Aspergillus fumigatus pre-exposure worsens pathology and improves control of Mycobacterium abscessus pulmonary infection in mice.

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Mutations in this chloride channel lead to mucus accumulation, subsequent recurrent pulmonary infections and inflammation, which in turn cause chronic lung disease and respiratory failure. Recently, rates of non-tuberculous mycobacterial (NTM) infections in CF patients have been increasing. Of particular relevance is infection with Mycobacterium abscessus, which causes a serious, life-threatening disease and constitutes one of the most antibiotic-resistant NTM species. Interestingly, an increased prevalence of NTM infections is associated with worsening lung function in CF patients who are also co-infected with Aspergillus fumigatus. We established a new mouse model to investigate the relationship between A. fumigatus and M. abscessus pulmonary infections. In this model, animals exposed to A. fumigatus and co-infected with M. abscessus exhibited increased lung inflammation and decreased mycobacterial burden, compared with mice infected with M. abscessus alone. This increased control of M. abscessus infection in co-infected mice was mucus-independent, but dependent on both transcription factors, T-box 21 (Tbx21) and RAR-related orphan receptor gamma t (RORγt), master regulators of Type I and Type 17 immune responses, respectively. These results implicate a role for both Type 1 and Type 17 responses in M. abscessus control in A. fumigatus co-infected lungs. Our results demonstrate that A. fumigatus, an organism found commonly in CF patients with NTM infection, can worsen pulmonary inflammation and impact M. abscessus control in a mouse model.
INTRODUCTION

The genus *Mycobacterium* encompasses numerous organisms, some of which are emerging opportunistic pathogens. In particular, non-tuberculous mycobacteria (NTM) are a diverse group of environmentally ubiquitous organisms, which cause a wide spectrum of disease in humans. NTM most commonly cause disease in people with structural lung abnormalities, including cystic fibrosis (CF) patients and patients with primary ciliary dyskinesia, with average prevalence rates of 20% and 10%, respectively (1). Rates of NTM infection in CF patients are reported to be increasing (2). In addition, older female patients without underlying lung disease appear to be at higher risk of NTM infection, in particular those with a tall and thin body habitus, scoliosis, pectus excavatum and mitral valve prolapse (3). The prevalence of chronic lung disease due to NTM is increasing and, in many areas of the US, exceeds that of *Mycobacterium tuberculosis*.

The most common NTM causing pulmonary infection is the *M. avium complex*, but other species, including *M. abscessus*, are becoming more common in CF patients. *M. abscessus* infection accounts for up to 50% of NTM infections in some CF cohorts, and is associated with decrease in lung function in CF patients (2). In addition, it constitutes one of the most clinically virulent and antibiotic-resistant NTM species (4). Treatment can be rendered difficult by the discordance that often occurs between in vitro antibiotic susceptibility tests and clinical effectiveness (1). Treatment options are limited, with a treatment failure rate reported at ~50% for antibiotic treatment, and lung resection sometimes being the only alternative (5).

Mucus accumulation and inhibition of lung antimicrobial peptides due to high ion concentration are thought to underlie the increased predisposition of CF patients to
Numerous pathogens can persist in the lungs of CF patients and contribute to the decline in respiratory function (6). Co-infections between NTM and other characteristic CF pathogens are common; *Pseudomonas*-derived genes have been identified in the genome of *M. abscessus* (7). Furthermore, studies show that *Pseudomonas aeruginosa* and *M. abscessus* frequently coexist in the lung environment (8). Similarly, *A. fumigatus* can colonize the lungs in CF patients and cause allergic bronchopulmonary aspergillosis (6), and its presence is associated with increased risk of NTM infection (2). It is not clear whether this association occurs because these CF patients have more severe lung disease, or because of specific interactions between these pathogens.

In this context, common CF co-infections may alter the course of NTM disease, and the nature and mechanisms behind such relationships have not been studied in depth. *A. fumigatus* has been shown to induce type 17 cytokines (including the signature cytokine Interleukin(IL)-17) and neutrophilia (9), which has been associated with inflammation and impaired immune resistance in a mouse model (10). Type 17 responses are beneficial for *A. fumigatus* persistence, given that they inhibit the Type 1 responses required to control infection and promote biofilm formation (9, 11). Given the importance of Type 1 immunity in mycobacterial control, a pre-existing *A. fumigatus* infection could affect mycobacterial containment and impact disease pathology. In addition, neutrophils have been shown to promote *M. abscessus* biofilm formation, thereby promoting bacterial persistence (12). *A. fumigatus* has also evolved other strategies to evade the immune response, among which is Toll-like receptor 2-dependent stimulation of IL-10 secretion (13). Previous work has demonstrated that peripheral blood mononuclear cells (PBMC) from CF patients, in comparison to PBMC
from healthy controls, secreted increased IL-10 levels when exposed to *A. fumigatus* antigens (13). Upon blockade of IL-10, Type 1 responses were enhanced, suggesting that IL-10 may play a role in inhibiting *A. fumigatus* T cell responses in CF (14). This raises the question of whether such inhibition of the immune response by *A. fumigatus* may affect concomitant immunity to NTM infections and their associated pathology.

In this work, we used a mouse model to study the effect of prior infection with *A. fumigatus* infection on challenge with *M. abscessus*. In this model, animals exposed to *A. fumigatus* and co-infected with *M. abscessus* exhibited increased lung inflammation and decreased mycobacterial burden, when compared to mice infected with *M. abscessus* alone. The increased control of *M. abscessus* infection was mucus-independent, but dependent on the presence of both transcription factors T-box 21 (Tbx21) and RAR-related orphan receptor gamma (RORγ-t). As these transcription factors are the master regulators of Type I and Type 17 immune responses, respectively, this suggests a role for Type 1 and Type 17 responses in *M. abscessus* control in co-infected hosts. Together, our data provide novel insights into how *A. fumigatus*, an organism frequently found in CF patients, affects the pathology and control of the opportunistic NTM pathogen *M. abscessus*.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 (B6) animals were purchased from Taconic. *Ifnγ−/−* mice on the B6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). *Il17ra−/−* (15), *Stat6−/−* (16), *Tbx21−/−* (17), *Rorc−/−* (18), and *Tbx21−/−Rorc−/−* mice, all on the B6 background, were bred and maintained in the animal facility at the University of
Pittsburgh and Washington University in St. Louis. Stat1−/− mice were a kind gift from Dr. John Alcorn (University of Pittsburgh). Experimental mice were age- and sex-matched and used between the ages of 6-8 weeks. All mice were used following the National Institutes of Health guidelines for housing and care of laboratory animals and in accordance with University of Pittsburgh and Washington University in St. Louis Institutional Animal Care and Use Committee guidelines. All efforts were made to minimize suffering and pain as described in these approved protocols.

**Experimental infections**

*M. abscessus* strain L948 (ATCC 19977) was grown in Middlebrook 7H9 broth containing 0.05% Tween-80 to mid-log phase and frozen in 1 mL aliquots at -80°C. For *M. abscessus* infections, animals underwent oropharyngeal infection with 2x10^4 colony forming units (CFU) of bacteria using the tongue-pull method (19). Briefly, mice were anesthetized with 3% isoflurane, suspended by their front incisors, and the tongue was extended using forceps. The bacterial suspension was pipetted into the trachea, and the tongue was held until normal breathing resumed. For 14 day experiments, mice underwent infection with *M. abscessus* on day 0. For 30 day experiments, mice underwent repeat infections with *M. abscessus* on day 0, 7, 14, and 21. Lung bacterial burden was established by plating out organ homogenates on 7H10 agar plates.

*A. fumigatus* Fresenius (ATCC 42202) was grown on potato agar dextrose medium. For co-infection with *A. fumigatus*, mice underwent single oropharyngeal infection with 2.5x10^7 *A. fumigatus* conidia 3 days prior to first *M. abscessus* challenge as described above.

**Lung single-cell preparation and detection of cytokine-producing cells by ELISpot assay**
Lung suspensions from *M. abscessus*-infected mice were prepared as described previously (21) and were used in ELISpot assays as described below. Antigen-specific interferon-gamma (IFN-γ) producing and IL-17-producing cells were analyzed by ELISpot assay. MultiScreen-HA filter plates (Millipore, Billerica, MA) were coated with antibodies to IL-17 (R&D Systems, Minneapolis, MN). Single cell suspensions were added to the plate at a starting concentration of $1 \times 10^5$ cells/well and doubling dilutions made. Cells were cultured overnight in the presence of $1 \times 10^6$ irradiated splenocytes, 10 µg/mL heat killed *M. abscessus*, and 10 U/mL recombinant mouse IL-2 (eBioscience, San Diego, CA). The following day, biotinylated IL-17 antibody (eBioscience, San Diego, CA) was added and incubated overnight. Plates were developed by incubation with streptavidin-alkaline phosphatase (Vector Labs, Burlingame, CA) for two hours, followed by incubation with NBT/BCIP (Sigma-Aldrich, St. Louis, MO). Spots were enumerated using a CTL-ImmunoSpot analyzer (CTL, Shaker Heights, OH) and the frequency and total number of responding cells calculated as described before (21).

**Surface and cytokine staining using flow cytometry**

Lungs were collected at specified time points, digested with collagenase D (3 mg/mL; Roche Applied Science, Penzberg, Germany) in DMEM for 1h at 37°C, pressed through 100 µM cell strainers (BioExpress, Kaysville, UT) and treated with ammonium chloride to lyse red blood cells. Single cell suspensions were incubated in incomplete Dulbecco’s Modified Eagle Media (iDMEM) with 10 percent FBS, PMA (50 ng/mL; Sigma-Aldrich), ionomycin (750 ng/mL; Sigma-Aldrich) and GolgiPlug (Becton Dickinson Pharmingen, Franklin Lakes, NJ) for 4 hours at 37°C. Cells were treated with 100 µL of a 1:100 dilution of Fc Block (anti-CD16/CD32, BD Biosciences, San Jose, CA) before
surface staining for CD3, CD4, CD44, MHC class II, CD11c, CD11b and TCR γδ. Cells were then fixed and permeabilized using the Cytofix/Cytoperm fixation permeabilization kit (BD Biosciences) before staining intracellularly for IL-17 and IFN-γ. Stained cells were acquired on an LSRII (BD Biosciences) flow cytometer, and results were analyzed using FlowJo (Treestar, Ashland, OR).

**Gene expression analysis**

To analyze gene expression, lungs were placed in TRIzol reagent (Life Technologies, Carlsbad, CA), homogenized and processed according to the manufacturer’s protocol. One microgram of RNA was used to synthesize cDNA (iScript; Bio-Rad, Hercules, CA). Real-time PCR primers for *Il17a* and hypoxanthine guanine phosphoribosyl transferase (*Hprt*) were purchased from Applied Biosystems (Foster City, CA) and used with TaqMan Universal PCR Master Mix (Applied Biosystems). PCR was performed on a Bio-Rad CFX96 Real-Time System.

**Histologic Data**

Lungs from infected mice were inflated with 10% neutral buffered formalin and paraffin embedded. Lung sections were stained with hematoxylin and eosin stain and processed for light microscopy. Slides were scored by one of the authors (T.D.O.), who was blinded to the sample groups. Every field in the entire lung was observed with a light microscope and collections of cells representing inflammatory nodules were counted.

**Generation of Bone Marrow-derived Macrophages (BMDM)**

BMDM were generated from the bone marrow of C57BL/6 mice. Cells were extracted from femurs and 1x10⁷ cells were plated with 10 mL of complete Dulbecco’s...
Modified Eagle Media (cDMEM) supplemented with 20 ng/mL mouse recombinant granulocyte macrophage-colony stimulating factor (mrGM-CSF) (Peprotech, Rocky Hill, NJ). Cells were cultured for 3 days at 37°C in 5% CO2, after which an additional 10 mL of cDMEM containing 20 ng/mL mrGM-CSF was added. On day 7, the adherent cells were collected by scraping after centrifugation, counted and plated for subsequent assays.

**In vitro *M. abscessus* killing assay**

For killing assays, 5x10^5 BMDMs were plated in 24-well plates, rested overnight and then pre-treated with iDMEM, 100 μg/mL zymosan or curdlan in iDMEM for 24 h. BMDMs were then infected with *M. abscessus* at a Multiplicity of Infection (MOI) of 1 for 48 h. At the end of the culture period, macrophages were washed twice with phosphate-buffered saline (PBS), lysed by a 5 min incubation with 0.05% sodium dodecyl sulfate (SDS) in PBS. Following SDS neutralization with 10% bovine serum albumin in PBS, intracellular *M. abscessus* burden was determined by plating of serial dilutions on 7H10 agar (BD, Franklin Lakes, NJ) plates.

**Detection of nitrites by the Griess reaction**

Culture supernatants were assessed for nitrite production using the Griess Reagent System Kit (Promega, Madison, WI), according to the manufacturer’s instructions.

**Statistical analysis**

Differences between the means of multiple experimental groups were analyzed using one-way ANOVA with Tukey’s post-hoc test unless otherwise indicated. For all other analyses, we used the two-tailed Student’s t-test. Differences were considered
significant when p≤0.05. For all figures, data represent mean ± SD. All analyses were performed using GraphPad Prism Software (GraphPad Software, La Jolla, CA).

RESULTS

Prior *A.fumigatus* infection worsened lung pathology and improved *M.abscessus* control in an acute model of co-infection

*A. fumigatus* commonly colonizes the lungs in CF patients (6), and is associated with an increased frequency of NTM infection (2). The contribution of *A.fumigatus* infection to *M.abscessus* disease, however, has not been studied. We therefore addressed the effect of *A.fumigatus* co-infection on *M.abscessus* control and associated lung pathology using a mouse model. We hypothesized that the *A.fumigatus*-induced immune response may alter *M.abscessus*-driven pathology and control. Given that the IL-17 response is critical in promoting *A.fumigatus*-driven inflammation and host susceptibility (10), we initially determined the kinetics of IL-17 induction in the lungs of *A.fumigatus*-infected mice. When B6 mice were infected with *A.fumigatus*, lung IL-17-producing lymphocytes accumulated by day 4, where the majority of the IL-17-producing cells were found to be CD4+ T cells and γδ T cells (Fig 1A-C). The accumulation of IL-17-producing cells in the infected lung also coincided with induction of *Il17* mRNA between day 1 and 4 post infection (Fig 1D). These data suggest that *Il17* is induced by day 4 after *A.fumigatus* infection.

Therefore, in subsequent experiments, mice were first infected with *A.fumigatus* followed by initial challenge with *M.abscessus* 3 days later, to coincide with the peak of the *A.fumigatus*-induced IL-17 response. Following *M.abscessus* infection, at 14 days,
co-infected mice exhibited improved *M. abscessus* control (Fig 2A). Similar to
*A. fumigatus*-only infected mice, *A. fumigatus* and *M. abscessus* co-infected mice cleared
*A. fumigatus* infection, as evidenced by failure to amplify *A. fumigatus* 18S rRNA, absence of gomori methenamine silver staining, and failure to culture *A. fumigatus* from lung homogenates (data not shown). Interestingly, co-infected mice, displayed increased lung inflammation and pathology, as evidenced by significantly more inflammatory foci in comparison to mice infected with *A. fumigatus* and *M. abscessus* alone, respectively (Fig 2B, 2C). These foci were composed predominantly of eosinophils and histiocytes and were associated with increased mucus accumulation as evidenced by Periodic acid-Schiff (PAS) staining (Fig 2D). Flow cytometry showed the presence of significantly more neutrophils, alveolar macrophages, and MHC class II activated alveolar macrophages in the co-infected mice, compared to responses in the lungs of mice infected with *M. abscessus* alone (Fig 2E-G). The accumulation of lung neutrophils and alveolar macrophages in mice infected with *A. fumigatus* alone was not significantly different when compared to co-infected mice, suggesting that the increased accumulation of inflammatory myeloid cells was likely driven by *A. fumigatus* infection.

*A. fumigatus*-induced *M. abscessus* control in co-infected mice is Stat6-independent.

As *A. fumigatus* infection caused mucus accumulation in airways, we next studied whether increased mucus may impair *M. abscessus* attachment to lung epithelial cells, thus decreasing bacterial burden in co-infected mice. We challenged *A. fumigatus*-infected mice with *M. abscessus* and assessed lung bacterial burden 1 hour following infection. However, *M. abscessus* burden did not significantly differ between control and *A. fumigatus*-infected mice, suggesting that the effects of *A. fumigatus* on *M. abscessus*
control occur at a later point during infection (Fig 3A). We further tested the importance of mucus production on *A.fumigatus*-induced *M.abscessus* control by infecting *Stat6<sup>−/−</sup>* mice, which are unable to produce mucus in response to antigen challenge (23). Co-infected *Stat6<sup>−/−</sup>* mice were able to control *M.abscessus* to an extent similar to C57BL/6 mice (Fig 3B), indicating that mucus production does not directly impact *M.abscessus* control.

*A.fumigatus* enhances control of *M.abscessus* through a *Tbx21* and *Rorc*-dependent mechanism

As *A.fumigatus* infection induced lung IL-17-producing cells following infection (Fig 1), we then studied the accumulation of IL-17 and IFN-γ-producing cells upon co-infection with *M.abscessus*. We found that co-infected mice showed enhanced accumulation of IL-17-producing and IL-17/IFN-γ-co-producing CD4<sup>+</sup>CD44<sup>+</sup> T cells (Fig 4A-B). Similarly, co-infected mice had increased numbers of IL-17-producing γ<sup>δ</sup> T cells (Fig 4C). There was a trend towards increased IL-17/IFN-γ-co-producing γ<sup>δ</sup> T cells, but this was not statistically increased in co-infected mice, when compared with the singly infected mice (Fig 4D). CD4<sup>+</sup>CD44<sup>+</sup> T cells producing IFN-γ alone mirrored more closely the responses observed in *M.abscessus*-infected mice (Fig 4E). Furthermore, while co-infected mice harbored higher numbers of *M.abscessus*-specific IL-17-producing cells in their lungs, there was no significant increase in accumulation of *M.abscessus*-specific IFN-γ-producing cells (Fig 4F, and data not shown). Thus, our data show that T cells producing IL-17 and IFN-γ accumulate in lungs of co-infected mice. We then further determined the immune mechanisms responsible for improved *M.abscessus* control in co-infected mice. First, we infected *Il-17ra<sup>−/−</sup>* mice with *M.abscessus* alone or co-infected
with *A. fumigatus* and *M. abscessus*. While lung bacterial burden in *Il-17ra*−/− mice infected with *M. abscessus* alone did not differ from *M. abscessus*-infected B6 mice, we found that *Il-17ra*−/− co-infected mice had higher lung bacterial burden, when compared to co-infected B6 mice (Fig 5A). This indicates that IL-17RA signaling is partially involved in *A. fumigatus*-induced *M. abscessus* control. Because interferon signaling has been established to be important for anti-NTM responses and control (24), we next determined the effect of STAT-1 deficiency on *A. fumigatus*-induced *M. abscessus* control. *Stat1*−/− mice demonstrated increased bacterial burden upon *M. abscessus* single infection, and decreased control of *M. abscessus* in co-infected mice, when compared to B6 mice (Fig 4A). These results indicate that interferon signaling via the STAT-1 pathway is required for the protection observed in co-infected mice. As multiple mechanisms may simultaneously contribute to *M. abscessus* control in co-infected mice, we next co-infected *Tbx21*−/−, *Rorc*−/− or *Tbx21*−/−*Rorc*−/− mice, which lack Type 1, Type 17, or both Type 1 and Type 17 responses, respectively. We found that, individually, each transcription factor was dispensable for *A. fumigatus*-induced *M. abscessus* protection at 14 days (Fig 5B). However, co-infected mice lacking both transcription factors failed to improve protection against *M. abscessus* challenge (Fig 5B). On histologic analysis, *Rorc*−/− mice and *Tbx21*−/−*Rorc*−/− mice exhibited higher numbers of inflammatory foci compared to B6 co-infected mice and *Tbx21*−/− co-infected mice (Fig 5C), as well increased lung pathology (Fig 5D-E). Together, these results suggest a role for both Type 1 and Type 17 responses in control of *M. abscessus* in *A. fumigatus* co-infected mice.

*Prior A. fumigatus* infection worsened lung pathology and improved *M. abscessus* control in a chronic model of co-infection.
Our data show that *A. fumigatus* infection worsens lung pathology upon subsequent *M. abscessus* infection in an acute (14 day) model of co-infection. Thus, we extended the duration of infection to determine whether similar findings were observed in a chronic co-infection model which would better mimic infection in CF patients.

When B6 mice were co-infected with *A. fumigatus* and *M. abscessus*, at 30 days no bacterial burden could be detected (data not shown). To create a chronic model of infection, B6 mice were initially infected with *A. fumigatus*, then over the next 30 days were serially infected 4 times with *M. abscessus*. A control group of B6 mice were also serially infected 4 times with *M. abscessus* alone over the 30 days. Similar to the results of the 14 day experiment described above, chronically co-infected mice exhibited improved clearance of *M. abscessus* (Fig 6A), and increased inflammation (Fig 6B-D) as exhibited by increased number of inflammatory foci. These results thus demonstrate that prior *A. fumigatus* infection can worsen lung pathology even in a chronic model of *M. abscessus* co-infection.

**Fungal antigens improve in vitro killing of *M. abscessus***

A potential mechanism leading to improved control of *M. abscessus* and increased pathology in *A. fumigatus* infected mice could be through activation of macrophages to induce *M. abscessus* killing. To further investigate this, bone marrow-derived macrophages (BMDMs) were generated and treated with fungal products, including zymosan and curdlan, following which they were infected with *M. abscessus*. Interestingly, similar to IFN-γ-treated macrophages, both zymosan and curdlan treatment improved *M. abscessus* control in macrophages (Fig 7A), and this was associated with
increased activation of macrophages, as measured by iNOS accumulation in supernatants (Fig 7B). These studies indicate that fungal antigens may potentiate macrophage activation, which could, in turn, improve *M. abscessus* control in co-infected mice while also worsening lung pathology.

**DISCUSSION**

One of the hallmarks of CF is the presence of recurrent pulmonary exacerbations due to infections, which are associated with a decline in lung function (25, 26). CF patients often harbor a variety of pathogens in their lungs, and how such infections interact with each other to affect lung function is still unknown. Several recent studies showed that *A. fumigatus* colonization is frequently associated with *M. abscessus* infection (2, 27, 28). This association may be due to more advanced disease that predisposes patients to *M. abscessus* infection, but also to host-pathogen interactions. *M. abscessus*-infected CF patients are more likely to have previously received IV antibiotics for other infections, and to have *A. fumigatus* isolated from their sputa (26). Thus, frequent antibiotic treatment for bacterial infections could lead to the generation of an ecologic niche that enables survival of both *A. fumigatus* and *M. abscessus* in CF patients. However, one infection also may directly affect the other through host-intrinsic mechanisms, such as modulation of immune responses.

Using a mouse model of co-infection between *A. fumigatus* and *M. abscessus*, we sought to determine the effect of *A. fumigatus* on lung pathology and protection in *M. abscessus* infection. Upon infection with *M. abscessus*, previous *A. fumigatus* infection worsened lung pathology and improved immune control of *M. abscessus*. Using several gene-deficient mouse strains, we showed that the improved control was partly IL-17RA and STAT1 signaling-dependent. In addition, when both T-bet and RORγ-t, the master
transcription factors for the Type 1 and Type 17 responses, respectively, were absent, A.fumigatus-induced protection was lost. These data suggest that the Type 1 and Type 17 pathways, or innate immune cells expressing T-bet and RORγ-t, can act together to control M.abscessus infection in co-infected mice. This is in accordance with previous findings in M.tuberculosis infection, where IL-23 was shown to compensate for IL-12p70 deficiency and to stimulate the induction of M.tuberculosis-specific Type 1 and Type 17 cells (29). In addition, innate immune cell activation may contribute to enhanced M.abscessus control in co-infected mice, as in vitro stimulation of macrophages with the fungal β-glucan products zymosan and curdlan promoted enhanced macrophage activation and M.abscessus killing. β-glucans can signal through a variety of immune receptors, including complement receptor 3 (CR3), Toll-like receptor (TLR) 2/6, and dectin-1, which is thought to be the main β-glucan receptor on leukocytes (30). Previous studies have found that macrophages can become activated in response to β-glucans, increasing tumor necrosis factor-α and inducible nitric oxide synthase (iNOS) expression in a Myeloid differentiation primary response gene 88 (MyD88) and Nuclear Factor Kappa B (NF-κB) dependent manner (30-32). Thus, both innate and adaptive immunity may contribute to M.abscessus containment in co-infected mice.

Our mouse model provides novel insights into human findings showing that co-infection of NTM and A.fumigatus is associated with lung function decline in CF (2). We observed enhanced immune control of M.abscessus in mice co-infected with A.fumigatus; however this came at the expense of increased lung pathology. In patients with cystic fibrosis, mucus accumulation and ineffectiveness of lung antimicrobial peptides prevent infections from being readily cleared (6). Thus A.fumigatus and
M. abscessus co-infection in a CF patient who is unable to easily clear infection could lead to a cycle of inflammation and lung damage, contributing to a decline in pulmonary function observed in studies (2). Even with antimicrobial treatment and eventual clearance of NTM, it is conceivable that CF patients co-infected with A. fumigatus and M. abscessus could experience a decline in lung function due to prolonged lung damage during a year-long treatment course. PBMCs from CF patients secreted increased amounts of IL-10 compared to healthy controls when exposed to recombinant A. fumigatus antigens (14). Blockade of IL-10 resulted in enhanced type 1 responses, suggesting a role of IL-10 in inhibiting A. fumigatus T cell responses in CF (14). This could represent an adaptive mechanism in CF patients in response to a prolonged cycle of lung inflammation and damage due to chronic infection with A. fumigatus and other organisms. In the current study, we have established an acute and chronic model of A. fumigatus and M. abscessus coinfection in wild-type B6 mouse. Although co-infection in B6 mice does not recapitulate all the features of human chronic lung inflammation seen in CF patients, the in vivo model described here provides a new platform to understand the host immune parameters of pathogens such as A. fumigatus and M. abscessus, that often coexist in CF patients (2). Future studies could refine the establishment of a chronic co-infection model which will enable the delineation of the immune factors that limit pathogenesis and/or drive chronic co-infection seen in human CF patients.

In summary, our results demonstrate that A. fumigatus, a CF-prevalent fungal organism, can worsen pulmonary pathology and inflammation, and improve M. abscessus control early during M. abscessus infection. This improved control was partly IL-17RA and STAT1-signaling dependent, and dependent on both T-bet and...
These findings also provide novel insight into the immune mechanisms regulating clearance of *M. abscessus* in a mouse model. The long-term effects of *A. fumigatus* co-infection on *M. abscessus* control and pathology, as well as the clinical significance of these findings, may be elucidated in future studies. Future work should include studying the effects of *A. fumigatus/M. abscessus* co-infection in an immunocompromised or CF animal model, which would allow for impaired clearance of pathogens and chronic prolonged infection. It would be important to determine whether prolonged co-infection would result in deterioration in lung function, as has been observed in some human cohorts. Previous work has demonstrated that chronic *A. fumigatus* infection is a risk factor for pulmonary exacerbations in CF patients (33). Currently, the indications for treatment of *A. fumigatus* colonization in CF patients are unclear and based on limited data. Thus, it would be useful to determine whether treatment of *A. fumigatus* may impact the course of *M. abscessus* infection and prevent deterioration of lung function. Finally, the precise downstream effects of the master transcription factors T-bet and RORγt, that regulate *M. abscessus* clearance in our model, should be further clarified.

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FIGURE LEGENDS

**Figure 1.** *A. fumigatus* infection results in early IL-17 responses in the lung.

C57BL/6 (B6) mice were challenged with $1 \times 10^7$ *A. fumigatus* conidia and flow cytometry and PCR of lung performed at time points ranging from 1 to 34 days. (A) The total number of lung IL-17+ cells, (B) CD4+ IL-17-producing cells, or (C) lung TCR $\gamma\delta$+ IL-17+ cells was determined by flow cytometry. (D) The expression of *Il-17* mRNA was determined by RT-PCR of lung tissue; hypoxanthine guanine phosphoribosyl transferase (*Hprt*) expression was used as a control. (n=6).

**Figure 2.** *A. fumigatus* co-infection worsens lung pathology and enhances *M. abscessus* control in acute infection.

*A. fumigatus*-infected (Mabs+Af) B6 mice were challenged with $2 \times 10^4$ cfu of *M. abscessus* after 3 days and control (Mabs) mice with $2 \times 10^4$ cfu of *M. abscessus* alone. A group of mice only received *A. fumigatus*-infection (Af). Naïve uninfected B6 mice were also included (-). (A) Lung bacterial burden was assessed 14 days post infection (dpi) by plating. (B) Pulmonary inflammation was assessed on 14 dpi formalin-fixed paraffin-embedded (FFPE) lung sections stained with H&E 100X magnification. (C) Inflammatory foci were counted on H&E stained lung sections. (D) PAS stain of FFPE lung sections to assess mucus and glycogen was performed and assessed at 200X.
magnification. (E) The number of neutrophils (CD11b+GR1+), (F) alveolar macrophages (CD11c+, high autofluorescence) and (G) MHCII+ alveolar macrophages (MHCII+) CD11c+, high autofluorescence) were determined in lungs of co-infected mice 14 dpi using flow cytometry. n=5 for all groups. *p≤0.05, **p≤0.01 ***p≤0.001

**Figure 3. A. fumigatus enhances M. abscessus control via a mucus-independent mechanism.**

*A. fumigatus*-infected (Mabs+Af) B6 mice were challenged with 2x10⁴ cfu of *M. abscessus* after 3 days, and control (Mabs) mice were infected with 2x10⁴ cfu of *M. abscessus* alone. (A) Lung bacterial burden was assessed 1 hour following *M. abscessus* challenge by plating. *A. fumigatus*-infected (Mabs+Af) B6 and Stat6−/− mice were challenged with 2x10⁴ cfu of *M. abscessus* after 3 days, and control (Mabs) B6 mice and Stat6−/− mice were infected with 2x10⁴ cfu of *M. abscessus* alone. (B) Lung bacterial burden was assessed 14 dpi by plating. n=3-5 for all groups. *p≤0.05, ***p≤0.001, NS - not significant.

**Figure 4. T cells producing IL-17 and IFN-γ accumulate in the lung of co-infected B6 mice.**

*A. fumigatus*-infected (Mabs+Af) B6 mice were challenged with 2x10⁴ cfu of *M. abscessus* after 3 days, and control (Mabs) mice were infected with 2x10⁴ cfu of *M. abscessus* alone. A group of mice only received *A. fumigatus*-infection (Af).

(A-D) The number of IL-17-producing and IL-17/IFN-γ-co-producing CD4⁺CD44⁺ and CD3⁺TCRγδ⁺ T cells were determined in the lungs of infected mice on 14 dpi by flow cytometry. (E) The number CD4⁺CD44⁺ T cells producing IFN-γ were determined
in the lungs of mice on 14 dpi by flow cytometry. (F) The number of IL-17-producing, 
*M. abscessus*-specific T cells were determined in the lungs of infected mice 14 dpi using 
antigen-driven ELISpot assay. *n*=3-5 for all groups. *p*≤0.05, **p*≤0.01, ***p*≤0.001. NS 
- not significant.

**Figure 5.** *A. fumigatus*-induced *M. abscessus* control is IL-17RA/STAT1-signaling 
and Tbx21/Rorc-dependent.

*A. fumigatus*-infected (Mabs+Af) B6, *Il17ra*<sup>−/−</sup> and *Stat1*<sup>−/−</sup> mice were challenged with 2x10<sup>4</sup> 
cfu of *M. abscessus* after 3 days. B6, *Il17ra*<sup>−/−</sup> and *Stat1*<sup>−/−</sup> were treated with 2x10<sup>4</sup> cfu of 
*M. abscessus* (Mabs) alone. (A) Lung bacterial burden was assessed 14 dpi by plating. 
*A. fumigatus*-infected (Mabs+Af) B6, *Tbx21*<sup>−/−</sup>, *Rorc*<sup>−/−</sup> and *Tbx21*<sup>−/−</sup>*Rorc*<sup>−/−</sup> mice were 
challenged with 2x10<sup>4</sup> cfu of *M. abscessus* after 3 days, and B6 mice with 2x10<sup>4</sup> cfu of 
*M. abscessus* (Mabs) alone. (B) Lung bacterial burden was determined 14 dpi. (C) 
Inflammatory foci were counted in H&E stained lung sections. (D-E) Pulmonary 
inflammation was assessed on 14 dpi FFPE lung sections stained with H&E at 100X magnification. Representative lung pathology is shown from (D) B6 control mice (Mabs), 
and *A. fumigatus*-co-infected B6 mice (Mabs+Af); and (E) *A. fumigatus*-co-infected 
*Tbx21*<sup>−/−</sup> mice, *Rorc*<sup>−/−</sup> mice, and *Tbx21*<sup>−/−</sup>*Rorc*<sup>−/−</sup> mice. *n*=3-5 for all groups. *p*≤0.05, 
**p*≤0.01, ***p*≤0.001.

**Figure 6.** *A. fumigatus*-associated lung pathology and improved *M. abscessus* 
control in co-infected mice persists in chronic infection.

*A. fumigatus*-infected (Mabs+Af) B6 mice were challenged with 2x10<sup>4</sup> cfu of 
*M. abscessus* after 3 days and control (Mabs) mice with 2x10<sup>4</sup> cfu of *M. abscessus* alone. 
Both group then received 3 additional weekly infections with 2x10<sup>4</sup> cfu *M. abscessus*. 
(A) Lung bacterial burden was assessed 30 dpi by plating. (B) Pulmonary inflammation was assessed at 30 dpi by FFPE lung sections stained with H&E 100X magnification. (C) Inflammatory foci were scored on H&E stained lung sections. n=4-5 for all groups. **p≤0.01.

**Figure 7. Fungal antigens improve in vitro killing of *M. abscessus*.**

IFN-γ, zymosan and curdlan-treated or control BMDMs were infected with *M. abscessus* (MOI 1) for 48 hours. IFN-γ was utilized as a positive control. (A) The number of viable bacteria within BMDMs was determined by plating and (B) nitrite levels in the supernatants were determined using GREIS assays. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

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Figure 5. Monin et al
Figure 6. Monin et al
Figure 7. Monin et al