# RECENT ADVANCES IN THE STUDY OF THE HOST-FUNGUS INTERACTION

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# RECENT ADVANCES IN THE STUDY OF THE HOST-FUNGUS INTERACTION

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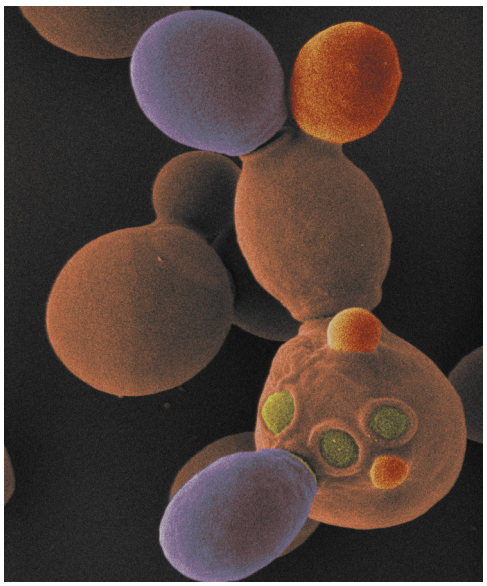

Scanning electron microscopic image of *Candida parapsilosis* budding yeast cells (Orange: freshly shaped daughter cells, Green: bud scars, Blue: daughter cells after cytokinesis), Magnification: 30000x.
(Pedro Leão, Csaba Papp, Leonardo Nimrichter, Attila Gácser)

Fungal infections represent nowadays a significant burden on the healthcare system of most of the countries, and are among the infections with the highest mortality rates. This has fostered the study of the interaction of these organisms with the human host. The outer most layer of a fungal cell is the cell wall, and together with the secreted components into the extracellular compartment, are the first lines of contact with the host cells. This interaction is critical for tissue adhesion, colonization and damage. In addition, these fungal extracellular components will define the outcome of the interaction with the host immune cells, leading either to the establishment of a protective antifungal immune response or to an immune-evasive mechanism by the fungal cell. On the other hand, our immune system has effectively evolved to deal with fungal pathogens, developing strategies for cell eradication, burden control, or antigen presentation from the innate branch to the adaptive immune response. Here, we provide a series of comprehensive review papers dealing with both aspect of the interaction fungus-immune cells: the role of virulence factors and cell wall components during such interaction, and the recent advances in the study of cellular receptors in the establishment of a protective anti-fungal immune response.

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# Table of Contents

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EDITORIAL
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# Editorial: Recent Advances in the Study of the Host-Fungus Interaction

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## The Editorial on the Research Topic

## Recent Advances in the Study of the Host-Fungus Interaction

Fungal infections are worldwide distributed and are associated with high morbidity rates, and in immunocompromised populations, are usually life-threatening conditions. The host-fungus interaction is critical for tissue adhesion, colonization and damage. Here, we provide a series of comprehensive review and original papers dealing with both aspects of this interaction: the role of virulence factors and cell wall components during contact with host cells, and the recent advances in the study of the antifungal immune response.

The fungal cell wall, along with the capsule and secreted components, is a relevant organelle during interaction with the host, and is the source of most of the pathogen-associated molecular patterns (PAMPs) recognized by immune cells. Melanin is one of its components found in many human pathogens, and plays a significant role in protecting the fungal cell against extracellular insults, including damage by immune cells. Here, Nosanchuk et al., provide an updated view of melanin synthesis and interaction with other wall components, with emphasis in the information derived from new analytical approaches.

Dermatophytes are the most frequent fungal pathogens causing human infections. Although most of them are related to superficial and not harmful infections, they can cause chronic dermatophytosis in immunocompromised population. Here, de Sousa et al., explore the mechanism underlying this infection, but in immunocompetent patients. Their results indicate that macrophages and neutrophils from patients with the chronic disease showed bias to generate a strong anti-inflammatory response. Since this observation was not found in patients with superficial skin infections, these data stress a differential host-dermatophyte interaction in both superficial and deep tissues.

*Paracoccidioides* spp. is the etiological agent of paracoccidioidiomycosis, a relevant systemic fungal infection in South America. The disease is mostly associated to rural workers, malnutrition, poor hygiene and chronic alcohol consumption and smoking. Here, de Oliveira et al. summarize the most important finding regarding cell surface adhesins, highlighting their role in virulence. Furthermore, authors explore the importance of the morphological switching during host colonization and tissue damage, as the filament to yeast transition is accompanied with changes in the cell wall and surface molecules that have a direct impact on the host-fungus interaction. As a new therapeutic and prophylactic approach to control paracoccidioidiomycosis, Bueno et al., describe the use of polyclonal antibodies against acidic glycosphingolipids to opsonize *P. brasiliensis* yeast cells. This strategy successfully increased fungal phagocytosis and had a positive impact in the murine model of intratracheal infection, with lower fungal burdens and tissue damage.

*Histoplasma capsulatum* is the causative agent of a worldwide distributed systemic disease that often affects lungs. As in the case of other fungal pathogens, phagocytic cells play a key role in controlling *H. capsulatum* yeast cells, being readily phagocytosed upon detection. Pitangui et al.

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demonstrate that, upon phagocytosis, *H. capsutalum* yeast cells aggregate around the nucleus of murine alveolar macrophages, forming a crown-like structure that stimulates macrophage apoptosis, thus describing a new mechanism to evade control by these immune cells.

Systemic cryptococcosis, candidiasis, and aspergillosis account for most of the opportunistic infections caused by fungi. Infections associated to *Cryptococcus neoformans* often affect immunocompromised patients; while the closely related species *Cryptococcus gattii* mainly affects healthy individuals and displays a decreased tropism to the central nervous system. Leopold Wager et al. describe the most relevant finding about the immune response stimulated by *Cryptococcus* spp., with especial emphasis in phagocytic cells. They discuss the survival and escaping mechanisms evolved for these pathogens to survive the macrophage strike, pointing out to the capsule presence as a mechanism to prevent phagocytosis, and phospholipase B as one of the mechanisms to survive within phagosomes. Finally, they also discuss the main PAMPS and receptors that allow recognition of *Cryptococcus* by immune cells and the potential of immunomodulatory therapies for cryptococcosis control. Reinforcing the central role of macrophages in *Cryptococcus* control, DeLeon-Rodriguez and Casadevall discuss the strategies involved in the survival of *C. neoformans* within the phagolysosome, with special emphasis in the observation that the outcome of the yeast-macrophage interaction depends on the extent of the damage to the phagosomal membrane.

Invasive aspergillosis is also considered as a common infection in immunocompromised patients, and it is usually caused by *Aspergillus fumigatus*. Here, Espinosa and Rivera present a thoroughly review about the receptors involved in the immune recognition of this pathogen, along with the relevance of most of the innate immune cells during the *Aspergillus*-host interaction.

Systemic candidiasis is one of the most frequent nosocomial fungal infections and is often associated to immunosuppression, tissue colonization and damage by *Candida albicans*. This organism forms part of the human microbiota, and has to adapt to several environmental conditions prevailing in human mucosa. Here, Prieto and Pla, using fluorescent *C. albicans* cells demonstrate that this organism rapidly adapts to the mouse gut, increasing the cell fitness once they proliferated for long colonizing periods; a fact that should be taken in consideration during candidiasis treatment. In addition of this, *C. albicans* needs to adhere to host tissues and cells in order to colonize and invade. Hoyer and Cota put together most of the relevant information generated in the last 20 years about one of the most studied adhesins family in *C. albicans*, the ALS family; with emphasis in the structural determinants that control ligand binding, aggregation and invasion of host cells.

Members of the *Candida parapsilosis* complex are other fungal species usually associated to candidiasis. Estrada-Mata et al., demonstrate that these organisms have subtle differences in the cell wall organization when compared to *C. albicans*, which are likely related with a differential recognition when incubated with human peripheral blood mononuclear cells. At difference of *C. albicans*, *C. parapsilosis sensu lato* stimulates high cytokine levels in a *O*-mannan dependent mechanism. Moreover, Tóth et al. show that two *C. parapsilosis* strains stimulate a similar phagocytic response, but differences in the human macrophage migration and cell engulfment were observed; underlining the fact of heterogeneity in the host-fungus interaction given only by different isolates of one fungal species. Finally, authors demonstrate that secreted lipases play a significant role precluding macrophage uptake.

## AUTHOR CONTRIBUTIONS

Both authors wrote and approved the manuscript.

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REVIEW
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# Fungal Melanin: What do We Know About Structure?

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The production of melanin significantly enhances the virulence of many important human pathogenic fungi. Despite fungal melanin's importance in human disease, as well as melanin's contribution to the ability of fungi to survive in diverse hostile environments, the structure of melanin remains unsolved. Nevertheless, ongoing research efforts have progressively revealed several notable structural characteristics of this enigmatic pigment, which will be the focus of this review. These compositional and organizational insights could further our ability to develop novel therapeutic approaches to combat fungal disease and enhance our understanding of how melanin is inserted into the cell wall.

**Keywords: melanin, fungi, structure, cryptococcus, fungal virulence**

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## INTRODUCTION

Melanins rank as one of the great natural pigments as they are synthesized by members of all biological kingdoms, including a wide array of fungi, bacteria, and helminths that cause disease in humans (Nosanchuk and Casadevall, 2003b). Melanins are polymerized from phenolic and/or indolic compounds forming negatively charged, hydrophobic pigments of high molecular weight (White, 1958). We have previously reviewed the broad contributions of melanin to fungal pathogenesis (Gomez and Nosanchuk, 2003; Nosanchuk and Casadevall, 2003b, 2006), which includes melanin's capacity to alter cytokine responses, decrease phagocytosis, and reduce the toxicity of microbicidal peptides, reactive oxygen species, and antifungal drugs as well as to play a significant role in fungal cell wall mechanical strength.

Despite the profound impact of melanin on fungal diseases as well as the abundance of the polymer in the world's biomass, the structure of melanin remains poorly defined. Classical biophysical methodologies cannot be applied to decipher the structure of melanin because this polymer is insoluble in aqueous or organic fluids and any attempt at solubilization disrupts its structure. Although melanins have ordered local structures their long-range organization is amorphous, and consequently their structures cannot be solved by X-ray crystallography. Melanins are typically dark in color (usually black or brown), acid resistant and bleached by oxidizing agents (Nicholaus et al., 1964; Prota, 1992; Butler and Day, 1998). The inability to define melanin based on solution-state or crystallographic techniques has prompted the use of alternative approaches to their structural characterization, including electron paramagnetic resonance (EPR) spectroscopy that capitalizes on the presence of a stable organic free radical signature (Enochs et al., 1993).

Melanin in fungi, bacteria and helminths is produced via the polyketide synthase pathway or catalyzed by phenoloxidases [reviewed in (Wheeler and Bell, 1988)]. Melanins formed by

the polyketide synthase pathway are called dihydroxynaphthalene [DHN] melanins. A variety of diverse enzymes, including phenoloxidases, tyrosinases, catecholases, and laccases, can generate melanins. Mammalian melanin is synthesized by a tyrosinase (Sanchez-Ferrer et al., 1995). Eumelanin formation is catalyzed by phenoloxidases from DOPA substrates. We have utilized *Cryptococcus neoformans* extensively in our studies of fungal melanin because this yeast-like fungus requires the addition of exogenous phenolic substrates to form eumelanin via laccase, and thus melanization can be closely observed by altering the quantity and type of substrate. Notably, disruption of genes essential for melanin production in *C. neoformans* results in both a reduction in fungal dissemination (Noverr et al., 2004) and lethality (Salas et al., 1996) in murine infection models. Similarly, disruption of genes associated with melanin synthesis in *Aspergillus fumigatus* results in attenuation (Heinekamp et al., 2012). Moreover, we have previously shown that chemical or antibody inhibition of *C. neoformans* melanization results in a reduction in fungal burden and prolonged survival in a murine cryptococcosis model (Nosanchuk et al., 2001; Rosas et al., 2001).

Since melanin is believed to contribute to fungal virulence by reducing the pathogen's susceptibility to killing by host antimicrobial mechanisms and by influencing the host immune response to infection, melanin and melanin synthesis pathways are indeed potential targets for antimicrobial drug discovery. Hence, a deeper understanding of melanin structure will facilitate the identification of innovative approaches to target this enigmatic polymer. This review discusses our current knowledge on the structure of fungal melanin.

## FUNGAL MELANIN AND THE CELL WALL

The fungal cell wall is a complex and dynamic construct of branched polysaccharides (particularly β-linked glucans), mannoproteins and proteins (Nimrichter et al., 2005; Latge and Beauvais, 2014). Fungal melanin is typically located within the cell wall, but the distribution and quantity varies widely between species. *C. neoformans* melanin is first detectable along the plasma membrane and fills throughout the cell wall over time (Nosanchuk and Casadevall, 2003b). In contrast, melanin can be found along the outer regions of the cell wall and/or clustered on the cell wall surface of several other pathogenic fungi, including *Candida albicans* (Morris-Jones et al., 2005; Walker et al., 2010), *Aspergillus* sp. (Rosas et al., 2000b; Bayry et al., 2014), *Sporothrix schenckii* (Morris-Jones et al., 2003), *Fonsecaea pedrosoi* (Franzen et al., 1999, 2006), *Paracoccidioides brasiliensis* (Gómez et al., 2001; Taborda et al., 2008), *Coccidioides* sp. (Nosanchuk et al., 2007), and *Histoplasma capsulatum* (Nosanchuk et al., 2002). These cited publications and other reports utilizing electron microscopic techniques have indicated that the layers or clusters of melanin are formed by granules of the polymer.

The most detailed study of fungal melanin localization was achieved using a combination of scanning electron and atomic force microscopy of melanin extracted from *C. neoformans* (i.e., melanin "ghosts") (Eisenman et al., 2005). This work revealed that cryptococcal melanin is formed by a complex of different sized spherical particles ranging from 40 to 130 nm in diameter. The ovoid nature of the particles was consistent with that reported for eumelanin produced by the cuttlefish *Sepia officianalis* (Clancy and Simon, 2001) and supportive of the granules observed in melanin in *Hortaea werneckii* (Kogej et al., 2007) and *F. pedrosoi* (Franzen et al., 2006). Additionally, the particulate nature of this organization suggested a mechanism by which macromolecules can pass through the melanin, which appears in many images as an impenetrable layer. In fact, studies using size exclusion methods or nuclear magnetic resonance cryoporometry have revealed that there are pores in melanin layers (Eisenman et al., 2005; Jacobson and Ikeda, 2005). The NMR cryoporometry results indicated pores that were mainly 1–4 nm in diameter and less commonly ∼30 nm (Eisenman et al., 2005). Significantly, pore sizes become smaller with increased cell age consistent with increased amounts of melanin deposited in the cell wall.

The pore formation is also significant as these pathways provide a mechanism for macromolecular transport in terms of the formation of layers of melanin rather than a single, uniform polymeric mass. Transmission electron microscopy of thin cross-sections of fungi, especially *C. neoformans*, revealed layers of melanin within the cell wall, with individual layers that are similar in dimension to that of individual melanin particles (Eisenman et al., 2005). X-ray diffraction studies using purified fungal melanins have confirmed the presence and further elucidated details of these layers (Casadevall et al., 2012). For instance, X-ray diffraction revealed a consistent presence of a basic stacked planar sheet structure in melanins isolated from *C. neoformans, Wangiella dermatitidis*, *A. niger,* and *Coprinus comatus* (a common mushroom), and these data were similar to those observed studying other natural melanins. Interestingly, stacking differences varied, such that the stacking distance of melanin layers in *Sepia officinalis* (cuttlefish) melanin was 3.46 Å compared to 4.15 Å for *W. dermatitidis* or 4.45 Å for *A. niger*, and *C. neoformans* stacking was 4.39 Å. The differences in stacking in these fungi may be due to subtle variations in composition as well as differences in the other structures within their individual cell walls. Nevertheless, this consistent finding of layering suggests that X-ray diffraction may be an additional means to define melanins. Moreover, it further raises the question of whether cell wall constituents influence the deposition and organization of melanin in fungi.

## FUNGAL VESICLES: "MELANOSOMES"

The discovery of fungal vesicle transport through the complex fungal cell (Rodrigues et al., 2007) provided an explanation for melanin deposition within the cell wall. Several studies have now shown that diverse fungi produce heterogeneous extracellular vesicles that contain lipids, carbohydrates and proteins (Rodrigues et al., 2007, 2008, 2014; Albuquerque et al., 2008, 2012; Eisenman et al., 2009; Oliveira et al., 2009, 2010; Panepinto et al., 2009; Kmetzsch et al., 2011; Rizzo et al., 2014; Peres da Silva et al., 2015; Vargas et al., 2015), many of which

are associated with fungal virulence. To reach the extracellular space, intracellularly synthesized macromolecules are targeted to the cell surface for release to the extracellular milieu (Wickner and Schekman, 2005). Notably, vesicle secretion enhances cryptococcal virulence in a murine disease model (Panepinto et al., 2009). However, these vesicles can also be captured within the cell wall. Laccase is a component of *C. neoformans* vesicles (Rodrigues et al., 2008) and vesicle melanization has been confirmed (Eisenman et al., 2009). Although there were variations in vesicle size, a population of melanized vesicles was observed with comparable diameters to those measured in *C. neoformans* melanin (Eisenman et al., 2009). Fungal melanosomes were subsequently described in *C. albicans* (Walker et al., 2010). Hence, it appears that laccase-loaded vesicles can be methodically trapped within the cell wall where they form into layers of melanin.

In combination with these observations, there is now overwhelming evidence from several independent groups that fungal melanization occurs in a specialized vesicle that is analogous to the mammalian melanosome (Franzen et al., 2008; Eisenman et al., 2009; Walker et al., 2010). In hindsight, the need for melanization in a vesicle in *C. neoformans* is obvious because the reaction is catalyzed by a single enzyme that generates a plethora of highly reactive, toxic intermediates that self-react to create melanin. Melanization in vesicles explains much of the biology of fungal melanin: morphology of relatively uniform microspheres with dimensions similar to those of extracellular vesicles (resulting from synthesis therein), the presence of aliphatic components in melanin ghosts resulting from early steps of synthesis in vesicles [described below and in (Zhong et al., 2008)], and budding through melanin (Nosanchuk and Casadevall, 2003a), wherein these vesicles can be simply displaced laterally for the daughter cell to emerge.

## CHITIN: A MELANIN ANCHOR

The mechanism(s) for localizing laccase-loaded vesicles to the cell wall have not been resolved; however, several studies suggest that chitin is a primary effector for melanin deposition within the fungal cell wall. Chitin is a long-chain polymer comprised by subunits of β(1,4)-linked N-acetylglucosamine, which is commonly cross-linked to diverse cell wall proteins and polysaccharides. The molecular composition of specific forms of chitin can affect intramolecular and intermolecular interactions of lipid bilayers (Fang et al., 2001), which may facilitate chitin-vesicle engagement. In 1970, Bull reported that melanin was "associated particularly with the chitin" in cell wall fractions of *A. nidulans* (Bull, 1970). This first recognition of the interplay between chitin and melanization in *A. nidulans* has been followed by additional findings in other fungi consistent with the importance of chitin-melanin interactions. For example, deletion of the chitin synthesis WdCHS4 gene in the black fungus *Exophilia (Wangiella) dermatitidis* resulted in a significantly reduced ability to deposit melanin within the cell wall as demonstrated by the accumulation of extracellular pigment (Wang et al., 1999). Mutations of *C. neoformans* chitin synthases, chitin regulatory genes and chitin deacetylases (Banks et al., 2005; Walton et al., 2005; Baker et al., 2007) impede melanization of the cell wall with concomitant detection of melanin in the medium and agar. Additionally, the inhibition of chitinases by methylxanthines results in a 'leaky melanin' phenotype in *C. neoformans* (Tsirilakis et al., 2012). *C. albicans* produces granular melanin (Morris-Jones et al., 2005), and deletion of chitin synthase inhibits melanization along the cell wall with concomitant accumulation of melanin particles within the yeast cells (Walker et al., 2010). The effects of defects in chitin that result in either secretion of melanin in *E. dermatitidis* and *C. neoformans* or accumulation of melanin granules in *C. albicans* are consistent with the requirement for vesicle interaction with the chitin. It is noteworthy that an intimate association between chitin and melanin has been described in marine invertebrates (Hwang et al., 2013) as well as insects (Stavenga et al., 2012), suggesting that this scheme for the anchoring of these macromolecular structures extends to other species.

It is highly probable that additional diverse constituents are involved in the localization and maintenance of melanin within the complex cell wall structure. For instance, comparative analyses of *F. pedrosoi* cells cultivated with or without the DHN melanin-specific inhibitor tricyclazole (5-methyl-1,2,4-triazol[3,4] benzothiazole) indicated that melanin was involved in cross-linking diverse cell wall compounds (Franzen et al., 2006).

## MICROANALYTICAL CHARACTERISTICS OF FUNGAL MELANIN

As melanin is insoluble, information on melanin structure gleaned in the prior century derives largely from spectroscopic analyses of melanin and characterization of melanin degradation products (Wakamatsu and Ito, 2002). HPLC microanalysis approaches have been particularly useful in the characterization of both pheomelanin and eumelanins (Wakamatsu and Ito, 2002; Wakamatsu et al., 2002). For instance, oxidation of melanized *C. neoformans* cells from cultures or infected mice revealed that both melanins contained PTCA and PDCA (Williamson et al., 1998; Liu et al., 1999), which indicates that cryptococcal melanin is formed of 3,4-dihydroxyphenylalanine (DOPA) oligomers or polymers.

Antibodies (Rosas et al., 2000a; Youngchim et al., 2004; Urán et al., 2011) and peptide ligands (Nosanchuk et al., 1999) have been generated to fungal melanin. Immunofluorescence studies utilizing some of these reagents reveal that there is diffuse, homogeneous binding along the surface of melanins isolated from different fungi, which suggests that there are conserved repeating units serving as epitopes to react with these reagents. Additionally, the peptides that bind melanin are highly positively charged (Nosanchuk et al., 1999), consistent with the finding that melanin is negatively charged (Nosanchuk and Casadevall, 1997; Eisenman et al., 2007; Frases et al., 2011). Moreover, the most reactive melanin-binding peptides are comprised of several aromatic amino acids (Nosanchuk et al., 1999), suggesting that

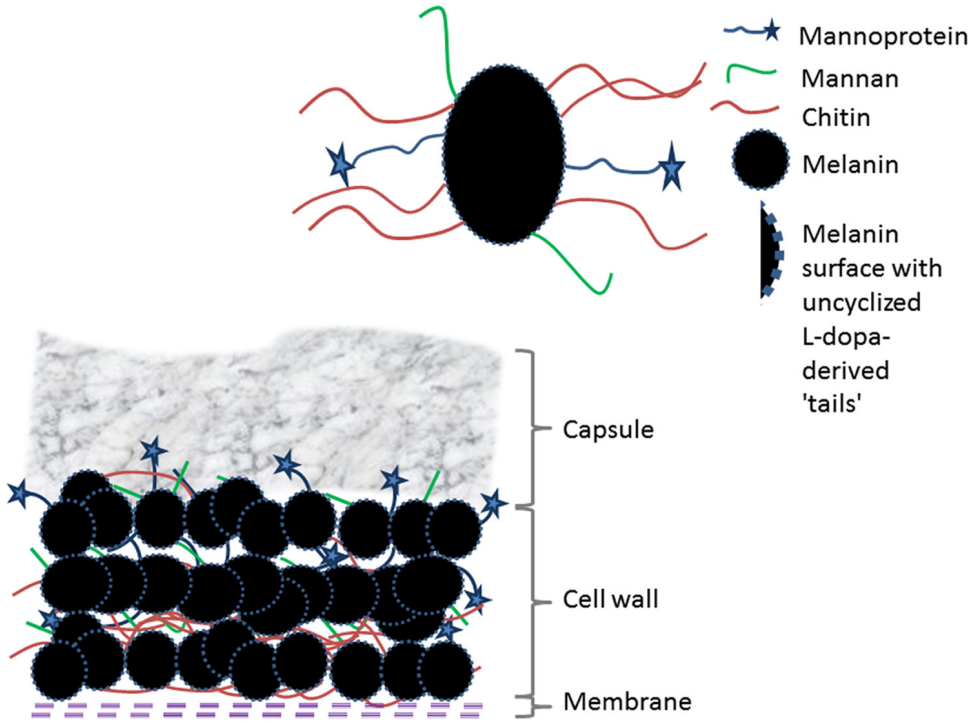


**FIGURE 1 | A conceptualization of melanin organization and identified interactions with macromolecular structures in the cell wall of *Cryptococcus neoformans*.**

similar aromatic and positively charged structures are present on melanin.

## RECENT INSIGHTS FROM NEW ANALYTIC APPROACHES

The molecular structure of fungal melanin remains unknown, but significant insights have recently been obtained using advanced nuclear magnetic resonance (NMR) and imaging techniques. By exploiting the requirement of *C. neoformans* for exogenous phenolic substrates to form melanin, pigments generated using natural $^{12}C$ or stable-isotope enriched $^{13}C$ forms of L-DOPA were subjected to high-resolution solid-state magic-angle spinning (MAS) $^{13}C$ NMR to reveal a rich assortment of chemical bonding patterns consistent with alkane, alkene, alcohol, ester, and indole functional groups (Tian et al., 2003). These initial insights have been pursued with site-specific $^{13}C$-enriched substrates to deduce that developing fungal melanins incorporate additional non-L-DOPA constituents, such as aliphatic groups consistent with triglycerides or phospholipids, and that these are components capable of facilitating interactions between melanin and structures within the fungal cell wall (Zhong et al., 2008). Notably, this latter result is in full accord with the above-mentioned proposal that melanin forms a spatially expanded rather than a discrete layer within the cell wall.

These findings of aromatic and aliphatic structures were followed by a detailed examination of the cell wall and pigment architecture in *C. neoformans* melanin using 2D $^{13}C$-$^{13}C$ correlation solid-state NMR methods (Chatterjee et al., 2015). Consistent with the concept of cell wall constituents comprising a scaffold for the pigment, NMR analyses revealed that the aliphatic moieties of *C. neoformans* melanin included polysaccharide and chitin constituents. It is notable that the chemically resistant melanized *C. neoformans* cell walls exhibit a plethora of proximal and bonded $^{13}C$-$^{13}C$ pairs comprising an aliphatic scaffold consisting of an intimately associated composite of glucan, chitin, mannan, mannoprotein, and phospholipid. In fact, the NMR data support complex architectural networks that include uncyclized aliphatic structures, closely interacting indole-indole pairs, and covalently bound pyrrole-chitin pairs. Moreover, during melanin synthesis, our spectroscopic evidence indicates an early (by day 4) aliphatic scaffold that subsequently incorporates the aromatic components (by day 14). This process is in accord with the increase in negative cell charge (Nosanchuk and Casadevall, 1997; Eisenman et al., 2007; Frases et al., 2011) and reduction in porosity (Eisenman et al., 2005) that occur during aging in a melanizing *C. neoformans* yeast cell.

Fundamental structural differences among melanins derived from L-DOPA, methyl-L-DOPA, epinephrine, and norepinephrine precursors have been demonstrated by $^{13}C$ and $^{1}H$ MAS NMR (Chatterjee et al., 2012). For example, the melanins generated with epinephrine and norepinephrine are observed as thinner by TEM (Garcia-Rivera et al., 2005) and MAS NMR revealed that these melanins also have lower aromatic-to-aliphatic ratios than the more robust melanins

formed from L-DOPA and methyl-L-DOPA (Chatterjee et al., 2012). Additionally, the MAS NMR data showed that the EPR signal used historically to define melanin is correlated with the presence of prominent aromatic resonances and that the negative charge of the polymer can be associated with the presence of polar oxygenated aliphatic molecular structures (Chatterjee et al., 2012).

High-field, two-dimensional NMR of $^{13}C$- and $^{15}N$-enriched materials was further used to demonstrate that both fungal melanin and synthetic eumelanins share a common indole-based aromatic core (Chatterjee et al., 2014). This investigation provides new information about the supramolecular organization of melanin. For example, Double Cross Polarization and Proton Assisted Insensitive Nuclear Cross Polarization (PAIN-CP) NMR revealed four magnetically distinct indole-like $^{13}C$-$^{15}N$ pairs (Chatterjee et al., 2014) that suggest multiple modes of polymeric assembly involving DHICA and DHI building blocks. The formation of heterogeneous oligomers and polymers is also consistent with assembly of melanins via multiple polymerization pathways.

## CONCLUSION

Although the molecular structure of fungal melanin remains enigmatic, significant progress has been made in understanding particular aspects of its macro- and microstructure during the past 20 years. A representation of our current view of melanin in *C. neoformans* is summarized in **Figure 1**. The increased interest in the structure of melanin has been driven in large part by a remarkable increase in the incidence of diseases due to melanotic fungi. The identification of melanosomes has opened up rich avenues for research that have expanded our appreciation of localization and production of cell wall melanin. The application of NMR techniques has revealed that a chemically resistant aliphatic matrix is assembled prior to significant deposition of indole-based pigments, showing that cell wall composites could serve as a supporting scaffold that fosters eumelanin buildup and presenting opportunities to map out this framework as well as define interlayer stacking interactions and melanin-cell wall interactions. All together, these advances provide a broad platform to gain new insights leading to innovative approaches to combat fungal diseases in which melanin plays a role in pathogenesis. Moreover, these findings can translate into enhanced ability to combat pigment disorders such as melanoma, respond to environmental disasters such as radioactive spills, and generate novel therapeutics such as melanin nanoparticles to ameliorate radiation injury.

## AUTHOR CONTRIBUTIONS

JN, RS, and AC contributed equally.

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# Chronic widespread dermatophytosis due to *Trichophyton rubrum*: a syndrome associated with a *Trichophyton*-specific functional defect of phagocytes

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Dermatophytes are agents of typically benign superficial infections. However, an increasing number of severe infections in immunocompromised hosts has been reported. We aimed to understand the factors underlying the existence of a cohort of patients presenting with chronic widespread dermatophytosis (CWD) due to *Trichophyton rubrum*, but with no signs of immunodeficiency. Their disease is usually recurrent and difficult to manage. Fourteen patients meeting the following criteria for CWD were studied: *T. rubrum* culture-proven skin lesions of $\geq$10 cm in at least one dimension; the involvement of at least three non-contiguous localizations of $>$1 year's duration; and no predisposing conditions. For comparison, we also studied 13 acute *Tinea pedis* patients. Macrophages and neutrophils were isolated and tested for *T. rubrum* conidia phagocytic and killing activity. $H_2O_2$, NO, and pro- and anti-inflammatory cytokine release were measured. All experiments were run with age- and sex-matched healthy donors' cells in parallel. CWD patients' macrophages and neutrophils presented with reduced *T. rubrum*–phagocytic and killing abilities, and reduced $H_2O_2$ and NO release when compared with those of healthy donors. CWD patients' macrophages secreted lower levels of the proinflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, but enhanced levels of the anti-inflammatory cytokine IL-10. Neutrophil secretion closely followed this unbalanced pattern. In contrast, responses to the positive controls zymosan, lipopolysaccharide, and phorbol myristate acetate were comparable with those of healthy donors. The same experiments were performed with macrophages and neutrophils from the acute *Tinea pedis* patients and showed no differences when compared with the matched healthy donors. Patients with CWD have a *T. rubrum*-related functional deficiency of phagocytes and may represent a distinct clinical entity in the complex spectrum of the *Trichophyton*–host interaction.

**Keywords: innate immunity, immunodeficiency, dermatophytosis, macrophages, neutrophils, *Trichophyton rubrum***

## Introduction

Dermatophytes are agents of typically benign superficial infections, of which the non-inflammatory, scaly lesions of toe webs due to *Trichophyton rubrum* are known as the most common example (Seebacher et al., 2008). However, there are numerous reports of severe and occasionally life-threatening dermatophytic infections in the increasing population of immunocompromised patients, showing that dermatophytoses may pose a more serious threat to these patients (Marconi et al., 2010). Recently, the immune–genetic background underlying deep (invasive) dermatophytic infections afflicting some members of consanguineous families in Northern Africa has been elucidated (Lanternier et al., 2013). This syndrome was associated with an autosomal recessive CARD9 deficiency that accounts for the patients' failure to limit the invasiveness of dermatophytes such as *T. rubrum* and *T. verrucosum*. CARD9 is an adaptor protein downstream to several immune receptors such as dectin-1, dectin-2, and mincle, which recognize fungal structures and are critical for antifungal Th-17 responses (Lionakis and Holland, 2013). On the other hand, there are also frequent reports of patients presenting with recurrent or chronic widespread dermatophytosis (CWD) of the skin which, although not invasive, is difficult to manage (Sentamilselvi et al., 1997–1998; Vittorio, 1997; Gorani et al., 2002; Cordeiro et al., 2006; Seyfarth et al., 2007; Balci and Cetin, 2008; Kong et al., 2015).

Patients with acute superficial dermatophytosis are able to mount cell-mediated immune (CMI) responses against the causative agent, which has been associated with resolution of the infection (Hanifin et al., 1974; Hay and Shennan, 1982). In contrast, those who suffer from chronic or recurrent infections are unable to develop a CMI response (Hanifin et al., 1974; Hay and Shennan, 1982). The reasons for this inability are not yet known. Here, we describe a series of dermatophytosis patients with a distinct clinical presentation, chronic, or recurrent widespread involvement, in whom we detected a deficiency of phagocytes (macrophages and neutrophils) to handle its apparently single causative agent, *T. rubrum*.

## Materials and Methods

### Patients and Controls

From January 2013 to March 2014, patients with CWD and no known predisposing conditions referred to our admission service were invited to participate in the study. Written informed consent was obtained from all patients prior to blood collection, and the study was approved by the Human Experimentation Ethics Committee of the Hospital das Clínicas, Universidade de São Paulo in Brazil (#0837/10). Patients were diagnosed with CWD if they had *T. rubrum* culture-proven dermatophytosis involving at least three non-contiguous localizations, with lesions (typically well-delimited plaques) of $\geq$10 cm in at least one of its extensions, for more than 1 year. Onychomycosis was not considered in the criteria. Bilateral involvement of the feet was considered as a single localization. Patients should not have presented with any condition that could potentially interfere with their immune system (pregnancy, immune-mediated, or inflammatory conditions, infectious diseases [including HIV and hepatitis C virus], diabetes mellitus, Cushing's syndrome, alcoholism, and topical or systemic treatments featuring immunosuppressive drugs). No patients had any potential occupational/professional risk for dermatophytosis, such as prolonged contact with water, working in warm/humid environments, or the use of special clothes. A second part of the study involved the recruitment of individuals presenting with *Tinea pedis* (*Tp*), the most common and benign form of dermatophytosis due to *T. rubrum* in Brazil (Costa-Orlandi et al., 2012). From March to June 2014, a total of 13 patients with *Tp* were enrolled using the same exclusion criteria as described above for the CWD patients.

For both studies, healthy donors that were age- ($\pm$3 years) and sex-matched with the CWD and *Tp* patients served as controls. All experiments were run with the matched control's cells in parallel.

### *Trichophyton rubrum* Conidia Preparation

*Trichophyton rubrum* ATCC28188 was streaked onto potato dextrose agar plates to isolate individual colonies for 12 days. Colonies were cultured in a shaking incubator for 72 h at 30°C in potato broth for the *in vitro* assays. The conidia were filtered to remove hyphae and washed with phosphate buffered saline (PBS) before use. For fluorescence labeling, washed conidia were labeled with carboxyfluorescein succinimidyl ester (CFSE, 100 μg/mL; Life Technologies, Eugene, OR, USA) for 30 min at 25°C, followed by extensive washing.

### Macrophage and Neutrophil Interaction with *T. rubrum* Conidia

Human monocyte-derived macrophages and human neutrophils were obtained from peripheral blood mononuclear cell (PBMC) leukocytes, as described previously (Böyum, 1968; Calvi et al., 2003). Specifically, for the generation of macrophages, human PBMCs were isolated by centrifugation over a Ficoll–Paque$^{TM}$ PLUS (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) gradient. Monocytes were purified by adherence on gelatin-coated plates for 1 h, followed by extensive washing to remove non-adherent cells. After at least 12 h of incubation, monocytes (day 1) were harvested and then differentiated into day 4 and day 7 macrophages via culturing in Roswell Park Memorial Institute (RPMI) medium with 10% fetal calf serum (FCS). Viability was >95%, as determined by trypan blue dye exclusion. The macrophages were then plated the night before use, while neutrophils were plated on the same day in 24-well plates at a density of $3 \times 10^5$ cells/well in RPMI–10% heat-inactivated FCS. For neutrophil isolation, following centrifugation over Ficoll–Paque, the granulocytes were isolated from the bottom part of the tube containing red cells, using dextran and saline as previously described (Böyum, 1968). This yielded neutrophils with >93% purity and $\geq$90% viability by trypan blue dye exclusion. For the *in vitro* binding and cytokine assays, unlabeled or CFSE-labeled *T. rubrum* conidia were added to the cells, as indicated, and incubated for 30 min at 37°C. In some experiments, unlabeled or fluorescein isothiocyanate-labeled zymosan (25

particles/cell; Thermo Fisher Scientific) and mannan (1 mg/mL; Sigma–Aldrich Co., St Louis, MO, USA) were added alone as indicated. Unbound particles were removed by washing. The medium was replaced, and the cells were cultured for either further 3 h at 37°C and 5% $CO_2$ for the analysis of tumor necrosis factor (TNF)-α or 18 h for the analysis of the other cytokines. After the 3-h incubation period, supernatants were stored at −80°C until cytokine determination, while the cells were lysed in 3% (volume/volume) Triton® X-100, and the cell-associated fluorescence was measured as the mean florescence intensity (Filtermax-F5; Molecular Devices LLC, Sunnyvale, CA USA). Cytokine release was not influenced by the presence of the fluorescent label on the fungal particles (not shown). Lipopolysaccharide (LPS; Sigma–Aldrich Co.) was used as the positive control for cytokine release. For the killing assays, macrophage and neutrophils ($3 \times 10^4$ cells) were co-cultured with non-opsonized *T. rubrum* conidia at a 5:1 ratio in 96-well plates for 3 h at 37°C and 5% $CO_2$. The phagocytes were then washed to remove unbound conidia and lysed in Triton® X-100, as previously described (Horwitz and Silverstein, 1980). The supernatants' serial dilutions were plated in duplicate in Petri dishes with 15 mL of Sabouraud agar and incubated for 5 days at 28°C. Colonies were counted manually.

### $H_2O_2$ Measurement

$H_2O_2$ release by phagocytes was measured with the horseradish peroxidase–phenol red oxidation method described previously (Calvi et al., 2003). Briefly, phagocytes co-cultured with *T. rubrum* conidia or stimulated with 100 nM of phorbol-12-myristate-13-acetate (PMA; Sigma–Aldrich Co.) were centrifuged at 200 *g* for 10 min at 4°C and were re-suspended in 1 mL of phenol red buffer containing 50 μg/mL of horseradish peroxidase type II (Sigma–Aldrich Co.). Aliquots of 100 μL were then transferred to 96-well culture plates and were incubated for 1 h at 37°C and 5% $CO_2$. The reaction was stopped by the addition of 10 μL of 1N NaOH, and the absorbance was read at 620 nm (Micro-ELISA reader, Filtermax-F5). The absorbance was transformed into a standard curve of $H_2O_2$ that was serially diluted from 3.2 to 200.0 μM $H_2O_2$/mL.

### NO Measurement

Supernatants were diluted 1:10 in deionized water and NO measurement was performed through chemiluminescence reaction between ozone and NO generated by the reduction of the sample with vanadium chloride in acid at 95°C, using a Nitric Oxide Analyzer Model 208A (Sievers Instruments Inc., Boulder, CO, USA) as described (Alves et al., 2002). The assay was standardized by a calibration curve of nitrate (0.05–20.0 μM/mL) obtained from sodium nitrate.

### Cytokine Measurements

Supernatants harvested from phagocyte–*T. rubrum* co-cultures were measured for interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α using a cytometric bead array (BD, Franklin Lakes, NJ, USA). Detection limits were, respectively, 7.2, 2.5, 3.6, 3.3, 1.9, and 3.7 pg/mL.

### Statistical Analysis

The Wilcoxon signed-rank test was used to compare the two patient groups (CWD and *Tp*) with the respective paired control groups (GraphPad Prisma; GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

## Results

### Patients' Clinical Characteristics

A total of 14 patients with CWD of the skin were diagnosed (10 males and 4 females). There were two Afro-Brazilians, one Asian, and the remaining patients were of mixed ethnic origin. None of the patients were from consanguineous families. The patients' age range was 22–61 years (mean: 46 years). Lesions were present for >3 years in all patients (range: 3–16 years), except for one patient with 1 year of lesions. As shown in **Figure 1**, patients had typical erythematous, slightly elevated, scaly, and well-delimited lesions over different, non-contiguous body segments, particularly those of glabrous skin. Small satellite lesions were also frequent. The distribution of the lesions was as follows: feet (bilateral) in nine patients; hands (usually bilateral) in seven patients; crural in seven patients; legs in six patients; trunk in six patients; buttocks in five patients; arms in three patients; axillas in three patients; neck in two patients; and ears in two patients. In addition, ten patients had concomitant onychomycosis. A history of atopy (asthma) was evident in only two patients. With the exception of the youngest patient, who had a twin brother with chronic *T. rubrum* lesions (who refused to participate in the study), no other patients reported having close relatives with similar manifestations. All patients reported previous antifungal treatments, either topical or systemic, some of which included terbinafine; compliance to the treatments, however, could not be assessed. Four patients reported apparent resolution with relapse after some months, while seven reported no or only partial improvement and three provided no reliable information. No other fungi (*Candida* sp. and dermatophytes) were isolated from the lesions. CWD patients' laboratory screening was within normal limits, including glycemic levels, hemoglobin levels, white blood cell counts, and liver and renal function tests.

### Phagocytic and Killing Abilities against *T. rubrum* of Macrophages and Neutrophils from CWD Patients

Macrophages from CWD patients presented with a slight but significantly lower ability to phagocytize *T. rubrum* compared with age- and sex-matched healthy controls, while the ability to phagocytize zymosan particles was similar (**Figure 2A**). *T. rubrum* phagocytosis was significantly blocked by mannan in both groups. In presence of the polysaccharide, phagocytosis by CWD macrophages remained lower than in controls. The lower phagocytic ability was reflected by the lower killing ability

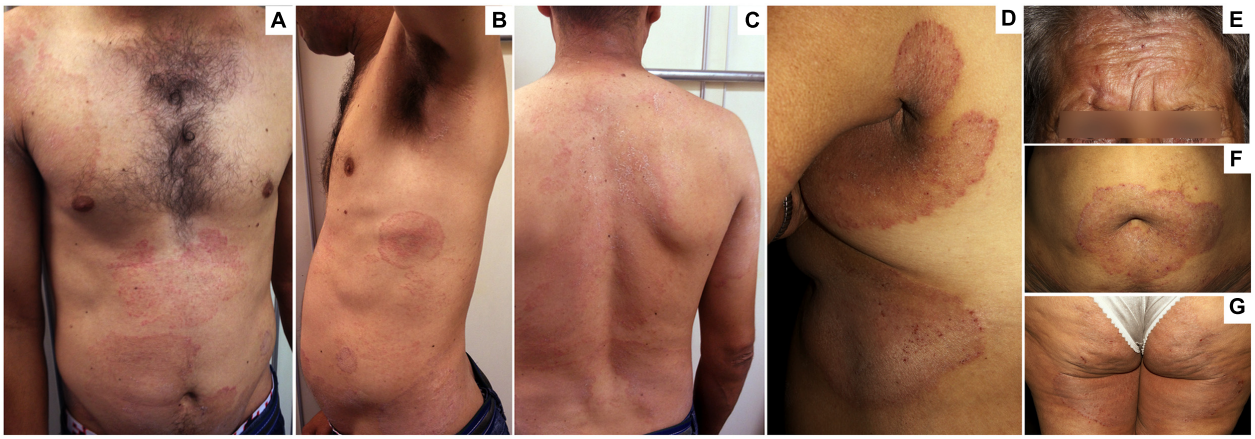


**FIGURE 1 | Clinical aspects of chronic widespread dermatophytosis (CWD) due to *Trichophyton rubrum*. (A–C)** Several well-delimited, scaly, and slightly erythematous plaques in the abdomen **(A–C)**, thorax **(A,B)**, shoulders **(A)**, arms **(B)**, neck **(C)**, and back **(C)** in a 36-year-old man with a 4-year history of dermatophytosis. **(D–G)** Well-delimited, scaly, and erythematous plaques in the face **(E)**, the periumbilical **(F)**, and submammary **(D)** regions; as well as the armpits **(D)**, thighs **(D)**, and buttocks **(G)** in a 60-year-old woman with a 5-year history of dermatophytosis.

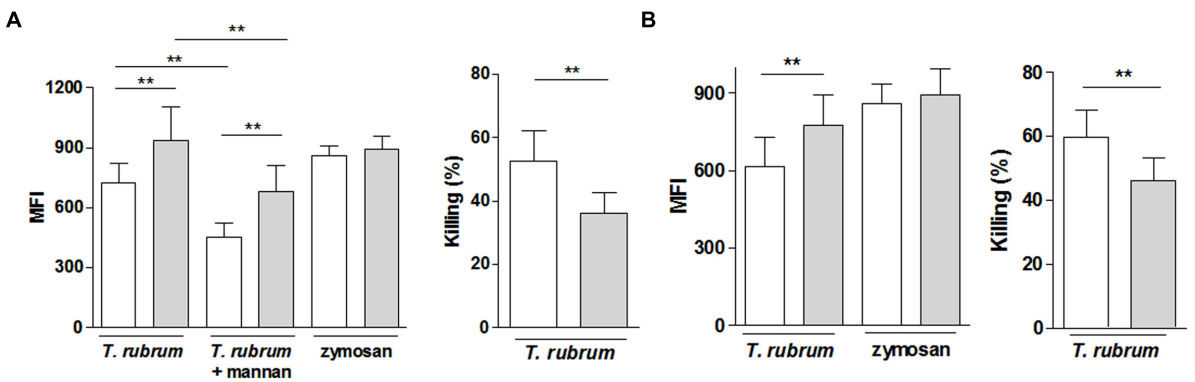


**FIGURE 2 | Reactivity of phagocytes (A, macrophages; B, neutrophils) from patients with CWD (clear bars) and age- and sex-matched healthy controls (gray bars) to *T. rubrum*.** Phagocytosis of CFSE-labeled *T. rubrum* conidia was assessed by fluorometry as the mean florescence intensity (MFI). Killing activity is shown as the % of the CFU counts in the control wells (conidia suspension in absence of phagocytes). Phagocytosis, $n = 14$; microbicidal activity, $n = 10$. Results are shown as the mean ± SEM. $^{**}P < 0.01$.

of CWD macrophages (**Figure 2A**). Experiments conducted in parallel showed the same modest but significant deficiency in the phagocytic and killing abilities of the CWD patients' neutrophils (**Figure 2B**).

## Release of the Cytotoxic Molecules $H_2O_2$ and NO by Macrophages and Neutrophils Challenged with *T. rubrum*

Chronic widespread dermatophytosis patients' macrophages released slightly but significantly less $H_2O_2$ and NO in the presence of *T. rubrum* than those of the control individuals (**Figure 3A**). Again, the non-specific ability to release these molecules was intact in the patients' macrophages, as shown by their preserved response to PMA. $H_2O_2$ release by CWD patients' neutrophils showed similar results: there was a decrease in the presence of *T. rubrum*, but there was also a preserved response to PMA (**Figure 3B**).

## Cytokine Secretion by Macrophages and Neutrophils Challenged with *T. rubrum*

The supernatants of macrophages and neutrophil co-cultures with *T. rubrum* were collected and evaluated for proinflammatory (IL-1β, IL-6, IL-8, IL-12p70, and TNF-α) and anti-inflammatory (IL-10) cytokines. With the exception of IL-12p70, where its levels were always below the detection limit (1 pg/mL), both macrophages and neutrophils secreted substantial levels of these cytokines (**Figure 4**). However, consistent with the lesser anti-*T. rubrum* activity of CWD patients, the amounts of proinflammatory cytokines released by CWD macrophages upon *T. rubrum* challenge were significantly lower than those of the matched controls, whereas IL-10 release was significantly increased. On the other hand, the amounts elicited in the positive control wells (LPS) and in the non-stimulated wells (medium only) were similar between CWD patients and controls for every cytokine. Neutrophils followed a similar pattern: there

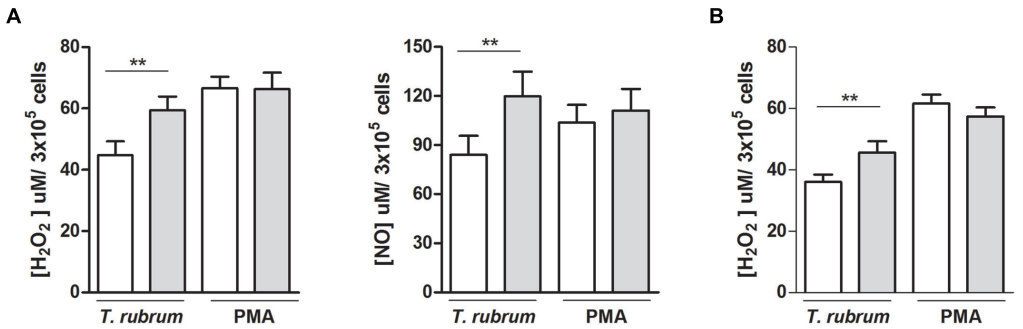


**FIGURE 3 | NO and $H_2O_2$ release by phagocytes (A, macrophages; B, neutrophils) from patients with CWD (clear bars) and age- and sex-matched healthy controls (gray bars) in the presence of *T. rubrum* or PMA.** NO and $H_2O_2$ release in culture supernatants were measured by fluorometric assays. $H_2O_2$, $n = 14$; NO, $n = 9$. Results are shown as the mean ± SEM. $^{**}P < 0.01$.

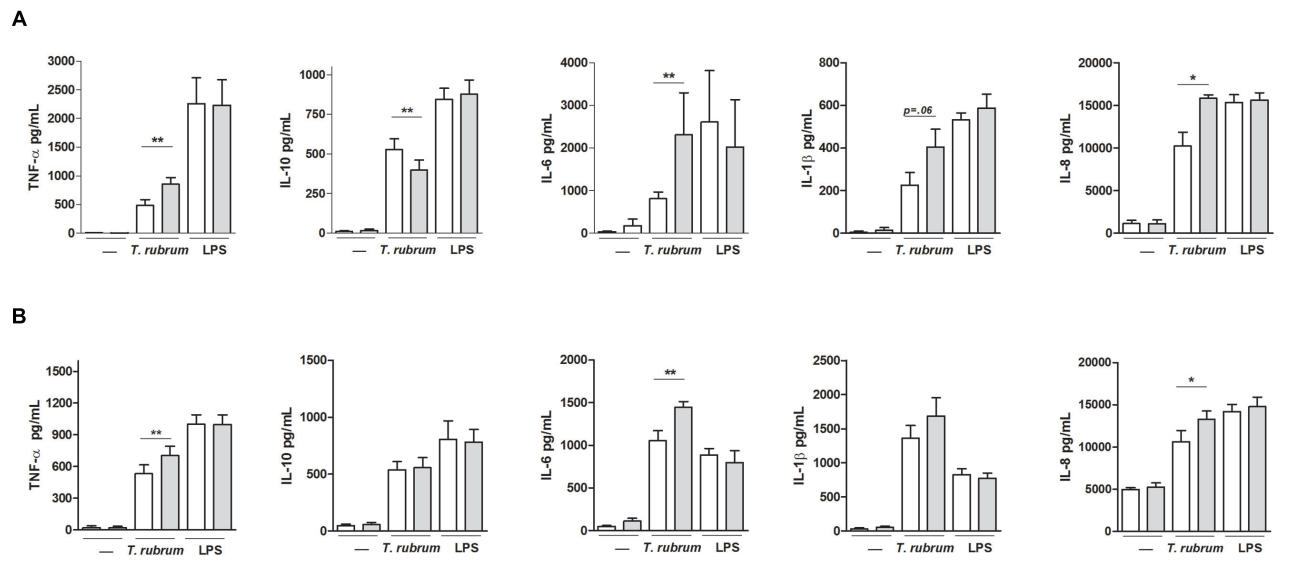


**FIGURE 4 | Cytokine levels in the supernatants of (A) macrophages and (B) neutrophils from patients with CWD (*n* = 8, clear bars) and age- and sex-matched healthy controls (gray bars) cultured in the presence of *T. rubrum* conidia, LPS, or medium alone (–).** Supernatants were collected after 3 h (TNF-α) and 18 h (IL-1β, IL-6, IL-8, and IL-10), and cytokine levels were measured by CBA. Results are shown as the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

was reduced release of the proinflammatory cytokines upon *T. rubrum* challenge (except for IL-1α, which reduction did not reach statistical significance, and IL-10, that was not increased), and no differences between patients and controls with PMA or medium alone were noted.

### Macrophage and Neutrophil Responses of Acute *Tp* Patients

Subsequently, the same series of experiments were performed with phagocytes isolated from *Tp* patients and age- and sex-matched healthy controls. Results of these experiments are shown in Supplementary Tables S1 (macrophage responses) and S2 (neutrophil responses). Both macrophages and neutrophils from *Tp* patients exhibited a similar ability to phagocytize, kill, and release $H_2O_2$, NO, and cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) upon challenge with *T. rubrum* as controls. There were also no differences between the patients and controls' responses in the positive control wells (zymosan, LPS, or PMA).

## Discussion

We were interested in understanding the factors underlying the existence of a distinct cohort of patients presenting with longstanding and widespread dermatophytosis due to *T. rubrum*, but with no other clinical evidence of immunodeficiency. Remarkably, their disease was refractory to apparently appropriate treatments. Here, we showed that macrophages and neutrophils from these patients presented with reduced phagocytic and killing abilities, as well as with disturbed immunoregulatory properties (cytokine secretion), upon challenge with *T. rubrum* conidia. These findings may help

explain why some individuals develop recalcitrant and widespread disease due to a fungus that typically causes a localized and easily manageable superficial infection. Of note is that despite the chronicity and extension of the skin involvement, in all patients it exhibited little inflammation and no evidence of invasion of deeper tissues.

Earlier studies reported that resolution of an acute episode of a dermatophyte infection was associated with the development of a specific CMI response that protected, at least partially, against reinfections (Jones et al., 1973). Further *in vitro* and *in vivo* studies demonstrated that these responses were predominantly Th-1 mediated (Miyata et al., 1996; Koga et al., 2001; Brasch, 2009; Santiago et al., 2014), although recent evidence in experimental models of dermatophytosis also pointed to Th-17 responses (Cambier et al., 2014). Chronic infections, on the other hand, were associated with a lack of CMI responses (Hanifin et al., 1974; Hay and Shennan, 1982). Importantly, there are few and conflicting data regarding predisposing genetic traits in chronic *Tp* (Svejgaard et al., 1983; Ahmed et al., 1985). Although patients with CWD are frequently described in the literature (Sentamilselvi et al., 1997–1998; Vittorio, 1997; Gorani et al., 2002; Cordeiro et al., 2006; Seyfarth et al., 2007; Balci and Cetin, 2008; Kong et al., 2015), it is not yet known why they are unable to mount CMI responses and resolve the infection. Our present findings may provide some clues to this issue. Both neutrophils and macrophages are actively attracted to the site of acute *T. rubrum* infection in patients and experimental models of dermatophytosis (Hay et al., 1988; Brasch and Sterry, 1992; Brasch, 2009), although only neutrophils penetrate the epidermis and interact with *T. rubrum* (Hay et al., 1988). Monocyte-derived macrophages are an important cellular component in the dermis inflammatory infiltrate of dermatophytosis (Hay et al., 1988; Brasch and Sterry, 1992), thus playing a role in the induction (or lack thereof) of effective CMI responses. The main antigen-presenting cell (APC) in the epidermis is the Langerhans cell (LC), which increases in number in the dermatophyte-infected epidermis and also interacts with *T. rubrum* (Brasch et al., 1993). As the study of patients' epidermal LCs was not possible, we focused on their precursors, peripheral blood monocytes (Ginhoux et al., 2006). Both human neutrophils and monocyte-derived macrophages are normally able to phagocytize and kill *T. rubrum* (Calderon, 1989). The participation of reactive oxygen species in this killing has been demonstrated (Calderon, 1989). NO also exhibits toxic activity against a range of fungi, including *T. rubrum* (Regev-Shoshani et al., 2013). In our chronic CWD patients, both phagocytes presented with reduced rates of phagocytosis and killing activity when challenged with *T. rubrum*, as compared with the age- and sex-matched controls. The macrophages' phagocytosis was partially blocked by mannan, which was expected since mannose receptors are important in the recognition of fungi harboring mannan in their cell walls; however, even after blockade, the phagocytosis index was lower in CWD patients than in controls. These findings are consistent with the reduced peroxide and NO release by both CWD patients' neutrophils and macrophages. It is important to note that the functional impairments in the presence of *T. rubrum* were frequently partial, with values around 20–30% below those of the matched healthy individuals, while the responses to the positive control stimuli (zymosan particles or PMA) were fully preserved. This suggests a partial but specific deficiency, consistent with the limited clinical severity of the disease (e.g., the absence of invasion) and the lack of susceptibility to other fungi (e.g., *Candida* sp.); however, it was apparently strong enough to slow the elimination of the fungus from the epidermis and hinder the capture of antigens for subsequent processing, presentation, and elicitation of the CMI response, which is in agreement with the chronic and widespread behavior of the infection.

This mechanism is reinforced by the altered pattern of cytokines released by the CWD patients' phagocytes. Macrophages secreted lower levels of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, although they enhanced levels of the anti-inflammatory cytokine IL-10 in response to *T. rubrum* but not to LPS. Neutrophils' secretion closely followed this unbalanced pattern. IL-1β is a key cytokine that drives a proinflammatory state and instructs adaptive immune responses (Dinarello, 2009); this latter function was due to the cytokine's ability to induce the differentiation of monocytes into macrophages with both enhanced phagocytic and antigen-presenting functions (Schenk et al., 2014). In addition, IL-1β has recently been shown to be a key mediator in the IFN-γ-induced control of *T. rubrum* proliferation in an experimental model of dermatophytosis (Baltazar et al., 2014). In addition to its systemic proinflammatory activity, IL-6 is engaged along with IL-1β in the establishment of Th-17 responses (Dinarello, 2009). Furthermore, IL-8 and TNF-α are also potent inducers of systemic inflammation and chemoattractants to neutrophils; in addition, TNF-α is crucial for APC activity (de Luca and Gommerman, 2012). Conversely, IL-10 has the ability to counteract most of these proinflammatory activities. Thus, the reduced levels of the proinflammatory cytokines with Th-1/Th-17-inducing properties, associated with the enhanced IL-10 release by macrophages, likely adversely affected the induction of an effective CMI response.

Two additional points from the present work deserve to be stressed. First, the observed immunological abnormalities were specific to patients with CWD, in the sense that the same experiments were carried out with the cells of patients with *Tp* alone; these experiments showed no differences when compared with the age- and sex-matched healthy controls. Second, it is possible that the reduced killing activity of the CWD phagocytes could be related to the impaired phagocytosis. Although the phagocytes' killing mechanisms of *T. rubrum* are not well defined, in many pathogenic fungi phagocytosis and killing are only partially related events as killing mechanisms may also occur in the extracellular milieu through the release of stressing molecules (Amulic et al., 2012). Moreover, as shown by Campos et al. (2006) in an *in vitro* mouse model of *T. rubrum* infection, the ingested conidia could survive and elongate inside the phagocytes, leading to the death of these cells (Campos et al., 2006). Thus, *T. rubrum* killing by phagocytes would eventually depend more on the release of extracellular toxic components, an important point that warrants further studies.

Collectively, these immunological data strongly support the widespread and recurrent nature of this infection, as illustrated in **Figure 1**. Although some authors defined *Tp* as the primary site of lesions and the reservoir in chronic *T. rubrum* infections (based on local epithelial features that would hinder eradication of the fungi; Zaias and Rebell, 1996), six of our 15 patients lacked *Tp*. Thus, concomitant chronic *Tp* infection, although frequent, does not seem to be a prerequisite for CWD. Also, a "chronic trichophytosis syndrome" has been proposed in Croatian patients due to *T. mentagrophytes var. interdigitalis*, which predominantly involves the feet (Gregurek-Novak et al., 1993). As in our patients, this chronic trichophytosis syndrome was associated with decreased leukocyte phagocytosis and digestion, although in this case, the defect was non-specific, as it was demonstrated using only sheep erythrocytes as a target.

Regularly viewed as a well-adapted and low-virulence pathogen (Dahl and Grando, 1994), the *T. rubrum* human–host interaction appears to be rather complex, giving rise to a spectrum of diseases depending on the quality of the host's defenses. This spectrum ranges from the benign and localized *tinea* on the one hand, where the host's defenses appear to be intact, to the life-threatening systemic infections on the other, as seen in CARD9-deficient (Lanternier et al., 2013) or severely immunocompromised patients [e.g., cirrhotic patients (Marconi et al., 2010)]. Other clinical entities would stand in between, such as the deep infections in immunocompromised patients (AIDS, hematological malignancies, and solid organ transplant recipients), who are still able to avoid systemic spread, possibly owing to preserved innate immune responses (Tsang et al., 1996; Franco, 2001; Smith et al., 2001). We propose that our series of patients could represent a distinct clinical entity – namely, chronic or recurrent widespread dermatophytosis – secondary to a subtle and apparently *Trichophyton*-specific innate immune deficiency, which also stands in between the two poles.

Chronic widespread dermatophytosis needs further characterization, such as by identifying the molecular pathways underlying its phagocyte dysfunction and its possible genetic traits. An association with mutations in the *CARD-9* gene seems unlikely due to the lack of familial segregation in all but one of the 14 patients, as well as to the less severe, non-invasive behavior of the CWD. Another interesting aspect would be to study the patients' epithelial LCs. Regarding the patients' response to treatment, preliminary results of the follow up of our CWD patients indicated that only half of them showed an initial good response to terbinafine; they are now being followed for recurrences. The other half of our patients persisted with residual *T. rubrum*-positive lesions (Santana GB, Bezerra TA, and Moraes-Vasconcelos D, unpublished data).

## Conclusion

As for the insights provided by the study of the immune mechanisms underlying the different outcomes of the host–*Candida* interaction (Lionakis and Holland, 2013), the study of the also rather complex *Trichophyton*–host interaction may uncover additional mechanisms associated with either protection or susceptibility to fungal infections.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00801

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REVIEW
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# *Paracoccidioides*-host Interaction: An Overview on Recent Advances in the Paracoccidioidomycosis

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*Paracoccidioides brasiliensis* and *P. lutzii* are etiologic agents of paracoccidioidomycosis (PCM), an important endemic mycosis in Latin America. During its evolution, these fungi have developed characteristics and mechanisms that allow their growth in adverse conditions within their host through which they efficiently cause disease. This process is multi-factorial and involves host–pathogen interactions (adaptation, adhesion, and invasion), as well as fungal virulence and host immune response. In this review, we demonstrated the glycoproteins and polysaccharides network, which composes the cell wall of *Paracoccidioides* spp. These are important for the change of conidia or mycelial (26°C) to parasitic yeast (37°C). The morphological switch, a mechanism for the pathogen to adapt and thrive inside the host, is obligatory for the establishment of the infection and seems to be related to pathogenicity. For these fungi, one of the most important steps during the interaction with the host is the adhesion. Cell surface proteins called adhesins, responsible for the first contact with host cells, contribute to host colonization and invasion by mediating this process. These fungi also present the capacity to form biofilm and through which they may evade the host's immune system. During infection, *Paracoccidioides* spp. can interact with different host cell types and has the ability to modulate the host's adaptive and/or innate immune response. In addition, it participates and interferes in the coagulation system and phenomena like cytoskeletal rearrangement and apoptosis. In recent years, *Paracoccidioides* spp. have had their endemic areas expanding in correlation with the expansion of agriculture. In response, several studies were developed to understand the infection using *in vitro* and *in vivo* systems, including alternative non-mammal models. Moreover, new advances were made in treating these infections using both well-established and new antifungal agents. These included natural and/or derivate synthetic substances as well as vaccines, peptides, and anti-adhesins sera. Because of all the advances in the PCM study, this review has the objective to summarize all of the recent discoveries on *Paracoccidioides*-host interaction, with particular emphasis on fungi surface proteins (molecules that play a fundamental role in the adhesion and/or dissemination of the fungi to host-cells), as well as advances in the treatment of PCM with new and well-established antifungal agents and approaches.

**Keywords:** ***Paracoccidioides brasiliensis,*** ***Paracoccidioides lutzii*****, fungi–host interaction, paracoccidioidomycosis, *Paracoccidioides* pathogenicity and virulence**

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## INTRODUCTION

It is estimated that about 1.2 billion people worldwide suffer from fungal diseases. Some of these are invasive or chronic and difficult to diagnose and treat. It is estimated that 1.5 to 2.000.000 people die of fungal infections each year, surpassing those who die from other causes (Denning and Bromley, 2015). In Latin America, the rich diversity of biomass and climates provides a rich range of habitats for different microorganisms, including these pathogenic fungi responsible for endemic mycoses and that have an important impact on public health: histoplasmosis, coccidioidomycosis, and paracoccidioidomycosis (PCM; Colombo et al., 2011).

The *Paracoccidioides* spp. specie complex is dimorphic fungi that are the etiologic agents of PCM. This is the most important systemic mycosis in Latin America with Brazil, Venezuela, and Argentina being the countries with the greatest number of patients. Non-autochthonous cases have been described outside endemic areas in patients who have lived in or visited Latin America. Brazil, which accounts for 80% of the cases, concentrated the occurrence in its southeastern, southern, and Midwestern regions. PCM is an endemic mycosis that is responsible by the highest cause of mortality and the eighth most important cause of mortality from chronic infectious diseases reaching rates of 1.65 deaths per 106 inhabitants (Coutinho et al., 2002; Bocca et al., 2013). According to Martinez (2010), an estimated 3,360 new cases per year reflect the fatality and mortality rates attributed to PCM in Brazil. Through epidemiological surveys it's known that PCM occurs throughout Brazil (Blotta et al., 1999; Bellissimo-Rodrigues et al., 2011; Loth et al., 2011; de Souza et al., 2014; Vieira et al., 2014). Recent eco-epidemiological studies (**Table 1**) have been demonstrating the occurrence of the PCM in different regions of the Brazil, warning the scientific community about the importance of this disease to the country.

The *Paracoccidioides* genus is composed of thermally dimorphic fungi classified in the *Onygenales* order and in the *Ajellomycetaceae* family (Untereiner et al., 2004). Currently, due to advances in phylogenetic studies of different *Paracoccidiodes* isolates, this genus is divided into two species: *P. brasiliensis* and *P. lutzii*, being the first divided into three different phylogenetic species, S1, PS2, and PS3 (Matute et al., 2006; Carrero et al., 2008; Marini et al., 2010). Agents of systemic mycoses, such as *P. brasiliensis* and *P. lutzii*, express factors that facilitate their survival in severe conditions inside the host cells and tissues, and as such, benefit the disease's development (Casadevall and Pirofski, 1999). The successful colonization of host tissues by the fungus is thus a complex event, usually involving various regulatory mechanisms of cellular homeostasis and the expression of different virulence factors during infection that allows the fungi to cause this systemic mycosis with deep extension in the host organism.

The PCM fungi primarily infects male peasants, between 30 and 60 years of age (Svidzinski et al., 1999; Villa et al., 2000), that are mostly represented by rural workers in the endemic areas (Franco, 1987). Poor hygiene, malnutrition, smoking, and alcohol consumption are considered risk factors for the manifestation of the disease (Silva-Vergara et al., 2000). The inhalation of the fungus conidia or mycelial propagules is the most common transmission mode that allows the fungi to reach the lungs (which are the primary infection site).

The clinical forms of PCM were classified based on the relationship between clinical aspects and the natural history of the disease. Then, the infection is related to the patient without signals and symptoms of the disease but with a positive paracoccidioidin skin test reaction. Patients with PCM disease were divided between acute/subacute form (juvenile type), that mainly affects children and young adults presenting more disseminated lesions, and chronic form (that primarily affect adult men) generally found in oral mucosa, airways, and lung lesions (Bocca et al., 2013; Marques, 2013; Martinez, 2015).

Because of its importance, this review will summarize all the recent discoveries on *Paracoccidioides*-host interaction with particular emphasis in fungi surface proteins, which play a fundamental role in the adhesion and/or dissemination of the fungi to host-cells. The goal is to point out that there are multi-factorial processes involving host–pathogen interactions as well as fungal virulence and host immune response. Finally, this review will also focus in the recent advances in drug discoveries and treatments of PCM.

## THE ADHESION PROCESS IN PCM

The interaction of host and parasite is a complex event in which the host is under pressure to develop resistance while the parasite tries to evade and adapt to the host's immune response and thus survive in the host's environment (Tronchin et al., 2008; Sironi et al., 2015).

Paracoccidioidomycosis can be acquired through inhalation of infectious propagules, which then lodge in the alveoli from which they spread to other organs. The fungi developed mechanisms (such as adhesion to host cells), to avoid entrapment within mucus and their elimination by mucigen cilliary cells (Filler and Sheppard, 2006; Hernández et al., 2010). Therefore, their effective adherence contributes to higher speed invasion of host cells, allowing for evasion of the immune system, establishment of the infection, and in the case of *Paracoccidioides* spp., lead to different clinical manifestations (Singer-Vermes et al., 1994; de Oliveira et al., 2015). Another important fact is that *P. brasiliensis* is able to form biofilm *in vitro*, which opens up new possibilities in understanding the infection process of these fungi, since biofilm formation is a condition that provides for the pathogen's protection against drugs and the host's immune system (Sardi et al., 2015).

Some differences in the degree of adherence have been observed for *Paracoccidioides* spp. regarding the manner in which they enter different cell types. This is perhaps related to changes in the cell wall composition (Telles-Filho, 1987; Puccia et al., 2011). Hanna et al. (2000) observed differences in adhesion capacity to Vero cells of four *P. brasiliensis* strains. Additionally, successive subcultures of *P. brasiliensis* resulted in their attenuation or loss of virulence (Brummer et al., 1990), that can be re-established

**TABLE 1 | Occurrence of paracoccidioidomycosis in the Brazilian territory raised by eco-epidemiological studies.**

| State | Region | Period | Number of cases | Gender/age | Reference |
|---|---|---|---|---|---|
| Parana | Western | 2008–2009 | 102 | 72 male and 30 female/18–81 years | Loth et al., 2011 |
| Sao Paulo | Southeast | 1960–1999 | 1.000 | 858 male and 142 female/03–80 years | Bellissimo-Rodrigues et al., 2011 |
| Amazon | North | 1997–2012 | 2.163 | 1.951 males and 211 females/03–81 years | Vieira et al., 2014 |
| Sao Paulo | Southeast | 1988–1996 | 584 | 492 males and 92 females/05–87 years | Blotta et al., 1999 |
| Rio Grande do Sul | South | 1966–2009 | 123 | 104 males and 17 females/02–97 years | de Souza et al., 2014 |

after passage in animals (San-Blas et al., 1977; Castaneda et al., 1987) or epithelial culture cells (Andreotti et al., 2005).

Recently de Oliveira et al. (2015) found significantly higher capacity for adhesion to pneumocytes by *P. brasiliensis* compared to *P. lutzii*. They also demonstrated that *P. brasiliensis* is more virulent than *P. lutzii*, using an *in vivo* model. This supports the fact that adherence and virulence are closely related in *Paracoccidioides* spp. and reinforces the importance of adhesion in the infection process of these fungi.

*Paracoccidioides* spp. recognizes several of the host molecules such as components of the extracellular matrix (ECM). ECM is basically composed by collagen, elastin fibers, glycosaminoglycans (GAGS), proteoglycans (PG), fibronectin, laminin, heparan sulfate, nidogen/entactin, hyaluronate, chondroitin sulfate, and collagens subtypes I, III, IV, and V (Dunsmore and Rannels, 1996; Mendes-Giannini et al., 2006; Balestrini and Niklason, 2015). These can serve as a substrate for the attachment of colonizing microorganisms (Chagnot et al., 2012). Comparing the adhesion to ECM components of two species of *Paracoccidioides*, de Oliveira et al. (2015) demonstrated that *P. brasiliensis* adheres more to fibronectin in contrast to *P. lutzii* that showed more tropism to type I and IV collagen.

Different studies reinforced the importance of the fungus interactions with ECM proteins during the adhesion process. González et al. (2008b) evaluated *Paracoccidioides* conidia treated with laminin, fibronectin, and fibrinogen in mice experimental PCM. They observed that the treatment with all ECM proteins, especially laminin and fibrinogen, induces a less severe pathology, with a decrease of chitin content in the lungs. In a different study, André et al. (2004) treated yeasts of *Paracoccidioides* with laminin and they to observed that this treatment induces a less severe pathology.

Several studies in the *Paracoccidioides* genus have been conducted to characterize the adhesion process, revealing that *Paracoccidioides* spp. synthetizes several molecules, known as surface adhesins, that are involved, directly or indirectly, in the interaction with host cells and in the *in vitro* biofilm formation (Mendes-Giannini et al., 2000; Hernández et al., 2010; Sardi et al., 2015).

*Paracoccidioides* spp. adhesins are widely studied using *in vitro* and *in vivo* approaches of different forms of *Paracoccidioides* spp. in order to identify and characterize these molecules. The understanding of the adhesion process provides a better understanding of the disease as well-bringing new possible targets for therapeutics (Gonzalez et al., 2005; Tomazett et al., 2005; Pereira et al., 2007; de Oliveira et al., 2015).

Several *Paracocccidiodes* spp. adhesins have been found to have a multifunctional role, being primarly involved in metabolic pathways and later found in fungus cell walls and/or secreted and mediating fungus adhesion (Karkowska-Kuleta and Kozik, 2014). The transport of these molecules (together with antigenic components and other molecules that can interact with the host's cellular immune system such as α-galactosyl), to the fungal cell wall and to the extracellular compartment, can be mediated by vesicles produced by the fungus as described by Vallejo et al. (2011, 2012). Recently, Peres da Silva et al. (2015) described the presence of RNA in extracellular vesicles secreted by *Paracoccidioides* spp., which might interfere in both fungi and host gene expression, modulating the host–pathogen interaction.

The gp43 is a glycoprotein that is the most studied molecule from *Paracoccidioides* spp. Due to its importance in the host–pathogen interactions, including the adhesion process. The gp43 was the first described *P. brasiliensis* adhesin, and it binds to laminin and fibronectin. *In vitro* studies showed that treatment with gp43-purified protein is able to reduce *Paracoccidioides* adhesion, showing that this protein is one of the mediators of fungus adhesion to host epithelial cells and internalization (Vicentini et al., 1994; Hanna et al., 2000). This interaction also occurs with macrophages cells. Silenced strains for gp43 are less adherent or internalized by activated macrophages (Almeida et al., 1998; Torres et al., 2013).

A 32 kDa hydrolase (PbHAD32) was found in *Paracoccidioides* spp. cell wall extracts from mycelial and yeast forms and is able to bind to laminin, fibronectin, and fibrinogen and act as an adhesin in the initial stage of *Paracoccidioides* spp. adhesion. An increase of PbHAD32 is observed during the transition from conidia to yeast or mycelial form (González et al., 2005, 2008a; Hernández et al., 2012). Hernández et al. (2010) using antisense RNA (aRNA) technology performed PbHAD32 silencing and the knockdown of this gene resulted in morphological changes in the yeast form. Furthermore, a decrease of the adherence of both yeast and conidia forms to epithelial lung cells (A549) in a knockdown strain (*PbHAD32 aRNA)* was observed. *In vivo* analysis demonstrated a significant increase in the survival rate of mice challenged with *Paracoccidioides PbHAD32 aRNA* when compared to the wild type *Paracoccidioides* (Hernández et al., 2010, 2012).

The 30 kDa protein was first identified in a *Paracoccidioides* proteomic study before and after mice infection, where a significant increase of its expression was observed after mice infection and was also characterized as a laminin ligand (Andreotti et al., 2005). Later, da Silva et al. (2013) sequenced this protein and identified it as belonging to the 14-3-3 protein

family characterized as small multifunctional proteins present in eukaryotic cells. During infection, Pb14-3-3 accumulates in the *P. brasiliensis* cell walls. The treatment with recombinant protein, promotes the inhibition of *P. brasiliensis* adhesion to epithelial lung cells, demonstrating its importance in this process (da Silva et al., 2013).

In a study conducted by Donofrio et al. (2009) to identify fibronectin-binding adhesins from *P. brasiliensis,* a 54 kDa protein was highly expressed in different strains when cultured in a medium supplemented with blood. The sequencing of this protein identified it as enolase (PbEno), a well-known glycolytic enzyme. Later, it was found that surface expressed PbEno also binds to laminin, type I and IV collagen, plasminogen, and fibrinogen (Nogueira et al., 2010; Marcos et al., 2012). The participation in the adhesion process was evaluated using anti-54 kDa polyclonal antibodies in which the inhibition of *P. brasiliensis* adhesion to epithelial lung cells *in vitro* was demonstrated (Nogueira et al., 2010). In addition, during infection and when cultivated with sheep blood, an increase of PbEno in cell walls was observed, demonstrating its role in fungus–host interaction (Marcos et al., 2012). The ability of enolase to interact with plasminogen has already been related to the pathogen's invasive capacity. It is mediated by lysine-dependent binding, degrading ECM, and promoting invasion (Ghosh and Jacobs-Lorena, 2011). Sequence analysis showed that PbEno has similar internal motif responsible for plasminogen binding in *Paracoccidioides* spp. and the recombinant PbEno binding to plasminogen in the presence of a lysine, suggesting that PbEno also plays a role in the invasion process (Marcos et al., 2012).

Triosephosphate isomerase (TPI) is a glycolytic enzyme described as a protein that is able to react with the sera of PCM patients. The localization of TPI was detected in the cytoplasm and in the cell wall of the yeast phase of *P. brasiliensis* (da Fonseca et al., 2001). Pereira et al. (2007) produced an anti-TPI polyclonal antibody and used it to treat *P. brasiliensis* and observe the influence of this treatment in the interaction of the fungi with epithelial cells. After the treatment, they observed that the antibody inhibited the adhesion of *P. brasiliensis*. These findings showed the involvement of the TPI in the cell adhesion acting as an adhesin.

Malate synthase is an enzyme from the glyoxylate pathway and in *Paracoccidioides* spp. is also required in allantoin degradation pathway (Zambuzzi-Carvalho et al., 2009). However, besides the metabolic role, PbMLS is found in fungus cell walls and is characterized as an adhesin able to bind to fibronectin as well as types I and IV collagen (da Silva Neto et al., 2009). In a study of intermolecular interactions of PbMLS, de Oliveira et al. (2013) found that PbMLS present in cell walls interact with other adhesins such as enolase and TPI and this interaction could enhance the adhesion ability.

GAPDH (glyceraldehyde 3-phosphate dehydrogenase) is a well-known protein from the glycolysis pathway, however, this protein can also act as virulence factor for some pathogens, including fungal pathogens (Karkowska-Kuleta and Kozik, 2014; Marcos et al., 2014). In *P. brasiliensis*, GAPDH expression is increased during the mycelium-yeast transition and was found in cell wall and extracellular vesicles (Barbosa et al., 2004; Longo et al., 2014). *Paracoccidioides* spp. GAPDH is able to bind to host ECM components laminin, fibronectin, and type I collagen. This molecule seems to play a role in the initial steps of infection once *in vitro* assays demonstrated the inhibition of adhesion and infection of *P. lutzii* to pneumocytes after fungus incubation with anti-GAPDH antibody or cell treatment with recombinant GAPDH (Barbosa et al., 2006).

Another protein involved in the process of cell adhesion and tissue invasion/dissemination is the fructose 1,6 bisphosphate aldolase (ALD) protein that interacts with plasminogen. The antibodies anti-ALD and the recombinant protein were able to reduce the interaction between macrophages and *Paracoccidioides* (Chaves et al., 2015).

Recently, de Oliveira et al. (2015) evaluated gene expression of differents adhesins as 14-3-3, ENO, gp43, MLS, GAPDH, and TPI after mice infection by *P. brasiliensis* and *P. lutzii*. They could observed that 14-3-3 and enolase were the most expressed adhesins and also that *P. brasiliensis* express in higher levels adhesins than *P. lutzii*. Besides this, this study demonstrated that the virulence of its species is related to its adhesion capacity with *P. brasiliensis* being more virulent than *P. lutzii*.

The adhesion is an universal prerequisite for pathogens to efficiently deploy their repertoire of virulence (Krachler and Orth, 2013). In summary, the attachment of *Paracoccidioides* spp. to host cells is mediated by adhesins present at the fungal surface and that this is a critical step in PCM, acting as an essential virulence factor for *Paracoccidioides* spp. **Figure 1** summarizes the affinity of each adhesin to different ECM components: laminin, fibronectin, type I collagen, type IV collagen, plasminogen, and fibrinogen.

Based on all that was presented in this section, what is needed are new approaches that aim for the discovery of new molecules or further investigation of the already known molecules. This holds especially true for studies that evaluate strategies to block the adhesion in order to try and discover how the fungi modulated itself to cause the infection and how we can avoid the infection and prevent the PCM.

## MORPHOLOGICAL SWITCHING AND PATHOGENICITY OF *Paracoccidioides* spp.

Different polysaccharides, proteins, lipids, and melanin compose the complicated structure of fungi cell walls. An incisive way for pathogens to respond, adapt, and survive in new niches of infection can be found in the alterations in the expression of surface-exposed molecules under different environmental conditions (Karkowska-Kuleta and Kozik, 2015).

The genus *Paracoccidioides* comprises of thermally dimorphic fungi that grow as saprophytic mycelium at environmental temperature (e.g., 26°C; San-Blas, 1993). Produces infective conidia or mycelial (M) fragment propagules that are inhaled by the host. When the propagules reach the pulmonary alveolar epithelium of a mammalian host (exposed to temperature higher than 37°C), they transform into the parasitic yeast (Y) form

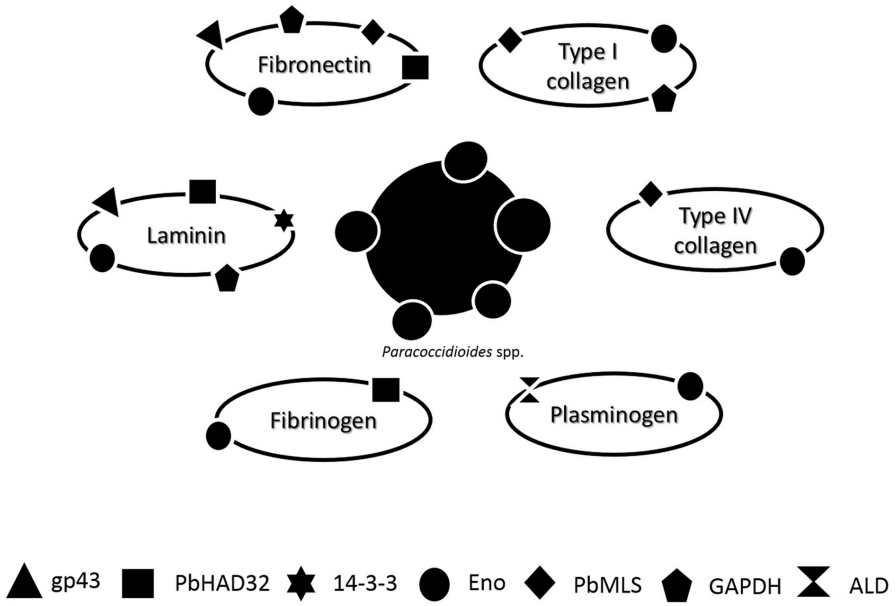


**FIGURE 1 | Schematic representation of the affinity of different *Paracoccidioides* spp. adhesin [gp43, hydrolase (PbHAD32), 14-3-3, Enolase (ENO), Malate synthase (MLT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 1,6 bisphosphate aldolase (ALD)] to different ECM components: laminin, fibronectin, type I collagen, type IV collagen, plasminogen, and fibrinogen.**

causing different clinical manifestations. This phenomenon can also be reversibly triggered *in vitro* by changing the temperature from 26 to 37°C (San-Blas et al., 2002).

The glycoproteins and polysaccharides network that composes the cell wall of *Paracoccidioides* spp., similar to many other fungi, is important for the protection against environmental stresses (De Groot et al., 2005) while promoting its virulence. The morphological switch, a mechanism for allowing the pathogen to adapt and thrive inside the host (Nemecek et al., 2006), is obligatory for the establishment of the infection. This seems to be related to pathogenicity, since isolates incapable of undergoing the morphological transition are less virulent (Maresca and Kobayashi, 2000).

The fungal cell wall synthesis is performed by glucan synthases (Sorais et al., 2010). The phenotypic switch entails changes in the composition of the fungal cell walls with a predominance of β-1,3 and β-1,6-glucan and carbohydrates in mycelial form, while in yeast form there is a prevalence of α-1,3-glucan and chitin (Puccia et al., 2011; Free, 2013). Changes may occur in the quantity and the spatial arrangement of these polysaccharides (San-Blas et al., 1994). This occurs in order to ensure fungal survival within the host (Tavares et al., 2015). α-1,3-glucan is correlated with the level of virulence (Hogan and Klein, 1994), hiding immunostimulatory β-glucans that could be detected by the host phagocytic cells (Klein and Tebbets, 2007), assisting its evasion of the host's immune responses (Rappleye et al., 2007).

For *Paracoccidioides* spp. the temperature is an essential factor involved in dimorphism and is preceded by several molecular changes (Boyce and Andrianopoulos, 2015). This is a characteristic shared with other dimorphic fungi such as *Histoplasma capsulatum*, *Blastomyces dermatitidis* (Medoff et al., 1987), *Coccidioides immitis, Sporothrix schenckii,* and *Penicillium marneffei* (Klein and Tebbets, 2007). The growth of yeast from the mycelium is initiated from the time of thermal change reaching 50% of changes after 48 h from the start of the process. The multiple developments into yeast budding occurs after 5 days (Salazar and Restrepo, 1985; Goldman et al., 2003). The reverse has also been reported and studies show that 48 h following the temperature change, 50–60% of the cells showed conversion mycelium (Goldman et al., 2003; Nunes et al., 2005).

However, in *Paracoccidioides* genus the conidia- or mycelium-to-yeast transition is blocked by exposure of *Paracoccidioides* spp. to female hormones, such as estrogen, via steroid-binding proteins (Loose et al., 1983; Aristizábal et al., 1998). This gives the PCM the peculiarity of affecting more men compared to the number of women (Shankar et al., 2011b).

Regarding the evaluation of molecular mechanisms and gene expression in both morphological states, several studies have been conducted using different strategies such as expressed sequence tags (ESTs) libraries, microarrays, analysis of genes expressed during the stages of mycelium and yeast, as well as those differentially expressed in transition and proteomics. Several efforts have been made to understand the morphological alterations, including those depending on the factors of temperature and the presence of female hormones. There are many studies focusing on genes that are regulated during mycelium-to-yeast (M-Y) transition (Felipe et al., 2005; Bastos et al., 2007; Parente et al., 2008; Muñoz et al., 2014). Although a number of genes that govern the phase transition are known, how these genes fit into a larger network of regulated genes remains poorly explored (Gauthier, 2015).

Transcriptional analysis of 6,022 assembled groups demonstrated that the mycelial cells have a more aerobic metabolism in comparison to the yeast phase, with greater expression of genes of citrate cycle such as succinyl-CoA dehydrogenase and isocitrate synthase suggesting a metabolic shift to oxidative phosphorylation. In contrast the yeast phase displays slanted energy metabolism for the production of alcohol by fermentation, presenting the glyoxylate pathway (into anaerobic metabolism) as being more active. This is demonstrated by analysis of transcription in yeast and mycelia which is consistent with low oxygen levels found in infected tissue (Felipe et al., 2005).

A biochip carrying of 4,692 genes from *P. brasiliensis* was used to trace gene expression in different points of the morphological transition (5–120 h). Among the various genes identified, some encoding enzymes are involved in the catabolism of amino acids, signal transduction, protein synthesis, cell wall metabolism, genome structure, response to oxidative stress, growth control and development (Nunes et al., 2005).

Proteomic analysis during phase conversion of *P. brasiliensis* demonstrated quantitative differences correlated with transcripts levels. The mycelia phase protein profile showed 18 overexpressed proteins involved in cell defense, energy, and protein fate. During M-Y transition, 33 proteins were upregulated, most of them belonging to the glycolytic pathway. Some glycolytic enzymes such as enolase and fosfoglucomutase begin to accumulate during the transition (M-L) and maintain high levels in the yeast phase. It is therefore another sign of the global reorganization of carbohydrate metabolism that occurs during morphological change (Rezende et al., 2011).

N-*linker* glycans are involved in glycoprotein folding, intracellular transport, secretion, and protection from proteolytic degradation (Nagai et al., 1997). In *Candida albicans* it has been shown to be involved in cell wall integrity as well as in the fungus–host interaction (Mora-Montes et al., 2007). Dos Reis Almeida et al. (2014) showed that the treatment of *Paracoccidioides* with tunicamycin, responsible for blocking the N-*linked* glycosylation of α-1,4 amylase, interfere in the transition for both Y-M and M-Y, since the α-1,4 amylase is responsible for biosynthesis of α-1,3 glucan the major cell wall glucan of the yeast form.

Phosphatidic acid and diacylglycerol produced by a phospholipase D1 participates in the morphological transition of *C. albicans* (Hube et al., 2001). A similar finding was seen in *P. brasiliensis* in which up-regulation of phospholipase was found in M-Y transition (Soares et al., 2013).

Thermal dimorphism may occur as a result of a specialized heat shock response triggering a cellular adaptation to high temperatures (Lambowitz et al., 1983). Matos et al. (2013) demonstrated the involvement of HSP90 during the dimorphism of *P. brasiliensis* using pharmacological approaches. HSP90 is required for the transition from non-infective to infective forms but not for Y-M transition. This protein is also highly transcribed under *in vitro* oxidative stress. HSP90 is a chaperone that binds and stabilizes calcineurin. It also competes with calmodulin for the $Ca^{2+}$/calmodulin docking site in calcineurin interfering with the activation of the latter (Imai and Yahara, 2000). So, it was suggested that HSP90 acts synergistically with calcineurin in the control of cell differentiation (Matos et al., 2013). Other proteins of HSP family such as HSP70, HSP80, and HSP88, were down-regulated in mycelial cells treated with estradiol (Shankar et al., 2011b) suggesting that this hormone impairs the favorable expression of genes necessary for adaptation to a change of temperature (Nicola et al., 2008).

In spite of several studies suggesting the potential role of estradiol in dimorphism of *Paracoccidioides*, the exact mechanism that leads to such genetic modulation resulting in differences in disease rates, remains unknown (Tavares et al., 2015). Hormones act as messenger molecules, leading to regulation of gene expression through receptor-mediated interactions that mediate this interaction and the subsequent functional response to the presence of the hormone (Shankar et al., 2011a).

*In vivo* studies have shown that female mice, especially at estrus, reach a higher clearance of yeast and restraint of fungal proliferation as compared to male mice (Aristizábal et al., 1998, 2002; Sano et al., 1999). Pinzan et al. (2010) revealed remarkable influences of gender on experimental PCM, which could be partly attributed to interference of female hormones on the immune response triggered by a *P. brasiliensis* infection. Estradiol promotes protective responses to this infection, IL-12, IFN-γ, and TNF-α cytokines (Calich et al., 1998; Kashino et al., 2000) correlated with resistance to female infection. On the contrary, infected male produces IL-10 (Benard et al., 2001; Oliveira et al., 2002; Romano et al., 2002) which plays an important role in antigen specific immunosuppression of PCM (Pinzan et al., 2010).

Shankar et al. (2011b) using microarray technology to evaluate the *P. lutzii* transcriptional response to a fixed concentration of estradiol during 9 days, revealed that the chitin synthase 1 gene (CHS) was down-regulated in response to estradiol at earlier time points. Nunes et al. (2005) identified a positive modulation of chitin synthases and down-regulation of chitinases in the M–Y transition, while Bastos et al. (2007) detected two chitinases over-expressed in the dimorphic transition. In fact, the yeast cell wall is mainly constituted of chitin (37–48%), compared to the mycelium form (7–18%; Kanetsuna et al., 1969).

High levels of gene expression may occur during this process. Hernández et al. (2011b) showed increased expression of HSP90, AOX, and GS1 (glucan synthase-1) throughout the entire yeast to mycelium germination and αGS (glucan synthase α) for the opposite. The HSP90 was up regulated early in the transition suggesting their involvement in the initial contact of the fungus with the host and the modifications necessary to adapt within the same. The AOX (alternative ubiquinol oxidase) gene acts by reducing the reactive oxygen species and correlates with metabolic activation required to obtain carbon and energy owing to the non-phosphorylative nature of the alternative respiratory pathway to the morphological changes (Gessler et al., 2007; Hernández et al., 2011a). AOX is present in early stages of M-Y transition and plays an important role in intracellular redox balance. Furthermore, it is the only enzyme in *P. brasiliensis* that is not present in its mammalian hosts therefore it is a promising target for therapy (Martins et al., 2011).

TNF-α is a cytokine related with anti-microbicidal activity in IFN-γ activated macrophages stimulating NO production. Gonzalez et al. (2004) illustrated that TNF-α activated macrophages are capable of inhibiting the conidia to-yeast transition in *P. brasiliensis* by an NO-independent pathway; acting as a fungicidal and/or fungistatic mechanism against *P. brasiliensis* conidia.

Cyclic AMP (cAMP) is the regulatory component of a well-characterized signaling pathway implicated in a variety of cellular processes among fungal species (Fernandes et al., 2005). The importance of the cAMP-signaling pathway in the control of morphological changes and pathogenicity of various fungi has been reported (Medoff et al., 1987; Cho et al., 1992; Borges-Walmsley and Walmsley, 2000). During morphological transition the cAMP levels increase transiently in the early stages (<24 h) and progressively in the subsequent stages (>120 h; Chen et al., 2007). Moreover, the transition may be modulated by exogenous cAMP (Paris and Duran, 1985; Chen et al., 2007), suggesting possible involvement of cAMP in the dimorphic transition.

Understanding how different cell types recognize both yeast and mycelial and how each cell type is activated in accordance with the morphology is important, however, the likely consequences of this activation probably differ according to cell type (e.g., in macrophage or in epithelial cell; Jacobsen et al., 2012). Inhibitors targeting the morphological transition from mycelium-to- yeast are an interesting choice to attempt to control the *Paracoccidioides* infection. As discussed above the change to yeast form is essential for the establishment of infection, and thereby inhibitors of this can prevent the infection. **Table 2** summarizes the main works related to dimorphism of *Paracoccidioides* spp. that focused on specific genes and proteins.

## HOST CELLS MANIPULATION BY *Paracoccidioides* spp.

The ability of pathogens to colonize their hosts is highly dependent on mechanisms that may allow the pathogen to break the physical and immunological barriers imposed by the host. In order to avoid rapid clearance of the organism, pathogens act quickly and effectively on adhesion to host cells.

The capacity of cells to interact with each other in an orderly manner depends on multiple adhesive interactions between cells and their adjacent extracellular environment, mediated by cell adhesion molecules (Miyoshi and Takai, 2008; Troyanovsky, 2009). These function as cell surface receptors that can trigger physical and biochemical signals that regulate a great numbers of functions such as cell proliferation, gene expression, differentiation, apoptosis, and cell migration and are used as a gateway to some pathogens (Finlay and Falkow, 1997; Guttman and Finlay, 2009; Han et al., 2010; Voltan et al., 2013).

Many pathogenic microorganisms have the ability to induce its internalization in epithelial cells, forcing the activation of phagocytosis mechanism. Specific extracellular signals can stimulate their cytoskeleton rearrangement in the contact site with the microorganism, making cells to behave like a "phagocyte unprofessional" (Swanson and Baer, 1995; Lim and Gleeson, 2011), in a process that involves integrins and the cytoskeleton (Mendes-Giannini et al., 2004; Feriotti et al., 2013). *Paracoccidioides* spp. invasion affects the structure of the cytoskeleton of pulmonary epithelial cells and keratinocytes, interfering with morphological aspects of actin, tubulin and cytokeratin (Mendes-Giannini et al., 2004; Peres da Silva et al., 2011).

The capacity of fungal invasion to mammalian cells is specific to some fungi and there is still a lacuna in the understanding of this process (Tsarfaty et al., 2000; Wasylnka and Moore, 2002; Mendes-Giannini et al., 2004). The signaling pathways that control the morphological changes in *P. brasiliensis*, as well as the cellular signals upon interaction with the host cell are also not well-understood. Mendes-Giannini et al. (2004) showed that treatment with cytochalasin D and colchicine reduced the invasion by *P. brasiliensis*, indicating the functional involvement of microfilaments and microtubules in this process.

Some studies evaluated the role of adhesins in the invasion process of *Paracoccidioides* and it was observed that gp43 may also participate in the cytokeratin degradation leading to the loss of the filamentous characteristics that can facilitate the invasion of the host (Mendes-Giannini et al., 1990; Puccia and Travassos, 1991; Tacco et al., 2009; Puccia et al., 2011). The 14-3-3 adhesin is also known to have the capacity to cause structural modifications in the host cells influencing in the polymerization of the cytokeratin microfilaments of actin (Andreotti et al., 2005; Mendes-Giannini et al., 2006; da Silva et al., 2013).

The Rho GTPase family of proteins is known to regulate the dynamic organization of the cytoskeleton and membrane traffic physiological processes such as cell proliferation, motility, polarity, and growth (Sinha and Yang, 2008). The Rho-GTPase is able to down-regulate genes related to chitin and glucans biosynthesis. Rho-GTPase, FKS (β-1,3 glucan synthase), and AGS1 (coding α-1,3 glucan synthase) were down-regulated on *Paracoccidioides* estradiol samples treated. They indicate that this hormone promotes a transcriptional modulation of the cell wall, remodeling related genes (Shankar et al., 2011b; Tavares et al., 2015). Rho GTPases have been extensively studied in human fungal pathogens and have a set of interacting proteins to orchestrate their activation in the cells (Yamochi et al., 1994). Cdc42, a member of the Rho GTPase, was characterized as a convergence point in the signal transduction and are involved in multiple signaling pathways including receptor tyrosine kinases and cytokines, heterodimeric G proteins, as well as physical and chemical stress. In *P. brasiliensis* plays a role in morphology of yeasts cells since the knockdown PbCDC42 showed decrease in cell size and more homogenous cell growth and this provided a higher phagocytosis and decreased virulence (Almeida et al., 2009).

In mammalian cells, the Rho GTPases are also the center of a complex signaling pathway that plays an important role in adhesion. The activation of tyrosine kinase (PTK) receptors stimulate Rho GTPase which in turn activates the Ras pathways and MAPKs (Sinha and Yang, 2008). Monteiro da Silva et al. (2007), showed significant inhibition of fungal invasion after pre-treatment of epithelial cells with genistein, a specific inhibitor

**TABLE 2 | Summary of studies related to dimorphism of *Paracoccidioides* genus.**

| Condition | Approach (isolate) | Target | Observation | Reference |
|---|---|---|---|---|
| Mycelium-to-yeast transition | Pharmacologycal tools – inhibition with geldanamycin (*P. brasiliensis)* | Hsp90 | Up-regulated | Matos et al., 2013 |
| Mycelium-to-yeast<br>Yeast-to-mycleium | Real-time reverse transcription-polymerase chain reaction | Hsp90/AOX/GS1<br>αGS | Up-regulated<br>Up-regulated | Hernández et al., 2011b |
| Mycelium-to-yeast transition | Pharmacologycal tools – inhibition with CsA, a calcineurin inhibitor cyclosporine A (*P. brasiliensis)* | Calcineurin | Transition to the yeast form was blocked before the blastoconidial budding stage | Campos et al., 2008 |
| Mycelium-to-yeast transition | Transcriptional profiling and pharmacological tools – 4-HPPD inhibitor (*P. brasiliensis*) | 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD) | Up-regulated<br>Inhibit growth and differentiation to the pathogenic yeast phase | Nunes et al., 2005 |
| Mycelium-to-yeast | Pharmacologycal tools – inhibition with benzohydroxamic acid – inhibitor of AOX (*P. brasiliensis)* | AOX | Delayed the M-Y transition | Martins et al., 2011 |
| Yeast-mycelium germination and mycelium/conidia-to-yeast transition | Antisense RNA technology (*P. brasiliensis*) | AOX | Delayed the Y-M transition | Hernández et al., 2015 |
| Mycelium-to-yeast transition | Transcriptional response to 17-β-estradiol treatment (*P. brasiliensis)* | Chitin synthase | Down-regulated in response to estradiol | Nunes et al., 2005 |
| Mycelium-to-yeast transition | 1007 ESTs from a transition cDNA library (*P. lutzii*) | Two chitinases | Up-expressed | Bastos et al., 2007 |
| Mycelium-to-yeast transition | Gene expression in the presence or absence of 17-β-estradiol (*P. lutzii*) | Rho-GTPase components FKS1 and AGS (coding α-1,3-glucan synthase) | Down-regulated | Shankar et al., 2011b |
| Mycelium-to-yeast and yeast-to-mycelia | Pharmacological approach – using tunicamycin (TM) | N-glycosylation | TM treatment interferes the transition in both directions by interference in the activity of α-1,4 amylase (involved in the biosynthesis of α-1,3 glucan) | Dos Reis Almeida et al., 2014 |
| Mycelium-to-yeast | Real-time (*P. brasiliensis*) | Phospholipase (PLB) | Up-regulation in mycelial cells | Soares et al., 2013 |

of PTK located on the plasma membrane of the epithelial cells. These results suggest that the inhibition of PTK is important in signal transduction during early events in the adhesion and invasion processes of *P. brasiliensis* in epithelial cells.

Apoptosis is a highly regulated physiological process of cell death required for the development and homeostasis of multicellular organisms by eliminating individual cells without inducing an inflammatory response. The enabling or prevention of apoptosis can be a critical step in the development of infectious processes. The process of apoptosis is characterized by typical changes in the symmetry of the plasma membrane, chromatin condensation, nuclear fragmentation, DNA cleavage, cell disintegration, and formation of apoptotic bodies (Strasser et al., 2011).

Programmed cell death has been observed as a response to a variety of infections and can be mediated by a variety of virulence determinants encoded by pathogens. The modulation induced by pathogens pathways responsible for cell death in the host favor the elimination of cells of the immune system or avoidance of host defense response that attempt to act in eliminating the infection (Weinrauch and Zychlinsky, 1999).

The ability of the pathogen to induce apoptosis in phagocytes may be an important virulence factor, since it reduces the host's defense mechanisms (Ashida et al., 2011). *P. brasiliensis* and other fungi can induce the apoptosis of phagocytes to acquire advantages, allowing intracellular survival in epithelial cells (Cacere et al., 2002).

*Paracoccidiodes brasiliensis* induces apoptosis when it invades epithelial cells or phagocytes, which benefits its intracellular survival (Souto et al., 2003; Mendes-Giannini et al., 2004, 2005; Verícimo et al., 2006; Ketelut-Carneiro et al., 2015). Silva et al. (2008), showed that *P. brasiliensis* induces apoptosis of macrophages by expression of caspase-2, 3 and 8, but also found that it induces the expression of genes that encode inhibitors of apoptosis proteins, such as caspase-8 and Fas-L inhibitors. Caspases-2 and 8 are responsible for the transduction of signals for cleavage of other caspases, such as caspase-3, which leads to apoptosis induction (Silva et al., 2008).

*Paracoccidiodes brasiliensis* may modulate apoptosis of epithelial cells A549 by the expression of apoptotic molecules such as Bcl-2, Bak, and caspase-3, confirming the inducing of apoptosis by the fungus which can then survive and spread to other parts of the body (Del Vecchio et al., 2009). More recently, Silva et al. (2015), showed that the 14-3-3 and gp43 adhesins has strong influence in this process. Campanelli et al. (2003) demonstrated that apoptosis mediated by Fas-FasL and CTLA-4 engagement are involved in modulating the immune response in patients infected with PCM. Souto et al. (2003) demonstrated in experimental PCM, a considerable increase in apoptosis in the infection site. Cacere et al. (2002) studied the role of apoptosis

in PCM using peripheral blood mononuclear cells of patients with the PCM disease, noting that apoptosis induced by gp43 was lower in controls than in peripheral blood mononuclear cells of patients.

All of these studies demonstrated that *Paracoccidioides* spp., during its evolution, has developed mechanisms that allows the fungi, since it is inhaled in its mycelial form, to survive in a hostile host environment. During the interaction, using its adhesins, *Paracoccidioides* spp. adheres to host ECM components and cells, manipulates the cell cytoskeleton, invades the cytoplasmic compartment, and can then induce the cell apoptosis, which gives it its capacity to evade the immune system and spread within the host organism causing systemic mycosis, as summarized in **Figure 2**.

## ADVANCES IN ANIMAL EXPERIMENTATION FOR THE STUDY OF THE PCM

The use of animals in research is essential for studies of host–fungal interaction, pathogenesis, treatment mechanisms, immunological aspects, or studies that imply the response of a complex organism. It is important to consider the experimental animal type, age, sex, and routes of inoculation (Conti-Diaz et al., 1959; Iabuki and Montenegro, 1979; Defaveri et al., 1982). The firsts studies described guinea-pigs (de Brito and Netto, 1963), hamsters (Iabuki and Montenegro, 1979), and rabbits (Conti-Diaz et al., 1959) as models to study PCM, however, most of the animals do not develop the disease until a long time after being infected (Linares and Friedman, 1972).

After many decades of investigation, murine animal models are considered the gold standard for *in vivo* studies to simulate the *Paracoccidioides* spp. infection. The establishment of a pulmonary PCM was described in murine models using the intratracheal route after about 30 days of infection. Antibodies were detected 15–60 days after infection, however, were not observed after 360 days (Defaveri et al., 1982). Intranasal route was efficient to develop fatal acute pulmonary or chronic pulmonary and disseminated PCM by using different inoculum concentrations. The development of this model of infection is useful to study treatment (fatal acute pulmonary) and to understand immunological aspects of the disease (chronic; Brummer et al., 1984). The dissemination occurs through a hematogenous route and affects mainly the lungs, secondly liver, thirdly the lymph-nodes and finally within the spleen with the formation of granuloma (Bedoya et al., 1986). The classification of nine different congenital strains of mice were realized after infecting these animals using an intraperitoneally route with *P. brasiliensis*. These mice were classified according to the susceptibility to infection as very resistant, intermediate, and sensitive. The study demonstrated that the susceptibility to *P. brasiliensis* infection of the different animal strains was not dependent on the inoculum concentration. In addition, male mice were generally more susceptible to the infection than females (Calich et al., 1985). In the infection of susceptible mice, high numbers of viable yeasts in different organs were found, however, low fungal burden were observed in all examined organs of resistant animals representing the regression of the infection (Singer-Vermes et al., 1993).

Once the infection model was established to study the efficacy of traditional medicines, new drug candidates or drug combinations could be evaluated (Lefler et al., 1985; McEwen et al., 1985; Restrepo et al., 1992), as well as to study immunologic aspects (Defaveri et al., 1989; Calich and Kashino, 1998; Kashino et al., 2000; Pinto et al., 2006).

Because of the advances in medical technology the number of animals in the research increased and many acts and laws were created in different countries to control ethical issues and to minimize the pain to animals during experimentation (Doke and Dhawale, 2015). Since 1959, the use of animals during scientific experiments has been a debated from which the three Rs theory was created. This theory proposes for the **R**eduction of the number of animals used in an experiment, the **R**efinement of the experiment to animal welfare, and the **R**eplacement of animals by using alternative methodologies (Russell and Burch, 1959).

The use of alternative models like tissues or cell cultures, computer or mathematical analysis (*in silico* testing), and imaging/analyzing techniques are suggested to obtain preliminary data before the start of *in vivo* assays. However, in many cases the research requires information about the response of one whole and complex organism (Balls, 2002; Arora et al., 2011; Doke and Dhawale, 2015).

Considering the need for animal experimentation and the restriction in the use of mammalian animals because of ethical issues, researchers developed alternative animal models. Invertebrates represent a good alternative for *in vivo* assays, because of their short life cycle, small size, evolutionary conservation of the innate immune response between invertebrates and mammals, and low cost. A large number of animals can be used per experiment and until now, no ethical problems limit their use (Mylonakis et al., 2007; Lionakis, 2011; Wilson-Sanders, 2011; Glavis-Bloom et al., 2012; Arvanitis et al., 2013).

Different invertebrate model have been used to study fungal diseases. The fly, *Drosophila melanogaster* (Alarco et al., 2004; Lionakis and Kontoyiannis, 2012), the nematode, *Caenorhabditis elegans* (Pukkila-Worley et al., 2009, 2011; Muhammed et al., 2012); the insect *Galleria mellonella* (Fuchs et al., 2010; Mesa-Arango et al., 2013; Firacative et al., 2014; Maurer et al., 2015) were reported as being used to study fungal virulence factors and to identify novel antifungal compounds.

The use of alternative animal models to study PCM was firstly described by Thomaz et al. (2013). In this study, *G. mellonella* model was used to study *P. lutzii* that was able to kill larvae at 25 and 37°C. Moreover, melanization and granuloma-like structures were observed. Recently, because of the new classification of two distinct species (*P. brasiliensis* and *P. lutzii*), a comparative study of the virulence was developed. Both species cause a hemocyte decrease and kill *G. mellonella* in a similar way. However, *P. lutzii* has higher adhesion ability to hemocytes and this could be attributed to the higher expression of the gp43 gene (Scorzoni et al., 2015). To study the importance of the adhesins for the virulence of *P. brasiliensis* and *P. lutzii*, the treatment of

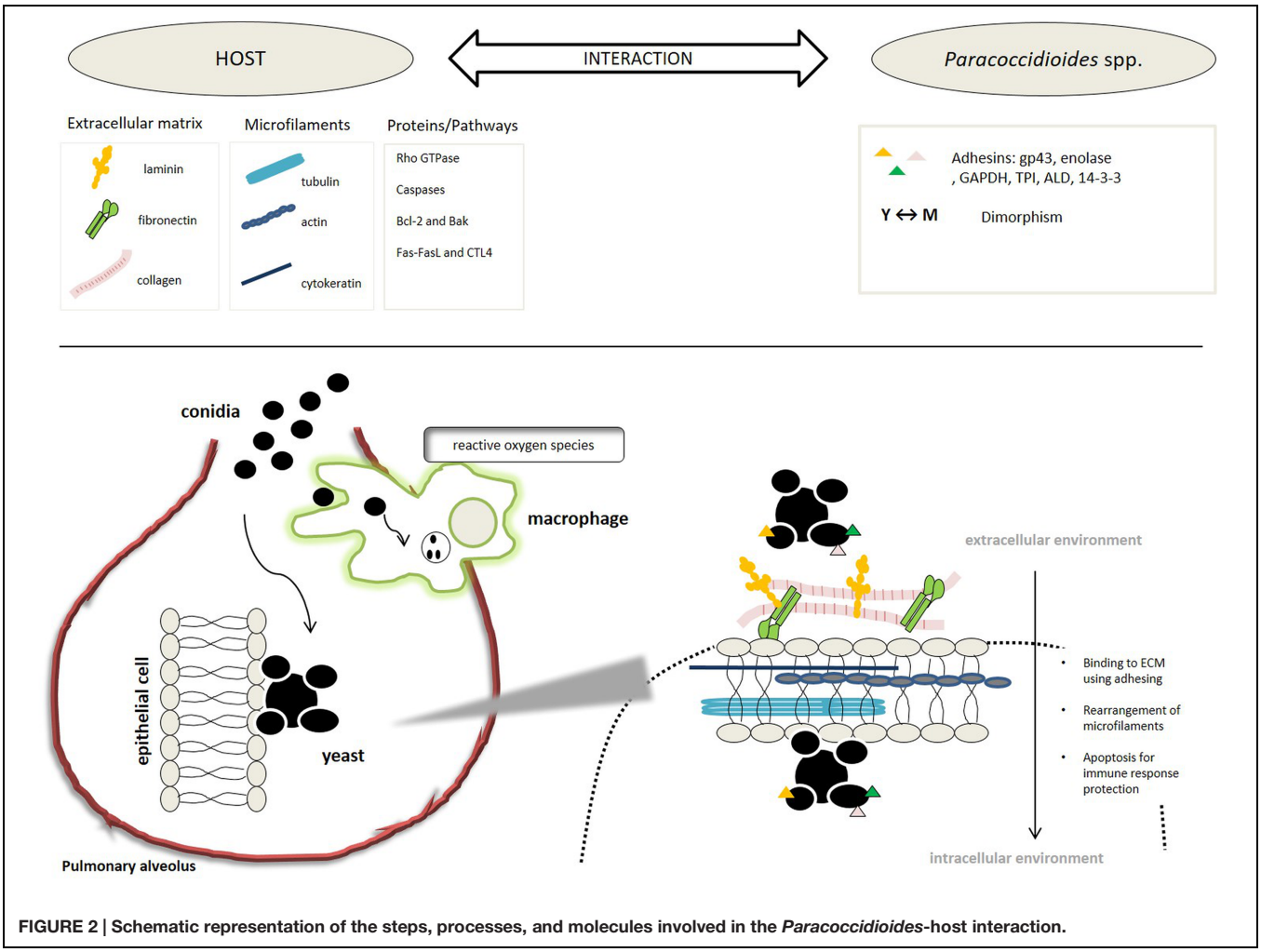


**FIGURE 2 | Schematic representation of the steps, processes, and molecules involved in the *Paracoccidioides*-host interaction.**

*Paracoccidioides* spp. with antibodies to block two adhesins (14-3-3 and enolase) caused a decrease in the death of the larvae (de Oliveira et al., 2015).

Despite the evolutionary distance between invertebrate and mammalian models, recent works describe the correlation response to infection between these models (Brennan et al., 2002; Brunke et al., 2015; Desalermos et al., 2015). Considering these evidences, invertebrate models are a good alternative for preliminary studies to investigate *Paracoccidioides* spp. virulence, as well as new treatment and immunological aspects of the infection.

## FACING THE PROBLEM: THROUGH THE DIAGNOSTIC TO TREATMENT OF PCM

The diagnosis of PCM is based on clinical and laboratory findings. In the acute or juvenile form of the disease, the skin lesions are often present. On the other hand, in the chronic or adult form, the lung is mainly affected. In this case, it is indicated by a radiography of the organ, which exhibits a pattern that resembles a butterfly wing, characterized by bilateral and symmetrical reticulonodular infiltrate in the two upper thirds of the lungs (Barreto et al., 2012). In the laboratory, the direct microscopic examination of the material collected from lesions or tissue is made to observe the fungi, especially its typical multiple budding aspect, as well as the culture to observe the thermal dimorphism, but the fungus has slow growth (Mendes-Giannini and Fusco-Almeida, 2013; Benard and Mendes-Giannini, 2014).

Furthermore, the diagnosis of disease can be made using serological methods. The counter-immunoelectrophoresis (CID) and double immunodiffusion (IDD) are the reactions most used in reference centers (Vidal et al., 2014). In these cases, the 43 kDa glycoprotein (gp43), which is secreted during the infection, is the main antigen detected. The values of titers correlate with the severity of the disease and efficacy of the treatments. In addition, the negativity or stabilization at dilution 1:2 or less indicates the disease cure (Abreu e Silva et al., 2013). However, differences in the antigenic composition, probably related to phylogenetic peculiarities of the two species, should be considered in the diagnosis of PCM (Batista et al., 2010).

When detection by microscopy and serology fail, an alternative can be the use of molecular techniques as polymerase

chain reaction (PCR) can be used (due to its greater sensitivity). Several studies have been designed with specific primers to target the genes of the *Paracoccidioides* species. For example, primers for the gp43 antigen were developed to identify *P. brasiliensis* DNA (Gomes et al., 2000). A set of primers for PbITS1s and PbITS3a genes was also used for the detection of the fungus by PCR (Buitrago et al., 2009). Another study reported the use of the primer OPG18, which generates two specific DNA fragments (0.72 and 0.83 kb) for *P. brasiliensis* (San-Blas et al., 2005). Finally, Motoyama et al. (2000) showed that the use of fungal universal primers to target 5.8S and 28S rDNA genes followed by more specific primers (OL3 and UNI-R) for PCR resulted in good identification of *Paracoccidioides* spp.

Recently, Nobrega de Almeida et al. (2015) proposed the use of MALDI TOF MS for *Paracoccidioides* spp. identification. In this study, they analyzed 22 strains, belonging to the two species of the genus. All of the strains were correctly identified. MALDI TOF MS is an interesting tool because of its possibility to adapt to routine laboratories and because the results achieved by this study brings benefits in the clinical and laboratorial studies allowing for the identifying of differences between the diseases caused by this genus.

Besides all well-established methodologies to diagnose the PCM, the diagnostic is not an easy subject. The observation of the fungi in clinical specimens, and growth and reversion to mycelium phase, is difficult in clinical labs. Because of this, until recent times, the serological diagnosis was the most commonly used, since molecular approaches are expensive in countries that PCM occur. However, several recent studies in the characterization of different isolates of *Paracoccidioides* spp. bring difficulties to the serological diagnosis.

Gegembauer et al. (2014) for example, demonstrated that serum from PCM patients infected with *P. brasiliensis* is not able to recognize any antigen from the cell-free preparations of *P. lutzii*, however, serum from patients infected with *P. lutzii* is able to recognize both antigens from *P. lutzii* and *P. brasiliensis*. This means that *P. lutzii* serum is more complex antigenically presenting species-specific antigens and common antigens shared with *P. brasiliensis*. Queiroz Júnior et al. (2014) analyzed the protein/glycoprotein profiles of exoantigens from two clinical isolates of *P. brasiliensis* and three of *P. lutzii* with differences between the species observed. *P. lutzii* exoantigens were different from each other showing high species-specific antigens variability, while *P. brasiliensis* isolates exoantigens present similar protein profiles.

Because of these difficulties in the identification and diagnostic of the PCM with incidence of false negative results (da Silva et al., 2015), this is a public health problem as the number of notifications of the disease can be higher than the numbers we currently have today. In addition, the correct identification of the infection can lead to an efficient treatment. Because of this, new efforts in the identification of serological markers is extremely necessary and one of the great challenge in the study of PCM.

Many drugs are useful in treating PCM. Ribeiro in 1940 suggested the initial treatment with sulfapyridine. Later, Lacaz, and Sampaio proposed the use of amphotericin B in 1958. Barbosa e Vasconcelos, in 1973, recommended the use of a combination of trimethoprim-sulfamethoxazole. Around 1979, Negroni suggested the use of ketoconazole. Restrepo, in 1987 suggested itraconazole and more recently, in 2007, the use of voriconazole was suggested by Queiroz-Telles (Cavalcante et al., 2014).

The treatment depends on the severity of the disease, type of antifungal agent, and the time of use. Despite the limited information on studies with different therapies, the itraconazole therapy is the first choice to control the mild to moderate clinical forms. Since 1987, many groups developed studies with azoles antifungals, which showed a reduction in the symptoms, and that they arrested the progression of the PCM (Negroni et al., 1987a,b,c; Restrepo et al., 1987). However, itraconazole therapy is not easily available in most of the endemic regions. Consequently, the therapy consisting of a trimethoprim-sulfamethoxazole combination (daily for 12 months for mild cases and for 24 months in moderate clinical infections) is a useful option. On the other hand, for severe cases, amphotericin B therapy is the best choice. In case of PCM of the central nervous system, the treatment should be with fluconazole or voriconazole therapy daily for 3–6 months, with a maintenance dose daily for 6–12 months. This is because both have a good penetration through the blood brain barrier (Marques, 2012). Today, ketoconazole is little used for the treatment of this infection because of its severe side effects (hepatotoxicity, loss of libido, inhibition of cortisol production etc; Ferreira, 2009).

The possibility of triazole derivatives interacting with several drugs has to be kept in mind such as antihistamines, antacids, H2 receptor blockers, barbiturates, cyclosporine, diphenylhydantoin, digoxin, cisapride, and rifampicin, among others, as well as the well-known side effects and toxicity (nephrotoxicity, myocardial toxicity, myelotoxicity, etc.) related to amphotericin B which will sometimes require discontinuation of therapy (Ferreira, 2009). In the last 30 years there have been efforts at improving AmB preparation, however, the high costs, neglected clinical data, and alternative antifungal therapies have led to the use of this therapy as a second-line therapy (Laniado-Laborín and Cabrales-Vargas, 2009).

More recently, Rodríguez-Brito et al. (2010) evaluated the susceptibility of *P. brasiliensis* (both at their mycelial and yeast phase), to caspofungin, an antifungal drug of the echinocandin class. For the yeast phase, they found that caspofungin was able to inhibit the growth in 20–65%, while in the mycelial, 75–82%. This variation in their susceptibility is related to the amount of cell wall β-1,3-glucan, that the caspofungin target, which is more pronounced in the mycelial than in the yeast phase of the fungi. These results are interesting and new studies in the use of this drug in the treatment of PCM should be made, especially in studies using combinations of caspofungin with other antifungal drugs to increase their inhibitory capacity.

There are not many reports in the literature about resistant yeasts of *Paracoccidioides* spp. to antifungal therapies. There is a study however, that relates clinical and *in vitro* resistance to ketoconazole and trimethoprim-sulfamethoxazole. In this study, they have found that patients infected with *P. lutzii* had good responses to trimethoprim-sulfamethoxazole, while those infected with *P. brasiliensis* relapsed with the same

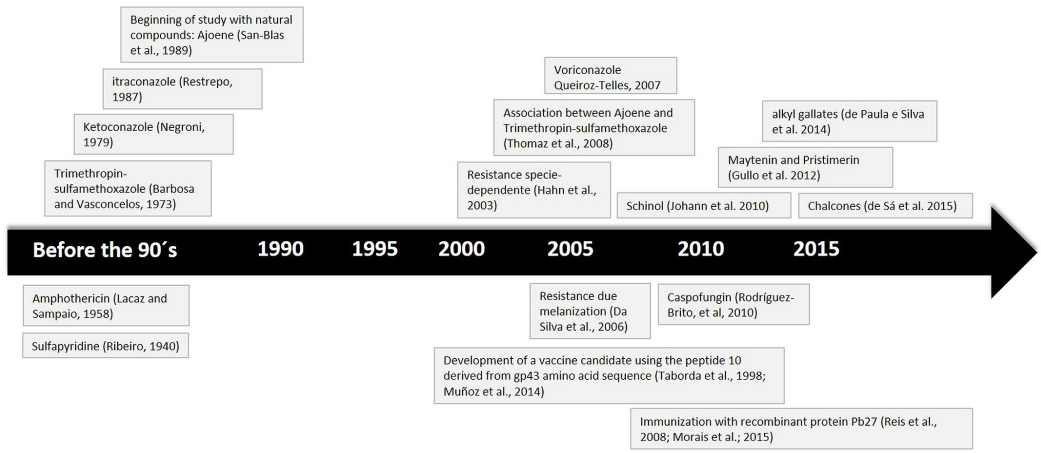


**FIGURE 3 | Time line based on studies that brings development and new insights on treatment and active biomolecules against PCM.**

drug administration (Hahn et al., 2003). In a different way, another study verified that the melanization process decreased susceptibility to antifungal agents, particularly amphotericin B, what can lead to resistance (da Silva et al., 2006).

Due to these facts, new drugs that are safer, more effective, cheaper and with shorter periods of therapy, seem warranted for the treatment of PCM. In this sense, many groups have been developing new alternatives treatment.

One trend is the study of natural and semi-synthetic compounds with great biological activity. In 1989 there began the evaluation of the antifungal activity of Ajoene, a compound derived from ethanolic garlic extracts. These inhibited the growth of *P. brasiliensis* by affecting the integrity of the fungal cytoplasmic membrane (San-Blas et al., 1989). The same authors discussed the possible involvement of ajoene on the sulphydryl metabolism of *P. brasiliensis*, inhibiting the effect on the yeast cells but not on the mycelial cultures (San-Blas et al., 1993). Alterations was observed in phospholipid, fatty acid proportions, phosphatidylcholine, and phosphatidylethanolamine in both phases and reduced saturated fatty acids in the yeast phase, with a corresponding increase in the unsaturated components (San-Blas et al., 1997). Two studies evaluated the antifungal effect of the ajoene in murine models were published; one showing a significant reduction in the levels of antibodies at the 10th week of treatment (Maluf et al., 2008). The other showed a positive additive effect when ajoene therapy was used in association with antifungal drugs (sulfametoxazol/trimethoprim) and a protective proinflammatory immune response (Thomaz et al., 2008).

Martins et al. (2009) showed that *Paracoccidioides* spp. isolates were susceptible to curcumin, a compound produced by the rhizome of *Curcuma long*, and which presented more inhibition effect than the antifungal agent, fluconazole. Johann et al. (2010a) found that the extract from *Schinus terebinthifolius* presented strong antifungal activity against *P. brasiliensis* isolates. Another study from the same authors showed that two compound isolates from the extract of *Schinus terebinthifolius*, schinol, and a new biphenyl compound, had antifungal activity against *P. brasiliensis* isolates. Schinol presented a synergistic effect when combined with Itraconazole (Johann et al., 2010b).

The 6-quinolinyl and quinolinyl N-oxide chalcones, specifically those named 4c and 4e, presented strong activity against *P. brasiliensis.* Histopathological analysis and a progression score of the disease in mice showed that the 4c compound was able to control inflammation and resolved the infection with better results than treatment with Itraconazole and 4e, while avoiding granuloma formation and preservation of lung tissue (de Sá et al., 2015).

Gullo et al. (2012) evaluated natural and semi-synthetics compounds such as maytenin and pristimerin and observed excellent minimum inhibitory concentration against different isolates of *P. brasiliensis*. In the same way, de Paula et al. (2014) evaluated the antifungal activity of the Alkyl gallates, which presented important biological activity reported by the literature, against different fungi species, including different isolates of *P. brasiliensis* and *P. lutzii*. They observed that these molecules presented important relations between the structure and activity, and that the decyl gallate have special activity against *Paracoccidioides* species.

Clinical and experimental data indicate that cell-mediated immunity plays a central role in host defenses against infection by *P. brasiliensis*, whereas high levels of specific antibodies and polyclonal activation of B cells are associated with more severe forms of disease (Cano et al., 1998).

The gp43 contains epitopes capable of producing a cellular immune response in guinea pigs (Rodrigues and Travassos, 1994) and human patients (Saraiva et al., 1996). The sensitivity of the immune response in mice to gp43 occurs by proliferation of CD4 + Th1 (Travassos et al., 1995). These epitopes stimulate CD4 + Th1 lymphocytes, which produce interferon (IFN-$\gamma$), which has the function of stimulating the formation of granulomas that may contain yeasts (Brummer et al., 1988). However, the contribution of each subtype of T cell in the immune response of the host

depends on the genetic patterns and an immunity with a balance of CD4/CD8 regulating the secretion of cytokines of the Th1 and Th2 type, which correlates with resistance of the host to infection by *P. brasiliensis* (Chiarella et al., 2007).

A 43 kDa glycoprotein (gp43) has 416 amino acids, where a specific stretch of 15 amino acids designated as P10, is recognized by T lymphocytes in mice and humans. The protective effect of P10 is related to inducing an immune response of Th1 dependent IFN-γ- dependent on isogenic mice (Taborda et al., 1998).

Isogenic mouse strains immunized with P10, developed lung infection 200 times less intense than the unimmunized animals (Taborda et al., 1998). Iwai et al. (2003) using TEPITOPE software verified the probability of caucasian HLA-DR recognizing different peptides. They verified that P10, a promiscuous peptide, was an important vaccine candidate for use in humans (Iwai et al., 2003). This peptide could be associated with drugs commonly used for the treatment of PCM and presented an additive effect in the experimental model using BALB/c mice. This demonstrates the capacity of peptide P10 to be useful for reducing the treatment time of this mycosis (Marques et al., 2006). Besides this, Magalhães et al. (2012) demonstrated the potential use of primed dendritic cells (DCs) with P10 as a vaccine that can protect the host against the development of PCM or treating a well-established disease.

According to studies developed in the last years, innate immune system and DCs play an important role in the resolution of *Paracoccidoides* spp. and other dimorphic fungal infections (Thind et al., 2015). DCs play a crucial role in the detection of pathogens, trigger an initial response of the host, as well as instruction to the adaptive immune response. It is also known that DCs play an important role in the induction of effector T cells that *P. brasiliensis* infection control and has been shown that *P. brasiliensis* induces regulatory DCs in susceptible mice. This, in turn, promotes IL-10 production and contributes to the infection susceptibility (Ferreira et al., 2007). Recently, Dos Santos et al. (2011) demonstrated that *P. brasiliensis* infection stimulates migration of DC and, bone marrow-derived DC, when stimulated by *P. brasiliensis,* migrate to the lymph nodes and activate a T-cell response. These studies open up new perspectives since the understanding of the regulation of the DC migration allow for the development of tools to efficiently activated a T-cell response aiding in the control of PCM.

The latest research about alternative therapies presented the immunization in murine models with rPb27, a recombinant protein of *P. brasiliensis,* showing its protective effect against the PCM and its important ability to prevent pulmonary fibrosis (Reis et al., 2008; Fernandes et al., 2011a,b; Morais et al., 2015).

The **Figure 3** we present a time line based on studies that bring development and new insights into the treatment and use of active biomolecules against PCM.

The treatment of mycosis is a great challenge for science, and in the PCM, this is not different. The problem is even more challenging because since 2006, no antifungal was approved. Of the drugs available today, many cannot be orally administered, have high toxicity, display cases of resistance, and present drug interactions. Thus, the development of new antifungal therapies has become an increasingly challenging problem mainly because of their growing resistance. This issue leads to a search for new antimicrobial agents that have different mechanisms to effectively combat infections and that do not contribute to the resistance of the pathogens that may complicate any therapy (Krachler and Orth, 2013).

## CONCLUSION REMARKS

The advances in PCM studies bring us a better knowledge of how the interaction with the host was constructed during its evolution enabling the fungi to evade from host human immune system and remain in the organisms causing a mycosis with a high incidence in Latin America. This disease is a great public health issue that, with agricultural expansion, has an increasing occurrence area that may affect many more people in the future. This expansion is an alarming problem since the detection of the disease is difficult depending on the isolate, the patient, and the fact that the treatment of the PCM is difficult given the limited arsenal available against it.

The studies we present in this review are evidence of a great effort in the search for knowledge of the PCM and its etiologic agents, *P. brasiliensis* and *P. lutzii,* in the last years. The details of the *Paracoccidioides*-host interaction, the advances in the use of animal models to study the disease, and the discovery of new treatment methods and anti-*Paracoccidioides* agents, reveal a promising future in combating this disease.

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# Antibodies Against Glycolipids Enhance Antifungal Activity of Macrophages and Reduce Fungal Burden After Infection with *Paracoccidioides brasiliensis*

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Paracoccidioidomycosis is a fungal disease endemic in Latin America. Polyclonal antibodies to acidic glycosphingolipids (GSLs) from *Paracoccidioides brasiliensis* opsonized yeast forms *in vitro* increasing phagocytosis and reduced the fungal burden of infected animals. Antibodies to GSL were active in both prophylactic and therapeutic protocols using a murine intratracheal infection model. Pathological examination of the lungs of animals treated with antibodies to GSL showed well-organized granulomas and minimally damaged parenchyma compared to the untreated control. Murine peritoneal macrophages activated by IFN-γ and incubated with antibodies against acidic GSLs more effectively phagocytosed and killed *P. brasiliensis* yeast cells as well as produced more nitric oxide compared to controls. The present work discloses a novel target of protective antibodies against *P. brasiliensis* adding to other well-studied mediators of the immune response to this fungus.

**Keywords: *Paracoccidioides brasiliensis*, paracoccidioidomycosis, glycosphingolipids, polyclonal antibodies, nitric oxide**

## INTRODUCTION

The agents of Paracoccidioidomycosis (PCM) are a complex group of fungi within the Paracoccidioides genus comprised of four distinct phylogenetic lineages known as PS2, PS3, S1, and Pb01-like (Matute et al., 2006; Teixeira et al., 2009). PCM is a systemic granulomatous disease initiated by the inhalation of Paracoccidioides sp. conidia that subsequently transform into yeast forms in the lungs. Paracoccidioides sp. grows in the yeast form at human physiological temperature and in the mycelial form at 25°C (Franco et al., 1993). PCM is endemic in a broad region from Mexico to Argentina, although ∼80% of diagnosed patients are in Brazil. Most patients are rural workers but cases also occur in urban centers, especially those located along routes used by migrant workers (Restrepo, 1985; McEwen et al., 1995). Among the fungal diseases, PCM is the prevalent systemic mycosis in Latin America, with the highest mortality rate among the systemic mycoses in Brazil (Prado et al., 2009).

Previous studies have identified different glucans related to dimorphism in Paracoccidioides brasiliensis (Kanetsuna and Carbonell, 1970; Kanetsuna et al., 1972), antigenic glycoproteins including the major diagnostic antigen gp43 (Puccia et al., 1986), galactomannans (San-Blas et al., 2005), brassicasterol, phospholipids (Pereira et al., 2010; Longo et al., 2013), and glycolipids (Puccia et al., 2011). Glycosphingolipids (GSLs) are important molecules in fungi, involved in adhesion, cell recognition, cell differentiation, signal transduction, and regulation of cell proliferation (Barreto-Bergter et al., 2004, 2011; Nimrichter et al., 2005; Bertini et al., 2007). GSLs from Saccharomyces cerevisiae and other species are structurally different from those of mammals mainly due to the presence of inositolphosphoceramide (IPC) as the core structure (Dickson and Lester, 2002). Acidic and neutral GSLs have both been identified in yeast and mycelium forms of P. brasiliensis (Toledo et al., 1995).

Bertini et al. (2007) used 31 sera from PCM patients to determine their reactivity to acidic GSLs Pb-1 and Pb-2 by ELISA. Only the Pb-1 antigen, which has a Galf residue, was reactive with the PCM patients' sera. Pb-2, which lacks the Galf residue, was not recognized. The titer of antibodies to Pb-1 increased after the start of antifungal treatment and decreased after 5 months of treatment; thus, the Pb-1 ELISA is a potentially clinically useful test to evaluate patient response to therapy. The authors also investigated the role of GSLs in the differentiation and colony formation of P. brasiliensis, Histoplasma capsulatum, and Sporothrix schenckii using three monoclonal antibodies (mAbs) against fungal GSLs: mAb MEST-1 directed to residues of β-D-galactofuranose linked to mannose, mAb MEST-2 directed to glucosylceramide, and mAb MEST-3 directed to Pb-2. These mAbs exerted a strong inhibitory activity on growth, differentiation and colony formation of these fungi. Experiments, however, using mAb MEST-2, showed no significant inhibition of CFUs or effect in the fungal dimorphism (Toledo et al., 2010). On the other hand, addition of purified human antibodies, directed to GlcCer, inhibited cell budding and growth of Cryptococcus neoformans (Rodrigues et al., 2000).

Therapeutic vaccination with fungal antigens or passive transfer of antibodies can boost the cell immune response and add to the protective effect of chemotherapy, eventually counteracting a relapsing disease and reducing fibrotic sequels. Both the innate immune response and the adaptive immunity are important for the antifungal protective effect (Travassos and Taborda, 2012).

The first evidence of antibody-mediated protection against *P. brasiliensis* was shown with the passive transfer of two murine mAbs against a glycoprotein of 70 kD which is recognized by 96% of sera from PCM patients (de Mattos Grosso et al., 2003). Administration of these mAbs led to a significant reduction in the CFUs and the number and size of granulomas in the lungs of experimentally infected mice. Studies on the effect of mAbs to the major diagnostic antigen gp43 provide additional insights into the role of antibody protection in PCM (Travassos and Taborda, 2012).

Given the potential role of GSLs in the virulence of *P. brasiliensis*, we investigated the effect of anti-acidic GSLs polyclonal antibodies on the infection clearance, using experimental prophylactic and therapeutic models and, we have found that at least to polyclonal antibodies to GSL in both models studied are protective to PCM experimental.

# MATERIALS AND METHODS

## Fungal Strain

Virulent *P. brasiliensis* Pb18 yeast cells were maintained by weekly passages on solid Sabouraud medium (Gibco) at 37°C and were used after 7–10 days of growth. Before experimental infection, the cultures were grown in Sabouraud Broth at 37°C for 5 days (Buissa-Filho et al., 2008). The fungal cells were washed in phosphate-buffered saline (PBS; pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions was assessed by 0.4% Trypan Blue (Sigma) exclusion staining and was always higher than 90% (Taborda et al., 1998).

## Extraction of GSLs

Crude lipid mixtures were extracted from *P. brasiliensis* yeast cells by homogenization using a mixer, three times with 200 mL of 2-propanol/hexane/water (IHW, 55:20:25, v/v/v, upper phase discarded), and twice with 200 mL of chloroform/methanol (CM, 2:1, v/v). The five extracts were pooled, dried on a rotary evaporator, dialyzed against distilled water, lyophilized, suspended in chloroform/methanol/water (30:60:8, v/v/v). Acidic glycolipids from the crude lipid extract were purified by ion exchange chromatography on DEAE-Sephadex A-25 (GE-Healthcare). The elution of the samples was performed following protocols I and II. In protocol I, GSLs were eluted from DEAE-Sephadex A-25 with five volumes of the following solvents (Carlo Erba): (a) $CHCl_3$:$CH_3OH$:$H_2O$ (30:60:8, v/v/v); (b) $CH_3OH$; (c) Sodium acetate 0.2% in methanol; (d) sodium acetate 0.6% in methanol. Fractions corresponding to the neutral glycolipids were eluted in the first solvent and the acidic fraction was eluted with the third solvent. In protocol II, the fraction of acidic glycolipids was purified by column chromatography on Silica Gel 60 (Merck) using five solvents: (a) $CHCl_3$:$CH_3OH$ (8:2, v/v); (b) $CHCl_3$:$CH_3OH$ (6:4, v/v); (c) $CHCl_3$:$CH_3OH$ (4:6, v/v); (d) $CHCl_3$:$CH_3OH$ (2:8, v/v), and (e) $CH_3CHOHCH_3$:$C_6H_{14}$:$H_2O$ (55:20:25, v/v/v). The purity was checked by high resolution thin layer chromatography (HPTLC; Merck) developed in the solvent $CHCl_3$:$CH_3OH$:$CaCl_2$ 0.02% at 60:40:9 (v/v/v). HPTLC plates were sprayed with 90% acetone in primuline (Sigma) and visualized under ultraviolet light. Compounds were revealed with 0.5% orcinol (Sigma), in 3 M sulfuric acid ($H_2SO_4$; Straus et al., 1993).

## Animal Use and Ethics Statement

BALB/c, 6 to 8-week-old male, mice were bred at the University of São Paulo animal facility under specific pathogen-free conditions. All animals were handled in accordance with good animal practice as defined by the rules of the national animal welfare bodies. The Animal Care and Use Committee of the University of São Paulo approved all *in vivo* testing.

## Polyclonal Antibodies to GSL

Polyclonal antibodies were raised in BALB/c mice by four immunizations with 50 μg of purified GSLs in Incomplete Freund Adjuvant, intraperitoneally. The animals were bled 24 h before the immunizations, to collect the pre-immune serum. ELISA was used to analyze the immune sera. Polyclonal antibodies obtained from the animals were purified by affinity chromatography using a protein-A column, according to the manufacturer's direction (Thermo Scientific, Netherlands). Protein-A tightly binds IgG2a, IgG2b, and IgG3, while it binds weakly to IgG1 and does not bind IgM. The polyclonal antibodies were dialyzed and concentrated by AMICON system with total concentration being determined by Nanodrop 1000. ELISA was also used to titer anti-GSL antibodies. The control polyclonal serum was generated in the same manner, except that bovine serum albumin (BSA-Sigma) was used as the immunogen.

## Intratracheal Infection of BALB/c Mice

BALB/c mice were inoculated intratracheally (i.t.) with virulent *P. brasiliensis* Pb18. Mice were anesthetized intraperitoneally (i.p.) with 200 μl of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (União Química Farmacêutica, Brazil). For inoculation, the mouse's neck was hyperextended, the trachea was exposed at the level of the thyroid, and $3 \times 10^5$ yeast cells in PBS were injected i.t. using a 26-gage needle. Incisions were sutured with 5-0 silk.

## Protective Effects of Polyclonal Antibodies to Acidic GSL

We studied two protocols: prophylactic and therapeutic. In the prophylactic protocol, mice were treated with 1 mg antibodies to GSL i.p. and then infected 24 h later with *P. brasiliensis*. These mice were euthanized 15 and 30 days after infection. For therapeutic protocols, animals were first infected i.t. and then immunized i.p. 30 days later with 1 mg of polyclonal antibodies to GSL. These mice were euthanized at either 45 or 60 days after intratracheal infection. In both therapeutic and prophylactic protocols, experimental groups included mice that received 1 mg of antibodies to BSA as a control for the polyclonal antibodies.

## Fungal Burden in Organs of Infected Mice

Fungal burdens were determined by CFUs counting. Sections of lungs were removed, weighed, and homogenized in 1ml of PBS. Samples (100 μl) were plated on solid brain heart infusion (BHI) medium supplemented with 4% fetal calf serum (Vitrocell, Brazil), 5% *P. brasiliensis* (strain 192)-spent culture medium, and 100 IU/ml streptomycin-penicillin (Sigma–Aldrich, USA). Petri dishes were incubated at 37°C and colonies were counted after 7 days.

## Histopathology

Sections of murine lungs were fixed in 10% buffered formalin and embedded in paraffin for sectioning. The sections were stained by the Gomori-Grocott method for fungal cells detection and were examined microscopically.

## Cytokine Detection

Sections of excised lungs were homogenized in 2 ml of PBS in the presence of protease inhibitors: benzamidine HCl (4 mM), EDTA disodium salt (1 mM), N-ethylmaleimide (1 mM), and pepstatin (1.5 mM) (Sigma). The supernatants were assayed for IL-4, IL-10, IL-12, TNF-α, and IFN-γ using enzyme-linked immunosorbent assay (ELISA) kits (BD OpTeia, San Diego, CA, USA). The detection limits of the assays were as follows: 7.8 pg/ml for IL-4; 31.25 pg/ml for IL-10, 62.5 pg/ml for IL-12, 15.6 pg/ml for TNF-α, and 7.8 pg/ml for IFN-γ, as previously determined by the manufacturer.

## Phagocytosis Assay

*In vitro* phagocytosis experiments were carried out with the J774.16 macrophage-like cell line. *In vitro* phagocytosis was performed according to our established protocol (Taborda and Casadevall, 2001, 2002) with minor modification. Cells were plated in 96-well tissue culture plates (TPP, Switzerland) at a density of $10^5$ cells per well, stimulated with 50 U/ml recombinant murine IFN-γ (BdBiosciences, USA) and incubated at 37°C overnight. The medium in each well was then replaced with the following additions: (1) acidic glycolipids purified, at 100 and 200 μg/ml; (2) antibodies from mouse serum immunized with GSL, purified by Protein A; (3) polyclonal antibodies to GSL purified by Protein A, at 100 μg/ml, with the concentration determined by ELISA; (4) polyclonal antibodies to BSA, at 100 μg/ml. *P. brasiliensis* cells were added at a ratio of 5:1 macrophages/yeast cells and then incubated at 37°C for 6, 12, and 24 h. The wells were then washed several times with sterile PBS, fixed with cold absolute methanol, and stained with a 1/20 solution of Giemsa (Sigma–Aldrich, USA). Phagocytosed yeasts were counted by light microscopy at 400× magnification. The phagocytic index (PI) is defined as $PI = P \times F$, where $P$ is the percentage of macrophages with internalized yeasts and $F$ is the average number of yeast cells per macrophage. Experiments were carried out in triplicate and five to eight different fields were counted.

## Antifungal Activity of Macrophages

After incubation of the macrophages, the viability of the *P. brasiliensis* yeast cells was determined by plating supernatants on BHI agar supplemented with 4% FCS and 5% spent culture medium of *P. brasiliensis* strain192. CFUs were counted after 7 days of incubation at 37°C.

## Production of Nitric Oxide

The levels of nitric oxide metabolite (nitrite) in the culture supernatant of macrophages challenged with opsonized yeasts were determined by Griess reaction (Pick and Keisari, 1981). All determinations were performed in triplicate.

## Statistical Analysis

Results were analyzed using GraphPad 5.0 software (GraphPad Inc., San Diego, CA, USA). Statistical comparisons were made

by analysis of variance (one-way ANOVA) followed by Turkey–Kramer posttest and student's *t*-test. All values were reported as means ± standard errors of the mean (SEM). *P*-values of $<0.05$ indicated statistical significance.

# RESULTS

## Extraction of GSLs

To purify the GSLs, the crude lipid extract was subjected to ion exchange chromatography on DEAE-Sephadex A-25 (protocol I) and A-25 and sequentially in Silica gel 60 (protocol II). The purity was checked by high-resolution chromatography (HPTLC); representative gels of extracted GSLs by protocol I and II are shown in **Figure 1**. For comparison of chromatographic mobility, in the protocol I we used a neutral glycolipid (CMH) standard whereas protocol II used CMH as well as acidic GSL standards.

## Phagocytosis Assay

The capacity of J774.16 macrophage-like cells for the phagocytosis of *P. brasiliensis* yeast was assessed using different conditions in 6, 12, and 24 h co-cultures with macrophages activated by IFN-γ 24 h before the assays. At all time intervals assessed, incubation in the presence of total serum with polyclonal antibodies against acidic GSL (100 μg/ml) along with the other antibodies present in total serum, significantly enhanced yeast cell uptake by J774.16 ($p = 0.009$), when compared to control groups, cells that received polyclonal antibodies to BSA at 100 μg/ml or only the acidic glycolipids at 100 and 200 μg/ml (**Figure 2A**). It is noteworthy that the total serum contains various kinds of antibodies, not only against GSL, and that these antibodies might induce macrophage-like phagocytosis of *P. brasiliensis* yeasts. However, as can be seen in **Figure 2A**, only the purified GSL antibodies, at all studied times, significantly increased the phagocytosis index, suggesting that only the polyclonal antibody against GSL has the ability to induce phagocytosis in this cell line. The specificity of the polyclonal antibodies was tested by adding glycolipids during phagocytosis, which resulted in a partial inhibition of the process (data not shown).

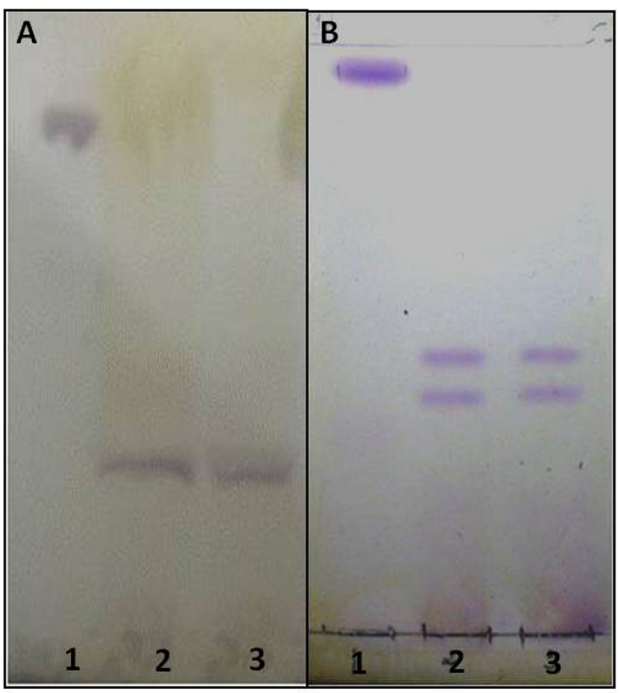


**FIGURE 1 | High resolution thin layer chromatography (HPTLC) of acidic GSLs purified from the crude lipid extract of *Paracoccidioides brasiliensis*.** The solvents used were $CHCl_3$:$CH_3OH$:$CaCl_2$ 0.02% in the proportions 60:40:9 (v/v/v). Revelation with 0.5% orcinol in 3 M sulfuric acid ($H_2SO_4$). **(A)** - (1) Standard CMH, (2) purified acidic GSL (3) purified acidic GSL. **(B)** - (1) Standard CMH, (2) purified acidic GSL Standard Acidic, (3) purified acidic GSL **(B)**. The glycolipids purple stained.

## Antifungal Activity of Macrophages

The fungicidal effects of J774.16 cells on *P. brasiliensis* was determined in co-cultures using different experimental media. The macrophage-like cells were lysed and the lysates plated onto agar; CFUs were counted after 7 days of incubation at 37°C. It may be observed in **Figure 2B** that the addition of polyclonal antibodies and total serum reduced the viability of the yeast *P. brasiliensis* internalized by the macrophage-like cells, as compared to the control groups. Therefore the polyclonal antibodies to GSL effectively killed *P. brasiliensis*.

## Production of Nitric Oxide

Nitrite levels were detected in culture supernatants of macrophage-yeast co-cultures using a Griess assay. The levels of nitric oxide are intrinsically related to the phagocytosis index. As shown in **Figure 2C** increased nitric oxide was produced in macrophage-like cells incubated with total immune serum and with purified anti-acidic GSL polyclonal antibodies at all incubation times (6, 12, and 24 h), as compared to the control groups. These data are compatible with the results obtained in the phagocytosis and killing assays.

## Fungal Burden in Organs of Infected Mice

Both treatment approaches reduced the fungal burden in animal groups that received polyclonal antibodies against acidic GSL when compared to control groups receiving polyclonal antibodies against BSA. The prophylactic group, immunized 24 h before infection and examined 15 and 30 days afterwards, showed a significant reduction in the fungal burden evaluated by CFUs, $p = 0.0001$ (15 days) and $p = 0.0129$ (30 days), as shown in **Figure 3A**. Moreover the histopathological analyses showed only a few yeasts in the lungs (**Figure 3B**. Slide B2).

In the therapeutic groups, examined 45 and 60 days after infection with *P. brasiliensis* and with established disease in the animals, CFUs decreased in the lungs, $p = 0.0001$ (45 days) and

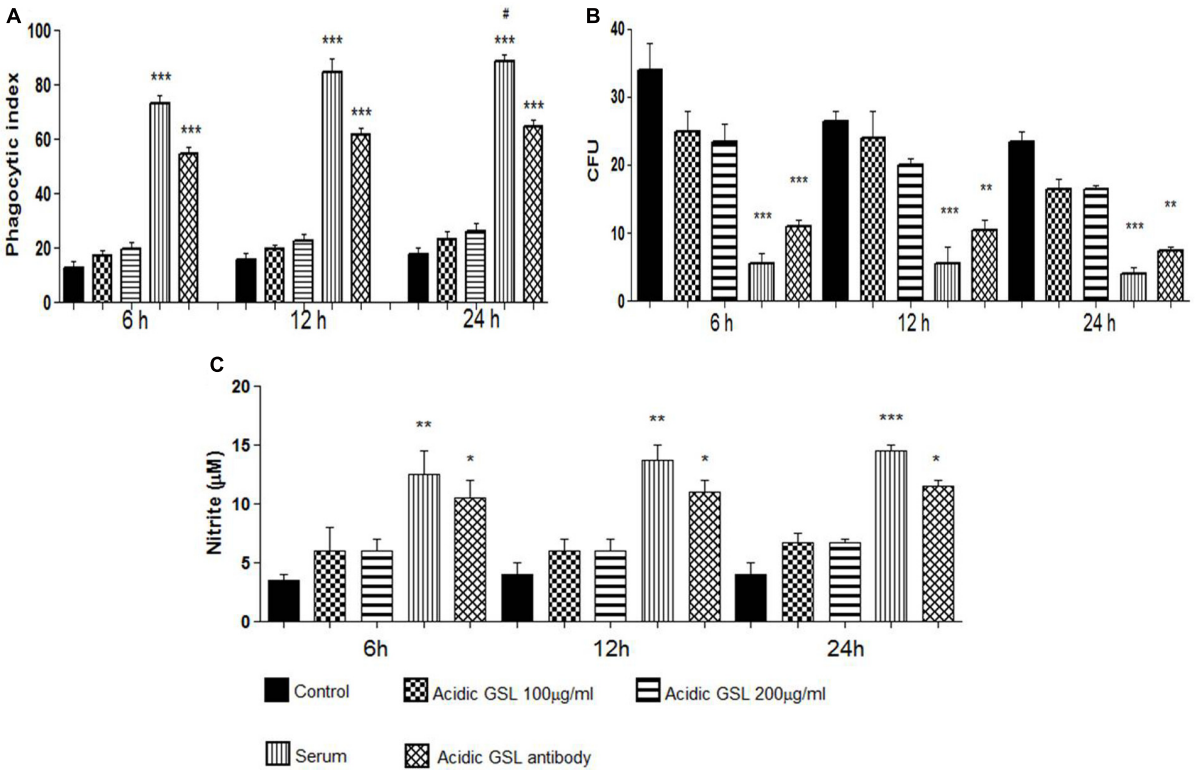


**FIGURE 2 | Experiments *in vitro:* (A) Phagocytosis of yeast cells by J774.16 cells in the presence of polyclonal antibodies to acidic GSL, polyclonal antibodies to BSA (control), total serum with different polyclonal antibodies and purified acidic GSLs, 100 and 200 μg.** Each bar represents the average of three measurements. Experiments were done in triplicate, and different fields were counted. Significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$). **(B)** Killing assay using peritoneal macrophages in the phagocytosis assay. Polyclonal antibodies against BSA (control), purified acidic GSL, total serum with different polyclonal antibodies, polyclonal anti-acidic GSL antibody. The columns represent the mean average of three readings. Significant values compared to controls, ***$p < 0.0001$, **$p < 0.01$. **(C)** Nitric oxide formed in J774.16 cells cultured with *P. brasiliensis* yeasts. Polyclonal antibodies to acidic GSL, polyclonal antibodies to BSA (control), total serum with different polyclonal antibodies, purified acidic GSLs. Nitrite was determined by Griess reagent. Significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$). $^{\#}p < 0.05$ (student's *t*-test).

$p = 0.006$ (60 days) according to **Figure 4A**. No yeasts were detected in the liver and spleen (data not shown).

## Histopathological Analyses

At day 15 after infection, the lungs of the control animals pre-treated with polyclonal antibodies to BSA showed intense infiltrations of inflammatory cells with numerous foci of proliferating fungal cells (**Figure 3B**. Slide A1). In contrast, the mice treated with polyclonal antibodies to acidic GSLs displayed significantly less lung tissue infiltration, few intact yeast cells, and large areas with preserved architecture (**Figure 3B**. Slide A2). After 30 days, the lungs of animals treated with polyclonal antibody to BSA showed compact and loose granulomas with many yeast in the lung tissue (**Figure 3B**. Slide B1). Although it is possible to observe yeasts inside lung granulomas, in animals of the prophylactic group after 30 days, the CFUs did not account for many yeasts, thereby suggesting that they were not viable (**Figure 3B**. Slide B2). The therapeutic protocol also resulted in enhanced protection against tissue damage induced by *P. brasiliensis*. Beneficial results were observed 45 days after infection in the treated group (**Figure 4B**. Slide A2) when compared to the control mice (BSA; **Figure 4B**. Slide A1). As shown in **Figure 4B**. Slides B1 and B2, 60 days after infection, in both protocols, there was reduced fungal burden in the lung tissue. Lung architecture remained largely preserved, well-organized granulomas and a little damage to the lung tissue of mice treated with anti-GSL polyclonal antibodies. The group that received polyclonal antibodies to BSA, however, showed loose granulomas and damage to the lung tissue.

## Cytokine Detection

Cytokine levels were measured in the lung tissues of i.t. infected mice treated with polyclonal antibodies against either BSA or acidic GSLs. As shown in **Figure 5** (prophylactic group) and **Figure 6** (therapeutic group), in the former, animals immunized with polyclonal antibodies against acidic GSL had higher levels of IFN-γ after 15 days ($p = 0.0328$) of infection compared with mice receiving polyclonal antibodies against BSA. There were no significant differences in the levels of IL-4 and TNF-α. However, IL-10 was statistically different between the groups ($p = 0.0027$).

In the therapeutic protocol, animals immunized with polyclonal antibodies against acidic GSL, showed significantly

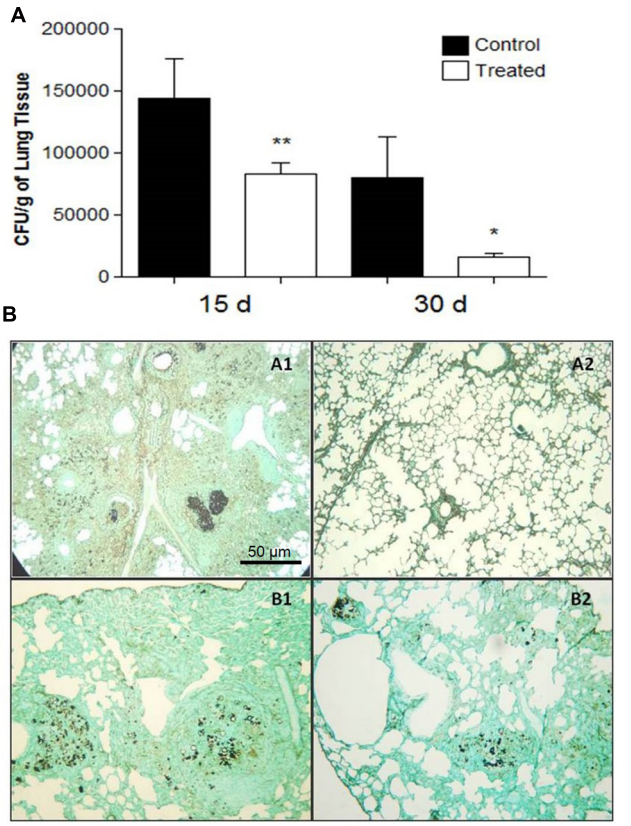


**FIGURE 3 | (A)** Colony forming units (CFU) in the lungs from BALB/c mice that received 1 mg of polyclonal antibodies against acidic glycolipids and 1 mg anti-BSA polyclonal antibodies (control) 24 h before infection with Pb18 and sacrificed after 15 and 30 days (prophylactic protocol). Significant values comparing the lungs of treated and control groups. $*p < 0.05$, $**p < 0.01$. **(B)** Representative lung sections: Prophylactic protocol. 24 h prior to infection, mice received polyclonal antibodies to acidic GSLs. Histopathological sections of murine lungs 15 (A1, A2) and 30 (B1, B2) days after i.t. infection. (A1, B1) Lungs from control mice; (A2, B2) Histopathological sections after i.t. infection. Photographs of sections were taken at 100× magnification.

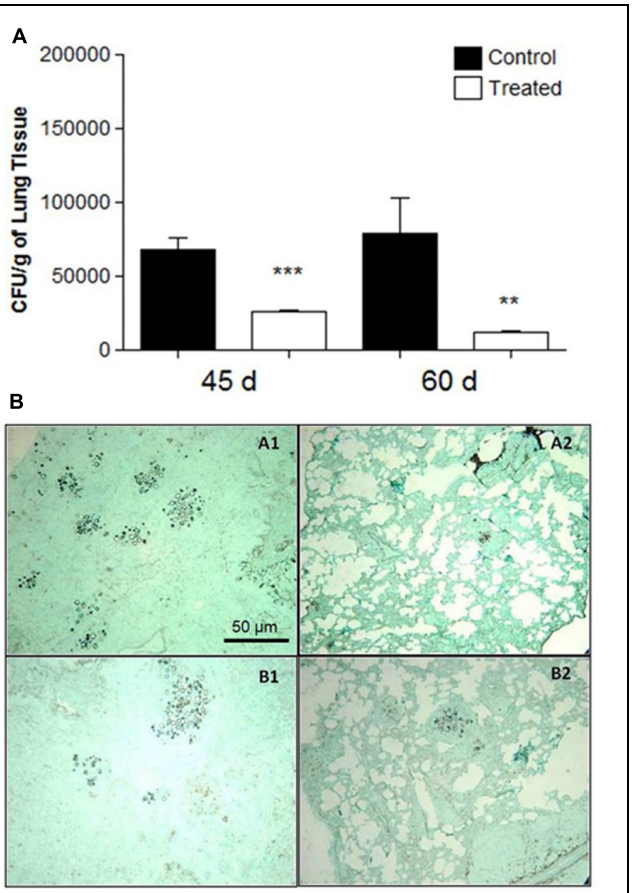


**FIGURE 4 | (A)** Colony forming units (CFU) in the lungs from BALB/c mice that received 1 mg of polyclonal antibodies against acidic glycolipids and 1 mg anti- BSA polyclonal antibodies (control) 30 days after infection with Pb18 and sacrificed after 45 and 60 days (therapeutic protocol). Significant values comparing the lungs of treated and control groups. $**p < 0.01$, $***p < 0.0001$. **(B)** Representative lung sections: therapeutic protocol. 3 days after i.t. infection, mice received polyclonal antibodies to acidic GSLs. Controls received polyclonal antibodies to BSA. Representative histopathological sections 45 days after i.t. infection: (A1) Lung from control mouse; (A2) Lung from treated mouse. After 60 days of i.t. infection: (B1) Control; (B2) Treated. Photographs of sections were taken at 100× magnification.

higher levels of IFN-γ ($p = 0.0083$) in animals sacrificed after 60 days of infection with *P. brasiliensis* and of IL-12 ($p = 0.0085$) after 45 and 60 days of infection.

## DISCUSSION

Studies on antibody immunity against fungal pathogens have revealed that experiments with polyclonal sera may not conclusively validate or disprove the capacity of antibody to effectively modify disease pathogenesis. This quandary arises due to the fact that polyclonal serum may consist of a mixture of protective, irrelevant, or even detrimental antibodies, and the relative quantity of each can dictate whether or not a protective effect is measured (Casadevall, 1995). The polyclonal antibodies also differ in isotype quantity and specificity, which further complicates the assessment of efficacy. Batista et al. (2014) showed that GSLs from *P. brasiliensis* affected the function of human monocytes and dendritic cells, interfering with antigen presentation. Toledo et al. (2010) demonstrated that mAbs against the *P. brasiliensis* glycolipid antigen, had a strong inhibitory activity *in vitro* on differentiation and colony formation of *P. brasiliensis*, *H. capsulatum,* and *S. schenckii*. Regardless, on the present work, the polyclonal antibodies that we generated to acidic GSLs purified from *P. brasiliensis* demonstrated protective responses in our *in vitro* and *in vivo* infection models. Most significantly, the polyclonal antibodies to acidic GSLs were protective using both the therapeutic and prophylactic protocols in our murine model of intratracheal *P. brasiliensis* infection. The fungal burden was reduced in the lungs of all groups studied, 15, 30, 45, and 60 days after infection. The lung sections from the anti-GSL antibody treated groups

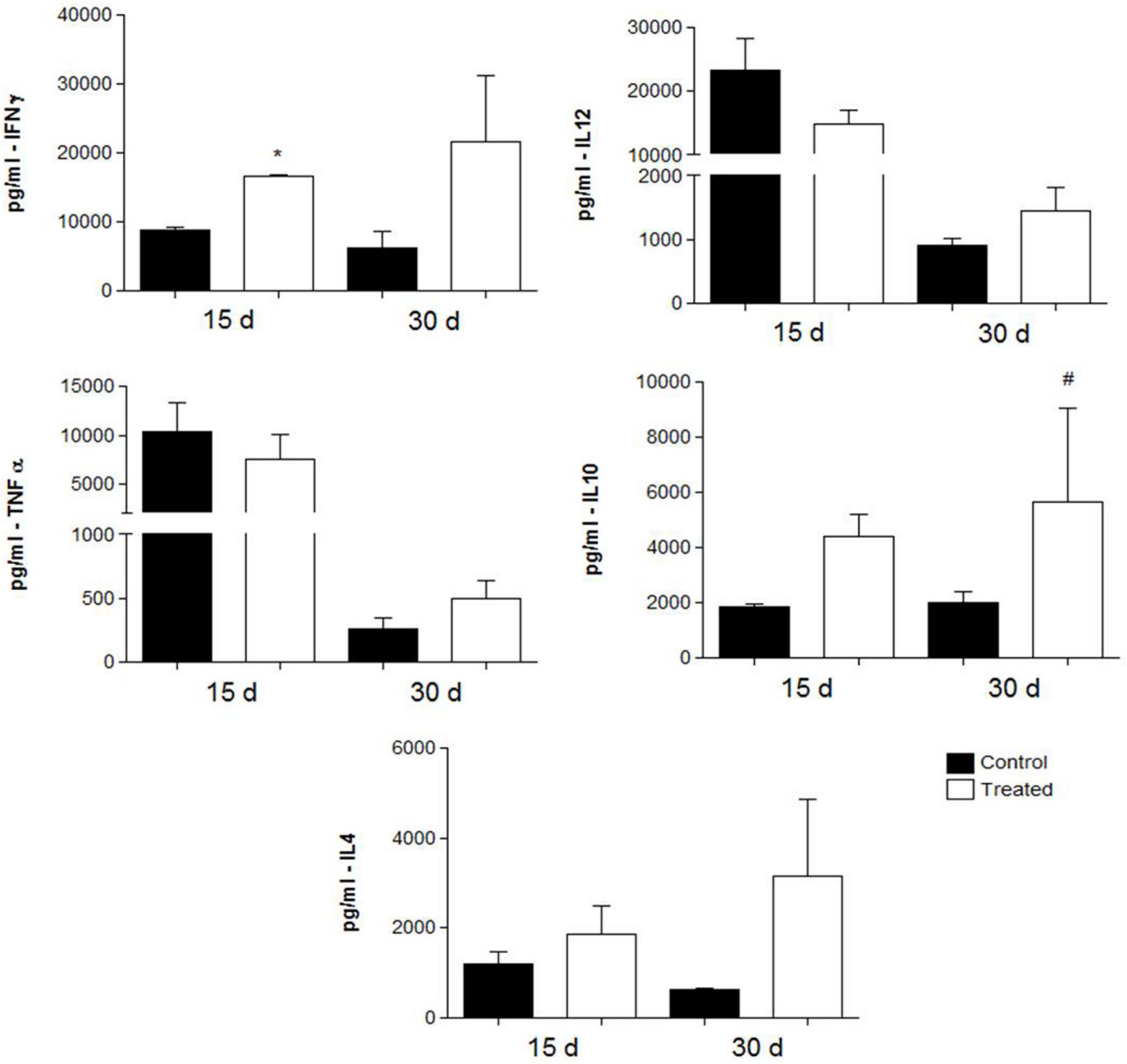


**FIGURE 5 | Cytokine levels in the lungs obtained from BALB/c mice that received either 1 mg of polyclonal antibodies against acidic glycolipids or 1 mg anti-BSA polyclonal antibodies (control) 24 h before infection with Pb18 and sacrificed 15 or 30 days post-infection (prophylactic protocol).** $^{*}p < 0.05$, $^{\#}p < 0.05$ (student's *t*-test).

exhibited well-organized granulomas and less histopathological damage.

In human PCM, high antibody titers against the major antigens expressed by the yeast *P. brasiliensis* correlate with active disease and the decline in antibody levels is consistent with a response to antifungal therapy and clinical improvement (Restrepo et al., 2008). Patients with clinical forms of the disease usually receive long-term treatment, to allow control of the clinical manifestations of mycosis and to avoid relapses (Del Negro et al., 2000; Shikanai-Yasuda et al., 2006).

The effects of six different IgG2a and IgG2b were evaluated in an i.t. infection of *P. brasiliensis* and the mAbs 19G, 10D (IgG2a), and 3E (IgG2b) significantly reduced lung CFUs. In a phagocytosis assay using peritoneal and alveolar macrophages most anti-gp43 mAbs increased significantly the phagocytosis index, with mAb 3E demonstrating the most impressive effect. The reactivity of mAb 3E was directed to an epitope within the sequence NHVRIPIGYWAV of gp43 shared with β–1,3-glucanases of a few other fungal species (Travassos et al., 2007; Buissa-Filho et al., 2008).

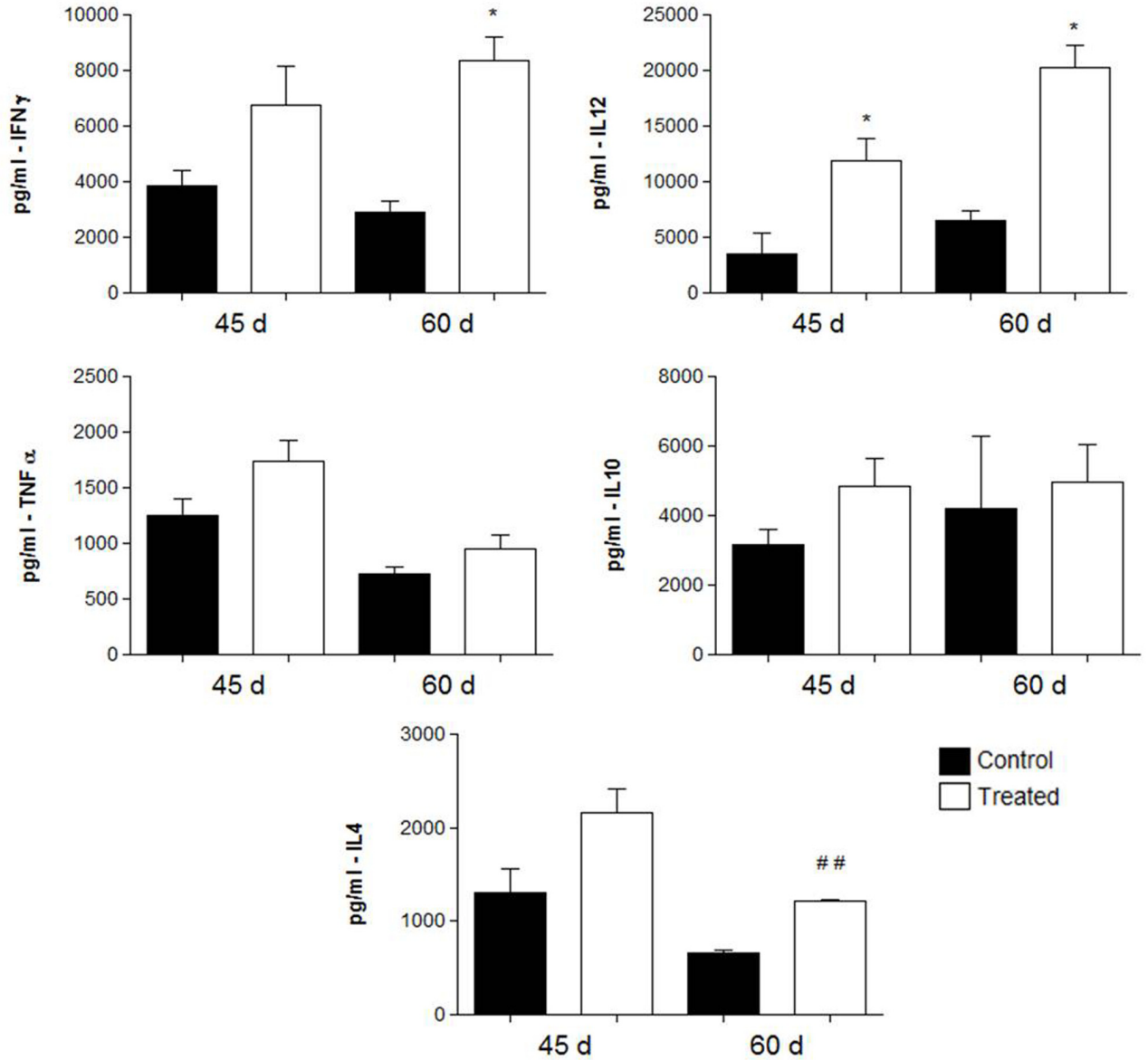


**FIGURE 6 | Cytokine levels in the lungs obtained from BALB/c mice that received either 1 mg of polyclonal antibodies against acidic glycolipids or 1 mg anti- BSA polyclonal antibodies (control) 30 days after infection with Pb18 and sacrificed 45 and 60 days post-infection (therapeutic protocol).** $^{*}p < 0.05$, $^{\#\#}p < 0.005$ (student's *t*-test).

Thomaz et al. (2014) showed that mAbs against *H. capsulatum* hsp60, (4E12 and 7B6, of different isotypes), were biologically active against *P. lutzii*. Both isotypes enhanced *P. lutzii* phagocytosis *in vitro*. Passive administration of the mAbs prior to intratracheal infection of *P. lutzii* in mice significantly reduced the fungal burden in pulmonary tissue.

On testing peritoneal macrophages activated by IFN-γ and incubated with polyclonal antibodies to acidic GSLs, an enhanced fungicidal activity of *P. brasiliensis* yeast forms was observed. Killing internalized yeasts occurred simultaneous with nitric oxide production. The involvement of NO in *P. brasiliensis* killing is inhibited by IL-10 (Moreira et al., 2010). The effectiveness of anti-acidic GSL antibodies *in vivo* reflected the three–fourfold ratio of IFN-γ to IL-10 in the immunized mice. The effect the nitric oxide in experimental cryptococcosis was also related to the modulation of cytokine expression, underscoring the interdependency of cellular and humoral defense mechanisms (Rivera et al., 2002).

The cytokine profiles in both protocols (prophylactic and therapeutic) showed a mixed activation of Th1 and Th2 cytokines, while similar results were observed previously by Buissa-Filho et al. (2008), using a protective mAb against gp43 of *P. brasiliensis*. In our prophylactic protocol, we observed a trend toward increased levels of IFN-γ and IL-10, whereas

in the therapeutic protocol IFN-γ IL-4 and IL-12 were more prominent. The role of IFN-γ mediating activated macrophages in PCM was previously documented, in which murine peritoneal macrophages and immortalized cells activated by IFN-γ displayed enhanced fungicidal activity (reviewed by Buissa-Filho et al., 2008 and Taborda et al., 2015). In our present results, polyclonal antibodies against acidic glycolipids enhance IFN-γ in both protocols; however, the best results were observed in the therapeutic protocols. As to the clinical relevance, therapeutic protocols would indeed be an ideal approach for the administration of antibodies for combating PCM.

In the present work, polyclonal antibodies directed to fungal acidic GSLs were shown to exert immune protection against *P. brasiliensis* in an intratracheal infection model.

## AUTHOR CONTRIBUTIONS

RB and LT – these two authors contributed equally to this work - laboratory experiment. JM, CS, and MP - laboratory experiment - glycolipids purification and analysis. JN and LT - senior researcher - analysis and discussion of results. CT - mentor.

## ACKNOWLEDGMENTS

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ORIGINAL RESEARCH
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# An Intracellular Arrangement of *Histoplasma capsulatum* Yeast-Aggregates Generates Nuclear Damage to the Cultured Murine Alveolar Macrophages

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*Histoplasma capsulatum* is responsible for a human systemic mycosis that primarily affects lung tissue. Macrophages are the major effector cells in humans that respond to the fungus, and the development of respiratory disease depends on the ability of *Histoplasma* yeast cells to survive and replicate within alveolar macrophages. Therefore, the interaction between macrophages and *H. capsulatum* is a decisive step in the yeast dissemination into host tissues. Although the role played by components of cell-mediated immunity in the host's defense system and the mechanisms used by the pathogen to evade the host immune response are well understood, knowledge regarding the effects induced by *H. capsulatum* in host cells at the nuclear level is limited. According to the present findings, *H. capsulatum* yeast cells display a unique architectural arrangement during the intracellular infection of cultured murine alveolar macrophages, characterized as a formation of aggregates that seem to surround the host cell nucleus, resembling a "crown." This extranuclear organization of yeast-aggregates generates damage on the nucleus of the host cell, producing DNA fragmentation and inducing apoptosis, even though the yeast cells are not located inside the nucleus and do not trigger changes in nuclear proteins. The current study highlights a singular intracellular arrangement of *H. capsulatum* yeast near to the nucleus of infected murine alveolar macrophages that may contribute to the yeast's persistence under intracellular conditions, since this fungal pathogen may display different strategies to prevent elimination by the host's phagocytic mechanisms.

**Keywords: *Histoplasma capsulatum*, host-pathogen interactions, intracellular arrangement, nucleus, alveolar macrophages**

## INTRODUCTION

Many studies have been performed to elucidate the interaction between the dimorphic fungus *Histoplasma capsulatum* and host macrophages, specifically to determine the role played by the components of the host's cell-mediated immunity and the evasion mechanisms used by the pathogen. In some conditions, in contrast to their usual function of eliminating deleterious microorganisms, macrophages give rise to a favorable environment for the survival and reproduction of the *H. capsulatum* yeast phase, which is the parasitic-virulent morphotype of this fungus (Medeiros et al., 2002; Tagliari et al., 2012). *H. capsulatum* has been described as a facultative intracellular pathogen, and it is almost exclusively found within host-parasitized cells (Wu-Hsieh et al., 1998; Hilty et al., 2008). Once the pathogen has been phagocytosed, several immunological factors can modulate the course of the infection (Allen and Deepe, 2005).

According to Newman et al. (2011), the destruction of alveolar macrophages and their subsequent ingestion by other immune cells are events that promote the propagation of the infection to different organs during the acute stage of primary histoplasmosis. Thus, it is clear that the interaction between the macrophage and *H. capsulatum* is a decisive step in the occurrence of yeast dissemination into host tissues.

Apoptosis of phagocytes in the initial stage of infection by *H. capsulatum* activates CD4+ and CD8+ T cells, both of which partially act as a defense mechanism for the host. Hsieh et al. (2011), described that apoptosis induced by the infection is an important immune function recognized by the antimicrobial host response mainly in the defense against phagosome-enclosed pathogens. Hence, inhibition of apoptosis modulates the inflammatory response and also interferes in the outcome of the infection process. According to Allen and Deepe (2005), IL-4 and IL-10 production are enhanced when apoptosis is inhibited, with the release of these cytokines exacerbating the fungal infection. This result occurs because the apoptosis of macrophages, which is induced early in a pulmonary infection by *H. capsulatum*, releases IL-10, which inhibits apoptosis of neighboring macrophages, enabling and delimiting the intracellular residence of *H. capsulatum* yeast (Deepe and Buesing, 2012).

Nuclear fragmentation is a morphological cellular alteration associated with apoptosis (Deepe and Buesing, 2012); thus, nuclear damage in host cells can be characterized as a cellular effect that contributes to the pathogenesis of histoplasmosis. Glukhov et al. (2008) reported that bacterial endotoxins induce nuclear DNA damage in human mononuclear cells, which is associated with the infectious process and disease manifestation. However, knowledge of the DNA fragmentation induced by microorganisms is limited. Hence, it is necessary to investigate the behavior of nuclear envelope proteins during infection.

Nuclear envelope proteins promote a functional link between support structures, cytoplasmic compartments, and nucleoplasmic compartments. These proteins have been identified as components of the LINC complex (nucleoskeleton and cytoskeleton linker), which are specific to the outer and inner nuclear membranes. The LINC complex is composed primarily of SUN nuclear proteins and by Nesprin, although other envelope proteins, such as Emerin, can also be identified. An illustration of the LINC complex organization can be found in Haque et al. (2010). These proteins play important roles in the positioning, migration and maintenance of nuclear architecture (Ostlund et al., 2009; Taranum et al., 2012). In addition, this complex is critically important because the blade and associated proteins play a role in modulating gene expression (Martins et al., 2012).

In general, Nesprin binds to actin and several other motor proteins of the microtubule network. In the inner nuclear membrane, SUN-domain proteins (SUN1, SUN2, and SUN3) bind to the blade in the nucleoplasm. Moreover, the Emerin protein characterized as a transmembrane protein may be associated with microtubules in the outer nuclear membrane, and can bind to the nuclear lamina when it is located in the inner nuclear membrane. Thus, the LINC complex passes through the perinuclear space and connects the components of the cytoskeleton with the nuclear lamina (Crisp et al., 2006; Martins et al., 2012). The labeling of nuclear envelope proteins in host cells could contribute to the characterization of the behavior of these proteins during the course of an *in vitro* infection.

The mechanisms by which *H. capsulatum* interacts with macrophages and evades host immune defenses have been well documented. However, this is the first report that attempts to characterize the interaction pattern and the nuclear damage of parasitized host cells after the internalization of *H. capsulatum* yeast in order to verify the correlation of these yeast cells with host cell integrity. Apoptosis assays were performed as well as the staining of nuclear envelope proteins in host cells infected with the fungus. Our study highlights the intracellular behavior and the effects induced by *H. capsulatum* at a nuclear level in cultured infected alveolar macrophages.

## MATERIALS AND METHODS

### Fungal Growth Conditions

*H. capsulatum* strains EH-315 and 60I were used. The EH-315 strain was isolated from a naturally infected bat and was deposited in the *H. capsulatum* Culture Collection of the Fungal Immunology Laboratory of the Department of Microbiology and Parasitology, from the School of Medicine, National Autonomous University of Mexico (UNAM) (www.histoplas-mex.unam.mx). This collection is registered in the database of the World Federation for Culture Collections under the number LIH-UNAM WDCM817 (www.wfcc.info/ccinfo/index.php/collection/by_id/817/). The 60I strain was isolated from a human clinical case and was deposited in the collection of the Clinical Mycology Laboratory of the Faculty of Pharmaceutical Sciences, UNESP, Brazil. Yeasts were grown in brain–heart infusion (BHI-broth) (Difco Laboratories, Detroit, MI, USA) and supplemented with 0.1% L-cysteine and 1% glucose, at 37°C, for 24 h, and with rotary agitation (100 rpm). Dispersed *H. capsulatum*

yeast cells were washed three times with phosphate-buffered saline (PBS), followed by low-speed centrifugation for 1 min at 600 × *g* to remove large yeast clumps. Suspensions of single yeast cells were separated for counting with a hematocytometer.

## Macrophage Cultures

Murine alveolar macrophages, AMJ2-C11 cell-line, were cultured overnight at 37°C on coverslips placed in the well-bottom of 24-well plates (TPP®, Trasadingen, Switzerland) using Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Cultilab, Campinas, SP, Brazil).

## Ethics Statement

Rabbits were used for antibody production. They were processed exactly as outlined in the experimental protocol recommended by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences of Araraquara—UNESP (reference number: 10/2011/CEUA/FCF), which was approved for this study. All efforts were made to minimize suffering in all animal procedures.

## Immunoglobulin to Cell-Free Antigen of *H. capsulatum*

*H. capsulatum* cell-free antigen, a rich solution of cell wall associated antigens, was prepared as described previously by Sá-Nunes et al. (2005). Protein concentration was quantified using the Bradford method (BioRad Laboratories Inc., Hercules, CA, USA). To prepare a polyclonal antibody raised against cell-free antigen of *H. capsulatum,* rabbits were inoculated by intradermal injection of 1.0 mL of the cell-free antigen mixed with 1.0 mL of complete Freund's adjuvant. Subsequent injections of this antigen with incomplete Freund's adjuvant were given weekly for a period of 4 weeks, and thereafter monthly, for a period of 3 months. The rabbits were bled at the 7th day after the last dose. The immunoglobulin fraction of each rabbit anti-serum was separated by precipitation with ammonium sulfate and stored at −70°C.

## Infection Rate of *H. capsulatum* in Alveolar Macrophages Detected by Colony Forming Units (CFU)

For this assay, a reference strain from the American Type Culture Collection (ATCC), G-217B, was compared with strains EH-315 and 60I. The infection rate of each strain was estimated using the AMJ2-C11 alveolar macrophage cell-line (ATCC, CRL-2456). The assay was performed in 24-well plates (TPP®) containing $10^5$ AMJ2-C11 macrophages per well, as described by Sardi et al. (2012). Each cultured macrophage monolayer was infected with 500 μL of yeast inoculum ($1 \times 10^6$ yeasts/mL) and plates were incubated at 37°C for 0, 7, 15, 30, 60, 120, 180, and 300 min (5 h). After each incubation time, a macrophage monolayer was washed three times with sterile PBS to remove released yeast cells. Then, the AMJ2-C11 cells were detached at 37°C for 2 min using trypsin-EDTA (Gibco Life Technologies, Carlsbad, CA, USA) diluted in PBS. Subsequently, 100 μL of each infected macrophage suspension was plated on supplemented BHI-agar (Difco) and incubated at 37°C, for 24–72 h. After incubation, fungal colonies were counted and the CFU/mL was estimated for each strain tested, corresponding to the number of *H. capsulatum* yeast cells that was able to infect the alveolar macrophage monolayer at each incubation time. For each assay, a control for yeast cell viability was performed in which yeast cells were maintained with trypsin-EDTA for 2 min and Trypan blue solution was added afterward to detect viability. *H. capsulatum* infection rate curves were constructed based on the data of each strain incubated at the different times. Tests were set up in triplicate in two independent assays.

The interaction between alveolar macrophages and *H. capsulatum* yeast were also monitored by conventional Giemsa staining and indirect fluorescence.

## Indirect Immunofluorescence

Samples of infected macrophages were maintained under the optimal culture conditions for a 5 h incubation. The infected monolayers were fixed with 4% paraformaldehyde, washed in PBS, and permeabilized in 0.5% Triton X-100 for 30 min. Polyclonal anti–*H. capsulatum* antibody was added for a 1 h incubation at room temperature, and unbound antibodies were removed by washing with PBS. Alexa Fluor®594-conjugate goat anti-rabbit IgG (Invitrogen-Molecular Probes, Eugene, OR, USA) was added and incubated for 1 h at room temperature and, subsequently, fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich, St Louis, MO, USA) was added with 1 h of incubation at 37°C. All nuclei were stained using 4′,6-Diamino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA). The infected and non-infected macrophages were washed three times with PBS and analyzed under fluorescence microscopy. All the images were acquired by the IN Cell Analyzer 2000 System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Additionally, the percentage of the infected macrophage population and the number of yeast cells per macrophage were determined using Investigator IN Cell 1000 Workstation software (GE Healthcare Bio-Sciences Corp.). This software includes an accurate analysis module that allows reaching reliable results to measure the morphology and the fluorescence intensity of user-defined nuclear and cytoplasmic compartments. Thus, cells can be classified into subpopulations by applying one or more filters, according to one or two user-selectable fluorescence or morphological events. For the analysis, the AMJ2-C11 alveolar macrophages were counted as cells based on some parameters, such as cells fluorescence intensity, nuclei fluorescence intensity, cells area, and nuclei area. To measure yeast cells they were assumed as being "organelles," and the following parameters were considered, organelles mean area, organelles total area, organelles number per macrophages, organelles fluorescence intensity. The final results were automatically obtained in a worksheet detailing the measures by well, by field, and by cell, regarding the indicated parameters as numerical values. The assay was performed in duplicate.

## Infection Rate of *Histoplasma capsulatum* in Alveolar Macrophages Detected by Flow Cytometry

For this assay, AMJ2-C11 macrophage monolayers containing $10^5$ macrophages per well were formed in 24-well plates (TPP®). After, 500 μL ($1 \times 10^6$ yeasts/mL) of each inoculum of *H. capsulatum* was stained with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 min. Stained *H. capsulatum* strains were added to their respective macrophage monolayer, and the plates were incubated at 37°C, 5 h. After the incubation time, the monolayers were washed three times with sterile PBS, and macrophages were detached at 37°C for 2 min using trypsin-EDTA (Gibco Life Technologies) diluted in PBS. Macrophage suspensions were harvested in Eppendorf tubes and centrifuged at 600 × *g*, 4°C. Supernatants were removed and PBS was added to each Eppendorf tube before cell counting by flow cytometry (BD FACSCanto Becton Dickinson, San Diego, CA, USA). For the analyses, we considered parameters related to the size (size forward scatter—FSC), granularity (granularity side scatter—SSC) and fluorescence of 10,000 cells per tube. The results were determined through the fluorescence intensity (FI) of yeast cells labeled with CFSE as estimated by BD FACSDiva software. Gates of specific population were viewed and analyzed by dot-plot. These data allowed one to determine the percentage of infected alveolar macrophages and discriminate the infectivity of different strains of *H. capsulatum*. Non-infected AMJ2-C11 alveolar macrophages, fluorescein-labeled yeast, and unlabeled yeast were used as negative controls in the assay. Assays were performed in three biological replicates and two technical replicates.

## Comet Assay

AMJ2-C11 macrophages, in 24-well plates, were infected with *H. capsulatum* strains EH-315 or 60I using a standardized suspension of $1 \times 10^6$ yeasts/mL and incubated at 37°C for 5 h. Non-infected macrophages were used as a negative control. The alkaline version of the comet assay (single cell gel electrophoresis) was performed as described by Singh et al. (1988). Duplicate slides were prepared and stained with ethidium bromide. We screened 50 AMJ2-C11 macrophages per sample with a fluorescence microscope (Carl Zeiss GmbH, Oberkochen, Germany) equipped with a 515–560 nm excitation filter, a 590 nm barrier filter, and a 40× objective. The level of DNA damage was assessed by an image analysis system (TriTek CometScore, version 1.5; TriTek Corp., Sumerduck, VA, USA), and the DNA percentage in comet tail was obtained for each treatment. Additionally, the percentage of the macrophage population that showed DNA damage was determined.

## TUNEL Assay

DNA fragmentation in infected macrophages was evaluated using TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining following the protocol recommended by the manufacturer (Roche Diagnostics, Penzberg, Germany), which has the feature of specific labeling of fragmented DNA sequences that occur during the process of apoptosis. Infection was performed with *H. capsulatum* strains EH-315 and 60I in AMJ2-C11 macrophages cultured in 96-well plates. The macrophages were incubated with a standardized suspension ($1 \times 10^6$ yeasts/mL) of *H. capsulatum* EH-315 or 60I and infection was allowed for 30 min, 2 h, and 5 h. Non-infected macrophages were used as a negative control. After each incubation time, macrophages were PBS washed and fixed in 4% paraformaldehyde for 1 h at room temperature. Samples were washed three times with cold PBS and incubated with 200 μL permeabilization solution (0.05 M Tris, 0.02 M CaCl2, and 2.5 mg/mL proteinase K) for 15 min at room temperature. After further washing with cold PBS, free reactive sites of the macrophage monolayer on the coverslips were blocked with 200 μL of a solution containing 3% bovine serum albumin and 20% fetal bovine serum in PBS at 37°C for 1 h. Then, the monolayers were washed three times with cold PBS and incubated with the components of the "TUNEL" mixture (dUTP solution containing the enzyme FITC-conjugated and "terminal deoxynucleotidyl transferase") at 37°C, for 1 h, in a moist chamber under darkness. During the incubation period, the 3′ ends of the apoptotic DNA fragments were incorporated into the FITC-labeled nucleotides. This reaction was catalyzed by terminal transferase. After incubation, three washes were performed with cold PBS, and 100 μL of 1% paraformaldehyde was added per well. Analysis of DNA fragmentation in macrophages was conducted to compare the EH-315 and 60I strains, using non-infected macrophages as a negative control. Images were captured using the IN Cell Analyzer 2000 System for light microscopy and were analyzed by Investigator IN Cell 1000 Workstation software (GE Healthcare Bio-Sciences Corp.). The results were evaluated using as parameter the fluorescence intensity emitted by the nucleus in each condition tested.

## Labeling of the Nuclear Envelope Proteins SUN2, Nesprin2, and Emerin

AMJ2-C11 macrophages, cultured in 24-well plates (TPP®), were infected with a standardized suspension of $1 \times 10^6$ yeasts/mL of *H. capsulatum* strains EH-315 or 60I at 37°C for 5 h. Nuclear envelope proteins were marked in either infected or non-infected AMJ2-C11 macrophages (negative control).

Initially, infected and non-infected macrophage samples were fixed with 4% paraformaldehyde, washed with PBS and permeabilized with 0.5% Triton X-100 for 30 min. Then, blocking was performed with 2.5% bovine serum albumin, 1% non-fat milk, and 8% fetal bovine serum. Primary anti-*H. capsulatum* antibody was added for 1 h. Unbound antibodies were removed by washing in PBS, then, Alexa Fluor®594-conjugate goat anti-rabbit IgG (secondary antibody) was added for 1 h. Afterward, in another staining series, anti-SUN2 antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-Nesprin2 antibody (Abcam, Cambridge, UK), or anti-Emerin antibody (Abcam) obtained in mice was added as a primary antibody, and macrophage samples were incubated overnight. Unbound antibodies were removed by PBS washing and a secondary Alexa Fluor®488-conjugate goat anti-mouse IgG

antibody was added for 1 h. All nuclei were DAPI stained. The infected and non-infected macrophages were then washed three times with PBS and analyzed under confocal laser scanning microscopy (Leica TCS SP5 Confocal Microscopy System). The assay was performed in duplicate.

## Statistical Analyses

Data were analyzed using Origin 7.0 (Origin Lab. Corporation, Northampton, MA, USA). ANOVA was used to compare groups in CFU and TUNEL assays with the Bonferroni post-test. *P* was calculated by Student's *t*-test and $P \leq 0.001$ were considered statistically significant. For the comet assay, the infected and non-infected macrophages (control) were compared using the Kruskal–Wallis test and the associated Dunn post-test with $P \leq 0.05$ were considered as statistically significant.

# RESULTS

## Infection Rate of *Histoplasma capsulatum* in AMJ2-C11 Alveolar Macrophage Cell-Line Detected by CFU

The infection rate of *H. capsulatum* yeasts was assessed with the *H. capsulatum* reference strain G-217B and strains EH-315 and 60I. As seen in **Figure 1**, strains EH-315 and 60I developed higher infection rates than the G-217B reference strain. Additionally, the EH-315 strain always exhibited the highest AMJ2-C11 macrophage infection rate at all times studied. **Figure 1** shows the number of yeast cells that infected the alveolar macrophages through the CFU/mL counting with these three fungal strains. The results suggested that at 120 min of contact, between alveolar macrophages and strain EH-315, the number of yeast cells in the macrophages increased. At 180 min, the infection rate declined, and it increased again at 300 min. With regard to the strain 60I, the number of yeast cells was increased at 60 min and at 120–180 min, the infection rate declined. Similarly to strain EH-315, the infection rate of strain 60I increased again at 300 min.

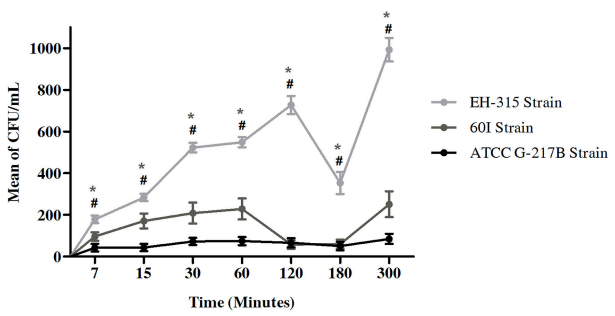


**FIGURE 1 | Infection rate assays of AMJ2-C11 alveolar macrophage with *H. capsulatum* strains G-217B, EH-315, and 60I.** Rates of *H. capsulatum* yeasts were considered using CFU/mL. Scores given are the mean ± S.D. and statistics were performed by Two-way ANOVA with the Bonferroni post-test. *$P < 0.001$ for EH-315 vs. 60I strain and #$P < 0.001$ for EH-315 vs. G-217B strain.

## Observation of *Histoplasma capsulatum* in AMJ2–C11 Macrophages

Infection of macrophages by *H. capsulatum* yeast (strains EH-315 and 60I) was also evaluated using Giemsa staining. This methodology was very useful for analyzing how yeasts interact with host macrophages (**Figure 2**). However, this staining did not provide accurate localization of yeast cells within phagocytes.

Indirect fluorescence microscopy, using images of the IN Cell Analyzer, revealed several intracellular yeasts in infected macrophages. An interesting finding was detected with this methodology, where intracellular *H. capsulatum* yeast cells aggregated in an architectural shape apparently surrounding the macrophage nucleus, resembling a "crown" (**Figure 3**). Moreover, additional analyses with Investigator IN Cell 1000 Workstation software showed that strain EH-315 infected 95% of the macrophage population with an infection multiplicity up to 30 cells per macrophage, whereas strain 60I infected 86% of macrophages with up to 24 yeast cells per macrophage.

## Flow Cytometry Assay

Results were expressed as FI of yeast labeled with CFSE and correspond to the fluorescence data of 10,000 cells per tube. To quantify the percentage of yeast cells bound to or within AMJ2-C11 macrophages, combination of two gates were applied to yeast cells and AMJ2-C11 cell-line (**Figure 4**). After using these combined gates, immediately the percentage of yeast cells interacting with AMJ2-C11 alveolar macrophages was

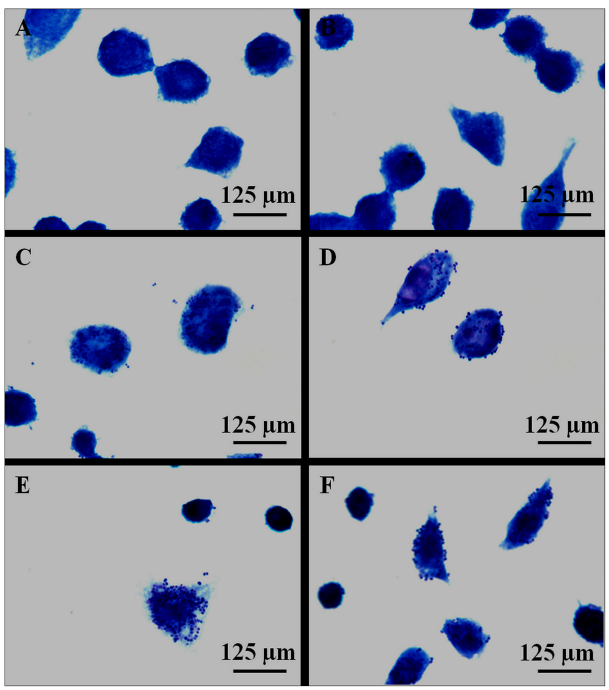


**FIGURE 2 | Giemsa staining of AMJ2-C11 macrophages after 5 h of *H. capsulatum* infection. (A,B)** Control of non-infected macrophages incubated with PBS. **(C,D)** Macrophages infected with *H. capsulatum* strain EH-315. **(E,F)** Macrophages infected with *H. capsulatum* strain 60I. The results are representative of two assays.

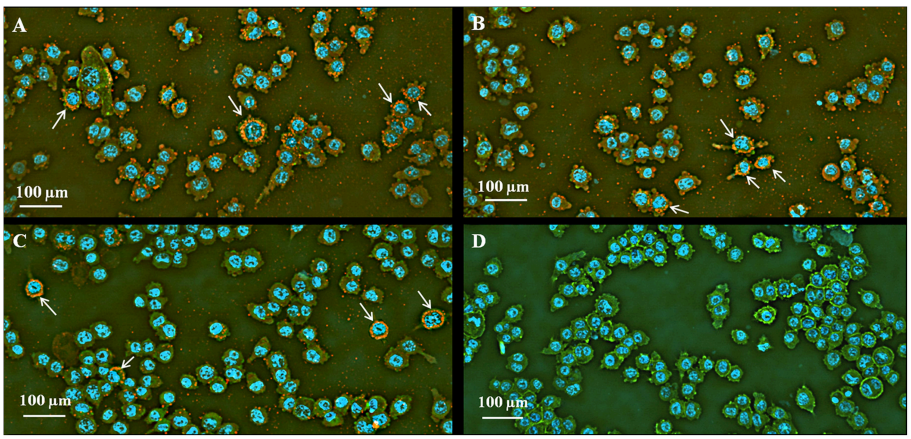


**FIGURE 3 | Effect of *H. capsulatum* yeast cells on the infection of AMJ2-C11 macrophages.** Macrophages infected with *H. capsulatum* yeast cells were incubated at 37°C for 5 h (see details in Section Materials and Methods). **(A,B)** AMJ2-C11 macrophages infected with the EH-315; **(C)** AMJ2-C11 macrophages infected with 60I; and **(D)** non-infected AMJ2-C11 macrophages. Indirect immunofluorescence: FITC-phalloidin in green showing the macrophage cytoplasm; Alexa Fluor®594 in red to yellow staining *H. capsulatum* yeast cells; DAPI in blue staining macrophages nucleus. Images were obtained using IN Cell Analyzer light microscopy. The results are representative of two assays. Arrows indicate the architectural arrangement of yeast-aggregates apparently surrounding the nuclei of the phagocytic cells.

determined. Regarding the profile of *H. capsulatum* infection in alveolar macrophages, strains EH-315 and 60I showed high infection rates in AMJ2–C11 macrophages. Moreover, both strains have a similar potential for infection because they are able to infect murine alveolar macrophages at rates of 98.34 and 96.52%, respectively, after 5 h of infection. The results represent the average of three independent assays set up in triplicate.

## Comet Assay

A similar pattern of DNA fragmentation was observed in infected AMJ2–C11 macrophages by comet assay, when *H. capsulatum* strains EH-315 and 60I were tested. Typical images of the comet assay showing DNA fragmentation in the tail are presented in **Figure 5A**. In the analysis of macrophage nuclear fragmentation by *H. capsulatum*, DNA damage corresponded to the percentage of DNA in the tail of the comet, and the results demonstrated that, for strains EH-315 and 60I, macrophage DNA damage was 10.67 ± 0.91% and 10.78 ± 1.31%, respectively; whereas 1.75 ± 0.18% of DNA damage was associated with the non-infected macrophages used as a negative control. Significant differences ($P < 0.05$) were found when macrophages infected with each strain were statistically compared with their respective negative controls (**Figure 5B**).

In addition, the comet assay data also revealed that 86.71% of the macrophage population infected with strain EH-315 showed DNA damage. Similarly, strain 60I induced DNA damage in 81.98% of infected alveolar macrophages. There was no statistically significant difference between the percentages of macrophages undergoing DNA damage induced by the EH-315 or 60I *H. capsulatum* strains. However, significant differences ($P < 0.05$) were found between each macrophage population infected with a fungal strain compared with its respective non-infected control, which revealed 34.41% of DNA damage (**Figure 5C**).

## TUNEL

**Figure 6A** shows images obtained by the IN Cell Analyzer for the TUNEL assay that was used to quantify macrophage apoptosis. The measurement of apoptotic nuclei was performed by the release of nuclear fluorescence intensity detected with the TUNEL method; thus, as the fluorescent labeling increases more damage is detected. DNA fragmentation was detected in AMJ2-C11 macrophages infected with *H. capsulatum* strain EH-315 or 60I at 30 min, 2 h and 5 h post-infection. The infection of macrophages with *H. capsulatum*, after 2 and 5 h, resulted in more apoptotic cells than the non-infected controls, and the number of apoptotic nuclei obtained from macrophages infected with each fungal strain was similar, as shown in **Figure 6B**.

## Labeling of the Nuclear Envelope Proteins SUN2, Nesprin2, and Emerin

Confocal microscopy was used to generate 3D images of infected macrophages labeled with anti-SUN2, anti-Nesprin2 and anti-Emerin antibodies. A diffuse distribution of these proteins was found outside on the nuclear envelope, with a similar pattern for non-infected macrophages, as shown in **Figure 7**. In addition, data from confocal microscopy also indicate that the nuclear fragmentation induced after infection, which was demonstrated by the comet and TUNEL assays, may occur as the result of the architectural conformation displayed by *H. capsulatum*, in which yeast cells appear to surround the macrophage nucleus after 5 h of infection (**Figure 7** and Videos S1–S6). Moreover, during infection the formation of large phagosomes within *H. capsulatum*-infected macrophages was noted (**Figure 7B**). These events were not found in non-infected macrophages.

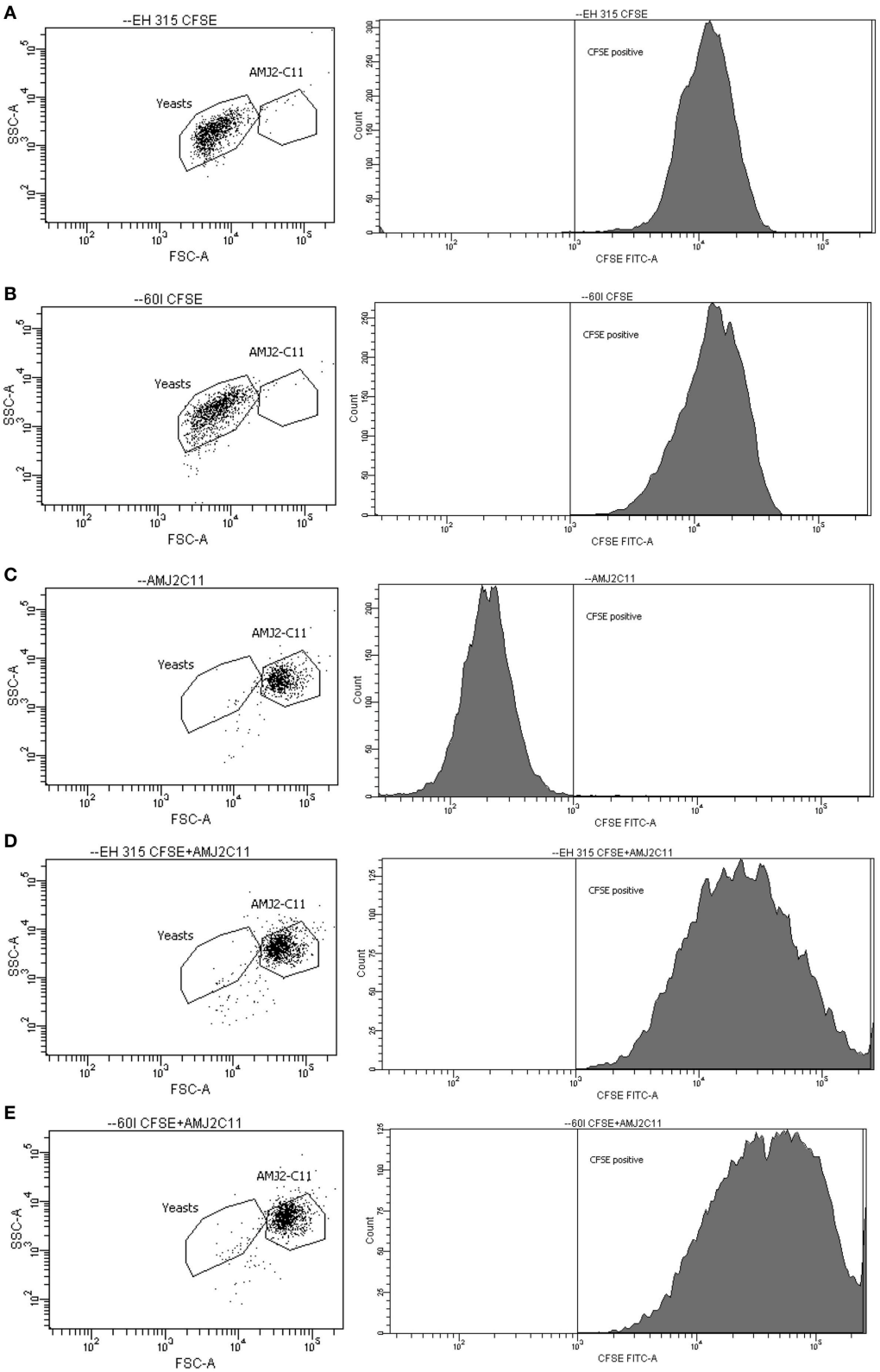


**FIGURE 4 | Dot-plot and histogram profiles of AMJ2-C11 alveolar macrophages and CFSE-labeled *H. capsulatum* yeast cells, at 5 h post-infection.** **(A,B)** Population of labeled yeast cells (EH-315 and 60I strain, respectively); **(C)** specific gate for AMJ2-C11 alveolar macrophages; and **(D,E)** Infected alveolar macrophages by EH-315 and 60I strain, respectively. The cells were analyzed by flow cytometer BD FACSCanto.

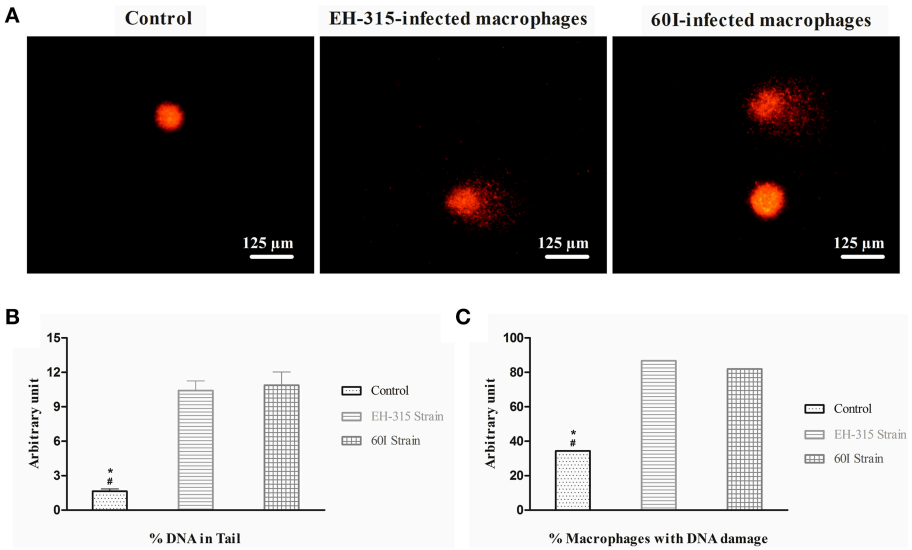


**FIGURE 5 | Comet assay data of AMJ2-C11 macrophages infected with *H. capsulatum* yeasts. (A)** Images were acquired using a fluorescence microscope equipped with a 515–560 nm excitation filter and a 590 nm barrier filter. **(B)** Evaluation of nuclear fragmentation in AMJ2-C11 macrophages infected with *H. capsulatum*. Control (non-infected macrophages). Values are representative of the percentage of fragmented DNA present in the comet's tail. **(C)** Percentage of macrophage population showing DNA damage in infected and non-infected (control) cells. Scores given are the mean ± SD. Data were analyzed by Kruskal-Wallis test with Dunn's post-test. *$P < 0.05$ when control vs. strain EH-315 was compared; #$P < 0.05$ when control vs. strain 60I was compared.

## DISCUSSION

Interactions of pathogenic fungi with host tissues are essential factors in the pathogenesis of mycoses (Tronchin et al., 2008). *H. capsulatum* infects different host cells, such as neutrophils, macrophages, dendritic cells, and epithelial cells.

The present study demonstrated particular characteristics of the interaction between *H. capsulatum* yeast cells and cultured murine alveolar macrophages. We compared two virulent *H. capsulatum* strains isolated from different sources, EH-315 and 60I, based on their behavior and potential infection for AMJ2-C11 alveolar macrophages. The EH-315 strain developed higher virulence (LD50 $3 \times 10^5$ yeasts/mL) when compared to the 60I strain (LD50 $3 \times 10^8$ yeasts/mL) under experimental conditions using an LD50 assay in male BALB/c mice (ML Taylor, personal communication).

We evaluated the ability of the EH-315 and 60I strains to infect alveolar macrophages when compared with the ATCC strain G-217B using CFU analysis of *H. capsulatum* yeast at 7–300 min (5 h) post-infection. The results demonstrated that the three strains of *H. capsulatum* have distinct efficiency for infecting alveolar macrophages. Strain EH-315 developed a better ability to infect macrophages than strains 60I and G-217B. However, regarding the efficacy of the CFU assay, other researchers have described the inconvenience and limitations of this method. According to Berkes et al. (2012), several factors contribute to non-optimize microorganisms plating that routinely reaches only 30% effectiveness for *H. capsulatum*, as the CFU number is generally lower than the number of viable yeasts plated.

Few studies have associated pathogen virulence with the ability to infect host cells or to adhere to abiotic surfaces. Thewes et al. (2008) performed phenotypic screening to compare the SC5314 strain of *Candida albicans*, which is invasive and highly virulent, with the strain ATCC 10231, which is non-invasive and less virulent. Their findings highlight that strain ATCC 10231 caused less damage to fibroblasts and epithelial cells when compared to SC5314. According to those authors, biological properties that influence adherence and invasion to host cells are critical attributes of *C. albicans* in colonization and disease progression, and the results of this study demonstrated that virulence had a direct influence on the ability of this fungus to colonize, damage, and invade host tissues. Furthermore, Sepúlveda et al. (2014) compared *H. capsulatum* strains with distinct genotype and virulence and noted that strain G-217B exhibited delayed response to the virulence effect, as in macrophage damage and cytokine production when compared to other strains. Likewise, Sahaza et al. (2015) highlighted that lung inflammatory responses, in regard to cytokine profile and lung-granuloma formation, varied in intensity and time when two different virulent *H. capsulatum* strains from distinct phylogenetic species, EH-46 (LAm A) and G-217B (NAm 2), were used. Our data corroborated those reported by Sepúlveda et al. (2014) and Sahaza et al. (2015), as strain G-217B showed delayed infection potential against host cells.

In the current study, the infection profile of *H. capsulatum* strains on alveolar macrophages over a period of 5 h was characterized by a variable behavior (increases and decreases) in the yeast infection rate of alveolar macrophages. We hypothesize

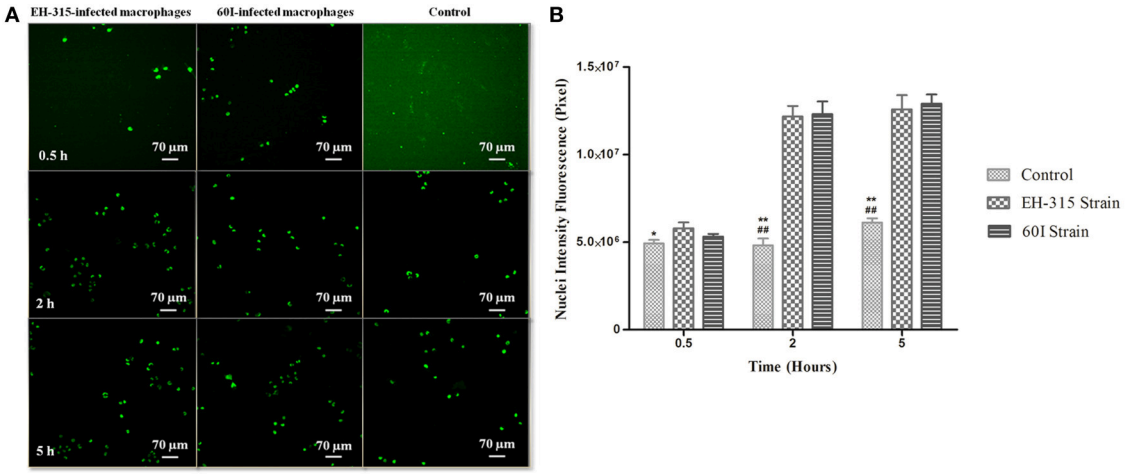


**FIGURE 6 | TUNEL staining of alveolar macrophages after *H. capsulatum* infection. (A)** DNA fragmentation in the nucleus of AMJ2-C11 macrophages infected with *H. capsulatum* yeasts. Images were obtained after 0.5, 2, and 5 h after infection of alveolar macrophages with *H. capsulatum* strains EH-315 or 60I. Infected alveolar macrophages were compared with non-infected macrophages incubated with PBS (control). Each photomicrograph was processed by IN Cell Analyzer light microscopy using a 20X power field. The results are representative of two assays. **(B)** Detection of nuclear fluorescence intensity derived from DNA fragmentation in AMJ2-C11 macrophages infected with *H. capsulatum* strain EH-315 or 60I and non-infected macrophages (control). TUNEL-positive macrophages are represented by the nuclear fluorescence intensity with values derived from analysis of images using Investigator IN Cell 1000 Workstation software. Scores given are the mean ± S.D and statistics were performed by Two-way ANOVA with the Bonferroni post-test. $^{*}P < 0.05$ and $^{**}P < 0.001$, control vs. strain EH-315, $\#\#P < 0.001$, control vs. strain 60I.

that this profile occurs as a result of the dynamic interactions between yeast and macrophage membrane receptors.

According to our results, macrophage infections were also monitored at 5 h by Giemsa staining and indirect fluorescence. As we mentioned before, Giemsa staining did not provide well-defined yeast localization within phagocytes, whereas quantification of the macrophage population that was effectively infected with *H. capsulatum* yeasts was successfully achieved by flow cytometric methodology. For these infection assays, the 5 h post-infection time was selected based on a previous kinetic study that showed the largest number of yeast cells interacting with alveolar macrophages at this time-point of infection. Once the CFSE-labeled yeasts had interacted with alveolar macrophages, the percentage of infected cells containing yeast cells was accurately quantified. It is important to note that flow cytometry has been employed to quantify several fungal infections (Chang et al., 1998; Berkes et al., 2012).

*H. capsulatum* is a pathogen that commonly survives within macrophages by developing several intracellular evasion mechanisms (Strasser et al., 1999; Sebghati et al., 2000). Microscopic images obtained by indirect immunofluorescence assays showed a singular pattern of *H. capsulatum* in the infected macrophages under *in vitro* conditions, which was similar for the two fungal strains tested. Interestingly, the yeast cells of both strains were able to form aggregates in the cytoplasm of the infected macrophages with an apparent distribution surrounding the macrophage nucleus after 5 h of infection. These findings could be related to a new strategy for fungal intracellular survival.

Based on the potential for infection displayed by both strains of *H. capsulatum* in alveolar macrophages, it became necessary to evaluate the genotoxic potential of this fungus in AMJ2-C11 macrophages to identify the ability of *H. capsulatum* to induce damage to DNA in host cells. According to Yang et al. (2011), genotoxic agents chemically interact with the genetic material and cause oxidative changes or disruptions in the DNA molecule.

The results of comet and TUNEL assays showed that the two strains of *H. capsulatum* (EH-315 and 60I) caused significant damage to the nuclear DNA of the AMJ2-C11 macrophages after 5 h of infection when compared to non-infected macrophages. Nuclear fragmentation is characterized as a cellular alteration associated with apoptosis (Deepe and Buesing, 2012). In this context, several studies have shown that *H. capsulatum* yeast cells induce apoptosis in different host cell-lines, including macrophages (Allen and Deepe, 2005; Lin et al., 2005; Deepe and Buesing, 2012). According to Das et al. (1999), apoptosis allows the host to develop an effective response against infectious diseases such as tuberculosis.

A study conducted by Del Vecchio et al. (2009) demonstrated the ability of a dimorphic fungus, *Paracoccidioides brasiliensis,* to induce apoptosis in A549 epithelial cells after 24 and 48 h of infection using the TUNEL assay to assess DNA fragmentation. Previous studies have reported that strains of *C. albicans* also induce apoptosis of macrophages after 30 min of infection under *in vitro* conditions. According to the authors, the ability of *C. albicans* to induce apoptosis may modulate a standard anti-inflammatory immune response in the host (Gasparoto et al., 2004). Moreover, polysaccharides of *Cryptococcus neoformans* also induce apoptosis in macrophages under *in vitro* and*in vivo* conditions, compromising the host immune response (Villena et al., 2008).

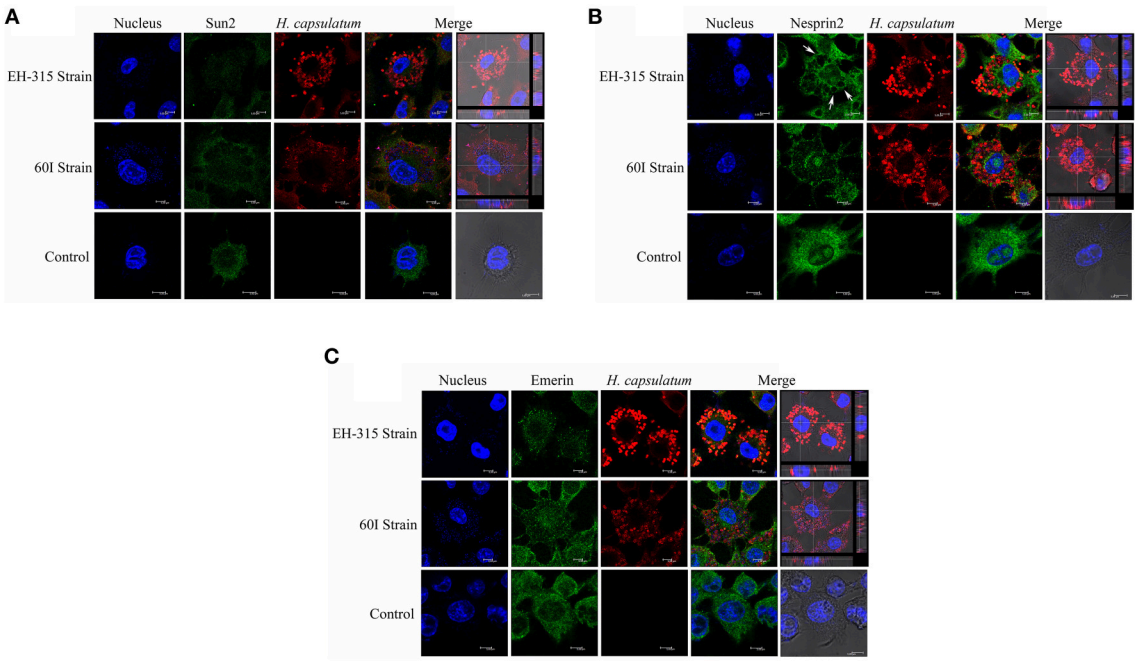


**FIGURE 7 | Labeling of nuclear envelope proteins SUN2, Nesprin2, and Emerin in *H. capsulatum* infected alveolar macrophages.** In Panels **(A–C)** show confocal scanning microscopy images of representative AMJ2-C11 macrophages infected with *H. capsulatum* strains EH-315 and 60I at 37°C for 5 h. Non-infected macrophages were processed as a control. **(A)** AMJ2-C11 stained for SUN2, **(B)** Nesprin2, and **(C)** Emerin as indicated in the figure. The arrows show phagosome formation where several yeast cells are present. DAPI was used for nuclear staining (blue). Alexa Fluor®488 and Alexa Fluor®594 conjugates were used as secondary antibodies to reveal nuclear proteins (green) and yeast cells (red), respectively. Bright field images were merged with Alexa Fluor®594 and DAPI stain with orthogonal z stack sections after 5 h of macrophage-yeast infection. The assay was performed twice.

According to our findings, the apoptosis of infected macrophages could be related to the formation of *H. capsulatum* aggregates that extend throughout the cytoplasm and display a conformational architecture that arrange themselves near to the macrophage nucleus. This conformational aggregation of yeast cells could form in the intracellular environment and remain within the macrophages, causing damage to the nucleus of the host cell and producing DNA fragmentation. This can be explained by the high percentage of infected macrophages with yeast-aggregates after 5 h of infection, which was similar to the percentage of macrophage suffering DNA damage induced by both strains. This new structural arrangement could be associated with the ability of *H. capsulatum* yeasts to prevent elimination by the immune system (Pitangui et al., 2012). The preference of *H. capsulatum* yeast cells to form intracellular aggregates became understandable when a high number of yeasts appeared as linked to each other, during infection of murine alveolar macrophages (**Figure 7** and Videos S1–S6).

Confocal microscopy images were used to determine if the effects on host cell nuclei induced by *H. capsulatum* aggregates could change the behavior of the nuclear membrane proteins SUN2, Nesprin2, and Emerin. The analyses revealed that infected macrophages with yeast-aggregates surrounding the macrophage nuclei did not show disruption in the organization of the nuclear lamina that underlies the nuclear envelope, given that the staining of nuclear proteins showed a very similar distribution to that seen in non-infected macrophages. Meinke et al. (2011) reported that disruption of SUN2 or Nesprin2 prevents nuclear movement. In the present study, it was possible to observe diffuse distribution of these proteins outside on the nuclear envelope in both infected and non-infected macrophages.

To date, a few studies have described Nesprin isoforms (Zhang et al., 2007; Morris and Randles, 2010; Randles et al., 2010) as a product generated by the alternative splicing of genes encoding Nesprin1 and Nesprin2. These isoforms vary in size, but they contain a common C-terminal region (Randles et al., 2010) and play important roles in cellular organization, especially in positioning the nucleus and other organelles. Nesprin isoforms appear in different subcellular fractions, including outer and inner nuclear membranes associated with organelles such as mitochondria, Golgi complex, sarcoplasmic reticulum and in the plasmatic membrane, where the isoforms form a network connecting these structures to the actin cytoskeleton (Zhang et al., 2007; Morris and Randles, 2010). Therefore, Nesprin can be found away from the nucleus (Gough et al., 2003), which is consistent with the results obtained in this study, where we observed diffuse presence of this protein in the cytoplasm of infected and non-infected macrophages. However, according to Randles et al. (2010), it is difficult to determine whether other isoforms are also present due to the absence of specific antibodies.

Emerin was also found to be diffuse throughout the cytoplasm of non-infected and *H. capsulatum* infected macrophages.

Conversely, a study using host cells infected with herpes simplex virus 1 found an irregular distribution of Emerin, as opposed to a uniform alignment on the nuclear membrane, which appeared like bubbles on the surface of the outer nuclear membrane (Leach et al., 2007). Recently, Ho et al. (2013) reported that Emerin regulates gene expression by modulating actin polymerization in the cytoplasm.

According to our results, it is necessary to emphasize that *H. capsulatum* yeast-aggregates were able to cause damage in the nuclear DNA and induce apoptosis in alveolar macrophages after 5 h of infection. This damage to the DNA of macrophages while the yeast cells are not located inside the core could be a fungus strategy for the facilitation of its persistence throughout the host infection. This finding has never been previously described. Hence, the intracellular arrangement and the occurrence of effects induced by *H. capsulatum* yeast-aggregates during the infection could promote the survival of the pathogen in the hostile conditions of the intracellular environment while also contributing to host tissue damage.

## AUTHOR CONTRIBUTIONS

NS, CS and AF conceived and designed the study. NS, JS, AV, CS, JS, RS and FS performed the experiments and analyzed the data. RS, FS and CS collaborated with reagents/materials/analysis tools. All authors read and approved the final manuscript. NS and AF wrote the paper with contributions from GR, MT and MM.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01526

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REVIEW
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# *Cryptococcus* and Phagocytes: Complex Interactions that Influence Disease Outcome

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*Cryptococcus neoformans* and *C. gattii* are fungal pathogens that cause life-threatening disease. These fungi commonly enter their host via inhalation into the lungs where they encounter resident phagocytes, including macrophages and dendritic cells, whose response has a pronounced impact on the outcome of disease. *Cryptococcus* has complex interactions with the resident and infiltrating innate immune cells that, ideally, result in destruction of the yeast. These phagocytic cells have pattern recognition receptors that allow recognition of specific cryptococcal cell wall and capsule components. However, *Cryptococcus* possesses several virulence factors including a polysaccharide capsule, melanin production and secretion of various enzymes that aid in evasion of the immune system or enhance its ability to thrive within the phagocyte. This review focuses on the intricate interactions between the cryptococci and innate phagocytic cells including discussion of manipulation and evasion strategies used by *Cryptococcus,* anti-cryptococcal responses by the phagocytes and approaches for targeting phagocytes for the development of novel immunotherapeutics.

**Keywords: cryptococcosis, *Cryptococcus neoformans*, *Cryptococcus gattii*, *Cryptococcus*, fungal immunity, innate immune response, medical mycology**

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## *Cryptococcus neoformans* AND *C. gattii*

Cryptococcosis is a disease predominantly caused by the species *Cryptococcus neoformans* and *C. gattii* in humans and other mammals (Kwon-Chung et al., 2014). *Cryptococcus* sp. are generally encountered in the environment. *C. gattii* is commonly found in tropical and subtropical regions, however, the species is expanding its reach as evidenced by the recent outbreak in Vancouver Island, British Columbia, Canada and the Pacific Northwest of the United States (reviewed in Hole and Wormley, 2012; Kwon-Chung et al., 2014). Until recently, *Cryptococcus* sp. have been categorized into only these two species and subcategorized by serotype (A–D) and molecular type (VNI-VNIV, and VGI-VGIV; reviewed in Kwon-Chung et al., 2014). Currently, seven species of *C. gattii*/*C. neoformans* have been identified based on genotyping studies that have revealed significant genetic diversity within the species complex (Hagen et al., 2015). For the purposes of this review to ensure accurate reference to strains used in the discussed studies, we will refer to the organisms based on their previous *C. neoformans* (serotype A and D) and *C. gattii* (serotype B and C) nomenclature unless otherwise specified.

Interestingly, despite sharing 80–90% genomic identity (Kavanaugh et al., 2006), *C. neoformans* and *C. gattii* affect different patient populations and with different disease manifestations. The

infectious propagule of both species is inhaled into the lungs and the infection is often controlled by the host's immune system (Park and Mehrad, 2009). *C. neoformans*, however, predominantly causes disease in immunocompromised individuals, including AIDS patients and those undergoing immunosuppressive therapies, resulting in pneumonia and has a predilection to disseminate to the central nervous system (CNS) leading to life threatening meningoencephalitis (Powderly, 1993; van der Horst et al., 1997; Saag et al., 2000; Husain et al., 2001; Liu et al., 2012; Kwon-Chung et al., 2014; Sorrell et al., 2015). *C. gattii* principally causes disease in healthy individuals with no discernable underlying condition (Marr et al., 2012; Lizarazo et al., 2014). However, the idea that *C. gattii* is a primary pathogen is being questioned as anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies have been detected in *C. gattii* infected patients but not in samples from healthy donors (Saijo et al., 2014; Kwon-Chung and Saijo, 2015). Thus, anti-GM-CSF autoantibodies could indicate an underlying immunodeficiency that predisposes patients to *C. gattii* infection. This demonstrates a need for more comprehensive screening before classifying a patient as "immunocompetent" and further examination of the *C. gattii* patient population. Another striking difference between these two species is that *C. gattii* shows little penchant for dissemination to the CNS compared to *C. neoformans* (Ngamskulrungroj et al., 2012). Comparison of two virulent strains of *C. neoformans* (strain H99) and *C. gattii* (strain R265) revealed that even though *C. gattii* can cross the blood-brain barrier (BBB), *C. neoformans* grew 10–100 times faster in naïve mouse blood and serum (Ngamskulrungroj et al., 2012). This suggests that these two species have a different specificity for their target organ that is not yet fully elucidated.

## IMMUNE RESPONSE

The host has efficient mechanisms for combating these debilitating fungi. Initiation of a pro-inflammatory Th1-type immune response, characterized by interleukin-2 (IL-2), IL-12, IL-18, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), is protective against *Cryptococcus* (Huffnagle et al., 1991; Aguirre et al., 1995; Huffnagle, 1996; Kawakami et al., 1996, 1997a; Zhang et al., 1997, 2009; Huffnagle and Lipscomb, 1998; Olszewski et al., 2010). Additionally, generation of an anti-inflammatory Th2-type immune response, characterized by IL-4, IL-5, IL-13, and the immunoregulatory cytokine IL-10, is associated with exacerbation of disease (Huffnagle et al., 1998; Arora et al., 2005, 2011; Milam et al., 2007; Muller et al., 2007; Chen et al., 2008; Jain et al., 2009; Osterholzer et al., 2009b). In addition, a Th17-type response characterized by IL-17A, IL-21, IL-22, IL-6, and TGF-β (Korn et al., 2007, 2009; Onishi and Gaffen, 2010) has been shown to contribute to anti-cryptococcal immune responses (Zhang et al., 2009; Murdock et al., 2014). However, IL-17A and signaling through the IL-17A receptor is not required for clearance in a protective model of cryptococcosis (Wozniak et al., 2011a).

Experimental studies in mice with certain *C. neoformans* strains, including the highly virulent strain H99, results in a Th2-type polarized response which is not protective (Huffnagle et al., 1998; Milam et al., 2007; Muller et al., 2007; Chen et al., 2008; Xiao et al., 2008; Osterholzer et al., 2009b). However, studies using a *C. neoformans* strain H99 that was genetically engineered to express and secrete murine IFN-γ (H99γ; Wormley et al., 2007) showed that inoculation of mice with strain H99γ results in protective Th1-type responses and IL-17 production, increased leukocyte infiltration and production of pro-inflammatory cytokines/chemokines (Wormley et al., 2007; Wozniak et al., 2009, 2011a). In addition, immunization of mice with H99γ yields 100% protection against subsequent challenge with WT *C. neoformans* H99, providing evidence that vaccination against *C. neoformans* is possible (Wormley et al., 2007; Wozniak et al., 2009; Hardison et al., 2010, 2012). Furthermore, studies examining T cell depleted mice showed that mice immunized with H99γ and challenged with WT *C. neoformans* during T cell depletion were also protected against challenge, showing that immune-compromised hosts could be protected (Wozniak et al., 2011b). The majority of studies utilize *C. neoformans* as a model organism to assess protection via Th1 and Th17-type responses, however, several studies have suggested that protection against *C. gattii* employs a different mechanism. It has been shown that infection with *C. gattii* leads to suppression of host's immune responses, including decreased leukocyte recruitment and pro-inflammatory cytokine production (Dong and Murphy, 1995; Wright et al., 2002; Cheng et al., 2009). This could provide an indication as to how *C. gattii* is able to affect immunocompetent individuals, while *C. neoformans* predominantly causes disease in the immunocompromised populations.

It is crucial that the host immune responses will eradicate or limit the spread of fungal pathogens from the lung before they are able to disseminate. The host relies on pulmonary innate immune cells as its first line of defense against *Cryptococcus* sp. These include the phagocytic macrophages, dendritic cells (DCs) and neutrophils. When the yeast-like cell or basidiospore is encountered by the phagocyte, the phagocytic cell will engulf the invading organism forming a phagosome. Ideally, the phagosome will mature, acidify following fusion with a lysosome (forming the phagolysosome), and lead to the destruction of the fungus. *Cryptococcus* sp. have evolved a number of defense mechanisms and virulence factors that allow it to either survive in the phagosome or avoid phagocytosis altogether (reviewed in Kronstad et al., 2011; Kwon-Chung et al., 2014; Alspaugh, 2015). The capsule of *Cryptococcus* is a complex and elaborate structure composed of glucuronoxylomannan (GXM), galactoxylomannan (GalXM) and, to a lesser degree, mannoproteins and is anchored to the cell wall, which is composed of chitin, chitosan (acetylated chitin), glucans and glycoprotein (Doering, 2009; O'Meara and Alspaugh, 2012). The capsule is dynamic and dramatically increases in thickness during infection or when cultured in host inducing conditions (RPMI, 37°C). Additionally, the capsule functions to mask cryptococcal pathogen associated molecular patterns (PAMPs) that can be recognized by pattern recognition receptors (PRRs) on host cells, allowing *Cryptococcus* to evade the immune system by preventing phagocytosis and can aid

in providing protection against reactive oxygen species and antimicrobial peptides (Zaragoza et al., 2008). Opsonization of *Cryptococcus* with anti-capsular antibodies is required for phagocytosis by immune cells due to the anti-phagocytic properties of the capsule (reviewed in Kozel, 1993). The ability to form "titan cells" is another anti-phagocytic mechanism utilized by *Cryptococcus*. In the pulmonary environment, *C. neoformans* cells can attain a large cell size varying from 50 to 100 μm in diameter with varying degrees of capsule thickness making them resistant to phagocytosis (Okagaki et al., 2010; Zaragoza et al., 2010; Okagaki and Nielsen, 2012). Melanin production by laccase is another virulence factor of *C. neoformans* and *C. gattii* which serves to protect the organism from oxidative stresses within the phagolysosome by breaking down host substrates into reactive intermediates that can harm the host (Zhu and Williamson, 2004; Panepinto and Williamson, 2006). One example of this is the iron oxidase activity of laccase in the phagolysosome that reduces potentially toxic Fenton reactants, thus protecting *Cryptococcus* from the antifungal activity of alveolar macrophages (Liu et al., 1999). Other virulence factors include the ability to grow and proliferate at mammalian body temperature (37°C) and extracellular enzymes including laccases, urease, and phospholipases (reviewed in Kronstad et al., 2011; Almeida et al., 2015). In this review, we discuss interactions between *Cryptococcus* sp. and innate phagocytic cells and how these interactions determine the outcome of disease.

# MACROPHAGES

## Macrophage Activation

Resident alveolar macrophages which live within the lung alveolar airspaces are critical to host defense as the first line of defense against pulmonary pathogens (Fels and Cohn, 1986). Alveolar and infiltrating macrophages phagocytose and kill invading pathogens and are capable of presenting antigen to activated T cells to stimulate an adaptive immune response in immunocompetent individuals. Interestingly, macrophages have a dynamic plasticity which allows them to respond to changes in their cytokine microenvironment and alter their activation phenotype (Porcheray et al., 2005; Stout et al., 2005; Gratchev et al., 2006; Sica and Mantovani, 2012; Davis et al., 2013; Mantovani et al., 2013; Leopold Wager and Wormley, 2014). Macrophage activation phenotype is broadly classified as classical (M1) or alternative (M2) based on cytokine production, extracellular receptor expression and secreted by-products (Stout and Suttles, 2004; Mosser and Edwards, 2008; Sica and Mantovani, 2012). Immune cells, including natural killer (NK) cells, $CD8^+$ T cells, and $CD4^+$ T-helper 1 (Th1)-type cells (Mosser and Edwards, 2008) respond to the pathogen by secreting inflammatory cytokines, including IFN-γ which signals macrophages to polarize toward an M1 phenotype (Mantovani et al., 2004; Mosser and Edwards, 2008; Martinez et al., 2009; Hussell and Bell, 2014). The markers commonly used for identification of M1 macrophages include inducible nitric oxide synthase (iNOS), chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11, IL-12, and suppressor of cytokine signaling 3 (SOCS3; reviewed in Mosser and Edwards, 2008; Murray and Wynn, 2011; Leopold Wager and Wormley, 2014). M1 macrophages mediate host defense against microbial pathogens via the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively; Ding et al., 1988). The enzyme iNOS acts on the substrate L-arginine to produce nitric oxide (NO), which has anti-cryptococcal properties (**Figure 1**; Alspaugh and Granger, 1991; Arora et al., 2011; Hardison et al., 2012; Davis et al., 2013; Leopold Wager et al., 2014, 2015). Conversely, the enzyme arginase-1 (Arg-1) is a hallmark marker of M2 macrophage activation and competes with iNOS for this substrate producing L-ornithine and urea (Bogdan et al., 2000). The Arg1 to iNOS ratio is often considered an indicator of macrophage polarization phenotype as conditions leading to the induction of M2 macrophages inhibit M1 macrophage activation (Takeda et al., 1996; Van Dyken and Locksley, 2013).

The induction of M2 macrophage activation is mediated by cytokines including IL-4 and IL-13 (Mosser and Edwards, 2008; Mantovani et al., 2013; Van Dyken and Locksley, 2013). It was recently shown that cryptococcal Ssa1, a heat shock protein 70 homolog, is secreted by *C. neoformans* and promotes early fungal growth and a shift to M2 macrophage activation by stimulating early production of IL-4 and IL-13 (Eastman et al., 2015). M2 macrophages contribute to the suppression and regulation of inflammatory responses and play a pivotal role in wound-healing, but are not antimicrobial against *C. neoformans* (Arora et al., 2005, 2011; Mosser and Edwards, 2008; Martinez et al., 2009; Hardison et al., 2010, 2012; Murray and Wynn, 2011; Davis et al., 2013; Van Dyken and Locksley, 2013; Leopold Wager and Wormley, 2014; Leopold Wager et al., 2014, 2015). *C. neoformans* cells can survive in M2 macrophages by using them as a protective niche to evade recognition and killing by the host (reviewed in Johnston and May, 2013). Immune cells including Th2-type $CD4^+$ T cells, basophils, eosinophils, mast cells and group 2 innate lymphoid cells (ILC2s) are sources of IL-4 and/or IL-13 (Martinez et al., 2009; Murray and Wynn, 2011; Walker and McKenzie, 2013; Walker et al., 2013). Recently, it was shown that chitin, an integral part of the cryptococcal cell wall, indirectly stimulates the production of IL-5 and IL-13 and can trigger IL-4 production, resulting in M2 macrophage activation (Reese et al., 2007; Van Dyken et al., 2014). IL-4 can stimulate increased expression of mannose receptor (MR or CD206), the first described M2 macrophage activation-associated marker (Stein et al., 1992). The commonly associated markers of M2 macrophage activation also include arginase1 (Arg1), found in inflammatory zone 1 (FIZZ1, also known as resistin-like α or Relm-α), and chitinase and chitinase-like molecules such as YM1 (Chi3l3) and YM2 (Mosser and Edwards, 2008; Murray and Wynn, 2011).

Interestingly, macrophages, which are polarized toward an M1 phenotype in the presence of IFN-γ, can re-polarize to an M2 phenotype when stimulated with IL-4. Similarly, M2 polarized macrophages can re-polarize toward a functional M1 phenotype in the presence of IFN-γ and are able to maintain functional anti-cryptococcal activity (Stout et al., 2005; Gratchev et al., 2006; Davis et al., 2013), signifying the critical importance of

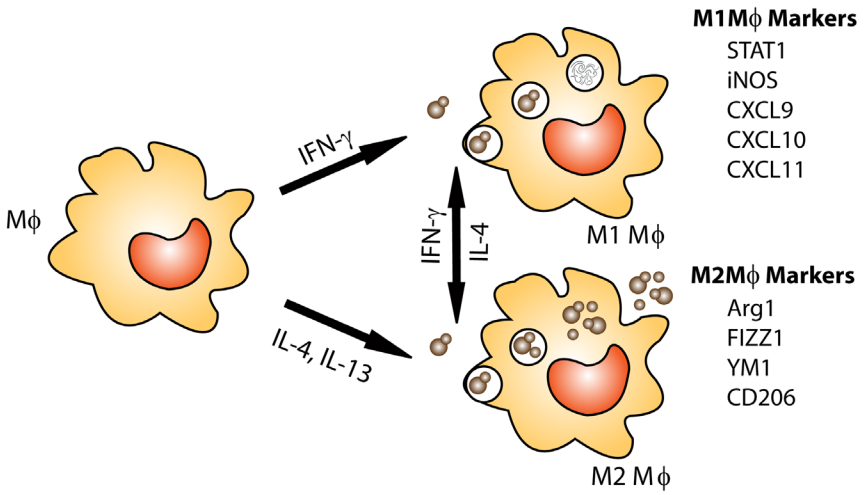


**FIGURE 1 | Macrophage activation: protection against *Cryptococcus* is associated with M1 or classically activated macrophages.** M1 macrophages are induced by exposure to the Th1 cytokine IFN-γ. M1 macrophages are efficient killers as they have increased nitric oxide (NO) and ROS production. M2 or alternatively activated macrophages are associated non-protective immune responses to *Cryptococcus*. M2 macrophages are not efficient killers and allow *Cryptococcus* to grow and proliferate within the macrophage and may lead to dissemination. In addition, macrophage phenotype is plastic, allowing for macrophages to switch between M1 and M2 phenotypes based on cytokines present in their environment.

the local cytokine milieu in driving macrophage polarization. IFN-γ production by Th1-type T cells and NK cells can stimulate M1 macrophage activation in a signal transducer and activator of transcription (STAT) 1-dependent manner (Hu et al., 2002). Studies utilizing the IFN-γ producing *C. neoformans* strain have illustrated that STAT1 signaling in macrophages is required for M1 macrophage activation and protection (Leopold Wager et al., 2014; Leopold Wager et al., 2015). More in-depth discussion of the macrophage polarization and *C. neoformans* infections has been recently published elsewhere (Leopold Wager and Wormley, 2014). Conversely, a clinical study recently showed that despite elevated IFN-γ levels and pro-inflammatory factors in the brains of two non-HIV cryptococcal meningitis patients, poor phagocytosis and macrophages polarized to the non-protective M2 phenotype was observed (Panackal et al., 2015). This establishes that macrophage polarization in response to *C. neoformans* in humans is quite complex and requires further study.

*Cryptococcus neoformans* is sensitive to the NO produced by M1 macrophages (Alspaugh and Granger, 1991; Aguirre and Gibson, 2000; Rivera et al., 2002; Muller et al., 2007; Osterholzer et al., 2009b; Stenzel et al., 2009; Zhang et al., 2009; Hardison et al., 2010). *C. neoformans* is thought to down-regulate macrophage iNOS RNA, thus mediating NO suppression (Kawakami et al., 1997b; Chinen et al., 1999). Inhibition of iNOS results in M2 macrophage activation, loss of anti-cryptococcal activity and progression of disease (Naslund et al., 1995; Arora et al., 2005, 2011; Xiao et al., 2008; Hardison et al., 2010; Leopold Wager et al., 2015). In addition, macrophages from iNOS deficient mice or from WT mice cultured with iNOS inhibitors are unable to control the intracellular proliferation of *C. neoformans*, even in the presence of intact ROS production (Leopold Wager et al., 2015). This reveals that NO, and not ROS, is the mechanism used by M1 macrophages to control *C. neoformans* in mice. However, how well this finding translates to human macrophages is a point of contention as the human iNOS gene is generally epigenetically silenced by CpG methylation, histone modifications and chromatin compaction (Gross et al., 2014). Interestingly, other studies have shown granulomas from pulmonary tissues in humans and non-human primates contain $iNOS^+$ macrophages (Facchetti et al., 1999; Mattila et al., 2013), suggesting that human macrophages are capable of NO production that could control cryptococcal growth. Further studies that would characterize the human macrophage response to *Cryptococcus* are required to comprehensively address this question.

## Survival and Replication in Macrophages

Following phagocytosis, cryptococci have been shown to proliferate in the macrophage phagosome (Tucker and Casadevall, 2002; Voelz et al., 2009), suggesting that these phagocytes are a preferred niche of the yeast where it can hide from recognition by the host's immune system (Johnston and May, 2013). A clinical study recently examined 65 clinical isolates of *C. neoformans* and demonstrated that strains with high rates of phagocytosis by macrophages with low intracellular proliferation *in vitro* coincided with higher CSF fungal burdens and, paradoxically, long-term survival of HIV patients (Sabiiti et al., 2014). The strains with high uptake rates were hypocapsular, had enhanced laccase activity, and were more resistant to patient antifungal treatment (Sabiiti et al., 2014). This study demonstrates that cryptococcal-phagocyte interactions are a major contribution to human clinical presentation and outcome.

In the phagosome, cryptococci are exposed to low pH, ROS and NO. Studies have shown that *C. neoformans* does not actively avoid acidification of the phagolysosome in macrophages,

but actually proliferate better in acidic rather than alkaline conditions (Diamond and Bennett, 1973; Levitz et al., 1999). Interestingly, live, but not heat killed, *C. neoformans* can induce premature removal of phagosome markers Rab5 and Rab11 (early phagosome markers; Smith et al., 2015). In contrast to previous studies, Smith et al. (2015) have recently shown that *C. neoformans* can hinder significant acidification of the phagosome, calcium flux and protease activity, rendering the phagosome permissive to cryptococcal proliferation in both the J774.1 macrophage-like cell line and human monocyte derived macrophages. This study also demonstrated that several virulence attenuated mutants are able to prevent phagosome maturation (Smith et al., 2015), suggesting that an unknown mechanism regulates this process. Studies have shown that incubation of murine macrophages with *C. neoformans* can result in host damage via activation of stress pathway including HIF-1α, receptor-interacting protein 1, apoptosis-inducing factor and mitochondrial depolarization (Coelho et al., 2015). A recent study by Davis et al. (2015) showed that live *C. neoformans* can cause lysosomal damage in bone marrow derived macrophages (BMM) which increases over time (Davis et al., 2015). Stimulation of BMMs with IFN-γ negated the lysosomal damage and increased killing of the yeast (Davis et al., 2015), suggesting that the induction of lysosomal damage is a potential survival strategy of *C. neoformans* that is counteracted when macrophages are activated to the M1 phenotype.

Phospholipase B, a known virulence factor of *Cryptococcus*, is a phospholipid modifying enzyme found at the cell surface (Djordjevic et al., 2005). This enzyme is active under acidic conditions at 37°C, as found in macrophage lysosomes. Deletion of *PLB1* in *C. neoformans* results in attenuated virulence, increased capsular diameter and size in the phagosome and in the lung (Evans et al., 2015). *PLB1* is also required for proliferation and survival in macrophages, as well as for dissemination to the CNS (Evans et al., 2015). Another cryptococcal protein, Fbp1, is critical for survival and proliferation in the macrophage phagosome and for dissemination from the lungs (Liu and Xue, 2014). Fbp1, along with its substrate inositol phosphosphingolipid-phospholipase C, is required for resistance to NO and dissemination (Liu and Xue, 2014).

*Cryptococcus neoformans* is capable of surviving even in a phagosome with ROS, in part due to absorption of ROS by the capsule (Zaragoza et al., 2008). In addition, the plasma membrane high-affinity Cch1-Mid1 calcium channel (CMC) of *C. neoformans* promotes cryptococcal survival during exposure to oxidative stress (Vu et al., 2015). Cch1 mutants have decreased survival in J774.1A macrophages, however, *C. neoformans* deficient in both Cch1 and Mid1 maintain resistance to the ROS, suggesting a compensatory mechanism in the yeast (Vu et al., 2015). *C. gattii*, however, has been shown to use a novel mechanism in response to ROS. The *C. gattii* Vancouver Island outbreak strain R265 induces to tubularization of its mitochondria, which can facilitate the growth of nearby "normal" *C. gattii* cells (Voelz et al., 2014). This enables the cryptococci to establish an intracellular niche in the macrophage, increasing pathogenesis. There was no observed correlation in *C. neoformans* between mitochondria tubularization and intracellular proliferation rate (Voelz et al., 2014), suggesting a unique mechanism of *C. gattii*.

## Trafficking Through the Blood Brain Barrier

Remarkably, *C. neoformans* is capable of non-lytic exocytosis, where the yeast cells burst out of the macrophage without lysing the phagocyte, again, preventing a response from the host (Alvarez and Casadevall, 2006; Johnston and May, 2010; Nicola et al., 2011). The ability of *Cryptococcus* to proliferate inside macrophages and then escape is the basis for one hypothesis as to how *C. neoformans* traffics across the BBB to cause meningoencephalitis in immunocompromised hosts. It has been posited that macrophages act as a "Trojan horse" carrying the yeast-like cells into the CNS while hidden from the immune system (Liu et al., 2012). Histological sections of *C. neoformans* infected brain tissue show cryptococci within macrophage-like cells either within or outside capillaries (Chretien et al., 2002). A second hypothesis is paracellular crossing of the BBB due to loss of integrity in the tight junctions or injury to the brain endothelium. These events compromise the BBB, leaving openings for the yeast cells to cross into the CNS (Ibrahim et al., 1995; Chen et al., 2003; Olszewski et al., 2004; Charlier et al., 2005). Third, the transcellular pathway is a hypothesis in which cryptococcal cells transmigrate via adherence to and internalization by the brain endothelium from the luminal surface (blood side) to the abluminal side (brain side) of the BBB (Chang et al., 2004; Liu et al., 2012; Sabiiti and May, 2012). The metalloprotease Mpr1 of *C. neoformans* is an extracellular protein that is required for migration across the BBB as it promotes adherence of the yeast cells to the brain endothelial cells (Vu et al., 2014). *C. neoformans* deficient in Mpr1 is able to survive in J774.1 macrophages (Vu et al., 2014), providing additional evidence for the transcellular pathway hypothesis.

Murine studies suggest that *C. gattii* does not traffic to the brain as readily as *C. neoformans* (Ngamskulrungroj et al., 2012), suggesting that dissemination to the CNS is not a primary target of *C. gattii*. *C. neoformans* is phagocytosed more efficiently by THP-1 macrophage-like human cells regardless of prior stimulation of the macrophage-like cells with IFN-γ (Sorrell et al., 2015). Remarkably, more *C. neoformans*-loaded macrophages traveled across the human brain endothelial cell line monolayer (a model of the BBB) than *C. gattii*-loaded macrophages (Sorrell et al., 2015). *C. neoformans* also had a higher rate of expulsion from the THP-1 macrophages (Sorrell et al., 2015), further validating the Trojan horse hypothesis. All hypotheses concerning trafficking of *C. neoformans* to the CNS are supported by strong evidence as discussed above. A likely explanation is that all mechanisms of trafficking are utilized by *C. neoformans* to gain access to this privileged site.

# DENDRITIC CELLS

Dendritic cells are innate cells that act as sentinels of the immune system. These cells are phagocytes, but also have the ability to present antigen to naïve T cells in order to direct

the adaptive immune response. Initial *in vitro* studies of DCs with *C. neoformans* showed that DCs are involved in detection, binding, phagocytosis, processing, antigen presentation, T cell activation, and killing of the organism (Bauman et al., 2000, 2003; Wozniak et al., 2006; Wozniak and Levitz, 2008). Early studies showed that Langerhans cells and myeloid DCs are necessary for the induction of protective immune responses against *C. neoformans* (Bauman et al., 2000).

Uptake of *C. neoformans* by DCs requires opsonization with either anti-capsular antibody or complement, due to the anti-phagocytic polysaccharide capsule surrounding the cryptococcal organisms (Kelly et al., 2005). In contrast to encapsulated strains, phagocytosis of acapsular mutant *C. neoformans* strains by DCs requires MR and FcγR II (Syme et al., 2002). Toll-like receptor 2 (TLR2) and TLR4 are not important in uptake of *C. neoformans* or activation of DCs by the fungus (Nakamura et al., 2006). Following phagocytosis by DCs, cryptococci translocate to the endosomal compartment followed by the lysosomal compartment, where they are killed by oxidative and non-oxidative mechanisms (Kelly et al., 2005; Artavanis-Tsakonas et al., 2006; Wozniak and Levitz, 2008; Hole et al., 2012). Studies examining DC lysosomal extract *in vitro* showed direct anti-cryptococcal activity (Wozniak and Levitz, 2008; Hole et al., 2012). Purified lysosomal enzymes, specifically cathepsin B, inhibit cryptococcal growth by cell wall damage followed by osmotic lysis of the cryptococcal cells (Hole et al., 2012).

During a cryptococcal infection in a mouse model, cryptococci are rapidly internalized by pulmonary DCs (Wozniak et al., 2006). These DCs increase surface expression of costimulatory molecules CD80 and CD86 as well as MHC II by D7 post-infection. DCs isolated from infected lungs present cryptococcal mannoprotein (MP) to MP-specific T cells and induced T cell activation and proliferation *ex vivo* (Wozniak et al., 2006). Furthermore, DC phagocytosis of cryptococcal MP in the presence of the appropriate adjuvant, such as the Th1-type inducing adjuvant CpG, induces production of protective Th1-type cytokines (Mansour et al., 2002; Dan et al., 2008a). Upon uptake of cryptococcal MP, DCs express markers of activation and maturation including MHC I and MHC II as well as CD40, CD80, and CD86 (Pietrella et al., 2005; Dan et al., 2008a,b). MP induces human DCs to secrete IL-12 and TNF-α, which are associated with the protective Th1-type immune response, and also leads to Iκβα phosphorylation (Pietrella et al., 2005). MP-loaded DCs are efficient stimulators of T cells resulting in CD4 and CD8 proliferation (Pietrella et al., 2005). Additional studies revealed that the interaction of *C. neoformans* with DCs, but not macrophages, induced the production of IL-12 and IL-23, two cytokines associated with protection against cryptococcosis (Kleinschek et al., 2010). Though macrophages are capable of presenting antigen to activated T cells, it is the DCs that are the most efficient antigen presenting cell type to present *C. neoformans* mitogen, and only a small number of DCs are needed for antigen presentation to T cells (Syme et al., 2002).

Dendritic cells and alveolar macrophages are required for protection against cryptococcal infection and are needed early in host defense (Osterholzer et al., 2009a). Depletion of DCs abrogated the T cell response in mice (Mansour et al., 2006). Depletion of $CD11c^+$ cells using a CD11cDTR mouse during cryptococcal infection lead to mortality within 6 days post-infection. Death was associated with neutrophilic bronchopneumonia and alveolar damage (Osterholzer et al., 2009a). The recruitment of monocyte-derived DCs into the lung appears to be dependent on the chemokine receptor CCR2. CCR2 KO mice infected with *C. neoformans* show impaired DC recruitment and the mice developed features of a Th2-type response including persistent infection, bronchovascular collagen deposition, and increased IL-4 production (Osterholzer et al., 2008). CCR2-dependent recruitment of DCs into *C. neoformans* infected lungs was due to increased recruitment of $Ly\text{-}6C^{high}$ monocytes that differentiate into $CD11b^+$ DCs in the lungs (Osterholzer et al., 2009a). These data suggest that CCR2 is required for the recruitment of DCs to the lungs and initiation of protective immune responses during a cryptococcal infection. (Osterholzer et al., 2008).

Cryptococcal capsule prevents phagocytosis of the organism (in the absence of opsonization by complement or antibody). In addition to its role in preventing phagocytosis by DCs and other phagocytes, the main capsular polysaccharide, GXM, has profound suppressive effects on immune responses (Yauch et al., 2006; Zaragoza et al., 2009). Cryptococcal capsule interferes with both DC activation and maturation (Vecchiarelli et al., 2003; Lupo et al., 2008; Grijpstra et al., 2009). In contrast, acapsular strains phagocytosed by DCs induce surface expression of MHC II and other costimulatory molecules, whereas the encapsulated strains do not induce activation unless opsonized by an anti-GXM antibody which is recognized by CD32 and CD16 (Vecchiarelli et al., 2003). Acapsular mutant strain cap56Δ induces human DC activation as seen by increased CD80 and CD86 surface expression (Grijpstra et al., 2009). In addition to affecting DC maturation, capsular material can influence DC gene expression of several cytokines and chemokines associated with protective responses as well as genes associated with antigen presentation (Lupo et al., 2008). Encapsulated strains induce a down-regulation of cytokine genes and inhibited the induction of the genes for cytokine and chemokine production as well as antigen presentation (Lupo et al., 2008).

Although the Th1-type immune response is typically associated with protective anti-cryptococcal responses, a Th2-type response may not always be detrimental under certain conditions. Experimental pulmonary infection of IL-4Rα KO mice with a *C. neoformans* serotype D strain resulted in higher fungal burden compared to WT mice (Grahnert et al., 2014). Additionally, IL-4Rα KO mice exhibited a defect in DC and macrophage recruitment due to a reduction in CCL2 and CCL20 chemokines. There was also a reduction in IFN-γ and NO production in the IL-4Rα KO mice (Grahnert et al., 2014). *In vitro* culture of DCs in the presence of *C. neoformans* and IL-4 resulted in increased IL-12 and reduced IL-10 production by the DCs (Grahnert et al., 2014). These data in conjunction with the increased fungal burden in the IL-4Rα KO mice suggest a beneficial role of IL-4Rα signaling early during infection, whereas

IL-4Rα signaling during the late phase of infection is detrimental (Grahnert et al., 2014).

Infection with virulent wild-type *C. neoformans* strains naturally induce a strong Th2-type immune response. Urease produced by *C. neoformans* directed a strong Th2-type immune response and lead to a significant increase in the amount of immature DCs that accumulated in the lung-associated lymph nodes (Osterholzer et al., 2009b). Using a tetramer for chitin deacetylase 2 (CDA2), Wiesner et al. (2015) were able to isolate *Cryptococcus*-specific Th2 $CD4^+$ T cells and found that chitin recognition via chitotriosidase leads to the induction of Th2 cells by DCs. Using multiple DC KO and DC depleted mouse models, Wiesner et al. (2015) also demonstrated that lung-resident $CD11b^+$ IRF4-dependent conventional DCs are responsible for the induction of Th2-type T cells. This indicates that DCs can have a protective or a non-protective role in the immune response to *C. neoformans*. The ability of DCs to react protectively or non-protectively suggests that these cells are heavily influenced by their environment, a fact that could be exploited for development of novel immunotherapies to combat cryptococcosis.

The interaction of DCs with *C. gattii* can differ dramatically from *C. neoformans*. Human monocyte-derived DCs can kill *C. gattii*, but this does not induce DC maturation (**Figure 2**; Huston et al., 2013). Even after uptake and processing of *C. gattii* by human DCs, they do not increase expression of MHC class II, CD86, CD83, CD80, and CCR7, which results in defects in T cell responses (Huston et al., 2013). In addition, DCs that kill *C. gattii* do not trigger a release of TNF-α, which is important for cryptococcal clearance (Huston et al., 2013). In mice infected with *C. gattii*, the DCs express much lower levels of surface MHC II and IL-12 or IL-23 transcripts and fail to induce effective Th1 and Th17 differentiation *in vitro* compared to mice infected with *C. neoformans* (Angkasekwinai et al., 2014). In a DC vaccine model for *C. gattii*, uptake of an acapsular mutant by DCs induced expression of costimulatory molecules and inflammatory cytokines (Ueno et al., 2015). Mice immunized intravenously with BMDCs pulsed with this acapsular mutant and challenged with *C. gattii* R265 showed significantly less pathology, reduced fungal burden, significantly increased survival compared to controls. Immunized mice had significantly increased lung and spleen lymphocytes producing IL-17A, IFN-γ, and TNF-α compared to non-immunized mice, and protection was significantly reduced IFN-γ KO mice (Ueno et al., 2015). Altogether, prevention of DC maturation by *C. gattii* could explain how this yeast causes disease in seemingly immunocompetent individuals.

## NEUTROPHILS

Neutrophils are phagocytes that are recruited to the lungs during cryptococcal infection and can kill *C. neoformans*. However, the importance of neutrophils in protective responses against cryptococcal infection is unclear. Human neutrophils, polymorphonuclear leukocytes (PMNs), have been shown to kill *C. neoformans in vitro* by both oxidative and non-oxidative mechanisms (Mambula et al., 2000). Treatment with inhibitors of the respiratory burst only partially reduced the anti-cryptococcal activity of human PMNs (Mambula et al., 2000). The non-oxidative anti-cryptococcal activity of human PMNs was shown to be mediated by calprotectin and defensins (Mambula et al., 2000). Mice lacking myeloperoxidase (MPO), an enzyme associated with neutrophil antimicrobial activity, succumb to the infection faster than WT mice upon infection with *C. neoformans* either intranasally or intravenously (Aratani et al., 2006). *In vitro*, neutrophils migrate toward cryptococcal cells using the complement C5a-C5aR pathway, and the interaction leads to phagocytosis and killing of *C. neoformans* and enhances activation of Erk and p38 mitogen activated protein kinases (MAPK) in neutrophils (Sun et al., 2016). Interestingly, inhibition of p38 MAPK pathway significantly decreased neutrophil migration and cryptococcal killing (Sun et al., 2016). Further studies showed that neutrophils can migrate into the brain microvasculature, phagocytose the cryptococcal cells before they are able to migrate into the brain parenchyma, and re-enter the circulation, effectively removing the cryptococci from the brain (Zhang et al., 2015b).

While neutrophils can kill *C. neoformans*, the pathogen can also modulate the neutrophil response. Cryptococcal capsular and cell wall components can inhibit neutrophil migration (Coenjaerts et al., 2001; Ellerbroek et al., 2004) and can inhibit the production of neutrophil extracellular traps (NETs; Rocha et al., 2015). In addition, melanized *C. neoformans* cells are able to abrogate the killing activity of neutrophils by interfering with sphingomyelin synthase (SMS) activity, which is required for cryptococcal killing by neutrophils (Qureshi et al., 2010, 2011). Cryptococcal cells can prevent neutrophil migration by secreting capsular components, which activates microglia to produce IL-8 (a neutrophil chemoattractant) and also reduces the expression of L-selectin (CD62L) on the neutrophil surface in humans with disseminated cryptococcosis, reducing neutrophil migration. Neutrophil endothelial rolling and production of surface expression of TNF-α receptor are also impaired due to cryptococcal capsular polysaccharide (reviewed in Urban et al., 2006).

Neutrophil depletion in mice during protective immune responses does not affect pulmonary fungal burden, indicating that neutrophils are not required for cryptococcal clearance (Wozniak et al., 2012). These data further support the observation by Mednick et al. (2003) that neutropenic mice given a pulmonary *C. neoformans* infection survived significantly longer than control mice with intact neutrophils, therefore indicating that neutrophils are not necessary for protective responses against cryptococcal infection. Furthermore, the presence of neutrophils in the lungs during cryptococcal infection can cause additional damage to the host (Osterholzer et al., 2009a).

In a rat model of *C. gattii* infection, there is an early recruitment of neutrophils into the lungs, but phagocytosis of cryptococci is not observed (Wright et al., 2002). *C. gattii* inhibits or fails to induce migration of neutrophils to the site of infection, thereby impeding an inflammatory response (Cheng et al., 2009). In addition, encapsulated *C. gattii* environmental strains that produced extracellular fibrils (which may be

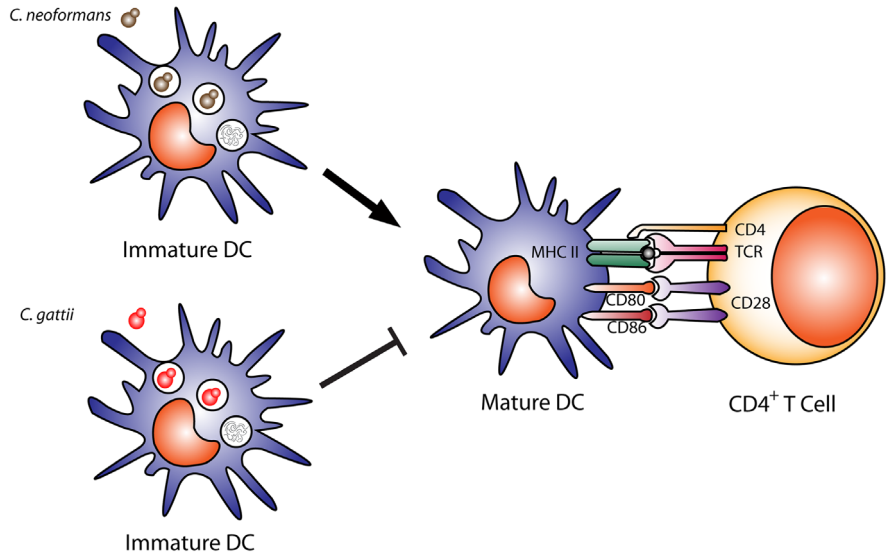


**FIGURE 2 | Dendritic cell cryptococcal killing and antigen presentation.** Immature dendritic cells efficiently phagocytose and kill opsonized *Cryptococcus neoformans* cells, leading to DC maturation, shown by upregulation of costimulatory molecules (MHC II, CD80, CD86) and antigen presentation to $CD4^+$ T cells. In contrast, *C. gattii* can also be phagocytosed and killed by DCs, but leads to a downregulation of DC maturation and expression of costimulatory molecules MHC II, CD80, CD86 and reduces antigen presentation to T cells.

important in cryptococcal cell communication or cryptococcal-host communication) were resistant to neutrophil killing, even when neutrophils produced NETs (Springer et al., 2010).

## PATTERN RECOGNITION RECEPTORS

In order for the phagocytic cell to take up a pathogen, it has to recognize that the pathogen is foreign. The cell does this by recognition of PAMPs via germline-encoded PRRs present either on the cell surface or within distinct intracellular compartments. These PRRs include TLRs, C-type lectin receptors (CLRs), NOD-like receptors and others. PRRs recognize a wide range of bacterial, fungal, and viral PAMPS, including lipopeptides, peptidoglycan, β-glucans mannan, and pathogen DNA and RNA (**Figure 3**).

One of the main virulence factors of *Cryptococcus* is the anti-phagocytic polysaccharide capsule (Zaragoza et al., 2009). TLR2 and TLR4 have been shown to recognize cryptococcal GXM (Shoham et al., 2001; Yauch et al., 2004, 2005). GXM was shown to bind to both TLR2 and TLR4 with the co-receptor CD14 but failed to activate the MAPK pathway and produce TNF-α (Shoham et al., 2001). TLR2 KO mice were more susceptible to cryptococcal infection than WT control mice; however, there was no difference in survival in C3H/HeJ mice which have a non-functional TLR4, compared to control mice (Yauch et al., 2004). Mice deficient in the adaptor molecule MyD88 (myeloid differentiation primary response gene 88), which is used by all TLRs except TLR3, exhibited a significant increase in mortality compared to WT mice and succumbed to a cryptococcal infection faster than the TLR2 KO mice, suggesting that MyD88 is required for protection against *C. neoformans* (Yauch et al., 2004; Biondo et al., 2005). Peritoneal macrophages from TLR2 KO or MyD88 KO mice exhibit reduced production of TNF-α when cultured with *C. neoformans in vitro* (Biondo et al., 2005). Furthermore, TLR2 KO and MyD88 KO mice showed decreased expression of TNF-α, IFN-γ, and IL-12p40 transcripts in the lungs, brain, and spleen during infection with *C. neoformans* (Biondo et al., 2005). Peripheral blood mononuclear cells (PBMCs) isolated from patients with cryptococcal meningitis exhibited a significant reduction in TLR2 expression compared to healthy control PBMCs and blocking TLR2 on PBMCs led to a reduction in IL-12p70 and IFN-γ when stimulated with *Cryptococcus* (Zhang et al., 2015a). While TLR2 and TLR4 can recognize cryptococcal GXM, these receptors may only play a minor role in protection to *C. neoformans* infection (Nakamura et al., 2006). What is clear is that MyD88 is necessary for protection against *C. neoformans,* suggesting that other TLRs are required.

TLR9 is an endosomal PRR that recognizes unmethylated pathogen CpG DNA following its ingestion and degradation by the immune cell. Uptake of *C. neoformans* leads to the recruitment of TLR9 to the fungal phagosome (Kasperkovitz et al., 2011), and TLR9 KO mice were more susceptible to cryptococcal infection than WT control mice (Wang et al., 2011). Mice treated with the TLR9 antagonist CpG-ODN 3 days before infection with *C. neoformans* strain 52D, a moderately virulent clinical isolate, had reduced fungal burden and pulmonary eosinophilia as well as increased IFN-γ production by $CD8^+$ T cells (Edwards et al., 2005). Cryptococcal DNA can activate DCs via TLR9 recognition to produce IL-12p40 and express CD40 (Nakamura et al., 2008). This activity was dependent on the methylation of the DNA as methylase treatment of the DNA led to a reduction of IL-12p40 produced by the DCs. Genetic ablation of TLR9 or MyD88 completely abrogated the

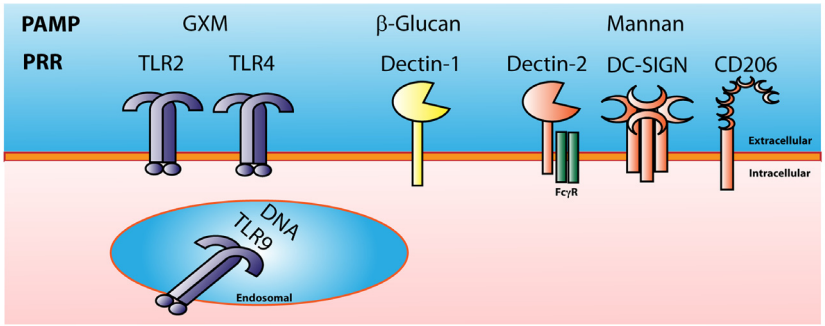


**FIGURE 3 | Cryptococcal Pattern Recognition Receptors.** *Cryptococcus* has multiple pathogen associated molecular patterns (PAMPs) that are recognized by many types of pattern recognition receptors (PRRs). GXM is recognized by TLR2 and TLR4. Cryptococcal mannan can be recognized by the C-type lectin receptors Dectin-2, DC-SIGN, and CD206 or mannose receptor. B-glucans found in the cryptococcal cell wall can be recognized by Dectin-1, and cryptococcal DNA can be recognized by the endosomal receptor TLR9.

effect of cryptococcal DNA on the DCs, demonstrating that DNA recognition and activation by DCs was dependent on TLR9 and MyD88 (Nakamura et al., 2008). Culture supernatants from *C. neoformans* are able to dampen the DC response to cryptococcal DNA (Yamamoto et al., 2011). The inhibitory effects of the supernatants were reduced by heat or trypsin treatment indicating that *C. neoformans* secretes proteinaceous molecules that suppress activation of DCs by cryptococcal DNA (Yamamoto et al., 2011). It was shown that the nucleic acid sequence for cryptococcal URA5 specifically activates DCs through a TLR9-mediated signaling pathway using a mechanism that is different than the canonical CpG motif that is associated with TLR9 signaling (Tanaka et al., 2012). Cryptococcal infection in TLR9 KO mice leads to decreased IFN-γ and TNF-α and an increase in IL-4 compared with WT mice (Zhang et al., 2010). The increased IL-4 in *C. neoformans*-infected TLR9 KO mice led to increased M2 macrophage activation markers arginase and FIZZ1 and a decrease in M1 macrophage marker iNOS compared to WT mice (Zhang et al., 2010). Ablation of TLR9 led to reduction of $CD11b^+$ DCs and CCL7 in the lungs during the afferent phase (week 1) and reduced the pulmonary accumulation of $CD11b^+$ DCs during the efferent phase [week 3; (Qiu et al., 2012)].

The fungal cell surface is covered in carbohydrates including β-glucans and mannan which are recognized by C-type lectin receptors (CLRs). The β-glucan CLR Dectin-1 has been shown to be important in protection to *Aspergillus*, *Candida,* and *Pneumocystis* infections (Steele et al., 2005; Saijo et al., 2007; Taylor et al., 2007). Dectin-1 has been shown to bind to the β-glucans found on cryptococcal spores (Giles et al., 2009), however, there was no significant difference in disease progression in Dectin-1 KO mice compared to WT mice during cryptococcal infection indicating that Dectin-1 may not be required for host defense to *C. neoformans* (Nakamura et al., 2007).

There are multiple receptors that recognize mannan including the CLRs Dectin-2, MR (CD206), and DC-SIGN. Dectin-2 has been shown to recognize mannan from multiple fungal organisms (Hardison and Brown, 2012). Dectin-2 KO BMDCs incubated with *C. neoformans* failed to produce IL-12p40 and TNF-α as well as failed to increase surface expression of CD86 and MHC II compared to WT BMDCs (Nakamura et al., 2015). In addition, Dectin-2 KO mice infected with *C. neoformans* exhibited higher levels of Th2-type cytokines which are associated with non-protective immune responses compared to infected WT mice (Nakamura et al., 2015). Both MR and DC-SIGN have been shown to recognize heavily mannosylated cryptococcal mannoproteins (Mansour et al., 2006). Human and murine DCs are able to recognize and capture cryptococcal MPs by a mannose receptor (CD206) mediated process (Mansour et al., 2006). MR KO mice succumb to *C. neoformans* infection significantly faster compared to WT mice (Dan et al., 2008a). Cryptococcal MPs in combination with TLR ligands enhanced production of proinflammatory cytokines and chemokines from DCs as well as enhanced MP-specific MHC II-restricted $CD4^+$ T-cell responses (Dan et al., 2008a). Increased surface expression of CD206, which is upregulated on M2 macrophages, results in increased phagocytosis but is accompanied by decreased intracellular killing and TNF-α production (Dan et al., 2008a).

## TARGETING MACROPHAGES AND DCS FOR VACCINES/IMMUNOTHERAPIES

As demonstrated in the above discussion, innate phagocytes play a pivotal role in the recognition of *Cryptococcus*, anti-fungal activity, and prevention of cryptococcosis. Identification of the specific PRRs utilized by innate immune cells to recognize *Cryptococcus* and lead to the yeast's internalization and destruction could provide a novel target for treatment. Studies designed to elucidate the host PRR must keep in mind that different immune cells could utilize different PRRs to recognize *Cryptococcus*. In addition, the PAMPs for *C. neoformans* and *C. gattii* may not be the same. More study is required to identify the specific PAMP and PRR combination that elicits protective immune responses.

As *C. gattii* tends to cause disease in immunocompetent individuals, traditional methods of vaccination that elicit a memory T cell response could prove fruitful, however, this

has not yet been achieved. High-risk groups for cryptococcosis caused by *C. neoformans*, including AIDS patients, are deficient in $CD4^+$ T cells. Nonetheless, recent evidence does suggest that development of a vaccine against *Cryptococcus* is feasible (reviewed in Leopold Wager and Wormley, 2015). It has been shown that $CD8^+$ T cells can compensate for the loss of $CD4^+$ T cells (Lindell et al., 2005), thus targeting this adaptive immune cell population is possible. Interestingly, immunization of mice with the IFN-γ producing *C. neoformans* strain H99γ leads to protection in B cell deficient mice (Wozniak et al., 2009) as well as WT BALB/c mice that are depleted of both $CD4^+$/$CD8^+$ T cells during the challenge phase (Wozniak et al., 2011b), suggesting that protection can be achieved in the absence of traditional adaptive immunity. A relatively new concept allows for the generation of "innate memory" or "trained immunity." This memory occurs in innate cells, including NK cells and monocytes/macrophages in response to *Candida albicans* or simply by "training" via exposure to β-glucans (Quintin et al., 2012). The "trained" monocytes/macrophages have enhanced cytokine recall responses when challenged with *C. albicans*. The protective responses in these cells are non-specific and confer heightened responses following secondary exposure to an antigen, including *C. albicans*. Altogether, this evidence is proof-of-concept that protection can be achieved in immunocompromised individuals.

A novel delivery platform utilizing glucan particles is a promising approach to vaccine design that targets innate phagocytes, including macrophages and DCs. The glucan particles are isolated from baker's yeast (*Saccharomyces*) and are purified, hollow, and porous cell wall shells composed mostly of β-1,3-glucan (reviewed in Levitz et al., 2015). These stimulate dectin-1 and other PRRs on phagocytes and induce the production of protective cytokines such as IFN-γ and IL-17A (Huang et al., 2009, 2010, 2012, 2013; reviewed in Levitz et al., 2015). The glucan particles can be loaded with proteins, siRNA, DNA, and other small molecules. Immunization with these particles loaded with synthetic peptides of *Coccidioides* results in elevated Th1 and Th17 responses following challenge with *Coccidioides posadasii* (Hurtgen et al., 2012). A recently published study loaded the glucan particles with soluble alkaline extracts from two different attenuated *Cryptococcus* strains, acapsular strain cap59 and strain cda123 which lacks cell wall chitosan. Immunization of mice with the cryptococcal-loaded glucan particles resulted in increased survival following challenge with *C. neoformans* Kn99 and was associated with Th1 and Th17 immune responses (Specht et al., 2015). In addition, mice immunized with glucan particles loaded with cap59 extracts were partially protected against challenge with *C. gattii* strain R265 (Specht et al., 2015). Thus, these studies have identified novel vaccine candidates in the alkaline extracts from *C. neoformans*, and suggest that administration via this delivery system is promising for novel vaccine development.

Immunodominant cryptococcal proteins have recently been identified from both *C. neoformans* and *C. gattii* (Chaturvedi et al., 2013, 2014). Intranasal immunization of mice with cryptococcal cell wall and/or cytoplasmic protein fractions from *C. gattii* or immunodominant protein fractions from *C. neoformans* extended survival following challenge of the respective *Cryptococcus* sp (Chaturvedi et al., 2013, 2014). In addition, certain mannoproteins have been shown to elicit vaccine-mediate immunity, extending survival of *C. neoformans* infected mice (reviewed in Chaturvedi and Wormley, 2013; Levitz et al., 2015). PBMCs from patients who have recovered from cryptococcosis are able to proliferate and produce pro-inflammatory cytokines when stimulated with mannoproteins (Levitz and North, 1997). These proteins and other highly conserved antigens, including heat shock proteins, β-glucan, and glycolytic enzymes, are potential targets for the development of subunit vaccines. Vaccines that utilize recombinant proteins must take into account the posttranslational modifications of these cryptococcal proteins and how that affects immunogenicity.

Specific targeting of macrophage phenotype also has the potential for development of vaccines that would target the host and not the organism, thus limiting selective pressure on the fungus. Treatment of macrophages with IFN-γ results in the M1, fungicidal phenotype, while stimulation with IL-4/IL-13 results in the M2, cryptococcal growth-permissive phenotype (Olszewski et al., 2010; Leopold Wager and Wormley, 2014). It was recently demonstrated that macrophages first polarized to an M2 phenotype with IL-4 can be repolarized to an M1 phenotype following IFN-γ treatment (Davis et al., 2013), demonstrating the plasticity of macrophages in response to their cytokine milieu. These repolarized macrophages are fully functional M1 macrophages, produce NO and are anti-cryptococcal (Davis et al., 2013). Additionally, immunization of mice with the *C. neoformans* IFN-γ-producing strain (H99γ) and challenge with WT *C. neoformans* H99 results in complete protection and M1 macrophage activation (Hardison et al., 2012). This macrophage polarization is occurring even without exogenous IFN-γ produced by the organism, providing evidence for memory T cell responses and possibly for "innate memory" in the macrophages. Recent studies have also shown that DCs are capable of polarizing to a pro-inflammatory DC1 phenotype and anti-inflammatory DC2 phenotype, which may also play a role in cryptococcal immune responses (Guiducci et al., 2005; Cook et al., 2012). Taken together, these studies suggest that targeting the polarization of macrophages and/or DCs toward an anti-cryptococcal phenotype could provide a novel mechanism for the induction of protective responses against *Cryptococcus*. Thus, these innate phagocytes that are critical for the development of protective responses against cryptococci could also be the key to development of novel vaccines and/or immunotherapies to prevent cryptococcosis.

## CONCLUSION

The interactions between the host and the pathogen are critical for early control of the infection and, thus, the ability of the host to clear the infection. *Cryptococcus* has developed numerous effective strategies to either evade the immune system

or to modulate the host cells allowing survival and replication within the phagocyte. There is still much to learn about how the host cells react to *Cryptococcus* and how they allow themselves to be manipulated by the fungus, as well as how the yeast expertly modulates and evades the immune system. In addition, there is an added complexity when the site of infection and the stage of disease is taken into account. Future research should aim to determine the roles of specific molecular interactions between *Cryptococcus* and phagocytes, and how these interactions either contribute to or prevent pathogenesis. A comprehensive understanding of these small, yet substantial interactions will undoubtedly result in effective anti-cryptococcal therapies that will likely be translatable to other intracellular infections which rely so desperately on the early responses of phagocytes.

## AUTHOR CONTRIBUTIONS

CW, CH, KW, and FW contributed to the writing, editing, and revision of the manuscript.

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REVIEW
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# *Cryptococcus neoformans*: Tripping on Acid in the Phagolysosome

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*Cryptococcus neoformans* (Cn) is a basidiomycetous pathogenic yeast that is a frequent cause of meningoencephalitis in immunocompromised individuals. Cn is a facultative intracellular pathogen in mammals, insects and amoeba. Cn infection occurs after inhalation of spores or desiccated cells from the environment. After inhalation Cn localizes to the lungs where it can be phagocytosed by alveolar macrophages. Cn is surrounded by a polysaccharide capsule that helps the fungus survive in vivo by interfering with phagocytosis, quenching free radical bursts and shedding polysaccharides that negatively modulates the immune system. After phagocytosis, Cn resides within the phagosome that matures to become a phagolysosome, a process that results in the acidification of the phagolysosomal lumen. Cn replicates at a higher rate inside macrophages than in the extracellular environment, possibly as a result that the phagosomal pH is near that optimal for growth. Cn increases the phagolysosomal pH and modulates the dynamics of Rab GTPases interaction with the phagolysosome. Chemical manipulation of the phagolysosomal pH with drugs can result in direct and indirect killing of Cn and reduced non-lytic exocytosis. Phagolysosomal membrane damage after Cn infection occurs both *in vivo* and *in vitro*, and is required for Cn growth and survival. Macrophage treatment with IFN-γ reduces the phagolysosomal damage and increases intracellular killing of Cn. Studies on mice and humans show that treatment with IFN-γ can improve host control of the disease. However, the mechanism by which Cn mediates phagolysosomal membrane damage remains unknown but likely candidates are phospholipases and mechanical damage from an enlarging capsule. Here we review Cn intracellular interaction with a particular emphasis on phagosomal interactions and develop the notion that the extent of damage of the phagosomal membrane is a key determinant of the outcome of the Cn-macrophage interaction.

**Keywords:** ***Cryptococcus neoformans*, macrophage, phagolysosomal membrane damage, pH, Interferon γ**

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## Cn AS A FACULTATIVE INTRACELLULAR PATHOGEN

*Cryptococcus neoformans* (Cn), a basidiomycetous pathogenic yeast, is a relatively frequent cause of meningoencephalitis in immunocompromised individuals (Horgan et al., 1990; Thinyane et al., 2015). Cn is ubiquitous in the environment, inhabiting soils (Currie et al., 1994; Gugnani et al., 2005; Randhawa et al., 2008) and human infection occurs when aerosolized spores or desiccated fungal cells enter the lung via inhalation where Cn encounters the first line of defense: the alveolar macrophage (Feldmesser et al., 2000).Macrophages play a critical role in the pathogenesis of

cryptococcosis, ranging from control of infection to possible roles in persistence, latency and extrapulmonary dissemination. Although historically Cn was divided into two varieties known as *neoformans* and *gattii,* genetic studies have subsequently separated these varieties into two species. The species *Cryptococcus gattii* has the potential to cause disease in immunocompetent individuals and animals (Stephen et al., 2002; Hoang et al., 2004). However, this review will only focus on Cn, since most of the macrophage interaction studies have been done with Cn. Cn is an facultative intracellular pathogen in such diverse hosts as mammals, amoebae (Steenbergen et al., 2001) and insects (Tenor et al., 2015; Trevijano-Contador et al., 2015), and employs various virulence factors to subvert cellular defense mechanisms. The manner in which Cn interacts with amoeba and macrophages is similar, suggesting that selection pressures in soil could lead to the emergence of particular traits that confer the capacity for virulence, thus making this microbe an accidental pathogen for mammals (Casadevall, 2012). In mammals, Cn was established to be a facultative intracellular pathogen *in vivo* and *in vitro* almost two decades ago (Feldmesser et al., 2001). In subsequent years, several groups have made major contributions to our understanding of the pathogenic strategy of Cn and those advances will be reviewed here.

The most distinctive feature of Cn is the expression of a large polysaccharide capsule that is a major virulence factor. The capsule functions in virulence through numerous mechanisms including preventing phagocytosis, quenching free radical bursts and interfering with immune responses (Bulmer and Sans, 1967; Zaragoza et al., 2008). Another mechanism by which Cn avoids phagocytosis is by the formation of titan cells, which prevent ingestion as a result of their enormous size (Okagaki et al., 2010; Zaragoza et al., 2010; Okagaki and Nielsen, 2012). The antiphagocytic function of the capsule is particularly relevant for intracellular pathogenesis since this process requires ingestion of the fungus by phagocytic cells. In the absence of opsonins, the capsule interferes with phagocytosis such that ingestion of encapsulated cells by macrophages is markedly lower (Macura et al., 2007). However, in the presence of capsule specific antibody and complement opsonins mediate efficient phagocytosis as described (Voelz and May, 2010). Although all encapsulated strains are opsonized by capsule binding antibodies, not all strains are efficiently opsonized by complement (Zaragoza et al., 2003). The mechanism for strain differences in complement opsonization involves differences in the geography of complement deposition in the capsule. If complement is deposited near the capsule surface, it is an effective opsonin, while complement deposition in the deeper layers of the capsule places complement component 3 in a location where it cannot interact with the complement receptor resulting in poor phagocytosis (Zaragoza et al., 2003). In addition, the capsule complement deposition pattern can be affected by the use of serum from different species, capsule size, and composition and the chronological age of the fungus (Young and Kozel, 1993; Gates and Kozel, 2006; Cordero et al., 2011). Therefore, the ability of Cn to increase its capsule size during infection is a mechanism that helps avoid complement-mediated phagocytosis.

Cn is able to survive and replicates at a higher rate inside macrophage than in the extracellular environment (Diamond and Bennett, 1973; Feldmesser et al., 2000). This ability of Cn to survive and replicate inside macrophages correlates with the virulence of clinical isolates, and is associated with dissemination via a Trojan horse hypothesis whereby Cn can cross the blood brain barrier inside macrophages (Charlier et al., 2009; Alanio et al., 2011). After infection, Cn can persist in the host in a latent state inside macrophages and multinucleated giant cells in granulomas. Cn in this latent state can emerge and cause disease if the host immune status change from immunocompetent to immunocompromised (Shibuya et al., 2005; Saha et al., 2007; Alanio et al., 2015). The macrophage-Cn interaction can have three major outcomes: (1) intracellular killing of Cn or control growth by the macrophage; (2) lysis of the macrophage and release of Cn; and (3) non-lytic exocytosis in which both the macrophage and Cn survive (**Figure 1**). In addition, the phenomenon of macrophage to macrophage transfer of Cn cells has been described *in vitro* (Alvarez and Casadevall, 2007; Ma et al., 2007; Stukes et al., 2014). Of these possibilities exocytosis is the most common outcome (Stukes et al., 2014). Depletion of alveolar macrophage in rats and mice shows that the role of macrophages during Cn infection varies with the host species. Rat macrophages controlled Cn intracellular growth and were more resistant to pathogen-mediated lysis. When rat lung macrophages were depleted the animals became more vulnerable (Shao et al., 2005). In contrast, murine macrophage served as a replicative niche for Cn and growth of Cn inside the macrophage can result in lysis of the macrophage (Shao et al., 2005). Differences in mouse strain susceptibility to Cn infection correlates with macrophage permissiveness for fungal intracellular replication (Zaragoza et al., 2007) but these differences are not well understood.

Acidification of the phagosome is an important macrophage antimicrobial mechanism but certain pathogens are able to adapt to overcome or exploit these acidic environments (Criscitiello et al., 2013). Pinpointing the mechanism by which Cn is able to survive within the harsh environment of the phagolysosome can provide insight to develop ways to reduce the intracellular replication of Cn and potentially prevent/treat disease. During infection, Cn experiences different pH levels and ability to survive in the host requires survival in the slightly alkaline environment of the blood and the cerebrospinal fluid, and the acidic environment of the phagolysosome. Consequently we will review the studies that explore the interactions of Cn in these diverse ranges of pH and phagosomal maturation.

## Cn LOCALIZATION INTO THE PHAGOLYSOSOME

The localization of the microbe in tissue can play an important role on the virulence and survival of the pathogen. In tissues, such as lungs, that mount granulomatous responses, Cn is often found inside macrophages or in close approximation to them

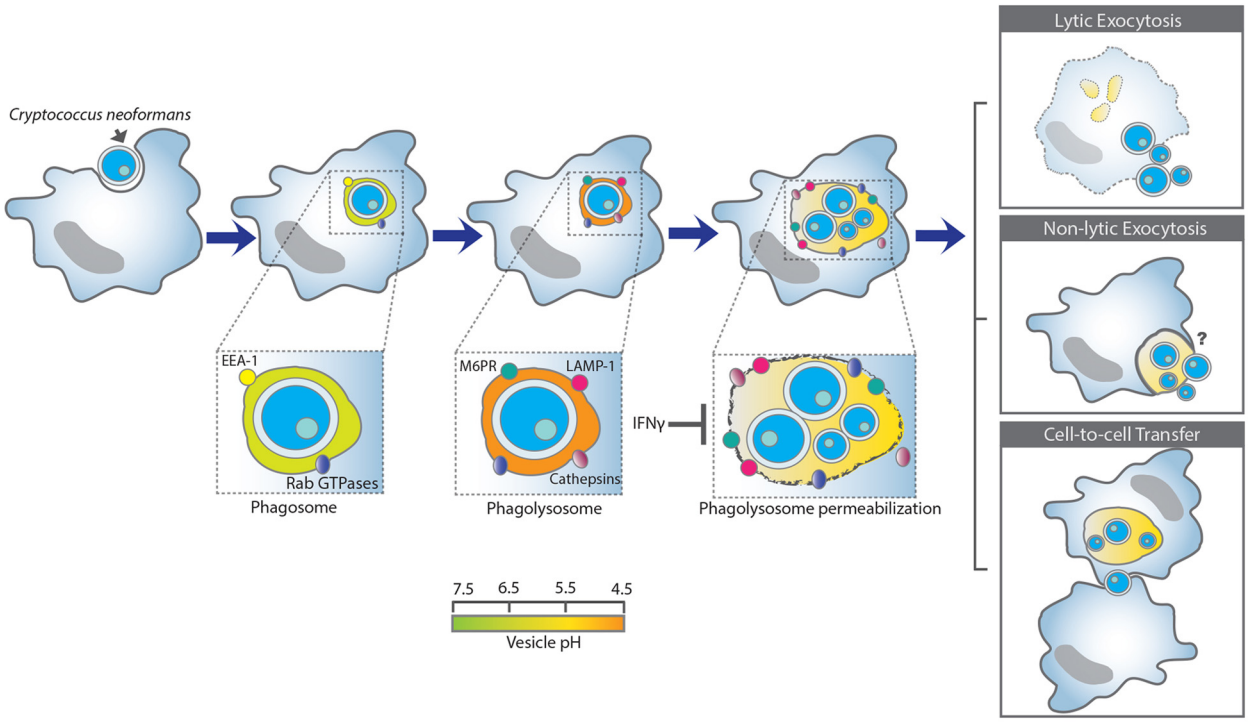


**FIGURE 1 | A schematic summary of the *Cryptococcus neoformans* and macrophage interaction.** Upon internalization Cn reside within phagosomes that mature into phagolysosomes by interacting and fusing with the early- and late- endosome, and lastly with the lysosome. Cn-containing phagolysosomes acidify shortly to around pH 4.3 but increased later to 5.3, which is less acidic than the normal phagolysosomal of pH 4.5 (Levitz et al., 1999). Phagolysosomal membrane permeabilization could contribute to the increase in pH, possibly by affecting the proton gradient require to maintain acidification and/or promoting leakage of contents into the cytoplasm and vice-versa. Phagosomal membrane damage favors Cn replication and survival. In contrast, IFN-γ reduces phagolysosomal membrane damage and promotes Cn killing (Davis et al., 2015). Phagosomal membrane integrity appears to be a key determinant of whether Cn lives or dies after ingestion. Intracellular Cn survival and replication can have three major outcomes: (1) lysis of the macrophage and release of Cn; (2) non-lytic exocytosis in which both the macrophage and Cn survive (non-lytic exocytosis can be complete or partial), and (3) cell-to-cell transfer in which one macrophage can pass a previously ingested Cn to another macrophage.

(Yamaoka et al., 1996; Kobayashi et al., 2001). To survive in their hosts, pathogenic microbes implement different strategies to avoid killing and degradation by the phagocytic cell, including inhibiting phagosome maturation (Clemens et al., 2000) or phagolysosome fusion (Horwitz, 1983), blocking phagosomal acidification (Horwitz and Maxfield, 1984) or escaping from the phagosome (Gaillard et al., 1987). By examining the localization of known endosomal and lysosomal markers, any inhibition of phagosome maturation or phagolysosome fusion during Cn infection can be observed. Studies done with Cn-infected human monocyte-derived macrophage and J774.16 macrophage-like cells showed co-localization of the yeast with the lysosomal-associated membrane protein (LAMP-1), a lysosomal marker (Levitz et al., 1999; Alvarez and Casadevall, 2006). Cn-containing phagosomes in murine bone marrow derived dendritic cells and monocyte derived human dendritic cells fused with the early endosome marker (EEA-1) and LAMP-1 as early as 10 minutes after incubation with Cn (Wozniak and Levitz, 2008). *In vivo* studies also showed that phagolysosomal fusion occurred by two hours after infection in the alveolar macrophage in a murine model (Feldmesser et al., 2000). Furthermore, studies with bone marrow derived dendritic cells expressing CD63-mRFP1 and Class II MHC-eGFP determined that these markers are recruited to Cn-containing vacuoles. Thus, class II MHC proteins are recruited before CD63 and CD63 recruitment is dependent on the acidification of the phagosome (Artavanis-Tsakonas et al., 2006). In contrast, LAMP-1 recruitment is independent of phagosome acidification (Artavanis-Tsakonas et al., 2006). Cryptococcal phagolysosomes have some characteristics of autophagomes such as manifesting LC3 (microtubule-associated protein 1 light chain 3 alpha) co-localization (Nicola et al., 2012).

Cn intracellular trafficking is conserved in *Drosophila melanogaster* S2 cells and J774.A1 cells, upon internalization by both cell types Cn co-localized with EEA-1, the late endosome marker, mannose-6-phosphatase receptor (M6PR), LAMP-1 and Cathepsin D, another lysosomal marker (Qin et al., 2011). Furthermore, surfactant protein D was shown to enhance phagocytosis of acapsular cryptococcal mutants by macrophages *in vitro* but the resulting Cn-containing phagolysosome shows a decrease in co-localization with LAMP-1 (Geunes-Boyer et al., 2009), suggesting that surfactant protein D influences Cn-containing phagosome maturation. Based on these studies, it was generally thought that Cn resided in the phagolysosome upon internalization by the macrophage and did not interfere

with phagosome maturation. However, new evidence based on the analysis of the Rab GTPases localization, which are early endosomes and phagosomes markers, revealed a different pattern for the interaction of these molecules with phagosomes containing live Cn, heat killed Cn or latex beads, suggesting that Cn influenced phagosome maturation. Rab 5 and 11 are recruited to the Cn-containing vesicle shortly after phagocytosis but their presence diminishes more rapidly in phagosomes containing live fungal cells than in those containing latex beads- or heat killed Cn-cells (Smith et al., 2015). However, the extent to which Cn affects phagosome maturation is unknown but it is likely that the effect is smaller than other pathogens, like *Mycobacteria tuberculosis*, which directly interfere with phagosome maturation by blocking fusion with late endosomes and lysosomes (Kelley and Schorey, 2003).

Phagosomal maturation and acidification are required for both the localization and optimal enzymatic activity of lysosomal proteases, respectively (Boya and Kroemer, 2008). Cn localization in the phagolysosome exposes the yeast to cathepsins, other hydrolases, and reactive oxygen species in the lysosomal lumens. Cathepsins are lysosome related proteases with a role in lysosomal protein recycling, Toll-like receptors signaling, extracellular matrix degradation, activation and inhibition of cytokines, antigen processing, and more recently have been implied in apoptosis. To activate apoptosis, cathepsins need to be released from the lysosomes into the cytosol via lysosomal membrane permeabilization (Conus and Simon, 2010). Lysosomal extracts purified from bone marrow derived dendritic cells can kill Cn in a dose dependent manner (Wozniak and Levitz, 2008). Analysis of purified lysosomal enzymes showed that Cathepsin B and Cathepsin L inhibited Cn growth, and that an inhibitor of Cathepsin B enzymatic activity surprisingly increased inhibition of Cn growth (Hole et al., 2012). These results suggested that Cn exposure to lysosomal proteases, specifically cathepsin B and L inhibited cryptococcal growth. *In vivo* analysis of Cathepsins B and Cathepsin L activity in Cn-containing phagolysosome has shown little evidence of enzymatic activity, but macrophages challenged with heat killed Cn show Cathepsin L activity (Smith et al., 2015). The discrepancy between these results might be attributed to the fact that in the first studies Cn was incubated *in vitro* with lysosomal extracts whereas in the later studies the analysis was done in infected cells. The Cn capsule confers resistance against reactive oxygen species (Zaragoza et al., 2008) and presumably protects the fungal cell during the lysosomal oxidative burst. However, as to whether the capsule also provides resistance against cathepsins and others proteases remain unclear. In this regard, the dense polysaccharide fibrillar network that surrounds the cell could provide protection by trapping cytotoxic enzymes but this mechanism remains to be shown.

In summary, the difference between cathepsin activity in phagosomes containing live and dead Cn suggests that Cn can affect cathepsin enzymatic activity but the mechanism is unknown. Given that phagosomes containing live Cn become progressively leaky during intracellular residence, one possible explanation for the discordance between the results obtained with live and dead Cn is that the negative cathepsin activity observed with the live cells is lost with a degraded phagosomal membrane.

## EFFECT OF pH ON THE Cn GROWTH (STUDIES INDEPENDENT OF HOST)

The response of Cn to pH is important in pathogenesis because the fungus goes from neutral to slightly alkaline conditions in extracellular body fluids to acidic conditions in the mature phagolysosomal compartment. An inverse correlation between growth of Cn and the pH of the growth medium was initially established more than 60 years ago (Mosberg and Mc, 1951; Howard, 1961), when several investigators reported that acidic milieus enhanced growth while alkaline conditions inhibited growth. More recent studies have confirmed those results by showing that Cn can grow in the pH range of 5–8 and also revealed that the optimal growth for Cn is at pH 5 (Levitz et al., 1997). These studies also established differences in the susceptibility of Cn strains to pH and a dependence on temperature such that fungal cells were less resistant at 37° to alkaline pH. However, a Cn strain deficient in the $Ca^{+2}$-regulated protein Calcineurin is more susceptible to alkaline pH, and become avirulent in a rabbit model of cryptococcal meningitis (Odom et al., 1997). Interestingly, a Cn strain lacking glycosphingolipid glucosylceramide was avirulent in a murine animal model when challenged intranasally but was virulent when the infection was done intravenously, suggesting that the lung environment controlled the infection. The glycosphingolipid glucosylceramide mutant was arrested in the S and $G_2$/M phase at pH 7.4 in 5% $CO_2$, but is not affected at pH 4 in 5% $CO_2$ (Rittershaus et al., 2006). One explanation for these observations is that during the intranasal infection the glycosphingolipid glucosylceramide mutant is not able to replicate in the alkaline extracellular environment of the lung. Those fungal cells that are ingested by the alveolar macrophage can replicate in the acidic environment of the phagolysosome but these are controlled by the formation of granulomas. In contrast, after intravenous infection the glycosphingolipid glucosylceramide mutant growth was also arrested in the alkaline environment of the blood, but was able to replicate once it invaded other organs and reside in abscess with an acidic environment optimal for the growth of the mutant (Rittershaus et al., 2006). These studies suggest that the ability of Cn to grow at different pH is important for pathogenicity.

The conserved Rim101 pathway mediates fungal response to extracellular neutral/alkaline pH, for reviews see (Davis, 2009; Cornet and Gaillardin, 2014). Extensive work has been done to identify the homologues of the Rim101 pathway and the role of Rim101 pathway during Cn–host interaction. Cn Rim101 mutants show a capsule defect, altered cell wall composition and increased susceptibility to different host induced stress stimulus (O'Meara et al., 2014). Recently, the Rim pathway was shown to be activated by the increase of pH, with the protein Rra1 functioning as a pH sensor, but others components of the pH sensing complex have not been identified (Ost et al., 2015).

Further studies of this pathway may shed light unto how Cn is able to modulate gene expression to survive between the different pH environments it encounter during host infection.

## Cn AND PHAGOLYSOSOMAL ACIDIFICATION

Phagosome maturation results in the acidification of the phagosomal lumen, creating an optimal environment for the proteases recruited to that site during the maturation process. In the next two sections, we review what is known about phagosomal acidification during Cn infection of phagocytic cells and the effect of chemical modulation of phagosomal acidification on the outcome of Cn infection. Initial studies measuring *in vivo* phagolysosomal pH done in rabbit alveolar macrophages after 24 h infection using fluorescein-labeled heat-killed cryptococcal cells revealed an average pH 5 and 5.2 for phagolysosomes containing heat killed Cn and fluorescein-labeled silica particles, respectively. They noted that approximately 2% of the phagolysosomes containing heat-killed Cn had a pH of 6.5, which never occurred with the silica particles (Nessa et al., 1997). The pH of Cn-containing phagolysosomes was also measured in monocyte-derived macrophage using live Cn and heat-killed Cn after 3 and 24 h infection. The pH of phagolysosomes containing heat-killed Cn remained stable over time, ranging from 5.2 to 5.1, but the pH of phagolysosomes containing live Cn increased from 4.3 to 5.3, which closely matches the optimal pH for fungal growth. The phagolysosomal pH of neutrophils infected with live Cn remained constant over a 3 h period hovering between 5.2 and 5.0 (Levitz et al., 1999). A more recent study suggests that live Cn, but not heat-killed Cn, could block acidification of the phagolysosome (Smith et al., 2015). The differences in these studies could reflect the use of different cell models, Cn strains, and/or technical approaches. Cn mutants in phospholipase B, Sec14 secretion system, urease expression, and the acapsular mutant maintained their ability to prevent acidification, suggesting that prevention of acidification occurred through an independent process that is unrelated to those virulence factors (Smith et al., 2015). However, a strain of Cn that overexpressed the antifungal resistance protein 1 (AFR1) delayed the acidification of the phagolysosome and resided in phagolysosomes with a lower degree of co-localization with Rab5-, Rab7- and LAMP2 (Orsi et al., 2009). In summary, Cn growth is affected by pH but the acidity of the phagolysosomal compartment does not appear to be a significant mechanism for microbial control and may in fact promote fungal replication.

## Cn AND CHEMICAL MANIPULATION OF PHAGOSOMAL ACIDIFICATION

Experiments done in the late 1990s showed that treatment of BV2 microglial cells with the weak bases, chloroquine, and ammonium chloride, enhanced the anticryptococcal activity of microglial cells. These weak bases act as lysosomotropic agents by accumulating in the lysosomal compartment (Villamil Giraldo et al., 2014). Similar effects were observed when microglial cells were treated with bafilomycin A1, an inhibitor of the vacuolar-type $H^+$-ATPases. These investigators also demonstrated an increase in the median survival time of mice treated with an intracerebral administration of chloroquine before challenge with a lethal dose of Cn (Mazzolla et al., 1997). Similar results were noted when treating human monocyte-derived macrophages (MDM) with chloroquine and ammonium chloride, which increased anticryptoccocal activity of the MDM independent of iron deprivation. Chloroquine enhancement of anticryptococcal activity was also observed with monocytes derived from HIV-seronegative and HIV-seropositive donors, and in a murine model of experimental cryptococcosis using immunocompetent and immunodeficient mice (Levitz et al., 1997). Chloroquine treatment increased the pH of phagosomes containing heat-killed Cn in a dose dependent manner showing pH values of approximately 5.2 at 1 μM, 6.5 at 10 μM, and 7.5 at 100 μM (Levitz et al., 1999). Interestingly, both chloroquine and quinacrine accumulate within Cn and directly inhibit its growth. Accumulation of chloroquine and quinacrine within Cn increased at physiological conditions, but the mechanism by which it exerts its anticryptococcal activity remains unknown (Harrison et al., 2000). Cn growth was also inhibited by ammonium chloride and bafilomycin A in a concentration dependent manner (Harrison et al., 2000). These results suggest that treatment of phagocytic cells with lysosomotropic agents had a direct effect on Cn as well as an alternative indirect effect by increasing the pH of the Cn-containing phagolysosome (Harrison et al., 2000; Weber et al., 2000).

Non-lytic exocytosis can occur after phagocytosis of Cn by macrophages, and results on the expulsion of viable Cn to the extracellular environment without the lysis of the macrophage. Blockage of phagosome maturation using Concanamycin A, an inhibitor of V-ATPase, reduced Cn non-lytic exocytosis (Ma et al., 2006). Macrophage treatment with bafilomycin A revealed a slight decrease in non-lytic exocytosis, while treatment with ammonium chloride and chloroquine significantly increase non-lytic exocytosis (Ma et al., 2006; Nicola et al., 2011; Qin et al., 2011). The mechanism for how pH affects non-lytic exocytosis remains unexplained.

## Cn AND PHAGOLYSOSOME MEMBRANE PERMEABILIZATION OR DAMAGE

As a consequence of lysosomal membrane permeabilization, cathepsins and other proteases are released from the lysosomal lumen into the cytosol where they can activate cell death (Boya and Kroemer, 2008). Phagolysosomal membrane damage was observed in alveolar macrophage of mice infected with Cn at 7 days post-infection (Feldmesser et al., 2000). Subsequent studies revealed that Cn-containing phagosomes pre-loaded with fluorescently labeled dextran showed diffusion of the fluorescent signal, indicative of leakage of phagosomal contents into the cytoplasm. Lysosomal membrane permeabilization was confirmed by demonstrating the inability of the Cn-containing

phagosome to maintain an acidic environment (Tucker and Casadevall, 2002). The mechanisms responsible for phagolysosomal permeability are unknown. Cn extracellular phospholipase activity was hypothesized to have a role on the degradation of the phagolysosomal membrane but this effect was not experimentally demonstrated (Cox et al., 2001). Nonetheless, Cn phagolysosomal damage is associated with cryptococcal replication and survival, but activation of macrophages with IFN-γ can reduce phagolysosomal damage (Davis et al., 2015). In Cn-infected THP-1 macrophage-like cells, phagolysosomal membrane permeabilization induces formation of the adaptor protein apoptosis-associated speck-like protein containing a CARD speck, suggesting that release of phagolysosomal content, including Cathepsin B, activates inflammasomes resulting in processing and release of IL-1β. Treatment with Cathepsin B inhibitor reduced IL-1β secretion implying that phagolysosomal damage is required for activation of the canonical caspase-8 inflammasome (Chen et al., 2015). Taken together, internalized Cn induces phagolysosomal membrane permeabilization and leads to host cell death in a manner dependent on inflammasomes activation.

## CONCLUSION AND PERSPECTIVE

During the preparation of this review, it was apparent that the literature is inconsistent with how it refers to the phagolysosome and the timing of its appearance. According to LAMP-1 staining, the Cn-containing phagosome fuses with the lysosome as early as one hour post incubation, implying that Cn resides inside the phagolysosome by one-hour post ingestion. To avoid confusion during this review, we used the term phagolysosome whenever we referred to studies that used experimental time of one-hour post incubation or longer. Readers should note that phagosomal maturation is a dynamic process that may vary between individual phagosomes. For example, EEA1 and LAMP-1 were each associated with some phagosomes at early time of macrophage infection but the number of phagosomes positive for these markers increased gradually with time (Wozniak and Levitz, 2008). Phagosomal acidification is a critical step during phagosomal maturation to allow phagosome-lysosome fusion and provide an optimal environment for the activity of antimicrobial enzymes. However, internalization of Cn results in a decrease of phagolysosomal pH shortly after ingestion, which is followed by an increase in phagolysosomal pH over time culminating in an inability of the Cn-containing phagolysosome to maintain the acidic pH as a result of membrane damage (Levitz et al., 1999; Tucker and Casadevall, 2002). Further studies are needed to determine the precise relationship between changes in pH in the Cn-containing phagolysosome, Cn growth and the onset of phagolysosomal membrane damage. Future studies should take in consideration the effect of phagolysosomal membrane permeabilization with regards to the acidification of the Cn-containing phagolysosome and determined causal and temporal relationships, if any. If phagolysosomal membrane permeabilization occurs only in a fraction of those Cn-containing phagolysosome, it is possible that will result in a gradient of pH values, as damage of the phagolysosomal membrane will result in neutralization of phagolysosome acidification by cytoplasmic contents.

Phagolysosome membrane damage can promote Cn growth after cell ingestion (Davis et al., 2015), presumably by disabling microbicide mechanisms or damaging the host cells through the spillage of vesicular content into the cytoplasm. The mechanism for Cn induction of phagolysosome membrane damage remains unknown but various hypotheses have been discussed in the literature. These include the notion that phagolysosomal membrane damage is a result of Cn replication and capsular growth that produce physical damage (Feldmesser et al., 2000). Secondly, secreted fungal proteins damage the phagolysosomal membrane directly. In this regard, extracellular phospholipase was suggested as a candidate for phagolysosomal membrane damage (Cox et al., 2001). Phospholipids induce enlargement of Cn capsule which also requires phospholipase B activity (Chrisman et al., 2011). It is possible that there is a synergistic effect that combines damage of the phagolysosomal membrane by the extracellular fungal phospholipase activity and physical damage of the phagolysosomal membrane by growth of the fungal capsule induced by the phospholipase products.

Cn intracellular residence was shown to result in damage to a variety of cellular systems including mitochondrial function (Coelho et al., 2015). The amount of damage incurred by the host cell may depend on the degree of cellular activation. In this regard, treatment of macrophage with IFN-γ was shown to protect the phagolysosomal membrane from damage and promoted the anti-fungicidal ability of the macrophages (Davis et al., 2015). Previous studies shows that IFN-γ also increased anti-fungicidal activity in rat alveolar macrophage and natural killer cells, and increase survival time in two murine models of Cn infection (Mody et al., 1991; Salkowski and Balish, 1991; Kawakami et al., 1995, 1996). IFN-γ administrations were shown to prolong mice survival when used as an adjuvant treatment in combination with amphotericin B in normal mice and SCID mice infected with Cn (Joly et al., 1994; Lutz et al., 2000; Clemons et al., 2001). IFN-γ has been used clinically as adjuvant treatment and was shown to improve in Cn clearance from the cerebrospinal fluid in HIV-positive patients with cryptococcal meningitis (Jarvis et al., 2012). These observations in rodents and humans suggest that interventions that promote phagosomal membrane integrity could have potential therapeutic applications.

In summary, it appears that the phagolysosomal membrane is a key battleground in the struggle between Cn and phagocytic cells. Damage to the membrane with loss of acidity and spillage of phagolysosomal contents into the cytoplasm favors the fungus and catalyzes as series of events that compromise the host cells and interfere with their ability to control infection. On the other hand, integrity of the phagolysosomal membrane is associated with control of infection. At this time the factors that tip the balance toward membrane damage or integrity are poorly understood and their elucidation is a research priority in the field. The understanding of this process is given additional urgency since therapeutic interventions to stabilize the phagolysosome may tip the balance to the benefit of the host.

## AUTHOR CONTRIBUTIONS

CD-R and AC collaborated to write the manuscript.

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REVIEW
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# First Line of Defense: Innate Cell-Mediated Control of Pulmonary Aspergillosis

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Mycotic infections and their effect on the human condition have been widely overlooked and poorly surveilled by many health organizations even though mortality rates have increased in recent years. The increased usage of immunosuppressive and myeloablative therapies for the treatment of malignant as well as non-malignant diseases has contributed significantly to the increased incidence of fungal infections. Invasive fungal infections have been found to be responsible for at least 1.5 million deaths worldwide. About 90% of these deaths can be attributed to *Cryptococcus, Candida, Aspergillus*, and *Pneumocystis*. A better understanding of how the host immune system contains fungal infection is likely to facilitate the development of much needed novel antifungal therapies. Innate cells are responsible for the rapid recognition and containment of fungal infections and have been found to play essential roles in defense against multiple fungal pathogens. In this review we summarize our current understanding of host-fungi interactions with a focus on mechanisms of innate cell-mediated recognition and control of pulmonary aspergillosis.

**Keywords: aspergillosis, innate cells, monocytes subsets, mechanisms of resistance, neutrophils, dendritic cells (DC)**

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## INTRODUCTION

Immunocompromised individuals comprise a growing population in today's world. In part, this is due to the increased use of immunosuppressive drugs as therapies for diverse disease states. Thus, a variety of patients are increasingly more susceptible to develop invasive fungal infections. *Aspergillus fumigatus* is the etiological agent of over 90% of the invasive aspergillosis (IA) cases and it is considered the most common inhaled fungal pathogen (Dixon et al., 1996; Hohl and Feldmesser, 2007; Lehrnbecher et al., 2010). Even with diagnosis and treatment, individuals suffering from IA rarely recover. Exposure to *A. fumigatus* spores is a daily event, and for most individuals exposure to this environmental fungus is without consequence (Ben-Ami et al., 2010). Immune responses to *A. fumigatus* are central in preventing IA, and are likely responsible for the absence of disease manifestations in people with an intact immune system. Several recent reviews have detailed the important contributions of adaptive immunity to antifungal defense (Wuthrich et al., 2012a; Rivera, 2014; Verma et al., 2015). In this review we will focus our discussion on the recognition of the pathogen, the role of the innate immune system in response to respiratory fungal infection, and how diverse innate cell populations orchestrate antifungal defense against *A. fumigatus*.

## *ASPERGILLUS FUMIGATUS* AND RELATED DISEASES

*Aspergillus fumigatus* is regarded as one of the most prevalent airborne fungal pathogens capable of causing severe to fatal invasive infections in immunocompromised individuals (Dixon et al., 1996; Hohl and Feldmesser, 2007; Lehrnbecher et al., 2010). Once inhaled, the conidia of *A. fumigatus* are small enough (2–3 microns) to enter the terminal respiratory airways, and reach the pulmonary alveoli (Ben-Ami et al., 2010). It is estimated that humans inhale several conidia per day, which are efficiently cleared by the pulmonary innate immune system (Margalit and Kavanagh, 2015). If not, they will germinate into hyphal structures, which can damage lung tissue (Dagenais and Keller, 2009). The innate immune system is the first line of defense against metabolically active and swelling conidia. Important innate cells in defense against aspergillosis include macrophages, neutrophils, monocytes and dendritic cells (Margalit and Kavanagh, 2015) (**Table 1**).

One of the most deleterious complications that can affect an immunocompromised individual is invasive aspergillosis (IA; Hohl and Feldmesser, 2007). Examples of susceptible immunocompromised patients include: those who are undergoing chemotherapy for acute leukemia, recipients of allogeneic haematopoietic stem cell transplants as well as solid-organ transplants, those under corticosteroid treatment for graft-vs.-host disease (GVHD), patients with aplastic anemias and prolonged neutropenia, patients that suffer from neutrophil defects such as chronic granulomatous disease (CGD), and patients suffering from advanced human immunodeficiency virus disease (HIV; Ben-Ami et al., 2010). Infection occurs primarily in the lungs of the patients, but dissemination to practically every organ can occur in the most severe of cases (Segal, 2009).

Some of the most prominent characteristics of IA include: filamentous growth in the pulmonary parenchyma, angioinvasion, intravascular thrombosis, tissue infarction, and haematogenous dissemination (Ben-Ami et al., 2010). Dissemination of aspergillosis to the central nervous system is a devastating effect of IA, which is characterized by the onset of seizures as well as other focal neurologic signs (Segal, 2009). IA has been found to be a leading cause of death among hematology patients (Latge, 1999). It is estimated to occur in 5–25% of acute leukemia patients, 5–10% after allogeneic bone marrow transplantation, and 0.5–5% after cytotoxic treatment of blood diseases as well as solid-organ transplantation (Latge, 1999). IA is also considered to be the main fungal infection found in cancer patients (Bodey et al., 1992; Wald et al., 1997; Kaiser et al., 1998; Lehrnbecher et al., 2010). The average incidences described are probably underestimations of the actual number of incidences since the diagnostic tests available are of low sensitivity (Bodey et al., 1992; Wald et al., 1997; Kaiser et al., 1998; Lehrnbecher et al., 2010; Brown et al., 2012).

*A. fumigatus* has also been shown to cause other diseases such as allergic bronchopulmonaryaspergillosis (ABPA) and aspergillomas (Latge, 1999). ABPA is the most severe allergic complication, (Latge, 1999) and it usually occurs in patients suffering from atopic asthma (1–2% develop ABPA) or cystic fibrosis (7–35% develop ABPA; Knutsen and Slavin, 1992; Moss, 2002). The disease manifests itself as a bronchial asthma that has transient pulmonary infiltrates, which may lead to proximal bronchiectasis and lung fibrosis (Cockrill and Hales, 1999; Moss, 2005). In the most severe of cases, ABPA can lead to respiratory failure and the fatal destruction of the infected lung (Knutsen et al., 2002; Moss, 2002, 2005). Aspergilloma, on the other hand, has been shown to occur in the preexisting lung cavities that have been caused by various lung disorders such as tuberculosis and sarcoidosis (Kirsten et al., 1992; Fujimura et al., 1998). Aspergilloma is characterized by a spheroid mass of hyphae that is embedded within a proteinaceous matrix in the external lining of the cavity (Latge, 1999).

**TABLE 1 | Summary of innate cell defense in *A. fumigatus* infection.**

| Cell type | Contribution to defense |
|---|---|
| Epithelial cells | Antimicrobial peptides such as lactoferrin, chitinase, and β-defensins<br>Production of pro-inflammatory cytokines (TNFα, GM-CSF, IL-8, and the β-defensins HBD2 and HBD9) |
| Alveolar macrophages | ROS and phagosomal acidification<br>Cytokine and chemokine production including neutrophil attractants CXCL1 and CXCL2 |
| Neutrophils | ROS generation via NADPH oxidase, lactoferrin production, NETosis, and through the release of antimicrobial proteases by degranulation |
| Eosinophils | Antimicrobial proteins present in their granules |
| Mast cells | Degranulate and release a variety of enzymes and bioactive substances, such as histamine and tryptase, that mediate pulmonary inflammation and airway constriction |
| Platelets | Damage the fungal cell wall upon exposure through the release microbicidal proteins stored in their granules |
| Natural killer cells | Release of perforins and cytokine production such as IFNγ |
| Conventional DCs | Function as an important regulator of the inflammatory response via IL-2 production |
| Plasmacytoid DCs | Type I IFN production, pET formation, and release of antifungal effector molecules such as zinc chelators like calprotectin and iron-binding proteins like lactoferrin |
| $CCR2^+$ inflammatory monocytes | Differentiate into inflammatory macrophages or into TIP DCs, which are capable of internalization and elimination of conidia<br>Shape the adaptive immune response toward Th1 instead of Th17 |

## RECOGNITION OF *ASPERGILLUS FUMIGATUS* BY INNATE CELL RECEPTORS

### C-Type Lectin Receptors (Dectin-1 and Dectin-2)

Upon inhalation, conidia mature and begin to swell, which leads to the loss of their RodA hydrophobic layer exposing the β-glucans in their cell wall (Aimanianda et al., 2009). β-glucans are recognized by the C-Lectin receptor, Dectin-1 (Hohl et al., 2005; Werner et al., 2009). Dectin-1 is expressed on macrophages, neutrophils, and dendritic cells (Werner et al., 2009). *In vitro*, it has been shown that Dectin-1 dependent alveolar macrophage production of cytokine and chemokines does not depend on the phagocytosis of the conidia, but on its morphology (Luther et al., 2007). Dectin-1 has been shown to be activated only in the presence of swollen, but not resting conidia (Gersuk et al., 2006; Aimanianda et al., 2009). Dectin-1 signals through Syk kinase, leads to the activation of NFκB, and the production of tumor necrosis factor (TNFα), IL-10, IL-6, IL-1α, granulocyte macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein α and β (MIP-1α, and MIP-1β; Hohl et al., 2005; Steele et al., 2005; Faro-Trindade et al., 2012). Dectin-1 has also been shown to have an important role in neutrophil recruitment (Werner et al., 2009). In Dectin-1 deficient mice, defects in neutrophil recruitment were due to unresponsive alveolar macrophages that were unable to produce chemoattractants (Werner et al., 2009).

Dectin-2, in contrast, recognizes α-mannans, which are found in the outer layer of the cell wall (Levitz, 2010; Sun et al., 2014). Dectin-2 has been shown to be mainly expressed on macrophages as well as dendritic cells (Sun et al., 2014). Detection of swollen conidia leads to the production of IL-1β, IL-10, IL-23p19, and TNFα via NFκB mediated by Syk (Sun et al., 2013, 2014). Blocking of Dectin-2 and Syk results in reduced conidial killing in macrophages differentiated from a human monocytic cell line (Sun et al., 2014).

### Toll-Like Receptors (TLRs)

Toll-like receptors (TLRs) are membrane receptors that have a leucine-rich extracellular domain that recognizes pathogen-associated molecular patterns (PAMPs) as well as an intracellular Toll/interleukin-1 receptor (TIR) domain needed for downstream signaling (Kawai and Akira, 2007). Once TLRs recognize the pathogen, the signaling cascade leads to the activation of NFκB and other transcription factors, which leads to cytokine and chemokine production (Kawai and Akira, 2007).

TLRs have been found to play important roles in recognition of *A. fumigatus* for host defense although there is conflicting data, which could be attributed to differences in experimental design (Steele et al., 2005). They are primarily expressed on the cell surface of monocytes, macrophages, and dendritic cells (Takeda and Akira, 2005). TLR1$^{-/-}$ murine bone marrow-derived macrophages had reduced amounts of IL-6, TNFα, CXCL2, and IL-12p40 in response to *A. fumigatus* conidia (Rubino et al., 2012). Cytokine production was diminished in TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR6$^{-/-}$ macrophages, but not in TLR3$^{-/-}$ or wild-type macrophages. In terms of survival, TLR1$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$ have been shown to be non-essential, and do not make immunocompetent mice more susceptible to *A. fumigatus* infection (Dubourdeau et al., 2006; Rubino et al., 2012). In another study performed *in vitro*, TLR2$^{-/-}$ murine alveolar macrophages (AMs) infected with *A. fumigatus* were found to have an impaired inflammatory response due to their deficiency in TNFα production (Steele et al., 2005). Because AMs were still able to produce some detectable TNFα, TLR2 was determined to be non-essential for TNFα production, but necessary for Dectin-1 mediated production of TNFα (Steele et al., 2005). When AMs were treated with Dectin-1 blocking antibody, there was an observed 80% decrease in cytokine production, which was consistent in both TLR2$^{-/-}$ and wild-type AMs (Steele et al., 2005). In addition, TLR2$^{-/-}$TLR4-/- mice, were found to have deficiencies in neutrophil recruitment compared to the single knockouts indicating that both receptors are required for an optimal immune response (Meier et al., 2003). TLR2 and TLR4 signaling requires the adaptor MyD88 adapter-like (Mal)/TIRAP in the myeloid differentiation primary response gene 88 (MyD88) dependent pathway (Horng et al., 2002). TLR4, on the other hand, can signal through another adaptor molecule, TIR-domain-containing adapter-inducing interferon-β (TRIF)-related adapter molecule (TRAM/TICAM-2), in the MyD88 independent pathway (Yamamoto et al., 2003).

In contrast to previous studies (Meier et al., 2003; Bellocchio et al., 2004; Rubino et al., 2012), other work has recently implicated a role for TLR3$^{-/-}$ in *A. fumigatus* infection. TLR3$^{-/-}$ mice were observed to have deficiencies in dendritic cell (DC) migration to the lymph node, which affected their ability to prime T cells (Carvalho et al., 2012). Consistent with this finding, TLR3$^{-/-}$ mice lacked the ability to produce a CD8$^{+}$ T cell response in response to *A. fumigatus* infection (Carvalho et al., 2012). TLR3 as well as TLR4 can signal through the adaptor protein TRIF (Kawai and Akira, 2007), and TRIF$^{-/-}$ mice displayed sustained inflammatory cell recruitment to the lungs in comparison to MyD88$^{-/-}$ and wild-type mice that were chemically immunocompromised to serve as models of IA (de Luca et al., 2010). In addition, TLR3-expressing lung epithelial cells were shown to activate indoleamine 2,3-dioxygenase, which is an interferon (IFN)- inducible enzyme that degrades the amino acid tryptophan and suppresses adaptive T cell immunity (de Luca et al., 2010).

TLR9 is expressed on a variety of cells such as macrophages and monocytes (Ramirez-Ortiz et al., 2008). During phagocytosis of *A. fumigatus*, TLR9 recognizes the exposed and unmethylated CpG DNA (Ramirez-Ortiz et al., 2008). TLR9$^{-/-}$ neutropenic mice exhibited a decreased inflammatory response compared to wild-type 2 days post infection, but was significantly increased 4 days post infection indicating an immunoregulatory role for TLR9 in *A. fumigatus* infection (Ramaprakash et al., 2009). Dectin-1 expression was also found to be decreased in TLR9$^{-/-}$ mice, which could explain why there is a delayed immune response since Dectin-1 is important for

recognition of *A. fumigatus* swollen conidia (Ramaprakash et al., 2009).

### Myeloid Differentiation Primary Response Gene 88 (MyD88)

MyD88, the universal adapter through which all TLRs except TLR3 signal, has been shown to play an important role early in the inflammatory response against *A. fumigatus* (Ramaprakash et al., 2009). MyD88$^{-/-}$ mice were shown to have delayed fungal clearance for the first 2 days, but were comparable to wild-type mice at about 3 days (Bretz et al., 2008). Early on, MyD88$^{-/-}$ lungs appeared to have more necrotic tissue, and using a fluorescent *A. fumigatus* strain, a deficiency in macrophage uptake was observed (Bretz et al., 2008). Also, there was decreased cytokine production of interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC/CXCL1), IFNγ, but increased amounts of TNFα and MIP-1α in MyD88$^{-/-}$ mice (Bretz et al., 2008). The normalization observed at day three indicates that there are alternative pathways involved in fungal clearance that are MyD88 independent (Margalit and Kavanagh, 2015). Recent studies further suggest that MyD88 signaling in defense against IA is crucially active on lung epithelial cells and is required for optimal production of neutrophil-recruiting chemokines (Jhingran et al., 2015).

## INNATE CELL SUBSETS AND THEIR ROLES IN DEFENSE AGAINST ASPERGILLOSIS

### Epithelial Cells

The airway epithelium is the first point of contact for fungal spores upon inhalation, which leads to the initiation of the innate immune response (**Figure 1**; Paris et al., 1997). The respiratory epithelium consists of a variety of cell types such as mucous-secreting goblet cells, ciliated cells, and most importantly, respiratory epithelial cells (Paris et al., 1997). Respiratory epithelial cells release a broad range of antimicrobial peptides such as lactoferrin, chitinase, and β-defensins (Alekseeva et al., 2009; Balloy and Chignard, 2009). The tracheobronchial epithelial cells, Type II alveolar epithelial cells, and endothelial cells have been shown to have the ability to internalize conidia, which are then trafficked to late endosomes for processing (Paris et al., 1997; Filler and Sheppard, 2006). In comparison to other phagocytosing cells such as macrophages and neutrophils, epithelial cells are less efficient in fungal elimination (Wasylnka and Moore, 2003). Respiratory epithelial cells also express recognition receptors such as CLRs and TLRs (Sun et al., 2012). Upon challenge of the human bronchial epithelial cell line with swollen *A. fumigatus* conidia, Dectin-1 expression was induced

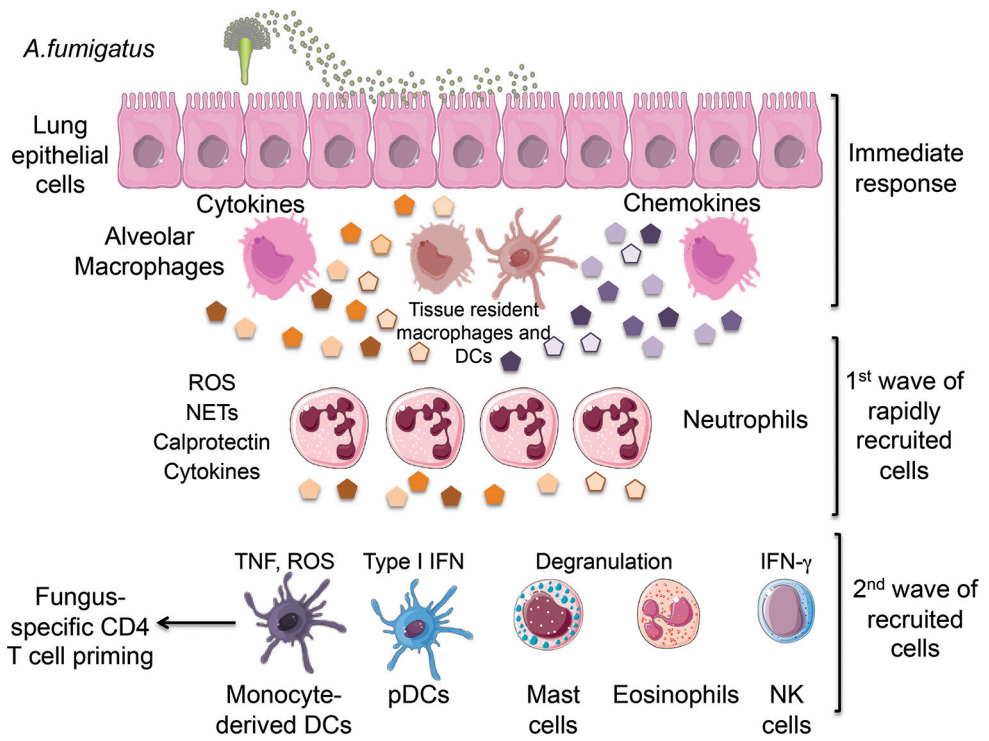


**FIGURE 1 | Inhalation of *Aspergillus fumigatus* (*A. fumigatus*) conidia leads to the initial recognition of infection by lung epithelial cells and tissue-resident innate cells including alveolar macrophages and dendritic cells.** This immediate response results in the production of chemokines that promote the rapid recruitment of neutrophils followed by the subsequent arrival of monocytes, pDCs, mast cells, eosinophils and NK cells. All of these innate cells cooperate in the elimination of fungal conidia by producing a combination of cytokines and protective factors. ROS, reactive oxygen species; NETs, neutrophil extracellular traps; TNF, tumor necrosis factor; IFN, interferon; pDCs, plasmacytoid dendritic cells. We would like to thank Servier Medical Art (http://www.servier.com) for figure graphics of immune cells.

in a TLR2-dependent manner, which induced the expression of ROS as well as TNFα, GM-CSF, IL-8, and the β-defensins HBD2 and HBD9 (Balloy et al., 2008; Sun et al., 2012). Dectin-1 blockade inhibited the expression of these factors indicating that airway epithelial cells require Dectin-1 for the upregulation of pro-inflammatory cytokines as well as antimicrobial factors (Sun et al., 2012).

## Alveolar Macrophages (AM)

Alveolar macrophages have been shown to uptake as well as kill conidia through two known mechanisms: Reactive oxygen species (ROS) generation and phagosomal acidification (Ibrahim-Granet et al., 2003; Philippe et al., 2003). ROS generation occurs in response to swollen but not resting conidia, which leads to the recruitment of cytosolic proteins ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1/Rac2 GTPase) to the plasma membrane where they form a complex with membrane-bound flavocytochrome units, $gp91^{phox}$ and $gp22^{phox}$, in order to form an active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Forman and Torres, 2002; Gersuk et al., 2006). In immunosuppressed mice through cyclophosphamide treatment, mice were found to be less susceptible to IA than $p47^{phox}$ deficient mice, which are defective in NADPH ROS generation and a model of CGD. In addition, mice transgenic for $p47^{phox}$ under the control of the human CD68 that allows for targeted NADPH oxidase expression on macrophages and monocytes had increased survival rates compared to the global knockout illustrating the importance of oxidative mechanisms (Grimm et al., 2013). Specifically in AMs, $p47^{phox}$ deficient AMs were unable to control the growth of phagocytosed conidia in contrast to wild-type AMs (Grimm et al., 2013).

AMs play an important role in the inflammatory response through the activation of PRRs and cytokine and chemokine production (**Figure 1**), which include neutrophil attractants such as macrophage inflammatory protein-2 (MIP-2/CXCL2) and CXCL1(Bhatia et al., 2011). During phagosomal acidification, a phagosome containing conidia fuses with a lysosome in order to form a phagolysosome, which leads to ATPase mediated acidification and activation of enzymes such as chitinases that leads to the degradation of the cell wall exposing ligands for pattern recognition receptors (PRR), TLRs, and Dectin-1 (Ibrahim-Granet et al., 2003; Kasperkovitz et al., 2010; Faro-Trindade et al., 2012).

*In vivo*, clodronate treatment has been used as a method of depletion of AMs (Philippe et al., 2003; Bhatia et al., 2011). Clodronate treated mice were shown to have higher fungal burdens than wild-type mice even though there was an increase in neutrophil recruitment, which can indicate that there is some form of communication between AMs and neutrophils since both seem to be needed in order to control the infection (Bhatia et al., 2011). The mechanism by which this occurs has yet to be fully elucidated and warrants further study. It has been suggested that AMs are able to eliminate low amounts of inocula, and that higher amounts warrant neutrophil activation and recruitment (Philippe et al., 2003). These findings are controversial since there is also evidence that AMs are dispensable in *A. fumigatus* infection, which could be attributed to their use of different strains of mice as well as their use of diverse strains of *A. fumigatus* (Mircescu et al., 2009).

## Neutrophils

*A. fumigatus* also produces immunosuppressive toxins such as gliotoxin and fumagillin, which affects neutrophil function by preventing the formation of a functional NADPH oxidase (Tsunawaki et al., 2004; Fallon et al., 2010). In a mutant strain of *A. fumigatus* in which the *gliP* gene is deleted, infected immunosuppressed mice through corticosteroid treatment had an attenuated virulence compared to non-immunosuppressed mice (Sugui et al., 2007). *GliP* catalyzes the first biosynthetic step in the synthesis of gliotoxin, and deletion prevents its synthesis as well as its effect on NADPH oxidase (Sugui et al., 2007). Neutrophils were found to be a primary target for gliotoxin since neutropenic mice did not differ in virulence when infected with the mutant compared to wild-type *A. fumigatus* (Spikes et al., 2008).

During neutrophil degranulation, azurophil granules expel fungicidal hydrolytic enzymes into the phagocytic vacuole (Segal, 2005). There are two predominant types of granules present in neutrophils: azurophil (primary) granules and specific (secondary) granules (Spitznagel, 1990; Segal, 2005). As mentioned, azurophils contain hydrolytic enzymes for killing and digestion of pathogens whereas specific granules serve as sources of replenishment for membrane components as well as limiting free radical reactions (Segal, 2005). Azurophil granules contain myeloperoxidase, cathepsin G, elastase, and proteinase 3 (Segal, 2005). Specific granules contain lactoferrin (binds and sequesters iron and copper), transcobalamin II, neutrophil gelatinase-associated lipocalin, and other membrane-associated proteins including flavocytochrome $b_{558}$ of the NADPH oxidase (Segal and Jones, 1979; Segal, 2005). NADPH oxidase derived ROS has been shown to promote degranulation and activation of these hydrolytic enzymes (Reeves et al., 2002; Feldmesser, 2006). Activation leads to an accumulation of ROS into the endocytic vacuole, which leads to the accumulation of potassium ions in the vacuole to compensate for the anionic charge from ROS (Reeves et al., 2002). The increase in ionic strength triggers the release of the granule proteins (Reeves et al., 2002). The importance of these granules is demonstrated in mice deficient in the serine protease cathepsin G and/or neutrophil elastase that succumb earlier to *Staphylococcal* and *Candida* infections, but are competent in ROS production (Reeves et al., 2002).

Neutrophils have been shown to be essential innate effectors in defense against *A. fumigatus* (Mircescu et al., 2009; Margalit and Kavanagh, 2015). Due to the high mortality rates of neutropenic mice and their inability to control fungal growth and hyphal formation, they are considered to be the most established model of IA (Stephens-Romero et al., 2005; Mircescu et al., 2009). Neutrophils employ various mechanisms in the elimination of *A. fumigatus* germinating spores such as: ROS generation via NADPH oxidase, lactoferrin production, and through the release of antimicrobial proteases by degranulation (**Figure 1**; Feldmesser, 2006; Sugui et al., 2007).

The importance of oxidative mediated conidiacidal activity by neutrophils is illustrated in patients suffering from CGD

(Grimm et al., 2011). These patients possess mutations in $p47^{phox}$, which leads to a defective ROS generation by NADPH oxidase (Grimm et al., 2011). *In vivo*, studies using NADPH oxidase deficient mice as a model for CGD demonstrated delayed recruitment of neutrophils as well as their inability to contain germinating conidia (Bonnett et al., 2006). Histology from the lung tissue samples of the mice showed hyphal structures as well as extensive damage to the lung tissue in contrast to their wild-type counterparts in both C57BL/6 and BALB/C backgrounds (Bonnett et al., 2006). *In vitro,* the addition of hydrogen peroxide and hypochlorous acid to the NADPH oxidase deficient cells prevented germination indicating the importance of ROS (Bonnett et al., 2006).

Neutrophil extracellular traps (NETs) are extracellular structures that are made of chromatin with proteins from neutrophilic granules attached including neutrophil elastase, myeloperoxidase, cathepsin G, lactoferrin, and gelatinase (Brinkmann et al., 2004). Chromatin is described as the backbone of NETs due to the ability of DNases, but not proteases to degrade it. NETs are formed in response to activation via IL-8, lipopolysaccharide (LPS), bacteria, fungi, or activated platelets (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007). Once activated, a subset of neutrophils begin a "suicide" program that leads to the their death and NETosis (Brinkmann and Zychlinsky, 2007). NETs require a respiratory burst, which has been experimentally supported by blocking ROS and preventing NET formation through the use of the oxidase inhibitor diphenylene iodonium (DPI; Brinkmann and Zychlinsky, 2007).

CGD patients, who are more susceptible to IA, have neutrophils that are unable to form NETs when stimulated with bacteria or phorbol myristate acetate (PMA; Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007). The importance of NET formation is controversial, because there is also evidence that neutrophil fungicidal activity is NET-independent (Margalit and Kavanagh, 2015). *In vitro*, human bronchoalveolar lavage neutrophils treated with micrococcal nuclease (MNase) in order to degrade chromatin and prevent NET formation were still capable of fungicidal activity, which indicates that killing is NET-independent (Bianchi et al., 2009). This observation was further supported by another group that treated neutrophils with cytochalasin-D in order to block phagocytosis, and found that neutrophil killing was abrogated (Bruns et al., 2010). This indicates that phagocytosis and not NET formation is the primary killing mechanism by neutrophils in response to fungi (Bruns et al., 2010). The mechanisms as to whether a neutrophil undergoes NETosis upon contact or phagocytosis of fungal elements remain unclear, but in regards to killing, antimicrobial peptides have been suggested to be one possible mechanism (Levitz et al., 1986; Bruns et al., 2010).

Human neutrophils have also recently been shown to produce NETs in response to *A. fumigatus* hyphal structures, but to a lesser extent in response to resting and swollen conidia (Bruns et al., 2010). An *A. fumigatus* mutant that lacks the hydrophobin RodA surface layer of swollen and resting conidia was shown to increase NET formation as compared to wild-type conidia (Bruns et al., 2010). This indicates that a NET inducing element is exposed during hyphal formation when the RodA layer is lost (McCormick et al., 2010). The RodA protein shields the conidia, and prevents the activation of an adaptive immune response (Bruns et al., 2010; McCormick et al., 2010). Another NET associated protein is calprotectin, which has been shown to chelate zinc ions, and inhibit growth of *A. fumigatus* (McCormick et al., 2010; Bianchi et al., 2011). Addition of zinc ions to culture medium was able to rescue the growth inhibition. (McCormick et al., 2010) The effect seen was not *A. fumigatus* specific. *In vitro, A. nidulans* growth was inhibited by blocking calprotectin through S100A9 blocking antibodies or in the S100A9 deficient mouse strain (Bianchi et al., 2011).

## Eosinophils and Mast Cells

In ABPA, there is enhanced eosinophil recruitment along with fungal enzymes that have been shown to contribute to epithelial damage. In contrast, there is evidence that eosinophils possess fungicidal activity due to the antimicrobial proteins present in their granules (Patterson and Strek, 2014). Eosinophils have recently been shown to play a role in defense against *A. fumigatus* (Lilly et al., 2014). Mice deficient in a high-affinity GATA-binding site in the GATA-1 promoter are depleted of eosinophils (not mast cells or platelets) have been shown to have deficiencies in fungal clearance and increase in fungal burden in comparison to their wild-type counterparts (Lilly et al., 2014). In addition, there was impaired production of cytokines and chemokines such as IL-6, IL-17A, GM-CSF, IL-1β, and CXCL1 (Lilly et al., 2014).

Mast cells have been shown to be key mediators of the pathophysiology of asthma (Bradding et al., 2006). They have been shown to localize in the bronchial smooth muscle bundles in patients with severe asthma such as ABPA patients, but not in normal subjects or those with eosinophilic bronchitis (Bradding et al., 2006). Using the RBL-2H3 cell line and bone marrow-derived mast cell cultures to examine mast cell function, *A. fumigatus* hyphae was shown to adhere to mast cells, and induced their degranulation in an IgE- independent manner unlike conidia and immature hyphae (Urb et al., 2009). Degranulation leads to the release of a variety of enzymatic proteins as well as bioactive substances such as histamine and tryptase, which are important in mediating pulmonary inflammation and airway constriction (Bradding et al., 2006). Although exposure to *A.fumigatus* leads to degranulation of mast cells, mast cells cannot inhibit their growth or metabolic activity (Urb et al., 2009).

## Platelets

Several studies have examined the role of platelets in *A. fumigatus* infection since development of IA leads to hyphal invasion of blood vessels, which can cause thrombosis as well as vascular infarction (Lopes Bezerra and Filler, 2004). *In vitro*, it has been shown that human platelets surround and adhere to hyphal structures as well as conidia, but are unable to phagocytose fungal spores (Christin et al., 1998). In contrast, other studies have indicated that it is *A. fumigatus*-derived serine proteases as well as gliotoxin that lead to platelet activation, and is not contact dependent (Speth et al., 2013). Platelet activation is characterized by the expression of CD62P and CD63 on the cell surface, which are released by α-granules and δ- granules (Perkhofer et al.,

2008; Rødland et al., 2010). Platelets incubated with fluorescein isothiocyanate-labeled cell walls lead to the loss of hyphal surface proteins and hyphal cell wall integrity as indicated by the loss of fluorescence via microscopy (Rødland et al., 2010). These results indicate a role for platelets in fungal containment by their ability to damage the fungal cell wall upon exposure through the release platelet microbicidal proteins stored in their granules.

## Natural Killer (NK) Cells

Natural Killer (NK) cells have been found to have fungicidal activity, but the mechanisms in which this occurs, are poorly understood (Bouzani et al., 2011; Schmidt et al., 2011, 2013). NK cells have been shown to be responsive to germinating but not resting conidia, and their release of perforins *in vitro* correlated with increased fungicidal activity (Schmidt et al., 2011). IFNγ produced by NK cells could also contribute to fungal clearance by preventing germinating conidia from growing into hyphal structures, which suggests that in addition to functioning as an immunoregulatory molecule, IFNγ can function as an antifungal effector against *A. fumigatus* directly (Park et al., 2009; Bouzani et al., 2011). AMs incubated with NK cells *in vitro* have also been shown to be more effective in killing the conidia than when incubated alone or with IFNγ deficient NK cells. (Park et al., 2009) In neutropenic models that have been depleted of NK cells, the mortality rate is doubled in comparison to neutrophil depleted mice with wild-type NK cell function further suggesting a contribution of NK cells to antifungal defense *in vivo* (Morrison et al., 2003). Although NK cells have been found to have antifungal activity, they do not seem to be essential. In our previous work, mice that lack all lymphocytes including NK cells, T cells, B cells, γδ T cells, iNKT cells, and innate lymphocytes (ILCs), $RAG^{-/-}$ $\gamma C^{-/-}$, do not develop IA upon infection (Espinosa et al., 2014). These results indicate that these lymphocytes are not required for defense against *A. fumigatus* infection.

## Dendritic Cells (DCs)

Dendritic Cells (DCs) have been shown to have multiple roles in response to *A. fumigatus* infection (Bhatia et al., 2011) (**Table 1**). DCs phagocytose conidia through PRRs including Dectin-1, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), complement receptor 3 (CR3), and FcγRIII (Bozza et al., 2002; Serrano-Gómez et al., 2005; Mezger et al., 2008). DCs also produce proinflammatory cytokines such as TNFα, IL-6, IL-12, IL-1α, and IL-1β in response to *A. fumigatus* (Bozza et al., 2002; Mezger et al., 2008; Morton et al., 2011). Differential cytokine production by DCs is observed when exposed to different forms of the fungus *in vitro* (Bozza et al., 2002). When exposed to conidia and hyphae, TNFα is produced. IL-12 is produced in response to conidia, and IL-4 and IL-10 in response to hyphae (Bozza et al., 2002). There are three major subtypes of DCs in the lung, conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (moDCs; Margalit and Kavanagh, 2015).

DCs also produce a variety of chemokines that are needed for the recruitment of a variety of innate and adaptive effector cells to aid in fungal elimination (Scapini et al., 2000; Gafa et al., 2007). DCs are recruited to sites of infection by the production of MIP-1α and MIP-1β by neutrophils (Scapini et al., 2000). *In vitro*, infection of human dendritic cells by *A. fumigatus* conidia triggers the secretion of chemokines for neutrophil and Th1 lymphocyte recruitment (Gafa et al., 2007). DCs release increased amounts of CXCL8, which results in neutrophil recruitment (Gafa et al., 2007). *A. fumigatus* infection resulted also in CCL3, CCL4, CXCL10, and CCL20 productions that induce the migration of effector memory Th1 cells (Gafa et al., 2007; Morton et al., 2011). Together these results indicate a dual role for DCs in the innate as well as adaptive immune response against *A. fumigatus* (Margalit and Kavanagh, 2015).

## Plasmacytoid Dendritic Cells (PDCs)

pDCs are known as Type I IFN producing cells in response to viral stimulation. They comprise about 0.2–0.8% of total peripheral blood mononuclear cells (PBMCs) in humans, and express TLRs 7 and 9 (Colonna et al., 2004). pDCs link the innate and adaptive immune systems by secreting IFNα and TNFα, and by differentiating into mature pDCs with upregulated major histocompatilibility complex (MHC) and costimulatory molecules capable of priming naive T cells (Colonna et al., 2004).

pDCs are a major source of type I IFN, but the role of pDCs as well as type I IFN has not been extensively examined in regards to fungal infections (Ramirez-Ortiz et al., 2011; Margalit and Kavanagh, 2015). Interferon αβ receptor deficient mice ($IFNAR^{-/-}$) have been shown to have a higher susceptibility to IA in comparison to wild-type mice, which is consistent with the finding that antibody (120G8) mediated depletion of pDCs make mice more vulnerable to *A. fumigatus* infection (Ramirez-Ortiz et al., 2011). When exposed to hyphae of *A. fumigatus,* human pDCs inhibited their growth, but did not kill the fungus (Ramirez-Ortiz et al., 2011). In addition, there are contrasting studies as to whether pDCs are capable of engulfing *A. fumigatus* conidia, which could be attributed to differences in experimental design for pDC isolation such as using CD303 as a marker for positive selection compared to CD123 (Ramirez-Ortiz et al., 2011; Lother et al., 2014). Apoptosis of pDCs is stimulated upon exposure to the release of cytotoxic molecules by *A. fumigatus* such as gliotoxin, and their death results in the release of antifungal effector molecules such as zinc chelators like calprotectin and iron-binding proteins like lactoferrin (Ramirez-Ortiz et al., 2011). Similar to neutrophils, pDCs have also recently been shown to form Dectin-2 mediated extracellular traps or pETs (plasmacytoid extracellular traps), which have been observed to form around *A. fumigatus* hyphae (Loures et al., 2015). Treatment with blocking antibodies against Dectin-2 led to decreased association of pDCs with hyphae in contrast to Dectin-1, which was similar to untreated pDCs exposed to *A. fumigatus* only (Loures et al., 2015). These results suggest that pDCs can recognize *A. fumigatus* via Dectin-2, which results in antifungal activity through pET formation.

## Conventional Dendritic Cells (cDCş)

cDCs as well as moDCs are typically identified by their high expression of the integrin CD11c and MHC class II. There are two types of cDCs: $CD103^{+}$ cDCs and $CD11b^{+}$ cDCs (Kopf

et al., 2015). They can be distinguished from each other by the markers CD207 (present on $CD103^+$ cDCs) and MER proto-oncogene tyrosine kinase (MerTK; present on $CD11b^+$ cDCs; Kopf et al., 2015). $CD11b^+$ cDCs share common markers with moDCs, but can be differentiated by using the marker for the Fc receptor CD64 that is present on moDCs (Kopf et al., 2015). The development of cDCs as well as pDCs is dependent on the FMS-like tyrosine kinase 3 ligand (Flt3L), which is demonstrated by the lack of $CD103^+$ DCs in Flt3L deficient mice (Ginhoux et al., 2009; Merad et al., 2013). Basic leucine zipper transcription factor ATF-like 3 (BATF3) is also required for their steady-state generation similar to CD8α DCs (Edelson et al., 2010), but under certain inflammatory conditions such as mycobacterial infection, other members of the BATF3 family have been shown to have compensatory roles (Tussiwand et al., 2012). In an allergic asthma house dust mite model, it was demonstrated that moDCs as well as $CD11b^+$ cDCs are important for sensitization, but $CD103^+$ DCs are dispensable (Plantinga et al., 2013). They were able to distinguish the contributions of the difference types of DCs by employing the use of Flt3L deficient mice as well as Langerin-diphtheria toxin receptor (DTR) mice (Plantinga et al., 2013). In Langerin-DTR mice, lung $CD103^+$ DCs and lymphoid tissue CD8α DCs are eliminated (Plantinga et al., 2013).

In a model of invasive aspergillosis, $CD103^+$ DCs were shown to play an important role in shaping a Th17 response through their IL-2 production via nuclear factor of activated T-cells (NFAT) signaling (Zelante et al., 2015). The absence of IL-2 in pulmonary $CD103^+$ DCs led to higher IL-17 production in comparison to T cells cultured with IL-2 competent DCs *in vitro* in response to *A. fumigatus* germlings (Zelante et al., 2015). The impact of IL-2 production by DCs was also assessed using mice lacking IL-2 in all tissues or lacking IL-2 in $CD4^+$ T cells as well as mice lacking IL-2 specifically in the $CD11c^+$ population ($CD11c^{cre}IL\text{-}2^{fl/fl}$; Zelante et al., 2015). Mice deficient in IL-2 in all tissues and in mice lacking IL-2 in $CD4^+$ T cells had significantly higher fungal burden, but had increased survival in comparison to $CD11c^{cre}$ $IL\text{-}2^{fl/fl}$. $CD11c^{cre}$ $IL\text{-}2^{fl/fl}$ expressed higher levels of IL-17 and IL-23, which led to a fatal hyperinflammatory Th17 response (Zelante et al., 2015). These results indicate that DCs can function as an important regulator of the inflammatory response upon fungal infection.

## $CCR2^+$ Inflammatory Monocytes ($CCR2^+$ Mo)

Macrophage and dendritic cell precursors (MDP) in the bone marrow give rise to $Ly6C^{hi}$ monocytes or inflammatory monocytes, which exit the bone marrow in a CC-chemokine receptor 2 (CCR2)- dependent manner in response to infection to the inflamed tissues (Serbina et al., 2008; Geissmann et al., 2010). They represent approximately 2–5% of circulating white blood cells in the bloodstream of a naïve mouse (Shi and Pamer, 2011). The absence of CCR2 leads to deficiencies in monocyte recruitment to the site of infection (Serbina et al., 2008; Shi and Pamer, 2011). CCR2 is also expressed by other cells such as hematopoietic stem cells (HSCs) as well as a subset of NK cells (Si et al., 2010). Once they reach infected tissues, $CCR2^+$ Mo can differentiate into inflammatory macrophages or into TNF and iNOS producing DCs (TIP DCs; Serbina et al., 2003; Auffray et al., 2009; Shi and Pamer, 2011). In the absence of inflammation, $CCR2^+$Mo can return to the bone marrow or to the spleen, which can function as a reservoir for circulating monocytes (Serbina et al., 2008; Geissmann et al., 2010; Shi and Pamer, 2011). Humans also have a similar counterpart, which is characterized by $CCR2^+$ $CD14^+$ $CD16^-$ expression (Geissmann et al., 2003).

CCR2 has two primary ligands, CC chemokine ligand 2 (CCL2) and CCL7 that have been shown to be important for monocyte recruitment although the mechanism of action has yet to be fully elucidated (Tsou et al., 2007; Shi and Pamer, 2011). In a theoretical model, it was hypothesized that CC-chemokine ligands establish gradients, which guide monocytes to the site of infection by their association with glycosaminoglycans (Proudfoot et al., 2003; Allen et al., 2007). The finding that amino acid substitutions in CCL2 affected monocyte recruitment supported this idea (Proudfoot et al., 2003). Other CC-chemokine ligands, CCL8 and CCL12, have also been shown to bind to CCR2, but did not have a significant role in monocyte trafficking (Tsou et al., 2007). Migration of monocytes is dependent on various integrin and adhesion molecules such as L-selectin (CD62L), P-selectin glycoprotein ligand 1 (PSGL1), lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin), macrophage receptor 1 (MAC1; also known as integrin αMβ2), platelet endothelial cell adhesion molecule (PECAM1), and very late antigen 4 (VLA4; also known as integrin α4β1; Ley et al., 2007). These molecules are necessary for proper rolling, adhesion, and migration of $CCR2^+$ Mo to infected tissues by a variety of different pathogens (Ley et al., 2007). $CCR2^+$ Mo have been shown to play important roles in bacterial, viral, protozoan, and fungal infections (Shi and Pamer, 2011).

In the context of *A. fumigatus* infection our previous studies found an essential role for $CCR2^+$ monocyte-derived dendritic cells in the activation of fungus-specific $CD4^+$ T cells. In these studies, the role of CCR2 was investigated using a depleter mouse strain, in which the CCR2 promoter drives the expression of the simian diphtheria toxin receptor (Hohl et al., 2009). Administration of diphtheria toxin (DT) leads to the transient depletion of monocytes in the blood, bone marrow, and peripheral tissues (Hohl et al., 2009). Depletion of $CCR2^+$ Mo before infection leads to decreased transport of fungal spores to the draining lymph nodes, which prevents *A. fumigatus*-specific $CD4^+$ T cell priming (Rivera et al., 2011). Depletion of $CCR2^+$ moDCs at later stages of infection, leads to a skewing from a Th1 to a Th17 response in the lung, which indicates that recruitment of monocytes has an influential role in shaping the adaptive immune response and are necessary to promote and sustain Th1 responses (Rivera et al., 2011). Studies with other fungal pathogens further support a conserved function for moDCs in shaping fungus-specific $CD4^+$ T cell responses (Traynor et al., 2002; Szymczak and Deepe, 2009; Ersland et al., 2010; Szymczak and Deepe, 2010; Wüthrich et al., 2012b).

In addition to their importance in shaping fungus-specific $CD4^+$ T cell responses, $CCR2^+$ Mo and moDCs are important direct effectors necessary for prevention of IA (Espinosa et al., 2014). In recent studies, we found that sustained depletion of $CCR2^+$ Mo results in the rapid development of IA. Our

studies suggest that $CCR2^+$ Mo and their derivative moDCs are important direct effectors of fungal spore killing. Moreover, they are necessary to sustain an inflammatory milieu in the lung and the antifungal activity of neutrophils (Espinosa et al., 2014; Caffrey et al., 2015). Altogether, these studies indicate that $CCR2^+$ Mo and their derivative moDCs are crucial, non-redundant cells in antifungal defense to *A. fumigatus* by acting both as direct innate effectors and by shaping the response of other innate and adaptive immune cells. The importance of monocytes in defense against IA is likely conserved in humans. Patients with monocytopenia are at increased risk for fungal infections (Vinh et al., 2010; Hsu et al., 2011). Moreover, $CD14^+CD16^-$ monocytes isolated from healthy allogeneic hematopoietic stem cell transplantation donors were shown to phagocytose conidia, and inhibit conidial germination while the $CD14^+CD16^+$ subset was able to produce cytokines (Serbina et al., 2009).

## CONCLUDING REMARKS

The detrimental impact of fungal infections to diverse patient populations across the globe is likely to continue to rise. The interactions of fungi with host innate cells crucially determine the outcome of infection with multiple innate cells subsets contributing essential protective functions. Future therapeutic interventions aimed at boosting innate immunity are likely to provide significant benefit and help improve the current detrimental outcomes associated with invasive aspergillosis and other invasive fungal infections.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# Distinct stages during colonization of the mouse gastrointestinal tract by *Candida albicans*

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*Candida albicans* is a member of the human microbiota, colonizing both the vaginal and gastrointestinal tracts. This yeast is devoid of a life style outside the human body and the mechanisms underlying the adaptation to the commensal status remain to be determined. Using a model of mouse gastrointestinal colonization, we show here that *C. albicans* stably colonizes the mouse gut in about 3 days starting from a dose as low as 100 cells, reaching steady levels of around $10^7$ cells/g of stools. Using fluorescently labeled strains, we have assessed the competition between isogenic populations from different sources in cohoused animals. We show that long term (15 days) colonizing cells have increased fitness in the gut niche over those grown *in vitro* or residing in the gut for 1–3 days. Therefore, two distinct states, proliferation and adaptation, seem to exist in the adaptation of this fungus to the mouse gut, a result with potential significance in the prophylaxis and treatment of *Candida* infections.

**Keywords: commensalism, adaptation, fitness, mouse gut, *Candida albicans***

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## Introduction

Fungal agents are an important cause of nosocomial infections, which are a primary health problem in several countries (Pfaller and Diekema, 2007; Alangaden, 2011). Their treatment, especially of systemic nosocomial infections is complicated by the limited number of antifungals available (Ostrosky-Zeichner et al., 2003) and the emergence of resistance to some of the most commonly used (Cowen et al., 2015). *Candida albicans* is the most frequent pathogenic fungus found in humans and the 4th most common cause of blood borne systemic nosocomial infections. This yeast is found as a harmless commensal in the human population, residing mainly in the gastrointestinal and vaginal tract. Under impairment of physical host barriers and/or alteration of immune defenses (Romani, 2004), *C. albicans* is able to translocate through the mucosa and gain access to internal organs, causing a systemic disease and organ failure (Mavor et al., 2005). Genetic evidence supports that *C. albicans* strains found in the bloodstream are genetically similar to those found in rectal isolates and that increased gut colonization is, as maybe expected, a risk for dissemination (Miranda et al., 2009). *C. albicans* infections are, therefore, mainly endogenous (Odds et al., 2006). Understanding the mechanisms that control the establishment of this microbe in host niches is essential for designing strategies to treat and prevent fungal infections (Pierce and Lopez-Ribot, 2013; Paul and Moye-Rowley, 2014).

The development of genetic tools for this organism (De Backer et al., 2000; Berman and Sudbery, 2002; Hernday et al., 2010; Vyas et al., 2015) has enabled the identification of several virulence genes, although the concept of virulence itself may be difficult to define for commensal microbes (Casadevall and Pirofski, 2003). Most virulence genes identified to date encode adhesion molecules,

metabolic enzymes, regulators of morphologic changes (such as the white-opaque) and members of signaling pathways (Mayer et al., 2013). Of particular interest is dimorphism, which is triggered in response to certain environmental factors, and that influences *C. albicans* ability to invade and proliferate within tissues and cope with immune cells (Saville et al., 2003, 2006; Gow et al., 2012; Jacobsen et al., 2012). These works have mainly used the mouse systemic model of infection and, more recently, alternative non-vertebrate models (Fuchs and Mylonakis, 2006; Arvanitis et al., 2013) that, unfortunately, do not mimic the natural route of *C. albicans* infections in humans. The development of models of gastrointestinal commensalism (Koh, 2013) has been a main advantage to analyze fungal factors influencing colonization. Although initial studies involved neonatal mice (de Repentigny et al., 1992), the most common system uses adult mice with a significant chemotherapy-driven microbiota reduction (Kinneberg et al., 1999; Mellado et al., 2000; Wiesner et al., 2001). These models have enabled the definition of the role that neutrophils have in the control of *C. albicans* dissemination (Koh et al., 2008) as well as provided experimental support for the role that certain yeast may have in the control and outcome of *C. albicans* colonization (Jawhara and Poulain, 2007). The development of these models has enabled to define the role that certain genes have on the commensal program promoting colonization via yet undefined regulatory circuits (White et al., 2007; Pierce and Kumamoto, 2012; Pande et al., 2013; Pérez et al., 2013; Prieto et al., 2014).

In this work we have addressed the temporal dependence of *C. albicans* adaptation to the mouse gastrointestinal tract. We have previously developed a dual labeled system that enables tracing populations in the gut via flow cytometry or standard viable colony counting (Prieto et al., 2014). Using this system and measuring competitive fitness between different fungal populations, we were able to show how *C. albicans* cells adapt to the commensal status in a timely fashion.

## Materials and Methods

### Strains and Growth Conditions

*C. albicans* strains used in this work were CAF2-GFP (COA6-3) and CAF2-dTOM2 (PPD7), both described in a previous work (Prieto et al., 2014). The only genetic difference between the two strains is the fluorescence label (GFP or RFP), which does not impose any loss of fitness to the population allowing the differentiation of each strain from a mixed population in colonies (Prieto et al., 2014). According to our nomenclature (**Table 1**), Ca-n refers to *C. albicans* cells grown *in vitro* (YPD), Ca-g refers to cells present in the gut and Ca-f to cells derived from stools (in a cohousing experiment). In some experiments, the genetic label of the cells (GFP, RFP) was swapped among the different physiological status (n, g, or f) to discard any role of the fluorescent label in terms of fitness. In these cases, all data have been plotted together as they were practically identical.

Ca-n population was obtained from an overnight (24 h) culture at 37°C in YPD liquid medium (2% glucose, 2% peptone, 1% yeast extract) of CAF2-GFP (COA6-3) or CAF2-dTOM2 (PPD7). These strains were obtained starting from fresh YPD plates derived from a −80°C glycerol stock and kept at 4°C for 2–4 weeks. Overnight cells were recovered by a low speed centrifugation (3000 r.p.m for 5 min) and resuspended in PBS. Different number of cells (see description of each experiment) in a volume of 100 μL were inoculated by gavage. Stools samples were plated in SD solid medium (2% glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base and 2% agar) plus amino acids and chloramphenicol (10 μg/mL) for colony counting.

### *In vivo* Procedures

The experiments involving animals performed in this work were carried out in strict accordance with the regulations in the "Real Decreto 1201/2005, BOE 252" for the Care and Use of Laboratory Animals of the "Ministerio de la Presidencia," Spain. The protocol was approved by the Animal Experimentation Committee of the University Complutense of Madrid (Permit Number: CEA 25/2012, BIO2012-31839-1). All efforts were made to minimize suffering. Mice euthanasia was performed by $CO_2$ inhalation following standard protocols (AVMA Guidelines for the Euthanasia of Animals: 2013 Edition). The number of animals used was minimized for ethical reasons.

Female mice C57BL/6 obtained from Harlan Laboratories Inc. (Italy) were used within an age of 7–10 weeks-old. Mice housing and other non-invasive procedures took place in the animal facility from the Medical School of the Universidad Complutense de Madrid. We used the protocol for studying commensal colonization that we have previously described (Prieto et al., 2014) with minor modifications. After 4 days of antibiotic pretreatment (2 mg/mL Streptomycin, 1 mg/mL Bacitracin, and 0.1 mg/mL Gentamicin) in drinking water, *C. albicans* cells were inoculated in a single gavage with a specific dose. In some experiments, a second inoculation was performed on days 2, 15, or 21 to establish a competition among different populations and *in vitro* cells. In the cohousing experiments, mice were maintained in the same cage without any artificial

**TABLE 1 | Nomenclature of *C. albicans* populations studied in this work.**

| Nomenclature | Origin | State | Description |
|---|---|---|---|
| Ca-n | *C. albicans* cells obtained under standard laboratory conditions | *In vitro* | Stationary overnight cells |
| Ca-g | *C. albicans* cells present in the gut | *In vivo* | Short term (2 days, Ca-gS) or Long term (>14 days, Ca-gL) after gavage |
| Ca-f | *C. albicans* cells derived from animal feces | *Ex vivo* | Ca-fS (Short term, 2 days) or Ca-fL (Long term, 21 days) refers to original state of the animal in the moment of cohousing |

inoculation. For all the experiments, colonization was assessed by measuring CFUs (Colony Forming Units) of *C. albicans* in freshly obtained stools. Fresh stool samples were collected from each individual on different days, mechanically homogenized in PBS to 40 mg/mL and viable cells were determined on SD-agar plates. Colonies were associated to a specific population in accordance to the colony color (red colonies, RFP expressing cells; white-greenish colonies, GFP expressing cells). Proportions of populations lower than ∼1:10,000 could not be distinguished on plates. The detection limit of our technique in competition experiments is ∼2500 cells/g stools, which is plotted in some figures. When fungal levels were below this limit, colonization was considered 0 for graphical representation. To normalize fungal levels with the levels at the intervention we used competition index (CI). For a population A vs. a population B, CI was calculated in the following way: $CI^{A/BDayX} = (CFU^{Ca-A}/CFU^{Ca-B})_{DayX}/(CFU^{Ca-A}/CFU^{Ca-B})_{Day1}$. The value of 2500 CFU/g stools was used for groups with colonization levels below detection limit to enable CI calculation.

### Statistical Analysis

All statistics have been calculated using GraphPad Prism 5 software. Linear regression was used to compare the decay of colonization of fungal populations tested after antibiotic removal on different days. To determine statistical differences among $\log_2$ CI values from different experiments and days, we performed One-Way ANOVA plus Bonferroni's multiple comparison tests to compare more than two groups and Student's two-tailed unpaired *t*-tests when there were just two groups. Data shown in figures are either each replicate and/or mean ± standard error.

## Results

### Equilibrium Levels of *C. albicans* in Gut Colonization are Independent of Fungal Dose

Treatment of mice with oral antibiotics has been a broadly used strategy to allow high *C. albicans* colonization levels after gastrointestinal inoculation (Wiesner et al., 2001; Koh, 2013). In these protocols, a high dose of *C. albicans* is normally given either by a single gavage or via drinking water *ad libitum*. Oral doses (about $10^7$ CFUs) are in the range of the levels later obtained from stools ($10^6$–$10^7$ CFUs/g stool). It is normally accepted that a high inoculum may help gut establishment of *C. albicans*. We tested this assumption by inoculating 5 groups of mice ($n = 2$) with 100 μL of serial dilutions of a $10^7$ CFUs/mL solution of stationary *C. albicans* phase cells. Mice therefore received $10^6$, $10^5$, $10^4$, $10^3$, or $10^2$ *C. albicans* cells in a single dose and stool fungal levels were determined in the following days. At day 1, CFUs obtained from stools correlated well with the inoculated dose and were roughly 10x higher (in CFUs/g) than the dose. At day 3, they all reached similar high colonization levels (7.3 ± 0.23, mean of Log (CFU/g) ± SEM), independently of the dose received (**Figure 1**). Ratios between stool concentration values at day 1 and dose inoculum was found to be very similar for all groups, 1.3 ± 0.15 [Log (CFU/g)/Log (CFU inoculated), mean ± SEM], suggesting that cells proliferated rather similarly during this period. This result indicates that the first 72 h after inoculation are critical for *C. albicans* to proliferate and attain stable stationary colonization levels which are then properly maintained in the next days. We hypothesized that during this period cells focus on proliferation, while at later stages, *C. albicans* may rely on adaptation to the new environment.

### *C. albicans* colonization Time Affects Fall of Fungal Levels after Antibiotics Removal

It is known that modification of the bacterial microbiota is needed to allow a stable colonization of *C. albicans* (Koh et al., 2008). Our colonization protocol is based on application of a specific antibiotic regime to the mice via the drinking water (Wiesner et al., 2001) and when removed, fungal loads decrease (Prieto et al., 2014) allowing efficient removal of *C. albicans* from the mouse gut. We wondered if this decline would occur similarly to gut-adapted or non-adapted populations of *C. albicans*. We used for this purpose cells obtained from *in vitro* standard laboratory growth conditions (that we call Ca-n, for non-adapted), and cells already present in the mouse gut (Ca-g, for gut adapted). We also distinguished between short-term

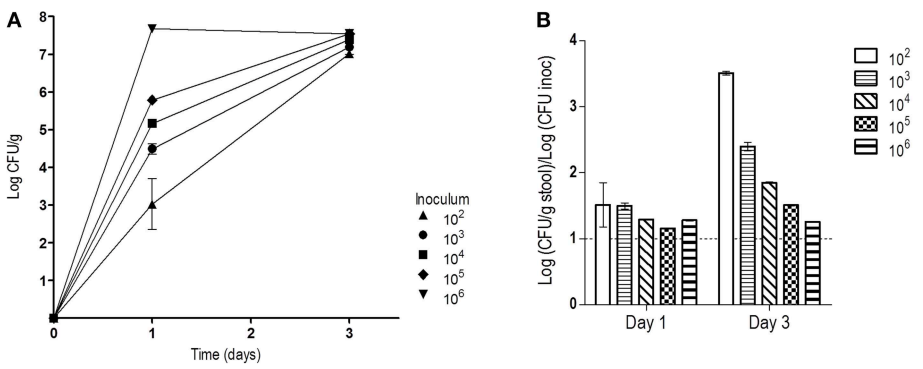


**FIGURE 1 | Influence of the inoculum dose in the colonization levels.** After four days of antibiotic treatment, different doses of *C. albicans* were orally given. **(A)** Fungal loads in stools (log CFU per gram, mean ± SEM) from each group ($n = 2$) on days 1 and 3. **(B)** Relation between levels detected in stools and dose on days 1 and 3 (mean ± SEM).

adapted (Ca-gS) cells for those present in the gut for 2 days and long-term adapted (Ca-gL) when they have established in the gut for 2 or more weeks (see **Table 1** and Materials and Methods). We therefore treated 3 groups of mice with antibiotics for 4 days and afterwards $10^7$ *C. albicans* cells were inoculated to each individual. Antibiotic therapy was removed from experimental groups at different time points: group 1 mice lacked antibiotics in drinking water immediately after inoculation (day 0), in group 2, antibiotic therapy was removed 2 days after inoculation (Ca-gS) while in group 3 cells were allowed to colonize for 23 days (Ca-gL). After oral antibiotics were removed, fungal loads on stools were analyzed at different days (0, 1, 8, 13, and 21). To assess the colonization decrease rate and be able to compare the behavior of different groups, we calculated linear regression of fungal loads (log CFU/g) at different time points (**Figure 2A**). Ca-gL group showed the lowest fall of colonization (0.117 log units per day), while groups Ca-gS and Ca-n displayed slopes of 0.263 and 0.209, respectively, roughly inversely correlating with colonization time. Actually, only Ca-gL group linear regression differed significantly from the other two groups ($p < 0.01$ for Ca-n and $p < 0.001$ for Ca-gS). Ca-gS and Ca-n did not show a significant different behavior. Since each group presents a different colonization level after antibiotic removal, we calculated ratios of fungal loads referring to the value attained at day 0 (**Figure 2B**). At day 1, all groups present similar $\log_2$ values close to 0, indicating that no important changes had affected the *C. albicans* populations yet. All groups experienced an important drop in fungal loads in the following days. This effect was less pronounced in Ca-gL group and, interestingly, much more in Ca-gS population. The latter was significantly different from the other two groups, on both days 8 and 13. However, on day 21 no significant differences among groups were observed.

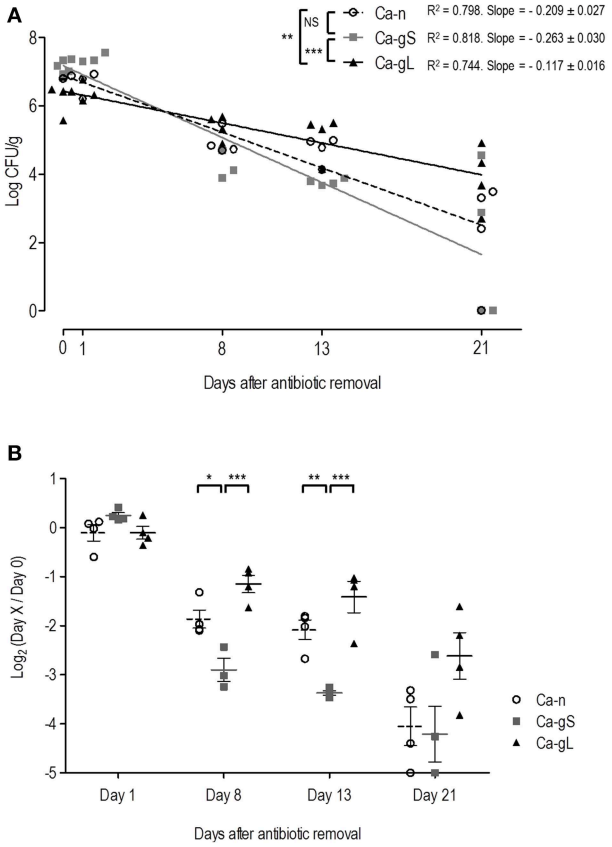


**FIGURE 2 | Decay of colonization of different *C. albicans* populations after antibiotic removal.** Antibiotic treatment was replaced with sterile water in each group ($n = 4$) on day 0 (Ca-n, open circles), day 2 (Ca-gS, gray squares) or day 23 (Ca-gL, black triangles) from the inoculation of $10^7$ cells of *C. albicans*. Each symbol represents data from an individual mouse. **(A)** Evolution of fungal loads in stools (log CFU per gram) along 21 days after the antibiotic treatment was removed. **(B)** Ratios of fungal levels (CFU per gram) in relation to the day when antibiotics was removed (day 0). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

## Long-term (But Not Short-term) Gut-adapted *C. albicans* Cells Displace Non-adapted Fungal Populations

In order to determine the fitness of different adapted *C. albicans* cells, we used our recently developed red or green fluorescent gene reporter system (Prieto et al., 2014) to distinguish between these populations. We used cells labeled with either GFP or RFP which were allowed to colonize mice thus generating Ca-gS and Ca-gL populations. Mice were inoculated by gavage with *C. albicans* cells (normally GFP-labeled) and after 2 or 15–21 days, a new Ca-n population was introduced (then RFP-labeled) to allow competition with already established Ca-gS and Ca-gL (respectively) present populations. At different times, the abundance of each population was determined in stool samples from every mouse (**Figure 3**). Different doses of Ca-n were tested to ensure that competition would not be critically dependent on the load (dose) of *C. albicans* inoculated.

We noticed that a Ca-n population easily maintains sustained levels of colonization in the gut when it competes with Ca-gS (**Figures 3A,C,E**). Although lower levels of Ca-n are detected in stools when inoculating low doses (**Figures 3A,C**), the pattern from the first day is rather constant. However, in the presence of a Ca-gL population, Ca-n is unable to establish and attain high colonization loads (**Figures 3B,D,F**). Only upon inoculation of a high dose ($10^7$ cells) of Ca-n cells, these cells were eventually able to establish in the gut. Notably, the colonization levels attained by Ca-n are lower to those found at day 1, which probably reflect transient passage through the gastrointestinal tract but not equilibrium levels (**Figure 3F**).

Since each strain is inoculated on different days (days 2, 15, or 21), it is difficult to compare the evolution of the populations in competitive fitness. We chose to use the proportions of strains determined in stool samples at day 1 as this value nicely correlates with the proportion in the mix inoculated in standard colonization competition assays in mice (**Figure S1**). We used colonization competition index (CI) (see Materials and Methods) as it actually reflects the ability of one population to persist over the other one. Using this parameter, we have analyzed whether the dose and/or stage of previous population influence the subsequent competition. While a high *C. albicans* dose ($10^7$ cells) helps Ca-n to reach higher fungal levels on day 1, it

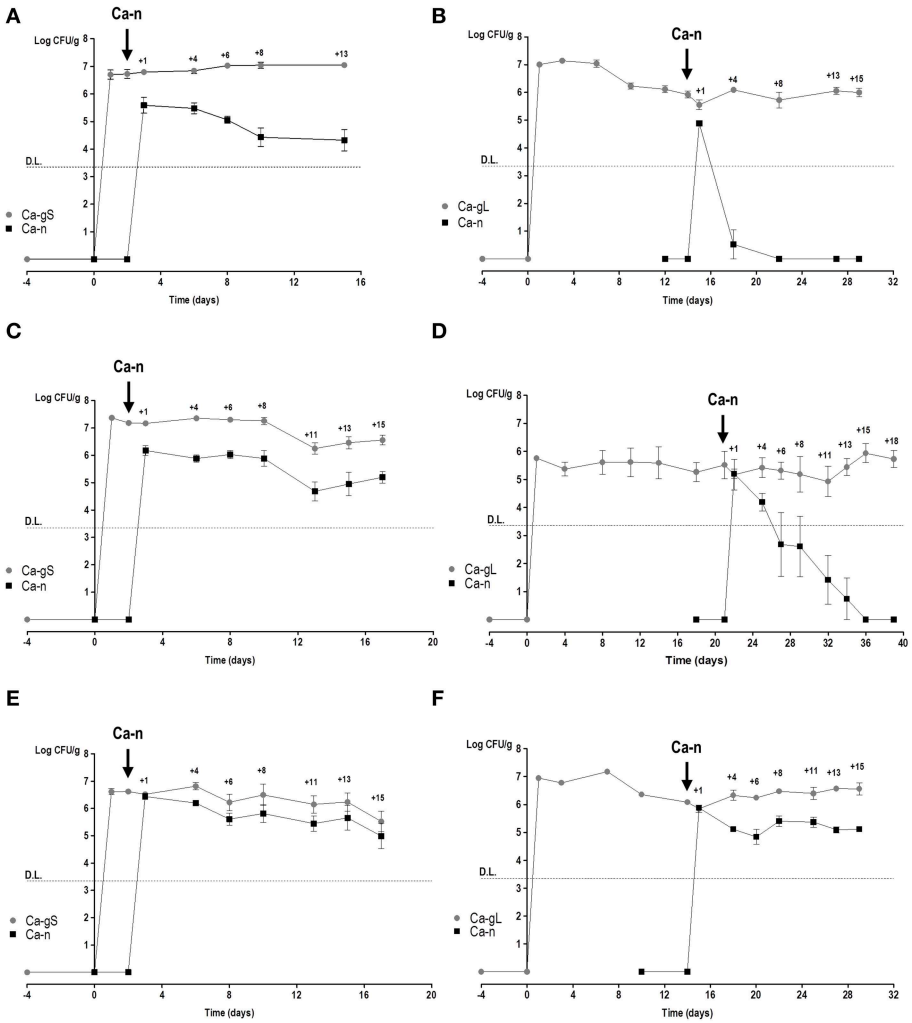


**FIGURE 3 | Competition in colonization among Ca-n and Ca-g *C. albicans* populations.** Competition between Ca-n and Ca-g populations was performed through inoculation of cells from *in vitro* culture in the day and doses indicated. Colonies were associated to a specific population in accordance to the fluorescent label. A value of 0 is attributed to non-detected-populations. Stool levels (log CFU per gram, mean ± SEM) are represented along the time. **(A)** Ca-gS vs. $10^6$ cells of Ca-n ($n = 3$); **(B)** Ca-gL vs. $10^6$ cells of Ca-n ($n = 7$); **(C)** Ca-gS vs. $5 \times 10^6$ cells of Ca-n ($n = 5$); **(D)** Ca-gL vs. $5 \times 10^6$ cells of Ca-n ($n = 5$); **(E)** Ca-gS vs. $10^7$ cells of Ca-n ($n = 6$); and **(F)** Ca-gL vs. $10^7$ cells of Ca-n ($n = 4$). DL refers to detection limit as explained in Materials and Methods section.

does not really impact the outcome of the evolution of the population levels as no statistically significant differences were detected in the $CI^{g/n}$ ratio at any dose administered, neither in short-term or long-term populations (**Figures 4A,B**). However, strikingly clear differences appear when comparing Ca-gS and Ca-gL groups, regardless the dose: the $CI^{g/n}$ was always higher for *C. albicans* cells that were in the gut for a long time, that is Ca-gL outcompetes more efficiently the new Ca-n population than Ca-gS (**Figure 4C**). Moreover, we only find values for $CI^{g/n}$ below 1 (negative as a logarithm) in the Ca-gS group. Remarkably, the mean of this index does not significantly changes within a group of mice over time (**Figure 4C**) with $\log_2$ values of 1.41 ± 0.36 (day 4), 2.31 ± 0.64 (day 8), 2.41 ± 0.64 (day 13), and 1.17 ± 0.45 (day 15) for short-term group; and 5.38 ± 0.69 (day 4), 4.54 ± 0.64 (day 8), 6.34 ± 0.49 (day 13), and 6.79 ± 0.73 (day 15) for Ca-gL group ($\log_2 CI^{g/n}$, mean ± SEM).

Collectively, these results demonstrate that Ca-gL cells show increased fitness over Ca-gS in competition experiments with a freshly inoculated *C. albicans* cell population, therefore reflecting different adaptation to the host.

## Gut-adapted *C. albicans* cells are Efficiently Maintained during Cohousing Experiments

In order to determine more precisely the behavior of gut-adapted populations, we performed competition experiments between different gut adapted *C. albicans* populations. For this purpose, we considered another type of adapted population, Ca-f (from feces-derived *C. albicans*, **Table 1**). We first determined the ability of antibiotic-treated mice to become colonized from

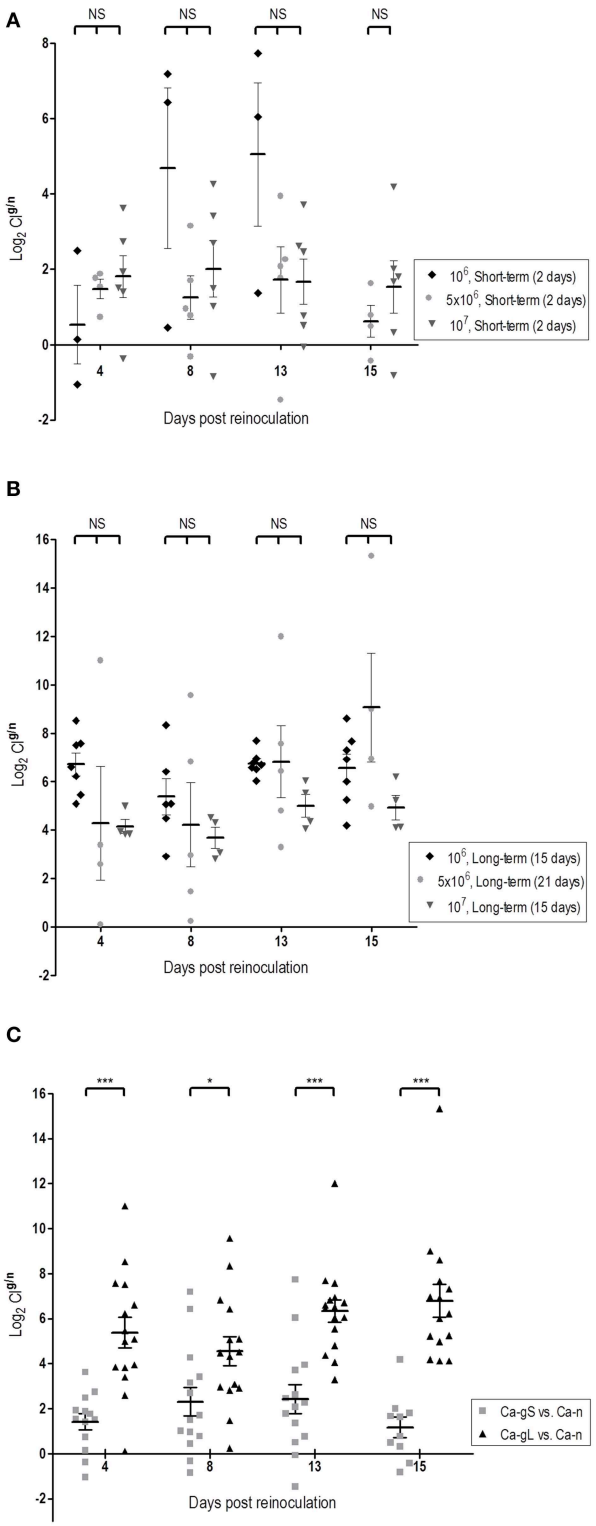


**FIGURE 4 | Dose and time influence in the colonization fitness.** Differences in fitness are determined through comparison of CI values ($log_2$). Each symbol represents data from an individual mouse. **(A)** Effect of dose among Ca-gS groups ($n = 3$–6). **(B)** Effect of dose among Ca-gL groups ($n = 4$–7). **(C)** Effect of the time past in the gut regardless the dose ($n = 14$–16). $^*p < 0.05$, $^{***}p < 0.001$.

stool samples; this possibility has been recently shown to occur and provide experimental evidence for horizontal transmission under specific experimental conditions (Cutler et al., 2011). We cohoused (same cage) two control mice (i.e., non-inoculated with *C. albicans*, only receiving PBS) with 8 mice inoculated with *C. albicans* (**Figure S2**). PBS-mice reached high fungal loads in stools as early as one day after the cohabitation (mean = 5.79, logCFU/g), although still not reaching the levels of *C. albicans*-mice (mean = 7, logCFU/g). At day 2, comparable fungal loads were found in both groups, PBS or *C. albicans*-inoculated (mean = 7.12 and 7.28, respectively). We therefore conclude that cohousing mice is an efficient and natural way to spread out *C. albicans* gut-adapted populations in mice under our conditions.

We then studied the behavior of short (Ca-fS) and long term (Ca-fL) Ca-f populations in competition with different Ca-g populations. For this purpose, 3 groups of mice were allowed to cohabit (same cage) together: Group 1 had no fungi and just received antibiotic treatment; Group 2 had colonizing *C. albicans* CAF2-dTOM2 on day 2 after gavage (Ca-gS) while Group 3 had *C. albicans* CAF2-GFP on day 21 (Ca-gL) after gavage (see **Figure S3**). The appearance of *C. albicans* cells from different origins was followed in the stools of the animal in the next 3 weeks. Given the above results, the source of exogenous (non-gavage mediated) colonization is presumed to be the stool population (either Ca-fS or Ca-fL). As shown in **Figure 5**, all mice presented both GFP and dTOM2-labeled strains in their stools. This result indicates that Ca-fS and Ca-fL are efficient to become established within mouse gut even in the presence of a Ca-g population. However, each population showed a different evolution. In group 1, as animals were devoid of fungi, competition is initially established between Ca-fS and Ca-fL. Both GFP and dTOM2 labeled cells were able to maintain constant levels ($\approx 10^5$ yeasts per gram, **Figure 5A**), indicating that a *C. albicans*-free gut niche is not restrictive enough to discriminate between those two populations. Identical levels achieved by each strain in the first day indicate that both populations were similarly accessible to mice. In group 2 mice, we also found $\approx 10^5$ CFUs/g stools of Ca-fL, while the previous endogenous population (Ca-gS) showed colonization over $10^6$ CFUs/g. For group 3, steady state levels for Ca-fS and Ca-gL populations were around $10^6$ and $10^7$ yeasts per gram, respectively. Interestingly, Ca-fS population peaked at day 1 showing values very similar to endogenous population (mean = 6.7 vs. 6.5, logCFU/g) and later showing a slight decrease. Group 2 involves competition between Ca-gS and Ca-fL (**Figure 5B**). The latter did not achieve really high colonization levels at day 1 (**Figure 5B**). However, it is evident that it is favored in competition since mean $\log_2 CI^{g/f}$ values were negative (**Figure 6A**), that is $-0.99 \pm 1.61$ (day 4), $-2.84 \pm 0.80$ (day 8), $-1.95 \pm 1.36$ (day 13) and $-3.42 \pm 1.89$ (day 15) (mean $\pm$ SEM). In group 3, Ca-fS is more efficient in immediate survival as it reached the highest loads at day 1, nevertheless it doesn't maintain this level during the rest of the experiment (**Figure 5C**). In contrast with the previous group, $\log_2 CI^{g/f}$ values were all positive with means around 4–5, confirming that Ca-fS population fails to outcompete Ca-gL. Taken together, this

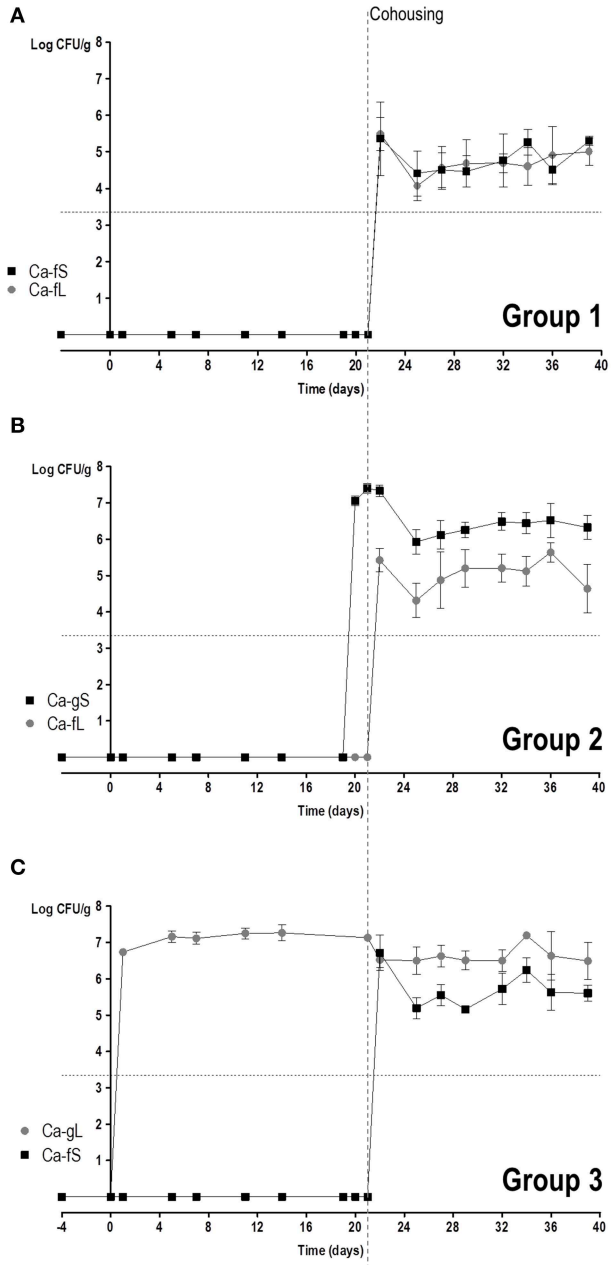


**FIGURE 5 | Competition in colonization among Ca-g and Ca-f *C. albicans* populations.** Competition between Ca-g and Ca-f populations was performed through putting together mice non-colonized (group 1) and mice already colonized for 2 or 23 days (groups 2 and 3, respectively). Colonies were associated to a specific population in accordance to the fluorescent label. Each symbol represents data from an individual mouse. Stool levels (log CFU per gram) are represented along the time. **(A)** Group 1: Ca-fS vs. Ca-fL ($n = 3$); **(B)** Group 2: Ca-gS vs. Ca-fL ($n = 3$); **(C)** Group 3: Ca-gL vs. Ca-fS ($n = 3$).

reveals that the population Ca-fL suffers a slight impairment in the early colonization stage (establishment and immediate survival) for which Ca-fS population is better adapted.

Regarding the second stage, Ca-fL population is conveniently prepared for persisting in the gut, while Ca-fS shows some difficulties competing with a population adapted to this stage (**Figures 5B,C**). In addition, calculated $CI^{L/S}$ show a similar competition between long-term and short-term, independently of the adapted-*C. albicans* source (**Figure 6B**). Although Group 3 presented always higher $CI^{L/S}$ means than group 2 (especially on day 4), the differences are not significant (for day 4, $p = 0.131$). However, we observe a reduction in fitness in short-term adapted populations (from either gut or feces) at all times compared with Ca-fL or Ca-gL, confirming an important difference between those populations and an advantage for long-term adaptation in persisting in the gut (**Figure 6A**).

## Discussion

Adaptation to host environment is a necessary trait for any commensal microbe. *C. albicans* is a member of the human microbiota and during the past few years, several efforts have been directed toward the identification of mechanisms responsible for its relationship with humans. The development of commensalism models, normally based on antibiotic-mediated microbiota reduction, is enabling the exploration of this essential aspect of the host-fungus interaction. Initial studies by Kinneberg et al. (1999), showed that inoculation with $10^3$ or $10^7$ *C. albicans* cells resulted in high colonization of the cecum after 3 or 7 days, but no information was provided for earlier time points. We show here that as early as 3 days after inoculation, steady levels of $10^7$ CFUs per gram in stools are usually attained starting from as low as 100 cells inoculated. Given the correlation between oral doses and stool levels and the increase in up to 5 logarithmic units, it seems that during this period *C. albicans* cells focus on proliferation. Since after 3 days we already observe steady colonization levels ($\sim 10^7$ CFU/g) we chose 2 days of colonization as representative of this period for comparative studies. We show here that populations that had colonized the intestine for a long time (15–21 days) (Ca-gL) display enhanced fitness over those that had been in the gut for few (2) days (Ca-gS) or *in vitro* cells (Ca-n). This is evidenced by their reduced kinetics of disappearance from the gut after removal of antibiotic therapy and by the increased competition *in vitro* cells compared with Ca-gS. In fact, a conclusion from our studies is that colonization levels do not correlate with fitness as Ca-n rapidly aquire high colonization levels but do still show decreased fitness. Therefore, we propose that initial stages of colonization in our experimental system are mainly responsible for attaining high fungal doses (replication period) but full adaptation (at least, in terms of competitiveness) is only achieved after a more prolonged time (adaptation). Similar behaviors are also observed when the adapted populations came from feces (through coprophagy). Using transcriptomal analysis, several genes have been shown to be differentially expressed in the gut (Rosenbach et al., 2010). Many of these genes were also expressed either during growth in exponential phase or post-exponential-phase, suggesting correlation with both its ability to grow quickly (characteristic of exponential phase) and the resistance to different types of stress (more prone to stationary phase) in accordance with the challenging environment (nutrient competition, pH, oxygen availability and microbial interactions)

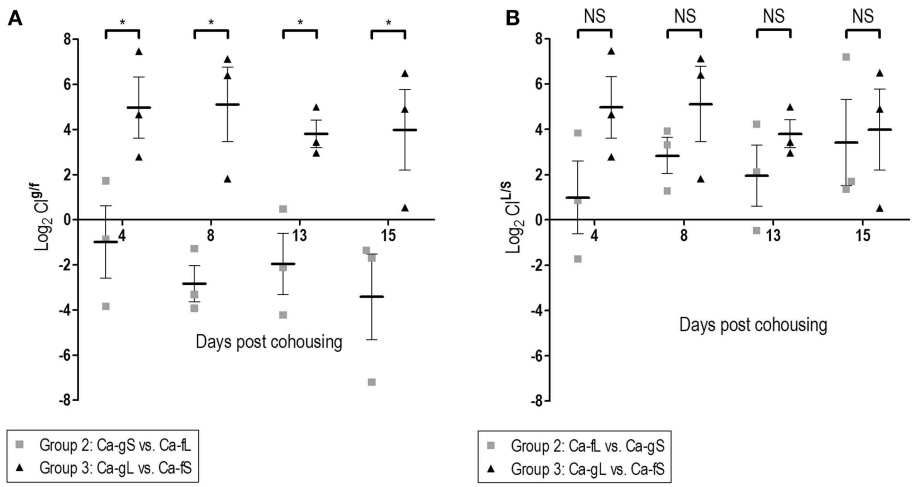


**FIGURE 6 | Dose and time influence in the colonization fitness.** Differences in fitness are determined through comparison of CI values ($log_2$). **(A)** Effect of the time past in the gut. **(B)** Effect of the source. *$p < 0.05$.

that cells encounter in this niche. Such a scenario is in accordance with recent data that indicate that identified transcription regulators of commensalism mainly affect functions involved in carbon and nitrogen metabolism (Pérez et al., 2013). In any case, in Rosenbach's study cells were harvested from cecum just 3 days after inoculation, so we would associate it with our Ca-gS population.

The existence of different stages in fungal colonization would, in addition, explain some observations from literature. For example, while MAPK signaling pathways are important in mouse gut colonization (Prieto et al., 2014), defects in Hog1 results in an immediate impairment to colonize mouse gut (especially when a competition assay with a wild type strain is performed), although others such as Mkc1 and Cek1 changes result in long terms defects. Moreover, it has been reported that *efh1* and *efg1* mutant cells develop higher gut establishment levels than the wild type strain and it is considered as colonization specific transcription factor (White et al., 2007; Pande et al., 2013; Pierce et al., 2013). This phenotype resembles what we observe in in the Ca-gS population, suggesting that these factors (and possibly Wor1) could play a role in this initial adaptation.

The existence of different stages for colonizing the gut has been proposed also for other microorganisms. For example, in *Vibrio cholerae* (Lee et al., 1999; Schild et al., 2007) an early period is essential for survival and multiplication *in vivo*, while prolonged period allows bacteria to live outside the host, where nutrients amounts are much lower. Host-induced shedding seems to be also a strategy used by the microbe for dissemination (Merrell et al., 2002; Almagro-Moreno et al., 2015). As *C. albicans* is a long-term permanent colonizer of the gut tract with no significant life outside the host ("after-hours"), our observations could reflect adaptation to host from domesticated laboratory strains. It has been reported that another gut-colonizer, *Salmonella typhimurium*, would derivate into two phenotypically different populations, one becoming pathogenic to improve the chances of the second one to colonize and outcompete the microbiota present in the gut (Stecher et al., 2007). In addition, different *C. albicans* levels in the gut have been proposed to be associated with the existence of hypothetical phenotypic variants (similarly than those from *S. typhimurium)* that appear during host colonization (Kumamoto, 2011). Such a scenario is in accordance with high fungal loads of *C. albicans* in the gut being a risk factor for developing a systemic infection and frequently associated with inflammatory bowel disease (Gerard et al., 2015). This interplay is multifactorial, as commensalism is not only a microbe-driven process but dependent on the immunological status of the host (Romani, 2004; Zelante et al., 2012) and even in immune competent mice, host factors (such as the mucins) can suppress virulence attributes (Kavanaugh et al., 2014).

In conclusion, we present here evidence that in *C. albicans* two different (at least, in terms of fitness) stages exist during the colonization of mouse gut with laboratory maintained strains of *C. albicans*. As this species is a non-habitual member of the mouse gut microbiota (Iliev et al., 2012), caution must be taken in interpreting these observations, but determining whether this occurs in its natural reservoir (humans) is crucial given its potential implications in the management of *C. albicans* infections.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00792

**Figure S1 | Correlation between strains composition in the inoculum and day 1 stool population.** Data from several competition experiments were collected. Each point correspond to one experiment that include some mice ($n = 3$–6). Percentage of RFP labeled strain in the mixed inoculum and percentage of the same strain in the stools samples on day 1 were plotted and lineal regression was performed.

**Figure S2 | Fungal loads in mice stools of naturally acquired *C. albicans* populations.** Eight mice out of ten were inoculated with *C. albicans* ($10^7$ cells) after four days of antibiotic treatment. The other two mice were inoculated with PBS and all of them were kept in the same cage. Fungal loads in stools (log CFU per gram, mean ± SEM) from each group are represented along the time.

**Figure S3 | Scheme of cohousing experiment.**

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REVIEW
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# *Candida albicans* Agglutinin-Like Sequence (Als) Family Vignettes: A Review of Als Protein Structure and Function

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Approximately two decades have passed since the description of the first gene in the *Candida albicans* ALS (agglutinin-like sequence) family. Since that time, much has been learned about the composition of the family and the function of its encoded cell-surface glycoproteins. Solution of the structure of the Als adhesive domain provides the opportunity to evaluate the molecular basis for protein function. This review article is formatted as a series of fundamental questions and explores the diversity of the Als proteins, as well as their role in ligand binding, aggregative effects, and attachment to abiotic surfaces. Interaction of Als proteins with each other, their functional equivalence, and the effects of protein abundance on phenotypic conclusions are also examined. Structural features of Als proteins that may facilitate invasive function are considered. Conclusions that are firmly supported by the literature are presented while highlighting areas that require additional investigation to reveal basic features of the Als proteins, their relatedness to each other, and their roles in *C. albicans* biology.

**Keywords: fungus, *Candida albicans*, gene family, Als proteins, adhesion, aggregation, attachment, invasion**

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## SETTING THE SCENE

*Candida albicans* can exist in its human host as a commensal, and under certain circumstances, cause disease. *C. albicans* is the principal cause of opportunistic mycoses worldwide (Pfaller and Diekema, 2007). Adhesion is important for establishing the *C. albicans*-host interaction. The adhesive role of Als proteins stimulated enthusiasm for their study.

The first *ALS* gene, *ALS1*, was detected in a differential hybridization screen in the pre-genome era of *C. albicans* research (Hoyer et al., 1995). The protein was named because of its similarities to *Saccharomyces cerevisiae* alpha-agglutinin, which promotes cell–cell contact during mating (sexual reproduction) of haploid yeasts (Lipke et al., 1989). The presence in *C. albicans* of additional genomic fragments that hybridized with *ALS1* sequences suggested the existence of a gene family (Hoyer et al., 1995, 1998a). Additional effort revealed the full nature of the ALS family in *C. albicans* (Gaur and Klotz, 1997; Hoyer et al., 1998b; Hoyer and Hecht, 2000, 2001; Zhao et al., 2007a), which proved to be essential for accurate assembly of the *C. albicans* genome sequence

**Abbreviations:** AFR, amyloid-forming region; Als, agglutinin-like sequence; CSH, cell surface hydrophobicity; CT, Als C-terminal domain; NT or NT-Als, Als N-terminal domain; PBC, peptide-binding cavity; T, Als Thr-rich domain; TR, Als tandem repeat domain.

(Braun et al., 2005). Cross-hybridization between *C. albicans ALS* sequences and genomic DNA from other *Candida* species suggested that similar genes are found in closely related fungi (Hoyer et al., 2001).

The novelty of coding tandem repeats in *ALS* genes figured largely into initial conceptual thinking about organization of the genes and their encoded proteins. For example, early descriptions of a typical Als protein reported three domains: the central tandem repeats, everything before the repeats (N-terminal domain), and everything after the repeats (C-terminal domain; **Figure 1A**). As investigations proceeded, Als proteins were described as including four domains: the N-terminal domain (NT or NT-Als; approximately amino acids 1–329 of the unprocessed protein), the T domain (T; approximately amino acids 330–433, ending just at the start of the tandem repeats), the central tandem repeats (TR), and the CT. Two notable sequence features prompted the idea that the NT and T domains should be considered separately: the Thr richness of amino acids 330–433 and the presence of a short sequence that has amyloid-forming propensity (approximately amino acids 325–329; Garcia et al., 2011). However, crystallographic analysis demonstrated that the AFR is a part of the NT structure (Salgado et al., 2011; Lin et al., 2014; **Figure 1B**), leaving open for question the best way to describe the domains of a typical Als protein. Because many manuscripts use the four-domain description of Als proteins, that convention is featured in **Figure 1** and throughout this review.

Over the years, as cell-biological observations about the Als family accumulated, the *C. albicans* research field also matured, providing new reagents and approaches for studying the Als family, as well as growing knowledge about numerous aspects of *C. albicans*–host interactions. Availability of the crystallographic structure of NT-Als (Salgado et al., 2011; Lin et al., 2014; **Figure 1B**) allows examination of the structural basis for Als function. This review manuscript critically interprets the literature in light of recent structural insights, as well as the abundance of new fungal genome sequences. The review is configured as a set of questions (vignettes) that focus on various properties of the Als proteins. Therefore, the review does not contain an exhaustive list of Als-related literature, but instead focuses on selected reports that shape the answer to the overall question "What do we really know about Als proteins and the mechanistic basis for their function?"

## WHAT GENES/PROTEINS ARE INCLUDED IN THE *ALS*/Als FAMILY?

When *C. albicans ALS1* was first described, a BLAST search of the non-redundant protein database produced one 'hit': *S. cerevisiae* alpha-agglutinin (now named Sag1; Lipke et al., 1989). We now know that there are many *ALS* genes in *C. albicans* and that *ALS* genes are present in other fungal species. Moreover, recent structural biology insights, coupled with long-standing functional observations, raise the question of whether Sag1 belongs to the Als family. These topics are reviewed here with the goal of defining the minimum features that are needed for including a protein in the Als family. Overall, the observations suggest that the Als family is more diverse than currently envisioned.

The composition of the ALS family is most clear for *C. albicans*, in which eight distinct loci are known (*ALS1* to *ALS7*, and *ALS9*; Hoyer et al., 2008). *ALS* gene names were assigned sequentially as the genes were discovered. Recombination between two contiguous loci (*ALS5* and *ALS1*) led to production of a novel open reading frame (ORF) in some strains; this locus was named *ALS51* to indicate its chimeric origin (Zhao et al., 2011). Each *ALS* locus encodes numerous alleles, with sequence variation occurring primarily in the tandem repeat and C-terminal domains (Zhang et al., 2003; Zhao et al., 2003, 2007c; Oh et al., 2005). Many sequence variants encoding the NT domain of Als5 have also been documented (Zhao et al., 2007c); such NT sequence variation may exist for other Als proteins, as well. Allelic variation caused confusion in providing a name for *ALS8*, which proved to be the same physical locus as *ALS3* (Zhao et al., 2004).

Based on *C. albicans* sequences, the consensus definition of Als proteins includes those with an NT domain, followed by the T, TR, and CT sequences (NT/T/TR/CT). The secretory signal peptide and GPI anchor addition sequence are key features that direct mature proteins to their localization in the cell wall, so also should be included in the consensus definition. The NT domain of Als proteins encodes conserved Cys residues that are key for folding of the protein, as well as the invariant positively charged amino acid (e.g., Lys59 in NT-Als3; Lin et al., 2014) located at the end of the PBC. These generalizations hold true for Als proteins in *Candida dubliniensis*, perhaps the closest relative of *C. albicans*. Sequence similarities and synteny analysis revealed that *C. dubliniensis* includes all *C. albicans* Als proteins except Als3 and Als5 (Jackson et al., 2009). *C. dubliniensis* has an extra Als protein that is not syntenic with those in *C. albicans*.

Unlike the initial BLAST search many years ago, a current BLAST search yields dozens of 'hits,' fueled by the availability of numerous fungal genome sequences. The sequence data provide a catalog of potential Als proteins much more readily than previous laborious methods such as cross-hybridization studies and amplification of sequences using degenerate primers (Hoyer et al., 2001). Butler et al. (2009) presented the best-known analysis of the Als family from the perspective of multiple fungal genomes. The study includes pathogens (*Candida tropicalis*, *Candida parapsilosis*, *Meyerozyma* (*Candida*) *guilliermondii*, *Clavispora* (*Candida*) *lusitaniae*) and non-pathogenic species (*Lodderomyces elongisporus*, *Debaryomyces hansenii*). Characterization of Als protein function in the pathogenic species is emerging, showing a role in adhesion and pathogenesis similar to the Als proteins in *C. albicans* (Bertini et al., 2015). The genomes of fungi that are important in biofuel production have been sequenced (e.g., *Scheffersomyces (Pichia) stipitis*, *Candida tenuis*, *Spathaspora passalidarum*) and Als proteins are predicted in them (Jeffries et al., 2007; Wohlbach et al., 2011; Maguire et al., 2013). Because Als proteins in these species are unlikely to mediate interactions with a mammalian host, studies to examine their function will provide novel insights.

As genome sequencing efforts advance, sequences are available for an ever-larger number of fungi (Grigoriev et al., 2014),

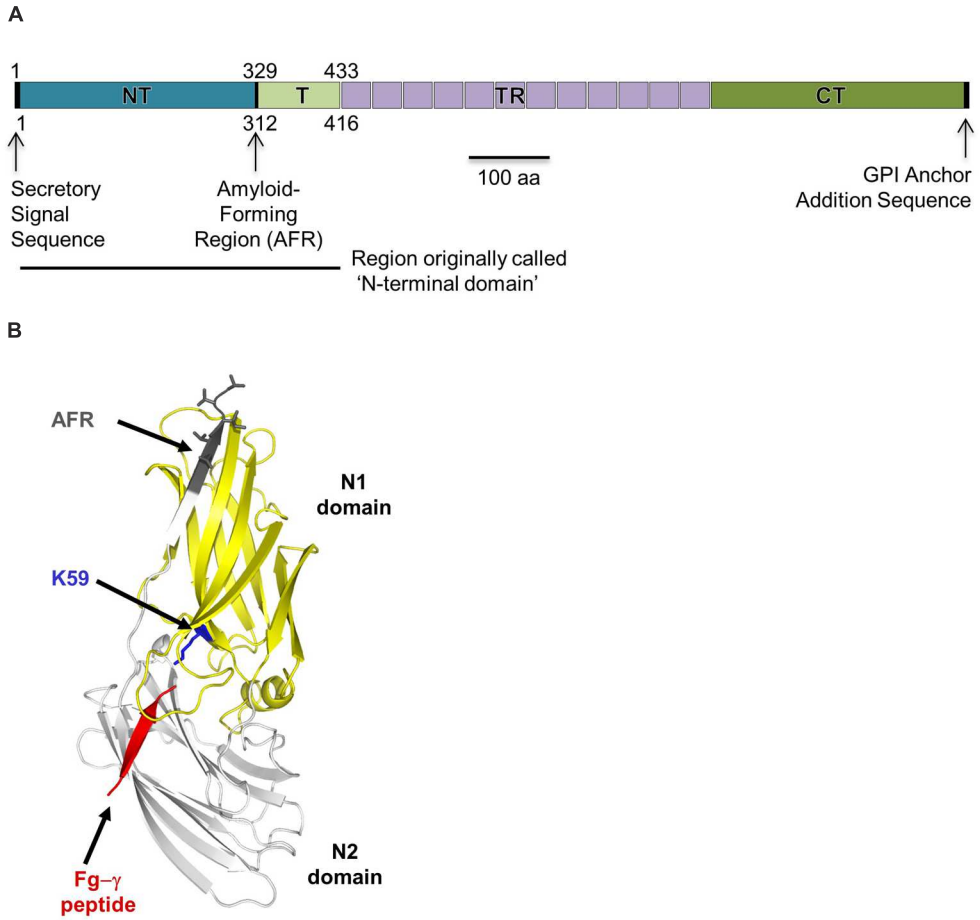


**FIGURE 1 | Als protein structure. (A)** Line drawing of a representative Als protein, using *C. albicans* Als3 as the example. A detailed schematic comparing the basic features of all *C. albicans* Als proteins was published previously (Hoyer et al., 2008). The various domains are labeled as they are discussed in this review: NT (also called NT-Als), T, TR, and CT. Early literature referred to the sequences N-terminal of the TRs as the 'NT'; this region is indicated by the solid line below the main drawing. Als proteins include a secretory signal sequence which is processed, so absent from the mature protein. Als proteins also encode a consensus sequence for GPI (glycosyl-phosphatidylinositol) anchor addition. The GPI anchor subsequently is processed and the mature protein linked to beta-1,6-glucan in the *C. albicans* cell wall (Kapteyn et al., 2000). Numbering schemes found in the literature may be confusing because some start at the initial Met (shown above the line drawing) while others start at the N-terminal amino acid of the mature protein, following cleavage of the secretory signal peptide (e.g., amino acid 18 of the unprocessed sequence in many of the Als proteins; shown below the line drawing). Clarifications are provided throughout the review to indicate whether the numbering scheme arises from the unprocessed (signal sequence present) or processed (cleaved signal sequence) protein. **(B)** X-ray crystallographic structure of the NT domain from Als9-2 in complex with the C-terminal peptide from fibrinogen-γ (red; Salgado et al., 2011) that fits into the protein's PBC. An invariant Lys residue (K59, using a numbering scheme for the processed protein; blue) at the end of the PBC recognizes the C-terminal carboxyl group of the peptide ligand. The overall fold of the protein involves eight conserved Cys residues that form four disulfide bonds. In the ligand-bound form of the protein, the AFR (gray) attaches to the NT-Als surface. The AFR is unattached to the NT-Als surface in protein molecules that do not have a ligand in the PBC.

as well as multiple isolates from the same species, providing the opportunity to compare strain diversity (Pryszcz et al., 2015). However, the rapid accumulation of fungal sequence data has outpaced the ability to refine genomic assemblies. Because *ALS* genes often contain extensive tracts of tandemly repeated sequences, they are extremely difficult to assemble correctly using automated methods. This same problem existed for *C. albicans*: accurate assembly of the *ALS* genes relied heavily upon laboratory experimentation to define the ORFs and corresponding physical loci (Braun et al., 2005). Descriptions of current *ALS* sequences derived from genome data provide the impression that the analysis is very precise, however, closer examination reveals incompletely assembled and misassembled ORFs that are not a solid foundation for such detailed conclusions. ORFs identified as unique sometimes lack a 5′ end or a 3′ end. Some ORFs lack both, existing only as tracts of *ALS*-like tandem repeats that are not joined to anything else. In most genome sequences, considerable effort will be required to answer even the most basic questions such as how many *ALS* loci are present.

Despite the need for follow-up experimentation, the genome sequence data provide sufficient information to indicate that while fungal species encode NT/T/TR/CT ALS genes like those in *C. albicans*, other variations exist. For example, some species have *ALS* genes with novel TR sequences of varying unit length

and composition. Some of the fungal genomes encode at least one NT/T/TR/CT Als protein and one that includes only NT and CT, suggesting the potential need to redefine the minimum features for including a gene in the ALS family. The NT/CT gene structure is more akin to *S. cerevisiae* Sag1 than *C. albicans* Als proteins (**Figure 1**). Closer examination of the NT domain of these proteins reveals a sequence that perhaps is also more like Sag1. The NT of Sag1 is predicted to include two immunoglobulin domains with three of the four disulfide bonds that are present in NT-Als (Grigorescu et al., 2000; Salgado et al., 2011). Functional analysis of Sag1 revealed that it binds the free C-terminal peptide of **a**-agglutinin, Aga2 (Cappellaro et al., 1994). Sag1 and other Sag1-like proteins include a positively charged amino acid (Arg) in a structurally equivalent position to the Lys residue located at the end of the PBC, suggesting that they also contain this cavity (Cota and Hoyer, 2015). Sag1 has resisted efforts to solve its structure (Grigorescu et al., 2000) so structural data are not available for comparison.

Sequence similarities and predicted structural similarities between Als and Sag1-like proteins raise the question of whether to consider them as part of the same family. Evidence to support the idea that the proteins belong to the same family includes the fact that the sequences share sufficient similarity to recognize each other using a simple BLAST search. However, while Als and Sag1-like proteins are also predicted to share similar structural features, they represent two functional 'extremes.' Als protein NT domains mediate adhesion to a broad range of ligands with moderate-to-low binding affinities, which facilitates their role in host-pathogen interaction (Donohue et al., 2011; Salgado et al., 2011). In contrast, the NT domain of Sag1-like proteins mediates cell–cell contact during the mating of haploid cells, and therefore ligand recognition must be very selective to maintain the integrity of the mating interaction. Binding between Sag1 and the C-terminal peptide of Aga2 occurs at a high affinity (Zhao et al., 2001). This separation between the proteins suggests that perhaps it is more appropriate to consider Als and Sag as different families, closely related by their similarities in sequence and structure. As new genome data emerge and functional analyses progress, it will become clear whether other Als and Sag proteins exist at the functional extremes described above or whether intermediate proteins exist (e.g., an Als-like protein with high affinity for a limited number of ligands). These data will provide additional evidence regarding classification of the overall group of proteins.

Another point to resolve in defining the ALS family across various fungal species is to assign a name for each gene. The Butler laboratory's extensive analysis of synteny between various fungal genomes provides a starting point for this discussion. The synteny analysis initially focused on two strains of *C. albicans* and a single isolate each of eight other species (Fitzpatrick et al., 2010), then was expanded to include a total of 13 species (Maguire et al., 2013). Results of the analysis are visualized easily using the Candida Gene Order Browser (http://cgob.ucd.ie) that highlights syntenic loci that are perhaps the most deserving of a name that is the same as a *C. albicans ALS* gene. However, conservation of protein function cannot be assumed from syntenic genes (Jackson et al., 2009) and regulation of syntenic genes may vary between species. Northern blot analysis of total RNA isolated from several different growth conditions suggested that *C. dubliniensis ALS* ORFs tend to be constitutively expressed, in contrast with the differential expression noted for many *C. albicans ALS* genes (Hoyer et al., 2001). Fortunately, most publications refer to *ALS* genes by the ID numbers assigned by the genome sequencing effort (e.g., Bertini et al., 2015). This practice avoids confusion by providing unambiguous reference to specific loci. Indeed, even though the literature appears to include considerable information about *C. albicans* Als proteins, their true functional relatedness is still relatively unexplored and the phenotypes observed may be heavily influenced by protein abundance and localization on the fungal cell surface rather than the true functional capabilities of the protein. Much of this review article examines these questions.

The list of *ALS* genes and Als proteins is certain to become longer and more diverse with the emergence of new genome sequencing data and functional insights. The current definition of Als proteins including the NT/T/TR/CT configuration will likely need to be broadened. Perhaps the minimum definition of an Als protein will only include the NT domain and sufficient structure to display it on the fungal cell surface. This definition would place more emphasis on protein function than on absolute number and configuration of Als domains. The ligand-binding activity of Als proteins is perhaps their most important function and is examined in detail in the subsequent section.

## HOW DO Als PROTEINS BIND LIGANDS?

The adhesive function of Als proteins is a major reason for studying them. Adhesion is an important feature of colonization, which provides the potential for disease development (Calderone and Braun, 1991). While it might appear easy to define the word 'adhesion,' its liberal use in the literature describes many different interactions. This diversity of interactions makes a precise definition elusive, especially for a molecule like an Als protein that has many different sticky interactions. For example, the process of Als proteins binding to each other might be called 'adhesion' in the literature, but could be more precisely described as 'aggregation.' Likewise, 'attachment' may be a better word choice to describe the non-specific interactions between *C. albicans* and abiotic surfaces (discussed below). Here, we define 'adhesion' as ligand binding with the goal of exploring the molecular mechanisms and structural features of the Als protein that are involved in this process.

Als proteins were demonstrated to function in adhesion by deletion of *ALS* genes from *C. albicans* or expression of *ALS* genes in *S. cerevisiae*, leading to reduction or gain of adhesive function, respectively (reviewed in Hoyer et al., 2008). Because deletion of *ALS3* provides the greatest loss of adhesive function among the ALS family, it gained considerable attention in the literature. Cell biology-based inquiry provided an extensive list of divergent binding partners for Als3 including human fibronectin, laminin, collagen, gp96, EGFR, HER2, N-cadherin, E-cadherin, fibrinogen, casein, equine ferritin, bovine serum albumin (BSA), and *Streptococcus gordonii* SspB (Gaur et al., 2002; Gaur and Klotz, 2004; Sheppard et al., 2004; Phan et al., 2007; Almeida et al.,

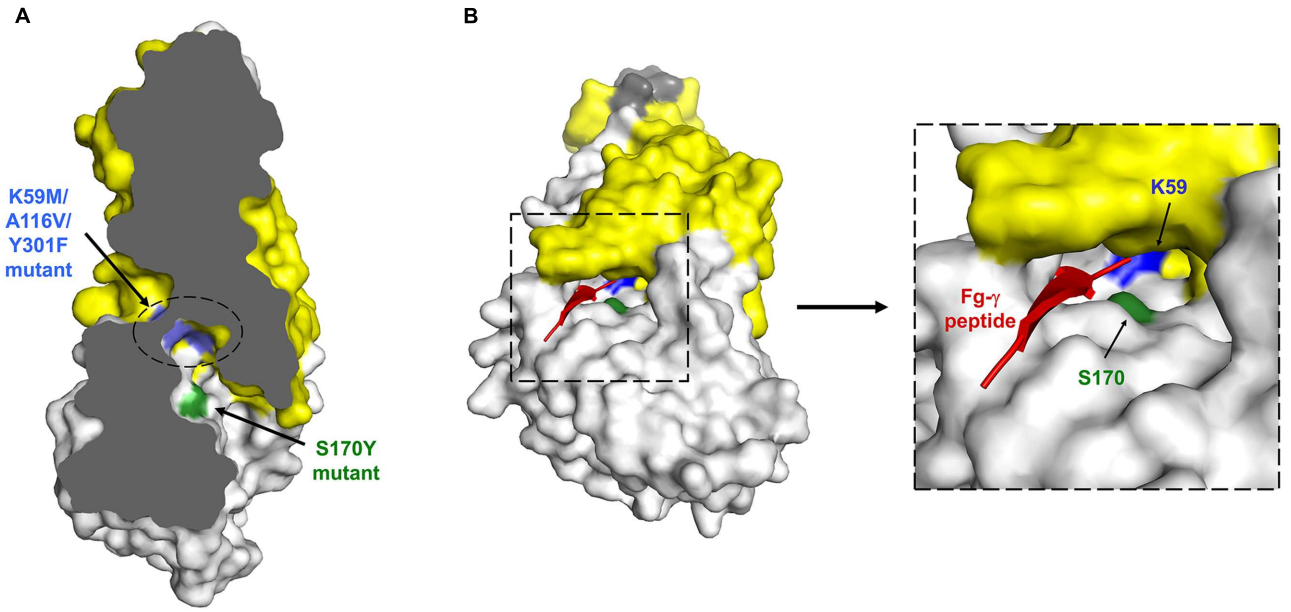


**FIGURE 2 | Schematics of NT-Als protein structure to illustrate the location of mutations used to deduce the ligand-binding mechanism.** **(A)** Cross-section of overall NT-Als3 structure highlighting the location of the PBC and key residues used in loss-of-function mutants. Note that the indicated mutations were introduced without altering NT-Als surface properties. Amino acid numbering reflects the processed (signal peptide removed) form of the protein (Lin et al., 2014). **(B)** Expanded PBC detail showing entry of a model peptide and location of amino acids included in the functional analysis using the structure of NT-Als9-2 (Salgado et al., 2011).

2008; Silverman et al., 2010; Liu et al., 2011; Zhu et al., 2012). The NT domain was implicated in much of the inferred protein–protein interactions (Loza et al., 2004; Sheppard et al., 2004; Zhao et al., 2006). Molecular modeling was used to conclude that the Als3 NT domain interacts with its binding partners by surface–surface interactions (Sheppard et al., 2004; Phan et al., 2007). However, the large number of proposed binding partners raises the question of how NT-Als can adapt to surfaces of so many structurally unrelated ligands to mediate relevant interactions. Solution of the molecular structures of the NT domains of Als1, Als3, and Als9-2 (Salgado et al., 2011; Lin et al., 2014) provided an Als protein model with atomic resolution that can reconcile these observations (**Figure 1B**). A discussion of how NT-Als structural data inform functional insights was communicated recently (Cota and Hoyer, 2015) and is presented briefly here.

The overall NT-Als fold is reminiscent of bacterial adhesins such as *Staphylococcus aureus* ClfA (clumping factor; Deivanayagam et al., 2002) and *Staphylococcus epidermidis* SdrG (Ponnuraj et al., 2003). Unlike the bacterial proteins, however, NT-Als contains a wide and flat cavity (PBC) between domains that can bury up to six C-terminal residues of peptides in an extended conformation (**Figure 1B**). The side chain amine of an invariant Lys at the end of this cavity (K59) establishes a salt-bridge with the C-terminal carboxylic acid of the incoming peptide. The peptide backbone forms extensive hydrogen bonds in parallel orientation to a β-strand (G2) from the second Ig domain. Water molecules mediate interaction with the A2 strand on the other side of the peptide. Water molecule number and arrangement are variable depending on the peptide ligand, and provide the ability for NT-Als to recognize a broad array of ligands. Thus, NT-Als has a novel mechanism to bind the flexible C terminus of proteins.

Als3 was selected as a model for mutational analysis to test structural hypotheses in a native *C. albicans* background (Lin et al., 2014). Mutations were designed to interfere with PBC function and also with function of the AFR that is located within the NT-Als domain. The role of the AFR in Als-mediated aggregation is discussed extensively in the literature and examined in subsequent sections below. PBC mutations involved altering either three amino acids (K59M, A116V, Y301F) or one amino acid (S170Y; **Figure 2**). The resulting mutations did not change the surface structure of Als3 and mutant proteins lacked peptide-binding capabilities *in vitro*. Mutations were made in *ALS3* constructs that were integrated into the *ALS3* locus of a *C. albicans* Δ*als3*/Δ*als3* strain. Immunolabeling with an Als3-specific monoclonal antibody (Coleman et al., 2009) demonstrated the presence of Als3 on the *C. albicans* surface in comparable quantities and location to those produced by a wild-type-Als3 control construct. The adhesive phenotype of the resulting strains was absolutely remarkable: strains with the targeted mutations had the adhesive phenotype of a null mutant strain in standard adhesion assays involving complex surfaces such as cultured and fresh human cells (Lin et al., 2014), as well as whole bacterial cells (*Streptococcus gordonii*; Hoyer et al., 2014). In other words, alteration of one or three amino acids and display of the mutant protein on *C. albicans* resulted in

the same phenotype as a strain on which no Als3 was displayed at all.

Collectively, these data provided a striking demonstration of the importance of the PBC to Als ligand-binding activity and its overall contribution to the phenotypes observed in standard adhesion assays. The idea that the PBC binds the free C-terminal end of proteins was reinforced because of the use in structural analyses of small synthetic peptides that readily fit into the PBC in that orientation. However, the mechanistic conclusion is also satisfying because of the strong conservation of K59 among *C. albicans* Als sequences and similar proteins identified in database searches. The positive charge of K59, positioned at the bottom of the PBC, is available to sink the net negative charge of the carboxyl end of a C-terminal peptide. Although purified proteins were key to deducing this mechanism, they provide a much more simple set of interactions than those involving whole Als proteins and a complex cell surface. Testing of the *C. albicans* PBC mutant strains with whole *Streptococcus gordonii* cells supported previously published work that showed SspB is an Als3 binding partner (Silverman et al., 2010; Hoyer et al., 2014). However, the C terminus of SspB is covalently linked to the bacterial cell wall, suggesting that Als3 must recognize other sequences in the large cell-surface SspB. Proteolytic action to create adhesion tethers (SspB-derived free C-terminal peptides that remain attached to SspB) was proposed as one hypothesis to explain the observations (Hoyer et al., 2014; Cota and Hoyer, 2015). Other hypotheses also exist and suggest that while considerable progress has been made toward understanding the mechanism by which Als proteins bind ligands, additional puzzles remain to be solved.

A discussion of the Als ligand-binding mechanism would not be complete without addressing the widespread notion that Als proteins also function as lectins, recognizing carbohydrate ligands. This information comes from multiple sources. Some sources may contain simple errors. Some may have improperly drawn conclusions from BLAST search data that reflected amino acid compositional similarities, rather than conservation of function. One experimental report exists that concludes a role for Als1 in binding fucose. These observations are discussed here.

BLAST searches are widely used because they provide quick information about the potential function of a newly characterized protein. A BLAST search of the non-redundant protein database using a *C. albicans* NT-Als sequence as the query leads to an alert for detection of a putative conserved domain called 'Candida_ALS_N superfamily.' Clicking on the alert leads the reader to this statement: 'This is likely to be the sugar or ligand binding domain of the yeast alpha-agglutinins.' This statement is problematic because it suggests lectin activity as the primary role for each protein, which is not true for either Als proteins or Sag1.

Databases within the *Candida* community, such as the Candida Genome Database (CGD; Inglis et al., 2012) also contribute to the conclusion that Als proteins bind carbohydrates. *C. albicans ALS* loci in CGD are appropriately annotated to reflect their protein-binding function, however, data for other *Candida* species are not developed fully and contain misleading information. *C. dubliniensis* locus Cd36_64220 is a useful example because it is syntenic with *C. albicans ALS9*. As expected, *C. albicans ALS9* is the best match for Cd36_64220 in a BLAST search. It would be reasonable to hypothesize that the proteins have similar function. However, *S. cerevisiae FLO1*, a large cell-wall lectin that binds mannose (Veelders et al., 2010) is listed as an ortholog for Cd36_64220. The Ser/Thr-richness and extensive tracts of repeated sequences in both Als proteins and Flo1 are likely responsible for these database searching 'hits' that are distractions, rather than indications of similar function.

One literature report suggests a role for Als1 in recognition of carbohydrate ligands (Donohue et al., 2011). The authors constructed a *S. cerevisiae* strain that secreted a soluble hexa-His-tagged NT/T Als1 protein. They applied the Als protein fragment to a glycan array and detected it with an anti-His antibody. Fucose-containing glycans were preferentially recognized. Fucosylated BSA was used in subsequent experiments to calculate the affinity of the interaction, but a BSA-alone control was not tested. Because Als proteins are known to recognize BSA (Klotz et al., 2004), the interaction between fucosylated BSA and the Als1 fragment may have indicated the affinity of the Als1–BSA interaction rather than the Als1–fucose interaction. Although the glycan array results suggest the possibility that the NT/T portion of Als1 can bind fucose, the mechanistic basis for this interaction remains unexplored. PBC involvement could be tested using structurally informed mutant proteins (Lin et al., 2014) and appropriate controls to support the conclusion of carbohydrate binding. The availability of structural data provides the opportunity to describe Als ligand-binding function at the molecular level. The abundance of published data that describe a role for Als proteins in peptide binding suggest that this function will outweigh any potential lectin activity and should be listed as the primary Als function in various reference databases.

## DO Als PROTEINS MEDIATE ATTACHMENT TO ABIOTIC SURFACES?

Questions about attachment of *C. albicans* to abiotic materials arise from a practical standpoint: *C. albicans* is able to form biofilms on the surface of implanted medical devices and attachment is an important initial step in biofilm formation. In addition to the role of Als proteins in binding peptide ligands, literature reports suggest that Als proteins are important for *C. albicans* attachment to abiotic surfaces. Although, this conclusion appears widely accepted, the mechanisms involved are still unclear. It is informative to separate the contribution of Als proteins to this function because many other cell wall proteins on the *C. albicans* surface [i.e., containing hydrophobic/amyloidogenic or glycosylated regions (Ramsook et al., 2010; de Groot et al., 2013)] could promote the same behavior. Several manuscripts were selected to represent the major viewpoints in this discussion (**Table 1**) and are detailed below.

### The Observations

Work by Aoki et al. (2012) has been cited as evidence that Als proteins mediate attachment to abiotic surfaces (de Groot et al., 2013; Demuyser et al., 2014). The authors used a

**TABLE 1 | Summary of key features from three published manuscripts that describe a role for Als proteins in attachment of *Candida albicans* to abiotic surfaces.**

| Manuscript | Aoki et al., 2012 | Finkel et al., 2012 | Garcia et al., 2011 |
|---|---|---|---|
| Yeast strain | *Saccharomyces cerevisiae* producing cell-surface Als NT/T/FLAG tag/alpha-agglutinin fusion proteins; one made for each Als protein | Wild-type Als proteins present on *C. albicans* cells grown to saturation in YPD, then released into fresh YPD; high levels of cell-surface Als1 | *S. cerevisiae* overproducing Als5 |
| Abiotic surface | Borosilicate glass, polypropylene, polyvinylchloride, polyurethane, polymethyl methacrylate, polytetrafluoroethylene, titanium | Fluxion flow chamber | Non-tissue-culture-treated polystyrene |
| Assay conditions | Cells washed and suspended in PBS | Cells resuspended in YPD | Cells washed and resuspended in TE |
| Quantification of adhesion | Plate 6M urea wash and count colonies | Capture image and count adherent cells | Absorbance at 570 nm to quantify retained crystal violet dye |
| Conclusion(s) | Yeast cells adhered to polypropylene, polyvinyl chloride and borosilicate glass, but not the other materials | *C. albicans* binds to PDMS channels but not to borosilicate glass; Als1 implicated by testing null mutant strain | Als5 promotes adhesion to polystyrene |
| Proposed adhesive mechanism | Ruled out hydrophobicity; implicated 'substrate recognition pockets' | Not specified | Amyloid-forming region |
| Other mechanisms to consider? | Non-specific protein adsorption | Non-specific adsorption of YPD proteins to silicone surface, followed by PBC-mediated Als adhesion | Hydrophobic interactions |

*YPD, yeast extract-peptone-dextrose medium; PBS, phosphate-buffered saline; TE, Tris-EDTA; PDMS, polydimethylsiloxane.*

constitutive promoter to drive production of a *S. cerevisiae* cell-surface fusion protein consisting of the NT/T region of Als proteins on a stalk composed of the C-terminal half of alpha-agglutinin. Fusion-protein-displaying yeast cells attached to polypropylene and polyvinyl chloride plastics, as well as borosilicate glass. No attachment was observed to polyurethane, polymethyl methacrylate, polytetrafluoroethylene, or titanium.

Finkel et al. (2012) sought to understand transcriptional regulation of *C. albicans* attachment to channels in a Fluxion flow cell, which has a borosilicate glass floor and polydimethylsiloxane (PDMS; silicone) walls. The authors observed that *C. albicans* attaches to the PDMS walls in the flow cells, but not to the borosilicate glass floor. A *bcr1/bcr1* strain showed reduced attachment to PDMS under flow conditions; testing of Bcr1 targets revealed attachment defects for an *als1/als1* strain. *C. albicans* growth conditions used for these assays produce high levels of Als1, but not Als3 (Coleman et al., 2010). This work suggested a role for Als1 in *C. albicans* attachment to PDMS.

Garcia et al. (2011) overproduced Als5 on the *S. cerevisiae* surface and evaluated the strain for biofilm formation in a polystyrene dish; conclusions were also drawn regarding the role of Als5 in attachment to polystyrene. Attachment was quantified by measuring crystal violet retained in each assay well. Micrographs were also captured. Micrographs showed greater numbers of attached cells for the Als5-producing strain compared to an empty vector control strain. These results suggested a role for Als5 in attachment to polystyrene.

## Attachment Mechanisms Proposed by the Authors

Aoki et al. (2012) used an assay that partitioned cells between water and *n*-octane to estimate hydrophobicity of the recombinant *S. cerevisiae* strains expressing Als fragments. Because they did not detect a positive correlation between these measurements and attachment data, the authors ruled out hydrophobicity as a potential mechanism for Als-mediated attachment. The authors concluded 'that ALS proteins bound to the abiotic surfaces mainly by a specific adhesion mechanism between the material and the substrate recognition pockets of the ALS proteins' although it is unclear how this interaction would occur. Finkel et al. (2012) implied that Als1 was directly involved in attachment to PDMS, but did not propose an attachment mechanism.

Garcia et al. (2011) suggested that the Als5 AFR (-IVIVATT-) is 'critical for. . . cell-substrate adhesion to polystyrene.' Their conclusion was based on decreased crystal violet retention by *S. cerevisiae* cells producing wild-type Als5 compared to cells producing an Als5 variant in which the AFR was mutated (-INIVATT-). However, micrographs show clearly that AFR mutation decreases cellular aggregation and overall cell abundance in the assay well. In other words, fewer cells are present in the well because of the reduction in the number of aggregated cells, rather than a reduction in the number of cells directly attached to polystyrene. Counting cells that are in direct contact with the polystyrene, rather than quantifying attachment using crystal violet (which cannot distinguish attachment from aggregation), could resolve these relationships.

## Could Hydrophobicity Be Involved in Als Attachment to Abiotic Surfaces?

Hydrophobicity has been invoked as a general property of Als proteins and bears additional discussion because of its potential to influence Als-mediated attachment to abiotic surfaces. Certainly, anyone who has ever attempted to collect *C. albicans* germ tubes by centrifugation has witnessed multiple phenomena (e.g., cellular aggregation, adsorption to the plastic

tube, resistance to sedimentation) that could be attributable in part to hydrophobicity. Different regions conserved in the Als architecture could promote these interactions.

Frank et al. (2010) used molecular modeling approaches to conclude that the Als tandem repeat units (TRs) have both hydrophilic (contributed by glycosylation) and hydrophobic (due to predicted exposed patches of amino acids) components. The authors hypothesized that the hydrophobic nature of the TRs allows Als5 to mediate binding to polystyrene. Beaussart et al. (2012) showed the hydrophobic character of the *C. albicans* germ tube surface by probing it with an AFM tip that was functionalized with hydrophobic groups. Compared to wild-type *C. albicans,* an *als1/als1 als3/als3* null strain had a significantly decreased interaction with the hydrophobic AFM tip. Cells analyzed in this study were required to stick to a hydrophobic surface, however, perhaps introducing biases in measurement. Overall, though, these observations suggest contributions of the Als proteins to hydrophobicity in the context of otherwise 'sticky' germ tubes. Because polystyrene is very hydrophobic (e.g., Curtis et al., 1983; Ryan, 2008), hydrophobicity may contribute to the observation that Als5 is involved in attachment to this surface (Garcia et al., 2011).

Investigations into the relationship between CSH and *C. albicans* attachment to polystyrene are not new for the field and served as a major focus for the laboratory of Kevin Hazen in the 1980s. CSH was initially investigated using a water/hydrocarbon partitioning assay until a polystyrene microsphere adhesion assay was developed to evaluate CSH of individual cells (Hazen and Hazen, 1987). Revisiting this literature in the context of current knowledge of cell surface localization and abundance of Als proteins yields striking parallels: the overall relative changes in CSH observed with different growth phases and growth media are highly similar to the abundance and localization of Als proteins on the *C. albicans* cell surface. For example, transfer of yeast cells from a saturated culture into fresh growth medium resulted in a sharp rise in CSH (Hazen and Hazen, 1988). These growth conditions produce large quantities of Als1 on the yeast cell surface (Coleman et al., 2010). Similarly, CSH is higher for *C. albicans* yeast cells grown at room temperature compared to 37°C; lower growth temperatures promote greater cell-surface quantities of Als4 (Coleman et al., 2012). Germ tubes are more hydrophobic than yeast cells, and they tend to be far more homogeneously coated in Als proteins than individual yeast cells in a culture population (Hazen et al., 1986; Coleman et al., 2009, 2010, 2012). While positive correlation does not necessarily indicate cause-and-effect, the relationship between Als protein localization and abundance and CSH of those cell types is consistent with the idea that Als proteins contribute to *C. albicans* CSH and, therefore, to attachment to abiotic surfaces.

## Other Mechanisms to Consider

Other interactions besides hydrophobicity could also contribute to the interactions between Als proteins and abiotic surfaces. Although the idea has not appeared in any published manuscripts, non-specific protein adsorption to solid surfaces may be involved in these interactions. An extensive literature exists discussing non-specific factors that mediate protein adsorption onto solid surfaces (e.g., Hlady and Buijs, 1996; Goebel-Stengel et al., 2011). Non-specific protein adsorption to solid surfaces could contribute to the observations of Aoki et al. (2012).

Specific interactions between the Als PBC and adsorbed proteins may also explain some of the published observations. For example, the work of Finkel et al. (2012) was conducted in YPD medium, which contains an abundance of protein fragments. Such proteins could efficiently coat surfaces like PDMS and provide anchoring points for the Als1 PBC, accounting for at least a part of the observed phenotype. This work would parallel introduction of medical devices into the body. Upon exposure to fluids such as serum or saliva, abiotic surfaces would quickly become coated with soluble proteins, such as serum albumin (Hawser and Islam, 1998) or salivary statherin (Johansson et al., 2000). PBC activity may work in conjunction with other mechanisms, but unlike hydrophobic/glycosylated contacts, it has the potential to provide specificity to the initial association of *C. albicans* with surfaces of different chemical compositions. Nonetheless, interaction with soluble proteins could also impair attachment to these materials, modulating the association of *C. albicans* with the host surfaces, the microbiota and different factors of the immune system. The interplay and relevance of these binding mechanisms remain to be characterized.

## Are All Als Proteins Equal in Attachment to Abiotic Surfaces?

Although the mechanism(s) of Als protein attachment to abiotic surfaces require(s) additional investigation, we can speculate whether these properties are unique to a subset of Als proteins or shared across the family. Aoki et al. (2012) tested each of the Als proteins and concluded that 'most of' them bound polypropylene, polyvinyl chloride and borosilicate glass. Finkel et al. (2012) tested null mutant *C. albicans* strains and implicated Als1 in adhesion to silicone, but ruled out Als3 because the null mutant failed to affect attachment in the flow assay. It is not surprising that Als3 failed to be implicated in attachment to silicone, because *C. albicans* does not produce Als3 under the growth conditions studied. Given the considerable similarity between Als1 and Als3 at the primary sequence level (Lin et al., 2014), it is likely that Als1 and Als3 have comparable silicone-attachment properties. Garcia et al. (2011) observed that wild-type *C. albicans* attaches to polystyrene, and commented on the potential for other Als proteins to show similar characteristics to those observed for Als5. Their explanation focused on identity between amyloid-forming sequences in the various Als proteins, leading to the conclusion that Als1 is likely to attach to polystyrene. However, the growth conditions used for the assay favor Als4 abundance on the *C. albicans* surface (Coleman et al., 2012). If attachment to abiotic surfaces involves a property that is common to the Als proteins (e.g., TRs), it is likely that they all may exhibit similar function. The availability of Als structural data and attribution of function to various Als structural features permits a mechanistic dissection and an understanding of what appear to be multiple factors that contribute to Als-mediated interaction with abiotic surfaces.

## DO Als PROTEINS INTERACT WITH EACH OTHER?

There are many literature reports that describe Als–Als interactions as the basis for *C. albicans* phenotypes important for colonization and subsequent pathogenesis. Here, we review some of these examples, with an emphasis on examining the mechanistic basis for the interaction between Als molecules.

Several manuscripts suggest that Als homotypic binding is mediated by the NT domain. Perhaps these first arose through hypotheses regarding the interaction of Als proteins with cadherins (Phan et al., 2007). Cadherins mediate homotypic binding via the N-terminal domain (Pokutta and Weis, 2007), possibly prompting extrapolation of that idea to Als proteins. Donohue et al. (2011) produced soluble NT/T from Als1, immobilized the protein on a CM5 chip and used surface plasmon resonance to measure its interaction with itself. Results suggested homotypic binding, further supporting the conclusion that Als NT domains bind to each other. Lipke et al. (2012) postulated a model of Als homotypic binding and mechanical stimulus (provided by an atomic force microscopy probe) that resulted in the formation of cell-surface amyloid nanodomains (**Figure 3A**). A key feature of this model is binding of one Als NT domain to another.

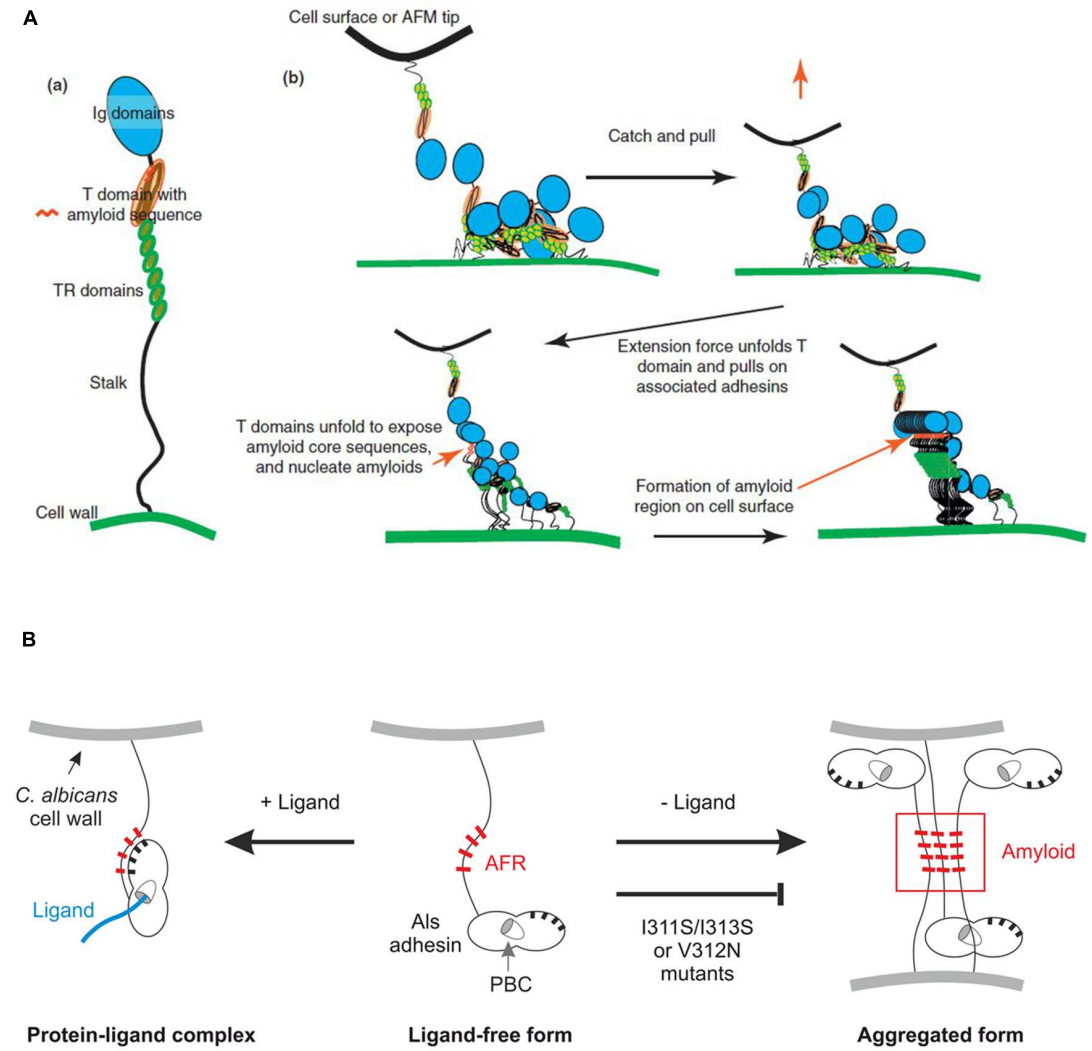


**FIGURE 3 | Models proposed to explain function of the AFR in Als protein interactions. (A)** Force-induced aggregation of Als proteins on the surface of the same cell from Lipke et al. (2012). Homotypic binding between NT domains of Als proteins is proposed to trigger force required to pull apart an Als protein, exposing the AFR for interaction with AFR sequences on other Als proteins. **(B)** Variable conformation of the AFR in relation to the NT domain of Als3 on the *C. albicans* cell surface based on Lin et al. (2014). Newly synthesized Als protein can either bind ligand via the PBC, which results in the AFR attaching to the NT domain surface (left) or use its free AFR to interact with others, forming protein and cellular aggregates (right). Note that the model in **(A)** and the model in **(B)** show different artistic interpretations of AFR placement, with **(B)** showing an exaggerated scale of the NT portion of the molecule (especially the AFR) to emphasize those interactions. **(A)** Reprinted from Lipke et al. (2012), with permission from Elsevier. **(B)** This research was originally published in Lin et al. (2014). Reprinted with permission from The American Society for Biochemistry and Molecular Biology.

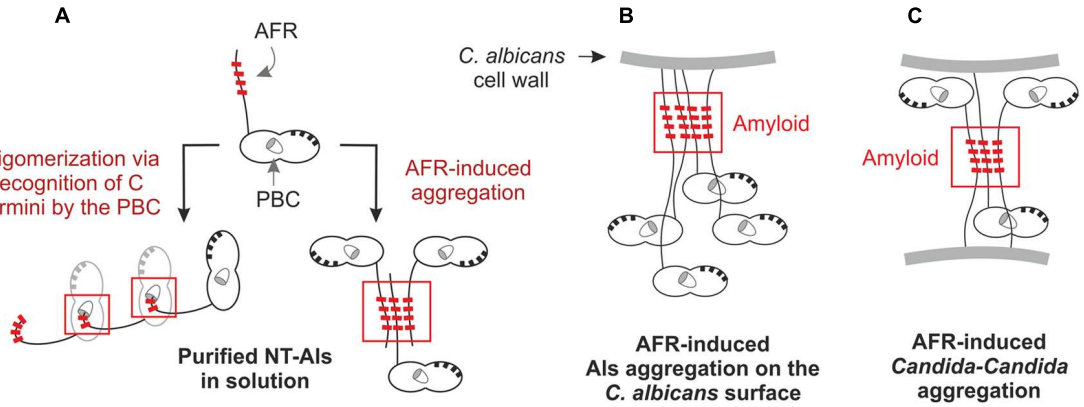


**FIGURE 4 | Mechanisms of NT domain interactions between purified proteins (A) and between mature, full-length Als proteins on the *C. albicans* cell surface (B,C). (A)** Purified NT-Als proteins may interact by two mechanisms. The first involves PBC-mediated recognition of the free C-terminal peptide, leading to oligomerization of the NT-Als molecules (left). The second mechanism involves aggregation mediated by the AFR (right). Because the NT domain is a small portion of the full-length, mature Als protein, PBC-mediated oligomerization of the proteins cannot explain aggregation between Als molecules on the *C. albicans* cell surface. These interactions are more likely attributable to the AFR **(B)**. The AFR of mature, full-length Als proteins can also promote Als–Als-mediated aggregation between different *C. albicans* cells **(C)**.

While current structural data support the idea that Als NT domains bind to each other, it is necessary to distinguish between the type of interactions that occur when working with purified Als NT domains from those that are possible for mature, full-length Als proteins displayed on the *C. albicans* surface. NMR and X-ray crystallography data indicate two possible mechanisms for interaction of purified Als NT domains, as described previously for Als9-2 and Als3 (Salgado et al., 2011; Lin et al., 2014; **Figure 4A**). One mechanism involves recognition of the flexible C terminus of one NT domain by the PBC of another, leading to oligomerization of NT-Als proteins. The other mechanism involves aggregation of NT-Als proteins via exposed AFRs. Analysis of a shortened version of the Als NT (sNT-Als) showed that a flexible C terminus, including the AFR, is necessary for the observed interactions. Removal of the AFR from the NT structure, leaving only the N1 and N2 domains, eliminates the self-complementary binding, resulting in soluble monomeric protein, even at high concentration (Lin et al., 2014). For mature, full-length Als proteins on the *C. albicans* surface, the C terminus is anchored to the cell wall, leaving the intermolecular association of different AFRs as the only mechanism for interaction of Als NT (**Figure 4B**). This activity is consistent with the aggregative properties proposed for the AFR (Lipke et al., 2012; Lin et al., 2014).

The AFR has been the subject of considerable study. Lipke et al. (2012) communicated an overall vision for importance of the AFR in Als interactions. AFR-mediated interactions play a large role in formation of Als protein aggregates. Although this phenomenon could be called 'adhesion,' here we attempt to clarify molecular mechanisms by distinguishing aggregative interactions from those involving ligand binding. The AFR mediates aggregative interactions that cluster Als proteins together (amyloid nanodomains) on the fungal cell surface (**Figure 3A** and **4B**). Considerable data have been offered to support this conclusion (reviewed in Lipke et al., 2012). Mutagenesis of the AFR inhibits these interactions, suggesting that they are AFR-mediated.

**Figure 4C** extends the AFR model to demonstrate how interaction between Als AFRs on the surface of different cells may result in *C. albicans* aggregation. Presumably, these amyloid-driven interactions may also occur between Als AFRs and AFRs in other proteins, on the surface of *C. albicans* or other cell types. AFR-mediated interactions explain fungal aggregation over biological surfaces (i.e., host cells or bacteria). At present, though, it is unclear how these interactions would promote attachment to abiotic surfaces, especially those that are not coated in protein (discussed above). AFR-mediated interactions may explain some of the Als–Als interactions reported in the literature such as complementary function of Als proteins in biofilm formation (Nobile et al., 2008). Als protein interaction with the *C. albicans* cell-surface adhesin Hwp1 may also be AFR-mediated.

Studying an Als3 molecule with a mutagenized AFR in *C. albicans* demonstrated the complexities in dissecting phenotypes and attempting to ascribe mechanistic interpretations to data. The AFR of Als3 was mutagenized to replace Ile residues with Ser, thereby destroying amyloidogenic potential (I311S/I313S; Lin et al., 2014). The mutant strain (Als3-afr) was tested in standard *in vitro* adhesion assays. Interpretation of data at face value suggested that the AFR both increased and decreased *C. albicans* adhesion. These data illustrate the assay-dependency of the results and the need to dig deeper to reconcile the observations. Adhesion assays where *C. albicans* cells interacted with monolayers of human cells suggested that the AFR inhibited adhesion while assays that involved interaction with freshly collected human cells in a shaking flask suggested that the AFR promoted adhesion. One common observation was that the *C. albicans* cells in which Als3-afr was produced were less aggregated compared to the

control strain. Results were interpreted to indicate that mutation of the AFR reduced aggregation of individual Als3 molecules on the *C. albicans* cell surface, freeing them to participate in adhesive interactions, rather than sterically hindering the PBC in a clump of Als3 proteins (**Figure 3**). Mutagenesis of the AFR also decreased aggregation between *C. albicans* cells, which lowered adhesion counts in the shaking flask assays. In these assays, any *C. albicans* that touches the mammalian cell is 'adhesion-positive' whether it is participating in a molecular interaction with the host cell surface, or just in contact because of its presence in a *C. albicans* aggregate. Overall results pointed to a role for the AFR in aggregation of Als3 molecules on the *C. albicans* surface, and its facilitation of formation of *C. albicans* multicellular aggregates. The literature on the function of the AFR is abundant, but to our knowledge no reports have been published so far describing the specific interaction of this region with a host cell or bacterial ligand.

Assays using purified protein also pointed to some other structural information regarding the AFR that has the potential to affect its function on the *C. albicans* surface (**Figure 3B**): the position of the AFR changes, depending on whether the PBC is in the ligand-bound or ligand-free form (Lin et al., 2014). In the ligand-free form, the AFR is dissociated from the surface of NT-Als3 and free to associate with other AFR sequences. When the NT-Als3 PBC binds a ligand, it undergoes a conformational change and the AFR becomes associated with the surface of the protein. This observation presents a novel opportunity to modulate Als3 activity because in the presence of higher-affinity ligands, it may be possible to shift the equilibrium of this reaction and decrease *C. albicans* aggregation. Decreased aggregation may have positive phenotypic effects such as making *C. albicans* cells more susceptible to the action of antifungal drugs. A higher-affinity ligand may serve as an anti-adhesion molecule, as well as the means to target drug delivery to the *C. albicans* surface.

Examples discussed in this section emphasize that Als proteins are multifunctional molecules with the potential to interact with other molecules or among themselves. Interactions among Als proteins can drive changes in the *C. albicans* surface or result in cellular aggregation that causes measurable differences in various phenotypic assays. Availability of NT-Als structural data has provided insight into the molecular mechanisms behind these interactions.

## ARE Als PROTEINS INTERCHANGEABLE?

This section addresses perhaps the most common question that arises when studying a protein family: are the various proteins interchangeable? In other words, can protein #1 replace the function of protein #2, suggesting they are functionally equivalent? Because Als proteins are a composite of many different functions (e.g., ligand binding, aggregation, attachment to abiotic surfaces), the answer may differ depending on which activity is considered. As detailed in the previous sections of this review, functions can be ascribed to different Als structural features. As we continue to dissect the Als molecule at the structural level, observations of functional equivalence can be used to derive new information such as identifying amino acids in the PBC that are responsible for ligand-binding specificity. Here, we examine published conclusions regarding functional equivalence and place them into a structural context.

Comparisons between the ligand-binding activity of Als1, Als3, and Als5 are the most useful literature observations relevant to the discussion of functional equivalence. The NT portion of the three proteins (amino acids 1–312 of the processed sequence) is 74% identical; Als1 and Als5 are 82% identical in this same region. Als1, Als3, and Als5 all bind to *Streptococcus gordonii* (Klotz et al., 2007; Silverman et al., 2010; Hoyer et al., 2014). Likewise, *S. cerevisiae* strains that overexpress *ALS1*, *ALS3*, or *ALS5* have similar ligand-binding profiles when tested against gelatin, fibronectin, laminin, epithelial, and endothelial cells (Sheppard et al., 2004). However, when the same set of strains is tested in a ferritin-binding assay, only Als3 is positive (Almeida et al., 2008). Using a diverse set of peptides, Klotz et al. (2004) demonstrated overlapping ligand-binding specificity for Als1 and Als5, but also demonstrated ligand-binding differences by identifying peptides that bound to one Als protein but not the other. Lin et al. (2014) identified fourteen amino acids in the NT-Als3 structure with side chains that interact with peptide ligands in the PBC. Eleven of these 14 amino acids are conserved in the PBC of NT-Als1; the three amino acids that are different may explain the ability of NT-Als3 to bind ferritin while NT-Als1 cannot. Comparisons between the 14 ligand-interacting amino acids in the PBC of NT-Als1 and NT-Als5 show four differences, which may account for the peptide-binding variation noted by Klotz et al. (2004). Collectively, these studies identify amino acids that could be mutagenized to demonstrate the structural features responsible for ligand-binding specificity.

Given the relatively large number of observations regarding Als ligand-binding function in the literature, it is surprising that there is little additional information that can be used in a discussion of Als protein functional equivalence. The sometimes-extreme allelic variability in the ALS family, coupled with a lack of detail regarding which allele or which portion of an Als protein was studied, conspire to complicate interpretation of published experiments. For example, two distinct forms of the NT domain of Als9 are known (named Als9-1 and Als9-2; 84% identical). Initial comparison of the proteins suggested that Als9-2 is more active in ligand binding than Als9-1 (Zhao et al., 2007a), so it is important to know which one was used in a specific experiment. There are also numerous sequence variants for the NT domain of Als5, leading to the potential for experimental results that are more or less similar to Als1 function (Zhao et al., 2007c). When testing mature proteins on the surface of a fungal cell, allelic variation in the numbers of copies of the TR sequence may also result in different functional conclusions (Oh et al., 2005). Finally, published manuscripts may not specify the portion of an Als protein that was studied. One common example is reference to the 'N-terminal domain' without an indication of whether this protein includes approximately 329 amino acids (now called NT) or 433 amino acids (NT/T; numbering based on the unprocessed

protein sequence; **Figure 1A**). Understanding whether the assayed protein included the AFR and/or was competent for self-complementation is key to interpreting experimental results in a structural context.

There are few comments in the literature about functional equivalence outside of the peptide-binding activity for Als proteins. One could imagine, however, that an Als feature like the AFR might be complemented readily by a diverse set of Als proteins. We may also find that CT domains are widely interchangeable, especially if their function is limited to providing a structural stalk to project the remainder of the Als protein away from the *C. albicans* surface. Therefore, the answer to the question about Als interchangeability is likely to vary depending on which function is considered.

## DOES Als PROTEIN ABUNDANCE AFFECT PHENOTYPIC CONCLUSIONS?

This question has the most straightforward answer of any asked in this review so far: yes. The relative abundance of Als proteins on the *C. albicans* cell surface can be evaluated using specific anti-Als monoclonal antibodies (reviewed in Cota and Hoyer, 2015). The quantity and localization of Als proteins varies naturally on the surface of wild-type *C. albicans* cells, providing disparate opportunities for the proteins to contribute to cellular phenotypes. In experimental constructs, Als protein levels may vary among strains and affect functional comparisons. Examples of the relationship between Als protein abundance and phenotype are discussed here.

Because of its generous quantities and widespread distribution on germ tubes (Coleman et al., 2009), Als3 is an ideal model for addressing the relationship between phenotype and protein abundance. A recent report described evaluating adhesive function of two *C. albicans* constructs that expressed the same, single *ALS3* allele (Lin et al., 2014). The first strain (1893; Δ*als3*/*ALS3*) was constructed by deleting one wild-type *ALS3* allele. The other strain (3464) was constructed by integrating the *ALS3* allele into a Δ*als3*/Δ*als3* background. Immunolabeling with a monoclonal antibody specific for NT-Als3 showed less intense fluorescence for strain 3464 compared to 1893. Strain 3464 showed lower adhesion to mammalian cells compared to 1893, even though the strains displayed the same wild-type Als3 protein. Data were consistent with the conclusion that less cell-surface Als3 resulted in lower adhesive capacity in phenotypic assays. A similar conclusion was observed in a study of Als protein contributions to biofilm formation (Nobile et al., 2008). In that work, strains with reduced gene dosage showed a lower capacity to form biofilms.

Presumably, as the abundance of Als protein decreases, a point will be reached where activity cannot be detected, the assay will be interpreted as 'negative,' and the Als protein will be concluded to lack the assayed function. Conversely, experimental approaches that feature protein overproduction may create artifacts of high abundance. Because Als proteins can interact with each other and with other *C. albicans* surface proteins (detailed above), packing them too densely on the cell surface could lead to phenotypes that wild-type *C. albicans* would not produce. *C. albicans* has determined which levels of proteins are 'just right' and while experimentally manipulating the system, researchers struggle to reproduce this effect.

The concept of protein abundance can also contribute to the discussion of Als protein functional equivalence (discussed above in Section "ARE Als PROTEINS INTERCHANGEABLE?") by explaining seemingly different phenotypic conclusions for very similar proteins. For example, did the adhesion assay produce a negative result because the Als protein cannot recognize the ligand or because there was not enough Als protein present for a measurable phenotype? Is one protein 'better' at mediating a particular function because its abundance and display more closely resemble wild-type levels or is the protein 'better' due to structural features that are not found in other Als proteins? Experimental controls that assess relative protein abundance are critical for accurate data interpretation.

Naturally low protein abundance for *C. albicans* Als7 has perhaps complicated efforts to determine whether the protein has adhesive function. At present, Als7 is the only Als protein in *C. albicans* for which adhesive function has not been documented. Attempts to assess adhesive function by overexpression failed to detect ligand binding, although cell-surface Als7 quantities could only be measured indirectly and appeared quite low (Sheppard et al., 2004). Deletion of *ALS7* in *C. albicans* led to increased adhesion of the mutant strain, an effect that still requires a molecular explanation (Zhao et al., 2007b). Study of Als7 is further complicated by a staggering number of allelic *ALS7* variants (Zhang et al., 2003), raising questions of whether assay results from a single allele would apply to them all. Recent structural solutions of the NT domain from three different Als proteins illustrated overall structure similarities that can be extrapolated to the remainder of the *C. albicans* Als family (Salgado et al., 2011; Lin et al., 2014). Among the Als proteins, Als7 has the largest amino acid variation in the PBC raising questions regarding its adhesive function. Ligand-binding analysis for Als7 might best be addressed using purified NT-Als7 and model peptides.

In contrast to Als7 that still lacks verification of adhesive function, published cell-biological experiments consistently demonstrate the importance of Als3 in many phenotypes including adhesion, biofilm formation and cellular invasion (Zhao et al., 2006; Phan et al., 2007; Nobile et al., 2008). It is unclear whether Als3 has unique structural features that allow it to perform these varied functions, or whether the relatively high Als3 abundance and widespread localization on the *C. albicans* cell surface simply provide greater functional opportunities for the protein.

Finally, it is worth noting that Als protein localization and abundance are different *in vitro* and *in vivo* (Coleman et al., 2009, 2012). Als1 localization on *C. albicans* cells recovered from *in vivo* animal models is more widespread than Als1 localization on cultured cells (Coleman et al., 2010). These immunolabeling observations likely explain other studies that demonstrated an *in vitro* biofilm formation defect for *C. albicans* strains lacking Als3, but wild-type biofilm for the same strain when tested *in vivo*

(Nobile et al., 2006). More widespread distribution of Als1 *in vivo* promoted biofilm formation, even in an *als3/als3* mutant strain. Analysis of Als protein abundance and localization on *C. albicans* cells recovered from animal models and clinical cases is a rich area for additional studies.

## WHAT STRUCTURAL FEATURE(S) OF Als PROTEINS MEDIATE(S) INVASION OF HOST CELLS?

Invasion refers to the process of a microbe entering a host cell. The invasin is a protein that promotes this process. Als proteins were first proposed to have invasin function when Sheppard et al. (2004) noted that *S. cerevisiae* cells that produced Als1, Als3, or Als5 on the surface were taken up in low numbers by cultured human umbilical vascular endothelial cells (HUVEC). Subsequent work suggested that binding of Als proteins (particularly Als3) to cadherins promoted endocytosis by cultured HUVECs and oral epithelial cell lines (Phan et al., 2007). A coating of the Als1 NT/T domains was able to promote endocytosis of latex beads by FaDu (pharyngeal carcinoma) epithelial cells, but not by HUVECs, suggesting Als1 also has invasin function, but perhaps is less effective than Als3. Despite their high degree of sequence identity in the NT/T domains, Als3 appears to have superior invasin function compared to Als1 or Als5. Analysis of invasin function for other Als proteins has not been reported. It is possible that given sufficient abundance and cell-surface distribution, other Als proteins could demonstrate invasin function.

Wachtler et al. (2012) also studied *C. albicans* invasion of epithelial cells and evaluated the contributions of various proteins. Their work featured TR-146 cells (human squamous carcinoma of the buccal mucosa) and sought to separate the effects of induced endocytosis from those of *C. albicans* active penetration into the mammalian cells. Induced endocytosis involves rearrangement of the host cell actin cytoskeleton and *C. albicans* internalization (Phan et al., 2007). Active penetration is a more forceful process, which involves pushing the hyphal tip through the host cell membrane, often passing through multiple contiguous host cells (Wachtler et al., 2012). Analysis of *als3/als3* mutant strains, use of cytochalasin D to inhibit microfilaments and the induced endocytosis process, and elimination of active penetration by killing *C. albicans* germ tubes with thimerosal, were used as complementary approaches to demonstrate that active penetration is the main mechanism that *C. albicans* uses to invade TR-146 cells. Wachtler et al. (2012) demonstrated a role for Als3 in both induced endocytosis and active penetration.

Adhesion is one possible characteristic that Als3 needs for either induced endocytosis or active penetration. Both processes require *C. albicans* to be in close proximity (even intimate contact) with the host cell. Adhesion to host cell proteins is one way to mediate that contact. In the context of active penetration, Wachtler et al. (2012) postulated that Als3 adhesion provides a foothold for the *C. albicans* germ tube. This strong anchorage of *C. albicans* to the host cell permits the force needed for the germ tube tip to penetrate host cell membranes. The Als3 PBC would likely provide this interaction since mutation of the PBC (leaving a fully formed wild-type Als3 surface exposed on *C. albicans*) eliminates Als3 adhesive function (Lin et al., 2014). While it is easy to envision a role for adhesion in the invasion process, it is unclear if Als3 features other than the PBC are also involved. For example, Als3 may have surface features that promote host cell invasive interactions more efficiently than the surface features of Als1 or Als5. Comparison of the structures of these proteins would identify candidates for mutational analysis.

Invoking adhesion as an important contributor to invasion leads to the question of what proteins serve as Als3 binding partners. While numerous cell-surface proteins likely could interact with the Als3 PBC and provide a firm foothold for active

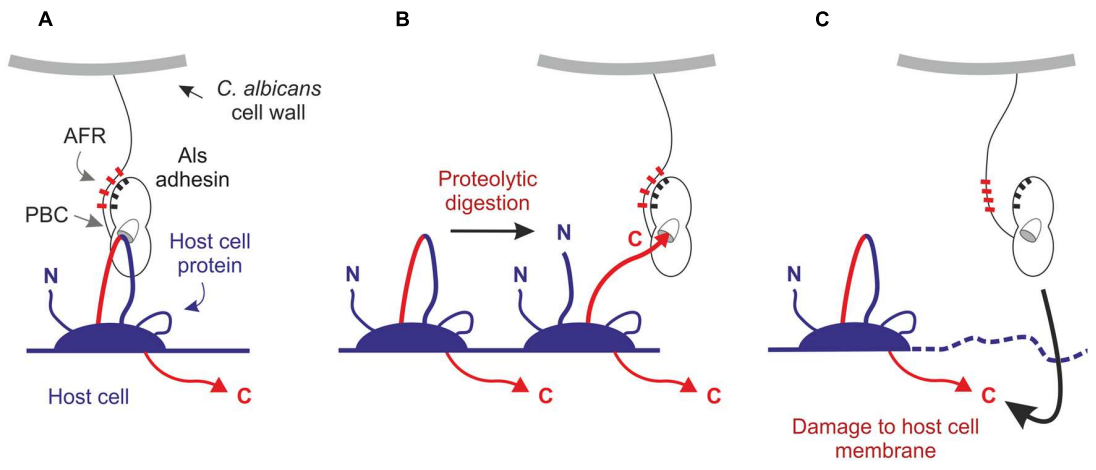


**FIGURE 5 | Potential mechanisms to explain the PBC-mediated adhesive/invasive interactions of Als3 with host cells. (A)** The Als3 PBC may interact with extracellular features of intact cadherins or other mammalian cell-surface proteins. **(B)** *C. albicans* may release proteases to facilitate partial digestion of cell-surface proteins, producing free C termini that are anchored to the host-cell membrane and competent for interaction with the Als3 PBC. **(C)** *C. albicans* may damage the host-cell membrane and promote translocation of Als3 into the host-cell cytoplasm where it may contact the C termini of membrane-anchored proteins.

penetration, cadherins were proposed to serve as the binding partner that promotes induced endocytosis (Phan et al., 2007). Work by Wachtler et al. (2012) supported this conclusion, but also identified an invasion mechanism that is independent of cadherin binding. Previous work demonstrated that Als3 ligand-binding function resides within the PBC and that the PBC prefers to bind free C-terminal peptides. Therefore, the Als3 PBC needs a way to contact the cadherin C terminus, which is located in the cytoplasm of the mammalian cell. These relationships set up an apparent contradiction and the need for alternative mechanisms to explain the ligand-binding interaction (**Figure 5**).

One possible explanation is that the Als3 PBC can bind other, yet undescribed, features on the extracellular portion of cadherins, or even bind other cell-surface proteins that promote invasion. A second possibility involves partial digestion of cadherins (Frank and Hostetter, 2007) by secreted proteases from *C. albicans* to generate extracellular C termini that remain anchored to the host cell membrane and become ligands for Als3. A third possibility is the translocation of Als3 through the host cell membrane as the initial event that leads to recognition of C termini. Damage of the cell membrane by *C. albicans* could promote exposure of cytoplasmic ligands for Als3.

Proposed explanations for published observations focus on Als3 PBC activity, but other features (such as the AFR) may also be involved in contacting host cell proteins and creating connections between fungal and mammalian cells. It is also helpful to note that published observations involve different cell lines and different cell types, and it is possible that mechanistic details for *C. albicans* invasion may vary among them. Adhesion and invasion assays using Als3 mutants produced in *C. albicans* and host cell lines with engineered cadherin molecules will clarify these relationships and provide the tools needed for detailed structural analyses of Als/cadherin complexes.

## EPILOGUE

Considerable progress has been made toward understanding the composition of the *C. albicans* ALS family and the function of its encoded proteins. Mechanistic explanations for Als protein function were elusive until recently and have been advanced by the availability of detailed NT-Als structural data. These data promote clarity in descriptions of Als function because function can be ascribed to specific structural features and precise words can be selected to describe the various interactions that Als proteins mediate. Because Als proteins are large molecules, many features remain to be examined at the structural level and placed into a functional context. Future investigations will also focus on understanding the boundaries of the ALS family and which genes from other species merit inclusion. Work in *C. albicans* provides the foundation for these more extensive explorations. As it has from the beginning, the ALS family provides a fertile area of inquiry with many fascinating questions to answer.

## AUTHOR CONTRIBUTIONS

LH and EC developed and wrote the manuscript. Each contributed to design and construction of the figures and table.

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# Members of the *Candida parapsilosis* Complex and *Candida albicans* are Differentially Recognized by Human Peripheral Blood Mononuclear Cells

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The systemic infections caused by members of the *Candida parapsilosis* complex are currently associated to high morbility and mortality rates, and are considered as relevant as those caused by *Candida albicans*. Since the fungal cell wall is the first point of contact with the host cells, here we performed a comparison of this organelle in members of the *C. parapsilosis* complex, and its relevance during interaction with human peripheral blood mononuclear cells (PBMCs). We found that the wall of the *C. parapsilosis* complex members is similar in composition, but differs to that from *C. albicans*, with less mannan content and more β-glucan and porosity levels. Furthermore, lectin-based analysis showed increased chitin and β1,3-glucan exposure at the surface of *C. parapsilosis sensu lato* when compared to *C. albicans*. Yeast cells of members of the *C. parapsilosis* complex stimulated more cytokine production by human PBMCs than *C. albicans* cells; and this significantly changed upon removal of *O*-linked mannans, indicating this wall component plays a significant role in cytokine stimulation by *C. parapsilosis sensu lato*. When inner wall components were exposed on the wall surface, *C. parapsilosis sensu stricto* and *C. metapsilosis*, but not *C. orthopsilosis,* stimulated higher cytokine production. Moreover, we found a strong dependency on β1,3-glucan recognition for the members of the *C. parapsilosis* complex, but not for live *C. albicans* cells; whereas TLR4 was required for TNFα production by the three members of the complex, and stimulation of IL-6 by *C. orthopsilosis*. Mannose receptor had a significant role during TNFα and IL-1β stimulation by members of the complex. Finally, we demonstrated that purified *N*- and *O*-mannans from either *C. parapsilosis sensu lato* or *C. albicans* are capable to block the recognition of these pathogens by human PBMCs. Together; our results suggest that the innate immune recognition of the members of the *C. parapsilosis* complex is differential of that reported for *C. albicans*. In addition, we propose that purified cell wall mannans can be used as antagonist to block specific receptors on innate immune cells.

**Keywords:** ***Candida parapsilosis*, *Candida albicans*, host-fungus interaction, cell wall, cytokine, mononuclear cells**

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## INTRODUCTION

Fungal infections are currently a burden for most of the health systems worldwide, and among them, superficial and invasive candidiasis are of special interest, since the latter has a mortality rate higher than 45% in infected patients (Brown et al., 2012). *Candida albicans* is the most frequent causative agent of candidiasis, being responsible of about 50% of total invasive candidiasis, while other members of the *Candida* genus, named emerging species, contribute together to the rest of the reported cases (Trofa et al., 2008). *Candida parapsilosis sensu lato* is a species that is mostly found in neonate patients, causing more than 33% of invasive candidiasis in this group (Pammi et al., 2013). It is a versatile yeast-like organism that, at difference of other pathogenic *Candida* species, can be found colonizing non-human organisms and inert material from the environment (Trofa et al., 2008). This organism is in fact a complex of three closely related species: *C. parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis* (Tavanti et al., 2005); which have subtle, but key differences in terms of virulence (Nemeth et al., 2013; Gago et al., 2014), drug sensitivity (Spreghini et al., 2012; Szenzenstein et al., 2013), and secretion of hydrolytic enzymes (Trevino-Rangel Rde et al., 2013).

The establishment of a protective anti-*Candida* immune response in the host relays on a proper activation of the innate immune branch, and significant efforts have been done to understand this host–pathogen interaction, using *C. albicans* as a model (Netea et al., 2015). In *C. parapsilosis sensu lato*, there is currently limited information about the mechanisms underlying its interaction with components of the innate immunity. Thus far, it has been demonstrated that galectin 3 expressed by human neutrophils drives an increased *C. parapsilosis* phagocytosis, but not when challenged against *C. albicans* yeast cells (Linden et al., 2013). In addition, human peripheral blood mononuclear cells (PBMCs) stimulated with heat-killed (HK) *C. parapsilosis sensu stricto* yeast cells produced lower Interleukin (IL) 1β, interferon γ, IL-17 and IL-22, but higher levels of IL-10, when compared to cells confronted with *C. albicans* (Toth et al., 2013). Despite this progress, there are not reports dealing with the interaction of immune cells with members of the *C. parapsilosis* complex.

The fungal cell wall contains most of the pathogen-associated molecular patterns recognized by pattern recognition receptors (PRRs) on innate immune cells, and again, the *C. albicans* cell wall is the best model thus far characterized (Díaz-Jiménez et al., 2012). This structure is composed of four main polysaccharides arranged in two well defined layers: the outermost layer composed of glycoproteins, bearing *N*- and *O*-linked mannans, and the components of the inner layer, chitin, β1,6- and β1,3-glucans (Díaz-Jiménez et al., 2012). The participation of this organelle in the activation of the innate immune response has been thoroughly studied. It is now well established that β1,3-glucan is normally hidden from the recognition of dectin-1 and TLR2, and if accessible, plays a major role in the induction of pro-inflammatory cytokines and phagocytosis by macrophages (Gantner et al., 2005; Gow et al., 2007; Heinsbroek et al., 2008). The *N*-linked mannans play also a significant role in both cytokine stimulation and macrophage uptake, via the mannose receptor (MR), dectin-2, and DC-SIGN (Netea et al., 2006; Mora-Montes et al., 2007, 2010; Cambi et al., 2008; McKenzie et al., 2010; Saijo and Iwakura, 2011). On the contrary, *O*-linked mannans play a dispensable role in both immunological processes, although it engages to TLR4 (Netea et al., 2006), a potent PRRs that plays a significant role in controlling bacteria (Miyake, 2007). Thus far, there is limited information about the cell wall of members of the *C. parapsilosis* complex, and the particular contribution of PRRs in the activation of cytokine production. Here, we performed a comparative study of the cell wall composition of *C. albicans*, *C. parapsilosis sensu stricto*, *C. orthopsilosis,* and *C. metapsilosis* and found that, although the composition is similar, the arrangement of the components has significant differences that impact their ability to activate human PBMCs. Moreover, we demonstrated that purified *N*- and *O*-linked mannans from either *C. albicans*, *C. parapsilosis sensu stricto*, *C. orthopsilosis* or *C. metapsilosis* are capable to block the recognitions of these pathogens by human PBMCs.

## MATERIALS AND METHODS

### Strains and Culturing Conditions

*Candida albicans* SC5314 (Gillum et al., 1984), *C. parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, and *C. metapsilosis* SZMC 1548 (Szenzenstein et al., 2013) were used in this study. Cells were propagated at 30°C in Sabouraud broth [1% (w/v) mycological peptone, 4% (w/v) glucose], and maintained in plates containing medium added with 2% (w/v) agar. For all the experiments here reported, 500 μL of overnight-grown cells were used to inoculated 100 mL of fresh medium and incubated at 30°C with shaking at 200 rpm, until reach the mid-log growth phase (typically 5–6 h). Cells were incubated at 56°C for 1 h for heat inactivation as reported (Mora-Montes et al., 2007). For all the cases, inactivation was confirmed by loss of fungal growth in Sabouraud medium at 30°C for 72 h. To remove *O*-linked mannans, cells were incubated overnight with 100 mM NaOH as described (Diaz-Jimenez et al., 2012). Under these conditions, more than 94% cells kept viability, as tested by CFU/mL before and after treatment with NaOH.

### Cell Wall Analysis

Cell homogenates were obtained in a Braun homogenizer, with 5 cycles of 30 s and cooling on ice in-between (Mora-Montes et al., 2007). The cell walls were pelleted by centrifugation and thoroughly washed with deionized water. Further wall cleansing was performed with hot 2% (v/v) SDS, 0.3 M β-mercaptoethanol, and 1 M NaCl as described (Mora-Montes et al., 2007). Freeze-dried cell walls were hydrolyzed by adding 2 M trifluoroacetic acid and boiling for 3 h; then, the acid was evaporated, and samples were suspended in deionized water. The hydrolysates were analyzed by High Performance Anion Exchange Chromatography coupled to Pulsed Amperometric Detection (HPAEC-PAD), using a gradient of sodium acetate in 150 mM NaOH (flow 0.5 mL/min) as follows: 0–5 min = 45–75 mM NaOH, 5.1–15.0 min = 90 mM NaOH,

15.1–17.0 min = 105 mM NaOH + 75 mM sodium acetate, 17.1–20.0 min = 75 mM NaOH + 150 mM sodium acetate, and 20.1–25.0 min = 45 mM NaOH, at a column temperature of 25°C. Applied potentials for detection by the amperometric pulse were: E1 (400 ms), E2 (20 ms), E3 (20 ms), and E4 (60 ms) of +0.1, −2.0, +0.6, and −0.1 V, respectively. Protein content was determined upon lyophilized cell walls were alkali-hydrolyzed (Mora-Montes et al., 2007), using the Bradford protein assay.

The cell wall porosity was assessed using the polycation method previously reported (De Nobel et al., 1990). Aliquots containing $1 \times 10^8$ cells were pelleted, the supernatant discarded and cells suspended in either 10 mM Tris-HCl, pH 7.4 (buffer A), buffer A plus 30 μg/mL poly-L-lysine (MW 30–70 kDa, Sigma Cat. No. P-2636) or buffer A plus 30 μg/mL DEAE-dextran (MW 500 kDa, Sigma Cat. No. D-9885). Then, cells were incubated at 30°C for 30 min, and shaking (200 rpm), pelleted by centrifuging, the supernatant saved, and further centrifuged before reading the absorbance at 260 nm. The relative cell wall porosity to DEAE-dextran was calculated as described (De Nobel et al., 1990).

The level of cell wall phosphomannan was determined by the ability of cells to bind the cationic dye Alcian Blue as described (Hobson et al., 2004).

## Analysis of Chitin and β1,3-Glucan Exposure at the Cell Surface

Cells were labeled with 1 mg/mL fluorescein isothiocyanate-wheat germ agglutinin conjugate (WGA-FITC; Sigma) (Mora-Montes et al., 2011) for chitin staining; while β1,3-glucan was labeled with 5 μg/mL IgG Fc-Dectin-1 chimera (Graham et al., 2006) for 40 min at room temperature, followed by incubating with 1 μg/mL donkey anti Fc IgG-FITC for 40 min at room temperature (Marakalala et al., 2013). In both cases, samples were examined by fluorescence microscopy using a Zeiss Axioscope-40 microscope and an Axiocam MRc camera. Pixels associated to 100 fluorescent cells were obtained with Adobe Photoshop$^{TM}$ CS6 and the following formula: [(total of green pixels-background green pixels) × 100]/total pixels.

## Ethics Statement

Universidad de Guanajuato, though the Ethics Committee, approved the use of human cells in this study (permission number 17082011). Blood samples from healthy adult volunteers were obtained upon information about the study was provided and written informed consent was signed.

## Human PBMCs-*Candida* Interaction

Human PBMCs were isolated by density centrifugation using Histopaque-1077 (Sigma) as reported (Endres et al., 1988). The immune cell-fungus interaction was performed in 96-well microplates with $5 \times 10^5$ PBMCs, in 100-μL RPMI 1640 Dutch modification (Sigma), and 100 μL with $1 \times 10^5$ fungal cells. When required, PBMCs were pre-incubated for 60 min at 37°C with either 200 μg/mL purified mannan, laminarin (200 μg/mL), anti-MR (10 μg/mL, Invitrogen, Cat. No. Mab-Hmr) or anti-TLR4 (10 μg/mL, Santa Cruz Biotechnology, Cat. No. sc-293072) prior to stimulation with yeast cells. Isotype matched, irrelevant $IgG_1$ antibodies (10 μg/mL, Santa Cruz Biotechnology, Cat. No. sc-52003) were used as controls for experiments assessing MR and TLR4. Despite all the reagents used for the pre-incubation experiments were negative to contamination by LPS (tested with the *Limulus* amebocyte lysate from Sigma) all reactions were performed in presence of 5 μg/mL polymyxin B (Sigma) (Schwartz et al., 1972). In all cases, the interactions were incubated for 24 h at 37°C with 5% (v/v) $CO_2$, plates were centrifuged for 10 min at 3000 × *g* and 4°C and supernatants saved and kept at −20°C until used. ELISA kits from Peprotech were used to measure the concentration of TNFα, IL-6, and IL-10; while IL-1β levels were measured using an ELISA kit from R&D Systems. Mock interactions with only human PBMCs incubated with RPMI 1640 Dutch modification were included as negative controls. For all the cases, the amount of cytokine quantified in the negative controls was subtracted before data analysis.

## *N*- and *O*-Linked Mannan Isolation

The *N*- and *O*-linked mannans were isolated, and purified using the endoglycosidase H and β-elimination strategy previously reported by our group (Mora-Montes et al., 2012). Absence of protein and other wall polysaccharides was confirmed by the Bradford method and HPAEC-PAD, respectively (Mora-Montes et al., 2012); while lack of bacterial and fungal contamination was assessed by negative detection of LPS and no growth in LB and Sabouraud broth for 72 h at 37 and 28°C, respectively.

## Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software. Cytokine stimulation using human PMBCs was performed in duplicate with six healthy donors, whereas the rest of the experiments were performed at least thrice in duplicate. Data represent cumulative results of all experiments performed and are showed as mean ± SD. The Mann–Whitney *U* test or unpaired *t*-test was used to establish statistical significance (see figure legends for details), with a significance level set at $P < 0.05$.

# RESULTS

## Members of the *C. parapsilosis* Complex have Similar Composition in the Cell Wall but Differ from that Present in *C. albicans*

In order to assess the proportions of the main cell wall components in the three members of the *C. parapsilosis* complex, cell walls where isolated, acid-hydrolyzed and the proportion of *N*-acetylglucosamine, mannose and glucose, the basic unit of chitin, mannan and β-glucan, respectively (Mora-Montes et al., 2007, 2010, 2012), were quantified by HPAEC-PAD. *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* showed similar levels of the three polysaccharides analyzed (**Table 1**), but when compared with *C. albicans*, the species belonging to the complex showed significantly less mannan and increased β-glucan content (**Table 1**). In terms of chitin content, only *C. metapsilosis* wall displayed higher content than

the *C. albicans* cell wall (**Table 1**). No differences were observed for protein content in the four yeast species analyzed (**Table 1**). Next, in order to confirm the lower mannan content in the members of the *C. parapsilosis* complex, we assessed the level of phosphomannan content and the wall porosity, parameters that suffer alterations depending on the status of the protein mannosylation pathways (De Nobel et al., 1990; Bates et al., 2005, 2006; Mora-Montes et al., 2007, 2010; West et al., 2013; Lopes-Bezerra et al., 2015). Interestingly, the cell wall from *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* showed lower phosphomannan content and higher porosity, when compared with *C. albicans* cells (**Table 1**). To assess whether the β1,3-glucan and chitin fibers in the members of the complex are underneath the mannan layer, as reported in *C. albicans* (Gow et al., 2007; Mora-Montes et al., 2011), yeast cells were labeled with the IgG Fc-Dectin-1 chimera (Graham et al., 2006), and the lectin-β1,3-glucan interaction revealed with FITC-conjugated IgG; whereas chitin was labeled with WGA-FITC. The analysis of the fluorescence associated to 100 cells indicated that, *C. parapsilosis sensu stricto*, *C. orthopsilosis,* and *C. metapsilosis* have β1,3-glucan and chitin more exposed the at the cell surface than *C. albicans* cells (**Figure 1**). However, when compared the three members of the complex, *C. orthopsilosis* displayed significantly higher levels of both polysaccharides at the wall surface than the other two species (**Figure 1**). As control, a similar experiment was conducted with HK yeast cells, where the components of the inner wall layer were artificially exposed at the cell wall surface and as consequence, higher labeling with the lectins was observed, with exception of *C. orthopsilosis*, where HK and live cells had similar content of both polysaccharides at the surface (**Figure 1**). Overall, these data indicate that the cell wall composition and organization among members of the *C. parapsilosis* complex differ to that described in *C. albicans*.

## *C. albicans* and *C. parapsilosis* sensu lato Differentially Stimulate Cytokine Production by Human PBMCs

The differences in composition and organization of the cell wall above described may suggest that the fungus-immune cell interaction differs between member of the *C. parapsilosis* complex and *C. albicans*. Thus, in order to get some insights about such interaction, yeast cells were co-incubated with human PBMCs and secreted cytokines levels were measured as a read out of such interaction. Live *C. albicans* cells barely stimulated the production of either TNFα, IL-1β, IL-6 or IL-10 (**Figure 2**); however, *C. parapsilosis sensu stricto*, *C. orthopsilosis,* and *C. metapsilosis* stimulated higher and similar levels of TNFα, IL-1β, and IL-10 (**Figure 2**). Although IL-6 stimulation was significantly higher during the interaction with any of the three members of the *C. parapsilosis* complex, *C. orthopsilosis* stimulated the highest IL-6 production, when compared with the other species analyzed (**Figure 2**). Next, we compared the cytokine profile stimulated with either live or HK cells with or without β-elimination treatment to remove *O*-linked mannans from the cell wall (Diaz-Jimenez et al., 2012). As previously reported for *C. albicans* (Gow et al., 2007), HK yeast cells stimulated significant levels of TNFα, IL-1β, IL-6, and IL-10, when compared to live cells (**Figure 3**). A subtle, not significant reduction was observed in the production of the cytokines upon stimulation of human PBMCs with β-eliminated HK *C. albicans* cells (**Figure 3**). Upon heat-inactivation, *C. parapsilosis sensu stricto* and *C. metapsilosis* stimulated increased levels of TNFα, IL-6 and IL-10, and these cytokine levels were similar when live yeast cells were *O*-deglycosylated, i.e., β-eliminated (**Figure 3**). No further changes in the cytokine stimulation were observed when this treatment was applied to HK cells (**Figure 3**). For stimulation of IL-1β, modest levels of this cytokine were obtained, being not as abundant as those stimulated with HK *C. albicans* cells, as reported (Toth et al., 2013), and displaying no significant changes even though cells were both HK and β-eliminated (**Figure 3**). For *C. orthopsilosis*, the heat killing and β-elimination did not affect production of TNFα, IL-1β, IL-6 and IL-10 by human PBMCs. Therefore, *C. parapsilosis sensu lato* is capable to stimulate a different cytokine production than that observed in *C. albicans*, when incubated with human PBMCs. Furthermore, these data indicate that *C. parapsilosis sensu stricto* and *C. metapsilosis* but not *C. orthopsilosis,* have a similar interaction with these immune cells.

## Role of PRRs in the Sensing of Members of the *C. parapsilosis* Complex

Next, in order to assess the importance of some PRRs during the fungus-immune cell interaction, human PBMCs were pre-incubated with antagonist of specific PRRs before the interaction with fungal cells. To evaluate the relevance of dectin-1 during this interaction, human PBMCs were pre-incubated with the specific antagonist laminarin (Maneu et al., 2011; Huang et al., 2012; Cohen-Kedar et al., 2014). Results showed in **Figure 4** indicate that stimulation of TNFα, IL-1β, IL-6, and IL-10

**TABLE 1 | Cell wall analysis of *Candida albicans* and members of the *C. parapsilosis* complex.**

| | Cell wall abundance | | | | | |
|---|---|---|---|---|---|---|
| **Organism** | **Chitin (%)** | **Mannan (%)** | **Glucan (%)** | **Phosphomannan content (μg)[a]** | **Porosity (%)[b]** | **Protein (μg)[c]** |
| *C. albicans* | 2.6 ± 1.2 | 33.0 ± 5.0 | 65.0 ± 3.3 | 127.2 ± 9.4 | 28.2 ± 8.7 | 125.7 ± 21.1 |
| *C. parapsilosis s. str.* | 5.2 ± 2.7 | 13.2 ± 6.8* | 81.6 ± 9.4* | 72.5 ± 7.5* | 60.9 ± 3.9* | 143.6 ± 47.3 |
| *C. orthopsilosis* | 4.9 ± 1.4 | 20.5 ± 1.3* | 74.6 ± 0.9* | 58.5 ± 12.6* | 57.2 ± 1.7* | 107.0 ± 20.7 |
| *C. metapsilosis* | 6.6 ± 1.3* | 21.7 ± 1.5* | 71.7 ± 2.7* | 85.4 ± 17.9* | 71.8 ± 13.1* | 97.3 ± 25.0 |

[a]*μg of Alcian Blue bound/$OD_{600}$ = 1;* [b]*Relative to DEAE-Dextran;* [c] *μg of protein/mg of cell wall; *P < 0.05, when compared to C. albicans.*

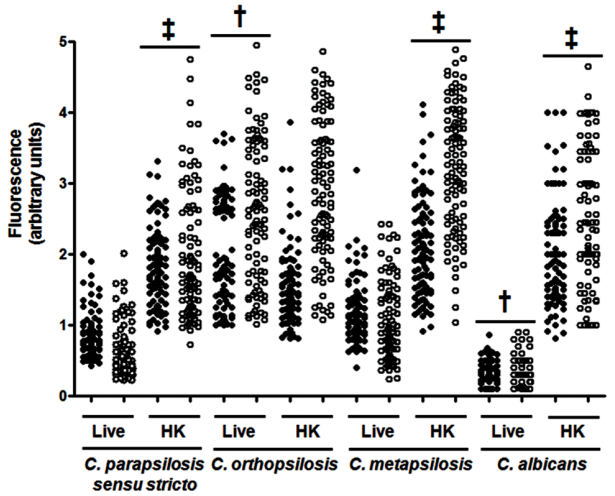


**FIGURE 1 | Analysis of β1,3-glucan and chitin exposure at the cell wall surface.** Live (L) yeast cells were incubated with IgG Fc-Dectin-1and then with FITC-conjugated IgG to label β1,3-glucan (closed circles). Alternatively, chitin was labeled with WGA-FITC (open circles). The fluorescence associated to each cell is plotted. In addition, the experiment was conducted with cells previously heat-inactivated (HK). Strains used are *Candida parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, *C. metapsilosis* SZMC 1548, and *C. albicans* SC5314. One hundred cells were analyzed for each group. ‡$P < 0.05$, when compared with the live cells; †$P < 0.05$ when compared to the other groups with live cells.

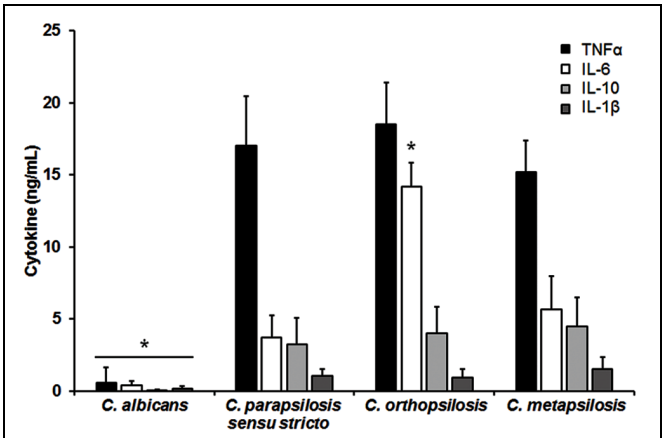


**FIGURE 2 | *Candida albicans* and members of the *C. parapsilosis* complex differentially stimulate the cytokine production by human PBMCs.** Human PBMCs were co-incubated 24 h with live yeast cells, the supernatant collected and used to quantify the cytokine levels. Strains used are *C. albicans* SC5314, *C. parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, and *C. metapsilosis* SZMC 1548. *$P < 0.05$, when compared with the cytokine level stimulated by other species.

by HK *C. albicans* cells was significantly dependent on the engagement of dectin-1 with β1,3-glucan, but not for live cells. When similar experiments were conducted with members of the *C. parapsilosis* complex, levels of TNFα, IL-6, and IL-10 stimulated with the three species were significantly reduced upon pre-incubation with laminarin, indicating a strong dependence on this receptor for their stimulation (**Figure 4**). At difference of *C. albicans*, stimulation of cytokines by live *C. parapsilosis sensu lato* cells was significantly sensitive to the presence of the dectin-1 antagonist. Interestingly, when human PBMCs were stimulated with *C. parapsilosis sensu lato* the IL-1β production was insensitive to the dectin-1 blocking, suggesting this receptor does not play a significant role for IL-1β stimulation by members of the *C. parapsilosis* complex.

To assess the relevance of TLR4 receptor during recognition of *C. parapsilosis sensu lato*, human PBMCs were pre-incubated with blocking anti-TLR4 antibodies before challenged with yeast cells. Results shown in **Figure 5** indicate that blocking of TLR4 has no impact on the cytokine production stimulated by live *C. albicans* cells. Similar experiments conducted with HK cells did not show significant differences in the levels of cytokine production (not shown). TNFα production stimulated by the three members of the *C. parapsilosis* complex was significantly reduced when TLR4 was blocked (**Figure 5**); while IL-6 stimulation was significantly diminished only when human PBMCs were co-incubated with *C. orthopsilosis* (**Figure 5**). Experiments conducted in presence of an irrelevant antibody of the same isotype gave no significant differences in the cytokine level (**Figure 5**). No significant changes where observed when IL-10 and IL-1β were quantified after pre-incubation with the TLR4 blocking antibodies (**Figure 5**). Therefore, TLR4 is required for TNFα production but not for IL-10 nor IL-1β, when human PBMCs are challenged with *C. parapsilosis sensu lato*. Furthermore, production of IL-6 was specifically affected when TLR4 engagement was disrupted in cells stimulated with *C. orthopsilosis*.

When the yeast-PBMC interaction was performed with human cells pre-treated with anti-MR antibodies, there were no significant changes in the cytokine production stimulated by *C. albicans* cells (**Figure 6**). However, TNFα, and IL-1β stimulation by *C. parapsilosis sensu lato* was significantly reduced upon MR blocking (**Figure 6**). IL-6 levels were only significantly reduced in anti-MR-pre-incubated human PBMCs stimulated with either *C. orthopsilosis* or *C. metapsilosis*, but not with *C. parapsilosis sensu stricto* (**Figure 6**); while blocking of MR only affected IL-10 production in cells stimulated with *C. orthopsilosis* (**Figure 6**). Control interactions with an irrelevant antibody did not show changes in the cytokine production stimulated by all tested yeast cells (**Figure 6**). Taken together, these results indicate the role of dectin-1, TLR4 and MR in the recognition of members of the *C. parapsilosis* complex and *C. albicans* is different.

## Purified Mannans from *C. albicans* and *C. parapsilosis sensu lato* are Capable to Mimic the Effect of Anti-TLR4 and Anti-MR Antibodies on Human PMBCs

Purified cell wall components from *C. albicans* have been previously used to block its proper recognition by human PBMCs (Mora-Montes et al., 2011), thus we aimed to isolate either *N*-linked or *O*-linked mannan using well-established protocols (Mora-Montes et al., 2012). Purified mannans from

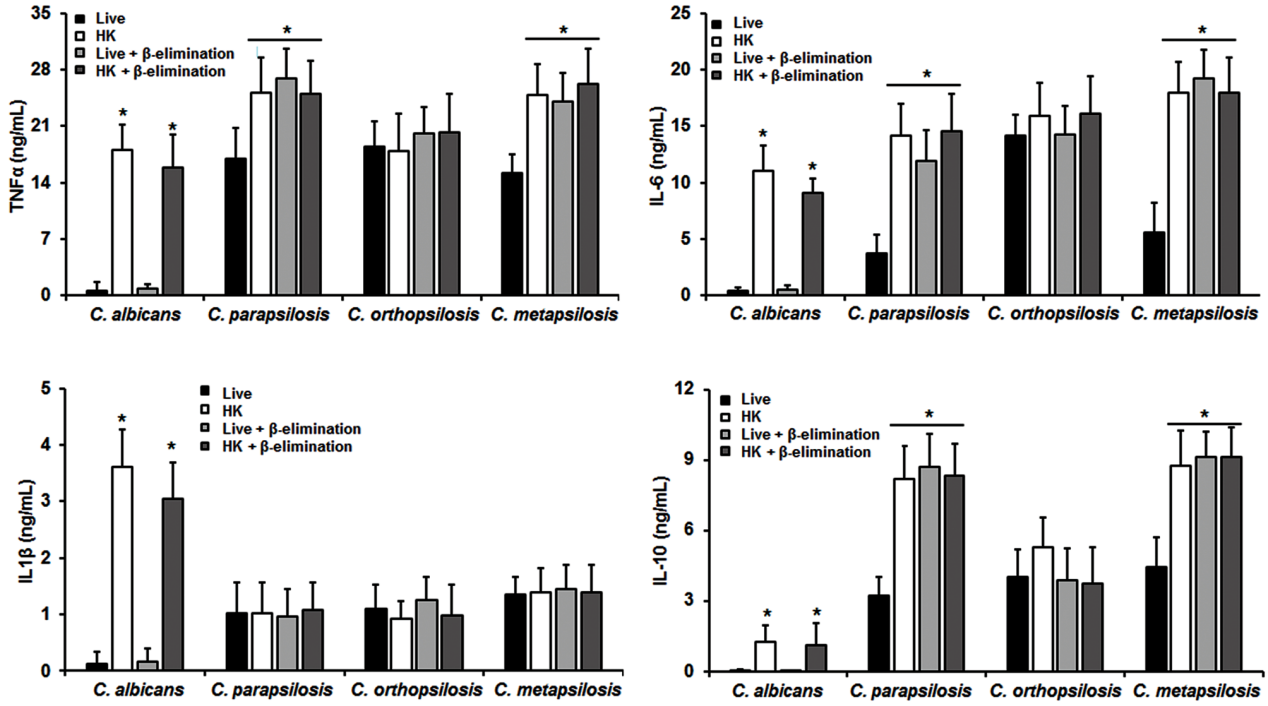


**FIGURE 3 | Cytokine production by human PBMCs stimulated with either live, heat-killed (HK) or β-eliminated yeast cells.** Human PBMCs were co-incubated 24 h with either live, HK, live and β-eliminated, or HK and β-eliminated yeast cells, supernatant collected and used to quantify the cytokine levels. Strains used are *C. albicans* SC5314, *C. parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, and *C. metapsilosis* SZMC 1548. *$P < 0.05$, when compared with live cells from the same species.

any of the three members of the *C. parapsilosis* complex or *C. albicans* were negative to fungal, bacteria and protein or other wall polysaccharide contamination, and did not stimulate any cytokine production when used at concentration up to 400 μg/mL (not shown). When human PBMCs were pre-incubated with 200 μg/mL *N*- or *O*-linked mannans from *C. albicans* and then stimulated with *C. parapsilosis sensu stricto*, TNFα stimulation was significantly reduced, but not IL-6 production (**Table 2**). Similar results were observed when mannans from either *C. parapsilosis sensu lato*, *C. orthopsilosis* or *C. metapsilosis* were used in the pre-incubation step (**Table 2**), or when mannans were used to block the proper recognition of *C. orthopsilosis* or *C. metapsilosis* (not shown). Therefore, *N*- and *O*-linked mannans are able to block the recognition of *C. parapsilosis sensu lato* as the anti-MR and anti TLR4 antibodies, respectively.

## DISCUSSION

Members of the *Candida* genus are frequently regarded as similar to *C. albicans*; however, it is possible to demonstrate they have both genetic and metabolic differences that can affect the interaction with the host (Butler et al., 2009). Here, our comparative analysis of cell wall composition showed that members of the *C. parapsilosis* complex displayed lower mannan quantity, with similar protein level, when compared to *C. albicans* cells. In order to explain these data, we hypothesize that the mannans displayed on the wall surface have lower content of mannose per oligosaccharide unit, i.e., they are shorter in *C. parapsilosis sensu lato*. Indeed, it has been demonstrated *C. parapsilosis* has shorter *N*-linked mannans than those from the *C. albicans* cell wall (Shibata et al., 1995). The presence of short mannans is likely to affect the cell wall porosity, offering higher ability to DEAE-dextran, a bulky polycation, to reach the plasma membrane with the consequent increment in nucleic acid leakage (De Nobel et al., 1990). Moreover, these shorter mannans could be responsible of the increased content of both chitin and β1,3-glucan exposed at the wall surface. Our hypothesis of shorter mannans on the surface of *C. parapsilosis sensu lato* cells is also supported by the reduced phosphomannan content, which is an indirect measurement of both *N*- and *O*-linked mannan length (Bates et al., 2005, 2006; Mora-Montes et al., 2007, 2010; West et al., 2013; Lopes-Bezerra et al., 2015). This lower content of cell wall mannan seems to be naturally compensated by increasing the β-glucan content. This is a well-documented compensatory mechanism driven by the activation of the calcineurin and the protein kinase C signaling pathways, where disruption of the synthesis of one cell wall component usually affects the levels of other components to avoid wall weakness (Bates et al., 2005, 2006; Mora-Montes et al., 2007; Walker et al., 2013). Our results also point out to subtle, but significant differences in the wall organization between members of the *C. parapsilosis* complex. In *C. orthopsilosis* all the β1,3-glucan and chitin is exposed in

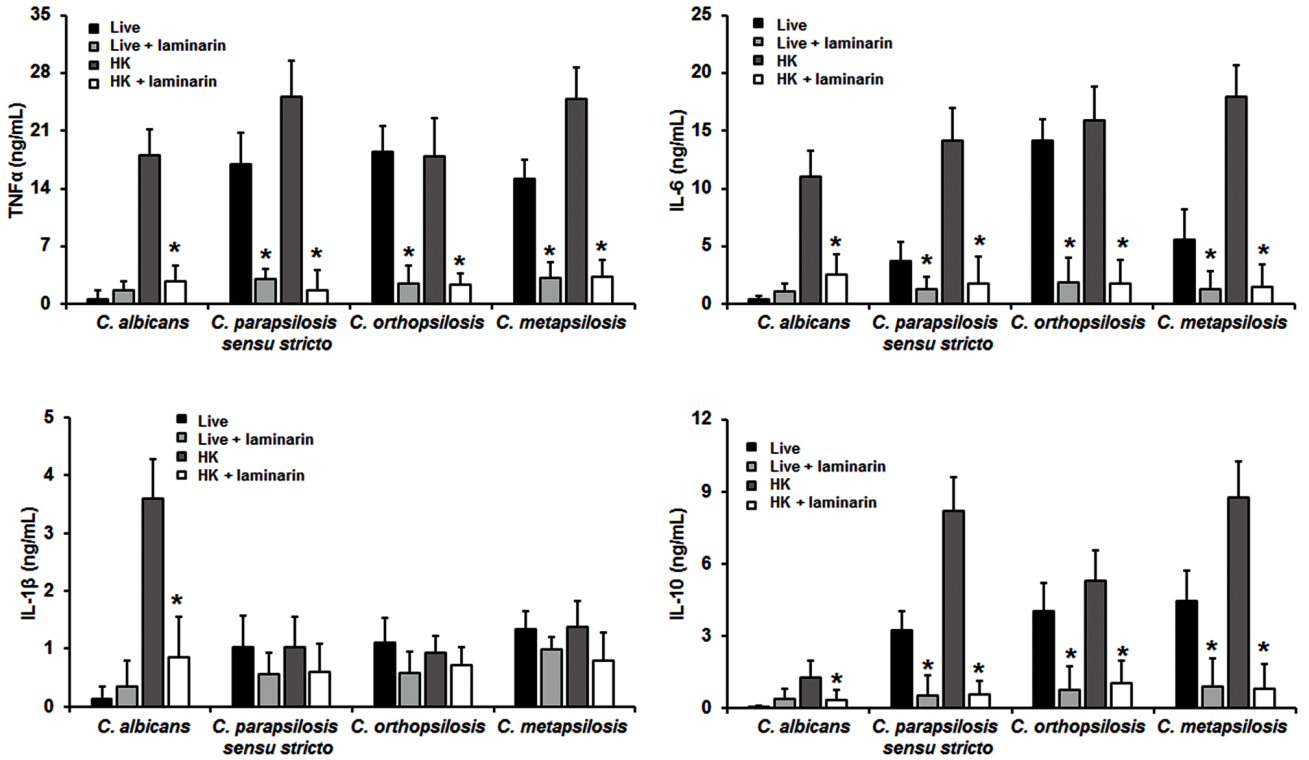


**FIGURE 4 | Cytokine production by human PBMCs pre-incubated with laminarin.** Human PBMCs were pre-incubated 60 min with 200 μg/mL laminarin before challenged for 24 h with either live or HK yeast cells, supernatant were collected and used to quantify the cytokine levels. Strains used are *C. albicans* SC5314, *C. parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, and *C. metapsilosis* SZMC 1548. *$P < 0.05$, when compared with untreated cells from the same organism.

live cells, which suggest its cell wall proteins have even shorter mannans than *C. parapsilosis sensu stricto* and *C. metapsilosis*, but with more glycosylation sites occupied per protein. Alternatively, it could also be possible that mannans attached to *C. orthopsilosis* wall proteins are larger than those on the surface of other members of the complex, occupying less number of sites in the proteins, making the inner wall polysaccharides highly exposed at the cell surface. Nevertheless, further experiments are required to provide a conclusive explanation to these observations.

Results presented here clearly demonstrate that the human PMBCs-*C. parapsilosis sensu lato* interaction is different to that previously characterized in *C. albicans*. While yeast cells of *C. albicans* are a poor stimulus for cytokine production, the three members of the *C. parapsilosis* complex induced a strong dectin-1-dependent cytokine production. This is in line with the higher cell wall β1,3-glucan content in live *C. parapsilosis sensu lato*, but not in *C. albicans*. It is noteworthy to mention that results obtained here with *C. albicans* cells contrast with those previously reported (Netea et al., 2006), where strong cytokine production was stimulated by yeast cells of this species. This apparent discrepancy relies in the fact that here we used live yeast cells, while in the previous work the main conclusions were based on HK organisms (Netea et al., 2006). When cells were heat killed we observed and increment in the chitin and the β1,3-glucan content, as previously reported (Gantner et al., 2005; Gow et al., 2007; Mora-Montes et al., 2011), and this correlated with a significant increment in cytokine production stimulated by *C. albicans*, *C. parapsilosis sensu stricto* and *C. metapsilosis*, stressing the importance of the dectin-1-β1,3-glucan interaction for a strong cytokine induction. In the case of *C. orthopsilosis*, we did not notice a significant increment in cytokine production upon heat killing, which support our result indicating most of the β1,3-glucan is naturally exposed on the *C. orthopsilosis* cell surface. Our results also suggest that *O*-linked mannans from yeast cells play a major role in the recognition of *C. parapsilosis sensu lato* than in *C. albicans*, where they are dispensable and redundant wall elements for cytokine stimulation by human PBMCs (Netea et al., 2006). It has been reported though, that *O*-linked mannans from *C. albicans* hypha have a major participation in cytokine stimulation by epithelial cells (Murciano et al., 2011), suggesting that the proposed redundant role during interaction with components of the immune system may be restricted to PBMCs. Here, we have evidence indicating that in the case of *C. parapsilosis sensu lato,* β-eliminated and HK cells stimulated similar cytokine levels, which suggest that upon removal of *O*-linked mannans, inner wall components are exposed and available to engage with PRRs, i.e., *O*-linked mannans have a role hiding inner wall components from immune receptors. Thus far, it has been reported that *N*-linked mannans mask the

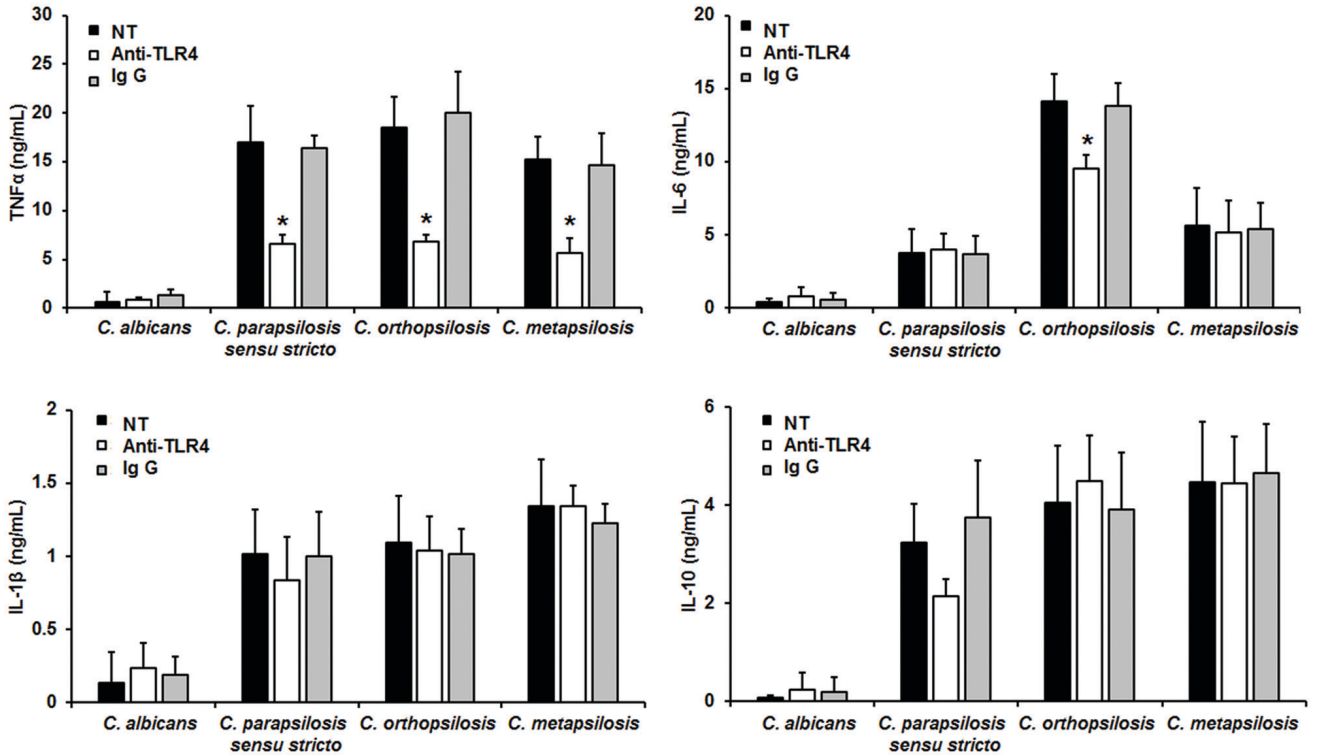


**FIGURE 5 | Cytokine production by human PBMCs pre-incubated with anti-TLR4 antibodies.** Human PBMCs were pre-incubated 60 min with 10 μg/mL either anti-TLR4 antibodies or $IgG_1$ before challenged for 24 h with live cells, supernatant were collected and used to quantify the cytokine levels. Strains used are *C. albicans* SC5314, *C. parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, and *C. metapsilosis* SZMC 1548. *$P < 0.05$, when compared with untreated cells from the same organism. NT, non-treated cells.

recognition of inner wall components and when disrupted, a significant cytokine production can be stimulated (Wheeler and Fink, 2006; Gow et al., 2007). Similar strategies, where a pro-inflammatory wall components is masked to avoid recognition by elements of the immune system, have been reported in *Histoplasma capsulatum* and *Aspergillus fumigatus*, where α-glucans and rodlet hydrophobins hide β1,3-glucan, respectively (Rappleye et al., 2007; Aimanianda et al., 2009). Therefore, it is feasible to conceive that in the case of *C. parapsilosis sensu lato*, both *N*- and *O*-linked mannans provide a disguising strategy to avoid recognition of β1,3-glucan by dectin-1. More experiments are required to demonstrate whether this structures are more abundant on the *C. parapsilosis sensu lato* cell wall or contain more mannose residues than those reported in *C. albicans*.

In this study we assessed the relevance of dectin-1, TLR4 and MR in the recognition of *C. parapsilosis sensu lato*. We chose these receptors because they are the main PRRs involved in the *C. albicans* immune recognition (Netea et al., 2002, 2006, 2015). TLR 4 is thus far the unique receptor for *C. albicans* *O*-linked mannans (Netea et al., 2006), and it is likely also participating in the recognition of the same cell wall component on *C. parapsilosis sensu lato*. The blocking experiments with either antibodies against receptors or laminarin, showed that TNFα stimulation by members of the *C. parapsilosis* complex, but not with *C. albicans* cells, depends on the engagement of TLR4, dectin-1 and MR with their ligands. These results contrast with our previous observation with β-eliminated cells, where TNFα levels upon disrupting the *O*-linked mannan-TLR4 interaction increased. The hypothesis to explain this apparent paradoxical response is that when the TLR4 receptor is blocked with anti-TLR4 antibodies, the *O*-linked mannans are still present on the surface of the fungal cell wall, hiding β1,3-glucan from the recognition by dectin-1. Interestingly, IL-6 production was only affected when human PBMCs were treated with anti-TLR4 antibodies and then stimulated with *C. orthopsilosis*, suggesting the *O*-linked mannans from this organism are different from those present on the surface of *C. parapsilosis sensu stricto* and *C. metapsilosis*. IL-10 production was strongly depend on engagement of dectin-1, as previously reported (Reid et al., 2009). It is noteworthy to mention that the IL-1β production by *C. parapsilosis sensu lato* was partially dependent on MR recognition, but not dectin-1 nor TLR4. This observation contrast with that reported earlier (Toth et al., 2013), where IL-1β stimulation by *C. parapsilosis sensu stricto* was dectin-1 dependent. Since the *C. albicans* immune recognition via dectin-1 is fungal strain-specific (Marakalala et al., 2013), it is feasible to conceive that this discrepancy can be explained because the genetic background of the strain used here (SZMC 8110) and in the previous report (GA-1) (Toth et al., 2013). In cells stimulated

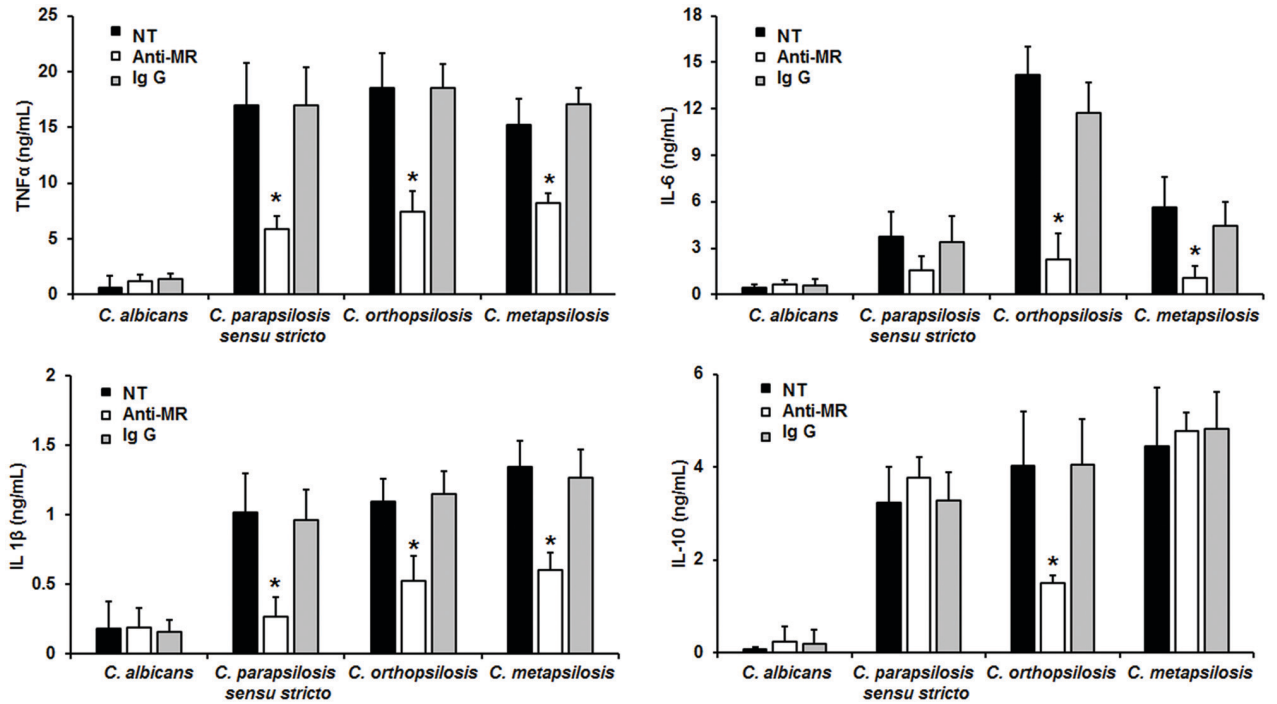


**FIGURE 6 | Cytokine production by human PBMCs pre-incubated with anti-MR antibodies.** Human PBMCs were pre-incubated 60 min with 10 μg/mL either anti-MR antibodies or $IgG_1$ before challenged for 24 h with live cells, supernatant were collected and used to quantify the cytokine levels. Strains used are *C. albicans* SC5314, *C. parapsilosis sensu stricto* SZMC 8110, *C. orthopsilosis* SZMC 1545, and *C. metapsilosis* SZMC 1548. *$P < 0.05$, when compared with untreated cells from the same organism. NT, non-treated cells.

by *C. albicans,* IL-1β production is dependent on the activation of dectin-1, TLR2 and MR (van de Veerdonk et al., 2009); thus, different PRRs or downstream signaling components after *C. parapsilosis sensu lato* recognition could be responsible of this difference.

It has been previously demonstrated that cell wall preparations or non-purified wall components, such as zymosan, are potent inductors of cytokines (Brown et al., 2003; Netea et al., 2006); while purified wall components are unable to stimulated cytokine production (Dennehy et al., 2008; van de Veerdonk et al., 2009), and can block cytokine production when immune cells are stimulated (Maneu et al., 2011; Mora-Montes et al., 2011; Huang et al., 2012; Cohen-Kedar et al., 2014). These observations have been the base to propose the co-stimulatory theory, where a strong cytokine response involves the engagement of not only one but two or more PRRs at once (Netea et al., 2015). Although it is likely mannans from *C. albicans* and *C. parapsilosis sensu lato* are different, here it was demonstrated that purified *N*- and *O*-linked mannan preparations do not stimulate cytokine production and are capable to block proper *Candida* recognition, in a similar way that anti-MR and anti-TLR4 antibodies, respectively. Thus, they can be a reliable and alternative tool to assess the contribution of these PRRs during the innate immune recognition of fungal cells.

Overall, our data clearly demonstrate that the current knowledge about *C. albicans* biology and interaction with the host cannot be extrapolated to other members of the genus. *C. parapsilosis sensu lato* displays significant differences in the cell wall that impact the recognition by human PBMCs, stimulating stronger cytokine production, via the increased exposure of β1,3-glucan at the cell surface. Finally, our results suggest different contribution of dectin-1, MR and TLR4 in the recognition of the

**TABLE 2 | Blocking of cytokine production by *N*- and *O*-linked mannans isolated from either *C. albicans* or *C. parapsilosis sensu lato* cell wall.**

| Cytokine stimulation by *C. parapsilosis sensu stricto* | TNFα (%) | IL-6 (%) |
|---|---|---|
| No mannan included | 100 | 100 |
| Pre-incubation with *C. albicans N*-linked mannan | 34.4 ± 4.3* | 90.4 ± 5.4 |
| Pre-incubation with *C. albicans O*-linked mannan | 43.8 ± 7.6* | 99.3 ± 3.2 |
| Pre-incubation with *C. parapsilosis s. st. N*-linked mannan | 39.4 ± 6.3* | 93.3 ± 4.3 |
| Pre-incubation with *C. parapsilosis s. st. O*-linked mannan | 40.3 ± 6.3* | 101.2 ± 4.3 |
| Pre-incubation with *C. orthopsilosis N*-linked mannan | 33.4 ± 9.3* | 101.4 ± 3.4 |
| Pre-incubation with *C. orthopsilosis O*-linked mannan | 42.3 ± 4.9* | 95.8 ± 4.2 |
| Pre-incubation with *C. metapsilosis N*-linked mannan | 35.6 ± 6.6* | 95.3 ± 5.5 |
| Pre-incubation with *C. metapsilosis O*-linked mannan | 43.8 ± 3.9* | 100.5 ± 6.3 |

*$P < 0.05$, when compared to stimulation with no mannan included.

members of the *C. parapsilosis* complex, which has to be taken in consideration when analyzing the *C. parapsilosis sensu lato*-host interaction.

## AUTHOR CONTRIBUTIONS

ML, AG, and HM-M conceived the study. EE-M, MN-A, LP-G, EM-M, and KC performed experiments. EE-M, MN-A, LP-G, EM-M, ML, KC, AG, and HM-M analyzed data. HM-M drafted the manuscript. EE-M, MN-A, LP-G, EM-M, ML, KC, AG, and HM-M revised and approved the manuscript.

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# Different *Candida parapsilosis* clinical isolates and lipase deficient strain trigger an altered cellular immune response

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Numerous human diseases can be associated with fungal infections either as potential causative agents or as a result of changed immune status due to a primary disease. Fungal infections caused by *Candida* species can vary from mild to severe dependent upon the site of infection, length of exposure, and past medical history. Patients with impaired immune status are at increased risk for chronic fungal infections. Recent epidemiologic studies have revealed the increasing incidence of candidiasis caused by non-albicans species such as *Candida parapsilosis*. Due to its increasing relevance we chose two distinct *C. parapsilosis* strains, to describe the cellular innate immune response toward this species. In the first section of our study we compared the interaction of CLIB 214 and GA1 cells with murine and human macrophages. Both strains are commonly used to investigate *C. parapsilosis* virulence properties. CLIB 214 is a rapidly pseudohyphae-forming strain and GA1 is an isolate that mainly exists in a yeast form. Our results showed, that the phagocyte response was similar in terms of overall uptake, however differences were observed in macrophage migration and engulfment of fungal cells. As *C. parapsilosis* releases extracellular lipases in order to promote host invasion we further investigated the role of these secreted components during the distinct stages of the phagocytic process. Using a secreted lipase deficient mutant strain and the parental strain GA1 individually and simultaneously, we confirmed that fungal secreted lipases influence the fungi's virulence by detecting altered innate cellular responses. In this study we report that two isolates of a single species can trigger markedly distinct host responses and that lipase secretion plays a role on the cellular level of host–pathogen interactions.

**Keywords:** ***Candida parapsilosis*, phagocyte response, secreted lipase, co-infection, live cell imaging**

**Abbreviations in figures:** LIP KO indicates the *Cp*ΔΔ*lip*1 − ΔΔ*lip2* lipase deficient strain, TRX represents Triton X-100.

## INTRODUCTION

*Candida* species are the most common etiological agents of systemic fungal infections (Gácser et al., 2005). Although *Candida albicans* is the leading *Candida* species responsible for bloodstream infections, a significant increase has been reported in the number of fungal invasions caused by *non-albicans Candida* (NAC) species (Guinea, 2014). *C. parapsilosis* is one of the most frequent NAC species found in the hospital environment and currently is the number one cause of neonatal candidemia (Chow et al., 2012; Pammi et al., 2013; Guinea, 2014; Quindos, 2014). Besides its association with nosocomial infections in children, this species also threatens adult patients with diminished immunity (Nosek et al., 2009). Despite the emerging relevance of *C. parapsilosis,* relatively little is known about the immune responses induced by this species. GA1 and CLIB 214 are two distinct *C. parapsilosis* clinical isolates that are the most frequently used model strains for biological and molecular characterization studies (Gácser et al., 2005; Holland et al., 2014). *C. parapsilosis* GA1 is a bloodstream isolate obtained in Hamburg, Germany, and primarily appears in a yeast form and produces smooth colonies on agar (Trofa et al., 2008; Pryszcz et al., 2013). *C. parapsilosis* CLIB 214 (ATCC 22019) was isolated from the feces of a patient in Puerto Rico, and rapidly forms pseudohyphae producing a rough, concentric colony phenotype on agar (Laffey and Butler, 2005; Nosek et al., 2009). To our knowledge, no comparison has been made between the virulence of these commonly used laboratory type strains.

The phagocytic cells of the innate immune system play a central role in host defense against invading microbes, including fungi. The phagocytic process can be separated into four distinct stages: (1) phagocyte aggregation at the site of infection, (2) recognition of pathogen associated molecular patterns (PAMPs) via receptors, (3) internalization of the foreign particles, and (4) digestion of ingested agents through phagosome maturation and activation of hydrolytic enzymes (Lewis et al., 2013; Rudkin et al., 2013). We recently investigated the phagocytosis of *C. parapsilosis* CLIB 214 focusing on migration and engulfment of these cells by macrophages, and compared the results to that occurring with *C. albicans* and *C. glabrata* (Tóth et al., 2014b). In the present study, one of our primary aims was to compare and define the interactions of *C. parapsilosis* GA1 and CLIB 214 with host effector cells.

One well-described virulence factor that promotes the pathogenesis of invasive candidiasis is the secretion of hydrolytic enzymes, such as lipases and proteinases (Gácser et al., 2007; Horváth et al., 2012). Presumed roles of microbial secreted lipases during an infection include host cell adhesion, lipid digestion for nutrient acquisition and triggering of inflammatory cascades (Trofa et al., 2008; Nguyen et al., 2011; Tóth et al., 2014a). Secreted lipase encoding genes have been identified in *C. parapsilosis* (Gácser et al., 2007). In contrast with *C. albicans*, the *C. parapsilosis* genome includes only four putative secreted lipases (Butler et al., 2009; Nguyen et al., 2011). However, the deletion of only two of the four lipase encoding genes (*LIP1* and *LIP2*), resulted in significantly decreased virulence as determined in both *in vitro* and *in vivo* infection models (Nagy et al., 2011; Trofa et al., 2011). In addition, the deletion mutant strain lacking both *LIP1* and *LIP2* (*Cp*ΔΔ*lip*1 − ΔΔ*lip2*) formed less complex and thinner biofilms when compared to the *C. parapsilosis* GA1 parental strain (Gácser et al., 2007). Studies that used human peripheral blood mononuclear cell-derived macrophages (PBMC-DMs; Tóth et al., 2014a) and dendritic cells (DCs; Nagy et al., 2011) reported *Cp*ΔΔ*lip*1 − ΔΔ*lip2* strain to be less virulent, as both types of primary cells killed lipase mutant cells at a higher ratio than wild type cells. Furthermore, the lack of the secreted component, increased the pro-inflammatory cytokine and chemokine expression levels, thus led to stronger inflammatory response (Nagy et al., 2011; Tóth et al., 2014a). The decreased virulence of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* was also reflected in the rate of reconstituted human tissue damage as the infected tissue was similar to the uninfected control and low LDH levels were measured (Gácser et al., 2007). Another study, using neonatal rats also confirmed these results, as low levels of organ fungal burdens were detected (Trofa et al., 2011).

Hence, the data show that secreted lipases play a role in *C. parapsilosis* virulence. Therefore, another major aim of the present work was to determine how secreted lipases influence the phagocytic process by examining the separate stages individually and if the presence of the wild type strain could complement the defective phenotype of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* and thus influence the phagocyte response.

Thus, in this study we compared the phagocytosis of CLIB 214 by macrophages to that of GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells using live cell imaging at defined stages of the phagocytic process: phagocyte migration, engulfment of fungal cells, and subsequent host cell damage. Additionally, co-infections with *Cp*ΔΔ*lip*1 − ΔΔ*lip2* and GA1 fungal cells were performed to determine whether secreted factors from wild type cells could complement the defects in the mutant yeasts.

## MATERIALS AND METHODS

### Preparation of *Candida parapsilosis* Strains

Wild type *C. parapsilosis* CLIB 214 and GA1 clinical isolates and the *LIP1–LIP2* deficient strain (*Cp*ΔΔ*lip*1 − ΔΔ*lip2*) were maintained on YPD solid medium at 4°C and prepared for experiments as described in our previous work (Tóth et al., 2014b). Prior to experiments, the *Candida* strains were cultured overnight in liquid YPD medium (1% yeast extract, 2% glucose, 2% peptone) at 30°C with shaking at 200 rpm. The cells were collected and washed three times with PBS (phosphate buffered saline), counted and diluted to the final concentration of $1 \times 10^8$/ml. UV-killed fungal strains were prepared using twenty exposures to 20 mJ/cm$^{-2}$ UV. In order to differentiate between fungal cells after the co-infection, 1 mg/ml FITC (Sigma, Dorset, UK) and 50 μg/ml CFW (Sigma, Dorset, UK) were used to label yeasts. FITC was dissolved in dimethyl sulfoxide (DMSO) and added to $10^8$/ml cells suspended in 0.05 M carbonate-bicarbonate buffer (pH 9.6). CFW was dissolved in distilled water and added to $10^8$/ml yeasts in PBS. *C. parapsilosis* cells were stained at room temperature in the dark for 10 min, followed by washing steps three times with PBS and suspended in 1 ml 1X PBS. In dual

yeast cell co-infection experiments, mixtures of GA1 and *LIP1–LIP2* yeast cells were used in which GA1 was labeled with FITC and the mutant with CFW. Experiments were simultaneously performed using GA1 yeast cells labeled with CFW and mutants with FITC. We found that there was no difference in our experiments whether the GA1 or lipase mutants were labeled with the alternate stain. Although we used both conditions in all experiments, we only reported out the condition where GA1 was labeled with FITC and the mutant with CFW.

## J774 Mouse Macrophage Cell Line Preparation and Staining

Dulbecco's modified Eagle's medium (DMEM; Lonza, Slough, UK) supplemented with 2 mM L-glutamine (Invitrogen, Paisley, UK), 10% fetal calf serum (FCS; Biosera, Ringmer, UK), and 200 U/ml penicillin/streptomycin (Invitrogen, Paisley, UK) was used in order to maintain the murine cell line. J774 cells were kept at 37°C, in the presence of 5% $CO_2$ prior to and during the experiments. For live video microscopy, similarly to our previous report, $1.2 \times 10^5$ macrophages were seeded on eight-well μ-slides (ibidi, Martinsried, Germany) and incubated at 37°C overnight prior to infection with *Candida* strains. For visualizing phagocytosis Lysotracker Red was used to label the acidic compartments of phagocytes. The original media was replaced with fresh, 300 μl pre-heated supplemented DMEM containing 1 μM Lysotracker Red DND-99 (Invitrogen, Paisley, UK) immediately before the experiments.

## Human PBMC-derived Macrophage Preparation and Staining

For the isolation of human peripheral blood mononuclear cells (PBMCs), a standard protocol was used (Rudkin et al., 2013) with modifications, under approval from the institutional review board of the University of Aberdeen and the University of Szeged. Following isolation, PBMCs ($7.5 \times 10^5$ cell/ ml) were then plated on eight-well μ-slides (ibidi, Martinsried, Germany) and incubated at 37°C, 5% $CO_2$ for 6–7 days in serum supplemented DMEM (Lonza, Slough, UK). Shortly before the experiment, similarly to the murine macrophage preparation, the media was replaced with 300 μl fresh, pre-heated supplemented and 1 μM Lysotracker Red DND-99 (Invitrogen, Paisley, UK) containing DMEM.

## Phagocytosis Assay and Live Cell Video Microscopy

In order to examine the phagocytic processes post infection, a previously described and standardized protocol was used accordingly (Tóth et al., 2014b). Live and UV-treated *C. parapsilosis* CLIB 214, GA1, and ΔΔ*lip*1 − ΔΔ*lip2* cells were added to Lysotracker red DND-99-stained (Invitrogen, Paisley, UK; 1 μM) $4 \times 10^5$ /ml J774 murine and $7.5 \times 10^5$ /ml human PBMC-derived macrophages on eight-well μ-slides (ibidi, Martinsried, Germany) in 300 μl volumes immediately before the video capture was initiated. During individual infections, the effector/target ratio was 3:1, while in the case of simultaneous infections the ratio of 1.5 GA1: 1.5 lipase mutant: 1 macrophage was used. Images were captured over a 6 h period with a CCD camera attached to an UltraVIEW VoX Spinning disc confocal microscope (PerkinElmer, Massachusetts, USA) using a 40X oil immersion objective. For the comparison of the *C. parapsilosis* GA1 and CLIB 214 clinical isolates, over a 100 J774 macrophages were monitored individually and evaluated from at least four separate experiments—with 20–40 macrophages analyzed per experiment, dependent on frequency of macrophage–*Candida* interactions in the respective videos. From PBMC-DM experiments, 60 macrophages were tracked from three separate experiments with 15–20 macrophages analyzed per experiment due to cell size. For the *C. parapsilosis* GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* comparison 250 macrophages were selected and monitored from five separate experiments, with 45–55 macrophages followed per experiment, dependent on frequency of macrophage–*Candida* interactions in the respective videos, in order to make clear assumptions from the co-infection studies.

For tracking and statistical analysis of both murine and human PBMC-derived macrophage migration Volocity 6.3 image analysis software (Improvision, PerkinElmer, Coventry, UK) was used. Similarly to our previous study, measurements included the macrophage migration toward *C. parapsilosis* cells, distribution of fungal cells per macrophages, prevalence of uptake events, engulfment time, average uptake of fungal cells by actively phagocytosing macrophages and post-ingestion rupture events of host cells.

High throughput analysis with Volocity software 6.3 allowed us to calculate the mean tract velocity of macrophages in the presence of different *C. parapsilosis* strains. The engulfment time was defined as the time difference between the establishment of the phagocyte-fungal cell contact and the phagocyte membrane enclosing around the fully ingested cell. Full engulfment events were determined when macrophages stretched toward fungal cells, the phagocyte membrane encircled around the yeast cell and the host cell regained its original shape. The prevalence of uptake represents the percentage of uptake events during defined time intervals. Individual events were defined by the starting point of the recognition of fungal cells by macrophages. The percentage of macrophages taking up specific numbers of fungal cell was referred to as distribution of fungal cells per macrophages. Average uptake is defined as the average number of fungal cells taken up by phagocytes that ingested at least one yeast cell over the whole incubation period. The percentage of dead macrophages relative to the visible macrophage population, that ingested at least one *C. parapsilosis* cell, represents post-ingestion macrophage rupture (PIR) events. Individual PIR events were defined as the disruption of membrane integrity that were visible up until the end of the 6 h of co-culturing period.

## LDH Assay to Determine Host Cell Damage

To determine the concentration of LDH released by macrophages, culture supernatants were collected 6 h after *C. parapsilosis* infection. As a postive control, cells were treated with 1% Triton X-100 solution. For LDH detection, the Cytotoxycity Detection kit (Takara Bio Europe/Clontech, France) was used according to the user's manual. Basal LDH

activity of human PBMC-derived macrophages may be due to the presence of undifferentiated monocytic cells or dead cells in the culture before the assay.

## Statistical Analysis

Tracking of individual macrophages and high-throughput migration analysis was achieved by Volocity software 6.3. Extracted data were used to calculate the mean track velocity of phagocytes cultured with *Candida* strains.

For the comparison of the calculated mean values of each of the aspects, student's unpaired, two-tailed *t*-tests were used and confirmed by One-way ANOVA analysis followed by a Bonferroni's multiple comparison *post-hoc* tests. GraphPad Prism v 5.0 software was used to determine statistical significance. Significant differences were considered at *p*-values of $\leq 0.05$. Data were pooled for migration analysis, engulfment time and LDH, thus SD-values are shown, percent phagocytosis, average uptake values and data for the fungal cell distribution/macrophages were evaluated as mean of means, thus SEM-values indicate error.

# RESULTS

## Comparison of *C. parapsilosis* GA1 and CLIB 214 Isolates

### Macrophage Migration Differs for *C. parapsilosis* CLIB 214 and GA1 Isolates

Murine macrophages were challenged with live or UV-killed *C. parapsilosis* GA1 or CLIB 214 cells at an effector/target ratio of 1:3 and the phagocytic process was monitored by live cell video microscopy for 6 h (Lewis et al., 2012; Okai et al., 2015). We first examined the migration of macrophages toward the two distinct isolates. As the majority of uptake events were detected during the early stages post infection, macrophages were tracked during the first 45 min. Interestingly, the track velocity of J774 macrophages in response GA1 was significantly lower (mean ± SD; 0.85 ± 0.37 μm/min, **Figure 1A**) than to CLIB 214 (1.02 ± 0.48 μm/min). UV-killing of CLIB cells led to a significant reduction in the velocity of macrophage migration (0.81 ± 0.38 μm/min). In contrast, the difference between live and dead GA1 (0.75 ± 0.31 μm/min) was not significant, and the track velocity was similar to that of UV-irradiated CLIB yeast cells. These differences are also evident in the macrophage tracking diagrams (**Figures 1B–E**). The tracking diagrams show the movement and distances traveled by individual phagocytes relative to their starting position. The tracked murine macrophages appeared to travel shorter distances toward both UV-killed CLIB 214 and GA1 cells (**Figures 1C,E**) than toward live CLIB 214 and GA1 yeasts (**Figures 1B,D**).

### CLIB 214 and GA1 Exhibit Altered Engulfment Dynamics during Macrophage Phagocytosis

In this study we further aimed to differentiate between the time taken to internalize GA1 and CLIB 214 by murine and human PBMC-derived macrophages. Differences were observable in phenotype between the two isolates of *C. parapsilosis* as shown on **Figure 2A**, as rapid pseudohypha formation was detectable in CLIB 214 but not with the GA1 strain. At the early stage of infection, *C. parapsilosis* GA1 does not form pseudohyphae, only slightly elongated blastospores. Engulfment time is defined as the time from the first phagocyte-fungal cell contact and the macrophage fully enclosing the bound cell. According to our analyses, both J774 phagocytes and the human PBMC-derived macrophages required significantly less time to internalize GA1 yeast cells (mean ± SD; 4.36 ± 1.94 and 3.77 ± 1.9 min for J774 and PBMC-derived macrophages, respectively, **Figures 2B,C**) compared to CLIB 214 cells (6.16 ± 3.43 and 8.16 ± 6.41 min). Furthermore, UV-treatment significantly decreased the engulfment time by J774 macrophages of GA1 and CLIB 214 yeast cells (3.05 ± 1.47 and 3.82 ± 2.32 min, respectively; **Figure 2B**). A similar trend for the engulfment of UV-irradiated cells was shown when using human primary macrophages.

### Uptake Rates for the *C. parapsilosis* Clinical Isolates

The majority of fungal cells were phagocytosed during the first hour post-infection by murine macrophages (**Figure 3**). We observed no difference between the overall uptake of GA1 and CLIB 214 cells for both the murine and primary macrophages even though the primary cells showed a higher capacity for phagocytosis (Supplementary Figure 1). In general, the total uptake pattern of contribution of individual macrophages to overall uptake appeared to be similar for both clinical isolates and no statistically significant differences were observed in the average uptake of yeasts by both types of phagocytes (Supplementary Figure 2).

### Host Cell Damage by CLIB 214 and GA1

In order to examine the impact of yeast cells on the host effector cells, macrophage damage was measured by the amount of lactate dehydrogenase (LDH) released into supernatant after co-incubation with *C. parapsilosis* cells. Culture supernatant was collected 6 h after interaction with J774 phagocytes and human PBMC-derived macrophages, respectively. No differences were detected between the two *C. parapsilosis* clinical isolates in terms of their ability to damage either J774 (**Figure 4A**) or human macrophages (**Figure 4B**). LDH results were also confirmed by quantifying post-ingestion macrophage rupture events (Supplementary Figure 3).

## Role of Fungal Lipase Secretion on Phagocytosis

### Comparison of *C. parapsilosis* GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip*2 Cells

Murine macrophages were infected with *C. parapsilosis* GA1 or *Cp*ΔΔ*lip*1 − ΔΔ*lip2* individually in a ratio of 3:1. In the case of co-infection, GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells were used in the ratio of 1:1, and the MOI was kept at 3:1 (representative image shown on **Figure 5**). In the mixed infections, yeast cells were either labeled with fluorescein isothiocyanate (FITC) or calcofluor white (CFW).

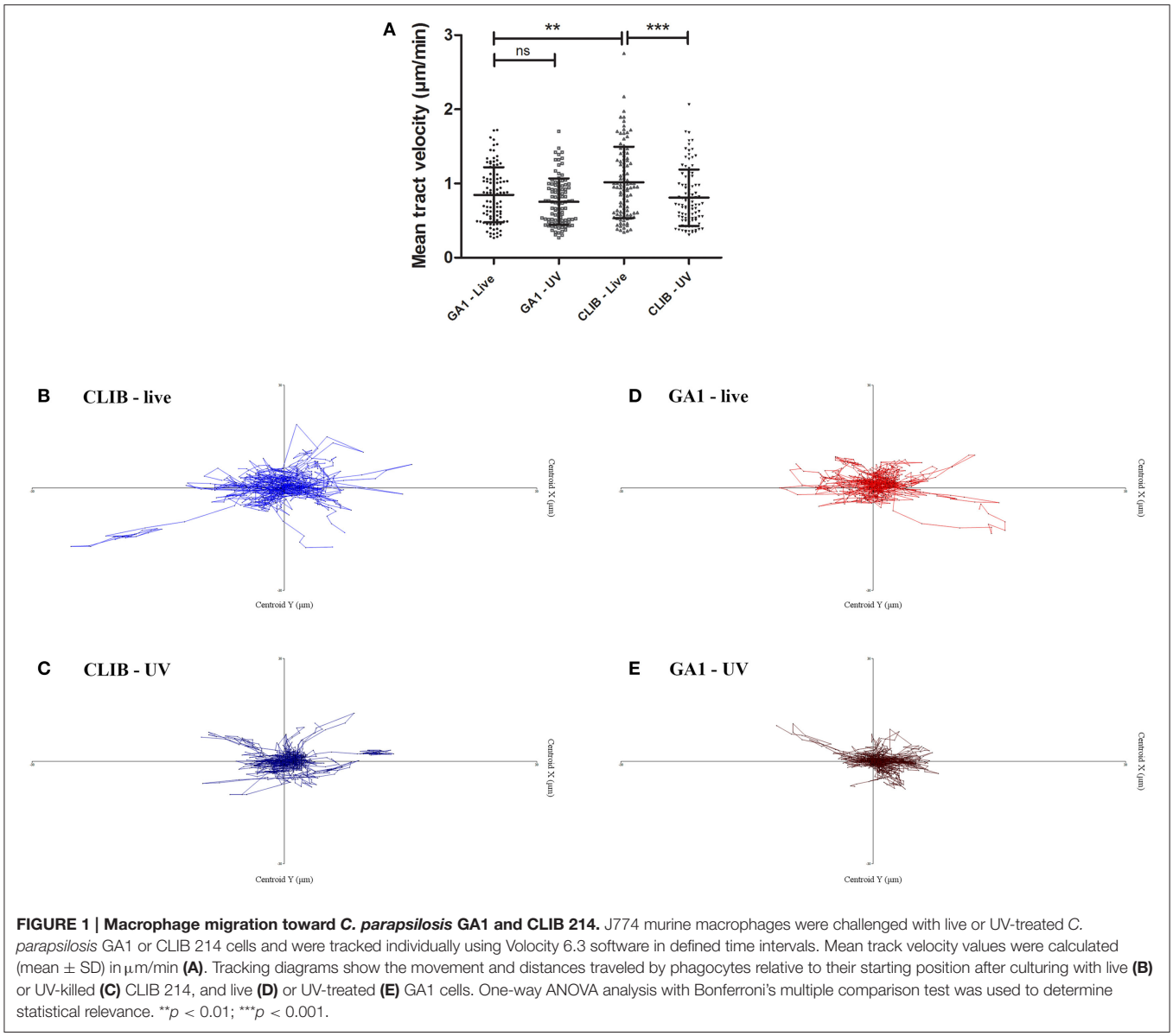


**FIGURE 1 | Macrophage migration toward *C. parapsilosis* GA1 and CLIB 214.** J774 murine macrophages were challenged with live or UV-treated *C. parapsilosis* GA1 or CLIB 214 cells and were tracked individually using Volocity 6.3 software in defined time intervals. Mean track velocity values were calculated (mean ± SD) in µm/min **(A)**. Tracking diagrams show the movement and distances traveled by phagocytes relative to their starting position after culturing with live **(B)** or UV-killed **(C)** CLIB 214, and live **(D)** or UV-treated **(E)** GA1 cells. One-way ANOVA analysis with Bonferroni's multiple comparison test was used to determine statistical relevance. $^{**}p < 0.01$; $^{***}p < 0.001$.

## Migration toward Wild Type and *Cp*ΔΔ*lip*1 − ΔΔ*lip*2 Mutant Strain

We assessed the migration of macrophages toward *Cp*ΔΔ*lip*1 − ΔΔ*lip2*, GA1 or mixed *Cp*ΔΔ*lip*1 − ΔΔ*lip2* and GA1 yeast cells during the first 45 min of co-incubation as above. The mean track velocity of J774 macrophages was significantly higher toward *Cp*ΔΔ*lip*1 − ΔΔ*lip2* (mean ± SD; 1.11 ± 0.28 µm/min; **Figure 6**) compared to GA1 (1.03 ± 0.28 µm/min). Interestingly, the macrophage migration was similar to that of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* when both GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* were present (1.11 ± 0.24 µm/min, **Figure 6**).

## Differences in Engulfment Time of GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip*2

We also assessed whether there were differences in the engulfment times of *C. parapsilosis* GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells by macrophages (**Figure 7**). Our results indicated, that more time was required for the full internalization of *Cp*ΔΔ*lip*1 − ΔΔ*lip*2 cells (mean ± SD; 3.76 ± 1.68 min; $p = 0.07$), in comparison with GA1 yeasts (3.09 ± 1.36 min). Mixed cultures of GA1 and lipase mutant yeasts had engagement times that were significantly increased relative to GA1 alone (3.948 ± 1.95 min, **Figure 7**), but were similar to the lipase mutants alone.

## Differences in the Phagocytosis of GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip*2

We further examined the overall uptake of *C. parapsilosis* GA1 and the lipase deficient mutant strain by J774 macrophages. Representative images of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* phagocytosis are shown on **Figure 8A**. In general, the overall uptake of mutant cells was more efficient than GA1 cells. Co-infection

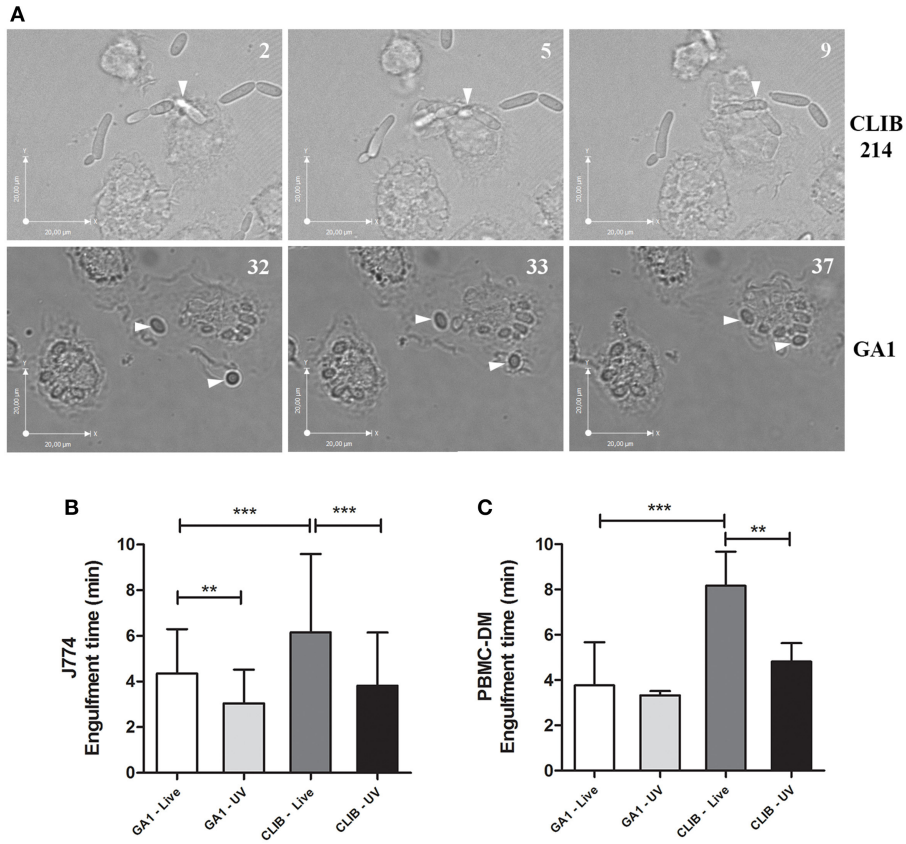


**FIGURE 2 | Engulfment time of GA1 and CLIB 214 by macrophages.** Representative images show the full internalization of *C. parapsilosis* CLIB 214 **(A, upper panel)** and GA1 cells **(A, lower panel)** by human PBMC-derived macrophages. Numbers at the upper right corner of pictures show the time (minutes) of observed individual events. Arrows show the fungal cells internalized by phagocytes. Diagrams show the average time (mean-values + SD) in minutes required for the full ingestion of live or UV-treated GA1 and CLIB 214 cells by J774 **(B)** and PBMC-derived macrophages **(C)**. One-way ANOVA analysis with Bonferroni's multiple comparison test was used to determine statistical significance. Significant differences were considered at *p*-values $^{**}p < 0.01$; $^{***}p < 0.001$. Scale bar: 20 μm.

of phagocytes with both strains (Supplementary Video 1) led to less effective overall phagocytosis as shown here by the intermediate values. Our results showed that, significantly more J774 macrophages contributed to the uptake of lipase deficient cells (mean ± SEM; 57.62 ± 4.95%) compared to their interactions with GA1 yeasts (33.90 ± 5.53%, **Figure 8B**). There was a significant reduction in uptake of yeast cells in conditions in which both *Cp*ΔΔ*lip*1−ΔΔ*lip2* and GA1 were present compared to the lipase mutant alone. A higher percentage of macrophages took up more than two or three *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells than GA1 cells (**Figure 8C**). The mean number of ingested yeast cells was significantly higher with *Cp*ΔΔ*lip*1 − ΔΔ*lip2* infection (mean ± SEM; 2.91 ± 0.25) compared to GA1 (1.79 ± 0.20) or co-infections with GA1 and lipase mutants (2.32 ± 0.20 **Figure 8D**). We found no difference in uptake kinetics for both types of strains either in case of single or co-infections (data not shown). No difference was detected between *Cp*ΔΔ*lip*1−ΔΔ*lip2* or GA1 cell preference of the individual macrophages and no differences were observed in *Cp*ΔΔ*lip*1−ΔΔ*lip2* or GA1 uptake, when both stains were present with the host cells (data not shown).

### Macrophage Damage by GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2*

As a measure of host cell damage, LDH released by J774 phagocytes was measured from culture supernatant 6 h post-infection. Similar levels of LDH were detected with either individual; or mixed wild type and lipase deletion strains. Thus, the lack of *LIP1* and *LIP2* genes did not affect host cell damage (**Figure 9**). Similar observations were made when comparing the *C. parapsilosis* strains in terms of post-ingestion macrophage rupture events (data not shown).

## DISCUSSION

In our previous study, we compared the phagocytic kinetics of three *Candida* species, *C. albicans*, *C. glabrata,* and *C. parapsilosis* CLIB 214 (Tóth et al., 2014b). Here we aimed to compare two well-described *C. parapsilosis* clinical isolates, GA1 and CLIB 214. In order to study the interaction between the innate immune system and *C. parapsilosis* clinical isolates on the cellular level, live cell imaging was used, which has previously been validated as a high-throughput image analysis tool for

quantitative analysis (Bain et al., 2014; Okai et al., 2015). We compared phagocyte migration, engulfment rate, overall uptake of fungal cells, and subsequent host cell damage after challenging murine and human PBMC-derived macrophages with fungal cells. Our results showed similar overall uptake rates for GA1 and CLIB 214 by both types of macrophages as fungal strains were ingested during the early phases of the co-incubation; no differences were detected in phagocytic activity, uptake kinetics or average uptake. However, significant differences were found in terms of macrophage migration and engulfment time. Recent publications have already used macrophage migration to differentiate between pathogenic strains, as it refers to recognition capabilities of either pathogenic PAMPs or microbial signaling molecules (Lewis et al., 2012; Ifrim et al., 2014). Thus, host cell migration rates might indicate virulence properties of pathogenic microorganisms. Interestingly, we determined that the mean velocity of macrophages significantly decreased when they were incubated with GA1 yeast cells compared to CLIB 214. In addition, UV-treatment of either strain led to decreased phagocyte migration, although this difference was not significant for GA1. This change in migration might be caused by different amounts of fungal signaling molecules released into the medium, thus differences between the metabolic activities of the two isolates. However, further investigations are needed to confirm this theory. Furthermore, our data suggested that significantly more time was required for the uptake of CLIB 214 cells in contrast with GA1, and UV treatment significantly decreased engulfment time of both strains. As we previously reported, even though *C. parapsilosis* CLIB 214 cells are able to form pseudohyphae rapidly post-infection, no correlation was found between the engulfment time and the length of pseudohyphae (Tóth et al., 2014b). Thus, the difference in the engulfment time of GA1 and CLIB 214 might suggest that this is due to differences between the cell wall PAMPs of the two isolates, rather than differences in terms of shape and length. Results based on LDH release and post-ingestion macrophage rupture events revealed that both *C. parapsilosis* isolates examined contributed equally to host cell damage.

Even though, *C. parapsilosis* GA1 and CLIB 214 isolates were phagocytosed similarly, dissection of the phagocytic stages revealed various host cell responses. These altered responses might suggest differences in the cell wall structure or composition and fungal signaling molecules released by these strains. However, further investigations are needed for confirmation. As both of these clinical isolates are commonly used laboratory strains for molecular characterization, this information might be taken into consideration when studying gene function.

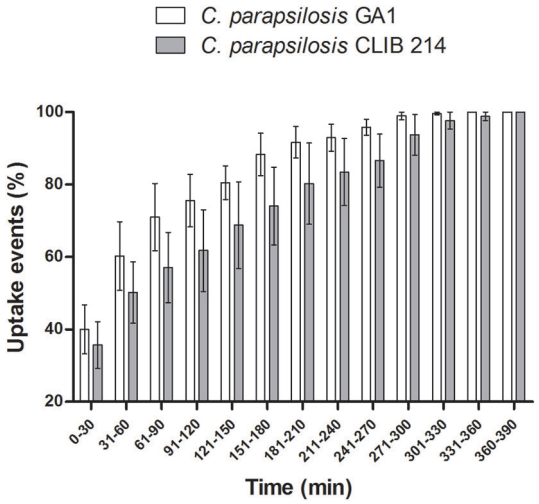


**FIGURE 3 | Uptake kinetics of GA1 and CLIB 214.** Percentage of uptake events (mean ± SEM) following the co-incubation of J774 murine phagocytes with live *C. parapsilosis* GA1 or CLIB 214 cells. Individual events are associated with the beginning of fungal cell recognition by macrophages. Data were analyzed using Two-way ANOVA with Bonferroni's post-test. No differences were found after the evaluation.

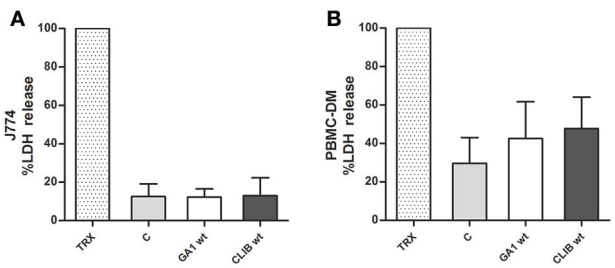


**FIGURE 4 | Macrophage damage by GA1 and CLIB 214.** Host cell damage (mean + SD) was measured by the amount of lactate dehydrogenase (LDH) released by murine J774 **(A)** and human PBMC-derived **(B)** macrophages following incubation with live *C. parapsilosis* GA1 or CLIB 214 cells. In order to reach maximum LDH release Triton X-100 (TRX) was used. The supernatant of fungal-free cultured phagocytes was used as control "C".

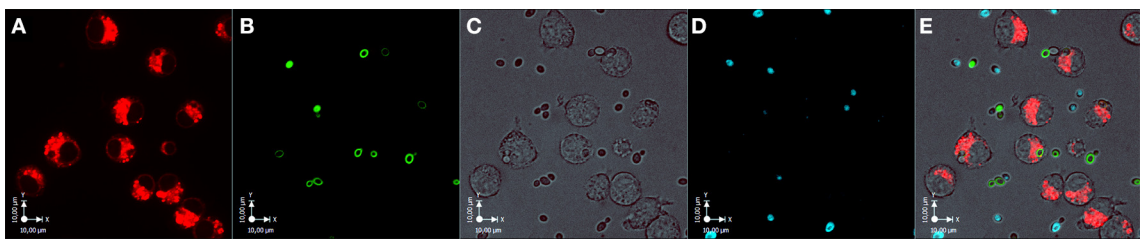


**FIGURE 5 | Infection of murine J774 macrophages with *C. parapsilosis* GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells, simultaneously.** The image series show four channels of the co-infection of J774 macrophages with GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells. Channel **(A)**, Lysotracker red stained acidic compartments of phagocytic cells; channel **(B)**, fluorescein isothiocyanate (FITC) stained *Cp*ΔΔ*lip*1 − ΔΔ*lip2* yeasts; channel **(C)**, Bright-field image; channel **(D)**, Calcofluor white (CFW) stained GA1 cells; channel **(E)**, merged image. Images were taken immediately after infection ($T = 0$ min). Scale bar: 10 μm.

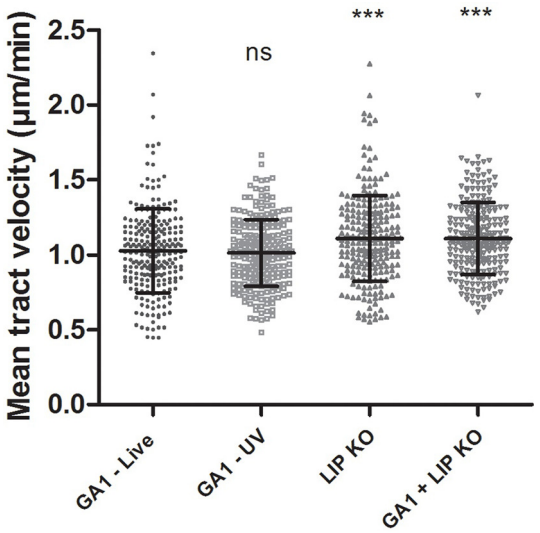


**FIGURE 6 | Macrophage migration toward GA1, *Cp*ΔΔ*lip*1 − ΔΔ*lip2* or GA1, and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells.** J774 murine macrophages were followed for 6 h after co-culturing with GA1 or *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells individually, and after GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* simultaneous infections. Phagocyte mean track velocity values (mean ± SD) are shown in μm/min. One-way ANOVA analysis with Bonferroni's multiple comparison test was used to determine statistical relevance. Significance was determined relative to the wild type. Different gray symbols represent the mean tract velocity of individual macrophages. ***$p < 0.001$.

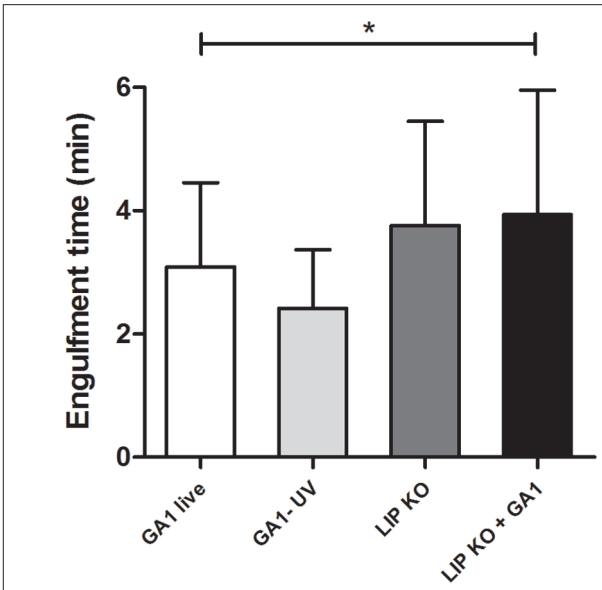


**FIGURE 7 | Time required for the engulfment of GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2*.** Diagrams show the average engulfment time (mean + SD in minutes) of live and UV-killed GA1 and LIP KO cells by J774 murine macrophages. Data were analyzed using One-way ANOVA with Bonferroni's post-test. Statistical significances were considered at *p*-values *$p < 0.05$.

Secreted hydrolytic enzymes play a critical role in host invasion by fungi (Ghannoum, 2000; Borst and Fluit, 2003; Schaller et al., 2005). Secreted lipases of *Candida* species are associated with host cell adhesion, tissue damage and inflammatory response induction (Hube et al., 2000; Trofa et al., 2009). While *C. albicans* is known to have 10 lipase encoding ORFs, only two lipase genes (*LIP1–LIP2*) have been identified in *C. parapsilosis* (Hube et al., 2000; Stehr et al., 2004; Gácser et al., 2007). Both *C. parapsilosis* GA1 and CLIB 214 are known to produce extracellular lipases. Although, the previously established *C. parapsilosis* lipase deficient strain is only available on a GA1 background, thus when studying the function of lipases during an infection, we compared these strains only. The virulence properties of the *C. parapsilosis* lipase deficient strain (*Cp*ΔΔ*lip*1 − ΔΔ*lip2*) have already been investigated. According to our previous findings, the deletion mutant was killed more efficiently, however, phagocytosed similarly by macrophages when compared to the wild type (Tóth et al., 2014a). As shown previously, examining the component stages of the phagocytic process can provide insights into host–pathogen interactions on the cellular level and therefore, reveal differences in the recognition, overall uptake, and engulfment processes (Tóth et al., 2014b; Bain et al., 2015). In this study we examined the phagocyte response toward the *Cp*ΔΔ*lip*1 − ΔΔ*lip2* lipase mutant and the parental strain GA1. Numerous preceding studies have used co-infection methods to gain insight into virulence attributes (Silva et al., 2011; Bou Ghanem et al., 2012; Xu et al., 2014). Thus, in order to investigate whether the absence of extracellular lipase in the *Cp*ΔΔ*lip*1 − ΔΔ*lip2* yeast cells could be overcome with the secretion of lipases by wild type yeasts during an infection, murine macrophages were challenged with wild type and lipase mutant yeasts individually, and simultaneously. In general, the overall macrophage uptake of mutant cells was more effective, as a higher percentage of phagocytes ingested a greater number of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells compared to the wild type yeasts. In correlation with the uptake rates, murine macrophage migration was increased toward mutant cells, indicating enhanced recognition of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* compared to wild type cells. These results suggest that, the lack of secreted lipase results in decreased virulence on the cellular level. Although, our current results are in contrast with a previous study on *Cp*ΔΔ*lip*1 − ΔΔ*lip2* and GA1 phagocytosis comparison (Tóth et al., 2014a), this is not the first report revealing differences in *Cp*ΔΔ*lip*1 − ΔΔ*lip2* phagocytosis. Nagy et al. have used dendritic cells to study the virulence properties of *C. parapsilosis* secreted lipases and also found increased phagocytic efficiency (Nagy et al., 2011). Interestingly, the engulfment time of the mutant cells was prolonged. This data might suggest that, deficiency in lipase secretion might have led to modified cell wall structure or alterations in the expressed PAMPs or alterations in the level of other potential secreted signaling molecules. Similarly to our previous report on host cell damage by *Cp*ΔΔ*lip*1 − ΔΔ*lip2*, we found no difference in murine macrophage damaging capacity compared to the wild type.

In conditions when both fungal strains were present, a significant reduction was observed in overall phagocytosis of yeast cells compared to the lipase mutant alone. Thus, lipase deficiency can be partially restored with the presence of the wild type cells that further confirms the role of fungal extracellular lipases during host–pathogen interactions. No difference was

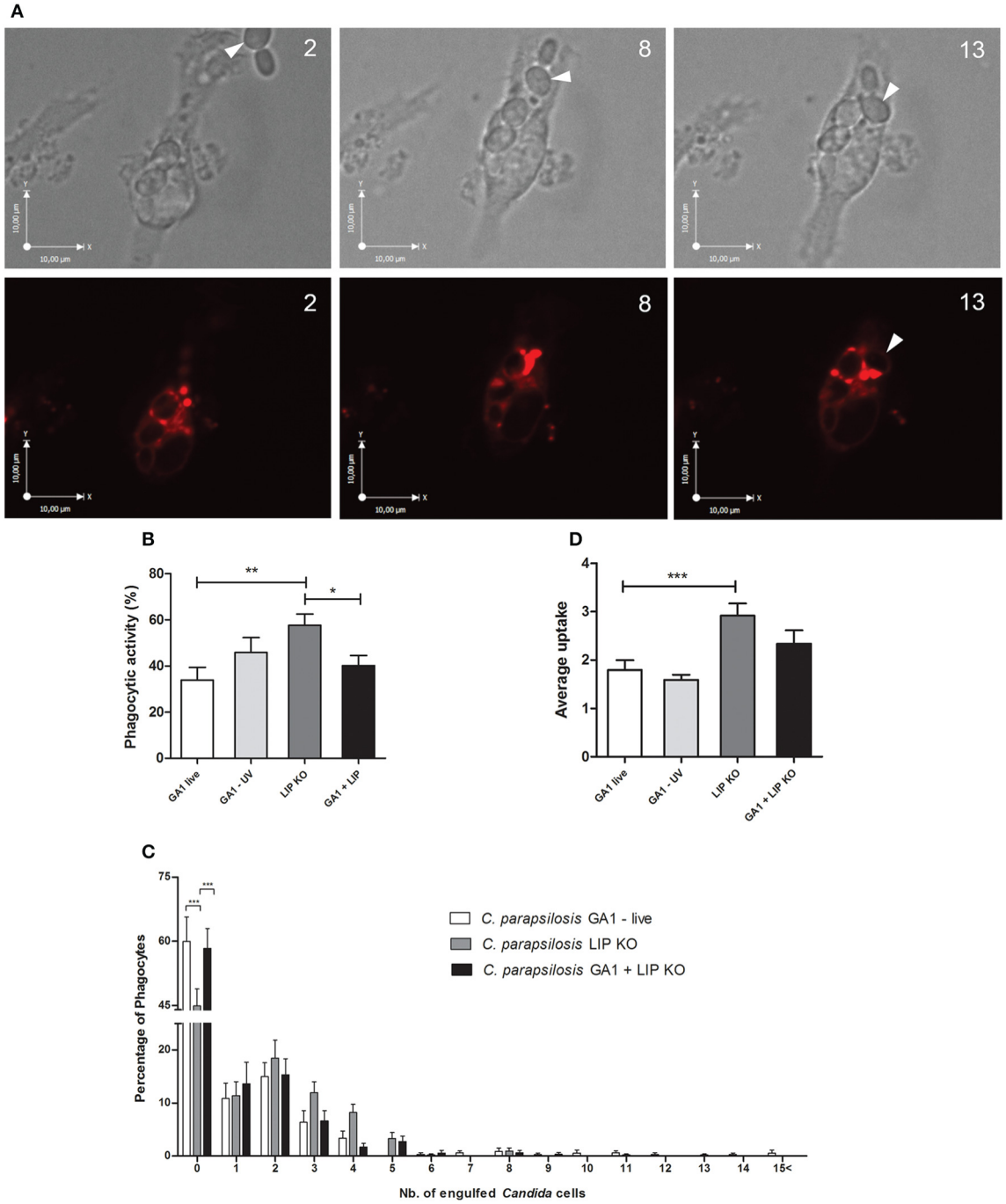


**FIGURE 8 | Overall uptake of GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2* by J774 macrophages.** On Panel **(A)** bright field and fluorescent microscopic images show the internalization of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells (arrows) by a J774 murine macrophage. Phagocyte was stained with Lysotracker Red. Numbers indicate the duration of time (min) from recognition until the complete ingestion of cells. Scale bar: 10 μm. Panels **(B–D)** represent the overall uptake of fungal cells showing phagocytic activity (mean + SEM; **B**), defined number of fungal cells taken up by phagocytes (mean + SEM; **C**) and average uptake (mean + SEM; **D**). Data for phagocytic activity and average uptake were analyzed using One-way ANOVA with Bonferroni's post-test. Two-way ANOVA analysis with Bonferroni's multiple comparison test (column to column comparison) was applied to determine significant differences between the distribution of fungal cells per macrophages. Significant statistical differences: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

detected in yeast cell preference when macrophages were incubated with both types of fungal cells further supporting our hypothesis on secreted component deficiency complementation. The presence of wild type secreted lipases might had a direct influence on fungal cell uptake regardless of the yeast cell types present in the media. However, co-infection with GA1

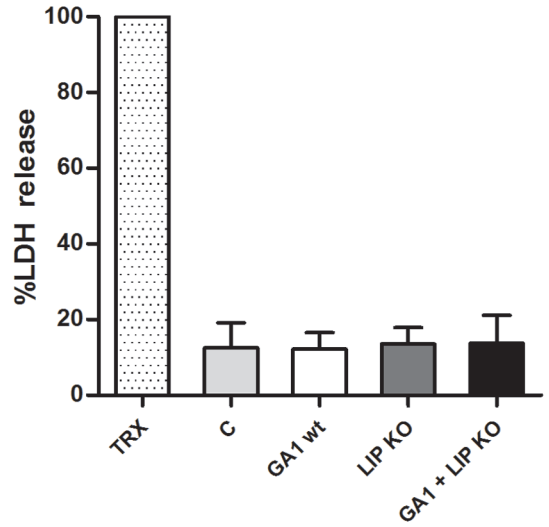


**FIGURE 9 | Host cell damage by GA1 and *Cp*ΔΔ*lip*1 − ΔΔ*lip2*.** LDH release (percentage of mean + SD) was measured to determine macrophage damage 6 h after co-incubation with *Cp*ΔΔ*lip*1 − ΔΔ*lip2*, GA1 or mixed *Cp*ΔΔ*lip*1 − ΔΔ*lip2* and GA1 yeast cells. TRX: Triton X-100 treated macrophages; C: fungal-free cultured phagocytes.

and lipase mutant yeast cells increased the engulfment time similarly to *Cp*ΔΔ*lip*1 − ΔΔ*lip2*. This result can be explained by the above mentioned hypothesis on an additional deficiency of *Cp*ΔΔ*lip*1 − ΔΔ*lip2* that cannot be rescued with the presence of secreted lipases. Interestingly, co-infection of macrophages with both strains led to significantly increased macrophage migration. As shown above, the presence of the *Cp*ΔΔ*lip*1 − ΔΔ*lip2* cells increased the average track velocity markedly. A possible explanation for this phenomenon is that in case of co-infection, the presence of the mutant cells might have unmasked a potential immune evasion ability in wild type cells. The presence of both strains simultaneously had no further effect on host cell damage compared to the wild type.

This study confirmed the role of secreted lipase in virulence, as lack of the secreted component resulted in decreased virulence by altering the dynamics of phagocytosis. Our results further showed that in terms of uptake, lipase deficiency is partially restored when the wild type strain is present, which further exemplifies the role of secreted lipases on phagocytosis.

In this report we have shown that two distinct isolates of a single species are able to trigger significantly different host responses and that fungal lipase secretion plays an important role in host–pathogen interactions on the cellular level.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01102

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