Phosphate Induces a Morphological Shift that Enhances Vascular Dissemination of *Cryptococcus neoformans*

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SUMMARY:

Phenotypic heterogeneity is a common microbial strategy to improves fitness in fluctuating environments. The fungal pathogen *Cryptococcus neoformans* exhibits dramatic size heterogeneity: it varies from 2-100 μm in diameter during mammalian infection. Following pulmonary invasion, cells enlarge to $>30 \mu\text{m}$ diameter, then decrease over disease course. Extrapulmonary organs, particularly the brain, contain uniformly small cells, implying that that morphotype is important for dissemination. To test this hypothesis, we isolated size-based *ex vivo* cell populations directly from mouse lungs. Small *ex vivo* cells readily disseminated compared with other *ex vivo* populations, small beads, and *in vitro*-grown small cells. The latter two groups are close in size to small *ex vivo* cells, suggesting that while size is important, fungal-specific elements also drive extrapulmonary dissemination. We found that mannose exposure facilitates host cell interaction and organ uptake. Phosphate induces small cell formation. This demonstrates how environmental cues shift phenotypic heterogeneity to drive pathogenesis.

INTRODUCTION:

Phenotypic heterogeneity within microbial populations promotes fitness in fluctuating and complex host environments (Weigel and Dersch, 2018). Heterogeneity arises among isogenic microbial populations as cells communicate, divide labor, and stochastically alter gene expression. Microbial responses to host microenvironments can also lead to phenotypic heterogeneity, even within single foci of infection (Davis, 2018). For example, microbial cells at the perimeter of infection foci may be more open to attack from immune cells, while the core may be depleted of nutrients and oxygen (Davis et al., 2015; Kowalski et al., 2019). Phenotypic heterogeneity can also influence which cells in a localized population are more likely to spread to new sites within a host, a phenomenon that significantly impacts the likelihood and trajectory of cancer metastasis (San Juan et al., 2019). However, due to the bottlenecks involved, we

understand little about how phenotypic heterogeneity influences the spread of infectious microbes within a host.

In this study, we used the model human fungal pathogen *Cryptococcus neoformans* to address the role that phenotypic heterogeneity plays in intra-host dissemination. A ubiquitous saprophytic fungus associated with trees and bird guano (Emmons, 1955; Lazéra et al., 1996), *C. neoformans* is generally harmless, with human exposure thought to be as high as 70% by age five (Goldman et al., 2001). Infection follows inhalation of a fungal cell or spore (Maziarz and Perfect, 2016), and in immunocompromised individuals *C. neoformans* disseminates via the bloodstream and/or lymphatics to almost any organ in the body (Lin et al., 2015; Maziarz and Perfect, 2016). *C. neoformans* cells rapidly proliferate in the central nervous system, and the resulting fungal meningoencephalitis drives cryptococcosis patient mortality, killing ~180,000 people each year worldwide (Rajasingham et al., 2017).

C. neoformans is an excellent model organism to study the impact of phenotypic heterogeneity on intra-host dissemination. Two of its primary virulence attributes—cell and capsule size—vary considerably during infection and are readily observable via microscopy and flow cytometry (Li and Nielsen, 2017). Isolates belonging to the *Cryptococcus* genus exhibit a high degree of cell and capsule size diversity, and morphological heterogeneity strongly impacts *C. neoformans* virulence: increased capacity for morphological variation among isolates correlates with worse clinical outcome (Fernandes et al., 2018).

Cell and capsule size are finely tuned to a multitude of environmental signals and impact stress resistance, immune recognition, and overall pathogenesis (Casadevall et al., 2019; O'Meara and Alspaugh, 2012). Cells grown in nutrient replete laboratory media are morphologically homogenous (5–10 μm in total (cell + capsule) diameter) and poorly encapsulated (O'Meara and Alspaugh, 2012), while fungal cells proliferating in the lungs are morphologically heterogeneous (1–100 μm in total diameter) and encapsulated, although capsule thickness varies considerably (Wang and Lin, 2015; Zaragoza, 2011). In the host, the polysaccharide capsule shields immunostimulatory microbe-associated molecular patterns in the fungal cell wall, suppresses phagocytosis, and protects against oxidative stress (Casadevall et al., 2019; O'Meara and Alspaugh, 2012).

The best understood *C. neoformans* morphotype is the polyploid ($\geq 4C$) “titan” cell, measuring 10–100 μm in cell body diameter (Dambuza et al., 2018; Hommel et al., 2018; Okagaki and Nielsen, 2012b; Okagaki et al., 2011; Trevijano-Contador et al., 2018; Zaragoza et al., 2010; Zhou and Ballou, 2018). Titans are more resistant to phagocytosis and oxidative

stress and skew immunity toward a non-protective Th2 response (Crabtree et al., 2012; Okagaki and Nielsen, 2012b; Okagaki et al., 2010).

While titans contribute considerably to cryptococcal pathogenesis, they are not the only morphotype present during infection, accounting for a minority of cryptococcal cells within the lungs (Zhou and Ballou, 2018). Tissue-specific differences in cryptococcal morphology also manifest in human patients and animal infection models (Denham et al., 2017; Rivera et al., 1998; Xie et al., 2012). Titan cells are rarely observed outside of the lungs, and overall cell and capsule sizes tend to be substantially smaller in extrapulmonary sites, including the brain (Charlier et al., 2005; Denham et al., 2017; Rivera et al., 1998; Xie et al., 2012). We previously observed that median cell and capsule size within the lungs of mice inoculated with the highly virulent KN99 strain of *C. neoformans* decreased from >30 μm to ~10 μm over the course of infection, correlating with extrapulmonary dissemination (Denham et al., 2017). Thus, we hypothesize that smaller morphotypes are more likely to disseminate.

Most infection models—including intravenous dissemination models—use *C. neoformans* yeast cells collected from nutrient replete laboratory media as the inoculum, failing to capture the phenotypic heterogeneity generated during lung infection (Mukaremera et al., 2019; Ngamskulrungrong et al., 2012b; Sabiiti et al., 2012). In this study, we accounted for phenotypic heterogeneity generated during lung infection by isolating cells directly from infected lung tissue and sorted them according to size. We then intravenously challenged naïve mice with those freshly isolated cryptococcal cells to determine the influence of morphology on bloodstream dissemination.

RESULTS:

***Cryptococcus* strain-dependent morphological transitions during pulmonary infection correlate with dissemination.**

Cryptococcus spp. isolates vary in their capacity for morphological variation (Fernandes et al., 2018). To more broadly assess morphological shifts during infection, we inoculated ~8-week-old female B6 mice with a panel of common *Cryptococcus* spp. reference strains (2.5×10^4 cells / mouse). At 3 days post-inoculation (dpi) and 17 dpi, we homogenized lungs and measured fungal cell body and capsule size (**Figures 1A and 1B**). Morphogenesis varied widely among *Cryptococcus* isolates. While KN99 (*C. neoformans*) and Bt63 (*C. neoformans*) cells shifted smaller between 3 and 17 dpi, R265 (*C. deuterogattii*) and WM276 (*C. gattii*) continued to increase in size—largely due to increased capsule thickness. Smaller median cell

size at 17 dpi correlated with increased extrapulmonary burden (**Figures 1C and 1D**). These data suggest that among *Cryptococcus* isolates, the emergence of small cells (<10 μm) within the lungs is a strong predictor of extrapulmonary dissemination.

We also measured cell and capsule size in the blood, liver, spleen, and brains of mice inoculated with KN99 cells (**Figure 1E**). The range of cell size in the blood and liver was surprisingly broad considering that murine microcapillaries can be as small as 3.5 μm . Although rare, we did find fungal cells in the blood measuring 50 μm across. However, median total diameter in the blood at 17 dpi was 12.72 μm , which was noticeably smaller than earlier timepoints in the lungs (27.21 μm at 3 dpi and 18.71 μm at 14 dpi) indicating that entry into the blood may still be a size-limiting bottleneck. In contrast, cell size in the spleen and brain was far more restricted. The vast majority of cells in the spleen were <15 μm , while cells in the brain rarely exceeded 10 μm in total diameter. These data suggest that the cell/capsule size profile generated within the lungs is a strong predictor of extrapulmonary dissemination, and that multiple bottlenecks may restrict vascular dissemination.

Inoculum size alters the rate at which *C. neoformans* populations shift toward smaller cell size in the lungs and disseminate to the brain.

Lower initial cell density increases the frequency of titan cell formation *in vitro* (Trevijano-Contador et al., 2018) and *in vivo* (Okagaki et al., 2010). We hypothesized that reducing the number of cells in the inoculum would increase the frequency of larger cells early in lung infection and slow the rate of small cell formation (**Figures S1A, S1B, and S1C**). We inoculated mice with 10^3 , 10^4 , or 10^5 KN99-mCherry cells / mouse and employed the flow cytometry forward scatter-area (FSC-A) parameter as a high-throughput assay to estimate relative changes in cell size at 3, 7, 10, 14, and 17 dpi.

Consistent with our hypothesis, inoculating mice with fewer fungal cells increased fungal cell size at 3 dpi, decreased the rate of cell size reduction in the lungs (**Figures S1A, S1B, and S1C**), decreased lung fungal burden (**Figure S1D**), and decreased dissemination to the brain at 17 dpi (**Figure S1E**). Therefore, lower initial fungal cell density slows the rate at which fungal cells shift smaller in the lungs and disseminate to the brain.

Populations of cells isolated from the lungs according to size display differences in their cell surface features and are differentially bound by soluble host factors.

When we compared the labor-intensive microscopy-based measurements of cell size with flow cytometry-based measurements, we still observed a fungal size shift over the course

of infection (**Figure S1A**). Thus, we sought to isolate KN99-mCherry cells from infected lung tissue (14 dpi) via fluorescence-activated cell sorting (FACS) according to size (FSC-A), characterize them, and directly test their ability to disseminate through the vasculature. We sorted cross-sections of the fungal cell size distribution corresponding to the smallest (small *ex vivo*: median total diameter of 7.56 μm), middle (mid *ex vivo*: median total diameter of 14.04 μm), and largest (large *ex vivo*: median total diameter of 21.80 μm) ~20% of the population (**Figure 2A**). We further confirmed that we isolated distinct populations based on cell and capsule size (**Figure 2B**) and that fungal cells sorted from each gate were also equivalently viable (**Figure 2C**).

In addition to the differences in cell and capsule size among the three populations, we observed differences in DNA ploidy measured by 4',6-diamidino-2-phenylindole (DAPI) staining (**Figure 2D**). Haploid cells cultured in minimal yeast nitrogen base (YNB) medium +/- benomyl served as controls for 1C and 2C DNA content; benomyl inhibits cell division, trapping cells at 2C. Cells with >2C content are considered titan cells. Only the large *ex vivo* population contained an appreciable number of titan cells (mean: 21.4% >2C) (**Figure S2**). Mid *ex vivo* cells averaged 74.2% 1C and 22.8% 2C, while small *ex vivo* cells averaged 89.2% 1C and 10.0% 2C.

Morphological changes in *C. neoformans* are often accompanied by alterations in cell surface architecture and immune recognition. We screened the binding rate (% total fungal cells bound) of a panel of soluble receptors to small, mid, and large *ex vivo* cells (**Figure 2E**). Large *ex vivo* cells were more likely to be bound by complement protein C3 than mid, or small *ex vivo* cells. In contrast, small and mid *ex vivo* cells were more likely to be bound by lung surfactant protein D (SPD). SPD binding to *C. neoformans* is deleterious for the host, potentially because SPD agglutinates fungal cells and disrupts fungal clearance by macrophages (Geunes-Boyer et al., 2012). We saw uniform and low-level binding of IgA and IgM class antibodies, and little to no binding by mannose-binding lectins.

Differences in host factor binding suggested that cell surface architecture may differ among the sorted populations. We used two lectins to probe the exposure of microbial features in different layers of the cell wall: wheat germ agglutinin (WGA), which recognizes chitin in the inner-most layers of the cryptococcal cell wall and concanavalin A, which binds mannose on mannoproteins in the outer cell wall and inner capsule. There were no significant differences in chitin exposure as measured by flow cytometry (**Figure 2F**). However, mannose was significantly more exposed on small *ex vivo* cells compared with mid and large *ex vivo* cells and cells grown in non-capsule inducing minimal YNB medium (**Figure 2G**).

We visualized mannose exposure pattern via microscopy and characterized staining as negative, punctate (one or more distinct staining puncta), diffuse (staining covers the observable cell surface), or both punctate and diffuse (**Figure 2H**). Mannose was more likely to be exposed both at specific puncta and diffusely across the surface of small *ex vivo* cells. Mannose exposure can correspond to recent bud scars, which may explain the punctate staining in our analysis (Panepinto et al., 2007). The high prevalence of diffuse mannose exposure on small cells may be due to their relatively thin capsules. Altogether, these results suggest that cell and capsule size heterogeneity correlates with heterogeneity in cell surface architecture and immune recognition.

Small *ex vivo* cells disseminate to extrapulmonary organs at a higher rate than mid and large *ex vivo* cells.

To directly address the impact of fungal cell and capsule size on dissemination, we intravenously inoculated naïve mice with small, mid, and large *ex vivo* cells (10^5 cells / mouse). With blood flow from the tail vein running directly to the heart and then lungs for reoxygenation, the lungs harbor the first major bed of microcapillaries fungal cells are likely to encounter following tail vein inoculation. Therefore, sorting fungal cells from infected lung tissue prior to tail vein inoculation roughly replicates the fate of fungal cells had they entered the bloodstream from the lungs. Consistent with our hypothesis, a greater number of small *ex vivo* cells reached the liver, spleen, and brain by 3 hours post-inoculation (hpi) than mid and large *ex vivo* cells (**Figure 3A**). In contrast, the majority of mid and large *ex vivo* cells remained in the lungs at 3 hpi, possibly because they failed to navigate the lung microcapillaries as well as small *ex vivo* cells. We observed the same trends in fungal burden at 3 dpi, indicating that small *ex vivo* cells are well-capable of proliferating in extrapulmonary tissues (**Figure 3A**).

Although brain fungal burden was equivalent in mice inoculated with mid and large *ex vivo* cells at 3 hpi, there were more mid *ex vivo* cells in the brain by 3 dpi. We asked whether the reduced brain fungal burden in mice inoculated with large *ex vivo* cells was due to inefficient blood-brain barrier crossing or reduced proliferation in the brain. To estimate blood-brain barrier crossing *in vivo*, we intracardially perfused mice at 3 hpi to drive out circulating fungal cells and normalized the fungal burden in perfused mice to nonperfused mice inoculated on the same day. While perfusion did not significantly affect brain fungal burden in mice inoculated with small or mid *ex vivo* cells, perfusion drove nearly all of the large *ex vivo* cells from the brain (**Figure 3B**). In contrast, fungal burden post-intracranial inoculation was independent of initial fungal cell

size (**Figure 3C**), suggesting that while large *ex vivo* cells are poorer at reaching the brain and crossing the blood-brain barrier, they are not deficient at proliferating in the brain.

Macrophages, but not platelets or neutrophils, clear small *ex vivo* cells from the vasculature to reduce brain dissemination.

As small *ex vivo* cells most efficiently disseminated to extrapulmonary organs, we examined the mechanisms governing their dissemination profile more closely. Most small *ex vivo* cells that made it past the lungs at 3 hpi were cleared in the liver and spleen (**Figure 3A**). The liver plays a major role in clearing microbes and microbial components from the blood (Macpherson et al., 2016). Recently, intravital imaging revealed that liver macrophages clear *C. neoformans* cells from the blood stream (Sun et al., 2019). However, the inoculum for those studies consisted of *C. neoformans* cells cultured *in vitro*. Our cell surface characterization revealed that small *ex vivo* cells differ from cells cultured *in vitro* (YNB medium), mid *ex vivo*, and large *ex vivo* cells, which may influence liver macrophage recognition of *C. neoformans* cells.

To address the role of macrophages in clearing small *ex vivo* cells from the bloodstream, we depleted macrophages from the liver, spleen, and bone marrow of mice with clodronate liposomes 48 hours prior to inoculation (**Figures S3A and S3B**). Macrophage depletion reduced small *ex vivo* cell fungal burden in the liver, while increasing fungal burden in the lungs, kidneys, and brain at 1 dpi (**Figure 3D**). These results corroborate the findings of Sun *et al.*, in that liver macrophages play a critical role in filtering *C. neoformans* from the blood (Sun et al., 2019). However, we also found that small *ex vivo* cells grow rapidly in the liver compared with *in vitro*-grown cells or other *ex vivo* populations (**Figure 3E**), suggesting that attempts by the host to clear *C. neoformans* cells by sequestering them in the liver may backfire.

Besides macrophages, platelets and neutrophils are among the most abundant immune cell-types present in the blood that contribute to microbial clearance. Platelets can recognize either complement protein bound to microbes or microbial features directly to initiate microbial clearance, especially in the spleen (Broadley et al., 2016). Platelets don't appear to interact with encapsulated *C. neoformans*, or mediate the clearance of *in vitro*-grown *C. neoformans* cells from the blood (Sun et al., 2019). We also did not see any effect on fungal dissemination when platelets were depleted prior to inoculation with small *ex vivo* cells (**Figures S4A, S4B, and S4C**).

It's unclear whether neutrophils play a predominantly protective or detrimental role during cryptococcosis, though there is evidence neutrophils can clear *C. neoformans* from the

blood (Sun et al., 2016; Zhang et al., 2016). In an intravenous infection model using *in vitro*-grown fungal cells as the inoculum, neutrophil depletion greatly increased fungal burden in the lungs and brain (Sun et al., 2016; Zhang et al., 2016). However, neutrophil-depletion prior to inoculation with small *ex vivo* cells slightly increased lung fungal burden, but did not affect fungal burden in other organs, indicating that the small *ex vivo* cells are more resistant to neutrophil-mediated clearance than typical *in vitro*-grown fungal cells (**Figures S4D, S4E, and S4F**).

Macrophage recognition of small *ex vivo* cells is partially mediated by mannose exposure.

We predicted that liver macrophages may recognize the high levels of exposed mannose on the surface of small *ex vivo* cells. We isolated liver macrophages from healthy ~8-week-old B6 mice (Li et al., 2014) and co-cultured them with 6 μ m plastic beads, minimal (YNB) medium-grown cells, and sorted *ex vivo* populations at a multiplicity of infection of 2:1. Half of the wells also received pretreatment with exogenous mannan, with the prediction that it would reduce macrophage association when mannan recognition is critical. After 4 hours we washed away free fungal cells, leaving only the fungal cells bound or internalized by macrophages, which we define as “macrophage association”.

Approximately 60% of macrophages had associated with 6 μ m plastic beads by 4 hours, indicating that the liver macrophages still displayed phagocytic capacity *ex vivo* (**Figure 3F**). Additionally, 17.67% of liver macrophages associated with small *ex vivo* cells, which was more than twice the frequency of mid and large *ex vivo* cells. Pretreatment with exogenous mannan prior to infection reduced macrophage association with small *ex vivo* cells by ~40%, but did not affect association with mid and large *ex vivo* cells, YNB-grown cells, or beads. These data suggest that liver macrophages recognize small *ex vivo* cells in part through exposed mannose, and that mannose exposure could contribute to fungal clearance in the liver.

Liver macrophages and other macrophages that sample the bloodstream are subject to shear force, which can affect recognition and phagocytosis of microbes and other foreign matter (Broadley et al., 2016). We assessed whether mannose exposure on small *ex vivo* cells affects vascular clearance *in vivo* by intravenously injecting either 400 μ g of exogenous mannan or vehicle (PBS) 2-3 minutes prior to intravenous inoculation with 10^5 small *ex vivo* cells. 10 minutes later, we perfused mice to drive out non-recirculating fungal cells and plated organs for colony forming units to estimate fungal burden. If mannose recognition contributed to fungal clearance in a given organ, we expected to see reduced fungal burden in mice that received

exogenous mannan, compared with the vehicle control. We repeated this experimental design with additional fungal cell wall and capsule components (GXM, β -glucan, chitin), as well as polyinosinic acid (poly(I)), which is a scavenger receptor ligand (Pearson et al., 1993).

Although mannan did reduce fungal clearance in the liver and kidneys by ~20% and in the spleen by ~75% (**Figure 3G**), it was not the only fungal ligand that influenced vascular clearance. Poly(I) reduced clearance to a strikingly similar extent as mannan. The purified capsule polysaccharide GXM reduced clearance in a similar extent in the liver, spleen, kidneys at roughly the same level (~40% reduction). β -glucan and chitin, on the other hand, did not appreciably affect fungal clearance, possibly because of their buried position beneath the capsule. These data indicate that clearance of small *ex vivo* cells is not dependent on a single cell surface ligand and that there are tissue-specific dependencies on various fungal features that mediate vascular clearance.

Enhanced small *ex vivo* cell dissemination is not entirely dependent on size.

We next assessed whether small *ex vivo* cells disseminate more efficiently due to their size alone, or if there were other contributing factors. We inoculated mice via the tail vein with either large *ex vivo* cells, small *ex vivo* cells or size-matched inert polystyrene beads. Large *ex vivo* cells disseminated similarly to size-matched 25 μ m beads; the majority resided in the lungs at 3 hpi (**Figure 4A**). Additionally, more 6 μ m beads disseminated past the lungs to reach the liver and spleen compared with 25 μ m beads. However, small *ex vivo* cells reached the liver and spleen at an even higher frequency, indicating that absolute size influences fungal dissemination through the vasculature, but it is not the only contributing factor.

We next examined *in vitro* conditions that could reproduce the small *ex vivo* cell morphotype and dissemination profile. Previously, we observed that cells grown in capsule-inducing medium (CAP) shifted toward smaller cell and capsule size if they were supplemented with conditioned medium (CM) from cells grown in non-capsule inducing medium (yeast nitrogen base + 2% glucose, or YNB) (Denham et al., 2017). YNB medium contains vitamins, nitrogen, and glucose but not amino acids. YNB-grown cells suppress capsule formation, and are typically <10 μ m in diameter. CAP medium consists of 10% Sabouraud's dextrose broth—made from digested animal tissue—buffered to pH 7.4. CAP medium induces capsule growth and is sufficient for cell division. We collected CM by filtering supernatant from saturated YNB-grown cultures. In this case, CM consists of unspent nutrients and soluble fungal factors secreted during growth in non-capsule inducing conditions.

We compared the dissemination profile of small *ex vivo* cells to cells grown in three *in vitro* populations: (1) YNB-grown cells (2) CAP-grown cells, and (3) CAP-grown cells supplemented with CM (CAP+CM). We also subjected the *in vitro*-grown populations to the same sorting process as *ex vivo* cells by sorting YNB, and CAP+CM-grown cells from the “small” FSC-A gate (YNB cells: 5.64 μ m median total diameter; CAP+CM cells: 6.48 μ m median total diameter; few YNB or CAP+CM cells fell in the “mid” or “large” FSC-A gates) and sorting the CAP-grown cells from the “mid” FSC-A gate (12.11 μ m median total diameter; few CAP-grown cells fell into the “small” or “large” FSC-A gates) (**Figure 4B**).

The prior culture conditions and morphology of cells in the inoculum heavily impacted dissemination. Only CAP+CM cells replicated the dissemination profile of small *ex vivo* cells in terms of fungal burden at 3 hours and 3 days post-inoculation (**Figure 4C**). Despite being roughly the same size as small *ex vivo* cells, YNB-grown cells displayed similar fungal burden to CAP-grown cells at 3 hours, and lower fungal burden in extrapulmonary organs than CAP, CAP+CM, and small *ex vivo* cells at 3 days. Therefore, both absolute fungal cell size, and the physiological state of cells entering the blood influence the outcome of vascular dissemination.

Cells sorted from infected host tissue or cells grown in host-like medium survive better in the blood than cells grown in nutrient replete medium.

The caveat to using fungal burden as a readout for dissemination is that we can’t easily distinguish between the ability of cells to reach certain organs, and their ability to survive. We hypothesized that YNB-grown cells may not be as primed for survival in the bloodstream as cells sorted from infected host-tissue or cells grown under stressful host-like conditions (CAP medium).

To address that hypothesis, we examined the survival of *in vitro*- and *ex vivo*-sorted cells in whole mouse blood, liver macrophage co-culture (multiplicity of infection of 1:1), and DMEM as a medium control (96-well plates, 37 °C, 5% CO₂, static). We assessed fungal cell survival as a percentage of input at 0-, 24-, and 48-hour timepoints. Liver macrophages suppressed *in vitro* and *ex vivo* cell growth equivalently (**Figures 4D and 4E**). However, the *ex vivo* populations and *in vitro* populations from host-like conditions survived better in blood than YNB-grown cells (**Figure 4F**), with large and mid-*ex vivo* populations faring the best. These results support the idea that size and physiology are critical for bloodstream dissemination: small *ex vivo* cells combine the stress resistance required for increased survival in the blood with the smaller cell size required for widespread dissemination via microcapillaries.

Phosphate acquisition genes are upregulated in capsule-inducing medium and suppressed following conditioned medium supplementation.

CAP+CM-grown cells resemble small *ex vivo* cells in terms of cell + capsule size and vascular dissemination profile, so we hypothesized that they might suitably model small cell formation in the lungs. We analyzed the transcriptome of cells grown in YNB, CAP, and CAP+CM medium to identify genes that might facilitate the transition to small cell size in host-like capsule inducing conditions. We also analyzed the transcriptome of small, mid and large *ex vivo* cells.

Comparing the transcriptome of CAP, CAP+CM, and the *ex vivo* populations to YNB-grown cells demonstrated that the YNB-grown transcriptome is vastly different from each of these populations (**Figure 5A**). Core sets of 699 and 291 genes were up- and down-regulated respectively among the *ex vivo* populations and CAP/CAP+CM populations relative to YNB-grown cells. There were also 1085 up-regulated and 1297 downregulated genes shared only among the *ex vivo* populations relative to YNB-grown cells, demonstrating that the transcriptome within the host environment is still quite distinct from even the host-like *in vitro* conditions. Among the *ex vivo* populations, it is notable that small *ex vivo* cells were the most transcriptionally distinct (**Figure S5A**). 928 genes were differentially expressed in small vs mid *ex vivo* cells, and 2,365 genes were differentially expressed in small vs large *ex vivo* cells. For comparison, only 93 genes were differentially expressed in mid vs large *ex vivo* cells (**Figure S5A**).

Gene ontology term (GO-term) enrichment analysis revealed that phosphate ion transport was upregulated in CAP-grown cells vs YNB-grown cells (**Figure 5C**), and downregulated in CAP+CM vs CAP-grown cells (**Figure 5D**). In other words, phosphate acquisition genes are induced when cells enter capsule-inducing conditions (CAP medium), and then suppressed following supplementation with CM.

We observed the same trends when analyzing the expression of specific genes involved in phosphate acquisition and storage (**Figure 5E**). When *C. neoformans* cells are starved of phosphate, the phosphate acquisition transcription factor, Pho4, translocates to the nucleus where it promotes its own gene expression and the expression of genes involved in phosphate acquisition and storage. These include phosphate transporters: *PHO84*, *PHO89*, and *PHO840*; phosphatases: *APH1* (secreted/vacuolar acid phosphatase) and *APH4* (predicted intracellular acid phosphatase); and *VTC4* (vacuolar transport chaperone involved in processing polyphosphate). Pho4-dependent genes were upregulated in CAP vs YNB-grown cells and then suppressed in following CM supplementation—although many phosphate acquisition genes still

remained more highly expressed in CAP+CM-grown cells compared with YNB-grown cells. Phosphate acquisition genes were also upregulated *in vivo* compared with YNB-grown cells, suggesting that phosphate acquisition could be similarly linked to morphological shifts in the host (**Figure 5E**).

Phosphate acquisition genes were also upregulated in the *ex vivo*-sorted populations relative to YNB-grown cells, suggesting that the host lung environment is phosphate-limiting to a degree. However, there were no differences in phosphate acquisition gene expression among the three *ex vivo* populations. We limited our transcriptomic profiling of *ex vivo* cells to just one timepoint (14 days post-inoculation). Had we profiled gene expression over time, we may have observed changes more reflective of available phosphate concentrations in the lungs.

Phosphate and acidic pH are sufficient to drive *C. neoformans* populations toward smaller morphotypes following growth in alkaline and host-like conditions.

We hypothesized that phosphate might be a critical nutrient that facilitates the emergence of small cells in CAP+CM media. Conditioned medium consists of unspent YNB medium and soluble fungal cell derived factors (Albuquerque et al., 2014). YNB medium is defined, so we screened every component individually (including phosphate) to determine which components are sufficient to induce small cell formation when cells are grown in CAP medium. We also tested two soluble fungal-derived factors that accumulate during growth in YNB medium: exo-GXM (Denham et al., 2017) and the quorum sensing-like peptide Qsp1 (Homer et al., 2016; Lee et al., 2007).

We grew cells for 24 hours in YNB medium, then sub-cultured 10^5 cells/mL into CAP medium for another 24 hours. We then sub-cultured CAP-grown cells 1:1 into fresh CAP medium with 10% of the final volume being a YNB medium component, fungal-derived factor, or water as the vehicle control. After a final 24-hour period, we measured cell size (FSC-A) by flow cytometry.

Conditioned YNB medium and fresh YNB medium were sufficient to stimulate a shift toward smaller cell size (**Figures 6A and 6B**). Among the YNB medium components, only phosphate was sufficient to stimulate a shift toward smaller cell size (**Figure S5A**), and did so in a concentration-dependent manner (**Figures 6A and 6B**). Phosphate-supplemented cells decreased both total (cell+capsule) diameter (**Figure 6C**) as well as cell (**Figure S6A**) and capsule (**Figure S6B**) size individually. Phosphate was also sufficient to induce a shift toward smaller size for cells grown in titan cell-inducing medium (5% Sabouraud's, 10% fetal calf serum, 15 μ M sodium azide, pH 7.4, 5% CO₂, 37 °C) (**Figure S5B**).

We previously reported that exo-GXM may trigger the formation of smaller cells (Denham et al., 2017). However, we were unable to reproduce those results (**Figure S5A**), possibly due to differences in exo-GXM preparation, but more likely because we previously solubilized exo-GXM in phosphate buffered saline. Qsp1 is a cell-density dependent signaling peptide that suppresses the formation of titan cells (Trevijano-Contador et al., 2018). However, the addition of Qsp1 did not affect cell size in this model (**Figures S5A and S5B**).

The Pho84 and Pho840 phosphate transporters are phosphate/H⁺ symporters, and *C. neoformans* therefore requires a proton gradient to efficiently transport phosphate into the cell (Kretschmer et al., 2014). In alkaline pH, *C. neoformans* struggles to efficiently import phosphate and upregulates phosphate acquisition machinery (Kretschmer et al., 2014; Lev et al., 2017). *C. neoformans* releases metabolites that acidify the local microenvironment and increase nutrient uptake (Himmelreich et al., 2003; Wright et al., 2002). For instance, cryptococcomas in the brain acidify to levels as low as pH 5.5 (Himmelreich et al., 2003; Wright et al., 2002).

We measured lung pH in mice inoculated with *C. neoformans* throughout infection, and found that pH in the lungs decreased from ~7.4 to as low as ~6.3 between 3 and 17 dpi (**Figure 6D**). Phosphate did not induce significant shifts in FSC-A-measured cell size when CAP medium was buffered to 6.3, potentially because phosphate availability in CAP medium buffered to pH 6.3 is not as limiting as pH 7.4 (**Figures 6A and 6B**). However, we did measure a modest, but significant decrease in total (cell+capsule) diameter when pH 6.3 CAP-grown cells were supplemented with phosphate (**Figure 6C**), which was mostly due to a decrease in capsule thickness (**Figure S5**). These data indicate that cell body and capsule size enlarge when phosphate is limiting due to starvation, alkaline pH, or both. When phosphate availability increases due to higher extracellular concentrations or decreased extracellular pH, *C. neoformans* cells shift toward smaller morphotypes.

Limiting phosphate acquisition genetically suppresses morphogenesis *in vivo*.

Since phosphate was sufficient to trigger cell and capsule size reduction *in vitro*, we hypothesized that limiting phosphate acquisition *in vivo* would alter morphogenesis, perhaps delaying the appearance of small morphotypes. To test this hypothesis, we intranasally inoculated mice with wild-type KN99, or either of two independently constructed KN99:*pho4*Δ strains, which lack the gene encoding the transcription factor *pho4*. Without *pho4*, *C. neoformans* cells fail to adequately upregulate genes involved in phosphate acquisition when available phosphate is limiting, and become hypersensitive to alkaline conditions. The *pho4*Δ

mutant is attenuated for growth in the lungs but more severely attenuated for dissemination to the brain, due to its poor survivability in alkaline blood (Lev et al., 2017). Our findings replicated the work from Lev *et al.*, in that the *pho4Δ* mutant cells were attenuated for proliferation in the lungs (**Figure 6E**) and dissemination to the brain (**Figure 6F**). We also found that cell and capsule size of *pho4Δ* mutant cells populating the lungs was static and less heterogeneous relative to wild-type cells (**Figures 6G, S6C, and S6D**). Most *pho4Δ* mutant cells remained between 10 and 20 μm in total (cell + capsule) diameter over 17 days (**Figure 6G**), with many of them also exhibiting irregular budding (**Figures 6H**). Total median wild-type (cell + capsule) diameter was far larger than the *pho4Δ* mutant at day 3 (wild-type: 26.13 μm; *pho4Δ* #1: 14.55 μm; *pho4Δ* #2: 14.17 μm) and smaller than the *pho4Δ* mutant at day 17 (wild-type: 10.25 μm; *pho4Δ* #1: 13.87 μm; *pho4Δ* #2: 15.10 μm), indicating that phosphate acquisition mediated by *pho4* is required for the full range of fungal morphogenesis during infection, including the shift toward smaller morphotypes (**Figure 6G**).

DISCUSSION:

We found that phenotypic heterogeneity is dynamic and supports *C. neoformans* dissemination from the lungs. Capsule growth and titan cell formation during early stages of lung infection enhance fungal resiliency, and as fungal proliferation continues, smaller cells with greater potential for extrapulmonary dissemination emerge and/or survive. These smaller morphotypes are phenotypically and transcriptionally distinct and can more easily navigate microcapillaries to reach sites such as the brain. To our knowledge, these experiments were the first to directly test the influence of *in vivo*-generated phenotypic heterogeneity on *C. neoformans* dissemination, and the first to analyze the transcriptome of discrete *C. neoformans* subpopulations infecting the lungs.

C. neoformans morphology is important at multiple stages of disease progression. For example, inhalation of spores rather than yeast cells may accelerate dissemination, because dendritic cells more readily phagocytose spores and quickly traffic them to draining lymph nodes (Walsh et al., 2019). Spores exhibit elevated pathogenicity compared to genetically identical yeast cells (Walsh et al., 2019). On yeast cells, the degree of encapsulation also altered the trajectory of disease progression through initial interactions with macrophages in a zebrafish infection model (Bojarczuk et al., 2016). Titan cells, in addition to being resistant to phagocytosis, decrease the phagocytosis of smaller cells, potentially facilitating smaller cells' dissemination (Okagaki and Nielsen, 2012a).

The dynamics of phenotypic heterogeneity *in vivo* varied widely among *Cryptococcus* isolates. Isolates that shifted toward smaller morphotypes (KN99 and Bt63) exhibited greater extrapulmonary fungal burden than isolates that remained morphologically static (52D) or those that shifted toward larger morphotypes as disease progressed (R265 and WM276). Absolute size is likely not the sole reason for the difference in dissemination among these isolates. For instance, R265 cells shifted larger over the course of lung infection, but R265 cells also die more quickly in blood than KN99 cells (Ngamskulrungrroj et al., 2012b). The capacity for morphological heterogeneity is likely more important than any given morphological feature, such as capsule thickness, cell body size, or titan formation, as different morphologies will hold advantages at different stages of disease progression (Fernandes et al., 2018), allowing for a bet hedging strategy during infection.

Our data also indicate that size alone does not explain the disseminating ability of small *ex vivo* cells. Namely, cells originating from more stressful environments such as host lungs or host-like *in vitro* conditions were better primed for survival and dissemination *in vivo*. Poorly encapsulated small cells grown in nutrient replete YNB medium survived worse in blood and failed to disseminate as well as small encapsulated *ex vivo* cells or small encapsulated cells generated *in vitro* (CAP+CM) (**Fig. 4**). This suggests that efficient bloodstream dissemination requires that cryptococcal cells adopt smaller cell and capsule morphologies, along with stress resistance that will prime them for survival in the blood (Alanio et al., 2015; Ngamskulrungrroj et al., 2012a). These findings will be important to consider when optimizing future models of fungal dissemination.

Cell size heterogeneity correlates with heterogeneity in cell surface architecture. Smaller cell body size correlated with thinner capsule size and greater mannose exposure *in vivo*. Prior data supporting the importance of mannose recognition by the host during cryptococcosis is contradictory. Mannose receptor deficient mice are more susceptible to *C. neoformans* infection (Dan et al., 2008). However, mannose recognition was dispensable for murine, but not primary human, macrophage phagocytosis of *C. neoformans* and *C. gattii* cells *in vitro* (Lim et al., 2018). There is also conflicting evidence as to whether mannose-binding lectin (MBL) deficiency predisposes individuals to cryptococcal infection (Eisen et al., 2008; Fang et al., 2015). Despite the increased mannose exposure on small *ex vivo* cells, we did not see significant MBL-binding to cryptococcal cells. This may be due to the fact that MBL is most abundant in serum, and we analyzed fungal cells from perfused lung tissue. We did find increased surfactant protein D (SPD) binding of small and mid *ex vivo* cells compared with large *ex vivo* cells, and SPD can bind to mannose-rich microbial features (Sahly et al., 2002). Our results indicate that the

importance of mannose recognition depends on a given fungal cell's morphotype: the addition of exogenous mannan reduced macrophage association with small *ex vivo* cells, but not large *ex vivo* cells or small YNB-grown cells. Increased mannose exposure may actually present a fungal cell fitness cost that comes with reduced size. Smaller capsules and more recent bud scarring may leave more mannose (and potentially other) residues exposed for recognition by phagocytes.

Nutrient availability, particularly phosphate, is sufficient to shift toward smaller cell and capsule morphologies. Out of all the YNB medium components, only phosphate was sufficient to induce the shift toward smaller cell and capsule size *in vitro*. Also, suppressing phosphate acquisition by deleting the *pho4* gene limited phenotypic heterogeneity *in vivo*: neither large nor small morphotypes appeared in mouse lungs. Phosphate is essential for cell homeostasis and growth, and its acquisition is tightly controlled in microbes that absorb fluctuating levels of phosphate from their surroundings (Köhler et al., 2020; Lev and Djordjevic, 2018). Phosphate is also critical for *Candida albicans* morphogenesis (hyphal formation), stress resistance, and tissue invasion (Ikeh et al., 2016; Liu et al., 2018).

Phosphate acquisition is also essential for survival in the blood and dissemination to the brain (Lev et al., 2017). *Cryptococcus* relies on a proton gradient to import many different nutrients, including phosphate, into the cell (Lev and Djordjevic, 2018). Thus, *C. neoformans* survives poorly in the alkaline (pH 7.4) bloodstream when its ability to upregulate phosphate acquisition machinery is impaired (Lev et al., 2017). Our results suggest that increased phosphate availability also impacts dissemination at an even earlier stage by mediating the emergence of small *C. neoformans* morphotypes within the lungs. We observed an order of magnitude decrease in lung pH over the course of infection, which could also enhance acquisition of phosphate and other nutrients (Lev et al., 2017) or independently repress capsule growth (Farhi et al., 1970; O'Meara and Alspaugh, 2012).

Since *pho4* mutants cannot form either large or small cells *in vivo*, it leads to the question of whether small cells are daughters of large cells such as titans. Zhou *et al* (Zhou et al., 2020) reported small cells, termed "titanides," that are titan daughter cells. We can obtain small cells *in vitro* without inducing titan cells beforehand (**Fig. 6**). Since phosphate-induced small cell formation is pH-dependent (**Fig. 6A-C**), there could be multiple pathways for small cell formation. Moreover, cell division is required for small cell formation *in vitro*, since if we do not add fresh growth medium when inducing small cells *in vitro*, the population size does not shift (**Fig. 6B**). In other ways, titanides are similar to our "small" cells, particularly their hypothesized roll in dissemination. Small cells are also about the same size as "drop" cells (Alanio et al.,

2015). These cells are of similar size as small cells but were isolated due to high non-cytoplasmic levels of the glutathione stain CMFDA, which measures oxidative stress response, and are not viable (Alanio et al., 2015).

Many studies identify fungal cell ploidy as a key driver of adaptation, in-host survival, pathogenicity (Gerstein et al., 2015; Okagaki and Nielsen, 2012b; Selmecki et al., 2015). Of our *ex vivo* populations, only large cells contain >2N cells (**Fig. S2**), and even within the large cell population, the majority of cells are 2N, which could be either cells in the G2 phase of mitosis or smaller titan cells. Medium cells display the DNA content profile of actively growing haploid yeast cells (Todd et al., 2018). Thus, we think that comparisons between small and medium *ex vivo* cells are likely independent of ploidy.

There are some notable limitations to our study. For example, we did not define the subpopulations within the lungs based on clear biological criteria, but rather sorted cross-sections of the population of fungal cells proliferating within the lungs. Small *ex vivo* cells correspond with the size of disseminated cells in the brain, but medium and large cells are based on arbitrary size cutoffs designed to distinguish them from small cells. Clearer definitions of fungal subpopulations within the lungs and analysis of their dissemination would greatly enhance our understanding of systemic cryptococcosis. For example, multiple groups have also documented the appearance of unusually small “micro” (~1 µm), sometimes tear-dropped shaped cells *in vivo* that are somewhat similar in shape to the small *ex vivo* cells in this study (Feldmesser et al., 2001; Fernandes et al., 2018; Zaragoza, 2011). Properties of these “micro-cells” are largely unknown and they could be very young, metabolically dormant, or aneuploid cells (Dambuza et al., 2018; Feldmesser et al., 2001; Fernandes et al., 2018; Zaragoza, 2011). Despite limited characterization, they likely represent the smallest cryptococcal cells seen during infection. Though some of the cells sorted from infected lung tissue did present these phenotypes, we did not attempt to further classify micro-cells.

Furthermore, *C. neoformans* faces multiple bottlenecks during dissemination, including escaping the lungs. *C. neoformans* can escape the lung mucosa to access the bloodstream by crossing lung epithelial cells, or via the Trojan horse mechanism within macrophages (Denham and Brown, 2018). We focused on extrapulmonary organ entry rather than examining lung escape by smaller cells directly. However, our small cells are more likely to be phagocytosed, which may lead to increased Trojan horse dissemination. We also find that in late infection (17 dpi), the total diameter of cells in the blood shows a size distribution similar to the lungs (**Fig. 1E**), suggesting that pulmonary escape may be less of a bottleneck than previously thought.

Phosphate was sufficient to induce size reduction of *C. neoformans* cells. Since phosphate is released from damaged cells (Gabow et al., 1982), we propose that small cells could form as a progressing *C. neoformans* infection damages the host tissue, thus priming the invading fungi for dissemination as tissue barriers are breaking down. Conditioned and fresh YNB medium induced a greater size shift than phosphate alone, particularly at pH 6.3. Combinations of different nutrients may also be sufficient to induce a size shift. Shifts toward smaller cell and capsule size can be mediated by signals in addition to nutrient availability. For example, Zhao *et al.* recently demonstrated that alleviation of genotoxic stress, such as that seen during immune-mediated oxidative bursts, activates meiosis genes in polyploid titan cells such that they begin to bud smaller haploid cells (Zhao et al., 2020).

Overall, we determine that a small, phosphate-inducible morphotype impacts a critical step in *C. neoformans* pathogenesis: dissemination from the lungs to the brain. Our results are consistent with observations in humans (Fernandes et al., 2018), in that phenotypic heterogeneity strongly influences clinical outcome. Small *C. neoformans* cells are found in the brains of human patients with cryptococcal meningitis (Xie et al., 2012). The emergence and/or survival of smaller morphotypes within the lungs results in a subpopulation of fungal cells primed for vascular dissemination that survive in the blood and are better able to enter organs than their larger counterparts.

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AUTHOR CONTRIBUTIONS:

S.T.D. and J.C.S.B. conceived and designed the experiments. S.T.D., B.B., K.Y.C., and M.A.W. performed the experiments. S.T.D., B.B., and J.C.S.B. analyzed the data. S.T.D. and J.C.S.B. wrote the paper. S.T.D., B.B., K.Y.C., M.A.W., and J.C.S.B. edited the paper.

DECLARATION OF INTERESTS:

The authors have no competing interests to declare.

Figure 1: Morphological transitions during pulmonary infections correlate with fungal dissemination. (A) Total (cell+capsule) diameter, cell body diameter, and capsule thickness measurements at 3 and 17 days post-intranasal inoculation (dpi) of mice (Mann-Whitney U test; N=4 mice per timepoint, 57-65 fungal cells measured per mouse at 3 dpi and 117-266 fungal cells per mouse at 17 dpi). Solid lines in the violin plots indicate the median and dotted lines mark quartiles. (B) Representative India ink images of fungal cells quantified in 1A. (C) Fungal burden in mice at 17 dpi (One-way ANOVA and uncorrected Fisher's LSD). The bar graph represents the mean. (D) Nonlinear regression of mean extrapulmonary (liver+spleen+brain) fungal burden and median total (cell+capsule) diameter. (E) Total diameter in KN99-infected lungs at 3, 10, and 14 dpi and total diameter in KN99-infected organs at 17 dpi (Mann-Whitney U-test; N=4 mice per timepoint; fungal cells measured per mouse in the lungs: 275, blood: 38, liver: 72, spleen: 43, brain: 72). Solid lines in the violin plots indicate the median and dotted lines mark quartiles. All panels in Figure 1: ns: not significant *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 2: Sorting and characterization of fungal populations from infected lungs. (A) FACS gating scheme for sorting KN99-mCherry cells from infected mouse lungs into populations of "small", "mid", and "large" cells using forward scatter-area (FSC-A) to approximate size. (B) Representative India ink images of cells sorted from "small", "mid", and "large FSC-A gates and cell/capsule size measurements (N=97-120 fungal cells). Solid lines in the violin plots indicate the median and dotted lines mark quartiles. (C) Cell viability post-sort quantified by colony-forming unit (CFU) count (N=4). (D) Representative flow plot of DNA-content measured with DAPI staining. YNB (minimal YNB medium). (E) Percentage of total cells bound by complement (C3), surfactant protein D (SPD), mannose binding lectins A and C (MBL-A; MBL-C), IgA, and IgM. (F) Representative flow plots and quantification of wheat-germ agglutinin (WGA) stained cells to determine chitin exposure. (G) Representative flow plots and quantification of concanavalin A (ConA) stained cells to determine mannose exposure. (H)

Representative images and characterization of mannose exposure pattern after staining with ConA (N=50 fungal cells). All comparisons in Figure 2 were analyzed using one-way ANOVA and uncorrected Fisher's LSD (ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). All bar graphs display the mean.

Figure 3: Small *ex vivo* cells disseminate to extrapulmonary organs at a higher rate than larger cells, with mannose recognition by macrophages contributing to their clearance from the vasculature. (A) Fungal burden, measured by colony forming unit counts (CFU), at 3 hours post-inoculation (hpi) and 3 days post-inoculation (dpi) in mice intravenously-inoculated with small, mid, or large *ex vivo* cells (One-way ANOVA and uncorrected Fisher's LSD). (B) Fungal burden in mice inoculated with sorted *ex vivo* fungal cells and perfused at 3 hpi. Fungal burden was normalized to inoculated, but non-perfused control mice (Mann-Whitney U-test). (C) Fungal burden at 3 dpi in mice intracranially inoculated with sorted *ex vivo* fungal cells (One-way ANOVA and uncorrected Fisher's LSD). (D) Fungal burden at 1 dpi in mice pretreated with either clodronate or vehicle control (PBS) liposomes and then inoculated with small *ex vivo* cells and (Unpaired t test). (E) Fungal burden in the livers of mice (N=4-6) inoculated with *ex vivo* fungal cells or fungal cells harvested from minimal YNB (YNB) medium at 3 and 72 hpi. Points indicate the mean and the error bars indicate standard deviation. (F) Percentage of primary cultured murine liver macrophages with physically associated plastic beads or fungal cells (stained with Direct Yellow 96) after 4 hours. Dashed lines indicate 10-minute pretreatment with exogenous mannan (One-way ANOVA and uncorrected Fisher's LSD; N=2263-3197 total macrophages counted across 3 independent replicates). (G) Fungal burden in mice intravenously treated with indicated ligand 2-3 minutes prior to intravenous inoculation with small *ex vivo* fungal cells. Mice were perfused 10 minutes post-inoculation and fungal burden was normalized to vehicle (PBS) treated controls (Unpaired t-test; N=6 mice per group). All panels: ns: not significant * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. All bar graphs display the mean.

Figure 4: Small cells generated under host-like conditions *in vitro* replicate the dissemination profile of small *ex vivo* cells. (A) Percentage of total plastic beads or live cells in the indicated organ 3 hours post-intravenous inoculation (hpi). (B) Representative India ink images and quantification of cell and capsule size (N=100 fungal cells). YNB (minimal YNB medium); CAP (capsule inducing medium); CAP+CM (capsule inducing medium supplemented with 10% conditioned medium). (C) Fungal burden, measured by colony forming units (CFU), in

mice at 3 hpi. and 3 days post-inoculation (dpi). **(D)** Fungal cell survival in Dulbecco's modified Eagle's medium (DMEM) and **(E)** liver macrophage co-culture (multiplicity of infection of 1:1) measured as a percentage of input. **(F)** Fungal cell survival in mouse blood measured as a percentage of input. All comparisons in Figure 4 were analyzed using one-way ANOVA and uncorrected Fisher's LSD (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). All bar graphs display the mean.

Figure 5: Expression of phosphate acquisition genes correlate with cell and capsule size changes *in vitro* and are upregulated *in vivo*. **(A)** Venn diagram of genes upregulated or downregulated in different conditions relative to YNB-grown cells (YNB: minimal YNB medium; CAP: capsule-inducing medium; CAP+CM: capsule inducing medium supplemented with 10% conditioned medium). **(B)** Comparisons of the total number of differentially regulated genes among *in vitro*-grown populations. **(C)** Top-ten most significantly enriched gene ontology (GO) terms representing genes upregulated in CAP vs YNB-grown cells ($-\log_{10}$ (p-value)). Phosphate ion transport was #46. **(D)** Top-ten most significantly enriched GO-terms representing genes downregulated in CAP+CM vs CAP-grown cells ($-\log_{10}$ (p-value)). **(E)** Select phosphate acquisition and storage RNA-seq gene expression relative to YNB-grown cells (adjusted p values; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). All bar graphs display the mean and error bars the standard deviation.

Figure 6: Phosphate is sufficient to drive *C. neoformans* toward smaller morphotypes in alkaline host-like conditions and is required for morphogenesis *in vivo*. **(A)** Cell size measured using flow cytometry forward scatter (FSC-A) after growth in CAP-medium and supplementation with the indicated factor (YNB: minimal YNB medium; CAP: capsule-inducing medium; CAP+CM: capsule inducing medium supplemented with 10% conditioned medium). FSC-A median fluorescence intensity (MFI) was normalized to control (10% H₂O)-treated cells (one-way ANOVA and uncorrected Fisher's LSD). Bar graphs display the mean. **(B)** Representative flow cytometry plots for conditions in Figure 6A. **(C)** Total (cell+capsule) diameter measurements representing conditions in Figure 6A (Mann-Whitney U-test; N=200 fungal cells). Solid lines in the violin plots indicate the median and dotted lines mark quartiles. **(D)** pH in *C. neoformans* strain KN99-infected and mock-infected mouse lungs at 3, 10, and 17 days post-inoculation (dpi) (N=4 mice; one-way ANOVA and uncorrected Fisher's LSD; error bars represent standard deviation). All panels: ns: not significant * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. **(E)** Lung fungal burden in mice at 3, 10, and 17 dpi (One-way ANOVA and

uncorrected Fisher's LSD). The bar graph represents the mean. **(F)** Brain fungal burden in mice at 17 dpi (One-way ANOVA and uncorrected Fisher's LSD). The bar graph represents the mean. **(G)** Total (cell+capsule) diameter measurements at 3, 10, and 17 dpi of mice (Mann-Whitney U test; N=6 mice per timepoint; 50, 100, and 150 wild-type cells measured per mouse at days 3, 10, and 17 respectively. 50, 50, and 100 *pho4Δ* mutant cells measured per mouse at days 3, 10, and 17 respectively). **(H)** Representative India ink images of fungal cells quantified in 6G.

Supplemental Figure 1: Inoculum size alters the rate at which *C. neoformans* populations shift toward smaller cell size in the lungs and disseminate to the brain. **(A)** We inoculated mice with the indicated number of KN99-mCherry cells. The line graph shows the forward scatter-area (FSC-A) measurements at the indicated days post-inoculation (dpi), which estimates relative fungal size in the lungs (N=6 mice per timepoint). Points indicate the mean and the error bars indicate standard deviation. **(B)** Representative flow cytometry plots from mice inoculated with 10^4 KN99-mCherry cells / mouse. **(C)** Quantification of the area under the curve for the curves displayed in Figure S1A. Bar graphs display the mean fluorescence intensity and error bars indicate standard deviation. **(D)** Lung fungal burden in the same mice described in Figure S1A and quantified by colony forming units (CFU). **(E)** Brain fungal burden in mice at 17 dpi quantified by CFUs. Bar graphs display the mean. All comparisons in Figure S1 were analyzed using one-way ANOVA and uncorrected Fisher's LSD (ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Supplemental Figure 2: DNA content of *ex vivo* *C. neoformans* populations. Percentage of cells with 1C, 2C, and >2C DNA content in the indicated population. Values correspond to the representative flow cytometry plot in Figure 2D. Bar graphs display the mean.

Supplemental Figure 3: Macrophage depletion efficiency in the liver and spleen. **(A)** Representative flow cytometry plots displaying the frequency of CD45⁺, F4/80⁺ macrophages in the liver and spleen after treatment with either PBS (control) liposomes or clodronate liposomes. **(B)** Quantification of macrophage depletion measured as the ratio of macrophages in clodronate-treated mice to PBS-treated mice.

Supplemental Figure 4: Depletion of platelets or neutrophils does not affect the dissemination of small *ex vivo* cells. **(A)** Fungal burden in mice, measured by colony forming

units (CFU), following treatment with PBS (control) or anti-GPIIb α antibody to deplete platelets prior to intravenous inoculation with 10^5 small *ex vivo* cells / mouse. **(B)** Platelet count (K: units) in the blood before and after treatment with PBS (control) or anti-GPIIb α antibody. **(C)** Quantification of platelet depletion in Figure S4B. **(D)** Fungal burden in mice treated with PBS (control) or anti-Ly6G antibody to deplete neutrophils prior to intravenous inoculation with 10^5 small *ex vivo* cells / mouse. **(E)** Representative flow cytometry plots displaying the frequency of CD45⁺, Ly6C⁺, Ly6G⁺ neutrophils in the blood after treatment with either PBS (control) or anti-Ly6G antibody. **(F)** Quantification of neutrophil depletion measured as the ratio of neutrophils in anti-Ly6G antibody-treated mice to PBS-treated mice. All comparisons in Figure S4 were analyzed using unpaired t-tests (ns: not significant * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). All bar graphs display the mean.

Supplemental Figure 6: Screen for factors that induce cell size reduction. **(A)** Fungal cell size measured by forward scatter-area (FSC-A). Cells were cultured in capsule inducing medium (CAP medium) buffered to pH 7.4 or 6.3 before subculture (1:1) in fresh medium with 10% of the final volume being the indicated supplement solubilized in H₂O (CM: conditioned medium; GXM: glucuronoxylomannan; YNB: minimal YNB medium; PBS: phosphate buffered saline). **(B)** Fungal cell size measured by forward scatter-area (FSC-A) of cells cultured in titan cell medium and subcultured as described in Figure S5A. All comparisons in Figure S5 were analyzed using one-way ANOVA and uncorrected Fisher's LSD (ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). All bar graphs display the mean and error bars indicate standard deviation.

Supplemental Figure 5: Phosphate acquisition genes are upregulated in *ex vivo*-sorted cells. **(A)** Comparisons of the total number of differentially regulated genes among *ex vivo*-sorted populations. **(B)** Select phosphate acquisition and storage RNA-seq gene expression relative to YNB-grown cells (adjusted p values; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). All bar graphs display the mean and error bars the standard deviation.

Supplemental Figure 7: Phosphate is sufficient to induce cell body diameter and capsule thickness reduction in alkaline capsule-inducing medium and is required for morphogenesis *in vivo*. **(A)** Cell body diameter and **(B)** capsule thickness of cells cultured in capsule inducing medium (CAP medium) buffered to pH 7.4 or 6.3 before subculture (1:1) in fresh medium with 10% of the final volume being the indicated supplement solubilized in H₂O

(CM: conditioned medium; YNB: minimal YNB medium). Solid lines in the violin plots indicate the median and dotted lines mark quartiles. All comparisons in Figure S6 were analyzed using Mann-Whitney U-tests; N=200 fungal cells (ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). **(C)** Cell body diameter and **(D)** capsule thickness measurements at 3, 10, and 17 days post-intranasal inoculation (dpi) of mice (Mann-Whitney U test; N=6 mice per timepoint; 50, 100, and 150 wild-type cells measured per mouse at days 3, 10, and 17 respectively. 50, 50, and 100 *pho4Δ* mutant cells measured per mouse at days 3, 10, and 17 respectively).

METHODS:

Fungal strains and growth media

The following strains were used in this study: KN99α (Brown lab stock which was also the background for mutant strains), Bt63 (Brown lab stock, NCBI:txid1295841), 52D (ATCC 24067), R265 (ATCC MYA4093), WM276 (ATCC MYA-4071).

The following growth media were used in this study: YNB medium (yeast nitrogen base without amino acids [Difco catalog no. 291940], 2% glucose), CAP medium (10% Sabouraud's dextrose [Difco catalog no. 238230], buffered with 50 mM HEPES (pH 7.4) or MES (pH 6.3)), Titan cell medium (5% Sabouraud's dextrose, 10% fetal calf serum (GenClone catalog no. 25-550), 15 μM sodium azide, 50 mM HEPES (pH 7.4)).

To screen for factors that facilitated shifts in cell size, we picked single colonies from *C. neoformans* cells streaked on YPAD agar and cultured them overnight (12-18 hours) in YNB medium at 30 °C. We then sub-cultured those cells into YNB medium, CAP medium, or titan cell medium for 24 hours at an initial cell density of 10^5 cells/mL. YNB- and CAP-grown cells were cultured at 37 °C, while titan cell medium-grown cells were cultured at 37 °C and 5% CO₂. At this stage, conditioned medium (CM) was collected from the 24-hour YNB cultures by pelleting the fungal cells and filtering (0.22 μm pore size) the supernatant. 450 μl of the CAP and titan cell media cultures were sub-cultured into 450 μl fresh media and 50 μl (10% of the final volume) of one of the following supplements solubilized in H₂O: 10% H₂O (vehicle control), CM, 1% CM, 50 μg GXM, 50 μM Qsp1 peptide (Peptide 2.0: NFGAPGGAYPW), 50 μM Qsp1 scrambled peptide (Peptide 2.0: AWAGYFPGPNG), 10% 1XPBS (phosphate-buffered saline), 10% YNB medium; the following YNB medium supplements were added to a final concentration that equaled their concentrations in YNB medium: 2% glucose, 4.531 nM folic acid, 8.186 nM biotin, 250.6 nM copper sulfate pentahydrate, 531.4 nM riboflavin, 602.4 nM potassium iodide,

839.5 nM calcium pantothenate, 971.3 nM Sodium molybdate dihydrate, 1.186 μ M thiamine hydrochloride, 1.233 μ M ferric chloride hexahydrate, 1.458 μ M p-aminobenzoic acid, 1.945 μ M pyridoxine hydrochloride, 2.477 μ M zinc sulfate heptahydrate, 2.649 μ M manganese sulfate monohydrate, 3.249 μ M niacin, 8.087 μ M boric acid, 11.10 μ M inositol, 901.1 μ M calcium chloride dihydrate, 1.711 mM sodium chloride, 4.154 mM magnesium sulfate heptahydrate, 7.348 mM potassium phosphate monobasic, and 37.8mM ammonium sulfate. The supplemented cultures were incubated for another 24 hours at 37 °C, following which 400 μ l was aliquoted to estimate cell size by flow cytometry and another 400 μ l was aliquoted to measure cell and capsule size (see “fungal cell size measurements”) in India ink (Higgins catalog no. 44201).

824 **Mouse infection models and fungal burden analysis**

825 For intranasal inoculations, ~8-week-old female C57BL/6NJ mice (Jackson Laboratory)
826 mice were anesthetized with ketamine/dexmedetomidine hydrochloride (Dexdomitor) delivered
827 intraperitoneally. They were then suspended by their front incisors on a horizontal strand of
828 thread. Unless otherwise indicated, mice were inoculated intranasally with 2.5×10^4
829 *Cryptococcus* cells in 50 μ l of 1XPBS using a micropipette. The inoculum was placed dropwise
830 onto a nasal flare before being inhaled by mice. Ten minutes later, mice were intraperitoneally
831 administered the reversal agent atipamezole (Antisedan).

832 For intravenous inoculations, ~8-week-old female C57BL/6NJ mice (Jackson
833 Laboratory) were warmed under a heat lamp before being placed in a restraint. Mice were
834 inoculated via the tail vein with 10^5 *C. neoformans* cells or beads in 200 μ l of 1XPBS using 28-
835 gauge x 12.7 mm syringes. In order to competitively inhibit host interactions with fungal
836 components *in vivo*, we administered 400 μ g of GXM (see “GXM isolation), mannan (Sigma-
837 Aldrich catalog no. M7504), β -glucan (Millipore Sigma catalog no. 1048288), the chitin monomer
838 N-acetyl glucosamine (Vector Laboratories S-9002), or polyinosinic acid (Poly(I)) (Sigma-Aldrich
839 catalog no. 26936-41-4) in 200 μ l of 1XPBS 2-3 minutes prior to inoculation with fungal cells.

840 For intracranial inoculations, ~6-week-old female C57BL/6NJ mice (Jackson
841 Laboratories) were anesthetized with ketamine/dexmedetomidine hydrochloride as described
842 above. They were inoculated intracranially with 10^3 *C. neoformans* cells in 30 μ l of 1XPBS via a
843 26-gauge 1/2-inch needle. Following inoculation, mice were intraperitoneally administered the
844 reversal agent atipamezole (Antisedan; ~0.0125 mg/g).

845 Nonperfused mice were euthanized by CO₂ asphyxiation and cervical dislocation. Mice
846 that were intracardially perfused at time of death were anesthetized with isoflurane and perfused

in a nonrecirculating fashion before cervical dislocation. Fungal burden was assessed by excising organs and homogenizing them in 5 mL 1XPBS, washing the probe in between samples: 30 seconds 10% bleach, 45 seconds 70% EtOH, and 10 seconds sterile H₂O. Ten-fold serial dilutions of organ homogenate were plated on Sabouraud's agar containing 10 mg/ml gentamicin and 100 mg/ml carbenicillin and stored at 30°C for 3 days before counting CFUs.

All animal procedures were approved by the University of Utah Institutional Animal Care and Use Committee.

Fungal cell size measurements

C. neoformans cells harvested from laboratory growth medium or infected mouse lungs were fixed in 2% paraformaldehyde for 20 minutes. To visualize capsule, 4 µl of India ink with 4 µl of cell suspension on a microscope slide. At least 10 successive images were taken starting at one edge of the coverslip and moving across to the opposite side, as smaller cells tended to drift to the edge of the coverslips. Total diameter was measured from one edge of the capsule to the other. Cell body diameter was measured from one edge of the cell wall to the other. Capsule thickness was calculated as follows: capsule thickness = (total diameter – cell body diameter) / 2. The total number of cells counted for a given experiment is indicated in the figure legends.

FACS isolation of fungal cells

Mice were intranasally inoculated with *C. neoformans* KN99-mCherry (see “Mouse infection models”). At 14 dpi, lungs from 3 mice were excised and placed in 15 mL of 1XPBS. The three lungs were homogenized together using a mechanical tissue homogenizer. The homogenate was filtered first through a 70 µm cell strainer and then through a 40 µm cell strainer to prevent downstream clogging of the flow cytometer. Cells were centrifuged at 2195xg for 10 min. The supernatant was discarded and the pellet was resuspended in ~1mL 1XPBS. The suspension was filtered once more through a 70 µm cell strainer and was resuspended at ~1x10⁷ cells/mL in 1XPBS for FACS (BD FACS Aria). The FACS gating scheme is represented in **Figure 2**.

Isolation of polystyrene beads from mouse organs

Mice were intravenously administered (see “Mouse infection models”) 10⁵ green fluorescent polystyrene beads (Polysciences, Inc. catalog no. 17156-2 and 18241-2), and 3 hours later the indicated organs were harvested. Organs were homogenized with a mechanical tissue homogenizer in 5 mL tissue lysis buffer (100 mM Tris-Cl pH 8.0, 200mM NaCl, 5mM

EDTA, 0.2% (w/v) SDS), washing the probe in between samples: 15 seconds H₂O, 15 seconds 70% EtOH, and 15 seconds fresh H₂O.

Beads from each organ were concentrated via differential centrifugation. The homogenates were poured into a fresh 50 mL conical. The homogenization tubes were washed with 30 mL of H₂O and combined with the respective homogenized sample. The samples were filtered through cell strainers (70 µm pore size), after which another 10 mL of H₂O was passed through each strainer. The samples were centrifuged at 4480xg for 40 minutes. The supernatant was aspirated to the 5 mL mark and the samples were transferred to fresh 15 mL tubes containing 2 mL of 100% Percoll (GE Healthcare catalog no. 17-0891-01). Water was added to the 10 mL mark. The tubes were vortexed to mix and allowed to settle for 5 minutes before centrifugation at 1120xg for 45 minutes. The supernatant was aspirated to the 2 mL mark. 10 mL of H₂O was added to the samples before centrifuging at 4480xg for 20 minutes. The supernatant was aspirated and resuspended in 200-1000 µl 1XPBS for flow cytometry. Entire samples were analyzed to determine the number of beads per organ.

Fungal cell staining and flow cytometry analysis

Between 10⁵ and 10⁶ *C. neoformans* cells harvested from laboratory growth medium or infected mouse lungs were pelleted and fixed in 2% paraformaldehyde for 20 minutes. Fixed cells were pelleted and washed twice with 1XPBS. Cells were then resuspended in 1XPBS and stained with the desired antibodies/lectins/fluorescent dye.

To estimate DNA content, cells were stained for 10 minutes (room temperature) in 0.3 µg/mL 1XPBS+0.1% triton X-100. Mid-log phase fungal cells grown in YNB medium +/- 80 µg/mL benomyl (Agilent catalog no. PST-1245) for 24 hours were used to set 1C and 2C gates. Untreated mid-log phase fungal cells display two DAPI peaks (1C and 2C), while benomyl prevents cell division and traps cells at 2C.

To estimate fungal microbial associated molecular pattern exposure, cells were stained at room temperature with 5 µg/mL fluorescein-conjugated wheat-germ agglutinin (WGA, Vector Laboratories catalog no. FL-1021) for 15 minutes to detect exposed chitin or 50 µg/mL fluorescein-conjugated concanavalin A (ConA, Vector Laboratories catalog no. FL-1001) for 5 minutes to detect exposed mannose.

To estimate the percentage of cells bound by soluble host factors, we used the following antibodies: α-complement C3 (primary: mAb 11H9, ThermoFisher catalog no. MA1-40046; secondary: Mouse anti-Rat IgG2a, FITC, ThermoFisher catalog no. 11-4817-82), α-surfactant protein D (primary: polyclonal, Abcam catalog no. ab203309; secondary: Donkey anti-Rabbit

IgG, AlexaFluor 405, Abcam catalog no. ab175651), α -mannose binding lectin A (primary: mAb 8G6, Hycult catalog no. HM1035; secondary: Mouse anti-Rat IgG2a, FITC, Thermofisher catalog no. 11-4817-82), α -mannose binding lectin C (primary: mAb 14D12, Abcam catalog no. ab106046; secondary: Mouse anti-Rat IgG2a, FITC, Thermofisher catalog no. 11-4817-82), α -IgM (mAb II/41, FITC, BD Biosciences catalog no. 553437), α -IgA (mAb 11-44-2, FITC, SouthernBiotech catalog no. 1165-02). Cells were stained with primary antibodies (1:50 dilution) for 30 minutes on ice. When a secondary antibody was required, primary antibody labeled cells were pelleted, washed twice with 1XPBS and stained with the secondary antibody (1:50 dilution) for 20 minutes on ice.

After staining, cells were pelleted and washed twice with 1mL 1XPBS before being resuspended in 200-400 μ L of 1XPBS for flow cytometry.

GXM isolation

GXM was isolated as described previously (Wozniak and Levitz, 2009) with slight modifications. *C. neoformans* (KN99) cells were grown in 100 mL for 3 days at 37°C in YNB medium (YNB+2% glucose). Cells were centrifuged for 15 minutes at 12,000xg. The supernatant was filtered (0.45 μ m pore size) and 50 mL of supernatant was transferred to a 250 mL bottle. ~150 mL of 95% EtOH was added to the samples in 4-5 aliquots, mixing after each addition. Samples were stored at 4°C overnight to precipitate GXM. Samples were centrifuged for 30 minutes at 15,000xg, 4°C. The pellets were resuspended in 0.2M NaCl to a concentration of about 10 mg/mL (~50 mL per 250 mL bottle). Samples were transferred to a clean beaker stirred until samples were completely resuspended. 3 mg hexadecyltrimethylammonium bromide (CTAB) per 1mg precipitate was slowly added with stirring and low heat. Once solubilized, the samples were removed from heat and 250 mL of 0.05% CTAB was slowly added with stirring. The samples were moved to fresh 250 mL bottles and centrifuged at for 2 hours at 11,000xg, 4°C. The supernatant was discarded and the pellet was washed in 150 mL of 10% EtOH. The samples were centrifuge for 20 minutes at 15,000xg, room temperature. The supernatant was discarded and the pellets were resuspended in 50 mL of 1M NaCl. The samples were transferred to a clean beaker and stirred until completely resuspended. Approximately 2 volumes of 95% EtOH was added dropwise with stirring to precipitate the GXM while leaving the CTAB in solution. The samples were centrifuged in fresh 250 mL bottles for 20 minutes at 11,000xg, room temperature. The supernatant was discarded and the precipitate was dissolved in 2M NaCl. The resulting solution was placed in a snake-skin dialysis cassette with a 3,500 molecular weight cutoff (Thermo Fisher Scientific catalog no. 68035) and dialyzed

overnight against 1M NaCl. The samples were subsequently dialyzed against distilled H₂O for 14 days at 4°C. The distilled H₂O was changed every 1-2 hours for the first 2 days, and then 4-5 times a day for the next 6 days. For the last 2-6 days, the water was changed 2-3 times a day. The dialyzed samples were lyophilized and stored at -80 °C.

Cell-type specific depletion in mice

Hepatic and splenic macrophages were depleted from mice using clodronate liposomes. Mice were intravenously administered 200 µl of clodronate liposomes (Liposoma BV catalog no. C-010) or PBS control liposomes (Liposoma BV catalog no. P-010) 48 hours prior to inoculation with *C. neoformans* cells. Macrophage depletion efficiency in the liver and spleen was assessed on the day of inoculation via flow cytometry. Macrophages were identified as CD45+ (anti-CD45-eFluor450, eBiosciences catalog no. 48-0451-82) and F4/80+ (anti-F4/80-APC, eBiosciences catalog no. 17-4801-80).

Neutrophils were depleted from mice using anti-Ly6G antibodies. Mice were intraperitoneally administered 200 µg of anti-Ly6G (clone 1A8 BioXcell catalog no. BP0075-1) antibodies or PBS control at 24 and 2 hours prior to inoculation with *C. neoformans* cells. Neutrophil depletion efficiency in the blood was assessed on the day of inoculation via flow cytometry. Neutrophils were identified as CD45+ (anti-CD45-eFluor450, eBiosciences catalog no. 48-0451-82), CD11b+ (anti-CD11b-APC, eBiosciences catalog no. 17-0112-82), and Ly6G+ (anti-Ly6G-FITC, eBiosciences catalog no. 11-5931-82).

Platelets were depleted from mice using anti-GPIIb/IIIa antibodies. Mice were intravenously administered 80 µl of anti-GPIIb/IIIa antibodies (Emfret catalog no. R300) or PBS control 24 hours prior to inoculation with *C. neoformans* cells. Platelet depletion efficiency was determined by Hemavet 950FS (Drew Scientific Group) analysis of blood collected by cheek bleed 10 minutes prior to anti-GPIIb/IIIa administration and on the day of inoculation.

Liver macrophage isolation and infection

Liver macrophages were isolated from 8- to 10-week-old female C57BL/6NJ mice (Jackson Laboratory) according to established procedures (Li et al., 2014). Isolated liver macrophages were resuspended in complete DMEM (Dulbecco's Modified Eagle's Medium, GenClone catalog no. 25-500) supplemented with 10% fetal bovine serum (FBS, (GenClone catalog no. 25-550) and 100 U/mL Penicillin/Streptomycin and seeded into a T-25 flask at a density of 8×10^6 cells/flask. Macrophages were allowed to settle and adhere for 4 hours in a mammalian tissue culture incubator (37 °C, 5% CO₂). Non-adherent cells were then removed

from the dish by gently washing 3 times with 1XPBS (without divalent cations), leaving adherent macrophages. The media was replaced with 5 mL complete DMEM, and the cells were rested for 4 days. The media was replaced on the day after initial seeding, and then as needed.

Fungal infection and association with liver macrophages was assessed using the following procedures. The media was aspirated from rested liver macrophages and the cells were washed once with 1XPBS. Macrophages were lifted from the plate by treating them with 1mL Accutase (Corning catalog no. 25-058-CI) for ~5 minutes, tapping the plate to dislodge cells. 1 mL complete DMEM was added to the Accutase-treated cells, which were then centrifuged at 300xg for 5 minutes. Liver macrophages were resuspended cells in 0.5-1 mL complete DMEM and live cells were counted by mixing trypan blue (HyClone catalog no. SV30084.01) with cell suspension at a 1:1 ratio and counting clear cells on a hemocytometer. 40,000 live cells/well were seeded into 96-well plates in complete DMEM. The liver macrophages were allowed to settle and adhere overnight.

The next day, the inoculum was prepared by resuspending *C. neoformans* cells in 500 µl of 0.01% Direct Yellow 96 (AK Scientific 61725-08-4), vortexing briefly to mix, and incubating for 5 minutes on the benchtop. *C. neoformans* cells stained with Direct Yellow 96 were pelleted, washed once with 1 mL 1XPBS, and resuspended at a concentration of 8×10^6 cells/mL in serum-free DMEM. The supernatant was aspirated from the macrophages seeded in the 96-well plates and was replaced with 200 µl of serum-free DMEM. 10 µl of the inoculum (8×10^4 cells) was added to each well, resulting in a multiplicity of infection of 1:1. Macrophages and *C. neoformans* cells were co-cultured for 4 hours in a mammalian tissue culture incubator (37 °C, 5% CO₂). The medium was then aspirated from each well, and the cells were washed three times with 100 µl of 1XPBS to remove non-macrophage associated fungal cells. The remaining cells were fixed in 100 µl of 4% paraformaldehyde for 10 min. The paraformaldehyde was then removed and the cells were washed twice with 100 µl of 1XPBS and stained with 100 µl of DAPI (Sigma Aldrich catalog no. 28718-90-3) working solution (600 nM DAPI; 0.1% Triton X-100; in 1XPBS) for 5 minutes. The DAPI working solution was then removed and cells were washed twice with 100 µl of 1XPBS. Finally, 100 µl of 1XPBS was added to the wells to prevent desiccation. Cells were imaged on a Nikon widefield microscope, and the number of macrophages with associated *C. neoformans* cell(s) were counted (green-fluorescent due to Direct Yellow 96). Six images were taken per well in the same relative positions and every imaged macrophage was scored as having a physically associated fungal cell(s) or not, resulting in 2263-3197 macrophages counted per experimental condition across three independent replicates.

In order to assess liver macrophage killing of *C. neoformans* cells, liver macrophages were seeded in 96-well plates and infected with *C. neoformans* cells as described above. The plates containing *C. neoformans* and macrophages were incubated for 24 or 48 hours. After the desired time, the supernatant was collected from each well and transferred to a microcentrifuge tube. 200 µl of sterile distilled water was then added to each well in order to lyse the macrophages for 40 minutes at 37°C. The contents of each well were then mixed to collect every *C. neoformans* cell and combined with the respective supernatant now in a microcentrifuge tube. The wells were rinsed with 200 µl sterile PBS and added to the respective microcentrifuge tube. Serial dilutions of each of the well contents were plated on YPAD agar and incubated at 30°C for 2-3 days before counting CFUs. CFUs quantified at each timepoint were normalized to the CFUs in the inoculum in order to calculate the “% of input”. Each macrophage killing experiment was repeated on three different days with each experimental condition being performed in duplicate wells.

Fungal survival in blood

We collected whole blood from 8- to 10-week-old female C57BL/6NJ mice (Jackson Laboratory) to assess *C. neoformans* survival in blood. Mice were anesthetized with isoflurane, and whole blood was collected by cardiac puncture using 28-gauge x 12.7 mm syringes pre-coated with 0.5M EDTA. Blood was immediately transferred to tubes containing heparin to achieve a final concentration of 30 units of heparin per mL of blood. 100 µl of blood was added to individual wells of a 96-well plate. 100 *C. neoformans* cells in 5 µl of 1XPBS were used to inoculate each well. The plates were incubated in a mammalian tissue culture incubator (37 °C, 5% CO₂) without shaking. At the indicated timepoint, the well contents were transferred to fresh microcentrifuge tubes. The wells were washed with 100 µl of 0.05M EDTA, which was then added to the respective microcentrifuge tube. The entire contents of the microcentrifuge tubes were plated on YPAD agar and incubated at 30°C for 2-3 days before counting CFUs. CFUs quantified at each timepoint were normalized to the CFUs in the inoculum in order to calculate the “% of input”. Each experiment was repeated on three different days with each experimental condition being performed in duplicate wells.

RNA isolation, sequencing, and analysis

RNA sequencing data is publicly available at NCBI GEO (Accession number: GSE152784). *C. neoformans* cells were harvested from laboratory media and from infected mouse lungs for RNA isolation and sequencing. Three replicates were sequenced for each

sample with replicates being defined as follows. Approximately 10^7 cells were harvested from the indicated *in vitro* growth condition on three separate days. At least 10^6 *ex vivo* cells were isolated per sort gate each day (see “FACS isolation of fungal cells”). *Ex vivo* cells were isolated from the pooled lung homogenate of three mice. *Ex vivo* sample populations were isolated on two consecutive days and pooled for RNA isolation and sequencing, with the end result being that each sequenced *ex vivo* replicate represents fungal cells isolated from pools of six mice.

RNA was isolated using phenol-chloroform extraction, followed by Qiagen RNeasy column-based cleanup. Fungal cells were pelleted, flash frozen in liquid nitrogen, and lyophilized until completely dry. Lyophilized cells were resuspended in 750 μ l of TRIzol (ThermoFisher Scientific catalog no. 15596026) inside screw-cap microcentrifuge tubes. Approximately 50 μ l of 1-mm and 150 μ l of 0.5mm zirconium beads (BioSpec Products catalog no. 11079110z and 11079105z) were added to each sample and placed in a Biospec Products mini bead beater to lyse (12 x 2 min beating pulses, using sample blocks stored at -20 °C to prevent overheating). 150 μ l of chloroform was added to the lysed samples and incubated for 2-3min at room temperature. Samples were centrifuged for 15 minutes at 12,000 x *g*, 4°C. The aqueous phase was transferred to a fresh tube and combined with 5-10 μ g RNase-free glycogen as a carrier. 0.375 mL of isopropanol was added to each sample before incubating at least ~20 minutes on ice. The samples were centrifuged for 15 minutes at 12,000 x *g*, 4°C. The supernatant was discarded, and the isopropanol was removed by pipetting. The samples were then briefly air-dried before resuspension in 350 μ l of RLT buffer (Qiagen RNeasy) and 350 μ l 70% EtOH. At this junction, samples were run through Qiagen’s RNAeasy kit (Qiagen catalog no. 74104) with on-column DNase treatment.

cDNA libraries were generated with the NEBNext Ultra II Directional RNA Library Prep with rRNA Depletion Kit (New England BioLabs catalog no. E7760) by the High Throughput Genomics Core at the University of Utah. Sample libraries were sequenced on the Illumina NovaSeq 6000. One set of samples (primarily the *ex vivo* small, medium, and large cells) were stored by the High Throughput Genomics Core at -80°C for six months prior to library construction and showed more degradation products than replicates stored at -80°C for one month. We therefore limited our analysis and interpretation of the data from these samples.

The *Cryptococcus neoformans* var. *grubii* H99 genome and gene feature files were downloaded from Ensembl Fungi release 46 and the reference database was created using STAR version 2.7.2c with splice junctions optimized for 150 base pair reads (Dobin et al., 2013). Optical duplicates were removed from the paired end FASTQ files using clumpify v38.34 and reads were trimmed of adapters using cutadapt 1.16 (Martin, 2011). The trimmed reads were

aligned to the reference database using STAR in two pass mode to output a BAM file sorted by coordinates. Mapped reads were assigned to annotated genes in the gene feature file using featureCounts version 1.6.3 (Liao et al., 2013). The output files from cutadapt, FastQC, Picard CollectRnaSeqMetrics, STAR and featureCounts were summarized using MultiQC to check for any sample outliers (Ewels et al., 2016). Differentially expressed genes were identified using a 5% false discovery rate with DESeq2 version 1.24.0 (Love et al., 2014). Differentially expressed genes were run through FungiDB Gene Ontology (GO) Enrichment analysis, selecting for “biological process” with a p-value cutoff of 0.05 (Basenko et al., 2018; Stajich et al., 2012). Venn Diagrams were made with software from the Van de Peer group at Ghent University (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

Lung pH measurements

To measure lung tissue pH, mouse lungs were excised and immediately pierced with a micro pH electrode (Orion (ThermoFisher Scientific) catalog no. 9863BN). The electrode was completely embedded in the lung tissue before taking pH readings. Two readings were taken per mouse. One reading was taken from the left lobe of the lungs and a second reading was taken from one of the right lobes. These two readings were averaged to estimate lung pH for a given mouse.

Construction of the KN99:*pho4*Δ strains

The KN99:*pho4*Δ (*CNAG_06751*Δ) strains were constructed via homologous recombination-based replacement of the *pho4* genomic sequence with a nourseothicin resistance marker. The KN99:*pho4*Δ #1 strain was obtained from the KN99 gene deletion library (Fungal Genetics Stock Center, 2015 Madhani plates). The KN99:*pho4*Δ #2 strain was generated by PCR amplification of the nourseothicin resistance marker and ~1,000 base pair flanking regions from the KN99:*pho4*Δ #1 strain (primers used to amplify the knock-out construct: Forward: 5' AAAACGGCTGAAGGCTCGTTCT3', Reverse: CTTCTGCAAGGTGAAGTTCACG). The resulting *pho4* knock-out cassette was used to transform wild-type KN99 cells via biolistic transformation as described previously (Davidson et al., 2000). Putative transformants were PCR-verified by confirming the absence of the *pho4* open reading frame (Primers to verify the absence of the open reading frame: Forward: 5'CCATCTCAGATACCAACTCGCC', Reverse: 5'CAATTTGCTGAGAGCCATAGGC3') and the integration of the knock-out construct at the correct site (Primers to verify the correct 5' integration site: Forward: 5'AGAGCTATATGGTATGACGAAC3', Reverse:

5'TGTGCTGATCATCCGATGCCAC3' and the correct 3' integration site: Forward
5'TGTGGAGGATGGTGGGGAATAG3', Reverse: 5'GGACGTGAGCCAATAAGTTCCT3'.

Statistics

All statistical analyses, with the exception of RNA sequencing analysis, were performed with GraphPad Prism 8 software. Hypothesis-driven experimental comparisons were analyzed by unpaired t-test or one-way ANOVA and uncorrected Fisher's LSD if the data held a Gaussian distribution and a Mann-Whitney U-test if the data were not Gaussian. *In vitro* experiments were replicated three times unless otherwise stated. *In vivo* (murine infection) experiments were replicated twice unless otherwise stated.

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1348

A. Gating scheme:

C.

B.

D.

E.

F.

G.

H.

Mannose exposure pattern

A. Upregulated vs MIN

Downregulated vs MIN

B.

C.

Upregulated in CAP vs YNB

D.

Downregulated in CAP+CM vs CAP

E.

Genes involved in phosphate acquisition and storage