

White-Opaque Switching of *Candida albicans* Allows Immune Evasion in an Environment-Dependent Fashion

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Candida albicans strains that are homozygous at the mating type locus can spontaneously and reversibly switch from the normal yeast morphology (white) to an elongated cell type (opaque), which is the mating-competent form of the fungus. White-opaque switching also influences the ability of *C. albicans* to colonize and proliferate in specific host niches and its susceptibility to host defense mechanisms. We used live imaging to observe the interaction of white and opaque cells with host phagocytic cells. For this purpose, we generated derivatives of the switching-competent strain WO-1 that express green fluorescent protein from a white-specific promoter and red fluorescent protein from an opaque-specific promoter or vice versa. When mixed populations of these differentially labeled white and opaque cells were incubated with human polymorphonuclear neutrophils (PMNs) on a glass slide, the neutrophils selectively phagocytosed and killed white cells, despite frequent physical interaction with opaque cells. White cells were attacked only after they started to form a germ tube, indicating that the suppression of filamentation in opaque cells saved them from recognition by the PMNs. In contrast to neutrophils, dendritic cells internalized white as well as opaque cells. However, when embedded in a collagen matrix, the PMNs also phagocytosed both white and opaque cells with similar efficiency. These results suggest that, depending on the environment, white-opaque switching enables *C. albicans* to escape from specific host defense mechanisms.

The yeast *Candida albicans* is a commensal microorganism in the orogastrintestinal and urogenital tracts of most healthy people, but it can also cause superficial infections of the skin and mucosae as well as life-threatening disseminated infections, especially in immunocompromised patients. Morphological transitions play an important role in the biology of *C. albicans* and in the interactions of the fungus with its host. In response to various environmental signals, *C. albicans* changes its growth mode from the budding yeast form to filamentous growth, which facilitates tissue invasion (1). Strains that have become homozygous at the mating type locus (*MTLa/a* or *MTL α / α*), either by mitotic recombination or by loss of one copy of chromosome 5 and duplication of the homologous chromosome, can also switch from the normal white yeast form to an elongated cell form termed “opaque” (2, 3). Opaque cells are the mating-competent form of *C. albicans*; they can mate with other opaque cells to produce recombinant progeny (4). Switching between the white and opaque phases occurs spontaneously at a relatively low frequency, but it can also be induced by certain environmental signals (5–10).

White and opaque cells differ not only in their mating competence but also in the expression of many genes that are unrelated to mating, including adhesins and metabolic genes, suggesting that the two cell types are adapted to different environments within a mammalian host (11–13). Indeed, opaque cells are better colonizers of the skin, but they are much less virulent than white cells in a mouse model of disseminated candidiasis (14, 15). Opaque cells do not readily undergo filamentation under most conditions that promote hyphal formation in white cells, which may result in a reduced ability to invade tissues (16). In addition, opaque cells have been reported to be more susceptible than white cells to killing by polymorphonuclear neutrophils (PMNs) or cell-free oxidants, and they also stimulated more superoxide generation by PMNs (17). As neutrophils are important first-line host defense cells in the prevention of disseminated candidiasis (18), an in-

creased susceptibility to killing by these phagocytes may contribute to the reduced virulence of opaque cells during systemic infections. On the other hand, white but not opaque cells release a chemoattractant for PMNs, indicating that opaque cells may in fact avoid being assaulted by neutrophils (19). Interestingly, white cells have also been found to be more efficiently phagocytosed than opaque cells by other types of innate immune cells, *Drosophila* hemocyte-derived S2 cells and mouse macrophage-derived RAW264.7 cells (20). Therefore, it remains unclear if and when white-opaque switching may enable *C. albicans* to escape from specific components of the host immune system.

In this work, we used live imaging by video microscopy to study the interaction of mixed populations of white and opaque cells of *C. albicans* with two types of phagocytic cells that play important roles in the primary host defense against microbial infections, neutrophils and dendritic cells (DCs). Our results demonstrate that neutrophils and dendritic cells differ in their ability to recognize the two types of cells and that, depending on the environment, white-opaque switching may enable *C. albicans* to evade attack by the host’s innate immune system.

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TABLE 1 *C. albicans* strains used in this study

Strain	Parent	Relevant genotype ^a	Reference or source
WO-1	WO-1		21
WOP4G41A and -B	WO-1	<i>OP4/OP4/op4::P_{OP4}-GFP-SAT1-FLIP</i>	This work
WOP4G42A	WOP4G41A	<i>OP4/OP4/op4::P_{OP4}-GFP-FRT</i>	This work
WOP4G42B	WOP4G41B	<i>OP4/OP4/op4::P_{OP4}-GFP-FRT</i>	This work
WOP4R21A and -B	WO-1	<i>OP4/OP4/op4::P_{OP4}-RFP-SAT1-FLIP</i>	This work
WOP4R22A	WOP4R21A	<i>OP4/OP4/op4::P_{OP4}-RFP-FRT</i>	This work
WOP4R22B	WOP4R21B	<i>OP4/OP4/op4::P_{OP4}-RFP-FRT</i>	This work
WOP4G42WH11R21A	WOP4G42A	<i>OP4/OP4/op4::P_{OP4}-GFP-FRT WH11/wh11::P_{WH11}-RFP-SAT1-FLIP</i>	This work
WOP4G42WH11R21B	WOP4G42B	<i>OP4/OP4/op4::P_{OP4}-GFP-FRT WH11/wh11::P_{WH11}-RFP-SAT1-FLIP</i>	This work
WOP4G42WH11R22A	WOP4G42WH11R21A	<i>OP4/OP4/op4::P_{OP4}-GFP-FRT WH11/wh11::P_{WH11}-RFP-FRT</i>	This work
WOP4G42WH11R22B	WOP4G42WH11R21B	<i>OP4/OP4/op4::P_{OP4}-GFP-FRT WH11/wh11::P_{WH11}-RFP-FRT</i>	This work
WOP4R22WH11G21A	WOP4R22A	<i>OP4/OP4/op4::P_{OP4}-RFP-FRT WH11/wh11::P_{WH11}-GFP-SAT1-FLIP</i>	This work
WOP4R22WH11G21B	WOP4R22B	<i>OP4/OP4/op4::P_{OP4}-RFP-FRT WH11/wh11::P_{WH11}-GFP-SAT1-FLIP</i>	This work
WOP4R22WH11G22A	WOP4R22WH11G21A	<i>OP4/OP4/op4::P_{OP4}-RFP-FRT WH11/wh11::P_{WH11}-GFP-FRT</i>	This work
WOP4R22WH11G22B	WOP4R22WH11G21B	<i>OP4/OP4/op4::P_{OP4}-RFP-FRT WH11/wh11::P_{WH11}-GFP-FRT</i>	This work

^a *SAT1-FLIP*, the *SAT1* flipper cassette; *FRT*, FLP recombination target sequence.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at -80°C . The strains were subcultured separately in the white and opaque phases at room temperature on agar plates containing Lee's medium, pH 6.8 (22), and 5 $\mu\text{g/ml}$ phloxine B, which selectively stains opaque colonies pink (23). Strains were routinely grown in YPD liquid medium (10 g yeast extract, 20 g peptone, 20 g glucose per liter) at 30°C in a shaking incubator. For selection of nourseothricin-resistant transformants, 200 $\mu\text{g/ml}$ nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. To obtain nourseothricin-sensitive derivatives in which the *SAT1* flipper cassette was excised by FLP-mediated recombination, transformants were grown overnight in YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) without selective pressure to induce the *MAL2* promoter controlling *caFLP* (*Candida*-adapted *FLP*) expression. One hundred to 200 cells were then spread on YPD plates containing 10 $\mu\text{g/ml}$ nourseothricin and grown for 2 days at 30°C . Nourseothricin-sensitive clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 200 $\mu\text{g/ml}$ nourseothricin as described previously (24).

Plasmid constructions. The previously described plasmid pGFP70 (25) contains a *Candida*-adapted green fluorescent protein reporter gene (*GFP*) (26) under the control of the *OP4* promoter and *URA3* as a selection marker. A *Sall*-*PstI* fragment from pOPT1G22 (27) was inserted between the same sites in pGFP70, thereby introducing a *BglII* site behind the *GFP* stop codon and substituting the dominant *caSAT1* (*Candida*-adapted *SAT1*) selection marker for *URA3* in the resulting plasmid, pOP4G2. An *ApaI*-*BglII* fragment from pOP4G2 containing the *P_{OP4}-GFP* fusion was then cloned together with a *BglII*-*XhoI* fragment from pNIM6 (9) containing the *TEF3* transcription termination sequence in *ApaI*/*XhoI*-digested pCZF1M2 (9) to generate pOP4G3. The *OP4* downstream region was amplified with the primers OPS21 (5'-CTTTAGTTAA TGCCCGCGGTCAAGCTGCCTC-3') and OPS8 (5'-TACTTGAGCTCT GCAACACTTCTTGCTCTTT-3'), and the *SacII*/*SacI*-digested PCR product was used to replace the *CZF1* downstream region in pOP4G3, yielding pOP4G4 (Fig. 1A, top). A *WH11* promoter fragment from pGFP68 (28) and a fragment containing the *WH11* downstream region, which was amplified with the primers WHS15 (5'-GAGTGAGTAACCG CGGTTGAGTTGAAGTC-3') and WHS16 (5'-CTTGAGCTCAGTGT TAGTGATACAGTC-3'), were used to replace the *OP4* flanking sequences in pOP4G4 to generate pWH11G2. The red fluorescent protein gene (*RFP*) was amplified from plasmid pCaADH1- γ EmRFP (29) with the primers RFP1 (5'-ATATGTCGACAAAATGGTTTCAAAGGTGAA GAAG-3') and RFP2 (5'-ATATAGATCTTATTTATATAATTCATCCAT

ACC-3') and used to replace *GFP* in pOP4G4 and pWH11G2, thereby producing pOP4R2 and pWH11R2, respectively.

Strain construction and analysis. *C. albicans* strains were transformed by electroporation (24) with the gel-purified inserts from plasmids pOP4G4, pWH11G2, pOP4R2, and pWH11R2 described above. The correct integration of each construct and subsequent excision of the *SAT1* flipper cassette were confirmed by Southern hybridization using the flanking sequences as probes. In each case, two independent series of strains (A and B) were generated.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* strains was isolated as described previously (24). The DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, transferred by vacuum blotting onto a nylon membrane, and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with an Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, United Kingdom) according to the instructions of the manufacturer.

Isolation of neutrophils and dendritic cells. Neutrophils were isolated from human blood samples by using polymorphPrep solution from Progen. The isolation was carried out according to the instructions of the manufacturer. Viability was assessed by trypan blue staining. The cells were suspended in RPMI 1640 medium containing 5% fetal calf serum (FCS) and stored on ice until usage. Bone marrow-derived dendritic cells (BMDCs) were generated in 7- to 9-day cultures as described previously (30). Briefly, bone marrow cells were flushed out of both hind legs of 10- to 12-week-old female BALB/c mice. After erythrocyte lysis, the cell suspension was precultured in a 10-cm cell culture dish for 2 h at 37°C in RPMI 1640 medium (supplemented with nonessential amino acids [NEAAs], 5% FCS, 2 mM L-glutamine, 50 μM β -mercaptoethanol, 50 $\mu\text{g/ml}$ gentamicin, 250 U/ml granulocyte-macrophage colony-stimulating factor [GM-CSF], 100 U/ml interleukin-4 [IL-4]) to eliminate adherent macrophage-like cells. Subsequently, the nonadherent cells were transferred to 6-well cell culture plates at a concentration of 3×10^6 cells per well and cultured at 37°C in supplemented BMDC medium (see above) for 7 to 9 days (exchange of medium at day 3; transfer to new culture plates at day 6). Cell lines secreting murine GM-CSF or IL-4 were kindly provided by Thomas Blankenstein.

Time-lapse video microscopy. Experiments were carried out as described by Behnsen et al. (31), with some modifications. Fresh colonies of *C. albicans* were washed once in phosphate-buffered saline and once in RPMI 1640 medium containing 5% FCS. For the experiments on glass slides, 1.25×10^6 cells of each phase (opaque and white) were mixed with 5×10^5 purified viable neutrophils in 200 μl RPMI 1640 medium con-

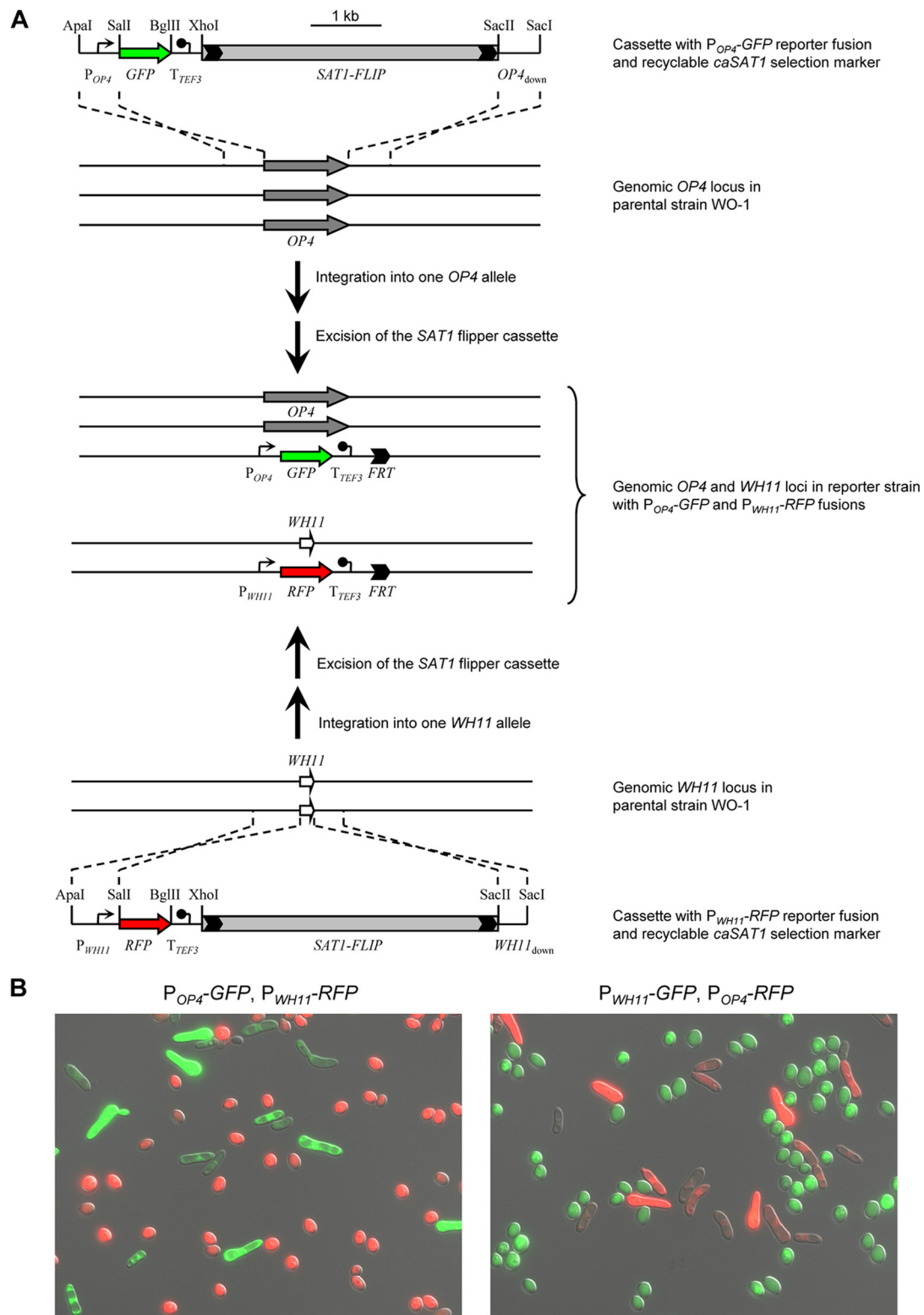


FIG 1 Generation of phase-specifically labeled *C. albicans* strains. (A) Schematic of the sequential integration of P_{OP4} -GFP (top) and P_{WH11} -RFP (bottom) reporter gene fusions into the genomic *OP4* and *WH11* loci of the parental wild-type strain WO-1 with the help of the recyclable *SAT1* flipper cassette (*SAT1*-FLIP) to generate strains WOP4G42WH11R22A and -B (see also Table 1). Strains WOP4R22WH11G22A and -B, which contain P_{OP4} -RFP and P_{WH11} -GFP reporter fusions, were generated in an analogous fashion. The parental strain WO-1 is trisomic for chromosome 1 and contains three copies of *OP4*, which is located on this chromosome (9). The genomic *OP4* and *WH11* coding sequences are indicated by the gray and white arrows, respectively; the cloned upstream (P_{OP4} and P_{WH11}) and downstream ($OP4_{down}$ and $WH11_{down}$) sequences served for integration of the reporter cassettes into the respective genomic loci by homologous recombination. T_{TEF3} , transcription termination sequence of the *TEF3* gene; *FRT*, FLP recombination target sequence. Relevant restriction sites used for the generation of the reporter cassettes (see Materials and Methods) are indicated. (B) Phenotype of white and opaque cells of the reporter strains. Cells from white and opaque colonies of strains containing the indicated reporter fusions were mixed and observed by fluorescence microscopy. The pictures show an overlay of transmission and epifluorescence micrographs with GFP and RFP filter settings and demonstrate the phase-specific expression of *GFP* and *RFP* in white and opaque cells of the reporter strains. (Left) Strain WOP4G42WH11R22B; (right) strain WOP4R22WH11G22A.

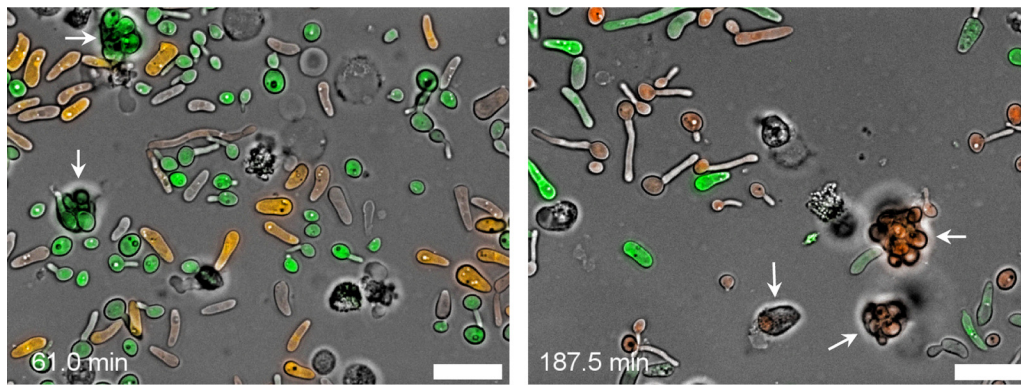


FIG 2 Interaction of white and opaque cells with human neutrophils on a glass slide. (Left) Neutrophils that were coincubated with a mixture of *GFP*-expressing white cells and *RFP*-expressing opaque cells of strain WOP4R22WH11G22B. Two neutrophils that have exclusively phagocytosed white-phase cells are labeled with arrows. (Right) Neutrophils that were coincubated with a mixture of *RFP*-expressing white cells and *GFP*-expressing opaque cells of strain WOP4G42WH11R22B. Three neutrophils that have exclusively phagocytosed white-phase cells are labeled with arrows. Pictures were taken from Videos S1 (left) and S2 (right) in the supplemental material; time points are indicated. Bars, 20 μ m.

taining 5% FCS. The suspension was poured in a glass chamber, and video microscopy was performed for 5 h at 37°C with two frames per minute using an Olympus BX61 microscope with a $\times 60$ LUMPLFL W/IR (numerical aperture, 0.9) lens, together with the cellR software (version 2.1) from Olympus Biosystems. For the experiments within a collagen matrix, 1×10^7 yeast cells of each phase (opaque and white) were mixed with 5×10^5 purified viable neutrophils in 66 μ l minimal essential medium and 133 μ l type I collagen stock solution (Vitrogen-100; Nutacon) to a final collagen concentration of 1.7 mg/ml and poured in a glass chamber. The chamber was incubated for 20 min at 37°C to allow polymerization of the matrix and subsequently used for video microscopy. The experiment with DCs was performed as described above for the neutrophils on glass slides, except that the coincubation was for 6 h with one frame per minute. All experiments were performed with two independent cultures of the *C. albicans* strains, and each coincubation was observed over time at four different positions. Scale bars in all videos are 20 μ m.

RESULTS

Generation of phase-specifically labeled *C. albicans* strains. To uncover differences in the interaction of neutrophils and dendritic cells with white and opaque cells of *C. albicans*, we performed coincubation experiments with the phagocytes and mixed white/opaque cell populations instead of studying the interaction with white and opaque cells separately, thus allowing the immune cells to encounter the two different forms of the pathogen simultaneously. Although white and opaque cells usually can be well distinguished under the microscope by their different morphologies, we decided to use differentially labeled white and opaque cells to facilitate the recognition of the two yeast forms by video microscopy during phagocytosis. For this purpose, we sequentially introduced the *GFP* reporter gene under the control of the opaque-phase-specific *OP4* promoter and the *RFP* reporter gene under the control of the white-phase-specific *WH11* promoter into the corresponding endogenous genomic loci using a recyclable nourseothricin resistance marker for the selection of transformants (Fig. 1A). In addition, we also generated complementary strains expressing *GFP* from the *WH11* promoter and *RFP* from the *OP4* promoter to exclude the (unlikely) possibility that any differences between the two cell types were caused by the expression of *GFP* or *RFP*. This strategy had the advantage that white and opaque cells of the same reporter strain rather than different derivatives of a particular strain could be compared. The *MTL α / α*

wild-type strain WO-1, in which white-opaque switching was originally discovered and which has been widely used to study this developmental program, was used as the parent for the construction of the reporter strains. Two independent series of strains were generated (Table 1) and used in subsequent experiments. After verifying the correct genomic configurations of the reporter strains, cells in the white and opaque phases were isolated, mixed, and observed by fluorescence microscopy. As can be seen in Fig. 1B, white and opaque cells of the reporter strains exhibited the expected phenotypes and appeared green or red in the respective phases.

Interaction of white and opaque cells with human neutrophils. To study the interaction of white and opaque cells with PMNs, freshly isolated human neutrophils were incubated with mixed white/opaque cell populations of the labeled *C. albicans* strains on glass slides and observed by video microscopy. Under these conditions, the neutrophils selectively phagocytosed cells in the white phase, whereas internalization of opaque cells was rarely observed, despite frequent physical contact (Fig. 2; see Videos S1 and S2 in the supplemental material). Therefore, the fact that opaque cells do not secrete a chemoattractant for neutrophils cannot be the sole explanation for their ability to prevent phagocytosis by these host defense cells. Another possible reason could be the larger size and elongated morphology of opaque yeast cells compared with white yeast cells. However, the culture conditions employed in these experiments promoted the yeast-hyphal switch in white cells, which formed germ tubes that became more and more elongated over time. Even white cells with germ tubes that were considerably longer than whole opaque cells were efficiently phagocytosed by the neutrophils (Fig. 3; see Video S3 in the supplemental material), indicating that the size of opaque cells is not the limiting factor that precludes phagocytosis.

Close inspection of all phagocytosis events observed in videos from independent experiments revealed that all white cells that were taken up by the neutrophils had started to germinate, whereas white yeast cells without a recognizable germ tube were not taken up, even after repeated contact with a neutrophil. An illustrative example is provided in Video S4 in the supplemental material, representative pictures of which are shown in Fig. 4A. The neutrophil that is highlighted in Fig. 4A could be followed

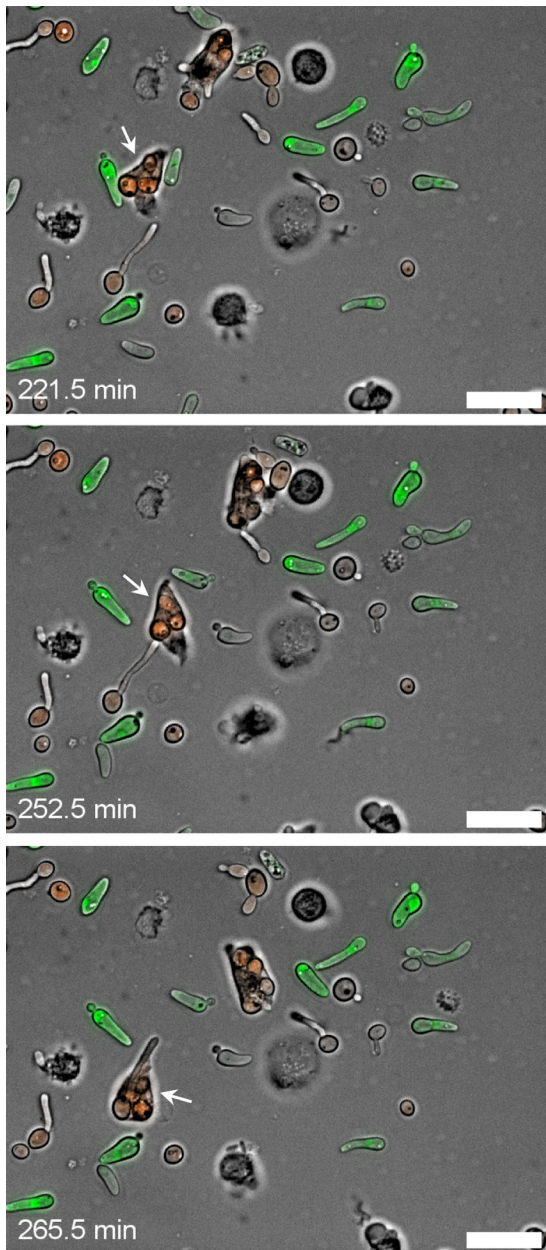


FIG 3 Neutrophils can phagocytose large cells. (Top) A neutrophil (marked with a white arrow) that has taken up several *RFP*-expressing white cells but does not phagocytose opaque cells despite close physical contact; (middle) the same neutrophil attacking a white cell with a large germ tube, which is followed by phagocytosis of the complete cell (bottom). Strain WOP4G42WH11R22A was used for this experiment. For more details, see Video S3 in the supplemental material, from which the pictures were taken at the indicated time points. Bars, 20 μ m.

over the whole time course of the experiment. It frequently and repeatedly interacted with all types of *C. albicans* cells, often forming extensions that seemed to probe the surface of white and opaque cells, but phagocytosed only germinating white cells, while finally leaving opaque cells and white cells without germ tubes alone. It can also be seen in Video S4 in the supplemental material that when the neutrophil had reached the end of its life span and disintegrated after many phagocytosis events (Fig. 4Ai), most of

the internalized *C. albicans* cells appeared to have been destroyed, indicating efficient killing by the neutrophil. From a total of 495 phagocytosis events analyzed in independent experiments with all four differentially labeled *C. albicans* strains, uptake of an opaque cell was observed only six times (1%), whereas the remaining 489 internalized fungal cells (99%) were germinating white cells (Fig. 4B). Therefore, under the experimental conditions of these assays, neutrophils appear to predominantly recognize fungal structures that are selectively expressed on germ tubes/hyphae of *C. albicans* but not on the surface of opaque cells.

Interaction of dendritic cells with white and opaque *C. albicans* cells. Dendritic cells are another important type of host defense cells that provide a linkage between the innate and adaptive immune system by phagocytosing microbial pathogens and presenting their antigens to other immune cells. They recognize and phagocytose both yeast and hyphal forms of *C. albicans* (32), but their behavior toward opaque cells has not been studied so far. We therefore examined the interaction of DCs with mixed populations of differentially labeled white and opaque cells under the same conditions as in the experiments with the neutrophils. Interestingly, the DCs did not distinguish between white and opaque cells and phagocytosed both cell types, albeit also with some preference for white cells. The DCs quickly started to take up *C. albicans* cells from the beginning of the coinubation, so that DCs with internalized fungi were already seen when video microscopy was initiated (see Videos S5 and S6 in the supplemental material). Often, individual DCs contained mixtures of white and opaque cells (Fig. 5A), and from a total of 96 phagocytosed cells counted in 21 DCs, 28 were opaque cells, 5 were white cells without a recognizable germ tube, and 63 were white cells with germ tubes (Fig. 5B).

Neutrophils phagocytose both white and opaque cells when embedded in a collagen matrix. In a previous study, it was observed that neutrophils selectively phagocytosed *Aspergillus fumigatus* conidia but not *C. albicans* yeast cells (which did not germinate under the experimental conditions of that study) when coinubated with both fungi on a glass surface (31). However, when coinubated with the two types of fungal cells within a collagen matrix (a three-dimensional [3D] environment, as opposed to the two-dimensional [2D] environment on a glass slide), the neutrophils phagocytosed the *C. albicans* yeast cells instead of the *A. fumigatus* conidia, indicating that the environment influenced the recognition of different pathogens by the neutrophils (31). We therefore investigated whether neutrophils would also interact in a different manner with white and opaque cells of *C. albicans* when embedded in a collagen matrix. Interestingly, we found that under these conditions the neutrophils also recognized and phagocytosed the opaque cells, in addition to white cells with and without germ tubes. An example is shown in Fig. 6A and the corresponding video (see Video S7 in the supplemental material). Among 319 phagocytosis events counted in four independent experiments, opaque cells were phagocytosed 131 times (41%), germinating white cells were phagocytosed 173 times (54%), and white yeast cells without a recognizable germ tube (whose proportion in the population declined over time due to the induction of germ tube formation) were phagocytosed 15 times (5%) (Fig. 6B). Therefore, under appropriate conditions, neutrophils can also efficiently recognize and phagocytose opaque cells.

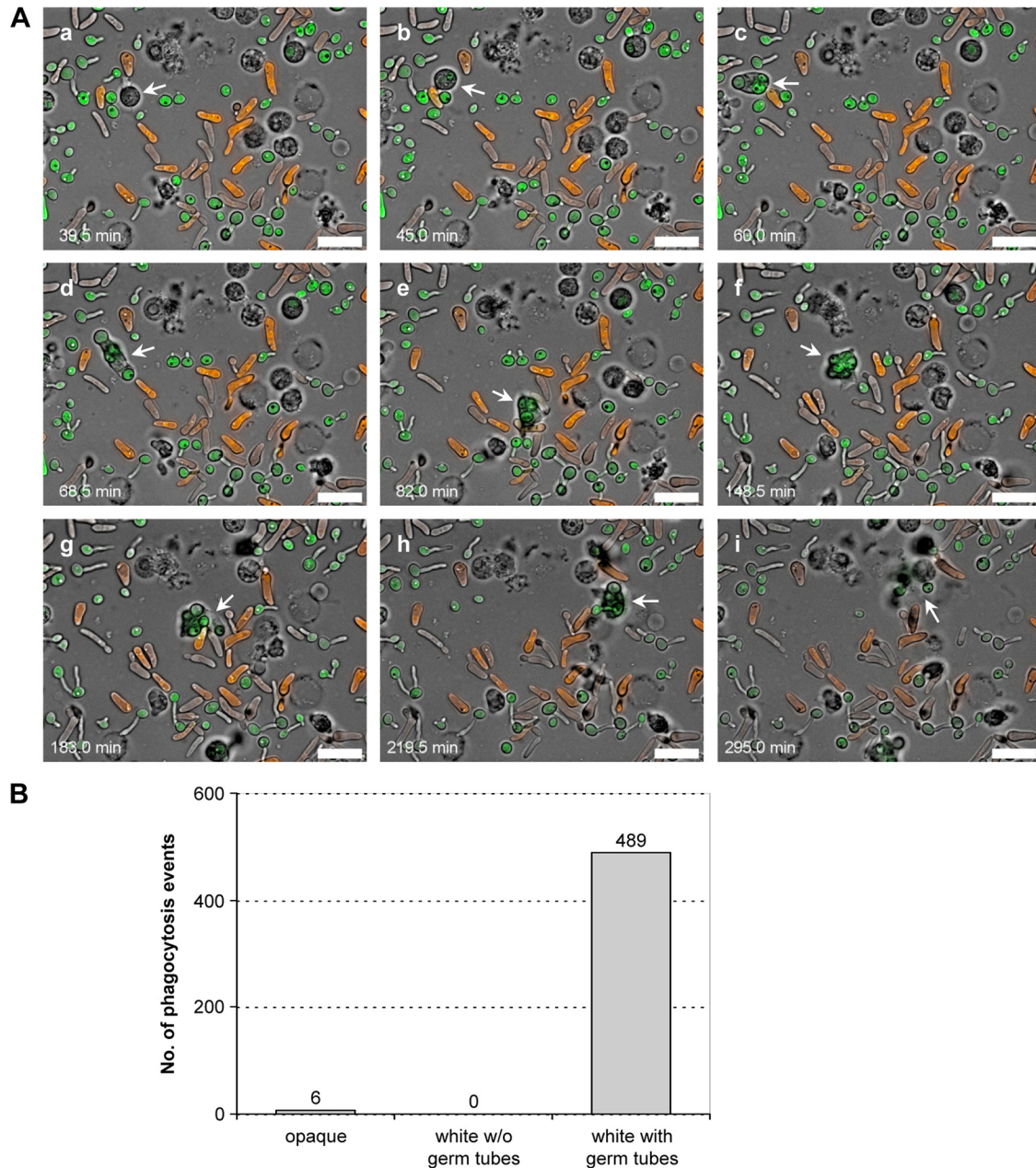


FIG 4 Neutrophils selectively phagocytose germ tube-forming white cells. (A) A series of pictures from Video S4 in the supplemental material (time points are indicated) in which the behavior of a single neutrophil (marked by the white arrow) can be followed over time. After the neutrophil becomes active and gets in physical contact with surrounding *GFP*-expressing white and *RFP*-expressing opaque cells (a), it takes up a germinating white cell (b), which is followed by phagocytosis of additional white cells with germ tubes (c and d). The neutrophil then repeatedly contacts a group of surrounding opaque cells without phagocytosing these cells (e). Instead, two germ tube-forming white cells, which can be seen above the neutrophil in panel e, are ingested (f), while another white cell without a recognizable germ tube (below the neutrophil in panel f) is frequently contacted but not phagocytosed. The neutrophil then pushes an opaque cell aside to attack and ingest another germ tube-forming white cell (g) and migrates on and phagocytoses an additional germinating white cell (h). When the neutrophil finally disintegrates, most of the phagocytosed *C. albicans* cells apparently have been destroyed (i). Strain WOP4R22WH11G22B was used in this experiment. Bars, 20 μ m. (B) Frequency of phagocytosis of opaque cells and white cells with and without a germ tube. A total of 495 phagocytosis events by 118 neutrophils were analyzed. Data are from four independent experiments including all four differentially labeled *C. albicans* strains and two biological replicates for each strain.

DISCUSSION

It is currently not understood why *C. albicans* has integrated a morphological switch into its life cycle to undergo sexual development. In contrast to the model yeast *Saccharomyces cerevisiae*, where haploid **a** or **α** cells derived from diploid **a/ α** cells can im-

mediately mate with cells of the opposite mating type, *C. albicans* cells that have become homozygous for the mating type locus (*MTLa/a* or *MTL α / α*) first have to switch from the normal yeast morphology (termed white in this context) to the opaque cell form in order to become mating competent (33, 34). It is com-

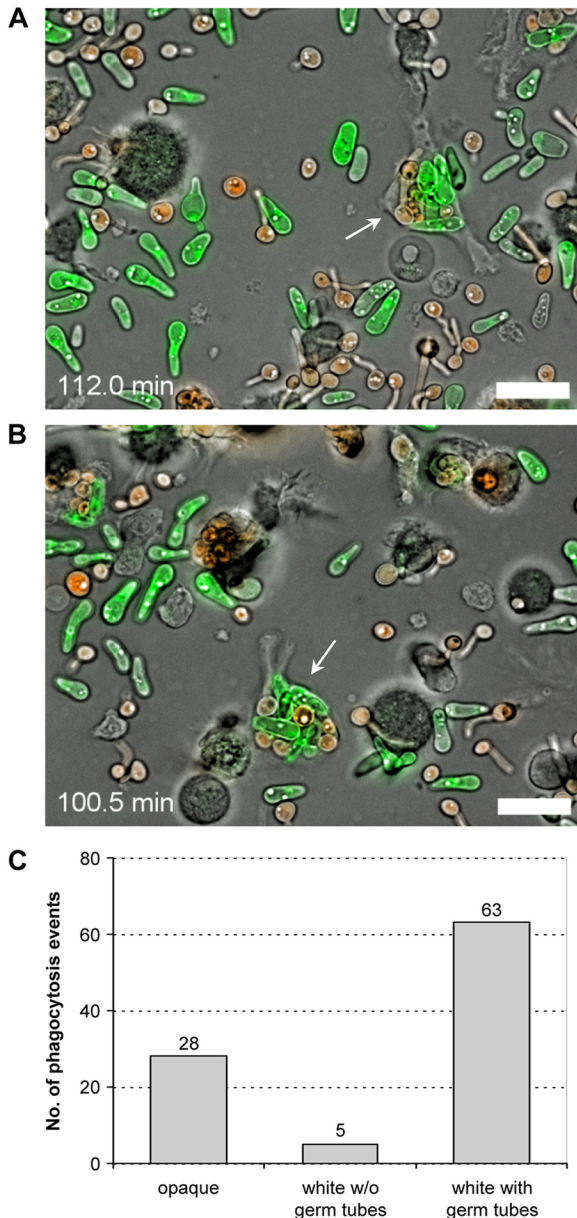


FIG 5 Dendritic cells phagocytose both white and opaque cells. (A, B) The pictures are from two biological replicates of one experiment (see Videos S5 and S6 in the supplemental material; time points are indicated) in which DCs were coincubated on glass slides with a mixture of *RFP*-expressing white cells and *GFP*-expressing opaque cells of strain WOP4G42WH11R22A. The arrows indicate DCs that have taken up several white and opaque cells. Bars, 20 μ m. (C) Frequency of phagocytosis of opaque cells and white cells with and without a germ tube. A total of 96 phagocytosis events by 21 DCs were analyzed in the experiment described above.

monly assumed that white-opaque switching has evolved in *C. albicans* as an adaptation to the specific requirements of life in human and warm-blooded animal hosts. The phase-specific expression of adhesins and metabolic genes may allow opaque cells an efficient colonization of host niches to which white cells are less well adapted and where mating may occur. Previous studies have also indicated that white-opaque switching affects the interaction of *C. albicans* with the host immune system. It has been shown that

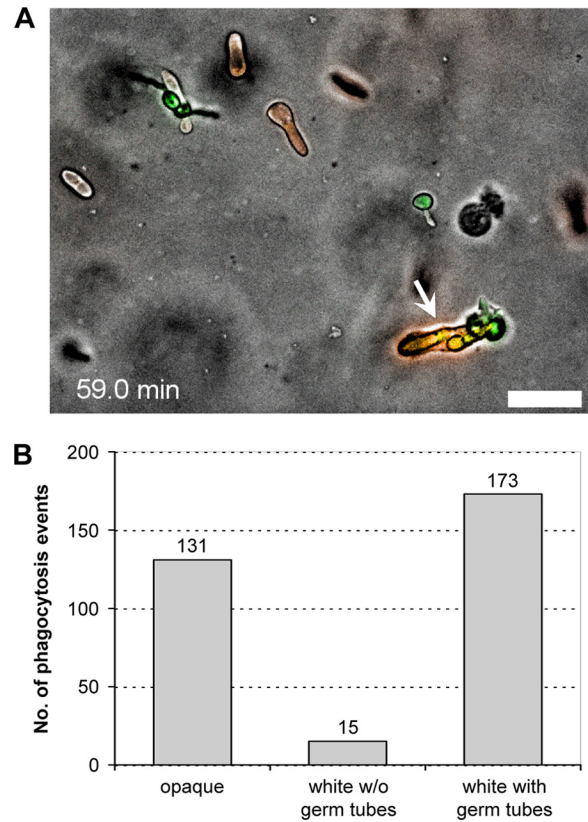


FIG 6 Neutrophils phagocytose both white and opaque cells upon coincubation in a collagen matrix. (A) The picture was taken from Video S7 in the supplemental material at the indicated time point. The arrow shows a neutrophil that has taken up two *GFP*-expressing white cells and three *RFP*-expressing opaque cells of strain WOP4R22WH11G22B. Bar, 20 μ m. (B) Frequency of phagocytosis of opaque cells and white cells with and without a germ tube. A total of 319 phagocytosis events by 274 neutrophils were analyzed. Data are from four independent experiments with two biological replicates.

opaque cells are less efficiently phagocytosed than white cells by *Drosophila* S2 cells and mouse macrophages (20), suggesting that switching to the opaque phase may be an immune evasion mechanism. In humans, neutrophils play a major role in the prevention of *Candida* infections. The finding that opaque cells do not produce a chemoattractant for neutrophils that is secreted by white cells pointed to the possibility that opaque cells can also avoid detection by these important first-line host defense cells (19). However, earlier work had shown that opaque cells are in fact more susceptible than white cells to killing by neutrophils and also stimulate the production of reactive oxygen species, which is one mechanism by which these phagocytes destroy invading pathogens, more strongly than do white cells (17). The findings of our present study may explain these conflicting results, and they also provide more detailed insight into the differential interaction of the various morphological forms of *C. albicans* with cells of the host immune system. Similar to the observations made with *Drosophila* S2 cells and mouse macrophages (20), we found that neutrophils selectively phagocytosed white cells and largely ignored opaque cells when coincubated with both cell types on a glass slide. In contrast, Kolotila and Diamond reported that white and opaque cells were phagocytosed with equal efficiency by neutrophils, and opaque cells were even more efficiently killed than white

cells because of their increased susceptibility to reactive oxygen species (17). Of note, these authors used opsonized white and opaque cells that were incubated with PMNs for 1 h at 37°C in tubes under shaking. Opsonization may facilitate recognition of opaque cells by neutrophils, and we indeed also found increased phagocytosis of opaque cells when the experiments were performed in culture medium containing active FCS instead of heat-inactivated FCS, although germinating white cells were still much more efficiently phagocytosed (91%) than opaque cells (7%) and nongerminating white yeast cells (2%) (unpublished data). In addition, coincubation under shaking, as was done in the experiments by Kolotila and Diamond (17), may have altered the behavior of the neutrophils compared to their behavior in our experiments on glass slides, similar to the different results that we obtained when neutrophils and *C. albicans* cells were coincubated within a collagen matrix. Therefore, the environment plays a major role in the recognition of the different morphologies of *C. albicans*. Although both the surface of a glass slide and a collagen matrix are artificial environments, similar environmental differences (e.g., between mucosal surfaces and deep tissue) are likely to exist *in vivo*, and switching to the opaque phase may allow immune evasion in some, but not all, host niches. Our results also demonstrate that opaque cells cannot avoid recognition by all types of phagocytic cells. In contrast to human neutrophils, *Drosophila* S2 cells, and mouse macrophages, DCs efficiently phagocytosed both white and opaque cells.

An important finding of our study was that during the coincubation on glass slides, white cells were attacked by the neutrophils only after they had started to germinate, indicating that under these conditions the neutrophils recognized a structure that is selectively expressed on the hyphal form of *C. albicans*. Phagocytes engage multiple receptors that bind different molecules on the fungal surface (35). Apparently, the involvement of specific receptors depends on the environment, because opaque cells were not recognized on a glass surface but efficiently phagocytosed when encountered within a collagen matrix. In line with these observations, it was previously found that carboxyfluorescein succinimidyl ester-labeled, nongerminating *C. albicans* yeast cells were inefficiently phagocytosed by PMNs and a peritoneal macrophage-derived cell line on a glass surface but efficiently phagocytosed within a collagen matrix, while the reverse was true for *A. fumigatus* conidia (31). Our results are also in agreement with those of a previous report in which the interactions of yeast and filamentous forms of *C. albicans* with neutrophils were compared (36). In that study, *C. albicans* filaments, but not yeast cells, induced the targeted motility of human neutrophils, and the neutrophils engulfed only filaments and germ tubes but did not phagocytose yeast cells. However, neutrophils can also kill *C. albicans* by extracellular (neutrophil extracellular trap formation) mechanisms, and another study found that hyphae and yeast cells were killed with equal efficiency (37).

An unresolved question is which hypha-specific structure is recognized by the neutrophils under conditions in which they do not phagocytose opaque cells and nongerminating yeast cells. An attractive candidate is the pH-regulated antigen Pra1, which is predominantly expressed in a highly glycosylated form on the surface of *C. albicans* hyphae, but not yeast cells, and bound by the $\alpha_M\beta_2$ receptor on PMNs. Pra1 also acts as a chemoattractant for PMNs, and deletion of *PRA1* decreased PMN migration and PMN adhesion to and killing of *C. albicans* (38, 39). It has also been

reported that dendritic cells can recognize *C. albicans* by an $\alpha_M\beta_2$ -independent mechanism (39), which would explain our finding that dendritic cells phagocytosed opaque cells under conditions in which they were not attacked by neutrophils. However, forced expression of *PRA1* in opaque cells did not result in enhanced phagocytosis by neutrophils (our unpublished data), although we cannot exclude the possibility that Pra1 was not properly expressed on the surface of the transformed opaque cells. Hyphae express many other cell surface proteins that are not found on yeast cells. One of these, Hyr1, confers even increased resistance to killing by neutrophils and other phagocytes (40, 41). Therefore, it remains to be established which hypha-specific antigen(s) and corresponding receptor(s) on neutrophils are responsible for the selective phagocytosis of germinating *C. albicans*.

Another open question is why the neutrophils phagocytosed opaque cells within the three-dimensional environment of the collagen matrix but not on the two-dimensional glass surface. Previous experiments have shown that the change in behavior of the neutrophils is immediate; i.e., after leaving the 3D environment, neutrophils immediately change into 2D mode (31). This would be too fast to explain it by any significant change in surface composition. We speculate that the altered behavior of the neutrophils is dependent on the sensing of the 3D environment, which has been shown to influence cell physiology (42, 43). On the other hand, it is also possible that a surface structure that is recognized by the neutrophils may be specifically presented on opaque cells within the 3D environment.

The switch to hyphal growth is a major virulence determinant of *C. albicans* that facilitates tissue invasion. As opaque cells do not undergo filamentation under most conditions that induce hypha formation in white cells, it is tempting to speculate that switching to the opaque form may render *C. albicans* less aggressive toward its host but at the same time allow escape from the host immune system in certain host niches, where mating and genetic exchange may then occur undisturbed.

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