Bet-hedging antimicrobial strategies in macrophage phagosome acidification drive the dynamics of *Cryptococcus neoformans* intracellular escape mechanisms

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Abstract

The fungus *Cryptococcus neoformans* is a major human pathogen with a remarkable intracellular survival strategy that includes exiting macrophages through non-lytic exocytosis (Vomocytosis) and transferring between macrophages (Dragotcytosis) by a mechanism that involves sequential events of non-lytic exocytosis and phagocytosis. Vomocytosis and Dragotcytosis are fungal driven processes, but their triggers are not understood. We hypothesized that the dynamics of Dragotcytosis could inherit the stochasticity of phagolysosome acidification and that Dragotcytosis was triggered by fungal cell stress. Consistent with this view, fungal cells involved in Dragotcytosis reside in phagolysosomes characterized by low pH and/or high oxidative stress. Using fluorescent microscopy, qPCR, live cell video microscopy, and fungal growth assays we found that the that mitigating pH or oxidative stress abrogated Dragotcytosis frequency, that ROS susceptible mutants of *C. neoformans* underwent Dragotcytosis more frequently. Dragotcytosis initiation was linked to phagolysosomal pH and oxidative stresses and correlated with the macrophage polarization state. Dragotcytosis manifested stochastic dynamics thus paralleling the dynamics of phagosomal acidification, which correlated with the inhospitality of phagolysosomes in differently polarized macrophages. Hence, randomness in phagosomal acidification randomly created a population of inhospitable phagosomes where fungal cell stress triggered stochastic *C. neoformans* non-lytic exocytosis dynamics to escape a non-permissive intracellular macrophage environment.

Introduction

Cryptococcus neoformans is a pathogenic yeast that can reside in the phagolysosome of host macrophages[1]. Macrophages are critical cells in the pathogenesis of cryptococcosis, being involved in the containment of, and extrapulmonary dissemination of infection[2]. Macrophages are also involved in the pathogenesis of latent infection where the organism can survive for a long time in granulomas. Hence, *C. neoformans* survival in macrophages is critical for both the persistence and dissemination of infection. The yeasts, inhaled from the environment, manage intracellular survival by modifying the phagolysosomal environment in their own favor. The capsule of *C. neoformans* is comprised of several subunits including glucuronic acid and glucuronoxylomannan (GXM) which act as weak acids capable of buffering the environment to pH ~5[3,4]. *C. neoformans* also produces urease which disrupts phagolysosomal acidification by breaking down urea into carbon dioxide and ammonia, a weak base[5]. Additionally, *C. neoformans* are capable of exiting host macrophages through lytic exocytosis, non-lytic exocytosis (Vomocytosis)[6–8], or lateral cell-to-cell transfer (Dragotcytosis)[9].

We recently observed that macrophages use a bet hedging strategy in phagosomal acidification to maximize the antimicrobial properties of acidic pH for controlling the growth of ingested microbes[10]. By increasing the diversity of possible phagolysosomal pH, a population of macrophages will optimize its chances to inhibit pathogen growth based on pH alone. We also noticed that even small perturbations in this system could disrupt the bet-hedging strategy and that different pathogens employ various strategies to tip the odds in their own favor. *C. neoformans* interferes with the stochastic modulation of phagosomal pH by capsule buffering and urease activity[3,5]. Phagosomal pH varies with macrophage polarization such that alternate activated M2 macrophages are less hostile[10], in terms of pH, to ingested *C. neoformans* yeasts.

A fascinating aspect of the interaction with *C. neoformans* with macrophages is the phenomenon of fungal cell transfer between two macrophages, a process we have recently termed 'Dragotcytosis'[9]. Dragotcytosis results from the coordinated exocytosis and phagocytosis events between adjacent macrophages[9]. We also noted that Dragotcytosis is favorable to *C. neoformans* as macrophages with Dragotcytosis blockaded yield more colony forming units after 24 h than those who were not blockaded. Finally, we note that Dragotcytosis is an active process, only occurring with live *C. neoformans*[6]. When taken together, these findings suggest Dragotcytosis is triggered by *C. neoformans* for a purpose beneficial to the yeast. However, it is unknown what specifically triggers Dragotcytosis or how transferring between macrophages confers a benefit Notably, previous observations have established that phagolysosomal pH can modulate non-lytic exocytosis frequency, supporting the idea that pH has an important and complex role in the regulation of this system[11,12].

In addition to phagolysosomal pH there are other stressors in the phagosome that could conceivably affect the rate of Dragotcytosis. The oxidative burst and the release of reactive oxygen species into the phagolysosome are also potentially important. Both ROS generation and nitric oxide synthase (NOS) activity are upregulated in M1 macrophages in response to phagocytosed pathogens[13–15]. NOS

metabolizes arginine into nitric oxide and citrulline. Conversely, Arginase (Arg), upregulated in M2 macrophages) hydrolyzes arginine to ornithine and urea. Increased activity of either Arg or NOS will consume available arginine, resulting in lower efficacy of the other. Thus, M2 polarized macrophages have lower NOS activity and a less significant oxidative burst, which is thought to be one of the main reasons M2 macrophages are less effective at killing pathogens.

In this study, we analyzed the consequences of macrophage polarization and phagolysosomal stress on *C. neoformans* pathogenesis and outcome. We hypothesized that macrophages containing *C. neoformans* phagolysosomes that cause significant stress to the yeast by acidity and/or oxidative stress would be more likely to result in Dragotcytosis events than other *C. neoformans* containing phagolysosomes. In this regard, we tested whether the more fungicidal environment in M1 macrophages was more likely to trigger Dragotcytosis than the more permissive intracellular environment of M2 macrophages. Our goal was evaluating the hypothesis that Dragotcytosis is a fungal driven mechanism for escape of a hostile environment. We found that both hostile pH and reactive oxygen species (ROS) in the phagolysosome drive Dragotcytosis and use modelling to show the potential increasing benefit of Dragotcytosis events as a function of resident phagolysosome hostility.

Materials and Methods

Cell Strains and Culture Conditions

Cryptococcus neoformans species complex serotype A strain H99 was originally obtained from John Perfect (Durham, NC) and *C. neoformans* H99 actin-GFP created by the May lab[16]. Culture stocks were stored at 80 °C. Frozen stocks were later streaked onto YPD agar and incubated at 30 °C. Liquid suspensions of cryptococcal cultures were grown in YPD overnight at 30 °C. Cryptococcal cultures were heat killed by incubating at 65 °C for 1 h. Mutant strains were obtained from a previously published knockout library[17].

Bone marrow derived macrophages (BMDMs) were harvested from 6 week old C57BL/6 female mice from The Jackson Laboratory from hind leg bones and were differentiated by seeding in 10 cm tissue culture treated dishes in DMEM with 10% FBS, 1% nonessential amino acids, 1% penicillin-streptomycin, 2 mM Glutamax, 1% HEPES buffer, 20% L-929 cell conditioned supernatant, 0.1% beta-mercaptoethanol for 6 days at 37 °C and 9.5% CO_2. BMDMs were used for experiments within 5 days after differentiation. BMDMs were activated with 0.5 ug/mL LPS and 10 ng/mL IFN-γ for M1 polarization or 20 ng/mL IL-4 for M2 polarization for 16 h prior to experiments.

Phagolysosomal pH measurement

Phagolysosomal pH was measured using ratiometric fluorescence imaging involving the use of pH-sensitive probe Oregon green 488 as described in prior studies[5]. Briefly, Oregon green 488 was first

conjugated to mAb 18B7 using Oregon Green 488 Protein Labeling Kit (ThermoFisher). BMDMs were plated at a density of 1.25×10^5 cells/well on 24-well plate with 12 mm circular coverslip. Cells were activated with 0.5 µg/ml LPS and 100 U/ml IFN-γ or 20 ng/mL IL-4 as previously described at 37 °C in a 9.5% CO_2 atmosphere overnight. Prior to infection, 2 d old live H99, heat killed H99, or anti-mouse IgG coated polystyrene bead (3.75×10^6 cells or beads/ml) were incubated with 10 µg/ml Oregon green conjugated mAb 18B7 for 15 min. Macrophages were then incubated with Oregon green conjugated mAb 18B7-opsonized particles in 3.75×10^5 cryptococcal cells or beads per well. Cells were either centrifuged immediately at 350 x *g* for 1 min and incubated at 37 °C for 10 min or incubated at 4 °C for 30 min to synchronize ingestion and allow phagocytosis. Extracellular cryptococcal cells or beads were removed by washing three times with fresh medium, a step that prevents the occurrence of new phagocytic events. As an additional safeguard against new phagocytic events fresh media was supplemented with AlexaFluor 568 conjugated mAb 18B7 for 1 h to label extracellular particles. Samples were collected at their respective time points after phagocytosis by washing the coverslip twice with pre-warmed HBSS and placing it upside down on a MatTek petri dish (35 mm with 10 mm embedded coverslip well) with HBSS in the microwell. Images were taken by using Olympus AX70 microscopy with objective 40x at dual excitation 440 nm and 488 nm, and emission 520 nm. Images were analyzed using MetaFluor Fluorescence Ratio Imaging Software. Fluorescence intensities were used to determine the ratios of Ex488 nm/Ex440 nm that were converted to absolute pH values using a standard curve where the images are taken as above but intracellular pH of macrophages was equilibrated by adding 10 µM nigericin in pH buffer (140 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and appropriate buffer ≤ pH 5.0: acetate-acetic acid; pH 5.5-6.5: MES; ≥ pH 7.0: HEPES. Desired pH values were adjusted using either 1 M KOH or 1 M HCl). The pH of buffers was adjusted at 3-7 using 0.5-pH unit increments.

Cryptococcal Capsule Measurements

Capsule measurements were acquired by measuring exclusion zones on India Ink slides and phase contrast microscopy. To determine differences between polarized macrophage incubations, *C. neoformans* were harvested after being ingested by macrophages for 24 h. Extracellular yeasts were first removed by washing the cells 3 times with 1 mL HBSS. Macrophages were lifted from their plates, centrifuged at 350 x *g* for 10 min, and resuspended in 1 mL distilled H_2O. Cells were then passed through a 27 ¾ gauge needle 10 times and left incubating for 20 total min to ensure lysis. After lysis *C. neoformans* were pelleted via centrifugation at 2300 x *g* for 5 min and resuspended in 50 µL of PBS. Slides were prepared using 8 µL of cell mixture and 1.5 µL India ink, then imaged on an Olympus AX70 at 20x objective. Capsules and cell bodies were measured using a previously published measuring program[18].

NOS and Arginase Activity Measurements

BMDMs were seeded at 10^6 cell/well in 6-well treated tissue culture plates and activated overnight for M0, M1, or M2 polarization as previously described. Prior to infection, 2 d old live H99 (10^6 cells/well) were incubated with 10 µg/ml mAb 18B7 for 10 min. Macrophages were then incubated with opsonized

particles at MOI 1. Cells were either centrifuged immediately at 350 x *g* for 1 min or incubated at 4 °C for 30 min to synchronize ingestion and cultures were incubated at 37 °C for 10 min to allow phagocytosis. Extracellular cryptococcal cells were removed by washing three times with fresh medium, a step that prevents the occurrence of new phagocytic events. After 24 hours, cell supernatant was collected and the BMDMs were lysed with distilled water and 10 passages through a syringe with 23G needle. The supernatant was tested for NO levels via Greiss reagent kit (Millipore-Sigma G4410). Cell lysates were tested for arginase activity with arginase activity assay kit (Millipore-Sigma MAK112).

Cryptococcus neoformans Viability and Growth Inhibition

BMDMs were seeded (10^6 cells/well) in 6-well tissue culture plates. The cells were activated overnight (16 h) with IFNγ (0.02 µg/mL) and (0.5 µg) LPS for M1, IL-4 (20 ng/mL) for M2, or unstimulated for M0. The cells were then infected with a 2 d culture of *C. neoformans* opsonized with 18B7 (10 µg/mL) at an MOI of 1 for 2 or 24 h. At the respective timepoint, the supernatant was collected and total yeast cells as well as GFP positive yeast were counted by hemocytometer and fluorescence microscopy using an Olympus AX70. *C. neoformans* ingested by adherent macrophages were imaged on a Zeiss Axiovert 200M inverted scope in a live cell incubator chamber. Viability was calculated as the proportion of yeast cells expressing GFP-actin.

The collected, conditioned supernatant was then pelleted at 2300 x *g* for 5 min to remove debris and extracellular crypto. Supernantants were seeded with 5 x 10^4 cell / mL of *C. neoformans* and absorbance (600 nm) as well as fluorescence (GFP 488/510) was read at 0 and 24 h. Samples were prepared on glass slides with India Ink negative stain and imaged with an Olympus AX70. Growth was characterized by increase in OD600 and viability was measured as the proportion of GFP positive cells.

Dragotcytosis Frequency Measurements

BMDMs were seeded (5 x 10^4 cells/well) in MatTek dishes. The cells were activated overnight (16 h) with IFNγ (0.02 µg/mL) and (0.5 µg) LPS for M1, IL-4 (20 ng/mL) for M2, or unstimulated for M0. Cells in the MatTek dish were infected with Uvitex 2B (5 µm/mL) stained and 18B7 (10 µg/mL) opsonized *C. neoformans* at an MOI of 3 for 1 h, then supplemented with 2 mL fresh media and 18B7 mAb. In the case of drug trials this fresh media was also supplemented with bafilomycin A1 (100 nM), chloroquine (6 µm), fluconazole (20 µg / µL), or amphotericin B (0.5 µg / µL). MatTek dishes were then placed under a Zeiss axiovert 200M 10X magnification, incubated at 37 °C or 30 °C and 9.5% CO_2, and imaged every 2 min for a 24 h period. Images were then manually analyzed to identify ingested yeast cell outcomes.

Modelling

To simulate the effect of Dragotcytosis on a population of *C. neoformans* in phagolysosomes we generated 10,000 hypothetical phagolysosomal pH values based on the distribution of observed

phagolysosomal pH in M1 polarized bead containing macrophage phagolysosomes. Each value < 4 was replaced one time by randomly determining a new phagolysosomal pH from the same distribution to simulate a Dragotcytosis event from the initial macrophage to a random new one.

Data Analysis for Stochastic Signatures

Discrimination of deterministic vs. stochastic dynamics was achieved using the previously characterized permutation spectrum test[19] and the methods outlined in our previous work[10]. In short, processed datasets are aligned in a vector and separated into subgroups by a scanning window of 4 units. Each of the overlapping four-unit segments are then assigned a value from 0 to 3 based on their relative size (the largest value being 3 and the least being 0) in what is referred to as an ordinal pattern. The frequencies for each pattern are calculated for the entire dataset. Determinism is characterized by observations of ordinal patterns that do not exist (forbidden patterns), whereas stochastic dynamics are characterized by every ordinal pattern existing at a non-zero frequency. In this work, the original data is the time interval between the initiation of each instance of host cell escape within an experiment.

qPCR

BMDMs were seeded at 10^6 cell/well in 6-well treated tissue culture plates and activated overnight for M0, M1, or M2 polarization as previously described. Prior to infection, 2 d old live H99 or inert beads (10^6 particles/well) were incubated with 10 µg/ml mAb 18B7 for 10 min. Macrophages were then incubated with opsonized particles at MOI 1. Cells were either centrifuged immediately at 350 x *g* for 1 min or incubated at 4 °C for 30 min to synchronize ingestion and cultures were incubated at 37 °C for 10 min to allow phagocytosis. Extracellular cryptococcal cells were removed by washing three times with fresh medium, a step that prevents the occurrence of new phagocytic events. After 24 hours, BMDMs were resuspended in TRIzol reagent and frozen at -80°C. Total RNA was isolated from frozen cell cultures using the TRIzol reagent per the manufacturer's suggestions (InVitrogen, Carlsbad, CA). The RNA was precipitated in isopropanol and then subjected to additional purification using the RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was reverse transcribed to generate cDNA using a First-Strand Synthesis kit (Amersham) and random hexamers as primers. The cDNA was then used as the template for quantitative PCR using an iCycler Thermal Cycler Real-Time PCR machine (Bio-Rad, Hercules, CA). The products of PCR amplification were detected with Syber green fluorescent dye and the relative expression of each gene of interest expressed with reference to that of glyceraldehyde phosphate dehydrogenase (GAPDH). The PCR products were analyzed on Tris-Acetate-EDTA agarose gels to confirm its correct size.

Macrophage Preference Assay

To ascertain whether *C. neoformans* preferred residence in macrophages as a function of host cell polarization we carried out cell transfer experiments in macrophage populations consisting of mixed M0, M1, and M2 polarized cells. BMDMs were seeded at 3 x 10^4 cells per well in a 24 well tissue culture plate loaded with circular coverslips then activated overnight for M1 polarization as previously described.

Three separate populations of BMDMs were seeded in dishes large enough to account for an additional 3 x 10^4 cells per well per condition and activated overnight for M0, M1, or M2 polarization as previously described. The initial population of M1 BMDMs on coverslips was infected with 6 x 10^4 18B7 opsonized *C. neoformans* yeasts per well for 1 h. The remaining three populations of uninfected BMDMs were stained with CellTracker CMFDA (Green). After the 1 h infection the three labeled populations of BMDMs were lifted with cellstripper and added to the unlabeled, infected M1 coverslips so that each coverslip had two populations of macrophages: 1. Unlabeled and infected M1 and 2. Labeled but uninfected M0, M1, or M2. The coverslips were then incubated for 24 h at 37 °C and 9.5% CO_2 for 24, 48, or 72 h. The cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and coverslips were mounted on slides with ProLong Gold Antifade Mountant (Thermofisher) and imaged on an Olympus AX70. For each field of view the total number of each macrophage population was counted along with how many infected macrophages in each population. To correct for floating cells and ensure a proper count of total cells, a hemocytometer was used to enumerate the total number of floating cells in each sample. We assumed any floating cells were previously evenly distributed across the original sample and thus used the percent of floating cells to estimate the number of missing cells in any given sample using the formula below:

$$T_G' = \left\lfloor \frac{T_G}{1 - \frac{SN_G}{I_G}} \right\rfloor$$

T_G = Total green positive cells counted on a given field of view

SN_G = Total number of green cells in supernatant per hemocytometer

I_G = Total amount of green cells plated initially

Modeling Dragotcytosis Dynamics

A population of 40,000 cells was generated consisting of 10,000 cells each of infected M1, uninfected M1, uninfected M2, and uninfected M0 macrophages. During each iteration of Dragotcytosis each infected cell had a random chance of experiencing Dragotcytosis or Vomocytosis based on observed frequencies *in vitro*. A Vomocytosed yeast was considered removed from the modeling experiment as we did not have an accurate way to model reuptake, and a Dragotcytosed yeast was randomly transferred to one of the four types of macrophage cells with no bias introduced. 10 rounds of sequential Dragotcytosis were modeled and the entire experiment was replicated 100 times.

Statistical analysis

Specific statistical tests for each experiment are denoted in the figure legends with tests for multiple hypotheses. When comparing frequency of host cell exit strategies, we compared mutant strains to the wild-type strain with a test of equal proportions and Bonferroni multiple hypothesis correction.

Results

Macrophage Polarization State Affects Frequency of Host Escape Events

Given our prior findings showing different acidification dynamics in differently polarized macrophages[10], we investigated whether macrophage polarization effected the frequency of Dragotcytosis. First, to ensure that our results were not confounded by differing population densities of macrophages resulting in higher or lower frequencies of events, we compared Dragotcytosis frequency to macrophage density and found no correlation (**Supplemental Figure 1**). Analysis of the pH distribution in cryptococcal phagosomes, M2 macrophages are more hospitable that M1 macrophages *C. neoformans,* having a higher proportion of macrophages at optimal growth pH (~5.5) and few phagolysosomes at inhibitory ranges (≤ 4) (**Figure 1**A). Using M0 macrophages as a baseline measure of Dragotcytosis frequency, we find that M1 polarization increases Dragotcytosis frequency while M2 polarization completely abrogates Dragotcytosis. (**Figure 2**, **Supplemental Figure 2**). This result correlates the frequency of Dragotcytosis with the relative hospitality of each type of macrophage phagolysosome. When taken in context with the previously reported insight that Dragotcytosis is an active process which is beneficial for *C. neoformans* survival[9], these data suggest that Dragotcytosis is more likely to be initiated in hostile phagolysosomes.

Cryptococcus neoformans Capsule and Cell Body Size are Unaffected by Polarization

The *C. neoformans* capsule is one of the most important virulence factors and determinants of infection outcome and a powerful modulator of phagolysosomal pH[3]. Capsule growth could modulate phagolysosomal pH by mechanically stressing the phagolysosomal membrane as it enlarges, causing the phagolysosome to become leaky[20]. Several aspects of cryptococcal pathology and phagolysosomal outcome are correlated to capsule size including buffering potential[3,20]. To investigate whether the changes in pH in differently polarized macrophages were due to capsule differences, we measured capsule sizes after infection of differently polarized macrophages. We found that the average and median capsule sizes did not appreciably differ between populations of *C. neoformans* ingested by differently polarized macrophages, nor did the average and median cell body sizes (Supplemental Figure 3). Furthermore, if phagolysosomal pH was modulated by capsule size, we would expect to observe a correlation between cell radius and phagolysosomal pH. We found no indication of a correlation between particle size and phagolysosomal pH, even if we began measuring clusters of multiple particles within a single phagolysosome (**Supplemental Figure 4**).

Macrophage Polarization Modulates Oxidative Response and Affects C. neoformans Viability

To determine whether the macrophage oxidative response varied between polarization states in a manner that would support our hypothesis on the triggers of Dragotcytosis frequency, we measured NOS and Arg1 activity between macrophage populations. M2 macrophages had the lowest NOS activity and the highest Arg1 activity, while M1 macrophages had the highest NOS activity with the lowest Arg1 activity. (**Figure 1**B and C). This data supports the notion that M2 macrophage phagolysosomes are more

hospitable for *C. neoformans.* Additionally, by comparing enzymatic activity between macrophages infected or uninfected with *C. neoformans* we found that infection lowers NOS activity in M0 and M2 populations while increasing Arg1 activity in M1 populations (**Figure 1**B and C). Each of these changes would result in more hospitable phagolysosomes for *C. neoformans*.

We used GFP expression behind an actin promoter as a surrogate for viability for internalized *C. neoformans* in macrophages. Whether or not GFP negative cells are truly "dead" is a philosophical question beyond the scope of this paper, but a cell that does not express actin would certainly be incapable of division. Thus, we explored viability in terms of fungistatic rather than fungicidal qualities. We found that, at two hours post infection, *C. neoformans* ingested by M1 or M0 macrophages were less viable than those in M2 macrophages (**Figure 1**D). Additionally, we investigated the viability of extracellular *C. neoformans* and found that at 24 h post infection extracellular yeasts cultured with M1 macrophages are still inhibited (**Figure 1**E and F). This is consistent with prior reports that macrophages can inhibit extracellular *C. neoformans* cells[21,22]. We hypothesize this inhibition is due to the buildup of NO in the extracellular media. We found no extracellular crypto at 2 hours post infection consistent with our observations that exocytosis occurs hours after ingestion.

Finally, to investigate whether *C. neoformans* infection modulates macrophage polarization during infection, we performed qPCR on RNA harvested from differently polarized macrophages 24 h after infection. We found that *C. neoformans* infection alone, in M0 macrophages, skews toward M2 polarization (**Figure 1**G). This effect is augmented in M2 macrophages activated beforehand with IL-4 suggesting that *C. neoformans* may actively skew macrophages toward the most hospitable state. We did not see such synergism when macrophages were stimulated with IFNγ and LPS, or when macrophages were not stimulated.

Phagolysosome pH is Associated with Dragotcytosis Frequency

To probe whether phagosomal pH modulated Dragotcytosis frequency rather than other downstream effects of polarization, we altered phagosomal pH in M1 macrophages with various drugs. Chloroquine is a weak base that localizes to phagolysosomes and can be used to alkalize macrophage phagolysosomes *in vitro*, and at 6 µM buffers phagolysosomes to a pH optimal *C. neoformans* growth. Bafilomycin A1 is a V-ATPase inhibitor which prevents protons from being pumped into the organelle. Alkalization of M1 cryptococcal phagosomes with either drug abrogated Dragotcytosis (**Figure 2**), consistent with the notion that phagolysosomal acidification triggers lateral cell transfer. Interestingly, treatment with sub-inhibitory concentrations of Fluconazole (20 µg / µL) and Amphotericin B (0.5 µg / µL) also reduced the frequency of Dragotcytosis.

Disrupting Macrophage Anti-Fungal Function Lowers Dragotcytosis Frequency

If Dragotcytosis is triggered by fungal cell stress, then further disrupting the macrophages' ability to inhibit *C. neoformans* would be expected to lower its overall frequency. We investigated this using two

methodologies. First, we infected macrophages with *C. neoformans* induced to grow large capsules. The sheer size of cell as well as the anti-phagocytic properties of the capsule are known to inhibit macrophage uptake and promote fungal survival. We found that capsule induced *C. neoformans* Dragotcytosed less frequently and Vomocytosed dramatically more often compared to wild-type, resulting in no overall change when comparing both types of events together (**Figure 2**A). Next, we infected macrophages with wild-type *C. neoformans* at 30 ^{o}C, a temperature optimal for the fungus and suboptimal for the macrophage. We again found that Dragotcytosis was abrogated (**Figure 2**A). Taken together these results support the hypothesis that Dragotcytosis is induced when *C. neoformans* are stressed or overwhelmed by host macrophage defenses. Finally, we investigated whether melanized *C. neoformans* Dragotcytosed at different frequencies as melanin is known to protect from phagolysosomal stressors[23–25]. We found that melanized yeast underwent Dragotcytosis at a reduced rate (**Figure 2**A). This especially raised the possibility that Dragotcytosis could be triggered by general cellular stress within the phagolysosome rather than pH specifically.

Oxidative Stress Modulates Dragotcytosis Frequency

To further probe whether oxidative stress had a role in triggering host cell escape, we investigated several mutant *C. neoformans* strains deficient in various oxidative stress mitigation pathways including: Superoxide Dismutases 1 and 2 (SOD1, SOD2), Catalases 1 and 3 (Cat1, Cat3), Thiorexodin 1 (TXN), and Glutathione Peroxidases 1 and 2 (GPx and GPx2)[17]. ΔSOD1, ΔCat1, ΔCat3, ΔTXN, ΔGPx, and ΔGPx2 strains of *C. neoformans* underwent Dragotcytosis at an increased frequency compared to the wild-type parental strain KN99, while Dragotcytosis frequency in ΔSOD2 and ΔTXN2 remained unchanged (**Figure 2B**). Overall, these data support the notion that Dragotcytosis is triggered by a stressed yeast, and that increasing stress on the yeast results in increased Dragotcytosis.

Time to Host Exit Initiation is Stochastic for Exocytosis Events

If Vomocytosis and/or Dragotcytosis are triggered by hostile pH and final pH is determined stochastically from a normal distribution[10], then these host cell exocytosis events should also display stochastic dynamics resembling those of the upstream pH trigger. To explore whether the stochasticity of phagolysosome acidification is preserved in this system, we measured the time at which each process occurs throughout a series of videos of macrophages infected with *C. neoformans.* We found that while the time of exocytosis events was not normally distributed, these events were triggered stochastically with no forbidden ordinal patterns observed (**Figure 3**A and B). The absence of forbidden ordinals implies that the process is not deterministic, ruling out chaotic dynamics.

Simulation Data Suggests Dragotcytosis at low pH Benefits Cryptococcal Cells

We hypothesized that triggering Dragotcytosis, or non-lytic exocytosis, in phagolysosomes of low pH would benefit *C. neoformans* cells by lowering the total number of yeasts within inhibitory phagolysosomes. The argument for non-lytic exocytosis is intuitive, in that the yeast leaves the phagolysosome altogether and could only result in fewer inhibitory phagolysosomes. Dragotcytosis

however is more nuanced as there is no obvious way for *C. neoformans* to know whether the new phagolysosome will be more or less hostile to the yeast. To explore this hypothesis, we modeled phagolysosomal pH distributions based on observed pH measurements of bead containing BMDM phagolysosomes and the established bet-hedging strategy of host macrophages in silico. We found that if a *C. neoformans* yeast were to trigger Dragotcytosis even just a single time if their original phagolysosome dropped to pH < 4, it would be enough to drop the proportion of inhibitory phagosomes from ~15% to ~1% (**Figure 3**C and D). The actual effect on *C. neoformans* survival would be even more pronounced since Dragotcytosis events are not limited to one event per yeast in vitro/vivo. We then investigated how beneficial Dragotcytosis would be at a range of pH cutoffs, assuming that an ingested *C. neoformans* would be equally likely to migrate into an M0, M1, or M2 macrophage. In the context of pH, the benefit of a Dragotcytosis event increases steadily the further away from an optimal pH the original phagolysosome is (**Figure 3**E).

Cryptococcus neoformans Does not Display an Inherent Bias for Destination Macrophages

While there is no obvious and apparent method for *C. neoformans* to specifically target M2 macrophages as the Dragotcytosis destination, such a system could still be possible. The donor and acceptor macrophages maintain physical contact during the Dragotcytosis process, and an immune synapse could transduce signals detectable by the ingested yeast. To investigate whether *C. neoformans* shows a preference during Dragotcytosis we compared the rates of transfer from infected M1 macrophages to uninfected M1 or M2 macrophages. If there is a preference, then we would expect to observe an increased amount of *C. neoformans* residing in M2 macrophages 24 HPI; if there were no preference, we would expect to see equal transfer into M1 and M2 macrophages. Instead, we see roughly an equivalent amount of transfer into M1 and M2 macrophages with slightly less transfer into M0 macrophages (**Figure 4**A and B). Based on a model of Dragotcytosis dynamics, we expect it would take approximately 4 to 5 sequential transfer events before we would be able to detect differences in population based on random chance alone (**Figure 4**C).

Proposed Model for Triggering Dragotcytosis

Dragotcytosis requires live *C. neoformans* and its frequency is modulated by variables that effect stress on the fungal cell (Supplemental Table 1). We synthesize these observations into a model whereby stress in the phagolysosome, resulting in fungal cell damage, triggers a program for exiting the host cell. We know that exocytosis is associated with actin flashes[7,26] and posit that stressed cells activate a program that manipulates the host cell to promote a choreography of events resulting in transit from the phagolysosome to outside of the host cell (Figure 5). If these events lead to exocytosis at the cell membrane region in contact with an adjoining macrophage, then non-lytic exocytosis becomes Dragotcytosis. Given that non-lytic exocytosis has been observed in mammalian, fish, insect, and protozoal cells the exit program is likely to target conserved functions in eukaryotic phagocytic cells[6,8,27–29]. As to whether apposition of two macrophages induces changes in the infected cell that are sensed by the fungal cell is unknown and a subject for future investigation.

Discussion

Dragotcytosis is a cellular process by which *C. neoformans* yeasts within a host macrophage phagolysosome transfer to another, proximal macrophage without lysis of the initial host[6,8,9,11]. During recent investigations into the receptors used in Dragotcytosis and into the phagolysosomal acidification dynamics of macrophages, we found a correlation between phagolysosomal hospitality and the frequency of Dragotcytosis and suggesting that Dragotcytosis is beneficial to, and triggered by, ingested *C. neoformans*[9,10]. This led us to hypothesize that fungal discomfort in the phagosome was a trigger for cell-to-cell transfer and in this study, we demonstrate that cellular stress on *C. neoformans* leads to increased Dragotcytosis frequency.

Previous studies have shown that M2 macrophages and Th2 skewed immune responses are more permissive to *C. neoformans* infection[30–33]. Our initial observation that M2 macrophages have, on average, higher pH phagolysosomes compared to M1 macrophages when containing inert particles offers a partial explanation of why these macrophages are more hospitable. Specifically, M2 macrophage populations had the fewest phagolysosomes at an inhibitory pH (pH < 4) and the most phagolysosomes in an optimal pH range (5 < pH < 6) for fungal growth[20]. Additionally, we noticed that *C. neoformans* underwent fewer Dragotcytosis events when residing in M2 macrophage phagolysosomes compared to yeast cells resident in M1 macrophages. From this correlation, we hypothesized that if the phagolysosomal pH dropped too low for an ingested *C. neoformans* to counter with its polysaccharide capsule or urease activity[3,5,34,35], then it triggered either non-lytic exocytosis or Dragotcytosis as an escape mechanism that would bring the yeast cell to the less acidic extracellular environment or into a phagolysosome of more hospitable pH, respectively.

Since phagolysosomal pH is stochastically determined with a distribution in which most phagosomes have pH > 4, it is likely that *C. neoformans* residing in a phagosome with pH < 4 would find a more hospitable home after a Dragotcytosis event[10]. Consequently, we probed how Dragotcytosis could modulate the effectiveness of the macrophage acidification bet hedging strategy via in silico modeling and found that even a single Dragotcytosis event triggered for *C. neoformans* residing in low pH phagolysosomes would significantly increase the likelihood of finding a less acidic phagosome. These findings could also help explain how *C. neoformans* replicates faster inside macrophages *in vivo* and *in vitro* than in the extracellular space, which has been attributed to faster replication in the acidic pH of the average phagosome[36–38]. Despite not all phagosomes being conducive to faster fungal growth, sequential Dragotcytosis events could lead *C. neoformans* to find phagolysosomes more permissive to rapid growth during an infection. These results fit well with the known data of *C. neoformans* infection in human macrophages. Human macrophage phagolysosomes acidify with different dynamics than those of mice. For example, human M2 macrophage phagolysosomes acidify to a lower pH than mouse macrophage phagolysosomes[39] and, perhaps as a result, humans are markedly resistant to *C. neoformans* infection which poses little threat to most immune competent hosts. Additionally, human macrophages undergo both Vomocytosis and Dragotcytosis with much greater frequency than mouse macrophages *in vitro*, consistent with the hypothesis that both processes are triggered by fungal residence in hostile phagolysosomes.

To probe deeper into whether there was a causal association between average phagolysosomal pH of M1 macrophages and the frequency of Dragotcytosis, we treated macrophages with either chloroquine or bafilomycin A1, keeping phagolysosomal pH near the optimal growth range of *C. neoformans* and comparable to the observed average phagolysosomal pH of M2 macrophage populations. We found that both chloroquine and bafilomycin A1 drastically reduced Dragotcytosis events, supporting the hypothesis that cellular stress is the stimulus for Dragotcytosis. We are confident that the drug treatments themselves did not have a direct effect on *C. neoformans* growth, virulence, or survivability at the concentrations used but rather modulated the host macrophage response. Chloroquine does not significantly reduce *C. neoformans* growth or viability unless ingested by macrophages and at concentrations higher than used in these experiments[40]. Similarly, at high concentrations Bafilomycin A1 has antifungal[41] and even inhibits *C. neoformans* melanization[42] but the concentrations used here are much lower than those associated with antifungal effects[43]. We also treated cells with sub-inhibitory concentrations of fluconazole and amphotericin B, to explore whether these drugs would also stress the yeast in a way that promotes Dragotcytosis[44]. Interestingly, both antifungal drugs reduced the overall frequency of Dragotcytosis possibly because the concentrations were still too disruptive to fungal metabolism and the yeast was too compromised to transfer. In this regard, both drugs affect fungal membrane ergosterol integrity and fungal lipids may be required for the Dragotcytosis process. Alternatively, ergosterol disruption is not sensed by the fungal machinery that triggers Dragotcytosis.

To further investigate the oxidative trigger hypothesis, we characterized Dragotcytosis frequencies of several *C. neoformans* strain KN99 mutants from a knockout library during infection. We selected a variety of potential candidates based on known pathways relevant to our cellular stress hypothesis. We found that SOD1, Cat1, Cat3, TXN, GPx, and GPx2 deficient mutants underwent Dragotcytosis drastically more frequently than the wild-type parental strain, supporting the notion that Dragotcytosis is triggered by the accumulation of oxidative stress. Interestingly, knocking out SOD2 and TXN2 did not increase frequency of Dragotcytosis which may reflect the nature of the phagolysosomal oxidative stress, as SOD1 and SOD2 are known to have different efficacy against different oxidative stresses[45].

If oxidative stress triggers Dragotcytosis, then reducing stress within the phagolysosome should reduce the frequency of Dragotcytosis. Melanin is known to protect *C. neoformans* from oxidative stress[46]. Melanized *C. neoformans* triggered Dragotcytosis at a lower frequency compared to non-melanized *C. neoformans*. The cryptococcal capsule is known to protect against both pH and ROS in macrophages. Macrophages infected with *C. neoformans* with large capsules manifested Dragotcytosis at a reduced rate, though Vomocytosis occurred at an increased rate. It is less clear whether Vomocytosis is triggered by the yeast or host as heat killed *C. neoformans* have been observed to undergo Vomocytosis, albeit at a drastically reduced rate[7], raising the question of what could trigger one pathway opposed to the other. When macrophage infection was done at a temperature optimal for *C. neoformans* and suboptimal for macrophages: 30 ^{o}C we again found that Dragotcytosis frequency was reduced.

We next considered whether *C. neoformans* induced a bias toward M2 macrophages as acceptor macrophages in the Dragotcytosis process. Logically it would benefit the yeast as M2 macrophages were found to be more permissive, but a mechanism by which the yeast could sense interaction with an M2 macrophage and initiate the Dragotcytosis process all from within the phagolysosome is difficult to imagine. Thus, we designed an experiment in which infected M1 macrophages would be seeded with labelled and uninfected M1 or M2 macrophages and we would compare the rate of transfer. If there was a preference, we should see an unequal rate of transfer between the two populations. Instead, we observed equal rates of transfer into both macrophage types. We expect that, given enough time, the distribution would shift to favor M2 macrophages by random chance. However, this was not observed due to the infrequency of sequential macrophage-to-macrophage transfers by a single cryptococcal cell during the first 24 hours of experimental observation. Thus, we find it more likely that *C. neoformans* initiates a transfer without prior knowledge of the receiver macrophage and instead would continue transferring until a hospitable phagolysosome is discovered by chance.

Dragotcytosis was stochastically initiated, reflecting the dynamics of phagolysosome acidification[10]. However, unlike phagosomal acidification, which manifested a normal distribution, the distribution of Dragotcytosis as a function of time was skewed away from normality. Therefore, while phagolysosomal pH contributes to triggering Dragotcytosis, it is likely not the only important trigger, a hypothesis supported by previous observations that inhibiting pH buffering via urease mutation did not increase Dragotcytosis events as would be expected if the process was triggered by pH alone[5]. Instead, Dragotcytosis may be triggered by an accumulation of diverse sources of cellular stress encountered in the phagolysosome. Whereas the endpoint of phagolysosomal acidification represents a bet hedging strategy by macrophages to control ingested microbes[10], *C. neoformans* also appears to be employing a bet hedging strategy in exocytosis that is triggered by the bet-hedging strategy in macrophage phagolysosome acidification. In fact, *C. neoformans* may engage in its own bet hedging strategy wherein Dragotcytosis is triggered past a certain threshold of stress in which it becomes more likely that the ingested yeast will migrate to a more hospitable phagolysosome. This results in the interesting situation whereby a defensive strategy by the host cells is co-opted for survival by the fungal cell. Hence, chance outcomes in the phagolysosome trigger events that produce chance outcomes in macrophage exocytosis, implying connectivity between these two cellular processes with regards to their system dynamics. Stochastic processes are random and thus inherently unpredictable, suggesting a fundamental unpredictability to cellular processes that could extend to making host-microbe interactions not predictable.

In summary, our results consistently show that conditions that increase or decrease *C. neoformans* oxidative stress are associated with enhanced and reduced Dragotcytosis, respectively. However, it may be impossible to narrow down Dragotcytosis triggers to one specific stressor. The drugs that inhibit phagosome acidification can also inhibit autophagy, and vice versa, making it difficult to parse out the effects of only one of these systems at a time. Even the generation of reactive oxygen species is innately tied to these processes, and phagolysosomal pH itself, with the concentration of ROS in the phagolysosome increasing alongside pH with previously measured concentrations of 50 µM $O_2^{\bullet-}$ at pH 7.4 and 2 µM at pH 4.5[13]. We find it more likely that a combination of cellular stresses triggers

Dragotcytosis and our findings suggest that initiation of Dragotcytosis is a downstream product of overwhelming stress in the phagolysosome. We note how the normally distributed phagolysosomal pH following phagocytosis randomly includes some phagolysosomes that are inhospitable to fungal cells which, in turn, triggers exocytosis with its own stochastic dynamics with a unique distribution, as other factors contribute to the exit phenomenon. Hence, stochastic dynamics in phagosomal acidification beget stochastic dynamics in non-lytic exocytosis, implying a fundamentally unpredictable host-microbe interaction at the level of cellular organelles. Such unpredictability at the cellular level is a likely contributor to our inability to predict the outcome of host-microbe interactions at the organismal level[47].

Acknowledgements

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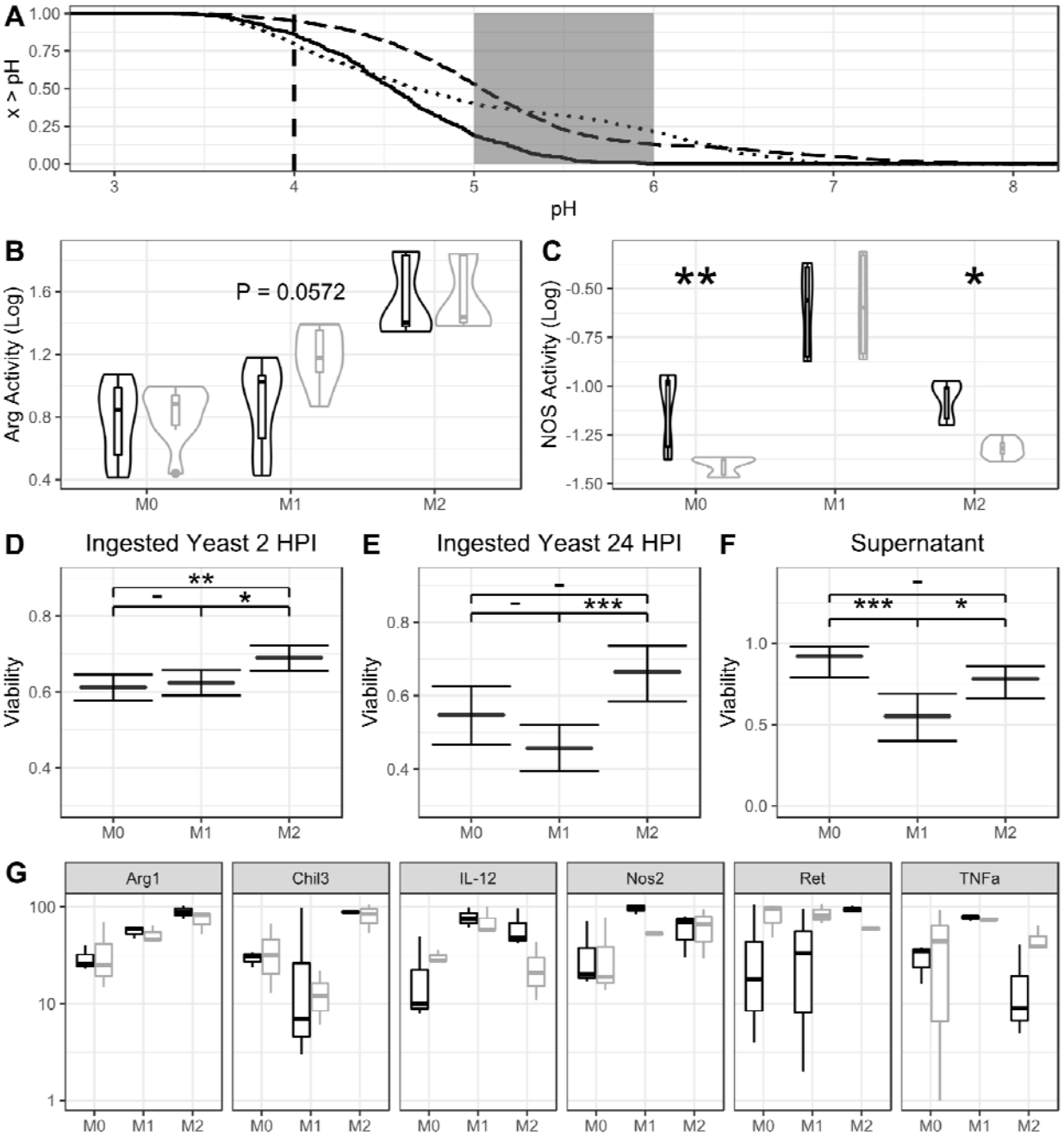
684 **Figures**



685

686 **Figure 1**. pH and Oxidative response in BMDM populations. **A.** Inverse empirical cumulative distribution
687 functions for bead containing phagolysosome pH data measured in M0 (dotted), M1 (solid), and M2
688 (dashed) macrophage populations. The dashed line at pH 4 represents the point at which pH inhibits *C.*
689 *neoformans* replication while the gray area between pH 5 and 6 represents the optimal growth pH for *C.*
690 *neoformans*. Hospitality of each population is estimated by the number of phagolysosomes within each
691 of these regions. **B.** Arg-1 activity of differently polarized BMDMs infected (gray) or uninfected (black)
692 with *C. neoformans*. M2 have the highest overall activity, and activity is promoted with infection in M1
693 populations. **C.** NOS activity of differently polarized BMDMs infected (gray) or uninfected (black) with *C.*

694 *neoformans*. M1 have the highest overall activity, and activity is decreased with infection in both M0
695 and M2 populations. Significance determined by 2-way ANOVA with Tukey's HSD comparisons. **D**.
696 Viability of *C. neoformans* ingested by macrophage populations after 2 h. **E.** Viability of *C. neoformans*
697 ingested by macrophage populations after 2 h. **F.** Viability of extracellular *C. neoformans* after 24 h
698 incubation with macrophages. **G.** qPCR data from differently polarized BMDMs uninfected (black) and
699 infected (gray) with *C. neoformans* 24 HPI. Values are normalized to the respective M0 uninfected fold
700 induction. *, **, *** represent $P < 0.05$, 0.01, and 0.001, respectively. ANOVA with Tukey comparisons
701 were used in panels B and C while a test of equal proportions with Bonferroni correction was used in
702 panels D and E.

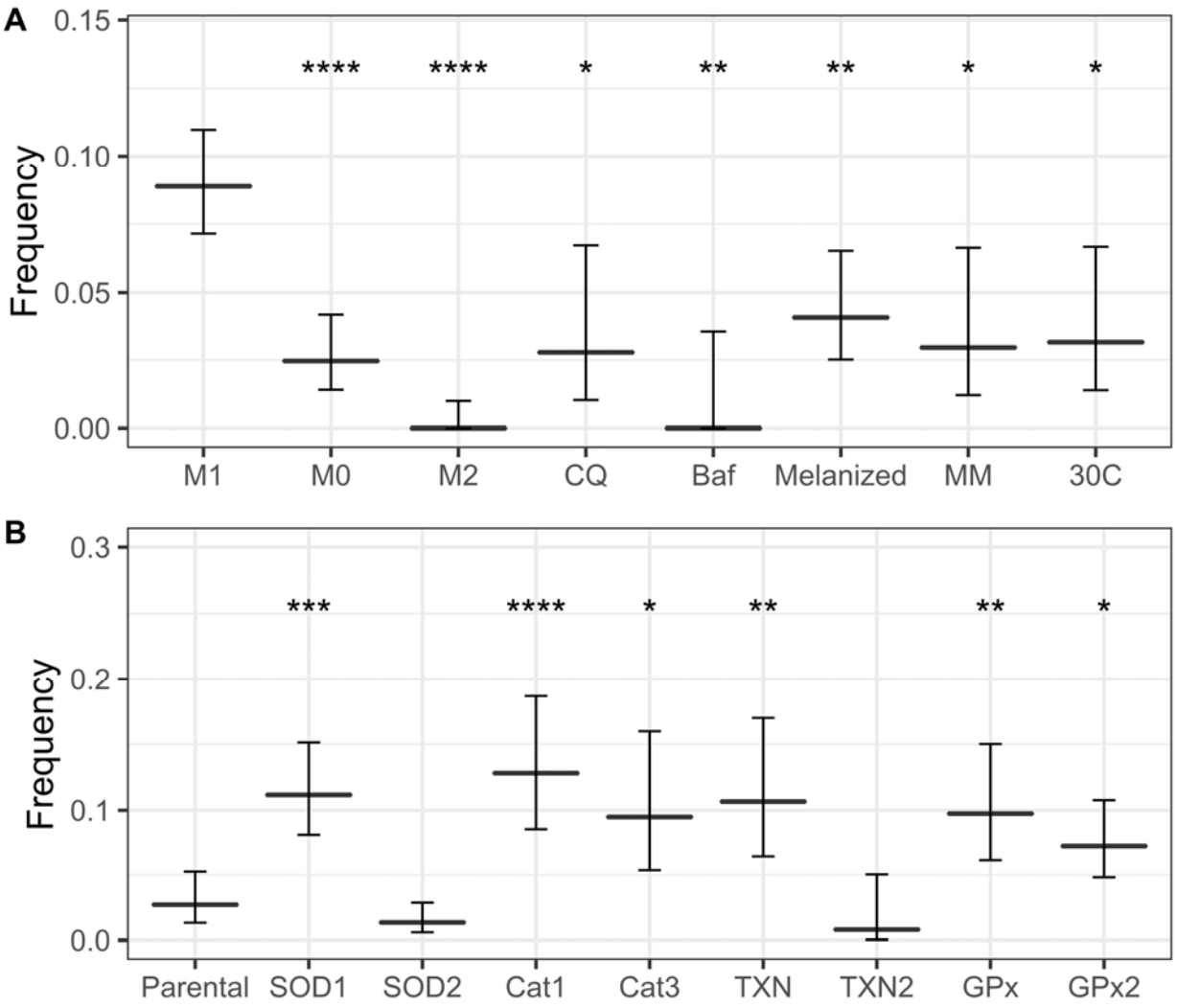
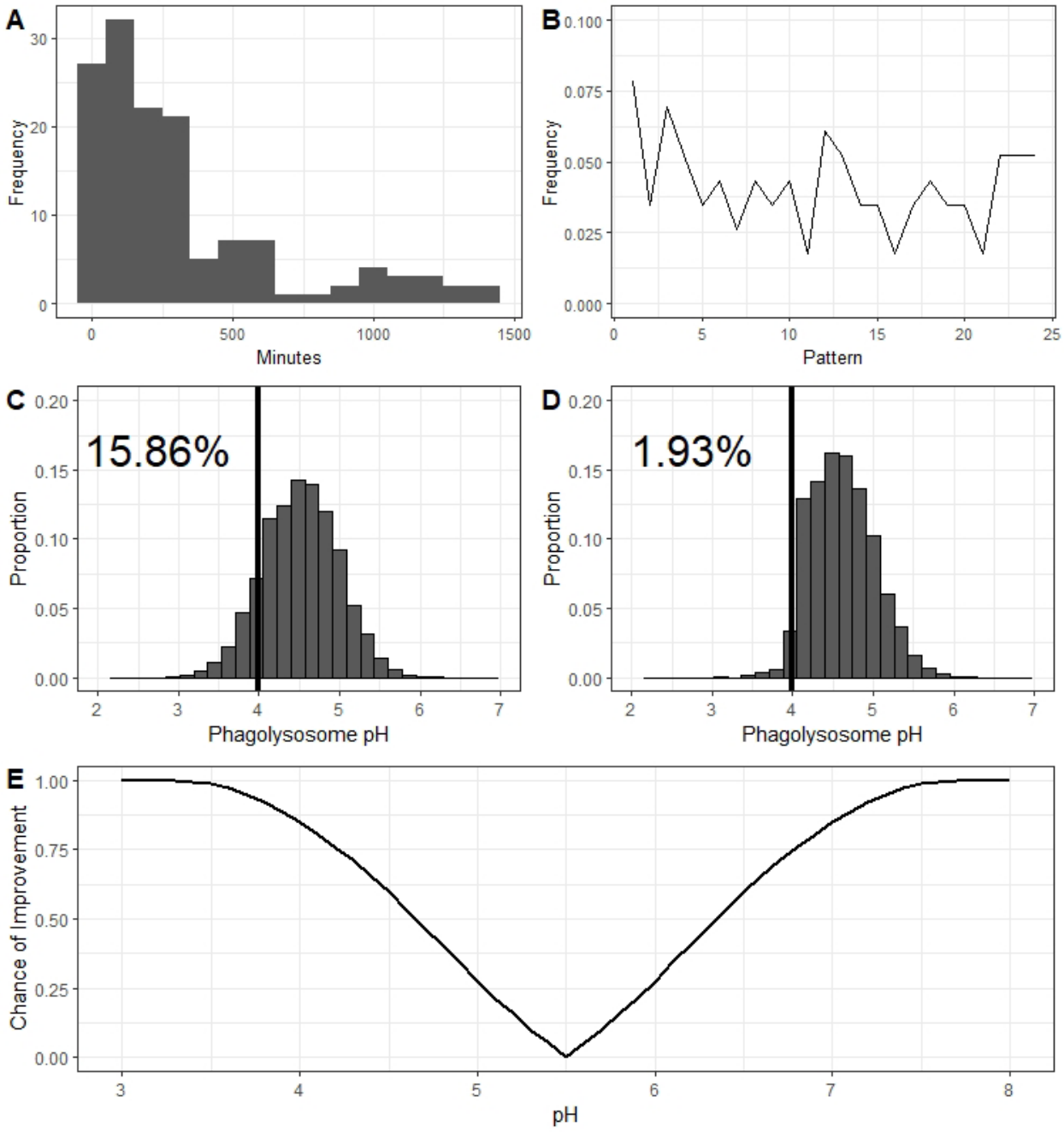


Figure 2. Dragotcytosis frequencies of *C. neoformans* ingested BMDMs under various conditions. **A.** *C. neoformans* strain H99 frequencies of Dragotcytosis. M0 and M2 macrophages have lower frequencies of Dragotcytosis overall compared to M1. Alkalizing the phagolysosomes of M1 macrophages with chloroquine (Chloro) or bafilomycin A1 (BafA), protecting from ROS with melanin (Melanized), stimulating capsule growth with minimal media conditions (MM), and inhibiting macrophage function with low temperature (30C) also abrogate Dragotcytosis frequency. **B.** *C. neoformans* strain KN99α Dragotcytosis frequencies among infected macrophages. Knocking out genes directly involved in oxidative stress mitigation increases frequency of Dragotcytosis. Graphs depict means with 95% confidence intervals. *, **, ***, **** denote $P < 0.05$, 0.01, 0.001, and 0.0001 via test of equal proportions compared to M1 with Bonferroni correction for multiple hypotheses.



714

715 **Figure 3**. Dynamics of Dragotcytosis. **A.** Distribution of the times at which *C. neoformans* yeasts initiated
716 host cell exit strategies. Both Vomocytosis and Dragotcytosis events are represented here. Bin widths
717 are set to 100 and the data depicted spans 139 samples from 12 experiments. **B.** Ordinal pattern
718 analysis for the intervals between events. Intervals were gathered and analyzed within experiments and
719 total proportions of individual ordinal patterns summed between experiments. **C.** Hypothetical
720 phagolysosomal pH distributions if *C. neoformans* particles were to undergo no Dragotcytosis events. **D.**
721 Modelled phagolysosomal pH distributions if *C. neoformans* particles were to undergo one Dragotcytosis
722 event if their resident phagolysosomal pH is < 4. Even a single round of low pH triggered Dragotcytosis
723 drastically shifts the distribution to the right resulting in a greater proportion of *C. neoformans*
724 hospitable phagolysosomes. Labelled percent values reflect the proportion of hypothetical

phagolysosomes inhibitory to *C. neoformans* (pH < 4, black vertical line) in each scenario. **E.** Hypothetical chance of an ingested *C. neoformans* to find a more hospitable phagolysosome in the context of pH assuming equal likelihood of migrating to an M0, M1, or M2 macrophage.

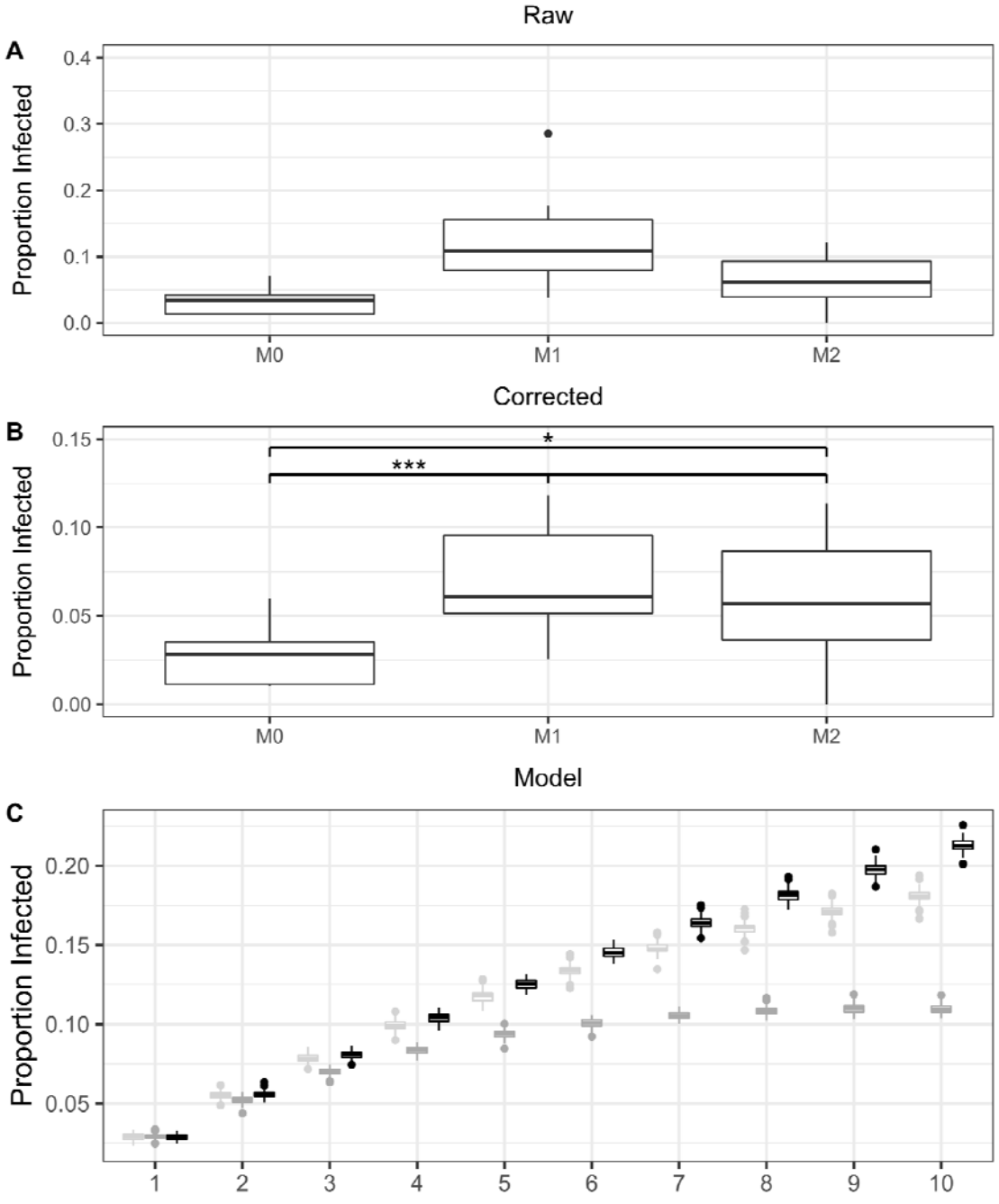
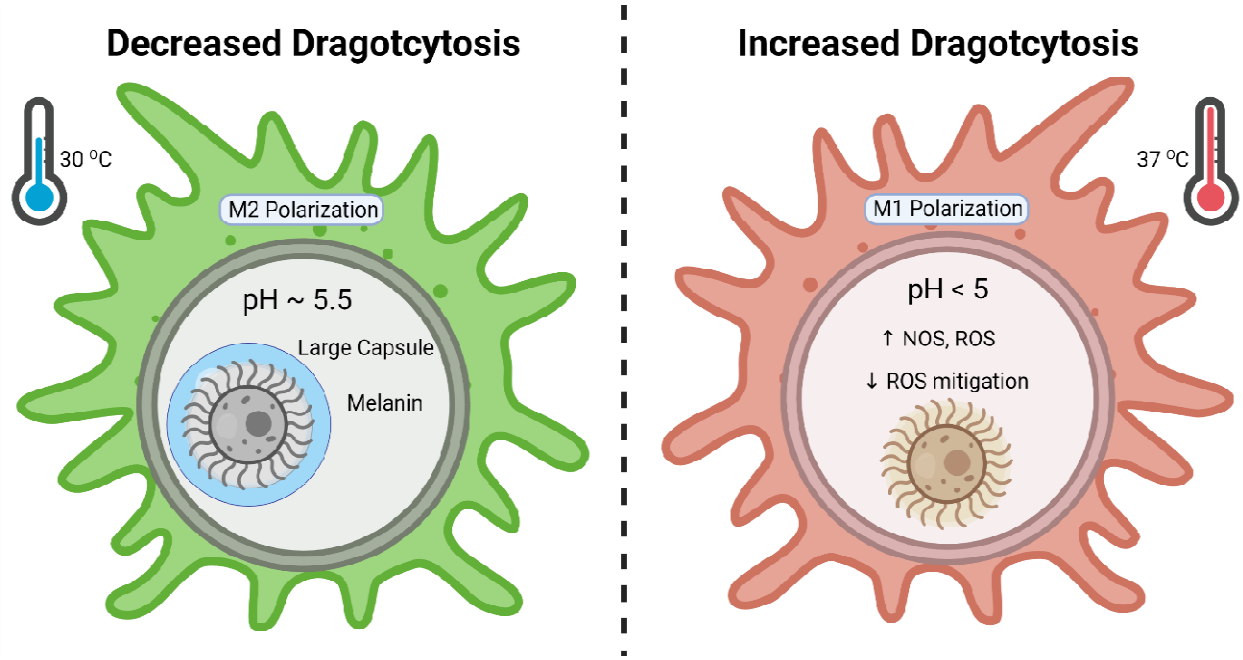


Figure 4. Comparisons of labelled macrophage populations and the proportion of which have ingested *C. neoformans* 24 hours post infection. **A.** The uncorrected proportion of adherent macrophages containing *C. neoformans* at the end of the infection. **B.** The proportion of macrophages containing yeast after correcting for floating cells. Both the proportion of labelled cells compared to the original population of infected M1 macrophages as well as the frequency of labelled cells which ingested *C. neoformans* remained consistent throughout the time course. *, *** signify $P < 0.05$ and 0.001 via

735 ANOVA with Tukey multiple comparisons. **C.** A model depicting expected proportions of infected cells
736 based only on random chance after sequential Dragotcytosis events for M0 (light gray), M1 (dark gray),
737 and M2 (black) macrophages.



738

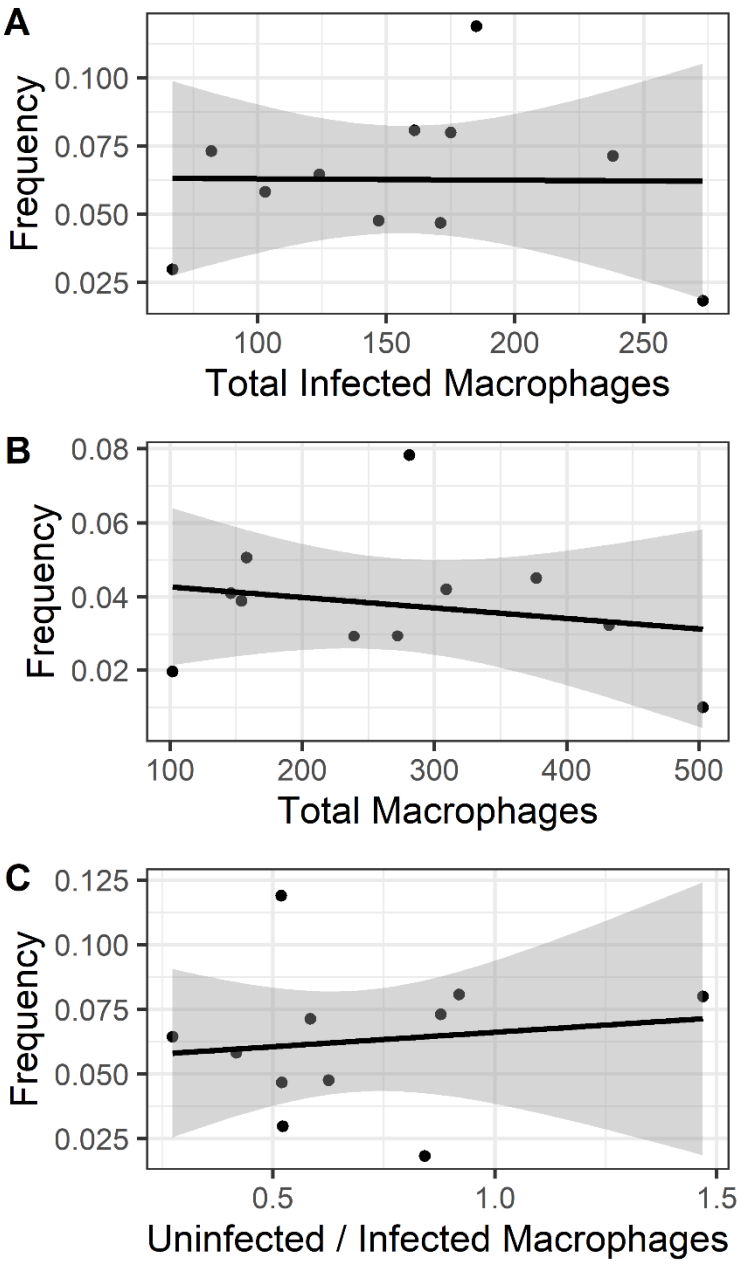
739 Figure 5

740 Graphical summary of conditions with effects on Dragotcytosis frequency. Figure was created with
741 BioRender.com.

742 Supplemental Table 1. Summary of variable effects on Dragotcytosis frequency.

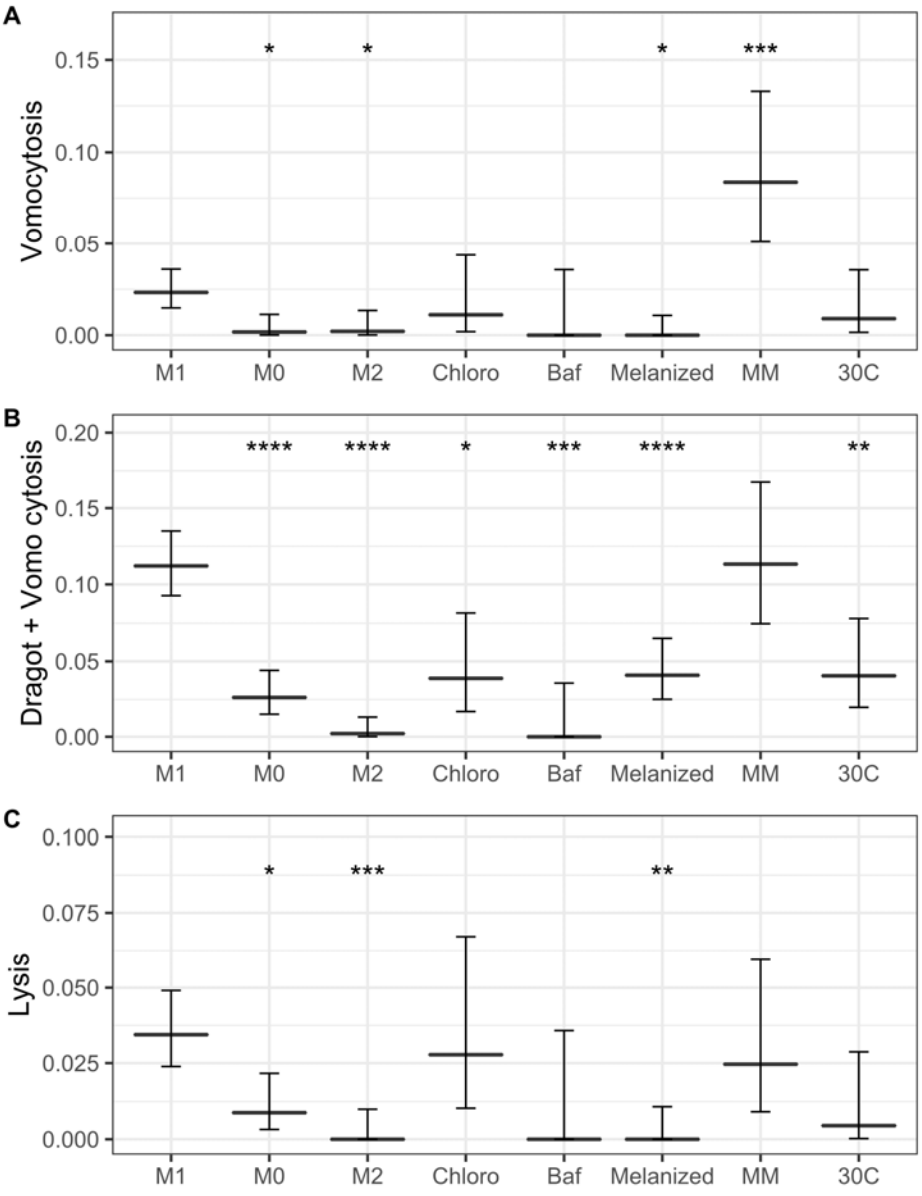
Frequency	Variable	Effect
Increase	Macrophage M1 Polarization	Enhanced antifungal activity (increased NO)
	Decreased Phagosome pH	Increased phagolysosome fungistatic mechanisms Reduced fungal growth
	CN Superoxide 1 Deficiency	Enhanced fungal susceptibility to oxidants
	CN Catalase 1 and 3 Deficiency	Enhanced fungal susceptibility to oxidants
	CN Thioredoxin Deficiency	Enhanced fungal susceptibility to oxidants
	CN Glutathione Peroxidase Deficiency	Enhanced fungal susceptibility to oxidants
Decrease	Macrophage M2 Polarization	Reduced antifungal activity
	Increased Phagosome pH	Reduced phagolysosome fungistatic mechanisms Enhanced fungal replication
	Bafilomycin A1 and Chloroquine Treatment	Increases phagolysosomal pH, reduced phagolysosome fungistatic mechanisms
	Fluconazole and Amphotericin B Treatment	Disrupts fungal ergosterol processes
	Melanin	Reduced fungal susceptibility to oxidants
	CN Urease Deficiency	Decreased phagosomal pH, increased intracellular CN replication, disrupted phagolysosomal membrane, increased apoptotic macrophages[5]
	Increased Capsule Size	Increased buffering potential, reduced macrophage phagocytosis efficiency
	Temperature (30 ^{o}C)	Reduced macrophage function

743

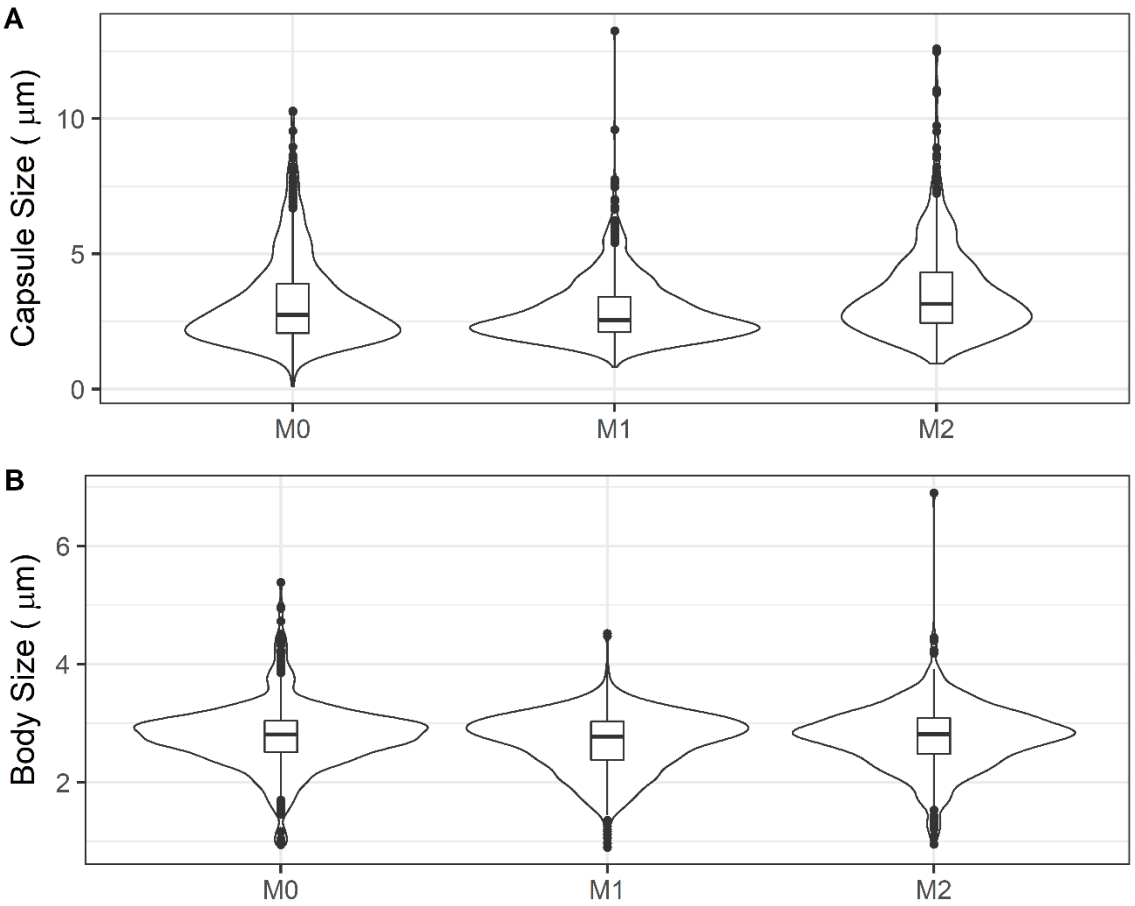


744

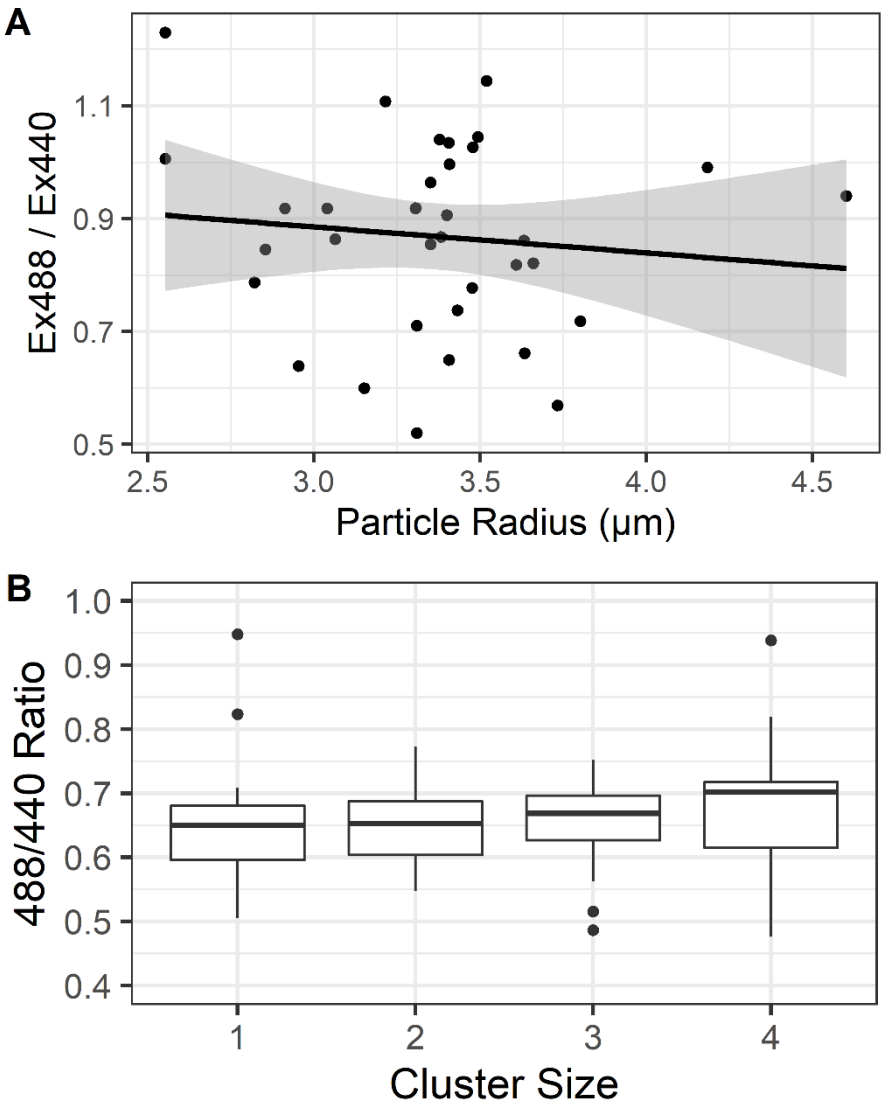
745 **Supplemental Figure 1.** Frequency of Dragotcytosis events of ingested *C. neoformans* H99 yeasts
746 according to population size of infected or total macrophages. **A.** Dragotcytosis frequency did not
747 correlate with total number of infected macrophages. **B.** Dragotcytosis frequency did not correlate with
748 the density of total macrophages. **C.** Dragotcytosis frequency did not correlate with the proportion of
749 uninfected to infected macrophages.



Supplemental Figure 2. Event frequencies of *C. neoformans* strain H99 ingested by BMDMs under various pH related conditions. **A.** Vomocytosis frequency among infected macrophages. **B.** Combined frequency of Dragotcytosis and Vomocytosis among infected macrophages. **C.** Lysis frequency among infected macrophages. All conditions have similar frequency. Event frequencies of wild-type KN99 strain and mutant *C. neoformans* after ingestion by M1 polarized BMDMs. *, **, ***, **** signify $P < 0.05$, 0.01, 0.001, and 0.0001 via test of equal proportions, respectively. Bonferroni correction was applied for multiple hypotheses.



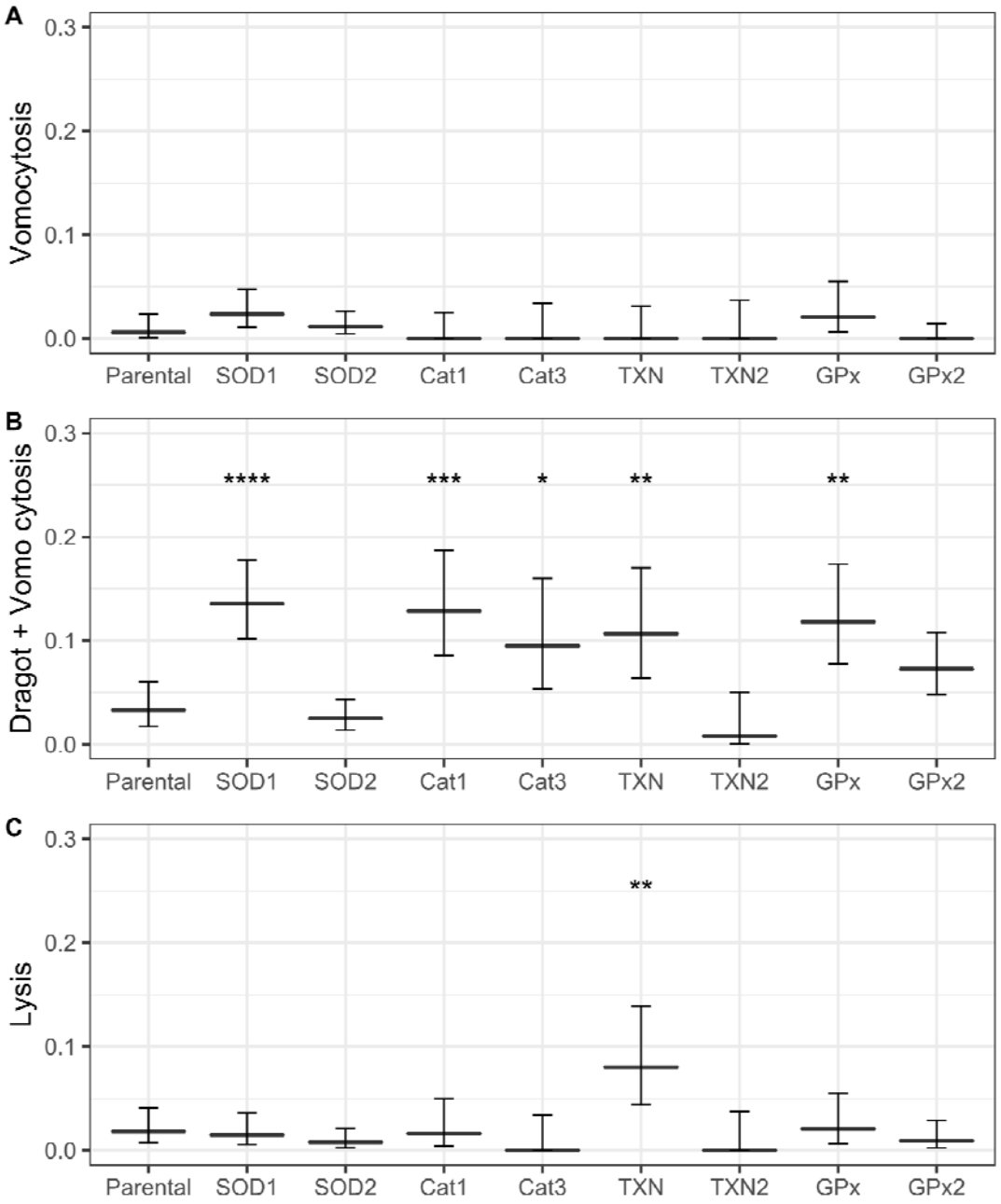
Supplemental Figure 3. Capsule and cell body size of *C. neoformans* isolated 24 hours after ingestion by BMDMs. Capsules and cell bodies are measured by preparing and imaging India Ink slides and a previously published[18] measuring code. No significant differences were found between the polarization states of host macrophages.



763

764 **Supplemental Figure 4**. Fluorescence ratio of ingested particles as a measurement of phagolysosomal
765 pH compared to total volume of ingested particles. **A.** Fluorescence ratio of ingested *C. neoformans.* We
766 found no significant correlation. **B.** Fluorescence ratio of ingested inert latex beads of 0.6 µm diameter.
767 Even with a cluster of four particles within a single phagosome we did not detect a threshold at which
768 size alone disrupts the phagolysosomal pH.

769



Supplemental Figure 5. Event frequencies of *C. neoformans* strain KN99α and knockout mutants ingested by M1 BMDMs. **A.** Vomocytosis frequency among infected macrophages **B.** Combined Dragotcytosis and Vomocytosis frequency among infected macrophages. **C.** Lysis frequency among infected macrophages. Graphs depict means with 95% confidence intervals. *, **, ***, **** signify $P <$ 0.05, 0.01, 0.001, and 0.0001 via test of equal proportions, respectively. Bonferroni correction was applied for multiple hypotheses.