Intravital Imaging Reveals Divergent Cytokine and Cellular Immune Responses to *Candida albicans* and *Candida parapsilosis*

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**ABSTRACT** *Candida* yeasts are common commensals that can cause mucosal disease and life-threatening systemic infections. While many of the components required for defense against *Candida albicans* infection are well established, questions remain about how various host cells at mucosal sites assess threats and coordinate defenses to prevent normally commensal organisms from becoming pathogenic. Using two *Candida* species, *C. albicans* and *C. parapsilosis*, which differ in their abilities to damage epithelial tissues, we used traditional methods (pathogen CFU, host survival, and host cytokine expression) combined with high-resolution intravital imaging of transparent zebrafish larvae to illuminate host-pathogen interactions at the cellular level in the complex environment of a mucosal infection. In zebrafish, *C. albicans* grows as both yeast and epithelium-damaging filaments, activates the NF-κB pathway, evokes proinflammatory cytokines, and causes the recruitment of phagocytic immune cells. On the other hand, *C. parapsilosis* remains in yeast morphology and elicits the recruitment of phagocytes without inducing inflammation. High-resolution mapping of phagocyte-*Candida* interactions at the infection site revealed that neutrophils and macrophages attack both *Candida* species, regardless of the cytokine environment. Time-lapse monitoring of single-cell gene expression in transgenic reporter zebrafish revealed a partitioning of the immune response during *C. albicans* infection: the transcription factor NF-κB is activated largely in cells of the swimbladder epithelium, while the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) is expressed in motile cells, mainly macrophages. Our results point to different host strategies for combatting pathogenic *Candida* species and separate signaling roles for host cell types.

**IMPORTANCE** In modern medicine, physicians are frequently forced to balance immune suppression against immune stimulation to treat patients such as those undergoing transplants and chemotherapy. More-targeted therapies designed to preserve immunity and prevent opportunistic fungal infection in these patients could be informed by an understanding of how fungi interact with professional and nonprofessional immune cells in mucosal candidiasis. In this study, we intravitally imaged these host-pathogen dynamics during *Candida* infection in a transparent vertebrate model host, the zebrafish. Single-cell imaging revealed an unexpected partitioning of the inflammatory response between phagocytes and epithelial cells. Surprisingly, we found that *in vivo* cytokine profiles more closely match *in vitro* responses of epithelial cells rather than phagocytes. Furthermore, we identified a disconnect between canonical inflammatory cytokine production and phagocyte recruitment to the site of infection, implicating noncytokine chemoattractants. Our study contributes to a new appreciation for the specialization and cross talk among cell types during mucosal infection.
Fungal species of the genus Candida are commensals on mucosal surfaces in healthy human hosts but cause both invasive and mucosal candidiasis when immune defenses are compromised (1, 2). While Candida albicans is the species most commonly isolated from patients, infections due to C. parapsilosis are increasing, especially in neonates born prematurely (3–5). In healthy hosts, Candida is maintained as a commensal through the defenses of professional immune cells and the barrier functions of the mucosal epithelium. When these defenses are compromised, mucosal candidiasis ensues (1, 6). Understanding how host cells at mucosal surfaces interact with fungal cells and how they coordinate their antifungal defenses will inform our attempts to prevent both systemic and mucosal disease (7, 8).

The mucosal epithelium is a complex environment, and protection from mucosal candidiasis requires the combined actions of several cell types. In addition to their barrier functions, epithelial cells respond to Candida by inhibiting Candida growth with antimicrobial peptides and recruiting immune effector cells with alarmins and proinflammatory cytokines (9–12). Among immune cells, neutrophils play key roles in defense at mucosal surfaces and in preventing dissemination of C. albicans (13, 14). In vitro, neutrophil/epithelial cross talk provides protection from C. albicans (15–17). However, neutrophil activity must be tightly controlled, as evidenced by its role in worsening symptoms of vulvovaginal candidiasis (18–20). Monocytes/macrophages are essential for establishing protective immunity to disseminated infection, but their role in mucosal infection is not completely clear (21–25). Evidence from mouse and zebrafish models points to the redundancy of macrophages in mucosal C. albicans infections (26, 27). However, macrophages have been shown to protect against other fungi in mucosal infection (28–31). C. parapsilosis is known to interact with macrophages and monocytes in vitro, but the roles of phagocytes in controlling C. parapsilosis infection have not yet been explored in any live vertebrate infection model.

Epithelial cells and patrolling phagocytes are the first host cells to detect pathogens and signal to coordinate defenses against mucosal candidiasis (6, 32, 33). In vitro experiments with single cell types have shown that epithelial cells and phagocytes differ with respect to inflammatory signaling during challenge by C. albicans and C. parapsilosis. Epithelial cells from oral and intestinal sources (the oral cell lines SCC15 and TR146 and the primary human enterocyte cell line H4) respond in vitro to C. albicans by producing proinflammatory cytokines but produce little cytokine response to C. parapsilosis (15, 34, 35). On the other hand, professional innate immune cells, including human peripheral blood mononuclear cells, murine peritoneal macrophages, and the murine macrophage cell line J774.2, produce proinflammatory cytokines in response to both heat-killed C. albicans and C. parapsilosis (36–38). These contradictory results make it difficult to predict how the different cell types in mucosal tissues coordinate defense against these opportunistic fungal pathogens, so we sought to measure immune responses in a tractable vertebrate mucosal infection model.

In vitro experiments are limited to a few host cell types, and in vivo imaging in mammalian models is technically difficult (39–41). Complex signaling interactions between different host cell populations during mucosal Candida albicans infection were hinted at in studies using in vitro models with two or more host cell types (16, 17) and have been further elucidated using fluorescence-activated cell sorting of infected mouse tissue (9, 42, 43). Although these studies have shed light on the signaling roles and interactions of various host cell types with C. albicans, there remain significant gaps in our knowledge about the dynamics and cell type specificity of immune responses in the host, especially with respect to infections with other clinically important Candida species, such as C. parapsilosis. To further explore these in vitro and in vivo findings using intravitral imaging, we turned to the zebrafish swimbladder mucosal model, which mimics many aspects of mammalian infection (27, 44). The swimbladder is a natural site...
of fungal infection initiation in the fish that shares functional, anatomical, ontological, and transcriptional similarities to the lung (45–54). We compared the mucosal immune responses to two clinically relevant *Candida* species in an environment containing multiple host cell types, measuring several aspects of the immune response, including pathway activation, cytokine production, and innate immune recruitment. While *C. albicans* activated nuclear factor kappa B (NF-κB) signaling and elicited a strong proinflammatory cytokine response at this mucosal site, the host inflammatory response to *C. parapsilosis* was muted, similar to what has been found *in vitro* for epithelial cells. Live single-cell imaging suggests that NF-κB activation and tumor necrosis factor alpha (TNF-α) upregulation occur in different cellular subsets. Interestingly, the inflammatory cytokine response was not predictive of phagocyte behavior, as neutrophils and macrophages were recruited to and attacked both *Candida* species. Nevertheless, neutrophils were essential for protection from only *C. albicans* and not *C. parapsilosis*, confirming their known role in attacking hyphae. The differential immune responses to the two species reveal a disconnection between chemokine production and phagocyte recruitment. Single-cell intravital imaging further suggests that there is tissue-specific activation of NF-κB and TNF-α expression in mucosal candidiasis.

**RESULTS**

*C. albicans* causes lethal infection, but *C. parapsilosis* does not. *C. parapsilosis* and *C. albicans* are opportunistic pathogens that live commensally on mucosal surfaces of healthy humans and elicit different reactions from immune and epithelial cells *in vitro* (34, 35). To explore the relative virulence of these two fungal species in the mucosal setting in a live vertebrate host, we modified the zebrafish swimbladder infection model previously developed in our laboratory (27, 44, 55). We performed infection with a larger inoculum of 50 to 100 yeast cells to promote morbidity without immunocompromising the host (Fig. 1A). Both *Candida* species grew readily in the swimbladder, with *C. albicans* covering about twice as much area as *C. parapsilosis* by 24 h postinfection (hpi) (Fig. 1B). In the high-inoculum infection of immunocompetent fish used in this study, the swimbladder remained fully inflated and appeared healthy in the first hours after infection (Fig. 1C). However, within 24 hpi, signs of disease were apparent, with swimbladders becoming partially (Fig. 1D) or completely (Fig. 1E) deflated. Over time, the swimbladder could become greatly distended (Fig. 1F), and in *C. albicans* infections, hyphae sometimes breached the swimbladder epithelium, a factor predictive of fish death (27, 56). *C. parapsilosis* infection caused no mortality within 4 days postinfection (dpi), while *C. albicans*-infected animals began to perish at 2 dpi and reached 20% mortality by 4 dpi (Fig. 1G). Thus, in these high-inoculum infections, only *C. albicans* caused patterns of disease leading to mortality that were similar to those previously seen in immunocompromised fish and in a mixed fungal-bacterial infection (27, 56).

Zebrafish infected with *C. albicans* produce higher levels of inflammatory cytokines than *C. parapsilosis*-infected fish. Because we saw differences in the severity of the infections, we expected to find different inflammatory responses to the two *Candida* species. We measured changes in the expression of six inflammation-associated cytokines at 24 hpi (Fig. 2). In *C. albicans* infection, expression was significantly elevated above control levels for all 6 cytokines and higher than that observed in *C. parapsilosis* infection for 4 of 6 cytokines. In contrast, in *C. parapsilosis*-infected fish, the median levels of cytokine expression were not significantly elevated above controls. Thus, *C. albicans* evokes a stronger whole-fish cytokine response than *C. parapsilosis* during *in vivo* mucosal infection, demonstrating that there are important differences in the immune response at this early time point, hours before mortality is observed.

The local inflammatory signaling pattern mirrors whole-fish cytokine levels. The whole-fish quantitative PCR (qPCR) data showed overall cytokine responses but did not give us any spatial information about inflammatory signaling or indicate the cell types involved. In the zebrafish, local immune activation and cytokine signaling by epithelial tissue and innate immune cells can be imaged in real time in the live host.
Two key signaling components activated by Candida are NF-κB and TNF-α (44, 57–61). TNF-α expression is activated downstream of NF-κB and other signaling pathways and is implicated in protective cross talk between polymorphonuclear cells and the oral epithelium (17, 62).

To detect activation of NF-κB at the infection site, we used a transgenic zebrafish line, Tg(NF-κB:EGFP), that reports on pathway activity in multiple cell types and is activated in the swimbladder upon oral infection (44, 63). [The current zebrafish genetic nomenclature uses colons to indicate the following organization for transgenic fish lines: _Tg_(regulatory sequence: coding sequence).] Imaging of infected fish at 24 hpi revealed significant induction of NF-κB in _C. albicans_-infected fish but only basal levels of activity in _C. parapsilosis_-infected fish (Fig. 3A to D). As expected, we found green fluorescent protein (GFP) expression in several tissues, but not the swimbladder, under homeostatic conditions (63). To visualize local cytokine expression, we used _TgBAC(tnfα:GFP)_ reporter fish (64). Again, we saw significant activation of _tnfα:GFP_ in only _C. albicans_ and not _C. parapsilosis_ infections (Fig. 3E to G).

Intriguingly, despite the well-characterized connections between NF-κB and TNF-α, our in vivo imaging revealed differences in the spatial patterns of NF-κB activation and expression of TNF-α during _C. albicans_ infection. NF-κB:EGFP fluorescence was more diffuse (Fig. 3C), while _tnfα:GFP_ expression was more punctate and visible mainly near _C. albicans_ yeast and hyphae (Fig. 3H). These patterns of activity were especially interesting because previous work has shown that, in addition to the resident phago-
Fig. 4E and 5

Both near and distant from the area at the back of the swimbladder containing fungi immediately after dissection revealed GFP-positive (GFP) cells that are also intermingled with yeast and hyphae (both near and distant from the area at the back of the swimbladder containing fungi immediately after dissection revealed GFP-positive (GFP)

Transgenic fish (5/7), but only a few were motile in transgenic fish (5/7), but only a few were motile in

Cells that are GFP positive (GFP) cells were motile in time-lapse experiments that were intermingled with yeast and hyphae (time-lapse experiments that were intermingled with yeast and hyphae (Fig. 4C and D). This is again illustrated in a representative z-slice (Fig. 4D). The morphology and location of these cells are consistent with those of phagocytes.

To further characterize these cells displaying immune activation, we assessed their motility by crossing Tg(NF-κB:EGFP) or TgBAC(tnfα:GFP) fish with mpeg1:dtTomato (mpeg1:dtTomato) reporter fish and using time-lapse imaging to view the shape, behavior, and identity of GFP-fluorescing cells in infected fish. We found in time-lapse experiments that mpeg1:dtTomato + macrophages were occasionally doubly positive for NF-κB:EGFP or tnfα:GFP (6/43 for NF-κB:EGFP and 7/35 for tnfα:GFP) (see also Movies S1 and S2 in the supplemental material). Cells that are GFP + are outlined and were monitored for more than 16 min (Fig. 4Ei to Fii and Fig. 4Fi to Fiii). In TgBAC(tnfα:GFP) fish, all GFP + cells (7/7) were also dtTomato +, indicating that they are macrophages, while this was the case for only a minority of GFP + cells in Tg(NF-κB: EGFP) fish (5/57) (Fig. 4Eii and Fig. 4Fii). Many GFP + cells were motile in tnfα:GFP transgenic fish (5/7), but only a few were motile in NF-κB:EGFP transgenic fish (3/57) (Fig. 4Eiii and Fig. 4Fiii). This indicates that while TNF-α expression in the swimbladder

C. albicans (C. albicans) elicits higher levels of cytokine expression than C. parapsilosis (C. parapsilosis). Zebrafish were infected at 4 dpf as described in the legend of Fig. 1. At 24 hpi, total RNA was extracted from groups of 9 to 14 fish. Gene expression levels were determined by qPCR relative to mock-infected fish using the 2^−ΔΔCt method. Data are from 11 independent experiments. Notations above each bar indicate the significance of the difference between experimental treatments and vehicle-injected controls. Notations above the brackets indicate if there was a difference between C. parapsilosis- and C. albicans-infected fish. Statistics are described in Materials and Methods (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant [P > 0.05]). Abbreviations: saa, serum amyloid A gene; tnfα, tumor necrosis factor alpha gene; il-10, interleukin-10 gene; ccl2, C-C motif chemokine ligand 2 gene; cxcl8, C-X-C motif ligand 8 gene; il-6, interleukin-6 gene.

Signaling patterns differ in macrophages and epithelial tissue. While live imaging of transgenic fish at low resolution narrowed the location of signaling to the infection site, it did not allow us to identify which cell types were activated and contributing to swimbladder fluorescence. Because of the differences in NF-κB and TNF-α patterns, we reasoned that the two signaling components might be activated in different cell types. To examine cellular activation at high resolution and distinguish between fluorescence within the swimbladder and fluorescence in overlying tissue, we dissected swimbladders from C. albicans-infected zebrafish using a method previously developed in our laboratory (55). Imaging of Tg(NF-κB:EGFP) zebrafish swimbladders immediately after dissection revealed GFP-positive (GFP +) cells of the epithelial layer both near and distant from the area at the back of the swimbladder containing fungi (Fig. 4A and B). This is also illustrated in a single representative slice by outlining fluorescent cells and adding tissue landmarks (Fig. 4B). In TgBAC(tnfα:GFP) zebrafish, GFP-positive cells were not seen in the epithelial layer, but many GFP-positive cells were intermingled with yeast and hyphae (Fig. 4C and D). This is again illustrated in a representative z-slice (Fig. 4D). The morphology and location of these cells are consistent with those of phagocytes.

To further characterize these cells displaying immune activation, we assessed their motility by crossing Tg(NF-κB:EGFP) or TgBAC(tnfα:GFP) fish with mpeg1:dtTomato (red macrophage [65]) reporter fish and using time-lapse imaging to view the shape, behavior, and identity of GFP-fluorescing cells in infected fish. We found in time-lapse experiments that mpeg1:dtTomato + macrophages were occasionally doubly positive for NF-κB:EGFP or tnfα:GFP (6/43 for NF-κB:EGFP and 7/35 for tnfα:GFP) (Fig. 4E and F; see also Movies S1 and S2 in the supplemental material). Cells that are GFP + are outlined and were monitored for more than 16 min (Fig. 4Ei to Fii and Fig. 4Fi to Fiii). In TgBAC(tnfα:GFP) fish, all GFP + cells (7/7) were also dtTomato +, indicating that they are macrophages, while this was the case for only a minority of GFP + cells in Tg(NF-κB: EGFP) fish (5/57) (Fig. 4Eii and Fig. 4Fii). Many GFP + cells were motile in tnfα:GFP transgenic fish (5/7), but only a few were motile in NF-κB:EGFP transgenic fish (3/57) (Fig. 4Eiii and Fig. 4Fiii). This indicates that while TNF-α expression in the swimbladder

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is limited to macrophages, NF-κB signaling is activated in both macrophages and other cells likely to be epithelial.

Large, nonmotile cells in Tg(NF-κB:EGFP) fish, such as cell 2 (Fig. 4Eiii, yellow dotted outline), were enhanced green fluorescent protein positive (EGFP⁺) but dTomato negative (dTomato⁻), suggesting that they are not macrophages. In fact, the position and behavior of such cells suggest that they reside in the swimbladder epithelial layer, consistent with what is observed in dissected swimbladders (Fig. 4A and B). In TgBAC-tnfα:GFP fish, some stationary cells, such as cell 4 in the time-lapse image (Fig. 4Fiii, yellow dotted outline), were interacting with Candida and were identified as macrophages based on their mpeg1:dTomato expression. These time-lapse data thus indicate that TNF-α-expressing cells are more likely to be motile macrophages, while NF-κB is most frequently activated in nonmotile cells with epithelial morphology.

Neutrophils are recruited to infection and attack both C. albicans and C. parapsilosis. The activation of NF-κB and expression of TNF-α at the infection site in C. albicans-infected fish, combined with the qPCR data showing that the chemokines CXCL8 and CCL2 were upregulated only in C. albicans infection, suggested that phagocytes might be recruited only to C. albicans infections. We measured neutrophil recruitment using the Tg(mpx:mCherry)uwmm7Tg fish line, which has been characterized...
to express red fluorescence almost exclusively in neutrophils (66). To our surprise, we found increased neutrophil recruitment compared to mock infections (11 neutrophils/fish) for both *C. parapsilosis* (25/fish) and *C. albicans* (50/fish) infections (Fig. 5A to D).

Because of the different cytokine milieus elicited by the two fungal species, we reasoned that there might be differential interactions of neutrophils with each fungal
species at the infection site. We examined z-stack images slice-by-slice and catalogued interactions between neutrophils and Candida (Fig. 5E to G). In C. albicans infection, significantly more neutrophils per fish were involved in interactions with the fungus, although this is not surprising considering their greater numbers in C. albicans-infected swimbladders (Fig. 5H). Interactions in which neutrophils had ingested C. parapsilosis (Fig. 5E, blue arrows) or C. albicans (Fig. 5G, blue arrows) yeast cells or were wrapped around C. albicans hyphae ("frustrated phagocytosis") (Fig. 5F, yellow arrows) were counted as phagocytosis. When all neutrophils interacting with Candida were considered together, similar percentages were engaged in phagocytosis in C. parapsilosis (H11011 65%) and C. albicans (H11011 72%) infections (Fig. 5I). Thus, despite the lower numbers of neutrophils in C. parapsilosis infection and the differing cytokine environment, neutrophils had similar levels of activity against each fungal species.

Dimorphic switching of C. albicans is considered an important virulence trait, although little is known about how different morphotypes interact with immune cells in vivo. In the swimbladder, C. albicans injected as yeast switches rapidly to hyphal growth within the first 3 hpi (55, 56), and here we found that C. parapsilosis remains in
the yeast form throughout the infection period. Neutrophils were found interacting more often with \textit{C. albicans} hyphae than with yeast, which could be due to the large number of hyphal segments present (Fig. 5J). Overall, these data are consistent with the known activities of neutrophils against \textit{C. albicans} hyphae and yeast \textit{in vitro} (67–70). In summary, neutrophils are recruited to and actively interact with fungal cells of both \textit{Candida} species, despite the nearly undetectable levels of inflammatory cytokine production in \textit{C. parapsilosis} infection.

**Macrophages are recruited to infections with both \textit{Candida} species.** Although patrolling macrophages play an important role in the initiation of inflammation through the production of cytokines and are essential for controlling invasive candidiasis, they are thought to play a redundant role in mucosal \textit{Candida} infection (23, 26, 27, 71–74). Nevertheless, we observed a significant \textit{C. albicans}-specific induction of \textit{ccl2}, which suggested that macrophages would be recruited only upon \textit{C. albicans} infection. To our surprise, we found increased numbers of macrophages in the swimbladders of both \textit{C. parapsilosis}-infected and \textit{C. albicans}-infected fish (medians of 3 macrophages for mock-infected fish, 6 for \textit{C. parapsilosis}-infected fish, and 9 for \textit{C. albicans}-infected fish) (Fig. 6A to D).

Patterns of macrophage interaction with \textit{Candida} cells were remarkably similar to those of neutrophils. We found more macrophages interacting with the pathogen in \textit{C. albicans} infections (median of 5 macrophages per fish) than in \textit{C. parapsilosis} infections (median of 2 per fish) (Fig. 6E). As was the case for neutrophils, similar percentages (around 60%) of macrophages interacting with the two pathogens were engaged in phagocytosing them (Fig. 6F). Macrophages, like neutrophils, were found interacting with \textit{C. albicans} hyphae more often than with yeast (Fig. 6G). Thus, macrophages are recruited to infections with both \textit{Candida} species, and although they are found in lower numbers than neutrophils, they interact with and phagocytose both species.

**Functional neutrophils are required for protection from \textit{C. albicans} but not \textit{C. parapsilosis} infection.** High levels of neutrophil engagement suggested to us that these cells play an important role in the immune response to both \textit{Candida} species in the swimbladder model. We were interested to see if neutrophilic inflammation is protective, as in the murine oral infection models, or damaging, as in human vulvovaginal infection (18, 75). To block neutrophil function, we employed the transgenic fish line \textit{Tg(mpxmCherry-2A-Rac2D57N)} (D57N), a model of leukocyte adhesion deficiency in which neutrophils are present but defective in extravasation and phagocytosis (76–79). In the low-dose swimbladder infection model, neutrophils in D57N zebrafish fail to migrate into the \textit{C. albicans}-infected swimbladder, and this makes the fish susceptible to invasive disease (27). When infected with higher doses of \textit{C. albicans}, D57N zebrafish exhibited nearly 100% mortality by 4 dpi, compared to only 50% mortality in their wild-type (WT) siblings (Fig. 7A). Surprisingly, survival rates for D57N fish infected with \textit{C. parapsilosis} were not significantly different from the nearly 100% survival found in their WT siblings, despite the lack of neutrophil recruitment that was expected in this fish line (Fig. 7A and Movie S3). \textit{C. albicans}-infected D57N fish had more-severe infections than their WT siblings, with extensive growth of filaments that often breached the swimbladder epithelium.

We reasoned that inactivation of neutrophils could alter cytokine signaling through opposing mechanisms: greater damage to epithelial and other tissues could release damage-associated molecular patterns and provoke higher expression levels of inflammatory cytokines, or, alternatively, the absence of neutrophils at the site of infection could eliminate their contribution to amplification of the inflammatory response (80). Surprisingly, we found that D57N fish had nearly identical levels of \textit{tnfa} and \textit{cxcl8} (Fig. 7B) as well as \textit{saa}, \textit{il-10}, and \textit{il-1β} (Fig. S1) expression compared to their WT siblings when infected with \textit{C. albicans}. Levels of these cytokines were also similar in both WT and D57N infections with \textit{C. parapsilosis}. These data suggest that neutrophil inactivation does not have a strong overall net effect on inflammatory signaling.
DISCUSSION

*Candida albicans* and *Candida parapsilosis* are opportunistic yeast pathogens that live as commensals of healthy people but breach epithelial barriers to cause serious illness in immunocompromised patients. To understand how fungi breach this barrier, it is important to study the interactions between *Candida* cells and host defenses at mucosal surfaces in the intact host. By modeling mucosal *Candida* infection in the transparent larval zebrafish, we were able to visualize interactions between host immune cells, epithelial cells, and fungal pathogens in four dimensions (4D) in the live host. We discovered that mucosal infection by *C. albicans*, but not *C. parapsilosis*, caused significant mortality, activated NF-κB signaling, and evoked a strong local...
proinflammatory response. Despite the differential abilities of the two species to activate inflammatory pathways, infections with both species stimulated the recruitment of neutrophils and macrophages that actively attacked the fungi. Overall, our findings suggest that the contrasting immune responses to the two species of Candida in the swimbladder more closely resemble in vitro epithelial cell responses than in vitro mononuclear phagocyte responses, suggesting an important role for the epithelium in the overall inflammatory response.

The lack of C. parapsilosis virulence in the zebrafish is consistent with what has been seen in other infection models. This is the case for disseminated and mucosal disease in mice (81) as well as in vitro challenges with epithelial cells (34, 35, 82). Although C. parapsilosis is a common commensal fungus (5, 83), its virulence is usually associated with the hospital setting, and it is thought that predisposing conditions, such as epithelial damage or barrier breach by medical interventions, lead to disseminated infection (14, 83). In zebrafish models of C. albicans infection, penetrating hyphae are closely associated with mortality, and yeast-locked strains have limited virulence (27, 56, 84). Hyphal growth has also been clearly implicated in epithelial destruction in vitro and in mouse disease models (85–88). Thus, while the inability of C. parapsilosis to cause mortality in the absence of neutrophil function may be due to any number of differences between the two species, the lack of filamentous growth and expression of genes coregulated with the hyphal switch (such as candidalysin) are likely to be major determinants of differential virulence (89, 90).

Infection with C. albicans, but not with C. parapsilosis, elicited strong proinflamma-
Phagocyte recruitment and activation are often associated with proinflammatory cytokine and chemokine production, but we observed recruitment and active engagement of both macrophages and neutrophils without significant cytokine elicitation in *C. parapsilosis* infection (111–113). Several noncytokine chemoattractants, such as reactive oxygen species, lipids, and secreted fungal molecules, are associated with fungal infection in mouse and zebrafish infection models (12, 75, 114–120). Thus, phagocyte recruitment in *C. parapsilosis* infection may be the result of noncytokine signals, underlining the potential importance of these alternative chemoattractants.

Although *C. albicans* and *C. parapsilosis* are two of the most common causes of systemic fungal infections, the risk factors for the two species differ. In humans, neutropenia is a major risk factor for disseminated *C. albicans* infection, but only a small percentage of *C. parapsilosis* cases involve neutrophil depletion (5, 83). Likewise, immunosuppressed mice are highly susceptible to *C. albicans* but not *C. parapsilosis* disseminated infection (121, 122). These differences are reflected in the experiments presented here, which show that neutrophils are not required for immunity to *C. parapsilosis* infection, in contrast to the previous finding that neutrophils are essential for protection from *C. albicans* mucosal infection (27). This difference may indicate that neutrophils are important in controlling hyphal growth of *C. albicans* but redundant for managing *C. parapsilosis*, whose yeast-only morphology may be contained by the
remaining phagocytes (27, 123). Indeed, in the zebrafish, neutrophils and macrophages interacted with both hyphae and yeast of C. albicans, consistent with results from in vitro neutrophil and macrophage challenges (124–126). C. parapsilosis yeast and pseudohyphae are readily engulfed and killed by phagocytes in vitro, while engulfment of C. albicans requires longer times that vary with hyphal size and orientation (127–132). Although macrophages are known to provide protection from disseminated candidiasis, our recent work and that of others indicate that macrophages are redundant with respect to protection from mucosal C. albicans infection (23, 26, 27). In our higher-dose model, macrophages were recruited in significant numbers, activated NF-κB, expressed TNF-α, and interacted with both Candida species. It is intriguing that macrophages upregulate TNF-α upon C. albicans but not C. parapsilosis infection, suggesting that epithelium-macrophage cross talk or damage-induced signaling regulates cytokine production.

Overall, our work points to the unique characteristics of the zebrafish model (ease of live imaging and availability of transgenic lines) for discovery of previously unattainable information about host-pathogen interactions in vivo. Our comparison of host responses to two Candida species indicates that, unlike C. albicans, C. parapsilosis does not cause strong inflammatory responses or invasive disease at this mucosal site. We found a disconnect between inflammatory responses and phagocyte recruitment/activity that emphasizes the need for further study of signaling molecules that act on innate immune cells. Finally, imaging of single-cell patterns of gene activation paints a more complex picture of cell type-specific signaling during mucosal candidiasis. In sum, the tissue-specific aspects of the host response against Candida species are important and understudied aspects of disease that will benefit from future studies in zebrafish, mammalian hosts, and more complex in vitro challenge systems with more cell types.

MATERIALS AND METHODS

Candida strains and growth conditions. Candida strains used in this study are listed in Table S1 in the supplemental material. Candida was maintained in YPD medium (20 g/liter peptone, 10 g/liter yeast extract; Difco) containing 2% glucose and glycerol (30%) at −80°C and then grown on YPD agar plates at 30°C. Single colonies were picked into 5 ml YPD liquid and grown at 30°C overnight on a rotator wheel (New Brunswick Scientific). Prior to injection into zebrafish swimbladders, Candida cultures were washed three times in phosphate-buffered saline (PBS), counted on a hemocytometer, and resuspended in 5% polyvinylpyrrolidone (PVP) (Sigma-Aldrich) in PBS at a concentration of 5 × 10⁶ cells/ml.

Animal care and maintenance. Adult zebrafish were held in recirculating systems (Aquatic Habitats) at the University of Maine Zebrafish Facility, under a 14-h/10-h light/dark cycle and a water temperature of 28°C; they were fed Hikari micropellets (catalogue number HK40; Pentair Aquatic Ecosystems). Zebrafish strains used in this study are described in Table S2. Spawned eggs were collected and reared at 28°C; they were fed Hikari micropellets (catalogue number HK40; Pentair Aquatic Ecosystems). Zebrafish were sorted for the presence of mCherry in neutrophils (D57N) or its absence (WT siblings). To obtain heterozygous offspring with consistent fluorescence levels, heterozygous transgenic fish were crossed with opposite-sex AB fish, and progeny were sorted for the presence of mCherry in neutrophils (D57N) or its absence (WT siblings). To obtain heterozygous offspring with consistent fluorescence levels, Tgf(NF-κBEGFP) or TgBAC(mfnGFP) fish were crossed with opposite-sex AB fish, and embryos were screened on a Zeiss AxioVision VivaTome microscope (Carl Zeiss Microscopy, LLC) for basal GFP expression before injection. mpeg1:Gal4:UAS:nfsB:mCherry embryos were obtained by crossing Tg(mpeg1:Gal4:UAS:nfsB) (65) fish with opposite-sex Tg(UAS-E1b:TNR:mCherry)y:c264Tg (66) fish.

Zebrafish infections. Zebrafish infections were carried out by glass needle injection into the swimbladder as previously described (55). Briefly, zebrafish at 4 dpf were anaesthetized with Tris-buffered tricaine methane sulfonate (160 μg/ml) (Tricaine; Western Chemicals, Inc., Ferndale, WA) and injected with 4 nl PVP alone or PVP containing 5 × 10⁷ yeast cells/ml of C. albicans or C. parapsilosis. Infected fish were placed in individual wells of a 96-well glass-bottom imaging dish (Greiner Bio-One, Monroe, NC) and screened for an inoculum of 50 to 100 yeast cells on a Zeiss AxioVision VivaTome microscope. For survival curves, injected fish that passed screening were held for 4 days postinjection and monitored daily for survival.

Fluorescence microscopy. For imaging, fish were anaesthetized with Tricaine, immobilized in 0.5% low-melting-point agarose (Lonza, Switzerland) in E3 containing Tricaine, and arranged in a 96-well glass-bottom imaging plate. Images were made on an Olympus IX-81 inverted microscope with an FV-1000 laser scanning confocal system (Olympus, Waltham, MA), using a 20×/0.7-numerical-aperture objective.
microscope using a 20x objective and grease (Dow Corning, Midland, MI) at the corners of the coverslip prevented crushing and deflation of the microscope slide and covered with an 18- by 18-mm no. 1.5 coverslip. Preapplied dabs of high-vacuum grease were transferred to 0.4% low-melting-point agarose in PBS on a 25- by 75- by 1.0-mm step (New England BioLabs, Ipswich, MA). RNA was eluted in 20 l of nuclease-free water and stored at −80°C. cDNA was synthesized from 500 ng of RNA per sample using iScript reverse transcription (RT) supermix for RT-qPCR (Bio-Rad, Hercules, CA), and a no-RT reaction was performed for each sample. qPCR was carried out using SsoAdvanced universal SYBR green supermix (Bio-Rad), in 10-μl reaction mixtures, using 1 μl cDNA per reaction and a 0.3 μM primer concentration, on a CFX96 thermocycler (Bio-Rad). Threshold cycle (CT) values and dissociation curves were analyzed with Bio-Rad CFX Manager software. The change in gene expression was normalized to the ΔΔCT method (135). Primers (Integrated DNA Technologies) are listed in Table S3.

Image analysis. The percentage of the swimbladder covered by Candida at 24 hpi was determined using Fiji software (ImageJ environment (136)) applied to maximum-projection images from stacks of 15 to 25 z-slices. Images were taken with identical acquisition settings to ensure comparability. The swimbladder area was delineated, and the percent coverage of Candida fluorescence above a set threshold (corresponding to background fluorescence) was calculated. Images of the swimbladder areas of Tg(mNF-kBEGFP) and TgBAC(tnfαGFP) fish were analyzed using Fiji software. Images covered the swimbladder from midline to skin in 5-μm z-slices. The number of slices per image ranged from 12 to 22, depending on the size of the fish. Time-lapse images were processed in Fiji using descriptor-based registration (137). Neutrophils and macrophages were outlined and counted in Fluoview (Olympus), from images taken at 24 hpi.

Statistical analysis. Statistical analyses were carried out using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA). All significant differences are indicated in the figures. When data failed to pass the D’Agostino-Pearson test for normal distribution of data, for which the number of samples was too small to determine normality, nonparametric statistics were used (Fig. 1B, Fig. 2, Fig. 3D and E, Fig. 5H, Fig. 6D and E, and Fig. 7B). Kaplan-Meier survival curves were subjected to a log rank (Mantel-Cox) test, and Bonferroni correction was then used to determine statistical differences between pairs of treatments (Fig. 1G and Fig. 7A). NF-κB activation, TNF-α expression, macrophage recruitment, and qPCR results were analyzed using the Kruskal-Wallis test by ranks and Dunn’s test for multiple comparisons (Fig. 2, Fig. 3D and E, Fig. 6D, and Fig. 7B). Neutrophil recruitment data were normally distributed, so analysis of variance (ANOVA) with Tukey’s test for multiple comparisons was used (Fig. 5D). To compare Candida burdens and phagocyte interactions, we used the Mann-Whitney test (Fig. 1B, Fig. 5H, and Fig. 6E). Fisher’s exact test was used to compare the neutrophils and macrophages engaged in phagocytosis of the two Candida species (Fig. 5I and Fig. 6F). Paired t-tests were used to compare interactions of phagocytes with C. albicans hyphae and yeast (Fig. 5J and Fig. 6G).

Ethics statement. All zebrafish studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (138). All animals were treated in a humane manner and euthanized with Tricaine overdose according to guidelines of the University of Maine IACUC, as detailed in protocol number A2015-11-03.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00266-19.

MOVIE S1, AVI file, 15.4 MB.
MOVIE S2, AVI file, 0.5 MB.
MOVIE S3, MOV file, 1.6 MB.
FIG S1, PDF file, 0.7 MB.
TABLE S1, PDF file, 0.02 MB.
TABLE S2, PDF file, 0.02 MB.
TABLE S3, PDF file, 0.02 MB.

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