Live Imaging of Disseminated Candidiasis in Zebrafish Reveals Role of Phagocyte Oxidase in Limiting Filamentous Growth

Kimberly M. Brothers, Zachary R. Newman, and Robert T. Wheeler
Department of Molecular and Biomedical Sciences and Graduate School of Biomedical Sciences.
University of Maine, Orono, Maine 04469

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Candida albicans is a human commensal and a clinically important fungal pathogen that grows in both yeast and hyphal forms during human infection. Although Candida can cause cutaneous and mucosal disease, systemic infections cause the greatest mortality in hospitals. Candidemia occurs primarily in immunocompromised patients, for whom the innate immune system plays a paramount role in immunity. We have developed a novel transparent vertebrate model of candidemia to probe the molecular nature of Candida-innate immune system interactions in an intact host. Our zebrafish infection model results in a lethal disseminated infection model that shares important traits with disseminated candidiasis in mammals, including dimorphic fungal growth, dependence on hyphal growth for virulence, and dependence on the phagocyte NADPH oxidase for immunity.

Using this model, we combined intravital imaging with gene knockdown to show for the first time that NADPH oxidase is required for regulation of C. albicans filamentation in vivo. The transparent and easily manipulated larval zebrafish model promises to provide a unique tool for dissecting the molecular basis of phagocyte NADPH oxidase-mediated limitation of filamentous growth in vivo.

Candida albicans is a human commensal that causes life-threatening invasive infections in immunocompromised patients. Disseminated candidiasis is the 4th leading infection in hospitalized patients in the United States, and despite antifungal therapy, the mortality associated with candidemia can reach 30 to 40% (62). Innate immunity is a key mediator of resistance to disseminated infection in both mice and humans, and defects in professional innate immune cells predispose individuals to invasive candidemia (5, 26, 68).

The zebrafish larva is a unique and powerful model for noninvasively visualizing and understanding the interactions of pathogens with the innate immune system (17, 48, 56). Notably, zebrafish have similar signaling through Toll-like receptors to that in humans, express similar cytokines, and have macrophages, neutrophils, dendritic cells, mast cells, eosinophils, T cells, and B cells (78). The delayed development of T cells and B cells, which do not mature until approximately 30 days postfertilization, permits a natural focus on innate immunity in embryonic and larval infection models. A larval model of candidiasis offers several advantages compared to other recently described models of zebrafish infection with C. albicans. Specifically, the adult zebrafish candidemia model does not permit real-time visualization of infection or morpholino (MO)-directed gene knockdown, both of which are techniques available with the larval host (19). Also, while others have described a localized embryonic zebrafish infection model (19), it is not clear if and how these infections parallel human diseases of clinical importance, such as disseminated candidiasis. A larval zebrafish model of disseminated candidiasis offers a clinically relevant disease in a transparent and small host that relies on innate immunity and can be perturbed by antisense morpholino-mediated gene knockdown (43, 48, 87). These advantages have been exploited by others to identify novel immune mediators of infectious disease (78, 79) and provide a unique opportunity to address the molecular nature of in vivo interactions between C. albicans and immune cells in the context of a live host.

One key mediator of innate immunity to C. albicans is the phagocyte NADPH oxidase complex, also referred to as NOX2 or the phagocyte oxidase, which is expressed in both macrophages and neutrophils and is defective in chronic granulomatous disease (6). NADPH oxidase catalyzes the production of microbicidal reactive oxygen and nitrogen species (ROS and RNS), in addition to driving changes in ion concentrations and phagosomal redox state (6, 72, 74). The NADPH oxidase is required for neutrophils to inhibit the C. albicans yeast-hypha switch in vitro (47), although its role in regulating C. albicans morphology in vivo has not been tested. Other in vitro experiments have linked the fungal oxidative stress response to both cellular defense against oxidative attack and inhibition of the yeast-hypha transition (1, 3, 15). However, the ultimate effect of ROS in vivo is unclear because it has also been reported that mild oxidative stress can enhance polarized growth in a thioredoxin-regulated manner in vitro (24, 59). The nature of oxidative stress in vivo has been explored during mouse candidemia...
describe the consequences of phagocytosis of pathogens throughout the host, proliferating, and killing the host. We have disseminated fungal infections.

To image and understand host-fungus interactions in the context of a live host, we developed the zebrafish larva as a transparent vertebrate model of disseminated candidiasis. We show that this infection faithfully reproduces many aspects of candidemia in mammalian hosts, with C. albicans disseminating throughout the host, proliferating, and killing the host. We describe the consequences of phagocytosis of C. albicans in the intact host and describe how this event can lead to a temporary impasse in which neither host cells nor fungi perish immediately. We also demonstrate that the activities of fungal and host factors are conserved in this infection model, including a dependence on fungal hyphal growth for virulence and a host requirement of NADPH oxidase for resistance to infection.

Finally, we exploited the power of this model to noninvasively visualize the cellular impact of the loss of NADPH oxidase activity. We found that the host NADPH oxidase is the major cause of oxidative stress in C. albicans during infection and is of vital importance in both limiting fungal proliferation and limiting filamentous growth.

Materials and Methods

Zebrafish care and maintenance.

All zebrafish were kept in recirculating systems (Aquatic Habitats, Apoika, FL) at the University of Maine Zebrafish Facility. Water temperature was kept at 28°C. All zebrafish care protocols and experiments were performed in accordance with NIH guidelines under Institutional Animal Care and Use Committee (IACUC) protocol A2009-11-01. Larvae were grown at a density of 50/dish in 10-cm petri dishes containing 60 ml egg water (deionized water with 60 ml/g liter Instant Ocean salts [Spectrum Brands, Mentor, OH]). The fish strains used are described in Table S2 in the supplemental material. Egg water was supplemented with 0.00003% methylene blue for the first 24 h to prevent microbial growth. Larvae were cleaned by changing of the water daily. Zebrafish were not grown in water containing 1-phenyl-2-thiourea (PTU) to prevent pigmentation, because this was not necessary for viewing fungus-immune interactions and anecdotal evidence of our own and others (49) suggests the possibility that PTU could alter infection dynamics. All experiments were performed in accordance with NIH guidelines under Institutional Animal Care and Use Committee (IACUC) protocol A2009-11-01. Larvae were maintained in 0.5% egg water, and homogenized with a few minor changes. Four to eight larvae per treatment were induced with phorbol 12-myristate 13-acetate (PMA), and four to eight other larvae were not. Tenfold serial dilutions were made in penicillin-streptomycin stock solution, and 10 nl of PBS or C. albicans suspension at 1 × 10^6/ml in PBS was microinjected through the otic vesicle into the hindbrain ventricle (to achieve a dose of approximately 10 CFU).

Fluorescence microscopy.

An Olympus IX-81 inverted microscope with an FV-1000 laser scanning confocal system was used for confocal imaging (Olympus). Objective lenses with powers of ×40/0.16 numerical aperture (NA), ×10/0.4 NA, ×20/0.7 NA, ×40/0.9 NA, and ×40/0.75 NA were used. Fish were anesthetized in Tris-buffered tricaine methane sulfonate (tricaine; 200 μg/ml) (Western Chemicals, Inc., Fredens, WA). For infection, 5 to 10 nl of PBS or C. albicans suspension at 1 × 10^6/ml in PBS was microinjected through the otic vesicle into the hindbrain ventricle (to achieve a dose of approximately 10 CFU).

Enumeration of fungal burdens.

Larvae infected with C. albicans were incubated at 28°C during infection. At each time point, 5 or 10 living larvae were anesthetized in tricaine, collected in 100 μl of egg water, and homogenized with a hand-held Kontes microgrinder (Research Products International, Inc., Mt. Prospect, IL). Four hundred to 900 μl of penicillin-streptomycin stock solution (Lonza, Walkersville, MD) was added to bring the final volume to 500 μl to 1 ml. Tenfold serial dilutions were made in penicillin-streptomycin stock solution, and 100 μl of each dilution was plated in triplicate onto YPD agar. Plates were incubated for 24 h at 37°C. Viable colonies were counted, and burdens were calculated per fish. Fungal burdens were graphed as CFU per fish. Error bars represent standard deviations. Student’s t-test was used to determine statistical significance. Three independent experiments were conducted.

Respiratory burst assay.

The respiratory burst assay was performed using H_2DCF-DA (dihydrodichlorofluoresceindiacetate) as described previously (38), with a few minor changes. Four to eight larvae per treatment were induced with phorbol 12-myristate 13-acetate (PMA), and four to eight other larvae were not.
induced for each treatment group (control MO or p47\textsuperscript{phox} or p91\textsuperscript{phox} MO). A Synergy2 plate reader (Biotek, Winooski, VT) was used to measure fluorescence every hour for a total of nine measurements. Excitation and emission wavelengths were 485 nm and 528 nm, respectively. Differences in the ratios (induced/uninduced) of control and p47\textsuperscript{phox} or p91\textsuperscript{phox} morphants were examined using bootstrapped confidence intervals obtained from 1,000 replicates, using the PopTools add-in for Microsoft Excel. The degree of significance was determined by observing whether 95%, 99%, and 99.9% confidence intervals overlapped.

**Imaging and quantification of fungal morphology in crushed fish.** To image fungal morphology without interference of melanophores and internal organs, we euthanized individual fish and placed them onto microscope slides, removed all excess water, and added a coverslip. This immediately crushed the fish into a thin aqueous layer without compromising the fungi. Crushed fish were imaged by epifluorescence microscopy on an Olympus IX-81 microscope using IP Lab (version 3.0; Scanalytics) or on a Zeiss Axiosvert microscope using Axiosvision (release 4.7; Carl Zeiss Microimaging, Thornwood, NJ). For each fish, fields were imaged to document all of the fungi in the entire fish. For analysis, images were overlaid in Photoshop (Adobe Inc., San Jose, CA). An illustration of the methodology is shown in Fig. S6 in the supplemental material. Regions with well-separated fungal cells were annotated by tracing filamentous (hyphal or pseudohyphal) cells (in one layer) and yeast cells (in another layer). For each annotated layer, the area was calculated by ImageJ (National Institutes of Health). For each fish, percent filamentous (hyphal or pseudohyphal) growth was calculated based on the number of countable cells. For any areas that had too dense a concentration of fungi for reliable counting, the percent filamentation for the image field was calculated and the uncountable area was divided accordingly into yeast and filamentous growth.

**RESULTS**

**Hindbrain ventricle infection route leads to lethal disseminated candidiasis in zebrafish larvae.** Several routes of infection (immersion, caudal vein, Duct of Cuvier, and hindbrain ventricle) were attempted to establish a disseminated infection in transparent zebrafish larvae. We initially attempted bath infection with up to 10^8 C. albicans yeast cells/ml of water. These bath infections of fish aged to 3 to 6 days postfertilization resulted in no mortality and no fungal invasion past the gastrointestinal tract. To date, our attempts at infection of the yolk have resulted in universal lethality within 24 h, while common cardinal vein infections yielded yolk-focused infections, similar to what was recently published (19). When these infected fish were examined, however, there was little to no apparent dissemination. Thus, these methods did not immediately offer a good model for disseminated candidiasis, which is the lethal form of human candidiasis. We also attempted to inject fish at 2 to 3 days postfertilization via the caudal vein, but our attempts resulted in too much lethality, even using buffered saline as a control. This was probably due to the relatively large bore needle required to inject C. albicans yeast (3 to 5 \( \mu \text{m} \) in diameter). In contrast, our initial infections of the hindbrain of prim25-stage fish embryos yielded a disseminated Candida albicans infection in which the fungus replicated rapidly and killed over half of the fish within the first 2 days postinfection.

Utilizing the hindbrain infection route, we found that C. albicans disseminated throughout the fish, and both yeast and filamentous fungi were found as far away as the tail (Fig. 1A and insets). Fish were microinjected in the hindbrain ventricle with 10 to 20 yeast cells/fish, as determined retrospectively by counting viable CFU from homogenates immediately following infection (Fig. 1B). After infection, the fungi proliferated rapidly, and by 24 h postinfection (hpi), they were at 100-fold greater numbers per surviving fish (Fig. 1B). By 48 hpi, the fungal burden in surviving fish dropped precipitously, to only five times that of the initial injection amount. The remaining live C. albicans and fish continued to survive for several days, although we did not examine fungal burdens beyond 6 days postinfection to determine how long the infected fish remained colonized. In parallel with fungal burden measurements, we also monitored fish for mortality. In over 20 experiments performed, we found that 49% ± 16% of infected fish succumbed to infection within 5 days postinfection, with the majority of mortality in the first 48 hpi (Fig. 1C). To determine if the mortality was pathogen specific, we also infected fish with heat-killed C. albicans and prototrophic Saccharomyces cerevisiae. Neither dead C. albicans nor live S. cerevisiae caused mortality (see Fig. S1 in the supplemental material).

It is notable that the fungal burdens shown in Fig. 1B were measured in surviving fish and do not include fish that succumbed to the infection; this methodology eliminates problems associated with assaying fungal burdens within dead fish, which disintegrate over time or permit the postmortem growth of fungi. When we looked at fungal burdens of dead fish, we found a much higher burden per fish than the value for live fish (see Fig. S2 in the supplemental material). The high fungal burden at 24 hpi shown in Fig. 1B includes both fish that died by 48 hpi and fish that cleared the infection. Examination of fungal burdens within individual fish at 24 hpi demonstrated a wide range of fungal burdens per fish. This suggests that many of the fish with high fungal burdens that were still alive at 24 hpi succumbed to the infection by 48 hpi, while fish with lower burdens likely cleared the infection.

*C. albicans* switches rapidly to hyphal growth but reverts to yeast form after immune infiltration. To visualize the extent of filamentation during infection, we noninvasively imaged the morphological form of *C. albicans* for the first 24 hpi. We found that the infecting yeast-form cells germinated within the first 6 h, but by 24 hpi, they reverted to yeast-form growth in most of the surviving fish (Fig. 2A). To reinforce these qualitative results, we scored individual fish for the presence of hyphae and found that 70% ± 8% of fish had at least one hyphal cell at 6 hpi, but this number was sharply decreased, to 27% ± 11%, by 24 hpi (Fig. 2B). In heavily infected fish that were moribund and likely to soon succumb to infection, we often found extensive filamentation that eventually broke through the skin of the fish (see Fig. S3A and Movie S1 in the supplemental material). In many cases, we also found blocked blood vessels (see Fig. S3B and Movie S2) and tissue damage throughout the fish.

To assess if the immune response could explain the fungal switch to yeast-form growth, we visualized fungal cells and the immune response noninvasively. We used transgenic *fli1:EGFP* fish with EGFP-expressing macrophage-like cells and endothelium (45, 65) and *mpx:GFP* fish with EGFP-expressing neutrophils (66). We infected these fish with *C. albicans* expressing yCherry (a version of mCherry that is codon optimized for *Candida* spp.) and coimaged fungi and innate immune cells. We found that macrophage-like cells and neutrophils phagocytosed yeast-form *C. albicans* and wrapped themselves around filamentous fungi. Figure 2C and Movie S3 in the supplemental material show several EGFP-positive neutrophils and several EGFP-negative macrophage-like cells that have phagocytosed *C. albicans* yeast. Time-lapse visualization of phagocytosis in *fli1:EGFP* larvae (Fig. 2D; see
Movie S4) also shows apparent chemotaxis of a phagocyte toward extracellular fungi.

The dynamics of immune infiltration and changes in fungal morphology are consistent with the idea that immune activity plays an important role in controlling both fungal proliferation and morphogenesis. However, it is notable that the immune system was not able to control proliferation and morphogenesis in all infected fish, such that by 1 day postinfection, the larvae were either moribund with extensive fungal hyphae or relatively healthy with a majority of yeast-form cells. We asked if the ability to control filamentous growth in the first 24 hpi is predictive of overall survival by imaging infected fish at 24 hpi and following their survival until 48 hpi. We found that fish that had restrained filamentous growth by 24 hpi (yeast-only group) all survived to 48 hpi, whereas fish with filamentous fungi (yeast and filament group) suffered significantly higher mortality between 24 hpi and 48 hpi (0% ± 0% versus 63% ± 15%; P = 0.002) (see Fig. S4 in the supplemental material). This suggests that the ability to control fungal morphogenesis within the first 24 hpi allows fish to survive disseminated infection.

**Fate of C. albicans within phagocytes.** After establishing that macrophage-like cells and neutrophils both engulf *C. albicans* during infection, we asked how the fungi fare inside phagocytes. In vitro, in the absence of most natural soluble and membrane-bound modulators of macrophages and neutrophil activity, *C. albicans* germinates inside macrophages but not inside neutrophils (16, 42, 71). The presence of these cues in vivo may lead to different phagocyte activity in the intact host, as macrophage and neutrophil activity in vitro can be enhanced by extracellular matrix contacts and by the presence of cytokines (16, 76). In fact, using our in vivo model, we found two distinct patterns of phagocyte interaction with *C. albicans* in the first several hpi that differ from what has been described in vitro. Neutrophils (EGFP positive in *mpx*::GFP transgenic fish and containing cytoplasmic granules) phagocytosed a limited number of *C. albicans* yeast cells (between 1 and 3) and remained highly motile postphagocytosis (Fig. 2C, E, and F and Table 1). On the other hand, macrophage-like cells (EGFP positive in *fli1*::EGFP transgenic fish, EGFP negative in *mpx*::GFP transgenic fish, and lacking cytoplasmic granules) phagocytosed more *C. albicans* yeast cells (up to 9) and had greatly
reduced motility postphagocytosis (Fig. 2A and D and Table 2). We refer to these macrophage-like cells as macrophages, because they exhibit overall characteristics most similar to what has been described for macrophages (49) and dissimilar to those described for neutrophils, the other major phagocytic cells present at this time of development (46).

We found that phagocytosis can lead to an impasse inside macrophages in which a macrophage inhibits germination of...
C. albicans but does not kill the fungus. This situation can last for at least 4 h (Fig. 2E; see Movie S5 in the supplemental material). In this particular case, there were more fungi in the macrophage at the end of the time-lapse experiment (10 versus 8), even though there were no further phagocytic events. Increases in the number of yeast cells in a macrophage, in the absence of additional phagocytosis, were observed in two other cases (Table 2). These data suggest that fungi not only remain viable but are able to divide within macrophages. In a total of seven time-lapse experiments, we visualized 77 macrophages with ingested yeast cells. Contrary to our expectations based on in vitro reports, we witnessed germination of only one yeast cell within a macrophage (among a total of 324 yeast cells followed for an average of 3.5 h) and did not observe a single event in which the fungus germinated and burst the phagocyte. By following fungal cytoplasmic yCherry fluorescence as an indicator of fungal viability, as described previously (84), we found no killing of C. albicans within macrophages. We did observe macrophages with filamentous fungi within them, which may be due to phagocytosis of small hyphae, as described previously (27).

Neutrophils were difficult to monitor over longer periods due to their rapid motility. To obtain a snapshot of fungal fate within neutrophils, we scored three-dimensionally reconstructed image stacks of EGFP+ neutrophils in mpx:GFP larvae at 3 to 6 hpi (Fig. 2C; see Movie S3 in the supplemental material). Among 87 neutrophils and 67 phagocytosed fungi observed in 3 independent experiments, we witnessed two cases of a fungus losing membrane integrity and being destroyed (as assessed by release of cytoplasmic yCherry fluorescence). In both cases, we observed red fluorescence, which is confined to the fungal cytoplasm in intact fungi (Fig. 2F), throughout the phagosome (Fig. 2G; see Movie S6). In each case, the phagosomal red fluorescence persisted within the phagosome for over 1 h. This suggests that when a neutrophil damages a C. albicans cell, some cellular contents (including yCherry fluorescent protein) can be released into and persist within the phagosome for an extended period. The rarity of these events, coupled with the length of time that the dissipated yCherry protein remained visible, argues that fungi are not always rapidly killed by neutrophils in vivo. Neutrophils roamed in and out of the site of infection, and many did not phagocytose fungi (Table 1). These empty innate immune cells may play an indirect role in inhibiting fungal growth or may act as a backup system in case fungi escape from within phagocytes. We saw no cases of filamentous fungi within neutrophils, although we did observe neutrophils wrapping themselves around C. albicans filaments.

In sum, we found 267 fungi killed within neutrophils but 324 fungi killed within macrophages, suggesting that neutrophils may have a greater capacity to kill C. albicans than that of macrophages in the intact host. The rarity of killing events observed within neutrophils does not exclude the possibility that a larger proportion of fungi phagocytosed by neutrophils may eventually have been killed at a time after our imaging, because we were unable to follow individual interactions over time. Yet these in vivo time-lapse and time course data reveal the prevalence of a previously unappreciated outcome of C. albicans-host interaction: an impasse between fungus and phagocyte.

Table 1. Fate of C. albicans within neutrophils

| Expt no. | No. of PMN without fungi inside | No. of PMN with fungi inside | Mean no. of fungi per PMN | Total no. of fungal cells | No. of killed fungi |
|---------|--------------------------------|-----------------------------|--------------------------|--------------------------|---------------------|
| 1       | 17                             | 20                          | 1.6                      | 32                       | 0                   |
| 8       | 32                             | 8                           | 2.25                     | 18                       | 1                   |
| 9       | 38                             | 10                          | 1.7                      | 17                       | 1                   |

* There were no germinating fungi observed during the time course in any experiment. PMN, polymorphonuclear leukocytes.

Table 2. Fate of C. albicans within macrophages

| Expt no. | No. of macrophages | Mean time (h) of visualization | Mean no. of fungi per macrophage | Total no. of fungal cells | No. of germinating fungi |
|----------|--------------------|-------------------------------|---------------------------------|--------------------------|-------------------------|
| 1        | 4                  | 2.25                          | 4.5                             | 18                       | 0                       |
| 2        | 2                  | 5                             | 4.5                             | 9                        | 1                       |
| 3        | 10                 | 4.9                           | 3.7                             | 37                       | 0                       |
| 4        | 34                 | 3.5                           | 5.1                             | 173                      | 0                       |
| 5        | 16                 | 2.1                           | 3.5                             | 56                       | 0                       |
| 6        | 4                  | 2.8                           | 4.25                            | 17                       | 0                       |
| 7        | 7                  | 3.6                           | 2                               | 14                       | 0                       |

* The host zebrafish genotype was flt1/EGFP.

** Yeast-hypha switch factors regulate virulence in larval zebrafish candidemia. To establish if similar genetic mechanisms regulate fungal virulence in this model and other infection models, we tested the virulence of the efg1ΔΔ cph1ΔΔ mutant, a hypofumigatus strain of C. albicans with reduced virulence in the mouse, fly, nematode, adult zebrafish, and moth models of infection (14, 18, 52, 64). We found that the efg1ΔΔ cph1ΔΔ mutant had a reduced capacity to replicate within and kill zebrafish larvae. The hypofumigatus fungus had significantly reduced virulence (P < 0.0001) (Fig. 3A). In addition, infections with mutant fungi resulted in a reduced fungal burden (P < 0.001) (Fig. 3B). The efg1ΔΔ cph1ΔΔ mutant retained its ability to grow in a filamentous form as pseudohyphae (Fig. 3C), as reported previously for oral and disseminated mammalian infections (8, 67). Although the transcription factors encoded by these genes regulate a number of genes (32), these data provide a further indication that hyphal growth, seen early during infection and in larvae that succumbed to infection, plays an important role in virulence in zebrafish larval candidemia. This reinforces the parallels between disseminated infections in zebrafish and mice.

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### Table 2. Fate of C. albicans within macrophages

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| 5        | 16                 | 2.1                           | 3.5                             | 56                       | 0                       |
| 6        | 4                  | 2.8                           | 4.25                            | 17                       | 0                       |
| 7        | 7                  | 3.6                           | 2                               | 14                       | 0                       |

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Phagocyte NADPH oxidase is required to limit C. albicans filamentation in larval zebrafish candidemia. We did not visualize any events in which fungi burst through phagocytes and saw very few in which fungi were killed (lost cytoplasmic fluorescence) within phagocytes (Tables 1 and 2). The inability of fungi to kill immune cells could result from a failure of the fungi to germinate at the reduced temperature of 28°C or could be due to immune regulation of fungal growth. To understand if the phagocyte NADPH oxidase plays a role in maintaining this impasse between macrophages and yeast, we imaged yCherry-expressing fungi within control and p47phox morphant fish. We found that between 6 and 24 hpi, filamentation increased in p47phox morphants but decreased in control morphants (Fig. 5A). To quantify this, we completely imaged individual fish at a high resolution by confocal microscopy and scored the number of C. albicans filaments per fish. Using this measure to compare control and p47phox morphants, there was no significant difference in the percentages of fish with filamentous fungi inside at 6 hpi (70% ± 14% versus 81% ± 8%, respectively; P = 0.32), but there was a significantly greater percentage of p47phox morphants with filamentous fungi inside at 24 hpi (27% ± 11% versus 92% ± 3%, respectively; P < 0.001). To examine this more closely and to eliminate potential artifacts due to pigment cells or tissue depth, we crushed fish between slide and coverslip, imaged fungal cells, and quantified the percentage of fungal growth in filamentous form (as described in Materials and Methods and illustrated in Fig. S6 in the supplemental material). When the ratio of filaments to yeast cells was quantified at 24 hpi, we found a significantly
higher percentage of filamentous growth in p47<sup>phox</sup> morphants (P < 0.005) (Fig. 5B). Thus, in the absence of NADPH oxidase activity, there is significantly increased hyphal growth that accompanies greater mortality.

**Phagocyte NADPH oxidase is required to generate oxidative stress in vivo.** Based on the important role played by the phagocyte NADPH oxidase in immunity and the overall importance of oxidative stress in infection (21), it could be predicted that *C. albicans* would be subjected to oxidative stress during infection. However, it is not known when or where phagocyte NADPH oxidase-dependent oxidative stress is felt during infection. To visualize the spatiotemporal dynamics of oxidative stress in *C. albicans* during infection, we engineered a dual reporter strain we called WT-OXYellow (Fig. 6A). This strain has constitutive expression of yCherry (from the *ADH1* promoter) and oxidative stress-inducible expression of EGFP (from the *CTA1* promoter). WT-OXYellow was created by integrating the P<sub>*ADH1*</sub>-yCherry construct into the *ADH1* locus of a *CTA1-GFP* strain recently created and characterized by the Brown laboratory (31). Upon infection of control morphants, there was little promoter activity at 6 hpi, but the oxidative stress response increased dramatically by 24 hpi (Fig. 6B, top four panels). We found widespread activation of EGFP expression in many tissues (including brain, skin, muscle, and heart), but we were not able to examine EGFP expression in blood because there were few fungi in the blood and they were moving too fast for high-resolution imaging. Because both EGFP and yCherry are cytoplasmic, the ratio of red to green fluorescence in micrographs should reflect the relative induction of the oxidative stress response. Quantitation of pixel values along lines bisecting two yeast-form cells demonstrated that red and green fluorescence did colocalize in the fungal cytoplasm in control morphants (Fig. 6C, top two panels). Stress-induced promoter activity was variable from cell to cell, as assessed by visual inspection (Fig. 6B) and by quantification of the ratio of green to red fluorescence within individual cells.
(Fig. 6D). Strikingly, when p47phox morphants were infected with WT-OXYellow, there was almost no detectable activity from the oxidative stress-inducible CTA1 promoter. This was the case when we examined images (Fig. 6B, lower four panels), quantified the data in lines pixel by pixel (Fig. 6C, lower two panels), and quantified the ratio of average green fluorescence to average red fluorescence on a cell-by-cell basis (Fig. 6D). The strong induction of phagocyte NADPH oxidase-dependent oxidative stress between 6 hpi and 24 hpi is consistent with the idea that this immune mechanism contributes to the NADPH oxidase-dependent reduction in fungal filaments seen over the same period (Fig. 5).

**DISCUSSION**

We have exploited unique advantages of a new transparent Candida-zebrafish infection model to provide the first noninvasive, real-time tracking of Candida-immune interaction in a vertebrate host. Our results suggest that in vivo interactions between innate immune cells and C. albicans are more complex than in vitro observations would lead one to believe. For the first time, we were able to visualize the cellular impact of loss of host phagocyte NADPH oxidase activity on both host and pathogen, finding that it is the primary cause of oxidative stress in fungi and that it limits filamentous growth. The accessibility of this new model of candidemia promises to provide further insight into the effects of host and pathogen manipulation on the innate immune response and virulence.

The novel and uniquely positioned vertebrate model of candidemia we describe shares important characteristics of mouse candidemia yet has distinct advantages compared to previously described models of candidiasis in zebrafish. On the pathogen side, in both zebrafish and mice C. albicans disseminates throughout the host, causes lethality, grows as both yeast cells and hyphae, and requires Efg1p, a master regulator of the yeast-hypha transition, for full virulence (51, 52, 70). On the host side, the zebrafish and mammalian immune responses involve both macrophages and neutrophils, and immunity to the infection is strongly dependent on the phagocyte NADPH oxidase (4–6, 16, 26, 76). The larval model we describe has greater versatility than a recently reported adult zebrafish model (19), in which it is not possible to either perturb gene function with morpholinos or image the infection noninvasively by confocal microscopy. In addition, our larval zebrafish model of disseminated candidiasis, in contrast to the localized larval infections described previously (19), allows a direct comparison to disseminated disease in mammals, which is clinically the most lethal form of the disease.

The zebrafish larval model derives advantages compared to mammalian systems from its small size and transparency, permitting high-throughput screens, chemical genetic screens, and noninvasive whole-animal visualization of host-pathogen inter-
action (2, 48, 56, 78, 79). Invertebrate models of candidiasis have similar advantages but lack specialized cells analogous to vertebrate immune cells (e.g., macrophages, neutrophils, dendritic cells, eosinophils, basophils, T cells, and B cells). Furthermore, in contrast to our zebrafish host, Drosophila and Caenorhabditis become permissive hosts for C. albicans infection only when genetic mutants with compromised immune systems are utilized (18, 34). Notably, anatomical and immune similarities among vertebrates have empowered translation of findings from zebrafish into progress on human disease (11, 17, 39, 54, 79, 82).

Exploiting the transparency of the zebrafish larva, we non-invasively imaged C. albicans inside macrophages and neutrophils and found that phagocytosis can result in a standoff wherein fungi survive and divide but neither germinate nor lyse the host cell. This temporary impasse is not typically seen in vitro, where phagocytosed C. albicans rapidly germinates within and lyses macrophages or, conversely, is rapidly destroyed by neutrophils (16, 33, 42, 53, 71). Our relatively short-term observations (on the order of hours) do not yet answer whether the impasse results in a type of long-term intracellular proliferation like that observed for Mycobacterium marinum, which would be a novel virulence strategy for C. albicans. The apparently enhanced ability of macrophages to block germination may be due to soluble factors and extracellular matrix attachments that are present in vivo but not in vitro (9, 16, 44, 79).

FIG. 6. C. albicans is subject to NADPH oxidase-dependent oxidative stress in vivo. (A) Schematic of dual-fluorescence oxidative stress reporter WT-OXYellow. C. albicans expressing EGFP from the CTA1 promoter was transformed with the P_{ADH1} yCherry plasmid to drive constitutive expression of yCherry. For panels B to D, one-cell fertilized eggs were injected with 2.5 ng of control or p47phox morpholino and infected with 10 to 15 CFU of WT-OXYellow at the prim25 stage. (B) Fungi infecting p47phox morphants exhibit little to no EGFP fluorescence, indicating no oxidative stress to the Candida. Infected control and p47phox morphants were imaged by confocal microscopy at 6 and 24 hpi. Bar = 10 μm. (C) Infected control and p47phox morphants were imaged by Vivatome microscopy, and line histograms of red and green fluorescence were compared. Yeast cells within controls (top) had correlated green and red fluorescence, indicative of cytoplasmic fluorescence. In contrast, yeast cells within p47phox morphants (bottom) displayed only red fluorescence and no corresponding green fluorescence. (D) Quantitation of confocal images of individual yeast cells and filament segments taken at 24 hpi was performed using ImageJ software to determine the average fluorescence per pixel as described in Materials and Methods. There was a significantly higher average green/red ratio at 24 hpi for control morphants than that for p47phox morphants (P < 0.0001; Student’s t test) and that for controls at 6 hpi (P < 0.0001; Student’s t test). Box plot whiskers represent the 1.5 interquartile range either below or above the lower or upper quartile, respectively. The results are representative of two independent experiments.
There could be host-specific factors, such as intrinsically greater activity of macrophages or reduced germinative capacity of *C. albicans*, within zebrafish. However, zebrafish and mammalian macrophages are functionally comparable (17, 48, 49, 56, 78), and the uncontrolled filamentation observed in p47phox morphants argues against an inherently impaired capacity for germination. The presence of live *C. albicans* persisting in phagocytes suggests the intriguing possibility that like other fungi, bacteria, and viruses, *C. albicans* may use phagocytes to disseminate within the host (20, 28, 48, 86).

This is the first study to track cellular host responses to fungal infection within the host over time, quantifying the number of fungi per immune cell, fungal division, and fungal death during the early hours of infection. Although the larval zebrafish model has been exploited to qualitatively describe immune cell migration and behavior in infection (13, 22, 25, 37, 38), our study is one of only a few studies to quantify host-pathogen interaction events (23, 79). Our findings of more phagocytosis exhibited by macrophages than by neutrophils are similar to what has been described qualitatively for phagocytosis of *Listeria* spp. (50), *Escherichia coli* (46), *Staphylococcus aureus* (63), *Burkholderia* spp. (81), and *Pseudomonas aeruginosa* (13). Only one other report has quantified phagocytosis by zebrafish macrophages and neutrophils in *vivo* (23), and similar to our data, that study found that macrophages phagocytosed more mycobacteria than neutrophils did. Thus, larval zebrafish macrophages apparently have a greater phagocytic role than neutrophils for Gram-negative, Gram-positive, acid-fast, and fungal pathogens. However, the overall role of macrophages in immunity to *C. albicans* remains to be tested. Fortunately, two recently described transgenic fish lines (30, 36) should permit the specific ablation of macrophages to test this previously inaccessible question.

Our data provide the first *in vivo* evidence in any infection model that the phagocyte NADPH oxidase regulates filamentation of *C. albicans* within the intact host. This is consistent with *in vitro* observations that neutrophils and macrophages lacking NADPH oxidase activity have a reduced ability to kill *C. albicans* and fail to inhibit germination (33, 47). Neutrophils exhibit filament-biased killing and growth inhibition (85) and are therefore likely contributors to inhibition of filamentous growth in the zebrafish host. The increased hyphal growth in p47phox morphants, in which *C. albicans* does not appear to be under oxidative stress, clarifies conflicting *in vitro* observations. It suggests that during infection, host-derived reactive oxygen species tend to inhibit (15) rather than activate (24, 59) the yeast-hypha switch. NADPH oxidase-generated reactive oxygen and nitrogen species may directly inhibit filamentation (6).

Other, more recently described responses that require this enzyme complex may also be involved, including alteration of the phagosomal redox state (72), modulation of phagosomal ion concentrations (74), inhibition of phagocyte inflammatory responses (58, 69, 75), and neutrophil extracellular trap production (10). It will be informative to combine morpholino-mediated gene knockdown and *in vivo* visualization in the zebrafish larvae to determine which NADPH oxidase-dependent mechanism(s) is required to inhibit filamentation in *vivo*.

Our findings of heterogeneous levels of CTA1 promoter activity, obtained using noninvasive *in vivo* imaging, provide complementary data to those of Brown and colleagues (31), who imaged the *CTA1-GFP* reporter strain in fixed histological sections from mice with disseminated candidiasis. Time-lapse studies with fluorescently marked immune cells in zebrafish, combined with morpholino-mediated gene knockdown, could address both the molecular basis for heterogeneity in observed oxidative stress in *vivo* and its direct consequences on fungal morphogenesis.

The mammalian host is endothermic, with normal temperatures of 36.8 ± 0.4°C for humans (55) and activity-dependent temperatures for mice of 36 to 37°C, with occasional dips in body temperature down to 28°C (73). Zebrafish grow at a wide range of temperatures, from 22 to 33°C, and ideally at 28°C (83). This difference in host temperature may be less of an issue for organisms such as *C. albicans* that grow and are pathogenic at a range of temperatures (34). Notably, although filamentous growth of *C. albicans* is enhanced at higher temperatures, we and others have observed fungal germination *in vivo* at 28°C and have implicated a role of filamentation in virulence in model hosts living at 28°C (14, 18, 19, 64). Furthermore, the intrinsic capacity of *C. albicans* to germinate within fish is apparently quite strong, because we found extensive filamentation upon reduction of phagocyte NADPH oxidase activity. In addition, preliminary results suggest that infected larvae maintained at 33°C versus 28°C have no major differences in *C. albicans* morphogenesis, fungal load, or fish mortality (data not shown). Taken together, these data suggest that despite the lower infection temperature, there is still good conservation of virulence and immune mechanisms. In fact, because zebrafish are ectothermic and can grow at temperatures between 18°C and 33°C, this model offers a unique opportunity to independently test the role of temperature in controlling *C. albicans* morphogenesis and virulence *in vivo*.

This novel model is a promising new resource that will enable fine-scale dissection of molecular mechanisms that direct *Candida*-host interaction. As we demonstrate, intravital imaging combined with morpholino gene knockdown, transgenic fish lines, and unique pathogen reporter strains can yield new insights into long-standing questions of innate immunity to fungal infection.

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