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The *Drosophila* Toll Pathway Controls but Does Not Clear *Candida glabrata* Infections

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The pathogenicity of *Candida glabrata* to patients remains poorly understood for lack of convenient animal models to screen large numbers of mutants for altered virulence. In this study, we explore the minihost model *Drosophila melanogaster* from the dual perspective of host and pathogen. As in vertebrates, wild-type flies contain *C. glabrata* systemic infections yet are unable to kill the injected yeasts. As for other fungal infections in *Drosophila*, the Toll pathway restrains *C. glabrata* proliferation. Persistent *C. glabrata* yeasts in wild-type flies do not appear to be able to take shelter in hemocytes from the action of the Toll pathway, the effectors of which remain to be identified. Toll pathway mutant flies succumb to injected *C. glabrata*. In this immunosuppressed background, cellular defenses provide a residual level of protection. Although both the Gram-negative binding protein 3 pattern recognition receptor and the Persephone protease-dependent detection pathway are required for Toll pathway activation by *C. glabrata*, only GNBP3, and not psh mutants, are susceptible to the infection. Both *Candida albicans* and *C. glabrata* are restrained by the Toll pathway, yet the comparative study of phenoloxidase activation reveals a differential activity of the Toll pathway against these two fungal pathogens. Finally, we establish that the high-osmolarity glycerol pathway and yapsins are required for virulence of *C. glabrata* in this model. Unexpectedly, yapsins do not appear to be required to counteract the cellular immune response but are needed for the colonization of the wild-type host. The *Journal of Immunology*, 2013, 190: 000–000.

*Candida* species are the most common cause of invasive fungal disease in humans. Morbidity caused by disseminated candidiasis continues to increase despite the availability of new antifungal drugs and has not changed much in the past two decades (1, 2). *Candida albicans* remains the predominant cause of disseminated candidiasis, accounting for more than half of all cases. However, *Candida glabrata*, which is involved in mucosal and bloodstream infections, has emerged as the second most common cause of invasive candidiasis in multiple countries (3, 4). *C. glabrata* systemic infections are difficult to treat because of their natural resistance to echinocandins and azole derivatives, especially to fluconazole, which is the classical antifungal treatment effective on *C. albicans* (3–5). Despite its name, *C. glabrata* is phylogenetically more closely related to *Saccharomyces cerevisiae*, a nonpathogenic haploid yeast, than to *C. albicans* (6). Indeed, it is mostly a monomorphic yeast, a major difference with the dimorphic *C. albicans*. Thus, a current challenge is to understand what makes *C. glabrata* virulent when compared with the nonvirulent yeast *S. cerevisiae* and the opportunistic pathogen *C. albicans*. Mammalian models are not optimal to identify virulence factors on a large scale because of low throughput, cost and labor-intensive procedures, and ethical issues. Furthermore, the study of the innate immune mechanisms, which are crucial for the outcome of systemic antifungal defense (7), are hampered in mammals by the adaptive immune response that is superimposed onto the innate immune response. Nevertheless, several *C. glabrata*-specific virulence factors have been identified such as adhesins and cell-bound proteases (8–14).

Invertebrates provide interesting alternative models to study microbial virulence factors and host immune responses (15, 16). *Drosophila melanogaster*, a small fly that is easy to rear, benefits from a century of genetic investigations. Its innate immunity has been widely studied during the past two decades (for a review, see Ref. 17). *D. melanogaster* is an excellent tool for the identification of novel virulence genes, for medium-throughput screening of antifungal molecules against *Candida* species, and for investigating pathogenicity of *C. albicans* (18–20).

Flies deficient for the evolutionarily conserved Toll signaling pathway are sensitive to fungal infections (18, 21, 22). The Toll pathway of *D. melanogaster* controls the systemic antifungal and the Gram-positive antibacterial host response, whereas immune deficiency (IMD), a second NF-kB–like pathway, is involved in the host response against Gram-negative bacteria infection. The Toll receptor is activated by the Spätzle cytokine, following its
proteolytic maturation into an active ligand by the Spätzle-processing enzyme (23). Gram-negative binding protein 3 (GNBP3) binds to fungal β-(1,3)-glucan and activates a proteolytic cascade that ultimately matures Spätzle through the Spätzle-processing enzyme (22, 24). However, D. melanogaster does not solely rely on pattern recognition receptors (PRRs) to detect infections. The host protease Persephone (PSH) is able to sense the enzymatic activity of microbial virulence factors, namely, some proteases, and subsequently triggers the Toll pathway through the transcription factor Dorsal-related immunity factor (DIF) (22, 25). The systemic immune response of D. melanogaster consists of the transcriptional induction of tens of genes, antimicrobial peptide genes such as Drosomycin, Diptericin, and Cecropins being highly expressed. These peptides are produced by the fat body, a functional equivalent of the mammalian liver, and subsequently secreted into the hemolymph. A complementary facet of the Drosophila host defense is the cellular immune response. Sessile and circulating hemocytes phagocytose microorganisms present in the hemolymph. Finally, a septic wound triggers immediate reactions that depend on proteolytic cascades, including the deposition of melanin at the wound site, which is thought to constitute another arm of host defense. Several steps of the melanization reactions are catalyzed by phenoloxidase (PO), which becomes active after the cleavage of its prodomain after the activation of dedicated proteolytic cascades (26–29). Of note, independently of its Toll-dependent function, the GNBP3 PRR is also required for the activation of PO after a fungal challenge and is required for the proteolytic cascades (26–29). Although, independently of its Toll-dependent function, the GNBP3 PRR is also required for the activation of PO after a fungal challenge and is required for the agglutination of C. albicans cells in the hemolymph (30). Based on its ability to form complexes that include PO and a serpin, it has been proposed that GNBP3 may muster the formation of an attack complex upon the detection of β-(1,3)-glucans (30).

In this study, we develop D. melanogaster as a model to investigate C. glabrata pathogenesis. We find that C. glabrata kills only Toll pathway immunocompromised flies. Like in mammals, wild-type Drosophila contain but are unable to clear C. glabrata infections (10). Although C. glabrata infections are detected by the fungal PRR of Drosophila, the known Toll-independent functions of GNBP3 do not appear to be required for host defense. C. glabrata cells are ingested by hemocytes, yet phagocytosis appears to be an essential host mechanism against fungi only in Toll pathway mutant flies. Finally, we demonstrate that C. glabrata strains mutated for the yapsin virulence factors or the high-osmolarity glycerol (HOG) pathway are hypovirulent, thus establishing Drosophila as a suitable model to identify and study novel C. glabrata virulence factors.

Materials and Methods

Microbial strains

Gram-positive bacteria used in this study included Micrococcus luteus (CIP A270; kind gift from H. Monteil, University Louis Pasteur, Strasbourg, France). Gram-negative bacteria used in this study included Escherichia coli. Yeasts strains are listed in Supplemental Table I. Bacteria and yeasts were grown overnight in liquid cultures of Luria-Bertani broth (37°C) and yeast extract/peptone/dextrose broth (29°C), respectively, and collected by centrifugation. Yeast titers were obtained by determining the CFUs. Infected flies were homogenized in PBS supplemented with 0.1% Tween and serially diluted. Hemolymph of infected flies was collected in PBS and treated the same way. Dilutions were plated on yeast extract/peptone/dextrose broth agar plates containing either hygromycin or streptomycin for selection and incubated overnight.

In vivo phagocytosis assays

Four thousand cells of C. glabrata were injected in flies. In vivo phagocytosis assays were then performed as previously described (24).

Cell culture

Drosophila S2 cells were cultured in Schneider’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, penicillin, and streptomycin.

In vitro phagocytosis assays

Drosophila S2 cells (1 × 10^5) were plated in eight-well latex slides in 150 μl Schneider’s medium with 10% FBS. Fifty microliter C. glabrata preparation at 5 × 10^6 cells/ml was added to each well containing S2 cells and incubated for 2 h at 25°C. After coinoculation, the cell culture medium was aspirated and cells were fixed with 2% formaldehyde in PBS, washed with 1× PBS, and blocked with 2% BSA in PBS for 2 h. The cell surfaces of C. glabrata were detected with primary Abs raised against whole Candida (a kind gift from Karl Kuchler in Vienna). Primary Abs were visualized with Cy3-conjugated goat anti-rabbit (Zymed Laboratories). In a second step, to visualize engulfed yeasts that were protected from binding the Ab by phagocytes, we permeabilized cells in BSA and Triton X-100 (0.1%), and then incubated them again with the same primary Ab. The subsequent procedure was identical except that primary Abs were visualized with Alexa 488 goat anti-rabbit Ab (Invitrogen). In each case, C. glabrata cells were considered phagocytosed if they were visualized only with the Alexa 488 goat anti-rabbit secondary Abs (green). DNA was visualized with DAPI. Slides were mounted in Vectorshield medium (Vector Laboratories) and were examined with a fluorescent Zeiss axioscope microscope (Carl Zeiss, Göttingen, Germany). Slides were kept at 4°C for storage. Images generated were processed using Adobe Photoshop CS (Adobe Systems) and analyzed using ImageJ plug-in RVB profiler.

Hemolymph extraction

Hemolymph was collected from the flies using thin glass capillaries mounted on the Nanoject apparatus (Drummond Scientific) and added to PBS supplemented with protease inhibitors (Complete mixture tablets; Roche). Protein concentration was determined using a Bradford assay.

Saturation of phagocytosis

For experiments in which phagocyte function is ablated, surfactant-free red, 0.3-μm diameter carboxylate-modified latex beads (Interfacial Dynamics) were washed in PBS and used 4× concentrated in PBS (corresponding to 5–10% solids), and 69 nl was injected 18–24 h before septic injury. The efficiency of the procedure in blocking phagocytosis was assessed using fluorescent-labeled bacteria or yeast and Trypan blue (data not shown), as previously described (32, 37).
Western blot

All hemolymph extracts, containing 10 mg protein, were equilibrated in Laemmli solution and denatured at 95°C for 5 min before loading on a 12 or 8% SDS-PAGE. After SDS-PAGE, proteins were blotted to Hybond ECL nitrocellulose membranes (Amersham) and blocked for 2 h at room temperature in 5% fat dry milk (Bio-Rad) in TBST (0.1%). Blots were incubated overnight at 4°C with the anti-PO Abs in 0.5% fat dry milk in TBST. After washing with TBST, the blots were incubated for 2 h at room temperature with the HRP-conjugated donkey anti-rabbit secondary Ab (Amersham). After washing with TBST, blots were detected by ECL according to the manufacturer’s instructions.

Agglutination and measurement of PO activity

Agglutination and PO activity assays were performed as described previously (30).

Killing of microbes and immunolocalization

Killing of microbes and immunolocalization were performed as previously described (24).

Data processing and statistical analysis

Data are representative of at least three independent experiments (n ≥ 3 in each experiment) that yielded similar results unless stated otherwise in this article. Results from quantitative analyses are expressed as mean ± SD of the data from at least three independent experiments, unless stated otherwise in this article. Statistical analyses were performed using Student t test, and p < 0.05 was considered significant. Statistical significance of survival experiment was calculated using the product limit method of Kaplan and Meier using the log-rank test (GraphPad PRISM4 software), and p < 0.05 was considered significant. All data significantly different from control values are marked with asterisks, and p values are given in the figure legends.

Results

The Toll pathway plays an essential role in host defense against injected C. glabrata

We first injected C. glabrata, C. albicans, and S. cerevisiae in wild-type flies. Three reference wild-type strains of C. glabrata and C. albicans hardly killed flies, and there was no significant difference in survival rates with flies infected with three strains of S. cerevisiae (Fig. 1A and data not shown). The few flies that were dying apparently did not succumb to the fungal infection, because the microbial titer had not significantly increased in those deceased flies (data not shown). Next, we determined that flies deficient for Toll pathway activation, namely, MyD88 mutants, were sensitive to C. glabrata and C. albicans, whereas wild-type flies were not at all susceptible to C. glabrata (Fig. 1A and data not shown). The few flies that were dying apparently did not succumb to the fungal infection, because the microbial titer had not significantly increased in those deceased flies (data not shown). Next, we determined that flies deficient for Toll pathway activation, namely, MyD88 mutants, were sensitive to C. glabrata and C. albicans, whereas wild-type flies were not at all susceptible to C. glabrata (Fig. 1A and data not shown).

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To assess how the fly detects C. glabrata, we first checked that GNBP1, which is required for sensing Gram-positive bacteria, was not involved in host defense against this fungus (survival and Drosomycin expression data not shown). Next, we monitored the survival of psh, GNBP3hades, and psh-GNBP3hades mutant flies (Fig. 1G). As previously reported for C. albicans, we found that GNBP3hades and psh-GNBP3hades mutant flies succumbed like MyD88 flies, whereas psh displayed only a weak susceptibility phenotype (22). Indeed, Drosomycin induction was impaired in GNBP3hades and psh-GNBP3hades mutant flies (Fig. 1H). However, in contrast with infections with C. albicans, Drosomycin expression was also significantly decreased in psh mutant flies. The observation that GNBP3hades, and not psh, mutants succumb rapidly to C. glabrata, even though Drosomycin expression is affected to similar levels in both mutants, suggests that GNBP3 may play other roles in the immune defense that, as reported previously (30), are independent of its function in Toll pathway activation.

The known Toll-independent functions of GNBP3 may not be involved in the host defense against C. glabrata

We have previously reported that C. albicans are agglutinated in a GNBP3-dependent manner (30). We therefore tested whether C. glabrata cells are agglutinated after incubation with hemolymph collected from wild-type or GNBP3hades mutant flies. As shown in Fig. 2A, we did not detect any significant aggregates as had been observed previously with C. albicans (30).

GNBP3 is also required for the activation of the proteolytic cascade that turns pro-PO into an active PO enzyme, which catalyzes the melanization reactions. We found that PO activity was induced by C. glabrata, but to a lower extent, as compared with C. albicans (Fig. 2B). In Dif mutants, PO was unexpectedly activated to similar levels as wild-type flies by C. glabrata, in contrast with challenges with other microbes (28, 30). We also found that pro-PO was poorly cleaved into active PO after a C. glabrata challenge, as compared with M. luteus and C. albicans (Fig. 2C). As expected, pro-PO cleavage was inhibited in MyD88 mutants. Finally, we also failed to detect any sensitivity of CG3066 mutants...
to a C. glabrata challenge, in which the PO activating enzyme is mutated (data not shown).

The cellular arm of the immune response provides an additional layer of defense against C. glabrata

We determined that hemocytes phagocytose living C. glabrata in vivo (Fig. 3A), as previously shown for C. albicans (30). Similar findings were established with S2 cultured cells, which originate from embryonic macrophages (data not shown). Interestingly, we still did find ingested C. glabrata in fly hemocytes 7 d after the beginning of the infection (Supplemental Fig. 3A).

It has been reported that C. glabrata is not killed by mammalian macrophages and actually grows inside them (8, 10). To determine whether hemocytes may constitute an intracellular niche in which...
C. glabrata can proliferate while being protected from the systemic immune response, we impaired the phagocytic activity of hemocytes by the prior injection of nondigestible latex beads. Our prediction was that the survival of wild-type flies would not be affected and that the C. glabrata titer would decrease. Indeed, we did not observe a significant change in the survival curves of C. glabrata–challenged wild-type flies preinjected with latex beads as compared with PBS preinjected controls (Fig. 3B). However,

FIGURE 2. Agglutination and melanization of C. glabrata. (A) Agglutination of yeast cells in Drosophila hemolymph. Hemolymph was extracted from wild-type (wt) or GNBP3hades mutant (hades) flies and incubated with C. glabrata. BSA was used as a control. The GNBP3-dependent agglutination of C. albicans has been previously published (30). Data are representative of at least three independent experiments. (B and C) C. glabrata and melanization. Enzymatic activity was measured after C. albicans and C. glabrata challenge. Enzymatic activity is expressed as mean ± SD of the data from three independent experiments (n = 20 in each experiment, *p < 0.05, **p < 0.01). Immunoblotting experiment showing PPO cleavage in its active PO form (C). PPO cleavage is induced at 4 h after challenge with M. luteus, C. albicans, and to a lesser extent with C. glabrata. PPO cleavage is blocked in MyD88 mutant flies. Data are representative of three independent experiments. ns, Not significant.

FIGURE 3. Phagocytosis of C. glabrata (C. g). (A) In vivo phagocytosis of C. glabrata by Drosophila hemocytes. Adult Drosophila carcasses were dissected and fixed 45 min after C. glabrata injection. Hemocytes are shown in yellow. In each experiment, yeasts cells were first stained in red before permeabilization and then in green. Ingested cells can only be stained in green, whereas yeasts on the outside are stained by both Abs. DNA was stained with Hoechst 33258 and hemocytes with Cy5 (yellow). Data are representative of three independent experiments (n = 15). Original magnification ×2000. (B) Phagocytosis in adult flies plays an essential role in host defense of Toll mutant flies. Flies were either preinjected with latex beads (LXB) or PBS and subjected to a septic injury with C. glabrata. Data are expressed as mean of the data from three independent experiments (n = 20 in each experiment). LXB preinjected MyD88 mutant flies were significantly more susceptible to infection than PBS-injected flies (***p < 0.001). (C) In vivo proliferation assay of wild-type (wt) and MyD88 mutant flies (MyD88). C. glabrata cells were retrieved from crushed whole flies. Before the injection of 10⁴ yeast cells, flies were either preinjected with LXB or PBS. Graph shows the log CFUs of C. glabrata per single fly, and data are representative of three independent experiments (n = 15). Original magnification ×2000. (D) Inactivation of phagocytosis by injections of LXB leads to increased Toll pathway activation after C. glabrata infection. Flies were either preinjected with LXB or PBS and subjected to a septic injury with C. glabrata. Drosomycin mRNA rate were then quantified 24 h postinfection. **p < 0.005. Data are expressed as mean of the data from four independent experiments (n = 15 in each experiment). CI, Clean injury; M. l, M. luteus.
we found that *C. glabrata* was still able to persist in phagocytosis-impaired wild-type flies (Fig. 3C). The somewhat decreased titer as compared with controls might be accounted for by a stronger induction of the Toll pathway (Fig. 3D), suggesting that the host more easily detects *C. glabrata* yeasts when hemocytes can no longer ingest them. Actually, the finding that *MyD88* mutants in which phagocytosis is blocked succumb more rapidly to *C. glabrata* than phagocytosis-competent *MyD88* flies (Fig. 3B) strongly suggests that hemocytes form a remaining defense in Toll pathway-deficient flies. In keeping with this observation, the *C. glabrata* titer reached a lethal threshold around 2 d postinfection (Fig. 3B, 3C).

Drosophila as a model to study *C. glabrata* interactions with its host

*C. glabrata* is pathogenic to immunodeficient patients; yet, only a few animal models that lead to *C. glabrata*-induced death are available to date (e.g., see Ref. 11). In this study, we asked whether flies might be useful to identify virulence factors of this pathogenic yeast. *C. glabrata* is auxotrophic for NAD*. *C. glabrata* is able to grow in the bladder and urinary tract of patients because of its ability to synthesize NAD* from external metabolites such as nicotinamide ribosides (NRs) through the use of either the NR kinase (Nrk1) or the nicotinamidase Pnc1 (43, 44). We found that *nrk1* and *pnc1* mutants are as virulent as control wild-type *C. glabrata* when introduced into *MyD88* flies (Fig. 4A), which suggests that NAD* may be available in the hemocoel of the fly. Next, we asked whether yapsins might be required for virulence in our model. The 11 *C. glabrata* yapsins are secreted GPI-anchored aspartyl proteases. Yapsins, especially YPS1 and YPS7, are required for the integrity of the cell wall, control of the level of adhesion, and survival within macrophages (8). We found that both a *yps1-yps7* double-mutant strain and a *yps1-11Δ* strain, in which all 11 yapsin genes have been deleted, were less virulent in *MyD88*-immuno deficient *Drosophila* (Fig. 4A). The *yps1-yps7* phenotype was, however, variable in some experiments when compared with that of *yps1-11Δ*. We next assessed whether the *C. glabrata* yapsins might be required to counteract the cellular response mediated by host hemocytes that forms an essential defense in Toll pathway-deficient flies (Fig. 3B). Following this hypothesis, the *yps1-11Δ* *C. glabrata* strain should become as virulent as the wild-type *C. glabrata* strain in Toll mutant flies that are deprived of a functional phagocytic system. The virulence of the *yps1-11Δ* strain was enhanced in *MyD88* mutants that were preinjected with latex beads, but, however, not to the level observed with wild-type *C. glabrata* in similarly pretreated *MyD88* flies (Fig. 4B). Thus, yapsins are still required as virulence factors in *MyD88*-immunosuppressed flies that also lack a cellular immune response, implying that yapsin function is not required to counteract phagocytosis. We next investigated further the pathogenesis of the less virulent *yps1-11Δ* *C. glabrata* strain by assessing the hemolymph titer in *MyD88* flies. Interestingly, the yapsin mutant strain initially proliferated in *MyD88* mutant flies but was subsequently controlled (Fig. 4C). Importantly, unlike the wild-type strain, the persistence of the *yps1-11Δ* *C. glabrata* strain was abolished in wild-type flies, suggesting that the *yps1-11Δ* strain is more sensitive to the action of the Toll pathway.

**FIGURE 4.** *C. glabrata* (*C. g*) mutants for virulence factors. (A) Survival of *Drosophila* to wild-type, yapsin, and NR salvage pathway mutant *C. glabrata* infection. *MyD88* mutant flies succumbed to *nrk1Δ* or *pnc1Δ* at a similar rate than to wild-type *C. glabrata* infection. *MyD88* mutant flies succumbed more slowly to a challenge with either *yps1Δyps7Δ* or *yps1-11Δ* than with wild-type *C. glabrata* (*p* < 0.05 for comparison with the corresponding *MyD88* flies infected with the wild-type *C. glabrata* strain). (B) *C. glabrata* yapsins are not required to counteract phagocytosis (*p* < 0.01). (C) The proliferation of the yapsin mutant strain is controlled by both wild-type and *MyD88* mutant flies. *C. glabrata* were retrieved from the hemolymph. Graphs show the log CFUs per single fly. All *MyD88* mutant flies infected with the wild-type *C. glabrata* strain or the *yps1-11Δ* *C. glabrata* strain were dead after days 2 and 7, respectively; hence the lack of data for subsequent time points. Data are representative of three independent experiments (pool of *n* = 15 in each experiment) that yielded similar results. (D) *C. glabrata* yapsins do not induce the PSH pathway. *Drosomycin* mRNA quantification 24 h after *C. glabrata* challenge. Results from quantitative analyses are expressed as mean ± SD of the data from 20 flies. (E) Survival of *Drosophila* to wild-type ATCC2001 or HOG mutant *C. glabrata* infection. *MyD88* mutant flies succumbed less rapidly to a challenge with either *sho1A* or *pbs2A* than with wild-type *C. glabrata*. *p* < 0.05 when compared with *MyD88* mutant flies infected with the wild-type *C. glabrata* strain. (A, B, E) Flies were challenged with a thin needle previously dipped in a pellet of *C. glabrata*. Survival was monitored at 29°C. The survival rate is expressed in percentage. The survival results are the mean of three to four independent experiments for each strain (*n* ≥ 20 in each experiment).
We have previously reported that the *Drosophila* PSH protease activates the Toll pathway when proteolytically matured by secreted fungal virulence factor (22) (Fig. 5). We therefore next assessed whether the *C. glabrata* yapsins were required to elicit the PSH pathway. However, we did not detect any decrease in the *Drosophila* PSH protease or its cleavage products in a wild-type *C. glabrata* strain (Fig. 4D).

The HOG pathway triggers adaptation of yeasts through intracellular accumulation of osmolytes. It has been reported that *C. glabrata* strains mutated for the HOG pathway showed severe defects under high-osmolarity conditions (45). Sho1 and Sln1 are cell-surface membrane proteins that redundantly detect and activate two independent signaling branches that converge on the Pbs2 kinase, which subsequently phosphorylates the downstream MAPK HOG1 (Supplemental Fig. 3B). Gregori et al. (45) have demonstrated that the ATCC2001 strain lacks the Ssk2 kinase gene, which renders the Sln1 branch nonfunctional. Therefore, the mutation of the cell-surface sensor of the unique functional branch, Sho1 (sho1Δ), causes osmosensitivity similar to that obtained with the mutation of the downstream MAPKK Pbs2 (pbs2Δ). Interestingly, *MyD88* mutant flies infected with ATCC2001-sho1Δ or with the ATCC2001-pbs2Δ strains were killed at the same slow rate as compared with *MyD88* flies infected with the wild-type ATCC2001 strain (Fig. 4E). The ssk2 gene is functional in the BG2 strain, another wild-type strain, resulting in a functional redundancy between the Sho1 and Sln1 activating branches of the HOG pathway (45). Interestingly, we found that only *pbs2* mutant *C. glabrata*, and not *sho1* or *ssk2* mutants, were less virulent when injected into *MyD88* flies (Supplemental Fig. 3C), a situation that mirrors that described for osmosensitivity in the BG2 strain. These findings in two independent strains make it unlikely that the effects we observe are due to second-site mutations, and thus allow us to conclude that the HOG1 (Supplemental Fig. 3B). Gregori et al. (45) have demonstrated that the ATCC2001 strain lacks the Ssk2 kinase gene, which renders the Sln1 branch nonfunctional. Therefore, the mutation of the cell-surface sensor of the unique functional branch, Sho1 (sho1Δ), causes osmosensitivity similar to that obtained with the mutation of the downstream MAPKK Pbs2 (pbs2Δ). Interestingly, *MyD88* mutant flies infected with ATCC2001-sho1Δ or with the ATCC2001-pbs2Δ strains were killed at the same slow rate as compared with *MyD88* flies infected with the wild-type ATCC2001 strain (Fig. 4E). The ssk2 gene is functional in the BG2 strain, another wild-type strain, resulting in a functional redundancy between the Sho1 and Sln1 activating branches of the HOG pathway (45). Interestingly, we found that only *pbs2* mutant *C. glabrata*, and not *sho1* or *ssk2* mutants, were less virulent when injected into *MyD88* flies (Supplemental Fig. 3C), a situation that mirrors that described for osmosensitivity in the BG2 strain. These findings in two independent strains make it unlikely that the effects we observe are due to second-site mutations, and thus allow us to conclude that the HOG pathway is required for *C. glabrata* virulence in our *Drosophila* infection model. Altogether, these results suggest that *Drosophila* is a suitable model to identify some virulence factors of *C. glabrata*.

**Discussion**

In this study, we deciphered the pathogenesis of *C. glabrata* infection, an increasingly common cause of morbidity and mortality in humans, in the model host *Drosophila*. As compared with other fungi, *C. glabrata* presents some specific features in this model. We discuss the unique and common attributes of *C. glabrata*, and examine the value and limitations of *D. melanogaster* as a model to study *C. glabrata* pathogenesis.

**C. glabrata persist even in wild-type flies**

Flies deprived of a functional Toll pathway succumb to *C. glabrata* infection, thus highlighting again the primary role of the Toll pathway in systemic antifungal host defense (18, 21, 22, 46). The inoculum size of *C. glabrata* we used did not induce a high mortality in wild-type flies in this systemic infection model for at least a period of 2 wk (data not shown). Remarkably, although the Toll pathway is an essential antifungal defense mechanism, wild-type flies are not able to clear *C. glabrata* infections within a fortnight. This persistence is reminiscent of the situation observed in mice in which *C. glabrata* persists up to 4 wk without killing the immunocompetent animal (47). These observations suggest either that *C. glabrata* is able to evade or withstand the host immune response or, alternatively, that a dynamic equilibrium is established between *C. glabrata* proliferation and the host-induced antifungal response. It has been shown that *C. glabrata* survives and multiplies in mammalian macrophages (8, 10). In *Drosophila*, *C. glabrata* is detected within hemocytes up to 7 d postinfection. It remains to be determined whether these yeasts had been freshly engulfed or had been phagocytosed early on during the infection and had been able to persist. It is, however, unlikely that they are able to proliferate in hemocytes because we did not detect a qualitatively distinct number of ingested *C. glabrata* after 1 or 7 d (Supplemental Fig. 3A). Furthermore, it is unlikely that hemocytes constitute a major niche in which yeasts proliferate or at least persist because we have shown that phagocytosis is indeed an efficient host defense mechanism against *C. glabrata* infections, at least in a Toll pathway mutant background. In addition, *C. glabrata* still persists, although at decreased levels, in phagocytosis-impaired wild-type flies (Fig. 3C). Macrophages are ultimately damaged by ingested *C. glabrata* (10). Whether *Drosophila* hemocytes are also finally lysed at later stages of the *C. glabrata* infection remains unknown. We note, however, that no

**FIGURE 5.** PO is differentially activated through the Toll pathway by *C. glabrata* and *C. albicans*. Pro-PO is cleaved into active PO by the MP1 protease, which is itself activated by the MP2 protease, an activation that is negatively regulated by the 27A serpin (SPN27A) (27–29). Pro-PO activation is directly triggered by GNB3P, which actually binds pro-PO and PO (30). PO activation also requires the activation of the Toll pathway, which regulates the expression of unknown factors required for pro-PO cleavage (X, Y) (28). PO activation by *C. albicans* requires DIF, through the expression of X, whereas PO activation by *C. glabrata* is not dependent on DIF. We propose in this study that in this latter case, a DIF/Dorsal heterodimer controls the expression of a distinct factor (Y).
hematopoiesis has been described in adult Drosophila. Thus, the observation of phagocytosed yeasts inside hemocytes at least up to 7 d after an immune challenge demonstrates that not all hemocytes are lysed at this stage.

The persistence of C. glabrata in immunocompetent flies demonstrates that the Toll-mediated immune response is not effectually fungicidal and might have a fungistatic effect, although, as discussed earlier, a dynamic equilibrium cannot be excluded. Tens of genes are activated on stimulation of the Toll pathway, with the antimicrobial peptides being strongly upregulated. Yet, no efficient effectors against yeasts have been described. Drosomycin and Metchnikowin are active only on filamentous fungi (48, 49). Moreover, in vitro fungicidal assays failed to reveal any activity of Drosomycin on C. albicans and C. glabrata yeasts (50, 51). In vivo, the ubiquitous overexpression of Drosomycin or Metchnikowin in spätzle Toll mutant flies did not rescue their susceptibility phenotype to C. albicans infection (data not shown). The effect of CecropinA has not been tested in vivo, and CecropinA activity in vitro remains controversial. It has been previously reported that the yeast S. cerevisiae and the filamentous fungi Metarhizium anisopliae were susceptible to CecropinA (52). However, other in vitro analyses have established that Drosophila CecropinA is inactive against S. cerevisiae, C. glabrata, and C. albicans, but effective against some filamentous fungi (Neurospora crassa, Fusarium oxysporum, Fusarium culmorum) (50, 51).

In this study, we have shown that the IMD pathway key mutants were resistant to C. glabrata infection and that Dif mutant flies were hardly affected by the infection, yet our preliminary data indicate that the induction of CecropinA is blocked in these mutants, in keeping with similar results obtained with other microbial challenges (31, 40). It is therefore unlikely that this antimicrobial peptide plays a major role in the host defense against C. glabrata. Overall, our results clearly point out the importance of an intact and functional Toll pathway to counteract C. glabrata infection, but its effectors remain to be discovered.

Altogether, our data indicate that C. glabrata is able to persist in wild-type flies and that this phenomenon depends on yapsins (Fig. 4C). The observation that phagocytosis is not required for C. glabrata persistence opens the possibility that the ability of this opportunistic yeast to persist in its host over long periods is not directly linked to its capacity to proliferate in vertebrate macrophages ex vivo.

Attributes of the Drosophila host response to C. glabrata

C. glabrata is detected both through the GNB3 and PSH arms of the fungal detection system. Although β-(1,3)-glucans are the ligands of GNB3 (24), the C. glabrata protease that activates Toll through PSH remains undefined as we have shown in this article that yapsins are not involved in this process. This function might be achieved through a subtilisin-like protease identified in the genome (T. Gabaldon, personal communication).

An unexpected finding was the almost normal resistance of psh mutants to a C. glabrata challenge as compared with GNB3- or psh double mutants, even though both affect Toll signaling to the same extent. This is reminiscent of the C. albicans case as exposed in the introduction to this article (30). However, agglutination and melanization cannot account for this disparity in the survival phenotypes as regards C. glabrata. One interesting possibility is that GNB3 might function as an opsonin because, in contrast with the situation for C. albicans (30), phagocytosis is a relevant host defense in Toll pathway mutants. Another hypothesis would be that GNB3 musters an effective attack complex on C. glabrata (30).

C. glabrata is thought to evade detection by the immune system in mammals (10), which may account for its persistence. In flies, C. glabrata is efficiently detected by the systemic surveillance apparatus. However, it is not agglutinated, as is the case for C. albicans, and it does not fully trigger the PO cascade, a phenomenon that might be linked to the limited role of DIF in triggering this cascade, as discussed later. Overall, C. glabrata hardly eludes immune detection in Drosophila.

A peculiarity of the C. glabrata infection model regards the role of the DIF transcription factor. It is not required for PO activation, unlike the C. albicans case (30), and the susceptibility of this mutant to a C. glabrata challenge was variable. This is likely linked to a redundancy with the Dorsal transcription factor. We envision two possibilities. In the first model, the Toll pathway would be activated differently after a C. glabrata challenge as compared with a C. albicans challenge, for instance, by modifying the mode of activation of the Toll receptor by amplitude or frequency modulation (53). A second model is that the set of effectual effector genes may vary between C. glabrata and C. albicans, and that their promoters have different requirements in terms of the actual NF-kB dimers that promote their transcription.

For instance, in the case of the PO cascade, it is hypothesized that the Toll pathway regulates the expression of an activator of the PO apparatus. It has been reported in larvae that Toll-dependent antimicrobial production by the fat body requires the Spätzle ligand secreted by hemocytes (54). However, a role of phagocytes in the induction of the systemic humoral Toll response in adult flies has never been proved. Our observations that wild-type flies present no susceptibility phenotype to C. glabrata infection upon phagocytosis blockade allow us to exclude a role of phagocytosis in Toll pathway activation.

We have observed in this study that C. glabrata remains present in the hemocytes of adult Drosophila up to 7 d postinfection and that hemocytes form an essential defense in Toll pathway-deficient flies. Yapsin proteases are required for the yeast proliferation in mammalian phagocytes (8) but do not counteract the cellular response in Drosophila. Had this been the case, then yapsin mutants would have displayed a full virulence phenotype in phagocytosis-impaired flies (55). This rescue of the virulence phenotype has been observed for another C. glabrata mutant we have identified (A. Taravaud, J. Bourdeaux, and D. Ferrandon, unpublished observations). It will be interesting to determine whether the C. glabrata catalase is required for the pathogen to withstand the cellular immune response, because it is currently unknown whether Drosophila hemocytes produce an oxidative shock to kill ingested microbes (56). The existence of an oxidative burst can also be tested on the host side by reducing genetically the expression of the Dual oxidase gene or that of the NADPH oxidase gene in hemocytes.

In conclusion, we have developed a model in which C. glabrata kills its host likely as a result of its unchecked proliferation in the body cavity. Interestingly, a strain presenting an attenuated virulence displays only a limited growth in the hemolymph. This
model is qualitatively distinct from current mouse models in which fungal load is monitored in several organs. The identification of several attenuated strains validates this model for further large-scale screens that can be achieved only in Drosophila. It will also be interesting to test mutants affecting the C. glabrata–specific genes that are absent in other Candida species, as well as potential virulence strategies unique to this pathogen. This may include C. glabrata tolerance to high copper concentrations (57), this ion being present in the hemolymph because it is required for PO activity. The challenge will then be to determine whether the virulence factors identified by these approaches will be relevant to an understanding of C. glabrata pathogenesis in patients. Indeed, the Drosophila model is adequate to identify “public” virulence factors, that is, general virulence factors that are required for pathogenicity independently of the host in opposition to “private” virulence factors, such as thermotolerance for mammalian hosts, which involves the calcineurin pathway in C. glabrata (14). Thus, it remains to be determined whether the HOG pathway belongs to the “public” or to the Drosophila “private” category of virulence factors. In support of the former possibility, we note that HOG pathway mutants in Cryptococcus neoformans displayed an attenuated virulence in mammals (58).

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