Fungal antioxidant pathways promote survival against neutrophils during infection

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Filamentous fungi are a common cause of blindness and visual impairment worldwide. Using both murine model systems and in vitro human neutrophils, we found that NADPH oxidase produced by neutrophils was essential to control the growth of Aspergillus and Fusarium fungi in the cornea. We demonstrated that neutrophil oxidant production and antifungal activity are dependent on CD18, but not on the β-glucan receptor dectin-1. We used mutant A. fumigatus strains to show that the reactive oxygen species–sensing transcription factor Yap1, superoxide dismutases, and the Yap1-regulated thioredoxin antioxidant pathway are each required for protection against neutrophil-mediated oxidation of hyphae as well as optimal survival of fungal hyphae in vivo. We also demonstrated that thioredoxin inhibition using the anticancer drug PX-12 increased the sensitivity of fungal hyphae to both H2O2- and neutrophil-mediated killing in vitro. Additionally, topical application of PX-12 significantly enhanced neutrophil-mediated fungal killing in infected mouse corneas. Cumulatively, our data reveal critical host oxidative and fungal anti-oxidative mediators that regulate hyphal survival during infection. Further, these findings also indicate that targeting fungal anti-oxidative defenses via PX-12 may represent an efficacious strategy for treating fungal infections.

Introduction

Pathogenic fungi, such as Aspergillus and Fusarium species, can cause lethal pulmonary and systemic disease in immune-suppressed individuals, including those with HIV infection (1, 2). These organisms are also a major cause of infectious blindness and corneal ulcers in immunocompetent individuals, and in contrast to individuals with systemic and pulmonary fungal infections, there is no indication that fungal keratitis patients are other than fully immunocompetent (3, 4). In the hot and humid southeastern United States, fungal infections of the cornea account for up to 35% of all corneal ulcers (5, 6). Globally, fungal infections of the cornea account for up to 65% of corneal ulcers, with estimates of 80,000 total cases and 10,000 cornea transplants per year due to fungal infections in India alone (7–12). Other risk factors for disease in the USA, Britain, and Europe include contact lens wear, as illustrated by a 2005–2006 fungal keratitis outbreak associated with a lens care product (13). A. flavus, A. fumigatus, F. solani, and F. oysporum are the main etiologic agents of fungal keratitis (14). These organisms are prevalent in vegetative matter and suspended in air, and are inoculated into the corneal stroma via traumatic injury associated with agricultural work (14). Current treatment with topical antymycotics is often ineffective, with up to 60% of cases requiring corneal transplantation (3, 14, 15).

Neutrophils are the predominant cell type infiltrating fungus-infected lungs and corneas, and contribute to tissue destruction by release of proteolytic enzymes and reactive oxygen and nitrogen species (13–17). Our recent studies characterizing fungus-infected human corneas in India showed that neutrophils constitute greater than 90% of cellular infiltrates in corneal ulcers in patients infected for less than 7 days and more than 70% total infiltrate at later stages of infection (18). Similarly, neutrophils are the first cells recruited to the corneal stroma in murine models of Aspergillus and Fusarium keratitis (19, 20), indicating that neutrophils are the main effector cells required for killing fungal hyphae. A role for neutrophils in control of fungal infection is also suggested by the increased incidence of systemic and pulmonary fungal infections in patients with neutropenia (2).

Neutrophils produce NADPH oxidase (NOX), which catalyzes the conversion of molecular O2 to superoxide anion (O2–) with the release of ROS and protons into the extracellular space (17, 21). Individuals with inherited defects in NOX such as in chronic granulomatous disease (CGD) exhibit an increased incidence of bacterial and fungal infections, supporting the concept that the specific expression of NOX by neutrophils is required for killing of fungi (22). However, even though it is the hyphal stage of these organisms that is invasive, most studies on CGD patients and transgenic mice with mutations in NOX genes have focused only on the role of NOX in killing conidia (23–26). Infected human corneas and lungs exhibit primarily hyphal stages of fungal growth, and conidia are rarely detected. Given that hyphae are significantly larger in size, and are not readily phagocytosed, they are likely killed through distinct mechanisms not required for anti-conidial defenses, and a recent study suggests that NOX is not required to control the growth of all filamentous fungi (27).

In the current study, we examined the role of ROS in killing Aspergillus and Fusarium hyphae by human neutrophils and in a murine model of fungal keratitis. We show that hyphae activate neutrophil NOX through CD18 and that NOX activation is essential for killing hyphae. In addition, utilizing mutant A. fumigatus strains, we show that the ROS-sensing transcription factor Yap1, the ROS-detoxifying enzyme superoxide dismutase, and the Yap1-regulated thioredoxin antioxidant pathway, but not catalases or fungal secondary metabolites such as gliotoxin are required for resistance to oxidation by neutrophils. Last, using pharmacologic inhibitors of thioredoxin, we provide proof of concept that tar-

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2012;122(7):2482–2498. doi:10.1172/JCI63239.
Targeting fungal anti-oxidative stress responses can enhance fungal clearance from infected tissues and may represent a new avenue for treatment of fungal infections.

**Results**

Neutrophils have an essential role in regulating fungal growth in the cornea. To examine the role of neutrophils in fungal keratitis, we used two complementary approaches: systemic depletion of neutrophils from immune-competent C57BL/6 mice and adoptive transfer of neutrophils into Cxcr2<sup>−/−</sup> and Cd18<sup>−/−</sup> mice. In the first approach, neutrophils were depleted from transgenic C57BL/6 mice expressing eGFP downstream of the lysozyme promoter (LysM) by i.p. injection of a neutrophil-specific monoclonal antibody (NIMP-14), while control mice were given rat isotype antibody. Injection of 400 μg NIMP antibody on day 1 resulted in significantly decreased neutrophils in peripheral blood smears at 0, 24, and 48 hours after infection (S.M. Leal Jr., unpublished observations). After 24 hours, corneas were infected with conidia (40,000 in 2 μl) isolated from the *A. fumigatus* strain Af-dsRed, which constitutively expresses the red fluorescent protein dsRed under control of the glyceraldehyde dehydrogenase promoter. Subsequently, RFP<sup>+</sup> fungal growth and eGFP<sup>+</sup> neutrophil infiltration were assessed in live corneas. We also examined the effect of neutrophils on corneal opacity.

**Figure 1**

Neutrophil depletion enhances fungal growth during corneal infection. (A) Transgenic C57BL/6 mice with neutrophil-specific eGFP expression downstream of the lysozyme promoter (LysM) were depleted of neutrophils (Neuts) with neutrophil-specific NIMPR-14 antibody (i.p.) and infected with 40,000 Af-dsRed conidia. Eyes were imaged at 24 and 48 hours after infection for neutrophil infiltration (eGFP), fungal growth (dsRed), and corneal opacity (BF). In addition, PASH stains were performed on 5-μm sections of corneas at 48 hours after infection. (B) MetaMorph software was used to quantify neutrophil infiltration (eGFP emission) and (C) fungal dsRed expression. (D) At 4 and 48 hours after infection, eyes were homogenized and plated on SDA plates and CFU quantified by direct counting. MetaMorph software was utilized to quantify (E) corneal opacity area and (F) total corneal opacity (described in detail in Supplemental Figure 1). Three independent experiments (n = 5) were performed. *P < 0.05.

Original magnification, ×20 (eye images); ×400 (histology).
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A) C57BL/6, Cxcr2−/−, No Neut, Cxcr2−/−, i.v. Neuts

B) Fungal dsRed

C) Corneal opacity, area

D) Corneal opacity, total

E) C57BL/6, No Neut, Cd18−/−, i.v. Neuts

F) Fungal dsRed

G) eGFP+ Neuts

H) Corneal opacity, area

I) Corneal opacity, total
Figure 2

Neutrophil adoptive transfer restricts fungal growth during corneal infection. (A) C57BL/6, Cxcr2+/–, and Cxcr2–/– mice were infected with 30,000 Af-dsRed conidia. At 24 hours after infection, one group of fungus-infected Cxcr2+/– mice were injected i.v. with 4 × 10⁸ BMNs from C57BL/6 (B6) mice. At 24 hours after infection, corneas were imaged for cellular infiltration, fungal growth, and corneal opacity. At this time point, mice were euthanized, eyes were fixed in formalin, and 5-μm corneal sections were PASH stained. (B) MetaMorph software was used to quantify fungal dsRed expression, (C) corneal opacity area, and (D) total corneal opacity in infected corneas. (E) Similar to Cxcr2+/– mice, C57BL/6 and Cxcr2+/– mice were infected with Af-dsRed conidia, and at 24 hours after infection one group of infected Cxcr2+/– mice were given 4 million adoptively transferred BMNs isolated from a LysM-eGFP mouse (eGFP–Neut) and eyes were imaged at 48 hours. (F) Fungal dsRed expression, (G) eGFP–neutrophil infiltration, (H) corneal opacity area, and (I) total corneal opacity were quantified using MetaMorph software. Three independent experiments (n = 5) were performed. *P < 0.05. Original magnification, ×20 (eye images); ×400 (histology).

Similarly, C57BL/6 neutrophils transferred to Cd18+/– mice during infection conferred a protective response with increased migration to the cornea and lower fungal load. Figure 2, E and F, shows that fungal dsRed expression at 48 hours was significantly increased in Cd18+/– mice compared with C57BL/6 mice. However, Cd18−/– mice given syngeneic BMNs from transgenic C57BL/6 LysM-eGFP mice exhibited significantly decreased fungal dsRed expression (Figure 2F) along with significantly increased eGFP–neutrophil infiltration (Figure 2G) and increased corneal opacity (Figure 2, H and I).

Together, these findings demonstrate that both Cxcr2 and Cd18 regulate neutrophil recruitment to fungus-infected corneas and that neutrophils have an essential role in controlling fungal growth at this site.

Neutrophil NOX activity is required for control of fungal growth during corneal infection. NOX is an enzyme complex required for reduction of molecular O₂ to O₂– (31). Superoxide is the limiting reagent in subsequent reactions, leading to the transient synthesis of ROS with greater oxidative and fungal killing potential (21), and individuals with CGD due to impaired NOX function are unable to control microbial infections and often succumb to filamentous fungal infections in the lung (2, 32).

Cybb−/– mice lack the gene encoding the NOX subunit gp91phox and thus do not express a functional NOX complex. These mice are also more susceptible to Aspergillus lung infections (23). To determine the role of NOX in fungal keratitis, we infected Cybb−/– mice with Af-dsRed, and ROS levels in the corneas were measured at 48 hours after infection after intrastromal injection of carboxyfluorescein diacetate (CFDA), which emits green fluorescence upon oxidation. Fungal dsRed expression and corneal opacification were measured by image analysis as described above. Figure 3, A and B, shows CFDA activity in C57BL/6 and Cybb−/– corneas at 48 hours after infection; however, total fluorescence was significantly lower in Cybb−/– corneas (there was no fluorescence in naive corneas injected with CFDA; S.M. Leal Jr., unpublished observation). Conversely, fungal dsRed expression and CFU (Figure 3, C and D) were elevated in Cybb−/– compared with C57BL/6 corneas, indicating impaired fungal clearance in Cybb−/– corneas. Interestingly, Cybb−/– corneas had more severe disease than in C57BL/6 mice, with significantly higher total corneal opacity scores (Figure 3, E and F), which correlated with increased neutrophil infiltration and formation of microabscesses and the presence of intact fungal hyphae (Figure 3G). As there is no defect in the ability of Cybb−/– neutrophils to migrate to the cornea, it is likely that these findings represent “frustrated” neutrophils that are unable to kill hyphae but can still recruit neutrophils to this site. Very similar results were found when mice were infected with other pathogenic Aspergillus and Fusarium species that cause keratitis (Supplemental Figures 2–4), indicating that ROS has a more general role in inhibiting growth of filamentous fungi.

To ascertain directly whether the impaired fungal killing in Cybb−/– mice is due to NOX that is specifically produced by neutrophils, we infected Cd18−/– mice with Af-dsRed as described above and injected BMNs from C57BL/6 or Cybb−/– mice i.v. 2 hours after infection. Corneas were imaged 24 hours after infection. Figure 3, H and I, shows that fungal dsRed expression was significantly reduced following adoptive transfer of C57BL/6 neutrophils, whereas mice given Cybb−/– neutrophils had the same fungal dsRed expression as mice not receiving neutrophils. These data clearly demonstrate that NOX-dependent ROS production by neutrophils is essential for inhibiting fungal growth in the cornea.
Figure 3
Neutrophil NOX is required for control of *A. fumigatus* fungal growth during corneal infection. (A) C57BL/6 mice and *Cybb*−/− mice were infected with 40,000 *A. fumigatus* strain Af-dsRed conidia. Eyes were imaged at 48 hours after infection for ROS-mediated CFDA dye oxidation, fungal dsRed expression, and corneal opacity. (B) CFDA dye oxidation, (C) fungal dsRed expression, (D) CFU, (E) corneal opacity area, and (F) total corneal opacity were quantified after infection. (G) 5-μm sections of 48-hour-infected fungal corneas were stained with PASH or neutrophil-specific NIMP antibody. (H) *Cd18*−/− mice were infected with *A. fumigatus* strain Af-dsRed conidia. At 2 hours after infection, one set of mice were left untreated, the second set received an i.v. injection of 4 × 10^6 BMNs isolated from C57BL/6 mice, and a third set received the same number of neutrophils isolated from *Cybb*−/− mice. (I) At 24 hours after infection, eyes were imaged and fungal dsRed expression quantified using MetaMorph software. Three independent experiments (n = 5) were performed. *P < 0.05. Original magnification, ×20 (eye images); ×400 (histology).
iNOS and RNS are not essential for control of fungal growth. The superoxide produced by NOX can be converted to ROS or alternatively can react with NO produced by the enzymatic cleavage of arginine by iNOS, and can form the highly reactive nitrogen species (RNS) peroxynitrite (ONOO–) (21). If iNOS activity is high and significant amounts of NO are produced, the end products of NOX activity will shift from production of ROS to production of RNS.

To test the hypothesis that RNSs are required for control of fungal growth during fungal keratitis, we injected C57BL/6 mice systemically with 1400W, which is an irreversible pharmacologic inhibitor of iNOS. In addition, the role of iNOS was examined in gene-knockout mice.

Figure 4 shows that there were no significant differences in corneal opacification or fungal CFU between iNOS–/– and C57BL/6 mice, or between untreated and 1400W-treated C57BL/6 mice. These findings indicate that in contrast to NOX and ROS, neither iNOS nor RNS have an essential role in control of fungal growth during corneal infection.

Human and murine neutrophil-mediated killing of A. fumigatus, A. flavus, and F. oxysporum hyphae is dependent on NOX, but not iNOS or myeloperoxidase. As neutrophil NOX is required for control of fungal growth in vivo, but both conidia and hyphae are present during corneal infection, we developed a neutrophil-hypha coinubcation assay to determine which neutrophil mediators are required to limit the growth of fungal hyphae. AF-dsRed, A. flavus eGFP, and F. oxysporum FoxL-GFP conidia were incubated in 96-well plates for 6 hours to allow time for germination and hyphal growth. For human neutrophil studies, neutrophils were isolated from the peripheral blood of normal volunteers and added to each well at a 16:1 ratio in the presence of pharmacologic inhibitors of NOX, iNOS, or myeloperoxidase (MPO) (NOX: diphenyliodonium [DPI], apocynin [Apo]; iNOS: aminoguanidine HCl [Agd], 2-methyl-2-thiopseudoureasulfate [SMT]; MPO: indomethacin [Indo], 4-aminobenzoic hydrazide [4-AH]). For mouse neutrophil studies, neutrophils were isolated from the peritoneal cavities of C57BL/6, Cybb–/–, and iNOS–/– mice and incubated with fungal hyphae as described above. Fungal growth was observed by fluorescence microscopy and measured by fluorescence spectroscopy.

Figure 5A shows hyphal growth of A. fumigatus and A. flavus, and Supplemental Figure 6A shows hyphal growth of F. oxysporum after 16 hours incubation in RPMI. However, in the presence of normal human neutrophils, fungal growth was clearly reduced, indicating that normal human neutrophils inhibit hyphal growth. However, addition of the NOX inhibitor DPI resulted in hyphal growth levels similar to that with RPMI alone, which is consistent with NOX-dependent killing. Figure 5B shows quantification of the growth of A. fumigatus exposed to human neutrophils for 16 hours in the presence or absence of inhibitors. Neutrophil-mediated killing was inhibited in the presence of the NOX inhibitors DPI and Apo, but not iNOS or MPO inhibitors.

As a complementary approach, we examined the role of NOX and iNOS in Cybb–/–, iNOS–/–, and C57BL/6 mouse neutrophils. Importantly, ROS was detected after A. fumigatus incubation with C57BL/6, but not Cybb–/–, neutrophils (Supplemental Figure 5), indicating that most of the ROS was dependent on NOX. In contrast, there was no difference in NO production between Cybb–/– and C57BL/6 neutrophils, and ROS production by iNOS–/– neutrophils was not significantly different from that by C57BL/6 neutrophils, while iNOS–/– neutrophils produced minimal NO (Supplemental Figure 5).

As shown in Figure 5C, A. fumigatus growth in the presence of C57BL/6 neutrophils was lower than with RPMI alone. In contrast, fungal growth after incubation with Cybb–/– neutrophils was...
significantly higher than after incubation with C57BL/6 neutrophils and similar to that after incubation with no neutrophils, and is consistent with impaired ROS activity and fungal killing. However, fungal growth was not significantly different between iNOS–/– and C57BL/6 neutrophils (Figure 5D). Similar results were obtained for A. flavus (Figure 5, E–G) and F. oxysporum (Supplemental Figure 6, B–D). Taken together, these data indicate that inhibition of A. fumigatus, A. flavus, and F. oxysporum hyphal growth by human and mouse neutrophils is dependent on NOX and ROS production but not iNOS or MPO.

ROS production and fungal killing are dependent on CD18 but not dectin-1. Activation of NOX is regulated by the physical separation of membrane and cytoplasmic components of the complex. However, following cellular activation by pathogen recognition or cytokine receptors, p47⁷⁷³ in the cytoplasm is phosphorylated and translocated along with other cytoplasmic NOX components to the membrane, thereby forming the functional enzyme (33). Dectin-1 and CD18 recognize fungal cell wall β-glucan and can activate NOX (19, 34, 35), and dectin-1–/– and Cd18–/– mice exhibit impaired fungal clearance in cornea infection models (19), suggesting a possible role for these receptors in neutrophil-mediated fungal killing. To examine whether these cell surface receptors mediate NOX activation and ROS production by neutrophils following exposure to fungal hyphae, we loaded naive BMNs with the ROS-sensitive dye CFDA, incubated them with A. fumigatus hyphae for 1 hour, and measured intracellular ROS-mediated CFDA dye oxidation using flow cytometry. Figure 6A shows that 91.5% of C57BL/6 neutrophils and 94.2% of dectin-1–/– neutrophils exhibited high levels of

Figure 5
NOX but not iNOS or MPO is required for human and mouse neutrophils to control hyphal growth. Fungal conidia were cultured in SDB media for 6 hours in 96-well plates. Neutrophils were added to each well containing hyphae, and the cells were coincubated for an additional 16 hours. In certain experiments, the NOX inhibitors (DPI, Apo), iNOS inhibitors (SMT, AgD), or MPO inhibitors (Indo, 4-AH) were added to the wells. (A) Af-dsRed and eGFP-expressing A. flavus (70-GFP) were cultured either alone in PBS or RPMI or coincubated with 2 × 10⁵ human neutrophils in RPMI or the same number of neutrophils in RPMI plus DPI. Fungal fluorescence was imaged at 16 hours after incubation. Original magnification, ×400. (B) Af-dsRed was coincubated with neutrophils as described above for 16 hours, plates were washed and stained with calcofluor white, and fungal chitin content determined via fluorometry. Tx, treatment. (C and D) To examine the role of NOX in mouse neutrophil-mediated killing of fungal hyphae, we grew Af-dsRed conidia as described above for 6 hours and coincubated them with thioglycolate-elicited peritoneal neutrophils from WT C57BL/6, (C) Cybb–/–, and (D) iNOS–/– mice. At 16 hours after infection, chitin content was quantified using fluorometry. (E) Similarly, A. flavus (70-GFP) neutrophil-hypha coinoculation assays were performed with human neutrophils and (F) Cybb–/– mice and (G) iNOS–/– mice. Three independent experiments were performed. *P < 0.05.
ROS, compared with 31.4% of Cd18 knockout neutrophils, indicating that ROS production is dependent on CD18. Figure 6B shows mean fluorescence intensity quantification of these data.

We also examined the role of neutrophil-expressed CD18 and dectin-1 in mediating killing of fungal hyphae by coincubating A. fumigatus hyphae with purified thioglycolate-elicited peritoneal neutrophils from C57BL/6, Cd18 knockout, or dectin-1 knockout mice. Figure 6C shows significant hyphal growth at 16 hours after infection when hyphae were incubated in RPMI alone but not in PBS. These parameters were used as positive and negative controls. Hyphae coincubated with either C57BL/6 or dectin-1 knockout neutrophils showed limited growth, whereas hyphae coincubated with Cd18 knockout neutrophils showed significant hyphal growth similar to that observed with RPMI alone (Figure 6C). Taken together, these findings identify a major role for CD18, but not dectin-1, in neutrophil NOX activation, ROS production, and killing of A. fumigatus hyphae.

A. fumigatus antioxidant resistance to human neutrophils is dependent on superoxide dismutase and Yap1, but not catalases, gliotoxin, or LaeA-regulated secondary metabolites. As neutrophil NOX is essential for control of fungal growth in the cornea, and NOX is required for both human and mouse neutrophil-mediated control of hyphal growth, we hypothesize that anti-oxidative mediators produced by Aspergillus and Fusarium will scavenge ROS or inhibit NOX and thereby impair neutrophil killing.

The transcription factor Yap1 is activated in A. fumigatus exposed to oxidative conditions and regulates production of intracellular antioxidants and enzymes that convert ROS into less reactive products (36). For example, A. fumigatus superoxide dismutases (SOD1/2/3) convert superoxide to the less-reactive hydrogen peroxide (37), whereas A. fumigatus catalases (CatA/1/2) convert hydrogen peroxide to H2O (38). In addition to ROS-catabolizing enzymes, filamentous fungi including A. fumigatus produce secondary metabolites such as gliotoxin and fumagillin, which inhibit NOX and are controlled by the master transcriptional regulator protein LaeA (39–41). We therefore examined the susceptibility of A. fumigatus strains with mutations in the Yap1 transcription factor (Δyap1), superoxide dismutases (Δsod1/2/3), catalases (ΔCatA, Δcat1/2), or NOX-inhibiting secondary metabolites (ΔgliP, ΔgliZ, ΔlaeA) to human neutrophils.

To determine whether Yap1 is required for hyphal survival in the presence of human neutrophils, we incubated Δyap1 A. fumigatus hyphae with 1 × 10⁴ human neutrophils, which are not sufficient to kill wild-type Aspergillus (2 × 10⁴ neutrophils were used above), and measured fungal biomass by calcofluor white binding as before. Figure 7, A–E, shows that all WT and mutant strains grew similarly after 16 hours in RPMI medium, suggesting that there are no intrinsic growth defects in the mutant strains. Figure 7A shows that the relative fluorescence unit (RFU) level of WT Dal hyphae grown in the presence of 1 × 10⁴ human neutrophils was not significantly different from that of those grown in RPMI alone, indicating that WT Dal hyphae were not killed by 1 × 10⁴ human neutrophils. In contrast, the RFU level of Δyap1 mutants (derived from WT Dal) incubated with the same number of neutrophils was significantly lower than that of WT Dal, which is consistent with increased susceptibility of Δyap1 mutants to killing by neutrophils. Inhibition of neutrophil NOX using the inhibitor DPI resulted in significant growth of the Δyap1 mutant strain in the presence of neutrophils, indicating that Yap1 is required for optimal antioxidant defense against NOX-mediated oxidative stress. Similarly, the RFU level of the Δsod1/2/3 mutant was significantly lower than that of the WT Ku80 strain, and DPI treatment enabled Δsod1/2/3 mutant growth in the presence of neutrophils (Figure 7B). However, there were no significant differences in RFU/fungal growth between Δcat1 or Δcat1/Δcat2 double mutants and WT G10 (Figure 7C), between ΔgliP and WT B-5233 A. fumigatus strains (Figure 7D), or between ΔgliZ or ΔlaeA mutants and WT Af293 (Figure 7E).

Taken together, these data show that the anti-oxidative pathways regulated by the transcription factor Yap1 and SOD1/2/3 are essential for optimal fungal growth in the presence of human neutrophils, whereas CatA, Cat1, Cat2, gliotoxin, and LaeA-regulated secondary metabolites have either a redundant or no role in blocking neutrophil-mediated killing.

Yap1 and SOD1/2/3 are required for fungal survival in vivo. Since the A. fumigatus transcription factor Yap1 and SOD1/2/3 regulate the survival of hyphae in the presence of human neutrophils, we next examined the role of these mediators in corneal infection. WT or mutant conidia were injected into the corneal stroma of C57BL/6, A. fumigatus Cd18 knockout, and dectin-1 knockout mice.
C57BL/6 mice, and corneal disease and fungal survival were examined as described above. We found that there was a significantly lower CFU level in corneas infected with Δyap1 mutants compared with those infected with WT Dal (Figure 8A). These mutants also induced significantly less corneal opacity than WT Dal (Figure 8, B–D). Similarly, the Δsod1/2/3 mutant CFU level was significantly lower than that in WT Ku80 (Figure 8E), although there was no detectable difference in corneal opacity (Figure 8, F–H).

In contrast to Δyap1 and Δsod1/2/3 mutants, and consistent with the human neutrophil studies, mice infected with ΔcatA or Δcat1/2 showed no significant differences in CFU compared with WT Dal (Figure 8, B–D). Similarly, the Δsod1/2/3 mutant CFU level was significantly lower than that in WT Ku80 (Figure 8E), although there was no detectable difference in corneal opacity (Figure 8, F–H).

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Fungal thioredoxin mediates resistance to human neutrophils, and oxidative stress and is required for fungal survival during corneal infection. Exposure of A. fumigatus to oxidative stress activates the transcription factor Yap1, which upregulates genes involved in anti-oxidative processes (36, 42). During oxidative stress, the Yap1-dependent proteins allergen aspf3 and peroxiredoxin Prx1 are highly upregulated. These are putative thioredoxin peroxidases (peroxiredoxins) in the thioredoxin antioxidant pathway (36), and their peroxidatic cysteine residues reduce H2O2 to H2O and are then oxidized and nonfunctional (43, 44). Subsequently, thioredoxin protein reduces peroxiredoxins to their functional state, allowing further detoxification of H2O2 (44). Thioredoxin is then further reduced by the enzyme thioredoxin reductase using H+ equivalents from NADPH+ (44).

Our bioinformatic analysis identified 5 putative thioredoxins encoded in the A. fumigatus genome (Afu5g11320/aspf29, Afu6g10300/aspf28, Afu3g14970, Afu8g01090, and Afu4g09090), two of which are known human allergens, suggesting high expression during infection (45). To inhibit total thioredoxin function in A. fumigatus, we utilized the anticancer drug PX-12 (2-[(1-methylpropyl)dithio]-1H-imidazole), which binds to the active site of thioredoxin and inhibits its ability to mediate redox reactions with target proteins and ultimately quench ROS (46–48). Figure 9A shows that A. fumigatus hyphae survived incubation with sublethal doses of human neutrophils; however, upon inhibition of total thioredoxin protein with 10 μM or 100 μM PX-12, fungal growth was decreased, indicating that thioredoxin is required for hyphae survival. To determine whether PX-12 sensitizes hyphae to ROS, we grew conidia for 6 hours in SDB and incubated them with hydrogen peroxide and PX-12 (1 μM). Figure 9B shows that 5 mM and 10 mM H2O2 killed hyphae in the presence or absence of PX-12, whereas 1 mM H2O2 alone had no effect. However, in the presence of 1 μM PX-12, this dose of H2O2 had antifungal activity. These findings demonstrate that Aspergillus thioredoxin mediates resistance to neutrophil and H2O2 oxidative stress.

Pharmacokinetic studies during a recent PX-12 phase I clinical trial revealed that PX-12 given at 400 mg/m2/d for 72 hours is safe to administer to patients (49). However, to determine whether PX-12 exhibits cytotoxicity to immune cells and cornea-specific C57BL/6 mice, and corneal disease and fungal survival were examined as described above. We found that there was a significantly lower CFU level in corneas infected with Δyap1 mutants compared with those infected with WT Dal (Figure 8A). These mutants also induced significantly less corneal opacity than WT Dal (Figure 8, B–D). Similarly, the Δsod1/2/3 mutant CFU level was significantly lower than that in WT Ku80 (Figure 8E), although there was no detectable difference in corneal opacity (Figure 8, F–H).
cell types, we incubated several human myeloid and cornea cell lines for 16 hours with a dose-response curve of PX-12, at which point the release of lactate dehydrogenase (LDH) into the surrounding medium was assayed. In these assays, LDH release in the presence of PX-12 was not significantly different from that with medium alone, indicating there was no cytotoxic effect of PX-12 (S.M. Leal Jr., unpublished observations).

To determine whether thioredoxin also impairs neutrophil-mediated killing in vivo, we infected C57BL/6 mice with Af-dsRed and gave them 8-μl eyedrops of 3-mM PX-12 or vehicle at 0 and 6 hours after infection. Mice were sacrificed at 24 hours after infection and imaged. Figure 9C shows no difference in corneal opacity between vehicle and PX-12 treatment; however, a significant reduction in fungal dsRed and CFU (Figure 9, D and E) was observed in PX-12–treated mice. Taken together with in vitro findings with human neutrophils and H2O2, these data clearly demonstrate that A. fumigatus thioredoxin proteins function as antioxidants during infection, impair neutrophil-mediated fungal killing, and are required for fungal growth during infection of the cornea.

Figure 8
Yap1, SOD1/2/3, but not catalases or secondary metabolites mediate fungal growth during corneal infection. (A) C57BL/6 mice were infected with 40,000 A. fumigatus conidia isolated from either Δyap1 or the WT Dal1 strain, and fungal CFU were quantified at 4 and 48 hours after infection. (B) Eyes were imaged at 24 and 48 hours after infection. (C) Corneal opacity area and (D) total corneal opacity were quantified. (E) C57BL/6 mice were infected as described above with either the Δsod1/2/3 or the WT Ku80 strain, and fungal CFU were quantified at 4 and 48 hours after infection. (F) Eyes were imaged at 24 and 48 hours after infection. (G) Corneal opacity area and (H) total corneal opacity were quantified. (I) C57BL/6 mice were infected as described with ΔcatA, Δcat1/2, or the WT G10 strain, and CFU were quantified in infected corneas after infection. (J) C57BL/6 mice were infected as described with ΔgiliZ, gliZR, ΔlglA, laeA-R, WT A1293 or ΔgiliP, gliPR, WT B-5233, and CFU were quantified in infected corneas after infection. Three independent experiments (n = 5) were performed. *P < 0.05.
Discussion

In this study, we identified the molecular interactions between neutrophils and fungal hyphae that result in fungal death. Conclusions from these studies are illustrated in the context of neutrophil oxidase production and antioxidants produced by hyphae (Figure 10). Unlike most leukocytes, neutrophils exhibit pronounced extracellular microbicidal activity and are likely required to kill all genera and species of pathogenic filamentous fungi (17). In addition, the anti-oxidative defense mechanisms shown to be important in this study are highly conserved (50); therefore, results from the current study are very likely relevant to fungal infections of other tissues in addition to other fungal pathogens.

Both dectin-1 and CR3 (CD11b/CD18) recognize fungal β-glucan (51, 52); however, we identified an essential role for CD18, but not dectin-1, in ROS production and killing of A. fumigatus hyphae in vitro. These findings are consistent with observations that CR3 is the major receptor on human neutrophils for β-glucan (35, 53) and, more recently, that CR3 induces ROS production in response to A. fumigatus hyphae (54). The role of CD18 in neutrophil-mediated hyphal killing in vivo could not be determined in the current model due to the requirement for CD18 for neutrophil extravasation (55). However, we have shown that dectin-1–/– mice exhibit defects in fungal killing in vivo (34, 56). In addition, we and others have shown that dectin-1 is required for neutrophil NOX activation in response to conidia and macrophage-mediated phagocytosis of conidia (57, 58). It is therefore likely that during infection, dectin-1 on macrophages and neutrophils mediates killing of conidia, whereas CD18 on neutrophils mediates killing of hyphae.

The requisite roles for CD18 and dectin-1 in fungal killing can potentially be explained by enhanced CR3 surface expression on neutrophils compared with macrophages (59). Resting neutrophils and macrophages express CR3 on their surface; however, activation of neutrophils, unlike macrophages, results in progressive degranulation, CD11b translocation to the cell surface, and CD11b/CD18 heterodimer (CR3) formation (60). Alternative explanations include differences in the ability of CD18 and dectin-1 to induce granule exocytosis and translocate gp91/p22phox to the cell surface (60) or differential CD18 and dectin-1 recognition of distinct β-glucan conformations variably expressed in hyphae and conidia (61). Both CR3 and dectin-1 signal through spleen tyrosine kinase (Syk), which mediates downstream activation of serine-threonine kinases (MAPKs: ERK, p38, JNK) and can potentially mediate p47phox phosphorylation and NOX activation (33, 62). Studies in our laboratory are currently addressing these hypotheses.

In addition to the role of CD18, we reported that Tlr4–/–, but not MD-2–/– or Tlr2–/–, mice exhibit increased Aspergillus and Fusarium growth during fungal keratitis (20, 56), indicating an MD-2–independent role for TLR4. However, Tlr4–/– neutrophils killed A. fumigatus hyphae in vitro as efficiently as control neutrophils (S.M. Leal Jr., unpublished observations), indicating no role for TLR4 in hyphal killing. As TLR4 binds A. fumigatus galactomannan and mediates o-linked mannose-dependent recognition of Candida albicans (63, 64), it is possible that these sugar residues are not expressed on living hyphae in sufficient number or the proper conformation to activate neutrophil TLR4. It is
The fungal cytoplasm and along with other ROS derivatives (H$_2$O$_2$, etc.) mediates oxidation of essential cytoplasmic proteins and lipids, leading to death of fungal hyphae. Using NOX-deficient Cybb–/– mice and adoptive transfer of Cybb–/– neutrophils, we demonstrate that neutrophil-specific expression of NOX and O$_2^•$ production are essential for controlling the growth of A. fumigatus in vivo. In addition, we demonstrated using specific inhibitors and gene-knockout neutrophils that NOX is required for human and murine neutrophils to kill A. fumigatus, A. flavus, and F. oxysporum hyphae. However, in contrast to NOX, we found no role for either iNOS or MPO in killing Aspergillus or Fusarium hyphae. Similar findings were obtained in experimental A. fumigatus lung infections in which NOX but not MPO or iNOS were found to be required to control fungal growth (21, 23, 25, 68). Our findings are also in agreement with the enhanced susceptibility of CGD patients, but not MPO-deficient patients, to filamentous fungal infections (2, 32). However, our findings differ from a study showing no role for NOX in killing A. nidulans in vitro (27). Given that iNOS is required for optimal intracellular killing of Candida and Cryptococcus yeasts (69, 70), and that MPO is also possible that, since TLR4 is also activated by endogenous molecules released from lysed cells (65), host-mediated inflammatory products such as heat shock proteins induce TLR4 activation and TLR4-dependent fungal killing in vivo. Similar to the role of TLR4 in fungal killing in vivo (56), MD-2 is not required for TLR4 activation by dead cells (6, 7, 66).

CD11b/CD18 activation results in translocation of cytoplasmic NOX components to the plasma membrane and formation of a functional NOX enzyme (33). As illustrated in Figure 10, NOX subsequently produces short-lived superoxide (O$_2^•$) within nanometer proximity to the fungal cell wall (33). O$_2^•$ can oxidize cell wall components directly or be converted to the more stable H$_2$O$_2$, and both can enter the fungal cytoplasm through porins and anion channels on the fungal plasma membrane (21, 67). H$_2$O$_2$ can also be converted extracellularly by MPO into hypohalous acids, which are likely too short-lived to enter the fungal cytoplasm or react with iNOS-derived NO forming ONOO$^•$ (21). Our finding that the fungal cytoplasmic antioxidant thioredoxin and superoxide dismutases, but not catalases, are essential for hyphal survival suggests that during infection, neutrophil-derived O$_2^•$ enters the fungal cytoplasm and along with other ROS derivatives (H$_2$O$_2$, etc.) mediates oxidation of essential cytoplasmic proteins and lipids, leading to death of fungal hyphae.

Using NOX-deficient Cybb–/– mice and adoptive transfer of Cybb–/– neutrophils, we demonstrate that neutrophil-specific expression of NOX and O$_2^•$ production are essential for controlling the growth of A. fumigatus in vivo. In addition, we demonstrated using specific inhibitors and gene-knockout neutrophils that NOX is required for human and murine neutrophils to kill A. fumigatus, A. flavus, and F. oxysporum hyphae. However, in contrast to NOX, we found no role for either iNOS or MPO in killing Aspergillus or Fusarium hyphae. Similar findings were obtained in experimental A. fumigatus lung infections in which NOX but not MPO or iNOS were found to be required to control fungal growth (21, 23, 25, 68). Our findings are also in agreement with the enhanced susceptibility of CGD patients, but not MPO-deficient patients, to filamentous fungal infections (2, 32). However, our findings differ from a study showing no role for NOX in killing A. nidulans in vitro (27). Given that iNOS is required for optimal intracellular killing of Candida and Cryptococcus yeasts (69, 70), and that MPO is

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Figure 10
Oxidative stress responses at the neutrophil-hypha interface. Cat, catalase; GR, glutaredoxin; GSH, glutathione; GSSG, dimeric glutathione; GTR, glutathione reductase; HOCI, hypochlorous acid; ox, oxidized; red, reduced; TR, thioredoxin reductase.
required for optimal responses to *Candida* (68), it is possible that these enzymes are more effective against intracellular yeast rather than extracellular hyphae.

Figure 10 also illustrates fungal antioxidant pathways. Oxidation by neutrophils induces nuclear translocation of Yap1, as well as upregulation of the H$_2$O$_2$-catabolizing thioredoxin-dependent peroxiredoxins following oxidative stress (44). Subsequently, peroxiredoxins are cyclically reduced to their functional state by thioredoxin protein, which is itself reduced by thioredoxin reductase (44). The importance of thioredoxin protein in fungal antioxidant defense is evidenced by (a) elevated *A. fumigatus* thioredoxin reductase expression found after exposure to normal versus CGD neutrophils (72); and (b) the essential role of thioredoxin in antioxidant responses in *A. nidulans* (73). In the current study, bioinformatic analysis revealed 5 putative thioredoxins in the *A. fumigatus* genome, including the human allergens aspf29 and aspf28, which are highly expressed during human infection (45). We constructed a single-knockout strain of aspf29 and did not observe a difference in fungal survival in vivo, likely due to redundancy by the other 4 putative thioredoxins encoded in the *A. fumigatus* genome (S.M. Leal Jr., unpublished observations). We therefore utilized PX-12 to inhibit all 5 putative thioredoxins and showed that thioredoxin is required for hyphal survival in the presence of neutrophils in vitro and that PX-12 enhances H$_2$O$_2$-mediated killing (Figure 9 and ref. 48). Further, we found that topical application of PX-12 was sufficient to restrict fungal growth in vivo, thereby indicating that this or similar compounds that target the thioredoxin pathway can block fungal thioredoxins and inhibit the predominant antioxidant defense utilized by these pathogens during infection.

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**Table 1**

Fungal strains utilized in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. fumigatus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF-BP</td>
<td>Keratitis clinical isolate; Bascom Palmer Eye Institute</td>
<td>WT</td>
</tr>
<tr>
<td>AF-dsRed</td>
<td>AF293-1: ΔpyrG::gpdA::dsRed::pyrG</td>
<td>dsRed fluorescence</td>
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<tr>
<td>Dal/C1005</td>
<td>WT: CBS144-89; aspergillus clinical isolate</td>
<td>WT</td>
</tr>
<tr>
<td>Ku80/C107</td>
<td>CEA10: Δku80</td>
<td>WT</td>
</tr>
<tr>
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<td>CEA10: Δasod1::B::asod2::TR-P::asod3::G</td>
<td>No Yap1 synthesis</td>
</tr>
<tr>
<td>Δasod1::2/3</td>
<td>CEA10: Δasod1::B::asod2::TR-P::asod3::G</td>
<td>No SOD1/2/3 synthesis</td>
</tr>
<tr>
<td>G10</td>
<td>Dal: Δnia</td>
<td>WT</td>
</tr>
<tr>
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<td>G10: ΔcatA::phioR</td>
<td>No CatA</td>
</tr>
<tr>
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</tr>
<tr>
<td>AF293</td>
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<td>WT</td>
</tr>
<tr>
<td>ΔgliZ</td>
<td>AF293: ΔgliZ::pyrG pyrG</td>
<td>No gliotoxin (GliZ)</td>
</tr>
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<td>gliZ</td>
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<td>GliZ reconstituted</td>
</tr>
<tr>
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<td>AF293: ΔlaeA::pyrG pyrG</td>
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</tr>
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<td>LaeA reconstituted</td>
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<tr>
<td>B-5233</td>
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<td>WT</td>
</tr>
<tr>
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<td>No gliotoxin (gliP)</td>
</tr>
<tr>
<td>gliP</td>
<td>B-5233: gliP::Af</td>
<td>gliP reconstituted</td>
</tr>
<tr>
<td><strong>A. flavus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-302</td>
<td>Keratitis clinical isolate; Aravind Eye Hospital</td>
<td>WT</td>
</tr>
<tr>
<td>70-GFP</td>
<td>A. flavus 70: gpd::eGFP</td>
<td>eGFP fluorescence</td>
</tr>
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<td>NRR3557</td>
<td>WT: environmental isolate</td>
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</tr>
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<td>NRR3557: pyrG ΔlaeA::Af</td>
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</tr>
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<td>laeA-R</td>
<td>NRR3557: pyrG ΔlaeA::Af phyG</td>
<td>LaeA reconstituted</td>
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<tr>
<td><strong>F. oxysporum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8996</td>
<td>Keratitis clinical isolate; Cleveland Clinic</td>
<td>WT</td>
</tr>
<tr>
<td>FoxL-RFP</td>
<td>Plant pathogen; gpd::dsRed2</td>
<td>dsRed fluorescence</td>
</tr>
<tr>
<td>FoxL-GFP</td>
<td>Plant pathogen; gpd::GFP</td>
<td>GFP fluorescence</td>
</tr>
</tbody>
</table>
secondary metabolites may mediate fungal survival in patients, but not mice, due to the more chronic nature of human disease — typically weeks to months — compared with our acute murine model of fungal keratitis.

In conclusion, we have identified superoxide production by neutrophil NOX as the optimal effector for hyphal killing and shown that superoxide dismutase and thioredoxin are the key antioxidant agents used by hyphae during infection. One approach to exploiting these findings is to block the antioxidant activity of the fungus, thereby increasing hyphal sensitivity to oxidative stress and decreasing the threshold required for neutrophils to kill hyphal during infection. As noted earlier, PX-12 blocks hyphal resistance to ROS, and as PX-12 has successfully completed two phase I clinical safety trials for cancer therapy (46, 47), it is possible that targeting fungal thioredoxin by PX-12 or related compounds could be utilized to treat a broad range of fungal infections.

Methods

Source of mice. C57BL/6 mice (6–12 weeks old) and Cbyb<sup>−/−</sup> mice were purchased from The Jackson Laboratory. Cd18<sup>−/−</sup> mice were provided by Claire Doerschuck (University of North Carolina, Chapel Hill, North Carolina, USA), and Cbx2<sup>−/−</sup> mice were provided by Richard Ransohoft (Cleveland Clinic, Cleveland, Ohio, USA). Dectin-1<sup>−/−</sup> mice were provided by Yoichiro Ikawara (Tokyo University, Tokyo, Japan). All mice used in this study were on a C57BL/6 background.

Fungal strains, media, and growth conditions. Table 1 lists the genotype and phenotype of all strains utilized in this study. A. fumigatus and A. flavus strains used in this study were cultured on Vogel’s minimal medium (VMM) with or without 2% agar unless otherwise stated. F. oxysporum and F. solani strains were cultured on Sabouraud dextrose medium. To visualize fungi in the transparent murine cornea, we utilized fluorescent strains of Aspergillus or Fusarium species expressing either RFP or GFP. Af-dsRed constitutively expresses enhanced monomeric dsRed protein (pgdA promoter driven; pyrG1 selection marker) (56). The strain A. flavus 70-GFP constitutively expresses GFP (pgdA promoter driven; niaD selection marker; provided by Rajah Rajasekaran, USDA, New Orleans, Louisiana, USA) (76). The strains FoxL-RFP and FoxL-GFP are F. oxysporum bopcentis strains constitutively expressing either RFP or GFP, which were provided by Seogchan Kang (Pennsylvania State University, University Park, Pennsylvania, USA). The A. fumigatus strain Af-BP was isolated from a keratitis patient at Bascom Palmer Eye Institute and provided by Darlene Miller (University of Miami, Miami, Florida, USA) (56). The A. flavus strain TN-302 was isolated from a keratitis patient at Aravind Eye Hospital, Tamil Nadu, India, and provided by Lalitha Pragai. The F. oxysporum strain 8996 was isolated from a keratitis patient at the Cole Eye Institute, Cleveland Clinic (20). For our studies on gliotoxin, June Kwon-Chung (NIAID, Bethesda, Maryland, USA) provided A. fumigatus strains B-5233 (WT), AgliP, and gliPR (77). Additionally, Nancy Keller (University of Wisconsin, Madison, Wisconsin, USA) provided the A. fumigatus strains AF293 (WT), AgliZ, gizIR, A laeA, and alsA, as well as the Aspergillus flavus strains A. flavus NRRRL3357 (WT), A laeA, and A laeB (78, 79). For ROS scavenging studies, we used A. fumigatus strains G10 (WT), ΔcatA, and ΔcatA/1, and Ku80 (WT), Aox1/2,3 and Dal (WT), Δap1 (36–38).

Mouse model of Aspergillus and Fusarium keratitis. Aspergillus strains were cultured for 2–3 days on VMM in 25-cm<sup>2</sup> tissue culture flasks. Fresh conidia were disrupted with a bacterial L-loop, harvested in 5 ml PBS, and filtered through sterile, PBS-soaked cotton gauze in a 10-ml syringe to obtain pure conidial suspensions. Conidia were quantified using a hemocytometer and adjusted in PBS to a final stock solution: A. fumigatus, 20,000 conidia/μl; A. flavus, 20,000 conidia/μl; F. oxysporum, 25,000 conidia/μl. Subsequently, mice were anesthetized with 1.25% 2,2,2-tribromoethanol. The corneal epithelium of anesthetized mice was abraded using a 30-gauge needle, through which a 2-μl injection containing conidia was released into the corneal stroma using a 33-gauge Hamilton syringe (total: A. fumigatus, 40,000 conidia; A. flavus, 40,000; F. oxysporum, 50,000). Mice were examined daily under a stereomicroscope for corneal opacification, ulceration, perforation, and fungal fluorescent protein expression. At set time points, animals were euthanized by CO<sub>2</sub> asphyxiation, and eyes were either placed in 10% formalin and embedded in paraffin and sectioned at 5-μm intervals or excised and placed in 1 ml sterile saline and homogenized for quantitative culture. For detection of neutrophils in mice, 400 μg of anti-mouse neutrophil antibody (NIMP-R14) was injected at day 1 prior to infection. To detect ROS production in the mouse cornea during fungal infection, 2 μl of 12.5-ng/ml CFDA (Invitrogen) was injected into the stroma of anesthetized mice and eyes imaged under standard GFP filters after 10 minutes incubation at 25°C. Subsequently, mice were euthanized, and eyes processed accordingly. To inhibit RNS production during corneal infection, 500 μg of the irreversible iNOS inhibitor 1400W was injected i.p. into C57BL/6 mice at 6, 24, and 48 hours after infection. To inhibit thioredoxin in vivo, PX-12 (Sigma-Aldrich) was dissolved at 3 mM in a proprietary eye drop formulation provided by Alcon and applied topically at 0 and 6 hours after infection. All animals were bred under specific pathogen–free conditions and maintained according to institutional guidelines.

Neutrophil-specific adoptive transfer mouse model used to study fungal killing. In vivo Cbx2<sup>−/−</sup> and Cd18<sup>−/−</sup> mice were anesthetized as described above, and conidia from a fluorescent fungal strain were injected into the corneal stroma strain (30,000 Af-dsRed conidia). At 2 hours after infection, 4 million naive BMNs isolated from the femurs and tibias of donor mice were injected i.v. into recipient mice. At 24 hours after infection, infected mice were euthanized, and fungal growth in the cornea was imaged using a fluorescence stereomicroscope. The level of fluorescence emitted from infecting fungi was quantified using image analysis software (MetaMorph, Molecular Devices; described below) and used as a measure of fungal growth during infection.

Imaging and quantification of light reflected or emitted from infected mouse corneas. Mice were euthanized by CO<sub>2</sub> asphyxiation and positioned in a 3-point stereotactic mouse restrainer. Corneal opacity (brightfield [BF]), fungal proliferation (RFP/GFP), cellular infiltration (GFP), and CFDA dye oxidation (GFP spectra) were visualized in the intact cornea using a high-resolution stereo fluorescence MZFLIII microscope (Leica Microsystems) and Spot RT Slider KE camera (Diagnetics Instruments). All images were obtained using the same Spot Advanced Software under the same magnification (×20), exposure (BF, 0.4 seconds; RFP, 10 seconds; eGFP, 2 seconds), gain (BF, 1; RFP/eGFP, 4/16), and gamma (BF/RFP/eGFP, 1.85) parameters. MetaMorph imaging software was used to quantify the percent area of opacity and the integrated corneal opacity as described in the legend to Supplemental Figure 1.

Quantification of Aspergillus CFU. For assessment of fungal viability, whole eyes were homogenized under sterile conditions in 1 ml PBS using the Mixer Mill MM300 (Retsch) at 33 Hz for 4 minutes. Subsequently, serial log dilutions were performed and plated onto bacteriologic-grade Sabouraud dextrose agar (SDA) plates (BD). Following incubation for 24 hours at 37°C (Aspergillus) or 30°C (Fusarium), the number of CFU was determined by direct counting.

Identification of fungal growth patterns and neutrophil recruitment into the cornea. Eyes were enucleated and fixed in 10% formalin in PBS (Fisher) for 24 hours. Five-micrometer sections from the center of the cornea (as determined by noncontiguous iris morphology) were cut and stained with PASH for identification of fungi and inflammatory cell recruitment. To detect infiltrating neutrophils, sections were immunostained using...
monoclonal rat anti-mouse neutrophil IgG (NIMP-R14, Abcam) and Alexa Fluor 488–tagged rabbit anti-rat IgG (Invitrogen). All histology slides were imaged and shown at an original magnification of x400.

**Isolation of human neutrophils from peripheral blood.** Human neutrophils were isolated from normal, healthy donors using Ficoll-Paque Plus (GE) density centrifugation. Peripheral blood (20 ml) was obtained, and rbc were separated from whole blood via incubation at 1 g for 20 minutes with 3% dextran in PBS (Sigma-Aldrich). The top clear layer containing leukocytes was transferred to a fresh 50-ml conical tube and 10 ml Ficoll-Paque Plus was underlain. The cell suspension was centrifuged at 500 g for 20 minutes at 4°C to separate mononuclear cells from neutrophils and the remaining rbc. The overlying plasma and PBMC layers were aspirated, and the neutrophil/rbc pellet was resuspended in RBC Lysis Buffer (eBioscience) (8.3 g NH₄Cl, 1 g KHCO₃, 0.09 g EDTA/1 l ddH₂O), incubated at 37°C for 10 minutes to lyse remaining rbc, and spun at 300 g for 5 minutes at 4°C. The lysis procedure was repeated as needed to obtain sufficient rbc lysis in cell preparations. Subsequently, cells were washed twice in PBS and ultimately resuspended in RPMI plus l-glutamine without phenol red (HyClone). The neutrophil cell suspension was counted using a hemocytometer, and samples were collected by Cytospin and stained by Wright-Giemsa (Fisher). Using this approach, neutrophils were routinely found to be greater than 97% of the final cell preparation.

**Isolation of peritoneal and bone marrow–derived murine neutrophils.** To isolate peritoneal neutrophils, mice were injected with 1 ml 4% thioglycolate 16 and 6 hours; F. oxysporum species) from fluorescent or non-fluorescent Aspergillus species and F. solani species. Purified conidia (12,500/well for F. solani) were harvested, washed twice in PBS in 50-ml conical tubes, and resuspended in RPMI plus l-glutamine without phenol red (Hyclone). The resulting cell suspension routinely yielded greater than 95% neutrophils. To obtain BMNs, mice were euthanized by CO₂ asphyxiation, and femurs and tibias were removed, cleaned, and centrifuged at 5,000 g for 45 seconds at 4°C. Centrifuging rbc were lysed in 5 ml RBC Lysis Buffer, and remaining bone marrow cells were pipetted onto a discontinuous Percoll gradient (GE) of 52%, 69%, and 78% that fluoresces upon oxidation (CFDA, Invitrogen), and NO was assayed by flow cytometry as described above.

**3. Results**

**Fungi were grown for 4 hours at 37°C in 200 μl SDB in 96-well plates as described above, and H₂O₂ was added at final concentrations of 1 mM–10 mM in the presence of PX-12 (0–100 μM) for an additional 2 hours. After 16 hours, fungal growth was examined by phase-contrast microscopy, stained with calcofluor white, and quantified by fluorometry as described above.

**Statistics.** Statistical analysis was performed for each experiment using 1-way ANOVA with a Tukey post hoc analysis (Prism, GraphPad Software). A P value less than 0.05 was considered significant.

**Study approval.** All animals were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research; and protocols were approved by the Case Western Reserve University IACUC. The protocol for the use of human peripheral blood from normal healthy volunteers was approved by the Institutional Review Board of University Hospitals of Cleveland. Informed consent was obtained from each volunteer.

**Acknowledgments**

This work was supported by NIH grants F31 EY019841 (to S.M. Leal Jr.), R01 EY018612 (to E. Pearlman), P30 EY011373 (to E. Pearlman), and R21 AI074846-02 (to M. Momany). These studies were also supported by a Research to Prevent Blindness Medical Student Fellowship (to S.M. Leal Jr.), and unrestricted grants from the Research to Prevent Blindness Foundation and the Ohio Lions Eye Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would like to thank Brandon Leibell and the Case Western Reserve University visual sciences core facility managers for excellent technical assistance.

Received for publication February 6, 2012, and accepted in revised form May 9, 2012.

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