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Cryptococcus neoformans Host Adaptation: Toward Biological Evidence of Dormancy

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ABSTRACT Cryptococcosis is an opportunistic infection due to the ubiquitous yeast Cryptococcus neoformans. This yeast interacts closely with innate immune cells, leading to various fates, including fungal persistence within cells, making possible the dissemination of the yeast cells with monocytes via a Trojan horse strategy. In humans, the natural history of the infection begins with primoinfection during childhood, which is followed by dormancy and, in some individuals, reactivation upon immunosuppression. To address the question of dormancy, we studied C. neoformans infection at the macrophage level (in vitro H99-macrophage interaction) and at the organ level in a murine model of cryptococcosis. We analyzed the diversity of yeast adaptation to the host by characterizing several C. neoformans populations with new assays based on flow cytometry (quantitative flow cytometry, multispectral imaging flow cytometry, sorting), microscopy (dynamic imaging), and gene expression analysis. On the basis of parameters of multiplication and stress response, various populations of yeast cells were observed over time in vivo and in vitro. Cell sorting allowed the identification of a subpopulation that was less prone to grow under standard conditions than the other populations, with growth enhanced by the addition of serum. Gene expression analysis revealed that this population had specific metabolic characteristics that could reflect dormancy. Our data suggest that dormant yeast cells could exist in vitro and in vivo. C. neoformans exhibits a huge plasticity and adaptation to hosts that deserves further study. In vitro generation of dormant cells is now the main challenge to overcome the limited number of yeast cells recovered in our models.

IMPORTANCE Cryptococcus neoformans is a sugar-coated unicellular fungus that interacts closely with various cells and organisms, including amoebas, nematodes, and immune cells of mammals. This yeast is able to proliferate and survive in the intracellular environment. C. neoformans causes cryptococcosis, and yeast dormancy in humans has been suggested on the basis of epidemiological evidence obtained years ago. By studying an in vitro model of yeast-macrophage interaction and murine models of cryptococcosis, we observed that yeast cells evolve in heterogeneous populations during infection on the basis of global metabolic activity. We compared the growth ability and gene expression of yeast cells belonging to various populations in those two models. We eventually found a population of yeast cells with low metabolism that fit some of the criteria for dormant cells. This paves the way for further characterization of dormancy in C. neoformans.

Cryptococcus neoformans is a basidiomycetous opportunistic yeast present in the environment. This fungus can survive predation by various organisms, ranging from protozoans to metazoans, through ready-made virulence traits (1). C. neoformans interacts closely with unicellular organisms (amoebas, paramecia) (2–4) and with cells dedicated to the innate immune response (macrophages or dendritic cells) exhibiting various propensities of phagocytosis and intracellular killing (5, 6). Intracellular persistence in immune cells provides advantages to the fungus by allowing escape of the immune response and subsequent dissemination (7, 8). In humans, contamination usually begins with the inhalation of basidiospores or desiccated yeast cells (9). Primoinfection occurs in childhood with unrecognized pneumonia (10). Yeast cells are then controlled by the immune system through granuloma formation (11) and adaptive immunity with production of antibodies against C. neoformans proteins (10). From then on, until immunosuppression occurs, the yeast cells remain invisible and dormant, as demonstrated epidemiologically (12, 13), and the infection is latent (14). When or if immunodeficiency occurs, yeast cells reactivate and replicate probably in the lung and disseminate through the bloodstream to different organs. Crossing of the blood-brain barrier leads to meningocencephalitis, the most common and severe clinical presentation (9, 15–17).

The pathophysiology of the infection has been studied experimentally in rats that naturally control C. neoformans lung infection and more extensively in mice that are highly susceptible to infection. The fate of the infection depends on both host and microbial factors. Virulence factors influence the outcome of infection in the murine model of cryptococcosis (18) but also in vitro...
and in humans (19). Microbial adaptation to the host is complex and has been studied as a whole in the lungs by histopathology (20) and by transcriptome analysis upon early infection (21). More recently, a particular adaptation to hosts was uncovered with the formation of titan cells in the lungs of infected mice (22, 23). These extraordinary enlarged yeast cells have specific properties that promote disease by preventing phagocytosis and clearance from the lung (24). However, we still do not know whether they really play a role in the persistence of the infection in vivo. Although extensive studies of C. neoformans-macrophage interactions and cryptococcosis have been performed with mice, no tangible biological evidence of dormancy has been brought to light. Our hypothesis is that populations of yeast cells with various metabolic states coexist in tissues after infection, with some yeast cells being dormant. We took advantage of assays and models previously implemented in the laboratory (19, 25, 26) to test this hypothesis and developed tools to analyze yeast cells at the single-cell level or after sorting of a low number of specific cell populations. In the lungs of infected mice and upon macrophage interaction, we uncovered a subpopulation of yeast cells exhibiting low metabolic activity and delayed growth rescued by the addition of serum that fit the criteria of a dormant phenotype.

**RESULTS**

Implementation of a new flow cytometry assay to analyze multiplication and stress responses simultaneously in *C. neoformans*. To study *C. neoformans* adaptation to different environments, we first developed an assay that combined multiplication and stress response analyses.

Multiplication was quantified on the basis of the decrease in calcofluor (CALCO) fluorescence intensity over time with the appearance of a population with a lower CALCO fluorescence intensity (CALCO<sub>med</sub>−<sub>low</sub>; daughter cells) and the persistence of the highly stained population (CALCO<sub>high</sub>; mother cells) (19). The proportion of daughter cells increased over time in standard yeast extract-peptone-dextrose (YPD) cultures, inside J774 cells, and in the lungs of infected mice (Fig. 1A).

The stress response was evaluated with 5-chloromethyl-1fluorescein diacetate (CMFDA), which measured a glutathione-dependent phenomenon, as shown by a preliminary experiment with diethyl maleate, a glutathione-depleting agent (see Fig. S1A in the supplemental material). *C. neoformans* cells in stationary phase (baseline, H0) harbored basal fluorescence that increased over time with various stresses (water, hydrogen peroxide, fresh medium, and heat) (Fig. 1B). Of note, after 24 h of incubation in hydrogen peroxide, the CMFDA fluorescence level was comparable to that of unstained condition (Fig. 1B, dark green histogram, right upper panel), with culture revealing that all of the yeast cells were dead (data not shown). The level of CMFDA fluorescence depended on the stimulus, with yeast nitrogen base (YNB) medium reducing the lowest and hydrogen peroxide the highest stress response (Fig. 1B; see Fig. S1B).

The viability of yeast cells was assessed on the basis of Topro-3 iodide (TOPRO) fluorescence intensity (Fig. 1C), allowing exclusion of dead cells in all subsequent experiments.

**Dynamics of *C. neoformans* host adaptation.** We studied yeast cell adaptation to host in terms of yeast multiplication and stress responses, looking for changes over time in vitro (J774 macrophages) and in vivo (lungs of mice) (Fig. 2).

A population of daughter yeast cells was observed as soon as 2 h after interaction with J774 cells and 15 h in lungs (Fig. 2A and C) and increased over time (Fig. 2B and D). An increase in CMFDA fluorescence intensity was observed in *C. neoformans* cells after 2 h of interaction with J774 cells and after 7 h in the lungs of infected mice, with the majority of the population remaining CMFDA<sub>high</sub> over time. However, a subpopulation with a lower CMFDA fluorescence level (CMFDA<sub>med</sub>−<sub>low</sub>, black arrows) appeared at 24 h in J774 cells and at 30 h in lungs and increased over time. This phenomenon affected first mother cells (24 h) and then daughter cells (48 h) in J774 cells and both populations at 30 h in lungs.

Subsequent experiments were done with mice inoculated 7 days before, on the basis of the assumption that dormant cells should already be present and in sufficient number for further analysis. *C. neoformans* multiplication and stress responses were then assessed in the brains, lungs and spleens of 6 OF1 mice (Fig. 3) and analyzed individually by classical flow cytometry (Macsquant analyser; Miltenyi Biotec). Mother cells were not observed in the brains, were rare in the spleens, and were present in the lungs of 4/6 OF1 mice (Fig. 3, gated population). The stress response pattern differed among the lungs, spleens, and brains but looked specific for each organ with several circumscribed populations in the lungs of 5/6 mice. Of note, under the same conditions, no mother cell population was detected in the organs of BALB/c mice; this is potentially related to their more acute infection (data not shown).

We thus decided to explore morphological and biological features of the viable yeast cells present in the lungs of infected mice by multispectral imaging flow cytometry (Imagestream<sup>®</sup> and pooled lung homogenates (Fig. 4 and 5; see Fig. S2 to S5 in the supplemental material). After determination of the analysis strategy (see Fig. S2), including an optical control, we defined nine populations of yeast cells (CALCO high, medium, and low) with various (high, medium, and low) levels of CMFDA fluorescence intensity on the CMFDA/CALCO dot plot (Fig. 4A and B; see Fig. S3A and B). Daughter cells always represented less than 20% of the three CMFDA populations. Mother cells represented more than half of the CMFDA<sub>high</sub> population and less than 40% of the others (Fig. 4B). The global analysis of morphological features (54 algorithms) revealed five clusters composed mainly of daughter cells, mother cells, CMFDA<sub>high</sub>, CMFDA<sub>med</sub>, and CMFDA<sub>low</sub> populations (Fig. 4C). The largest yeast cells were composed mostly of mother cells (Fig. 4C, cluster h, red outline), but a subpopulation of mother cells was small yeast cells (see Fig. S3C and D). Daughter cells were composed mostly of small yeast cells (Fig. 4C, cluster h, pink outline) and yeast cells with a specific morphology (Fig. 4C, cluster b, red outline). Apart from mother and daughter cells, CMFDA<sub>low</sub> populations harbored specific morphological features (Fig. 4C, cluster c, red outline).

**Analysis of specific *C. neoformans* populations.** To go further into the analysis of the different populations, we focused first on the CMFDA<sub>high</sub>/mother cell population (Fig. 5). Two patterns of CMFDA fluorescence were visualized by Imagestream<sup>®</sup> (Fig. 5A) and microscopy (Fig. 5B), with some cells with peripheral extra-cellular fluorescence (designated “surrounded”) and others intracellular fluorescence (“intracellular”). These two populations were delineated on the basis of the modulation (texture) and the area (size) dot plot algorithms (Fig. 5C).

The majority of mother cells were found to have intracellular CMFDA. Yeast cells with a surrounded CMFDA were composed of similar proportions of mother and daughter cells (see Fig. S4 in the supplemental material), and some yeast cells exhibited a pe-
cular morphology by Imagestream X (Fig. 5B) and interference contrast microscopy (Fig. 6A). These cells (Drop Cn in Fig. 6) were small (5.80 ± 0.80 μm) with an enlarged cell wall and a well-defined round refringent vesicle. This population was composed of a higher proportion of mother cells than C. neoformans cells with a regular morphology (Reg Cn in Fig. 6) (see Fig. S4B). Drop C. neoformans cells represented less than 25% of each CMFDA population (Fig. S4C). Compared to regular C. neoformans cells, drop C. neoformans did not harbor a well-organized nucleus (4′,6-diamidino-2-phenylindole [DAPI] staining), had a low RNA content (SYTO 85) and mitochondrial activity (Mito-tracker) and exhibited a complete retraction of the cytoplasm around the central vesicle (MDY64, Fig. 6B and C). These observations suggested that drop C. neoformans were dead yeast cells with low transcriptional and mitochondrial activity, and disorganized cytoplasm and nucleus architectures, despite having been initially selected in the TOPROlow (viable) population. This was confirmed by dynamic imaging after observation that drop C. neoformans cells from lung homogenates were unable to bud over 48 h (data not shown).

Additional experiments showed that the relative proportion of the different yeast cell populations in terms of multiplication and stress response (see Fig. S5A to D), and the proportion of drop C. neoformans (see Fig. S5A) varied among C. neoformans strains, when comparing H99 and two previously characterized clinical isolates (19).
Specific phenotype of the CMFDAQlow population. Cell sorting was implemented to focus on and compare the CMFDAQlow population to the other populations recovered from both models of host interaction (24-h incubation with macrophages or lungs recovered on day 7 after inoculation of OF1 mice) and to monitor yeast cells in stationary phase all stained under the same conditions (Fig. 7) and monitored visually by fluorescence microscopy (see Fig. S6 in the supplemental material). We wondered whether the sorted populations harbored specific phenotypes in terms of growth capacity and transcriptional activity.

Growth curves analysis revealed important differences among the various populations. Indeed, the CMFDAQlow population was unable to grow when recovered from mice (mice_CMFDAQlow) or exhibited delayed growth when recovered from macrophages (macrophage_CMFDAQlow) in YPD compared to that of other populations and control yeast cells (Fig. 7). Addition of fetal calf serum (FCS) restored growth for the population of mice_CMFDAQlow and markedly accelerated growth of all of the populations, including that of control yeast cells. Of note, the growth of the CMFDAQmed and CMFDAQhigh populations was delayed in mice, but not in macrophages, compared to that of control yeast cells (Fig. 7B and D), while both populations behaved similarly under each condition (macrophages or mice).

The expression of 37 selected genes in the above-described populations was performed by real-time quantitative PCR (Fig. 8). Hierarchical clustering of yeast cell populations revealed that the CMFDAQmed and CMFDAQhigh populations were closely related under both the macrophage and mouse conditions. Overall, four groups/populations were delineated: mice_CMFDAQhigh/med, macrophage_CMFDAQhigh/med, mice_CMFDAQlow, and macrophage_CMFDAQlow. Of note, the CMFDAQhigh population clustered with control yeast cells (data not shown). Hierarchical clustering of genes revealed four clusters, with two clusters of particular interest, cluster 1 (C1) and C4 (Fig. 8; see Fig. S7 in the supplemental material). C1 was composed of genes known to be upregulated in the lungs during early infection (21). C4 included genes that were expressed more in the macrophage_CMFDAQlow population. Finally, PCK1 downregulation and COX1 upregulation were observed only in the mice and macrophage_CMFDAQlow population.

DISCUSSION

Rapid overview of the published literature on C. neoformans and cryptococcosis clearly shows the complexity and the diversity of the host-yeast interplay. On the host side, a number of organisms are potentially exposed to C. neoformans (27, 28), variable susceptibility to infection (29, 30) and clinical presentation (31) are reported in humans, and differences in the interaction between yeast and immune cells are well established (5, 32, 33). On the fungal side, the diversity is also noticeable on several levels: genome (34), genotypes (35), capsule structure (36, 37), production of other virulence factors (38, 39), interaction with hosts cells (19, 40, 41), virulence in mice (19, 41), and microevolution during infection (5, 42, 43). Fungal adaptation to specific host environments has also been demonstrated (21, 25, 44, 45) without a focus on specific subpopulations of yeast cells, except for the titan cells that have been initially reported in the lungs of infected mice (22, 23).

There is no consensus on the definition of dormancy/quiescence. Most often, dormant cells are characterized by a low met-
abolic activity sometimes undetectable under normal laboratory conditions (46) and the ability to be resuscitated by adequate stimuli (47). Dormancy has already been described in a wide range of organisms, including bacteria (46) (Bacillus spp., Clostridium difficile, Chlamydia spp., Vibrio spp., Pseudomonas spp.), mycobacteria (Mycobacterium tuberculosis [47–49]), apicomplexan parasites (in particular, Plasmodium spp. [50]), and fungi (Saccharomyces cerevisiae [51], Schizosaccharomyces pombe [52], Aspergillus fumigatus [53], or Neurospora crassa [54] spores). In C. neoformans, dormancy has only been demonstrated epidemiologically (12) and evoked for basidiospores (55).

We hypothesized that metabolically different subpopulations of yeast cells coexist in organs upon infection with the ambition to identify a population of yeast cells with low metabolic activity through a combination of selected reagents and techniques (dyes respecting yeast viability, multispectral imaging flow cytometry, dynamic imaging, single-cell real-time quantitative PCR protocols, cell sorting, growth curves).

The approach used was first to quantify yeast cell multiplication and stress response on the basis of CALCO and CMFDA staining, respectively. CALCO staining was used to track mother cells (CALCOhigh, i.e., those that were inoculated), allowing us to compare stress responses in mother and daughter cells (19). CMFDA has previously been used in mammalian and fungal cells to quantify glutathione (56–58) or as a long-term cell tracker since it preserves viability (59). Glutathione is a nonenzymatic defense against oxidative stress. We showed here that C. neoformans responded to various stresses by increasing glutathione content. Exposure to protracted or violent stress (hydrogen peroxide) emptied the pool of glutathione in C. neoformans, as it does in S. cerevisiae (60, 61).

An intriguing observation was the surrounded and not cytoplasmic pattern of CMFDA fluorescence associated with yeast cells with a specific morphology (drop C. neoformans) in the lungs. The peripheral extracellular staining could be related to either excretion of glutathione or diffusion of the host’s glutathione during infection or sample processing. However, the use of various dyes evaluating metabolic activity and cell architecture, as well as dynamic imaging, strongly suggested that drop C. neoformans cells were mostly dead mother cells. This conclusion does not contradict the negative TOPRO staining, since negative DAPI and SYTO 85 staining suggested complete loss of nucleic acids in the drop C. neoformans. It is noteworthy that yeast cells looking like drop C. neoformans cells are brought out as typical cells of C. neoformans in many publications.

In addition to differences in morphology, analysis of multiplication and stress responses revealed the coexistence of several subpopulations of yeast cells that evolved over time in the host. The proportions of the various cell populations also varied with the host (OF1 versus BALB/c), tissue (lung versus brain), model (in vitro versus in vivo), and C. neoformans strain (H99 versus well-characterized clinical isolates). This clearly underscores the fact that findings on global C. neoformans adaptation to various environments should be interpreted with caution.

Sorting of the various populations of yeast cells may thus help in understanding C. neoformans biology in the host. The commonly accepted pathophysiology is that most cases of cryptococcosis result from the reactivation of dormant yeast cells without

**FIG 3** C. neoformans stress response and multiplication depends on individuals (outbred OF1 mice) and tissues. Seven days after inoculation with 10^5 CALCO-stained yeast cells (H99), mice were sacrificed and their organs (brains, lungs, and spleens) were ground. Yeast cells were stained for stress response and viability by CMFDA/TOPRO assay. Flow cytometry analysis was performed after exclusion of the TOPROhigh population (dead yeast cells) (MacsQuant analyzer; Miltenyi Biotec). The C. neoformans brain and spleen profile was homogeneous in terms of multiplication and stress response, whereas two profiles were observed in lungs. A well-defined CALCOhigh/CMFDAhigh population (black gate) was observed in some mice (M1 and M2, M3, and M5) and not in others (M4 and M6).
precise knowledge on their site of residence. We focused here on the lung because it was the only body site where well-delineated populations composed of enough yeast cells to allow further analysis were observed.

We assumed that dormant cells would be metabolically poorly active. The population that met this criterion was the CMFDAlow population that included less than 20% of the dead yeast (C. neoformans) cells. The delayed or absent growth of the CMFDAlow population undergoing serum starvation (YPD) was partially rescued by the addition of FCS, a phenotype described for dormant cells of various origins (mammalian fibroblasts, mammalian cancer cells, stem cells, bacteria, and S. cerevisiae) (62).

This suggested that the CMFDAlow population could be composed predominantly of dormant cells.

Zaragoza and Nielsen evoked the possible role of their titan cells in dormancy (63). It is interesting that, in our studies, the biggest cells corresponding most probably to titan cells were composed almost exclusively of mother cells in the lungs (see Fig. S3D in the supplemental material), confirming data obtained from in vitro culture (23). Titan cells are composed of the three CMFDA populations, thus suggesting that some of them (CMFDAlow) could be dormant. The original report on the multiplication of titan cells under standard culture conditions (Sabouraud agar plates) without the need for additional growth factors (23) does not contradict this finding since the titan cells were not sorted on CMFDA fluorescence.

The limited number of cells after sorting (Table 1) led us to implement a transcriptional gene analysis based on the adaptation of single-cell PCR assays. We were not able to analyze the expression of all of the genes in the mice_CMFDAlow cells under standard culture conditions (Sabouraud agar plates) without the need for additional growth factors (23) does not contradict this finding since the titan cells were not sorted on CMFDA fluorescence.

The limited number of cells after sorting (Table 1) led us to implement a transcriptional gene analysis based on the adaptation of single-cell PCR assays. We were not able to analyze the expression of all of the genes in the mice_CMFDAlow cells because of the limited number of cells recovered from lungs. However, the finding that a cluster of genes (C1, Fig. 8) known to be upregulated during lung infection (21) was upregulated only in yeast cells recovered from lungs (mice_CMFDAmid, mice_CMFDAhigh) was a means to validate the technical and biological approach. The major difference between the CMFDApopulations and the other populations, whatever the condition, was modulated expression of the PCK1 (decrease) and COX1 (increase) genes. The phosphoenolpyruvate carboxykinase 1 (Pck1) protein plays a major role in gluconeogenesis, which is known to impact yeast cell sur-

![Multispectral imaging flow cytometry](image_url)
vival (64). Decreased PCK1 expression (macrophage_CMFDAlow and mice_CMFDAlow) was associated with increased expression of ATG9 and VPS13 (genes involved in autophagy [65, 66]) (macrophage_CMFDAlow), suggesting that the CMFDAlow population may not need activation of the gluconeogenesis pathway and could use autophagy to survive starvation. Toffaletti et al. suggested that COX1 (encoding mitochondrial cytochrome c oxidase subunit 1) was upregulated during the stress response induced by high temperature (39°C) (67) and differentially expressed in well-characterized clinical isolates in correlation with intramacrophagic proliferation (19). In C. neoformans, mutations in various mitochondrial genes affect the respiration rate, the response to oxidative stresses, and the ability to survive low-oxygen conditions (68, 69), and this independently of glutathione activity. In C. gattii, enhanced mitochondrial activity and modifications of mitochondrial morphology were found to be related to an increased proliferation rate and increased virulence (41, 70). COX1 upregulation in the CMFDA	extsuperscript{low} population (glutathione-dependent stress response) could reflect increased mitochondrial activity that could contribute to maintenance of viability in hostile environments.

Finally, the proof of concept described herein needs to be translated to in vitro assays to generate large quantities of these low stress responder yeast cells. A basic description of dormancy would then be possible.

**MATERIALS AND METHODS**

**C. neoformans strains and cell line.** C. neoformans strain H99 (serotype A, MATa, haploid; kindly donated by J. Heitman, Duke University, Durham, NC) was used as the reference in all of our experiments (71). Two C. neoformans clinical isolates (AD1-83a and AD1-07a, serotype A, MATa/H9251) were selected on the basis of their virulence characteristics (19). They were previously recovered from the cerebrospinal fluid of patients enrolled in the CryptoA/D study (31) and were responsible for single infections (one isolate/one genotype/one infection). Before each experiment, yeast cells were first cultured on Sabouraud agar medium and then subcultured in 10 ml of liquid YPD at 30°C at 150 rpm for 22 h (standard YPD culture). The J774.16 cell line (J774; American Type Culture Collection) was used to study the interaction of C. neoformans isolates with macrophages. Cells were maintained at 37°C in the presence of 5% CO	extsubscript{2} in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (fresh medium) (all from Invitrogen). Cells were used between 10 and 35 passages.

**Reagents and C. neoformans labeling.** The white dye CALCO (Fluorescent brightener 28; Sigma) was used to monitor yeast multiplication. Staining was performed before incubation with macrophages (19) or inoculation into mice. TOPRO (Invitrogen) diluted to 10 μM in phosphate-buffered saline (PBS) was used to stain nucleic acids of cells with a disrupted membrane (dead yeast cells) and then analyzed in the allophycocyanin (APC) channel. CMFDA (CellTracker Green CMFDA; Life Technologies) is a dye that needs two enzymatic steps and glutathione to acquire fluorescence properties (fluorescein isothiocyanate [FITC]).

**FIG 5** Morphological and fluorescence features of the Calcoh/CMFDAhigh H99 population in the lungs of outbred OF1 mice. (A) Multispectral imaging flow cytometry (ImagestreamX; Amnis) was used to picture each event of the CALCO/CMFDAOH population in five channels: BF (transmitted light), yellow (Cy3, capsule), green (CMFDA, stress response), blue (CALCO, multiplication), and red (TOPRO, viability). Different patterns of morphology and CMFDA fluorescence were observed. (B) The CALCO/CMFDAhigh population of yeast cells from lung homogenates was also observed by classical fluorescence microscopy. CMFDA fluorescence was observed surrounding the cell wall CMFDAOH (white dotted arrows in panel A), or within the yeast cell cytoplasm (CMFDAOH, white arrows in panel A). (C) Multispectral imaging flow cytometry based on the modulation algorithm (texture) and the area (size) of the yeast cells easily discriminated CMFDAOH and CMFDAOH cells. The CMFDAOH yeast cells were composed of regular and drop C. neoformans cells.
FIG 6 Morphological features and metabolic activity revealed that the drop *C. neoformans* (Drop Cn) cells in the lungs of outbred OF1 mice were dead yeast cells. Yeast cells from lung homogenates were observed by interference contrast microscopy. (A) Examples of cells composing a subpopulation of yeast cells with a typical morphology (small size \([3.80 \pm 0.80 \mu m, n = 12]\), thick cell wall, and one well-defined round refringent vesicle) are shown. (B) Nucleus morphology (DAPI, blue), RNA content (SYTO85, orange), and mitochondrial activity (Mitotracker, orange) were assessed in yeast cells previously stained with CMFDA (green). Drop *C. neoformans* cells were devoid of nucleus (regular shape and regular DAPI fluorescence), RNA, and mitochondrial activities, in contrast to regular *C. neoformans* (Reg Cn) cells. (C) Lipid membrane layers (MDY64, green) were assessed in unstained yeast cells. Drop *C. neoformans* cells harbored a complete retraction of the cytoplasm around the central refringent vesicle.

channel). It is commonly used to quantify glutathione in mammalian cells (56, 57) and has been employed for long-term tracking of *C. neoformans* in vivo (59). The stock solution in anhydrous dimethyl sulfoxide was diluted to 10 \(\mu M\) in PBS. Preliminary experiments showed that combining TOPRO and CMFDA for 30 min of incubation at 37°C without agitation was efficient at *C. neoformans* concentrations ranging from 10^6 to 10^7 cells/ml and in the presence of tissue debris. CALCO staining was always performed with living cells prior to the use of all other stimuli, whereas the CMFDA/TOPRO assay followed them. The various dyes did not alter yeast cell viability (data not shown).

In specific experiments, yeast cells were subjected in vitro to various stimuli to test the glutathione-mediated stress response, washed twice, and then subjected to the CMFDA/TOPRO assay at different time points. The stimuli included (i) pure water; (ii) hydrogen peroxide (Sigma) adjusted to 2 mM in water (72); (iii) fresh medium or YNB, which represents a nonoptimal culture medium for yeast cells on the basis of preliminary experiments (data not shown); (iv) heating at 65°C for 2 h, which killed 99.9% of the yeast cells. All stimuli were tested in duplicate. Diethyl malate (Sigma) adjusted to 100 \(\mu M\) in sterile water was used to deplete the *C. neoformans* glutathione stock (56) and assess glutathione-dependent CMFDA staining.

E1, a murine IgG1 anti-capsular polysaccharide monoclonal antibody (MAB) was used as an opsonin (73). Polyclonal rabbit anti-capsular antibodies together with cyanine 3 (Cy3) or R-phycocerythrin (PE)-labeled goat anti-rabbit IgG (anti-IgG–Cy3 or anti-IgG–PE) (Invitrogen) were used to stain the capsule of *C. neoformans* at 1:100 for 30 min of incubation. The polyclonal sera were obtained by a method of immunization of rabbits with whole heat-killed *C. neoformans* cells adapted from published procedures (74). Additional dyes were used as follows to characterize metabolic activities or yeast cell organization: (i) mitochondrial activity with Mitotracker Orange CMTMros (Invitrogen) at 40 nM (30 min of incubation at 37°C) (41), (ii) RNA content with SYTO85 (Invitrogen) at 5 \(\mu M\) (30 min of incubation at 37°C), (iii) lipid membrane layer staining with MDY64 (Invitrogen) at 10 \(\mu M\) (3 min of incubation at room temperature [RT]) (23), and (iv) nucleus morphology with DAPI (Invitrogen) at 1:5,000 (5 min of incubation at RT) (23).

**In vitro and in vivo models to study yeast host adaptation.** (i) Fungal cell isolation after *C. neoformans*-macrophage interaction. J774 cell suspension (10^6 cells in fresh medium per well of a 24-well culture plate) were incubated at 37°C in 5% CO\(_2\), for 48 h. On the day of the experiment, an E1 MAB- and CALCO-stained *C. neoformans* suspensions, both in fresh medium at the desired concentrations, were added to the J774 cell monolayer and incubated at 37°C and 5% CO\(_2\) for 2 h (*C. neoformans*/J774 ratio of 5:1). Nonadherent extracellular yeast cells were then removed by washings with PBS, and incubation was stopped (iH2) or prolonged for 24 h (iH24). At the end of the incubation time, nonadherent yeast cells were removed by washing with PBS and intracellular yeast cells were recovered with a cell scraper and 0.05% sodium dodecyl sulfate (Sigma) in ice-cold RNase-free water (Invitrogen) (lysis solution) over the macrophage layer. The yeast cell suspension was then centrifuged and washed twice with PBS.

(ii) Fungal cell isolation from organs of infected mice. Experimental infections were performed with 6- to 8-week-old outbred OF1 (Charles Rivers Laboratories) or BALB/c (Janvier Labs) male mice.

The inoculum was prepared in sterile saline from CALCO-stained yeast cells after standard YPD culture. A 10^6-cell/ml *C. neoformans* suspension was inoculated intravenously (10^6 cells/mouse). For specific experiments, mice were sacrificed at early time points (30 min and 7, 15, and...
microscopy techniques. Interferential contrast microscopy (DMI LB2 microscope; Leica) and epifluorescence microscopy (Axio Scan; Carl Zeiss) were used to analyze the morphology and fluorescence features of yeast cells recovered from lung homogenates before and after staining and pictured with an Axiocam MRm camera (Carl Zeiss).

Yeast multiplication was assessed by dynamic imaging (Nikon Bioscience). Series of images were taken by phase contrast microscopy at 5-min intervals and fluorescence microscopy (DAPI filter) every 10 min for 48 h at ×40 magnification.

(ii) 
**Classical flow cytometry analysis.** To better characterize yeast cell populations in terms of multiplication and stress responses, fluorescence intensity was quantified with a MacsQuant Analyzer with the MacsQuantify Software 2.0 (Miltenyi Biotec) and analyzed with FlowJo 8.7 software (Tree Star, Inc.). Aggregates were excluded by gating relevant events in the forward scatter/side scatter (FSC/SSC) contour plot. PE−high (yeast cells) and APC−low (viable yeast cells) populations were then gated, and the DAPI/FITC channel dot plot was analyzed for multiplication (CALCO/DAPI channel) and stress responses (CMFDA/FITC channel).

(iii) 
**Multispectral flow cytometry analysis.** To connect cell morphology and fluorescence features we used ImageStream® with the INSPIRE software (Amnis Corporation). Suspensions were adjusted to 10^7 cells in 80 μl, and 10,000 cells were recorded at ×40 magnification in five different channels, including the bright-field (BF) channel and four fluorescence channels (channel 3, 560 to 595 nm [E1-Cy3, yeast cells]; channel 2, 470 to 560 nm [CMFDA]; channel 7, 430 to 505 nm [CALCO]; channel 11, 660 to 720 nm [TOPRO]). Data analysis was performed with the IDEAS software (Amnis Corporation) after fluorescence compensation procedures. The first step was the definition of a mask that delineated the relevant pixels in each picture. Fifty-four algorithms (calculations made for each event within a defined mask) were then available to analyze size, texture, location, shape, or signal strength.

Using basic algorithms, unfocused events, tissue debris, yeast cell aggregates, and dead yeast cells were excluded (see Fig. S1 in the supplement-
Heat map of gene expression analysis (n = 37) of different CMFDA populations by real-time quantitative PCR. CMFDA populations of yeast cells recovered from macrophages (MP) or murine lungs (MO) were studied. After lyophilization, yeast cells were lysed and primer-specific reverse transcription and quantitative PCR were performed. The targets selected (n = 37) were genes involved in growth, stationary phase, resistance to oxidative stress, autophagy, adaptation to the lung environment, and capsule and chitin formation. For each gene, the fold change compared to the ACT1 and GAPDH genes was normalized between 0 and 1. The genes for which amplification failed are depicted in grey. MO_CMFDAmed and MO_CMFDAhigh from mice clustered together and apart from the other populations. All of the macrophage populations except MP_CMFDA low clustered together. The pattern of expression of MP_CMFDAlow and MO_CMFDAlow was different from that of the other populations and between them, except for PCK1 and COX1 (red arrows). Four clusters of genes can be differentiated, with C1 specifically composed of genes involved in lung adaptation during infection (21) and C4 composed of genes expressed more in the MP_CMFDA low population.

### TABLE 1 Distribution of the various CMFDA populations studied after 24 h of macrophage interaction and in lungs of infected outbred mice

| Population         | Mean no. of yeast cells (10^5) ± SD for analysis of: | | |
|--------------------|---------------------------------|---------------------|---------------------|
|                    | Growth curves                   | Gene expression     | |
|                    | Macrophages                     | Lung homogenate     | Macrophages         | Lung homogenate |
| CMFDA low          | 5.8 ± 0.1                       | 0.7 ± 0.2           | 8 ± 1.6             | 1.1 ± 0.6       |
| CMFDA med           | 12.8 ± 2.5                      | 2.5 ± 1.5           | 17.4 ± 2.1          | 4.6 ± 0.3       |
| CMFDA high          | 38.8 ± 3.9                      | 0.6 ± 0.2           | 26.5 ± 0.7          | 0.9 ± 0.1       |
| CMFDA high CALCO low | 27.0 ± 7.1                     | NDb                 | 27.3 ± 3.5          | ND              |

a Two independent experiments were performed with macrophages and lung homogenates (21 and 42 pooled lungs recovered 7 days after inoculation).  
b ND, not done.
nal material). For multiplication and stress response analyses, gates were determined on the basis of the CALCO/CMFDA dot plot considering the intensity algorithm and optical control of the events pictured in the gates. For each population of interest, the geometric mean was calculated with the IDEAS software. From the whole data set and each algorithm, data were then normalized between 0 and 1, with 0 as the minimal value and 1 as the maximal value. Schematic representation was performed with the open-source genomic analysis software MeV v4.6.1 (The TM4 Development Group) obtained from http://www.tm4.org (75). Hierarchical clustering was performed by complete linkage clustering and Pearson correlation.

**Analysis of specific C. neoformans populations.** (i) Sorting of the C. neoformans cell population on the basis of CMFDA fluorescence intensity. On the basis of the discovery of variable C. neoformans stress responses upon host interaction, cell sorting was used to better characterize the phenotypes of these populations with the FACS Aria II (BD Biosciences).

Yeasts cells obtained after 24 h of incubation with macrophages were sorted on the basis of the CALCO/CMFDA dot plot while yeast cells obtained from 21 to 42 mice inoculated 7 days before were sorted on the basis of the SSC/CMFDA dot plot. Aggregates, cell debris (PElow population), and dead yeast cells (APChigh population) were then excluded. Three populations were sorted as CMFDAhigh, CMFADmedium, and CMFADAlow for both conditions, with an additional population of CMFDAhigh, CMFADmedium, and CMFADAlow for the yeast cells recovered after macrophage interaction (Fig. 7A and C). The purity of each sorted population was assessed by fluorescence microscopy (see Fig. S6 in the supplemental material) Independent duplicate experiments were performed and used for determination of growth kinetics and gene expression analysis.

A yeast cell sample harvested from a standard YPD culture, stained at the same time, and stored under the same conditions as the experimental samples was used as a control (control yeast cells).

(ii) Growth curves determination. The growth kinetics of each population were analyzed. After sorting and centrifugation at an RCF of 2,000, the populations were resuspended in sterile YPD containing 10% penicillin/streptomycin (YPDps) at the desired concentration on the basis of the CALCO/CMFDA dot plot. Aggregates, cell debris (PElow population), and dead yeast cells (APChigh population) were then excluded. Three populations were sorted as CMFDAhigh, CMFADmedium, and CMFADAlow for both conditions, with an additional population of CMFDAhigh CALCOmed+low for the yeast cells recovered after macrophage interaction (Fig. 7A and C). The purity of each sorted population was assessed by fluorescence microscopy (see Fig. S6 in the supplemental material) Independent duplicate experiments were performed and used for determination of growth kinetics and gene expression analysis.

A yeast cell sample harvested from a standard YPD culture, stained at the same time, and stored under the same conditions as the experimental samples was used as a control (control yeast cells).

(iii) Sample preparation for quantitative real-time PCR. After sorting, the different C. neoformans populations were washed carefully in RNase-free water to remove PBS salts, flash-frozen in liquid nitrogen, and stored at −80°C. Control yeast cells cultured in YPD medium were processed in parallel. All experiments were performed with the SuperScript III Platinum CellsDirect One-Step quantitative reverse transcription-PCR kit (Invitrogen), which is classically used for single-cell PCR analyses. The day before RNA extraction, the samples were lyophilized overnight. One microliter of lysis enhancer and 10 μl of resuspension buffer were added to the lyophilized samples and incubated 10 min at 75°C. A 1.6-μl volume of DNase buffer and a 5-μl volume of DNase I were then added, and the mixture was incubated for 5 min at 25°C and then inactivated with 4 μl of EDTA and incubation at 70°C for 10 min. RNA samples were then stored at −80°C.

(iv) Quantitative real-time PCR. We based our selection on the genes that were homologues between H99 and S. cerevisiae (77). All H99 genes for which the S. cerevisiae gene description contained the keywords growth, stationary phase, starvation, and oxidative stress were first selected. Additional H99 genes involved in capsule and cell wall formation and some regulated during early pulmonary infection (21) were added (see Table S1 in the supplemental material). The coding sequence of the selected H99 genes was then retrieved from the Broad Institute website (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html). The sequences of 90 different predesigned locked nucleic acids octamer probes of the human Universal ProbeLibrary set (Roche) were used as the probe library. If a probe matched the selected gene, the corresponding primers were designed with the dedicated ProbeFinder Software (Roche Universal ProbeLibrary). At the end, 37 assays (see Table S2) were implemented for real-time quantitative PCR, including 33 for nuclear genes, 1 for a ribosomal RNA gene (18S rRNA), 1 for a mitochondrial gene (COX1), and 2 for reference genes (ACT1 and GAPDH).

Quantitative reverse transcription-PCR that allows primer-specific reverse transcription (50°C for 15 min) and quantitative PCR amplification on the basis of probe detection (95°C for 2 min and 45 cycles of 95°C for 15 s and 60°C for 30 s) was performed in a total volume of 10 μl including 2 μl of a 1:10 dilution of each RNA samples.

Each RNA sample was normalized with the geometric mean of the quantification cycle of the corresponding ACT1 and GAPDH gene expression (78). Fold changes for each sample were calculated according to Pfaffl (79). For hierarchical clustering analysis with MeV v4.6.1, the data were normalized as described above.

**Ethics statements.** This study was carried out in strict accordance with the French and European regulations on the care and protection of laboratory animals (EC directive 86/609, French law 2001–486 issued on 6 June 2001). Animals were housed at the Institut Pasteur animal facilities, which are accredited by the French Ministry of Agriculture to perform experiments with live mice (accreditations A 75 15–27 and B 75 15–05, issued on 12 November 2004 and 22 May 2008, respectively). The protocol was approved by the Institut Pasteur Animal Care Committee (number 03/144) and performed in compliance with the NIH Animal Welfare Insurance (number a5476–01 issued on 27/02/2007). All efforts were made to minimize suffering during animal handling and experimentation. Animals were housed seven per cage in our animal facilities and received food and water ad libitum. Mice were euthanized by CO₂ inhalation.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02580-14/-/DCSupplemental.

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