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High Efficiency Opsonin-Independent Phagocytosis of *Candida parapsilosis* by Human Neutrophils

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Abstract

Candida species are associated with invasive fungal infections, and *C. parapsilosis* has become increasingly prevalent. As key antifungal effector cells, the function of human neutrophils confronting *C. parapsilosis* was investigated. We hypothesized that interaction between neutrophils and *Candida* species may not be uniform. Opsonins were omitted from these studies to understand the antifungal mechanisms at their most basic level. Human neutrophils underwent phagocytosis of *C. parapsilosis* with much higher efficiency than *C. albicans*. Immunofluorescence assays with β-glucan specific antibody detected more surface exposed β-glucan on *C. parapsilosis* than *C. albicans*. However, blockade of the β-glucan receptor, Dectin-1, reduced phagocytosis of *C. albicans* but not *C. parapsilosis*. Inclusion of excess β-glucan, mannan, or chitin also had no effect on phagocytosis of *C. parapsilosis*. Consistent with the difference in phagocytosis, neutrophils mediated damage to *C. parapsilosis* but not *C. albicans* in assays of residual metabolic activity. *C. parapsilosis* was more sensitive to oxidative stress, and inclusion of antioxidant in toxicity assays decreased neutrophil mediated damage, suggesting that generation of reactive oxygen species contributes to the mechanism of toxicity. These data suggest that the interaction between neutrophils and *Candida* species is not uniform and may partially account for differences observed in the epidemiology and natural history of infections caused by these species.

Keywords

Neutrophil; Phagocytosis; *Candida*; Dectin-1; β-glucan

INTRODUCTION

Candida species are a prevalent cause of invasive fungal infections, currently the fourth leading organism in hospital acquired bloodstream infections in the United States [1]. *Candida albicans* is associated with the majority of these infections and possesses a number of specific virulence attributes that have been the subject of intensive study [2]. *Candida parapsilosis* has been considered a less virulent species and historically has been associated with a small proportion of bloodstream infections. However, this organism has increased in prevalence in recent years. *C. parapsilosis* is a common cause of bloodstream infection in neonates [3-5], and has overtaken *C. albicans* in frequency of hospital-acquired infection in

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some hospitals worldwide [4, 6, 7]. Recent studies have identified the importance of secreted lipase in the virulence of this organism and have documented its ability to damage tissue in vitro [8, 9].

The neutrophil is well-recognized for its important role in host defense against invasive fungal infections, and various neutrophil functions have been studied using *C. albicans* and other fungi as targets [10, 11]. The interaction between neutrophils and *C. parapsilosis* has received considerably less attention. Because of the increasing prevalence of *C. parapsilosis* in invasive candidiasis and the central role of neutrophils in host defense, we sought to more clearly understand the specific attributes of human neutrophils confronting this species. In this report, we focus on phagocytosis as an important initial step in this interaction and on the mechanisms of neutrophil-induced toxicity to each species. Opsonization of pathogenic organisms with specific antibody or complement plays a key role in this process *in vivo*, and is well known to alter the dynamics of phagocytosis under *in vitro* conditions [12-14]. In an effort to understand the interaction between neutrophils and yeast with the least number of confounding variables, we conducted these studies in the absence of opsonins. We also investigated the role of carbohydrates present in the fungal cell wall in the phagocytosis process.

MATERIALS AND METHODS

Organisms and media

C. albicans strains used in this study include a laboratory strain, Ca3153A [15, 16], and a clinical isolate from an infant with urinary candidiasis, Ca-4 [17]. *C. parapsilosis* strains included three independent clinical isolates colonizing premature infants in a previously reported study, Ro18, Ro29 and Ro75 [18]. Starter cultures for phagocytosis and toxicity assays were grown 16 h at 37°C with vigorous agitation in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose). Cultures were predominantly (>99%) yeast forms following this incubation.

Preparation and pretreatment of neutrophils

Following review and approval by the Institutional Review Board, human neutrophils were isolated by density gradient centrifugation from peripheral blood of healthy adult volunteers. Briefly, leukocytes were separated from whole blood on Histopaque-1077 (Sigma) by density gradient centrifugation. Neutrophils were further purified by dextran sedimentation and hypotonic lysis of contaminating erythrocytes. Cells were adjusted to 5×10^6 cells/mL in HBSS + Ca/Mg, and were >95% neutrophils as determined by Wright-Giemsa stain. In selected experiments, neutrophils were pretreated with various reagents to study the specifics of the yeast-neutrophil interaction. To evaluate the requirement for actin polymerization, neutrophils were preincubated with cytochalasin D (10 μg/mL) at 4°C for 30 min with constant mixing. The role of β-glucan was studied by pretreating neutrophils with excess β-glucan or with blocking monoclonal antibodies to Dectin- 1. A β-glucan solution (10 mg/ml, Sigma, from barley) was prepared using described methods to maximize solubility [19]. Briefly, β-glucan was dissolved in 1N NaOH by heating to 60°C, diluted to 0.5 mg/ml in HBSS, and corrected to neutral pH with HCl. A vehicle control was used for comparison in these experiments, prepared identically to the β-glucan solution but omitting the β-glucan. Neutrophils were incubated with β-glucan or vehicle control for 30 min at 4°C with constant mixing prior to inclusion in the phagocytosis assay. Dectin-1 receptor was blocked with specific antibody (GE2, generously provided by Gordon Brown) [20] by incubation with 10 μg/mL of antibody for 20 min at 4°C. To investigate the role of other carbohydrate receptors in phagocytosis, neutrophils were pretreated with mannan (Sigma, from *Saccharomyces cerevisiae*) or chitin (Sigma, from crab shells). Mannan was

solubilized in HBSS and preincubated with neutrophils (10 mg/ml or 1 mg/ml) for 30 min at 4°C with constant mixing. Solubility of chitin is limited in aqueous solution, so solutions were prepared according to the described method [21]. Briefly, chitin was suspended in HBSS with 8% NaOH and 4% Urea (w/v) and sonicated for 30 min. The solution was incubated at 4°C overnight with rotation and incubated at −20°C for 1 hour. A 1:100 dilution was performed into HBSS to achieve 0.1 mg/ml, and the pH was adjusted using HCl. A vehicle control was used for comparison in these experiments, prepared identically to the chitin solution but omitting the chitin. Neutrophils were incubated with chitin or vehicle control for 30 min at 4°C with constant mixing prior to inclusion in the phagocytosis assay.

Phagocytosis assay

Candida species were washed in Hank's Balanced Salt Solution (HBSS), enumerated on a hemacytometer, and adjusted to a final concentration appropriate to provide the desired multiplicity of infection (MOI). In selected experiments, yeast were heat killed at 65°C for 90 min. Yeast were labeled with FITC at 10 μg/mL in the dark with rotation for 30 minutes at 37°C followed by extensive washes with HBSS. Once neutrophils were prepared and pretreated when indicated, equal volumes of *Candida* yeast and neutrophils were combined at ratios appropriate to provide a MOI ranging from 10 to 100 (10 to 100 yeast per neutrophil). Cells were pelleted at 500 × *g* for 2 minutes, incubated on ice for 30 min to give a pool of cells with surface-bound *Candida*, then at 37°C for 30, 60 or 90 min to allow phagocytosis. Cells were pelleted at 500 × *g* for 2 min and resuspended in 20 μL HBSS. 2.5 μL of ethidium bromide (100 μg/mL) was mixed with 2.5 μL of the sample on the surface of a microscope slide and examined by fluorescence microscopy. Intracellular *Candida* were differentiated from extracellular by retention of green fluorescence. A minimum of 100 neutrophils were counted, and the percent that had undergone phagocytosis of yeast was calculated. In some experiments, the number of intracellular yeast within neutrophils undergoing phagocytosis was calculated and reported as 1, 2, 3, or 4+ yeast per cell.

Indirect immunofluorescence assay and flow cytometry for β-glucan

C. albicans and *C. parapsilosis* yeast were harvested from overnight culture and washed with PBS. Yeast cells were blocked with 3% bovine serum albumin (BSA) at 22°C for 30 min, then incubated with 15 μg/ml BF-Div, a mouse IgM monoclonal antibody specific for both β-(1-3)- and β-(1-6)-linked glucan [22] for 30 min at 22°C. Cells were washed with PBS and incubated with an appropriate FITC-conjugated secondary antibody for 30 min at 22°C. Controls included yeast cells incubated in 3% BSA only or with secondary antibody only. Cells were washed and examined by fluorescence microscopy or by flow cytometry with a BD Biosciences FACSCanto instrument.

Neutrophil-induced toxicity assay

Candida species were washed in HBSS + Ca/Mg with 0.1% (w/v) glucose. Human adult neutrophils were prepared as described above. In selected experiments, neutrophils were preincubated with cytochalasin D (10 μg/mL) at 4°C for 30 min with constant mixing. Cells were enumerated on a hemacytometer and combined at varied effector to target (E: T) ratios in a 96 well dish in 60 μl total volume with appropriate controls. After a 60 min incubation at 37°C, plates were centrifuged at 1600 × *g* and the supernatant was carefully aspirated from the wells. Neutrophils were lysed by agitation in 0.02% (v/v) Tween-20 in water, pH 11.0 [23] and incubated at 37°C for 15 min. The centrifugation and lysis steps were repeated, and lysis confirmed by light microscopy. Aspirated supernatants were plated on YEPD, confirming that only trivial numbers of yeast cells (< 2% of inoculum) were lost in this procedure. Toxicity to *Candida* was measured by residual metabolic activity, using (2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT, Sigma) assay as described [24]. Briefly, XTT was freshly prepared in Dulbecco's PBS, heated at

60°C for 30 min and filtered. Coenzyme Q (Sigma) was added, and the solution was added to cells at concentrations of 0.5 mg/ml XTT and 40 mg/ml Coenzyme Q. Plates were incubated at 37°C for 1 h and the intensity of the colorimetric reaction, reflecting metabolic activity, was measured by optical density at 450 nm (OD450) using an automated plate reader. Individual experiments were performed in triplicate at minimum, and a minimum of 5 individual neutrophil donors were studied in independent experiments. Percent residual XTT activity was calculated as Mean OD450 (Yeast + neutrophils) / Mean OD450 (Yeast alone). To study the contribution of reactive oxygen species (ROS) in neutrophil-mediated toxicity to *Candida* yeast, toxicity assays were conducted in the presence of the ROS scavenger, N-acetylcysteine, at concentrations of 1, 2, and 4 mM in HBSS. Percent residual XTT activity was calculated as above for each concentration of N-acetylcysteine.

Hydrogen peroxide (H_2O_2) toxicity assay

Candida species were washed 3 times in HBSS and enumerated on a hemacytometer. 1×10^6 cells/well were inoculated into 96 well plates containing HBSS+ 0.1% glucose with and without 1mM H_20_2 in triplicate. Plates were incubated at 37°C for 1 hour. Hydrogen peroxide induced toxicity was measured by residual XTT activity as described above. Percent residual XTT activity was calculated as Mean OD450 (Yeast + H_20_2) / Mean OD450 (Yeast alone).

Statistical analysis

Comparisons of phagocytosis and induced toxicity among different *Candida* species and strains and among various neutrophil treatments were made by analysis of variance (ANOVA). Between group comparisons were made by Newman-Keuls test, and p values < 0.05 were considered significant.

RESULTS

Phagocytosis of *Candida* species by human neutrophils

To compare the relative phagocytosis efficiency of *C. albicans* and *C. parapsilosis* yeast forms by human neutrophils, phagocytosis assays were conducted using FITC-labeled *C. albicans* and *C. parapsilosis* yeast and neutrophils isolated from healthy adult volunteers in the absence of opsonins. Heat killed organisms gave more uniform and reliable labeling with FITC and thus were used preferentially in these experiments. Live organisms behaved similarly (see below). To determine the optimal incubation time to allow phagocytosis to occur, yeast and neutrophils were coincubated for 30, 60, and 90 minutes. No increase in phagocytosis of any strain was detected at incubations longer than 30 minutes (data not shown), so the 30 minute time point was used in all subsequent experiments. Phagocytosis assays were conducted at multiplicity of infection (MOI) ranging from 10 to 100. Two strains of *C. albicans* (laboratory strain, Ca3153A and a clinical isolate, Ca-4) and 3 strains of *C. parapsilosis* (clinical isolates) were studied. In all cases and at all MOIs, phagocytosis was much more efficient for neutrophils incubated with *C. parapsilosis* than *C. albicans* (Fig. 1A, $p < 0.001$). Increasing MOI above 10 increased phagocytosis efficiency for *C. albicans* strain Ca3153A ($p < 0.02$) but not for Ca-4 ($p > 0.16$). Increasing MOI above 10 increased phagocytosis efficiency for *C. parapsilosis* strains Ro18 ($p < 0.006$) and Ro29 ($p < 0.002$) but not Ro75 ($p > 0.46$).

To confirm that the difference in phagocytosis efficiency between *C. albicans* and *C. parapsilosis* was not an artifact related to the heat-killing process, phagocytosis assays were conducted with live *C. albicans* and *C. parapsilosis* yeast at an MOI of 100. Again, significantly higher rates of phagocytosis were seen with all *C. parapsilosis* strains relative to *C. albicans* (Fig. 1B). *C. albicans* strain Ca3153A showed somewhat higher phagocytosis

efficiency when using heat-killed vs. live yeast (p=0.001), whereas phagocytosis rates between heat-killed and live yeast of strain Ca-4 were no different (p=0.99). Heat-killed yeast also improved phagocytosis rates for *C. parapsilosis* strains Ro18 (p=0.006) and Ro29 (p=0.005), but not Ro75 (p=0.39). Although some minor differences in phagocytosis efficiency between live and heat-killed yeast were observed in some strains, in all cases *C. parapsilosis* maintained higher phagocytosis rates with both live and heat-killed yeast than *C. albicans* ($p < 0.001$).

In addition to the higher rates of phagocytosis noted with *C. parapsilosis*, neutrophils with intracellular yeast were qualitatively noted to have a higher yeast burden with *C. parapsilosis* than with *C. albicans*. To quantify this observation, the number of yeast per neutrophil undergoing phagocytosis was counted for each strain. A single, representative strain of *C. albicans* (Ca3153a) and *C. parapsilosis* (Ro18) is depicted in Fig. 1C. The percentage of neutrophils undergoing phagocytosis that had 4 or more yeast per cell was significantly higher for *C. parapsilosis* than for *C. albicans* at a MOI of 100 or 50 (p=0.0001 for each comparison). There was a trend toward higher yeast burden with *C. parapsilosis* at a MOI of 10 as well (p=0.056).

Finally, because actin polymerization is known to be required for phagocytosis, we wished to demonstrate this requirement in our assays. Neutrophils were preincubated with cytochalasin D prior to exposure to yeast. Cytochalasin D treatment reduced phagocytosis to 8% or less in all strains (Fig. 1D).

Role of β-glucan/Dectin-1, mannan and chitin in phagocytosis of *C. albicans* and *C. parapsilosis*

The β-glucan receptor, Dectin-1, has recently been shown to be expressed on neutrophils and to have a role in phagocytosis of *C. albicans* [20]. To assess whether this receptor was involved in the enhanced phagocytosis activity of *C. parapsilosis*, exposure of β-glucan on the cell surface of *C. albicans* and *C. parapsilosis* yeast was assessed by indirect immunofluorescence assay (IFA). Additionally, Dectin-1 blocking antibody and soluble glucan were used to determine the role of this receptor in phagocytosis. The β-glucan specific monoclonal antibody, BFDiv [22], was used to label β-glucan in live *C. albicans* and *C. parapsilosis* yeast cells by IFA (Fig. 2). This antibody binds to both β-(1-3)- and β-(1-6)-linked glucan. Exposure of β-glucan as detected by antibody binding was not uniform for *C. albicans*. Only occasional yeast showed detectable fluorescence, and in the majority of cells where fluorescence was detected, it was faint and localized to punctate regions of the cell (arrows in Fig. 2A). Labeling of *C. parapsilosis* yeast with the antibody was much more consistent and brighter, however binding was also not uniform, with some regions of the cell surface brighter than others (Fig. 2B). To quantify the differential binding of β-glucan specific antibody, yeast cells stained in an identical fashion were analyzed by flow cytometry (Fig. 2C). Two *C. parapsilosis* (Ro18 and Ro29) strains were analyzed. The third strain (Ro75) had a propensity to clump into cellular aggregates and was therefore less amenable to flow cytometric analysis. Consistent with the IFA results, *C. parapsilosis* strains exhibited much increased fluorescence relative to *C. albicans*.

To determine whether the apparently increased surface exposure of β-glucan in *C. parapsilosis* relative to *C. albicans* contributed to the increased phagocytosis efficiency of the former, phagocytosis assays were conducted in the presence of excess β-glucan (Fig. 3A). Although β-glucan reduced phagocytosis of *C. albicans* (p = 0.04), excess β-glucan had no effect on *C. parapsilosis* phagocytosis. To investigate the role of the β-glucan receptor, Dectin-1, blocking antibody GE2 was coincubated with neutrophils prior to phagocytosis assays. Consistent with previous studies [20], blocking of Dectin-1 decreased the phagocytosis of *C. albicans* by 57% (p< 0.001) (Fig. 3B). In contrast, treatment of

neutrophils with this antibody resulted in a small, but statistically significant increase in phagocytosis of *C. parapsilosis* (p= 0.02).

To investigate the possible contribution of other fungal polysaccharides to the difference in phagocytosis efficiency between *C. albicans* and *C. parapsilosis*, assays were conducted in the presence of excess mannan (Fig. 3C) or chitin (Fig. 3D). Neither of these polysaccharides resulted in any detectable inhibition of phagocytosis in either species.

Toxicity to *Candida* species mediated by human neutrophils

To assess the capacity of human neutrophils to damage unopsonized *Candida* yeast, metabolic activity of the yeast following coincubation was measured using the colorimetric XTT metabolism assay. Traditional, plate-based methods to measure fungal killing by neutrophils were found to provide inconsistent results and to be unduly affected by the tendency of yeast cells to adhere to plastic or each other (data not shown). This inaccuracy has also been noted by others [25]. To circumvent these issues, we opted to use metabolic activity as a surrogate for toxicity to the yeast cells. In these assays, neutrophils and *Candida* yeast were coincubated, the neutrophils lysed, and residual metabolic activity of the yeast was measured by XTT assay. After preliminary experiments using varied effector: target (E:T) ratios, a ratio of 2 neutrophils to 1 yeast was selected for subsequent analyses, as it allowed maximal discrimination between species and strains. Mean residual metabolic activity of each strain, expressed as a percent of total metabolic activity of the same strain of *Candida* incubated in the absence of neutrophils, is shown in Fig. 4A. All strains of *C. parapsilosis* were more susceptible to neutrophil-induced toxicity than *C. albicans* ($p \leq 0.001$), and no statistically significant differences among strains of the same species were observed.

The efficient phagocytosis of *C. parapsilosis* described above required actin polymerization, as pretreatment of neutrophils with cytochalasin D disrupted phagocytosis activity. To determine the requirement for actin polymerization in neutrophil-induced toxicity to *C. parapsilosis*, cytochalasin D treated neutrophils were used in the XTT assay. The capacity of neutrophils to induce toxicity to *C. parapsilosis* was significantly inhibited by cytochalasin D (Fig. 4B), suggesting that the induced toxicity was an active process that likely requires phagocytosis.

Neutrophils can generate a number of antimicrobial effects, including reactive oxygen species (ROS) as well as non-oxidative mechanisms. The observation that *C. albicans* is more resistant to neutrophil-induced toxicity may reflect this species' resistance to phagocytosis. However, there may also be differences between these species in their intrinsic resistance to oxidative stress. To test this hypothesis, yeast forms of both species were treated with 1 mM hydrogen peroxide (H_2O_2), followed by XTT assay to determine residual metabolic activity (Fig. 5A). *C. albicans* yeast were more resistant than *C. parapsilosis* yeast to this form of oxidative stress ($p < 0.01$ for all strains). To investigate the contribution of oxidative mechanisms to the susceptibility of *C. parapsilosis* to neutrophil-mediated toxicity, assays were conducted in the presence of the ROS scavenger, N-acetylcysteine (Fig 5B). Although rates of phagocytosis were unaffected (data not shown), N-acetylcysteine decreased the toxicity of neutrophils in an apparently dose-dependent manner, achieving statistical significance in the case of *C. albicans* strain Ca3153A ($p = 0.009$) and *C. parapsilosis* strain Ro18 ($p = 0.02$). Similar trends were seen with the other strains, although statistical significance was not achieved. These data suggest that ROS play a role in neutrophil mediated toxicity, and the extent to which ROS contribute may vary among strains. Taken together, these data suggest that the resistance of *C. albicans* to neutrophil-mediated toxicity may be a combination of resistance to phagocytosis and

resistance to oxidative stress, while *C. parapsilosis* is more susceptible to both. Generation of ROS is likely to contribute to neutrophil-mediated toxicity of *C. parapsilosis*.

DISCUSSION

The importance of opsonins in efficient phagocytosis of *C. albicans* yeast has long been recognized. Lehrer and Cline reported greater than 90% phagocytosis of *C. albicans* yeast by neutrophils with human serum present, but when the serum was replaced by albumin or buffered salt solution, phagocytosis was “virtually absent” [26]. Likewise, Diamond *et al.* observed 5-8% ingestion of *C. albicans* by neutrophils when serum was absent [13]. Similar trends were observed more recently with the human monocytic cell line, THP-1 [12, 14]. Another study comparing phagocytosis rates among several fungal pathogens found similar rates of phagocytosis between *C. albicans* and *C. parapsilosis*, but all samples were opsonized with pooled human serum [27]. The two main sources of opsonins, antibody and complement, have the potential to create variability in assay systems. Antibody properties such as isotype, antigen specificity and binding affinity, as well as properties of the yeast cell target including abundance of cognate antigens and variability in their expression, may all impact the nature of phagocytosis. Likewise, the extent and efficiency of complement deposition on the yeast cell surface and the binding to complement receptors may not be uniform. Because opsonization inherently introduces additional complexities to the interaction between yeast and phagocyte, we conducted these experiments in the absence of opsonins. These conditions may also be relevant for premature infants, a group in which *C. parapsilosis* is particularly prevalent [3], and in whom low concentrations of antibody and key complement components have been reported [28]. Surprisingly, we detected a previously undescribed efficiency of phagocytosis with *C. parapsilosis* that is far greater than *C. albicans* under these conditions, and was associated with increased neutrophil-induced toxicity. Generation of ROS by the neutrophils is likely an important mechanism for the antifugal effect. The differences observed between these species may contribute to the increased virulence of *C. albicans* over *C. parapsilosis* in disseminated candidiasis.

We found ample, but nonuniform binding of the β-glucan antibody, BF-Div, to *C. parapsilosis*, but considerably less binding to yeast forms of *C. albicans*. These patterns are consistent with previously reported surface exposure of β-glucan in *C. albicans* as detected by a soluble form of the β-glucan receptor, Dectin-1, as probe [29]. The contribution of β-glucan and its receptor, Dectin-1, in interaction between human neutrophils and *C. albicans* has been the subject of recent study [20]. These authors demonstrated that treatment of neutrophils with the Dectin-1 blocking antibody, GE2, reduces phagocytosis of *C. albicans* yeast. Our data are consistent with this observation; however, despite apparently increased exposure of β-glucan on *C. parapsilosis*, receptor blockade with GE2 caused no reduction in phagocytosis nor did pretreatment with excess β-glucan. These data suggest that Dectin-1 is not required for the increased phagocytosis efficiency of *C. parapsilosis*. The binding of fungi by phagocytes is complex and is mediated primarily by a host of pattern-recognition receptors (PRRs), which recognize characteristic pathogen-associated molecular patterns (PAMPs) found on a wide range of microorganisms but absent in the host [11]. Individual fungal species can be recognized by several PRRs, and the outcomes of these interactions vary considerably [30]. The nature of these specific interactions likely explains the differences in phagocytosis between these two species. The receptor(s) important for the enhanced phagocytosis of *C. parapsilosis* is the subject of ongoing investigation.

Our finding of enhanced neutrophil-induced toxicity of *C. parapsilosis* relative to *C. albicans* is in agreement with others that found *C. parapsilosis* more susceptible to neutrophil killing by human [31] or murine [32] neutrophils. The latter study found that killing (based on colony forming unit counts) correlated with increases in toxicity, based on ^{51}Cr release from

labeled fungi. In contrast, Roilides *et al*. reported that *C. parapsilosis* was less susceptible to human neutrophil induced damage than *C. albicans* [33]. However, the neutrophils in this study were maintained in the absence of calcium and magnesium, which others have shown to be important for interaction of neutrophils with *C. albicans* [13]. In a study of murine macrophages, *C. parapsilosis* was again more efficiently killed than *C. albicans* [34]. The authors attributed the increased killing of *C. parapsilosis* to a relative decrease in stimulation of oxygen metabolism in the macrophages by *C. albicans*. Similar differences in macrophage killing of these two species were also reported by Brummer and Stevens [35]. Interestingly, macrophages were efficient at phagocytosis of both species, with *C. parapsilosis* rates only slightly higher than *C. albicans*. Phagocytosis of the two species was only modestly different whether serum was present or absent. This finding implies that the mechanism of phagocytosis by murine macrophages is intrinsically different from that of human neutrophils, perhaps through the use of differing receptors. Alternatively, murine macrophages may behave differently than human cells. Unlike the murine studies, the human monocytic cell line, THP-1, did not phagocytose *C. albicans* yeast efficiently in the absence of opsonins [12, 14].

The interaction between human neutrophils and *C. parapsilosis* is strikingly different than the interaction with *C. albicans*. Understanding these differences may help to explain the difference in virulence between these two species and provide insight in the molecular mechanisms of neutrophils which are varied and tailored toward the specific microbe faced. As *Candida* infections with non-*albicans* species increase, insights into the specifics at the host-fungus interface may aid in the design of novel therapeutic strategies in patients at risk.

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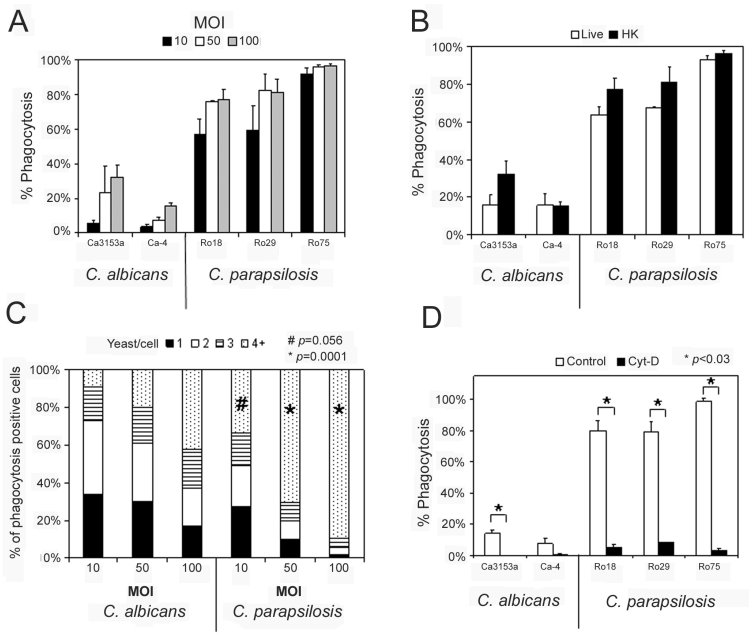

Fig. 1. Phagocytosis of *C. albicans* and *C. parapsilosis* by human neutrophils

C. albicans and *C. parapsilosis* yeast were labeled with FITC and combined with human neutrophils at the indicated MOI. After allowing phagocytosis to occur, cells were counterstained with ethidium bromide and examined by fluorescence microscopy. Neutrophils containing intracellular (green) yeast were scored as a percentage of total neutrophils. Error bars represent standard deviation. Each experiment was performed in triplicate at minimum, and data represent a minimum of three individual neutrophil donors. (A) Phagocytosis of heat-killed yeast at MOI = 10, 50, or 100. Phagocytosis of *C. parapsilosis* was significantly higher than *C. albicans* for all strains and at all MOIs ($p < 0.001$). (B) Phagocytosis of live vs. heat-killed (HK) yeast at MOI = 100. Phagocytosis of live *C. parapsilosis* was significantly higher than live *C. albicans* for all strains ($p < 0.001$). (C) Percentage of neutrophils undergoing phagocytosis that internalized 1, 2, 3, or 4+ yeast per cell. A single, representative strain of *C. albicans* (Ca3153a) and *C. parapsilosis* (Ro18) is depicted in the figure. The percentage of neutrophils that had phagocytosed 4+ yeast/cell was significantly higher for *C. parapsilosis* than *C. albicans* at MOI = 50 or 100 ($p = 0.0001$), and trended toward a higher percentage at MOI = 10 ($p = 0.056$). (D) Effect of cytochalasin D on phagocytosis. Neutrophils were pretreated with cytochalasin D and phagocytosis assays were conducted at MOI = 100 to maximize phagocytosis efficiency. As expected, cytochalsin D inhibits phagocytosis ($p < 0.03$). Because of the low baseline phagocytosis rate of Ca-4, statistical significance was not achieved.

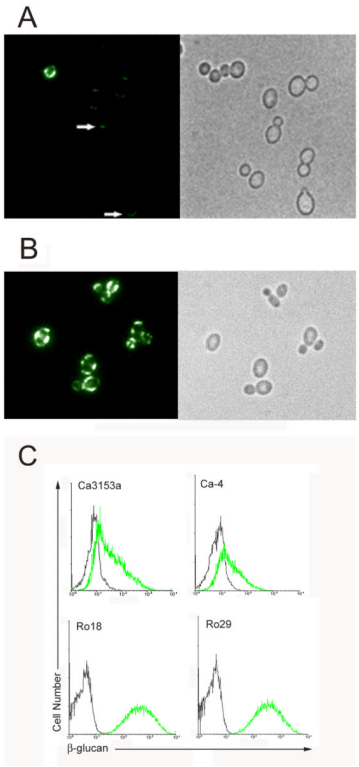

Fig. 2. Indirect immunofluorescence assay and flow cytometric analysis of surface exposed β-glucan

C. albicans (Ca3153a, Panel A) and *C. parapsilosis* (Ro18, Panel B) yeast incubated with the β-glucan specific monoclonal antibody, BF-Div. Antibody binding was detected with an appropriate FITC-labeled secondary antibody and viewed by fluorescence microscopy. All strains were tested and showed similar patterns. Representative strains are included in the figure. *C. albicans* yeast were not labeled uniformly by the antibody, with the majority of cells being either entirely negative or showing faint, localized fluorescence (arrows). In contrast, *C. parapsilosis* yeast were labeled more consistently and intensely by the antibody, but also with a nonuniform distribution. A phase contrast photomicrograph of the same microscopic field is included. To quantitate antibody binding, *C. albicans* (Ca3153a, Ca-4) and *C. parapsilosis* (Ro18, Ro29) yeast cells were labeled with β-glucan specific monoclonal antibody as described above and analyzed by flow cytometry (Panel C). The green line represents staining with BF-Div and the black line represents control yeast incubated with the secondary antibody only. Consistent with the IFA results, increased fluorescence intensity was seen with *C. parapsilosis* relative to *C. albicans*.

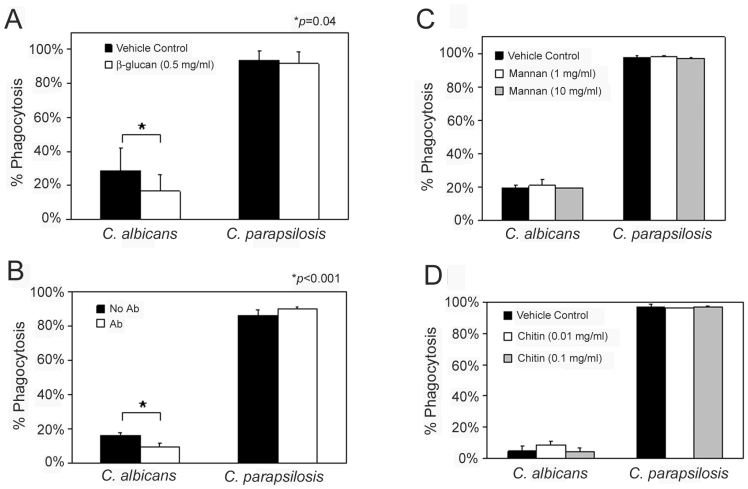

Fig. 3. Effect of excess carbohydrate and Dectin-1 blocking antibody on phagocytosis of *C. albicans* and *C. parapsilosis*

Neutrophils were preincubated with excess β-glucan (Panel A); blocking antibody, GE2 (Ab), to the β-glucan receptor, Dectin-1 (Panel B); excess mannan (Panel C); or excess chitin (Panel D) prior to incubation with *C. albicans* (Ca3153a) or *C. parapsilosis* (Ro75) yeast in phagocytosis assays. Percent phagocytosis was calculated as described in Fig. 1. Data are from independent experiments, each performed in duplicate at minimum, from a minimum of 2 individual donors. Error bars represent standard deviation. Excess β-glucan and treatment with GE2 resulted in a significant decrease in phagocytosis of *C. albicans*, ($p = 0.04$ for glucan, $p < 0.001$ for GE2), but not *C. parapsilosis*. Treatment with excess mannan or chitin had no effect on phagocytosis efficiency of either species ($p > 0.3$ for mannan, $p > 0.1$ for chitin).

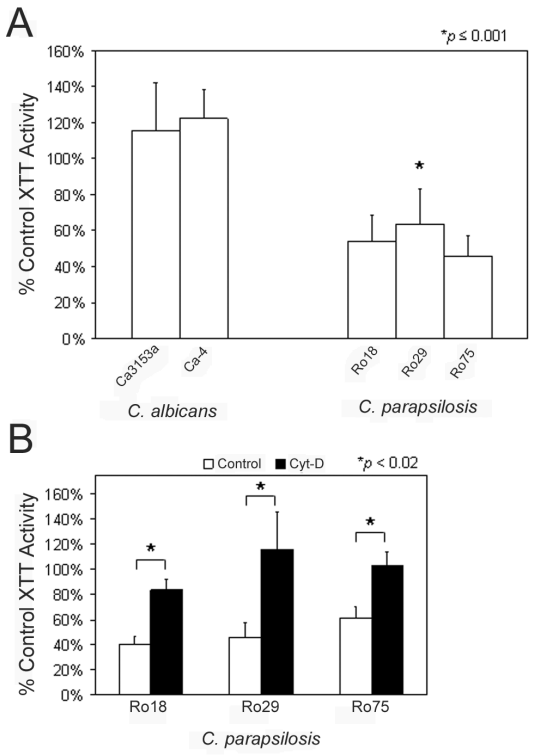

Fig. 4. Toxicity to *C. albicans* and *C. parapsilosis* mediated by neutrophils and effect of cytochalasin D

(A) *C. albicans* and *C. parapsilosis* yeast were combined with neutrophils. After an incubation period, neutrophils were lysed, and residual metabolic activity of the yeast was measured by XTT. Data are expressed as the percentage of XTT activity of yeast of the same strain incubated in the absence of neutrophils. Data are from 5 independent experiments with 5 individual donors, and each was performed in triplicate. Error bars represent standard deviation. Neutrophils induced significant toxicity to each *C. parapsilosis* strain but no detectable toxicity to *C. albicans* strains ($p \leq 0.001$ comparing each *C. parapsilosis* strain to each *C. albicans* strain). There were no significant differences among strains of the same species. (B) Neutrophils were pretreated with either cytochalsin D (Cyt-D) or buffer alone (Control) prior to inclusion in the toxicity assay with the 3 strains of *C. parapsilosis*. Data are expressed as the percentage of XTT activity of yeast of the same strain incubated in the absence of neutrophils and are from triplicate experiments. Error bars represent standard deviation. In each case, pretreatment of neutrophils with cytochalasin D resulted in significantly less toxicity than control ($p < 0.02$ for each strain).

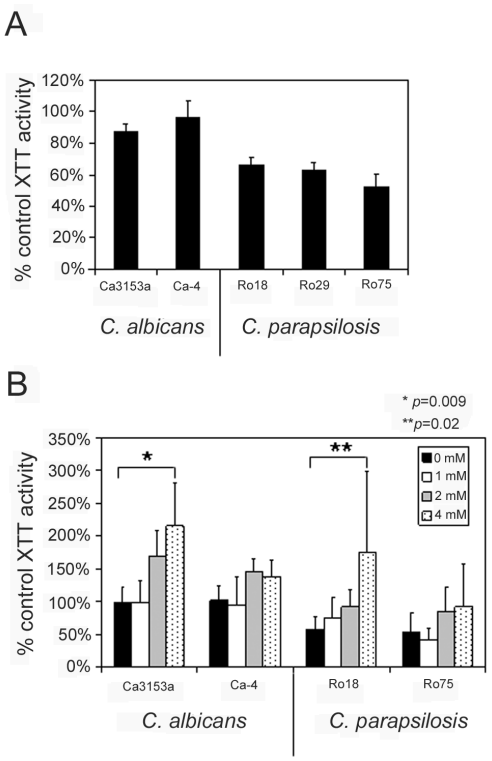

Fig. 5. Contribution of oxidative mechanisms to neutrophil-mediated toxicity

(A) To determine the inherent sensitivity of each species to oxidative stress, all strains were incubated in the presence of 1 mM H_2O_2, followed by XTT assay. Data are expressed as the percentage of XTT activity of yeast of the same strain incubated in the absence of H_2O_2. Error bars represent standard deviation. *C. parapsilosis* strains were significantly more sensitive to H_2O_2 than *C. albicans* ($p < 0.01$ for all strains). (B) Toxicity assays were conducted as described in Fig. 4 in the presence of varied concentrations of the ROS scavenger, N-acetylcysteine. Data are expressed as the percentage of XTT activity of yeast of the same strain incubated in the absence of neutrophils. Data are from 5 individual donors in a minimum of 4 individual experiments, and each was performed in triplicate. Error bars represent standard deviation. This agent led to a dose-dependent inhibition of toxicity, and statistical significance was achieved for *C. albicans* strain Ca3153A and *C. parapsilosis* strain Ro75 (* p = 0.009; ** p = 0.02). Because the quantity of neutrophils that could be obtained from an individual donor was limited, only two strains of each species were tested.