



Mitochondrial Reactive Oxygen Species Enhance Alveolar Macrophage Activity against *Aspergillus fumigatus* but Are Dispensable for Host Protection

Neta Shlezinger,^{a,b,c}  Tobias M. Hohl^{a,b}

^aInfectious Disease Service, Department of Medicine, Memorial Hospital, Memorial Sloan Kettering Cancer Center, New York, New York, USA

^bImmunology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, New York, USA

^cKoret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel

ABSTRACT *Aspergillus fumigatus* is the most common cause of mold pneumonia worldwide, and a significant cause of infectious morbidity and mortality in immunocompromised individuals. The oxidative burst, which generates reactive oxidative species (ROS), plays a pivotal role in host defense against aspergillosis and induces regulated cell death in *Aspergillus* conidia, the infectious propagules. Beyond the well-established role of NADP (NADPH) oxidase in ROS generation by neutrophils and other innate effector cells, mitochondria represent a major ROS production site in many cell types, though it is unclear whether mitochondrial ROS (mtROS) contribute to antifungal activity in the lung. Following *A. fumigatus* infection, we observed that innate effector cells, including alveolar macrophages (AMs), monocyte-derived dendritic cells (Mo-DCS), and neutrophils, generated mtROS, primarily in fungus-infected cells. To examine the functional role of mtROS, specifically the H₂O₂ component, in pulmonary host defense against *A. fumigatus*, we infected transgenic mice that expressed a mitochondrion-targeted catalase. Using a reporter of fungal viability during interactions with leukocytes, mitochondrial H₂O₂ (mtH₂O₂) was essential for optimal AM, but not for neutrophil phagocytic and conidiocidal activity in the lung. Catalase-mediated mtH₂O₂ neutralization did not lead to invasive aspergillosis in otherwise immunocompetent mice and did not shorten survival in mice that lack NADPH oxidase function. Collectively, these studies indicate that mtROS-associated defects in AM antifungal activity can be functionally compensated by the action of NADPH oxidase and by nonoxidative effector mechanisms during murine *A. fumigatus* lung infection.

IMPORTANCE *Aspergillus fumigatus* is a fungal pathogen that causes invasive disease in humans with defects in immune function. Airborne conidia, the infectious propagules, are ubiquitous and inhaled on a daily basis. In the respiratory tree, conidia are killed by the coordinated actions of phagocytes, including alveolar macrophages, neutrophils, and monocyte-derived dendritic cells. The oxidative burst represents a central killing mechanism and relies on the assembly of the NADPH oxidase complex on the phagosomal membrane. However, NADPH oxidase-deficient leukocytes have significant residual fungicidal activity *in vivo*, indicating the presence of alternative effector mechanisms. Here, we report that murine innate immune cells produce mitochondrial reactive oxygen species (mtROS) in response to fungal interactions. Neutralizing the mtROS constituent hydrogen peroxide (H₂O₂) via a catalase expressed in mitochondria of innate immune cells substantially diminished fungicidal properties of alveolar macrophages, but not of other innate immune cells. These data indicate that mtH₂O₂ represent a novel AM killing mechanism against *Aspergillus* conidia. mtH₂O₂ neutralization is compensated by other killing mechanisms in the lung, demonstrating functional redundancy at the level of host defense

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Address correspondence to Neta Shlezinger, neta.shlezinger1@mail.huji.ac.il, or Tobias M. Hohl, hohl@mskcc.org.

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in the respiratory tree. These findings have important implications for the development of host-directed therapies against invasive aspergillosis in susceptible patient populations.

KEYWORDS fungus, innate immunity, lung

A *Aspergillus* species are ubiquitous molds that form and disperse conidia (i.e., vegetative spores) into the air. These infectious propagules can penetrate deeply into the respiratory tract upon inhalation (1). In immunocompetent individuals, conidia are effectively cleared from the lungs by the respiratory innate immune system. Beyond the essential role of neutrophils and alveolar macrophages (AMs), monocyte-derived and plasmacytoid dendritic cells cooperate to prevent the germination of conidia into tissue-invasive hyphae (2–6). However, patients with a compromised innate immune function are at risk of developing invasive aspergillosis (IA). *Aspergillus fumigatus* accounts for about 65% of all invasive infections in humans, with ~300,000 annual cases and is the most frequently encountered *Aspergillus* spp. in pulmonary infections (7).

The generation of the oxidative burst is a signature event associated with the activation of phagocytic cells (8) and involves the assembly and formation of a functional NADPH oxidase enzyme on the phagosomal membrane (7, 9). The requirement of reactive oxidative species (ROS) production by NADPH oxidase to protect against invasive fungal infections is underscored by the high prevalence of *Aspergillus* infections in patients with chronic granulomatous disease (CGD), a rare hereditary disease, in which a defect in one of the subunits of NADPH oxidase leads to a defect in ROS production by phagocytes, resulting in severe recurrent bacterial and fungal infections (10).

Previous studies from our and other groups indicate that NADPH oxidase-deficient neutrophils have significant residual conidiacidal activity *in vitro* and *in vivo* (11–13). Furthermore, most CGD patients survive years without infection, despite ubiquitous exposure to *A. fumigatus* conidia (14). These observations led to the discovery of NADPH oxidase-independent fungicidal mechanisms, for example, nutritional immunity which relies on the sequestration of essential transition metals by host proteins (15–18). Notably, sequestration of iron and zinc by neutrophil lactoferrin and manganese chelation by neutrophil calprotectin play essential roles in host defense against fungal pathogens (13, 19–21).

However, NADPH oxidases are not the only intracellular source of ROS. Hence, alternate ROS-dependent killing pathways may contribute to the antifungal properties of innate immune cells. Over the past decade, studies have demonstrated that mitochondrial ROS (mtROS) play multifaceted signaling functions that orchestrate innate and adaptive immunity (22–28). These include macrophage phagocytosis, the assembly of inflammasomes, cytokine production, and antigen processing (29–31). Recent studies have implicated mtROS in bacterial clearance from infected macrophages and monocyte-derived dendritic cells through the recruitment of mitochondria to pathogen-containing phagosomes (32), consistent with the idea that mtROS can augment antibacterial defenses. In contrast to bacteria, mtROS have not been integrated into an *in vivo* model of host defense against fungal pathogens.

In this study, we explored the role of mtROS in antifungal immunity against *A. fumigatus*. By employing a fluorescent reporter of fungal viability, we quantified the role of mtROS in conidial phagocytosis and killing by cells of respiratory innate immune system with single-encounter resolution. Using wild-type and transgenic mice that expressed a mitochondrion-localized catalase (mCAT), we demonstrated mtROS generation in a range of innate immune cells in response to *Aspergillus* infection and found that the H₂O₂ component of mtROS was crucial for optimal alveolar macrophage antifungal activity. mtH₂O₂ regulated conidial uptake and killing by alveolar macrophages in an NADPH oxidase-independent and cell intrinsic manner. These results implicate a specific mtROS as a regulator of AM antifungal immunity and demonstrate functional redundancy with other oxidative and nonoxidative killing systems in the lung.

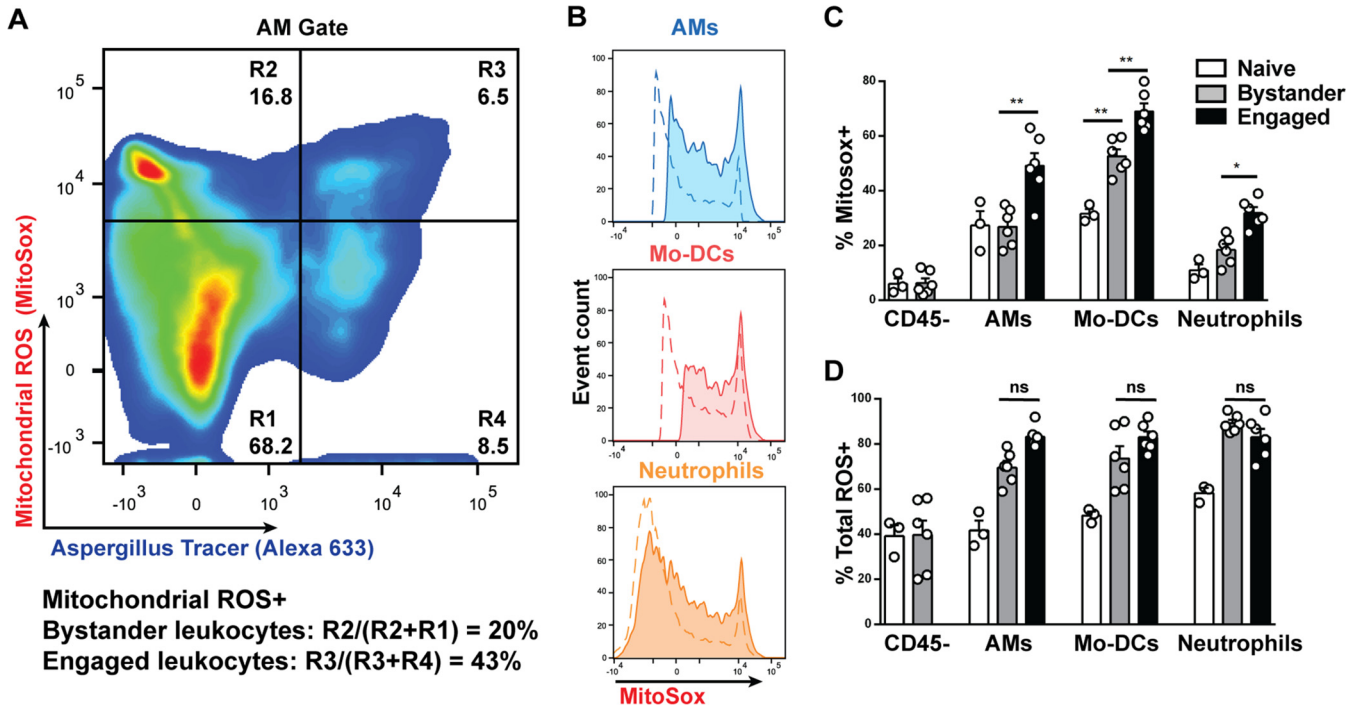


FIG 1 *A. fumigatus* conidia trigger mitochondrial ROS in lung leukocytes. C57BL/6 mice were challenged with 3×10^7 *A. fumigatus* FLARE conidia, and single-cell lung suspensions were analyzed for mitochondrial ROS (MitoSox) and total ROS (CM-H2DCFDA) 24 h postchallenge. (A) Mitochondrial ROS staining in AF633⁻ bystander and AF633⁺ fungus-engaged alveolar macrophages (AMs). The gates indicate the frequencies of mtROS-negative (R1 and R4) and mtROS-positive (R2 and R3) bystander (R1 and R2) or fungus-engaged (R3 and R4) AMs. (B) Histogram of mitochondrial ROS in bystander (broken line) or fungus-engaged (continuous line) alveolar macrophages, Mo-DCs, and lung neutrophils. (C and D) Mitochondrial ROS (C) and total ROS (D) in naive (white bars), bystander (gray bars), and engaged (black bars) CD45⁻ nonhematopoietic, alveolar macrophages, Mo-DCs, and neutrophils. Data shown represent means \pm SEM (error bars) from two independent experiments ($n=6$ for infected mice, $n=3$ for naive mice). Statistical analysis was performed with a one-way ANOVA ($P < 0.001$) followed by a *post hoc* Tukey HSD analysis. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

RESULTS

Leukocytes produce mtROS in response to phagocytosed *A. fumigatus* conidia.

To determine whether *Aspergillus* interactions stimulate mtROS production in lung leukocytes, C57BL/6 mice were challenged with *A. fumigatus* conidia, and mtROS was quantified in single-cell lung suspensions (Fig. 1A). Fungus-engaged and bystander lung leukocytes were distinguished by the use of fluorescent *Aspergillus* reporter (FLARE) conidia that were covalently labeled with the fluorescent tracer Alexa Fluor 633 (AF633) (12). Conidial uptake by alveolar macrophages (AMs) triggered mtROS induction, as judged by MitoSox staining of fungus-engaged cells (AF633-positive [AF633⁺]) compared with bystander AMs and with AMs isolated from naive mice (Fig. 1B to D). Similarly, conidial uptake triggered mtROS induction in lung monocyte-derived dendritic cells (Mo-DCs) and neutrophils (Fig. 1B to D). In contrast, mtROS induction was not observed in CD45⁻ nonhematopoietic cells isolated from *A. fumigatus*-infected lungs. These data demonstrate that conidium-leukocyte interactions induce, at least in part, mtROS induction during respiratory *A. fumigatus* challenge.

Mitochondrial ROS mediate AM phagocytic and fungicidal activity in the lung.

To examine the functional role of mtROS in antifungal immunity, we utilized well-characterized transgenic mice that expressed a mitochondrion-localized human catalase (mCAT^{Tg/+}) and thus neutralized mtH₂O₂ (33). mCAT^{Tg/+} mice exhibit significantly lower mitochondrial H₂O₂ levels, reduced age-related oxidative damage, and an extended life span compared to nontransgenic littermate controls (33). In the first series of experiments, we measured the leukocyte intrinsic role of mtH₂O₂ in conidial uptake and conidial killing by generating mixed bone marrow chimeric mice, in which irradiated CD45.1⁺ CD45.2⁺ recipients were reconstituted with a 1:1 ratio of CD45.2⁺ mCAT^{Tg/+} and CD45.1⁺ wild-type congenic bone marrow cells (see Fig. S1 in the

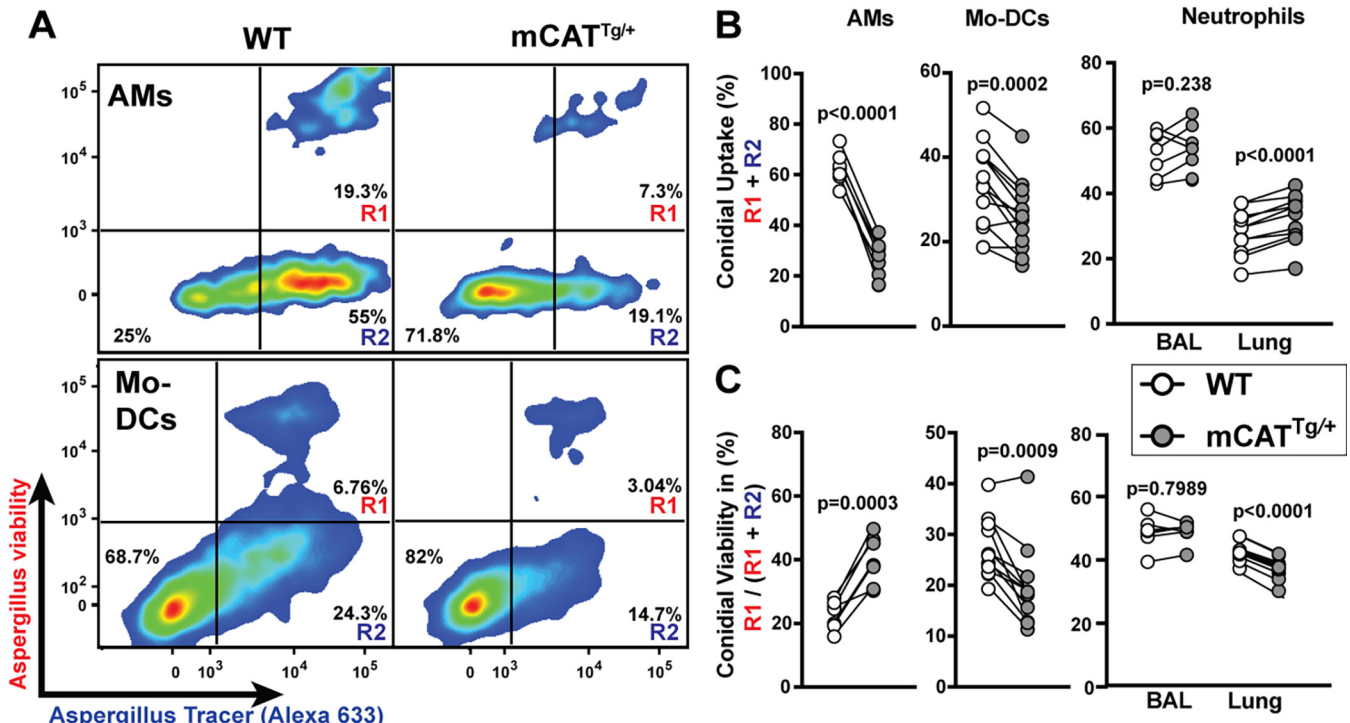


FIG 2 Impact of mtH_2O_2 neutralization on AMs, Mo-DCs, and neutrophil conidial uptake and killing in the lung. Mixed BM chimeric mice were infected with 3×10^7 FLARE conidia intranasally. (A) Representative plots showing WT or mCAT-expressing AMs and lung Mo-DCs harvested from mixed bone marrow chimeric mice 24 h postinfection (p.i.), analyzed on the basis of dsRed (*Aspergillus* viability) and AF633 (*Aspergillus* tracer) fluorescence. R1, leukocytes with live conidia; R2, leukocytes with dead conidia. (B and C) The scatterplots indicate conidial uptake (R1 + R2) (B) by and intraphagosomal conidial viability [R1/(R1 + R2)] (C) in indicated leukocyte subsets. The lines indicate paired data sets isolated from a single mixed chimeric mouse. $n \geq 9$ per group pooled from yeoindependent experiments. A paired *t* test was used for the statistical analysis.

supplemental material). The properties of CD45.2⁺ mCAT^{Tg/+} and CD45.1⁺ wild-type leukocytes were thus compared in the same host inflammatory environment (Fig. S2).

Mixed chimeric mice were infected with FLARE conidia that, in addition to the AF633 tracer fluorophore, also encoded a dsRed transgene which acted as a marker of fungal viability (12). The dsRed tracer fluorophore distinguished AF633⁺ fungus-engaged leukocytes that contained either live (dsRed⁺) or killed (dsRed⁻) conidia, as shown for AMs in Fig. 2A. The frequency of fungus-engaged AF633⁺ AMs (Fig. 2A, conidial uptake = R1 + R2) was vastly reduced in mCAT-expressing cells (mCAT^{Tg/+}, 35% ± 3%) compared to WT cells (70% ± 2%; Fig. 2B) at 24 h postinfection. Similar results were observed in mCAT-expressing Mo-DCs compared to wild-type (WT) Mo-DCs (Fig. 2A, lower panel, and Fig. 2B), consistent with a conidial uptake defect in mCAT^{Tg/+} AMs and Mo-DCs compared to wild-type counterparts isolated from the same inflammatory milieu. Moreover, the frequency of fungus-engaged AMs that contained live conidia was increased in mCAT^{Tg/+} AMs (38% ± 3%) compared to WT (22% ± 2%) AMs [Fig. 2A, intraleukocyte conidial viability = R1/(R1 + R2)], indicating an AM intrinsic defect in intracellular conidial killing when mtH_2O_2 were neutralized (Fig. 2C). Notably, mitochondrial catalase expression did not interfere with lung and bronchoalveolar lavage fluid (BALF) neutrophil conidiocidal activity (Fig. 2B and C). In fact, mCAT^{Tg/+} neutrophils exhibited a modest increase in conidial uptake and conidial killing. Surprisingly, mCAT^{Tg/+} Mo-DCs also exhibited improved conidial killing. Collectively, these findings indicate that mtH_2O_2 differentially regulated AM, Mo-DC, and neutrophil phagocytic and fungicidal activity during *A. fumigatus* infection, with an essential role for optimal AM antifungal activity.

To define the role of mtH_2O_2 on infectious outcomes following *A. fumigatus* infection, we compared the histopathology and mortality in mCAT^{Tg/+} mice and nontransgenic littermate controls. Lung histopathology of mCAT^{Tg/+} mice 3 days postinfection

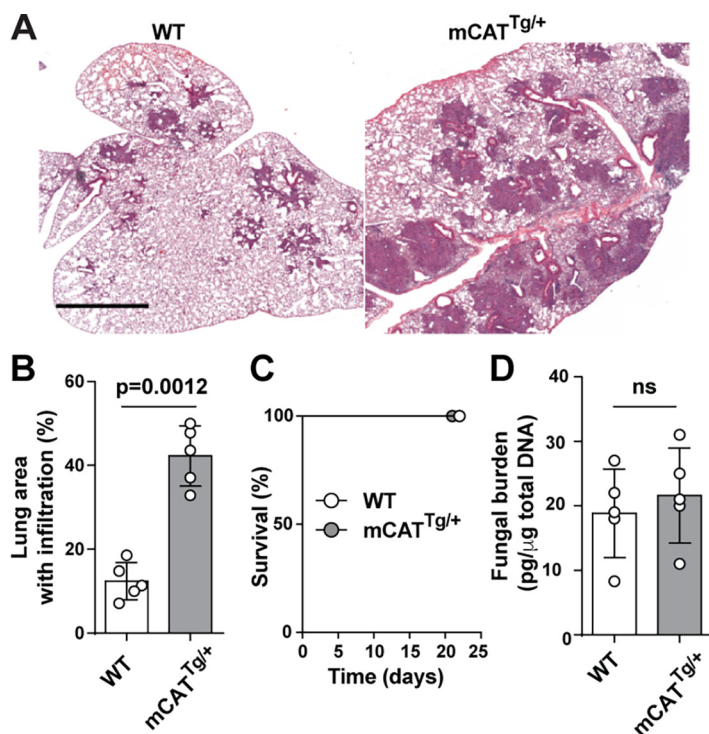


FIG 3 mtH₂O₂ neutralization regulates lung inflammation but is dispensable for *A. fumigatus* susceptibility. Immunocompetent C57BL/6 and mCAT^{Tg/+} mice were challenged with 8×10^7 Af293 conidia. (A) Representative micrographs of hematoxylin and eosin-stained lung sections from two independent experiments (bar, 2 mm). (B) Quantitative morphometric analysis of lung consolidation at day +3 postinfection. (C) Kaplan-Meier survival analysis. (D) Lung fungal burden (fungal DNA) at day +3 postinfection, according to murine genotype. Data shown represent means \pm SEM (error bars) ($n=5$ for panel B, $n=10$ for panel C, $n=6$ for panel D). For statistical analysis, *t* test was used for the data in panels B and D, and log rank (Mantel-Cox) test was used for the data in panel C.

revealed multifocal areas of necrosis and inflammation that affected >40% of the parenchyma, while control mice had moderate multifocal inflammation that involved ~10% of the parenchyma (Fig. 3A and B). Despite widespread inflammatory lesions in mCAT^{Tg/+} lung sections, evidence of conidial germination and hyphal tissue invasion was not observed (Fig. S3).

To determine whether catalase-dependent mtH₂O₂ neutralization influenced murine susceptibility to *A. fumigatus*, immunocompetent mCAT^{Tg/+} and littermate control mice were challenged with 8×10^7 *A. fumigatus* Af293 conidia and monitored for survival (Fig. 3C). Mice from both genotypes all survived the challenge, and consistent with this finding, the lung fungal burden at 3 days postinfection, as measured by fungal DNA content, was not affected by mitochondrion-targeted H₂O₂ neutralization (Fig. 3D). These findings indicate that mtH₂O₂ is not essential for protection against respiratory *A. fumigatus* challenge with the low virulence Af293 strain.

One possible explanation for these results is that products of NADPH oxidase can compensate for the mitochondrion-targeted catalase-catalyzed H₂O₂ breakdown. To test this hypothesis, we crossed the mCAT transgene to the *p91phox*^{-/-} background and generated mixed bone marrow chimeric mice to compare the cell intrinsic fungicidal activities of CD45.1⁺ *p91phox*^{-/-} and CD45.2⁺ *p91phox*^{-/-} mCAT^{Tg/+} leukocytes in the same lung (Fig. S1 and S2), as outlined above. Following infection with FLARE conidia, AM conidial uptake was reduced in *p91phox*^{-/-} mCAT^{Tg/+} AMs (34% \pm 5%) compared to *p91phox*^{-/-} (50% \pm 6%) counterparts at 24 h postinfection (Fig. 4A and B). In addition, intraleukocyte conidial viability was higher in *p91phox*^{-/-} mCAT^{Tg/+} AMs (60% \pm 2%) compared to *p91phox*^{-/-} (28% \pm 7%) counterparts that had intact mtH₂O₂

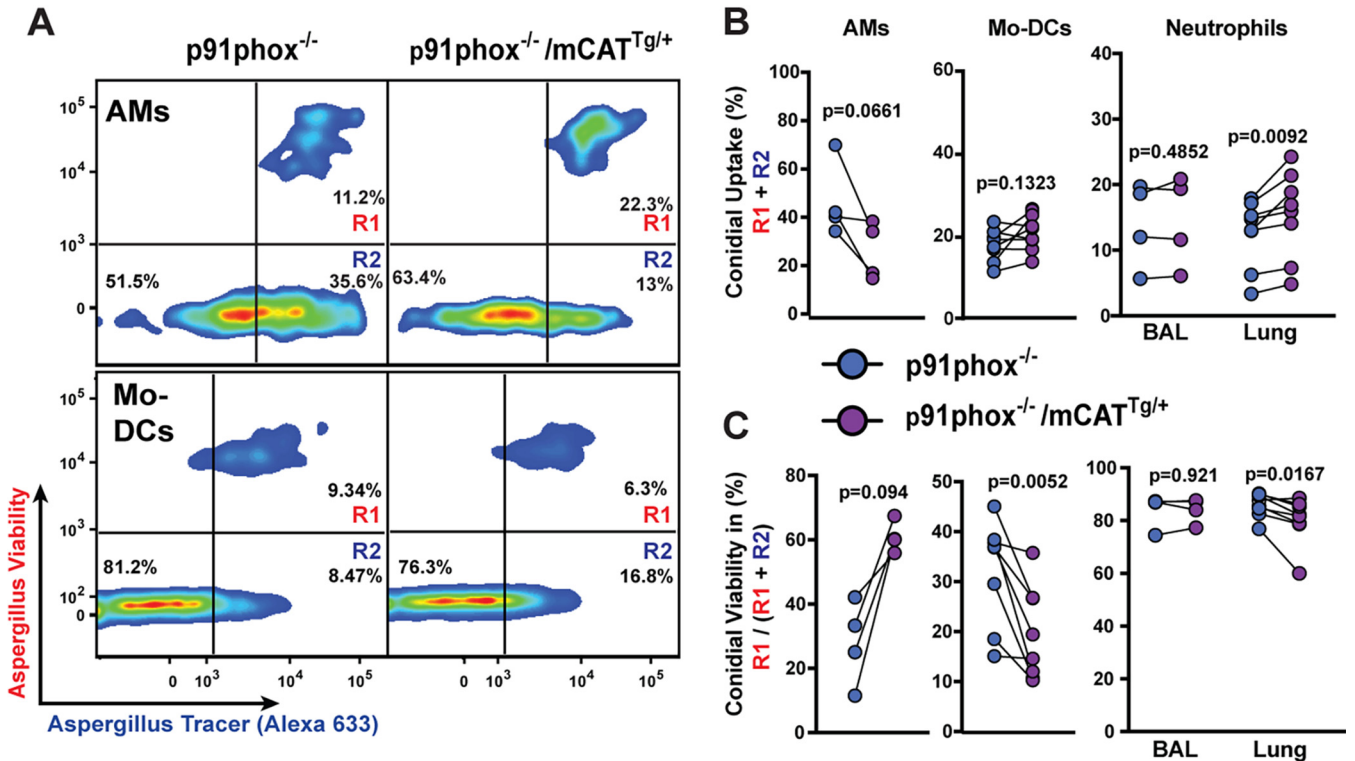


FIG 4 Impact of mtH₂O₂ neutralization on leukocyte conidial uptake and killing in NADPH oxidase-deficient mice. Mixed BM chimeric mice were infected with 3×10^7 FLARE conidia intranasally. (A) Representative plots showing *p91phox*^{-/-} or mCAT-expressing *p91phox*^{-/-} (mCAT^{Tg/+} *p91phox*^{-/-}) BAL fluid alveolar macrophages and lung Mo-DCs harvested from mixed bone marrow chimeric mice 24 h p.i., analyzed on the basis of dsRed (viability) and AF633 (tracer) fluorescence. R1, leukocytes with live conidia; R2, leukocytes with dead conidia. (B and C) The scatterplots indicate conidial uptake (R1 + R2) (B) by and intraphagosomal conidial viability [R1/(R1 + R2)] (C) in indicated leukocyte subsets. The lines indicate paired data sets isolated from a single mixed chimeric mouse. $n \geq 6$ per group pooled from two independent experiments. A paired *t* test was used for statistical analysis.

levels, though the difference did not reach statistical significance (Fig. 4C). The previously observed mCAT-dependent defect in conidial uptake by Mo-DCs was not observed in the context of NADPH oxidase deficiency (Fig. 4B). Mo-DC and neutrophil intracellular conidial killing in *p91phox*^{-/-} mice was slightly higher with mCAT transgene expression compared to nontransgenic counterparts, consistent with the idea that mtH₂O₂ did not contribute to conidiacidal activity in these cell types (Fig. 4C).

To determine whether mtH₂O₂ was essential for host defense in the context of NADPH oxidase deficiency, *p91phox*^{-/-} mCAT^{Tg/+} and nontransgenic *p91phox*^{-/-} control mice were challenged with 5×10^4 conidia and monitored for survival, lung pathology, and fungal burden (Fig. 5). In line with previous studies (10), *p91phox*^{-/-} mice succumbed to infection (Fig. 5A and B). Although mtH₂O₂ neutralization in the *p91phox*^{-/-} background further exacerbated lung inflammation, this finding was not associated with increased mortality and a higher lung fungal burden compared to *p91phox*^{-/-} mice (Fig. 5C and D).

DISCUSSION

In this study, we demonstrated that *A. fumigatus* infection induced mtROS production in a range of innate immune effector cells during pulmonary infection. In AMs, the mtROS constituent H₂O₂ contributed to fungicidal activity against conidia, since catalase-mediated neutralization of mtH₂O₂ diminished their antifungal activity *in vivo*. These findings mirror and extend a study of cultured macrophages, recently published by Hatinguais and colleagues (34). In this work, pharmacologic inhibition of the macrophage mitochondrial reverse electron transport chain reduced *Aspergillus* metabolic activity and regulated *Aspergillus*-triggered cytokine production *in vitro* (34).

Our findings clarify and expand previous studies that highlight context-specific roles

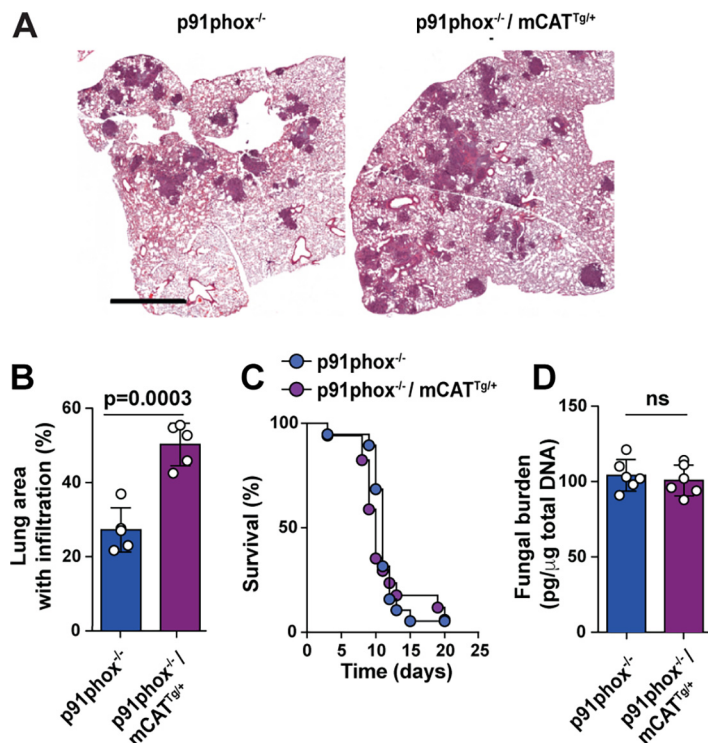


FIG 5 Impact of mtH₂O₂ neutralization on lung inflammation, survival, and fungal growth in NADPH oxidase-deficient mice. p91phox^{-/-} and mCAT^{Tg/+} p91phox^{-/-} mice were challenged with 5 × 10⁴ Af293 conidia. (A) Micrographs of lung sections stained with hematoxylin and eosin from two independent experiments (bar, 2 mm). (B) Quantitative morphometric analysis of lung consolidation at day +3 postinfection. (C) Kaplan-Meier survival analysis. (D) Lung fungal burden (fungal DNA) day +3 postinfection, according to murine genotype. Data shown represent means ± SEM (n = 5 for panel B, n = 19 for p91phox^{-/-} and n = 17 mCAT^{Tg/+} p91phox^{-/-} in panel C, and n = 6 for panel D). For statistical analysis, t test was used for the data in panels B and D, and log rank (Mantel-Cox) test was used for the data in panel C.

for AM function in host defense. In a prior study, clodronate liposome-mediated AM depletion did not facilitate the development of lethal invasive aspergillosis (3), while another study reported an increase in lung fungal burden in clodronate liposome-treated mice (35). Previous work suggested that AM fungicidal activity was similar in NADPH oxidase-sufficient and -deficient cells, raising questions about relevant alternative conidial killing mechanisms (12, 36). In support of this notion, many bacterial, protozoan, and fungal pathogens, including *Candida albicans*, *Cryptococcus neoformans*, and *A. fumigatus*, subvert the phagosomal oxidative burst by interfering with NADPH oxidase complex assembly or by utilizing detoxification mechanisms, yet phagocytes retain significant microbicidal activity *in vivo*, indicating the presence of additional antifungal effectors (37–42). This study provides evidence for mtH₂O₂ as a bona fide AM effector molecule against *A. fumigatus* conidia in the lung. The precise contribution and interdependence of NADPH oxidase and mtROS to AM anti-*Aspergillus* activity remain to be determined, though it is notable that both rotenone- or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)-treated macrophages *in vitro* (34) and mtH₂O₂-neutralized AMs *in vivo* both exhibit a clear trend to decreased antifungal activity when NADPH oxidase is either pharmacologically blocked (i.e., diphenyleneiodonium chloride [DPI]) or genetically ablated.

Previous studies showed that mitochondria regulate *Salmonella* and lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) signaling and bacterial killing in murine bone marrow-derived macrophages (BMDMs) (32). TLR activation resulted in the recruitment of mitochondria to macrophage phagosomes and in enhancement of mtROS production. A subsequent study corroborated these findings in macrophage-mediated defense against methicillin-resistant *Staphylococcus aureus* (MRSA) and

further revealed that, upon infection, endoplasmic reticulum (ER) stress stimulated mtROS production and delivery to bacterium-containing phagosomes via mitochondrion-derived vesicles (43). In agreement with our observations, expression of mitochondrial catalase or superoxide dismutase-2 (Sod2) depletion led to reduced macrophage bactericidal activity. However, it remained unknown whether mtROS constituents, individually or collectively, were essential for survival against systemic or compartmentalized infections has not been studied in animal models.

Using mCAT^{Tg/+} mice, we found that mtH₂O₂ neutralization did not reduce host survival or lung fungal burden in otherwise immunocompetent mice or in *p91phox*^{-/-} mice. These data supported the idea that mtH₂O₂ did not contribute substantially to fungal killing in innate immune cells beyond AMs, even in the absence of NADPH oxidase activity. We cannot exclude the possibility that mitochondrion-localized catalase may exhibit different degrees of mtH₂O₂ neutralization and downstream impact on effector functions in AMs compared to other immune cell populations.

While the requirement of AMs for protection against aspergillosis can be functionally compensated in murine models of disease, neutrophils are essential for host defense and have the capacity to inhibit germination and kill conidia and hyphae *in vivo* (3, 5, 12, 44, 45). In patients with functional or numeric neutrophil defects, the role of AMs in host defense against *A. fumigatus* is likely accentuated, given the loss of cellular redundancy (6). Thus, we were surprised that functional impairment of mCAT^{Tg/+} AMs in *p91phox*^{-/-} mice did not result in a higher lung fungal burden or in more rapid disease development. One possibility for this finding is that AM mtH₂O₂ is particularly relevant for host defense against conidia but less effective against hyphae and products of germination which are prevalent in *p91phox*^{-/-} mice (46). In this model, once hyphae are formed, fungal killing is driven primarily by neutrophils that were not affected by mtH₂O₂ neutralization (3, 47). A study of zebrafish larvae demonstrated that neutrophil killing required conidial germination, while macrophages inhibited conidial germination and attenuated neutrophil recruitment and neutrophil-mediated killing (47). This model may align with our data showing that mtH₂O₂ neutralization rendered AMs dysfunctional with regard to fungicidal activity, resulting in increased conidial uptake and killing by lung neutrophils.

The current study was conducted using the widely used Af293 strain, a clinical isolate with low virulence characteristics. It is possible that the role of mtH₂O₂ in host defense may be greater, equal, or diminished with other *A. fumigatus* strains (e.g., CEA10) that have the capacity to germinate more rapidly and act in a more virulent manner than Af293 yet trigger more pronounced neutrophilic inflammation in the murine lung as well (48). Collectively, experiments in this study and in reference 34 support the idea that mtROS components enhance alveolar macrophage antifungal activity and that this process can act against different *Aspergillus* strains (Af293 and CEA10). Future work should focus on integrating mtROS in pulmonary host defense in a different model of disease, e.g., in neutropenic or in corticosteroid-treated mice, and in the context of a range of clinical *A. fumigatus* isolates. In conclusion, our studies reveal that mtH₂O₂ is dispensable for protection against *A. fumigatus* infection in an immunocompetent and CGD model of *A. fumigatus* infection. However, mtROS do have a functional role in regulating and boosting AM antifungal activity against inhaled conidia. Further studies will be needed to address the *in vivo* role and full range of mtROS against a broad range of inhaled fungal and nonfungal pathogens.

MATERIALS AND METHODS

Mice, animal care, and ethics statement. C57BL/6 mice (Jackson Laboratories, strain 000664; CD45.2⁺), C57BL/6.SJL mice (Charles River Laboratories, strain 564; CD45.1⁺), C57BL/6.mCAT mice (mCAT^{Tg/+}; Jackson Laboratories, strain 016197; CD45.2⁺), and *p91phox*^{-/-} mice (Jackson Laboratories, strain 002365) were bred in the Memorial Sloan Kettering Cancer Center (MSKCC) Animal Vivarium. mCAT^{Tg/+} mice were crossed to *p91phox*^{-/-} mice to generate *p91phox*^{-/-} mCAT^{Tg/+} mice. Lethally irradiated (9.5 Gy) F1 progeny (from cross of C57BL/6 and C57BL/6.SJL strains) were reconstituted with 1×10^6 to 2.5×10^6 C57BL/6.SJL and C57BL/6.mCAT or *p91phox*^{-/-} and *p91phox*^{-/-}.mCAT BM cells, treated with enrofloxacin in the drinking water for 21 days to prevent bacterial infections, and rested for 6 to 8 weeks prior to

use. All animal experiments were conducted with sex- and age-matched mice and performed with approval from MSKCC Institutional Animal Care and Use Committee (protocol number 13-07-008). Animal studies were compliant with all applicable provisions established by the Animal Welfare Act and the Public Health Services Policy on the Humane Care and Use of Laboratory Animals.

***Aspergillus fumigatus* culture and infection model.** *A. fumigatus* Af293 and Af293-dsRed (12) strains were cultured on glucose minimal medium slants at 37°C for 4 to 7 days prior to harvesting conidia for experimental use. To generate FLARE conidia, briefly, 7×10^8 Af293-dsRed conidia were rotated in 10 μ g/ml Biotin XX, SSE in 1 ml of 50 mM carbonate buffer (pH 8.3) for 2 h at 4°C, incubated with 20 μ g/ml Alexa Fluor 633 succinimidyl ester at 37°C for 1 h, resuspended in phosphate-buffered saline (PBS) and 0.025% Tween 20 for use within 24 h (12). To infect mice with 30 to 60 million *A. fumigatus* cells, conidia were resuspended in PBS and 0.025% Tween 20 at a concentration of 0.6×10^9 to 1.2×10^9 /ml cells, and 50 μ l of cell suspension was administered via the intranasal route to mice anesthetized by isoflurane inhalation.

Reagents and antibodies. Chemical and cell culture reagents were purchased from Thermo Scientific and Gibco, unless noted otherwise. Voriconazole (Pfizer) was obtained from the Memorial Sloan Kettering Cancer Center pharmacy and dissolved in sterile PBS at 10 mg/ml and stored at -80°C . Fluorescent antibodies were from Tonbo or eBioscience.

Fungal growth and culture. All *A. fumigatus* strains were grown on glucose minimal media that contains 1% glucose, salt solution, and trace minerals (22). Conidia were harvested by flooding 5- to 10-day-old plates or slants with 10 ml PBS and 0.025% Tween 20, briefly vortexing sealed plates or slants, and by filtering the conidial suspensions twice through a 40- μ m nylon cell strainer. Resting conidia were labeled with Alexa Fluor 633 as previously described (10).

qPCR. Lung fungal burdens in animals were determined by quantitative real-time PCR (qPCR) using methods reported by Bowman et al. (49). Briefly, lungs were homogenized using 2.5-mm sterile beads in sterile saline with a bead beater homogenizer (Mini Bead beater; BioSpec, Bartlesville, OK). DNA was then extracted from 90 μ l of lung homogenate with the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Recovered DNA in 200 μ l of elution buffer was then stored at -80°C until analysis. DNA samples were analyzed in duplicate using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Inc., Foster City, CA) with primers and dually labeled fluorescent hybridization probes specific for the *A. fumigatus* 18S rRNA gene (GenBank accession no. [AB008401](#)). The cycle threshold (C_T) of each sample, which identifies the cycle number during PCR when fluorescence exceeds a threshold value determined by the software, was then interpolated from a six-point standard curve of C_T values prepared by spiking naive uninfected mouse lungs with 1×10^2 to 1×10^7 *A. fumigatus* Af293 conidia. Results were reported as *A. fumigatus* DNA conidial equivalents. Lungs from sham-infected mice were used as a specificity control in each experiment.

Analysis of infected mice. Mice were infected via the intratracheal route as described previously (2). Bronchoalveolar lavage (BAL) fluid and lung suspensions were prepared for flow cytometry as described previously (11). Briefly, lung digest and, if applicable, BAL fluid cells were enumerated and stained with fluorophore-conjugated antibodies for flow cytometric analysis on a BD LSR II. Neutrophils were identified as CD45⁺ CD11b⁺ Ly6C^o Ly6G⁺ cells, alveolar macrophages as CD45⁺ CD11b⁺ CD11c⁺ Ly6G⁻ siglecF⁺ cells, and Mo-DCs as CD45⁺ CD11b⁺ CD11c⁺ Ly6G⁻ Ly6C^{hi} major histocompatibility complex (MHC) class II⁺ cells.

Histology. After euthanasia, lungs were inflated with 1 ml of 10% neutral buffered formalin, removed en bloc after tracheal ligation, preserved in 10% neutral buffered formalin for 24 h at 4°C, and subsequently embedded in paraffin. Lung tissue sections (5 mm) were stained with hematoxylin and eosin (H&E) and Gomori's methenamine-silver (GMS).

ROS measurements. Total intracellular H₂O₂ levels and mitochondrial superoxide levels were measured as previously described (2, 32). Briefly, lung cell single-cell suspensions were washed with PBS and then incubated with MitoSox (to measure mitochondrial superoxide) and/or CM-H2DCFDA [5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester] (to measure total cellular H₂O₂) (Invitrogen) at 5 μ M final concentration in HBSS buffer for 15 to 30 min at 37°C. Cells were washed with warmed PBS three times, removed from plates with cold PBS containing 0.5 mM EDTA by pipetting, pelleted at 1,500 rpm for 5 min, immediately resuspended in cold PBS containing 1% fetal bovine serum (FBS), and subjected to fluorescence-activated cell sorting (FACS) analysis. Unstained controls were treated similarly, except that treatments and dyes were omitted. Flow cytometry data were analyzed using FlowJo software.

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software Inc.) and display experimental data of at least two independent experiments performed in triplicate, displaying the means and standard errors of means (SEM) throughout the manuscript, except for animal-related experiments, which display means \pm 95% confidence intervals. The following statistical tests were applied: comparisons between three or more groups, one-way analysis of variance (ANOVA); comparison between two groups, Student's *t* test; animal survival curves, log rank test. Statistical significance was accepted at $P < 0.05$.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.2 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 0.3 MB.

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