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Innate Immune Defense Against The Fungal Pathogen Aspergillus Fumigatus

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INNATE IMMUNE DEFENSE AGAINST THE FUNGAL PATHOGEN *ASPERGILLUS FUMIGATUS*

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ABSTRACT

The opportunistic fungal pathogen *Aspergillus fumigatus* is a ubiquitous mold that causes severe, invasive life-threatening infections in patients that are severely immunocompromised. Major risk factors include neutropenia, neutrophil dysfunction and immunosuppressive therapies. Recently, however, rising incidences of colonizing *A. fumigatus* have been reported in patients without classical risk factors. We sought out to elucidate non-classical pre-disposing conditions that may render a lung environment susceptible to fungal colonization as well as mechanisms involved in immune defense against the invading fungal pathogen.

Further defining a role for Dectin-1 in an *Aspergillus fumigatus* infection, we demonstrated a critical role for Dectin-1 dependent interleukin-22 (IL-22) in the clearance of *A. fumigatus.* Mice deficient in IL-22 demonstrated higher lung fungal burden after *A. fumigatus* challenge in the presence of impaired IL-1α, TNFα, CCL3/MIP-1a and CCL4/MIP-1b production, lower neutrophil recruitment, yet intact IL-17A production. Moreover, lung lavage fluid from *A. fumigatus*-challenged IL-22 deficient mice had compromised anti-fungal activity against *A. fumigatus* in vitro. Furthermore, we show that despite enhanced recruitment of inflammatory cells to the lungs of chlorine-exposed *A. fumigatus* challenged

mice, these cells demonstrated a profound impairment in generating superoxide. Following exposure to Cl_2, mice challenged with *A. fumigatus* demonstrated a significantly higher fungal burden in the presence of higher IL-6 and TNFα, but lower IL-17A and IL-22 in the lungs compared to mice that were not challenged with *A. fumigatus*.

Collectively, our results indicate that early innate lung defense against *A. fumigatus* is mediated by Dectin-1 dependent IL-22 production. Furthermore, a predisposing condition such as chlorine exposure prior to fungal colonization markedly impairs the antimicrobial activity and inflammatory reactivity of myeloid cells in the lung leading to increased susceptibility to opportunistic pathogens.

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I dedicate this dissertation to my parents. Without your never-ending support (mentally, emotionally and financially) I would never be where I am today. Thank you both for teaching me the value of hard work, dedication and perseverance. You always believe in me even when I don't believe in myself – thank you for never letting me give up… in anything. You both have provided me with so many opportunities for which I cannot begin to thank you enough. You are the epitome of a parent, role model and friend. I hope one day I can be as cool as you guys! I love you.

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Aspergillus fumigatus

Aspergillus species are ubiquitous soil molds that play a critical role in recycling carbon and nitrogen in the environment. The *Aspergillus* genus is one of the most common molds encountered by humans. There are roughly 650 species of *Aspergillus*, about 40 of which reported to be human pathogens [2]. *Aspergillus fumigatus* is responsible for 90% of all cases of invasive aspergillosis (IA) with *A. terreus, A. flavus and A. niger* being responsible for the majority of remaining cases of infection [1,3]. Invasive aspergillosis has a mortality rate greater than 50%, reaching 95% in certain situations [4-6]. A ubiquitous, saprophytic soil mold, *A. fumigatus* grows as multicellular branching hyphae. Reproduction occurs asexually as conidia, the reproductive spores, become airborne [10]. These small, hydrophobic conidia are produced in very large quantities and remain airborne for hours at a time [8,9]. In turn, humans inhale hundreds of airborne conidia on a daily basis [7]. Once resting conidia encounter an appropriate environment, they will begin to swell within hours followed by germination.

Aspergillus fumigatus Mechanisms of survival

Studies have shown that survival and virulence of *A. fumigatus* is multifactorial, involving structure, adapting to stressful environments and mechanisms for evading the host immune system.

Survival. *A. fumigatus* is a thermophilic fungus, allowing for growth at temperatures up to 55 degree Celsius and survival at more than 75 degrees Celsius [11, 15]. This allows for the fungus to sustain itself in decaying organic matter as well as infecting mammalian hosts. When exposed to high temperatures, cells become resistant by producing heat shock proteins (HSPs) [14]. The *A. fumigatus* cell wall also acts as a mechanism of virulence and defense against potentially hostile environments. The cell wall is composed primarily of polysaccharides, mainly beta-1,3-glucans, alpha-1,3-glucans, chitins and galactomannans [12,13]. *A. fumigatus* hyphae and conidia also possess a hydrophobic layer for further protectio. Furthermore, presence of sialic acids linked to galactose may play an important role in adhesion [8, 16].

Immune resistance and evasion. In addition to it's small size allowing for deposition in the lower respiratory zone [24] and thick cell wall resistant to lysis [22], *A. fumigatus* has other characteristics allowing for evasion of the host immune system. Actual pigmentation of *A. fumigatus* has been shown to limit neutrophil activation. Melanin on the cell surface of conidia can further protect conidia from ultraviolet light, enzymatic lysis and oxidation [16]. Furthermore, *A.*

fumigatus can bind Factor H, FHL-1 and C4BP on cell surfaces to dampen complement response [17, 25, 26]. *A. fumigatus* has also been reported to produce superoxide dismutases (SODs) and catalases that affect reactive oxygen species (ROS) production in macrophages and neutrophils [20, 21, 23]. *A. fumigatus* also has the capability of producing toxins during germination that can affect the synthesis of host proteins, DNA and RNA, or alter cell membranes [24,18,19].

Aspergillus fumigatus Mechanism of Infection

Inhalation of fungal spores, or conidia, is the primary route of *Aspergillus* infection. Spores range from 2-5 micron in size, making it easy to deposit themselves in both the upper and lower respiratory tract. If not cleared, these deposited conidia can reach the alveoli will germinate and invade the local tissue. This in turn leads to destruction of the tissue and respiratory failure. Invasive aspergillosis is primarily seen in the lungs of patients as invasive pulmonary aspergillosis (IPA) with 90% of cases being restricted to the lungs with inhalation as the primary route of exposure [27, 28]. Germination begins in the alveolar spaces and pulmonary epithelial cells and continues to invade the surrounding cells causing endothelial cell damage. Upon destruction of surrounding tissues, *A. fumigatus* hyphal fragments can then enter in to the blood vessels, becoming angioinvasive, and lead to dissemination [29].

Patient Susceptibility

Aspergillus fumigatus, the etiological agent of invasive aspergillosis (IA), is a ubiquitous mold that causes severe, invasive, life-threatening infections in patients who are severely immunocompromised. Major risk factors include neutropenia, neutrophil dysfunction, and immunosuppressive therapies [30]. Recent data from the Transplant Associated Infections Surveillance Network (TRANSNET), a network of 23 United States transplant centers, have shown that IA occurred in 43% of hematopoietic stem cell transplant (HSCT) recipients [31] and in 19% of solid organ transplant recipients [32] between March 2001 and March 2006. IA is also becoming more recognized in individuals with less severe levels of immunosuppression. This is increasingly observed in intensive-care unit (ICU) populations, often associated with such diseases as chronic obstructive pulmonary disorder (COPD), cirrhosis, alcoholism and post influenza infection; various postsurgical settings; and adults presenting with heterozygous chronic granulomatous disease [33]. Although antifungal drugs have been introduced, the development of antifungal drug resistance has become increasingly apparent [6, 29].

Spectrum of Disease

Despite constant exposure, a healthy host with a normally functioning immune system is able to successfully clear airborne conidia thus preventing infection by the microorganism. Of the patients that do succumb to *Aspergillus* infections, there is a broad spectrum of disease depending on the reaction of the host

immune system. Infections are considered under three categories: invasive infections, being characterized by growing hyphae infiltrating in organ tissues and a weak immune response; infections due to colonization of the microorganism on mucosal surfaces without infiltrating tissue; and hypersensitive allergic-type reactions, due to a prolonged overactive immune response. If there is a pre-existing cavity in the lung, such as from tuberculosis, a fungal ball (aspergilloma) may form. These have the potential to invade surrounding tissues, but usually remain asymptomatic and stable in size for years [34, 35]. Patients with cystic fibrosis (CF) or asthma are at risk for allergic bronchopulmonary aspergillosis (ABPA); the colonization of lungs with *A. fumigatus* [36]. This colonization results in enhanced hypersensitivity to *A.* fumigatus antigens as seen through mucus-blocked airways [34]. The most frequent clinical presentation at 90% manifesting from various underlying risk factors is invasive pulmonary aspergillosis, or IPA [42]. IPA is life threatening with a global mortality rate of 45% after 3 months of disease [32].

Traditional Risk Factors

In an immunocompetent host with no underlying disease, *A. fumigatus* conidia are cleared from the lung as normal debris. However, immunocompromised patients will develop potential life threatening disease based on their immune condition and pre-existing risk factors. Classically, an important predisposing defect causing susceptibility to IPA is a defect in neutrophils, or neutropenia. Most at risk are patients that have undergone hematopoietic stem cell transplant

(HSCT), followed by patients that have undergone solid-organ transplant due to the immunosuppressive treatments they are required to have to prevent rejection [37]. With a mortality rate of 36% at 12 weeks post-transplant, invasive aspergillosis tops invasive candidiasis and mucormycosis as the top invasive fungal infection in HSCT recipients [38]. Lung transplant patients also display an increased risk for invasive fungal infections (IFIs) with 15-35% of patients succumbing to IFIs. 44 – 63% of these lung transplant patients' IFIs are due to *A. fumigatus* infection [39, 40]. Other classical risk factors for IPA include HIV and corticosteroid treatment [1, 41].

Non-traditional Risk Factors

Recently, rising incidence of IPA in non-neutropenic patients have been observed. While classical risk factors causing susceptibility to IPA have been studied, it is necessary to expand knowledge on other risk factors that may less studied or not yet known. During ICU stays, critically ill patients are prone to developing disturbances in their immunoregulation, including immune paralysis, characterized by the deactivation of macrophages and an altered cellular response due to severity the of illness [126]. Risk factors including chronic obstructive pulmonary disease (COPD), prolonged use of steroids, advanced liver disease and diabetes can render patients susceptible to fungal infections [126].

Chronic obstructive pulmonary disease. Both patients with COPD and Graft versus Host disease (GVHD) have demonstrated an increasing incidence of IPA. These patients are treated with intravenous or inhaled corticosteroids. Corticosteroids affect the functions and distribution of neutrophils, monocytes and lymphocytes and directly stimulate *A. fumigatus* growth [124]. The pathogenesis of IPA in patients with steroid-associated immunosuppression compared to neutropenic patients is observed to be different. Mortality in neutropenic patients seems to be due to a high fungal burden whereas mortality in steroid-associated immunosuppression seems to be due to an adverse host inflammatory response [124, 125].

Chronic granulomatous disease. Chronic granulomatous disease, or CGD, is a rare disorder causing a defect in the NADPH oxidase complex in phagocytes. This defect is due to mutations in one of the four essential genes in enzyme; $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$. $gp91^{phox}$ and $p22^{phox}$ are stored in PMN secondary granules and translocated to the plasma or phagocytic membrane upon cellular activation. NADPH oxidase activation occurs when $p67^{phox}$ and $p47^{phox}$ translocate to this membrane-bound complex essentially leading to the reduction of oxygen to superoxide that further is converted to hydrogen peroxide by superoxide dismutase. In the presence of myeloperoxidase (MPO) in neutrophil primary granules, this hydrogen peroxide is converted to hypochlorous acid and then converted to chlorine. This whole in tact process is an essential upstream regulator of many antimicrobial proteins involved in innate immune host

defense. These phagocytes in turn have a defect in reactive oxygen species production, most likely due to disruption of respiratory burst in granulocytes and monocytes, resulting in an inability to kill microorganisms thus increasing susceptibility to bacteria and fungi [121]. CGD has a mortality rate of 2% per year with about one third of these deaths are caused by *Aspergillus* infection [144]. Patients with CGD have a 25-40% probability of *Aspergillus* infection [145] with *A. fumigatus* being associated with approximately 40% of CGD cases, with 17% actually developing IPA [119, 120]. CGD patients frequently suffer from opportunistic *Aspergillus* infections due to impaired oxidative functions of neutrophils [152]. This phenotype is further confirmed by mouse models for x-linked or autosomal CGD in $gp91^{phox-/-}$ [153] $p47^{phox-/-}$ mice [154].

Hyper IgE Syndrome. Autosomal dominant hyper IgE syndrome (AD-HIES), previously known as Job's syndrome, is a primary immune deficiency that is generally characterized by recurrent skin boils, cyst-forming pneumonias and high elevations of serum IgE. Other manifestations include eczema and mucocutaneous candidiasis. 20% of these patients develop IPA due to lung lesions caused by bronchiectasis or pneumatocyst formation with a 17% mortality rate [122]. Unlike CGD patients, AD-HIES patients do have normal functioning phagocytes that allow for inhibition of *A. fumigatus* growth. However, due to repeated bacterial infections causing pneumatocysts compounded with defective STAT3-dependent epithelial immunity, these patients may become more vulnerable to a secondary IPA infection [123].

Diagnosis and Treatment

Diagnosis. Diagnosis of IPA is made on the basis of compatible clinical findings, abnormal radiologic findings, and microbiologic confirmation or on the basis of histologic proof of tissue invasion by the fungus [118]. Diagnosis of IA becomes increasingly more challenging in the ICU due to the fact that most patients do not belong to a well-established risk group. The standard diagnostic tools were developed on hematologic disease patients. Positive results of a culture of respiratory specimen or positive findings of a microscopic examination is present in only 50% of patients with IA [118]. It has also proven to be difficult, due to the ubiquitous prevalence of *A. fumigatus,* differentiating whether the fungus has colonized or actually caused infection. Recently, lung CT has become one of the most important diagnostic tools [118].

Treatment. Amphotericin B has been the classical treatment of IA for a long time. However, a number of adverse side effects including nephrotoxicity, hypokalemia and fever associate with this treatment. New anti-fungal drugs with improved safety profiles, including voriconazole, posaconazole and echinocandins, have become available as alternatives over the past few years. More recently, voriconazole has replaced amphotericin B as the standard for treating IA. Difficulties have arisen in treatments with the onset of anti-fungal resistant strains fungal infections [116, 117].

Host Defense Against *Aspergillus fumigatus*

Host defense against *A. fumigatus* begins as soon as conidia are inhaled via physical barriers of the respiratory tract (i.e. nasal turbinates and branching patterns of the bronchial tree). The majority of inhaled particles will be trapped in surface fluid and removed by respiratory epithelial cell ciliary action [43]. Due to its small size, however, sometimes *A. fumigatus* spores will evade this initial defensive immune mechanism and travel down to the respiratory part of the lungs [24].

Immune Detection of A. fumigatus

Once inhaled conidia evade initial clearance mechanisms by the immune system, alveolar macrophages rapidly ingest and attempt to clear the pathogen. Conidia are also being recognized by both soluble and cell-bound pattern recognition receptors at this time. Organisms that escape alveolar macrophages will begin to germinate leading to the recruitment of and targeting by neutrophils. Studies have shown that macrophages handle *A. fumigatus* conidia more effectively while neutrophils are more effective against germinating *A. fumigatus* conidia and hyphae [71]. Once conidia have been recognized, cells will respond by producing a cascade of cytokines and chemokines to eventually lead to clearance of the invading pathogen.

Toll like receptors. Studies identifying the interactions between toll like receptors (TLRs) and *A. fumigatus* have been somewhat conflicting [45, 46, 49]. Initially, it was found that human monocytes, when challenged with *A. fumigatus* hyphal fragments, demonstrated a role for TLR4 in recognition, but not TLR2 [50]. Contrarily, a study a year later demonstrated that peritoneal macrophages required TLR2 in both mice and humans to recognize *A. fumigatus* while TLR4 was not essential [44]. Further studies went on to demonstrate that macrophages responded differently to conidia and hyphae. While TLR2 recognizes both conidia and hyphae, TLR4 only seems to recognize conidia [48]. Furthermore, while signaling through both TLR2 and TLR4 promote fungicidal activity, TLR2-dependent signaling promotes release of proinflammatory cytokines while TLR4-dependent signaling promotes anti-inflammatory cytokine production, specifically IL-10 [48]. These findings suggest that *A. fumigatus* conidia and hyphae express different cell wall components acting as pathogen associated molecular patterns, or PAMPs. While all these specific PAMPs have not been described, it seems clear that the host innate immune system recognizes 2 different PAMPs via TLR2 and TLR4. While TLRs seem to be involved in the recognition of *A. fumigatus*, studies suggest that while TLRs are essential in the absence of neutrophils, other pattern recognition receptors, or PRRs, like Dectin-1 may be more critical for innate defense against *A. fumigatus* in a more immunocompetent host.

Non-TLRs. Non-TLRs have also been shown to be important in recognizing *A. fumigatus*. As the cell wall of *A. fumigatus* contains galactomannan moieties, studies have identified and described mannose- or mannan-specific receptors as playing a role in the uptake of *A. fumigatus* by phagocytes [53]. C-type lectin DC-SIGN (dendritic-cell-specific, ICAM-3-grabbing non-integrin) has been shown to be involved in the binding of *A. fumigatus* conidia to macrophages and dendritic cells [52, 54, 55].

Dectin-1. Dectin-1 is a 43-kDA, type II transmembrane receptor and contains a single cytoplasmic immunoreceptor tyrosine activation motif [51]. Dectin-1 is characterized as a pattern recognition receptor that recognizes beta-1,3 glucans; a PAMP expressed on fungal cell walls [56, 57]. Dectin-1 is expressed in a variety of tissues; predominantly lung, liver and thymus [58, 59] and on myeloid cell lineages of neutrophils, monocytes, macrophages and dendritic cells [58]. Once activated by PAMP detection, Dectin-1 induces proinflammatory effects of NF-kB activation via the Syk kinase and the adaptor CARD9 [60, 61, 62, 56]. Dectin-1 has been known to play a role in innate host defense against opportunistic fungal pathogens such as *Pneumocystis carinii* [63] and *Candida albicans* [64]. These pathogens, like *A. fumigatus*, are a major cause of mortality in immunocompromised patients. Dectin-1 deficient mice display an increased susceptibility and increased fungal burden when challenged with *C. albicans* [65]. Furthermore, blocking Dectin-1 in a human neutrophil culture reduced binding and phagocytosis as well as reactive oxygen species (ROS) production. ROS is

critical in host defense against fungal pathogens as seen with CGD patients lacking neutrophils capable of producing ROS being highly susceptible to fungal infections. Human neutrophils have also been shown to have a reduction in *C. albicans* phagocytosis when Dectin-1 is blocked [64]. Dectin-1 expression is also important to dendritic cell immune function as blocking Dectin-1 decreases levels of dendritic cell TNF-a and interleukin-12 (IL-12) [64].

Innate Immune Response

Upon receptor recognition of *A. fumigatus* a cascade of innate immune mechanisms will begin in an effort to clear the invading pathogen. There has been growing evidence implicating IL-17A and IL-22 as important cytokines in multiple models of inflammation.

Dectin-1 and A. fumigatus. Similar to other opportunistic fungal infections, Dectin-1 has been reported to be critical in the clearance of *A. fumigatus* [51, 65]. Not only does blocking Dectin-1 on murine macrophages inhibit phagocytosis of *A. fumigatus* conidia, RAW264.7 macrophages have a defect in pro-inflammatory cytokine and chemokine production, such as MIP-2, interleukin-1a (IL-1α), and granulocyte colony-stimulating factor (G-CSF) when co-cultured with *A. fumigatus*. Mice deficient in Dectin-1 are highly susceptible to *A. fumigatus* lung infection due to a defect in inflammatory reactivity of alveolar macrophages and impaired recruitment and defense of neutrophils [64]. A role for Dectin-1 in the generation of Th17/IL-17A-mediated responses has been identified [65]. The

Dectin-1/Syk/CARD9 signaling axis promotes dendritic cell (DC) activation and the secretion of proinflammatory mediators, such as IL-23 [65]. Studies have shown that DCs activated by Dectin-1 produced IL-23 and converted Foxp3+ regulatory T cells (Tregs) to IL-17A-producing cells. In addition to being critical for the maintenance of the Th17 lineage [66] and IL-17A-producting cells [67,69], IL-23 is also a critical effector cytokine for the induction of IL-22 [68, 69].

Neutrophils and macrophages. Classically, host defense against *A. fumigatus* was thought to be mediated through initial phagocytosis of conidia by resident alveolar macrophages followed by neutrophil attack of the remaining hyphae and conidia. Recruited neutrophils were thought to act only on hyphae while the resident macrophages took care killing resting and swollen conidia [71]. Recent evidence, however, is suggesting that early recruitment of neutrophils to the site of infection is critical for host survival, having an essential role in killing germinating conidia [72, 73] while macrophages may not be as critical as once thought. This may be due to the relatively slow killing of larger conidia by alveolar macrophages [70]. Once *Aspergillus* begins to germinate and surpass macrophages as the first line of defense, neutrophils serve as the main defense against *Aspergillus.* Neutrophils are able to recognize invading *Aspergillus* via detection by Dectin-1, as blocking dectin-1 in a human neutrophil culture stimulated with zymosan reduces binding and phagocytosis as well as ROS production in a dose dependent manner [155]. Mechanistically, neutrophil killing of *A. fumigatus* may involve production of reactive oxygen species (ROS), the

use of neutrophil extracellular traps (NETs), release of microbicidal granules and phagocytic vacuoles [73, 74]. As demonstrated with CGD patients, the NADPH oxidase system in phagocytes is critical in host defense against *Aspergillus* [144]. Constituents of the fungal cell wall activate neutrophil NADPH oxidase. This activation coupled with activation of neutrophilic antimicrobial proteases in primary granules is essential for host defense against *Aspergillus* conidia and hyphae. Neutrophil inhibition of conidial growth does not require NADPH oxidase, but in turn requires lactoferrin. Lactoferrin, a secondary granule protein in neutrophils, is known to be capable of inhibiting *A. fumigatus* conidia growth via the chelation of iron, an essential growth factor for *A. fumigatus* [146]. Other secondary granule constituents, serine proteases, also contribute to neutrophil microbial killing [151]. Neutrophil elastase has also been implicated in host defense against microbial and fungal pathogens, as mice deficient in elastase are unable to clear invading pathogens [9]. Elastase is an important component of neutrophil extracellular traps (NETs) playing an important role in defense against *A. fumigatus* [151,152].

Dendritic and epithelial cells. Dendritic cells also play a critical role in innate host defense against *A. fumigatus*. Plasmacytoid dendritic cells (pDCs) have been reported to restrict growth of *A. fumigatus* hyphae, as well as secreting pro-inflammatory cytokine mediators IFNα and TNFα in response to hyphal stimulation [75]. Moreover, mice depleted in pDCs show an increased susceptibility to *A. fumigatus* than control mice. Inflammatory dendritic cells are

also an important source of TNFα during a persistent fungal infection [76]. Although not as studied, airway and alveolar epithelial cells play are important in host defense against *Aspergillus*. A human alveolar epithelial cell line can bind both *Aspergillus* conidia and hyphae as well as ingest conidia while generating IL-6 and CXCL8 in response [77, 78]. Furthermore, epithelial cells are responsible for secreting antimicrobial peptides that may play a role in the clearance of invading pathogens.

Interleukin-23. Discovered in 2000, IL-23 was an important breakthrough to further understand immune-mediated diseases. Previously unknown, the p19 protein was found to pair with the p40 subunit of IL-12 to form IL-23 [147, 148]. IL-23 was further discovered to activate T cells that differed in comparison to Th1 and Th2 cells in regards to their cytokine production. These T cells produced IL-17A and were thus termed “Th17” cells, further going on to regulate tissue inflammation and autoimmune responses [148-150]. Developing Th17 cells require IL-23 for the commitment of Th17 pathway, leading to enhanced IL-17A production as well as other Th17 cytokines [148].

Interleukin-17. Interleukin-17 (IL-17) is a proinflammatory cytokine that upregulates a number of cytokines and chemokines, leading to the recruitment of neutrophils to sites of inflammation [12]. Originally discovered in 1995 as a product of human T cells [79], IL-17 was found to induce the production of IL-6 and IL-8 while enhancing surface expression of the intracellular adhesion

molecule-1 (ICAM-1) by human fibroblasts. Several IL-17 family members have been described since then with IL-17A being the original family member described. Recently, a T-cell lineage (Th17 cells) has been identified as producing IL-17A [80, 81]. These cells are hypothesized to play an important role in the pathophysiology of respiratory diseases such as asthma, COPD, cystic fibrosis (CF), and lung transplant rejection. Patients with defects in Th17 cells are known to suffer from fungal infections like *C. albicans* and *Staphylococcus aureus* [82]. Depending upon the invading pathogen, Th17 cells will produce IL-17 and IFN-g in the presence of no IL-10 (*C. albicans*) or IL-17 along with IL-10 (*S. aureus*) [82]. Th17 differentiation is dependent upon IL-6, IL-23 and IL-1b in regards to either pathogen [83, 84]. IL-17 has been reported as being produced by gamma delta T cells, NKT cells, innate lymphoid cells and lymphoid tissue inducer (LTi) cells and is mediated by CD11b(+) Ly-6G(+) neutrophils during invasive fungal infections [85, 86]. Human IL-17A binds with it's receptor IL-17RA which in turn binds with IL-17RC to form heterodimers for optimal signaling [86]. IL-17RA is highly expressed in haematopoietic tissues and upon binding targets epithelial cells, endothelial cells, fibroblasts, macrophages and dendritic cells [86]. Upon signaling, IL-17A is important for the recruitment of neutrophils. Mechanistically still unclear, this process is essential in the clearance of invading pathogens [88]. In regards to an *A. fumigatus* infection, IL-23 dependent and Dectin-1 dependent IL-17A produced by neutrophils is essential for the clearance of *A. fumigatus* as neutralizing IL-17A in

a mouse model of infection impairs clearance and increases lung fungal burden [87].

Interleukin-22. In addition to being critical in the maintenance of Th17 cell lineages [66] and IL-17A production [67, 69], IL-23 is also critical for the induction of IL-22 [40, 68]. Interleukin-22 (IL-22) is a member of the IL-10 family of cytokines [89]. Mouse IL-22 shares structural similarities and 22% sequence homology with mouse IL-10 while human IL-22 shares 79% homology with mouse IL-22 and 25% homology with human IL-10 [91]. IL-22 signals through the IL-22 receptor (IL-22R), a class 2 receptor compromised of the subunits IL-22R1 (shared with IL-20 and IL-24) and IL-10R2 (shared with IL-10 and IL-26) [89, 93, 94]. IL-22 initially binds to IL-22R1, which promotes the complex binding to the IL-10R2 to further propagate downstream signaling [95]. Like other members of the IL-10 family, IL-22 signals via the Jak-STAT signal transduction pathway which leads to the phosphorylation of Jak1 and Tyk2 kinases and STAT1, 3 and 5 transcription factors [89, 91, 93]. Although pathways and functions have yet to be characterized, it is known that a soluble secreted receptor of IL-22 (IL-22-binding protein, also known as IL-22RA2) does exist [95]. IL-22, like IL-17A, is produced by many cell types, but unlike IL-17A targets a limited number of cells [96]. To date, only epithelial cells and keratinocytes have been identified as expressing the IL-22 receptor [90]. IL-22 has been reportedly produced by human natural killer (NK) cells as well as mouse NK1.1(+) cells as well as innate lymphoid cells in certain models [96]. Originally, IL-22 was found

to be produced by T cells and has sense been reported in a broad range of T cells [97]. In humans, Th1, Th22, Th17 as well as a population of gamma delta T cells are among T cell subsets that have since been reported to express IL-22. In mice, IL-22 is expressed highly in Th17, Tc17, innate-like gamma delta T cells and NKT cells [97]. Differentiation of murine naïve T cells to T helper lineages producing IL-22 require IL-6, IL-1b and TGF-β for differentiation along with IL-23 for critical maintenance of expansion and ultimate IL-22 expression. Similarly, human T helper subsets require TGF-β, IL-1b, IL-23 and the transcription factor Aryl Hydrocarbon Receptor (AhR) for IL-22 expression. Upon secretion, IL-22 targets epithelial cells that in turn produce a number of antimicrobial peptides, depending on the model of infection, that in turn aid in the clearance of invading pathogens. Stimulation of keratinocytes with IL-22 results in induction of genes encoding for S100A7, S100A8, S100A9, beta-defensin-2 and beta-defensin-3 [100, 101] while intestinal stimulation with IL-22 leads to induction of RegIII-β and RegIII-γ antimicrobial peptide production [98, 99].

IL-23, IL-17, IL-22 axis. Although often produced in a similar fashion, growing data in multiple models has shown that IL-17A, IL-22 and IL-23 do not necessarily function equally. In a *Citrobacter rodentium* infection, mucosal defense is more dependent on IL-23 than IL-17A [104]. In colitis, IL-23 deficiency is more effective at improving disease compared to IL-17A deficiency [103,102]. In fact, IL-17A may play a protective role in intestinal inflammation [105]. IL-22 has been shown to act as an anti-inflammatory agent in hepatitis

[107] and inflammatory bowel disease (IBD) [106]. On the other hand, IL-22 is thought to be a contributing factor to inflammation associated with psoriasis [108]. Taken together, IL-23, IL-17A and IL-22 often function in concordance together, but in certain models, the function of one may differ or be more important than the others.

IL-22 expression in non-Aspergillus models of inflammation. IL-22 expression is observed in multiple human infectious disease models of inflammation. In psoriasis and arthritis, higher concentrations of Th-17 cell produced IL-22 are observed in peripheral blood and tissues [111, 112], potentially playing a negative role of promoting pathological inflammation. Th22 produced IL-22 is also seen in higher concentrations in the skin of patients with atopic dermatitis [109]. Similarly, in models patients with inflammatory bowel disease display an increased concentration of IL-22 in peripheral blood and intestine [113]. As far as IL-22's role in intestinal inflammatory models, it is controversial whether IL-22 is playing a protective or pathogenic role, as IL-22 expression in mouse models seem to be anti-inflammatory while in human models expression seems to be pro-inflammatory [113]. *Leishmania donovani* infection is associated with increased Th17 IL-22 expression correlating with disease protection [110].

Mutations associated with IL-22. Patients suffering for hyper-IgE syndrome have a hypomorphic mutation in the STAT3 gene [114], a critical transcription

factor involved in IL-22 expression. These patients present with impaired Th17 differentiation and lower IL-22 production. These patients also suffer from severe and recurrent secondary infections in the skin, lung and intestine. Moreover, patients with chronic mucocutaneous candidiasis display impairment of IL-22 production due to a mutation that neutralizes IL-17A, IL-17F and IL-22 [115]. These mutations, although affecting multiple cytokine pathways, point to a potentially critical role for IL-22 aiding immunity against invading fungal pathogens.

DECTIN-1 DEPENDENT IL-22 CONTRIBUTES TO EARLY INNATE LUNG DEFENSE AGAINST *ASPERGILLUS FUMIGATUS*

by

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Abstract

We have previously reported that mice deficient in the beta-glucan receptor Dectin-1 displayed increased susceptibility to *Aspergillus fumigatus* lung infection in the presence of lower IL-23 and IL-17A production in the lungs and reported a role for IL-17A in lung defense. As IL-23 is also thought to control the production of IL-22, we examined the role of Dectin-1 in IL-22 production as well as the role of IL-22 in innate host defense against *A. fumigatus*. Here, we show that Dectin-1 deficient mice demonstrated significantly reduced levels of IL-22 in the lungs early after *A. fumigatus* challenge. Culturing cells from enzymatic lung digests *ex vivo* further demonstrated Dectin-1 dependent IL-22 production. IL-22 production was additionally found to be independent of IL-1β, IL-6 or IL-18, but required IL-23. The addition of recombinant IL-23 augmented IL-22 production in WT lung cells and rescued IL-22 production by lung cells from Dectin-1 deficient mice. *In vivo* neutralization of IL-22 in the lungs of WT mice resulted in impaired *A. fumigatus* lung clearance. Moreover, mice deficient in IL-22 also demonstrated higher lung fungal burden after *A. fumigatus* challenge in the presence of impaired IL-1α, TNF-α, CCL3/MIP-1α and CCL4/MIP-1β production, lower neutrophil recruitment, yet intact IL-17A production. We further show that lung lavage fluid collected from both *A. fumigatus*-challenged Dectin-1 deficient and IL-22 deficient mice had compromised anti-fungal activity against *A. fumigatus in vitro*. Although lipocalin 2 production was observed to be Dectin-1 and IL-22 dependent, lipocalin 2 deficient mice did not demonstrate impaired *A. fumigatus* clearance. Moreover, lung *S100a8*, *S100a9* and *Reg3g* mRNA expression was

not lower in either Dectin-1 deficient or IL-22 deficient mice. Collectively, our results indicate that early innate lung defense against *A. fumigatus* is mediated by Dectin-1 dependent IL-22 production.

Introduction

Aspergillus fumigatus, the etiological agent of invasive aspergillosis (IA), is a ubiquitous mold that causes severe, invasive life-threatening infections in patients that are severely immunocompromised. Major risk factors include neutropenia, neutrophil dysfunction and immunosuppressive therapies [19]. Recent data from the Transplant Associated Infections Surveillance Network (TRANSNET), a network of 23 United States transplant centers, has reported that IA occurred in 43% of hematopoeitic stem cell transplant (HSCT) recipients [20] and in 19% of solid organ transplant recipients [33] between March 2001 and March 2006. IA is also becoming more recognized in individuals with less severe levels of immunosuppression. This is becoming increasingly observed in ICU populations, often associated with such diseases as COPD, cirrhosis, alcoholism, post-influenza infection, various post-surgical settings and adults presenting with heterozygous chronic granulomatous disease (reviewed in [1]).

Our laboratory has had a long standing interest in pulmonary innate immune mechanisms involved in controlling *A. fumigatus*. We have previously demonstrated a central role for the beta-glucan receptor Dectin-1 in innate lung immune responses against *Aspergillus fumigatus* [38]. Mice deficient in Dectin-1 are highly susceptible to lung infection with *A. fumigatus* as a result of impaired inflammatory reactivity of alveolar macrophages and impaired recruitment of and defense by neutrophils [42]. Among several cytokines we have reported to be induced in a Dectin-1 dependent manner during *A. fumigatus* lung infection, we have identified IL-17A as being a critical mediator in host defense [42]. Dectin-1

deficient mice produced IL-17A at lower levels in the lungs after exposure to *A. fumigatus* and neutralization of IL-17A in WT mice resulted in a compromised ability to clear *A. fumigatus* from the lungs, indicating a strong requirement for this mediator in pulmonary defense against *A. fumigatus* [42]. In our most recent studies, we have identified neutrophils as a source of Dectin-1 dependent IL-17A production during lung *A. fumigatus* infection [43]. IL-17A production by neutrophils required the presence of IL-23, which we have previously reported to be produced in a Dectin-1 dependent manner in the lungs [42], and more recently, in a Dectin-1 dependent manner by DCs [43].

In addition to being critical for the maintenance of the Th17 lineage [23] and IL-17A-producing cells in general [27 40], IL-23 is also a critical effector cytokine for the induction of IL-22 [40 37], a class 2 α-helical cytokine of the IL-10 family of cytokines [34]. Although often produced in concert with each other, growing data in several models has found that IL-23, IL-17A and IL-22 do not necessarily function equally. For example, mucosal defense against the gut pathogen *Citrobacter rodentium* is more dependent on IL-23 than IL-17A [26]. In colitis, IL-23 deficiency is more effective in ameliorating disease than IL-17A deficiency [22 11]. In fact, IL-17A may be protective in intestinal inflammation [32]. With respect to IL-22, infection models with the Gram-negative bacteria *Klebsiella pneumoniae* [2] and *Citrobacter rodentium* [50] showed a requisite role for IL-22 in protection. However, although produced by Th17/IL-17A producing cells [29], IL-22 has been shown to act as an anti-inflammatory agent in hepatitis [47] and IBD [39]. Paradoxically, IL-22 is thought to be a contributing factor in inflammation

associated with psoriasis [49]. Collectively, IL-23, IL-17A and IL-22 often function in concordance with each other, but in certain models, the function of one may be more important than another. As we have previously identified a role for IL-17A *in vivo* during *A. fumigatus* lung infection [42], we questioned whether IL-22 was also required for innate immune-mediated defense against *A. fumigatus*.

Materials and Methods

Mice

C57BL/6NTac mice, 6 to 8 weeks of age, were purchased from Taconic Farms Incorporated (Germantown, NY). Dectin-1 deficient mice were generated on the 129/SvEv background as previously described [41], backcrossed 10 generations to the C57BL/6 background and bred at Taconic. IL-22 deficient mice [50] were provided by Dr. Wenjun Ouyang at Genentech and bred at UAB. Lipocalin 2 deficient mice [4] were provided by Dr. Yvonne Chan at the University of Pittsburgh. Mice were maintained in a specific pathogen free environment in microisolator cages within an American Association for Laboratory Animal Science-certified animal facility in the Lyons Harrison Research Building at the University of Alabama at Birmingham. Animal studies were reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC).

Preparation of *A. fumigatus*, *in vivo* challenge and lung fungal burden assessment

A. fumigatus isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5-7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate buffered saline supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40 μm nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. Mice were lightly anesthetized with isoflurane and administered

5-7 x 10^7 *A. fumigatus* conidia in a volume of 50 µl intratracheally. For lung fungal burden analysis, the left lungs were collected and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure™ yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI), which includes a DNAse treatment step to eliminate genomic DNA as previously reported [28]. Lung *A. fumigatus* burden was analyzed with real time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession number AB008401) ([5]) and quantified using a standard curve of *A. fumigatus* conidia (10^1 – 10^9) as previously described ([28]). Specifically, total RNA was isolated using the MasterPure™ kit from serial 1:10 dilutions of *A. fumigatus* conidia beginning with 10^9 and real time PCR amplification of *A. fumigatus* 18S rRNA was performed on each dilution. As a validation of the real-time PCR method, heat-killed *A. fumigatus* did not yield a signal by real-time PCR and were unable to grow on potato dextrose agar plates. In addition, no amplification controls (i.e. no reverse transcriptase included in the cDNA reaction) yielded a signal of <0.001% by real-time PCR, indicating that the DNAse treatment step was efficient at eliminating contaminating *A. fumigatus* DNA.

IL-22 neutralization

For *in vivo* IL-22 neutralization, WT mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia in 50 µl and 6 h thereafter, mice were administered 50 µg of goat anti-mouse IL-22 (R&D Systems) or goat IgG isotype control antibody. Twenty-four hours after challenge, mice were sacrificed, the left

lungs were collected and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure™ yeast RNA purification kit and lung *A. fumigatus* burden was analyzed with real time PCR measurement of the *A. fumigatus* 18S rRNA as described [42].

Lung cell isolation, culture, cytokine neutralizations and IL-23 stimulation

Mice were anesthetized with intra-peritoneal ketamine/xylazine and sacrificed by exsanguination 18 h post-infection. Both lungs were collected and minced in IMDM media (Sigma, St. Louis, MO) supplemented with 1% pen-strep-glut (Mediatech, Herndon, VA), 10% heat inactivated FBS (Invitrogen, Carlsbad, CA) and 0.4 mg/ml polymyxin B (Thermo Fisher), followed by incubation for 60 min with tissue culture-grade type IV collagenase (1 mg/ml; Sigma, St. Louis, MO) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70 μm and 40 μm nylon filters and red blood cells lysed with ACK buffer (Lonza, Walkersville, MD) to create lung cell preparations. For lung cell cultures, cells were enumerated on a hemacytometer and plated at 1 x 10^6 cells in a volume of 0.2 ml. Supernatants were collected after 24 h, clarified by centrifugation and stored at -80°C. IL-22 levels were quantified by ELISA [42]. In specific experiments, neutralizing antibodies were added to lung cells to assess the effects of cytokine neutralization on IL-22 production. For this, anti-mouse IL-1β, IL-6, IL-18 and IL-23 (all from R&D Systems) were added to lung cell cultures at a final concentration of 2-5 μg/ml for 24 h. Rat (IL-1β, IL-6, IL-18) or Goat (IL-23) isotype antibodies were added to lung cell cultures as a

control. Supernatants were collected after 24 h, clarified by centrifugation and IL-22 levels quantified by ELISA (R&D Systems). In specific experiments, recombinant murine IL-23 (R&D Systems) was added to lung digest cells at 1 or 10 ng/ml for 24 h. Supernatants were collected after 24 h, clarified by centrifugation and IL-22 levels quantified by ELISA (R&D Systems). For lung neutrophil analysis by flow cytometry, cells were washed and Fc receptors were blocked with Mouse BD Fc Block™ (BD Biosciences, San Diego, CA) at 4°C for 20 min. Thereafter, cells were stained with a single-color LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen) followed by labeling with CD11b+ and Ly6G+ (1A8 clone) (antibodies BD Biosciences) [43].

Analysis of lung lavage fluid anti-fungal activity

Wild-type, Dectin-1 deficient and IL-22 deficient mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia. Twenty-four hours post-infection, a bronchoalveolar lavage was performed as previously described [38 31]. Lavage fluid was centrifuged to remove inflammatory cells and live *A. fumigatus*. Fifty µl of clarified lavage fluid from each strain was incubated with 1 x 10^5 *A. fumigatus* conidia (in 150 µl of RPMI supplemented with 10% FBS and 1% pen-strep) for 4 h at 37°C. Thereafter, the contents of the well were subjected to total RNA extraction using the MasterPure™ yeast RNA purification kit and analyzed for *A. fumigatus* viability as described above. RNA was also extracted from lavage fluid to assess the presence of *A. fumigatus* after centrifugation, which

demonstrated negligible levels (4-5 logs below that quantified in 50 µl lavage fluid plus 1 x 10^5 *A. fumigatus* conidia).

Lipocalin 2, *S100a8*, *S100a9* and *Reg3g* analysis

C57BL/6 wild-type (WT), Dectin-1 deficient and IL-22 deficient mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 18 h after exposure, lungs were collected, homogenized in TRIzol reagent (Invitrogen) and total RNA isolated as per the manufacturer's instructions. Thereafter, RNA was transcribed to cDNA (iScript cDNA synthesis kit, Bio-Rad), and real-time PCR for *S100a8* (Mm00496696_g1; Applied Biosystems), *S100a9* (Mm00656925_m1, Applied Biosystems) and *Reg3g* (Mm00441127_m1, Applied Biosystems) and was performed (iQ Supermix, Bio-Rad). mRNA levels were normalized to *Gapdh* mRNA levels (primers/probe from Applied Biosystems) using the $2^{-(\Delta\Delta Ct)}$ method. For lipocalin 2 quantification, C57BL/6 wild-type (WT), Dectin-1 deficient and IL-22 deficient mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 24 h after exposure, the left lung was homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche), clarified by centrifugation and stored at -80°C. Supernatants from lung homogenates were analyzed for lipocalin 2 levels by ELISA (R&D Systems).

Statistics

Data were analyzed using GraphPad Prism Version 5.0 statistical software. Comparisons between groups when data were normally distributed

were made with the Student's t-test. Significance was accepted at a value of $P < 0.05$.

Results

IL-22 production after *A. fumigatus* challenge is dependent on Dectin-1.

As we have previously reported that Dectin-1 dependent IL-17A was a critical component of lung defense against *A. fumigatus* [42], we sought to determine whether IL-22 was also dependent on beta-glucan recognition via Dectin-1 and whether it was required for *A. fumigatus* host defense. Results in Figure 1A show that IL-22 was robustly induced in the lungs after *A. fumigatus* challenge (naïve lungs have undetectable IL-22, data not shown) and required Dectin-1 mediated recognition of *A. fumigatus* as mice deficient in Dectin-1 had severely compromised production of IL-22 in the lungs. We next collected lungs from C57BL/6 (WT) and Dectin-1 deficient mice (KO) 18 h after *A. fumigatus* challenge and subjected them to enzymatic digestion to determine whether single cell suspensions could replicate the differences in IL-22 levels observed in whole lung homogenates, as we have recently reported for IL-17A [43]. Upon *ex vivo* culturing of lung cells overnight, cells from Dectin-1 deficient mice had a > 8-fold reduction in IL-22 production compared to WT lung digest cells (Figure 1B). Thus, beta-glucan recognition via Dectin-1 mediates lung IL-22 production after *A. fumigatus* challenge.

IL-22 is required for early *A. fumigatus* lung clearance.

IL-17A is acknowledged to stimulate the antimicrobial immune effector functions of multiple cell types, including neutrophils, macrophages and epithelial

cells [12]. We have previously reported that neutralization of IL-17A compromised lung clearance of *A. fumigatus* [42]. However, as IL-22 appears to primarily activate epithelial cells [45 37], we questioned whether neutralization of IL-22, based on its limited cellular targeting, would have a significant affect on innate immune clearance of *A. fumigatus*. Results in Figure 2 show that neutralization of IL-22 in the lungs of C57BL/6 mice (Figure 2A) resulted in a > 5-fold increase in *A. fumigatus* lung burden by 24 h post-infection (Figure 2B). We confirmed this finding in mice deficient in IL-22, which demonstrated a > 8-fold increase in *A. fumigatus* lung burden by 24 h post-infection (Figure 3A). Despite having higher *A. fumigatus* lung burden, IL-22 deficient mice demonstrated significantly lower levels of multiple cytokines and chemokines previously implicated in lung host defense against *A. fumigatus*, including IL-1α, TNF-α, CCL3/MIP-1α and CCL/4MIP-1β [30 13] (Figure 3B). In turn, the lack of these proinflammatory cytokines and chemokines resulted in a > 7-fold reduction in CD11b+ Ly-6G+ neutrophils in the lungs of IL-22 deficient mice (Figure 3C). We also observe reductions in IL-12p40 and IL-12p70 (Figure 3D), although IFN-γ levels were unaffected (data not shown). IL-22 has also been shown to induce CXCL9/Mig production in the lungs during bacterial pneumonia [2] and CXCL9/Mig, as well as CXCL10/IP-10, have been reported to have direct antimicrobial activity [9], suggesting the possibility that IL-22-induced CXCL9/Mig or CXCL10/IP-10 could function as an innate effector molecule against *A. fumigatus*. However, IL-22 deficient mice exposed to *A. fumigatus* did not demonstrate a reduction in CXCL9/Mig or CXCL10/IP-10 (Figure 3E), therefore diminishing a role for these

molecules in IL-22-mediated defense against *A. fumigatus*. Intriguingly, IL-17A levels were significantly increased in the lungs of *A. fumigatus* challenged IL-22 deficient mice (Figure 3F), indicating that IL-17A production in the lungs is not dependent on IL-22. Thus, IL-22 is required for optimal clearance of *A. fumigatus* from the lungs.

Impaired anti-fungal activity in lung lavage fluid from *A. fumigatus*-challenged Dectin-1 deficient and IL-22 deficient mice.

Mice deficient in the beta-glucan receptor Dectin-1 have impaired IL-17A [43] and IL-22 (Figure 1) production in the lungs in response to *A. fumigatus* and neutralization of IL-17A [42] or IL-22 (Figure 2) compromises clearance of *A. fumigatus* from the lungs. As both IL-17A and IL-22 are efficient at eliciting soluble antimicrobial factors from epithelial cells [50], we hypothesized that defects in these factors would be reflected in the antifungal activity of lung lavage fluid. Results in Figure 4A show that lung lavage fluid from Dectin-1 deficient mice demonstrated poor anti-fungal activity compared to lung lavage fluid from WT mice. Lung lavage fluid from IL-22 deficient mice also showed compromised anti-fungal activity, although this was not at the level of Dectin-1 deficient mice. We observed both a Dectin-1 dependent and IL-22-dependent (Figure 4B) induction of the siderophore binding protein lipocalin 2, which can be induced by IL-17A and IL-22 [2]. As *A. fumigatus* requires iron for growth and encodes its own siderophores [35], we hypothesized that lipocalin 2 may act as an anti-fungal agent against *A. fumigatus* by limiting *A. fumigatus* iron acquisition. To our surprise,

mice deficient in lipocalin 2 were not more susceptible to *A. fumigatus* lung infection (Figure 4C). IL-22 has also been shown to induce other antimicrobial proteins [50]. Real-time PCR analysis of *Reg3g* mRNA expression indicated low induction in response to *A. fumigatus* (1.5 to 2-fold), although intact expression in Dectin-1 deficient and IL-22 deficient mice (data not shown). Similarly, *S100a8* and *S100a9* mRNA expression was induced 15 to 25-fold in response to *A. fumigatus*, but was not modulated in Dectin-1 deficient or IL-22 deficient mice (data not shown). Thus, one mechanism of susceptibility to *A. fumigatus* in the setting of Dectin-1 or IL-22 deficiency is a putative lack of or impairment in soluble factor(s) with anti-fungal activity, however this factor(s) does not appear to be lipocalin 2, S100A8, S100A9 or RegIIIγ.

IL-22 production by lung cells in response to *A. fumigatus* is independent of IL-1β, IL-6 and IL-18, but requires IL-23.

We have previously employed the culture system in Figure 1B to determine mechanisms associated with Dectin-1 dependent IL-17A production [43]. IL-22 is recognized to be produced by IL-17A-producing CD4 T cells (Th17 cells), although other cellular sources have been described [27 40]. Along with IL-17A production, cytokines such as IL-6, IL-23 and IL-1β have also been shown to be important for IL-22 production by multiple cell types [21]. In addition, IL-18 may synergize with IL-12 or IL-23 for IL-22 induction in NK cells [44 48]. We have recently shown that neutralization of IL-23 in lung cell cultures from *A. fumigatus*-challenged mice resulted in attenuated IL-17A production [43]. Moreover, IL-23p19

deficient mice have reduced IL-22 production in a murine model of gastrointestinal candidiasis [10]. Therefore, we questioned whether lung IL-22 production was similarly dependent on IL-1β, IL-6, IL-18 or IL-23 during *A. fumigatus* infection. We have previously reported that IL-6 and IL-1β were produced at lower levels by lung cells from Dectin-1 deficient mice [43], suggesting a possible role for these cytokines in lung cell IL-17A and IL-22 production during *A. fumigatus* infection. However, neutralization of IL-1β, IL-6 or IL-18 did not significantly reduce lung cell production of IL-22 (Figure 5A). Although IL-1β neutralization appeared to lower IL-22 production, this did not reach statistical significance (P = 0.0685). Once again, however, IL-23 was a key factor in IL-22 induction, as neutralization of IL-23 resulted in a 75% decrease in IL-22 production by lung cells (Figure 5A). Results in Figure 5B show that supplementing IL-23 in lung cell cultures resulted in increased IL-22 production, even in lung cells from Dectin-1 deficient mice. Thus, IL-22 production by lung cells from *A. fumigatus* challenged mice is partially dependent on IL-23 and IL-23 can restore IL-22 production in Dectin-1 deficient mice.

Discussion

With the explosion of IL-17A-related research over the last 5 years, studies have discovered that CD4+ T cells producing IL-17A can also produce the cytokine IL-22, a member of the IL-10 family of cytokines [24] . Analogous to that observed for IL-17A, additional cell types such as lymphoid tissue-inducer cells [40], NK cells [6] and γδ T cells [27] can also produce IL-22. Although IL-22 can act in both an anti-inflammatory [47] and pro-inflammatory [49] manner, IL-22 has been reported to play a major role in stimulating epithelial anti-microbial activity and host defense against multiple mucosal pathogens [50] including the fungal organism *C. albicans* [10]. To date, only epithelial cells and keratinocytes have been identified as expressing the IL-22 receptor [45]. We have previously identified a role for Dectin-1 dependent IL-17A in host defense against *A. fumigatus* [42 43]. As IL-17A may work in tandem with IL-22 [26 2 50] and IL-23 is reportedly required for IL-22 induction in several models [10 40 37], we extended our studies here to investigate the role of Dectin-1 in the induction of IL-22 and the role of IL-22 in *A. fumigatus* lung defense. In our initial studies, we were surprised at the magnitude of Dectin-1 dependency for IL-22 production in the lungs after *A. fumigatus* challenge. Both in lung homogenates and lung cell cultures from Dectin-1 deficient mice, IL-22 was produced at less than 10% of that produced by WT mice. Although we hypothesize that some of this is due to compromised IL-23 production in Dectin-1 deficient mice [43], the dependency of IL-22 on Dectin-1 during *A. fumigatus* exposure is more striking compared to that observed in IL-23 deficient mice systemically exposed to *Candida albicans*, which demonstrated IL-

22 levels that were still a third of WT levels [10]. Coupling this observation with our data indicating that IL-22 production by lung cells is reduced by three-fourths in the presence of IL-23 neutralization leads us to hypothesize that at sites of infection, additional mediators are likely involved in optimal IL-22 production (i.e. the remaining quarter to a third in both *C. albicans* and *A. fumigatus* infections). This does not appear to be IL-6, IL-1β or IL-18, as we show that neutralization of these cytokines had no effect on IL-22 production by lung cells from *A. fumigatus* exposed mice. As IL-23 signals through IL-12Rβ1 and IL-23Rα, it is thought to activate the STAT1, STAT4, STAT3 and STAT5 signaling pathways [14]. With respect to Th17/IL-17A responses, STAT3 activation is clearly favored by IL-23 [23]. Therefore, we can speculate that an additional mediator(s) may activate STAT3, possibly other STATs as well, and synergize with IL-23 for optimal lung IL-22 production. Current studies are underway to identify additional cytokines that may be involved in IL-22 production by lung cells.

To thoroughly examine the role of IL-22 in lung host defense against *A. fumigatus*, we employed two independent experimental designs: (i) neutralization and (ii) genetic deficiency. Neutralization of IL-22 as well as IL-22 deficiency both led to significantly compromised clearance of *A. fumigatus* from the lungs. The level of impairment in fungal clearance was also more apparent with *A. fumigatus* compared to a previous report with *C. albicans*, which demonstrated 2-fold changes in stomach (gastrointestinal infection) and kidney (systemic infection) burden (3 days post-challenge) when IL-22 was genetically deficient [10]. Neutralization of IL-22 in this model had little to no effect on *C. albicans* stomach

burden in C57BL/6 mice (8 days post-challenge) and only increased kidney burden by a third in Balb/c mice [10]. In contrast, our studies revealed that IL-22 neutralization resulted in a 5-fold increase in lung *A. fumigatus* burden whereas IL-22 genetic deficiency resulted in an 8-fold increase in *A. fumigatus* burden. There are many possibilities as to why differences were observed in our study vs. the *C. albicans* study. Clearly, these two pathogens are quite different in their tissue specificities and host defense requirements, thus it is possible that host defense against one organism may require IL-22 more than the other. Moreover, our studies investigated the role of IL-22 in early/rapid host defense against *A. fumigatus*, i.e. 1 to 2 days post-challenge, in contrast to the 3 to 8 day time course of the *C. albicans* infections. In addition, it is also possible that the role of IL-22 may be more evident, perhaps even more important, in such tissues as the lung and gut where the overwhelming majority of cells are epithelial cells and keratinocytes. Nevertheless, our studies point to an essential role for IL-22 at the earliest stages of *A. fumigatus* lung infection.

As mentioned previously, we have documented a role for IL-17A in *A. fumigatus* lung defense [42] and now extend this to IL-22. Dual roles for IL-17A and IL-22 have also been observed in lung infection with *K. pneumoniae* [17 2] and gut infection with *C. rodentium* [26 50]. Surprisingly however, IL-17A and IL-22 do not always play equal roles in host defense. Cutaneous infection with *Staphylococcus aureus* is worse in γδ T cell deficient mice and correlated with a lack of IL-17A, but not IL-22 production [7]. In models of oral infection [8] and skin infection [18] with *C. albicans*, IL-17A, but not IL-22, was required for defense.

Protective immunity to systemic infection with attenuated *Salmonella enterica* serovar *enteritidis* is associated with IL-22, but not IL-17 [36], while infection with *Borrelia burgdorferi* induces a potent IL-22 response, yet IL-17A is completely absent [3]. Finally, in *Listeria monocytogenes* infection, IL-17A is required for clearance [16], but not IL-22 [15], a finding also observed in *Francisella tularensis* infection [25]. However, during *A. fumigatus* lung infection, our studies indicate that both IL-17A and IL-22 are simultaneously required for host defense. IL-17A levels are significantly increased in the lungs of IL-22 deficient mice challenged with *A. fumigatus*, yet lung clearance is impaired. In turn, we have reported that IL-17A neutralization leads to impaired *A. fumigatus* lung clearance [42], although IL-22 levels were not affected by IL-17A neutralization (1,276 ± 105 pg/ml, n=10 vs. 1,148 ± 98 pg/ml, n=10 in lung homogenates for isotype and anti-IL-17A treated mice, respectively). Therefore, in a scenario where either IL-17A or IL-22 is absent, our data would suggest that the remaining response is not sufficient enough to compensate.

A well-documented role of IL-22 in the context of host defense is in the induction of the epithelial antimicrobial response. Initial studies examining the function of IL-22 showed that stimulation of epithelial cells and keratinocytes with IL-22 led to the induction of antimicrobial defense factors such as beta-defensins, S100 proteins and RegIII proteins [50]. IL-17A also has an acknowledged role in the induction of these factors as well [2] and IL-22 can often add to or synergize with IL-17A for the induction of the epithelial antimicrobial response. Recognizing that IL-22, along with IL-17A, can evoke this response in the lungs [2] led us to

determine whether functional defects existed in the lungs of Dectin-1 deficient and IL-22 deficient mice exposed to *A. fumigatus*. To this end, we demonstrated that clarified lung lavage fluid (i.e. that was free of live *A. fumigatus* and live host cells) from both Dectin-1 deficient and IL-22 deficient mice did not kill *A. fumigatus* as robustly as lung lavage fluid from WT mice. The defect in antifungal activity was more severe in lavage fluid from Dectin-1 deficient mice, which we hypothesize is a result of these mice having significant reductions in both IL-17A [42] as well as IL-22 (Figure 1). Despite compromised S100A8 and S100A9 expression in IL-22 deficient mice intragastrically infected *C. abicans* [10], we found that *S100a8* and *S100a9* mRNA expression was intact in the lungs of *A. fumigatus* exposed Dectin-1 deficient and IL-22 deficient mice (data not shown). In addition, *Reg3g* was not found to be statistically lower in the lungs (data not shown). In contrast, we did observe a reduction in the lung levels of lipocalin 2, a siderophore binding protein induced by IL-22 [2], in both Dectin-1 deficient and IL-22 deficient mice, suggesting a possible role for lipocalin 2 in *A. fumigatus* lung defense. However, lipocalin 2 deficient mice did not demonstrate and impairment in *A. fumigatus* lung clearance, indicating that lipocalin 2 does not appear to be playing a major role in the susceptibility of Dectin-1 deficient and IL-22 deficient mice to *A. fumigatus*. Although we did not see an effect of lipocalin 2 deficiency on *A. fumigatus* lung clearance, we can not exclude the possibility that other antimicrobial factors are compensating for the loss of lipocalin 2. For example, lactoferrin can mediate ROS-independent killing of *A. fumigatus* by neutrophils [46].

Current studies are underway to identify the Dectin-1 and IL-22 dependent soluble antifungal factors induced in the lungs during *A. fumigatus* infection.

In summary, we have identified a role for IL-22 in early innate immune responsiveness to *A. fumigatus* lung infection. Induction of IL-22 was significantly dependent on *A. fumigatus* recognition by the beta-glucan receptor Dectin-1. Both neutralization of and genetic deficiency in IL-22 compromised early clearance of *A. fumigatus* from the lungs. IL-22 was critical for both the induction of lung inflammatory cytokines and chemokines as well as the lung antifungal response. However, the Dectin-1 and IL-22 dependent lung antifungal response was independent of the known IL-17A and IL-22-associated antimicrobial factor S100 proteins, RegIIIγ and lipocalin 2, suggesting a separate, yet-to-be-characterized Dectin-1 and IL-22 dependent antifungal mechanism. As with our recent report on IL-17A [43], our data further suggests that soluble mediators in addition to IL-23 may also be playing a role in Dectin-1 dependent IL-22 production. However, as our data indicates that IL-17A and IL-22 are simultaneously needed for *A. fumigatus* lung clearance, and IL-23 is essential for the induction of both cytokines during *A. fumigatus* lung infection, IL-23 may be an effective immunotherapy for the treatment of IA in susceptible individuals. In conclusion, the current body of work adds depth to our understanding of the role Dectin-1 and the IL-23/IL-17A/IL-22 axis in innate lung defense against *A. fumigatus*.

Acknowledgements

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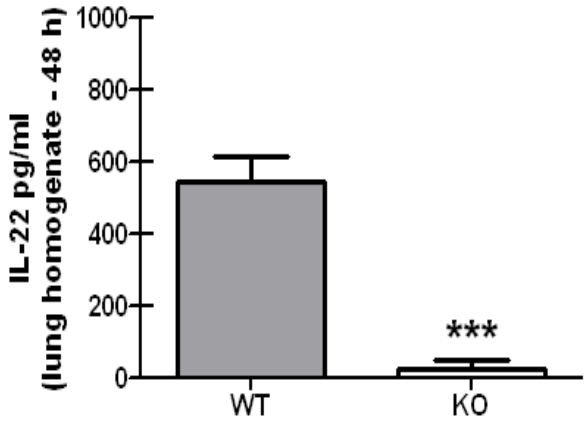
Figure 1. IL-22 production after *A. fumigatus* challenge is dependent on Dectin-1. (A) C57BL/6 wild-type (WT) and Dectin-1 deficient (KO) mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 48 h after exposure, IL-22 levels were quantified in lung homogenates by ELISA. Data are expressed as mean pg/ml + SEM. The Figure illustrates cumulative data from three independent studies (n = 5 mice/group for each study). *** represents a P value of < 0.001 (Unpaired two-tailed Student's t test). **(B)** C57BL/6 wild-type (WT) and Dectin-1 deficient (KO) mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 18 h after exposure, lungs were collected and enzymatically digested. Single cell suspensions were isolated and 1 x 10^6 cells were cultured for 24 h in a volume of 0.2 ml. IL-22 levels were quantified in clarified co-culture supernatants by ELISA. The Figure illustrates cumulative data from four independent studies. Data are expressed as mean pg/ml + SEM. *** represents a P value of < 0.001 (Unpaired two-tailed Student's t test).

A.


IL-22 pg/ml
(lung homogenate - 48 h)
1000
800
600
400
200
0
***
WT
KO

B.

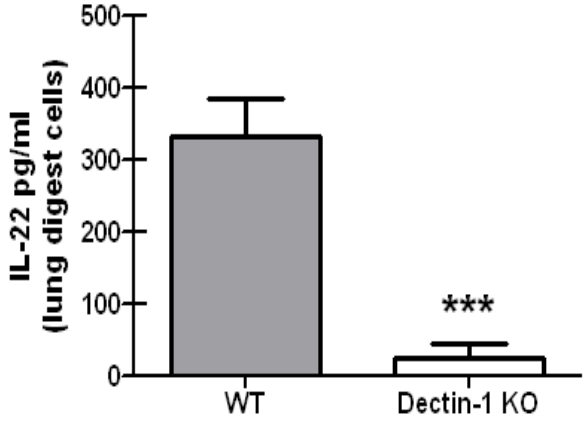
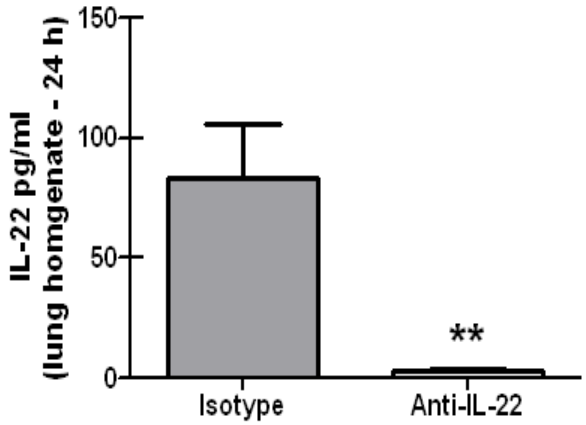

IL-22 pg/ml
(lung digest cells)
500
400
300
200
100
0
***
WT
Dectin-1 KO

Figure 2. Neutralization of IL-22 compromises early *A. fumigatus* lung clearance. (A) C57BL/6 wild-type (WT) mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia. Six hours after challenge, mice were administered 50 μg of goat anti-mouse IL-22 or goat IgG antibodies intratracheally. IL-22 levels were quantified in lung homogenates 24 h after challenge by ELISA. The Figure illustrates cumulative data from two independent studies (n = 5 mice per group per time point). Data are expressed as mean pg/ml + SEM. ** represents a P value of < 0.01 (Unpaired two-tailed Student's t test). **(B)** Real-time PCR analysis of *A. fumigatus* 18S rRNA levels in the lungs of WT mice administered anti-IL-22 or isotype control antibodies. The Figure illustrates cumulative data from two independent studies (n = 5 mice per group per time point). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM. ** represents a P value of < 0.01 (Unpaired two-tailed Student's t test).

A.


IL-22 pg/ml
(lung homgenate - 24 h)
150
100
50
0
**
Isotype
Anti-IL-22

B.

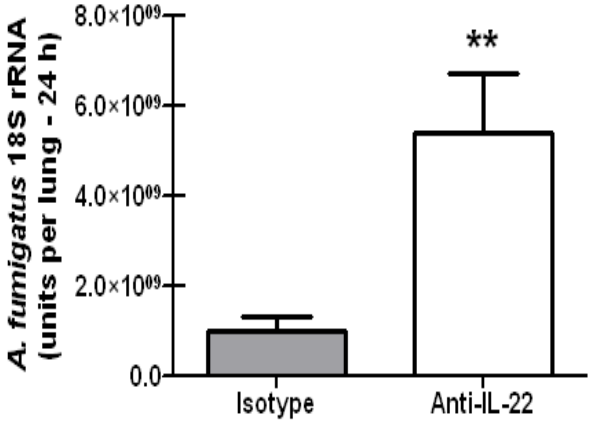

A. fumigatus 18S rRNA
(units per lung - 24 h)
8.0×10⁰⁹
6.0×10⁰⁹
4.0×10⁰⁹
2.0×10⁰⁹
0.0
**
Isotype
Anti-IL-22

Figure 3. IL-22 deficient mice have impaired *A. fumigatus* lung clearance. **(A)** C57BL/6 wild-type (WT) and IL-22 deficient (IL-22 KO) mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 24 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates cumulative data from three independent studies (n = 5 mice per group). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM. ** represents a P value of < 0.01 (Unpaired two-tailed Student's t test). **(B)** Levels of IL-1α, TNF-α, CCL3 and CCL4 and were quantified in lung homogenates collected 24 h post-infection by Bio-Plex. The Figure illustrates cumulative data from three independent studies (n = 5 mice per group per time point). Data are expressed as mean pg/ml + SEM. * and *** represent a P value of < 0.05 and 0.001, respectively (Unpaired two-tailed Student's t test). **(C)** Lung cells were isolated via bronchoalveolar lavage, Fc-blocked, stained with a live/dead staining kit and thereafter stained with fluorochrome-conjugated CD11b and Ly-6G. The Figure illustrates representative data from one of two independent studies. Data are expressed as absolute number of live cells in lung lavage fluid. * represents a P value of < 0.05 (Unpaired two-tailed Student's t test). **(D)** IL-12p40 and IL-12p70 were quantified in lung homogenates collected 24 h post-infection by Bio-Plex. The Figure illustrates cumulative data from three independent studies (n = 5 mice per group per time point). Data are expressed as mean pg/ml + SEM. * and ** represent a P value of < 0.05, and 0.01, respectively (Unpaired two-tailed Student's t test). **(E)** Levels of CXCL9 and CXCL10 and **(F)** IL-17A were quantified in lung homogenates collected 24 h

post-infection by ELISA. The Figure illustrates cumulative data from three independent studies (n = 5 mice per group per time point). Data are expressed as mean pg/ml + SEM. ** represents a P value of < 0.01 (Unpaired two-tailed Student's t test).

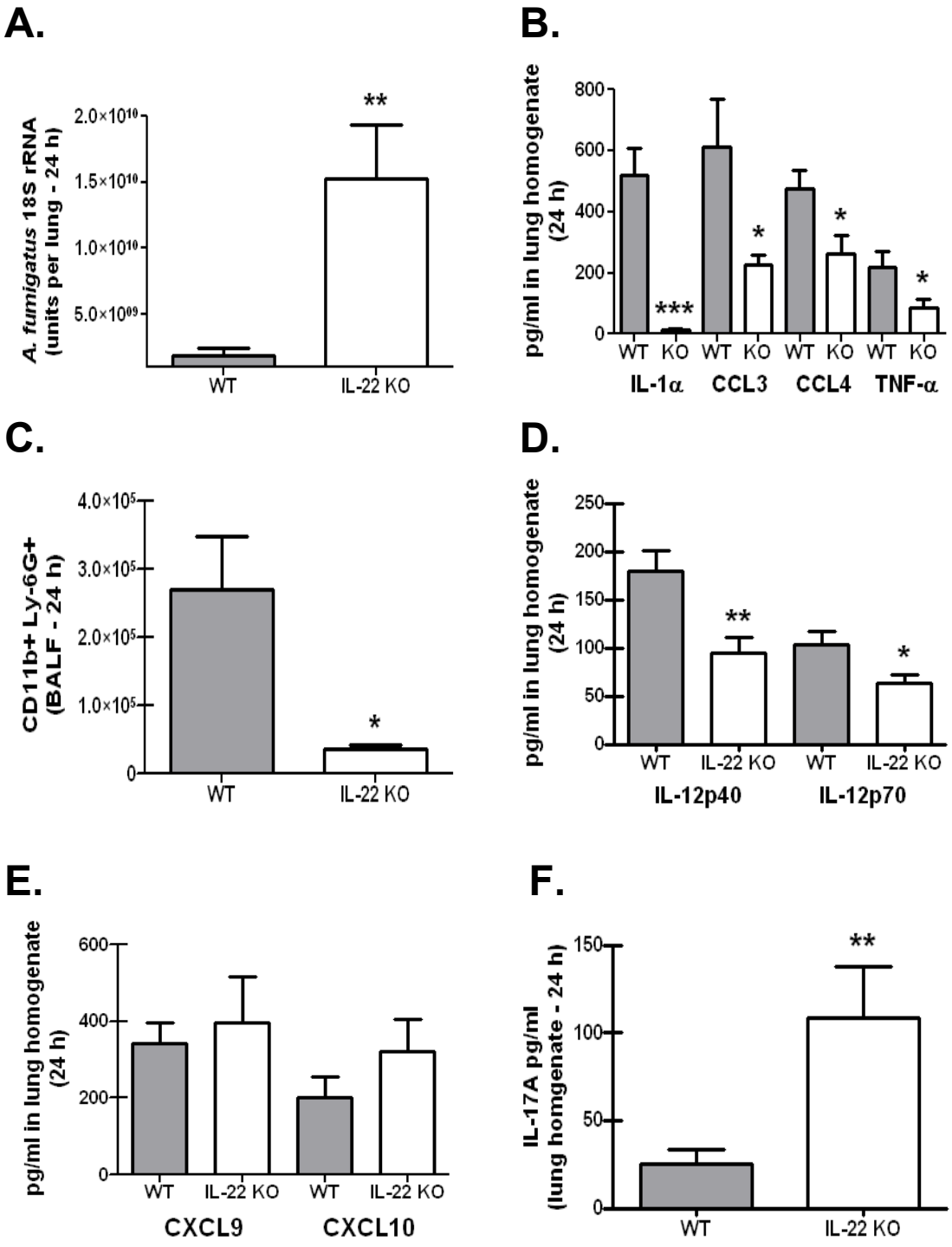
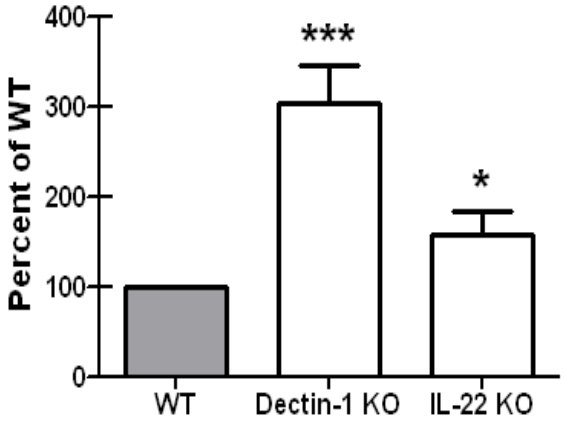

A.
A. fumigatus 18S rRNA (units per lung - 24 h)
2.0×10¹⁰
1.5×10¹⁰
1.0×10¹⁰
5.0×10⁰⁹
**
WT
IL-22 KO
B.
pg/ml in lung homogenate (24 h)
800
600
400
200
0
***
*
*
*
WT KO WT KO WT KO WT KO
IL-1α
CCL3
CCL4
TNF-α
C.
CD11b+ Ly-6G+ (BALF - 24 h)
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
*
WT
IL-22 KO
D.
pg/ml in lung homogenate (24 h)
250
200
150
100
50
0
**
*
WT
IL-22 KO
WT
IL-22 KO
IL-12p40
IL-12p70
E.
pg/ml in lung homogenate (24 h)
600
400
200
0
WT
IL-22 KO
WT
IL-22 KO
CXCL9
CXCL10
F.
IL-17A pg/ml (lung homgenate - 24 h)
150
100
50
0
**
WT
IL-22 KO

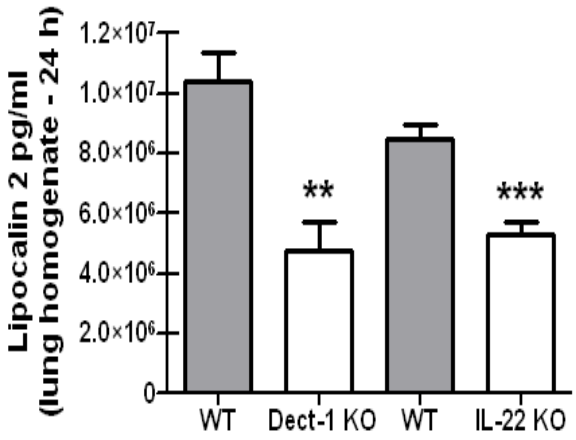
Figure 4. Impaired anti-fungal activity in lung lavage fluid from *A. fumigatus*-challenged Dectin-1 deficient and IL-22 deficient mice. (A) C57BL/6 wild-type (WT), Dectin-1 deficient (Dectin-1 KO) and IL-22 deficient (IL-22 KO) mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 24 h after exposure and bronchoalveolar lavage was performed. Lung lavage fluid was processed to remove cells and *A. fumigatus* and thereafter 50 µl of clarified lavage fluid from each strain was incubated with 1 x 10^5 *A. fumigatus* conidia (in 150 µl of RPMI supplemented with 10% FBS and 1% pen-strep) for 4 h at 37°C. Thereafter, the contents of the well were subjected to total RNA extraction using the MasterPure™ yeast RNA purification kit and analyzed for *A. fumigatus* viability. For each experiment, the percent above WT was calculated by dividing the *A. fumigatus* 18S rRNA units in Dectin-1 deficient and IL-22 deficient cultures by the *A. fumigatus* 18S rRNA units in WT cultures. WT values were set at 100. The Figure illustrates cumulative data from eight independent studies. * and *** represent a P value of < 0.05 and 0.001, respectively (Paired two-tailed Student's t test). **(B)** C57BL/6 wild-type (WT), Dectin-1 deficient and IL-22 deficient mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus* conidia and 24 h after exposure, lungs were collected, homogenized and lipocalin 2 levels quantified in clarified homogenates by ELISA. The Figure illustrates cumulative data from two independent studies with n = 4-5 per group. ** and *** represent a P value of < 0.01 and 0.001, respectively (Unpaired two-tailed Student's t test). **(C)** C57BL/6 wild-type (WT) and lipocalin 2 deficient mice (Lcn2 KO) mice were challenged intratracheally with 5-7 x 10^7 *A. fumigatus*

conidia and 24 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates cumulative data from two independent studies (n = 5 mice per group). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM.

A.


Percent of WT
400
300
200
100
0
***
*
WT
Dectin-1 KO
IL-22 KO

B.


Lipocalin 2 pg/ml
(lung homogenate - 24 h)
1.2×10⁷
1.0×10⁷
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
**
***
WT
Dect-1 KO
WT
IL-22 KO

C.

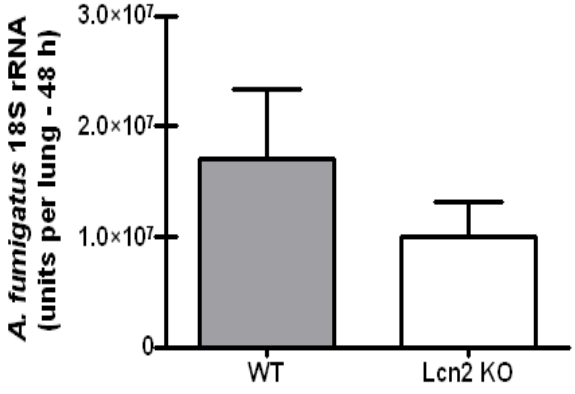
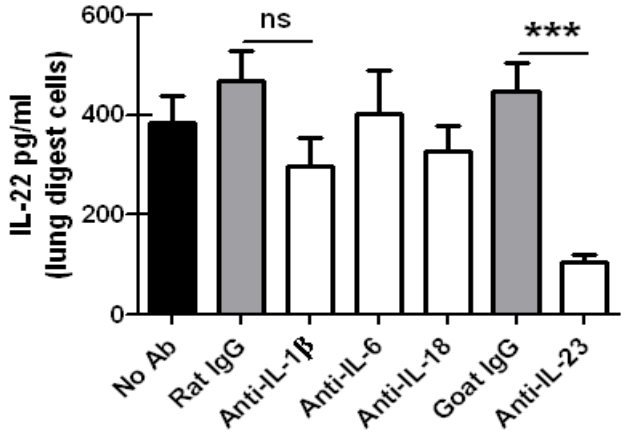
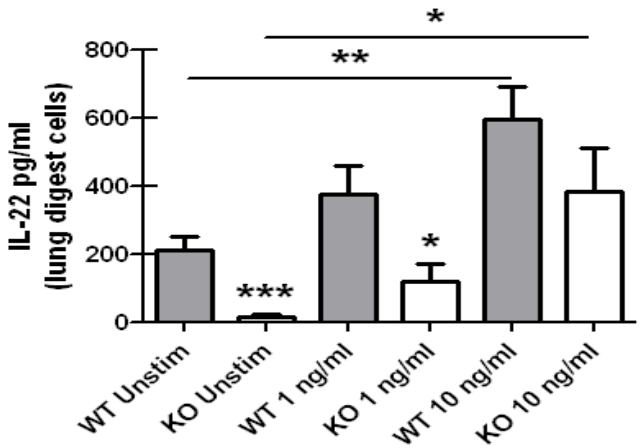

A. fumigatus 18S rRNA
(units per lung - 48 h)
3.0×10⁷
2.0×10⁷
1.0×10⁷
0
WT
Lcn2 KO

Figure 5. IL-22 production by lung cells in response to *A. fumigatus* is independent of IL-1β, IL-6 and IL-18, but requires IL-23. (A) Lung cells were isolated as described and 1 x 10^6 cells were cultured for 24 h in a volume of 0.2 ml. Neutralizing antibodies against IL-1β, IL-6, IL-18 and IL-23 were added at a final concentration of 2-5 μg/ml at the beginning of the culture. Rat (IL-1β, IL-6, IL-18) or Goat (IL-23) isotype antibodies were included as a control. IL-22 levels were quantified in clarified co-culture supernatants by ELISA. The Figure illustrates cumulative data from two independent studies with each condition (isotype, neutralizing antibody) run in triplicate. Data are expressed as mean pg/ml + SEM. *** represents a P value of < 0.001 (Unpaired two-tailed Student's t test). **(B)** Lung cells were isolated from WT and KO mice as described and 1 x 10^6 cells were cultured for 24 h in a volume of 0.2 ml. Recombinant murine IL-23 was added at 1 and 10 ng/ml at the beginning of the culture. Controls included lung cells cultured in the absence of IL-23. IL-22 levels were quantified in clarified co-culture supernatants by ELISA. The Figure illustrates cumulative data from three independent studies. Data are expressed as mean pg/ml + SEM. *, ** and *** represent a P value of 0.05, 0.01 and 0.001, respectively (Unpaired two-tailed Student's t test).

A.


IL-22 pg/ml
(lung digest cells)
600
400
200
0
ns
***
No Ab
Rat IgG
Anti-IL-1β
Anti-IL-6
Anti-IL-18
Goat IgG
Anti-IL-23

B.


IL-22 pg/ml
(lung digest cells)
800
600
400
200
0
*
**
***
*
WT Unstim
KO Unstim
WT 1 ng/ml
KO 1 ng/ml
WT 10 ng/ml
KO 10 ng/ml

CHLORINE GAS EXPOSURE INCREASES SUSCEPTIBILITY TO INVASIVE LUNG FUNGAL INFECTION

by

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Abstract

Chlorine (Cl_2) is a highly irritating and reactive gas with potential occupational and environmental hazards. Acute exposure to Cl_2 induces severe epithelial damage, airway hyperreactivity, impaired alveolar fluid clearance and pulmonary edema in the presence heightened inflammation and significant neutrophil accumulation in the lungs. Herein, we investigated whether Cl_2 exposure affected the lung antimicrobial immune response leading to increased susceptibility to opportunistic infections. Mice exposed to Cl_2 and challenged intratracheally 24 h thereafter with the opportunistic mold *Aspergillus fumigatus* demonstrated a > 500 fold increase in *A. fumigatus* lung burden 72 h post-challenge compared to *A. fumigatus* mice exposed to room air. Cl_2-exposed *A. fumigatus* challenged mice also demonstrated significantly higher lung resistance following methacholine challenge and increased levels of plasma proteins (albumin and IgG) in the bronchoalveolar lavage fluid. Despite enhanced recruitment of inflammatory cells to the lungs of Cl_2-exposed *A. fumigatus* challenged mice, these cells (> 60% of which were neutrophils) demonstrated a profound impairment in generating superoxide. Significantly higher *A. fumigatus* burden in the lungs of Cl_2 exposed mice correlated with enhanced production of IL-6, TNF-α, CXCL1, CCL2 and CCL3. Surprisingly however, Cl_2-exposed *A. fumigatus* challenged mice had a specific impairment in the production of IL-17A and IL-22 in the lungs compared to mice exposed to room air and challenged with *A. fumigatus.* In summary, our results indicate that Cl_2 exposure markedly

impairs the antimicrobial activity and inflammatory reactivity of myeloid cells in the lung leading to increased susceptibility to opportunistic pathogens.

Keywords: Inflammation, neutrophils, *Aspergillus fumigatus*, lung injury

Introduction

Exposure to chlorine (Cl_2), either through chemical disasters, such as railway spills, or passive exposure, such as inhalation of disinfectants, can result in profound changes in the lung. Even minute exposure to Cl_2 (1-5 ppm) can lead to acute changes in forced expiratory volume at 1 second (FEV1) and functional residual capacity (FRC) (45). It is known that the extent of symptoms leading up to lung injury can vary depending on factors such as exposure concentration, exposure duration, ventilation and host susceptibility (reviewed in (20)). While the most well-studied effects of Cl_2 exposure on lung physiology in humans have been in low-dose challenge studies or assessments within the first few days after acute exposure, the effects of Cl_2 exposure on long-term lung complications have also been reported. Workers in various industrial settings that are chronically exposed to low-dose Cl_2 reveal incidences of decreased pulmonary function, such as diffusing capacity and FEV/FVC ratios, over time (reviewed in (20)). Case reports have documented incidences of obstructive lung disease several years after a single Cl_2 gas exposure (47) (48). The development of irritant induced asthma, also called Reactive Airways Dysfunction Syndrome or RADS, is another reported long-term consequence of Cl_2 gas exposure (12).

A commonality between obstructive airway diseases, such as COPD and asthma, is that microorganisms may often contribute to the lung phenotype. With regards to COPD, lung exacerbations are often thought to be microbial in origin (50). Over a decade ago, the “vicious circle hypothesis” was proposed for COPD, which states that cigarette smoke impairs innate lung defenses allowing microbial

pathogens to become established in the lower respiratory tract, leading to chronic inflammation and lung destruction (49). In the context of asthma, exposure to or contamination of allergens with microorganism-derived compounds, such as lipopolysaccharide (LPS, from Gram-negative bacteria, (55)) or fungal cell wall components (chitin, beta-glucans; (59)), often leads to rapid lung responsiveness and reactions such as airway hyperreactivity. Moreover, reports indicate that among severe asthmatics, sensitivity to fungi range from 25% to over 70% (reviewed in (19)) and correlate with hospital/ICU admissions compared to asthmatics that do not require hospitalization (1). As Cl_2 gas exposure may lead to an obstructive lung disease (47) (48) or asthma-like phenotype (12) over time, it is conceivable that microbial colonization or infection may occur after Cl_2 gas exposure.

According to the Office of The Surgeon General, United States Army (58), Cl_2 gas exposure is of historical significance during wartime. Incidences of chronic bronchitis were thought to be common after World War I chlorine inhalant exposures, which were thought to occur in association with pulmonary infections. It was further documented that chronic or progressive illness was more likely to have resulted from a combination of inadequately treated complicating infections and cigarette smoking. Finally, bacterial superinfection was commonly noted within 5 days post-exposure to Cl_2 (58). These observations suggest that lung infections are a possible consequence of Cl_2 gas exposure. In the current study, we investigated the effects of Cl_2 gas exposure on the lung immune response to *Aspergillus fumigatus*. *A. fumigatus* is a ubiquitous mold inhaled daily by humans

that is normally cleared by the lung innate immune system. In susceptible individuals, however, *A. fumigatus* can cause life-threatening invasive fungal infections (invasive pulmonary aspergillosis, IPA) (30) (31) (2). We show here that Cl_2 gas exposure negatively affects cellular and inflammatory responses critical for the elimination of *A. fumigatus* from the lungs and results in significant increases of airway hyperreactivity and alveolar permeability to plasma proteins.

Materials and Methods

Mice

C57BL/6 male mice (8 week old, 20 g body weight) were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained in a specific pathogen free environment in microisolator cages within an American Association for Laboratory Animal Science-certified animal facility in the Bevill Biomedical Research Building II at the University of Alabama at Birmingham. Animal studies were reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC).

Chlorine (Cl_2) gas exposure

Mice were exposed to Cl_2 gas (400 ppm) in a cylindrical glass chamber for 30 minutes, as previously described (34) (52) (71) (67), and returned to room air. Continuous measurements of Cl_2 concentrations during the exposure were monitored with an Interscan Corporation (model RM34-1000 m) Cl_2 detector, connected to a data logger for data storage.

Preparation of *A. fumigatus*, *in vivo* challenge and lung fungal burden assessment

A. fumigatus isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5-7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate buffered saline supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40 μm

nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. Twenty-four hours post-Cl_2 exposure, mice were lightly anesthetized with isoflurane and administered 7 x 10^7 *A. fumigatus* conidia in a volume of 50 µl intratracheally. Briefly, mice are held in a vertical, upright position and the tongue is withdrawn from the mouth using forceps. A pipette is used to deliver the 50 µl inoculum to the caudal oropharynx in which normal breathing results in fluid aspiration into the lungs (41). Controls included mice exposed to Cl_2 and administered PBS intratracheally and mice exposed to air and then challenged with *A. fumigatus*. For lung fungal burden analysis, the left lungs were collected and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure™ yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI), which includes a DNAse treatment step to eliminate genomic DNA as previously reported (36). Total RNA was also extracted from serial 1:10 dilutions of live *A. fumigatus* conidia (10^1 – 10^9) and DNAse treated to form a standard curve. Lung *A. fumigatus* burden was analyzed with real time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession number AB008401 (11)) and quantified using a standard curve of *A. fumigatus* conidia as previously described (36). As a validation of the real-time PCR method, heat-killed *A. fumigatus* did not yield a signal by real-time PCR and were unable to grow on potato dextrose agar plates (36). In addition, no amplification controls (i.e. no reverse transcriptase included in the cDNA reaction) yielded a signal of <0.001% by real-time PCR, indicating that the

DNAse treatment step was efficient at eliminating contaminating *A. fumigatus* DNA (as DNA is not predicative of organism viability (27)).

Lung injury, inflammatory cell and lung function analysis

For lung injury analysis, 72 h post-*A. fumigatus* challenge, a bronchoalveolar lavage (BAL) was performed as previously described (34) (52) (71) (67). The BAL fluid was spun at 150 x g for 10 min at 4°C to pellet cells and cellular debris. Protein concentrations in cell free BALF samples were measured with the Micro BCA* Protein Assay Reagent Kit (Pierce, Rockford, IL) using the microtiter plate protocol as previously described (34) (52) (71) (67). Equal volumes of BAL fluid were separated by denatured SDS-PAGE (10%) and transferred to PVDF membranes and immunoblotted for murine albumin with using goat anti-mouse albumin (GeneTex, Irvine, CA) and anti-goat IgG-horseradish peroxidase (HRP; Santa Cruz Biotechnology, Dallas TX) or murine IgG using chicken anti-H+M+R IgG Fc (Abcam, Cambridge, MA) and rabbit anti-chicken IgY-H,L-HRP (Abcam, Cambridge, MA). Protein bands were revealed by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL) and exposed to X-ray films. For assessment of inflammatory cells, live and dead cells collected from BAL fluid were enumerated using trypan blue staining. Cell differential counts were determined from 300 live cells per cytospin slide, which were prepared using a cytospin centrifuge (Shandon, Pittsburgh, PA) and stained with Wright protocol (Kalamazoo, MI).

For measurement of airway reactivity and lung resistance and elastace, mice were anesthetized with an intraperitoneal injection of Nembutal (sodium pentobarbital; 50 mg/kg body weight), paralyzed with an intraperitoneal injection of Pavulon (pancuronium bromide; 2 mg/kg body weight) intubated, connected to a ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada), and ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2 ml, with a positive end-expiratory pressure of 3 cm H_2O. Newtonian resistance, total respiratory system resistance and elastance were recorded continuously, as previously described (61) (52). Baseline was set via deep inhalation. Increasing concentrations of methacholine chloride (0–40 mg/ml; Sigma-Aldrich, St. Louis, MO) were administered via aerosolization within an administration time of 10 seconds. Airway responsiveness was recorded every 15 seconds for three minutes after each aerosol challenge. Broadband perturbation was used, and impedance was analyzed via constant phase model.

Whole lung cytokine and chemokine analysis

Seventy-two hours post-*A. fumigatus* exposure, the left lung was excised homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche), clarified by centrifugation and stored at -80°C. Supernatants from lung homogenates were analyzed for protein levels of 23 cytokines and chemokines using Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) according to the manufacturer's instructions (61) (26). The data were analyzed

using Bio-Plex Manager software (Bio-Rad Laboratories). IL-23 and IL-22 levels were quantified by ELISA (R&D Systems) as previously described (61) (26)

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Assessment of rates of superoxide production by lung inflammatory cell levels

The rate of superoxide (O_2^-) generated by inflammatory cells in the BALF was measured by monitoring the reduction of cytochrome C (23) with a Shimadzu UV-2501PC spectrophotometer (Kyoto, Japan) at 37°C. A BAL was performed on air or Cl_2 exposed mice 72 h post-*A. fumigatus* challenge and lavaged inflammatory cells were resuspended in one ml PBS and kept at 4°C until the time of the experiment. One ml of buffer (10 mM Potassium Phosphate with 100μM DTPA at a pH of 7.4 and 50 nM of Cytochrome C (Sigma-Aldrich , St. Louis, MO) containing 2 x 10^6 cells was added to a spectrophotometer cuvette. The reference cuvette contained buffer and cytochrome C in the absence of cells. Both cuvettes were heated to 37°C. Absorbance at 550 nM was continuously recorded for 3 min. At this time, PBS or 100 ng of Phorbol 12-Myristate 13-Acetate (PMA, Sigma-Aldrich) in DMSO was added into the reference and measurement cuvettes respectively and absorbance was measured for approximately 5 min. Superoxide disumutase (SOD; 200 units Sigma-Aldrich) and catalase (250 units; Sigma-Aldrich) were then added in both cuvettes and absorbances were measured for an additional 5 min. Bone marrow-derived neutrophils were provided courtesy of Dr. Jaroslaw Zmijewski (Department of Medicine, UAB).

Statistics

Data were analyzed using GraphPad Prism Version 5.0 statistical software. Comparisons between multiple groups when data were normally distributed were made with one-way ANOVA and with Student's t-test when comparing two groups. Significance was accepted at a value of $P < 0.05$.

Results

Compromised lung microbial clearance after Cl_2 exposure.

Although the effects of Cl_2 gas exposure on lung inflammation, airway epithelial cell injury and pulmonary edema are well described (34) (52) (71) (67), the effects on lung antimicrobial responses has not been addressed. To determine whether Cl_2 gas exposure renders mice susceptible to infection, we exposed mice to 400 ppm Cl_2 for 30 min and returned them to room air. At this Cl_2 regimen, C57BL/6 mice develop significant airway hyperreactivity, which is still present seven days post exposure, but have mortality less than 10% (52). Moreover, it has been documented that in the setting of chlorine accidents, exposures may be as high as 400 ppm within 75 yards of the spill site for as long as 7 hours post-spill (60). In humans, concentrations above 400 ppm may be fatal (44) (29). Twenty-four hours thereafter, mice were challenged intratracheally with the opportunistic mold *Aspergillus fumigatus.* Results in Figure 1 show that 72 h after challenge, mice exposed to Cl_2 had a > 3-log increase in live fungal organisms compared to mice exposed to room air and challenged with *A. fumigatus*. Thus, Cl_2 gas exposure results in sustained impairment in lung microbial clearance.

Increased lung injury in Cl_2 exposed mice after microbial challenge.

To determine whether Cl_2 exposure followed by microbial challenge resulted in increased injury to the alveolar epithelium and microvascular

endothelium, we assessed the levels of serum albumin and IgG in BALF by western blotting (34). Representative blots are shown in Figures 2A and 2B and cumulative data of mean densitometry values presented in Figures 2C and 2D. We have previously reported that exposure of mice to 400 ppm of Cl_2 for 30 min leads to increased concentrations of albumin and IgG in the BAL at 30 min and 24 h post exposure (34). Data shown in Figure 2 indicate that at 72 h post exposure, BAL albumin and IgG are slightly elevated or at control levels, indicating that Cl_2 induced injury to the blood gas barrier was in the early stages of repair. *A. fumigatus* challenge resulted in significant increases in albumin (Figure 2A, 2C) and IgG (Figure 2B, 2D) levels in BALF compared to mice exposed to air. However, mice exposed to Cl_2 followed by *A. fumigatus* challenge demonstrated the highest amount of albumin (Figure 2A, 2B) and IgG (Figure 2C, 2D) in BALF suggesting that microbial infection post-Cl_2 exposure results in increased lung injury consistent with the development of pulmonary edema.

Impaired pulmonary function in Cl_2 exposed mice after microbial challenge.

As data in Figure 2 suggested increased lung injury in mice challenged with *A. fumigatus* after Cl_2 exposure, we determined the magnitude this translated into decrements in lung function. We show that mice challenged with *A. fumigatus* after Cl_2 exposure demonstrated higher total lung resistance (Figure 3A) and elastance (Figure 3B) prior to and following challenge with methacholine, as opposed to mice exposed to either Cl_2 or *A. fumigatus* alone. Intriguingly, major airway resistance, although trending higher, was not significantly increased

in mice challenged with *A. fumigatus* after Cl_2 exposure (Figure 3C). Thus, coupled with the lung injury data in Figure 2, lung infection after Cl_2 exposure increases airway and alveolar epithelial injury and impairs lung function consistent with the development of reactive airway disease syndrome.

Cl_2 exposed mice demonstrate intact lung myeloid cell recruitment, but impaired antimicrobial activity, after microbial challenge.

A component of the lung response to acute Cl_2 gas exposure is the recruitment of neutrophils in the lung interstitial and alveolar spaces (67). Although often viewed as a contributor to lung injury, neutrophils are essential for the clearance of pathogens from the lung, including *A. fumigatus* (6) (24) (53). We determined whether microbial challenge after Cl_2 gas exposure modulated the recruitment of inflammatory cells, including neutrophils. Mice were exposed to Cl_2 and *A. fumigatus* as described in Figure 1 and inflammatory cell numbers in lung lavage fluid were quantified by differential staining. Results in Figure 4A show that recruitment of myeloid cells in response to microbial challenge is not impaired by Cl_2 gas exposure. In fact, Cl_2 exposure followed by *A. fumigatus* challenge resulted in significantly higher recruitment of lymphocytes, monocytes and neutrophils to the lungs compared to Cl_2 exposure alone. However, as Cl_2 exposed mice were unable to clear *A. fumigatus*, yet neutrophils were present in high numbers, we determined whether the recruited inflammatory cells produced reactive oxygen species, which are essential for neutrophil antifungal activity against *A. fumigatus* (43) (22). Results in Figure 4B (representative data) and 4C

(cumulative data) show that despite functional migration to the lungs of Cl_2 exposed mice challenged with *A. fumigatus*, inflammatory cells had a profound impairment in ROS production as measured by cytochrome c reduction. In fact, bone marrow-derived neutrophils treated with PMA reduced cytochrome c 10-times faster than myeloid cells (approximately 60% neutrophils) in the BALF fluid of mice breathing air and challenged with *A. fumigatus* (data not shown). Thus, Cl_2 gas exposure negatively affects inflammatory cell/neutrophil antimicrobial activity in mice exposed to a pathogenic microbe.

Comprehensive assessment of proinflammatory cytokine and chemokine levels in the lungs of Cl_2 exposed mice after microbial challenge.

As stated earlier, Cl_2 gas exposure induces inflammatory changes in the lung, which is often characterized by the recruitment of neutrophils and induction of such cytokines as IL-6, TNF-α and CXCL1/KC (52) (56). We therefore questioned whether these proinflammatory mediators were modulated by Cl_2 after microbial challenge. Results in Figure 5 show that Cl_2 did not have a negative effect the production of IL-1α, IL-1β, IL-6 and TNF-α after *A. fumigatus* challenge (Figure 5A). Similarly, robust CXCL1/KC, CCL2/MCP-1 and CCL3/MIP-1α chemokine production was observed in Cl_2 exposed mice after microbial challenge (Figure 5B), most likely as a result of higher fungal burden in the Cl_2 exposed group (Figure 1). In contrast, the levels of IL-12p40, which is a component of the cytokines IL-12p70 and IL-23, was significantly decreased by Cl_2 gas exposure (Figure 5C). G-CSF is a potent growth factor for neutrophil survival and

mobilization from the bone marrow (18) and has been shown to augment neutrophil-mediated killing of *A. fumigatus* (35). Intriguingly, G-CSF levels after microbial challenge were not impaired by Cl_2 exposure (Figure 5C). Thus, Cl_2 gas exposure does not impair proinflammatory cytokine and chemokine production after microbial challenge, yet does inhibit the production of specific myeloid-derived cytokines, such as IL-12p40.

Impaired induction of neutrophil-derived IL-17A and IL-22 in Cl_2 exposed mice after microbial challenge.

The inflammatory cytokines IL-17A and IL-22 are essential for host defense against multiple viral and bacterial lung pathogens (21) (5) (33) (4). A major function of IL-17A and IL-22 in the lung is the induction of epithelial antimicrobial responses (4). We have previously reported that lung clearance of *A. fumigatus in vivo* also requires IL-17A (61) and IL-22 (26). Moreover, we have reported that the lung cellular source of IL-17A and IL-22 during acute *A. fumigatus* infection is neutrophils (62) (26). Finally, we have shown that optimal production of both IL-17A (62) and IL-22 (26) require IL-23, which is produced by dendritic cells in a Dectin-1 dependent manner. IL-23 is comprised of IL-12p19 and IL-12p40 (42). As IL-12p40 was lower in Cl_2 exposed mice after microbial challenge, we questioned whether Cl_2 gas targeted the IL-23/IL-17A/IL-22 axis while leaving other inflammatory axes (such as chemokine production) intact. Results in Figure 6A show that 72 h after challenge, despite having significantly higher *A. fumigatus* lung burden (Figure 1), mice exposed to Cl_2 had significantly

lower IL-17A and IL-22 levels in the lungs compared to mice exposed to room air and challenged with *A. fumigatus*. Lower IL-17A and IL-22 72 h after challenge are likely a result of an early impairment in IL-23 production, as mice exposed to Cl_2 had significantly lower IL-23 levels 24 h, but not 72 h, after challenge (Figure 6B). Thus, Cl_2 exposure impairs the ability of neutrophils to produce IL-17A and IL-22, a major function of which is to induce the epithelial antimicrobial response.

Discussion

Clinical evidence indicates that exposure to Cl_2 gas has the potential to induce a lung environment that is conducive to the development of infection (63). In fact, antibiotics are often prescribed to individuals exposed to Cl_2 as a prophylactic measure to control the development of infection (63). Assessing this experimentally, we show here that exposure to Cl_2 gas results in profound immune suppression leading to the development of an invasive fungal infection that is most often observed in individuals with defects in neutrophil function (6) (24) (53). Moreover, Cl_2 exposed mice challenged with *A. fumigatus* demonstrated greater lung injury and poorer lung function compared to Cl_2 exposure alone indicating that exposure to microbes following an exposure to Cl_2 may exacerbate Cl_2-associated toxicity.

We first asked the question of whether exposure of mice to a dose of Cl_2 that induces lung injury (34) (52) (71) (67) rendered mice more susceptible to microbial challenge. For these studies, we chose the opportunistic fungal organism *Aspergillus fumigatus*, as it is ubiquitous in the environment, inhaled daily by humans and causes infections only in those with severe immune suppression (30) (31) (2). Despite a Cl_2 exposure regimen that results in lung injury, but rarely mortality, this level of exposure had a dramatic effect on antimicrobial lung clearance mechanisms. We quantified *A. fumigatus* lung burden via real-time PCR measurement of *A. fumigatus* 18S rRNA in lung tissue, which is the most sensitive method for the determination of lung fungal burden in experimental aspergillosis (51). Employing a standard inoculum of 70 million

conidia, which is based on published reports employing inoculums at this concentration when assessing fungal clearance in animals that are not chemically or genetically immunosuppressed (7) (8) (25) as well as our own work (61) (26), it is impressive to note that three days after challenge, Cl_2 exposed mice had the equivalent of 27 million live organisms in their lungs compared to only 49,000 live organisms in the lungs of mice exposed to air. This inability of mice exposed to Cl_2 to clear *A. fumigatus* led to decrements in lung physiological measurements. More specifically, when compared to mice exposed to Cl_2 alone, lung resistance was enhanced in Cl_2 exposed mice challenge with *A. fumigatus* 166% at baseline which increased to 242% at the highest dose of methacholine challenge. Similarly, lung elastance was enhanced in Cl_2 exposed mice challenge with *A. fumigatus* 153% at baseline which increased to 224% after methacholine challenge. Further insight into decreased lung function was revealed by characterization of albumin and IgG levels in lung lavage fluid, which demonstrated much higher levels in Cl_2 exposed mice challenge with *A. fumigatus*. Collectively, Cl_2 gas exposure renders the lung susceptible to microbial infection and this combination leads to accentuated pulmonary edema, lung leakage and compromised respiratory mechanics.

Our data indicates that in addition to severely injuring the lung, Cl_2 gas exposure compromises neutrophil defense mechanisms leading to impaired antimicrobial immunity. Neutrophil deficiency/dysfunction is the hallmark predisposing factor for the development of invasive fungal infection caused by *A. fumigatus* (6), (24). However, recruitment of neutrophils to the lung is a hallmark

of Cl_2 gas exposure (16) (57) (71). Indeed, we observed that Cl_2 exposed mice challenge with *A. fumigatus* had much higher neutrophil levels than either air exposed mice challenge with *A. fumigatus* or mice exposed to Cl_2 alone. This heightened recruitment of neutrophils directly correlated with enhanced production of CXCL1/KC and CCL3/MIP-1α, two chemokines documented to play a critical role in the recruitment of neutrophils to the lungs during *A. fumigatus* infection (38) (39). Collectively, these observations suggest that Cl_2 gas exposure does not impair the generation of pro-neutrophil chemokine production nor does Cl_2 inhibit the ability of neutrophils to respond to chemotactic signals induce by a lung microbial infection.

Neutrophils kill *A. fumigatus* through multiple mechanisms, including oxidative mechanisms, such as ROS (43) and MPO (3), and non-oxidative mechanisms, such as lactoferrin (70), pentraxin 3 (28) and calprotectin (9). Of these, ROS, and specifically superoxide, is considered the most indispensable for the killing of *A. fumigatus* by neutrophils. Indeed, NADPH oxidase deficiency in humans, i.e. chronic granulomatous disease, is uniquely associated with the development of IPA (22). Moreover, mice deficient in the NADPH oxidase subunits gp91 or p47 are arguably the most susceptible mouse strain for the development for experimental *A. fumigatus* infection (43), (40). Although neutrophil recruitment was not impaired in Cl_2 exposed mice challenge with *A. fumigatus*, these mice had significantly higher lung burden compared to air exposed mice challenged with *A. fumigatus*. As neutrophils are required for the elimination of *A. fumigatus* from the lungs in humans and experimental animal

models (6) (24) (53), the probability was high that Cl_2 exposure results in defects in neutrophil-mediated defenses. Indeed, we further discovered that inflammatory cells (nearly two-thirds of which were neutrophils) isolated from the lungs of Cl_2 exposed mice challenged with *A. fumigatus* were incapable of producing reactive oxygen species. As macrophages from mice deficient in NADPH oxidase do not have impaired killing activity against *A. fumigatus* (40) (10), these results suggest that impaired ROS production by neutrophils contributes to impaired lung clearance of *A. fumigatus* in Cl_2 exposed mice. It would be important in future studies to determine the extent by which Cl_2 affects assembly of the NADPH oxidase complex in neutrophils. Although neutrophils are often documented to be part of the lung response to Cl_2 gas exposure, and thus deemed a harmful contributor to lung injury, experimental studies have shown that some therapeutic interventions after Cl_2 exposure may ameliorate lung injury and/or lung function without having a significant impact on neutrophil numbers (16) (52) (67). This would suggest that some aspects of Cl_2-induced lung injury are not mediated by neutrophils. In turn, our data suggests that after Cl_2 gas exposure, neutrophils have an ability to respond to chemokines, yet an inability to respond to microbes via the production of ROS. This defect in host defense leads to increased susceptibility to infection or long-term microbial colonization.

Dectin-1 is a 43-kDa, type II transmembrane receptor containing a single cytoplasmic immunoreceptor tyrosine activation motif and a single extracellular C-type lectin recognition domain, and is the predominant receptor in both humans (65) (64) and mice (54) for beta-1,3 glucans (13) (14), the major

component of the fungal cell wall. We have shown that mice deficient in Dectin-1 are highly susceptible to lung infection with *A. fumigatus* (61). We have further reported that a component of susceptibility in Dectin-1 deficient was lower lung production of IL-17A (61) (62) and IL-22 (26). Neutralization of either IL-17A (61) or IL-22 (26) results in impaired clearance of *A. fumigatus*, illustrating a critical role for IL-17A and IL-22 in pathogen elimination. As lung production of IL-17A and IL-22 in response to *A. fumigatus* was compromised by Cl_2 exposure, it can be postulated that Cl_2 affects Dectin-1 expression or signaling and neutrophil-mediated production of IL-17A and IL-22. Ongoing studies are examining these possibilities. Although multiple cell types may express the receptor for IL-17A (68) (69), epithelial cells represent the predominant cell type in the lung that respond to IL-17A (37). In contrast, the receptor for IL-22 has not been observed to be expressed by myeloid cells and is exclusively expressed on epithelial cells and keratinocytes (66). Both IL-17A and IL-22 stimulate multiple inflammatory and host defense/antimicrobial pathways in mucosal epithelial cells (4) (72). Furthermore, we have reported that clarified lung lavage fluid from Dectin-1 deficient (lower IL-17A and IL-22) and IL-22 deficient mice (lack IL-22) has an attenuated ability to limit the growth of *A. fumigatus in vitro* compared to lung lavage fluid from WT mice (26). Collectively, the lack of robust IL-17A and IL-22 production in the lungs of Cl_2 exposed mice after microbial challenge suggests that a component of Cl_2-induced immunosuppression is lower induction of soluble antimicrobial factors via the IL-17A and IL-22 axis. As IL-17A and IL-22 are essential effector cytokines that coordinate the immune response to multiple

lung pathogens, including *A. fumigatus* (61) (26), influenza (46) (33), *Pseudomonas aeruginosa* (15), *Klebsiella pneumonia* (4) and *Staphylococcus aureus* (17) (32), our finding has broad implications for lung host defense mechanisms impaired by Cl_2 gas exposure (and potentially other agents that target the respiratory tract).

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Figure 1. Compromised lung microbial clearance after Cl_2 exposure. C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 72 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates cumulative data from two independent studies (n = 5 mice/group for each study). Each circle represents an individual mouse. *** represents a P value of < 0.001 (Unpaired two-tailed Student's t test).

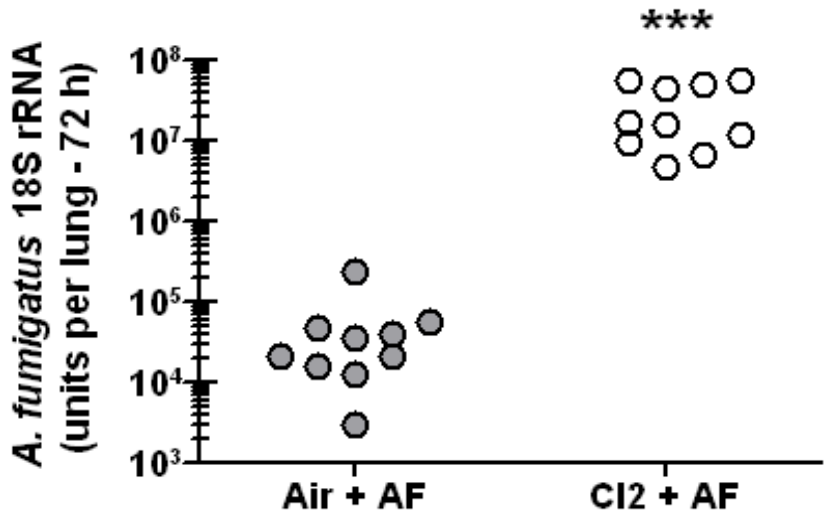

***
A. fumigatus 18S rRNA
(units per lung - 72 h)
10^8
10^7
10^6
10^5
10^4
10^3
Air + AF
Cl2 + AF

Figure 2. Increased lung injury in Cl_2 exposed mice after microbial challenge. (A) C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 72 h after exposure, a bronchoalveolar lavage was performed. Lung lavage fluid was clarified, subjected to SDS-PAGE and PVDF transfer and immunoblotted with anti-murine albumin or (B) anti-murine IgG (heavy chain, ~ 55 kDa). Positive bands were identified using an ECL western blot detection kit. The Figure illustrates a representative image. Lane 1, 2 3, 4 and 5 represent mice exposed to air, saline, Cl_2, *A. fumigatus* (AF) and Cl_2 + AF, respectively. (C) Cumulative data from three independent experiments (n = 6 per group). Optical density units were set at 1 for air-exposed mice and fold-changes calculated for the remaining groups. ** represents a P value of < 0.01 (Unpaired two-tailed Student's t test). (D) Cumulative data from three independent experiments (n = 5 per group). Optical density units were set at 1 for air-exposed mice and fold-changes calculated for the remaining groups. *** represents a P value of < 0.001 (Unpaired two-tailed Student's t test).

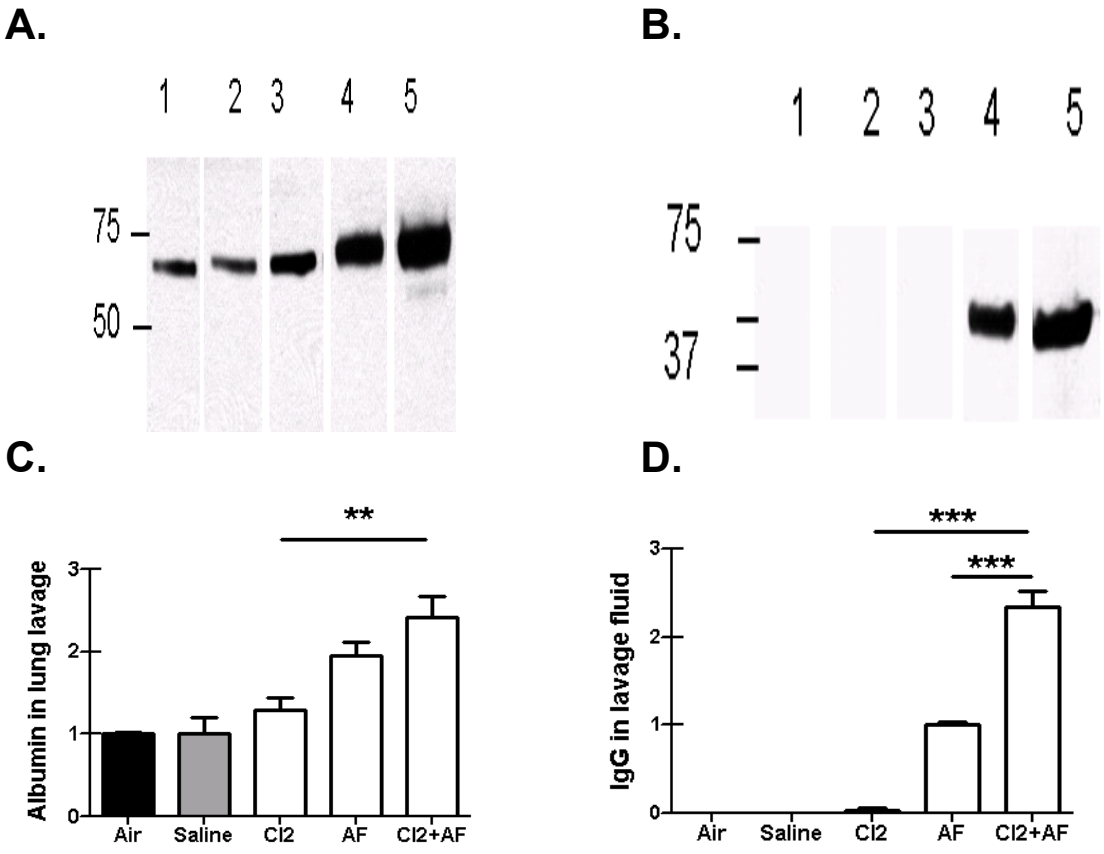
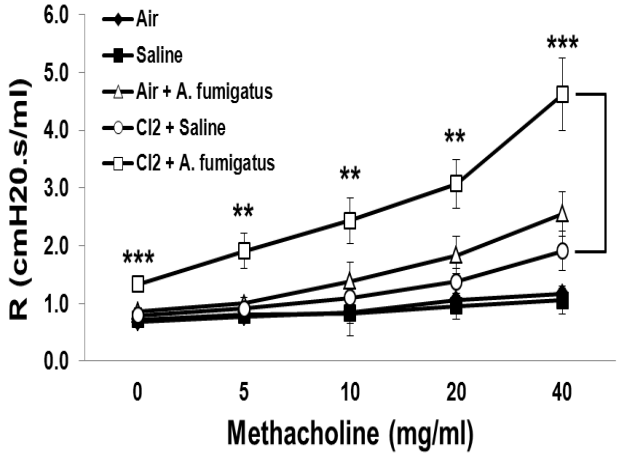
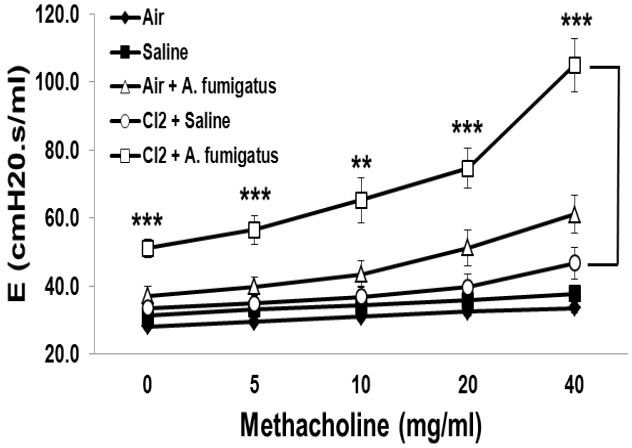

A.
1 2 3 4 5
75
50
B.
1 2 3 4 5
75
37
C.
**
Albumin in lung lavage
0
1
2
3
Air
Saline
Cl2
AF
Cl2+AF
D.
***
***
IgG in lavage fluid
0
1
2
3
Air
Saline
Cl2
AF
Cl2+AF

Figure 3. Impaired pulmonary function in Cl_2 exposed mice after microbial challenge. (A) C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 72 h after exposure, dynamic lung resistance, **(B)** tissue elastance and **(C)** airway resistance were assessed via mechanical ventilation using the flexiVent system. The Figure illustrates cumulative data from two independent studies (n = 5 mice per group per study). All graphs demonstrate mean values ± SEM. One-way ANOVA comparing all groups at each methacholine dose (0, 5, 10, 20 and 40) yielded P values of < 0.0001, < 0.0001, 0.0002, <0.0001 and < 0.0001 for dynamic lung resistance, 0.0014, 0.0051, 0.0058, 0.002 and < 0.0001 for tissue elastance and 0.0012, 0.0074, 0.0896, 0.1320 and 0.0299 for airway resistance. ** and *** represent a P value of < 0.01 and < 0.001, respectively (Unpaired two-tailed Student's t test; comparisons were performed between mice exposed to Cl_2 gas alone and mice exposed to Cl_2 gas followed by *A. fumigatus* challenge).

A.


Air
Saline
Air + A. fumigatus
Cl2 + Saline
Cl2 + A. fumigatus
R (cmH20.s/ml)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
**
**
**
***
0
5
10
20
40
Methacholine (mg/ml)

B.


Air
Saline
Air + A. fumigatus
Cl2 + Saline
Cl2 + A. fumigatus
E (cmH20.s/ml)
120.0
100.0
80.0
60.0
40.0
20.0
***
***
**
***
***
0
5
10
20
40
Methacholine (mg/ml)

C.

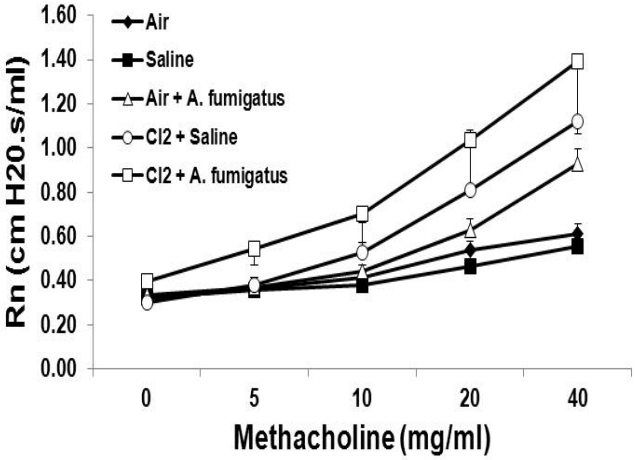
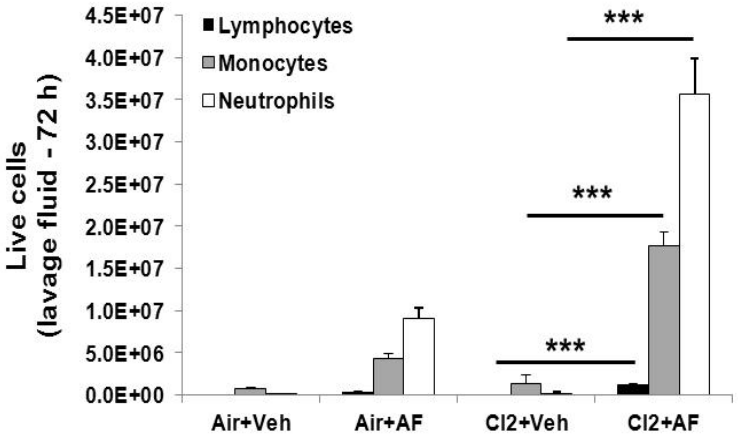
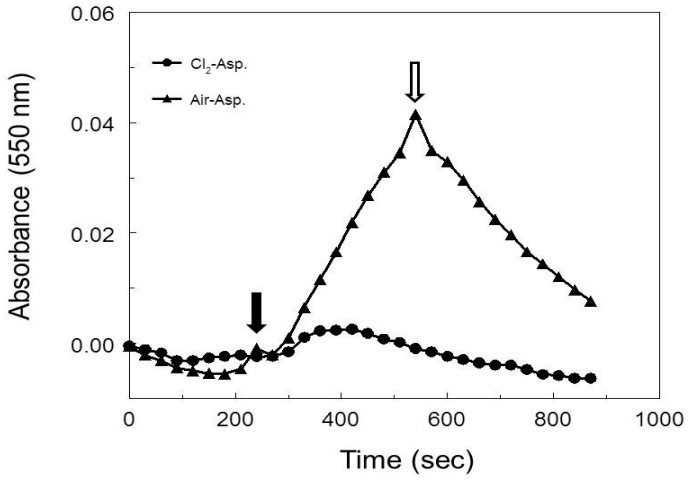

Air
Saline
Air + A. fumigatus
Cl2 + Saline
Cl2 + A. fumigatus
Rn (cm H20.s/ml)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
5
10
20
40
Methacholine (mg/ml)

Figure 4. Cl_2 exposed mice demonstrate intact lung myeloid cell recruitment, but impaired antimicrobial activity, after microbial challenge. **(A)** C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 72 h after exposure, a bronchoalveolar lavage was performed. Lung lavage cells were collected by centrifugation, differentially stained and enumerated. The Figure illustrates cumulative data from two independent studies (n = 5 mice per group). Data are expressed as mean live cells + SEM. One-way ANOVA comparing all groups yielded a P value of < 0.0001 for both monocytes/macrophages and neutrophils. *** represents a P value of < 0.001 when comparing Cl_2 + Veh and Cl_2 + AF groups (Unpaired two-tailed Student's t test). **(B)** C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 24 h after exposure, lung cells were isolated via a bronchoalveolar lavage. Measurement of reactive oxygen species was performed as described in the Methods. The black arrow represents the addition of PMA, the white arrow represents the addition of SOD. The Figure shows a representative plot from one of three independent experiments. **(C)** Cumulative data from three independent experiments measuring O_2^- formation. * represents a P value of < 0.05 (Unpaired two-tailed Student's t test).

A.


Live cells
(lavage fluid - 72 h)
4.5E+07
4.0E+07
3.5E+07
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
Lymphocytes
Monocytes
Neutrophils
***
***
***
Air+Veh
Air+AF
Cl2+Veh
Cl2+AF

B.


Absorbance (550 nm)
0.06
0.04
0.02
0.00
Cl2-Asp.
Air-Asp.
0
200
400
600
800
1000
Time (sec)

C.

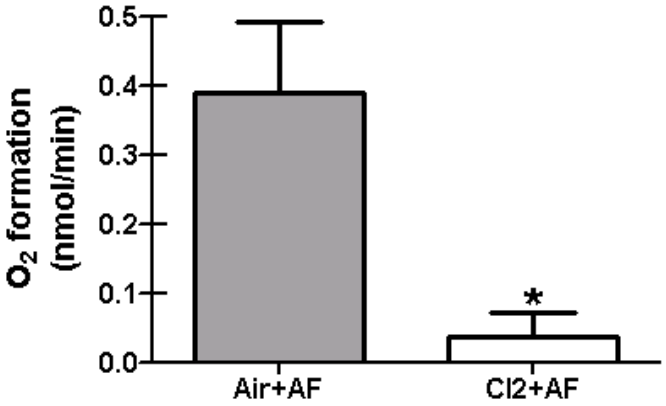
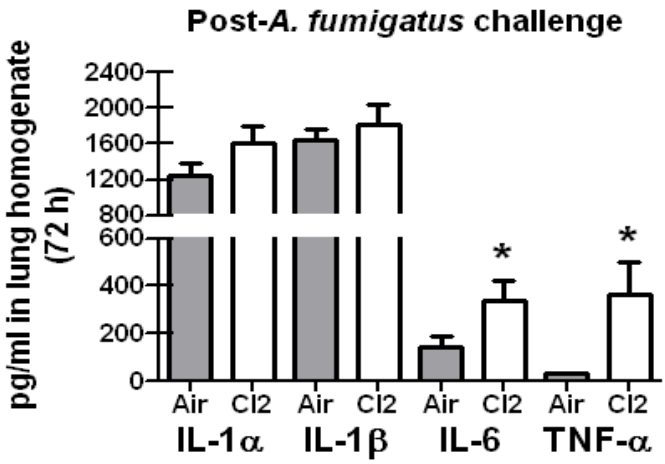
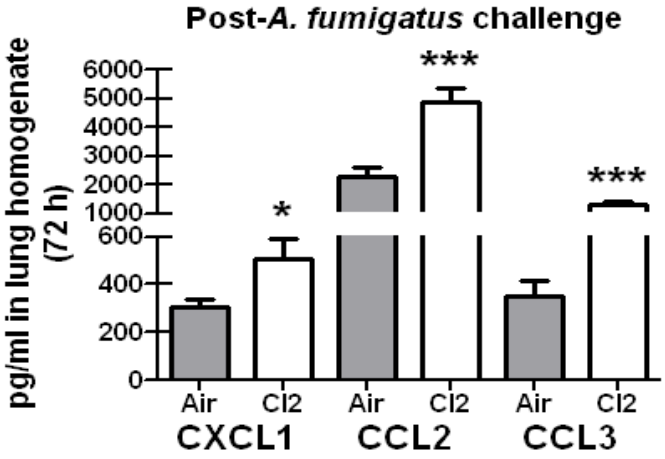

O2 formation
(nmol/min)
0.5
0.4
0.3
0.2
0.1
0.0
*
Air+AF
Cl2+AF

Figure 5. Comprehensive assessment of proinflammatory cytokine and chemokine levels in the lungs of Cl_2 exposed mice after microbial challenge. (A) C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 72 h after exposure, levels of **(A)** IL-1α, IL-1β, IL-6 and TNF-α, **(B)** CXCL1/KC CCL2/MCP-1 and CCL3/MIP-1α and **(C)** IL-12 p40 and G-CSF were quantified in clarified lung homogenates by Bio-Plex. The Figure illustrates cumulative data from two independent studies (n = 5 mice per group). Data are expressed as mean pg/ml + SEM. * and *** represent a P value of < 0.05 and 0.001, respectively (Unpaired two-tailed Student's t test).

A.


Post-A. fumigatus challenge
pg/ml in lung homogenate (72 h)
2400
2000
1600
1200
800
600
400
200
0
*
*
Air
Cl2
Air
Cl2
Air
Cl2
Air
Cl2
IL-1α
IL-1β
IL-6
TNF-α

B.


Post-A. fumigatus challenge
pg/ml in lung homogenate (72 h)
6000
5000
4000
3000
2000
1000
600
400
200
0
*
***
***
Air
Cl2
Air
Cl2
Air
Cl2
CXCL1
CCL2
CCL3

C.

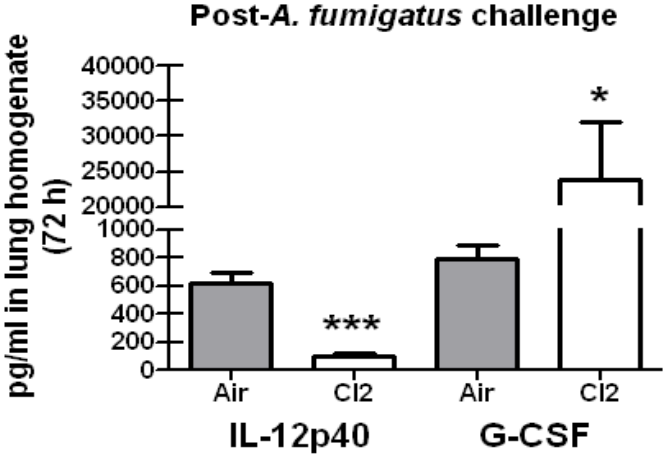
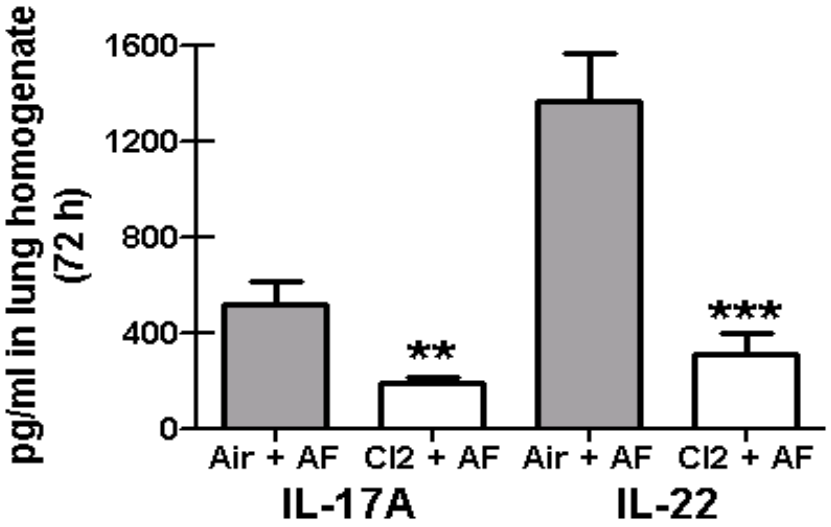
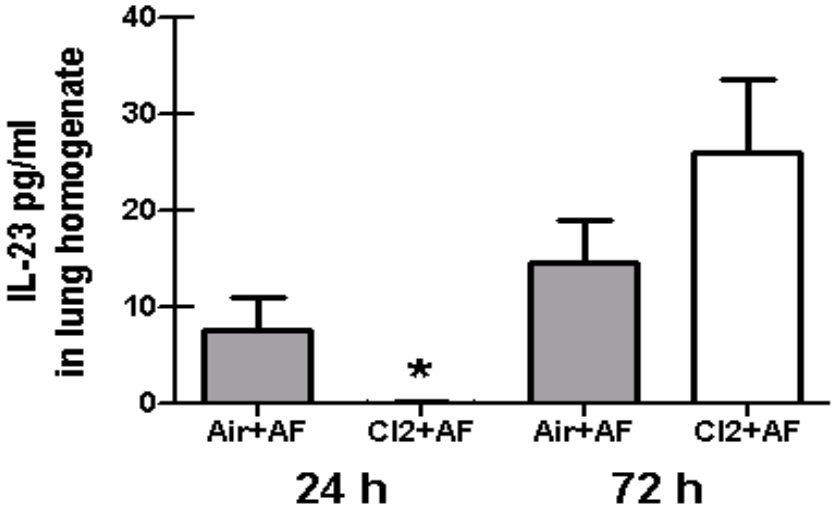

Post-A. fumigatus challenge
pg/ml in lung homogenate (72 h)
40000
35000
30000
25000
20000
1000
800
600
400
200
0
***
*
Air
Cl2
Air
Cl2
IL-12p40
G-CSF

Figure 6. Impaired induction of neutrophil-derived IL-17A and IL-22 in Cl_2 exposed mice after microbial challenge. (A) C57BL/6 mice were exposed to air or 400 ppm of Cl_2 gas for 30 min and returned to room air. Twenty-four hours thereafter, mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 72 h after exposure, levels of IL-17A and IL-22 or (B) IL-23 were quantified by Bio-Plex and ELISA, respectively. The Figures illustrate cumulative data from two independent studies (n = 5 mice per group). Data are expressed as mean pg/ml + SEM. *, ** and *** represent a P value of < 0.05, 0.01 and 0.001, respectively (Unpaired two-tailed Student's t test).

A.


pg/ml in lung homogenate
(72 h)
1600
1200
800
400
0
**
***
Air + AF
Cl2 + AF
Air + AF
Cl2 + AF
IL-17A
IL-22

B.


IL-23 pg/ml
in lung homogenate
40
30
20
10
0
*
Air+AF
Cl2+AF
Air+AF
Cl2+AF
24 h
72 h

DISCUSSION

Aspergillus fumigatus, the etiological agent of invasive pulmonary aspergillosis (IPA), is a ubiquitous mold that causes severe, invasive life-threatening infections in patients that are typically immunocompromised displaying neutropenia. With the rising incidences of IPA in non-neutropenic patients, it is becoming increasingly important to identify atypical risk factors and elucidate mechanisms of host defense involved in the clearance of *A. fumigatus*.

IL-22 is critical in host defense against *A. fumigatus*

Our lab has previously identified a role for beta-glucan receptor Dectin-1 as well as dectin-1 dependent IL-17A in host defense against *A. fumigatus* [65, 87]. As IL-17A may work in tandem with IL-22 [104, 99, 98] and IL-23 is reportedly required for IL-22 induction in several models [127, 69, 68], we extended our studies to investigate the role of Dectin-1 in the induction of IL-22 and the role of IL-22 in *A. fumigatus* lung defense. Our data suggests that not only is IL-22 dectin-1 dependent, as we previously saw with IL-17A, but IL-22 is critical for the clearance of *A. fumigatus* infection in the lungs, as mice deficient in IL-22 were not able to clear *A. fumigatus* compared to mice with intact IL-22 production. Intriguingly, neutralization of IL-22 did not affect levels of IL-17A in response to infection, suggesting that these cytokines, while equally important, may be functioning independent of each other.

The cellular source of IL-22 in an invasive *A. fumigatus* lung infection still remains unclear. Unpublished data from our lab suggests that thioglycollated-elicited neutrophils respond to *A. fumigatus* through IL-22 production. It has recently been seen that during a mouse model of colitis, neutrophils are responsible for the majority of IL-22 present in the colon [128]. While neutrophils may not account for all of the IL-22 produced in an invasive aspergillosis model, we hypothesize that neutrophils are a contributing cell source of IL-22. Future studies using IL-22 reporter mice will further elucidate potential cellular sources of IL-22 during IPA.

As we have identified a critical role for IL-22 in protection against the development of IPA, further understanding of how IL-22 is positively and negatively regulated after *A. fumigatus* exposure is necessary. As we have seen that neutralizing IL-23 in lung cell cultures from *A. fumigatus* exposed mice reduces IL-22 levels by 75%, it can be speculated that additional cytokines may be required for optimal IL-22 production. Several cytokines have been implicated in promoting IL-22 production by CD4+ T cells, specifically IL-6 and IL-21 as well as IL-1β [129]. However, we have excluded individual roles for IL-6 and IL-1β. The role of additional IL-1 family member cytokines in IL-22 production has not been investigated. IL-22 production has been shown to be induced by IL-18 in combination with IL-12 or IL-23 [129]. Unpublished data from our lab has shown that IL-18 is produced in a Dectin-1 dependent manner after *A. fumigatus* challenge with IL-18 knockout mice demonstrating impaired IL-22 production,

suggesting a role for IL-18 in the induction of IL-22 that needs to be further explored.

Recently, Medley et. al., have shown inter-regulation of Th17 cytokines and IL-36 cytokines in a mouse model of psoriasis. A recent paper by Gresnigt et. al., has also revealed that IL-36gamma is produced during an *A. fumigatus* infection in a dectin-1 and TLR4 dependent manner [130]. Taken together, we anticipate that IL-36 family members may be playing a role in IL-22 regulation.

Although we have shown that IL-22 production leads to critical antimicrobial peptide secretion via target epithelial cells, it still remains unclear as to what antifungal mechanisms are responsible for the clearance of *A. fumigatus*. Through real-time PCR, we have excluded that the typical IL-22 induced antimicrobial peptides are critical on their own in the clearance of *A. fumigatus*. Reg proteins and S100 proteins that are important in IL-22 mediated gut inflammation are not altered in the lung in response to *A. fumigatus* exposure at the mRNA level. While this suggests that these proteins may not be involved in IL-22 dependent host defense against *Aspergillus,* mRNA levels do not always correlate with gene function. While transcription seems to be functional, there may be a defect in gene function due to the lack of IL-22 that may be contributing to a defect in fungal clearance. As Lipocalin mRNA was altered, we did look further in to this gene's involvement in the clearance of *Aspergillus,* however, mice deficient in Lipocalin do not exhibit an increased fungal burden when compared to mice with intact Lipocalin. Further studies involving RNA sequencing can help further elucidate targets.

As we have determined a critical role for secreted IL-22 dependent antimicrobial factors necessary for *Aspergillus* clearance, we cannot limit these as products solely of the epithelium. As neutrophils are a known source of ROS and other antimicrobial factors, the defect we see in BALF killing of *Aspergillus* could very well be due to defects in antifungal secretory mechanisms of other cell types. Furthermore, unpublished data from our lab suggests that mice deficient in IL-22 have less recruitment of neutrophils in to the lungs when compared to mice with in tact IL-22.

Chlorine Exposure Leads to a Compromised Lung Environment

Development of IPA is classically thought to arise from immunosuppression leading to neutropenia. Non-traditional risk factors that don't render a patient neutropenic have, however, been observed. For example, a recent study in Spain identified 173 patients with COPD that were not neutropenic, yet 70 patients were classified as colonized with *A. fumigatus* with 48 patients having probable or proven IPA. Recent studies and clinical observations have suggested that fungal infections can occur in patients that aren't necessarily neutropenic or immunocompromised, but in patients that have some primary risk factor predisposing them to infection. With increasing incidences of *A. fumigatus* colonization in cystic fibrosis (CF) patients and patients with severe allergy [131], it is becoming clear that there may be other factors that compromise a lung environment that lead to opportunistic fungal colonization.

It is well known that chlorine exposure can have profound affects on lung physiology with case reports documenting incidences of obstructive lung disease several years after chlorine gas exposure [135, 136]. As chlorine gas exposure may lead to an obstructive lung disease, it is conceivable that, like COPD or CF, exposure to chlorine may set up a compromised lung environment appropriate for colonization of *A. fumigatus*. Knowing that IL-23 and Dectin-1 dependent IL-22 is critical for clearing an *A. fumigatus* infection, we questioned not only whether or not *A. fumigatus* colonization could occur with non-classical predisposing conditions, but how these key factors played a role in the fungal infection. We show that exposure to chlorine gas results in severe injury to the lung and when followed by *A. fumigatus* exposure, compromises neutrophil defense mechanisms leading to impaired antimicrobial immunity. Particularly interesting is the fact that chlorine gas exposure does not impair the ability of neutrophils to respond to chemotactic signals induced by microbial infection. Neutrophil recruitment is not only impaired, but as it is known with chlorine exposure, we saw an increase in neutrophil recruitment to the lung as compared to mice not exposed to chlorine. Although neutrophil recruitment was not impaired in chlorine-exposed mice challenged with *A. fumigatus,* these mice had significantly higher burden compared to air exposed mice challenged with *A. fumigatus*. Furthermore, an increased *A. fumigatus* burden correlated with decreased IL-22 production and impaired production ROS of formation of O_2, suggesting that chlorine gas exposure does compromise neutrophil antimicrobial activity.

Neutrophil Defense Mechanisms

As our data suggests that chlorine gas exposure leads to defective neutrophil antimicrobial defense, it still remains unknown as to how this occurs. Although previous reports suggest that *Aspergillus nidulans* neutrophil defense is independent of ROS [132], our lab has reported that neutrophils do make ROS in response to *A. fumigatus* in a dectin-1 dependent manner [87]. Intriguingly, chlorine exposure not only greatly inhibits fungal clearance, but also occurs in the presence of defective ROS production by neutrophils. This phenomenon not only suggests that ROS is involved in neutrophil mediated host defense against *A. fumigatus*, but also that a compromised lung environment, such as exposure to chlorine, may cause a defect in ROS production. That defect in ROS may, in turn, cause a blunted host immune response to fungal pathogens and lead to a defect in clearance. As a hallmark in chlorine lung exposure is recruitment of neutrophils to the lung [133, 134], a decrease in ROS production cannot be attributed to a decrease in neutrophils being recruited to the site of infection. Furthermore, we saw a heightened recruitment of neutrophils in response to chlorine exposure that directly correlated with enhanced production of CXCL1/KC and CCL3/MIP-1a. It can be hypothesized that chlorine exposure has a direct effect on neutrophil function, not neutrophil recruitment or response to chemotactic signaling. Further studies need to be done exploring the effect chlorine exposure has on the neutrophil itself.

Neutrophils, as the first line of defense, are able to attack and kill invading pathogens in a number of ways. Through phagocytosis, release of antimicrobial peptides and reactive oxygen species (ROS), neutrophils are able to clear invading pathogens. Neutrophils also implement the use of 'neutrophil extracellular traps' or NETs, where they expel chromatin to trap and kill pathogens. NET formation is dependent on the generation of ROS and NADPH oxidase, sometimes independent of NADPH oxidase. Neutrophil elastase and myeloperoxidase (MPO) are also necessary for NET formation [137-139]. As we see a defect in ROS production in neutrophils exposed to chlorine, it would be interesting to further explore whether or not a defect in NET formation is influencing fungal clearance.

Chronic granulomatous disease (CGD) is caused by congenital defects in five components of NADPH oxidase, responsible for catalyzing neutrophil respiratory burst [140, 141]. Patients with CGD often present with recurrent bacterial and fungal infections due to this defect. Moreover, mice deficient in the NADPH oxidase subunits gp91 or p47 are arguably the most susceptible mouse strain for the development of *A.* fumigatus infection [142]. It could be hypothesized that chlorine exposure impairs neutrophil NADPH oxidase ultimately leading to impaired fungal clearance, explaining intact neutrophil recruitment but defective neutrophil-mediated defense mechanisms. Macrophages defective in NAPDH oxidase do not have impaired killing against *A. fumigatus*, further suggesting that impaired neutrophil mechanisms contribute to impaired lung clearance of *A. fumigatus* in chlorine-exposed mice.

As we see with chlorine exposure, patients with COPD show a marked increase in neutrophil numbers being recruited in to the airways [143]. Although neutrophil recruitment in COPD patients is not impaired, the neutrophils that are being recruited to the airways do show intrinsic differences including reduced migratory accuracy due to fewer pseudopods as compared to healthy neutrophils. Further studies are needed to reveal what affect chlorine exposure is having on neutrophils once they are recruited in to the lungs. While chemokines responsible for neutrophil recruitment are produced in normal amounts in response to infection and neutrophils seem to be responding to these signals, it is not certain what defects these neutrophils may display or where these defects may be occurring during the neutrophil recruitment process. Furthermore, it is not certain how these neutrophils are getting in to the lung. While production of neutrophil recruited chemokines seems to be in tact, it is unclear whether or not neutrophil surface adhesion molecules like I-CAM are properly expressed. Furthermore, it is not known whether or not there is a defect in the lung endothelium. If neutrophils are entering the lung due more to a damaged endothelium and less to binding of adhesion molecules, it could be that these neutrophils were never be activated in response to an invading pathogen. Regardless of how neutrophils are getting in to the lung, it is clear there is a major defect in their defensive functions against *Aspergillus fumigatus.* Lactoferrin, a secondary granule protein in neutrophils, is known to be capable of inhibiting *A. fumigatus* conidia growth via the chelation of iron, an essential growth factor for *A. fumigatus* [146]. It could be possible that while neutrophils

are able to respond to chemotactic signals, once they get in to the lung, they are unable to secrete lactoferrin thus increasing host susceptibility to *A. fumigatus* tissue infiltration. The expression of chlorine, or a possible metabolite of chlorine may render part of the NADPH oxidase system defective leading to defective secretion of granule proteins, like lactoferrin or neutrophil elastase, required for immune defense against *Aspergillus fumigatus.*

In conclusion, we have identified a critical role for IL-22 in innate host defense against the etiologic agent of invasive pulmonary aspergillosis (IPA), *Aspergillus fumigatus*. IL-22 is potentially produced by neutrophils in an IL-23 dependent manner once fungal beta-glucan is recognized via the Dectin-1 receptor (Figure 1). *A. fumigatus* is an opportunistic fungus, often colonizing after predisposing conditions rendering a compromised lung environment. We sought out to further elucidate how IL-22 and other critical mechanisms responded to fungal infection following an atypical predisposing condition. We found that chlorine gas exposure results in a profound immune suppression leading to the development of invasive fungal infection. Chlorine exposed mice challenged with *A. fumigatus* demonstrated greater lung injury and poorer lung function in the presence of decreased IL-22 production that we have proven is necessary for *A. fumigatus* clearance. Furthermore, these mice demonstrated a defect in neutrophil ROS production, suggesting that chlorine gas exposure, while not impairing neutrophil recruitment, impairs the antimicrobial activity of neutrophils.

This defect in host defense leads to increased susceptibility to infection or long-term microbial colonization (Figure 2).

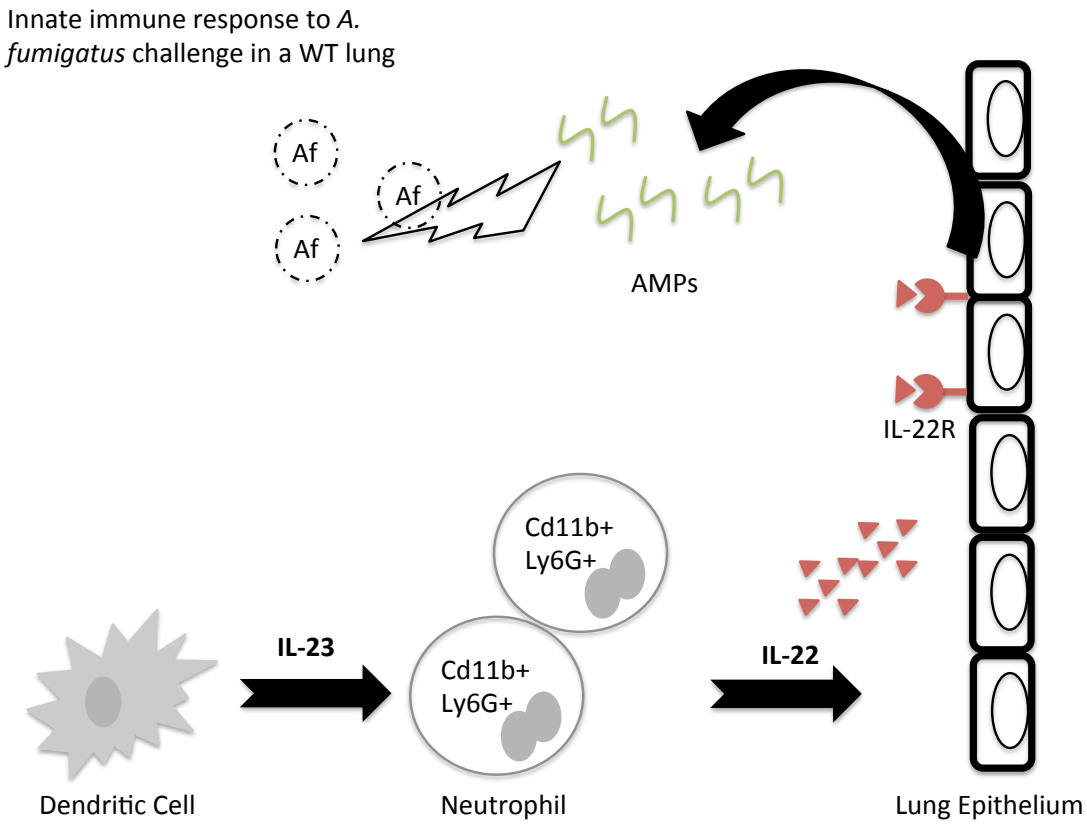


Figure 1. The innate immune response to *A. fumigatus* challenge. Upon Dectin-1 recognition of fungal beta-glucan exposed on the *A. fumigatus* surface, neutrophil chemoattractants will recruit neutrophils to the site of infection. Also dependent on dendritic cell produced IL-23, neutrophils will produce IL-22. IL-22 will target the epithelium to then secrete antimicrobial peptides that will target and clear the invading *A. fumigatus*.

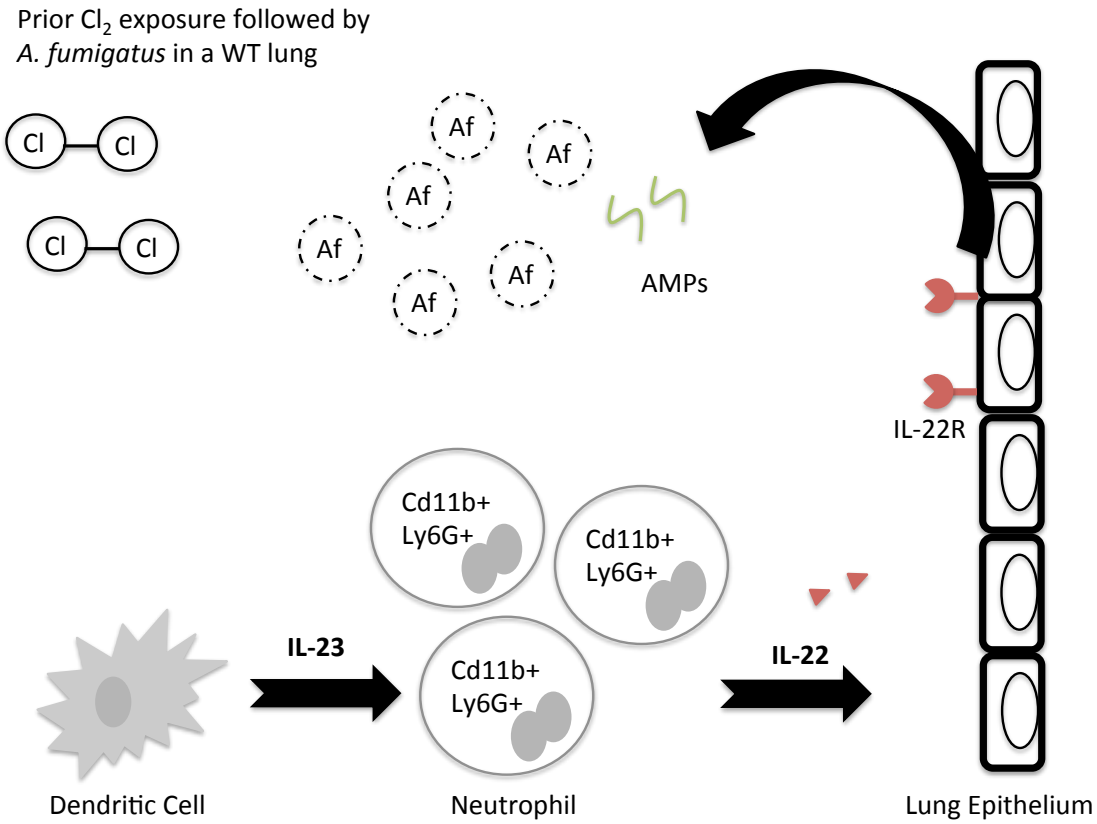


Figure 2. The innate immune response to *A. fumigatus* challenge following prior Cl_2 exposure. Following a prior exposure to Cl_2, Dectin-1 recognition of fungal beta-glucan remains functional. An increased recruitment of inflammatory cells is observed, but these cells are not functioning appropriately. A defective production in IL-22 leads to increased fungal burden.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: December 6, 2013

TO: CLAUDE HENRY STEELE III, Ph.D.
THT -422
(205) 996-9598

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Pulmonary Defense Against Aspergillus Fumigatus
Sponsor: NIH
Animal Project_Number: 140109031

As of January 8, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	498
Mice	C	498

Animal use must be renewed by January 7, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140109031 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: December 6, 2013

TO: CLAUDE HENRY STEELE III, Ph.D.
THT -422
(205) 996-9598

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 6, 2013.

Title: Pulmonary Defense Against Aspergillus Fumigatus
Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC)
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