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The Role of Galectin-3 in Phagocytosis of *Candida albicans* and *Candida parapsilosis* by Human Neutrophils

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SUMMARY

Candida albicans causes the majority of invasive candidiasis in immunocompromised adults while *Candida parapsilosis* is a leading cause of neonatal candidiasis. While much work has focused on how the immune system recognizes and responds to *C. albicans*, less is known about host interaction with *C. parapsilosis*. This study investigates the human neutrophil phagocytic response to these species. Neutrophils underwent phagocytosis of *C. parapsilosis* yeast and *C. albicans* hyphae much more efficiently than *C. albicans* yeast. Treatment of neutrophils with a galectin-3 (gal3) blocking antibody inhibited phagocytosis of *C. parapsilosis* yeast and *C. albicans* hyphae, but not *C. albicans* yeast. The majority of neutrophil gal3 was expressed intracellularly and was secreted from neutrophils after treatment with *C. parapsilosis* mannan. When neutrophils were treated with exogenous gal3, phagocytosis of both *C. albicans* and *C. parapsilosis* yeast increased. Exposure of neutrophils to *C. parapsilosis* yeast increased phagocytosis of *C. albicans* yeast and was inhibited by gal3 blocking antibody. Taken together, these data indicate that gal3 secreted from neutrophils may act as a proinflammatory autocrine/paracrine signal in neutrophil phagocytosis and suggest that gal3 has a unique role in neutrophil response to *C. parapsilosis* yeast and *C. albicans* hyphae distinct from *C. albicans* yeast.

INTRODUCTION

The *Candida* genus includes opportunistic pathogens that cause life threatening disease in immunocompromised individuals. Systemic infections with these fungi are associated with high morbidity and mortality rates, even with antifungal treatment (Bassetti *et al*., 2010, Benjamin *et al*., 2010). Historically, *C. albicans* has been the leading cause of invasive nosocomial fungal infections; however the incidence of infections involving non-*albicans* species has dramatically increased (Blyth *et al*., 2009, Bassetti *et al*., 2010, Falagas *et al*., 2010). Although *C. albicans* remains the most common cause of invasive candidiasis in immunocompromised adults (Pfaller *et al*., 2010), *C. parapsilosis* causes 15.5–67% of invasive candidiasis in premature newborn infants, often outranking *C. albicans* in neonates (Spiliopoulou *et al*., 2012). This species-dependent predilection to cause disease in specific patient groups may be due in part to differences in the patients' immune recognition and response to these specific pathogens. Understanding how an intact immune system recognizes and responds to these separate species will facilitate our understanding of unique patient susceptibilities.

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Traditionally, studies focusing on host-*Candida* interactions have used *C. albicans* as a model organism. Although substantially advancing our understanding of fungal host defense, the assumption that the immune system recognizes and responds to other *Candida* species similarly to *C. albicans* has recently been challenged. For example, we have shown that neutrophils phagocytose *C. parapsilosis* much more efficiently than *C. albicans* yeast (Linden *et al*., 2010) while others have shown that dendritic cells produce more fungipods when exposed to *C. parapsilosis* than when exposed to *C. albicans* or *C. tropicalis* (Neumann *et al*., 2010). In addition, macrophages preferentially phagocytose *C. glabrata* over *C. albicans* (Keppler-Ross *et al*., 2010) and phagocytose *C. glabrata* and *C. lusitaniae* more efficiently than *C. albicans* (Dementhon *et al*., 2012). These differences in cellular responses to different species taken together with the unique susceptibility of specific patient groups highlight the need to study fungal pathogenesis in a species dependent manner.

The host recognizes and responds to invading pathogens by recognizing specific pathogen associated molecular patterns (PAMPs). Fungal PAMPs are generally components of the carbohydrate rich cell wall and include chitin, β-glucan, and mannan structures. PAMPs are recognized by pathogen recognition receptors (PRRs) which are found on a wide array of effector cells and include C-type lectin receptors such as dectin-1, toll-like receptors, and integrins such as complement receptor 3 (CR3) (Netea *et al*., 2008, Netea *et al*., 2010). Recently, galectin-3 (gal3), an S-type (soluble) lectin receptor which recognizes specific β-(1-2) oligomannans (Fradin *et al*., 2000, Kohatsu *et al*., 2006), has emerged as an important co-receptor in distinguishing non-pathogenic from pathogenic fungi in macrophages (Jouault *et al*., 2006, Esteban *et al*., 2011). Gal3 plays a critical role in influencing immunity to many different types of microbial infections (Bernardes *et al*., 2006, Farnworth *et al*., 2008, Ferraz *et al*., 2008, Nieminen *et al*., 2008, Ruas *et al*., 2009). Found in numerous cell types, gal3 influences the function of innate immune cells including neutrophils, monocytes, macrophages, endothelial cells and epithelial cells (Henderson *et al*., 2009, Sato *et al*., 2009). This lectin contains a carbohydrate recognition domain (CRD) at the C-terminus (Seetharaman *et al*., 1998) while the N-terminus, also known as the regulatory domain, contains multiple repeats capable of oligomerization after CRD binding (Hsu *et al*., 1992, Ahmad *et al*., 2004).

In this study, we examine the role of gal3 in neutrophil phagocytosis of *C. parapsilosis* and *C. albicans*. We show that gal3 is involved in neutrophil phagocytosis of *C. parapsilosis* yeast and *C. albicans* hyphae, but not *C. albicans* yeast. In addition, we demonstrate that gal3 is secreted from neutrophils and present data that indicate this secreted gal3 acts as a proinflammatory autocrine/paracrine signal in phagocytosis. Taken together, these data suggest that gal3 has a unique role in the neutrophil response to *C. parapsilosis* yeast and *C. albicans* hyphae distinct from *C. albicans* yeast.

RESULTS

Neutrophils have unique phagocytic responses to *C. albicans* yeast compared to *C. parapsilosis* yeast

We have previously shown that neutrophils phagocytose *C. parapsilosis* yeast much more efficiently than *C. albicans* yeast (Linden *et al*., 2010). These results were confirmed by comparing phagocytosis rates of *C. parapsilosis* yeast to *C. albicans* yeast across a range of effector to target (E:T) ratios. Neutrophils underwent phagocytosis of *C. parapsilosis* yeast much more efficiently than *C. albicans* yeast at all E:T ratios (Figure 1A). To evaluate the kinetics, the time course of phagocytosis was investigated. Neutrophils were incubated with *C. parapsilosis* at an effector to target ratio (E:T) of one neutrophil to ten *C. parapsilosis* yeast (1:10). Neutrophils were incubated with *C. albicans* yeast at an E:T ratio of 1:50. Neutrophils were incubated with more *C. albicans* yeast than *C. parapsilosis* yeast in an

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effort to augment phagocytosis rates of the former so that conditions for kinetic comparisons would be as similar as possible. Even when neutrophils were incubated with five times more *C. albicans* yeast, neutrophils underwent phagocytosis of *C. parapsilosis* yeast much faster and more efficiently than *C. albicans* yeast (Figure 1B). The phagocytic process was also evaluated using scanning electron microscopy (SEM). Neutrophils had distinctly different morphological responses to the two species. When incubated with *C. albicans* for ten min, neutrophils exhibited membrane ruffling (Figure 1C), extrusion of large, arm-like protrusions towards single yeast (Figure 1D) and extension of finger-like projections around engulfed *C. albicans* yeast (Figure 1E). In response to incubation with *C. parapsilosis* for 2.5 min, neutrophils exhibited much less membrane ruffling and underwent phagocytosis of multiple yeast at the same time (Figure 1F–H). When incubation time was extended to 10 min with *C. parapsilosis* yeast, very few ongoing phagocytosis events were seen as would be predicted by the kinetics (Figure 1B), however, no membrane ruffling was observed (data not shown). *C. parapsilosis* yeast were more elongated and somewhat smaller than *C. albicans* yeast, (approximately 1×4 microns versus 4×6 microns respectively), which may also influence neutrophil phagocytic efficiency. Taken together however, these data demonstrate that neutrophils respond quite differently to these two *Candida* species.

Endogenous galectin-3 plays an important role in neutrophil phagocytosis of *C. parapsilosis* yeast and *C. albicans* hyphae, but not *C. albicans* yeast

To identify the neutrophil receptors involved in efficient phagocytosis of *C. parapsilosis*, blocking antibodies against neutrophils PRRs known to recognize different fungal cell wall components were screened for their ability to inhibit phagocytosis. Dectin-1 was excluded from the screen because dectin-1 blocking antibody was previously shown to have no effect (Linden *et al.*, 2010). Significant inhibition of *C. parapsilosis* phagocytosis was only seen with antibody directed against gal3 at an E:T ratio of 1:10 (Figure 2A). Treatment with the gal3 antibody did not inhibit the low frequency phagocytosis of *C. albicans* yeast at an E:T ratio of 1:40 (Figure 2B). Neutrophils were incubated with *C. albicans* yeast at a higher E:T ratio to increase the likelihood that differences in phagocytosis rates after antibody treatment could be observed.

Unlike *C. parapsilosis*, *C. albicans* has the capacity to produce true hyphae, a trait necessary for virulence (Lo *et al.*, 1997). Because macrophages have been shown to undergo phagocytosis of *C. albicans* yeast preferentially over *C. albicans* hyphae (Keppler-Ross *et al.*, 2010), we also compared neutrophil phagocytosis rates of *C. albicans* yeast versus *C. albicans* hyphae. To produce hyphal morphology, *C. albicans* was grown in medium 199 to promote germ tube formation (Figure 3A). *C. albicans* germ tubes were phagocytosed much more efficiently than *C. albicans* yeast, even when neutrophils were incubated with eight times more yeast than hyphal forms (Figure 2B). Neutrophils also underwent phagocytosis of *C. albicans* hyphae much more efficiently than *C. albicans* yeast across a range of E:T ratios (Figure 2C). Because of this difference, the role of gal3 in neutrophil phagocytosis of *C. albicans* hyphal forms was also evaluated. Treatment with the gal3 antibody significantly inhibited neutrophil phagocytosis of C. *albicans* germ tubes at an E:T ratio of 1:5 (Figure 2B). Again, neutrophils were incubated with more *C. albicans* yeast than hyphae to ensure that differences in phagocytosis rates after treatment could be observed. Neutrophil treatment with other blocking antibodies against TLR2, TLR4, TLR6, CR3 and dectin-1 also did not significantly affect phagocytosis of *C. albicans* hyphae. (Figure 2D). To exclude an effect of medium 199 itself on phagocytosis rates, *C. parapsilosis* strains were grown under the same conditions and evaluated for morphological changes (Figure 3A). *C. parapsilosis* strains grown under conditions used to induce *C. albicans* germ tubes did not exhibit morphological changes nor were phagocytosis rates significantly affected for the strains tested (Figure 3B). Phagocytosis of *C. parapsilosis* strains grown under germ tube inducing

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conditions was also still inhibited by treatment with the gal3 blocking antibody (Figure 3C). Similar results were seen among all strains tested (Figures 3C and 3D).

Neutrophils express galectin-3 intracellularly and secrete galectin-3 when stimulated

To determine the cellular location of gal3 in neutrophils, indirect immunofluorescence assays were performed with intact and permeabilized cells. A limited amount of gal3 was detected on the cell surface (Figure 4A) while the majority was detected intracellularly (Figure 4B). Treatment with *C. parapsilosis* yeast did not increase cell surface expression of gal3 (Figure 4C). Treatment did significantly increase CD66b expression, which is consistent with neutrophil activation and degranulation (Stocks *et al*., 1995, Fernandez *et al*., 2005, Hajkova *et al*., 2009). Increased cell surface expression of CD66b suggests that gal3 could be secreted during degranulation.

Supernatants of treated neutrophils were examined for the presence of secreted gal3 by western blot (Figure 4D). Because the structure and composition of fungal mannans differs among species and each induces unique immunological responses (Netea *et al*., 2006, McKenzie *et al*., 2010, Rizzetto *et al*., 2010), neutrophils were treated with mannan isolated from *C. albicans*, *C. parapsilosis* and *Saccharomyces cerevisiae*. Because the structure of mannan is also known to differ between the different morphological forms on *C. albicans* (Shibata *et al*., 2007), mannan was extracted from both *C. albicans* yeast and *C. albicans* hyphae. Neutrophils were treated with mannan instead of whole yeast because gal3 is known to adhere to *Candida* species expressing specific β-(1-2) oligomannan (Fradin *et al*., 2000, Kohatsu *et al*., 2006). Mannan treatment had no effect on neutrophil viability as determined by Trypan Blue exclusion (data not shown). Neutrophils were also treated with LPS, fMLP, and PMA to determine if gal3 secretion was a specific response to fungal stimuli. For densitometer measurements, secretion was expressed as relative density compared to the untreated control supernatants. Though there was some variation from donor to donor, treatment with *C. parapsilosis* mannan, *C. albicans* yeast mannan, *C. albicans* hyphae mannan, PMA and LPS all induced gal3 secretion relative to control as determined by western blot (data not shown). As determined by densitometry measurements, however, only *C. parapsilosis* mannan and LPS treatment induced a significant amount of gal3 secretion compared to the untreated control, and secretion induced by *C. parapsilosis* mannan was nearly double that of all other species tested. No significant difference was observed between *C. parapsilosis* and *C. albicans* mannan. Because LPS induced the greatest amount of gal3 secretion, we confirmed that all of our mannan preparations were LPS free (data not shown). These data indicate that *C. parapsilosis* mannan may have unique stimulatory properties capable of inducing gal3 secretion. However gal3 secretion is not unique to fungal stimuli, as treatment with LPS also induced gal3 secretion.

Exogenous galectin-3 increases phagocytosis of *C. parapsilosis* and *C. albicans* yeast

Because neutrophils secrete gal3 when stimulated with *C. parapsilosis* mannan and binding of gal3 to the neutrophil cell surface has been shown to increase phagocytosis (Fernandez *et al*., 2005, Farnworth *et al*., 2008), we determined whether the addition of exogenous gal3 could increase phagocytosis of both *C. parapsilosis* and *C. albicans* yeast. Neutrophils were treated with recombinant gal3 (rGal3) prior to the addition of yeast. Neutrophils were combined with *C. parapsilosis* yeast an E:T ratio of 1:5 and *C. albicans* yeast at an E:T ratio of 1:20 to ensure that differences in phagocytosis rates after treatment could be observed if present. Treatment with rGal3 increased phagocytosis rates of both *C. parapsilosis* and *C. albicans* yeast (Figure 5A), whereas pretreatment of rGal3 with the gal3 blocking antibody inhibited the increase in phagocytosis rates of both species (Figure 5B and 5C, respectively).

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Galectin-3 dependent increase in neutrophil phagocytosis requires recognition of the neutrophil cell surface via the carbohydrate recognition domain with a lesser role for the regulatory domain

In some biological systems, the ability of gal3 to augment the inflammatory response is dependent on oligomerization of the regulatory domain (Rabinovich *et al.*, 2002, Sato *et al.*, 2009). To determine if the CRD or the regulatory domain played a role in the increased phagocytosis of *C. parapsilosis* and *C. albicans* yeast, a series of experiments were performed using full length rGal3 (FL-G3), a truncated form of rGal3 (CRD-G3), and inhibitors of CRD binding (β-lactose, β-lac) and regulatory domain oligomerization (antibody, AB) (Figures 5D and 5E). Phagocytosis of *C. parapsilosis* at an E:T ratio of 1:5 (Figure 5D) and *C. albicans* at an E:T ratio of 1:20 (Figure 5E) was measured to ensure that differences in phagocytosis rates after treatment could be observed. For *C. parapsilosis*, both the FL-G3 and CRD-G3 increased phagocytosis relative to the untreated control. To further delineate the mechanism, both FL-G3 and CRD-G3 were pretreated with either β-lactose (β-lac) to inhibit CRD binding or a gal3 antibody (AB) that inhibits regulatory domain oligomerization prior to neutrophil treatment. When FL-G3 was pretreated with β-lactose, phagocytosis rates were similar to the untreated control, whereas pretreatment with blocking antibody had a less pronounced inhibitory effect. When CRD-G3 was pretreated with β-lactose, phagocytosis was inhibited compared to the CRD-G3 treated neutrophils. CRD-G3 pretreatment with the blocking antibody had no effect as expected given its lack of the regulatory domain. For phagocytosis of *C. albicans*, similar trends were observed. Treatment with both the FL-G3 and CRD-G3 increased neutrophil phagocytosis compared to the untreated control. When FL-G3 was pretreated with β-lactose, phagocytosis rates were similar to the untreated control, whereas pretreatment with the blocking antibody led to much less inhibition. When CRD-G3 was pretreated with β-lactose, phagocytosis was inhibited compared to the CRD-G3 treated neutrophils while pretreatment with the blocking antibody led to no inhibition. Taken together, these data indicate that gal3 mediated phagocytosis of *C. parapsilosis* and *C. albicans* is regulated by CRD binding to the neutrophil cell surface, and to a lesser extent, regulatory domain oligomerization.

Coincubation of *C. albicans* with *C. parapsilosis* increases neutrophil phagocytosis of *C. albicans* yeast

C. parapsilosis mannan induces a significant amount of gal3 secretion compared to untreated controls. This finding together with the observation that exogenous gal3 increases neutrophil phagocytosis of *C. albicans* led to the hypothesis that *C. parapsilosis* induced gal3 secretion will increase phagocytosis of *C. albicans* yeast. To test this hypothesis, labeled *C. albicans* were incubated either with neutrophils alone or in combination with unlabeled *C. parapsilosis* (Figure 6A). The total number of yeast was kept constant for each condition. When *C. albicans* was incubated in the presence of *C. parapsilosis*, phagocytosis of *C. albicans* significantly increased (Figure 6B). Pretreatment of neutrophils with blocking antibodies against gal3 or CR3 inhibited the increase in phagocytosis, but dectin-1 blocking antibody did not, suggesting that the presence of *C. parapsilosis* increases phagocytosis of *C. albicans* and is dependent on both gal3 and CR3. Unexpectedly, the presence of *C. albicans* inhibited phagocytosis of *C. parapsilosis* (Figure 6C), but the possibility that this result was due to steric interference by the large number of *C. albicans* vs. *C. parapsilosis* yeast cannot be excluded. Pretreatment with the gal3 blocking antibody inhibited phagocytosis of *C. parapsilosis* even further, but pretreatment with the CR3 blocking antibodies did not. Again, these data suggest that gal3 plays an important role in phagocytosis of *C. parapsilosis*, even in the presence of *C. albicans*.

Because the presence of *C. parapsilosis* increased phagocytosis of *C. albicans*, we tested whether neutrophils selectively underwent phagocytosis of one species over the other and if

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this was dependant on gal3. This experiment was designed to determine if gal3 increased phagocytosis overall or if it increased phagocytosis of a specific species. *C. parapsilosis* was labeled green, *C. albicans* was labeled orange, and both were combined with neutrophils (Figure 7A). Compared to neutrophils that contained only *C. albicans,* a significantly larger number of phagocytic neutrophils contained either *C. parapsilosis* alone or both *C. parapsilosis* and *C. albicans* (Figure 7B). Pretreatment with the gal3 antibody did not significantly change the distribution of the phagocytic contents. Taken together, these data suggest that neutrophils undergo phagocytosis of *C. parapsilosis* more efficiently than *C. albicans*, but the presence of *C. parapsilosis* activates phagocytic pathways that are not selective to *C. parapsilosis* alone.

DISCUSSION

Several recent studies have demonstrated that the immune system responds differently to individual *Candida* species, suggesting that fungal pathogenesis must be approached in a species specific manner. This study adds to this collection of work by demonstrating that neutrophils have distinctly different phagocytic responses to *C. parapsilosis* versus *C. albicans* yeast. Neutrophils undergo phagocytosis of *C. parapsilosis* yeast much more efficiently and faster than *C. albicans* yeast and also have different morphological responses. Neutrophils exhibit extensive membrane ruffling and produce long, arm like projections towards single *C. albicans* yeast reminiscent of dectin-1 mediated protrusions generated when neutrophils are exposed to unopsonized zymosan particles (Lee *et al*., 2003, Kennedy *et al*., 2007). In comparison, when neutrophils were confronted with *C. parapsilosis* yeast, no membrane ruffling was observed. Neutrophil membranes were, however, observed advancing over the surface of *C. parapsilosis* yeast in a process morphologically similar to phagocytosis of serum opsonized zymosan particles (Kennedy *et al*., 2007). In addition, neutrophils were observed engulfing multiple *C. parapsilosis* yeast at the same time. These differences in phagocytic efficiency and appearance may be due, in part, to gal3. For the first time, we demonstrate that neutrophils secrete gal3, and this secreted gal3 may act as a proinflammatory autocrine/paracrine signal responsible for augmenting neutrophil phagocytosis.

By performing a screen with blocking antibodies against neutrophil receptors known to recognize fungal PAMPs, gal3 was identified to be involved in efficient phagocytosis of *C. parapsilosis* yeast but not *C. albicans* yeast. Although dectin-1, CR3, and possibly TLR2 have been identified as receptors involved in neutrophil phagocytosis of *C. albicans* (Kennedy *et al*., 2007, van Bruggen *et al*., 2007, van Bruggen *et al*., 2009, Linden *et al*., 2010, Tessarolli *et al*., 2010, Li *et al*., 2011), treatment with blocking antibodies against these PRRs did not inhibit phagocytosis of *C. parapsilosis* yeast. Interestingly, gal3 also appears to play a role in phagocytosis of *C. albicans* hyphae which are also phagocytosed much more efficiently than *C. albicans* yeast. Although important in neutrophil phagocytosis, gal3 does not appear to function as a traditional membrane receptor; instead, it appears to augment phagocytosis by acting as a proinflammatory autocrine/paracrine signal. We propose that the exposure of neutrophils to *C. parapsilosis* yeast and *C. albicans* hyphae, but not *C. albicans* yeast, induces gal3 secretion which binds to the neutrophil cell surface, increasing neutrophil phagocytic efficiency. Because treatment with the gal3 blocking antibody only partially inhibited phagocytosis of both *C. parapsilosis* yeast and *C. albicans* hyphae, it is likely that multiple neutrophil receptors may have a role in these processes. Experiments using different combinations of blocking antibodies in conjunction with the gal3 blocking antibody may reduce phagocytosis rates even more and may elucidate other receptors and pathways involved in these phagocytic pathways.

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The effects of exogenous gal3 on neutrophil function have been well documented (Kuwabara *et al.*, 1996, Karlsson *et al.*, 1998, Sato *et al.*, 2002, Nieminen *et al.*, 2008); however, this study is the first to show that neutrophils secrete gal3 and this secretion augments phagocytosis. Several lines of evidence support this conclusion: 1) the majority of neutrophil gal3 is expressed intracellularly but can be secreted when stimulated, 2) *C. parapsilosis* mannan induces the most gal3 secretion from neutrophils among fungal stimuli tested, 3) exogenous gal3 increases phagocytosis of both *C. parapsilosis* and *C. albicans* yeast, 4) neutrophil exposure to *C. parapsilosis* increases phagocytosis of *C. albicans* and this increase is inhibited by a gal3 blocking antibody.

To the best of our knowledge, there is no molecular difference between endogenous and exogenous gal3. However, for endogenous gal3 to augment phagocytosis, it must first be secreted from stimulated neutrophils. Once secreted, this gal3 can further activate neutrophil effector functions, augmenting and increasing phagocytosis. In our system, exposure of neutrophils to *C. parapsilosis* yeast induces more gal3 secretion than exposure to *C. albicans* yeast. This secreted gal3 can then augment neutrophil phagocytosis of either target, which is consistent with the finding that exogenous gal3 increases phagocytosis of both species. Because *C. albicans* yeast did not induce gal3 secretion, gal3 was unavailable to augment phagocytosis of *C. albicans* yeast. However, secreted gal3 is apparently nonselective in its action, as exposure of neutrophils to *C. parapsilosis* increased phagocytosis of *C. albicans* yeast, and this increase was inhibited by gal3 antibody. We propose that the role of gal3 in neutrophil phagocytosis is determined by the ability of the target to induce gal3 secretion.

The mechanism of gal3 secretion from neutrophils remains unclear. Gal3 lacks a signal sequence and is secreted into the microenvironment by activated or damaged cells through a non-classical secretory pathway (Hughes, 1999). It has been proposed that gal3 can be secreted from activated or damaged cells by translocation, exocytosis, or vesicular transport. As neutrophil degranulation is a form of vesicular transport, gal3 secretion could be a result of neutrophil degranulation; however data supporting a specific mechanism of neutrophil gal3 secretion are inconclusive.

The neutrophil receptors involved in induction of gal3 secretion also remain to be defined. Although gal3 recognizes specific β-(1-2) oligomannans (Fradin *et al.*, 2000, Kohatsu *et al.*, 2006), it is unlikely that this recognition is responsible for initiating gal3 secretion for several reasons. Previous studies have demonstrated that *C. albicans* expresses β-(1-2) oligomannans (Fradin *et al.*, 2000) while *C. parapsilosis* does not (Suzuki, 1997). Additionally, because *C. parapsilosis* mannan induced more gal3 secretion than *C. albicans* mannan it seems improbable that β-(1-2) oligomannan expression on yeast plays a role in initiating gal3 secretion. It is also unlikely that dectin-1, TLR2, TLR4, TLR6 or CR3 play a role in secretion, as treatment with these blocking antibodies did not inhibit phagocytosis of *C. parapsilosis* yeast. If these receptors were necessary for gal3 secretion, treatment with the blocking antibodies should have inhibited gal3 secretion, therefore reducing phagocytosis. It seems probable, however, that several receptors may play a role in neutrophil gal3 secretion as LPS, a known TLR4 ligand, induced the most gal3 secretion. The involvement of additional receptors is also suggested by the observation that treatment with the gal3 blocking antibody inhibited phagocytosis of both *C. parapsilosis* yeast and *C. albicans* hyphae, yet only mannan extracted from *C. parapsilosis* yeast induced a significant amount of gal3 compared to the untreated control. Since the mannan from *C. albicans* hyphae was less stimulatory of galectin-3 secretion, the fungal ligand inducing galectin-3 secretion in response to *C. albicans* hyphae may be a cell wall protein. Indeed, many aspects of host immunity respond quite differently to *C. albicans* yeast versus hyphal morphologies (Gow *et al.*, 2012). Experiments investigating gal3 secretion specifically in conjunction with specific

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targets and specific blocking antibodies would be informative. More studies are needed to determine the mechanisms and receptors involved in neutrophil gal3 secretion.

The main neutrophil receptors for gal3 have been reported to be CD66a and CD66b (Feuk-Lagerstedt *et al.*, 1999). Interestingly, co-ligation of CD66a and CD66b with monoclonal antibodies increased neutrophil receptor clustering and integrin-mediated adhesion, suggesting that gal3 mediated activation of neutrophils may be mediated by gal3 ligation of these receptors (Stocks *et al.*, 1995, Ruchaud-Sparagano *et al.*, 1997). In addition, previous studies have shown that primed neutrophils treated with exogenous gal3 internalize the lectin at 37°C within 5 min (Nieminen *et al.*, 2005), indicating that internalization of gal3 may play a role in neutrophil activation. This internalization of gal3 may explain why we did not see an increase in gal3 surface expression after neutrophil treatment with whole *C. parapsilosis* yeast at 37°C for 15 min. Alternatively, because we did not cross-link gal3 to the cell surface prior to FACS analysis, bound gal3 may have been washed off and resulted in absence of gal3 staining. Gal3 may also exert its proinflammatory effect on neutrophil function by activating the p38 mitogen activated protein kinase (MAPK) pathway, an important signaling pathway in neutrophils (Fernandez *et al.*, 2005). Gal3 mediated activation of the p38 MAPK pathway or other important signaling pathways may be activating CR3, a receptor that requires activation for proper functioning. Gal3 mediated CR3 activation may explain the increase in neutrophil phagocytosis of *C. albicans* yeast when coincubated with *C. parapsilosis* yeast. This is supported by the observation that blocking antibodies against both gal3 and CR3 inhibited increased phagocytosis. The observation that CR3 blocking antibodies also inhibited the increase in *C. albicans* phagocytosis suggests that neutrophil contact with *C. parapsilosis* leads to CR3 activation, a necessary step in efficient functioning of this receptor (Hynes, 2002). Although a recent study has suggested that neutrophil recognition of *C. albicans* yeast requires CR3 activation via dectin-1 signaling (Li *et al.*, 2011), dectin-1 does not appear to play a role when *C. parapsilosis* is present. Such interactions between gal3 and CR3 are the focus of ongoing investigation.

The proinflammatory activity of gal3 has been shown to be dependent on oligomerization of the regulatory domain after ligand recognition by the carbohydrate recognition domain (CRD) (Liu *et al.*, 1995, Kuwabara *et al.*, 1996, Nieminen *et al.*, 2007, Karlsson *et al.*, 2009). Oligomerization can result in ligand cross-linking on the same cell surface, resulting in augmentation of many inflammatory responses. To determine the mechanistic role of the CRD and regulatory domain (RD) of gal3 in phagocytosis of *Candida* species, we treated neutrophils with both full length and truncated gal3. Full length gal3 contains both the CRD and RD of gal3, while the truncated form only contains the CRD. In addition, we also treated gal3 with β-lactose, a competitive inhibitor of the CRD. Data from these experiments indicated CRD binding to the neutrophil cell surface was sufficient to augment neutrophil phagocytosis. The RD also appears to play a role, as treatment of the FL rGal3 with a blocking antibody that inhibits RD oligomerization partially inhibited the increase in neutrophil phagocytosis. The RD of gal3 could influence phagocytosis through several different mechanisms including establishing cell surface receptor lattices, causing receptor clustering, and by bridging or cross-linking microbes to the neutrophil cell surface (Sato *et al.*, 2004). Further investigation into the exact mechanisms involved in gal3 dependant phagocytosis is still needed.

In addition to augmenting neutrophil phagocytosis, gal3 secreted from activated neutrophils may play an important role in host defense against invasive fungal disease by activating additional neutrophil effector functions, having direct antifungal activity, and playing a role in additional immune recruitment. Gal3 has been shown to play a role in neutrophil effector functions including ROS production, degranulation and increased phagocytosis and

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inhibition of apoptosis (Farnworth *et al.*, 2008, Forsman *et al.*, 2008, Karlsson *et al.*, 2009, Alves *et al.*, 2010, Fermino *et al.*, 2011). Gal3 has also been shown to kill *Candida* species expressing specific β(1,2) linked oligomannans, including *C. albicans* (Kohatsu *et al.*, 2006). Gal3 has also been implicated in integrin independent neutrophil recruitment to *Streptococcus pneumonia* infected lungs (Sato *et al.*, 2002, Farnworth *et al.*, 2008, Nieminen *et al.*, 2008) by acting as a bridging molecule between neutrophils and endothelial cells (Sato *et al.*, 2002, Gil *et al.*, 2006, Nieminen *et al.*, 2007). Understanding how neutrophil secreted gal3 influences host defense will further our understanding of fungal pathogenesis and may lead to the development of novel therapeutics.

EXPERIMENTAL PROCEDURES

Organisms

C. albicans strains used in this study were SC5314 and Ca3153A. *C. parapsilosis* strains included the clinical isolates Ro75-R1, Ro18-G3, Ro88-R2, and Ro98-R1 from colonized infants (Bliss *et al.*, 2008) and the clinically invasive isolate 15-72391-101, referred to here as JMB81 (Benjamin *et al.*, 2010). Strains were maintained on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). Overnight (ON) cultures were grown for 16 h at 37°C with vigorous agitation in YPD broth. *C. albicans* strain Ca3153A and *C. parapsilosis* strain Ro75-R1 were used for phagocytosis assays unless otherwise noted. To induce germ tube formation, yeast from washed ON cultures were incubated in medium 199 at 37°C. *C. albicans* strains were incubated for 60 min while *C. parapsilosis* strains were incubated for 90 min.

Calcofluor White staining

Strains grown in YPD or medium 199 were washed with HBSS, stained with 20 μg/ml Calcofluor White for 15 min at room temperature, washed again, and examined by fluorescence microscopy.

Neutrophil isolation

Human neutrophils were isolated from healthy adult peripheral blood by density gradient centrifugation as previously described (Linden *et al.*, 2010). Briefly, leukocytes were separated from whole blood using Histopaque-1077 (Sigma), dextran sedimentation and hypotonic lysis of contaminating erythrocytes. Cells were adjusted to the appropriate concentration in Hank's Balanced Salt Solution (HBSS) with Ca/Mg. All experiments involving human subjects were approved by the Institutional Review Board.

Phagocytosis assay with a single *Candida* species

Yeast from ON cultures were heat killed at 65°C for 90 min and labeled with fluorescein isothiocyanate (FITC). To induce germ tube formation, *C. albicans* yeast was incubated in medium 199 for 1 h at 37°C and then heat killed. Phagocytosis assays were performed as previously described (Linden *et al.*, 2010). Live organisms were also tested in phagocytosis assays with similar results (Linden *et al.*, 2010). Briefly, 5×10^5 neutrophils were combined with the number of yeast appropriate to achieve the desired effector to target ratio (E:T). Cells were pelleted and incubated at 37°C for 30 min unless otherwise noted. For select experiments, cell pellets were resuspended in ice cold HBSS to stop phagocytosis at indicated time points. To visualize phagocytosis, resuspended cells were combined with ethidium bromide (100 μg/ml) and examined by fluorescence microscopy. Intracellular yeast appeared green while external yeast appeared orange. To calculate percent phagocytosis, the number of neutrophils with internalized green yeast was divided by the total number of neutrophils. A minimum of 100 neutrophils were counted for each

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condition. Where indicated, neutrophils were treated with 25 μg of blocking antibodies against TLR2 (Clone TL2.1, Abcam), TLR4 (Clone HTA125, Abcam), TLR6 (Clone C585, Invitrogen) and gal3 (Clone B2C10, Genway) on ice for 30 min prior to the addition of yeast. To block the CR3 receptors, neutrophils were treated with a cocktail of antibodies against the lectin (Clone ICR44, eBioscience) and I domain of CD11b (Clone vim12, Invitrogen) as well as CD18 (Clone CBL158, Millipore) at 25 μg each. IgG1 and IgG2 antibodies were used as isotype controls. Neutrophils were also treated with 1–5 μg of human rGal3 (Biovision, Milpitas, CA) for 30 min on ice prior to addition of yeast. To block the effects of rGal3, 2 μg of rGal3 was pretreated with 25 μg gal3 blocking antibody before addition of neutrophils.

SEM Analysis of phagocytic neutrophils

Neutrophils (1×10^7) were combined with *C. albicans* strain Ca3153A or *C. parapsilosis* strain Ro98-R1 at an E:T ratio of 1:50 or 1:10 for 10 or 2.5 min, respectively. Cells were fixed in 2% v/v glutaraldehyde or 3% v/v paraformaldehyde for 30 min and transferred to 13mm isopore filters. Samples were rinsed with 0.1M sodium cacodylate, post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 30 min, and rinsed again. Rinsed samples were dehydrated in an ascending ethanol series from 10% to 100% ethanol. Samples were critical point dried using liquid CO_2 (Tousimis CPD), mounted and metal coated with 60/50 gold-palladium, and examined using a Hitachi S-5800 field emission scanning electron microscope (FESEM).

Phagocytosis assay with multiple *Candida* species

For phagocytosis experiments involving coincubation of *C. albicans* and *C. parapsilosis*, yeast were differentially labeled. When evaluating phagocytosis of *C. albicans* when coincubated with *C. parapsilosis*, *C. albicans* was labeled with cell tracker orange (Invitrogen) and then heat killed. Heat killed *C. parapsilosis* was left unlabeled. When evaluating phagocytosis of *C. parapsilosis* when coincubated with *C. albicans*, *C. parapsilosis* was labeled with cell tracker orange and *C. albicans* was left unlabeled. When incubated alone, neutrophils were combined at an E:T of 1:30 or 1:10 for *C. albicans* or *C. parapsilosis*, respectively. When coincubated, neutrophils were combined with *C. albicans* at an E:T ratio of 1:20 plus *C. parapsilosis* at an E:T ratio of 1:10. Phagocytosis assays were performed as described above, and external fluorescence of yeast was quenched with Trypan Blue. Where indicated, neutrophils were treated with 25 μg blocking antibodies against gal3 (Clone B2C10, Genway) and dectin-1 (Clone GE2, Abcam) on ice for 30 min prior to addition of yeast. To block the CR3 receptors, neutrophils were treated with a cocktail of antibodies against the lectin domain (Clone ICR44, eBioscience) and I domain of CD11b (Clone vim12, Invitrogen) as well as CD18 (Clone CBL158, Millipore) at 25 μg each.

For selective phagocytosis experiments, heat killed *C. albicans* were stained with ethidium bromide (100 mg/ml) for 10 min at room temperature and *C. parapsilosis* were stained with FITC as described above and extensively washed in 3% BSA/PBS. When incubated alone, neutrophils were combined at an E:T of 1:30 or 1:10 for *C. albicans* or *C. parapsilosis*, respectively. When coincubated, neutrophils were combined with *C. albicans* at an E:T ratio of 1:20 plus *C. parapsilosis* at an E:T ratio of 1:10. Phagocytosis assays were performed as described above and external fluorescence of yeast was quenched with Trypan Blue. To determine the percent of phagocytic neutrophils, neutrophils were scored as containing orange *C. albicans* only, green *C. parapsilosis* only, or both; divided by the total number of neutrophils with internalized yeast. Where indicated, neutrophils were treated with the gal3 blocking antibody.

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Immunofluorescence Assay

To determine cell surface and intracellular expression of gal3, neutrophils were fixed in 3.3% formalin or 3.3% formalin + 0.1% tween-20, respectively, for 30 min at room temperature. Fixed cells were blocked in 3% BSA/PBS and probed with anti-gal3 antibody (Clone B2C10, Genway), followed by the appropriate secondary antibody. Cells were examined by fluorescence microscopy.

FACS analysis

Neutrophils were treated with unlabeled, heat killed *C. parapsilosis* yeast at an E:T ratio of 1:10. Cells were pelleted and incubated at 37°C for 30 min. Untreated pelleted neutrophils incubated at 37°C for 30 min were used as controls. Cell suspensions were fixed in 3.3% formalin for 30 min, blocked in 3% BSA/PBS and probed with antibodies against TLR2 (Clone TL2.1, Abcam), TLR4 (Clone HTA125, Abcam), TLR6 (Clone C585, Invitrogen) and gal3 (Clone B2C10, Genway), CD18 (Clone CBL158, Millipore), CD11b (Clone ICR44, eBioscience), and CD66b (Clone 80H3, Gentex). Two separate antibodies against gal3 were used; one that recognized the CRD domain (Clone Gal397, Biolegend) and another that recognized the regulatory domain (Clone B2C10, Genway). IgG1 and IgG2 isotypes were used as controls. Appropriate secondary antibodies were applied, and cells were examined with a BD Biosciences FACSCanto instrument.

Western blot analysis and Densitometry Measurements

Neutrophils (1×10^7) were treated with 15 mg/ml mannan, 0.1 mg/ml fMLP, 2.5 μg/ml PMA, or 125 mg/ml LPS for 15 min at 37°C. Untreated neutrophils incubated for 15 min at 37°C were used as control. Whole cell neutrophil lysates were collected by lysing neutrophils in 5.8% Octyl β-D-glucopyranoside in PBS plus protease inhibitors on ice for 30 min. Mannan was extracted from *C. albicans* strain Ca3153A and *C. parapsilosis* strain Ro75-R1 grown in YPD at 37°C for 48 h and *S. cerevisiae* strain By5751a grown at 30°C for 48 h as previously described (van de Veerdonk *et al.*, 2009). Mannan extracts were tested for endotoxin using the *Limulus* Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit (Pierce) per manufacturer's instructions. To extract mannan from *C. albicans* hyphae, strain Ca3153A was grown in m199 at 37°C for at least 48 h. Cells were spun at $16.1 \times g$ for 10 min and supernatants were separated by SDS-PAGE, analyzed by western blot with gal3 antibody (Galectin-397) and an appropriate secondary antibody. Bands were visualized by chemiluminescence, and densitometry measurements were made using ImageJ software.

Statistics

For parametric variables, comparisons were made by ANOVA. Between-group comparisons were made by the Holm-Sidak test unless otherwise indicated. *P* values 0.05 were considered significant. Tests appropriate for nonparametric variables were used as indicated.

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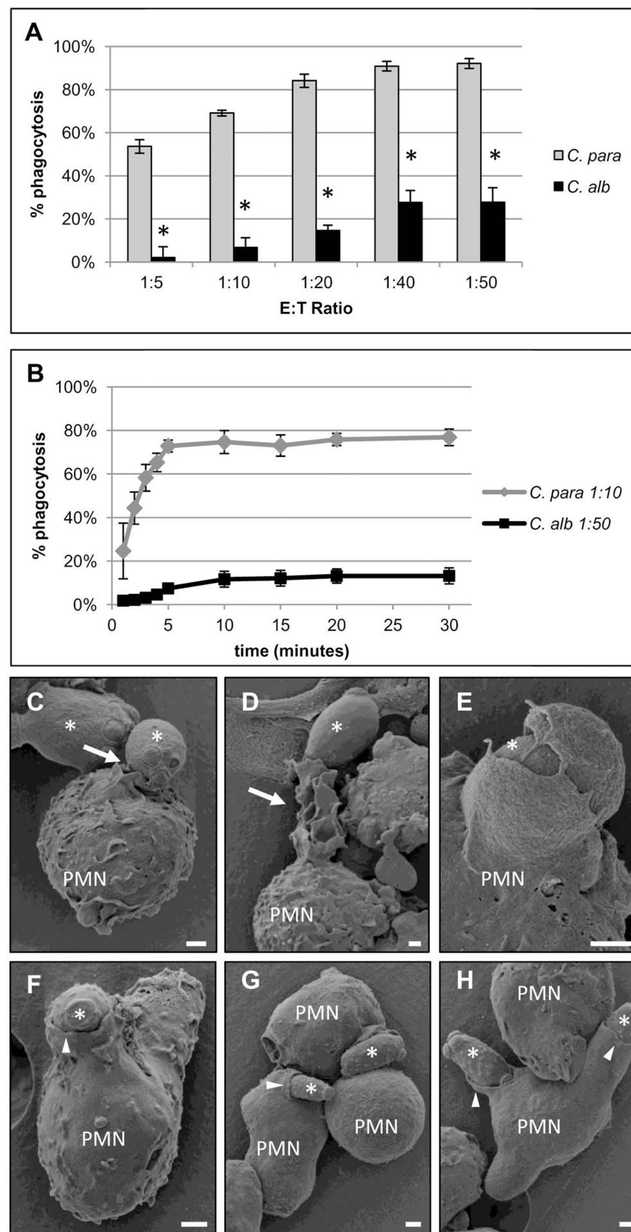

Figure 1. Neutrophils have different phagocytic responses to *C. parapsilosis* compared to *C. albicans* yeast

Results are mean ± SEM of at least three different neutrophil donors. **(A)** Neutrophil phagocytosis rates of *C. parapsilosis* (*C. para*) and *C. albicans* (*C. alb*) yeast at various effector to target (E:T) ratios. * *p* 0.05 comparing *C. para* and *C. alb* at that E:T ratio. % phagocytosis was calculated by dividing the number of neutrophils with internalized yeast by the total number of neutrophils. **(B)** Neutrophil phagocytosis rates of *C. parapsilosis* (*C. para*) at an E:T ratio of 1:10 or *C. albicans* (*C. alb*) at an E:T ratio of 1:50 at indicated time points. **(C–H)** Scanning electron microscopy (SEM) photomicrographs of fixed neutrophils after 10 min of incubation with *C. albicans* **(C–E)** or 2.5 min of incubation with *C. parapsilosis* **(F–H)**. Selected neutrophils are labeled (PMN) and yeast are indicated by asterisks. White arrows indicate neutrophil membrane ruffling and pseudopodia extending

towards *C. albicans* yeast. White arrow heads indicate neutrophil membranes smoothly advancing over *C. parapsilosis* yeast. Bar = 1 micron.

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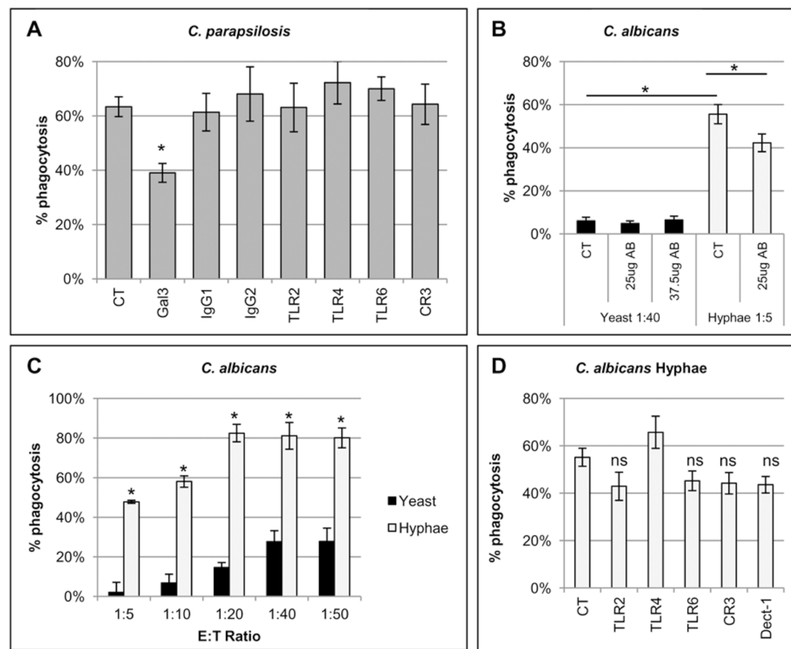

Figure 2. Endogenous gal3 plays a role in neutrophil phagocytosis of *C. parapsilosis* yeast and *C. albicans* hyphae

Results are mean ± SEM of at least three separate neutrophil donors. **p* 0.05 compared to untreated controls (CT) unless otherwise indicated. **(A)** Phagocytosis rates of *C. parapsilosis* after neutrophil treatment with 25 μg blocking antibodies at an E:T ratio of 1:10. Blocking antibodies were specific for gal3, toll-like receptor 2 (TLR2), TLR4, and TLR6. Three antibodies were used to block complement receptor 3 (CR3) including antibodies against CD11b domains and CD18. IgG1 and IgG2 were isotype controls. **(B)** Phagocytosis rates of *C. albicans* yeast or hyphal forms after neutrophil treatment with a blocking antibody against gal3 at indicated doses at an E:T ratio of 1:40 or 1:5, respectively. **(C)** Phagocytosis rates of *C. albicans* yeast or hyphae at various E:T ratios. * *p* 0.05 comparing yeast and hyphae at that E:T ratio. **(D)** Phagocytosis rates of *C. albicans* hyphae after neutrophil treatment with 25 μg blocking antibodies at an E:T ratio of 1:5. Blocking antibodies were specific for TLR2, TLR4, TLR6 and dectin-1 (Dect-1). Three antibodies were used to block complement receptor 3 (CR3) including antibodies against CD11b domains and CD18. ns=not significant. n=9 independent experiments for CT, TLR2, CR3 and Dect-1 and n=4 for TLR2.

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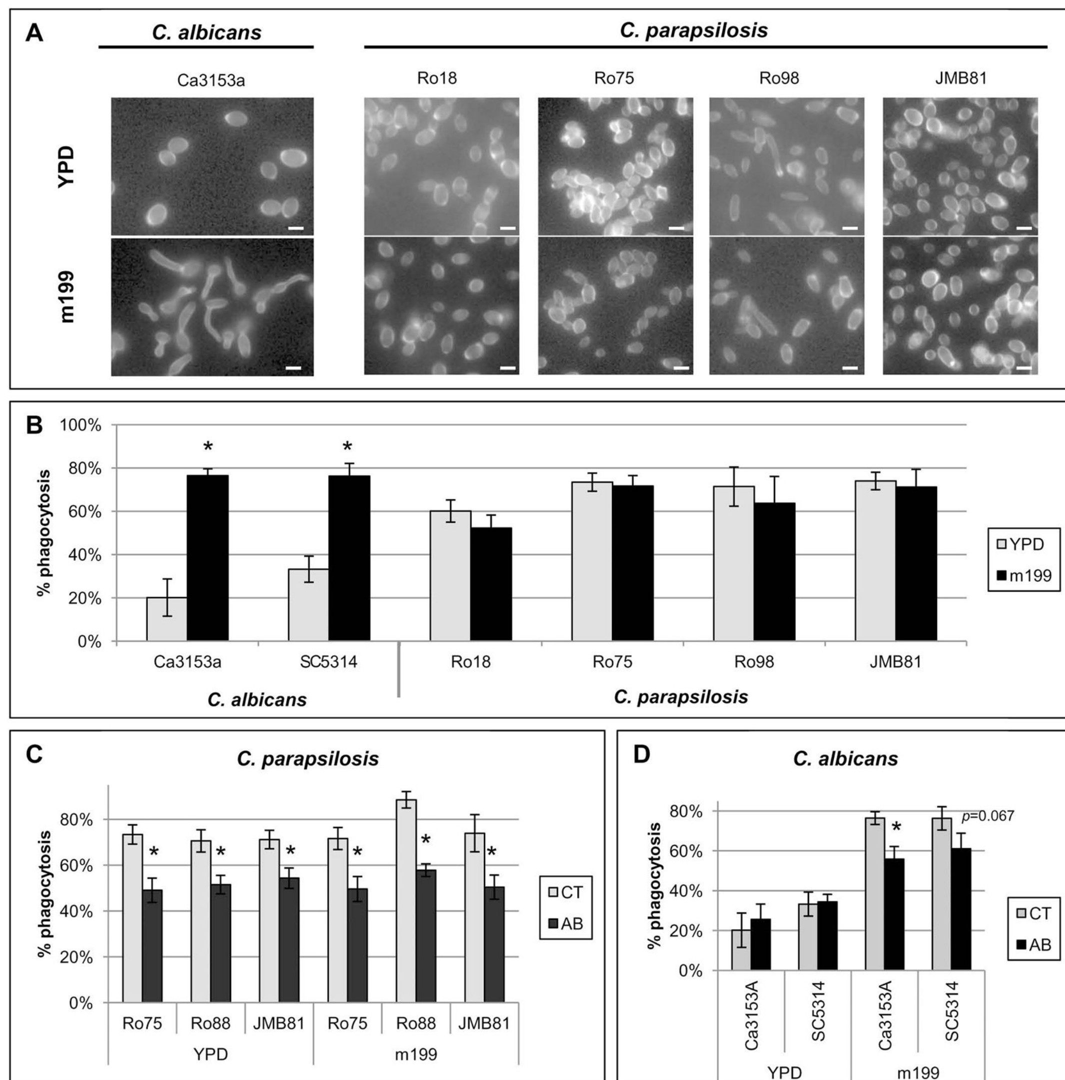

Figure 3. Evaluation of additional *C. albicans* and *C. parapsilosis* stains

Results are mean ± SEM of at least two separate neutrophil donors in three separate experiments. Between-group comparisons were made by the Fisher LSD test. * p 0.05. **(A)** Photomicrographs of *C. albicans* or *C. parapsilosis* strains grown as yeast in YPD broth or grown in medium 199 (m199) to induce germ tube formation. Cells were stained with Calcofluor White to facilitate imaging. *C. albicans* exhibited germ tube growth while *C. parapsilosis* strains exhibited no detectable morphological change. Bar = 4 microns. **(B)** Neutrophil phagocytosis rates of *C. albicans* and *C. parapsilosis* grown in YPD or m199 at an E:T of 1:10. **(C–D)** Neutrophil phagocytosis rates of selected *C. parapsilosis* strains **(C)** or *C. albicans* strains **(D)** grown in YPD or m199 at an E:T of 1:10. Phagocytosis rates of untreated neutrophils (CT) were compared to phagocytosis rates of neutrophils treated with gal3 blocking antibody (AB).

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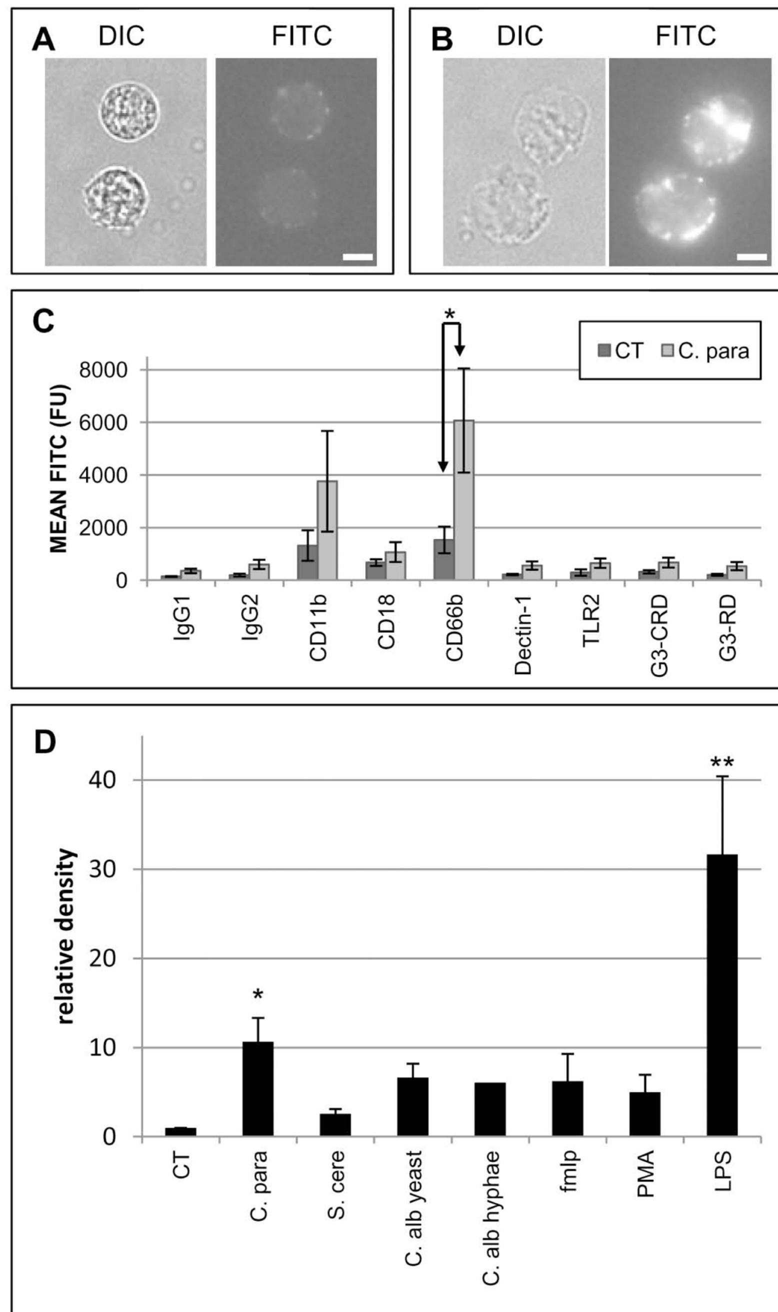

Figure 4. Neutrophils express galectin-3 intracellularly and secrete galectin-3 after stimulation

All results are representative of at least three different donors ± SEM. *p 0.05 and **p 0.005 compared to control. Surface **(A)** or intracellular **(B)** expression of gal3 in neutrophils by immunofluorescence assay. Bar = 5 microns. **(C)** Surface receptor expression of untreated neutrophils (CT) or neutrophils treated with whole *C. parapsilosis* yeast (C. para) by FACS analysis. Surface expression of CD11b, CD18, CD66b, dectin-1, TLR2, the gal3 carbohydrate recognition domain (G3-CRD), and the gal3 regulatory domain (G3-RD) were evaluated. Isotype control antibodies (IgG1 and IgG2) were used as controls. Expression was measured as arbitrary fluorescent units (FU) and expressed as mean FITC. **(D)** Gal3 secretion from neutrophils by western blot analysis and densitometric measurements.

Densitometric measurements are expressed as mean relative density compared to untreated controls of at least three separate donors, except *C. albicans* hyphae (two donors). Neutrophils were treated with mannan isolated from *C. parapsilosis* (*C. para*), *S. cerevisiae* (*S. cere*), *C. albicans* yeast (*C. alb* yeast) or *C. albicans* hyphae (*C. alb* hyphae); or fMLP, PMA and LPS.

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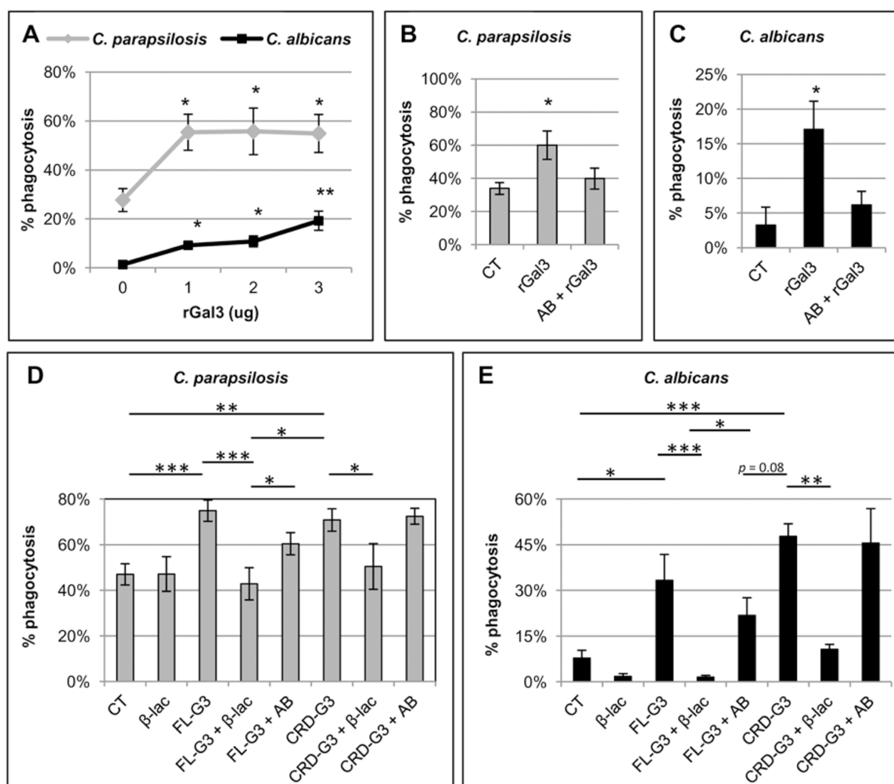

Figure 5. Exogenous galectin-3 augments phagocytosis of *C. parapsilosis* and *C. albicans*

Results are mean ± SEM of at least three different neutrophil donors. * *p* 0.05, ***p* 0.005, ****p* 0.0005 compared to untreated controls. **(A)** Phagocytosis rates of *C. albicans* and *C. parapsilosis* at an E:T ratio of 1:20 and 1:5, respectively, after neutrophil treatment with rGal3 at the indicated doses. Untreated neutrophils (0 μg) were used as a control. Phagocytosis of *C. parapsilosis* at an E:T ratio of 1:5 **(B)** and *C. albicans* at an E:T ratio of 1:20 **(C)** after treatment with 2 μg of rGal3 alone or after pretreatment with 25 μg of gal3 blocking antibody before adding neutrophils (AB + rGal3). Untreated neutrophils (CT) were used as control. Phagocytosis of *C. parapsilosis* yeast at an E:T ratio of 1:5 **(D)** and *C. albicans* yeast at an E:T ratio of 1:20 **(E)** was measured after treatment with 3 μg full length recombinant gal3 (FL-G3) or a truncated form of recombinant gal3 containing only the CRD domain (CRD-G3). FL-G3 and CRD-G3 were pretreated with either β-lactose (β-lac) to inhibit CRD binding or a gal3 antibody (AB) that inhibits regulatory domain oligomerization prior to neutrophil treatment.

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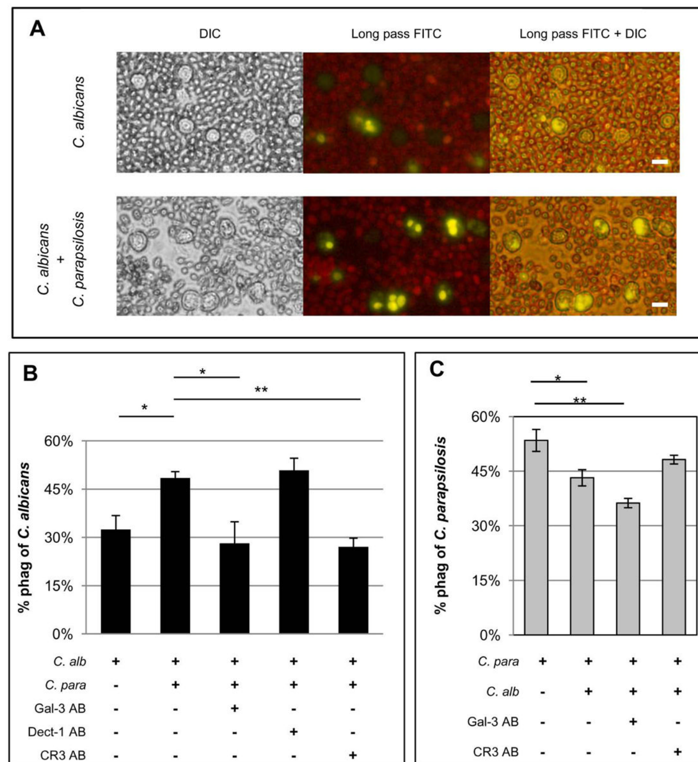

Figure 6. Exposure of neutrophils to *C. parapsilosis* increases phagocytosis of *C. albicans*

All images are representative fields of at least three different donors and results are mean ± SEM of at least three different donors. *p 0.05, **p 0.005. Bar = 10 microns. **(A)** Images of neutrophil phagocytosis of *C. albicans* labeled yellow when incubated alone (top panel) or in combination with unlabeled *C. parapsilosis* (bottom panel). **(B)** Neutrophil phagocytosis rates of *C. albicans* (*C. alb*) when incubated alone, coincubated with *C. parapsilosis* (*C. para*), or coincubated with *C. parapsilosis* after neutrophils were pretreated with blocking antibodies against gal3 (Gal-3 AB), dectin-1 (Dect-1 AB), or CR3 (CR3 AB). **(C)** Neutrophil phagocytosis rates of *C. parapsilosis* (*C. para*) when incubated alone, coincubated with *C. albicans* (*C. alb*), or coincubated with *C. albicans* after neutrophil pretreatment with blocking antibodies against gal3 (Gal-3 AB) or CR3 (CR3 AB).

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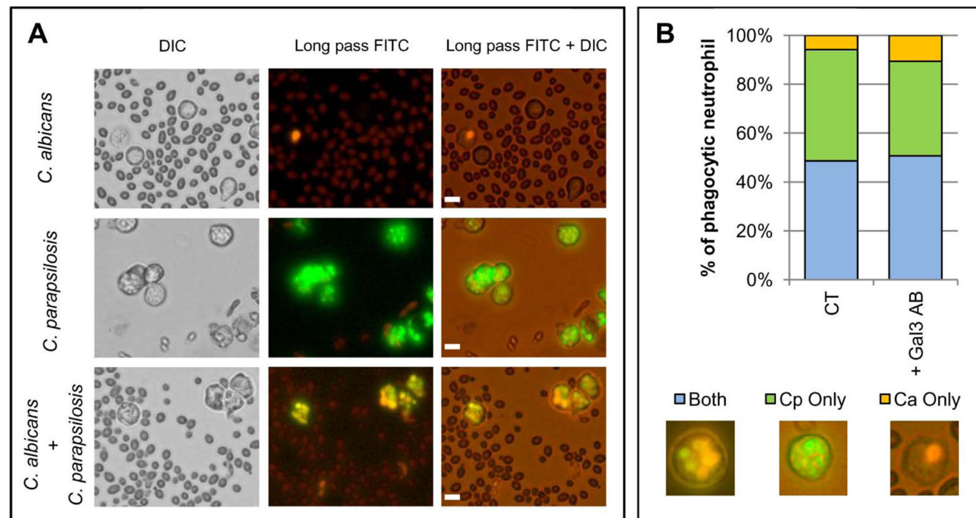

Figure 7. Neutrophils do not selectively phagocytose *C. parapsilosis* yeast over *C. albicans* yeast during coincubation

All images are representative fields of at least three different donors. Bar = 10 microns. **(A)** Images of neutrophil phagocytosis of *C. albicans* dyed orange with ethidium bromide alone (top panel), *C. parapsilosis* dyed green with FITC alone (middle panel), or both orange *C. albicans* and green *C. parapsilosis* together (bottom panel). **(B)** Percent of total phagocytic neutrophils containing only *C. albicans* (Ca) or *C. parapsilosis* (Cp) or both species together. Images under the graph represent neutrophils containing both *C. albicans* and *C. parapsilosis* (left panel), green *C. parapsilosis* only (middle panel), or orange *C. albicans* only (right panel). Phagocytic contents of untreated neutrophils (CT) and neutrophils pretreated with gal3 blocking antibody (+Gal3 AB) were evaluated.