Dectin-1 dependent IL-22 contributes to early innate lung defense against *Aspergillus fumigatus*¹

Melissa A. Gessner¹, Jessica L. Werner¹, Lauren M. Lilly¹, Michael P. Nelson¹
Allison E. Metz¹, Chad W. Dunaway¹, Yvonne R. Chan², Wenjun Ouyang³,
Gordon D. Brown⁴, Casey T. Weaver⁵ and Chad Steele¹

¹Department of Medicine, University of Alabama at Birmingham, Birmingham, AL
²Department of Medicine, University of Pittsburgh, Pittsburgh, PA
³Department of Immunology, Genentech, Inc., South San Francisco, CA
⁴Section of Infection and Immunity, Institute of Medical Sciences,
University of Aberdeen, Aberdeen, UK
⁵Department of Pathology, University of Alabama at Birmingham, Birmingham, AL

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Address correspondence to:
Chad Steele, Ph.D.
Associate Professor
Department of Medicine
School of Medicine
University of Alabama at Birmingham
1900 University Blvd, THT 437A
Birmingham, AL 35294
Ph: 205-996-9598
chadsteele@uab.edu
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. 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 hematopoietic stem cell transplant (HSCT) recipients and in 19% of solid organ transplant recipients 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. 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. 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. 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. In our most recent studies, we have identified neutrophils as a source of Dectin-1 dependent IL-17A production during lung A. fumigatus infection. 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, and more recently, in a Dectin-1 dependent manner by DCs.

In addition to being critical for the maintenance of the Th17 lineage and IL-17A-producing cells in general, IL-23 is also a critical effector cytokine for the induction of IL-22, a class 2 α-helical cytokine of the IL-10 family of cytokines. 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. In colitis, IL-23 deficiency is more effective in ameliorating disease than IL-17A deficiency. In fact, IL-17A may be protective in intestinal inflammation. With respect to IL-22, infection models with the Gram-negative bacteria Klebsiella pneumoniae and Citrobacter rodentium showed a requisite role for IL-22 in protection. However, although produced by Th17/IL-17A producing cells, IL-22 has been shown to act as an anti-inflammatory agent in hepatitis and IBD. Paradoxically, IL-22 is thought to be a contributing factor in inflammation associated with psoriasis. 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, 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, backcrossed 10 generations to the C57BL/6 background and bred at Taconic. IL-22 deficient mice were provided by Dr. Wenjun Ouyang at Genentech and bred at UAB. Lipocalin 2 deficient mice 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 10⁷ 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. Lung A. fumigatus burden
was analyzed with real-time PCR measurement of the \textit{A. fumigatus} 18S rRNA (GenBank accession number AB008401) \cite{5} and quantified using a standard curve of \textit{A. fumigatus} conidia ($10^1 - 10^9$) as previously described \cite{28}. Specifically, total RNA was isolated using the MasterPure™ kit from serial 1:10 dilutions of \textit{A. fumigatus} conidia beginning with $10^9$ and real-time PCR amplification of \textit{A. fumigatus} 18S rRNA was performed on each dilution. As a validation of the real-time PCR method, heat-killed \textit{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 \textit{A. fumigatus} DNA.

\textbf{IL-22 neutralization}

For \textit{in vivo} IL-22 neutralization, WT mice were challenged intratracheally with $5-7 \times 10^7$ \textit{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 \textit{A. fumigatus} burden was analyzed with real-time PCR measurement of the \textit{A. fumigatus} 18S rRNA as described \cite{42}.

\textbf{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^(-ΔΔCt) 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).
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. We have previously reported that neutralization of IL-17A compromised lung clearance of *A. fumigatus*. However, as IL-22 appears to primarily activate epithelial cells, 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 CCL4/MIP-1β (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 and CXCL9/Mig, as well as CXCL10/IP-10, have been reported to have direct antimicrobial activity, 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 antifungal activity, however this factor(s) does not appear to be lipocalin 2, S100A8, S100A9 or Regllly.

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. Analogous to that observed for IL-17A, additional cell types such as lymphoid tissue-inducer cells, NK cells, and γδ T cells can also produce IL-22. Although IL-22 can act in both an anti-inflammatory and pro-inflammatory manner, IL-22 has been reported to play a major role in stimulating epithelial anti-microbial activity and host defense against multiple mucosal pathogens including the fungal organism *C. albicans*. To date, only epithelial cells and keratinocytes have been identified as expressing the IL-22 receptor. We have previously identified a role for Dectin-1 dependent IL-17A in host defense against *A. fumigatus*. As IL-17A may work in tandem with IL-22 and IL-23 is reportedly required for IL-22 induction in several models, 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, 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. 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. With respect to Th17/IL-17A responses, STAT3 activation is clearly favored by IL-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. 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. 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. IL-17A also has an acknowledged role in the induction of these factors as well 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 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 as well as IL-22 (Figure 1). Despite compromised S100A8 and S100A9 expression in IL-22 deficient mice intragastrically infected *C. abicans*, 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, 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 defense.
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.

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, 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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References


Figure Legends

**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).

**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).

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).
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.

**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).
Gessner et al. Figure 1

A.

B.
Gessner et al. Figure 2

A.

B.
Gessner et al. Figure 3

A.  

\[ A. fumigatus 18S rRNA \]  

\[ \text{tissues per lung - 24 h} \]  

WT  

IL-22 KO  

**

B.  

pg/ml in lung homogenate  

(24 h)  

WT  

IL-1\(\alpha\)  

CCL3  

CCL4  

TNF-\(\alpha\)  

***  

*  

C.  

CD11b+ Ly-6G+  

(BALF - 24 h)  

WT  

IL-22 KO  

* 

D.  

pg/ml in lung homogenate  

(24 h)  

WT  

IL-12 p40  

IL-12 p70  

**  

* 

E.  

pg/ml in lung homogenate  

(24 h)  

WT  

IL-22 KO  

CXCL9  

CXCL10  

F.  

IL-17A pg/ml  

(lung homogenate - 24 h)  

WT  

IL-22 KO  

**
Gessner et al. Figure 4

A.  

B.  

C.  

A. fumigatus 18S rRNA  
(units per lung - 48 h)  

Lipocalin 2 pg/ml  
(tcad homogenate - 24 h)  

Percent of WT  

WT  Dectin-1 KO  IL-22 KO