of *A. fumigatus* on airway epithelial cells.

5.1 Summary of findings

In the introduction, we established the foundation for the project focused on the examination of the effects of *A. fumigatus* on airway epithelial cells. We summarized the major findings related to the recognition of the fungus by the epithelial cell, the mechanisms employed by the fungus in the host-fungal interaction, iron requirements of both species, and the role of the immune response during fungal infections. We described transcriptional and proteomics studies in airway epithelial cells and/or *A. fumigatus*, and reviewed the conceptual and experimental limitations. We hope in future studies more research will evaluate the response of epithelial cells, especially from the perspective of the connection between innate immunity and iron regulation.

In Chapter 2, we analyzed the transcriptional response of airway epithelial cells to *A. fumigatus* at 2, 6, and 12 hours post-infection. This is the first study to investigate the co-incubation of airway epithelial cells with *A. fumigatus* conidia at multiple time points. We captured the behavior of the host transcriptome at three stages of infection: resting conidia, swollen conidia, and germinating conidia. The variation in gene expression of airway epithelial cells at each time point highlights the importance of refined time points in the host-fungal interaction. Using Ingenuity Pathway Analysis, GOrilla, and Pantherdb software to characterize the differentially expressed genes, we showed changes in the expression of genes related to the immune response, iron regulation, hypoxia and oxidative stress, and fungal recognition. We compared our lists of differentially expressed genes with two previous studies to determine the similarities and differences in our data set. We found some similarities, but, due to different cell line usage and only one time point, there were comparative limitations. We also surveyed the data to discover the genes with the highest fold change in differential expression comparing untreated airway epithelial cells to cells treated with *A. fumigatus* conidia at each time point. To confirm the changes observed in the RNA-seq data, the up-regulation of IL8, IL6, and DMT1 in *A. fumigatus* exposed airway epithelial cells, we used qRT-PCR to quantify the expression of these genes. The results showed an increase in the relative expression of those genes and the change in DMT1 is groundbreaking. The increase in the expression of DMT1 in airway epithelial cells in response to *A. fumigatus* is an innovative finding, and future work should investigate the cause and motivation for this change. This study was the platform for the inclusion of components like DMT1 in the mathematical model of the host-fungal interaction described in Chapter 4.

In Chapter 3, we examined the effects of the cytokines IL-8, IL-1β, and TNF-α on the iron importers, DMT1 and TfR1 in airway epithelial cells. The motivation for this work was the lack of a comprehensive study investigating the effect of cytokines on iron related genes. Previous work either examined the prolonged effect of a cytokine on one iron importer, or preferentially focused on the response of DMT1. Using all possible combinations of the three recombinant cytokines, we treated airway epithelial cells to determine their individual and combined effects. qRT-PCR was employed to quantify the gene expression of DMT1and TfR1 at 0, 2, 4, and 6 hrs. post-treatment. We concluded cytokines have a significant influence on DMT1 leading to a drastic increase in its expression, while TfR1 had little to no change in response to cytokines, and these changes are most likely via an NF-κB dependent pathway. Since TNF-α upregulates the NF-κB pathway, we observed the effects on the iron importers. We compared our findings with previous work and discovered similarities in DMT1 expression but contradictory data for the findings related to TfR1. These differences may be attributed to the use of BEAS2B cells versus A549 cells and using 6 hrs. as the latest time point opposed to 24 hrs. We used the results from this study to generate biological rules governing the behavior of DMT1 in response to cytokines. These rules were incorporated into a mathematical model of the airway epithelial cell and added much needed information to understand the dynamics of the immune response and iron metabolism in the host in response to fungi. Since DMT1 and TfR1 varied in response to cytokines, the effect of cytokines on the iron importer genes in airway epithelial cells in response to fungus may also be diverse.

In Chapter 4, we constructed a mathematical model of a human bronchial epithelial cell and the cascade of changes on iron importers using the literature, RNA-seq data, and observations from Chapter 3. The model connects the HIF1-α and NF-κB pathways incorporating transcription factors to link together the immune response genes with iron importer genes. Using the polynomial dynamical systems framework, we constructed a model focused on oxidative stress and innate immunity based on available oxygen levels and the presence/absence of *A. fumigatus*. The model made several predictions including an increase in cytokine expression, no change in the expression of the transcription factor HIF1-α, a decrease in the expression of the iron importer TFRC, and an increase in DMT1 expression. The lack of change in HIF1-α is expected and if we exposed the cells for a longer period of time with *A. fumigatus* conidia, hypoxic environment is induced. The change in IL-6 and IL-8 has been shown in previous studies, but this work is the first to investigate the effect of *A. fumigatus* on IL-1β. The model predicted an increase in IL-1β expression and also in the growth factor gene, VEGFA. VEGFA has been studied in other host-pathogen interactions but not in the host-fungal interaction; therefore, this model prediction is novel. All of the listed model predictions were confirmed using qRT-PCR. In addition, to discern if it is truly the immune response which modifies iron import via TFRC during fungal infection, we used siRNA to reduce the efficiency of the IL-1β gene in airway epithelial cells and treated the cells with *A. fumigatus* conidia. The reduction in IL-1β expression lead to an increase in TFRC expression in the presence of *A. fumigatus* at 12 hrs., suggesting IL-1β has an inhibitory effect on TFRC during fungal exposure. This evidence supports the argument that the immune response is activated in response to fungus and causes downstream changes in iron regulation, in our case iron importers, for airway epithelial cells.

The mathematical model provides valuable insight into the effects of *A. fumigatus* on bronchial epithelial cells. Some may wonder why a mathematical model was constructed and why not focus solely on the changes to a subset of genes when a stimulus, such as fungus, is present. The

answer to this question is the model incorporates several findings and without inclusion of known or newly discovered interactions between components of the system, it is difficult to predict the dynamics of the system. If previous work has not examined a node of interest, one might not anticipate scientific discoveries. Although this project targeted DMT1 and TFRC, this model revealed original findings about other model components. With a model, the network components can be perturbed to simulate a wet lab experiment, and the results determine the validity and value of the model findings. An airway epithelial cell model is applicable to other research areas such as airway inflammation, asthma, hypoxia, and the influence of particulates. The model requires minimal modification for application to these and other areas of interests.

This dissertation used several methods including RNA-seq analysis, qRT-PCR, recombinant cytokine experiments, fungal treatment experiments, and mathematical modeling to elucidate information related to the effect of *A. fumigatus* on airway epithelial cells and the relationship between the immune response and iron regulation (Figure 5.1). Our findings establish solid preliminary evidence about the host-fungal interaction and support the value of examination of the role of airway epithelial cells.

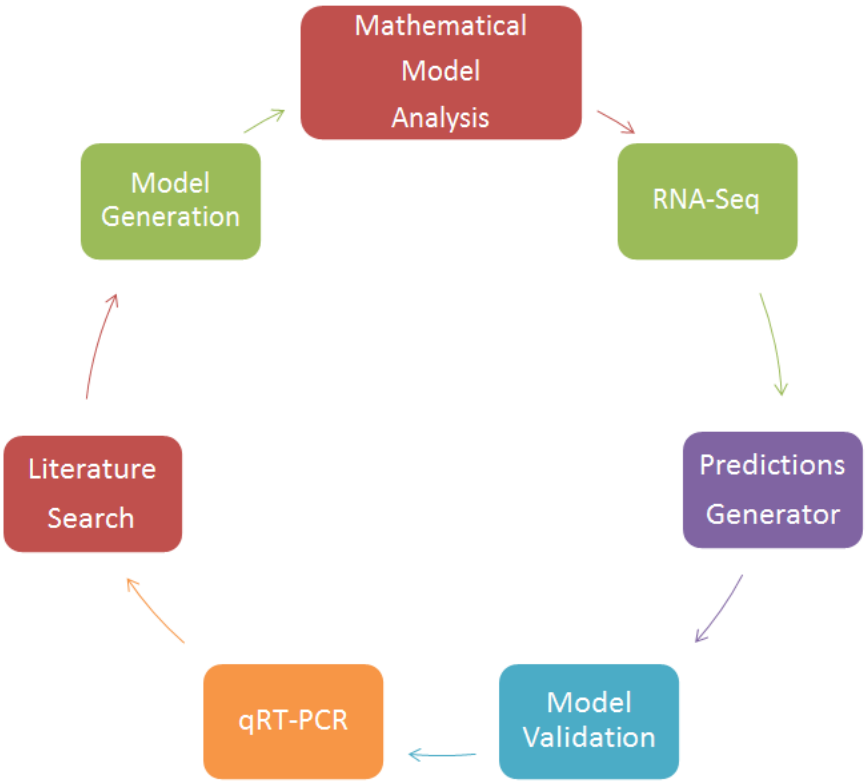

Figure 5.1: Summary of project methodology. The method includes an extensive literature search, creation of a mathematical model, RNA-seq analysis, generating model predictions, and using experimental techniques to confirm the predictions of the model.

5.2 Directions for future research

The host-fungal interaction is an exciting research area due to the multiple layers of complexity of involved organisms. The methodology used in this dissertation is applicable to other cell types and pathogens. Although we shed light on key mechanistic responses in airway epithelial cells to *A. fumigatus* using bioinformatics, computational modeling, and experimental biology, there are still additional areas of research needed. The availability of sequencing and visualization technologies expands the repertoire of information regarding the host-fungal interaction.

This dissertation focused solely on iron import, but it would be useful to expand the analysis to other iron regulatory components, such as the iron exporter and storage molecule. This allows for a larger picture of the distribution of iron inside and outside the cell during fungal infection. It helps generate a comprehensive idea of the motivation of the airway epithelial cell when responding to *A. fumigatus*. We also consider incorporation of the airway epithelial cell model with a current model of iron metabolism in breast cancer cells. This model describes the core intracellular iron network including import, export, and storage mechanisms. Many of the components in our mathematical model are important in other pathways, so model expansion is useful. Even though there are similarities and differences in the cell types, compiling a workable mathematical model of iron regulation is novel and beneficial for both the cell biology and fungal biology communities.

The significant changes in DMT1 expression are exciting from the immunological viewpoint. A future study can determine which transcription factors induced by cytokines work synergistically in the binding of the DMT1 transcript. An expansion of this concept is to examine the post-transcriptional, translational, and post-translational changes in airway epithelial cells post treatment with cytokines. Many iron components are regulated post-transcriptionally so this may or may not reduce the observed transcriptional response.

From the therapeutics perspective, drug resistance is a growing topic of interest for *A. fumigatus*. One future direction is to examine the effects of the drug, dexamethasone, on iron related components during *A. fumigatus* infection. It has been shown that dexamethasone increases *A. fumigatus* invasiveness, but perhaps that may be due to changes in iron levels causing the fungus to take iron from the only available source. Many drugs have unknown targets so the investigation of the change in iron levels, may suggest alternative therapeutic targets.

There are numerous research possibilities in this area of study ranging from the host side to the pathogen side. One can focus on another shared feature besides iron metabolism such as copper transport or even refine the methods used in this dissertation. I hope to continue working in the host-pathogen interaction field by incorporating both computational and experimental approaches to extract information about the cellular response to stimuli.