THE ROLES OF INTERLEUKIN-1 AND LEUKOTRIENE-B4 IN THE INNATE IMMUNE RESPONSE TO PULMONARY *ASPERGILLUS FUMIGATUS* INFECTION

by

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ABSTRACT

Aspergillus fumigatus is a ubiquitous environmental mold, and even though most individuals are regularly exposed to fungal spores, clinical invasive disease is a rare manifestation. However, in the growing population of individuals with weakened immune systems, for example due to prolonged corticosteroid treatment or chemotherapeutic interventions, *A. fumigatus* exposure can cause severe, invasive aspergillosis (IA). Overall, invasive fungal infections are estimated to kill at least 1.5 million people annually (Brown et al. 2012), with IA being the most common and deadly invasive respiratory fungal infection. Thus, it is critical to better understand the host-pathogen interactions after *A. fumigatus* exposure in order to develop novel treatment options which harness the power of the host's immune response. Defining key immunological events that are needed for the prevention of *Aspergillus* growth within the pulmonary environment of immune competent individuals is an essential step toward a better understanding of how the immune response is altered within the immune compromised populations that are at risk of developing IA. Utilizing an immune competent murine model of IA, we have shown that signaling through both the Interleukin-1 receptor, type I (IL-1RI) and the Leukotriene B4 receptor (BLT1) are both critical pathways for host resistance against IA through timely neutrophil recruitment which ultimately control fungal germination. More recently, we have found that different environmental and clinical strains of *A. fumigatus* lead to different inflammatory profiles as well as different disease pathology. Strains that are able to germinate within the lung environment are more virulent, and lead to enhanced lung damage, vascular leakage and inflammation. Furthermore, the more virulent strains induce neutrophil recruitment and subsequent fungal clearance that is dependent on the alarmin IL-1α, while clearance of the less virulent strains are independent of IL-1α signaling. With this research we will better understand the fungal component(s) that are important in virulence determination, which immune pathways are contributing to the different disease pathologies observed, as well as understand the mechanism through which a healthy immune system can resist *A. fumigatus* exposure on a daily basis.

CHAPTER ONE

INTRODUCTION

Aspergillus fumigatus is a ubiquitous mold that humans inhale on a daily basis. For the majority of the population, breathing in *A. fumigatus* conidia does not lead to disease. However, immune compromised individuals that lack a sufficient anti-*Aspergillus* immune response are at increased risk of developing invasive aspergillosis (IA), a disease of high morbidity and mortality. Due to diagnosis difficulties, a limited repertoire of antifungal drugs, and a lack of effective antifungal vaccines (Segal 2009; Brown et al. 2012) the mortality rate of IA remains between 30-70% (Steinbach et al. ; Cornillet et al. 2006; Upton et al. 2007; Garcia-Vidal et al. 2008; Thompson and Patterson 2008; Baddley et al. 2010). One area of research that has great potential to improve treatment options for IA is immunotherapy. Gaining an understanding of the immune pathways that mediate protection from invasive fungal growth in the lungs is a critical step toward understanding how these pathways are altered in different immune-compromised populations.

The purpose of this chapter is to provide a detailed overview of the key features of *A. fumigatus* that contribute to its ability to cause disease in immune compromised populations, as well as what is currently known concerning the host immune factors leading to host resistance against IA. Overall, the aim of this dissertation is to understand the innate immune pathways that mediate neutrophil recruitment to the airways and how these pathways impact overall disease outcome. The results presented here provide novel

insights into the roles that the Interleukin-1 (IL-1) and Leukotriene (LT) pathways play in limiting fungal growth in the pulmonary environment. Further, these results demonstrate the importance of considering heterogeneity among *A. fumigatus* isolates/strains when conducting studies to analyze pathogenicity and immunity to infectious disease.

Aspergillus fumigatus

Aspergillus species are filamentous fungi that are ubiquitous in the environment and found prevalently in soil and on decaying organic matter where they play an important role in recycling environmental carbon and nitrogen sources (Latgé 1999; Brakhage and Langfelder 2002). There are over 200 known *Aspergillus* species, however *A. fumigatus* accounts for greater than 90% of human infections (Bodey and Vartivarian 1989; Latgé 1999; Dagenais and Keller 2009; Krappmann 2016). The infectious propagules of *A. fumigatus* are the conidia, which are highly hydrophobic and dispersed readily into the air with little agitation (Kwon-Chung and Sugui 2013). *A. fumigatus* is thermotolerant and can withstand a wide range of environmental conditions and stresses, which is thought to contribute to its prevalence as an opportunistic pathogen compared to other environmental molds (Bhabhra and Askew 2005). Growth of *A. fumigatus* occurs between 12° and 65°C, which is thought to be above the upper temperature limit that facilitates eukaryotic growth (Tansey and Brock 1972; Kozakiewicz and Smith 1994; Bhabhra and Askew 2005). Although growth may be halted under conditions outside of this range, *A. fumigatus* conidia have the ability to survive under extreme conditions, including in temperatures up to 70°C, long-term freezing, and long-term dehydration

(Kozakiewicz and Smith 1994; Latgé 1999; Kwon-Chung and Sugui 2013). Moreover, *A. fumigatus* can survive in a pH range between 2.1 and 8.8 (Jensen 1931).

Under optimal conditions, conidia can undergo polarized growth and form tubular extensions called hyphae, which can continue to branch into a network of filaments known as a mycelium (Adams et al. 1998). From the mycelium, aerial branches of hyphae emerge that can subsequently develop into condiophores, which are the conidia-producing stalk structures of *Aspergillus* species (Adams et al. 1998). From the conidiophore, thousands of conidia can develop, disperse into the air, and once the conidia reach a suitable substrate this growth process can begin again (Adams et al. 1998). The small size of *A. fumigatus* conidia, ranging from 2-3 μm in diameter, allow them to easily gain access to the lower airways and it is thought that the high density of sialic acid residues on the outer surface of these conidia enable them to adhere to the epithelium more efficiently than other species of fungal conidia with a similar size (Wasylnka et al. 2001; Kwon-Chung and Sugui 2013). In individuals with a sufficient immune response, these conidia will be cleared from the body without causing significant disease; however, in individuals who are immune compromised or those with primary immunodeficiencies, conidia can germinate, form hyphae and lead to invasive disease with high levels of pulmonary damage and potential dissemination to other organs (Dagenais and Keller 2009).

Disease Spectrum

A. fumigatus is considered an opportunistic pathogen and typically causes disease in hosts with an underlying immune deficiency. Clinically, pulmonary aspergillosis can manifest as a spectrum of diseases depending on the host immune status. On one end of the spectrum, and the focus of this dissertation, is Invasive Aspergillosis (IA), which predominantly affects the severely immune compromised population (Soubani and Chandrasekar 2002; Dagenais and Keller 2009; Kosmidis and Denning 2014). Chronic pulmonary aspergillosis (CPA) occurs in individuals that are not severely immune compromised, but that have an underlying pulmonary condition such as chronic obstructive pulmonary disease (COPD) or prior tuberculosis infection (Kosmidis and Denning 2014). On the other end of the spectrum is allergic bronchopulmonary aspergillosis (ABPA), which is hypersensitivity to *Aspergillus* antigens and affects patients with asthma and cystic fibrosis (CF) (Soubani and Chandrasekar 2002; Patterson and Strek 2010; Kosmidis and Denning 2014). Below I will briefly discuss each of these forms of aspergillosis in terms of risk factors, diagnosis, treatment options and prognosis, with a major focus on IA.

Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA most commonly affects individuals with asthma or CF, and disease is thought to be driven by a shift in balance from a protective Th1 response to an allergic Th2 response (Patterson and Strek 2010). In these patients, there is a defect in conidial clearance from the airways which leads to fungal germination and antigen exposure. In

an allergic host this persistent antigen exposure leads to T cell activation, IgE production, eosinophilia and local inflammation which in turn leads to airway hyperresponsiveness and increased mucus production (Patterson and Strek 2010; Kosmidis and Denning 2014). Diagnosis of ABPA can be challenging and although there is no single confirmatory test, diagnostic criteria include consideration of clinical symptoms, serum total/*Aspergillus* specific-IgE levels, serum eosinophil counts, and skin testing for hypersensitivity to *Aspergillus* (Patterson and Strek 2010; Kosmidis and Denning 2014). Treatment of ABPA is critical to prevent permanent damage to the airways and bronchiectasis. Oral corticosteroids to dampen the immune response are the main treatment for patients with ABPA, however because steroids do not target fungal growth and antigen exposure, steroid treatment is normally needed long-term (Patterson and Strek 2010; Kosmidis and Denning 2014). Recently, use of antifungal drugs concurrently with corticosteroid treatment has been associated with improved lung function in ABPA patients (Patterson and Strek 2010; Chishimba et al. 2012; Kosmidis and Denning 2014; Moreira et al. 2014).

Chronic Pulmonary Aspergillosis (CPA)

CPA is a non- or semi-invasive form of aspergillosis which mainly manifests in non-immune compromised individuals that have underlying pulmonary disease, or in mildly immune compromised individuals (Kosmidis and Denning 2014; Schweer et al. 2014; Denning et al. 2016). There is a diverse set of risk factors that predispose individuals to the development of CPA, but for the most part it occurs in individuals possessing pulmonary cavities due to underlying structural damage, which can occur

from prior or concurrent mycobacterial infection, COPD, ABPA, pneumothorax, treated lung cancer or sarcoidosis (Franquet et al. 2000; Hafeez et al. 2000; Denning et al. 2003; Hasse et al. 2005; Kobashi et al. 2005; Kobashi et al. 2006; Kunst et al. 2006; Felton et al. 2010; Pena et al. 2011; Smith and Denning 2011; Denning et al. 2013; Kosmidis and Denning 2014; Schweer et al. 2014; Denning et al. 2016). Risk factors for mild immune suppression include alcoholism, corticosteroid use and diabetes (Binder et al. 1982; Tasci et al. 1999; Agusti et al. 2003; Denning et al. 2003; Schweer et al. 2014). CPA can manifest as simple aspergilloma, chronic cavitary pulmonary aspergillosis (CCPA), *Aspergillus* nodule and subacute invasive aspergillosis (SAIA) (Kosmidis and Denning 2014; Denning et al. 2016). An aspergilloma is a fungal mass comprised of fungal hyphae, extracellular matrix, mucus and cellular debris that forms within a pulmonary cavity (Kosmidis and Denning 2014; Denning et al. 2016). Symptoms of CPA include weight loss, fatigue, chronic cough, hemoptysis and breathlessness. A diagnosis of simple aspergilloma is made when a single cavity contains a single fungal ball that remains stable for a period of 3 months, with minor or a complete absence of symptoms (Kosmidis and Denning 2014; Denning et al. 2016). When multiple pulmonary cavities are present either with or without aspergilloma, along with the presence of pulmonary and/or systemic symptoms, then the patient is diagnosed with CCPA (Kosmidis and Denning 2014; Denning et al. 2016). A condition known as chronic fibrosing pulmonary aspergillosis (CFPA) can develop as an end result of untreated CCPA, which is extensive fibrosis that leads to significant loss of lung function (Kosmidis and Denning 2014; Denning et al. 2016). *Aspergillus* nodules are a less common manifestation of CPA and

are currently not well understood. Many times these nodules are discovered during a CT scan, having an appearance similar to malignant lesions and it is not until a biopsy is performed that it is revealed to be an *Aspergillus* nodule (Kosmidis and Denning 2014; Denning et al. 2016; Muldoon et al. 2016). SAIA, also referred to as chronic necrotizing aspergillosis, manifests in patients with mild immune suppression and is similar to CCPA, however disease progresses much more rapidly and tissue invasive hyphal growth can be observed upon biopsy (Kosmidis and Denning 2014; Denning et al. 2016).

Although ABPA and CPA are not the main focus of this dissertation, this disease spectrum demonstrates the diverse environments that *Aspergillus* can thrive in and how critical the immune host status is in the ability to prevent fungal disease.

Invasive Aspergillosis (IA)

IA is the most severe form of aspergillosis, mainly affecting individuals who are severely immune compromised and characterized by invasive fungal growth into lung tissue. There are different populations of individuals at risk for IA and the type of immune suppression dictates the pathology of disease (Berenguer et al. 1995; Balloy et al. 2005; Stergiopoulou et al. 2007; Dagenais and Keller 2009).

Risk Factors. The classic risk factor for developing IA is prolonged neutropenia associated with hematological malignancies, allogeneic hematopoietic stem cell transplantation (HSCT), solid organ transplant (SOT), and chemotherapy treatment (Gerson et al. 1984; Dagenais and Keller 2009; Nucci and Anaissie 2009; Bhatt et al. 2011; Kosmidis and Denning 2014). The risk of developing IA further increases with

repeated cycles of neutropenia and with concurrent corticosteroid treatment (Segal et al. 2002; Gregg and Kauffman 2015). Disease in neutropenic hosts is characterized by rapid fungal growth within the host, resulting in angioinvasion and potential dissemination to other organs (Dagenais and Keller 2009; Kosmidis and Denning 2014). IA in neutropenic patient populations progresses rapidly, within days to weeks (Kosmidis and Denning 2014). Disease in the non-neutropenic population is characterized by limited fungal growth without angioinvasion, but in the presence of robust inflammation and necrosis that result in high levels of tissue damage (Dagenais and Keller 2009; Kosmidis and Denning 2014). Unlike the neutropenic population, disease typically progresses more slowly in the non-neutropenic population, occurring over weeks to months (Kosmidis and Denning 2014). The most common risk factor for development of IA in non-neutropenic hosts is treatment with high-dose corticosteroids (Kosmidis and Denning 2014), although calcineurin inhibitors which are used in conjunction with corticosteroid treatment in SOT patients, have recently been implicated in increased IA susceptibility (Shah et al. 2013; Herbst et al. 2015; Shah et al. 2016). Although HSCT and SOT recipients can be neutropenic in the early stages of the transplant process, they also have an increased risk of developing IA later during transplantation while severely immune suppressed for the prevention/treatment of graft versus host disease (GVHD) or transplant rejection (Nucci and Anaissie 2009). Severe GVHD in itself is also a risk factor for the development of IA (Nucci and Anaissie 2009). Other non-neutropenic hosts that have an increased risk of developing IA include patients with AIDS, COPD, or liver failure, patients in the intensive care unit (ICU), and individuals with primary

immunodefiencies that alter antifungal effector functions (chronic granulomatous disease), pattern recognition receptor signaling pathways (mutations in Dectin-1 and CARD9), or inflammatory pathways (mutations in CXCL10 and STAT3) (Mylonakis et al. 1998; Mezger et al. 2008; Cunha et al. 2010; Vinh et al. 2010; Falcone et al. 2011; Ben-Ari et al. 2012; Dimopoulos et al. 2012; Tutar et al. 2013; Kosmidis and Denning 2014; Rieber et al. 2016). More recently, it has been recognized that patients in the ICU with severe influenza infection have a significant risk of developing IA (Garcia-Vidal et al. 2011; Crum-Cianflone 2016; van de Veerdonk et al. 2017). In rare instances, immune competent hosts have developed IA after exposure to a large amount of *Aspergillus* conidia (Meeker et al. 1991; Arendrup et al. 2006; Shimaoka et al. 2006; Butler et al. 2013; Kosmidis and Denning 2014; Ratermann et al. 2014).

Diagnosis. Diagnosis of IA has historically been problematic and delayed diagnosis is one of the factors that contribute to the high mortality rate of this disease. Symptoms are non-specific and patients are often initially treated unsuccessfully with antibiotics when they present with a fever (Barton 2013). Complicating this is the fact that *Aspergillus* is rarely able to be cultured from blood, and culture from the bronchoalveolar lavage (BAL) fluid or lung tissue is insensitive (Barton 2013). If culture is successful, further distinguishing between infecting or colonizing fungi is difficult (Barton 2013). Thus, diagnosis of IA is achieved through a combination of methods including culture methods, computerized tomography (CT) imaging, microscopy, histopathology, antigen detection and DNA detection through polymerase chain reaction (PCR) (Chamilos and Kontoyiannis 2006; Barton 2013). While CT imaging, microscopy

techniques and histopathology can be indicative of an invasive fungal infection, they lack specificity and differentiating between IA and other filamentous fungal infections can be difficult (Chamilos and Kontoyiannis 2006; Barton 2013). Furthermore, histopathology requires that a biopsy sample be available, however biopsy procedures are risky in these patients due to thrombocytopenia and other medical risk factors associated with being immune suppressed (Soubani and Chandrasekar 2002; Barton 2013). Methods of antigen detection include measuring fungal cell wall components such as galactomannan (GM) or 1,3 ß-D-Glucan (BDG) in the serum or BAL (Barton 2013). While GM is relatively specific for IA diagnosis, GM can only be detected in the serum upon angioinvasion which is more characteristic of IA in the neutropenic population than the non-neutropenic population (Barton 2013). A BDG-positive test lacks specificity and can be indicative of other invasive fungal infections including candidiasis and histoplasmosis (Barton 2013). Using PCR for the detection of *Aspergillus* nucleic acids has the advantage of being highly sensitive and more rapid than culture-based methods (Barton 2013; Lamoth and Calandra 2017). For nearly two decades, the use and validation of PCR in the diagnosis of IA was limited due to a lack of standardized techniques (White et al. 2010; Barton 2013; Lamoth and Calandra 2017). The development of the European *Aspergillus* PCR Initiative (EAPCRI) in 2006 led to the comparison of PCR protocols from 23 different centers, and the publication of recommendations to improve PCR-diagnosis of IA (White et al. 2010; White et al. 2011). Importantly, the majority of PCR testing has been done in neutropenic patients with hematological malignancies and the success of this method for diagnosis in non-angioinvasive forms of IA remains unknown (Lamoth and Calandra

2017). Altogether, diagnosis of IA is a complex process since no single method possesses sufficient sensitivity and specificity for a positive diagnosis. Because of this, multiple approaches must be utilized and many factors considered in the diagnosis of IA.

Treatment. Treatment options for IA are limited due to a limited repertoire of antifungal drugs and toxic side effects of those available. The main groups of antifungal used in the treatment of IA are the triazoles, the polyenes and the echinocandins (Walsh et al. 2008; Patterson et al. 2016). Primary recommended treatment is with the triazole voriconazole, which targets erogsterol biosynthesis, preventing fungal cell growth or inducing fungal cell death (Walsh et al. 2008; Patterson et al. 2016). Other triazoles approved for treatment of IA are isavuconazole, itraconazole and posaconazole (Walsh et al. 2008; Patterson et al. 2016). Drug-drug interactions are a serious problem with triazole treatments and review of the patient's concurrent medications should be cautiously evaluated (Walsh et al. 2008; Patterson et al. 2016). The polyenes and echinocandins are generally used as a second line of treatment, and it is thought that combined use of either triazoles or polyenes with echinocandins may have a synergistic effect during treatment, however this is not completely agreed on (Walsh et al. 2008; Patterson et al. 2016). Amphotericin B, a commonly used polyene, acts by binding ergosterol and forming ion channels that induce fungal cell death, and is associated with high levels of toxicity and severe side effects (Walsh et al. 2008; Patterson et al. 2016). Echinocandins, such as caspofungin, act by interfering with synthesis of the cell wall component 1,3-ß-glucan and ultimately disrupting the cell wall strength and osmotic balance (Walsh et al. 2008; Patterson et al. 2016). A minimum treatment period of 6-12

weeks and, if feasible, reducing or removing immune suppressive regimens are recommended in the treatment of IA (Walsh et al. 2008; Patterson et al. 2016). Most of the available antifungal drugs, with the exception of Amphotericin B and in some studies voriconazole, are fungistatic rather than fungicidal, suggesting that immune modulation to strengthen antifungal immunity in combination with these drugs may be of therapeutic value (Lewis and Graybill 2008). Immune modulation with colony stimulating factors such as granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), and potentially with Interferon-γ (IFN-γ) are recommended for the prevention of IA through the reversal of neutropenia and/or increasing the antifungal effector mechanisms of phagocytic cells (1991; Rowe et al. 1995; Roilides et al. 1998; Ezekowitz 2000; Stevens 2006; Walsh et al. 2008). Overall, early diagnosis of IA is critical to the success of treatment, and future work should explore combinatorial treatment avenues to increase the effectiveness of current treatment options.

Antifungal Resistance. Resistance to antifungal drugs is an emerging issue in *Aspergillus* species, however due to a lack of routine susceptibility testing of isolates, the true prevalence and impact of resistance during IA remains unknown (Arendrup 2014). Species besides *A. fumigatus* that in rare cases can cause IA, such as *A. terreus* and *A. flavus*, are known to have intrinsic resistance or reduced susceptibility to Amphotericin B (Arendrup et al. 2012; Arendrup 2014). Infections caused by isolates of *A. fumigatus* that are resistant to azole treatment have been reported in several countries. The main mechanism of azole resistance is through mutations in the *cyp51A* gene, which encodes

the target for azole binding, however it is thought that there are additional mechanisms such as overexpression of drug efflux pumps (Nascimento et al. 2003; Susan et al. 2009). Azole-resistant infections can develop in patients who were previously treated with azoles (Susan et al. 2009). Additionally, it is thought that environmental use of azoles in agriculture has contributed to the rise in resistant IA infections (Verweij et al. ; Mortensen et al. 2010). Overall, it is estimated that the prevalence of azole resistance in *Aspergillus* is 3-6% (Arendrup 2014).

Prognosis and Patient Outcome. Data from the last decade suggest that although the outcome of patients with IA has improved compared to data from the 1990's, overall mortality rates still remain between approximately 30-70% (Cornillet et al. 2006; Upton et al. 2007; Nivoix et al. 2008; Neofytos et al. 2009; Ramos et al. 2011; Garcia-Vidal et al. 2015). In the event of missed or delayed diagnosis, the mortality rate approaches 100% (Brown et al. 2012). One study comparing neutropenic and non-neutropenic IA cases over a 6-year period demonstrates that the non-neutropenic population has a significantly higher mortality rate (89%) than the neutropenic population (60%) (Cornillet et al. 2006). This was attributable to the fact that non-neutropenic patients were less likely to show symptoms and were less closely monitored for IA than neutropenic patients, leading to delayed diagnosis in which antifungal drug treatment is less effective (Cornillet et al. 2006). In this particular study, the GM serum test was equally sensitive in the neutropenic and non-neutropenic patients and mycological examination of BAL fluid was more sensitive in non-neutropenic patients (Cornillet et al. 2006). As non-neutropenic hosts are being recognized as a major population at risk for

IA, close attention to a wide range of risk factors should be considered and diagnostic screening performed in order to increase the likelihood of early diagnosis and successful treatment.

Murine Models of IA. In order to better understand the host and pathogen factors that contribute to the development and progression of IA, *in vivo* models of disease are critical. There are several murine models designed to mimic the immunosuppression found in human populations that are at increased risk for IA, including the neutropenic and chemotherapeutic models, corticosteroid immune-suppressed model, and the CGD model.

In the chemotherapy model of IA, leukocytopenia in mice is induced by using a combination of cyclophosphamide or other chemotherapeutic agents, with corticosteroid treatment to mimic treatment in humans (Smith et al. 1994; Stephens-Romero et al. 2005). Neutropenic models have also utilized the monoclonal antibodies RB6-8C5 and 1A8, however while 1A8 binds Ly6G, a marker specific for neutrophils, RB6-8C5 is less specific and binds both Ly6G and Ly6C, the latter of which is found on neutrophils, monocytes and some $CD8^+$ T cells (Daley et al. 2008; Carr et al. 2011). In each of these models, disease is characterized by high fungal burden and extensive pulmonary tissue invasion due to a lack of leukocyte recruitment to the site of infection (Balloy et al. 2005).

Corticosteroid-induced immunosuppression in mice is used to mimic the non-neutropenic patient population. Mice can be treated with a number of corticosteroids, which result in immunosuppression through several different mechanisms, including

increasing transcription of anti-inflammatory genes such as interleukin-10 (IL-10), IL-1 receptor antagonist (IL-1ra) and lipocortin-1 and through the inhibition of NF-κB activation (Barnes 1998; Barnes 2006). Ultimately, corticosteroid treatment results in the impairment of both macrophage and neutrophil antimicrobial activity (Schaffner 1985; Barnes 1998; Mircescu et al. 2009). Interestingly, it was shown that *in vitro* exposure of *A. fumigatus* and *A. flavus* to hydrocortisone resulted in significantly increased fungal growth, suggesting corticosteroid treatment of the host could be advantageous to the fungi (Ng et al. 1994). Similar to IA in non-neutropenic humans, disease in the corticosteroid murine model is characterized by tissue necrosis and high levels of tissue damage, with limited fungal growth (Balloy et al. 2005; Dagenais and Keller 2009).

Another model that is characterized by high levels of immune pathology is the CGD model of IA (Romani et al. 2008). CGD is a genetic disorder in which phagocytes are unable to generate reactive oxidants due to mutations in the gene coding for NADPH oxidase (Segal and Romani 2009). CGD patients are at high risk for recurrent bacterial and fungal infections, with IA being the most common cause of filamentous fungal infection in this population (Segal and Romani 2009). The CGD murine models were developed by genetic deletion of the p47 ($p47^{phox-/-}$) or gp91 ($gp91^{phox-/-}$) subunits of the NADPH oxidase complex, both of which result in mice with increased susceptibility to bacterial and fungal infections due to an inability to produce superoxide and related species (1995; Pollock et al. 1995).

Finally, the model used most commonly throughout this dissertation is the immune competent model, which utilizes a high dose inoculum in order to induce

disease. Different transgenic mouse models can be employed to test the contribution of specific cytokines and immune pathways in the host resistance against IA. Eventually this work can be used to determine how these pathways are altered in different immune compromised populations in order to identify novel targets for immunomodulatory therapeutic intervention.

Although each of these models have been invaluable in progressing our knowledge of fungal immunology, it is important to acknowledge the limitations and factors to carefully consider while analyzing murine studies of pathogenicity. One major factor to consider during interpretation of host-pathogen interactions during IA is the status of the host immune system. Importantly, utilizing multiple murine models can give a more complete understanding of the role of specific fungal factors or host factors in disease progression. For example, use of both the corticosteroid model and chemotherapeutic model have demonstrated that gliotoxin contributes to virulence and pathogenicity in corticosteroid-treated mice, but not in neutropenic mice (Bok et al. 2006; Cramer et al. 2006; Sugui et al. 2007; Kupfahl et al. 2008; Spikes et al. 2008). Furthermore, some strains of inbred mice are intrinsically more susceptible to IA than others, however this has emerged as a valuable tool to identify single nucleotide polymorphisms (SNPs) that result in enhanced susceptibility to IA (Zaas et al. 2008). In one specific study, authors compared ten inbred mouse strains with varied susceptibility to IA and were able to find an association between SNPs in the plasminogen gene and susceptibility to IA, which they further analyzed in a human cohort of HSCT patients (Zaas et al. 2008). Plasminogen SNPs in this specific human population were associated

with an increased susceptibility to IA, however more studies are needed to determine clinical implications of the finding (Zaas et al. 2008). Thus, although careful consideration should be taken when using different "wild-type" mouse strains, this type of platform can be useful for the identification of genetic factors associated with susceptibility to IA. Other important factors to consider when analyzing pathogenicity studies in murine models is the inoculum dose, route of inoculation and fungal strain used.

Aspergillus fumigatus: Fungal Factors Contributing to Pathogenesis

A. fumigatus possesses several important characteristics that contribute to its saprophytic lifestyle, and these traits are thought to contribute to its ability to adapt to and survive within the mammalian host. These fungal characteristics are discussed below.

Tolerant to Wide Range of Environmental Conditions

As described previously, *A. fumigatus* is considered thermotolerant and can survive a wide temperature range (Kozakiewicz and Smith 1994; Latgé 1999; Bhabhra and Askew 2005; Kwon-Chung and Sugui 2013). The optimal temperature for *A. fumigatus* is 37°C, which provides a fitness advantage for growth within the mammalian host (Kwon-Chung and Sugui 2013). Only a handful of genes have been identified in *A. fumigatus* that contribute to thermotolerant growth and virulence in a murine host, but future work aims to determine whether these genes and associated pathways can be targeted in order to inhibit growth at 37°C (Sales-Campos et al. 2013).

A. fumigatus has the ability to thrive under a broad pH range which is also thought to contribute to its ability to adapt to the host environment, especially the host phagolysosome (Jensen 1931; Dagenais and Keller 2009). The transcription factor PacC has been shown to be important in adaptation to alkaline conditions, such as that experienced within the host environment, and is required for the tissue invasive phenotype and full virulence of *A. fumigatus* in a neutropenic murine model (Dagenais and Keller 2009; Bertuzzi et al. 2014). In *A. nidulans* it was shown that PacC controls the production of several secreted proteases which may have a role in tissue invasion and virulence, however as described below, relevance of proteases *in vivo* has been difficult to study and remains to be determined (Bignell et al. 2005). Genetic deletion of *pacC* from *A. fumigatus* negatively affected 75 uncharacterized secreted gene products (Bertuzzi et al. 2014). Thus, exploration of these secreted gene products may reveal specific roles for PacC-dependent protease production that impacts tissue invasiveness and virulence during IA.

Although *A. fumigatus* is considered an obligate aerobe, it is capable of growth in oxygen levels as low as 0.1% (Park et al. 1992; Hall and Denning 1994; Grahl et al. 2011). Dynamic oxygen levels in compost heaps (Wang et al. 2007), the natural environment for *A. fumigatus*, likely contributed to the evolution of mechanisms to adapt to low oxygen stress. It has been shown in three distinct murine models that hypoxic microenvironments form within the lung during IA, and that the hypoxic fitness of *A. fumigatus* strains positively correlates with virulence in the corticosteroid model of IA (Grahl et al. 2011; Kowalski et al. 2016). Among other functions, the transcription factor

SrbA has been shown to be critical for *A. fumigatus* adaptation to hypoxia, and the *srbA*-genetic null mutant is completely avirulent in multiple murine models of IA (Willger et al. 2008). Early during infection with this mutant, fungal growth occurs within the pulmonary environment, however once a hypoxic environment forms during the progression of IA disease, the *srbA*-null mutant is unable to adapt and maintain invasive growth (Willger et al. 2008). In this case, it is thought that the growth defect is fungistatic rather than fungicidal and it is hypothesized that what effector cells do remain functional within the lung after immune-suppression are able to clear the "static" fungi (Willger et al. 2008). This is supported by the fact that neutropenic mice infected with the *srbA*-null mutant were able to clear all fungi from the lungs by 14 days post-infection (Willger et al. 2008). Conversely, CGD mice infected with the *srbA*-null mutant were unable to clear the fungi from the lungs, although disease progression was halted likely due to the inability of the mutant to thrive in the hyperinflammatory, hypoxic pulmonary environment (Willger et al. 2008). This strengthens the idea that a combination of antifungal drug regimens combined with immune-modulatory treatments may provide a benefit in the treatment of IA.

Properties of Resting Conidia

The small size of *A. fumigatus* conidia, ranging in size from 2.0-3.0 μm in diameter, allows them to bypass the mucociliary escalator and gain access to the lower airways (Kwon-Chung and Sugui 2013). The outer surface of conidia are covered in a hydrophobic proteinaceous layer, known as the rodlet layer, which serves to protect conidia from external assaults and also facilitates conidial dispersion in the environment

(Paris et al. 2003; Bayry et al. 2012; Kwon-Chung and Sugui 2013). This rodlet layer is an immunologically inert molecule and masks the fungal cell wall components that would otherwise induce an inflammatory response, thereby preventing immune recognition and activation (Aimanianda et al. 2009). Once the germination process is initiated in resting conidia the rodlet layer is shed, exposing the underlying polysaccharide cell wall components to the external environment (Aimanianda et al. 2009). Another component found on the surface of resting conidia is the lectin, FleA, which macrophages can recognize to mediate clearance of fungi prior to conidial swelling (Kerr et al. 2016).

A second protective component on the conidial surface is dihydroxynaphthalene (DHN)-melanin, which gives *A. fumigatus* conidia their characteristic gray-green pigment and protects the fungal genome from UV damage. DHN-melanin contributes to the hydrophobicity and negative charge of conidia, both of which impact the ability of conidia to bind to extracellular matrix proteins within the lung (Pihet et al. 2009; Bayry et al. 2014). Utilizing environmental and clinical isolates with mutations in genes of the melanin pathway, Pihet *et al*. demonstrated that melanin is required for the correct assembly of the different layers of the conidial cell wall and the absence of melanin resulted in a defect in the formation of the hydrophobic rodlet layer (Pihet et al. 2009). Furthermore, melanin acts as an ROS scavenger and protects *A. fumigatus* conidia from oxidative and hydrolytic damage from the host (Heinekamp et al. 2012). *A. fumigatus* mutants that produce non-pigmented conidia due to a mutation in the *pksP* gene were more susceptible to monocyte-killing *in vitro* and were less virulent in animal models of IA (Jahn et al. 1997). It was shown that upon macrophage engulfment of conidia lacking

functional *pksP*, increased phagolysosomal fusion and acidification occurred resulting in increased killing of conidia (Jahn et al. 2002). This suggests a role for genes involved in pigmentation with protection from host insults.

A. fumigatus also possesses other ROS scavengers that may contribute to protection of both conidia and hyphae against host defenses, including superoxide dismutases and catalases (Dagenais and Keller 2009). The contribution of these molecules in the pathogenesis of IA is controversial and remains an area of ongoing investigation.

Cell Wall Components

The cell wall of *A. fumigatus* provides structure and protection from external stress, and is comprised of interconnected polysaccharides that account for approximately 90% of the structure with glycoproteins scattered throughout the wall (Gastebois et al. 2009). The polysaccharide content is as follows: 20-35% ß-(1,3)-glucans, 35-46% α-(1,3)-glucans, 20-25% galactose polymers (galactomannan and galactosaminogalactan) and 7-15% chitin, but the fungal cell wall is a dynamic organelle and its composition changes under stressful conditions and during different stages of fungal growth (Gastebois et al. 2009; Latgé 2017). Cell wall plasticity and adaptability to stress are exemplified by compensatory mechanisms during antifungal drug treatment, in which decreases of chitin in the cell wall after drug exposure lead to increased levels of ß-glucan, and vice versa (Verwer et al. 2012). The core skeleton of the cell wall is composed of ß-(1,3)-glucan bound to chitin, galactomannan and ß-(1,3/1,4)-glucans (Gastebois et al. 2009; Latgé 2017). Embedded in this core structure, forming an

amorphous network are α-(1,3)-glucan, galactose polymers and glycoproteins (Gastebois et al. 2009; Latgé 2017). The cell wall is constantly exposed to the external environment, including the host environment when conidia enter the lungs. Several of these cell wall constituents are recognized as pathogen- associated molecular patterns (PAMPs) by the immune system and play a role in coordinating the immune response to *A. fumigatus* (Bozza et al. 2009). The most well-defined example of this is dectin-1 recognition of ß-(1,3)-glucan, but more studies are needed to identify other host receptors that mediate recognition of the remaining fungal cell wall components, such as chitin and galactosaminogalactan (GAG) (Dambuza and Brown 2015; Latgé 2017). Galactosaminogalactan (GAG) is exposed on germlings and on hyphal networks, and has been shown to have several immune-suppressive effects including concealing ß-glucan exposure and induction of the IL-1 receptor antagonist (IL-1ra) among others (Gresnigt et al. 2014). Determination of how these cell wall components act in concert with each other to stimulate immune recognition and identification of the pathways needed for their biosynthesis can provide novel immune and fungal therapeutic targets.

Secondary Metabolites

Secondary metabolites are so named for their dispensability during *in vitro* growth, compared to metabolites that are essential for growth produced by primary metabolism (Bennett and Bentley 1989). It is thought that these molecules evolved as a form of communication with surrounding microbial life and as a way to inhibit growth of competing microbes in the complex natural habitat of *A. fumigatus* (Brakhage 2013). *A. fumigatus* produces several secondary metabolites including gliotoxin, restrictocin,

verruculogen, fumagillin, helvolic acid, ergot alkaloids, and fumitremorgin which have an array of functions from inducing host cell death to inhibiting ciliary function (Dagenais and Keller 2009).

Of all the known secondary metabolites, only gliotoxin has been isolated from mice experimentally infected with *A. fumigatus* and from patients with IA (Lewis et al. 2005; Lewis et al. 2005). Gliotoxin is thought to be immune-suppressive through a number of mechanisms including inhibition of phagocytosis, ROS, mast cell activation, and T cell responses (Müllbacher and Eichner 1984; Eichner et al. 1986; Yamada et al. 2000; Yoshida et al. 2000; Tsunawaki et al. 2004; Stanzani et al. 2005; Niide et al. 2006). *In vitro* studies reveal that gliotoxin can induce apoptosis in a number of cell types, induce epithelial damage and slow ciliary beating, all of which may contribute to the successful proliferation of *A. fumigatus* (Braithwaite et al. 1987; Piva 1994; Amitani et al. 1995; Coméra et al. 2007). In a murine model of sublethal irradiation, injection of gliotoxin was shown to delay the recovery of immune cells, demonstrating immune-suppressive activity *in vivo* (Sutton et al. 1994). As previously stated, a role for gliotoxin during IA in murine models has only been demonstrated in non-neutropenic mice in which genetic mutants that do not produce gliotoxin are less virulent than wild type strains of *A. fumigatus* (Sugui et al. 2007; Spikes et al. 2008). The decreased virulence of the gliotoxin-mutant strain was associated with a decrease in neutrophil apoptosis at the site of fungal lesions, which could explain the full virulence of gliotoxin mutants in the neutropenic model (Spikes et al. 2008). One study comparing multiple clinical and environmental isolates of different *Aspergillus* species demonstrated that of four different

species, gliotoxin was not only detected most frequently in culture filtrates of *A. fumigatus* isolates, but it was detected at significantly higher levels in *A. fumigatus* when compared to isolates of other species (Kupfahl et al. 2008). This suggests a potential link between gliotoxin production and virulence of *A. fumigatus* as this is the most common species to cause human disease, however the exact contribution of gliotoxin to pathogenesis of IA in humans remains unknown.

Nutrient Acquisition and Uptake

Successful growth of *A. fumigatus* requires the degradation of surrounding nutrient sources, whether it be from plant sources or within the human host. *A. fumigatus* has many ways in which it can acquire and utilize nutrients from a given substrate, supporting its ubiquitous growth. Below these factors are discussed.

Proteases. The *A. fumigatus* genome encodes 111 proteases, 47 of which possess an endoplasmic reticulum signal sequence suggesting a potential for secretion from the cell (Sharon et al. 2009). These proteases have the capability to break down extracellular matrix components of the lung including elastin, collagen, laminin and fibronectin (Lee and Kolattukudy 1995; Ladarola et al. 1998; Dagenais and Keller 2009). Furthermore, an *A. fumigatus* serine protease was shown to be sufficient to induce respiratory damage when introduced into the lung, while an aspartic protease was shown to be actively secreted by developing germ tubes during tissue invasive growth in a neutropenic mouse model (Lee and Kolattukudy 1995; Ladarola et al. 1998). It is thought that these proteases contribute to virulence and host tissue damage, however there is no evidence

that definitively shows the role of specific proteases in virulence of IA. Determination of the role of fungal proteases in the pathogenicity of *A. fumigatus* has been technically difficult due to the high number of proteases encoded in the genome and likelihood of functional redundancy among them.

A more recent approach has been to identify transcription factors that control expression of multiple secreted proteases and genetically delete the transcription factor, rather than delete single proteases (Sharon et al. 2009). One such transcription factor is PrtT, and genetic deletion resulted in the reduced expression of six secreted proteases, reduced killing of epithelial cells *in vitro* and reduced hemolysis *in vitro (Sharon et al. 2009).* However, *in vivo* studies using two murine models of IA showed no difference in virulence between the mutant strain and the wild-type strain used (Sharon et al. 2009). The environment within the host during infection is more complex than the environment found *in vitro* and it is possible that these differences could account for the confounding results. It is possible that fungal secretion of proteases are inhibited by protease inhibitors found within the lung, or that epithelial cells within the host are better equipped to tolerate proteolytic insults (Sharon et al. 2009). Another consideration is that PrtT only controls 6 of the 111 proteases in the *Aspergillus* genome. Identification of other transcription factors that control expression of these proteases will help elucidate the contribution, if any, of fungal-induced proteolytic damage to the host during IA.

Carbon and Nitrogen Metabolism. Metabolism is a central component in the ability of fungi to adapt to a given environment and therefore is thought to contribute to virulence (Rhodes 2006). Carbon and nitrogen sources may vary widely in different

environments so utilization of non-preferential sources can provide a fitness advantage to *A. fumigatus*.

A. fumigatus utilizes glucose as a preferential carbon source for the production of ATP during aerobic respiration; however, in an infection setting carbon sources and oxygen likely become limited due to influx of host immune cells as well as fungal growth. *A. fumigatus* can also utilize acetate, ethanol, glycerol and lactate as non-preferential carbon sources (Beattie et al. 2017). A shift in metabolism is critical for *A. fumigatus* to be able to adapt to this changing microenvironment. Recently it has been shown that the carbon catabolite repression (CCR) system in *A. fumigatus*, regulated by CreA, is critical for the progression and maintenance of IA in the corticosteroid murine model (Beattie et al. 2017). CreA transcriptionally represses genes associated with catabolism of non-preferential carbon sources, as to maximize the use of the most energetically favorable carbon source available (Ries et al. 2016). Specifically in the context of IA, CreA-regulated CCR is dispensable in the early stages of infection as evidenced by the ability of the *creA*-null mutant to germinate in the airways. This is thought to be due to the availability in the airways of sufficient alternative carbon sources and oxygen for use in gluconeogenesis and oxidative phosphorylation (Beattie et al. 2017). However, as these sources are depleted in the progression of infection, CreA is essential for the ability to optimally utilize alternative carbon sources as metabolism is switched to a glycolytic- and fermentative-based metabolism (Beattie et al. 2017). Importantly, this study was conducted in the steroid model of IA in which steroid treatment alone led to an increase in alternative carbon and nitrogen sources, with a

concurrent decrease in preferred sources. More studies are needed to determine the details of carbon metabolism and the CCR system in other models of IA.

Ability to metabolize alternative sources of nitrogen is also thought to contribute to the pathogenicity of *A. fumigatus*. The preferred nitrogen sources for *A. fumigatus* are glutamate and glutamine, but it can also utilize ammonium, nitrate, urea and amino acids (Krappmann and Braus 2005). The transcriptional activator AreA controls expression of genes needed for utilization of nitrogen sources, however a role in virulence has not been shown (Hensel et al. 1998). Deletion of the *areA* gene from *A. fumigatus* did not impact growth rate, but did render the mutants unable to utilize some nitrogen sources (Hensel et al. 1998). In neutropenic mice challenged with *areA* mutants, disease progression was slowed but overall mortality remained the same when compared to the wild-type strain (Hensel et al. 1998). Interestingly, *areA*-null mutants recovered from lung tissue of neutropenic mice revealed a high rate of reversion to wild-type, suggesting a beneficial role for functional AreA in the progression of IA (Hensel et al. 1998).

Further exploration is needed to determine the available carbon and nitrogen sources available in the lungs and airway space, the kinetics of depletion of these sources from the lung in the different clinically relevant murine models of IA, and whether there is *A. fumigatus* strain-specific utilization of different carbon and nitrogen sources.

Immune Response to *A. fumigatus*

The mammalian respiratory tract is exposed daily to *A. fumigatus* conidia, however very rarely is disease initiated in the immune competent host. This supports that

the host defense mechanisms are fine-tuned in order to prevent invasive fungal growth in the pulmonary environment without inducing inflammatory pathways that can be damaging to the host. Primary mechanisms of immunity involve the physical features of the respiratory tract. If conidia bypass this primary immune defense system and enter the lung environment then innate and adaptive immunity will be activated.

Physical Barriers of the Respiratory Tract

The respiratory system is a highly branched structure, which facilitates the deposition of inhaled particles onto the airway surface liquid (ASL) that lines the airways (Knowles and Boucher 2002). ASL is comprised of two layers: the mucus layer and a mucus-free periciliary liquid layer (PCL) (Knowles and Boucher 2002). Mucus consists of mucin macromolecules and functions in trapping inhaled particles for removal from the lung. The PCL is found between the mucus layer and the ciliated epithelial cell surface, and contrary to the gel-like consistency of mucus, PCL is a low-viscosity liquid which encourages rapid beating of cilia (Knowles and Boucher 2002). Beating cilia contribute to the expulsion of trapped particles from the lung environment. Multiple secondary metabolites produced by *A. fumigatus* inhibit the rapid beating of cilia, potentially decreasing the effectiveness of the mucociliary escalator (Dagenais and Keller 2009).

Innate Immune Recognition of *A. fumigatus*

Due to the small size of *A. fumigatus* conidia it is possible for them to bypass this barrier and enter the alveoli, in which case the innate immune system will be employed.

Innate immunity begins with the recognition of *Aspergillus* through several soluble and cell-associated pattern recognition receptors (PRRs), which in turn results in cytokine and chemokine production that recruits effector cells to the lung.

Soluble Receptors. Within the airway lining are a number of soluble components that aid in the recognition of *A. fumigatus* conidia. Among these are collectins, a family of C-type lectin receptors (CLRs) that recognize carbohydrate moieties and include the surfactant proteins A and D (SP-A and SP-D) and mannose binding lectin (MBL) (Dagenais and Keller 2009; Park and Mehrad 2009). These receptors act as opsonins and have shown the ability to bind *A. fumigatus* and enhance phagocytosis and killing of conidia by innate effector cells (Madan et al. 1997; Allen et al. 1999; Neth et al. 2000; Allen et al. 2001). In animal studies, the role of MBL-deficiency has shown conflicting results. One study reported genetically deficient MBL mice were more resistant to IA, suggesting a deleterious role of MBL during disease, whereas another study showed that recombinant MBL attenuated disease and increased survival in mice with IA. Importantly, very different murine models were used in these studies, the first utilizing a systemic model of IA in which conidia are introduced directly into the bloodstream, and the second study using the corticosteroid model (Kaur et al. 2007; Clemons et al. 2010). Furthermore, MBL binding to *A. fumigatus* conidia resulted in activation of the alternative pathway of complement (Dumestre-Pérard et al. 2008). Studies involving the role of complement *in vivo* during IA are limited, however it has been shown that mice deficient in C5 displayed increased mortality compared to C5-sufficient animals (Hector et al. 1990). Additionally, *A. fumigatus* has mechanisms to counteract complement

activation including the production of a complement inhibitory factor and the binding of host-derived inhibitors which contribute to evasion of alternative pathway activation, as well as its decreased affinity for binding C3 compared to other species (Washburn et al. 1986; Washburn et al. 1990; Henwick et al. 1993; Behnsen et al. 2008; Vogl et al. 2008).

Another soluble opsonin involved in *A. fumigatus* recognition is pentraxin-3 (PTX3). Mice lacking PTX3 have increased susceptibility to IA, which was associated with a defect in conidial recognition by alveolar macrophages and dendritic cells (Garlanda et al. 2002). In humans undergoing HSCT, SNPs in *Ptx3* of donor cells were associated with increased risk of developing IA attributed to a defect in antifungal mechanisms of neutrophils (Cunha et al. 2014).

Cell-Associated Receptors. In the airways, *A. fumigatus* will encounter phagocytic cells possessing cell-bound receptors that assist in the recognition and phagocytosis of conidia. Of these, CLRs have been shown to be critical in the coordination of antifungal immunity. CLRs include DC-SIGN, Dectin-1, Dectin-2, the mannose receptor (MR), and Mincle (Drummond et al. 2011; Hardison and Brown 2012). DC-SIGN is found on alveolar macrophages and dendritic cells and has been shown to participate in the recognition and phagocytosis of *A. fumigatus* conidia *in vitro* (Serrano-Gómez et al. 2004). Dectin-1, Dectin-2 and Mincle signal through Syk which associates with the ITAM-like motif on Dectin-1 or with the ITAM-coupled adaptor FcRγ of Dectin-2 and Mincle. Syk signaling results in a CARD9-BCL10-MALT1 complex which ultimately activates NF-κB-dependent cytokine production to enhance antifungal immunity through development of protective Th1 and Th17 responses (Rogers et al. ;

Gross et al. 2006; Sato et al. 2006; LeibundGut-Landmann et al. 2007; Yamasaki et al. 2008; Hardison and Brown 2012; Strasser et al. 2012). Dectin-1 recognizes and binds ß-glucan, which is exposed once conidial swelling and germination occur (Hohl et al. 2005; Steele et al. 2005; Gersuk et al. 2006). In animal models, the role of Dectin-1 has varied with one study suggesting mice lacking Dectin-1 have increased mortality (Werner et al. 2009), while others do not see a difference in disease outcome whether Dectin-1 is present or absent (Jhingran et al. 2012). However, humans with genetic defects in the *card9* gene have an increased risk for the spontaneous development of IA that was associated with a defect in neutrophil recruitment to the site of infection (Rieber et al. 2016). Furthermore, a SNP in the gene encoding Dectin-1 was associated with increased susceptibility of IA development in patients undergoing allogeneic HSCT, suggesting a role for Dectin-1 in human disease (Cunha et al. 2010). Dectin-2 on dendritic cells can recognize *A. fumigatus* hyphae *in vitro* (Loures et al. 2015), but the specific roles of Dectin-2, and the other CLRs, *in vivo* during pulmonary IA remains to be determined.

Toll-like receptors (TLRs) are a family of receptors that recognize PAMPs and coordinate the innate immune response to infection. TLRs involved specifically in immunity to IA are TLR2, TLR4 and TLR9 (Park and Mehrad 2009). TLRs signal through the adaptor proteins MyD88 and TRIF to activate downstream NF-κB and pro-inflammatory cytokine production (Laichalk et al. 1996; Underhill and Ozinsky 2002; Vasselon and Detmers 2002; Kopp and Medzhitov 2003; Beutler 2004; Balloy et al. 2005). Many *in vitro* studies have been performed to demonstrate the importance of TLR2/4 signaling in recognizing and responding to *A. fumigatus*, yielding conflicting

results (Wang et al. 2001; Mambula et al. 2002; Marr et al. 2003; Meier et al. 2003; Netea et al. 2003; Bellocchio et al. 2004; Bellocchio et al. 2004; Balloy et al. 2005; Steele et al. 2005; Dubourdeau et al. 2006). Park and Mehrad comprehensively reviewed these results and attributed the inconsistencies to a number of differences in the models used, including differences in host cells used (species and cell type), fungal morphotype, and immunological readouts (Park and Mehrad 2009). *In vivo*, deficiency of TLR2 and TLR4 in neutropenic mice resulted in increased fungal burden, and increased mortality in mice lacking TLR4 however inconsistent results were seen in mice lacking TLR2 (Bellocchio et al. 2004; Balloy et al. 2005). More studies are needed to determine the role of TLRs in non-neutropenic animals. In a recent study, TLR9-dependent activation of calcineurin and downstream nuclear factor of activate T cells (NFAT) occurred upon phagocytosis of *A. fumigatus* conidia (Herbst et al. 2015). NFAT activation was Myd88-independent, rather occurring through Bruton's tyrosine kinase (Btk) activation and was needed for optimal production of TNF-α and early recruitment of neutrophils (Herbst et al. 2015). This study highlights an important clinically relevant pathway (TLR9-Btk-calcineurin-NFAT) since calcineurin inhibitors are commonly used in prevention of SOT rejection and in the treatment of GVHD after HSCT, and recently have been associated with increased susceptibility to IA (Herbst et al. 2013).

Overall, collaboration between CLRs, TLRs and potentially other yet to be discovered PRRs are required to generate an optimal inflammatory response against *A. fumigatus* (Romani 2011).

Immune Effectors Against *A. fumigatus*

Although macrophages and neutrophils are considered the main effector cells in host defense against *A. fumigatus*, the host response requires coordination between several cell types to elicit an optimal anti-*Aspergillus* immune response. Below these cells and their effector functions are discussed.

Epithelial Cells. The first step in the infectious process is adhesion of conidia to the host constituents. *A. fumigatus* conidia bind to airway epithelial cells, which are likely the first cells encountered by conidia, as well as components of the basal lamina (Croft et al. 2016). Although fungal components that mediate this binding have been determined, but little is known about the host receptors that are engaged by these fungal factors. One fungal factor mediating adherence is the secreted protein Fucose-specific Lectin A (FleA) which binds fucose residues found on epithelia and on mucins (Houser et al. 2013; Kerr et al. 2016). Purified FleA was sufficient to induce IL-8 production from human epithelial cells, and conidia genetically deficient in FleA were not phagocytosed by macrophages as efficiently as wild-type conidia, suggesting a role for this protein in the recognition of and pro-inflammatory immune response to *A. fumigatus* (Houser et al. 2013; Kerr et al. 2016). Other fungal cell wall components that can mediate adherence to airway epithelia are sialic acids, GAG, and a secreted protein named AfCalAp (Croft et al. 2016). Once recognition occurs, epithelial cells can engulf conidia in a process similar to phagocytosis in which they are trafficked to the phagolysosome for destruction (Wasylnka and Moore 2003; Croft et al. 2016). *In vitro*, most of the engulfed conidia are killed however it has been determined that some conidia can survive, germinate and

escape into the extracellular space (Wasylnka and Moore 2003). Although *in vitro* up to 30% of conidia can be engulfed by epithelial cells, the true relevance of this to disease outcome *in vivo* remains to be determined. Nevertheless, airway epithelial cells have the ability to produce an array of cytokines and chemokines which could contribute to the initial recruitment of leukocytes after recognition of *A. fumigatus*.

Macrophages. Alveolar macrophages are one of the main phagocytic cells of the respiratory tract. Upon recognition, conidia are phagocytosed by alveolar macrophages resulting in cytokine production that promotes leukocyte recruitment, including TNF-α, IL-1ß, MIP-1α, IL-6, and IL-8 (Mehrad et al. 1999; Cenci et al. 2001; Morrison et al. 2003; Phadke and Mehrad 2005; Dagenais and Keller 2009). Macrophages can be divided into two broad categories: classically activated macrophages (M1) or alternatively activated macrophages (M2) (Bhatia et al. 2011). M1 macrophages are induced by IFN-γ and are associated with upregulation of NOS2 and enhanced NO production needed to kill phagocytosed pathogens (Mosser and Edwards 2008). The prototypical activation marker for M2 macrophages is Arginase1 (Arg1) and these macrophages are associated with tissue repair processes and suppression of Th2-mediated immunity (Pesce et al. 2009; Bhatia et al. 2011). After challenge with *A. fumigatus*, alveolar and tissue macrophages were shown to take on an M2 phenotype with enhanced Arg1, but not NOS2, expression (Bhatia et al. 2011). Depletion of macrophages in this study resulted in increased fungal burden in infected mice, suggesting a protective role for M2 macrophages following *A. fumigatus* challenge (Bhatia et al. 2011). In another study, clodronate-induced alveolar macrophage depletion did not alter the survival rate of

A. fumigatus-challenged mice, suggesting that although these cells likely play a role in host defense against *A. fumigatus*, other leukocytes can potentially compensate for their loss *in vivo* (Mircescu et al. 2009).

Once phagocytosed, the conidia can be killed by the alveolar macrophage through ROS and phagolysosomal acidification, however RNS is insufficient to kill conidia (Washburn et al. 1987; Ibrahim-Granet et al. 2003; Philippe et al. 2003). It has been demonstrated that the *A. fumigatus* gene, *pksP*, which is involved in DHN-melanin production contributes to the inhibition of phagolysosome acidification (Thywißen et al. 2011). Interestingly, *A. terreus* interacts with macrophages quite differently than *A. fumigatus*. Specifically, *A. terreus* does not inhibit phagolysosome acidification like *A. fumigatus*, which results in decreased conidial germination and lower levels of phagosome escape and cytotoxicity to the macrophages. However, recombinant expression of an *A. nidulans*-derived homolog of PksP in *A. terreus* led to the inhibition of phagolysosome acidification which allowed increased germination, macrophage escape and cytotoxicity, and increased virulence *in vivo* (Thywißen et al. 2011; Slesiona et al. 2012). These studies could explain why some species of *Aspergillus* are associated with higher incidence of IA and warrants further exploration. Recent work has also shown that fungal germination within the macrophage phagosome induces necroptotic host cell death, which allowed lateral transfer of *A. fumigatus* to nearby macrophages to occur (Shah et al. 2016). If this programmed cell death was inhibited, then *A. fumigatus* was able to escape from the macrophage, leading to a defect in ability to control fungal

germination. Thus, the early fungal-macrophage interaction sets the stage for either host control of the *Aspergillus* or fungal escape from the macrophage and initiation of disease.

Inflammatory Monocytes/Dendritic Cells. Once monocytes leave the bone marrow, they are released into the blood stream and can migrate into tissue in steady state conditions or in response to inflammatory stimuli (Mosser and Edwards 2008). In response to *A. fumigatus*, CCR2+ inflammatory monocytes are rapidly recruited to the lung and differentiate into monocyte-derived dendritic cells (Mo-DC) after exposure to *A. fumigatus* (Hohl et al. ; Espinosa et al. 2014). These cells possess potent anti-*Aspergillus* activity, contribute to the fungicidal activity of neutrophils, are critical for induction and maintenance of Th1 CD4 T cell responses, and overall are indispensable for protection against IA (Hohl et al. ; Rivera et al. 2011; Espinosa et al. 2014).

Dendritic cells are positioned directly beneath the epithelium of the airways in order to survey the airway lumen for presence of antigen, which can then be presented to T cells to induce activation and proliferation (Holt et al. 2008). In this sense DCs are an important bridge between the innate and adaptive immune systems. DCs can be divided into plasmacytoid DCs (pDC), myeloid DCs (CD8α-) and lymphoid DCs (CD8α+) and it has been shown that these subsets respond very differently to stimuli. Pulmonary pDCs express TLR7, TLR9, Dectin-2 and Siglec-H and release high levels of TNF-α, IL-8 and IFN-α in response to stimuli (Demedts et al. 2006; Loures et al. 2015). Compared to mDCs, pDCs were very poor inducers of T cell proliferation (Demedts et al. 2006). A recent role for pDCs in innate immune recognition of *A. fumigatus* has been established. In this study, pDCs were shown to recognize *A. fumigatus* in a Dectin-2-dependent

manner, leading to high levels of TNF-α and IFN-α release, direct DC-mediated antifungal activity, and formation of pDC extracellular traps (Loures et al. 2015). Direct antifungal activity of pDCs was shown to be contact-independent as pDC lysates were sufficient to inhibit fungal metabolic activity, potentially through calprotectin which chelates zinc (Ramirez-Ortiz et al. 2011). Furthermore, pDC-depleted mice were hypersusceptible to development of IA (Ramirez-Ortiz et al. 2011). CD8α- and CD8α+ DCs were shown to have different functions in response to the yeast *Saccharomyces cerevisiae* (Backer et al. 2008). Although both CD8α- and CD8α+ DCs were able to take up *S. cerevisiae* in a Dectin-1 dependent manner and present antigen on MHC II to CD4+ T cells, only CD8α- DCs were able to present antigen on MHC I to CD8+ T cells (Backer et al. 2008). Moreover, only CD8α- DCs produced cytokines in response to *S. cerevisiae* (Backer et al. 2008). No studies have been conducted to compare the roles of CD8α- and CD8α+ DCs in response to *A. fumigatus*, however it is hypothesized that cross presentation of fungal antigens to CD8+ T cells preferentially occurs through CD8α- DCs (Backer et al. 2008).

Neutrophils. Neutrophils mature and, for the most part, reside in the bone marrow until they are recruited to the bloodstream via a G-CSF-CXCL1-CXCL2 gradient (Hasenberg et al. 2013). In the bloodstream, these cells are very short-lived with a half-life of only 6-11 hours (Hasenberg et al. 2013). Once in circulation, neutrophils recognize chemotactic signals from sites of inflammation and extravasate from the bloodstream to the site of tissue inflammation. Neutrophils have long been regarded as the main effector cell in host defense against IA and neutropenia remains a primary risk

factor in the development of disease. Neutrophils are known to interact with swollen conidia and hyphae of *A. fumigatus*, resulting in fungal killing by both oxidative and non-oxidative mechanisms (Diamond and Clark 1982; Levitz and Farrell 1990). In response to hyphae, *A. fumigatus* can attach to the hyphal surface and degranulate (Feldmesser 2006). Degranulation releases a number of toxic compounds including myeloperoxidase which contributes to oxidative killing, and a number of antimicrobial proteins such as cathepsins, defensins, and lactoferrin (Kolaczkowska and Kubes 2013). Furthermore, a study has shown that neutrophils form aggregates around *A. fumigatus* conidia and prevent fungal germination *in vivo*, potentially through an oxidative mechanism (Bonnett et al. 2006). Neutrophil extracellular trap (NET) formation occurs in response to *A. fumigatus in vitro* and *in vivo*, and it is proposed that within the host NET formation is fungistatic rather than fungicidal (Bruns et al. 2010).

B Cells. Due to daily exposure to *Aspergillus* conidia, serum IgG and IgM and BAL-derived IgA antibodies are present in individuals without aspergillosis (Bardana 1974; SchØNheyder et al. 1983). There are a number of molecules on the hyphal surface that act as B cell antigens, however the role of antibodies in host defense against *A. fumigatus* remains unknown (Hohl and Feldmesser 2007). B cell-deficienct, neutropenic mice were shown to be more resistant to IA, however administering serum to B cell-deficient mice enhanced their survival during IA (Montagnoli et al. 2003). More studies are required to determine the role of antibodies in host protection against *A. fumigatus*.

T Cells. Like antibody presence, T cell responses to *Aspergillus* are found in patients without aspergillosis. It has been shown *in vitro* that different fungal components have the ability to induce different Th cytokine responses from CD4+ T cells where secreted proteins induced Th2 cytokines, membrane proteins induced Th1/Treg cytokines, and glycolipids induced Th17 cytokines (Bozza et al. 2009). This indicates the importance of fungal germination stage and morphology in the induction of protective or detrimental adaptive responses.

Th1 CD4+ T cell responses are known to protect against IA in immune compromised hosts, while Th2 CD4+ T cell responses are associated with the development of ABPA and contribute to disease progression (Cramer et al. 2011). It is known that the production of IFN-γ from CD4+ T cells is protective, and adoptive transfer of these cells after bone marrow transplantation was protective against IA in both mice and humans (Cenci et al. 2000; Perruccio et al. 2005). Furthermore, in neutropenic mice, neutralization of Th2 cytokines was beneficial (Cramer et al. 2011).

Interestingly, nude mice which lack functional T cells, and SCID mice lacking B and T cells, do not show enhanced susceptibility to IA compared to wild type mice (Hohl and Feldmesser 2007). This demonstrates the critical role the innate immune system plays in host protection from the development of IA.

Inflammatory Mediators Important for Recruitment of Effector Cells

Recognition of *A. fumigatus* results in the induction of a number of cytokines, chemokines and inflammatory mediators that regulate the recruitment and activation of effector cells. Below I will briefly discuss these mediators with special attention to the

IL-1 and Leukotriene family members, which are the main focus of the research in this dissertation.

One of the earliest induced cytokines induced during IA is TNF-α (Chotirmall et al. 2013). Early after recognition of conidia TNF-α is released from alveolar macrophages leading to the expression of chemokines MIP-1α, MCP-1, and MIP-2, which in turn act to recruit effector cells to the site of infection (Mehrad et al. 1999; Chotirmall et al. 2013). A defect in TNF-α signaling has been shown to result in decreased neutrophil recruitment associated with increased fungal burden and increased mortality (Mehrad et al. 1999; Schelenz et al. 1999). Importantly, treatment with TNF-α antagonists is associated with increased incidence of aspergillosis in humans supporting an essential role of TNF-α in the prevention of IA development (Filler et al. 2005; Rychly and DiPiro 2005; Tsiodras et al. 2008).

Overall, Th1 cytokines are associated with protection and resistance from IA, while Th2 cytokines are thought to be detrimental to the host and associated with disease progression (Cenci et al. 1997; Cenci et al. 1999). Th1-associated cytokines IFN-γ and GM-CSF are able to enhance antifungal mechanisms of effector cells, such as ROS production, and increase the expression of CXCR3 ligands leading to cell recruitment to the site of infection (Roilides et al. 1993; Gaviria et al. 1999; Park et al. 2009). Conversely, Th2 cytokines such as IL-4 and IL-10 are associated with increased fungal burden and decreased survival in a murine model of IA (Cenci et al. 1999; Clemons et al. 2000). The Th1 response is suppressed by both of these cytokines and in non-

neutropenic immune compromised patients, increased serum levels of IL-10 were associated with development of IA and ultimately, death (Roilides et al. 2001).

A number of chemokines are produced during the host response to *A. fumigatus*. Chemokines can be grouped into 4 categories depending on their protein structure: CXC, CC, C, and CX_3C. The CC chemokines CCL2/MCP-1, CCL3/MIP-1α, and CCL4/MIP-3α are needed for recruitment of mononuclear cells to the site of *A. fumigatus* infection and an optimal antifungal immune response, as evidenced by increased mortality observed in the absence of these mediators (Mehrad et al. 2000; Blease et al. 2001; Morrison et al. 2003; Phadke et al. 2007). CXC chemokines can further be classified based on the presence or absence of an ELR amino acid motif. ELR+ CXC chemokines include the CXCR2 ligands CXCL2/MIP-2 and CXCL1/KC, important in neutrophil chemotaxis, while ELR- CXC chemokines include CXCR3 ligands CXCL9-11 (Zlotnik and Yoshie). In a murine model of IA, blockade of CXCR2 signaling resulted in increased mortality of mice that was associated with a defect in neutrophil recruitment to the lungs, while pulmonary overexpression of CXCL1 increased resistance to IA (Mehrad et al. 1999; Mehrad et al. 2002). Recently it was discovered that CXCR2-dependent neutrophil recruitment after exposure of mice to *A. fumigatus* occurs in an IL-1RI/Myd88- and CARD9-dependent manner that is temporally regulated (Jhingran et al. 2015).

IL-1RI Signaling

IL-1RI signaling and its role in host defense against IA is a main focus of this dissertation. Early work on this pathway determined there was no role for IL-1RI

signaling in immunity against *A. fumigatus* (Bellocchio et al. 2004), however more recent work including that of our lab suggests this is not the case.

IL-1 is a central component in the regulation of innate immunity and inflammation. The IL-1 family is comprised of eleven members: seven agonists, three receptor antagonists and an anti-inflammatory cytokine (Garlanda et al. 2013). This dissertation will focus solely on the three family members that bind the IL-1RI. IL-1α and IL-1ß are agonists that bind IL-1RI and result in pro-inflammatory signaling to enhance the immune response, while IL-1ra competitively binds IL-1RI in order to dampen the immune response (Garlanda et al. 2013). Once IL-1 binds to the IL-1RI, the IL-1 receptor accessory protein (IL-1RAcP) is recruited to form a heterodimer. This leads to recruitment of MyD88 through a TIR domain, IL-1R-associated kinase 4 (IRAK4), TNFR-associated factor 6 (TRAF6) and other intermediates that ultimately lead to activation of NF-κB and MAPK pathways (Sims and Smith 2010).

IL-1α and IL-1ß are both produced as pre-cursor proteins, each possessing pro-segments of approximately 100 amino acids, however they differ in their maturation process and the way in which they are secreted from cells. Pro-IL-1ß must be transcriptionally upregulated and is only active once cleaved by a caspase-1 or caspase-8 containing inflammasome into mature IL-1ß (Latz et al. 2013). Mature IL-1ß is then released from the cell through Gasdermin D-mediated pyroptosis where it can bind the IL-1RI in a paracrine or autocrine manner to initiate downstream signaling (He et al. 2015). Contrary to this, pro-IL-1α is constitutively expressed in cells where it can be either bound to DNA acting as a component of gene transcription, or found within the

cytosol (Garlanda et al. 2013). IL-1α can move between the nucleus and cytosol quickly upon different cell signaling events. It has been shown that once apoptosis is initiated, cytosolic IL-1α transfers to the nucleus and binds DNA as to prevent release from the cell and initiation of an inflammatory event (Cohen et al. 2010). Contrary to this, upon necrotic cell death IL-1α moves from the nucleus into the cytosol where it can then be released into the extracellular environment to initiate neutrophil recruitment and inflammation (Cohen et al. 2010; Rider et al. 2011). Cleavage of pro-IL-1α, which occurs through calpain and neutrophil-derived proteases, is not necessary for biologic activity but it is thought that mature IL-1α has enhanced activity compared to the non-cleaved form (Kim et al. 2013). IL-1α is considered an alarmin, an endogenous molecule released in the event of cellular and tissue damage that functions in induction of a rapid and robust response to an immunological threat (Bianchi 2007).

IL-1 signaling is highly inflammatory and can result in high levels of tissue damage if left unchecked. Because of this there are several negative regulators in the IL-1 pathway, including IL-1ra, the decoy receptor IL-1R2, and SIGIRR. IL-1ra is the only member of the IL-1 family to not be produced as a precursor, and is secreted from cells in a soluble form. IL-1ra binds the IL-1RI with a higher affinity than IL-1 cytokines, however does not recruit the IL-1RAcp and so cannot initiate downstream signaling (Garlanda et al. 2013). There is an intracellular pool of IL-1ra that is released upon cell death that can act to limit inflammation and tissue damage that accompanies inflammatory cell death events (Garlanda et al. 2013). Individuals born with genetic defects in IL-1ra suffer from autoinflammatory diseases characterized by local and

systemic inflammation, demonstrating the importance of regulating IL-1 signaling (Aksentijevich et al. 2009; Reddy et al. 2009). The decoy receptor IL-1R2 acts through many mechanisms to inhibit IL-1RI signaling. IL-1R2 binds to IL-1α and IL-1ß and recruits the IL-1RAcP, however has no TIR domain and so cannot initiate downstream signaling (Garlanda et al. 2013). IL-1R2 can also bind IL-1ra, however with a much lower affinity than it binds the agonists (Garlanda et al. 2013). When pro-IL-1ß is bound to IL-1R2, caspase-1 dependent cleavage into mature IL-1ß is inhibited (Smith et al.). Similarly, pro-IL-1α can bind IL-1R2 in the cytosol inhibiting IL-1α activity, however during necrosis caspase-1 can cleave IL-1R2 which leads to IL-1α separation from the decoy receptor and restoration of IL-1α activity (Zheng et al. 2013). Because IL-1R2 recruits IL-1RAcP to the decoy complex, it is also inhibiting pro-inflammatory IL-1RI signaling through sequestration of the accessory protein from IL-1RI complexes. Finally, SIGIRR is a receptor that is thought to contain 2 amino acid substitutions in the TIR domain, leading to non-conventional signaling (Sims and Smith 2010; Garlanda et al. 2013). Much less is known about the mechanisms utilized by SIGIRR and a ligand for this receptor has yet to be identified, but it was shown to inhibit NF-κB activation following IL-1RI signaling (Sims and Smith 2010; Garlanda et al. 2013).

In several different disease models, IL-1α and IL-1ß have been shown to have non-redundant inflammatory activities which is a central theme of this dissertation (Vonk et al. 2006; Chen et al. 2007; Rider et al. 2011; Al Moussawi and Kazmierczak 2014; Caffrey et al. 2015). The role of IL-1 as a central component of innate immunity is demonstrated by its conserved role in host defense against a broad range of pathogens,

spanning bacteria, viruses and fungi (Hultgren et al. 2002; Power et al. 2004; Deepe and McGuinness 2006; Vonk et al. 2006; Miller et al. 2007; Di Paolo et al. 2009; Gross et al. 2009; Hise et al. 2009; Kankkunen et al. 2010; Said-Sadier et al. 2010; Mijares et al. 2011; Tomalka et al. 2011; Barry et al. 2013; Johnson et al. 2013; Li et al. 2013; Pietrella et al. 2013; Tavares et al. 2013; Wuthrich et al. 2013; Al Moussawi and Kazmierczak 2014; Di Paolo et al. 2014; Gresnigt and van de Veerdonk 2014; Hung et al. 2014; Mao et al. 2014; Milora et al. 2014; Wellington et al. 2014; Yang et al. 2014; Amarilla et al. 2015; Bagaitkar et al. 2015; Caffrey et al. 2015; Karki et al. 2015; Altmeier et al. 2016; Gresnigt et al. 2016). Furthermore, it is implicated in autoinflammatory diseases, autoimmune diseases and sterile immunity (Chen et al. 2007; Cohen et al. 2010; Sims and Smith 2010; Rider et al. 2011; Zheng et al. 2013; Bagaitkar et al. 2015). The role of IL-1 in these inflammatory responses will be discussed in further detail in following chapters.

Leukotriene Signaling

Leukotrienes are lipid inflammatory mediators that are a product of arachidonic acid (AA) metabolism. Leukotrienes have long been implicated in asthma and allergic disease, and are also recognized for their critical role in innate immunity to infectious disease and neutrophilic inflammatory.

AA can yield a number of eicosanoid products that are involved with inflammatory processes, including prostaglandins, thromboxanes, and leukotrienes. For the purpose of this dissertation, I will focus mainly on the leukotrienes. The first step in the synthesis of leukotrienes is the release of AA from the membrane phospholipids, which occurs through phospholipase A_2 (PLA_2) (Sadik and Luster 2012). AA is then

converted to LTA_4 by the enzyme 5-lipoxygenase (5-LO), which is activated by the 5-LO-activating protein (FLAP) (Sadik and Luster 2012). LTA_4 is the substrate that can then be converted to any one of the leukotrienes. It has been shown that neutrophils preferentially form LTB_4, while macrophages, eosinophils, basophils and mast cells preferentially form the cysteinyl leukotriene (cys-LT), LTC_4 (Kanaoka and Boyce 2004). Other cys-LTs are LTD_4 and LTE_4.

CysLTs bind to two receptors: CysLT1 and CysLT2 (Kanaoka and Boyce 2004). CysLTs are known for their role in asthma and the allergic response and several successful drugs blocking the actions of these mediators have proven successful in the treatment of asthma. One study found that bone marrow derived DC production of LTC_4 contributed to the induction of Th2 cytokines in response to dust mite antigen (Kanaoka and Boyce 2004; Machida et al. 2004). CysLTs also impact vascular permeability in models of innate and adaptive immunity, recruitment and activation of eosinophils and mast cells, and pulmonary inflammation and fibrosis (Kanaoka and Boyce 2004).

There are two G-protein coupled receptors for LTB_4, BLT1 and BLT2, of which BLT1 is a high-affinity receptor and BLT2 is a low-affinity receptor (Toda et al. 2002). BLT1 is expressed mainly on leukocytes, while BLT2 is expressed ubiquitously (Tager and Luster 2003). Binding of LTB_4 to its receptor results in signal transduction that leads to inflammatory cell recruitment, cell activation, and increased inflammatory cell survival. The first discovered role for LTB_4 was its potent neutrophil chemoattractant activity (Ford-Hutchinson et al. 1980; Camp et al. 1983; Soter et al. 1983; Martin et al. 1989). LTB_4 also has the ability to direct monocyte, macrophage and eosinophil

chemotaxis (Tager and Luster 2003). Furthermore, LTB_4 induces integrin upregulation and activation on leukocytes, contributing to their migration from the vasculature into tissues (Tager and Luster 2003). Production of ROS, neutrophilic granule release and phagocytosis by neutrophils are all enhanced by LTB_4 signaling (Tager and Luster 2003). LTB_4 has the ability to prevent apoptosis of neutrophils (Tager and Luster 2003). Interestingly, dexamethasone treatment was shown to upregulate BLT1 expression in human neutrophils suggesting a mechanism by which glucocorticoids can prolong neutrophil survival (Stankova et al. 2002).

LTB_4 has been associated with IL-1 signaling in a lipid-cytokine-chemokine cascade that amplifies neutrophil recruitment in a murine model of arthritis (Chou et al. 2010). In response to the dimorphic fungi, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* leukotrienes were critical for optimal leukocyte recruitment and activation (Medeiros et al. 1999; Medeiros et al. 2004; Santos et al. 2013). No studies to date have investigated a role for leukotrienes in host defense against filamentous fungal infections such as IA. Given the inhibitory effects of corticosteroids on the production of eicosanoids (Sebaldt et al. 1990), and the high risk of IA development in corticosteroid-treated patients, these studies could provide novel insights into mechanisms that contribute to susceptibility of IA.

Research Objectives

One main objective of this dissertation is to determine the inflammatory mediators needed to initiate chemokine production and subsequent leukocyte recruitment

to the pulmonary environment following *A. fumigatus* challenge. By understanding the innate immune pathways that are critical in coordinating a host response against *A. fumigatus*, we can eventually gain an appreciation for how these pathways are altered in different susceptible patient populations which may facilitate pharmacological strategies to enhance immunity to *A. fumigatus*. Antifungal drug treatments are often ineffective on their own due to the fact that disease is already established in a host when treatment is started. Perhaps inducing an optimal anti-*Aspergillus* immune response through immune modulatory treatments, combined with the use of antifungal drugs may provide a benefit to these patients.

Overall the aims of this dissertation are to define the specific roles of IL-1α, IL-1ß and leukotrienes in the immune competent host response following *A. fumigatus* exposure. We hypothesize that signaling through IL-1RI and BLT1 contribute to the anti-*Aspergillus* response through timely recruitment of neutrophils to the pulmonary environment in order to prevent invasive fungal growth. Furthermore, we hypothesize that IL-1α and IL-1ß play distinct, non-redundant roles in host resistance to IA.

CHAPTER TWO

IL-1A SIGNALING IS CRITICAL FOR LEUKOCYTE RECRUITMENT AFTER PULMONARY ASPERGILLUS FUMIGATUS CHALLENGE

Contributions of Authors and Co-Authors

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IL-1ALPHA SIGNALING IS CRITICAL FOR LEUKOCYTE RECRUITMENT AFTER PULMONARY ASPERGILLUS FUMIGATUS CHALLENGE

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Abstract

Aspergillus fumigatus is a mold that causes severe pulmonary infections. Our knowledge of how *A. fumigatus* growth is controlled in the respiratory tract is developing, but still limited. Alveolar macrophages, lung resident macrophages, and airway epithelial cells constitute the first lines of defense against inhaled *A. fumigatus* conidia. Subsequently, neutrophils and inflammatory $CCR2^{+}$ monocytes are recruited to the respiratory tract to prevent fungal growth. However, the mechanism of neutrophil and macrophage recruitment to the respiratory tract after *A. fumigatus* exposure remains an area of ongoing investigation. Here we show that *A. fumigatus* pulmonary challenge induces expression of the inflammasome-dependent cytokines IL-1β and IL-18 within the first 12 hours, while IL-1α expression continually increases over at least the first 48

hours. Strikingly, *Il1r1*-deficient mice are highly susceptible to pulmonary *A. fumigatus* challenge exemplified by robust fungal proliferation in the lung parenchyma. Enhanced susceptibility of *Il1r1*-deficient mice correlated with defects in leukocyte recruitment and anti-fungal activity. Importantly, IL-1α rather than IL-1β was crucial for optimal leukocyte recruitment. IL-1α signaling enhanced the production of CXCL1. Moreover, $CCR2^{+}$ monocytes are required for optimal early IL-1α and CXCL1 expression in the lungs, as selective depletion of these cells resulted in their diminished expression, which in turn regulated the early accumulation of neutrophils in the lung after *A. fumigatus* challenge. Enhancement of pulmonary neutrophil recruitment and anti-fungal activity by CXCL1 treatment could limit fungal growth in the absence of IL-1α signaling. In contrast to the role of IL-1α in neutrophil recruitment, the inflammasome and IL-1β were only essential for optimal activation of anti-fungal activity of macrophages. As such, *Pycard*-deficient mice are mildly susceptible to *A. fumigatus* infection. Taken together, our data reveal central, non-redundant roles for IL-1α and IL-1β in controlling *A. fumigatus* infection in the murine lung.

Author Summary

Aspergillus spp. are ubiquitous in the environment, and even though individuals are regularly exposed to fungal spores clinical invasive disease is a rare manifestation. In contrast, individuals with weakened immune systems develop severe disease, such as invasive pulmonary aspergillosis (IPA). IPA is associated with extremely poor prognoses and unacceptably high mortality rates. Knowledge gained from understanding how

immunocompetent mammals control *Aspergillus* challenge will help develop new immunomodulatory strategies aimed at improving patient outcomes. It is well known that neutrophils and monocytes are crucial immune cells that act to limit fungal growth. Our work demonstrates a central role for the cytokine IL-1α in orchestrating the optimal recruitment of neutrophils and monocytes, whereas IL-1β and the inflammasome are more important in activation of anti-fungal activity of the monocytes. Moreover, our studies indicate that $CCR2^{+}$ monocytes are required for optimal production of IL-1α in the lungs of *A. fumigatus* challenged mice. Thus, our data highlight a crucial role of the IL-1 cytokine in mediating anti-fungal immunity which might be harnessed to treat clinical cases of IPA.

Introduction

The mold *Aspergillus fumigatus* is one of the leading causes of invasive fungal infections. It is the causative agent of severe pulmonary infections such as invasive pulmonary aspergillosis (IPA), a disease of high morbidity and mortality which affects immunocompromised individuals (Brown et al. 2012). IPA has been a disease of growing concern over recent decades due to an increase in the immunocompromised population, specifically caused by advances in immunosuppressive drugs and organ transplantation methods as well as chemotherapy treatments in cancer patients (Segal 2009). In addition, there is increasing evidence that IPA can sporadically develop in certain immunocompetent populations (Stevens and Melikian 2011). Currently there are no available vaccines for *A. fumigatus* and anti-fungal drugs have a modest rate of

success in limiting high mortality rates typically due to late diagnosis of IPA (Segal 2009; Brown et al. 2012; Hayes and Denning 2013). Moreover, the recent emergence of drug resistance has further limited treatment options in certain clinical cases and geographic areas (Verweij et al. 2007; van der Linden et al. 2011; Vermeulen et al. 2013).

The concentration of *Aspergillus* conidia in air samples ranges from 0.2-15 conidia/m^3 and on a daily basis an individual can inhale hundreds of conidia (VandenBergh et al. 1999). In most immunocompetent individuals the conidia are typically removed from the body by physical barriers encountered within the respiratory tract. However, if the conidia escape this primary immune barrier and enter the lung, they will be removed by alveolar macrophages and other resident leukocytes, such as $CCR2^+$ monocytes. Conversely, in an individual lacking a sufficient immune response, *Aspergillus* conidia are able to swell, germinate, and form hyphae, invading pulmonary tissue with the potential to disseminate systemically (Hohl et al. ; Park and Mehrad 2009). Our understanding of the inflammatory pathways necessary for an immunocompetent individual to maintain control of *A. fumigatus* while constantly being exposed to conidia is an ongoing area of investigation.

Control of *A. fumigatus* growth in the lung during invasive infection is highly dependent on rapid recruitment and activation of innate immune cells, including neutrophils (Mircescu et al. 2009), inflammatory monocytes (Hohl et al. ; Espinosa et al. 2014), NKT cells (Cohen et al. 2011), and plasmacytoid dendritic cells (Ramirez-Ortiz et al. 2011). The importance of appropriate activation of leukocytes in the control of *A. fumigatus* is highlighted by patients and mice with chronic granulomatous disease or

lacking NADPH oxidase subunits, being highly susceptible to developing IPA after *A. fumigatus* challenge (Pollock et al. 1995; Morgenstern et al. 1997; Ben-Ari et al. 2012). Furthermore, patients who become neutropenic after chemotherapy for a bone marrow transplant are at a higher risk for developing IPA (Torres et al. ; Wald et al. 1997; Perfect et al. 2001; Marr et al. 2002). In the murine model of *A. fumigatus* infection, CXCR2 and its ligands are important signaling components for neutrophil recruitment (Mehrad et al. 1999; Mehrad et al. 2002; Bonnett et al. 2006). In the absence of CXCR2 signaling during pulmonary *A. fumigatus* infection, there is a decrease in neutrophil recruitment along with a higher fungal burden and increased mortality rate, similar to a neutropenic model (Mehrad et al. 1999). Additionally, a role for CCR2 signaling has been shown to be necessary to promote recruitment and differentiation of inflammatory monocytes from the bone marrow into $CD11b^{+}$ dendritic cells upon *A. fumigatus* infection (Hohl et al. ; Espinosa et al. 2014). However, the exact sequence of events necessary for the expression of chemotactic molecules for optimal leukocyte recruitment has not been well elucidated.

In addition, it has been shown that polymorphisms in the Interleukin (IL)-1 gene cluster may be important in determining the susceptibility or resistance to IPA in humans (Sainz et al. 2008; Wójtowicz et al. 2015). The IL-1 gene cluster codes for two pro-inflammatory cytokines, IL-1α and IL-1β, as well as the IL-1 receptor antagonist (IL-1Ra) (Garlanda et al. 2013). All three of these IL-1 family members bind to the IL-1 receptor, type I (IL-1RI). IL-1α and IL-1β enhance the immune response while IL-1Ra competitively binds to IL-1RI, thereby preventing the binding of IL-1α and IL-1β

(Garlanda et al. 2013). Although IL-1α and IL-1β are both pro-inflammatory cytokines within the same IL-1 cytokine family, they differ in their maturation processes. IL-1α can be released as pro-IL-1α or mature IL-1α after calpain cleavage. In either form it can actively bind to IL-1RI and mediate downstream signaling (Garlanda et al. 2013; Kim et al. 2013). Conversely, IL-1β is first produced as inactive pro-IL-1β which must be cleaved by a caspase-1 containing inflammasome to yield the mature biologically active cytokine (Latz et al. 2013). After fungal exposure, IL-1β production has been linked to activation of the NLRP3 inflammasome (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Said-Sadier et al. 2010; Tomalka et al. 2011; Lei et al. 2013; Tavares et al. 2013; Mao et al. 2014). Mice lacking the NLRP3 inflammasome are highly susceptible to disseminated candidiasis (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009). However, the role of inflammasome activation by *A. fumigatus in vivo* is unknown.

The role of IL-1α in regulating the pulmonary inflammatory response after infectious challenge is much less understood and is an active area of research. Importantly, several studies have shown that IL-1α and IL-1β can have non-redundant roles in infection and inflammation. Specifically, it has been demonstrated that an increase of IL-1α correlated with early neutrophil recruitment, while IL-1β correlated with macrophage recruitment during later time points in a model of sterile inflammation (Chen et al. 2007; Rider et al. 2011). During pulmonary *Legionella pneumophila* infection IL-1α is essential for early neutrophil responses (Barry et al. 2013). In a systemic candidiasis model, IL-1α and IL-1β played non-redundant roles in anti-fungal immunity by enhancing anti-fungal activity of leukocytes and recruitment of neutrophils,

respectively (Vonk et al. 2006). However, the role(s) of IL-1 cytokines after challenge with the mold *A. fumigatus* remains to be fully defined.

Here, we delineate the differential roles of IL-1α and IL-1β after *in vivo* challenge with *A. fumigatus* and further define the sequence of events required for leukocyte recruitment after *A. fumigatus* challenge. Specifically, we observed, unlike the diseases caused by the yeast *Candida albicans* (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009), that the inflammasome is not essential for preventing severe invasive pulmonary aspergillosis, but does participate in initiating the full anti-fungal activity of leukocytes. In stark contrast, IL-1α signaling through IL-1RI is crucial for the control of pulmonary *A. fumigatus* infection through optimal leukocyte recruitment, which correlated with CXCL1 expression. $CCR2^{+}$ monocytes regulated the early expression of IL-1α and CXCL1, and promoted early neutrophil accumulation in the airways. Treatment of *Il1r1*-deficient mice with a chemokine known to enhance neutrophil recruitment enhanced immunity against pulmonary *A. fumigatus* infection. Thus, our studies define the specific sequence of events regulated by both IL-1α and IL-1β necessary for control of *A. fumigatus* growth and lung damage within the respiratory tract.

Results

Differential Temporal Expression Kinetics of IL-1 Cytokine Family Members After *Aspergillus fumigatus* Challenge.

To examine the early pulmonary inflammatory milieu induced after *A. fumigatus* challenge, bronchoalveolar lavage fluid (BALF) was collected 6, 12, 24, and 48 h after intratracheal (i.t.) instillation of ~$5x10^7$ conidia of the CEA10 strain of *A. fumigatus.* Of

note, both the inflammasome-dependent cytokines IL-1β (Figure 2.1A) and IL-18 (Figure 2.1B) were expressed within approximately 6 h after *A. fumigatus* challenge. In contrast, IL-1α (Figure 2.1C) and IL-1Ra (Figure 2.1D) were expressed in a linearly increasing manner during the first 48 h. Thus, *A. fumigatus* challenge results in temporally distinct expression of IL-1 cytokine family members.

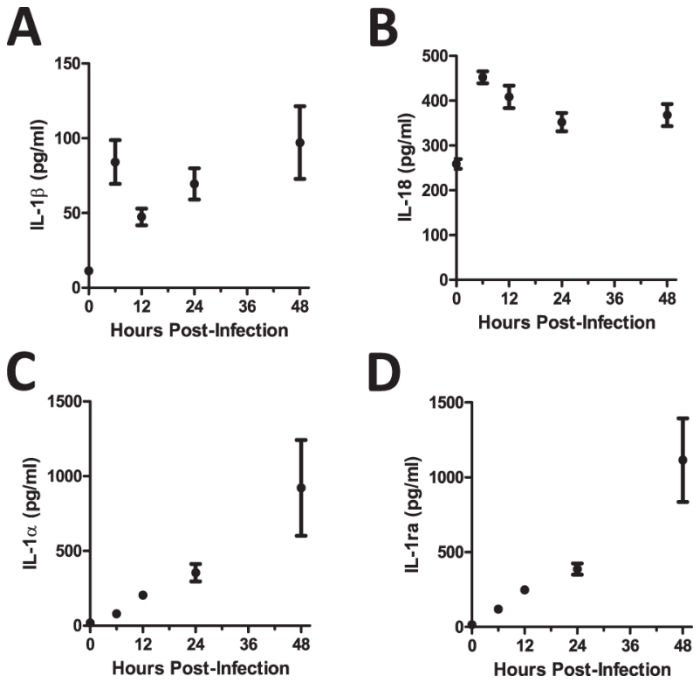


Figure 2.1. C57BL/6 mice show differential expression of IL-1α and IL-1ß after *A. fumigatus* infection. Mice were infected i.t. with $5x10^7$ CEA10 conidia and at indicated time-points, mice were euthanized, bronchoalveolar lavage fluid (BALF) collected, and lung tissue homogenized. IL-1β **(A)**, IL-18 **(B)**, IL-1α **(C)**, and IL-1Ra **(D)** levels in lung homogenate (IL-1α) and BALF (IL-1β, IL-18, and IL-1Ra) were measured using ProcartaPlex Mouse Cytokine & Chemokine 36-plex Immunoassay or ELISA. Data are representative of four mice per time point and two independent experiments. Each dot represents the mean ± one SEM.

Il1r1-Deficient Mice Are Highly Susceptible to Pulmonary *Aspergillus fumigatus* Infection.

In one cohort of human patients it has been shown that a complex polymorphism in the *Il1a*, *Il1b,* and *Il1rn* genes, which was associated with decreased IL-1 dependent inflammatory events, resulted in increased risk for the development of IPA (Sainz et al. 2008). Because of this prior clinical observation plus our finding that both IL-1α and IL-1β are produced in the lungs after *A. fumigatus* challenge (Figure 2.1), we questioned whether IL-1RI signaling was critical in the clearance of *A. fumigatus* from the lung. To globally test the role of IL-1 signaling in limiting *A. fumigatus* growth in the respiratory tract of mice, we challenged C57BL/6 and *Il1r1*-deficient mice with ~$5x10^7$ conidia of *A. fumigatus* CEA10 delivered via the i.t. route. Subsequently, control of *A. fumigatus* in the respiratory tract was assessed by histological analysis at 24, 48, and 72 h after instillation. Strikingly, Grocott-Gomori methenamine silver (GMS) staining of lung tissue from *Il1r1*-deficient mice revealed the presence of a significant fraction of germinating *A. fumigatus* conidia at 48 h that was not observed in C57BL/6 mice (Figure 2.2A). When the presence of germinating *A. fumigatus* conidia was quantified over the first 72 h, C57BL/6 mice displayed minimal germination that was ~4% at 48 h before resolving (Figure 2.2B); in contrast, *Il1r1*-deficient mice displayed a significant impairment in controlling *A. fumigatus* germination within 24 h (Figure 2.2B). By 48 h, the majority of fungal conidia in *Il1r1*-deficient mice were germinated (Figure 2.2B). High levels of germination were observed in the majority of *Il1r1*-deficient mice and this was associated with significant mortality in those mice (Figure 2.2C). To strengthen our conclusion that IL-1RI signaling was crucial for controlling *A. fumigatus* germination in

the lungs rather than a development issue in the *Il1r1*-deficient mice, we treated C57BL/6 mice intraperitoneally (i.p.) with 200 μg of hIL1ra, which antagonizes IL-1α and IL-1β, or placebo every 24 h starting one day prior to challenging mice with ~$5x10^7$ conidia of *A. fumigatus*. Lung tissue from hIL1ra-treated C57BL/6 mice revealed the presence of a significant fraction of germinating *A. fumigatus* conidia at 48 h, which was not observed in placebo treated C57BL/6 mice (Supplemental Figure 2.8). Taken together, these results strongly support the conclusion that IL-1RI signaling is critical for prevention of *A. fumigatus* strain CEA10 pulmonary proliferation and host damage.

Neutrophils and macrophages are widely acknowledged to be critical effector cells for clearing *A. fumigatus* from the lungs (Cramer et al. 2011). Assessing cellular recruitment via differential microscopic counting of cytospins stained with Diff-Quik from the bronchoalveolar lavage fluid at 12, 24, and 48 h post-challenge demonstrated a significant impairment in neutrophil recruitment at each time point analyzed, while macrophage recruitment was similar between C57BL/6 and *Il1r1*-deficient mice at early time points after *A. fumigatus* challenge, but were decreased by 48 h (Figure 2.2D). When inflammatory infiltrates within the BALF and lung parenchyma were assessed at 12, 24, and 36 h by flow cytometry a similar decrease in neutrophils in both compartments was observed in the *Il1r1*-deficient mice (Supplemental Figure 2.9A-B), while $CD11b^+$ macrophages (Supplemental Figure 2.9C), $CD11c^+$ alveolar macrophages (Supplemental Figure 2.9D), and $CD103^+$ dendritic cells (Supplemental Figure 2.9E) were found at similar levels as observed in C57BL/6 mice. We next questioned whether leukocyte recruitment was diminished in *Myd88*-deficient mice because it is the key signaling

adapter for IL-1RI, as well as TLRs (Kawai and Akira ; Garlanda et al. 2013) , and *Myd88*-deficient mice have an impaired ability to control pulmonary *A. fumigatus* growth (Bretz et al. 2008). Indeed, *Myd88*-deficient mice exhibited defective neutrophil recruitment 12 and 24 h after *A. fumigatus* instillation, but normal macrophage recruitment at these early time points (Supplemental Figure 2.10). Thus, mice deficient in *Il1r1* and *Myd88* are highly impaired in their ability to clear *A. fumigatus* from the lungs, which correlates with defects in early neutrophil recruitment to the lungs.

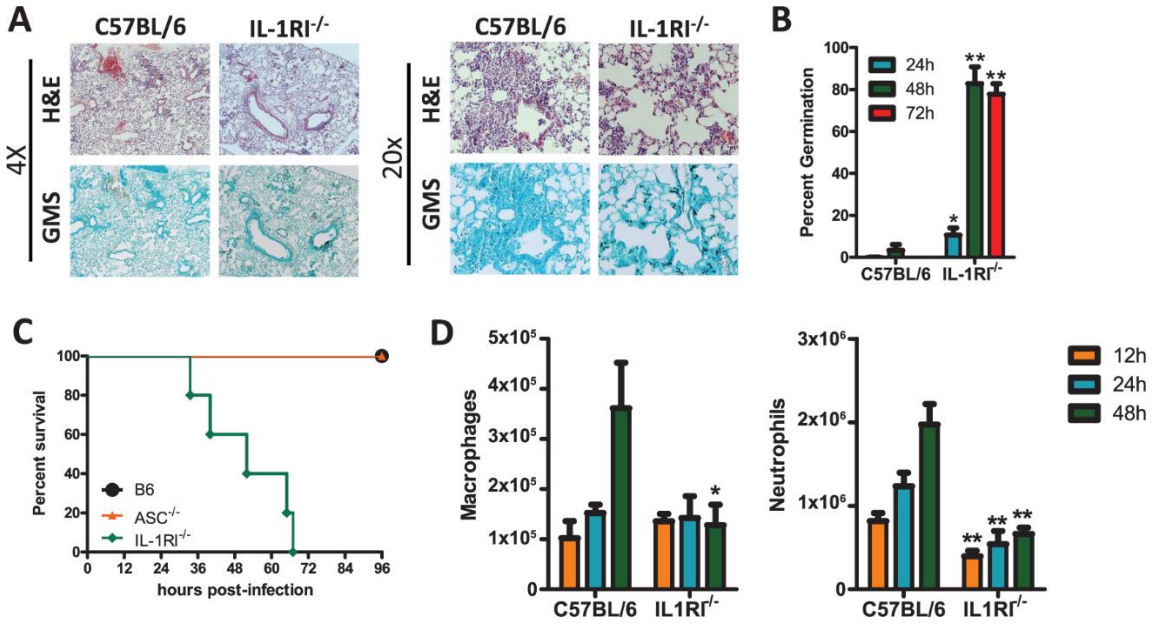


Figure 2.2. *Il1r1*-deficient mice are highly susceptible to *Aspergillus fumigatus* infection. Age-matched C57BL/6 or *Il1r1*-deficient mice were infected i.t. with $5x10^7$ CEA10 conidia and at indicated time-points, mice were euthanized, BALF collected, and lungs saved for histological analysis. **(A)** Formalin-fixed lungs were paraffin embedded, sectioned, and stained with H&E (top) or GMS (bottom) for analysis by microscopy. Representative lung sections from C57BL/6 and *Il1r1*-deficient mice infected with CEA10 for 48 h are shown using either the 4x (left) or 20x (right) objectives. **(B)** *A. fumigatus* germination rates were assessed over the first 72 h of infection by microscopically counting both the number of conidia and number of germlings in GMS-stained section. **(C)** Survival of C57BL/6, *Pycard*$^{-/-}$, and *Il1r1*$^{-/-}$ mice challenged i.t. with $1.5x10^7$ *A. fumigatus* (CEA10) conidia was then monitored for survival over the first 96 h (Mantel-Cox log-rank test, p=0.0002). Data are representative of 2 independent experiments at each time point consisting of at least 5 mice per group. **(D)** Total macrophage (left panel) and neutrophil (right panel) recruitment in the BALF was measured at 12, 24, and 48 h post-infection. Data are representative of at least 2 independent experiments at each time point consisting of 3-5 mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using Student's t-test (*$p < 0.05$; **$p < 0.01$).

Pycard-Deficient Mice Are Only Mildly Susceptible
to Pulmonary *Aspergillus fumigatus* Exposure.

It is well documented that IL-1β secretion requires the function of the inflammasome (Latz et al. 2013) and that both the inflammasome and IL-1β are

important in limiting systemic fungal infections (Deepe and McGuinness 2006; Vonk et al. 2006; Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Tomalka et al. 2011; Lei et al. 2013). Recent *in vitro* studies have shown that the NLRP3-ASC-Capase1 inflammasome can be triggered by *A. fumigatus* (Said-Sadier et al. 2010), but the *in vivo* relevance of this triggering during *A. fumigatus* infection remains unknown. Multiple inflammasome complexes exist, but ASC (*Pycard*) is a central adapter protein needed for maturation of IL-1β and IL-18 (Latz et al. 2013). Thus, to determine the role of the inflammasome after *A. fumigatus* challenge, we challenged C57BL/6 and *Pycard*-deficient mice with ~$5x10^7$ conidia of *A. fumigatus* CEA10 i.t.; subsequently, control of *A. fumigatus* in the respiratory tract was assessed by histological analysis at 24, 48, and 72 h after instillation. GMS staining of lung tissue from *Pycard*-deficient mice revealed the presence of germinating *A. fumigatus* conidia at elevated frequencies compared to C57BL/6 mice at 48 h (Figure 2.3A-B), but this phenotype was less severe than what was observed in *Il1r1*-deficient mice and did not result in murine mortality (Figure 2.2). When the presence of germinating *A. fumigatus* conidia was quantified over the first 72 h, C57BL/6 mice display minimal germination that was ~3% at 48 h (Figure 2.3B). *Pycard*-deficient mice displayed normal control of *A. fumigatus* germination at 24 h. However, by 48 h impaired control of *A. fumigatus* germination (~22%) was observed, but these mice were ultimately able to resolve the *A. fumigatus* challenge (Figure 2.3B). Because, neutrophils and macrophages are widely acknowledged to be critical effector cells for clearing *A. fumigatus* from the lungs (Cramer et al. 2011) and were diminished in the absence of IL-1RI signaling (Figure 2.2C), we next assessed inflammatory cell

recruitment in BALF via differential microscopic counting of cytospins stained with Diff-Quik from the bronchoalveolar lavage fluid at 12, 24, and 48 h after instillation. Interestingly, C57BL/6 and *Pycard*-deficient mice demonstrated equivalent neutrophil and macrophage recruitment at each time point analyzed (Figure 2.3C). Moreover, when the inflammatory infiltrates within the BALF and lung parenchyma were assessed at 12, 24, and 36 h by flow cytometry the number of neutrophils in the BALF and lung parenchyma, $CD11b^+$ macrophages, $CD11c^+$ alveolar macrophages, and $CD103^+$ dendritic cells in the lung parenchyma were found at similar levels in C57BL/6 and *Pycard*-deficient mice (Supplemental Figure 2.9). When we examined the expression of IL-1β in the BALF of *Pycard*-deficient mice, no expression of IL-1β at 12 h was observed while significant levels were detected in C57BL/6 mice (Figure 2.3D); however, when IL-1α was examined in the lung parenchyma we observed equivalent levels of cytokine expression (Figure 2.3E), suggesting that IL-1α signaling could still be activated in the *Pycard*-deficient mice.

Since *Pycard*-deficient mice did not demonstrate impaired leukocyte recruitment after *A. fumigatus* challenge, we next sought to quantitate the anti-fungal activity of macrophages from C57BL/6 and *Pycard*-deficient mice. Hyphal damage induced by macrophages was assessed using the XTT hyphal damage assay, which measures fungal cell metabolic activity as an indirect measure of fungal viability (Shepardson et al. 2013). C57BL/6 and *Pycard*-deficient bone marrow-derived macrophages induced similar hyphal damage when co-cultured with *A. fumigatus* under normoxic conditions (Figure 2.3F, yellow bars). Interestingly, a previous report demonstrated enhanced anti-fungal

activity of leukocytes against fungal hyphae under hypoxic conditions (Shepardson et al. 2013), which occurs within the lungs after *A. fumigatus* challenge and at sites of microbial infection (Nizet and Johnson 2009; Grahl et al. 2011). Intriguingly, time-points when hypoxia is observed also coincides with the recruitment of inflammatory monocytes to the site of infection (Espinosa et al. 2014). Thus, we sought to test the contribution of the inflammasome to the anti-fungal response of macrophages under hypoxic conditions. Similar to the previous findings (Shepardson et al. 2013), C57BL/6 bone marrow-derived macrophages displayed significantly enhanced anti-fungal activity when cultured in hypoxia (Figure 2.3F). In contrast, *Pycard*-deficient macrophages induced less hyphal damage when co-cultured with *A. fumigatus* under hypoxic conditions (Figure 2.3F, blue bars). Since activation of the inflammasome triggers the release of both IL-1β and IL-18 we next sought to assess which inflammasome-dependent cytokine was responsible for increasing the anti-fungal activity of macrophages in hypoxia. C57BL/6 macrophages were treated with an isotype control antibody, anti-IL1β antibody, or anti-IL18 antibody during the co-culture with *A. fumigatus* germlings. Subsequently, fungal damage was again assessed by an XTT assay. Macrophages treated with an isotype control antibody display increased anti-fungal activity in hypoxia. This increased anti-fungal activity was lost in the presence of a blocking anti-IL1β antibody, but not a blocking anti-IL18 antibody (Figure 2.3G). Collectively, these data demonstrate that mice deficient in *Pycard* are mildly impaired in their ability to clear *A. fumigatus* from the lungs, which correlated with *in vitro* defects in

the anti-hyphal activity induced by IL-1β in hypoxia, rather than inflammatory cell recruitment to the lungs.

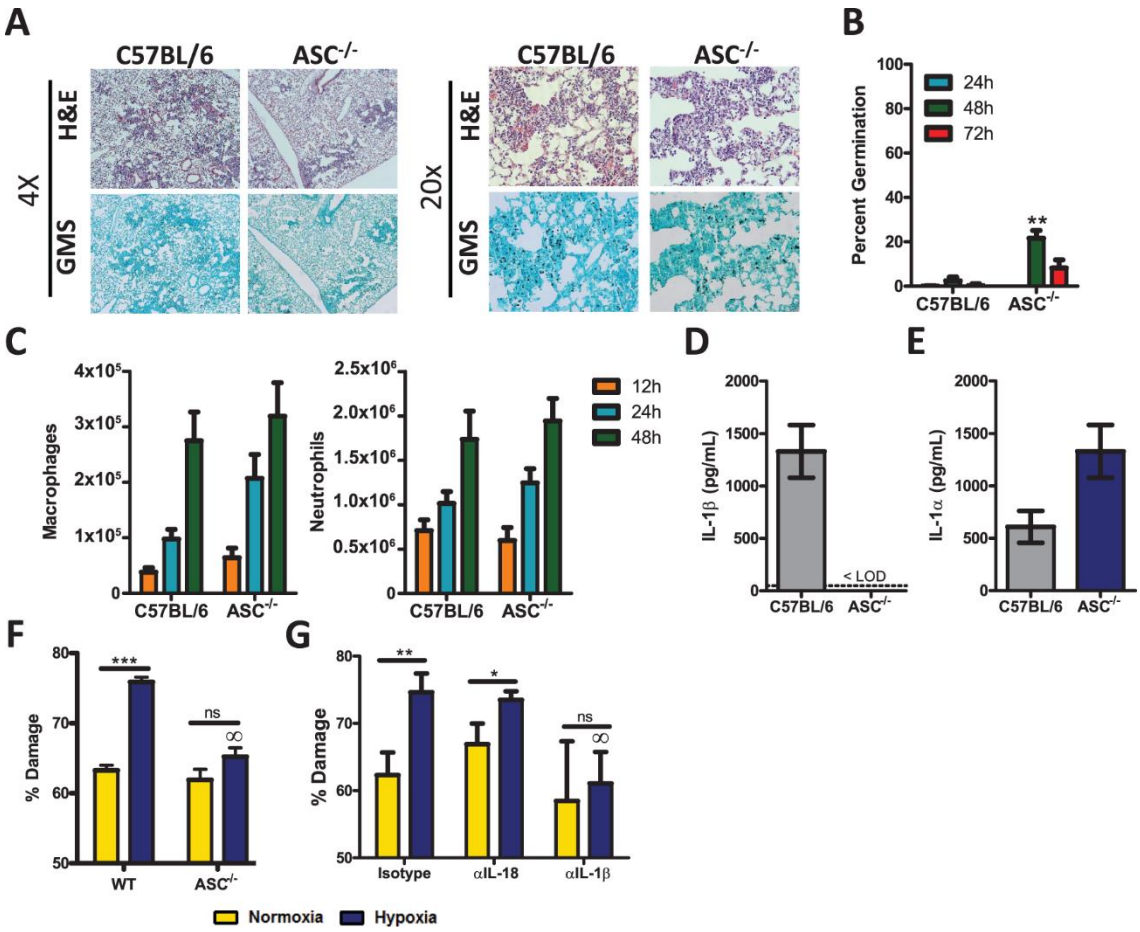


Figure 2.3. *Pycard*-deficient mice are mildly susceptible to *A. fumigatus* infection**.** Age-matched C57BL/6 or *Pycard*-deficient mice were infected i.t. with $5x10^7$ CEA10 conidia and at indicated time-points mice were euthanized, BALF collected, and lungs saved for histological analysis. **(A)** Formalin-fixed lungs were paraffin embedded, sectioned, and stained with H&E (top) or GMS (bottom) for analysis by microscopy. Representative lung sections from C57BL/6 and *Pycard*-deficient mice infected with CEA10 for 48 h are shown using either the 4x (left) or 20x (right) objectives. **(B)** *A. fumigatus* germination rates were assessed over the first 72 h of infection by microscopically counting both the number of conidia and number of germlings in GMS-stained section. **(C)** Total macrophage (left panel) and neutrophil (right panel) recruitment in the BALF was measured at 12, 24, and 48 h post-infection. **(D)** IL-1β levels in the bronchoalveolar lavage fluid (BALF) and **(E)** IL-1α levels in the lung parenchyma were assessed from C57BL/6 and *Pycard*-deficient mice infected i.t. 12 h prior with $5x10^7$ CEA10 conidia.

Figure 2.3 Continued

IL-1α and IL-1β levels in BALF were measured by ELISA. **(B-E)** All data are representative of at least 2 independent experiments at each time point consisting of 3-5 mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using Student's t-test (*$p < 0.05$; **$p < 0.01$). LOD = limit of detection. **(F-G)** The anti-fungal activity of bone marrow-derived macrophages (BMDM) were assessed *in vitro* using the previously described XTT assay (Shepardson et al. 2013). **(E)** BMDMs were obtained from C57BL/6 (WT) or *Pycard*-deficient (ASC) mice. An XTT assay was performed using BMDM from each mouse strain in both normoxic and hypoxic conditions. **(B)** BMDMs from C57BL/6 mice were obtained and incubated with isotype control antibody, IL-18 neutralizing antibody or IL-1ß neutralizing antibody. These BMDMs were then used in an XTT assay in both normoxic and hypoxic conditions. **(E-F)** Data are representative of four biological replicates in each group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using an one-way ANOVA with Bonferroni's post-test ($\infty p < 0.05$ compared to C57BL/6 cells under the same conditions).

Anti-IL-1α Treatment Impairs Pulmonary Recruitment of Leukocytes, Enhancing the Susceptibility of Mice to Pulmonary *Aspergillus fumigatus* Infection.

As *Il1r1*-deficient mice were much less able to control *A. fumigatus* germination than *Pycard*-deficient mice (Figure 2.2 & 2.3) and *Pycard*-deficient mice still produced IL-1α in the lungs (Figure 2.3D), we next sought to understand the role IL-1α played in the clearance of *A. fumigatus* from the lung. To determine the role of IL-1α after *A. fumigatus* challenge, we treated C57BL/6 mice i.p. with 40 μg of goat IgG or anti-IL1α 24 h prior to and 24 h after challenging mice with ~$5x10^7$ conidia of *A. fumigatus*. Control of *A. fumigatus* in the respiratory tract was assessed by histological analysis at 48 h after instillation. GMS staining of lung tissue from anti-IL1α treated C57BL/6 mice revealed the presence of germinating *A. fumigatus* conidia at significantly higher frequencies than seen in goat IgG treated C57BL/6 mice (Figure 2.4A & B). As leukocyte recruitment to the lungs was significantly impaired in *Il1r1*-deficient, but not

Pycard-deficient, mice following *A. fumigatus* challenge, we next assessed inflammatory cell recruitment to the BALF via differential microscopic counting of cytospins stained with Diff-Quik from the bronchoalveolar lavage fluid at 12, 24, and 48 h post-challenge. Interestingly, anti-IL1α treated C57BL/6 mice demonstrated reduced neutrophil recruitment at 24 h post-*A. fumigatus* challenge (Figure 2.4C). Additionally, treatment of *Pycard*-deficient mice with anti-IL1α significantly enhanced the susceptibility of those mice to *A. fumigatus* challenge, mirroring what was found in *Il1r1*-deficient mice (Supplemental Figure 2.11). Thus, blocking IL-1α in mice significantly impairs early neutrophil recruitment to the lungs early after *A. fumigatus* challenge resulting in impaired control of *A. fumigatus* germination in the lungs.

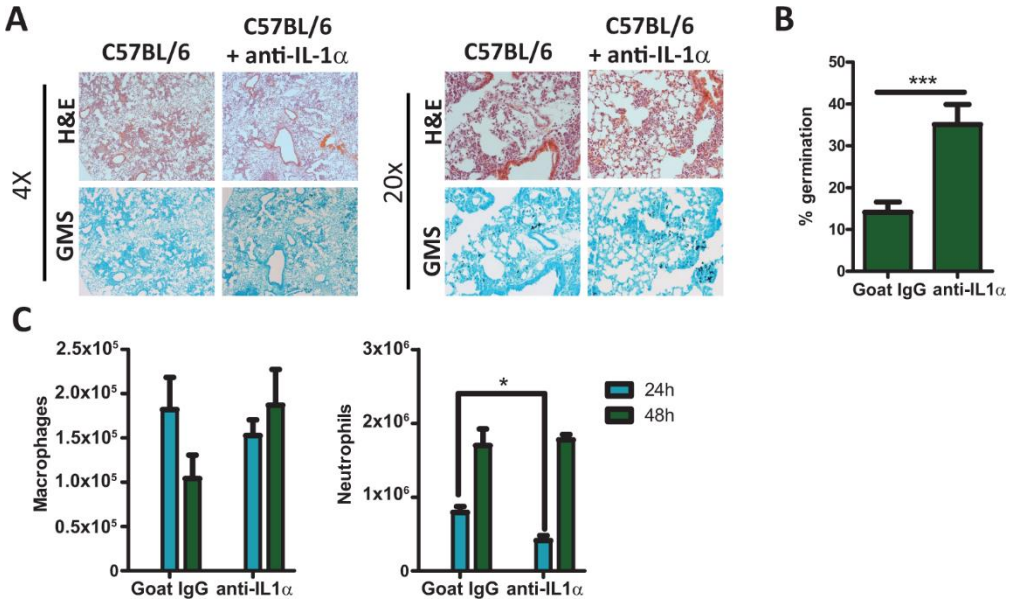


Figure 2.4. C57BL/6 mice treated with IL-1α neutralizing antibody were more susceptible to *Aspergillus fumigatus* infection. C57BL/6 mice treated with isotype control antibody or IL-1α neutralizing antibody were infected i.t. with $5x10^7$ CEA10 conidia. At the indicated time points mice were euthanized, BALF collected and lungs saved for histological analysis. **(A)** Formalin-fixed lungs were paraffin embedded, sectioned and stained with H&E (top) or GMS (bottom) for analysis by microscopy. Representative lung sections from C57BL/6 mice treated with isotype control antibody (left) or with anti-IL-1α antibody (right) and infected with CEA10 for 48 h are shown using either the 4x (left) or 20x (right) objectives. **(B)** *A. fumigatus* germination rates at 48 h after challenge was determined by microscopically counting both the number of conidia and number of germlings in GMS-stained section. **(C)** Total macrophage (left panel) and neutrophil (right panel) recruitment in the BALF was measured at 24 and 48 h post-infection via cytospins. Data are representative of two independent experiments consisting of 4-5 mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using a Student's t-test (*$p < 0.05$, ***$p < 0.001$).

IL-1α Signaling Enhances the Expression of CXCL1.

As both *Il1r1*-deficient mice and anti-IL1α treated C57BL/6 mice displayed significantly decreased cellular infiltration into the BALF (Figure 2.2D and 2.4C), we next sought to understand the roles that IL-1α, IL-1RI, and the inflammasome play in setting up the inflammatory milieu within the lungs. Thus, we challenged four cohorts of

mice, C57BL/6 treated with 40 μg of goat IgG, C57BL/6 treated with 40 μg of anti-IL1α, *Il1r1*-deficient, and *Pycard*-deficient, with ~5x10^7 conidia of *A. fumigatus.* Twenty-four hours after challenge, the inflammatory milieu in the lung parenchyma was assessed by a 12-plex multiplex cytokine assay. Anti-IL1α treatment, rather than *Pycard*-deficiency, largely mirrored the inflammatory cytokine defects found in the *Il1r1*-deficient mice (Figure 2.5) fitting with the biological outcomes of *A. fumigatus* challenge in those mice. Specifically, TNFα, CCL3, and CCL4 expression was not diminished in the absence of IL-1α, IL-1RI, or ASC (Figure 2.5). Interestingly, CXCL1 and G-CSF expression were significantly reduced in *Il1r1*-deficient mice. CXCL1 expression was almost entirely dependent on IL-1α signaling (Figure 2.5), while G-CSF expression trend to being dependent on both IL-1α and ASC in an additive manner (Figure 2.5). A similar trend, as observed with CXCL1, was seen with IL-6 and CCL2, but it did not reach significance (Figure 2.5). Thus, blocking IL-1α in mice significantly decreased the abundance of CXCL1 in the lungs, which correlates with the decreased neutrophil recruitment in *Il1r1*-deficient mice.

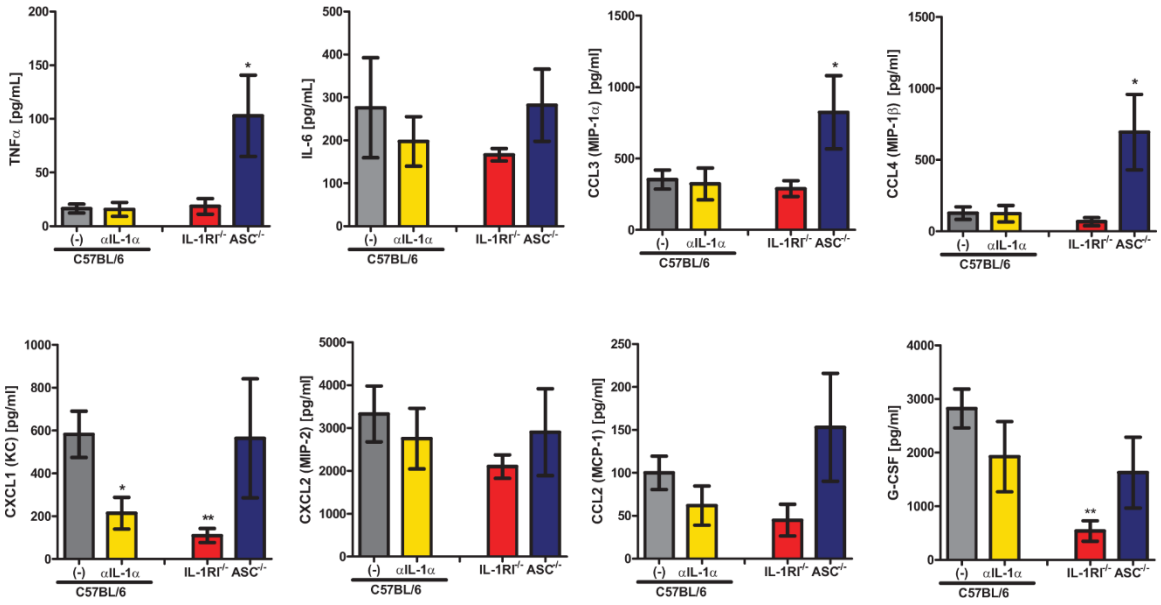


Figure 2.5. IL-1α signaling enhances expression of leukocyte recruiting chemokines. C57BL/6 mice treated with either isotype control antibody or IL-1α neutralizing antibody, *Il1r1*-deficient and *Pycard*-deficient mice were infected with $5x10^7$ CEA10 conidia and at 24 hours post-infection, mice were euthanized, BALF collected, and lung tissue homogenized. Cytokine and chemokine levels in the lung homogenates were measured using 12-plex multiplex Luminex assay, similar trends were observed in BALF. Data are representative of two independent experiments consisting of 4-5 mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using a Kruskal-Wallis one-way ANOVA with Dunn's post-test (*$p < 0.05$, **$p < 0.01$).

Absence of $CCR2^+$ Monocytes Results in Decreased IL-1α, CXCL1, and Neutrophil Recruitment.

As IL1α was necessary for optimal CXCL1 expression and neutrophil infiltration into the BALF (Figures 2.4C & 2.5), we next sought to identify potential cellular sources of IL-1α in response to pulmonary challenge with *A. fumigatus*. Within the lung of a naïve mouse several potential sources of IL-1α exist including: non-hematopoietic cells (epithelial and endothelial cells), alveolar macrophages in the airway spaces, and $CCR2^+$ monocytes within the lung parenchyma. During pulmonary *Mycobacterium tuberculosis*

infection two distinct populations of myeloid cells co-express IL-1α and IL-1β: inflammatory monocytes which are $CD11b^+$ $CD11c^-$ $Ly6c^+$ and monocytic dendritic cells which are $CD11b^+$ $CD11c^+$ (Mayer-Barber et al. 2011). In response to pulmonary *A. fumigatus* challenge, $CCR2^+$ inflammatory monocytes are rapidly recruited to the lung and give rise to monocyte-derived dendritic cells that play essential roles in innate defense against invasive aspergillosis (Espinosa et al. 2014). Interestingly, both $CCR2^+$ inflammatory monocytes and monocyte-derived dendritic cells show increased transcription of the *Il1a* gene at 48 h post-*A.fumigatus* challenge (Espinosa et al. 2014), but whether lung-resident $CCR2^+$monocytes could contribute to IL-1α production at early times after infection was not explored.

Thus, we challenged either C57BL/6 or CCR2-depleter mice (Hohl et al. ; Serbina et al. 2009), which had been treated one day prior with 250 ng of diphtheria toxin, with ~$5x10^7$ conidia of *A. fumigatus.* To confirm depletion of the $CCR2^+$ monocytes we quantified $CCR2^+$ inflammatory monocytes (identified as $CD45^+CD11b^+Ly6C^+Ly6G^-$) in the BALF and lung parenchyma 8 h after *A. fumigatus* challenge by flow cytometry. We found that diphtheria toxin had no effect on $CCR2^+$ monocytes in control animals, while CCR2-depleter mice treated with DT had no detectable $Ly6C^+$ inflammatory monocytes, in the BALF or lung parenchyma as expected (Figure 2.6A) (Espinosa et al. 2014). We found that IL-1α protein levels were significantly decreased when $CCR2^+$ monocytes were absent (Figure 2.6B) consistent with the idea that lung-resident $CCR2^+$ inflammatory monocytes are important for producing and/or inducing expression of this cytokine in the lung at early times after infection. Since blocking IL-1α in mice

significantly decreased the expression of CXCL1 in the lungs (Figure 2.5), we next asked whether CXCL1 protein levels were diminished in the lung parenchyma of the CCR2-depleter mice. We found that CXCL1 protein levels were also significantly decreased in the absence of $CCR2^+$ monocytes (Figure 2.6C). Thus, $CCR2^+$ monocytes are important regulators of the early expression of IL-1α and CXCL1. Consistent with the importance of these factors in promoting early neutrophil recruitment (Fig 2.2 and 2.4), diminished IL-1α and CXCL1 levels in CCR2-depleter mice correlated with diminished recruitment of neutrophils to the airways 8 h after *A. fumigatus* challenge (Figure 2.6D). Thus, $CCR2^+$ monocytes are important regulators of the early expression of IL-1α and CXCL1, which are required for optimal recruitment of neutrophils at early times after *A. fumigatus* challenge.

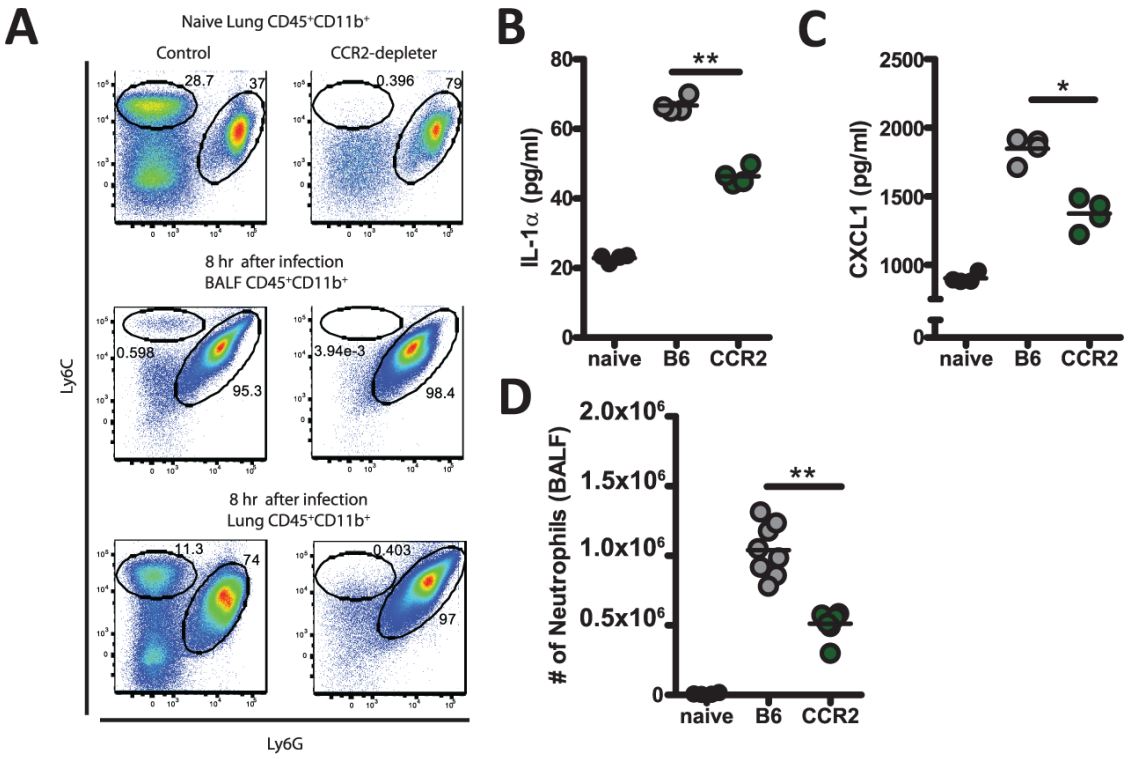


Figure 2.6. CCR2$^+$ monocyte regulate early IL-1α and CXCL1 expression. C57BL/6 or CCR2-depleter mice were treated i.p. with 250 ng of DT 24 h prior to challenge with 5x10^7 Af293 conidia. **(A)** Naïve C57BL/6 or CCR2-depleter mice or C57BL/6 or CCR2-depleter mice challenged eight hours prior were euthanized and the BALF and lung tissue collected for flow cytometric analysis to assess depletion of target cells by DT. Plots are gated on CD45$^+$ CD11b$^+$ cells and show Ly6c and Ly6g staining, which identify the CCR2$^+$ monocytes and neutrophils, respectively. **(B)** IL-1α and **(C)** CXCL1 protein levels in the lung parenchyma at 8 h post-challenge with 5x10^7 conidia of *A. fumigatus* strain Af293 were measured using ELISA assays. Bar graphs show the group means ± one SEM. **(D)** Eight hours post-challenge with 5x10^7 conidia of *A. fumigatus* strain Af293, neutrophils in the BALF were enumerated. Data are representative **(B-C)** or pooled **(D)** from two independent experiments consisting of 4 mice per group. Each symbol represents an individual mouse and the line represents the group mean. Statistically significant differences were determined using a one-way ANOVA with Bonferroni's post-test compared C57BL/6 mice (*$p < 0.05$, **$p < 0.01$).

CXCL1 Supplementation of *Il1r1*-Deficient Mice Enhances Neutrophil Recruitment and Resistance to Pulmonary *Aspergillus fumigatus* Challenge.

As both *Il1r1*-deficient mice and anti-IL1α treated mice displayed significantly decreased cellular infiltration into the BALF (Figure 2.2D and 2.4C) that correlated with decreased abundance of CXCL1 (Figure 2.5), we next sought to test whether immunotherapy which enhances neutrophil accumulation in the lungs, such as CXCL1 supplementation, could enhance control of *A. fumigatus* growth in the *Il1r1*-deficient mice. We challenged either C57BL/6 or *Il1r1*-deficient mice with ~$5x10^7$ conidia of *A. fumigatus*. Three hours after challenge mice were treated i.t. with either PBS or 0.5 μg CXCL1. As expected, *Il1r1*-deficient mice displayed a significant impairment in controlling *A. fumigatus* germination at 48 h when compared with C57BL/6 mice (Figure 2.7A-B). Provision of CXCL1 to *Il1r1*-deficient mice could partially rescue control of *A. fumigatus* germination in the lungs, while no enhancement in control of fungal growth was observed in the CXCL1 treated C57BL/6 mice (Figure 2.7A-B). Furthermore, provision of CXCL1 i.t. rescued the impairment of anti-IL1α treated C57BL/6 mice in controlling *A. fumigatus* infection (Supplemental Figure 2.12A). As expected, twenty-four hours after challenge the recruitment of neutrophils, but not macrophages, to the BALF in *Il1r1*-deficient mice was enhanced by the provision of CXCL1 (Figure 2.7C). Additionally, neutrophil recruitment to the BALF in anti-IL1α treated C57BL/6 mice was significantly enhanced (Supplemental Figure 2.12B). While CXCL1 provision enhanced neutrophil accumulation in the airways of *Il1r1*-deficient mice, we also sought to test whether the anti-hyphal activity of neutrophils was altered in the absence of IL-1RI

signaling but exogenous addition of CXCL1. Hyphal damage induced by neutrophils isolated from the bone marrow of respective mouse genotypes was assessed using the XTT hyphal damage assay (Shepardson et al. 2013). C57BL/6 bone marrow neutrophils induced robust hyphal damage when co-cultured with *A. fumigatus*, which was not further enhanced by treatment with 50 nM of CXCL1 (Figure 2.7D). Interestingly, *Il1r1*-deficient bone marrow neutrophils induced significantly less damage to *A. fumigatus* hyphae than was observed with C57BL/6 bone marrow neutrophils (Figure 2.7D). In contrast to the treatment of C57BL/6 bone marrow neutrophils, treatment of *Il1r1*-deficient bone marrow neutrophils with 50 nM of CXCL1 significantly enhanced the anti-hyphal activity of those cells (Figure 2.7D). When cell death and endothelial/epithelial cell leakage were assessed *in vivo* by lactate dehydrogenase (LDH) and albumin measurement, respectively, in the BALF both markers were increased in the absence of IL-1RI (Figure 2.7E-F). CXCL1 supplementation reduced both markers, but albumin levels were more dramatically reduced than LDH levels (71% versus 32%, respectively) (Figure 2.7E-F). Thus, provision of CXCL1 could significantly enhance neutrophil recruitment to the lungs in the absence of IL-1α signaling and enhanced the *in vitro* anti-hyphal activity of *Il1r1*-deficient bone marrow neutrophils, which together ultimately resulted in a partial repair of the *A. fumigatus* control mechanisms in the *Il1r1*-deficent lungs.

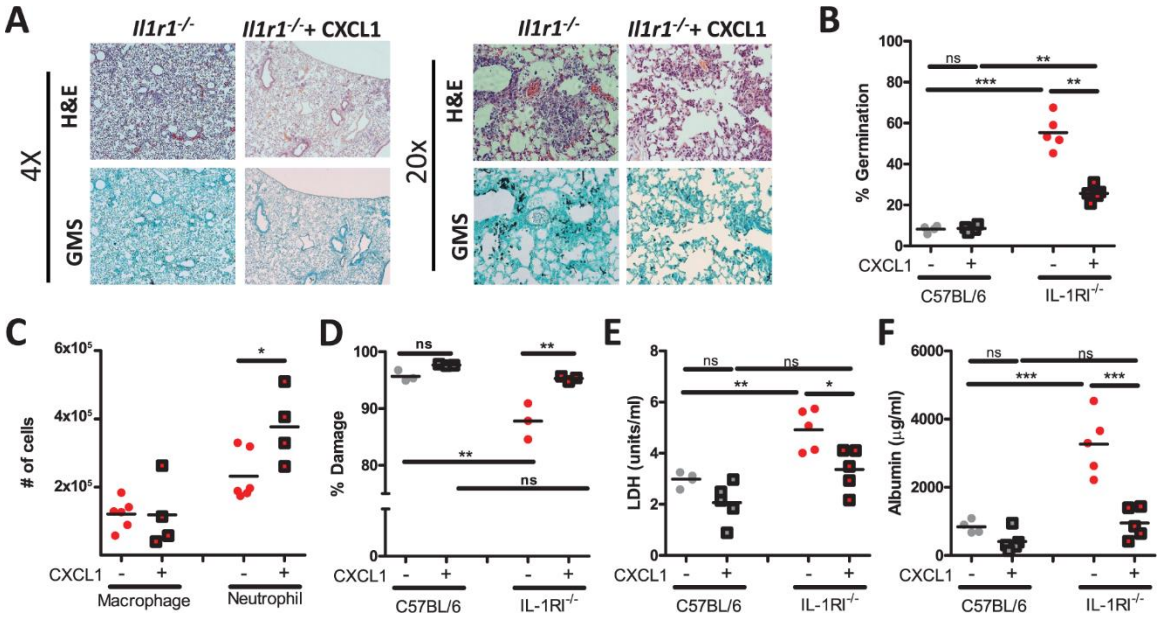


Figure 2.7. Treatment of *Il1r1*-deficient mice with CXCL1 partially increases resistance to *Aspergillus fumigatus* infection. C57BL/6 mice and *Il1r1*-deficient mice were challenged i.t. with $5x10^7$ CEA10 conidia. Three hours post-challenge mice were given 0.5 μg CXCL1 i.t. or PBS alone. Twenty-four hours post-infection, mice were euthanized, BALF collected, and lungs saved for histological analysis. **(A)** Formalin-fixed lungs were paraffin embedded, sectioned and stained with H&E (top) or GMS (bottom) for analysis by microscopy. Representative lung sections from *Il1r1*-deficient mice challenged with CEA10 for 48 h and treated with either PBS or CXCL1 are shown using either the 4x (left) or 20x (right) objectives. **(B)** *A. fumigatus* germination rates were assessed at 48 h of infection by microscopically counting both the number of conidia and number of germlings in GMS-stained section. Number of conidia and number of germlings were counted for each GMS-stained section to quantify the percent germination. **(C)** Macrophage and neutrophil recruitment in *Il1r1*-deficient mice 24 h post-challenge infected with *A. fumigatus* treated with PBS or CXCL1 given i.t. was determined via cytospins.**(D)** Bone marrow neutrophils from C57BL/6 and *Il1r1*-deficient mice were incubated with CEA10 germlings *in vitro* at a 10:1 ratio in normoxia for 2 h. The XTT assay was used to determine percent fungal damage. **(E)** Lung damage and **(F)** leakage were assessed by measuring LDH and albumin, respectively. Data is representative of at least two independent experiments consisting of three to five mice per group, except for the bone marrow neutrophil anti-hyphal XTT assay which is a single experiment which consisted of pooled bone marrow neutrophils from three mice done in triplicate. Each symbol represents an individual mouse or replicate and the line represents the group mean. Statistically significant differences were determined using a one-way ANOVA with Bonferroni's post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not significant).

Discussion

In this study, we uncover an essential function for IL-1RI in preventing fungal proliferation and host damage in murine lungs. We have demonstrated a novel dichotomy for the IL-1 cytokines in regulating the innate immune response induced by *A. fumigatus*. Specifically, IL-1α is required for initiating the correct inflammatory signals necessary for optimal leukocyte recruitment, while the inflammasome and IL-1β was necessary for optimal anti-fungal activity against fungal hyphae. We have elucidated that IL-1α plays the dominant role in activating IL-1RI signaling which results in amplified CXCL1 expression, which correlated with optimal leukocyte recruitment to the respiratory tract. $CCR2^{+}$ monocytes were important cells in regulating the early production of IL-1α, CXCL1, and neutrophil recruitment. Taken together, our data demonstrate that signaling through IL-1RI by both IL-1α and IL-1β was necessary for optimal control of *A. fumigatus* pulmonary challenge to prevent IPA development.

IL-1RI signaling was essential in resisting pulmonary *A. fumigatus* challenge in our studies, as demonstrated by *Il1r1*-deficient mice being unable to resist fungal growth resulting in significant mortality in those animals. This finding is consistent with results reported by Pearlman and colleagues who also found that *Il1r1* was needed to prevent the development of *A. fumigatus* induced keratitis (Leal et al. 2010). Moreover, van de Veerdonk and colleagues have recently shown that a polysaccharide fungal virulence factor, galactosaminogalactan (GAG), from *A. fumigatus* induces the expression of IL-1Ra, which antagonizes IL-1 signaling resulting in enhanced susceptibility to IPA (Gresnigt et al. 2014). Gresnigt *et al* demonstrated that GAG pretreatment of BALB/c

mice resulted in more fungal growth associated with impaired neutrophil recruitment, which was completely dependent on IL-1Ra expression (Gresnigt et al. 2014). However, the importance of GAG induction of IL-1Ra during a live pulmonary *A. fumigatus* infection remains unknown. GAG expression might actually be reduced at specific infection sites during *in vivo A. fumigatus* challenge because hypoxia, which occurs after *A. fumigatus* challenge (Grahl et al. 2011) has been observed to reduce GAG production (Shepardson et al. 2013). In general, the temporal and spatial dynamics of fungal cell wall PAMPs *in vivo* during an active infection is not fully understood and likely complicated by the heterogeneous nature of the lung and infection site microenvironments.

Downstream of IL-1RI the proximal signaling adapter to propagate IL-1 signaling is MyD88. Similar to our observation with *Il1r1*-deficient mice, Marr and colleagues (Bretz et al. 2008) and Hohl and colleagues (Jhingran A. *et al*, in press) have found the *Myd88*-deficient mice are more susceptible to *A. fumigatus* challenge. Additionally, impaired control of pulmonary histoplasmosis and disseminated candidiasis was observed in *Il1r1*-deficient and *Il1a/Il1b*-doubly deficient mice, respectively (Deepe and McGuinness 2006; Vonk et al. 2006). While our studies and the studies just discussed strongly support a role for IL-1RI signaling in limiting IPA, and other invasive fungal infections, Romani and colleagues have observed that *Il1r1*-deficient mice were more resistant to pulmonary *A. fumigatus* challenge (Bellocchio et al. 2004). The difference with our study is potentially due to the *A. fumigatus* strain studied, as Romani and colleagues have shown that different *A. fumigatus* strains have diverse abilities to induce

pathology and immune responses (Rizzetto et al. 2013). Importantly, the infection models studied are significantly different, with Romani and colleagues utilizing a cyclophosphamide-induced immunosuppression model with *Aspergillus* conidia delivered on 3 consecutive days intranasally (Bellocchio et al. 2004), while our studies used immunocompetent mice and a single dose of *Aspergillus* conidia given intratracheally. Additionally Bellocchio et al. (Bellocchio et al. 2004), reported that histological analyses in the *Il1r1*-deficient mice revealed "numerous fungal elements in the relative absence of signs of inflammatory pathology" which is consistent with the results we report here in our experimental model. Murine mortality in infectious disease models can result from direct pathogen mediated damage or immunopathogenesis, and it is unclear, in this regard, how our models differ. What appears to be clear, however, is that in the absence of IL-1RI signaling, *Aspergillus* proliferation increases *in vivo.* Taken together, all these findings demonstrate that the IL-1 signaling pathway is likely central for resistance to fungal diseases, but their role during immunosuppression and frequency of fungal exposure/quantity may differ, warranting further exploration of the IL-1 cytokine family in each clinically relevant model of IPA.

In further support of our observation, the protective role of IL-1 cytokines in anti-fungal immunity uncovered in our study using the murine model of *A. fumigatus* infection is likely to be operational in humans as indicated by genetic linkage studies. First, individuals with SNPs in the IL-1 gene cluster, which are associated with decreased IL-1 dependent inflammatory events, were at increased risk for the development of IPA (Sainz et al. 2008; Wójtowicz et al. 2015). Second, polymorphisms in the *CIAS1* gene

play a central role regulating inflammasome activity and IL-1β production, which can alter the risk of a subset of patients to developing recurrent vulvovaginal candidiasis (Lev-Sagie et al.). Third, macrophages from patients with chronic cavitary pulmonary aspergillosis (CCPA) had prolonged expression of *Il1a* and *Il1b* after *A. fumigatus* treatment when compared to healthy controls and SNPs in the *Il1b* and *Il1rn* loci are associated with susceptibility to developing CCPA (Smith et al.). Thus, targeting the IL-1 cytokine pathways in humans could be important in managing fungal infections.

In previous papers exploring the role of MyD88 during *A. fumigatus* (Bretz et al. 2008; Leal et al. 2010) it was shown that fungal growth was not controlled, but the mechanism impaired in the absence of IL-1RI and MyD88 signaling remains an open question. In this study and a parallel study by Jhingran *et al* (in press) it was demonstrated that MyD88 and IL1RI mediated signals are necessary for optimal leukocyte recruitment after pulmonary *A. fumigatus* challenge, which is needed for preventing the development of IPA. Analogously, in the *A. fumigatus* keratitis model both *Myd88*- and *Il1r1*-deficient mice demonstrated reduced cellular infiltrate early after inoculation (Leal et al. 2010). However, why the lack of IL-1RI or MyD88 signaling results in decreased cellular infiltrates was an open question. Interestingly, we found a decrease in the expression of the chemokine CXCL1 in *Il1r1*-deficient mice, which others have also observed in challenged *Myd88*-deficient mice (Bretz et al. 2008) (Jhingran A. *et al*, in press). CXCL1, together with CXCL2 and CXCL5, are ligands for CXCR2 and are key chemoattractants for recruitment of neutrophils. Administration of a blocking anti-CXCR2 antibody or genetic ablation of *Cxcr2* has been shown to

exacerbate mortality and delay neutrophil recruitment following pulmonary *A. fumigatus* challenge (Mehrad et al. 1999; Bonnett et al. 2006). Moreover, transient over-expression of CXCL1 in CC10-expressing lung epithelial cells resulted in significantly enhanced leukocyte accumulation and reduced fungal burden (Mehrad et al. 2002). Correspondingly, when we treated *Il1r1*-deficient mice with recombinant murine CXCL1 we observed significantly enhanced neutrophil accumulation. In addition, *Il1r1*-deficient bone marrow neutrophils displayed decreased anti-hyphal activity *in vitro,* which was restored by treatment with CXCL1. These data demonstrate that both IL-1RI and CXCL1 signaling is critical in not only enhancing neutrophil recruitment to the airways in the *Il1r1*-deficient mice, but also in inducing the optimal anti-hyphal state of the recruited neutrophils. While the mechanism behind CXCL1 mediated anti-hyphal activity in our model is unknown, neutrophils from *Cxcl1*-deficient mice have an impaired reactive oxygen response in a polymicrobial sepsis model (Liliang et al. 2014). Moreover, *Cxcl1*-deficient neutrophils stimulated with *Klebsiella pneumoniae* had reduced expression of $p67^{phox}$ and $p47^{phox}$ and reduced production of myeloperoxidase, nitric oxide, and hydrogen peroxide, which results in decreased killing of *Klebsiella pneumoniae* by the neutrophils (Batra et al. 2012). Finally, IL-8 has been shown to be important for priming the human neutrophils reactive oxygen burst (Swain et al. 2002). Thus, our data supports a model where IL-1RI signaling is critical for optimal neutrophil recruitment and activation of their anti-hyphal activity in part through the regulation of CXCL1 abundance. Further support for this conclusion comes from a recent analysis of mice with a myeloid deficiency of the transcriptional regulator HIF1α. Loss of myeloid

HIF1α results in severe susceptibility to the same strain of *A. fumigatus* utilized here in part through reduction in neutrophil recruitment. Importantly, loss of HIF1α resulted in decreased IL1-α and CXCL1 levels after *A. fumigatus* challenge similar to what we observed in our studies (Shepardson et al. 2014). Interestingly, other inflammatory pathways are also temporally regulating neutrophil recruitment after *A. fumigatus* challenge, because *Card9*-deficient mice had a late defect in neutrophil recruitment that was associated with a more global diminution of the inflammatory milieu (Jhingran et al. 2012). In addition to the early defect in neutrophil recruitment *Il1r1*-deficient mice also had decreased macrophage recruitment to the airways by 48 h post-inoculation. The reason for this is unknown at this time, but it is known that G-CSF deficient mice have monocyte defects and our cytokine analysis demonstrated that *Il1r1*-deficient mice had significantly lower level of G-CSF in the airways (Lieschke et al. 1994). Thus, further studies exploring the regulation of multiple neutrophil chemotactic pathways, such as CXCR2-, CCR1-, IL17-, leukotriene-, and complement-dependent pathways, and monocyte chemotactic pathways, such as G-CSFR- and CCR2-dependent pathways, are needed after pulmonary fungal challenge.

IL1RI, together with IL1RAcP, is the high-affinity receptor for both IL-1α and IL-1β (Garlanda et al. 2013). The maturation and secretion of IL-1α and IL-1β is known to be regulated by distinct proteolytic pathways dependent on calpain and caspase-1, respectively (Garlanda et al. 2013; Zheng et al. 2013). Numerous fungal pathogens have been shown to activate the inflammasome resulting in the production of IL-1β (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Said-Sadier et al. 2010; Tomalka et al. 2011;

Lei et al. 2013; Tavares et al. 2013; Mao et al. 2014). Importantly for our studies, others have demonstrated that the NLRP3-ASC-Caspase1 inflammasome could be activated by *A. fumigatus* (Said-Sadier et al. 2010), but the *in vivo* relevance of that finding was unknown. Control of *C. albicans* infection, which also activated the NLRP3-ASC inflammasome, was highly dependent on NLRP3 and IL-1β (Vonk et al. 2006; Gross et al. 2009; Hise et al. 2009; Joly et al. 2009). In sharp contrast, our current results indicate that the inflammasome only plays a modest role in the control of pulmonary *A. fumigatus* growth. In our experiments, neutrophil recruitment in mice lacking the inflammasome was completely normal, which is in contrast to *C. albicans* infection where mice deficient in IL-1β displayed a significant reduction in neutrophil recruitment (Vonk et al. 2006). Furthermore, antibody blockade of IL-1β during pulmonary *Histoplasma capsulatum* infection resulted in decreased survival associated with decreased recruitment of $Gr\text{-}1^+$ cells early and $CD4^+$ cells late to the lungs of challenged animals (Deepe and McGuinness 2006). Thus, we were surprised to observe such a dominant role for IL-1α in regulating early leukocyte recruitment following pulmonary *A. fumigatus* challenge, which correlates with its regulation of the chemokine CXCL1. In support of this finding, during sterile inflammation the importance of IL-1α in regulating neutrophil recruitment is unquestionable (Chen et al. 2007; Rider et al. 2011). It has also been demonstrated that IL-1α plays a critical role during murine *L. pneumophila* infection, initiating neutrophil recruitment and the inflammatory response early after infection (Barry et al. 2013). Others have previously shown that IL-1RI and MyD88 expression within a radioresistant population of cells was essential for optimal expression of CXCL1 and CXCL2 during *L.*

pneumophila infection (LeibundGut-Landmann et al. 2011). Interestingly, in their parallel study Hohl and colleagues found that IL-1RI/MyD88 signaling in a radioresistant cell population was necessary for optimal CXCL1 expression and neutrophil recruitment early after pulmonary *A. fumigatus* challenge (Jhingran A. *et al,* in press).

Because of the early importance of IL-1 cytokines in regulating the pulmonary anti-fungal immune response, non-hematopoietic cells (epithelial or endothelial cells) or lung-resident myeloid cells could represent potential sources of IL-1α and IL-1β after *A. fumigatus* challenge. During pulmonary *Mycobacterium tuberculosis* infection two distinct populations of myeloid cells co-express IL-1α and IL-1β: inflammatory monocytes which are $CD11b^{+}$ $CD11c^{-}$ $Ly6c^{+}$ and monocytic dendritic cells which are $CD11b^{+}$ $CD11c^{+}$ (Mayer-Barber et al. 2011). After pulmonary *A. fumigatus* challenge both inflammatory monocytes and monocytic dendritic cells are found in the lung parenchyma and both show increased transcription of the *Il1a* gene (Espinosa et al. 2014). Here our data demonstrates that $CCR2^{+}$ monocytes are at least one of the important cell types regulating the early expression of IL-1α and CXCL1, as well as neutrophil recruitment at 8 hpi. However, in the absence of $CCR2^{+}$ monocytes there is still a significant amount of IL-1α and CXCL1 produced in the lungs after *A. fumigatus* challenge, thus there are likely multiple sources of IL-1α that can regulate early pulmonary neutrophil accumulation. Moreover, by 48 h after infection CXCL1 levels and neutrophil recruitment to the lung is unaffected in CCR2-depleter mice (Espinosa et al. 2014), thus suggesting that distinct mechanisms of neutrophil recruitment are operational at various times after infection. This is supported by observation that *Myd88*-

deficient and *Card9*-deficient mice have early or late defects in neutrophil recruitment, respectively (Jhingran A. *et al,* in press and (Jhingran et al. 2012)).

In our experiments the inflammasome and IL-1β appear to regulate the anti-fungal activity of macrophages against hyphae, especially under hypoxic conditions. This enhancement of anti-fungal activity in hypoxic microenvironments is physiologically and clinically important because hypoxia can be generated within the lungs of mice with IPA (Grahl et al. 2011), which is coincident with inflammatory monocyte arrival to the lungs (Espinosa et al. 2014). Understanding how hypoxia can enhance the anti-fungal activity of macrophage in an inflammasome and IL-1β dependent manner will be important in understanding how macrophages limit fungal growth. Interestingly, a recent paper from Torres *et al* demonstrated that acidosis, which can be driven by hypoxia, resulted in increased IL-1β production in response to *P. aeruginosa* challenge (Torres et al. 2014). Perhaps somewhat surprisingly, in the absence of MyD88 anti-fungal activity against *A. fumigatus* conidia remains intact (Jhingran A. *et al,* in press). It has been shown in other fungal pathogens that IL-1β treatment of human peripheral blood leukocytes enhances their anti-fungal activity against *Paracoccidiodies brasiliensis* (Kurita et al. 2000; Kurita et al. 2005). Additionally, *Il1r1*- and *Nlrp3*-deficient macrophages have impaired antifungal activity against *P. brasiliensis* (Tavares et al. 2013). In contrast, during disseminated candidiasis *Il1a*-deficiency was associated with decreased anti-fungal activity of leukocytes (Vonk et al. 2006). Thus, studies designed to understand the differential dependencies of the IL-1 cytokines in regulating

leukocyte recruitment and anti-fungal activity during a range of fungal diseases and morphological forms are needed.

In addition to understanding the cellular source of IL-1α and IL-1β, understanding the inflammatory pathways leading to expression of IL-1α and IL-1β are essential to our understanding of resistance to IPA. In the absence of dectin-1 signaling there is decreased expression of both IL-1α and IL-1β (Steele et al. 2005; Werner et al. 2009). The loss of HIF1α in the LysM-expressing cells also resulted in decreased IL-1α levels after *A. fumigatus* challenge (Shepardson et al. 2014), which can be regulated by dectin-1 agonists such as β-glucan (Cheng et al. 2014). Pulmonary *A. fumigatus* infection results in significant tissue damage and cell death, but the exact type of cell death is not known. Moreover, the phenotype of cell death will be shaped by the hypoxic microenvironment found during IPA. The type of cellular death occurring *in vivo* during *A. fumigatus* will have important immunological impacts shaping the early IL-1α and IL-1β response because necrotic cell death favors IL-1α release while pyroptosis favors IL-1β release (England et al. 2014). Interestingly, *C. albicans* mutants with defects in inducing pyroptosis also demonstrated defects in inducing IL-1β secretion, but IL-1α release was not examined (Wellington et al. 2013). Additionally, understanding how deficiencies in PRR signaling alters the overall inflammatory response will be crucial as patients with SNPs in PRRs are known to have elevated risks for developing IPA (Romani 2011). Our data demonstrate that in the *Pycard*-deficient mice there are elevated levels of TNFα, CCL3, and CCL4. One explanation for observing elevated levels of TNFα, CCL3, and CCL4 could be the range or degree that PRRs are being

engaged in the *Pycard*-deficient mice and/or temporal and spatial dynamics of fungal cell wall PAMP engagement with PRRs *in vivo* that are not fully understood and further complicated by a PRR known to be engaged during infection now being absent.

It is well defined that prolonged corticosteroid treatment increases susceptibility of hosts to IPA (Lewis and Kontoyiannis 2009). Interestingly, dexamethasone induces the expression and release of IL-1RII (Colotta et al. 1993). IL-1RII is known to limit the activity of IL-1 cytokines and/or sequester IL-1α protein in the cytosol, preventing the cleavage of IL-1α by calpain (Zheng et al. 2013). Dexamethasone has also been shown to impair IL-1α and IL-1β secretion from human mast cells in response to *Pseudomonas aeruginosa* stimulation (Lin et al. 2002). Moreover, dexamethasone treatment of bronchoalveolar macrophages prior to treatment with *A. fumigatus* conidia significantly impaired their release of IL-1α (Kamberi et al. 2002; Brummer et al. 2003). Because we have uncovered such a prominent role for IL-1α in controlling pulmonary *A. fumigatus* challenge, future studies exploring the cleavage status of IL-1α and expression of IL-1RII in clinically relevant models are critical.

Finally, the importance of appropriate activation of leukocytes in the control of *A. fumigatus* is highlighted by patients with chronic granulomatous disease being highly susceptible to *A. fumigatus* (Pollock et al. 1995; Morgenstern et al. 1997; Ben-Ari et al. 2012). Interestingly, CGD patients or mice are typically in a hyperinflammatory state, which is linked to inflammasome activity and IL-1β expression (van de Veerdonk et al. 2010; de Luca et al. 2014). Further, blockade of IL-1 cytokines in $p47^{phox}$-deficient mice through treatment with hIL1ra results in improved control of *A. fumigatus* (de Luca et al.

2014). Together, these studies demonstrate that further exploration of the positive and negative regulators of IL-1 signaling during invasive fungal infections is needed. Moreover, it is critical that we continue to explore the regulation of the inflammatory response induced in each of the different subpopulations of hosts susceptible to developing invasive fungal infections in order to develop patient specific novel immunotherapeutic approaches that could complement treatment with anti-fungal agents.

Materials and Methods

Mice

C57BL/6J mice were bred in-house. *Pycard* (ASC)-deficient and *Myd88*-deficient mice were originally provided by Dr. Vishiva Dixit (Genentech) and Dr. Mark Jutila (Montana State University), respectively. *Il1r1*-deficient (Stock #003245) and C57BL/6 (Stock #000664) mice were originally purchased from Jackson Laboratories. Mouse strains were then bred in-house. The CCR2-depleter (CCR2-DTR) strain was generated on the C57BL/6 background as previously described (Hohl et al. ; Serbina et al. 2009). Control animals for $CCR2^{+}$ monocyte depletion experiments were sex- and age-matched, non-transgenic littermates. All mice were 8-10 weeks of age at the time of infection. All animal experiments were approved by the Montana State University or Rutgers University Institutional Animal Care and Use Committee.

Preparation of *Aspergillus fumigatus* Conidia

A. fumigatus strain CEA10 or Af293 was grown on glucose minimal media (GMM) agar plates for 3 days or Sabouraud dextrose agar (SDA) for 7-10 days at 37°C,

respectively. Conidia were harvested by adding 0.01% Tween 80 to plates and gently scraping conidia from the plates using a cell scraper. Conidia were then filtered through sterile Miracloth, were washed and resuspended in phosphate buffered saline (PBS), and counted on a hemacytometer.

Aspergillus fumigatus Challenge Pulmonary Model

Mice were challenged with *A. fumigatus* conidia by the i.t. route. Mice were anesthetized with 2.5% 2,2,2-tribromoethanol or a Ketamine/Xylazine solution given i.p.; subsequently, mice were challenged i.t. with ~5×10^7 *A. fumigatus* conidia in a volume of 100 µl. At the indicated time after *A. fumigatus* challenge, mice were euthanized using a lethal overdose of pentobarbital. Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs with 2 ml of PBS containing 0.05*M* EDTA. BALF was clarified by centrifugation and stored at -20°C until analysis. BAL cells were resuspended in 200 µl of PBS and total BAL cells were determined by hemacytometer count. BAL cells were subsequently spun onto glass slides using a Cytospin4 cytocentrifuge (Thermo Scientific) and stained with Diff-Quik stain set (Siemens) for differential counting. For histological analysis lungs were filled with and stored in 10% buffered formalin phosphate for at least 24 hours. Lungs were then embedded in paraffin and sectioned into 5-micron sections. Sections were stained with H&E and GMS using standard histological techniques to assess lung inflammatory infiltrates and fungal germination, respectively. For cytokine analysis lungs were homogenized in 2 ml of PBS. After clarification, lung homogenates were stored at -20°C until analysis.

Neutralizing Antibodies and Chemokine Reconstitution

For IL-1α neutralization studies, normal goat IgG control and anti-mIL-1α neutralizing antibody were purchased from R&D systems. IgG control or anti-mIL-1α neutralizing antibody were administered i.p. at 40 µg per mouse. Administration of neutralizing antibody was given every other day, beginning the day prior to *A. fumigatus* challenge. For CXCL1 reconstitution studies, recombinant murine CXCL1 was purchased from PeproTech. CXCL1 was administered i.t. at 0.1-0.5 µg per mouse and was given 3 hours after *A. fumigatus* challenge. For the hIL1ra studies, recombinant hIL1ra and the appropriate placebo (Amgen) were kindly provided by Dr. Charles A. Dinarello. The hIL1ra and placebo were administered i.p. at 200 mg per mouse given at -24, 0, and +24 h relative to *A. fumigatus* challenge.

$CCR2^+$ Cell Depletion Strategy

For depletions of $CCR2^+$ cells, CCR2-DTR mice and control littermates received 250 ng of diphtheria toxin i.p. one day prior to infection. Diphtheria toxin was purchased from List Biological Laboratories (Campbell, CA) and reconstituted at 1 mg/ml in PBS. Aliquots were stored at -80°C. The specificity and efficiency of depletion in the lung was confirmed by flow cytometry.

Quantification of Lung Damage and Leakage

To assess lung damage, bronchoalveolar lavage fluid was analyzed by measuring lactate dehydrogenase levels using a CytoTox 96 Cytotoxicity Assay (Promega) following the manufacturer's instructions. To assess vascular/pulmonary leakage,

bronchoalveolar lavage fluid was analyzed using an Albumin BCG Reagent Set (Eagle Diagnostics). A standard curve was made by diluting the calibrator in PBS. Then 100 μl of sample or standard was transferred to a 96 well flat-bottomed plate, mixed with 100 μl of BCG reagent, let sit at RT for 5 min and then read on a plate reader at 630 nm.

Lung Cell Isolation and Flow Cytometric Analysis

After collection of the BAL fluid, lung samples were minced in RPMI containing 100 units/ml of collagenase (Gibco) at 37°C for 60 minutes, followed by disruption through a 40-μm filter. After which, red blood cells were lysed using a Tris ammonium chloride solution. Staining of ~10^7 cells was performed in 200 μl of PBS containing 2% bovine serum and 2 mM EDTA. For analysis of leukocytes, antibody staining was both conducted at 4°C for 30 minutes. Phenotypic analysis of leukocytes was conducted using a panel of cell surface markers: CD11b, CD11c, Ly6g, Ly6c, 7/4, CD19, and I-A/I-E, as previously described (Hohl et al.). All antibodies used for analysis were purchased from Biolegend, BD Biosciences, eBioscience or Novus Biologicals. After staining, cells were washed and fixed with 1% paraformaldehyde in PBS. Fluorescent intensities were measured using an LSR (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Assay for Cytokine, Chemokine, and Soluble Receptor Secretion

Bronchoalveolar lavage fluid and lung homogenates from C57BL/6 mice challenged with *A. fumigatus* for 6, 12, 24, and 48 h were initially analyzed for cytokines and chemokines using ProcartaPlex Mouse Cytokine & Chemokine 36-plex (Affymetrix-

eBioscience). IL-1Ra levels were determined by ELISA (R&D Systems). Plates were read using a BioPlex 200 (Bio-Rad) or a SpectraMax Paradigm plate reader (Molecular Devices).

Growth of Bone Marrow-Derived Macrophages (BMDM)

Femurs and tibias from 8-10 week old mice were obtained and centrifuged to collect bone marrow. Cells were resuspended in media containing RPMI 1640, 2 mM L-glutamic acid, 50 mg/l gentamycin, 100 U/ml penicillin/streptomycin, 30% L929 cell supernatant, 20 % FBS and 0.0004% 2-ME. On day 3 fresh medium was added to the cultures. Cells were incubated for a total of 6 days at 37°C and 5% CO_2.

Isolation of Bone Marrow Neutrophils

Bone marrow neutrophils were isolated from femurs and tibias from 8-12 week old C57BL/6 and *Il1r1*-deficient mice as previously described (Shepardson et al. 2014). Briefly, single cell suspensions of bone marrow in HBSS containing 0.1% FBS and 1% glucose were resuspended in 3 ml of 45% Percoll (GE Healthcare). A discontinuous Percoll gradient was set-up consisting of (top to bottom) 3 ml 45%, 2 ml 50%, 2 ml 55%, 2 ml 62%, and 3 ml 81%. Gradients were then centrifuged for 30 min at 1600 x *g* in a Sorvall Legend Mach 1.6R benchtop centrifuge. Bone marrow neutrophils were collected from the 62%/81% border and washed with HBSS before counting and viability assessment.

In Vitro Fungal Damage Assay

An XTT assay was used to measure fungal metabolic activity as previously described (Shepardson et al. 2013). Bone marrow derived macrophages and CEA10 germlings were incubated together in normoxic or hypoxic conditions at a 10:1 (effector:target) ratio for 5 hours. Bone marrow neutrophils and CEA10 germlings were incubated together in normoxic conditions at a 10:1 (effector:target) ratio for 2 hours with or without 50 nM CXCL1 (Klesney-Tait et al. 2013). Following incubation, macrophages or neutrophils were lysed and the remaining fungi were incubated with 0.4 mg/ml XTT and 0.05 mg/ml coenzyme Q for 1 h and the optical density (OD) subsequently measured on a spectrophotometer at a wavelength of 450 nm. The percent fungal damage was defined by the equation: (1-[A_{450} of fungi with cells - A_{450} of cells alone] / [A_{450} of fungi alone]) * 100.

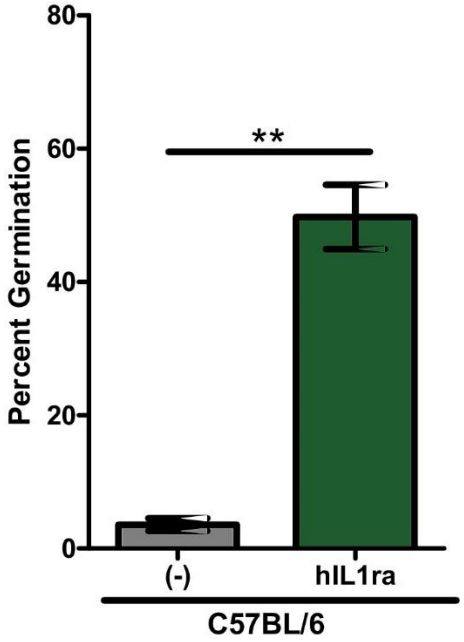
Statistical Analysis

Statistical significance was determined by a Student's t-test, one-way ANOVA using a Bonferroni post-test, or Kruskal-Wallis one-way ANOVA with Dunn's post-test through the GraphPad Prism 5 software as outlines in the figure legends.

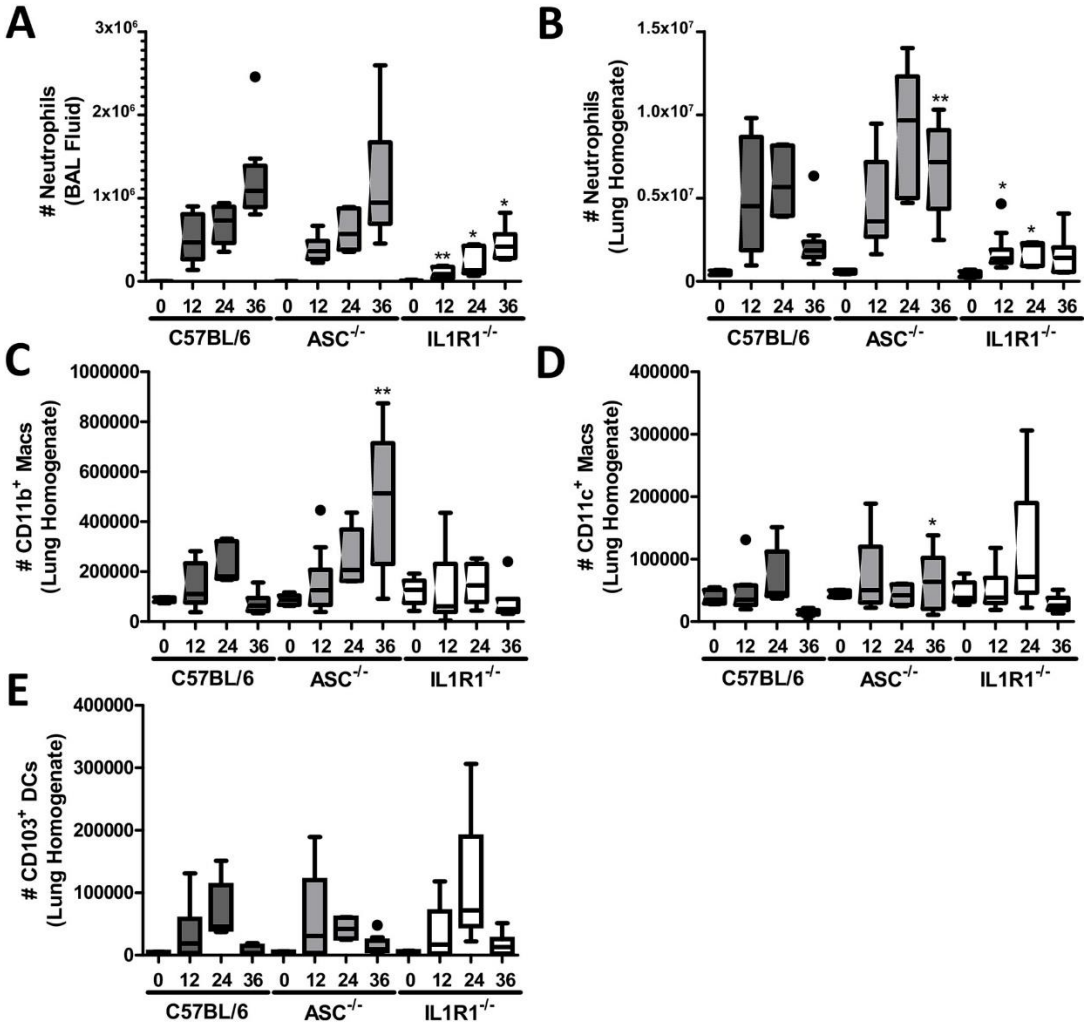
Acknowledgements

Thank you to Dr. Tobias Hohl (MSKCC) who provided many helpful suggestions and shared unpublished data related to this project. The authors wish to thank Dr. Vishva Dixit (Genentech) and Dr. Mark Jutila (Montana State University) for providing the original breeding pairs of the *Pycard*- and *Myd88*-deficient mice, respectively. Thank

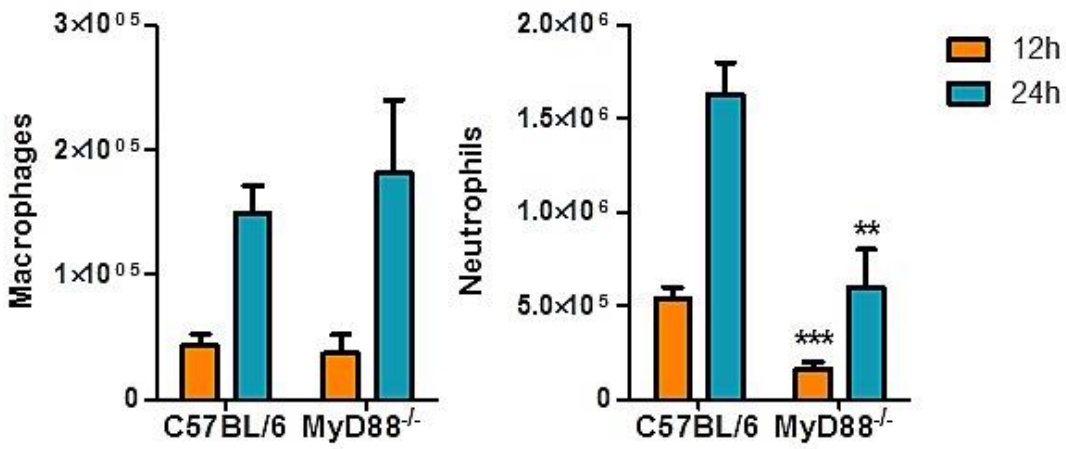
you to Dr. Charles Dinarello for providing the hIL1ra reagent and sharing the protocol for blocking IL-1α *in vivo*. Also, thanks to Drs. Jovanka Voyich, Matthew Taylor, and Blake Wiedenheft (Montana State University) for helpful discussion about this project and manuscript.



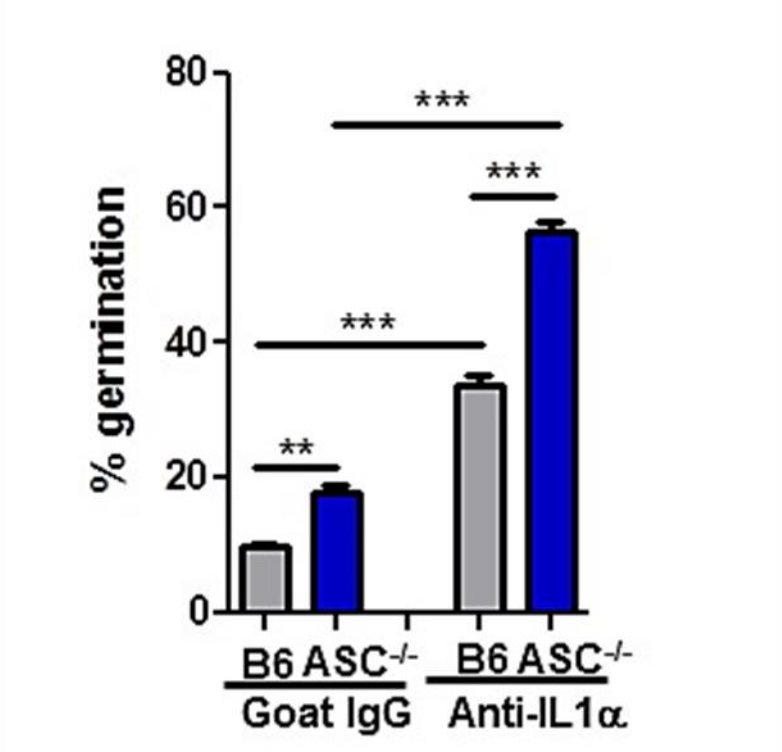
Supplemental Figure 2.8. hIL-1ra treatment results in impaired control of *Aspergillus fumigatus*. C57BL/6 mice were treated with recombinant hIL-1ra or placebo and infected i.t. with $5x10^7$ CEA10 conidia. Forty-eight hours post-infection, mice were euthanized, BALF collected, and lungs saved for histological analysis. Formalin-fixed lungs were paraffin embedded, sectioned, and stained with GMS for analysis by microscopy. *A. fumigatus* germination rates were assessed at 48 h of infection by microscopically counting both the number of conidia and number of germlings in GMS-stained section. Number of conidia and number of germlings were counted for each GMS-stained section to quantify the percent germination. Data are representative of one experiment consisting of 5 mice per group. The bar graph show the group means ± one SEM. Statistically significant differences were determined using a Student's t-test (**$p < 0.01$).



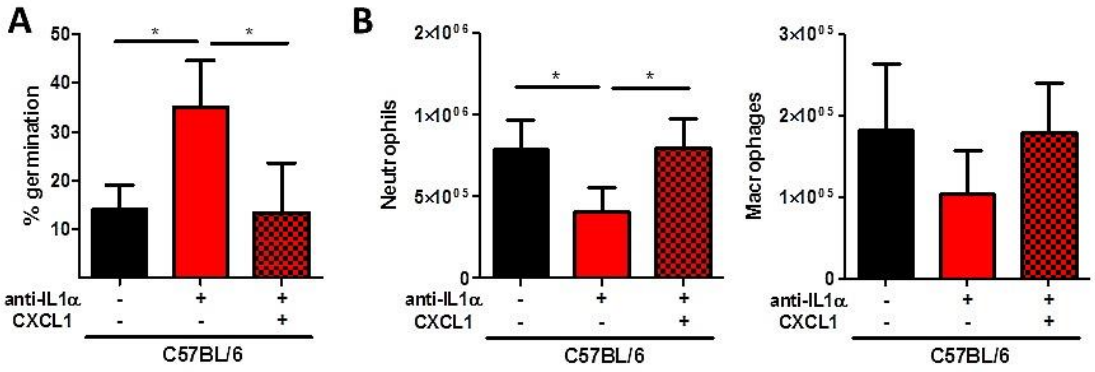
Supplemental Figure 2.9. Impaired neutrophil recruitment in the lung parenchyma of *Il1r1*-deficient mice. C57BL/6, *Il1r1*-deficient, and *Pycard*-deficient mice were challenged i.t. with $5x10^7$ CEA10 conidia. At 12, 24, and 36 h post-challenge, mice were euthanized, BALF and lungs collected for flow cytometric analysis of neutrophil, monocyte, and dendritic cell populations with the lungs, like previously done (Hohl et al.). (A) Neutrophils in the lungs were identified as being $CD45^+$ $Ly6g^+$ $7/4^+$. (B-E) Lungs were digested in collagenase to generate single cell suspensions, after which cells were stained for flow cytometric analysis. Lung neutrophils were identified as $CD45^+$ $CD11b^+$ $Ly6g^+$ $7/4^+$ (B), $CD11b^+$ macrophages were identified as $CD45^+$ $Ly6g^-$ $CD11b^+$ $CD11c^-$ $CD103^-$ (C), $CD11c^+$ macrophages were identified as $CD45^+$ $Ly6g^-$ $CD11b^+$ $CD11c^+$ $CD103^-$ (D), $CD103^+$ dendritic cells were identified as $CD45^+$ $Ly6g^-$ $CD11b^-$ $CD11c^+$ $CD103^+$ (E). Each time-point represents 8-10 mice pooled from two independent experiments. Data are presented as box and whisker plots with Tukey whisker and outliers displayed as dots. Statistically significant differences were determined using a one-way ANOVA with Bonferroni's post-test (*$p < 0.05$; **$p < 0.01$).



Supplemental Figure 2.10. *Myd88*-deficient mice are impaired in their ability to recruit neutrophils after *Aspergillus fumigatus* challenge. Age-matched C57BL/6 or *Myd88*-deficient mice were infected i.t. with $5x10^7$ CEA10 conidia and at indicated time-points, mice were euthanized, and BALF collected. Total macrophage (left panel) and neutrophil (right panel) recruitment in the BALF was measured at 12 and 24 h post-challenge. Data are representative of at least 2 independent experiments at each time point consisting of 3-5 mice per group. Bar graphs show the group means ± one SEM. Statistically significant differences were determined using Student's t-test (*$p < 0.05$; **$p < 0.01$).



Supplemental Figure 2.11. *Pycard*-deficient mice treated with IL-1α neutralizing antibody are highly susceptible to *Aspergillus fumigatus* infection. C57BL/6 or *Pycard*-deficient mice treated with isotype control antibody or IL-1α neutralizing antibody were infected i.t. with $5x10^7$ CEA10 conidia. Twenty-four hours post-infection mice were euthanized, BALF collected and lungs saved for histological analysis. Formalin-fixed lungs were paraffin embedded, sectioned, and stained GMS for analysis by microscopy. *A. fumigatus* germination rates were assessed 48 h after challenge by microscopically counting both the number of conidia and number of germlings in GMS-stained section. Data are representative of two independent experiments consisting of 4-5 mice per group. The bar graph show the group means ± one SEM. Statistically significant differences were determined using a one-way ANOVA with Bonferroni's post-test (**$p < 0.01$, ***$p < 0.001$).



Supplemental Figure 2.12. Intratracheally provision of CXCL1 to anti-IL1α treated C57BL/6 mice increases resistance to *Aspergillus fumigatus* infection. C57BL/6 mice were treated with goat IgG or anti-IL1α 24 h prior to and 24 h after i.t. challenge with $5x10^7$ CEA10 conidia. Three hours post-challenge half the anti-IL1α treated mice were given 0.5 µg CXCL1 in PBS or PBS alone given i.t. At 48 h post-infection mice were euthanized, BALF collected, and lungs saved for histological analysis. Formalin-fixed lungs were paraffin embedded, sectioned and stained with GMS for analysis by microscopy. (A) *A. fumigatus* germination rates were assessed 48 h after challenge by microscopically counting both the number of conidia and number of germlings in GMS-stained section. (B) Total macrophage (left panel) and neutrophil (right panel) recruitment in the BALF was measured at 24 h post-challenge. Data are representative of two independent experiments consisting of 3-5 mice per group. Bar graphs show the group means ± one SEM. Statistically significant differences were determined using a one-way ANOVA with Bonferroni's post-test (*$p < 0.05$).

CHAPTER THREE

ALARMIN(G) THE INNATE IMMUNE SYSTEM TO INVASIVE FUNGAL INFECTIONS

Contributions of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Conceived, designed, wrote, and edited the manuscript.

Co-author: Joshua J. Obar

Contributions: Conceived, designed, wrote, and edited the manuscript.

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ALARMIN(G) THE INNATE IMMUNE SYSTEM TO INVASIVE FUNGAL INFECTIONS

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Abstract

Fungi encounter numerous stresses in a mammalian host, including the immune system, which they must adapt to in order to grow and cause disease. The host immune system tunes its response to the threat level posed by the invading pathogen. We discuss recent findings on how interleukin (IL)-1 signaling is central to tuning the immune response to the virulence potential of invasive fungi, as well as other pathogens. Moreover, we discuss fungal factors that may drive tissue invasion and destruction that regulate IL-1 cytokine release. Moving forward understanding the mechanisms of fungal adaption to the host, together with understanding how the host innate immune system recognizes invading fungal pathogens will increase our therapeutic options for treatment of invasive fungal infections.

Introduction

Fungi are ubiquitous in nature and, for the most part, are harmless to the majority of individuals. However, it is estimated that 2 million cases of invasive mycoses are

reported worldwide each year (Brown et al. 2012). These invasive mycoses occur primarily in immunocompromised patients. Thus, in the absence of an adequate innate host defense, these opportunistic pathogens can infect the host and lead to disease. Furthermore, invasive fungal infections continue to be a rapidly emerging and serious threat because of the growing immunocompromised population and the emergence of drug resistance (Brown et al. 2012).

Candida spp. and *Aspergillus* spp. are known to cause approximately 30% of all invasive fungal infections (Brown et al. 2012). However, the environmental niche filled by *Candida* spp. and *Aspergillus* spp. substantially differs, which could drive evolution of distinct adaptation traits necessary for virulence. *Candida* spp. are found as a normal commensal component of the human skin, gastrointestinal tract and other mucosal surfaces (Underhill and Iliev 2014), whereas *Aspergillus* spp. are saprophytic molds found in the environment on decaying organic material (Park and Mehrad 2009). Hundreds of species exist within the *Candida* and *Aspergillus* genera, however only a handful have been shown to cause invasive mycoses in humans. Even though mortality rates for invasive fungal infections have significantly decreased in the past decade (Upton et al. 2007), mortality rates from IC and IA are unacceptably high, ranging anywhere from 20-50% due to limited diagnostic tools and lack of effective treatment options (Maertens et al. ; Steinbach et al. ; Gudlaugsson et al. 2003; Upton et al. 2007; González de Molina et al. 2012; Marr et al. 2015). Thus, novel therapeutic targets for anti-fungal drugs are desperately needed. Fungal factors driving adaption and growth in the mammalian host offer great potential as novel anti-fungal targets. In addition, tuning the

host inflammatory response to confer optimal host resistance is another potentially exciting avenue for limiting invasive fungal infections.

Innate immunity is essential for resistance against *A. fumigatus* and *C. albicans*. Patients with primary immunodeficiencies in the NADPH oxidase complex, STAT3 signaling pathway, CARD9 signaling pathway, IL-17 immunity, leukocyte adhesion deficiencies, and those with severe congenital neutropenia have been shown to be predisposed to developing invasive fungal infections (reviewed in (Lanternier et al. 2013) and (Wojtowicz and Bochud 2015)). Moreover, polymorphisms in numerous innate immune sensing and signaling pathways alter the susceptibility of transplant patients to developing invasive fungal disease (reviewed in (Wojtowicz and Bochud 2015), (Romani 2011) and (Smeekens et al. 2013)). In this review, we discuss the importance of interleukin-1 (IL-1) in tuning the inflammatory response in the context of invasive fungal disease. Moreover, we highlight the potential importance of this model broadly across the spectrum of infectious diseases. We highlight recent data which demonstrate that the mammalian innate immune system responds in a regulated manner that is tuned to the level of growth, virulence, and pathology induced by the fungal pathogen.

Alarmins/Damage-Associated Molecular Patterns (DAMPS) Versus Microbial-Associated Molecular Patterns (MAMPS)

The innate immune system provides an essential early response to microbial infection. Initial sensing of microbes has been well established to be mediated by a series of germline-encoded host pattern-recognition receptor (PRR) families, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), and RIG-I-

like receptors (RLRs), which can recognize conserved microbial structures termed microbial-associated molecular patterns (MAMPs). Examples of MAMPs of particular relevance to fungal pathogens include ß-1,3-glucan, chitin, mannans, mannoproteins, and unmethylated DNA. However, all fungi whether pathogenic to the host or not will express these MAMPs. To address the conceptual problem of pathogen versus commensal organism, Vance and colleagues have proposed that it is not just the MAMP that is critical for immune cell activation, but also its location within the host and/or cell, which they termed the "patterns of pathogenesis" (Vance et al. 2009). These early pathogenic signatures will instigate the early inflammatory response seen following fungal exposure. During the course of invasive fungal infections, fungal growth and invasion into the body, together with the host immune response will cause significant tissue damage, extracellular matrix destruction, and cell death at the site of infection. Tissue destruction and cell death during infection is highly inflammatory due to the release of damage-associated molecular patterns (DAMPs) or alarmins from the dying host cells (Bianchi 2007; Stephenson et al. 2016). These alarmins include both non-protein materials, such as ATP and uric acid, as well as proteins, which include IL-1α, IL-33, S100 proteins, and HMGB1. Thus, during invasive fungal infections initial inflammation will be regulated by PRRs, but if that response is insufficient to prevent invasive growth, alarmin release due to tissue destruction and/or host cell death will amplify the magnitude of the inflammatory response to attempt to regain control (Figure 3.1).

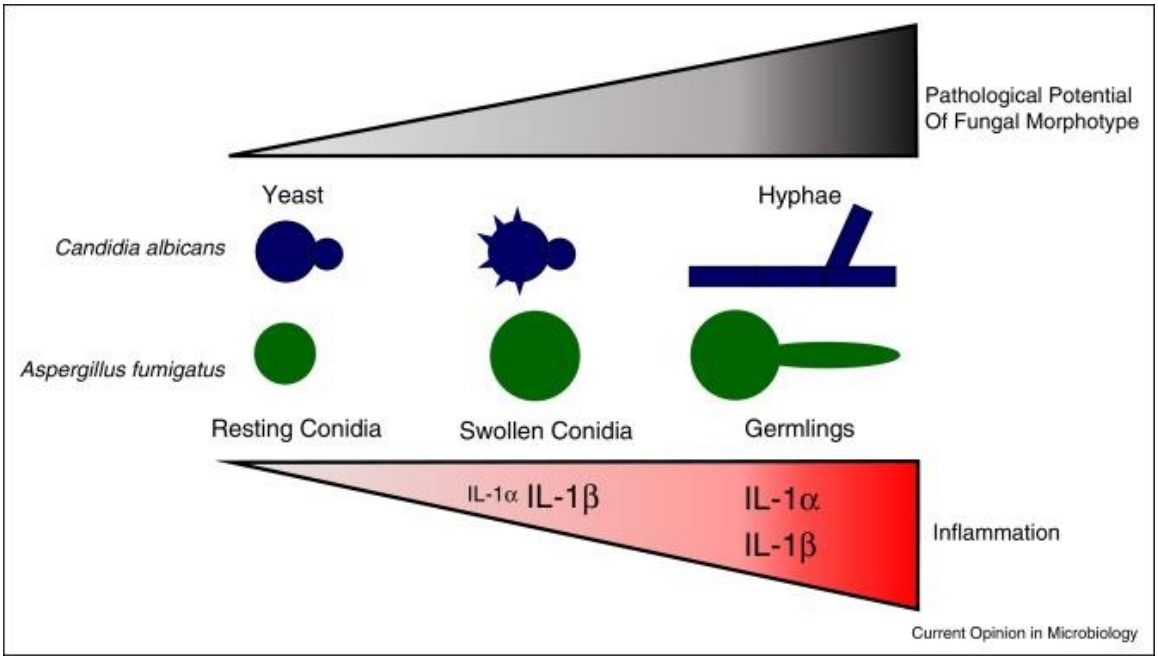

Figure 3.1. An escalating IL-1 inflammatory response regulates mammalian resistance to invasive, pathological fungal infection. In homeostatic conditions when the barrier to mucosal surfaces is intact, *C. albicans* and *A. fumigatus* can be found in a resting state on mucosal surfaces and in the airways, respectively. Once this primary immune barrier is breached, fungi can access nutrients necessary for growth. In the case of *C. albicans*, rearrangement of the cell wall occurs during phagocytosis, while in the case of *Aspergillus* spp. the cell wall architecture is changed upon conidial swelling. In either case, fungal MAMPs are revealed to the immune system resulting in the activation of an inflammasome-dependent immune response to clear the fungal threat. If the fungi continue to grow forming invasive hyphae and express hyphal-specific effectors, which may include GAG, secondary metabolites, and proteases, robust tissue pathology can result. This increased pathology drives elevated levels of pro-inflammatory cytokines and alarmin release, such as IL-1α, which intensify the innate immune response in hope of clearing the infection. Font size for each cytokine is indicative of their relative abundance.

Basics of the IL-1 Cytokines in Inflammation

The IL-1 gene cluster codes for the cytokines IL-1α and IL-1ß, as well as the IL-1 receptor antagonist (IL-1ra), all three of which can bind to the IL-1 receptor, type I (IL-

1RI) (Garlanda et al. 2013). While IL-1α and IL-1ß are pro-inflammatory cytokines, IL-1ra competitively binds the IL-1RI to dampen the immune response (Garlanda et al. 2013). Although IL-1α and IL-1ß belong to the same cytokine family, they rely on different proteases and cell death pathways for their secretion. IL-1ß is produced as an inactive precursor termed pro-IL-1ß, which must first be transcriptionally upregulated (Latz et al. 2013). Pro-IL-1ß is then cleaved by a caspase-1 or caspase-8 containing inflammasome (Latz et al. 2013). Ultimately, IL-1ß is released by pryoptotic cell death, which is mediated by gasdermin D (He et al. 2015; Kayagaki et al. 2015; Shi et al. 2015). Alternatively, pro-IL-1ß can be cleaved by neutrophil-derived proteases, such as elastase, cathepsin G, proteinase 3, and granzyme A, which might be important in neutrophil-rich inflammatory settings, such as those observed during later stages of invasive fungal infections (Netea et al. 2015). Unlike IL-1ß, pro-IL-1α is constitutively expressed in cells and can be released either as pro-IL-1α or mature IL-1α after calpain cleavage, but in either form it can bind to IL-1RI to initiate signaling (Garlanda et al. 2013; Kim et al. 2013). IL-1α is typically released during highly pathological situations, including necrotic and necroptotic cell death (Garlanda et al. 2013; Wallach et al. 2016). Biologically, IL-1α and IL-1ß can have different inflammatory activities in certain inflammatory settings (Chen et al. 2007; Rider et al. 2011; Barry et al. 2013), including during fungal infections (Vonk et al. 2006; Caffrey et al. 2015). This was most pointedly demonstrated by the work of Rider *et al* which demonstrated that IL-1α released by cells undergoing necrotic cell death is necessary for neutrophil accumulation to initiate a sterile inflammatory response, while later IL-1ß release promotes macrophage

accumulation (Rider et al. 2011). However, the distinct roles of IL-1α and IL-1ß during infections are only beginning to be appreciated.

Role of IL-1α and IL-1ß During Invasive Fungal Infections

Candida albicans

IL-1 signaling is essential for immunity against candidiasis, as immune competent *Il1r1*-deficient and *Il1a*/*Il1b*-deficient mice are both highly susceptible to *C. albicans* infection (Bellocchio et al. 2004; Vonk et al. 2006). Moreover, treatment of neutropenic mice with either recombinant IL-1α or IL-1ß was able to partially protect those mice (Van Wout et al. 1988). Interestingly, IL-1α and IL-1ß serve non-redundant roles in the immune response against systemic candidiasis (Table 1) (Vonk et al. 2006). Specifically, IL-1ß is needed for optimal neutrophil recruitment, whereas IL-1α was needed to enhance anti-fungal activity of neutrophils (Vonk et al. 2006).

When activated by *C. albicans*, pro-IL-1ß transcription is regulated through a Syk-Card9 pathway and IL-1ß release is dependent on the NLRP3 and NLRC4 inflammasomes (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Tomalka et al. 2011). Mice lacking either inflammasome are highly susceptible to disseminated candidiasis (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Tomalka et al. 2011). To date, the fungal PAMP driving inflammasome activation has largely remained elusive. Initially, NLRP3 activation in macrophages was thought to be dependent on the morphological switch of *C. albicans* from yeast to filamentous form, but is independent of the actual filament because both pseudohyphae and hyphae are capable of inducing IL-

1ß secretion (Joly et al. 2009). More recent data from screening multiple *C. albicans* genetic mutant libraries demonstrate that filamentation is not sufficient for inflammasome activation resulting in IL-1ß release and pyroptosis (Wellington et al. 2013; Wellington et al. 2014; O'Meara et al. 2015). These screens were able to identify both mutants that could filament, but not cause pyroptosis and mutants that were unable to filament, but could still drive pyroptosis. Interestingly, heat-killed *C. albicans* could not activate macrophage pyroptosis, but if the *C. albicans* that was previously phagocytized by macrophages for ~1h were heat-killed, they could induce robust pyroptosis (O'Meara et al.). Taken together, these data led Cowen and colleagues to postulate that *C. albicans* remodels its cell surface in response to macrophage phagocytosis. In an elegant experiment, Cowen and colleagues treated previously phagocytized *C. albicans* with the Endo H glycosidase prior to heat killing and observed a complete loss of pyroptosis, suggesting highly mannosylated surface proteins were required to induce macrophage pyroptosis (O'Meara et al.). In addition to this early pyroptotic cell death, *C. albicans* can also induce macrophage cell death in a pyroptosis-independent manner at later times that is highly dependent on the presence of fungal filaments, which likely are mechanically piercing the cell (Uwamahoro et al. 2014). Whether these two phases of macrophage cell death result in differential activation and release of IL-1α and IL-1ß has not been explored.

These prior studies focused largely on the interaction of *C. albicans* with macrophages. *C. albicans* is a normal member of the mucosal microbiota and, thus, would have to invade the epithelium to cause diseases. Thus, the transition of *Candida*

spp. from the yeast form into the filamentous form can be thought of as an important virulence determinant of the pathogen, enabling it to invade through the epithelium and grow within the host (Thompson et al. 2011; Yang et al. 2014), but likely would also cause significant tissue pathology due to destruction of the epithelium barrier and alter the inflammatory environment. Interestingly, in an *in vitro* oral candidiasis model in which human epithelial cells were infected with *Candida* spp. or *C. albicans* mutants that either can or cannot form hyphae, IL-1α expression was increased during the infections with *Candida* spp. that are able to form hyphae (Schaller et al. 2002; Villar et al. 2004; Jayatilake et al. 2007). However, a thorough dissection of this oral epithelium model with the *C. albicans* mutant libraries, as was done in the IL-1ß and pyroptosis studies discussed above, has not been undertaken to examine how IL-1α release is controlled. Taken together, these studies support our model that fungal morphotype can influence the release of alarmins, specifically IL-1α, by creating a high-threat environment in which a quick response by neighboring cells is critical, and prevention of collateral damage is not as important as clearing the threat.

Aspergillus fumigatus

IL-1 signaling is essential to control *A. fumigatus* growth, but controversy still exists on the relative importance of IL-1α and IL-1ß following *A. fumigatus* challenge (Table 1) (Bretz et al. 2008; Leal et al. 2010; Caffrey et al. 2015; Jhingran et al. 2015; Karki et al. 2015). Our group has shown that IL-1α was required for neutrophil recruitment after pulmonary challenge with *A. fumigatus*, while the inflammasome and IL-1ß were necessary for optimal fungicidal activity of macrophages (Caffrey et al.

2015). However, others have shown the inflammasome and IL-1ß are necessary for neutrophil recruitment following *A. fumigatus* challenge (Gresnigt et al. 2014; Karki et al. 2015). Both the NLRP3 and AIM2 inflammasomes work in concert to generate an optimal anti-*Aspergillus* response (Gresnigt et al. 2014; Karki et al. 2015). At least *in vitro*, activation of the NLRP3 inflammasome by *A. fumigatus* is greatest by hyphal fragments (Said-Sadier et al. 2010). However, compared to *Candida*, there is a lack of data concerning how distinct fungal morphotypes of *Aspergillus* spp. may relate to overall pathology and inflammation. It is important to note that in the above mentioned studies in which discrepancies concerning IL-1 dependency arise, different *A. fumigatus* strains and morphotypes were used (Gresnigt et al. 2014; Caffrey et al. 2015; Karki et al. 2015). Interestingly, it is rapidly emerging that different strains of *A. fumigatus* can induce both dramatically different inflammatory responses (Rizzetto et al. 2013; Amarsaikhan et al. 2014) and differing levels of virulence *in vivo* (Mondon et al. 1996; Rizzetto et al. 2013; Alshareef and Robson 2014). Interestingly, Rizzetto *et al* showed that the CEA10 isolate of *A. fumigatus* induced the greatest inflammation, which corresponded with its increased growth in immune competent mice (Rizzetto et al. 2013). This raises the possibility that different strains of *A. fumigatus* might undergo differential growth in the respiratory tract which will induce varying degrees of tissue pathology, resulting in the differential release of IL-1α and IL-1ß or generally alter the inflammatory response.

Table 3.1. Functions of IL-1α and IL-1ß during *C. albicans* and *A. fumigatus* infection.

	IL-1α	**IL-1ß**
C. albicans	Recombinant IL-1α increases survival in a neutropenic mouse model of invasive candidiasis (Van Wout et al. 1988)	Recombinant IL-1ß increases survival in a neutropenic mouse model of invasive candidiasis (Van Wout et al. 1988)
	Endogenous IL-1α needed for optimal antifungal activity of neutrophils against pseudohyphae, and Th1 response (Vonk et al. 2006)	Endogenous IL-1ß needed for recruitment of neutrophils, superoxide generation, and Th1 response (Vonk et al. 2006)
		NLRP3 and NLRC4-dependent IL-1ß production (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Tomalka et al. 2011)
A. fumigatus	IL-1α needed for neutrophil recruitment (Caffrey et al. 2015)	IL-1ß needed for optimal antifungal activity of macrophages (Caffrey et al. 2015)
		NLRP3- and AIM2- dependent IL-1ß production (Said-Sadier et al. 2010; Moretti et al. 2014; Karki et al. 2015)
		NLRP3-dependent neutrophil recruitment (Moretti et al. 2014; Karki et al. 2015)

Invasive aspergillosis is a spectrum of diseases in the clinic. In neutropenic hosts, pathology is driven by excessive fungal growth (Balloy et al. 2005), whereas in hosts immunosuppressed with steroids, patients with chronic granulomatous disease (CGD), or patients with cystic fibrosis, pathology is driven by an inflammatory response that appears out of proportion to hyphal growth in diseased tissues (Balloy et al. 2005;

Romani et al. 2008; Iannitti et al. 2016), which might alter the type of immunotherapeutic intervention one might consider (Figure 3.2). With this prior observation in mind, van de Veerdonk and colleagues explored whether immunotherapeutic intervention with anakinra to decrease inflammation could ameliorate IA in the murine model of X-linked CGD. Interestingly, blockade of IL-1 signaling using anakinra (hIL-1Ra) in the murine model of CGD significantly blunted IA severity (de Luca et al. 2014). Similarly, anakinra treatment of *Cftr*$^{-/-}$ mice, which are more susceptible to IA (Iannitti et al. 2013), also resulted in an amelioration of *Aspergillus*-induced mortality which was associated with decreased fungal growth and inflammation (Iannitti et al. 2016). In both cases, the authors have not specifically addressed whether excessive IL-1α and/or IL-1ß signaling was responsible for disease caused in these IA models. Interestingly, cells from CGD patients are known to secrete significantly greater amounts of IL-1α and IL-1ß in response to inflammatory stimuli (de Luca et al. 2014; Bagaitkar et al. 2015). Considering the earlier finding that neutropenic mice infected with *C. albicans* and subsequently treated with either recombinant IL-1α or IL-1ß were partially protected from disease (Van Wout et al. 1988), it will be intriguing to assess whether similar cytokine therapies might enhance protective immune responses in neutropenic models of IA. Overall, it is possible that *Aspergillus* spp. might induce varying degrees of tissue pathology in each of the clinically relevant models of IA, resulting in the differential release of IL-1α and IL-1ß and, therefore, may require unique therapeutic interventions.

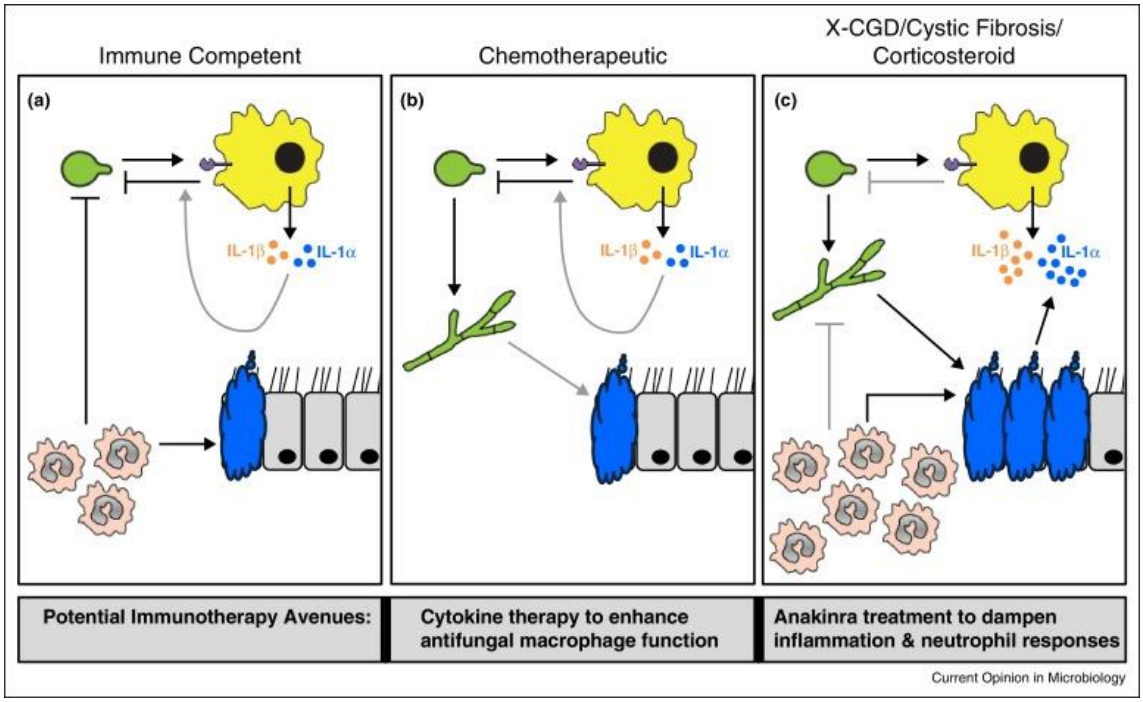

Figure 3.2. Differential tissue damage and IL-1 mediated inflammation in clinical models of IA will necessitate different immunotherapeutic interventions. (a) In immune competent hosts, germinating *Aspergillus* conidia are rapidly recognized by tissue-resident leukocytes, which results in the release of IL-1α and IL-1ß and the recruitment of neutrophils to the site of infection. Neutrophils then exert their powerful antifungal effects to limit *Aspergillus* growth. Additionally, IL-1α/β may work to enhance the antifungal functions of macrophages. **(b)** In neutropenic hosts, typically due to cyclophosphamide treatment, the lack of neutrophils allows extensive fungal growth. In these hosts, macrophages will still exert their antifungal function, but this is obviously not sufficient to prevent IA. In this population it would likely be advantageous to treat with inflammatory cytokines to further enhance monocyte/macrophage recruitment to the respiratory tract and/or their antifungal effector functions. **(c)** In CGD patients, cystic fibrosis patients, and individuals treated with high-doses of corticosteroids there is significant inflammation, neutrophil recruitment, and tissue damage associated with IA. However, due likely to impaired antifungal activity of the monocytes/macrophages and neutrophils in these populations, the host is unable to effectively clear the *Aspergillus* leading to continued inflammation. In these populations it has been shown to be advantageous to treat with anti-inflammatory compounds, such as anakinra, in order to limit inflammation, neutrophil recruitment, and tissue damage to the respiratory tract.

Fungal Targets That Contribute to Tuning Host Pathology, Cell Death, and IL-1 Immunity

During invasive fungal infections, the initial fungal morphotype that establishes an infection within the mammalian host likely is not the most inflammatory fungal morphotype. This is especially true with *A. fumigatus* as the conidia have a hydrophobin layer that is immunologically inert and is responsible for hiding the underlying carbohydrate cell wall from the immune system (Aimanianda et al. 2009). Upon seeding the environmentally exposed mucosa of the mammalian host, both *A. fumigatus* and *C. albicans* encounter the mucosal epithelium which provides a barrier against growth inside the host. It is recognized that invasion of the host is typically mediated by a transition to filamentous growth, which also corresponds with immunogenicity of the fungi. Thus, understanding both the molecular switch(s) for filamentous growth and its resultant changes in fungal biology will be essential for limiting tissue pathology and inflammation associated with invasive growth.

During the transition to filamentous growth, rearrangement of cell wall components is critical for immune activation through CLRs and NLRP3 inflammasome activation (Joly et al. 2009; Said-Sadier et al. 2010; O'Meara et al.). ß-1,3-glucans have been shown to induce both pro-IL1ß expression, as well as induce its maturation by the NLRP3 inflammasome (Kankkunen et al. 2010). More recently, highly mannosylated cell wall proteins in *C. albicans* were determined to induce macrophage pyroptosis through inflammasome activation (O'Meara et al.). Specific to *A. fumigatus*, a novel cell wall carbohydrate, galactosaminogalactan (GAG), has emerged as an important virulence

determinant that can also regulate IL-1 signaling and cell death (Fontaine et al. 2011; Gravelat et al. 2013; de Luca et al. 2014; Lee et al. 2015). GAG is not expressed by resting conidia, but is highly expressed on germtubes and hyphae (Fontaine et al. 2011), existing in both acetylated and deacetylated forms (Lee et al. 2016). GAG has several immunomodulatory functions. First, it masks ß-1,3-glucans from immune recognition in the hyphal cell wall (Gravelat et al. 2013; Beaussart et al. 2015). Second, GAG can induce IL-1ra, which inhibits IL-1 signaling and ultimately impairs neutrophil recruitment to the site of infection (de Luca et al. 2014), though others have not observed this (Lee et al. 2015). The reason for these discrepant findings remains to be elucidated, but the experimental design differed significantly between those studies. Specifically, Latgé and colleagues have exogenously treated mice with urea-soluble GAG (Fontaine et al. 2011; de Luca et al. 2014), while Sheppard and colleagues expressed the *A. fumigatus* Uge3 enzyme in *Aspergillus nidulans*, which typically cannot make GAG (Lee et al. 2015). Third, GAG contributes to resistance against NADPH-oxidase induced neutrophil extracellular traps (NETs) (Lee et al. 2015). Finally, GAG induces cell death in both epithelial cells and peripheral blood neutrophils, but the mechanism(s) of cell death induced remains inconclusive (Fontaine et al. 2011; Gravelat et al. 2013). GAG is critical for *A. fumigatus* adherence to epithelial cells, which is likely important for its induction of cell death (Gravelat et al. 2013; Beaussart et al. 2015). Moreover, it has recently been shown that the deacetylated form of GAG is necessary for adhesion and virulence (Lee et al. 2016). Thus, a greater understanding of fungal cell wall carbohydrate and glycoprotein expression, biosynthesis, and exposure on distinct fungal

morphotypes is necessary for understanding cell adhesion, invasive growth, cell death, and production of the IL-1 cytokine family during invasive fungal infections.

The founding infectious fungi must also rapidly adapt to the environmental and nutritional stresses of its mammalian niche to grow before the mammalian host can clear it. Among all eukaryotic organisms, cAMP and MAP kinase pathways are conserved and required for the transcriptional regulation of cell growth and differentiation processes in response to specific extracellular stimuli. In both *A. fumigatus* and *C. albicans* it is well established that MAP kinase and cAMP signaling are necessary for full virulence (Shapiro et al. 2011). Adaptation to neutral-alkaline pH environments that are encountered within the mammalian host is one environmental stress invasive fungi most overcome, and this has been shown to occur through Rim101/PacC signaling. The Rim101 mutant of *C. albicans* was unable to undergo the morphological change from yeast to hyphal growth when grown on basic media, and has been shown to have defects in cellular and tissue invasion *in vitro* and *in vivo* (Davis et al. 2000; Bensen et al. 2004; Sanchez et al. 2004; Villar et al. 2005). In an *in vitro* oral epithelium model of *C. albicans* infection, a Rim101 mutant was unable to induce as much cell damage, which corresponded with decreased expression of IL-1α, TNFα, and IL-8 compared to its parental or complemented strains (Villar et al. 2005). Interestingly, the PacC mutant of *A. fumigatus* did not have a defect in hyphal growth, but PacC-mediated signaling was crucial for *in vitro* epithelial cell destruction and tissue invasion in a neutropenic murine model of IA (Bertuzzi et al. 2014). *In vitro,* epithelial cell destruction by *A. fumigatus* occurred in a biphashic manner in which the early cell death (<16h) was dependent on

direct fungal-epithelial cell contact, whereas later cell death (>16h) was partially dependent on a secreted protease that is antipain sensitive (Bertuzzi et al. 2014). This later finding with the PacC-null mutant of *A. fumigatus* demonstrates that secreted factors that are expressed during hyphal growth are likely critical in host pathology and cell death, which will likely skew the inflammatory environment, including the IL-1 cytokines. In support of this, deletion of the transcription factor PrtT, which controls the expression of multiple *Aspergillus* proteases, resulted in an *A. fumigatus* mutant strain that was less cytotoxic to lung epithelial cells in *in vitro* assays (Sharon et al. 2009). While our understanding of the role of proteases in IA is limited, much more is known about the role of *Aspergillus* proteases in chronic airway disease and inflammation. Fungal proteases are sufficient to induce inflammation and allergic airway diseases (Millien et al. 2013). A protease isolated from *Aspergillus melleus* was sufficient to induce allergic airway diseases through a unique TLR4-dependent inflammatory pathway that included increased expression of both IL-1α and IL-1ß (Millien et al. 2013). During invasive *C. albicans* infections, secreted aspartyl proteinases (SAPs) have been identified and are associated with hyphal formation, tissue damage, and virulence (Naglik et al. 2003). Moreover, internalized Sap2 and Sap6 could mediate NLRP3 inflammasome activation in human monocytes leading to IL-1ß release (Pietrella et al. 2013). Sap2 was also sufficient to drive IL-1ß-dependent inflammation in a vaginitis model and contributed to the inflammation observed during *C. albicans*-induced vaginitis (Pericolini et al. 2015). Thus, many more studies examining the role of extracellular proteases, as

well as other secreted factors including lipases and secondary metabolites, are needed to understand invasive fungal disease, tissue pathology, cell death, and inflammation.

Is There a Universal Importance of Sensing Pathological Potential of Pathogens: Lessons from Bacteria And Viruses?

Bacteria

This idea of an escalating immune response based on the level of threat posed to the immune system has also been demonstrated in the context of bacterial infections. Both *Pseudomonas aeruginosa* and *Staphylococcus aureus* can cause highly inflammatory pulmonary disease resulting in substantial damage and destruction of the lung tissue. Highly virulent strains of *P. aeruginosa* possess a cytotoxin named ExoU that is known to cause epithelial damage and necrotic cell death (Finck-Barbancon et al. 1997; Hauser and Engel 1999). Infection with *exoU*-encoding strains results in neutrophil recruitment that is dependent on IL-1α, but when the *exoU* gene was deleted, neutrophil recruitment switched to be dependent on IL-1ß signaling (Al Moussawi and Kazmierczak 2014). Similarly, during infection with the highly virulent USA300 strain of *S. aureus*, it has been shown that bacterial toxins induce necroptosis of host cells, which largely contributes to the highly inflammatory pathology seen during *S. aureus*-induced pneumonia (Kitur et al. 2015). Thus, it appears that highly pathological bacterial strains that encode cytotoxic exotoxins can drive rapid necrotic host cell death which correlated with IL-1α release from the dying cells.

Viruses

New viral virions can bud from infected cells either in a cytopathic (lytic) or non-cytopathic manner. During cutaneous infection with cytopathic herpes simplex virus-1 (HSV-1), IL-1α is essential for leukocyte recruitment and restriction of viral dissemination (Milora et al. 2014). Likewise, in a model of disseminated adenovirus infection, cooperation between IL-1α signaling and the complement system were shown to induce neutrophil recruitment and subsequent clearance of virus-containing macrophages (Di Paolo et al. 2014). In this particular system, the presence of a mutated viral protease p23, in which the virus cannot induce endosomal rupture, IL-1α levels were significantly lower resulting in an overall reduced inflammatory response (Di Paolo et al. 2009). Finally, in pigs experimentally infected with the cytopathic porcine reproductive and respiratory syndrome virus (PPRSV), the degree of lung pathology observed 7 days after challenge correlated with IL-1α expression (Amarilla et al. 2015). This suggests that host cell damage and IL-1α release is a central part of activating the antiviral immune response to viruses that have cytopathic effects.

Concluding Remarks

As highlighted in this review, IL-1 signaling is a critical signaling hub in host defense against infectious disease, spanning fungi, bacteria and viruses. In the context of innate anti-fungal immunity, we propose a model in which the immune system responds in an escalating manner, based on the threat posed by the specific invading pathogen in accordance to its virulence and pathological potential (Figure 3.1). The first level of

protection lies in the physical barrier functions of the epithelial layer, which prevents the pathogen from gaining access to the body. Additionally, tissue sentinel immune cells patrol the epithelial barrier and can phagocytose and kill microbes with limited virulence potential, in an inflammasome and IL-1ß-dependent manner. However, when a pathogen takes on a highly virulent phenotype, in which it can rapidly adapt to the new environment, it can cause epithelial cell destruction, tissue damage, and cell death. In this latter scenario, IL-1α will be released from host cells to act as a rapid danger or alarmin signal to surrounding cells. In this "last resort" scenario, limiting the amount of tissue damage caused by the immune system is less important than clearing the threat posed by the pathogen to the host. Overall, by understanding all the factors that contribute to increased virulence and high levels of damage to the host, we can discover novel therapeutic strategies to combat a number of infectious diseases.

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CHAPTER FOUR

IL-1A SIGNALING IS CRITICAL FOR RESISTANCE AGAINST HIGHLY VIRULENT ASPERGILLUS FUMIGATUS STRAINS

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IL-1A SIGNALING IS CRITICAL FOR RESISTANCE AGAINST HIGHLY VIRULENT ASPERGILLUS FUMIGATUS STRAINS

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Abstract

Heterogeneity amongst *Aspergillus fumigatus* isolates results in unique virulence potential and inflammatory responses. How these isolates drive specific immune responses and how this affects fungal-induced immunopathology and disease outcome is unresolved. We demonstrate that the highly virulent CEA10 strain is able to rapidly germinate within the immune competent lung environment inducing greater lung damage, vascular leakage, and IL-1α release compared to the low virulent Af293 strain that does not germinate in this environment. Importantly, clearance of CEA10 was consequently dependent on IL-1α signaling in contrast to Af293. Our finding that early fungal conidia germination drives greater pathology and IL-1α dependent inflammation is supported by three independent experimental lines. First, pre-germination of Af293 prior to *in vivo* challenge drives lung damage and an IL-1α dependent neutrophil response. Second, the virulent EVOL20 strain, derived from Af293, is able to germinate in the airways, leading

to enhanced lung damage and IL-1α dependent inflammation and fungal clearance. Third, primary environmental *A. fumigatus* isolates that rapidly germinate in the airway conditions follow the same trend toward IL-1α dependency. Our data support the hypothesis that *A. fumigatus* phenotypic variation significantly contributes to disease outcomes.

Introduction

Aspergillus fumigatus is a ubiquitous mold whose conidia humans inhale on a daily basis. An individual with a sufficient immune response clears the conidia from the body without causing disease. In immune compromised populations, the anti-*Aspergillus* immune response is altered leading to a significant risk of developing invasive aspergillosis (IA). Patients at increased risk of developing IA include those receiving chemotherapy treatments for cancer as well as patients receiving immune-suppressive regimens for hematopoietic stem cell transplants or solid organ transplants (Segal 2009). Additionally, individuals with primary immunodeficiencies, such as chronic granulomatous disease, which alter antifungal effector pathways are highly susceptible to IA (Pollock et al. 1995; Morgenstern et al. 1997; Ben-Ari et al. 2012). In all these contexts, prolonged leukopenia or altered leukocyte function is a major risk factor for IA. For multiple reasons, which include difficulty in diagnosis and a limited repertoire of anti-fungal drugs for clinical use (Segal 2009; Brown et al. 2012), the mortality rate from IA remains between 30-50% (Steinbach et al. ; Upton et al. 2007; Garcia-Vidal et al. 2008; Thompson and Patterson 2008; Baddley et al. 2010). Thus, novel stand-alone or

adjunctive therapeutic options are sought after for treatment of IA. One line of research that holds great promise for improving IA outcomes is to modulate the host immune response (Peters et al. 1996; Liles et al. 1997; Bandera et al. 2008; Gresnigt and van de Veerdonk 2014; Caffrey and Obar 2016) .

Typically, in an immune competent individual, conidia are removed through mucociliary action and the physical barriers within the respiratory tract prevent conidia from entering the lung environment. If this primary barrier is bypassed, airway epithelial cells and lung-resident macrophages comprise the first line of defense against inhaled conidia, while neutrophils and inflammatory monocytes are then sequentially recruited to the site to prevent fungal growth (Espinosa and Rivera 2016; Obar et al. 2016). Defining the immunological events that are necessary for *A. fumigatus* conidia to be cleared from the lungs without excessive and immune-mediated pathology is a critical step toward understanding how the response is altered in different immune compromised populations. Ultimately, this understanding will be required for the development of immunomodulatory therapies for the treatment of IA.

Interleukin-1 (IL-1) has been shown to be important in host defense against numerous fungal infections, including histoplasmosis, candidiasis and aspergillosis (Van Wout et al. 1988; Bellocchio et al. 2004; Deepe and McGuinness 2006; Vonk et al. 2006; Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Moretti et al. 2014; Caffrey et al. 2015; Karki et al. 2015). Additionally, single-nucleotide polymorphisms in the IL-1 gene cluster have been shown to be associated with greater risks of patients developing IA (Sainz et al. 2008; Wójtowicz et al. 2015), while polymorphisms in the NLRP3 gene was

associated with recurrent vulvovaginal candidiasis in a subset of women (Lev-Sagie et al.). The IL-1 gene cluster codes for IL-1α and IL-1ß, as well as the IL-1 receptor antagonist (IL-1ra), all of which can bind to the IL-1 receptor, type I (IL-1RI) (Garlanda et al. 2013). In several different disease models, IL-1α and IL-1ß have been shown to have different inflammatory activities (Vonk et al. 2006; Chen et al. 2007; Rider et al. 2011; Al Moussawi and Kazmierczak 2014; Caffrey et al. 2015).

Following *A. fumigatus* challenge, IL1R1- and MyD88-dependent signals are necessary for optimal leukocyte recruitment and antifungal effector functions to prevent the development of IA (Bretz et al. 2008; Caffrey et al. 2015; Jhingran et al. 2015). However, there is controversy in the literature concerning the specific roles of IL-1α and IL-1ß during IA (Moretti et al. 2014; Caffrey et al. 2015; Karki et al. 2015). The differing conclusions drawn from these studies could be due to a number of factors, including different host immune status in the murine models used, different strains of fungi, and/or different morphology of the fungi used. Most notably, in these studies different *Aspergillus fumigatus* strains were used, CEA10 (Caffrey et al. 2015) and Af293 (Moretti et al. 2014; Karki et al. 2015). It has recently been shown that Af293 is significantly less virulent than CEA10 in the triamcinolone model of IA (Kowalski et al. 2016). Moreover, both the inflammatory response elicited by these strains, and the mortality associated with each strain significantly differed in immune competent mice (Rizzetto et al. 2013). However, the mechanism(s) behind how different inflammatory responses are induced by different *A. fumigatus* strains is unknown.

Here, we explore the role of IL-1α signaling in maintaining host resistance *A. fumigatus* using an array of strains and isolates. We demonstrate that *A. fumigatus* strains and isolates that can rapidly germinate within the lung environment induced higher levels of pulmonary damage, which corresponded with a requirement for IL-1α signaling for prevention of IA. In contrast, *A. fumigatus* strains and isolates that cannot germinate rapidly within the lungs of immune competent mice, drive lower levels of pulmonary damage and can be controlled through an IL-1α independent or alternative compensatory mechanism. Intriguingly, germlings of low virulent isolates are sufficient to induce greater lung pathology and an IL-1α dependent inflammatory response, which is likely due to increased cytotoxicity to macrophages. Thus, our data highlight a role for early pathogen adaptation and growth in the pulmonary environment in establishing which host inflammatory pathways are necessary to maintain resistance against this ubiquitous and increasingly important human pathogen.

Materials and Methods

Mice

C57BL/6J (Jackson Laboratory, Stock #000664), *Il1r1*$^{-/-}$ (Jackson Laboratory, Stock #03245), *Il1a*$^{-/-}$ (Horai et al. 1998), *Pycard*$^{-/-}$ (Mariathasan et al. 2004), and *Il1a/b*$^{-/-}$ (Miller et al. 2007) mice were bred in-house. All mice were 8-10 weeks of age at the time of challenge. All animal experiments were approved by either the Montana State University Institutional Animal Care and Use Committee or Dartmouth College Institutional Animal Care and Use Committee.

Preparation of *Aspergillus fumigatus* Conidia

Aspergillus fumigatus strains and isolates CEA10, Af293, 02-10, W72310, and EVOL20 were used for this study, origins of these strains and isolates have previously been reported (Kowalski et al. 2016). Each strain or isolate was grown on glucose minimal media (GMM) agar plates for 3 days at 37°C. Conidia were harvested by adding 0.01% Tween 80 to plates and gently scraping conidia from the plates using a cell scraper. Conidia were then filtered through sterile Miracloth, were washed and resuspended in phosphate buffered saline (PBS), and counted on a hemacytometer.

To make Af293 germlings for our sufficiency experiment, resting conidia of Af293 were incubated at 30°C in a shaking platform incubator for 8-9 hours in liquid glucose minimal media containing 1% yeast extract. At 8 hours, the conidia were checked microscopically for swelling/germ-tube formation and subsequently returned to the shaker for incubation until germ tubes emerged. Once germ tubes formed, sample was vortexed and transferred to a 10 ml tube containing 1.0 mm disruption beads (Research Products International Corp). The sample and beads were vortexed on the highest setting for 2 minutes and subsequently transferred to a dounce homogenizer to further break up any clumps of germlings. Sample was then re-counted on a hemacytometer since during the shaking process, the fungi began to stick to the walls of the flask, decreasing the concentration. Resting conidia concentration was then adjusted to the germling concentration, vortexed with beads and homogenized in a dounce homogenizer for consistency. C57BL/6 mice or *Il1α*$^{-/-}$ mice were infected with ~2.7x10^{7}

Af293 resting conidia or germlings, and at 12 hpi BALF was collected for analysis of damage, vascular leakage, and inflammatory cell recruitment.

Aspergillus fumigatus Pulmonary Challenge Model

Mice were challenged with *A. fumigatus* conidia by the i.t. route. Mice were anesthetized by inhalation of isoflurane; subsequently, mice were challenged i.t. with ~4 × 10^7 *A. fumigatus* conidia in a volume of 100 µl PBS. For the Af293 germling experiment, mice were challenged with ~2.7x10^7 resting conidia or germlings in a volume of 100 µl of liquid GMM containing 1% yeast extract. At the indicated time after *A. fumigatus* challenge, mice were euthanized using a lethal overdose of pentobarbital. Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs with 2 ml of PBS containing 0.05*M* EDTA. BALF was clarified by centrifugation and stored at -20°C until analysis. After centrifugation, the cellular component of the BAL was resuspended in 200 µl of PBS and total BAL cells were determined by hemacytometer count. BAL cells were subsequently spun onto glass slides using a Cytospin4 cytocentrifuge (Thermo Scientific) and stained with Diff-Quik stain set (Siemens) for differential counting. For histological analysis lungs were filled with and stored in 10% buffered formalin phosphate for at least 24 hours. Lungs were then embedded in paraffin and sectioned into 5-micron sections. Sections were stained with H&E and GMS using standard histological techniques to assess lung inflammatory infiltrates and fungal germination, respectively. Representative pictures of lung sections were taken using an Olympus BX50WI microscope with a QImaging Retiga 2000R camera. For cytokine analysis

lungs were homogenized in 2 ml of PBS. After clarification, lung homogenates were stored at -20°C until analysis.

Determination of *In Vivo* Germination of *A. fumigatus*

As described above, mice were challenged with *A. fumigatus* conidia by the i.t. route and at 12 hpi BALF was collected. BAL cells were spun onto glass slides using a Cytospin4 cytocentrifuge (Thermo Scientific) and stained with Diff-Quik stain set (Siemens) for differential counting. Fungal conidia/germlings could be visualized in these cytospins. The percent germination of each *A. fumigatus* strain or isolate was quantified by manual counting of 100-400 fungal conidia and germlings at 100X magnification using a standard upright microscope.

In vitro Germination Assays

The germination potential of each *A. fumigatus* strains or isolates was tested in either GMM + yeast extract, or lung homogenate medium. GMM is a minimal medium that contains 1% glucose in a base of salts and trace elements (Shimizu and Keller 2001). To make it a nutrient rich medium we added 0.5 g/L of yeast extract (Amerco). Finally, to mimic the nutrient availability in the lungs at the time of challenge we utilized lung homogenate. To make lung homogenate medium, lungs from a 6-12 week old C57BL/6J mice were homogenized through a 70μm filter in 2 mL of phosphate buffered saline (PBS). The lung homogenate was then clarified and stored at -20°C until use. For experiments the lung homogenate was diluted 1:4 in PBS prior to use. Experimental cultures were inoculated with 10^7conidia/ml in 2 ml of medium in glass 20ml disposable scintillation vials (VWR). Cultures were shaken at 300 rpm at 37°C in an Excella E24

shaking incubator (New Brunswick Scientific). Starting at 4h, the germination of *A. fumigatus* conidia was determined every 2h. To quantify *A. fumigatus* germination, a 200µl sample was collected and added to a microtube containing 1.0mm glass beads (Research Products International Corp). The microtube was then vortexed to disrupt any clumps of *A. fumigatus.* The solution was then placed on a glass slide and cover slipped at which point fungal germination was quantified manually using the 40× objective lens of an upright VWR microscope. A minimum of 100 conidia and germlings for each sample were counted.

Preparation of Bone Marrow Derived Macrophages

Bone marrow cells were eluted from tibias and femurs of 8–12 week old mice and cultured for macrophages in RPMI containing 20% FCS, 5 mM HEPES buffer, 1.1 mM L-glutamine, 0.5 U/ml penicillin, and 50 mg/ml streptomycin and supplemented with 30% (v/v) L929 cell supernatant (source of M-CSF). Bone marrow cells for macrophages were plated in a volume of 10 ml in 100 mm x 15 mm sterile polystyrene petri dish (Fisher Scientific, Catalog #FB0875712). Bone marrow cells from each mouse were split evenly into 5 plates. The medium was supplemented on day 3 with an additional 10 ml of macrophage medium. Adherent bone marrow-derived macrophages (BMDMs) were harvested on day 6 using ice-cold PBS.

In Vitro Activation of Bone Marrow Derived Macrophages with *Aspergillus fumigatus*

BMDM cells were washed with clear RPMI containing 10% FCS, 5 mM HEPES buffer, 1.1 mM L-glutamine, 0.5 U/ml penicillin, and 50 mg/ml streptomycin (TC

medium). Immediately prior to addition of BMDM to *A. fumigatus,* BMDM were stimulated with 1 µg/ml of LPS (Invivogen). Swollen conidia were generated by culturing resting conidia in TC media for 16 h at room temperature, followed by 2 hours at 37°C in a flat bottom 96 well plate. Germlings were generated by culturing resting conidia in TC overnight at 30°C and then shifting to 37°C for 2 h in a flat bottom 96 well plate. When ready the plates were centrifuged at 4000 rpm to pellet the *Aspergillus.* After centrifugation the medium was removed and BMDM cells were added on top of the swollen conidia or germlings of *A. fumigatus* in 0.2 ml TC medium at a density of 5 x 10^5 cells/ml into a 96-well plate. BMDM cells were stimulated for 24 h at 37°C, after which supernatants were collected and stored at -20°C until use.

ELISA Detection of IL-1α and Lactate Dehydrogenase
(LDH) Secretion by Bone Marrow Derived Macrophages

Commercially available ELISA kits for IL-1α (BioLegend) and LDH CytoTox 96® Cytotoxicity Assay (Promega) were used according to the manufactures' instructions. Plates were read using a SpectraMax Paradigm plate reader (Molecular Devices).

Quantification of Lung Damage and Leakage

To assess lung damage, bronchoalveolar lavage fluid was analyzed by measuring lactate dehydrogenase levels using a CytoTox 96® Cytotoxicity Assay (Promega) following the manufacturer's instructions. To assess vascular/pulmonary leakage, bronchoalveolar lavage fluid was analyzed using an Albumin BCG Reagent Set (Eagle Diagnostics). A standard curve was made by diluting the calibrator in PBS/EDTA. Then

100 µl of sample or standard was transferred to a 96 well flat-bottomed plate, mixed with 100 µl of BCG reagent, let sit at RT for 5 min and then read on a plate reader at 630 nm.

Statistical Analysis

Statistical significance was determined by a Mann-Whitney U test, one-way ANOVA using a Bonferroni post-test, or Kruskal-Wallis one-way ANOVA with Dunn's post-test through the GraphPad Prism 5 software as outlined in the figure legends.

Results

CEA10 and Af293 Strains of *A. fumigatus* Induce Differing Levels of Lung Damage and Inflammation

Previous reports have indicated variation in virulence amongst the commonly studied "wild-type" *Aspergillus fumigatus* strains, CEA10 and Af293 (Rizzetto et al. 2013; Kowalski et al. 2016). To examine the overall pathology induced by each of these strains in an immune competent model of invasive aspergillosis (IA), C57BL/6 mice were challenged with $4x10^7$ conidia of CEA10 or Af293. We noted that by 24 hours post-inoculation (hpi), lungs from mice challenged with CEA10 appeared to have more edema and, overall, appeared more damaged than lungs from mice challenged with Af293 (Figure 4.1A). Thus, we quantitatively assessed lung damage and vascular leakage by measuring lactate dehydrogenase (LDH) and albumin levels in the bronchoalveolar lavage fluid (BALF). Mice inoculated with CEA10 had significantly higher levels of LDH and albumin in their BALF than mice inoculated with Af293 by 12 hpi (Figure 4.1B). IL-1α is a cytokine that is constitutively-expressed in cells and is

typically released in highly pathological situations, such as during necrotic or necroptotic cell death (Garlanda et al. 2013; Wallach et al. 2016). Given the role of IL-1α in driving inflammation during highly pathological inflammatory responses, we wanted to know whether IL-1α protein levels were also differentially abundant after exposure to CEA10 or Af293. IL-1α protein levels were significantly higher in the lungs of CEA10-inoculated mice compared to Af293-inoculated lungs at 12 hpi (Figure 4.1C). Furthermore, using Spearman's rank-order correlation analysis, we found a positive correlation between the amount of IL-1α protein in the lung and the amount of LDH in the BALF following *A. fumigatus* exposure (Spearman's rho (r) = 0.8846, p = 0.0001 (Figure 4.1D). Values on the higher end of the correlation plot were obtained from mice inoculated with CEA10 (blue dots), whereas values on the lower end of the plot were from mice inoculated with Af293 (red dots), suggesting that the CEA10 strain is more pathological than the Af293 strain which corresponds to the release of IL-1α protein.

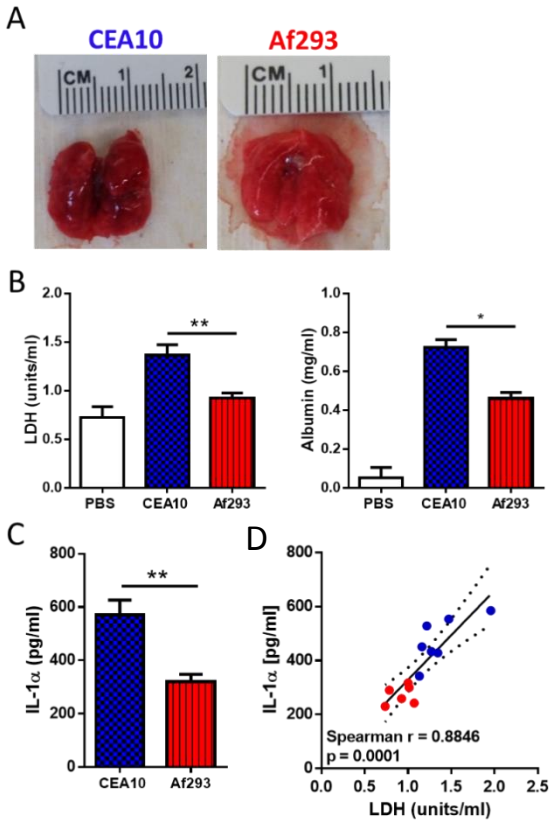

Figure 4.1. CEA10 and Af293 induce different levels of pulmonary damage. C57BL/6 mice were infected intratracheally (i.t.) with $4x10^7$ CEA10 or Af293 conidia. (A) At 24 hours post inoculation (hpi), mice were euthanized and lungs removed to observe gross pathology of lungs infected with CEA10 (left) or Af293 (right). (B) At 12 hpi, mice were euthanized, bronchoalveolar lavage fluid (BALF) collected, and lung tissue homogenized. Lung damage and leakage were quantified by measuring lactate dehydrogenase (LDH) and albumin levels in the BALF, respectively. (C) IL-1α levels were measured in the lung homogenate. (D) Spearman rank order correlation for LDH and IL-1α levels in mice infected with CEA10 (blue dots) or Af293 (red dots) (Spearman r = 0.8846; p = 0.0001). Data is representative of at least 2 independent experiments consisting of at least 5 mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences in (B) were determined using a one-way ANOVA with Bonferroni's post-test. Statistically significant differences in (C) were determined using Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$).

IL-1α-Dependent Neutrophil Recruitment and
Fungal Clearance is Fungal Strain Dependent

The specific roles of IL-1α and IL-1ß signaling during IA is controversial in the literature (Moretti et al. 2014; Caffrey et al. 2015; Karki et al. 2015). We have now shown that the CEA10 strain is hypervirulent and induced greater inflammation than Af293 in immune competent mice (Figure 4.1) (Rizzetto et al. 2013). Thus, to address whether the increase in IL-1α protein levels observed following CEA10 challenge was immunologically relevant we challenged C57BL/6, *Il1a*$^{-/-}$, *Pycard*$^{-/-}$, and *Il1r1*$^{-/-}$ mice with $4x10^7$ conidia of either CEA10 or Af293. Twelve hours after fungal challenge, BALF was collected to assess macrophage and neutrophil recruitment to the airways. *Il1r1*$^{-/-}$ mice had a defect in neutrophil recruitment when challenged with either CEA10 or Af293, whereas macrophage recruitment was normal (Figure 4.2A and 4.2B). Thus, IL-1 signaling is essential in driving the accumulation of neutrophils in the airways following *A. fumigatus* challenge in response to multiple *A. fumigatus* strains, similar to what has previously been observed (Caffrey et al. 2015; Jhingran et al. 2015). Interestingly, *Il1a*$^{-/-}$ mice only had a defect in neutrophil recruitment when inoculated with CEA10 (Figure 4.2A). In contrast, *Pycard*$^{-/-}$ mice were impaired in neutrophil recruitment to the airways only following inoculation with Af293 (Figure 4.2B). Taken together, these data demonstrate that the CEA10 strain induces IL-1α-dependent neutrophil recruitment, while Af293 drives inflammasome-dependent neutrophil recruitment early after challenge, clarifying the findings of the previously published work (Moretti et al. 2014; Caffrey et al. 2015; Karki et al. 2015).

Because neutrophils are widely acknowledged to be critical effector cells in mediating clearance of *A. fumigatus* from the lungs, we hypothesized that following CEA10 challenge control of fungal growth and tissue invasion would be IL-1α dependent, while Af293 would be controlled through an IL-1α independent manner. To test this, C57BL/6 mice and *Il1a*$^{-/-}$ mice were challenged with 4x10^{7} conidia of CEA10 or Af293. At 42 hpi lungs were collected for histological analysis to assess fungal growth by Grocott-Gomori's methenamine silver (GMS) staining. Following CEA10 challenge there was significantly greater fungal germination and tissue invasion in the absence of IL-1α signaling compared to C57BL/6 mice (Figure 4.2C). In contrast, after Af293 challenge both C57BL/6 and IL-1α-deficient mice were able to control fungal growth (Figure 4.2C). Although both CEA10 and Af293 are typically cleared from C57BL/6 lungs, mice challenged with CEA10 consistently had greater fungal growth at 42 hpi, approximately 10-20% of the stained fungi were germinated (Figure 4.2C), while almost no fungal germination (<2%) was observed following Af293 challenge at this time point (Figure 4.2C). Taken together, these results demonstrate that the CEA10 strain of *A. fumigatus* drives neutrophil recruitment and fungal clearance through an IL-1α dependent mechanism, whereas neutrophil recruitment and fungal clearance of the Af293 strain occurs in an IL-1α-independent manner.

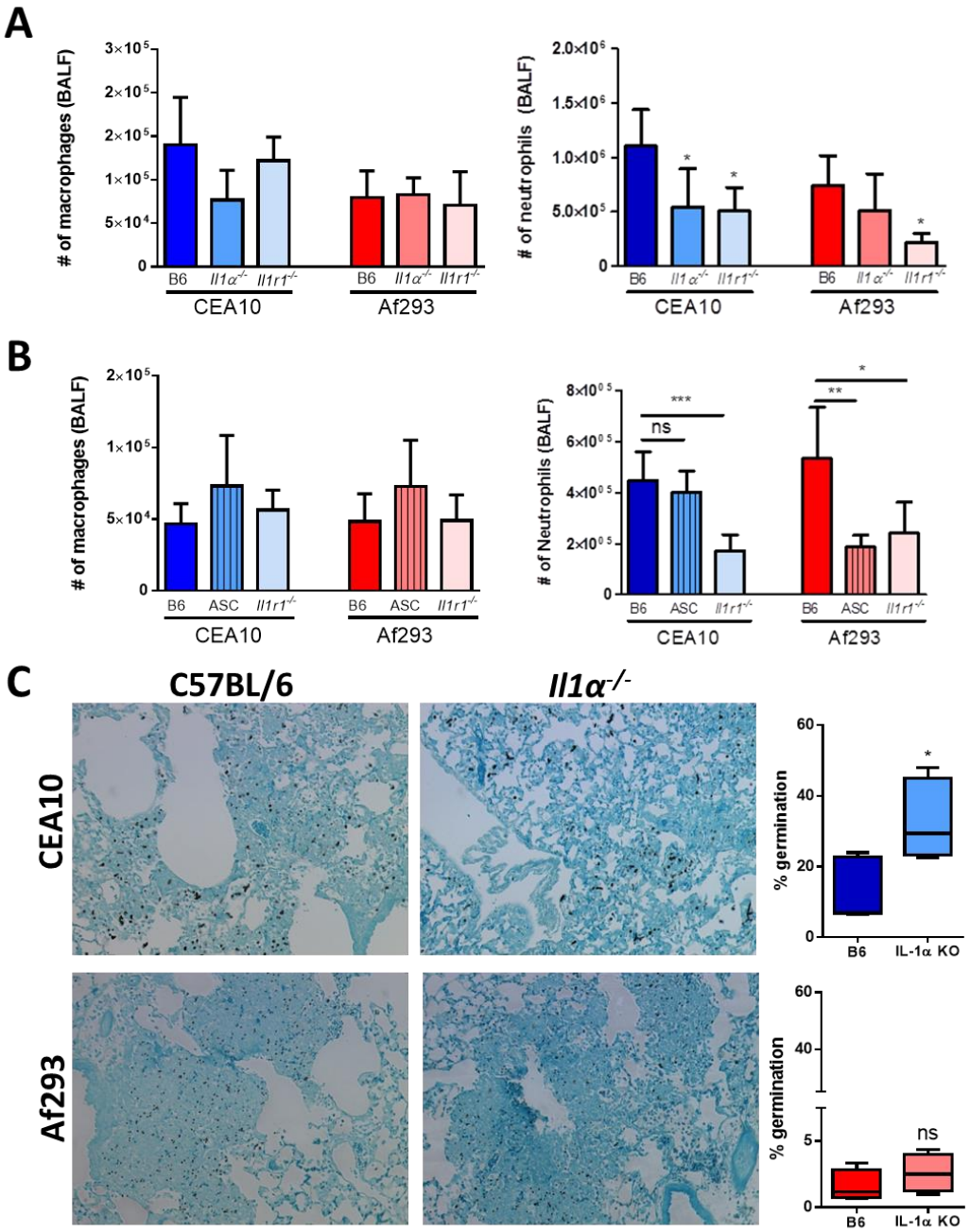

Figure 4.2. *Il1α*$^{-/-}$ mice are more susceptible to infection with CEA10, but not with Af293. (A, B) C57BL/6, *Il1α*$^{-/-}$, *Pycard*$^{-/-}$, or *Il1r1*$^{-/-}$ mice were infected with 4x10^{7} conidia of either CEA10 or Af293. At 12 hpi, mice were euthanized and BALF collected for quantification of macrophage and neutrophil recruitment to the airways. Data is representative of 1-2 experiments with 5-7 mice per group. Bar graphs show the group mean ± one SEM. (C) At 42 hpi, mice were euthanized and lungs saved for histological analysis. Formalin-fixed lungs were paraffin embedded, sectioned and stained with GMS for analysis by microscopy. Representative lung sections from C57BL/6 mice (left) or

Figure 4.2 Continued

Il1α$^{-/-}$ mice (right) infected with CEA10 (top) or Af293 (bottom) are shown using the 20× objective. *A. fumigatus* germination rates were determined by microscopically counting both the number of conidia and number of germlings in GMS-stained sections (C). Statistical significance in (A, B) was determined using a one-way ANOVA with Bonferroni's post-test. Statistical significance in (C) was determined using a Mann-Whitney U test (ns = not significant, *$p < 0.05$).

IL-1α is Produced by Radiosensitive Cells After *A. fumigatus* Challenge

To determine which cells contribute to the production of IL-1α after *A. fumigatus* challenge, we made a series of bone marrow chimeric mice. C57BL/6 and *Il1a/b*$^{-/-}$ mice were lethally irradiated then reconstituted with either C57BL/6 or *Il1a/b*$^{-/-}$ bone marrow intravenously to develop the following groups: C57BL/6 mice possessing C57BL/6 bone marrow, *Il1a/b*$^{-/-}$ mice possessing *Il1a/b*$^{-/-}$ bone marrow, C57BL/6 mice possessing *Il1a/b*$^{-/-}$ bone marrow, and *Il1a/b*$^{-/-}$ mice possessing C57BL/6 bone marrow. Mice were then rested for 6-8 weeks prior to challenge with $4x10^7$ conidia of CEA10. At 12 hpi, mice were euthanized and lungs collected to measure IL-1α levels. As expected, C57BL/6 mice possessing C57BL/6 bone marrow were able to produce IL-1α, while *Il1a/b*$^{-/-}$ mice possessing *Il1a/b*$^{-/-}$ bone marrow could not produce IL-1α in the lungs (Figure 4.3A). Interestingly, mice that were devoid of IL-1α in the radiosensitive cells had significantly decreased levels of IL-1α in the lungs, whereas mice lacking IL-1α in radioresistant cells had levels of IL-1α comparable to that of C57BL/6 mice (Figure 4.3A). These data show that radiosensitive cells are the major source of IL-1α after challenge with *A. fumigatus*.

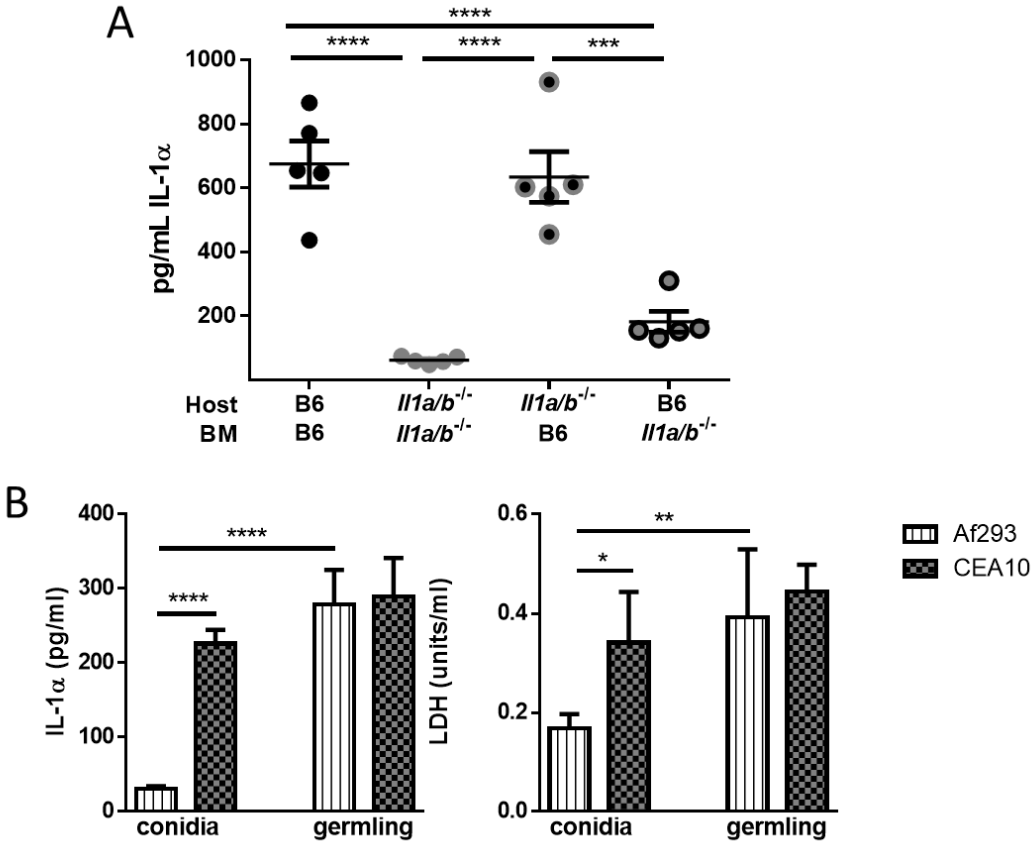

Figure 4.3. Cells of hematopoietic origin contribute to IL-1α production after challenge with *A. fumigatus in vivo* and *in vitro.* (A) Bone marrow chimeric mice were made by irradiating C57BL/6 and *Il1a/b*$^{-/-}$ mice, and reconstituting the mice with bone marrow from either C57BL/6 or *Il1a/b*$^{-/-}$ mice to develop the following groups: C57BL/6→C57BL/6, *Il1a/b*$^{-/-}$→ *Il1a/b*$^{-/-}$, B6→*Il1a/b*$^{-/-}$, and *Il1a/b*$^{-/-}$→C57BL/6. These mice were infected i.t. with 4x10^{7} CEA10 conidia and at 12 hpi, mice were euthanized and lung tissue homogenized to quantitate IL-1α levels (A) using ELISA. Data is representative of at least 2 independent experiments consisting of 5-7 mice per group. Each symbol represents an individual mouse and the line represents the group mean. (B) Bone marrow derived macrophages (BMDM) were incubated with swollen conidia or germlings of Af293 (red) or CEA10 (blue) for 18 hours and supernatant collected to quantify IL-1α and LDH levels. Data is representative of 2 independent experiments consisting of 6 biological replicates per group. Bar graphs show the group mean ± one SEM. Statistical significance in (A) was determined using a One-Way ANOVA with Tukey post-test. Statistical significance in (B) was determined using a two-way ANOVA with Tukey post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Aspergillus fumigatus Germlings are More Cytotoxic to Macrophages Resulting in Greater IL-1α Release

We have previously shown that following *A. fumigatus* inoculation, a large portion of IL-1α is originating from $CCR2^+$ monocytes (Caffrey et al. 2015). Thus, we next wanted to determine if Af293 and CEA10 were interacting differently with macrophages in an *in vitro* system. Swollen conidia or germlings of Af293 and CEA10 were incubated with bone marrow derived macrophages (BMDM) from C57BL/6 mice at a 10:1 ratio for 24 hours. Both swollen conidia and germlings of CEA10 induced robust release of IL-1α and LDH from BMDM (Figure 4.3B). Interestingly, Af293 swollen conidia induced low levels of IL-1α and LDH release from cells, but if Af293 germlings were incubated with the BMDM IL-1α and LDH release were comparable to CEA10 levels (Figure 4.3B). This demonstrates that different morphotypes of *A. fumigatus* isolates can drive specific IL-1α and damage responses from macrophages.

Early Germination of the CEA10 Strain of *A. fumigatus* within the Lung Environment Corresponds with its Greater Virulence and Pathology Potential

In *A. fumigatus* infection models, resting conidia are instilled into the airways where they can begin to swell and subsequently, begin polarized cell growth forming germlings and eventually hyphae if not controlled. *Aspergillus* hyphae are highly invasive and express an array of secondary metabolites and hydrolytic enzymes that could be highly damaging to the host (Dagenais and Keller 2009). Based on our observation that *A. fumigatus* germlings were highly cytotoxic and inflammatory when co-cultured with BMDM, we wanted to examine whether the initial growth of CEA10

and Af293 was different within the mouse lungs. In our BALF cytospins that were differentially stained we noted significant fungal material that was recovered from the airways (Figure 4.4). Interestingly, the fungal material recovered from the BALF was found to be in both conidial (white arrows) and germling (black arrows) forms (Figure 4.4). As early as 6 hours after challenge, CEA10 showed an increased ability to germinate within the airway when compared to Af293 (Figure 4.4). CEA10 is able to germinate more extensively under these conditions than Af293, which corresponds with its ability to induce high levels of lung damage and drive an IL-1α-dependent inflammatory response.

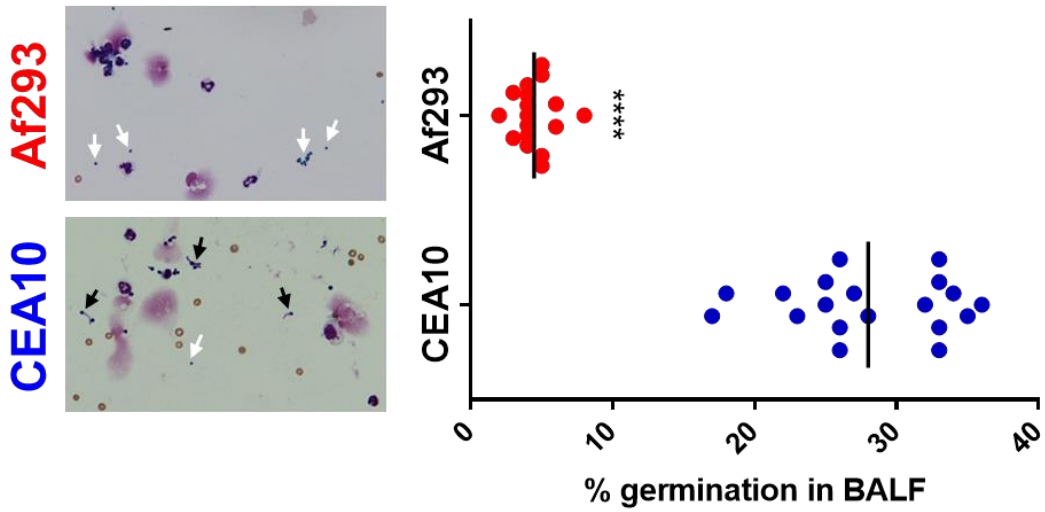

Figure 4.4. CEA10 germinates more efficiently than Af293 in C57BL/6 airways. C57BL/6 mice were infected i.t. with $4x10^7$ conidia of Af293 (top picture) or CEA10 (bottom picture). At 6 hpi, mice were euthanized and BALF collected. BALF was spun onto slides and stained with a Differential Quik Staining Kit. White arrows indicate conidia in the BALF, while black arrows indicate germlings. Fungal germination was quantified by counting conidia and germlings in the slides and represented as percent of fungal matter that was germinated. Each symbol represents an individual mouse and the line represents the group mean. Data is pooled from 3 independent experiments consisting of 5-8 mice per group. Statistical significance was determined using a Mann-Whitney U test (****$p < 0.0001$).

Heterogeneous Germination of *A. fumigatus* Isolates *In Vitro* in Lung Homogenate Medium

Based on our *in vivo* observation that CEA10 germlings were more prevalent in the airways at early times after instillation, we next sought to test whether the CEA10 strain was better able to utilize the nutrients found in the respiratory tract. It is well established that only certain carbon and nitrogen sources are able to trigger *Aspergillus* spp. growth (Hayer et al. 2013; Hayer et al. 2014). Since it is unclear which nutrients are available to the conidia upon instillation into the airways, we conducted our *in vitro* germination assay in mouse lung homogenate medium. In line with our *in vivo* data, CEA10 was able to germinate more extensively in the lung homogenate media compared to Af293 (Figure 4.5A). This is not caused by an inherent growth defect in the Af293 strain because it germinates as extensively as the CEA10 strain in nutrient rich medium (Figure 4.5B). Thus, our data support a role for inherent differences in nutrient sensing and/or utilization by *A. fumigatus* strains in the airways of immune competent mice for their germination, which corresponds with their pathological and inflammatory potential.

Our observation until now has been based on two commonly used reference strains of *A. fumigatus*. To extend this observation we screened a number of strains and isolates of *A. fumigatus* that we have previously studied (Kowalski et al. 2016), for their ability to germinate in lung homogenate medium. Interestingly, the EVOL20 strain of *A. fumigatus*, which is a hypervirulent strain recently generated by *in vitro* serial passaging of Af293 through low oxygen conditions (Kowalski et al. 2016), was able to germinate more extensively than its parental Af293 in lung homogenate medium (Figure 4.5C). Additionally, we found environmental isolates of *A. fumigatus* that had either Af293-like

germination potential (W72310) or CEA10-like germination potential (02-10) (Figure 4.5C). Thus, *A. fumigatus* isolates display significant heterogeneity in their ability to germinate in airway-mimicking conditions *in vitro* that may be an important determinant of fungal virulence in immune competent mice.

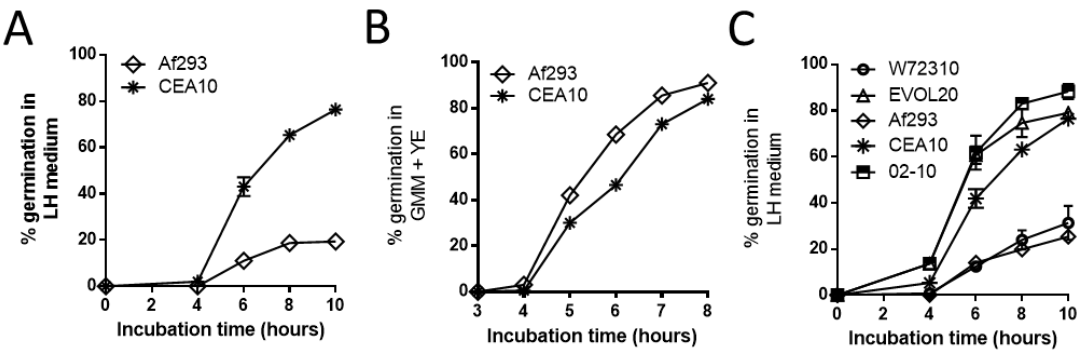

Figure 4.5. Heterogeneity in germination potential of *A. fumigatus* isolates in lung homogenate medium. (A) Lung tissue from C57BL/6 mice was homogenized in PBS and used as medium for *in vitro* germination assays. Lung homogenate (LH) was diluted 1:4 with PBS then inoculated with $2x10^7$ conidia of either CEA10 (blue) or Af293 (red). Beginning at 4 hours post-inoculation, germination was quantified every 2 hours by microscopically counting the number of conidia and germlings. Data is represented as the percent of fungal matter that was germinated. (B) To show that Af293 does not have an inherent germination defect, 2 ml of nutrient rich media containing 0.05% yeast extract (YE) was inoculated with $2x10^7$ conidia of Af293 (red) or CEA10 (blue) and germination assay performed. In (A/B) data is representative of at least 3 independent experiments consisting of 3 biological replicates per group. Each symbol represents the group mean ± one SEM. (C) Environmental isolates 02-10 (orange) and W72310 (gray), microevolved-Af293 strain EVOL20 (purple), and our "wild-type" reference strains CEA10 (blue) and Af293 (red), were used for germination assays in LH media as described in (A). Data in (C) is pooled from 3 independent experiments consisting of 3 biological replicates per group, except for EVOL20 which is pooled from 2 independent experiments consisting of 3 biological replicates per group. Each symbol represents the group mean ± one SEM.

Ability of *A. fumigatus* Strains and Isolates to Germinate in Lung Homogenate Medium Predicts *In Vivo* Airway Germination and IL-1α Dependent Fungal Clearance

To extend our work with the CEA10 and Af293 strains we next wanted to determine if ability of an unknown *A. fumigatus* isolate to germinate *in vitro* in lung homogenate medium could predict which isolates are hypervirulent and induce IL-1α dependent inflammation in an *in vivo* murine infection model. To do this, C57BL/6 mice were inoculated with $4x10^7$ conidia of either Af293, CEA10, W72310, 02-10, or EVOL20. At 12 hpi, BALF was collected to analyze fungal germination in the airways and levels of LDH and albumin in the airways. Strains and isolates that were unable to germinate in the lung homogenate medium (Af293 and W72310) were similarly unable to germinate within the airways (Figure 4.6A), whereas strains and isolates that germinated extensively in the lung homogenate medium (CEA10, 02-10, and EVOL20) were also able to germinate within the lung airways (Figure 4.6B, 4.6C). Moreover, in mice challenged with CEA10, 02-10, and EVOL20, significantly higher levels of LDH and albumin were present in the BALF (Figure 4.6E, 4.6F) compared to mice challenged with Af293 and W72310 (Figure 4.6D). To test whether the ability to germinate within the host is associated with an IL-1α-dependent immune response, C57BL/6 mice and *Il1a*$^{-/-}$ mice were infected with $4x10^7$ conidia of either Af293, CEA10, W72310, 02-10, or EVOL20. At 42 hpi, lungs were collected for histological analysis to determine fungal germination. GMS staining of lung sections demonstrated that mice lacking IL-1α signaling lost the ability to control fungal growth and tissue invasion only when inoculated with the strains and isolate that were able to rapidly germinate within the

airways (CEA10, 02-10, and EVOL20) (Figure 4.6G and Supplemental Figure 4.8). In contrast, mice deficient in IL-1α signaling that were inoculated with Af293 and W72310 were normal in their control of fungal growth and germination (Figure 4.6G and Supplemental Figure 4.8). Taken together, our data reveal a specific role of IL-1α signaling for the control of fungal germination in hosts inoculated with strains and isolates of *A. fumigatus* that rapidly sense and grow in the nutrient environment of the lung.

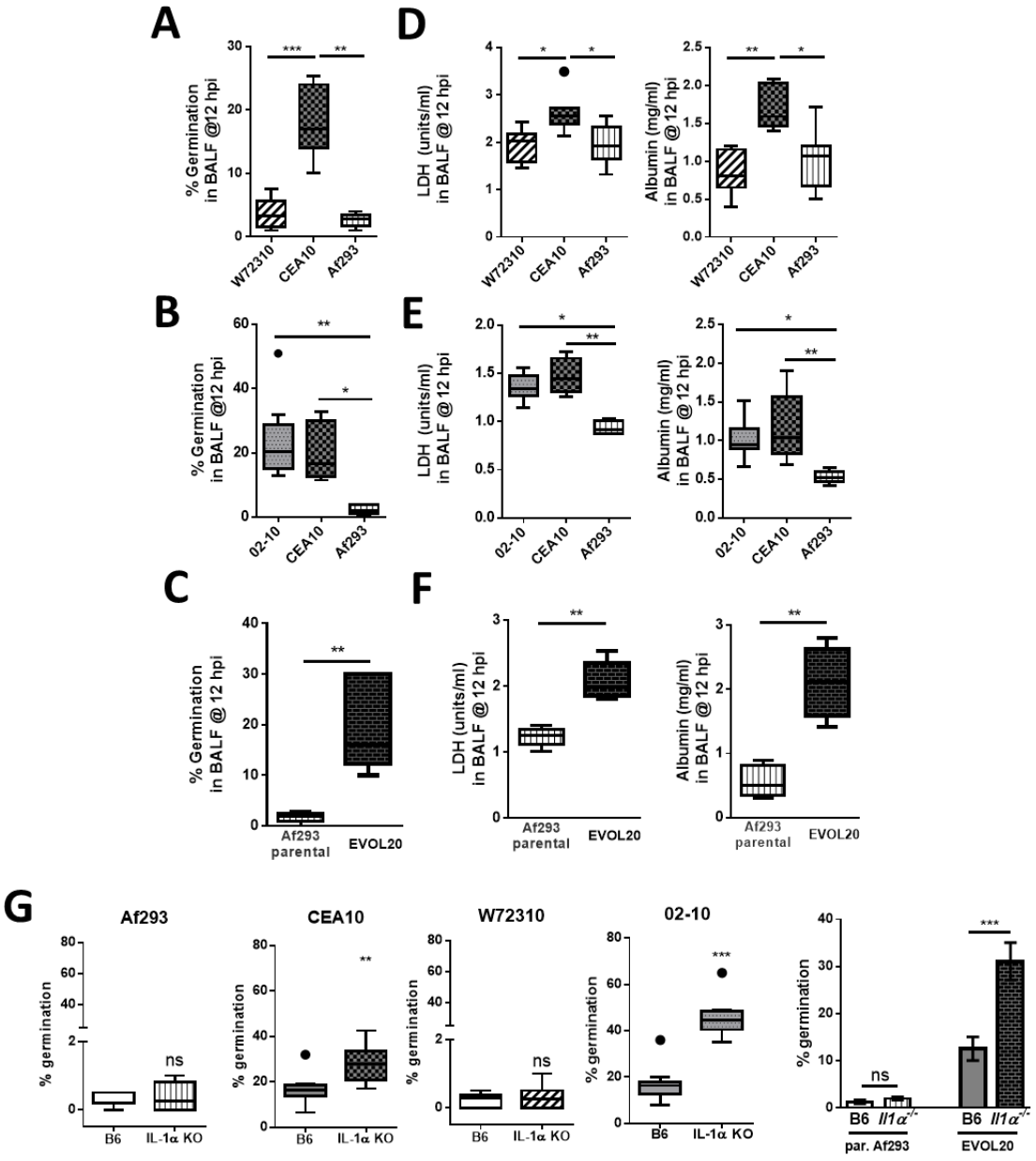

Figure 4.6. *A. fumigatus* isolates that are able to germinate within the airways induce greater lung pathology and IL-1α dependent control of fungal germination. C57BL/6 mice were infected with $4x10^7$ conidia of W72310 (A, D), 02-10 (B, E), or EVOL20 (C, F) along with our "wild-type" reference strains CEA10 and Af293. (A-F) At 12 hpi, mice were euthanized and BALF collected to quantify germination in the airways (A, B, C) and measured LDH and albumin levels to quantify lung damage and leakage, respectively (D, E, F). Data in (A-F) are representative of 2 independent experiments consisting of 5-9 mice per group. Data are presented as box and whisker plots with Tukey whisker and outliers displayed as dots. Statistical significance in (A, B, D, E) was

Figure 4.6 Continued

determined by Kruskal-Wallis one-way ANOVA with Dunn's post-test (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical significance in (C, F) was determined by a Mann-Whitney U test (**p < 0.01). (G) For histological analysis and quantification of germination at 42 hpi, C57BL/6 and *Il1α*$^{-/-}$ mice were infected with 4x10^7 conidia of (from left to right) Af293, CEA10, W72310, 02-10, or EVOL20. Formalin-fixed lungs were paraffin embedded, sectioned and stained with GMS for analysis by microscopy. *A. fumigatus* germination rates were determined by microscopically counting both the number of conidia and number of germlings in GMS-stained sections. Data for Af293, CEA10, W72310, and 02-10 are representative of at least 2 independent experiments consisting of 4-10 mice per group and are presented as box and whisker plots with Tukey whisker and outliers displayed as dots. Data for EVOL20 and the parental Af293 strain are pooled from 2 independent experiments consisting of 5-9 mice per group, and are presented as bar graphs showing the group mean ± one SEM. Statistical significance in (G) for Af293, CEA10, W72310 and 02-10 was determined by a Mann-Whitney U test (ns = not significant, **p < 0.01, ***p < 0.001). Statistical significance in (G) for EVOL20 and the parental Af293 strain was determined by a two-way ANOVA with Tukey's post-test (***p < 0.001).

Af293 Germlings Are Sufficient to Induce Greater Lung Pathology and Initiate an IL-1α Dependent Neutrophil Response

Our data strongly suggest that the presence of increased numbers of *A. fumigatus* germlings in the airways causes extensive lung pathology and an IL-1α-dependent inflammatory response. Because our analysis up to now has been correlative between the presence of fungal germlings and lung pathology, we next sought to address whether Af293 germlings are sufficient to induce greater lung pathology and IL-1α dependent inflammation. To do this, Af293 conidia were pre-germinated in liquid culture prior to instillation into mice. At the time of challenge, approximately 20-30% of all fungal matter in the culture initiated germ-tube formation. This level of growth is very similar to the level of airway germination observed with hypervirulent strains and isolates of *A. fumigatus* (Figure 4.6). C57BL/6 and *Il1a*$^{-/-}$ mice were challenged with 2.7x10^7 resting

conidia or germlings of Af293. At 12 hpi, leukocyte recruitment to the airways and lung damage were quantified. *Il1a*$^{-/-}$ mice challenged with Af293 resting conidia had normal macrophage and neutrophil recruitment to the airways (Figure 4.7A). In contrast, *Il1a*$^{-/-}$ mice challenged with Af293 germlings had a significant defect in neutrophil recruitment to the airways compared to C57BL/6 mice, while macrophage recruitment was not changed (Figure 4.7A). Moreover, Af293 germlings induced significantly greater lung damage as evidenced by elevated levels of both LDH and albumin in the BALF compared to Af293 conidia (Figure 4.7B). Together, these data demonstrate that Af293 germlings are sufficient to drive enhanced pulmonary damage which results in an IL-1α dependent inflammatory neutrophil response.

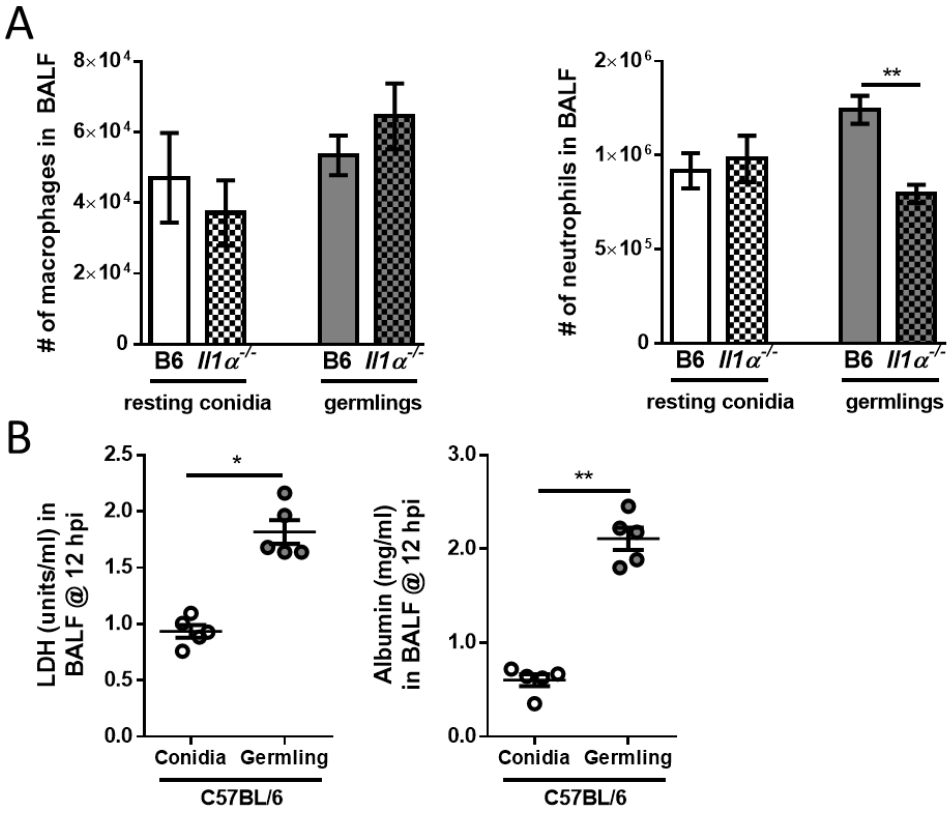

Figure 4.7. Af293 germlings are sufficient to enhance lung pathology and drive IL-1α dependent neutrophil recruitment *in vivo*. C57BL/6 and *Il1α*$^{-/-}$ mice were infected with 2.7x10^7 resting conidia (gray) or germlings (blue) of the Af293 strain of *A. fumigatus*. At 12 hpi, mice were euthanized and BALF collected to analyze (A) macrophage and neutrophil recruitment to the airways and (B) LDH and albumin levels. Bar graphs (A) show the group mean ± one SEM. Each symbol in (B) represents an individual mouse and the black line represents the group mean, ± one SEM. Data in (A, B) are representative of 2 independent experiments consisting of 5-8 mice per group. Statistical significance in (A) was determined using a two-way ANOVA with Tukey's post-test (**$p < 0.01$). Statistical analysis in (B) was determined using a Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$).

Discussion

In this study, we reveal an essential function of IL-1α in the control of fungal growth and tissue invasion following challenge with isolates of *A. fumigatus* previously

observed to be hypervirulent in a clinically relevant disease model. We observe that rapid fungal germination in the airways corresponds with the induction of high levels of pulmonary damage which results in IL-1α dependent control of fungal growth of hypervirulent isolates, while IL-1α was dispensable for the clearance of the less virulent isolates. Importantly, we demonstrated that *A. fumigatus* germlings are sufficient to induce excessive lung pathology, which then necessitates IL-1α signaling to prevent fungal growth and invasive disease. Taken together, our data show that initial fungal germination following fungal deposition in the airways is a critical determinant of virulence and the subsequent immune response induced. This further validates the importance of using multiple isolates in studies of pathogenesis and immunity, in order to fully understand the virulence potential of specific pathogens (Baba et al. 2002; Hsu et al. 2003; Voyich et al. 2005; Kobayashi et al. 2010; Planet et al. 2013; Rizzetto et al. 2013; Al Moussawi and Kazmierczak 2014; Parker et al. 2014; Kowalski et al. 2016; Keller 2017).

In order to cause disease, *A. fumigatus* must be able to sense, germinate, and grow within the lung microenvironment. Such growth, if left unchecked leads to tissue invasion, destruction, and dissemination. Our data demonstrate that *A. fumigatus* isolates that can rapidly germinate within the airways cause greater tissue damage leading to inflammatory cell death during which IL-1α is released. Following inoculation with these rapidly germinating *A. fumigatus* isolates, IL-1α signaling is essential for fungal clearance. In contrast, *A. fumigatus* isolates that do not germinate extensively within the airways are not able to penetrate the epithelial barrier and are likely rapidly cleared by

lung resident macrophages through an IL-1α independent mechanism. This concept is supported by work done in an oral candidiasis model (Schaller et al. 2002; Villar et al. 2004; Jayatilake et al. 2007). In oral candidiasis, the epithelial barrier is breached by the *Candida* spp. in order to cause disease. *In vitro,* incubation of human epithelial cells with *Candida* spp. that were able to undergo hyphal growth induced higher levels of IL-1α expression, which was dampened in *C. albicans* mutants that were defective in hyphal growth (Schaller et al. 2002; Villar et al. 2004; Jayatilake et al. 2007). Moreover, IL-1α plays a critical role in regulating host resistance in murine models of oral candidiasis (Altmeier et al. 2016; Schonherr et al. 2017). During oral candidiasis IL-1α is secreted by keratinocytes (Schonherr et al. 2017). In contrast, we show that radiosensitive cells, including macrophages, are the major cellular source of IL-1α following *A. fumigatus* challenge (Figure 4.2). This likely has to do with the epithelial structural differences between the tongue and lungs. Specifically, the tongue epithelium is highly stratified. LeibundGut-Landmann and colleagues have found that the depth of hyphal growth corresponds with IL-1α release in the candidiasis model (Schonherr et al. 2017). In contrast, the respiratory epithelium is a single cell deep to facilitate gas exchange. Thus, *Aspergillus* hyphae immediately enter the basement membrane where innate phagocytes are located, driving IL-1α release from those phagocytes.

The observation that hypervirulent fungal strains and isolates drive excessive cell death, pathology, and IL-1α dependent inflammation appears to be conserved across numerous types of mucosal pathogens (Finck-Barbancon et al. 1997; Di Paolo et al. 2009; Al Moussawi and Kazmierczak 2014; Di Paolo et al. 2014; Milora et al. 2014;

Amarilla et al. 2015; Kitur et al. 2015). Highly virulent *Pseudomonas aeruginosa* isolates express a cytotoxin, ExoU that has been found to induce necrosis and epithelial damage (Finck-Barbancon et al. 1997). Interestingly, ExoU-expressing isolates of *Pseudomonas* induced an immune response that was dependent on IL-1α signaling, whereas isolates lacking ExoU led to an immune response that was dependent on IL-1ß signaling (Al Moussawi and Kazmierczak 2014). In *Staphylococcus*-induced pneumonia it was shown that a highly virulent *S. aureus* isolate possessed toxins that caused necroptosis of host cells, which was shown to be a key factor in lung pathology and damage (Kitur et al. 2015). During infection with highly virulent *Mycobacterium tuberculosis* isolates, rapid growth of the bacteria within macrophages resulted in cell damage and lung necrosis that was mediated by innate immune sensing of a damage signal from dying cells, extracellular ATP (Amaral et al. 2014). Moreover, a specific genomic locus in *M. tuberculosis*, RD1, contributes to bacterial virulence, necrosis, and the secretion of mature IL-1α through induction of calcium influx (Hsu et al. 2003; Yang et al. 2014). In an infection model of porcine reproductive and respiratory syndrome viruses (PRRSV), animals that were infected with the highly pathogenic-PRRSV isolate showed enhanced IL-1α expression within the lung which correlated with high scores for lung pathology, compared to the low-virulent isolates (Amarilla et al. 2015). Together, these studies support our findings that the hypervirulent isolates of a given pathogen may induce an immune response that is highly dependent on IL-1α signaling due to an increase in inflammatory cell death. The results we show here present an opportunity to

reveal fungal factors that promote damage and highly pathological IL-1α responses in the host, and suggest that targeting hyphal-specific factors may be of therapeutic value.

Fungal germination is initiated immediately after instillation of the fungal conidia into the airways based on the nutrients present. How certain isolates can germinate better than other remains elusive, but it could be because of genetic differences between isolates or due to the environmental origins of the *Aspergillus* conidia. In this regards, our *in vitro* germination assay in lung homogenate medium consistently corresponded with our *in vivo* germination studies. This data suggest that the free nutrients in the lungs are sufficient to drive the *in vivo* growth differences. However, it is unclear which specific nutrients in the lungs/airways are driving growth of *A. fumigatus* isolates. Alternatively, a factor found within the lungs may directly inhibit germination of the less virulent isolates. More studies are necessary to determine the specific contributions of and mechanisms behind nutrient acquisition/utilization and other factors in the airways that lead to fungal isolate specific growth and pathology profiles.

Differences between how conidia and germlings interact with the respiratory epithelium and lung-resident macrophages are likely critical for establishing the early inflammatory response. Our data demonstrate the germlings of *A. fumigatus* are much more pathological to macrophages and drive greater IL-1α secretion than swollen conidia in general. However, it also appears that swollen conidia of the hypervirulent CEA10 strain are more damaging to macrophages. This suggests even in the presence of macrophages, hypervirulent *A. fumigatus* isolates may continue to grow either through increased nutrient sensing in the phagosome or increased resistance to the antifungal

effector functions of macrophages. Similarly, *C. albicans* drives pyoptosis-dependent and -independent cell death pathways in macrophages which contributes to the IL-1β response from macrophages (Wellington et al. 2013; Uwamahoro et al. 2014; Wellington et al. 2014; O'Meara et al. ; Altmeier et al. 2016), but its role in IL-1α secretion has not been explored. In *C. albicans* it is well established that within the macrophage phagosome the fungi encounter significant nutrient stress which must be dealt with in order to continue to grow and escape the macrophage (Lorenz et al. 2004; Barelle et al. 2006). Interestingly, Shah et al. have shown that *A. fumigatus* conidia from the CEA10 strain have the capability to germinate within the late phagosome of macrophages (Shah et al. 2016). This germination initiates necroptotic cell death of the macrophage, resulting in lateral transfer of hyphae to other macrophages in order to prevent hyphal escape and maintain control of fungal germination (Shah et al. 2016). The metabolic response that enables this growth in *A. fumigatus* is unknown, but in *C. albicans* amino acid catabolism is essential for continued growth necessary for phagosome escape and induction of pyroptosis (Vylkova and Lorenz 2014; Vylkova and Lorenz 2017).

Given the drastic differences in the level of virulence and the immune response induced by numerous *A. fumigatus* isolates, it will be important to identify central inflammatory hubs that are necessary for host resistance against an array of isolates in order to maximize any potential immunotherapeutic interventions to treat IA. Further studies are needed to examine the factors that contribute to fungal germination within the host, which could lead to novel therapeutic targets for the treatment of IA. Understanding the response to several clinically relevant isolates will expand our

understanding of the host-pathogen interactions during aspergillosis and give insights to the fungal factors that contribute to adaptation to the lung environment and allow disease to manifest in different patient populations.

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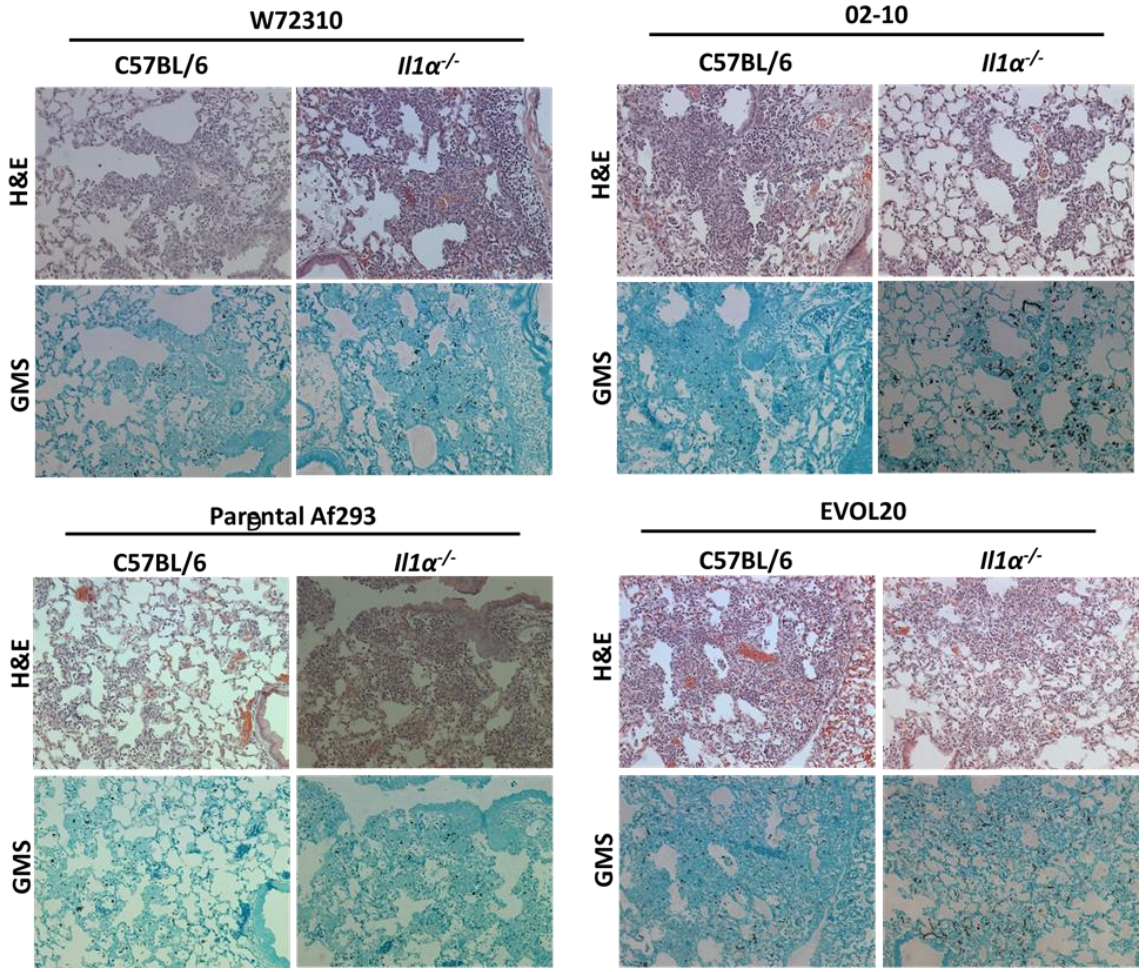
Disclosures

The authors have no conflict of interests to disclose.



Supplemental Figure 4.8. Histology for figure 4.6. For histological analysis and quantification of germination at 42 hpi (shown in Figure 4.6G), C57BL/6 and *Il1α*$^{-/-}$ mice were infected with 4x10^{7} conidia of W72310 (top left), 02-10 (top right), parental Af293 (bottom left), or EVOL20 (bottom right). Formalin-fixed lungs were paraffin embedded, sectioned and stained with H&E (top panel for each strain) or GMS (bottom panel for each strain) for analysis by microscopy, and representative images are shown using the 20x objective.

CHAPTER FIVE

HOST-DERIVED LEUKOTRIENE B4 IS CRITICAL FOR RESISTANCE AGAINST INVASIVE PULMONARY ASPERGILLOSIS

Contributions of Authors and Co-Authors

Manuscript in Chapter 5

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Contributions: Conceived, designed, and performed the experiments, as well as analyzed the data and wrote the paper.

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Contributions: Conceived and designed the experiments, analyzed the data, and wrote the paper.

Manuscript Information Page

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HOST DERIVED LEUKOTRIENE B4 IS CRITICAL FOR RESISTANCE AGAINST INVASIVE PULMONARY ASPERGILLOSIS

The following work is currently in progress to be submitted for publication.

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Abstract

Aspergillus fumigatus is a mold that causes severe pulmonary infections. Our knowledge of how immunocompetent hosts maintain control of fungal infections while constantly being exposed to fungi is developing, but still limited. It is known that timely neutrophil recruitment to and activation in the lungs is critical to the host defense against development of invasive pulmonary aspergillosis, but the inflammatory sequelae necessary remains ill-defined. Here, we show that leukotrienes and are produced following challenge with *A. fumigatus.* Moreover, host-derived LTB_4 is critical for leukocyte recruitment and resistance to pulmonary *A. fumigatus* challenge.

Background

Aspergillus fumigatus is a ubiquitous mold that causes severe infections, such as invasive pulmonary aspergillosis (IPA), in the immunocompromised population. Due to a combination of (i) difficulty in diagnosis, (ii) limited efficacy of antifungal drugs coupled with the emergence of drug resistance, and (iii) a lack of an effective vaccine

against *Aspergillus* spp., mortality rates of IPA are extremely high (Segal 2009; Brown et al. 2012). To this end, development of novel immunomodulatory strategies that can potentially be combined with current anti-fungal treatments is an active area of research.

On a day to day basis, inhaled spores are removed from the body through physical barriers encountered within the respiratory tract. If spores are deposited in the lung, resident alveolar macrophages and $CCR2^{+}$ monocytes, together with alveolar epithelial cells, phagocytose and kill the invading conidia (Espinosa and Rivera 2016; Obar et al. 2016). However, in individuals that lack this immune response, conidia are able to germinate and grow within the lung. These initial encounters are important in the recruitment and activation of neutrophils, inflammatory monocytes, NK cells, and CD4 T cells to further control the fungi within the lung (Cramer et al. 2011). Of these, neutrophils have long been recognized as one of the key effector cells necessary for resistance against *Aspergillus* infection and neutropenia is a risk factor for patients that will develop IPA (Park and Mehrad 2009).

Neutrophil recruitment and activation is a highly controlled process that is regulated by a number of different inflammatory mediators including C5a, PAF, fMLP, LTB_4, CXCR2 ligands, CCR1 ligands, TNFα, and IL-17 (Williams et al. 2011; Kolaczkowska and Kubes 2013). However, our understanding of the inflammatory mediators driving neutrophil accumulation and activation following *A. fumigatus* challenge remains incomplete. Following *A. fumigatus* challenge, IL-1RI/MyD88 signaling is essential for optimal production of CXCL1 that is necessary for early neutrophil recruitment through CXCR2 (Park and Mehrad 2009; Caffrey et al. 2015;

Jhingran et al. 2015). In addition, an unknown CARD9-dependent pathway is critical for late neutrophil accumulation following *A. fumigatus* challenge (Jhingran et al. 2015). Additionally, a TLR9/Btk/calcineurin/NFAT-dependent pathway regulates neutrophil accumulation during aspergillosis through its regulation of TNFα (Herbst et al. 2015). Thus, knowledge of other signaling pathways which contribute to the anti-*Aspergillus* neutrophil response is required. Because of the important role of LTB_4 in the early recruitment and activation of neutrophils in other inflammatory models (Sadik and Luster 2012), we asked whether LTB_4 was crucial in the neutrophil response following pulmonary *A. fumigatus* challenge. Here, we show that LTB_4 produced by host cells plays a critical role in the anti-fungal neutrophil response induced after pulmonary *A. fumigatus* challenge.

Methods

Mice

C57BL/6J (Stock #000664), C57BL/6NJ (Stock #005304), B6.129S2-*Alox5*^{tm1Fun} (*Alox5*$^{-/-}$; Stock #004155), and B6.129S4-*Ltb4r1*^{tm1Adl} (*Ltb4r1*$^{-/-}$; Stock #008102) were purchased from Jackson Laboratories. All mice were 8-10 weeks of age at the time of infection. All animal experiments were approved by the Montana State University Institutional Animal Care and Use Committee.

Preparation of *Aspergillus fumigatus* and Pulmonary Challenge Model

A. fumigatus strain CEA10 was grown and harvested as previously described (Caffrey et al. 2015). For infection, mice were anesthetized with isoflurane and

challenged i.t. with ~5 × 10^7 *A. fumigatus* conidia in 100 µl. At the indicated time after challenge, mice were euthanized using an overdose of pentobarbital. Samples were collected and analyzed for inflammatory cell recruitment, fungal growth, lung damage, and vascular/epithelial leakage as previously described (Caffrey et al. 2015).

Leukotriene Quantification

Lipids were extracted from BALF using a hot methanol extraction. Briefly, three parts HPLC-grade methanol were added to one part BALF sample. Samples were then vortexed for 30 seconds and placed into an 80°C water bath for 2 minutes. Tubes were spun at 14,000 RPM for 15 minutes and supernatant was collected then dried using a vacuum concentrator. Pellets were resuspended in HPLC-grade water in a volume equal to the starting volume of BALF sample. Extracted samples were then analyzed using EIA kits for LTB_4, cysteinyl leukotrienes (cysLT) (Cayman Chemical). Plates were read using a SpectraMax® Paradigm® plate reader (Molecular Devices).

Statistical analysis.

Statistical significance was determined by a Student's t-test, one-way ANOVA using a Bonferroni post-test, or Kruskal-Wallis one-way ANOVA with Dunn's post-test through the GraphPad Prism 5 software as outlined in the figure legends.

Results

Leukotriene Production After Pulmonary Challenge with *A. fumigatus*

In order to determine whether leukotrienes are expressed following *A. fumigatus* challenge, we challenged C57BL/6J wild-type mice with the CEA10 isolate of *A. fumigatus*. Throughout a time course of 6 to 48 hours post-infection (hpi), we collected bronchoalveolar lavage fluid (BALF) and measured leukotriene production. We found that LTB_4 and cysLT showed increased production after *A. fumigatus* challenge. The production of these inflammatory lipid mediators followed a similar trend early after infection in which their expression peaked at 6 hpi, followed by decreased levels at 12 hpi. At 24 and 48 hpi LTB_4 increased from the 12 hpi levels, while the cysLT levels continue to decrease (Supplemental Figure 5.4). These data demonstrate that both LTB_4 and cysLTs are synthesized following challenge of immunocompetent mice with *A. fumigatus*.

Ltb4r1$^{-/-}$ Mice Have a Defect in Inflammatory Cell Recruitment and Resistance to IPA

LTB_4 is known to be important in the recruitment of neutrophils in numerous inflammatory settings (Sadik and Luster 2012), but whether it is crucial in regulating the innate immune response following *A. fumigatus* challenge is unknown. To address whether LTB_4 was critical for neutrophil recruitment and resistance against IPA, we infected *Ltb4r1*$^{-/-}$ and C57BL/6NJ mice with *A. fumigatus*. At 12 hpi, we analyzed BALF via differential cytospins stained with Diff-Quik™ to assess early inflammatory cell

recruitment to the airways. Compared to the C57BL/6NJ mice, *Ltb4r1*$^{-/-}$ mice had a significant defect in neutrophil and eosinophil recruitment, while macrophage accumulation was similar (Figure 5.1A). Because the early recruitment of neutrophils is needed for host resistance to invasive *A. fumigatus* infection (Park and Mehrad 2009), we next addressed whether fungal growth was enhanced in the *Ltb4r1*$^{-/-}$ animals. At 24 hpi, Grocott-Gomori methenamine silver (GMS) staining of lung sections revealed the presence of an increased proportion of germinated *A. fumigatus* in *Ltb4r1*$^{-/-}$ mice compared with C57BL/6NJ mice (Figure 5.1B) demonstrating *Ltb4r1*$^{-/-}$ mice were highly impaired in their ability to clear the fungi (Figure 5.1C). Lastly, lung damage and endothelial/epithelial leakage induced by *A. fumigatus* challenge were assessed by quantifying LDH and albumin in the BALF, respectively. Albumin levels were significantly elevated in *Ltb4r1*$^{-/-}$ mice compared to C57BL/6NJ mice, indicating an increase in protein leakage from the vascular system (Supplemental Figure 5.5B). In contrast, LDH levels were only mildly elevated suggesting induction of similar degrees of cell damage (Supplemental Figure 5.5A). Taken together, these data demonstrate that LTB_4 signaling through its high affinity receptor LTB4R1 is important in mediating neutrophil and eosinophil recruitment to the airways, which was ultimately necessary for host resistance to *A. fumigatus* infection.

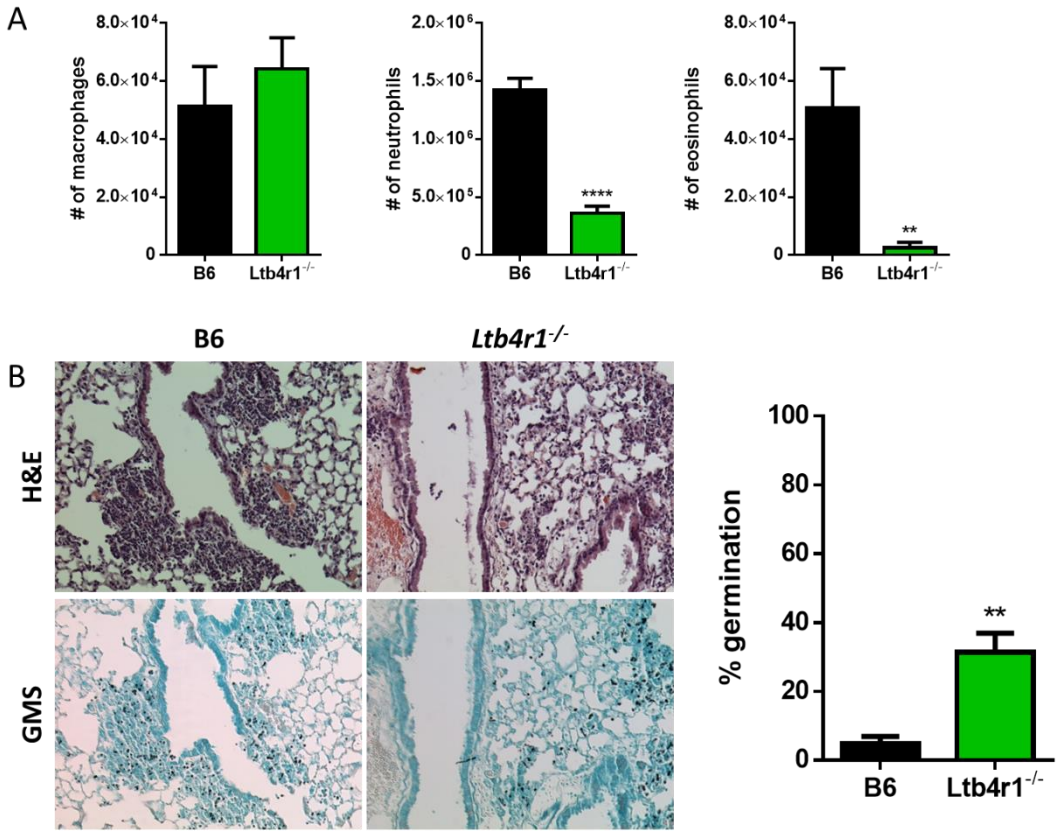

Figure 5.1. *Ltb4r1*-deficient mice have a defect in neutrophil and eosinophil recruitment and increased fungal burden after pulmonary *A. fumigatus* challenge. Age-matched C57BL/6NJ or *Ltb4r1*-deficient mice were infected intratracheally (i.t.) with 5×10^7 CEA10 conidia and at indicated time-points, mice were euthanized, bronchoalveolar lavage fluid (BALF) collected, and lungs saved for histological analysis. (A) Total macrophage (left panel), neutrophil (middle panel) and eosinophil (right panel) recruitment in the BALF was measured at 12 hours post-infection (hpi). (B) Formalin-fixed lungs were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) (top) or Grocott-Gomori methenamine silver (GMS) (bottom) for analysis by microscopy. Representative lung sections from C57BL/6NJ and *Ltb4r1*-deficient mice infected with CEA10 are shown using the 10x objective. (C) *A. fumigatus* germination rates were assessed at 24 hpi by microscopically counting the number of conidia and germlings in GMS-stained sections from C57BL/6NJ and *Ltb4r1*-deficient mice. (D) Lung damage (left panel) and leakage (right panel) were assessed at 24 hpi by measuring lactate dehydrogenase (LDH) and albumin levels in the BALF, respectively. Data is representative of at least two independent experiments consisting of five to eight mice per group. Bar graphs show the group mean ± one SEM (A and C). Each symbol in (D) represents an individual mouse and the line represents the group mean. Statistically significant differences were determined using Student's t-test (*p<0.05; **p<0.01; ***p<.001; ****p<.0001).

Alox5$^{-/-}$ Mice Are Impaired in Inflammatory Cell Recruitment and Resistance to IPA, But Do Not Present with Increased Vascular Leakage

A. fumigatus itself is known to be capable of producing eicosanoids (Noverr et al. 2002), which results in an infection system in which both the mammalian and fungal cells could be the source of bioactive LTB_4. Thus, to address whether LTB_4 production coming from the murine cells was necessary for host resistance to IPA, we challenged 5-lipoxygenase (*Alox5*$^{-/-}$) deficient mice with *A. fumigatus*. *Alox5*$^{-/-}$ mice cannot convert arachidonic acid to LTA_4 and, therefore, lack all leukotriene synthesis (Santos et al. 2013). After *A. fumigatus* challenge, inflammatory cell recruitment to the airways was quantified at 12 hpi via cytospins and Diff-Quik™ staining. Similar to what we found with the *Ltb4r1*$^{-/-}$ mice, *Alox5*$^{-/-}$ mice had a significant defect in both neutrophil and eosinophil recruitment, while macrophage accumulation remained largely similar to C57BL/6J (Figure 5.2A). This defect in neutrophil and eosinophil recruitment correlated with an impairment in the ability of *Alox5*$^{-/-}$ mice to control fungal growth within the lung, demonstrated by a significantly higher germination rate (Figure 5.2B, 5.2C). We also measured LDH and albumin levels in the BALF of the *Alox5*$^{-/-}$ mice to assess lung damage and vascular/epithelial permeability, respectively. Interestingly, unlike the *Ltb4r1*$^{-/-}$ mice, LDH and albumin levels in BALF from *Alox5*$^{-/-}$ mice were not significantly different than C57BL/6J levels (Supplemental Figure 5.5A-B). Together, these data indicate that leukotriene synthesis by host cells is critical for neutrophil and eosinophil recruitment, as well as resistance to IPA.

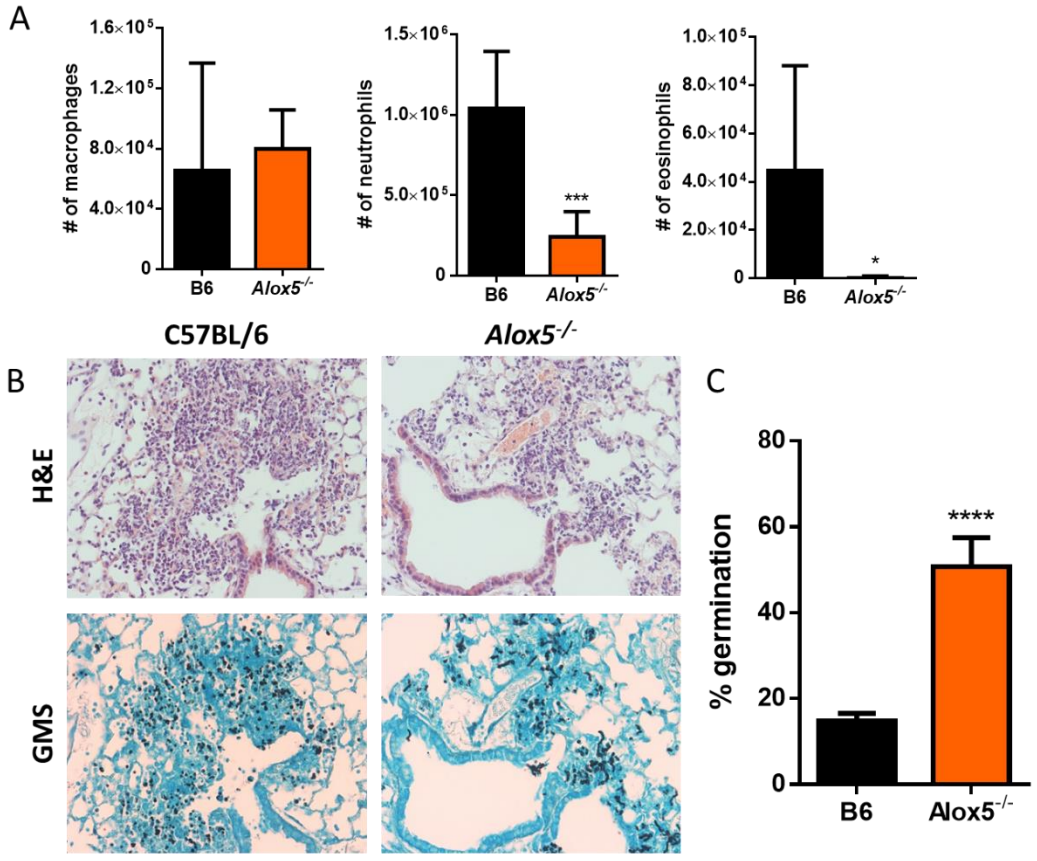

Figure 5.2. *Alox5*-deficient mice have a defect in neutrophil and eosinophil recruitment and increased fungal burden after pulmonary *A. fumigatus* challenge. Age-matched C57BL/6J or *Alox5*-deficient mice were infected intratracheally (i.t.) with 5×10^7 CEA10 conidia and at indicated time-points, mice were euthanized, (BALF) collected, and lungs saved for histological analysis. (A) Total macrophage (left panel), neutrophil (middle panel) and eosinophil (right panel) recruitment in the BALF was measured at 12 hpi. (B) Formalin-fixed lungs were paraffin embedded, sectioned, and stained with H&E (top) or GMS (bottom) for analysis by microscopy. Representative lung sections from C57BL/6J and *Alox5*-deficient mice infected with CEA10 are shown using the 10x objective. (C) *A. fumigatus* germination rates were assessed at 36 hpi by microscopically counting the number of conidia and germlings in GMS-stained sections from C57BL/6J and *Alox5*-deficient mice. (D) Lung damage (left panel) and leakage (right panel) were assessed at 36 hpi by measuring lactate dehydrogenase (LDH) and albumin levels in the BALF, respectively. Data is representative of at least two independent experiments consisting of five to eight mice per group. Bar graphs show the group mean ± one SEM (A and C). Each symbol in (D) represents an individual mouse and the line represents the group mean. Statistically significant differences were determined using Student's t-test (*p<0.05; ****p<.0001).

Radiosensitive Cells Contribute to 5-Lipoxygenase Activity Following Pulmonary *A. fumigatus* Challenge

To determine which cells contribute to the activity of 5-lipoxygenase after *A. fumigatus* challenge, we utilized a bone marrow chimerism approach. C57BL/6 mice were lethally irradiated then reconstituted with either C57BL/6 or *Alox5*$^{-/-}$ bone marrow intravenously to develop the following groups: C57BL/6 mice possessing C57BL/6 bone marrow and C57BL/6 mice possessing *Alox5*$^{-/-}$ bone marrow. Mice were then rested for 6-8 weeks prior to challenge with $4x10^7$ conidia of CEA10. At 36 hpi, mice were euthanized, BAL collected for analysis of leukocyte recruitment to the airways, and lungs saved for histological analysis to assess fungal growth by GMS staining. Compared to C57BL/6 mice possessing C57BL/6 bone marrow, C57BL/6 mice possessing *Alox5*$^{-/-}$ bone marrow had a significant defect in neutrophil and eosinophil recruitment to the airways at 36 hpi, while macrophage accumulation was not significantly altered (Figure 5.3A). Moreover, at 36 hpi, C57BL/6 mice possessing *Alox5*$^{-/-}$ bone marrow were impaired in their ability to control fungal germination compared with C57BL/6 mice possessing C57BL/6 bone marrow (Figure 5.3B). Taken together, these data suggest that radioresistant cells contribute to 5-lipoxygenase activity which is needed for neutrophil and eosinophil recruitment to the airways and control of fungal germination following pulmonary *A. fumigatus* challenge.

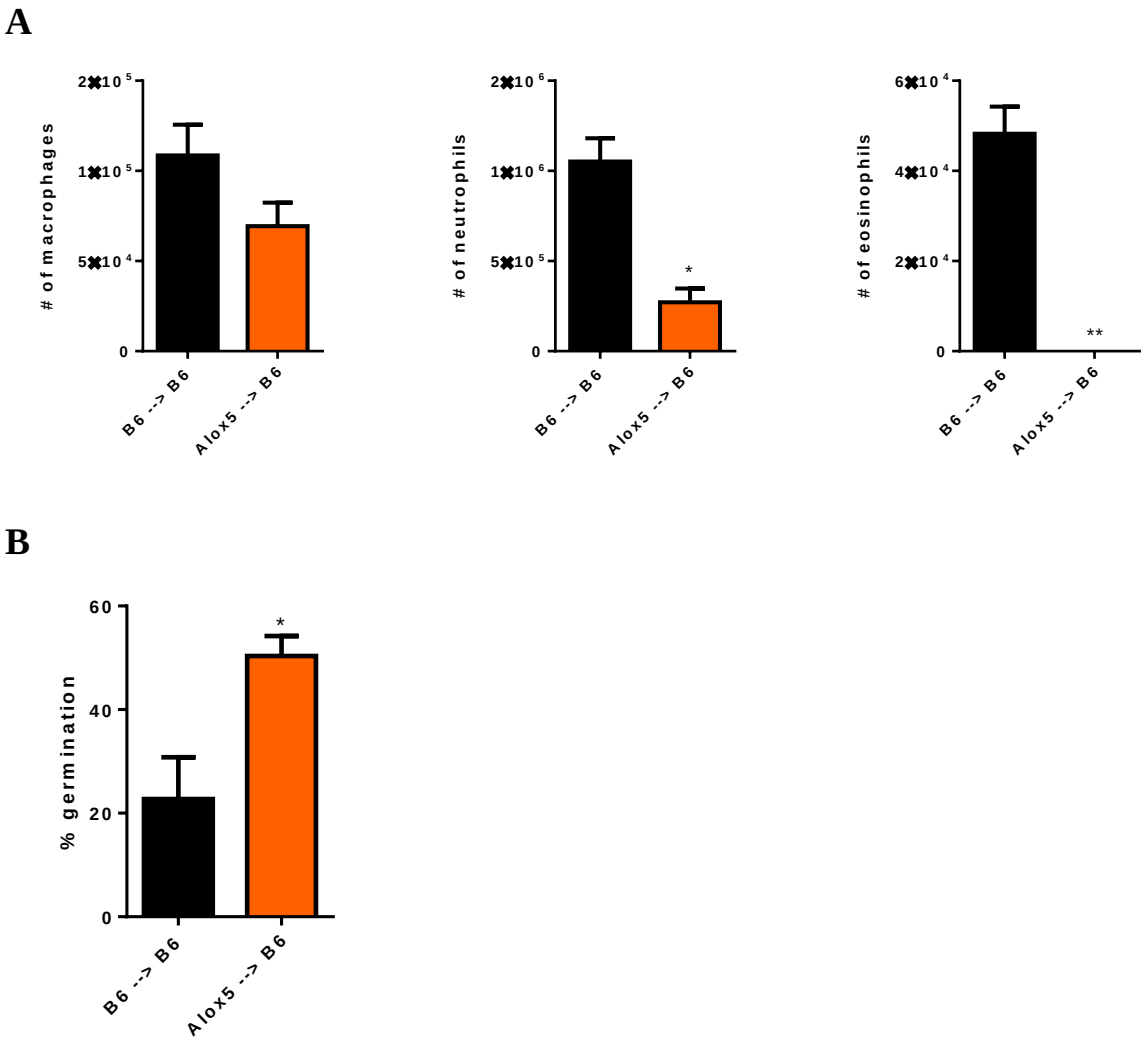

Figure 5.3. Radiosensitive cells contribute to 5-lipoxygenase activity after pulmonary *A. fumigatus* challenge. Bone marrow chimeric mice were made by lethally irradiating C57BL/6 mice and reconstituting the mice with bone marrow from either C57BL/6 or *Alox5*$^{-/-}$ mice. These mice were challenged i.t. with 4x10^7 conidia of CEA10 and at 36 hpi, mice were euthanized and (A) BALF collected for quantification of leukocyte recruitment to the airways and (B) lungs saved for histological analysis in which formalin-fixed lung were paraffin embedded, sectioned and stained with GMS for analysis by microscopy. Data is representative of 1 experiment with 4-5 mice per group. Bar graphs show the group mean ± one SEM. Statistical significance was determined using a Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$).

Discussion

This study reveals an essential role for host-derived LTB_4 in the recruitment of both neutrophils and eosinophils through its high-affinity receptor LTB4R1 following pulmonary challenge with the mold *A. fumigatus*. Ultimately, LTB_4 was crucial for host resistance to invasive disease. In addition to recruitment of neutrophils, it was recently shown that LTB_4-treated neutrophils have enhanced anti-fungal activity against *A. fumigatus* (Jones et al. 2016). Thus, LTB_4 will not only recruit neutrophils to the site of *A. fumigatus* infection, but also simultaneously activates their anti-fungal armory. While our data are the first to demonstrate a critical role for LTB_4 in regulating neutrophil recruitment following challenge with a mold, others have reported leukotrienes are critical for leukocyte recruitment and activation following infection with the dimorphic fungi, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* (Medeiros et al. 2004; Santos et al. 2013; Secatto et al. 2014). In these systems it is unknown how leukotriene synthesis is initiated, but with the yeast *Candida albicans* treatment of macrophages in vitro results in the synthesis of leukotrienes through a Dectin1-, Dectin2-, and MyD88-dependent pathway (Suram et al. 2010). Given that Dectin1, Dectin2 and MyD88 play a role in not only recognizing *A. fumigatus* (Steele et al. 2005; Sun et al. 2013; Caffrey et al. 2015; Jhingran et al. 2015), but a range of fungal pathogens, it is likely this pathway will be important in the induction of the inflammatory eicosanoid pathway universally following challenge with fungal pathogens.

While it is well known that neutrophils are one of the most important effector cells against *A. fumigatus* (Cramer et al. 2011), a recent study demonstrated a potential

role for eosinophils in limiting the development of IPA (Lilly et al. 2014). Our data demonstrate that LTB_4 is also critical for the recruitment of eosinophils to the airways of *A. fumigatus* challenged mice. In immunocompetent mice, the absence of eosinophils resulted in a defect in *A. fumigatus* clearance in vivo which did not correlate with a defect in recruitment or function of other inflammatory cells, but rather the potential direct anti-fungal activity of eosinophils (Lilly et al. 2014). In contrast, with repeated administration of *A. fumigatus* eosinophils were shown to have a detrimental effect on disease outcome and eosinophilia was associated with a decrease in neutrophil recruitment (O'Dea et al. 2014). Thus, more studies are needed to determine the exact role eosinophils play in different experimental models of IPA, and whether crosstalk between eosinophils and neutrophils regulate anti-fungal immunity.

Clinically, patients with chronic granulomatous disease (CGD) have a higher risk of developing invasive *Aspergillus* infections, which is mirrored in mice lacking the NADPH oxidases. In these mice, IPA is characterized by excessive inflammation and tissue damage (Petersen et al. 2002; de Luca et al. 2014). Interestingly, when $gp91^{phox}$-deficient mice were exposed subcutaneously to *A. fumigatus* there is an early neutrophilic response which is associated with elevated LTB_4 levels (Petersen et al. 2002). Thus, it will be intriguing whether limiting LTB_4 signaling can ameliorate IPA in $gp91^{phox}$-deficient mice, similarly to what was observed with anakinra treatment to limit IL-1 signaling (de Luca et al. 2014). This is plausible because in a *Mycobacterium tuberculosis* model, eicosanoid and IL-1 signaling regulate *M. tuberculosis* growth and pathogenicity (Mayer-Barber et al. 2014). Another patient population at high risk of

developing IPA is individuals receiving long-term corticosteroid treatment. Treatment with glucocorticoids leads to inhibition of PLA2 through the induction of lipocortin-1, blocking the conversion of phospholipids to arachidonic acid (Rhen and Cidlowski 2005). Given this link between glucocorticoid treatment and the arachidonic acid pathway, it would be interesting to see the overall impact the presence or absence of leukotrienes have on the outcome of disease during a corticosteroid model of IPA.

In conclusion, we have shown that during the inflammatory response of immunocompetent mice following pulmonary challenge with *A. fumigatus*, leukotriene production is induced. In the absence of all leukotriene synthesis, and specifically in the absence of LTB4R1 signaling, there is a critical defect in neutrophil and eosinophil recruitment to the airways which results in an elevated susceptibility of these mice to developing IPA. These data reveal that the leukotriene pathway may be a novel immunotherapeutic target for the treatment of IPA.

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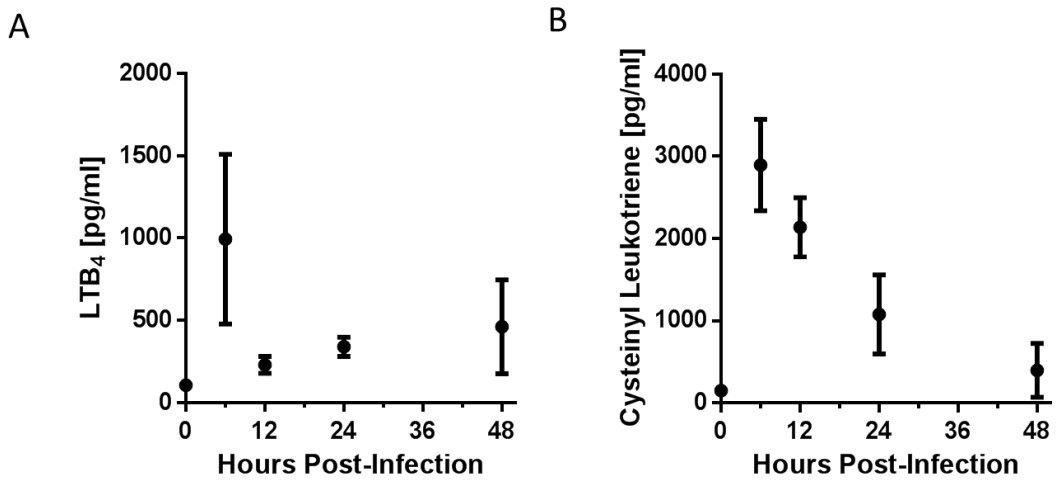
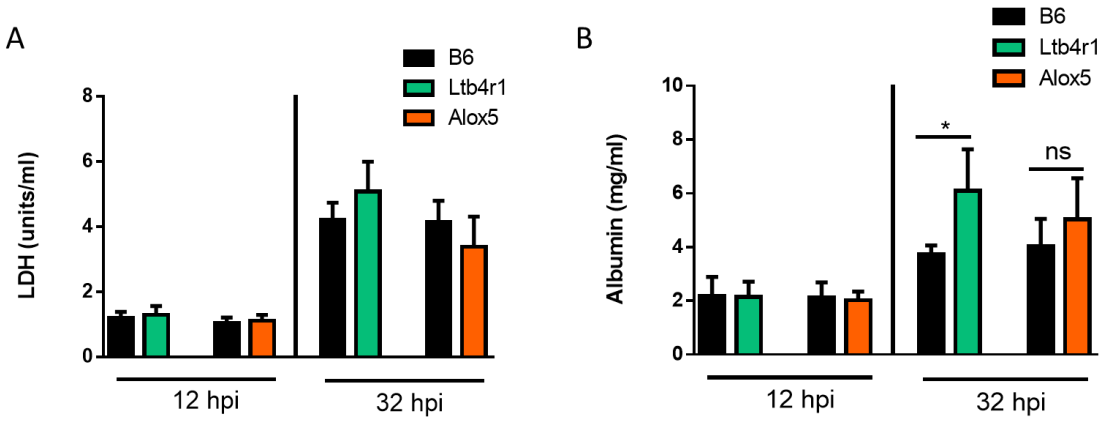


Supplemental Figure 5.4. C57BL/6J mice produce leukotrienes after pulmonary challenge with the CEA10 isolate of *A. fumigatus*. Mice were infected intratracheally (i.t.) with 5×10^7 CEA10 conidia and at indicated time-points, mice were euthanized and bronchoalveolar lavage fluid (BALF) collected. Lipids were then extracted from BALF using a hot-methanol extraction procedure, and LTB_4 (A) and cysteinyl leukotriene (B) levels in the extracted BALF samples were measured using Cayman Chemical enzyme immunoassay (EIA) kits. Data are representative of 5 mice per timepoint and 1 independent experiment. Each dot represents the mean ± one SEM.



Supplemental Figure 5.5. Enhanced vascular leakage in *Ltb4r1*-deficient mice, but not *Alox5*-deficient mice following pulmonary *A. fumigatus* challenge (A) Lung damage and (B) vascular leakage were assessed at 12 and 32 hpi by measuring lactate dehydrogenase (LDH) and albumin levels in the BALF, respectively. Data is representative of at least two independent experiments consisting of five to eight mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using Mann-Whitney U-test (*$p<0.05$; ****$p<.0001$).

CHAPTER SIX

DISCUSSION AND CONCLUSIONS

Due to increased numbers of invasive medical procedures being performed, such as bone marrow and solid organ transplants, and increased life span of these patients due to immune suppressive drugs, the population at risk for IA is continuously expanding (Low and Rotstein 2011). Although advancements in diagnostic tools and antifungal drug treatments have been made, IA remains a disease of high mortality thus necessitating the development of novel therapeutic options. One line of research that holds great promise for the development of novel treatment options in these patients is harnessing the immune system to combat invasive fungal infections. Only by understanding the immune pathways needed for optimal resistance to *A. fumigatus* in which fungal clearance occurs in the absence of excessive immune-mediated pathology, can the development of immunomodulatory treatment options for IA be successfully tested and implemented.

Neutrophils are a major effector cell population in host defense against IA. The overall goal of this dissertation is to define the immunological events that are necessary for the recruitment of neutrophils to the lungs after exposure to *A. fumigatus* in an immune competent animal. Given the different pathologies associated with IA in neutropenic and non-neutropenic populations, defining these immunological events can begin to reveal specific immune defects in different populations. A central hub that is critical for neutrophil recruitment across a wide range of disease models is IL-1RI

signaling (Chapter 3). Furthermore, in humans SNPs in genes coding for components of the IL-1 pathway are associated with an increased risk of developing invasive fungal infections (Sainz et al. 2008; Wójtowicz et al. 2015). Thus we sought to determine the specific role of IL-1 signaling during IA.

We hypothesized that IL-1RI signaling would be important in limiting invasive fungal growth in the pulmonary environment through initiating recruitment of neutrophils. To test this hypothesis we utilized mice genetically deficient in IL-1RI signaling. Our results demonstrate signaling through IL-1RI was critical for the induction of CXCL1 production, neutrophil recruitment to the airways early after *A. fumigatus* challenge, and the prevention of tissue invasive fungal growth. Absence of IL-1α, but not the inflammasome, led to a defect in CXCL1 production and neutrophil recruitment with an inability to control fungal germination at later time points after fungal challenge. This suggests that IL-1α and IL-1ß play non-redundant roles in host resistance against IA. Upon further investigation we found this IL-1α dependent immune response to be fungal strain-specific. Fungal strains that were able to rapidly germinate within the airways (CEA10, 02-10 and EVOL20) induced high levels of pulmonary damage, enhancing the release of alarmins such as IL-1α, which in turn led to rapid and robust recruitment of neutrophils. Conversely, strains that were unable to rapidly germinate in the airways (Af293 and W72310) induced lower levels of pulmonary damage and neutrophil recruitment was independent of IL-1α signaling. We did not specifically test if neutrophil recruitment and subsequent control of Af293 and W72310 germination is dependent on IL-1ß, but Af293 neutrophil recruitment was IL-1RI and

ASC-dependent (Chapter 4). Furthermore, mice deficient in IL-18 signaling show enhanced neutrophil recruitment compared to wild-type mice after challenge with Af293, rather than a defect in neutrophili recruitment (Appendix A, Figure A.1). Although this strongly suggests the occurrence of an IL-1ß-dependent immune response for non-germinating strains, further investigation is needed to confirm this.

It is currently unknown why some strains are able to germinate within the pulmonary environment and others cannot. One possibility is that some strains are able to sense and utilize nutrients more efficiently than other strains. In order to germinate within the lungs, *A. fumigatus* must be able to sense and utilize available carbon and nitrogen sources. Specifically, in the corticosteroid murine model, the carbon catabolite repression system controlled by CreA, although dispensable for early growth in the airways, was absolutely required for progression and maintenance of fungal growth (Beattie et al. 2017). In this case, corticosteroid treatment in the absence of fungal challenge resulted in a decrease in preferred sources glucose and glutamate, and a concomitant increase in non-preferred sources (Beattie et al. 2017). This suggests that in an immune competent host early after challenge preferred sources of carbon and nitrogen are available in the airways, which *A. fumigatus* can potentially utilize and conidial swelling can begin. With this swelling, exposure of PAMPs on the fungal cell wall occurs, leading to a rapid influx of immune cells to the site of fungal growth. The kinetics of nutrient depletion and limiting sources in our immune competent model remain to be elucidated, however competition between host immune cells and fungi is a probable occurrence. Thus, the ability to utilize alternative carbon and nitrogen sources

in the lung could provide *A. fumigatus* with a fitness advantage and is one explanation to the strain-specific fungal germination observed in our studies. Interestingly, there is a SNP present in CreA between Af293 and CEA10 that results in a single amino acid change (Fedorova et al. 2008). Similarly, AreA which is the transcriptional regulator of nitrogen metabolism in *Aspergillus* species, also has a SNP between Af293 and CEA10 resulting in a single amino acid change (Fedorova et al. 2008). Whether these amino acid changes result in functional differences in CreA and AreA between the two fungal strains remains to be determined, however this is notable given the significant differences observed in their *in vivo* germination.

Where initial fungal germination occurs within the pulmonary environment is an area of ongoing investigation. When conidia enter the respiratory tract, they are likely deposited in the airway surface liquid (ASL) found covering the epithelial lining in the airways. The top layer of the ASL is the mucus layer, which is composed of mucin glycoproteins, lipids, water, inorganic salts and proteins (Senel et al. 2015). The mucin glycoproteins are made up of a protein backbone with a large number of oligosaccharide side chains made of glucose, galactose and fucose (Senel et al. 2015). Given this composition, it is possible that *A. fumigatus* conidia are able to utilize nutrients in the ASL to initiate germination. Supporting this is the finding that mucin was sufficient as a sole carbon source in minimal media to support the *in vitro* germination of CEA10, 02-10, and EVOL20, but not Af293 and W72310 (Appendix B, Figure A.3). Interestingly, it has been shown that bacterial chitinases are capable of degrading mucin, suggesting the possibility that fungal chitinases could participate in the degradation of mucin in order to

liberate nutrients for fungal growth (Argueso et al. 1998; Sanders et al. 2007). A recent study revealed that extracellular proteases of *P. aeruginosa* were critical for the acquisition and utilization of acetate obtained from mucin degradation (Flynn et al. 2017). Additionally, the *A. fumigatus* lectin, FleA, was shown to bind fucosylated structures on mucin and on the macrophage cell surface, which enhanced recognition and phagocytosis of conidia (Kerr et al. 2016). It is hypothesized that fungi evolved FleA in order to aid in their growth on carbohydrate-rich substrates, however FleA contribution to nutrient utilization *in vivo* remains to be demonstrated (Kerr et al. 2016). Given the significant increase in fungal germination and increased virulence after challenge of immune-competent mice with FleA-deficient conidia, it can be postulated that this lectin plays a dominant role as a PAMP leading to rapid recognition of *A. fumigatus* conidia, rather than participating in nutrient utilization within a host. However, it will be interesting to determine if different *A. fumigatus* strains express varying levels of FleA, as this may provide a mechanism by which fungi can evade recognition by the host immune system, leading to enhanced virulence. FleA was present on both resting conidia and swollen conidia of *A. fumigatus*, albeit at significantly higher levels on resting conidia (Kerr et al. 2016). This study demonstrates a mechanism by which phagocytosis occurs prior to conidial swelling and cell wall exposure, raising the possibility that germination may occur within the phagosome, rather than in the ASL.

Upon recognition, *A. fumigatus* can be phagocytosed by macrophages, neutrophils, dendritic cells and other immune cells. The goal of phagocytosis is to kill the invading microbial threat through oxidative and non-oxidative mechanisms, including

ROS, RNS, phagosome acidification, and a number of antimicrobial peptides (Haas 2007). Microbes must have mechanisms to counteract host immunity in order to survive and adapt to the host environment. One mechanism is through induction of pyroptosis, a lytic form of cell death which *Candida* spp. have been shown to utilize to escape from within the macrophage phagosome (Uwamahoro et al. 2014). In response to phagocytosis, both *Candida albicans* and *Cryptococcus neoformans* undergo cell wall rearrangement exposing mannosylated glycoproteins that induce macrophage pyroptosis (O'Meara et al.). In addition to this early pyroptotic cell death, pyroptosis-independent macrophage lysis occurs at later times due to mechanical piercing of the macrophage by fungal filaments (Uwamahoro et al. 2014). Which host factor(s) is inducing this cell wall rearrangement is unknown, but there are several stressors present within the phagosome that could potentially activate fungal transcriptional networks critical for adaptability. One such stressor is nutrient deprivation as the phagosome is believed to be a nutrient-poor environment, however the exact nutrient composition of the phagosome remains unclear (Haas 2007; Seider et al. 2014). In *Mycobacterium tuberculosis*, phagocytosis induces a starvation response in which a shift away from glycolysis and towards the glyoxylate cycle occurs (McKinney et al. 2000). One study analyzed the long-term changes and adaptations acquired by *Mycobacterium bovis* after long-term infection of macrophages (Vázquez et al. 2014). The intracellular *M. bovis* that was adapted to the macrophage environment were better able to metabolize glucose, accumulated higher levels of neutral lipids, and exhibited increased survival within macrophages and in mice upon re-infection (Vázquez et al. 2014). Upon phagocytosis of *Candida galbrata* by

murine macrophages, it was shown that prolonged carbon starvation occurs and autophagy was critical to fungal survival within the macrophage (Roetzer et al. 2010). Additionally, when *C. albicans* is phagocytosed alternative metabolic pathways are induced which aid in fungal growth and escape from the macrophage (Lorenz et al. 2004; Barelle et al. 2006; Piekarska et al. 2006; Ramírez and Lorenz 2007; Ene et al. 2012). Interestingly, *C. albicans* is able to co-opt amino acid catabolism while in the phagosome, which leads to the generation of ammonia and neutralization of the pH found in the phagosome (Vylkova and Lorenz 2017). Mutants that were defective in their ability to neutralize the phagosome pH were unable to induce macrophage pyroptosis (Vylkova and Lorenz 2017). How *A. fumigatus* metabolic activity is altered within the phagosome is currently unknown, but it is likely that the ability to adapt to the phagosome environment is a trait that facilitates virulence during IA. Studies have shown that an *A. fumigatus* gene involved in melanin biosynthesis, *pksP*, was associated with the inhibition of phagolysosome fusion and acidification. *A. fumigatus* strains that have the *pksP* gene are able to inhibit phagolysosome acidification, which in turn allows the fungi to germinate and lyse the macrophage in order to escape the phagosome (Slesiona et al. 2012). Interestingly, *A. terreus*, which does not have a *pksP* ortholog, does not inhibit phagolysosome acidification and is unable to germinate within the phagosome and induce macrophage lysis (Slesiona et al. 2012). However, expression of an *A. nidulans*-derived *pskP* gene in *A. terreus* was sufficient to reverse this phenotype, allowing *A. terreus* to germinate within the phagosome and enhance macrophage cytotoxicity and virulence *in vivo* (Slesiona et al. 2012). It will be interesting to

determine if *pskP* is differentially regulated or expressed between germinating (CEA10, 02-10 and EVOL20) and non-germinating (Af293 and W72310) strains, and if this is a mechanism that contributes to the enhanced virulence in the former strains. Another study that supports a role for melanin in virulence of *A. fumigatus* utilized a wax moth model (Jackson et al. 2009). In this study authors performed random insertional mutagenesis in two strains of *A. fumigatus*, B5233 and Af293, to obtain different conidial color mutants (Jackson et al. 2009). Wax moth larvae were then infected with the conidial color mutants and it was found that all color mutants had enhanced virulence compared to the parental strain (Jackson et al. 2009). Although in this study there is no observed difference in germination rate between color mutants and the parental wild type strains, the germination assays were performed in nutrient rich YPD media (Jackson et al. 2009). We have shown that nutrient rich media is sufficient to facilitate germination of *A. fumigatus* strains that are unable to germinate within nutrient-poor environments (Appendix B, Figure A.2; Chapter 4). This warrants further exploration of melanin mutants to determine if germination rate is changed within a nutrient-limited environment such as the lung, and whether this contributes to overall virulence.

There is a possibility that in addition to nutrient utilization contributing to fungal germination *in vivo*, something in the pulmonary environment is actively inhibiting growth of some strains. Growth of the dimorphic fungal pathogen *Blastomyces dermatitidis* was inhibited by serum albumin through the binding of a fungal growth factor (Giles and Czuprynski 2003). *A. fumigatus* that lacks the RodA protein, which is a component of the cell wall that contributes to hydrophobicity, results in decreased

binding of conidia to albumin (Thau et al. 1994). Preliminary results have shown that albumin was sufficient to inhibit Af293 germination *in vitro*, but not CEA10 germination (Appendix B, Figure A.4). Whether Af293 conidia express more RodA protein compared to CEA10 is unknown, but this could explain the differential inhibition of germination observed *in vitro* in the presence of serum albumin. Although this does not explain the germination difference observed *in vitro* in minimal media, the contribution of host factors that may contribute to inhibition or facilitation of *A. fumigatus* germination *in vivo* remain to be defined.

We have shown that fungal germination is associated with enhanced pulmonary damage, alarmin release and virulence, but the fungal factor that is contributing to this damage remains unknown. *A. fumigatus* can produce a number of secreted molecules needed in their natural environment to kill competing or predatory microbes or to break down surrounding substrate for nutrient utilization (Dagenais and Keller 2009; Brakhage 2013; Hillmann et al. 2015). These secondary metabolites and proteases could possibly lead to tissue damage if secreted within a host and we hypothesized these molecules could contribute to the strain-dependent virulence observed in our model. We utilized fungal strains deficient in the secondary metabolites gliotoxin and fumagillin, Δ*gliP* and Δ*fumR* respectively, to test this hypothesis. Contrary to what we expected, we found an increase in the alarmin IL-1α in the lungs of mice challenged with CEA10 conidia deficient in gliotoxin or fumagillin, as well as no difference in LDH compared to wild type conidia (Appendix C, Figure A.5). Furthermore, in the absence of gliotoxin there was increased fungal germination in the lungs at 48 hpi (Appendix C, Figure A.6). The

reasons for this counterintuitive result is unknown, however it is possible that in the absence of one secondary metabolite, other secondary metabolite profiles are changed. One study found that gliotoxin could regulate the production of other secondary metabolites under specific laboratory conditions (Bok et al. 2006). In this study, gliotoxin did not regulate production of other secondary metabolites within the neutropenic lung, however it is possible that in the immune competent pulmonary environment, this could be different (Bok et al. 2006). LaeA is a transcription factor in *A. fumigatus* that regulates the production of several secondary metabolites and utilizing this strain could aid in determining the overall role of secondary metabolites in our immune competent model of IA. In neutropenic mice, loss of *laeA* resulted in decreased virulence associated with enhanced macrophage phagocytosis and neutrophil survival (Bok et al. 2005).

Fungal protease production could also lead to pulmonary damage and alarmin release. We have challenged mice with a Δ*prtT A. fumigatus* strain, which was shown to have reduced proteolytic activity, in order to test this hypothesis. In the absence of *prtT*, we observed a small, but statistically significant decrease in IL-1α production within the lungs at 12 hpi (Appendix C, Figure A.7). Importantly, germination in the airways was similar to the wild-type fungal strain (Appendix C, Figure A.7). More studies are needed to confirm a role of proteases in alarmin release and the development of IA in our model. Although we observed a significant decrease in IL-1α production in the absence of *prtT*, the difference compared to wild type conidial challenge was very small and does not account for all of the IL-1α production. PrtT only controls the production of 6 secreted

proteases in *A. fumigatus*, so it is possible that the other 105 proteases encoded within the genome are compensating for the loss of PrtT-controlled proteases or that the other proteases play a more dominant role in our model (Sharon et al. 2009). Furthermore, we only looked for phenotypic differences at 12 hpi, and a more thorough investigation of the kinetics following challenge with *prtT*-deficient conidia are needed to confirm our preliminary data.

In *C. albicans*, the *upc2* gene is involved in IL-1ß release and pyroptosis of bone marrow-derived macrophages (BMDM) (Wellington et al. 2013), but a role for this gene in IL-1α release has not been studied. *A. fumigatus srbA* is the ortholog of *C. albicans upc2*, so in order to test the hypothesis that SrbA is contributing to IL-1α production following *A. fumigatus* challenge, mice were inoculated with *srbA*-null conidia. At 24 hpi, there was a significant defect in IL-1α production in the absence of *srbA*-mediated signaling *(*Appendix C, Figure A.8). SrbA is a fungal transcription factor that controls expression of genes involved in a number of processes including hypoxia adaptation, ergosterol biosynthesis, iron metabolism, and carbon and nitrogen utilization (Willger et al. 2008; Blatzer et al. 2011; Chung et al. 2014). Interestingly, *srbA*-null and wild type *A. fumigatus* germinate to a similar extent in the pulmonary environment (Appendix C, Figure A.8), suggesting that *srbA*-dependent nutrient utilization and metabolism is unlikely to contribute to the early decrease in IL-1α production we observed. Ergosterol is structurally similar to mammalian cholesterol, which during atherosclerosis has been associated with IL-1 release (Chu et al. 2009; Duewell et al. 2010; Freigang et al. 2011; Freigang et al. 2013). Furthermore, in *C. albicans* the ergosterol biosynthesis pathway

contributes to macrophage pyroptosis (O'Meara et al. 2016). Thus we hypothesize that ergosterol may contribute to the production of IL-1α after challenge with *A. fumigatus* conidia. Supporting this, ergosterol crystals are sufficient to drive IL-1α release from BMDMs (Appendix C, Figure A.9), but further experiments are needed to confirm a specific role for ergosterol in driving IL-1α release within the host during IA. Fungal genetic mutants deficient in genes that are necessary for the terminal steps of ergosterol biosynthesis, such as *erg5* and *erg4a/erg4b*, can be utilized to determine the role that ergosterol plays in initiating an innate immune response after *A. fumigatus* exposure (Dhingra and Cramer 2017).

Other factors which we have not yet examined that could potentially contribute to enhanced pulmonary damage, alarmin release and virulence are fungal cell wall components. The cell wall of *A. fumigatus* provides structure and protection and is composed of a number of interconnected polysaccharides including ß-glucans, mannans, chitin and galactosaminogalactan (GAG) (Gastebois et al. 2009). GAG is only expressed when conidia begin to germinate and was shown to be required for epithelial cell injury and full virulence in two murine models of IA (Gravelat et al. 2013). Other studies have shown that GAG is anti-inflammatory, limiting neutrophil recruitment and inducing IL-1ra within the pulmonary environment (Fontaine et al. 2011; Gresnigt et al. 2014). Whether GAG induces other IL-1 cytokines *in vivo* is unknown and further studies are needed to determine whether GAG is a fungal factor that contributes to virulence or acts in an anti-inflammatory manner in our model of IA. Although a direct quantification comparing GAG levels in CEA10 and Af293 has not been made, scanning electron

micrographs of A293 and CEA10 show that Af293 possesses GAG (Gravelat et al. 2013), while this GAG-like structure is missing from CEA10 (Thammahong et al. 2017). Another cell wall component which we have not yet explored, ß-glucan, may contribute to the IL-1 response during IA. It was shown that blockade of Dectin-1, the receptor that recognizes ß-glucan, significantly reduced the amount of IL-1α and IL-1ß released by alveolar macrophages (Steele et al. 2005). Chitin is a fungal cell wall component shown to have both pro- and anti-inflammatory properties depending on the presence of other PAMPs and immunoglobulins (Becker et al. 2016). Specifically it was shown that IgG-opsonized chitin resulted in IL-1ra induction through a FcγR-Syk-PI3K-dependent manner, while IgG-opsonized chitin in the presence of Syk-independent PRR ligands induced high levels of IL-1ß from human peripheral blood mononuclear cells (Becker et al. 2016). Given the difference in morphologies and likely PAMP exposure of different fungal strains *in vivo*, it will be interesting to determine the strain-dependent contribution of chitin to the IL-1 response. Future work elucidating the contribution of fungal cell wall components to the IL-1 and damage response may reveal novel antifungal targets for therapeutic intervention.

One factor that this dissertation does not address, however future experiments should aim to determine, is how IL-1 contributes to disease establishment and progression in immune compromised populations. We hypothesize that similar to *C. albicans* infection in neutropenic mice, IL-1α and IL-1ß would be protective to the host by enhancing antifungal activity of effector cells that are present in the neutropenic host following *A. fumigatus* challenge (Van Wout et al. 1988). Conversely, in the non-

neutropenic host such as corticosteroid treated mice or CGD mice in which IA is characterized by high levels of immune-mediated pathology, excessive IL-1α and/or IL-1ß could be detrimental and exacerbate disease. In the murine model of CGD, it was shown that blockade of IL-1RI signaling with the receptor antagonist, anakinra, dampened inflammation and protected mice from invasive aspergillosis (de Luca et al. 2014). In cystic fibrosis (CF) patients, IA is rare, but disease is characterized by dysregulated inflammation and high levels of tissue damage. A recent study demonstrated that administration of the IL-1ra in CFTR-deficient mice could dampen inflammation due to aberrant NLRP3-inflammasome activity, resulting in a reduction in neutrophil recruitment to the lungs as well as significantly decreasing fungal burden (Iannitti et al. 2016). Moreover, it was shown that blockade of IL-1 signaling with the IL-1ra in corticosteroid-treated mice led to a decrease in hypoxia and pro-inflammatory mediators in the lungs, corresponding with a modest increase in survival (Gresnigt et al. 2016). In this specific study, while the increase in survival of IL-1ra treated mice was significant, the increased survival was very small compared to non-treated mice. We hypothesize that specifically targeting IL-1α signaling in this model may be more beneficial than targeting IL-1RI signaling. Our previous work has shown that IL-1ß is important in the optimal activation of leukocytes exposed to *A. fumigatus*, while IL-1α is critical for neutrophil recruitment (Chapter 2). In the corticosteroid-treated lung, enhancing antifungal activity of leukocytes through IL-1ß signaling while dampening neutrophilic inflammation through blockade of IL-1α could be of therapeutic value, however more studies are needed to explore this hypothesis. Although immune-mediated

factors are contributing to disease progression, there are also fungal factors that need to be addressed.

One of the fungal factors that is associated with establishment of IA is the ability to adapt to hypoxic environments (Willger et al. 2008). Hypoxia is a significant environmental pressure on *A. fumigatus* in the corticosteroid model of IA (Kowalski et al. 2016), which is the model that was shown to exhibit the greatest level of tissue hypoxia during infection (Grahl et al. 2011). CEA10 has a high hypoxic fitness value and accordingly is highly virulent in the corticosteroid model of IA; in contrast, 02-10 has a low hypoxic fitness value and displays low virulence in the corticosteroid murine model of IA (Kowalski et al. 2016). This is contrary to our results which demonstrate that CEA10 and 02-10 are both highly virulent and able to rapidly germinate within the airways of immune competent mice (Chapter 4). We hypothesize that early growth within the pulmonary environment and hypoxic fitness are separable traits that both contribute to IA disease establishment and progression. We hypothesize that hypoxia is a contributing factor at later time points following *A. fumigatus* challenge as it likely takes a significant amount of time and inflammation for the hypoxic microenvironment to develop within the lungs (Grahl et al. 2011). In contrast, fungal germination is likely determined immediately after instillation of the conidia into the airways based on the nutrients present, which is supported by our *in vitro* lung homogenate germination assay and *in vivo* airway germination data (Chapter 4). Additionally, the host immune status dictates host-derived factors that influence the establishment and progression of IA, such as hypoxia as was shown by Shepardson et al. (Shepardson et al. 2013). Hypoxia

enhances antifungal activity of leukocytes against *A. fumigatus*, but also enhances the exposure of ß-glucan on the fungal cell wall suggesting that a combination of both host-derived factors and fungal factors potentially contribute to the outcome of disease (Shepardson et al. 2013). Preliminary data show that two such factors are host-derived IL-1α and fungal hypoxic fitness (Appendix D, Figure A.10). Specifically, when corticosteroid-treated *Il1a*-deficient mice were challenged with CEA10, survival was prolonged for ~24-48 h compared to C57BL/6 mice, but ultimately survival rates were similar between both groups (Appendix D, Figure A.10). We hypothesize the delayed mortality in the absence of IL-1α was due to dampening of immune-mediated pathology, which needs to be confirmed in future studies by histological and immunological analysis. Ultimately, CEA10 is able to thrive within hypoxic environments (Kowalski et al. 2016), so although the immune response was dampened, CEA10 will be able to adapt to the hypoxic environment and continue to grow within the lung, causing the mice to succumb to infection. However, when corticosteroid-treated *Il1a*-deficient mice were challenged with 02-10, a strain which is unable to thrive in hypoxia, mortality rates were significantly lower compared to C57BL/6 mice (Appendix D, Figure A.10). This suggests that concomitantly dampening immune-mediated pathology and targeting fungal adaptation to the pulmonary environment are beneficial to host resistance to the development of IA. In this preliminary study, we only measured survival and more studies to test our hypotheses are necessary to determine the specific role of host-derived and fungal factors that influence the development and progression of IA.

In a murine model of arthritis, IL-1 and LTB_4 signaling were linked in a lipid-cytokine-chemokine cascade that was shown to orchestrate neutrophil recruitment (Chou et al. 2010). In this study, LTB_4 recruits neutrophils that express IL-1 and chemokines into the joint, which then act to further amplify neutrophil recruitment and sustain inflammation. Although in this dissertation we have demonstrated that both IL-1 and LTB_4 are critical components contributing to neutrophil recruitment and control of fungal germination following *A. fumigatus* challenge (Chapters 2, 4, and 5), it remains unknown whether these signaling molecules work dependently or independently of each other. In *Il1r1*-deficient mice, LTB_4 is produced at similar levels compared to C57BL/6 mice at 24 hpi (Appendix E, Figure A.11). Similarly, in *Ltb4r1*-deficient mice, IL-1α is produced at similar levels and IL-1ß at higher levels than C57BL/6 mice (Appendix E, Figure A.11). This suggests that these pathways act independently of each other, however a more complete analysis of the kinetics of these inflammatory mediators is needed to confirm this. Future directions should be aimed at whether these two pathways are interconnected and if so, which mediator is induced first. Furthermore, how exactly LTB_4 signaling contributes to disease in the non-neutropenic population is of interest given the negative impact that corticosteroids have on eicosanoid production (Sebaldt et al. 1990). Interestingly, in a model of CGD in which mice were injected intradermally with heat-killed *A. fumigatus* hyphae, robust neutrophilic inflammation that eventually became granulomatous developed and was associated with elevated levels of LTB_4 (Petersen et al. 2002). Whether this neutrophilic inflammation was dependent on LTB_4 signaling was

not elucidated, and studies in the lung to extend this finding to IA have not been performed.

In a preliminary study we have found that the leukotriene pathway in host resistance to IA is also a fungal strain-dependent phenomenon in which resistance to CEA10 is highly dependent on leukotriene signaling and resistance to Af293 is leukotriene-independent (Appendix E, Figure A.12). Interestingly, the CEA10 genome encodes a phospholipase that is predicted to be structurally similar to the *Pseudomonas aeruginosa* ExoU, which is absent in the Af293 genome (Cramer lab, unpublished data). This could provide an explanation to both the IL-1α and LTB_4-dependency of CEA10 given that ExoU in *P. aeruginosa* drives an IL-1α dependent immune response and also contributes to eicosanoid production *in vitro* and *in vivo* (Phillips et al. 2003; Saliba et al. 2005; Al Moussawi and Kazmierczak 2014). Phospholipase activity has also been associated with phagosome lysis and escape by the bacterial pathogen *Rickettsia prowazekii* (Ojcius et al. 1995).

Hypoxia has been shown to contribute to leukotriene formation by enhancing the expression of 5-lipoxygenase activating protein (FLAP), an enzyme required for 5-lipoxygenase activity and subsequent leukotriene formation (Gonsalves and Kalra 2010). In this model, hypoxia activated HIF-1α and enhanced binding of HIF-1α to hypoxia-response elements in the FLAP promoter (Gonsalves and Kalra 2010). HIF-1α was shown to be critical in host protection against pulmonary *A. fumigatus* challenge through the enhancement of both CXCL1 signaling and neutrophil survival in the lung (Shepardson et al. 2014). To determine if LTB_4 production *in vivo* following *A. fumigatus*

challenge is dependent on HIF-1α, Hif1α$^{fl/fl}$ x LysM-cre mice (Hif1$^{-/-}$) and wild-type (WT) mice were challenged with CEA10 or Af293 (Appendix E, Figure A.13). Hif1$^{-/-}$ mice challenged with CEA10 had reduced levels of LTB_4 in the BALF compared to their WT counterparts, while Hif1$^{-/-}$ and WT mice challenged with Af293 had equivalent levels of LTB_4 in the BALF (Appendix E, Figure A.13).. How HIF-1α is activated after *A. fumigatus* exposure and how it is enhancing LTB_4 production are unknown. Corticosteroid treatment was shown to inhibit HIF-1α translocation to the nucleus, where it binds to hypoxia response elements of target hypoxia response genes to initiate signaling (Shepardson et al. 2014). Due to the HIF-1α dependent production of LTB_4 (Appendix E, Figure A.13), it is possible that this is a pathway which contributes to enhanced susceptibility of corticosteroid treated patients to IA. Many questions remain to be explored in the role of the leukotriene pathway in host resistance to IA.

Overall, there is a fine balance in maintaining immune resistance to *A. fumigatus*. The detrimental outcome of a shift in immune balance in either direction is highlighted by the disease spectrum that *A. fumigatus* occupies. Understanding both the fungal factors that allow *A. fumigatus* to adapt to different host environments and the immune pathways that are needed to maintain resistance to *A. fumigatus*-induced disease are critical future directions for this dissertation. In conclusion, this dissertation has defined novel and key roles for IL-1RI and BLT1 signaling in host resistance to IA (Figure 6.1). IL-1α is critical for neutrophil recruitment against highly virulent fungal strains and the ability of *A. fumigatus* strains to rapidly adapt and germinate within the airways was associated with enhanced virulence in the immune competent model of IA. Further

investigation into the unique fungal strain factors and host-derived factors that are associated with rapid germination within the airways are needed to develop novel antifungal and immune-modulatory therapeutics.

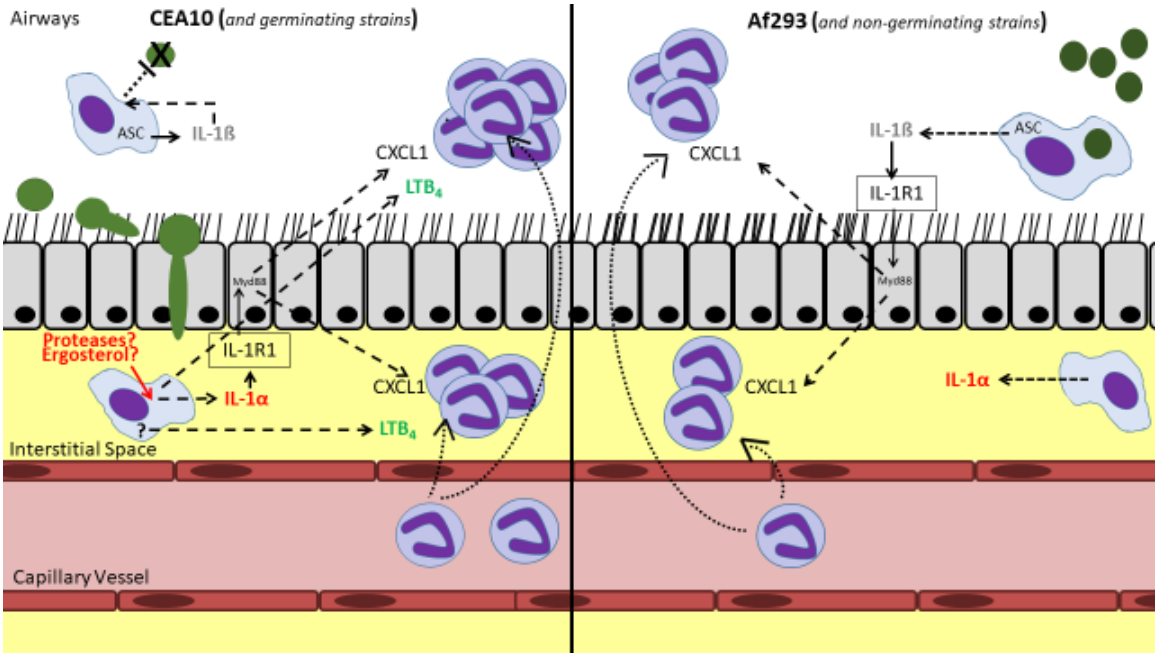


Figure 6.1. Summary model of dissertation research. Highly virulent strains of *A. fumigatus* that are able to rapidly germinate within the pulmonary environment induce IL-1α- and LTB_4-dependent immunity, while non-germinating strains induce IL-1ß dependent immunity. *A. fumigatus* strains that are deficient in PrtT-mediated signaling and SrbA-mediated signaling induce lower levels of IL-1α in the lungs. We hypothesize that when *A. fumigatus* germinates, proteases and ergosterol drive macrophage cell death and IL-1α release leading to enhanced pulmonary damage and virulence. Neutrophil recruitment and control of fungal germination is dependent on IL-1α. In response to the CEA10 strain of *A. fumigatus*, IL-1ß is released and required for optimal antifungal activity of macrophages. Alternatively, *A. fumigatus* strains that are unable to germinate and instead develop into swollen conidia, expose PAMPs to the immune system and induce inflammasome-dependent neutrophil recruitment and control of fungal germination. The role of IL-1α during IA caused by "non-germinating" strains remains undefined. Furthermore, CEA10 induces an LTB4-dependent immune response, while Af293 does not; however, whether this extends to all "germinating" and "non-germinating" strains remains to be determined. The exact cell type(s) that requires 5-LO activity for LTB_4 production and release is unknown, but these cells are known to be of hematopoietic origin.

APPENDICES

APPENDIX A

AF293 NEUTROPHIL RECRUITMENT OCCURS INDEPENDENTLY OF IL-18 SIGNALING

The studies in Chapter 4 demonstrate that IL-1α dependent neutrophil recruitment is fungal strain-specific. Fungal strains that were able to rapidly germinate within the airways (CEA10, 02-10 and EVOL20) induced high levels of pulmonary damage, enhancing the release of alarmins such as IL-1α, which in turn led to rapid and robust recruitment of neutrophils. Conversely, strains that were unable to rapidly germinate in the airways (Af293 and W72310) induced lower levels of pulmonary damage and neutrophil recruitment was independent of IL-1α signaling. Although Af293 neutrophil recruitment was IL-1α independent, it was still dependent on IL-1RI signaling as shown in Figure 4.2A-B. Furthermore, there was a defect in neutrophil recruitment following Af293 challenge in the absence of ASC (Figure 4.2B). Although this suggests a role for IL-1ß in Af293-induced neutrophil recruitment, ASC is a central adapter protein needed for maturation of both IL-1β and IL-18 (Latz et al. 2013). Therefore, we wanted to determine if *Il18*-deficient mice had a defect in neutrophil recruitment (Figure A.1). To do this, C57BL/6 mice or *Il18*-deficient mice were challenged with $4x10^7$ conidia of either CEA10 or Af293 intratracheally (IT). With both strains, *Il18*-deficient mice had significantly higher leukocyte recruitment to the airways when compared to C57BL/6 mice (Figure A.1). The reason for this enhanced cell recruitment is currently unknown, however it suggests that the ASC-dependent neutrophil recruitment observed after challenge with Af293 is dependent on IL-1ß rather than IL-18.

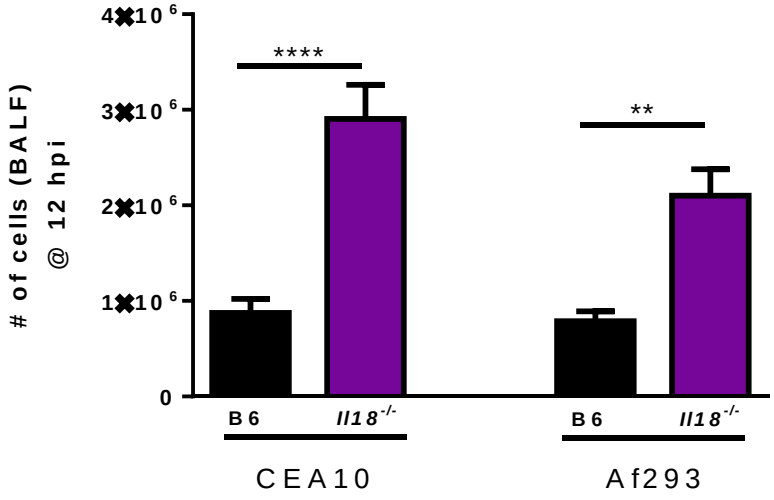


Figure A.1. *Il18*-deficient mice have enhanced leukocyte recruitment to the airways following *A. fumigatus* challenge. C57BL/6 or *Il18*-deficient mice were infected with $4x10^7$ conidia of CEA10 or Af293 IT. At 12 hpi, mice were euthanized and BALF collected for analysis of leukocyte recruitment to the airways. Total cell counts were determined through hemacytometer counts. Data are representative of at least 2 independent experiments consisting of 5-8 mice per group. Bar graphs show the group mean ± one SEM. Statistically significant differences were determined using a two-way ANOVA with Tukey's post-test (****$p < 0.0001$, **$p < 0.01$).

APPENDIX B

GROTWH OF *ASPERGILLUS FUMIGATUS IN VITRO*

In Chapter 4, we demonstrated that there is significant heterogeneity in virulence among *A. fumigatus* strains and isolates, which induce specific immune profiles in mice. Fungal strains that were able to rapidly germinate within the airways (CEA10, 02-10 and EVOL20) induced high levels of pulmonary damage and were more virulent than strains that were unable to rapidly germinate within the airways (Af293 and W72310), which induced lower levels of pulmonary damage. Why some strains are able to germinate to a greater extent than others within the pulmonary environment is unknown, but is an important future direction of this work. One possibility is that some strains are able to sense and utilize nutrients more efficiently than other strains. In order to germinate within the lungs, *A. fumigatus* must be able to sense and utilize available carbon and nitrogen sources. Figure A.2 supports the hypothesis that the heterogeneity in fungal strain germination is at least partially a nutrient-dependent occurrence. Af293, while able to germinate to a similar extent as CEA10 in liquid glucose minimal media (LGMM) containing 0.05% yeast extract (YE), germinated to a lesser extent when incubated in LGMM without YE (Figure A.2). Conversely, CEA10 was able to germinate to a similar extent whether YE was present or absent (Figure A.2).

When conidia enter the respiratory tract, they are likely deposited in the airway surface liquid (ASL) found covering the epithelial lining in the airways. It is possible that this is where initial germination of *A. fumigatus* occurs as the ASL consists of mucin glycoproteins (containing a large number of oligosaccharides), lipids, water, inorganic salts and proteins (Senel et al. 2015). Interestingly, a recent study showed that *Pseudomonas aeruginosa* can utilize mucin as a carbon source (Flynn et al. 2017). Thus,

we wanted to test if mucin was sufficient to facilitate *A. fumigatus* growth *in vitro* (Figure A.3). To test this, liquid minimal media was formulated with 1% mucin instead of 1% glucose, and a germination assay was performed. Mucin was sufficient to support robust germination of CEA10, 02-10 and EVOL20, but not Af293 or W72310. Whether mucin in the ASL *in vivo* is contributing to the germination of highly virulent *A. fumigatus* strains remains to be determined.

It is possible that in addition to the contribution of nutrient sensing and utilization to fungal germination, something in the pulmonary environment is actively inhibiting growth of some strains. Growth of the dimorphic fungal pathogen *Blastomyces dermatitidis* was inhibited by serum albumin through the binding of a fungal growth factor (Giles and Czuprynski 2003). Interestingly, preliminary data show that albumin is sufficient to inhibit Af293 germination *in vitro*, but has no impact on CEA10 germination (Figure A.4). *A. fumigatus* lacking the hydrophobic cell wall component, RodA, showed a decreased ability to bind to albumin (Thau et al. 1994). Thus, it is possible that Af293 has more RodA than CEA10, which could potentially enhance binding to serum albumin, but further testing is needed to dissect this. Albumin-mediated inhibition of Af293 would not explain the germination differences observed *in vitro* in minimal media, but it is possible that *in vivo*, host factors contribute to the inhibition of fungal germination.

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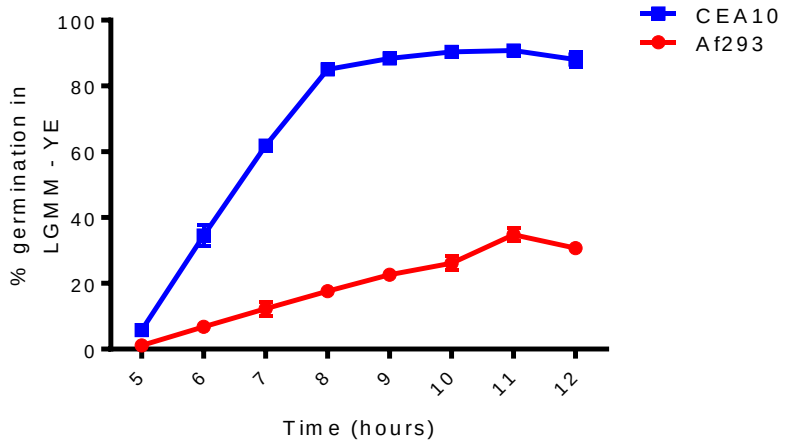

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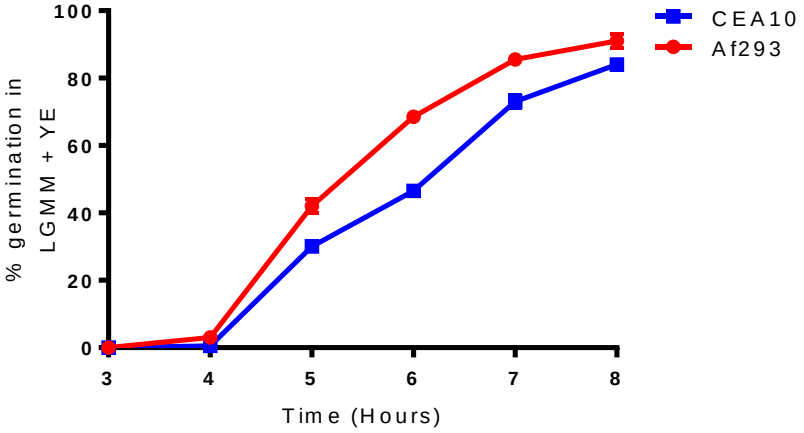

Figure A.2. Af293 germinates to a lesser extent in minimal media than CEA10. (A) Liquid glucose minimal media (LGMM) without yeast extract (YE) or (B) LGMM containing 0.05% YE was inoculated with $2x10^7$ conidia of either Af293 (red) or CEA10 (blue). Every hour, germination was quantified by microscopically counting the number of conidia and germlings. Data are represented as the percent of fungal matter that was germinated. Data are representative of at least 3 independent experiments consisting of 3 biological replicates per group. Each symbol represents the group mean ± one SEM.

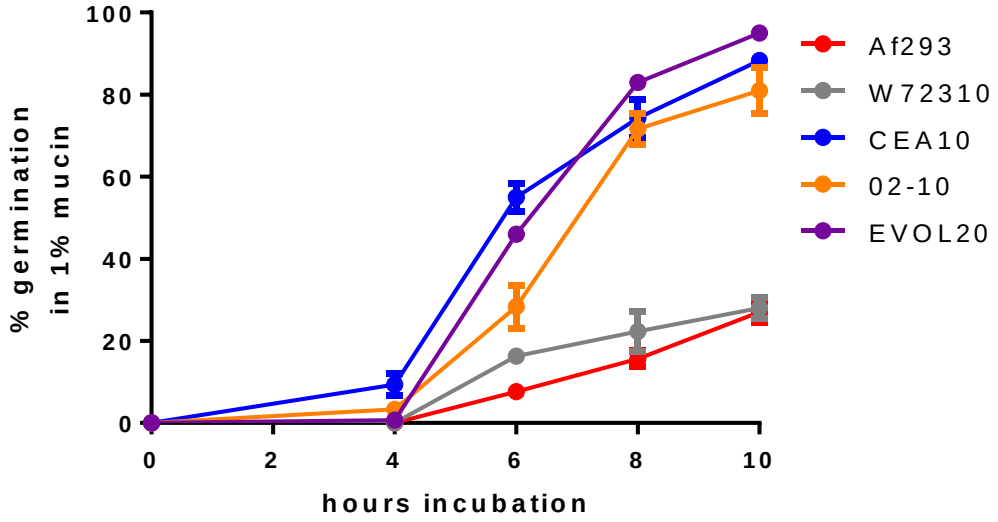

Figure A.3. *A. fumigatus* strains that rapidly germinate within the lung (CEA10, 02-10 and EVOL20) are able to germinate with mucin as the sole carbon source. LGMM without YE was made with 1% mucin, rather than 1% glucose and inoculated with $2x10^7$ conidia of either Af293, W72310, CEA10, 02-10, or EVOL20. Every two hours, germination was quantified by microscopically counting the number of conidia and germlings. Data are represented as the percent of fungal matter that was germinated. Data are representative of at least 2 independent experiments consisting of 3 biological replicates per group. Each symbol represents the group mean ± one SEM.

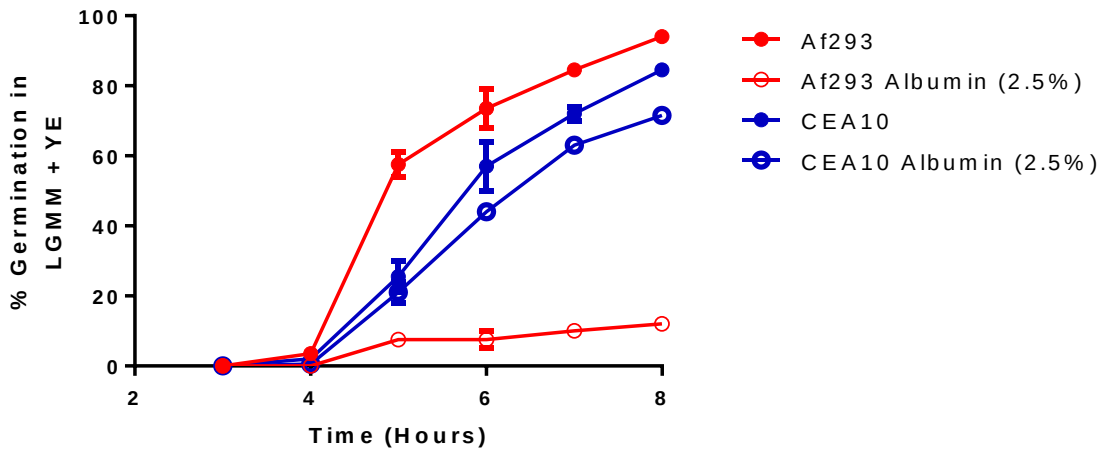

Figure A.4. Serum albumin inhibits Af293 germination in nutrient rich medium. LGMM containing 0.05% YE with (open circles) or without (solid circles) 2.5% Albumin was inoculated with $2x10^7$ conidia of either Af293 (red) or CEA10 (blue). Every hour, germination was quantified by microscopically counting the number of conidia and germlings. Data are represented as the percent of fungal matter that was germinated. Data are representative of at least 2 independent experiments consisting of 3 biological replicates per group. Each symbol represents the group mean ± one SEM.

APPENDIX C

FACTORS CONTRIBUTING TO IL-1A PRODUCTION

We have shown that fungal germination is associated with enhanced pulmonary damage and virulence, but the fungal factor that is contributing to this damage and alarmin release remains unknown. *A. fumigatus* can produce a number of secreted molecules such as secondary metabolites or proteases that we hypothesize could induce pulmonary damage and lead to cellular release of alarmins, such as IL-1α (Dagenais and Keller 2009). Secondary metabolites produced by fungi serve to kill competing microbes or predatory microbes (Brakhage 2013; Hillmann et al. 2015). Gliotoxin and fumagillin are both thought to contribute to virulence of *A. fumigatus* during IA (Dagenais and Keller 2009; Fallon et al. 2011). Gliotoxin-deficient *A. fumigatus* strains have been shown to be less virulent in the corticosteroid model of IA, but not in the neutropenic model (Bok et al. 2006; Cramer et al. 2006; Sugui et al. 2007; Kupfahl et al. 2008; Spikes et al. 2008). Fumagillin was shown to be immunosuppressive in a Galleria model by suppressing the killing ability of immune cells (Fallon et al. 2011). The contribution of these secondary metabolites to the IL-1 response and virulence in an immune competent model has not been determined. To test whether these fungal factors could contribute to damage and alarmin release, we utilized fungal strains deficient in the secondary metabolites gliotoxin and fumagillin, Δ*gliP* and Δ*fumR* respectively. C57BL/6 mice were infected with $4x10^7$ conidia of either Δ*gliP*, Δ*fumR,* or their wild-type counterparts CEA10 and KU80, respectively. The Δ*fumR* mutant was made in the KU80 deletion strain, which acts as a tool for high-throughput gene replacement in fungal genetics and is derived from the CEA10 background (da Silva Ferreira et al. 2006). KU80 is a strain that is deficient in non-homologous end-joining machinery, thus increasing homologous

recombination and enhancing the rate of genetic transformation (da Silva Ferreira et al. 2006). At 10-12 hpi, mice were euthanized, lungs homogenized to measure IL-1α, and BALF collected to measure LDH release. Unexpectedly, in the absence of both gliotoxin and fumagillin, IL-1α production in the lungs was enhanced, while no difference in LDH was observed (Figure A.5A-B). Moreover, in the absence of gliotoxin, there is a small but significant increase in fungal germination at 48 hpi (Figure A.6). Further exploration into the mechanism behind this enhanced IL-1α production and germination in the absence of secondary metabolites is needed.

Proteases are secreted by fungi for liberation of nutrients from a given substrate, and within the host environment may lead to tissue degradation, alarmin release and damage (Dagenais and Keller 2009). A recent study showed extracellular proteases from *P. aeruginosa* are critical in acquisition of nutrients from mucin degradation (Flynn et al. 2017). Our data suggest that some *A. fumigatus* strains are able to utilize mucin as a carbon source (Appendix B). Due to functional redundancy of the 111 proteases encoded within the *A. fumigatus* genome, testing the role of specific proteases in development of IA has been technically difficult (Sharon et al. 2009). A transcription factor, PrtT, was shown to control multiple *A. fumigatus* secreted proteases and a *prtT*-deficient strain had less proteolytic activity compared to the wild-type strain (Sharon et al. 2009). To determine if proteases contribute to IL-1α production and pulmonary damage, C57BL/6 mice were infected with $4x10^7$ conidia of Δ*prtT* or its parental strain, KU80. At 12 hpi, mice were euthanized and lungs homogenized to measure IL-1α levels and BALF collected to analyze LDH levels. There was a very small, but significant defect in IL-1α

production in the lungs in the absence of *prtT*, however LDH levels remained unchanged (Figure A.7A). Furthermore, early germination in the airways was equivalent between Δ*prtT* and KU80 (Figure A.7B). PrtT was shown to control expression of only 6 of the 111 proteases encoded by the *A. fumigatus* genome, which could account for such a small difference in IL-1α production between Δ*prtT* and KU80. We have only looked at 12 hpi for phenotypic differences in mice infected with Δ*prtT*, thus further studies are needed to determine the contribution of PrtT-controlled proteases to overall disease outcome.

In *C. albicans*, Upc2 contributes to pyroptosis and IL-1ß release from BMDMs, however contribution to IL-1α release has not been studied (Wellington et al. 2013). In *A. fumigatus*, the *upc2* ortholog is *srbA*. To test the hypothesis that *srbA* contributes to IL-1α production in our model, C57BL/6 mice were challenged with Δ*srbA* or CEA10 and at 24 hpi, IL-1α in the lungs was measured (Figure A.8A). There was a small, but significant defect in IL-1α release in the absence of SrbA-mediated signaling, however no difference in LDH was observed. Germination of Δ*srbA* and CEA10 strains within the airways were equivalent at 24 hpi (Figure A.8B). *A. fumigatus* SrbA is a transcription factor that controls expression of genes involved in hypoxia adaptation, iron metabolism, carbon and nitrogen utilization, and ergosterol biosynthesis (Willger et al. 2008; Blatzer et al. 2011; Chung et al. 2014). Of these pathways, ergosterol biosynthesis is of great interest to us in future studies. Ergosterol is structurally similar to mammalian cholesterol, which was shown to contribute to IL-1 release in atherosclerosis (Chu et al. 2009; Duewell et al. 2010; Freigang et al. 2011; Freigang et al. 2013). Supporting a role for ergosterol biosynthesis in IL-1α release during IA is the fact that ergosterol crystals

were sufficient to drive IL-1 release from BMDMs *in vitro* (Figure A.9). Future studies should aim to determine the role that ergosterol plays *in vivo* during IA, and whether this contributes to IL-1α release and virulence.

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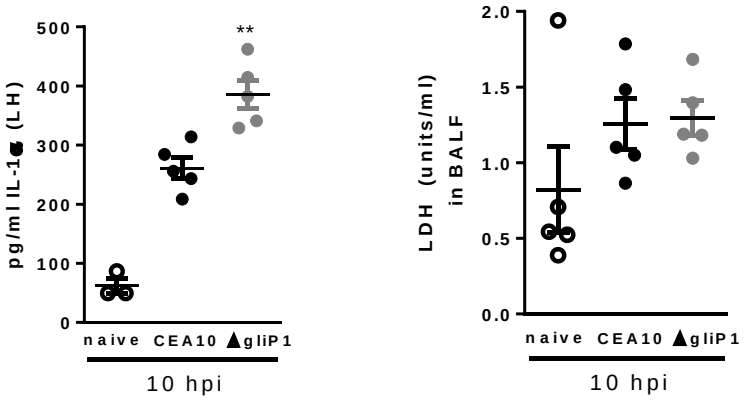

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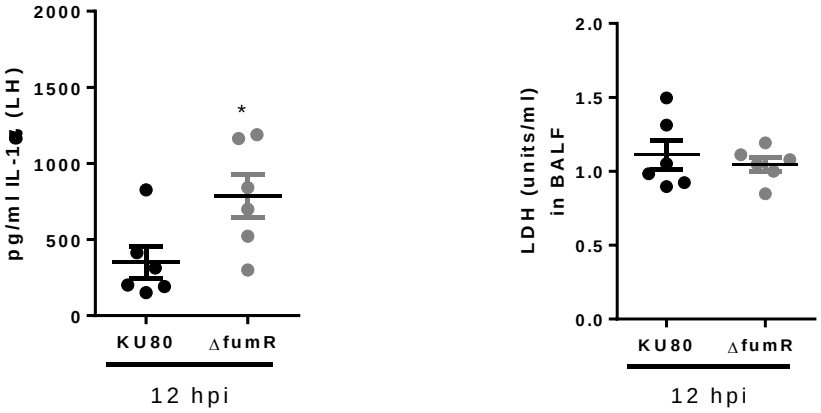


Figure A.5. Secondary metabolites gliotoxin and fumagillin do not drive IL-1α release *in vivo*. C57BL/6 mice were challenged with $4x10^7$ conidia of either (A) *ΔgliP* or CEA10, or (B) *ΔfumR* or KU80 IT. At 12 hpi mice were euthanized, BALF collected and lungs homogenized to measure LDH and IL-1α, respectively. Data are representative of 1-2 independent experiments, consisting of at least 5 mice per group. Each symbol represents an individual mouse and the line represents the group mean ± one SEM. Statistical significance was determined using a Mann-Whitney U-test (*$p < 0.05$, **$p < 0.01$).

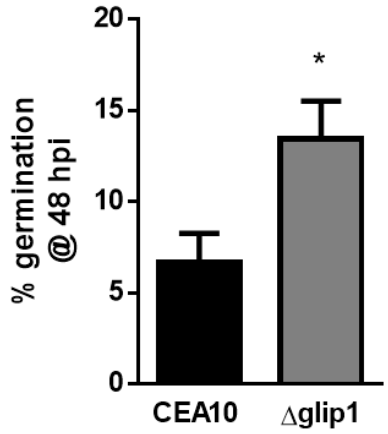


Figure A.6. Germination is enhanced *in vivo* in the absence of gliotoxin production. C57BL/6 mice were challenged with $4x10^7$ conidia of either *ΔgliP* or CEA10 IT. At 48 hpi, mice were euthanized and lungs saved for histological analysis. Formalin-fixed lungs were paraffin embedded, sectioned and stained with GMS for analysis by microscopy. *A. fumigatus* germination rates were determined by microscopically counting both the number of conidia and number of germlings in GMS-stained sections. Statistical significance in was determined using a Mann-Whitney U test (*$p < 0.05$).

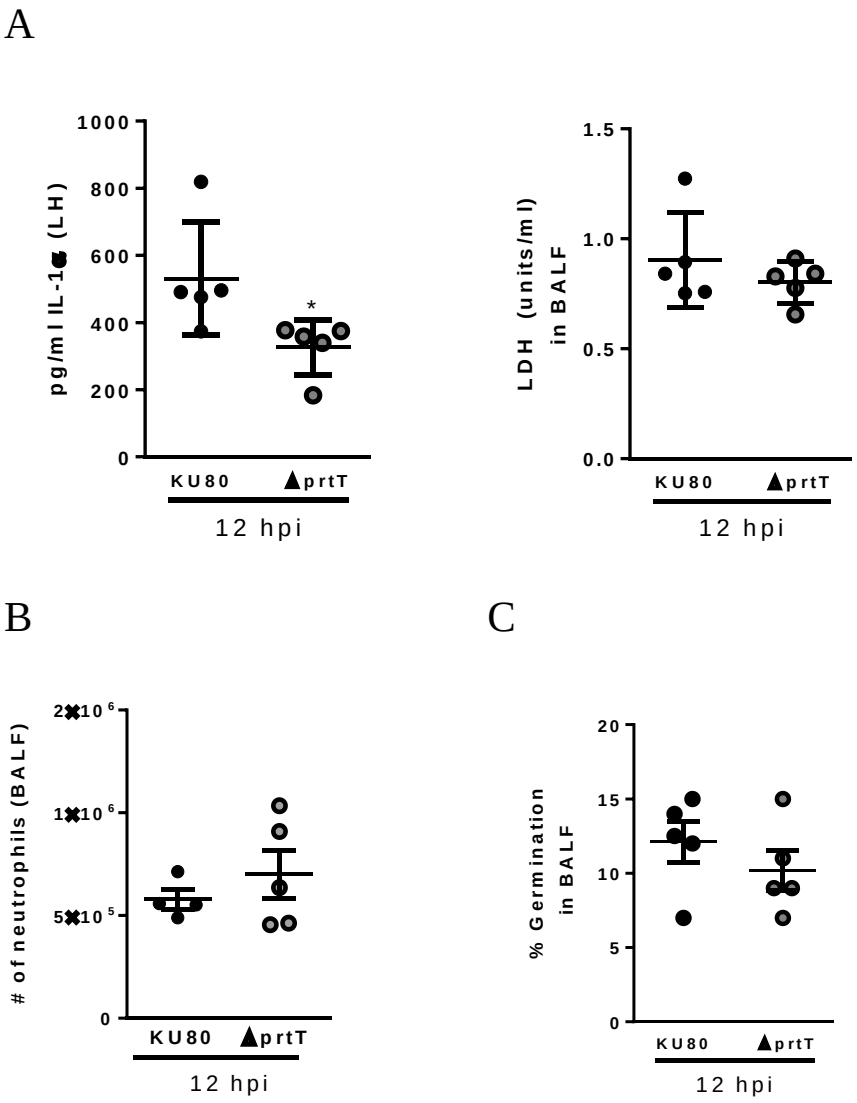


Figure A.7. *A. fumigatus prtT*-mediated signaling partially drives IL-1α release *in vivo*. C57BL/6 mice were challenged with $4x10^7$ conidia of either Δ*prtT* or parental strain, KU80 IT. At 12 hpi, mice were euthanized, BALF collected and lungs homogenized. (A) IL-1α in the LH and LDH in BALF was measured. (B/C) BALF was spun onto slides and stained with a Differential Quik Staining Kit. (B) Neutrophil recruitment to the airways was quantified and (C) fungal germination in BALF was quantified by counting conidia and germlings in the slides and represented as percent of fungal matter that was germinated. Each symbol represents an individual mouse and the line represents the group mean ± one SEM. Data is representative of 2 independent experiments consisting of 5-8 mice per group. Statistical significance was determined using a Mann-Whitney U test (*$p < 0.05$).

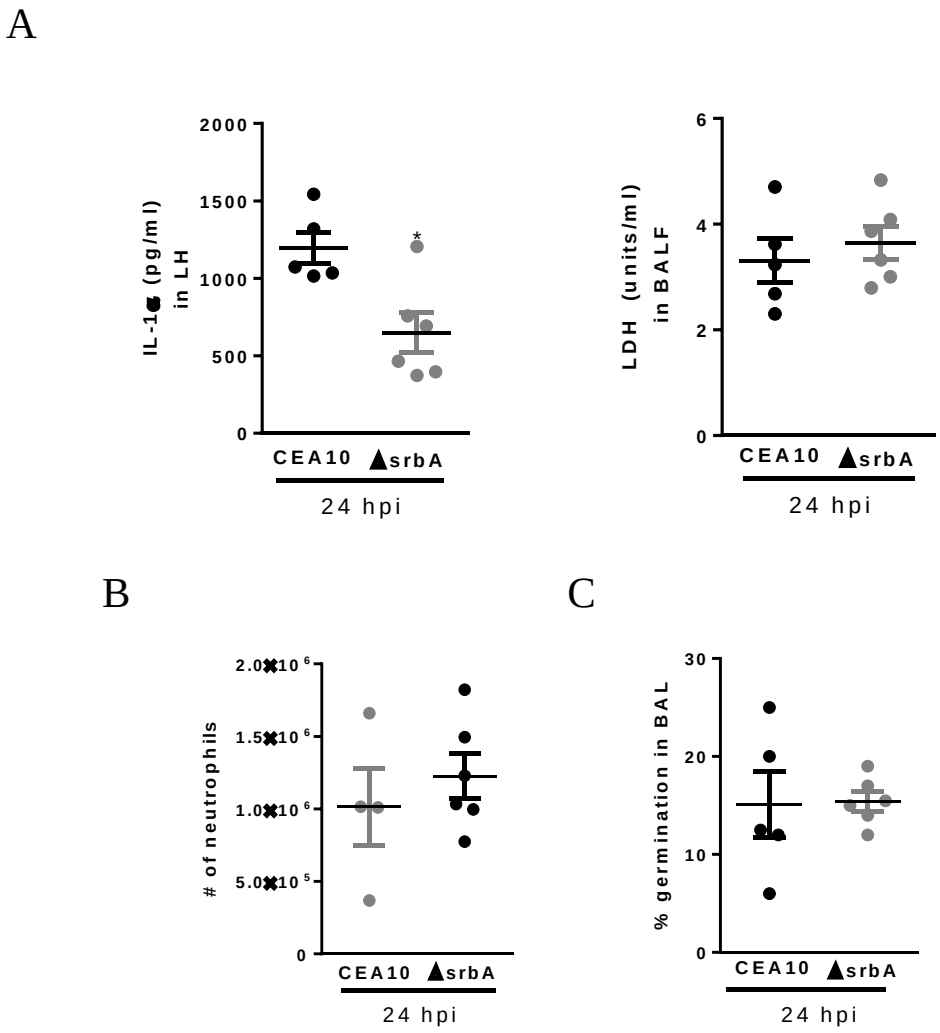


Figure A.8. *A. fumigatus srbA*-mediated signaling partially drives IL-1α release *in vivo*. C57BL/6 mice were challenged with $4x10^7$ conidia of either Δ*srbA* or parental strain, CEA10 IT. At 24 hpi, mice were euthanized, BALF collected and lungs homogenized. (A) IL-1α in the LH and LDH in BALF was measured. (B/C) BALF was spun onto slides and stained with a Differential Quik Staining Kit. (B) Neutrophil recruitment to the airways was quantified and (C) fungal germination in BALF was quantified by counting conidia and germlings in the slides and represented as percent of fungal matter that was germinated. Each symbol represents an individual mouse and the line represents the group mean ± one SEM. Data are representative of 1 experiment consisting of 5-6 mice per group. Statistical significance was determined using a Mann-Whitney U test (*$p < 0.05$).

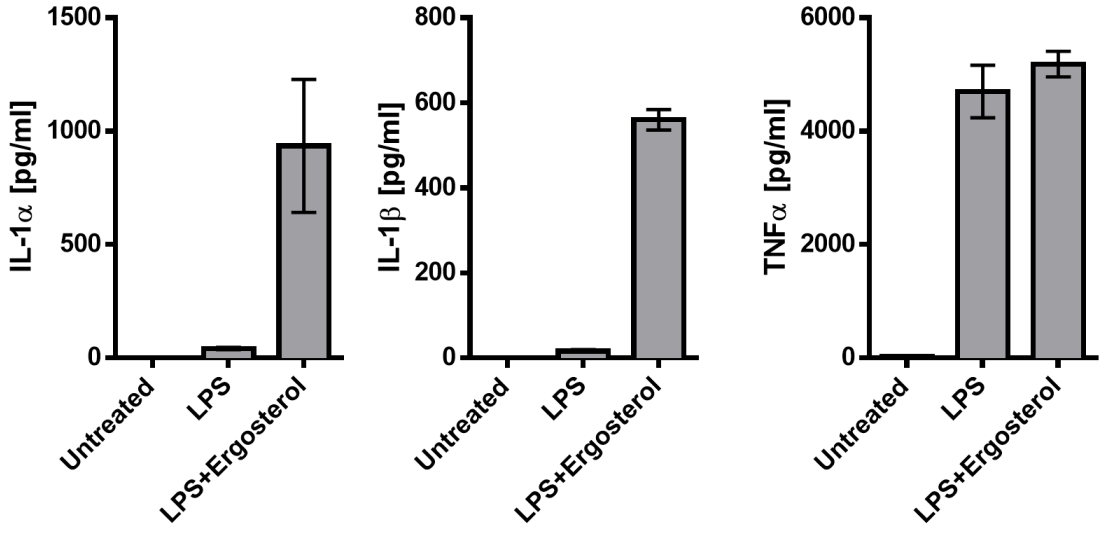


Figure A.9. Ergosterol is sufficient to drive IL-1 release from BMDMs. Bone marrow derived macrophages (BMDM) were incubated with media (untreated), LPS (1 μg/ml), or LPS+Ergosterol (4 mg/ml) for 24 hours and supernatant collected to quantify IL-1α, IL-1ß and TNF-α levels. Data are representative of 2 independent experiments consisting of 6 biological replicates per group. Bar graphs show the group mean ± one SEM.

APPENDIX D

HOST AND FUNGAL FACTORS CONTRIBUTE TO IA IN THE IMMUNE COMPROMISED HOST

In this thesis, we have demonstrated that IL-1 signaling is critical to host defense against hypervirulent strains of *A. fumigatus* in an immune competent host. However, we have not determined how IL-1 signaling contributes to the establishment and progression of IA in clinically relevant immune compromised models. We hypothesize that in the non-neutropenic host, IL-1 signaling could potentially be detrimental and contribute to the robust and pathological inflammation observed in these hosts. It has been shown in CGD mice, blockade of the IL-1RI with anakinra dampened excessive inflammation and protected mice from IA (de Luca et al. 2014). Furthermore, anakinra treatment of *A. fumigatus*-challenged, corticosteroid-treated mice dampened hypoxia in the lung as well as inflammation, and this was associated with a modest increase in survival (Gresnigt et al. 2016). When these mice were treated with both anakinra and an antifungal drug, survival was increased even further, suggesting that both host-derived factors and fungal-derived factors are contributing to disease outcome (Gresnigt et al. 2016).

It has been shown that hypoxic fitness is a fungal trait that determines virulence potential in a corticosteroid murine model of IA (Kowalski et al. 2016). In this study the 02-10 isolate had low hypoxic fitness and had low virulence in the corticosteroid model of IA (Kowalski et al. 2016). This is contrary to our results in an immune competent host, which demonstrates that 02-10 is highly virulent (Chapter 4). Differences in the formation of hypoxic microenvironments in the different models used could be a potential explanation to these conflicting results, as different modes of immunosuppression have been shown to induce varying degrees of hypoxia formation within the pulmonary environment during IA (Grahl et al. 2011).

We have performed a pilot study to begin to tease apart host-derived factors and fungal-derived factors that contribute to the development of IA in the immune compromised host. Specifically, corticosteroid-treated C57BL/6 or *Il1a*-deficient mice were challenged with $2x10^6$ conidia of either CEA10 or 02-10 and monitored for survival. Corticosteroid-treated *Il1α*-deficient mice challenged with CEA10 survived for approximately 24-48 hours longer than their C57BL/6 counterparts, but ultimately, mortality was similar between these groups (Figure A.10). We hypothesize the delayed morality is associated with dampened immune-mediated pathology in the early stages of infection due to the absence of IL-1α. Ultimately, CEA10 is able to thrive within hypoxic environments (Kowalski et al. 2016), and we hypothesize this is contributing to the high mortality of CEA10 in both C57BL/6 and *Il1a*-deficient mice in this study. Conversely, 02-10 is unable to thrive in hypoxia (Kowalski et al. 2016). When corticosteroid-treated *Il1a*-deficient mice were challenged with 02-10, mortality rates were significantly lower compared to C57BL/6 mice. This suggests treatment aimed both at dampening immune-mediated pathology and targeting fungal adaptation to the pulmonary environment could be beneficial to host resistance against development of IA. Importantly, this is only a pilot study and further histological and immunological analyses are required to confirm our hypotheses.

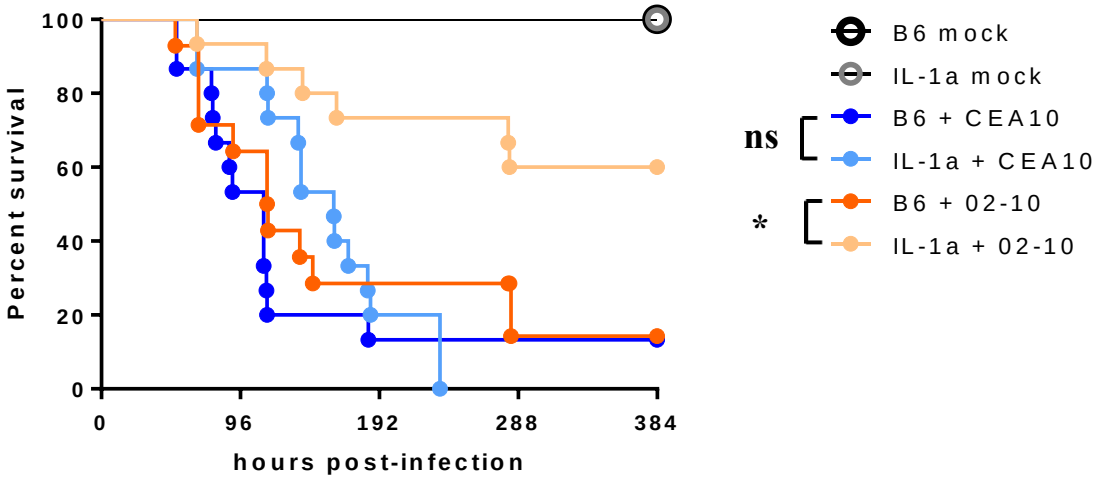


Figure A.10. *Il1a*-deficient mice are more resistant to the 02-10 isolate of *A. fumigatus* compared to C57BL/6 mice in the corticosteroid model of IA. C57BL/6 and *Il1a*-deficient mice were injected subcutaneously with 40 mg/kg of Kenalog-10. 24 hours post-Kenalog injection, mice were challenged IT with PBS (mock), CEA10, or 02-10. Mice were monitored daily using a humane endpoint scoring system (based on a combination of weight loss, inactivity, ruffled fur, and difficulty breathing), and when a total of 16 points was reached mice were humanely euthanized. Data are representative of 1-2 independent experiments with 14-15 mice per group. Survival was plotted on Kaplan-Meier curves, and statistical significance between curves determined using the Mantel-Cox log rank (ns = not significant, *$p < 0.05$).

APPENDIX E

LEUKOTRIENE SUPPORTING INFORMATION

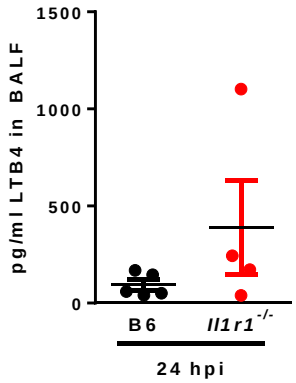
We have demonstrated in a brief report that leukotriene signaling is critical to resistance to IA in an immune competent host, but many questions remained to be answered. Both IL-1 and LTB_4 are necessary for timely neutrophil recruitment to the lung following challenge with *A. fumigatus* (Chapter 2, 4, and 5). Previous work in a murine model of arthritis linked IL-1 and LTB_4 signaling in a lipid-cytokine-chemokine cascade that was shown to orchestrate neutrophil recruitment to the joint (Chou et al. 2010). Whether these signaling mediators are linked in our model of IA, or whether they work independently of each other is an area of ongoing investigation. We have shown that in *Il1r1*-deficient mice following challenge with *A. fumigatus*, LTB_4 is produced at similar levels compared to C57BL/6 mice (Figure A.11A). Moreover, there is no defect in IL-1 production in *Ltb4r1*-deficient or *Alox5*-deficient mice following challenge with *A. fumigatus* (Figure A.11B). This suggests that these pathways act independently of each other, however a kinetic analysis is necessary to confirm this.

In a preliminary study we have found that similar to the IL-1α-dependent immune response being a fungal strain-specific occurrence, the leukotriene-dependent immune response also appears to be fungal strain-specific (Figure A.12). C57BL/6, *Ltb4r1*-deficient, and *Alox5*-deficient mice were challenged with either CEA10 or Af293. All mice infected with Af293 survived infection, regardless of whether leukotriene signaling was present or absent. Contrary to this *Alox5*-deficient mice challenged with CEA10 had increased mortality compared to C57BL/6 mice. Although a similar percentage of *Ltb4r1*-deficient and *Alox5*-deficient mice succumbed to infection (60% and 66%, respectively), survival of *Ltb4r1*$^{-/-}$ mice was not significantly different than C57BL/6

mice. This pilot experiment should be repeated to determine whether *Ltb4r1*$^{-/-}$ mice are more susceptible than C57BL/6 mice to CEA10 challenge, as data in Chapter 5 would support this hypothesis. Further studies are needed to determine why leukotriene-mediated immunity is fungal-strain dependent.

Additional studies are needed to determine how leukotriene signaling contributes to disease outcome in clinically relevant models of IA. We have shown that LTB_4 production *in vivo*, is dependent on HIF-1α following challenge with CEA10, but not Af293 (Figure A.13). HIF-1α is a transcription factor shown to be critical for host protection against *A. fumigatus* through enhancing CXCL1 production and neutrophil survival within the lung (Shepardson et al. 2014). Interestingly, corticosteroid treatment inhibited HIF-1α from localizing to the nucleus, where it binds to target genes to regulate transcription (Shepardson et al. 2014). Due to the HIF-1α dependent production of LTB_4 (Appendix A.13), it will be interesting to determine if this pathway contributes to enhanced susceptibility of corticosteroid treated patients to IA. Moreover, these data suggest that the susceptibility of *Hif1α*-deficient mice to IA might also be fungal strain-dependent. Future studies should examine the susceptibility to IA of Hif1a$^{fl/fl}$ x LysM-cre mice challenged with different *A. fumigatus* strains.

A



B

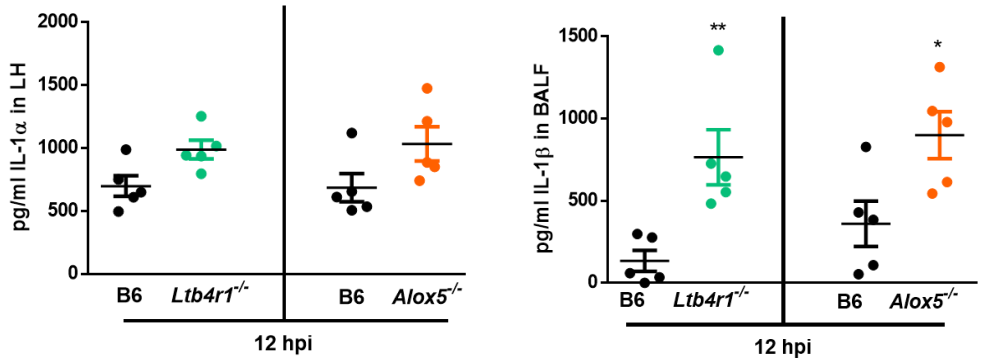


Figure A.11. LTB_4 and IL-1 are produced independently of each other *in vivo* following *A. fumigatus challenge*. (A) C57BL/6 and *Il1r1*-deficient mice or (B) C57BL/6 mice, Ltb4r1-deficient, or Alox5-deficient mice were challenged with $4x10^7$ conidia of CEA10 IT. (A) At 24 hpi, mice were euthanized, BALF collected, and LTB_4 protein levels measured. (B) At 12 hpi, mice were euthanized, BALF collected, and lungs homogenized. IL-1α protein levels were measured in the LH and IL-1ß protein levels were measured in the BALF. Data is representative of 1-2 independent experiments consisting of at least 5 mice per group. Statistical significance was determined using a Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$).

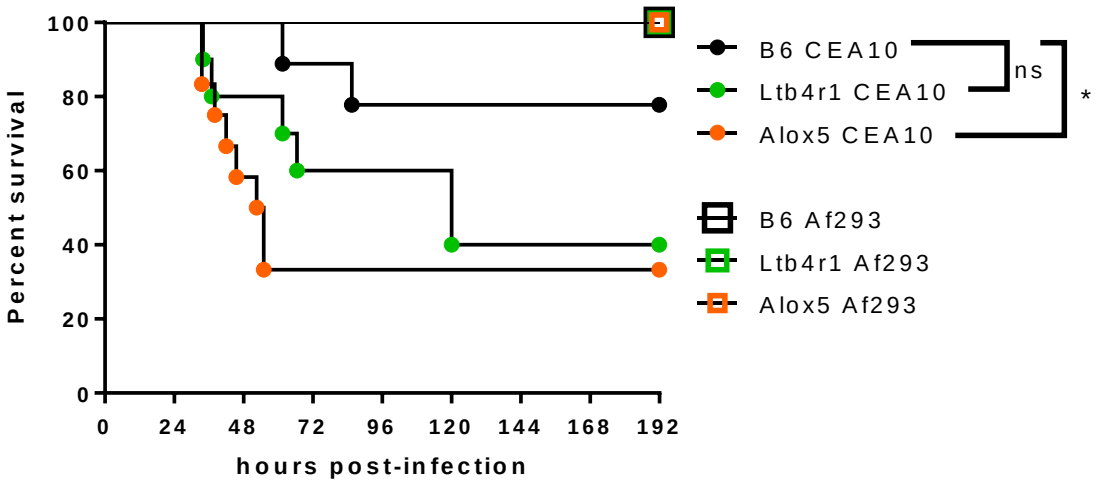


Figure A.12. Leukotriene-mediated immunity is fungal strain-dependent. C57BL/6, *Ltb4r1*-deficient and *Alox5*-deficient were challenged with $4x10^7$ conidia of CEA10 or Af293. Mice were monitored daily using a humane endpoint scoring system (based on a combination of weight loss, inactivity, ruffled fur, and difficulty breathing), and when a total of 16 points was reached mice were humanely euthanized. Data are representative of 1 experiment with 9-12 mice per group. Survival was plotted on Kaplan-Meier curves, and statistical significance between curves determined using the Mantel-Cox log rank (ns = not significant, *$p < 0.05$).

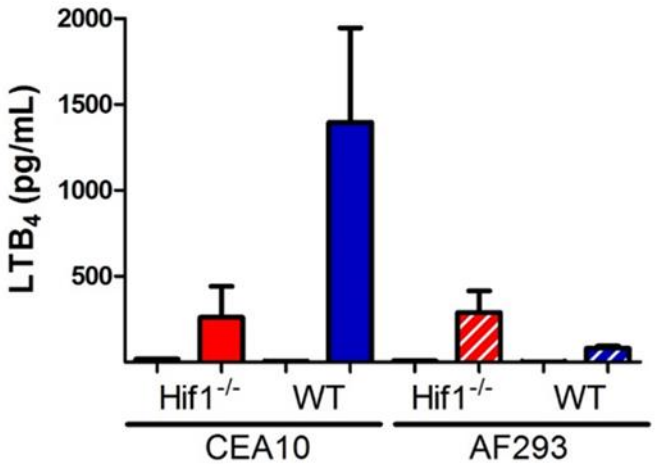


Figure A.13. LTB_4 production following *A. fumigatus* challenge is dependent on HIF-1α. C57BL/6 or myeloid-specific lysozyme-M cre-recombinase driven HIF-1α null mice ($Hif1^{-/-}$) were infected with either PBS, CEA10 or Af293 IT. At 8 hpi, mice were euthanized, BALF collected and LTB_4 measured. Data are representative of 1 experiment consisting of 4 mice per group for *A. fumigatus*-challenged mice and 1 mouse per group challenged with PBS.

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