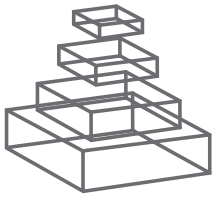
frontiers

RESEARCH TOPICS

SURFACE ARCHITECTURE OF FUNGAL PATHOGENS

Hosted by
Marcio Rodrigues

frontiers in MICROBIOLOGY



frontiers

Cover image provided by Ibbl sarl, Lausanne CH

ISSN 1664-8714
ISBN 978-2-88919-032-4
DOI 10.3389/978-2-88919-032-4

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

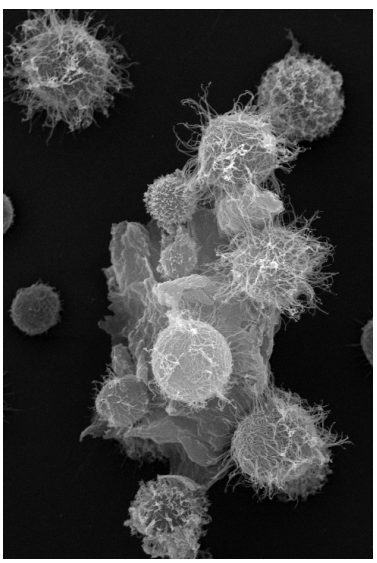
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

SURFACE ARCHITECTURE OF FUNGAL PATHOGENS

Hosted By
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil



Surface components in fungal cells include cell wall molecules and, in certain cases, capsular structures. In pathogens, these components are responsible for key events during interaction with the host. These events include recognition of pathogens by the immune system and generation of damage to host cells and tissues. The molecular nature of surface structures in fungi is vast and may include (glyco)proteins, polysaccharides, lipids and pigments. Many of them have been strictly associated with the antifungal immune response, as well as with steps of fungal adhesion and dissemination during interaction with host cells. For many fungal pathogens, surface composition and architecture are determinant for either disease progression or control. The diversity of the composition of the cell surface and its molecular architecture are believed to include targets for the action of new antifungals, as well as immunogens with potential to interfere with fungal diseases in favor of the host.

Image: Courtesy of Caroline L. Ramos

Table of Contents

frontiers in
MICROBIOLOGY

EDITORIAL
published: 02 March 2012
doi: 10.3389/fmicb.2012.00080

Surface architecture of fungal pathogens

Marcio L. Rodrigues*

Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil

**Correspondence: marcio@micro.ufrj.br*

Surface components in fungal cells include cell wall molecules and, in certain cases, capsular structures (**Figure 1**) and extracellular matrix components. In pathogens, surface molecules are responsible for key events during interaction with the host (Nimrichter et al., 2005). These events include recognition of pathogens by the immune system and generation of damage to host cells and tissues (Roeder et al., 2004). The molecular diversity of surface structures in fungi is vast and may include (glyco) proteins, polysaccharides, lipids, and pigments (Nimrichter et al., 2005). Many of them have been associated with the antifungal immune response, as well as with steps of fungal adhesion and dissemination during interaction with host cells. For many fungal pathogens, surface composition and architecture are determinant for either disease progression or control (Roeder et al., 2004; Nimrichter et al., 2005; Latgé, 2010; Figueiredo et al., 2011). The diversity of the composition of the cell surface and its molecular architecture are believed to reveal targets for the action of new antifungals, as well as immunogens with potential to interfere with fungal diseases in favor of the host (Nimrichter et al., 2005).

In the Research Topic "Surface Architecture of Fungal Cells," many of the important aspects related to structure and function of surface components of fungi were covered. It was, of course, impossible to discuss the same research topic for each important fungal pathogen. In addition, surface architecture of some of the most important fungal pathogens has been extensively reviewed before (Cassone, 1989; Poulain and Jouault, 2004; Ruiz-Herrera et al., 2006; Chaffin, 2008; Nather and Munro, 2008; Latgé, 2010). We therefore selected a group of pathogens as prototypes for the topic, and this list included *Cryptococcus neoformans, Histoplasma capsulatum, Paracoccidioides brasiliensis, Sporothrix schenckii, Pseudallescheria boydii, Aspergillus nidulans, Aspergillus fumigatus*, and *Colletotrichum gloeosporioides*. Components of the cell surface that were discussed in this topic included cell wall and capsular polysaccharides (Frases et al., 2011; Rodrigues et al., 2011; Zaragoza, 2011), peptidopolysaccharides (Lopes-Bezerra, 2011), proteins (Figueiredo et al., 2011; Guimaraes et al., 2011; Puccia et al., 2011), pigments (Zaragoza, 2011), and glycolipids (Barreto-Bergter et al., 2011; Nimrichter and Rodrigues, 2011). In this context, articles in this topic were focused on (i) how fungal molecules are assembled at the cell surface (Guimaraes et al., 2011; Lopes-Bezerra, 2011; Nimrichter and Rodrigues, 2011; Puccia et al., 2011; Zaragoza, 2011), (ii) how they impact the immune response (Figueiredo et al., 2011; Guimaraes et al., 2011; Lopes-Bezerra, 2011; Nimrichter and Rodrigues, 2011; Puccia et al., 2011; Rodrigues et al., 2011), (iii) the role of surface components in fungal physiology (Guimaraes et al., 2011; Lopes-Bezerra, 2011; Nimrichter and Rodrigues, 2011; Puccia et al., 2011; Zaragoza, 2011), (iv) their potential to work as target for preventive or therapeutic agents (Figueiredo et al., 2011; Guimaraes et al., 2011; Lopes-Bezerra, 2011; Nimrichter and Rodrigues, 2011; Puccia et al., 2011; Rodrigues et al., 2011), and (v) how to study structure and function of these molecules (Barreto-Bergter et al., 2011; Frases et al., 2011). The extensive work of each contributor resulted in a clear notion that surface molecules of fungal cells are essential to fungal pathogenesis, physiology, and immune recognition. Essentially, these articles strongly indicate that knowledge on structure and functions of surface molecules in fungi can be translated soon into the discovery of new diagnostic, therapeutic, and preventive alternatives.

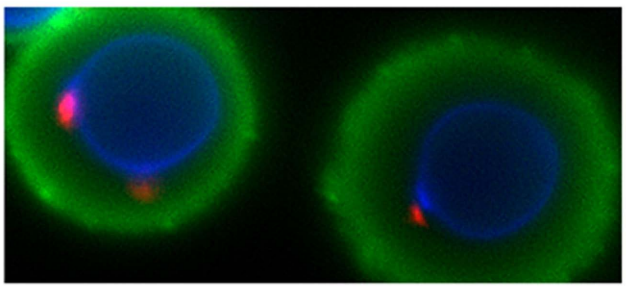


FIGURE 1 | Surface architecture of a fungal pathogen. This image illustrates how some of the surface components of the human pathogen *Cryptococcus neoformans* are distributed at the cell surface. Cell wall-associated chitin is stained in blue, chitooligomers are stained in red, and capsular polysaccharides are stained in green. Courtesy of Dr. Fernanda L. Fonseca.

ACKNOWLEDGMENTS

Marcio L. Rodrigues is supported by grants from the Brazilian agencies CAPES, FAPERJ, and CNPq. I am grateful to Dr. Maurizio Del Poeta for encouraging preparation of this Research Topic and to each of the authors that kindly contributed with their views about the architecture of the cell surface of fungal pathogens.

REFERENCES

Barreto-Bergter, E., Sassaki, G. L., and De Souza, L. M. (2011). Structural analysis of fungal cerebrosides. *Front. Microbiol.* 2:239. doi: 10.3389/fmicb.2011.00239

Cassone, A. (1989). Cell wall of *Candida albicans*: its functions and its impact on the host. *Curr. Top. Med. Mycol.* 3, 248–314.

Chaffin, W. L. (2008). *Candida albicans* cell wall proteins. *Microbiol. Mol. Biol. Rev.* 72, 495–544.

Figueiredo, R. T., Carneiro, L. A., and Bozza, M. T. (2011). Fungal surface and innate immune recognition of filamentous fungi. *Front. Microbiol.* 2:248. doi: 10.3389/fmicb.2011.00248

Frases, S., Viana, N. B., and Casadevall, A. (2011). Biophysical methods for the study of microbial surfaces. *Front. Microbiol.* 2:207. doi: 10.3389/fmicb.2011.00207

Guimaraes, A. J., De Cerqueira, M. D., and Nosanchuk, J. D. (2011). Surface architecture of *Histoplasma capsulatum*. *Front. Microbiol.* 2:225. doi: 10.3389/fmicb.2011.00225

Latgé, J. P. (2010). Tasting the fungal cell wall. *Cell. Microbiol.* 12,863–872.

Lopes-Bezerra, L. M. (2011). *Sporothrix schenckii* cell wall peptidorhamnomannans. *Front. Microbiol.* 2:243. doi: 10.3389/fmicb.2011.00243

Nather, K., and Munro, C. A. (2008). Generating cell surface diversity in *Candida albicans* and other fungal pathogens. *FEMS Microbiol. Lett.* 285, 137–145.

Nimrichter, L., and Rodrigues, M. L. (2011). Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials. *Front. Microbiol.* 2:212. doi: 10.3389/fmicb.2011.00212

Nimrichter, L., Rodrigues, M. L., Rodrigues, E. G., and Travassos, L. R. (2005). The multitude of targets for the immune system and drug therapy in the fungal cell wall. *Microbes Infect.* 7, 789–798.

Poulain, D., and Jouault, T. (2004). *Candida albicans* cell wall glycans, host receptors and responses: elements for a decisive crosstalk. *Curr. Opin. Microbiol.* 7, 342–349.

Puccia, R., Vallejo, M. C., Matsuo, A. L., and Longo, L. V. (2011). The *Paracoccidioides* cell wall: past and present layers toward understanding interaction with the host. *Front. Microbiol.* 2:257. doi: 10.3389/fmicb.2011.00257

Rodrigues, M. L., Nimrichter, L., Cordero, R. J., and Casadevall, A. (2011). Fungal polysaccharides: biological activity beyond the usual structural properties. *Front. Microbiol.* 2:171. doi: 10.3389/fmicb.2011.00171

Roeder, A., Kirschning, C. J., Rupec, R. A., Schaller, M., Weindl, G., and Korting, H. C. (2004). Toll-like receptors as key mediators in innate antifungal immunity. *Med. Mycol.* 42, 485–498.

Ruiz-Herrera, J., Elorza, M. V., Valentin, E., and Sentandreu, R. (2006). Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res.* 6, 14–29.

Zaragoza, O. (2011). Multiple disguises for the same party: the concepts of morphogenesis and phenotypic variations in *Cryptococcus neoformans*. *Front. Microbiol.* 2:181. doi: 10.3389/fmicb.2011.00181

Received: 29 December 2011; accepted: 16 February 2012; published online: 02 March 2012.

*Citation: Rodrigues ML (2012) Surface architecture of fungal pathogens. Front. Microbio. **3**:80. doi: 10.3389/fmicb.2012.00080*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 18 November 2011
doi: 10.3389/fmicb.2011.00225

Surface architecture of *Histoplasma capsulatum*

Allan J. Guimarães[1,2]*, Mariana D. de Cerqueira[3] and Joshua D. Nosanchuk[1,4]*

[1] Department of Microbiology and Imunology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA
[2] Departamento de Imunologia, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[3] Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[4] Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA

Edited by:
Michael H. Perlin, University of Louisville, USA

Reviewed by:
Bjorn Sandrock, University of Marburg, Germany
Jose M. Diaz-Minguez, University of Salamanca CIALE, Spain
Erika Kothe, Friedrich Schiller University, Germany

***Correspondence:**
Allan J. Guimarães, Departamento de Imunologia, Centro de Ciências da Saúde, Bloco I, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho 373, Rio de Janeiro 21941-902, Brazil.
e-mail: allan.guimaraes@phd.einstein.yu.edu;
Joshua D. Nosanchuk, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA.
e-mail: josh.nosanchuk@einstein.yu.edu

The dimorphic fungal pathogen *Histoplasma capsulatum* is the most frequent cause of clinically significant fungal pneumonia in humans. *H. capsulatum* virulence is achieved, in part, through diverse and dynamic alterations to the fungal cell surface. Surface components associated with *H. capsulatum* pathogenicity include carbohydrates, lipids, proteins, and melanins. Here, we describe the various structures comprising the cell surface of *H. capsulatum* that have been associated with virulence and discuss their involvement in the pathobiology of disease.

Keywords: *Histoplasma capsulatum*, surface, cell wall, architecture, proteins, carbohydrates

INTRODUCTION

Histoplasma capsulatum variety *capsulatum* (Hc) is the causative agent of classical histoplasmosis (Meloan, 1952; Kwon-Chung, 1972, 1975; Bradsher, 1996) and the fungus has a worldwide distribution. The fungus is heterothallic and compatible (+) and (−) mating types unite to form the ascomycetous perfect stage designated *Ajellomyces capsulatus*.

Hc is a dimorphic fungal pathogen with two distinct morphological forms, filamentous and yeast, depending on a variety of nutritional factors and temperature (Maresca and Kobayashi, 1989). Hc is found in nature primarily as a saprophytic mold, and exists in soils enriched with organic nitrogen sources, like animal excrements (Emmons, 1950, 1956a,b; Zeidberg et al., 1952; Alteras, 1966; Emmons et al., 1966; Disalvo et al., 1970; Smith, 1971a,b). Additionally, the fungus can grow as a mold when cultured on laboratory mediums at less than 35°C. The mold form is composed by hyaline septate hyphae that are 1–2.5 μm diameter. Moreover, hyphae produce two different hyaline asexual reproduction structures. Macroconidia are large, thick-walled, round, and 7–15 μm in diameter, typically tuberculate, knobby, or with short cylindrical projections, although they occasionally may be smooth. Microconidia are smooth-walled spherical, pyriform, or cigar shaped, ranging in size from 2 to 6 μm in diameter (Edwards et al., 1960; Pine, 1960; Berliner, 1973; Garrison and Boyd, 1977). In contrast, the pathogenic single, budding yeast-like form is predominately isolated from infected tissue specimens and occurs when the microbe is grown at ≥37°C on specific media (Smith, 1971a,b; Hay, 1992). Hc var. *capsulatum* are generally small yeast cells (2–4 μm in length), thick-walled and ovoid with a narrow base at the smaller end, whereas variants found predominantly in Africa (Hc variety *duboisii*) produced larger yeast cells (8–15 μm in length).

Infection typically occurs after disturbances in the environment which results in aerosolization of fungal propagules with subsequent inhalation of microconidia or hyphal fragments by a susceptible host (Guimaraes et al., 2006). Histoplasmosis is not generally associated with person to person spread of disease. However, vertical transmission was once observed in a human neonate (Kwon-Chung and Bennett, 1992). Rarely, histoplasmosis can be acquired by cutaneous inoculation of the fungus.

Histoplasmosis is a cosmopolitan fungal infection with areas of high endemicity. However, patients are usually unaware of their potential exposure (Wheat, 2003). Generally, the environmental conditions present in areas of high endemicity are a moderate climate with a relatively constant humidity (Maresca and Kobayashi, 1989). Endemic regions in North America are located in the Midwestern and Southeastern parts of the United States (USA), especially the Mississippi, Ohio, and Missouri river valleys, where 80% of the resident population has been shown to react to histoplasmin by skin testing (Ajello, 1971; Goodwin and Des Prez, 1978; Wheat, 1997). It is estimated that Hc is responsible for approximately half a million new human infections in the USA each year, making it the most prevalent pulmonary fungal pathogen (Cano and Hajjeh,

2001). In Latin America, the most prevalent areas of endemicity are present within Brazil, Venezuela, Ecuador, Paraguay, Uruguay, and Argentina (Borelli, 1970; Wheat, 1997, 2001). In Brazil, endemic areas are located in the Midwestern and Southeastern portions of the country (Zancope-Oliveira et al., 2005; Guimaraes et al., 2006), where the prevalence ranges from 4.4 to 63.1% and 3.0 to 93.2%, respectively (Londero and Ramos, 1978; Zancope-Oliveira et al., 2005; Guimaraes et al., 2006).

Exposure to Hc is exceedingly common for persons living within areas of high endemicity (Wheat and Kauffman, 2003). The clinical manifestations of disease range from asymptomatic infection or a mild influenza-like illness to a disseminated sepsis form that may involve virtually any tissue (Meloan, 1952; Csillag and Wermer, 1956; Goodwin and Des Prez, 1978; Wheat, 1994; Bradsher, 1996). These manifestations depend mainly on the magnitude of exposure (i.e., the number of fungal particles inhaled), the immunological status of the host, and the virulence of the infective strain, indicating that environmental and genetic factors regulate the manifestation of disease (Goodwin et al., 1981; Kauffman, 2007). The vast majority of infected persons have either no symptoms or a very mild illness that is never recognized as being histoplasmosis (Wheat et al., 2007). In fact, 95–99% of the primary infections are not recognized in immunologically normal hosts in endemic areas (Saliba and Beatty, 1960; Isbister et al., 1976; Goodwin et al., 1981). Although the majority of symptomatic infections follow primary exposures to Hc, reactivation of latent infection can result in significant disease, particularly in the setting of immunosuppression, such as individuals chronically receiving steroids or patients on chemotherapy (Kauffman, 2007). Furthermore, reactivation disease can be developed in liver transplant recipients with disease originating from latent infections in the transplanted organs (Limaye et al., 2000). Additionally, reactivation has increasingly occurred in patients receiving anti-cytokine therapies, especially inhibitors of gamma interferon (INF-γ) and tumor necrosis factor alpha (TNF-α; Deepe, 2005; Deepe et al., 2005; Scheckelhoff and Deepe, 2005). Individuals with advanced HIV disease are also at significant risk for severe infection due to primary disease or reactivation of latent lesions, and disseminated disease occurs in 95% of individuals with AIDS (Wheat, 1996). Additionally, in the setting of severe immunocompromised, Hc strains previously considered avirulent have been able to cause fatal disease (Davies et al., 1978; Wheat et al., 1990).

Hc is a model dimorphic pathogen for the study of invasive mycotic diseases. After inhalation, the fungal propagules reach the terminal bronchioles of the lung and deposits in alveoli, undergoing conversion to the pathogenic yeast form (Couto et al., 1994; Allendoerfer et al., 1997). As a facultative intracellular parasite, the interaction of Hc with macrophage cells is a critical component of the host response to infection (Newman, 2005) and is a complex and obscure phenomenon. Hc yeasts also have critical interactions with inflammatory neutrophils, and with dendritic cells (DCs) in the lung and other organs. Indeed, recent evidence suggests that DCs can restrict the differentiation of conidia into yeast (Newman, 2005; Newman et al., 2011) and may be the key antigen-presenting cells that initiate cell-mediated immunity (Deepe et al., 2008). Hc yeast cells must survive and/or subvert the hostile anti-microbial environmental within phagocytes (Allendoerfer et al., 1997), including fungicidal mechanisms dependent on hydrogen peroxide and products of the nitric oxide synthase (NOS) pathway (Eissenberg and Goldman, 1987). Yeast cells actively inhibit phagolysosomal fusion, thereby preventing exposure to the acidic hydrolytic enzymes of the lysosomes. Hc also prohibits accumulation of vacuolar ATPase, which is important for proton accumulation in phagosomes, and the fungus can actively alkalinize phagosomal pH to 6.5 (Strasser et al., 1999). Within the phagocytes, yeast may travel to hilar and mediastinal lymph nodes where they can gain access to the blood circulation for dissemination to various organs, such as liver and spleen (Wheat and Kauffman, 2003).

YEAST CELL SURFACE

The components of the yeast surface are the main interface of Hc to communicate with its environment and to interact with cells of the immune system. In particular, Hc yeast display several surface molecules involved in entry and survival within the host (**Figures 1** and **2**). The cell wall is essential to diverse aspects of Hc biology and pathogenicity (**Table 1**). As discussed, uptake of Hc yeast by macrophages provides a protected environment for yeast growth and replication. However, in promoting its phagocytosis, Hc must also subvert or avoid activating macrophage antifungal defenses.

CELL WALL CARBOHYDRATES

The cell wall is composed of about 80% of saccharides in dry weight (Bernard and Latg, 2001). The monosaccharide composition consists in glucose (Glc), which is the most abundant monosaccharide in filamentous and yeast cell wall, followed by mannose (Man), and galactose (Gal). Structurally, the sugars form a rigid polysaccharide structure that varies in composition ratios depending on the chemotype of the strain (Reiss, 1977; Reiss et al., 1977). Although different glycans comprise the structure; some fluctuations can occur in certain isolates from a single chemotype, such as shown with a major fibrillar chitin skeleton component, α-1,3-glucan, β-1,3-glucan, and soluble galactomannan (Kanetsuna et al., 1974).

The yeast and mycelia phases of Hc contain different chitin fibril arrangements within their cell walls (Kanetsuna, 1981). These structures are the foundation of the dynamic immunoreactive construction that comprises the Hc cell wall.

The α and β-glucans present in the cell wall also change during morphogenesis and have different biological roles (Domer et al., 1967; Domer, 1971). At room temperature, the production of β-glucan is favored, resulting in increased amounts of this glucans which is predominant in the fungal mycelial phase. Also, induction of 3-glucanase activity promotes a decrease of cell wall rigidity, resulting in elongation or budding (2, 3). The β-1,3-glucan consists of a linear β-1,3-glucosyl-linked backbone with β-1,6-glucosyl-linked side chains that vary in length and distribution, while forming a complex tertiary structure stabilized by interchain hydrogen bonding (Kanetsuna et al., 1974). This structure is antigenic and participates in the modulation of the host immune response (Gorocica et al., 2009).

A shift to 37°C favors α-1,3-glucan synthesis and low β-1,3-glucan synthetic activity during yeast growth. During phase transition from mycelia to yeast, the synthesis of α-glucans increases rapidly (Kanetsuna et al., 1972; Kanetsuna, 1981) and this effect is strictly dependent on temperature, with a lower synthesis at 20 than 37°C. Hc α-glucan contains α-1,3-glucosyl linear

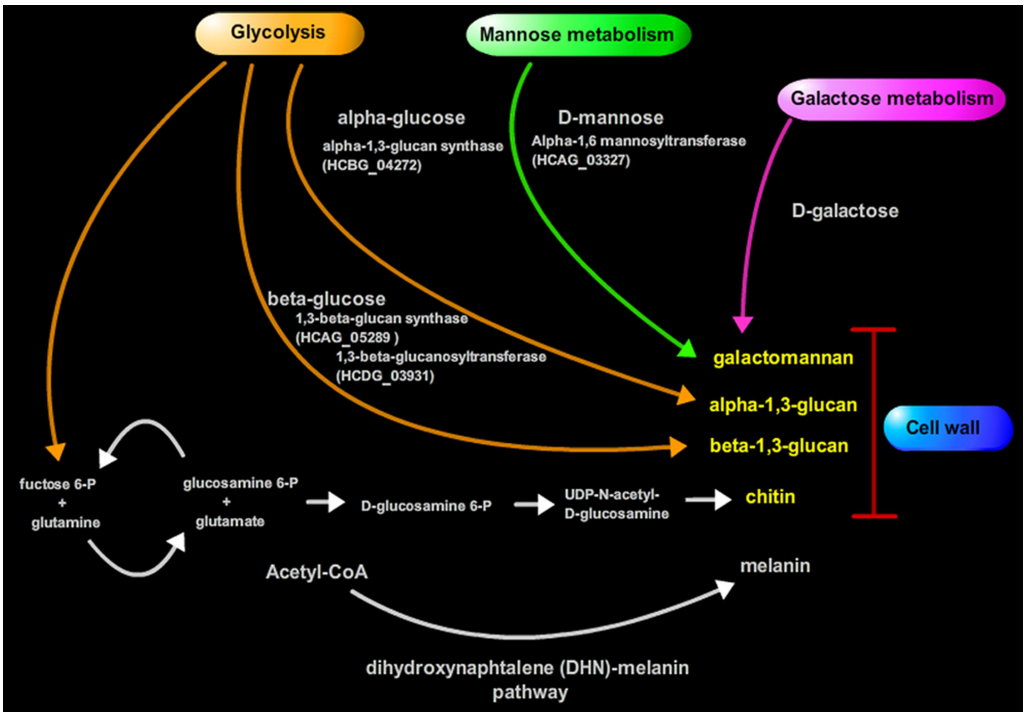


FIGURE 1 | Biosynthesis of important cell wall components of Hc.

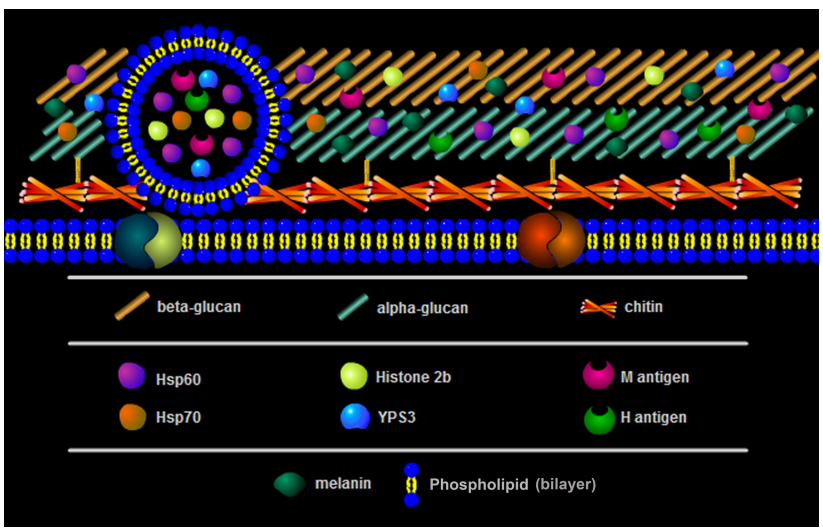


FIGURE 2 | Schematic model showing the composition of the Hc cell wall.

residues and topographically overlaps the β-glucan surrounding this last polymer in the yeast cell wall and it is considered relevant for Hc yeast virulence (Klimpel and Goldman, 1988; Rappleye et al., 2004).

Based on the α-1,3-glucan content of the yeast cell wall, Hc is classified as chemotype I or II (Domer et al., 1967; Domer, 1971). A strain is classified as chemotype I when the α-1,3-glucan is absent and it the fibers are entirely β-linked (Davis et al., 1977). A chemotype II cell wall contains a mixture of α and β-1, 3-glucans.

Chitin

The major cell wall component is an inner layer of chitin, a polysaccharide composed by polymers of glucans and β-1,4-*N*-acetyl-glucosamine (GlcNAc) residues (Kanetsuna et al., 1974). The chitin fibrils are considered to be the skeleton of the cell

Table 1 | Components of Hc cell surface and their role in pathogenicity/virulence.

Surface component	Role in virulence/pathogenicity
SURFACE CARBOHYDRATES	
Chitin	Maintenance of cell wall integrity and rigidity, as well as in resisting the extracellular environment (Ruiz-Herrera and Osorio, 1974); immunomodulatory; and immunosuppressing molecule recognized by dectin-1 (Mora-Montes et al., 2011) inducing activation of anti-microbial activities of macrophages and PBMCs, (Rementeria et al., 1997; Mora-Montes et al., 2011); bind diverse products in supernatant, such as polysaccharides such as *Cryptococcus neoformans* capsule or self-proteins like YPS3 (Bohse and Woods, 2005).
β-1,3-glucan	Major constituent of the cell wall of the filamentous phase of Hc (Davis et al., 1977); promotes inflammatory cell recruitment and production of pro-inflammatory cytokines including TNF-α (Figueiredo et al., 1993; Medeiros et al., 1999, 2004; Anjos et al., 2002). Dectin-1 and DC-SIGN (CD209) are receptors for β-1,3-glucan polymers (Brown, 2006).
α-1,3-glucan	Presence correlated with virulence (Kugler et al., 2000; Rappleye et al., 2007); regulates yeast proliferation inside host phagocytes (Kugler et al., 2000) by protecting the yeast within phagolysosomes (Eissenberg and Goldman, 1991), resulting in a state called intracellular latency (Eissenberg et al., 1996, 1997); Hc displaying α-1,3-glucans can persist for several weeks inside these cells and induce the formation of granulomas, which result in chronic infected tissues (Klimpel and Goldman, 1988); loss of α-1,3-glucan impaired Hc yeast proliferation within macrophages killing of cultured macrophages (Rappleye et al., 2004); *in vivo*, loss of α-1,3-glucan resulted in reduction in lung colonization (Marion et al., 2006); subverts the host immune mechanisms of recognition of cell wall components and contributes to yeast survival. By blocking the innate recognition of the fungal PAMP β-1,3-glucan by its PRR dectin-1 receptor on host phagocytes (Rappleye et al., 2007).
Gallactomannan	Involved in DTH with inhibition of macrophage migration factor release (Azuma et al., 1974; Reiss et al., 1974). In *Paracoccidioides brasiliensis,* it appears to be involved in the protection against its own serine-thiol protease, an enzyme associated with pathogen dissemination through the extracellular matrix (Matsuo et al., 2006).
Lectin-like components	Binds to a 68 KDa galactosylated surface molecule (mainly β-anomer) on murine macrophages (Taylor et al., 1998; Duarte-Escalante et al., 2003), and participates in macrophage activation, and regulation of phagocytosis (Maldonado et al., 1998). Also involved in agglutination of human erythrocytes (Taylor et al., 2004).
Mannoproteins	Highly antigenic and can lead to dendritic cell maturation and activation with pro-inflammatory cytokine production, (Pietrella et al., 2006). Also involved in host tissue adherence (Ross, 2002).
LIPIDS	
Ceramide monohexoside	Expressed in almost all fungal species (Barreto-Bergter et al., 2004); found in the mycelia and yeast phases of Hc (Barr and Lester, 1984; Barr et al., 1984) and appears to be required for fungal survival (Dickson and Lester, 1999).
Extracellular vesicles	Carry lipids, proteins, polysaccharides, and pigment-like structures involved in diverse processes including metabolism, cell recycling, signaling, and virulence (Yoneda and Doering, 2006; Rodrigues et al., 2007, 2008a; Albuquerque et al., 2008; Vallejo et al., 2011); some proteins found in Hc vesicles are pathogenic determinants and/or are involved in host–pathogen interactions. Hc extracellular vesicle may function as a "virulence bag," since virulence factors are concentrated within them and these molecules can modulate the host–pathogen interaction and immune response (Albuquerque et al., 2008; Rodrigues et al., 2008b; Casadevall et al., 2009; Oliveira et al., 2010).
PROTEINS	
Hsp60	Major ligand that mediates attachment of Hc to macrophage/monocyte CR3 integrin (CD11b/CD18; Long et al., 2003), resulting in phagocytosis (Long et al., 2003; Habich et al., 2006); it is also an immunogenic molecule, being a potential target for passive immunization therapy (Guimaraes et al., 2009, 2011a); contributes with cell wall changes that allow the pathogen to survive under stress conditions (Shaner et al., 2008); interacts with a large diversity proteins in both cytoplasmic and cell wall fractions, participating as a key regulator of several cellular processes, including amino acid, protein, lipid, and carbohydrate metabolism, cell signaling, replication, and expression of virulence associated proteins (Guimaraes et al., 2011c).
Hsp70	Recombinant Hsp70 elicits a cutaneous delayed-type hypersensitive response in mice; however, vaccination with Hsp70 did not confer protection against Hc infection (Allendoerfer et al., 1996); Hsp70 is highly expressed by the fungus when undergoing transition from mycelium-to-yeast (Kamei et al., 1992) and has its peak of expression at 37˚C (Shearer et al., 1987). Hsp70 synthesis increases soon after heat shock (Lambowitz et al., 1983; Shearer et al., 1987) and we demonstrated more interactions between Hsp70 and Hsp60 at elevated temperatures.
M antigen	Major diagnostic antigen of Hc as it elicits intense humoral and cellular immune responses (Hamilton et al., 1990; Deepe, 1994; Deepe and Durose, 1995; Hamilton, 1998; Zancopé-Oliveira et al., 1999; Guimaraes et al., 2006). Its cell surface localization makes the M antigen the most important catalase for detoxification of host derived peroxides, protecting the fungus against oxidative stress, and also makes this protein accessible to host immune cells and antibody (Guimaraes et al., 2008).

(Continued)

Table 1 | Continued

Surface component	Role in virulence/pathogenicity
H antigen	Involved in nutrient acquisition (Woodward and Wiseman, 1982) or modulation of cell wall architecture (Kruse and Cole, 1992; Deepe and Durose, 1995; Akiyama et al., 1998; Fisher et al., 1999; Fisher and Woods, 2000); vaccination with H antigen protected mice in a pulmonary histoplasmosis model (Deepe and Gibbons, 2001) associated with production of IFN-γ, GM-CSF, IL-4, and IL-10 by splenocytes and in parallel, there was major expansion of $CD4^+$ or $CD8^+$ cells in spleens of mice (Deepe and Gibbons, 2001).
H2B	Important in cell–cell signaling and modulation of the immunoresponse by the fungus (Nosanchuk et al., 2003); mAbs against this protein enhanced levels of IL-4, IL-6, and IFN-gamma in the lungs of infected mice. The mAbs increased phagocytosis of yeast by J774.16 cells through a CR3-dependent process and uptake of the opsonized yeast cells was associated with yeast cell growth inhibition and killing (Nosanchuk et al., 2003; Nosanchuk, 2005). The altered intracellular fate of the opsonized Hc yeast was characterized by significantly enchanced macrophage phagosome activation and maturation and also a reduced ability of the organism to regulate the phagosomal pH. Opsonization also increased antigen processing and reduced negative PD-1/PDL-1 co-stimulation in macrophages, resulting in more-efficient T-cell activation (Shi et al., 2008).
YPS3	YPS3 is able to bind chitin and it is linked to Hc virulence *in vivo* and associated with increased fungal burden in phagocyte-rich tissues, such spleen and liver (Bohse and Woods, 2007b).
Melanin	Hence, Hc cell wall melanin protects the fungus from a myriad of insults and the polymeric nature of the pigment enhances its structural strength. It protects against extremes in temperatures, radiation, and predation in the environment, free radicals, defensins, and other toxic responses within a host. Melanization of Hc protects the fungus against chemotherapy with amphotericin B or echinocandins (van Duin et al., 2002; Gomez and Nosanchuk, 2003).

wall, playing a structural role in maintaining cell wall integrity and rigidity, as well as in resisting the extracellular environment (Ruiz-Herrera and Osorio, 1974). The chitin layer is attached to the non-reducing end of the outer β-1,3-glucan chain by a β-1,4 linkage (Mol and Wessels, 1987), and as for the number of chitin-β-1,3-glucan linkages compared to the total number of linkages in the cell wall, the ratio is about one chitin–glucan bond per 8,000 hexose units. Experiments with appropriate mutants lacking such linkage, have shown that their cell wall are weaker and more prone to damage (Bulawa et al., 1995).

The immunoreactivity of Hc chitin with host cells has not been formally investigated. Nevertheless, several chitin-binding proteins had been described, such as RegIIIg (HIP/PAP), a C-type lectin expressed in the neutrophil-like Paneth cells of the small intestine (Cash et al., 2006), and FIBCD1, a calcium-dependent acetyl group-binding receptor that is also expressed in the gastrointestinal tract (Schlosser et al., 2009). The cells of the immune system seem to use dectin-1 to recognize *Candida albicans* (Mora-Montes et al., 2011) through chitin, but in Hc, the role of chitin in the interaction with the components of the immune system is unclear. However, it is possible that chitin functions as an immunomodulatory molecule, immunosuppressing, or activating anti-microbial activities of macrophages and PBMCs, as described in *C. albicans* (Rementeria et al., 1997; Mora-Montes et al., 2011). Intriguingly, Hc chitin can bind diverse products in supernatant, such as polysaccharides from the capsule of *Cryptococcus neoformans* or self-proteins like YPS3 (Bohse and Woods, 2005), described later in this essay. Hence, chitin plays diverse roles in interacting with host cells and diverse compounds in the local environment.

β-*1,3-glucan*

Surrounding chitin, there is a layer comprised of glucans polymers linked by a β-1,3 linkage. This polymer is a major constituent of the cell wall of the filamentous phase of Hc and in chemotype I Hc yeasts, where the glycan layer is entirely β-linked (Davis et al., 1977). Some biological functions have been attributed to the β-1,3-glucan, such as promotion of inflammatory cell recruitment to the site of infection and production of pro-inflammatory cytokines including TNF-α (Figueiredo et al., 1993; Medeiros et al., 1999, 2004; Anjos et al., 2002). Intriguingly, β-glucans possess many of the characteristics attributed to PAMPs and are known to be potent triggers of innate immunity (Brown, 2006). Dectin-1 and DC-SIGN (CD209) are receptors for β-1,3-glucan polymers (Brown, 2006). Dectin-1, a non-classical C-type lectin major non-opsonic β-glucan receptor, was one of the first PRRs identified that can mediate its own signaling or act synergistically with Toll-like receptors (TLR) to initiate specific responses to infectious agents. Dectin-1 has been shown to mediate inflammatory responses to fungi and facilitate pathogen clearance (Steele et al., 2005). Detection of glycosylated fungal components by this receptor occurs in DCs, neutrophils, natural killer, and subsets of T cells and can result in the induction of cellular responses, including ligand uptake by phagocytosis and endocytosis, DC maturation, respiratory burst, and synthesis of a number cytokines, including TNF, IL-10, IL-2, IL-23, and IL-6 as well as chemokines like CXCL2 (Brown, 2006; Dennehy and Brown, 2007). DC-SIGN, another C-type lectin involved in recognition of β-glucans, is expressed primarily by DCs (Koppel et al., 2005) and has been proposed to mediate engulfment of certain fungi (Cambi et al., 2003; Koppel et al., 2005; Serrano-Gomez et al., 2005).

Due to its immunogenicity and predominance in cell wall, β-glucans have been studied as a potential target for vaccination. In fact, a vaccine based on glucan-laminarin conjugate with the recombinant diphtheria toxoid CRM197 (Lam-CRM197) recently tested was able to induce a protective immunity against different pathogenic fungi, such as *C. albicans* and *A. fumigates in vivo* (Torosantucci et al., 2005; Bromuro et al., 2010). Limitation of

β-glucan exposure is one mechanism shaping the overall pathogenic potential of different medically important fungi including Hc. However, several membrane components, such as CR3 can interact with β-glucan (Kataoka et al., 2002). Consequently, the abrogation of the host immune response by blocking Dectin-1 may be circumvented by other β-glucan receptors during natural host interactions. However, certain Hc pathogenic strains mask β-glucan by an outer α-1,3-glucan layer.

α-1,3-glucan

Morphological transition from filamentous to yeast phase modifies the biosynthesis of the glucans pool, with the production of the α-1,3-glucan, which is a specific attribute of the Hc yeast phase (Kanetsuna et al., 1974; Klimpel and Goldman, 1988). α-1,3-Glucan is also common to most medically important fungi, including all the other dimorphic pathogenic species. However, the cell walls of Hc chemotype I strains lack the α-1,3-glucan polymer, but contain more chitin and less glucan than chemotype II (Domer et al., 1967; Domer, 1971). Some strains of Hc spontaneously produce variants lacking α-1,3-glucan that display reduced virulence (Kugler et al., 2000; Rappleye et al., 2007). Such phenomena can also be induced by successive laboratory passages of the Hc yeast (Klimpel and Goldman, 1988; Hogan and Klein, 1994). In other dimorphic fungi, spontaneous loss of α-1,3-glucan also correlates with reduced virulence, indicating this may be a conserved mechanism of fungal pathogenicity (Klimpel and Goldman, 1988; Hogan and Klein, 1994). In general, several studies have demonstrated that virulent Hc strains contain up to 1,000-fold more α-1,3-glucan than avirulent strains.

Most recently, the importance of α-1,3-glucan in chemotype II Hc virulence was examined in spontaneous mutants, and by RNA interference and traditional allelic replacement of the gene that encodes for α-1,3-Glucansynthase (*ags1*; Rappleye et al., 2007). Loss of α-1,3-glucan does not impair growth of Hc *in vitro*. However, the loss significantly impaired the proliferation of the yeast in macrophages *in vitro* and these yeast were less able to kill cultured macrophages (Rappleye et al., 2004). In an *in vivo* model, loss of α-1,3-glucan resulted in a substantial reduction in lung colonization, suggesting that α-1,3-glucan might be a virulence determinant designed specifically for survival and replication in murine respiratory infection models (Marion et al., 2006).

The regulation of α-1,3-glucan occurs upstream AGS-1, by the function of the *amy1* and *ugp1* gene products, respectively, an α-1,4-amylase involved in the synthesis of α-1,3-glucan (Marion et al., 2006) and an UTP-glucose-1-phosphate uridylyltransferase that synthesizes UDP-glucose monomers. As observed with AGS-1, loss of AMY1 and UGP1 function reduces the virulence of Hc, with attenuation of yeast to grow within or kill macrophages, and a reduced capacity to colonize murine lungs (Marion et al., 2006).

However, the exact mechanism for how α-1,3-glucans alters Hc pathogenesis remains poorly understood and, to date, there is no identified receptor to Hc cell wall α-1,3-glucans. In a *Pseudallescheria boydii* model, this carbohydrate seems to be important in phagocytic internalization, which stimulates the secretion of inflammatory cytokines trough the involvement of TLR2, CD14, and MyD88 (Bittencourt et al., 2006). As described above, the explanation for their modifying effect on virulence may primarily be due to their influence in the host–pathogen interactions achieved by subverting the host immune mechanisms of recognition of cell wall components and contributing to yeast survival. As the α-1,3-glucans are expressed as the most external cell wall layer, they block innate recognition of the fungal β-1,3-glucan by dectin-1 receptor on host phagocytes (Rappleye et al., 2007). Blockage of this receptor suppresses the production of pro-inflammatory cytokine TNF-α, consisting in an important virulence mechanism for Hc, but it also helps explain mechanistically the higher native pathogenicity of dimorphic fungi. Yeasts which also lack α-1,3-glucan have reduced capacity to cause significant disease. Consistent with this hypothesis, the parasitic forms of the dimorphic fungal pathogens each possess α-1,3-glucan and can cause disease even in the face of normal host immune function. However, this does not explain how chemotype I strains maintain virulence in the absence of α-1,3-glucans. Additional functions have been attributed to α-1,3-glucans, such regulation of yeast proliferation inside host phagocytes (Kugler et al., 2000) by protecting the yeast within phagolysosomes (Eissenberg and Goldman, 1991), thus resulting in a state called intracellular latency (Eissenberg et al., 1996, 1997). Hc displaying α-1,3-glucans can persist for several weeks inside these cells and induce the formation of granulomas, which result in chronically infected tissues (Klimpel and Goldman, 1988).

Galactomannans

Hc galactomannan–protein complexes have antigenic activities, and can induce delayed-type hypersensitivity (DHT) in guinea pigs with inhibition of macrophage migration factor release (Azuma et al., 1974; Reiss et al., 1974). It is noteworthy that fungal galactomannan complexes in the related dimorphic fungus *Paracoccidioides brasiliensis* appear to be involved in the protection of the organism against its own serine-thiol protease, an enzyme associated with pathogen dissemination through the extracellular matrix (Matsuo et al., 2006).

Lectin-like components

Interactions between carbohydrates and lectins are considered the basis of recognition of target particles by phagocytes (Sharon, 1984). The expression of lectins by pathogenic microorganisms has been correlated to the organism attachment and invasion to host tissues (Mendes-Giannini et al., 2000; Singh et al., 2011). Interestingly, Hc has components with lectin activity on the yeast surface that can bind to surface molecules on murine macrophages (Taylor et al., 1998; Duarte-Escalante et al., 2003). It has been shown that a lectin-like molecule plays a role in the binding to macrophage surface proteins suggesting that the specific receptor for histoplasmin components on macrophage could be an oligosaccharide–protein complex containing galactose (mainly β-anomer) as determinant, since enzymatic cleavage of galactosyl residues or a galactose *N*-acetyl-D-galactosamine compound reduced this interaction (Maldonado et al., 1994, 1998; Taylor et al., 1998). The macrophage surface ligand is a 68 kDa protein and seems to participate in macrophage activation and regulation of phagocytosis (Maldonado et al., 1998; Taylor et al., 1998). Hc also has the ability to bind and agglutinate human erythrocytes using this lectin-like component (Taylor et al., 2004).

Hc also expresses a 50 kDa lectin that recognizes sialic acid residues on laminin, a key component of the membrane basement protein (Mcmahon et al., 1995) and might function as a possible mechanism for dissemination or is involved in cell–cell interactions.

Other cell wall associated carbohydrates

Several additional carbohydrates contribute to the composition of the Hc cell wall. Mannans and mannosylated proteins are found in the cell wall of Hc and several fungi, and their importance has been best described in *C. albicans* (Lipke and Ovalle, 1998; Pietrella et al., 2008). These mannoproteins are highly antigenic and can lead to DCs maturation and activation followed by pro-inflammatory cytokine production, which drives a protective T-cell response (Pietrella et al., 2006). In addition, mannans have been implicated in host tissue adherence (Ross, 2002).

CELL WALL LIPIDS

Lipids have recently emerged as important bioactive molecules in fungi in addition to being critical structural components of cellular membranes. Several structurally and functionally distinct lipids have been characterized in fungi. Based on simple extractions with organic solvents and determination of the total lipid content in Hc cell wall fractions, earlier reports have shown that it corresponded to 3–10% of the cell wall dry weight (Macwilliam, 1970; Cox and Best, 1972). For example, ceramide monohexoside (CMHs) consists of a lipid (ceramide) moiety linked to a single sugar residue. This molecule is the simplest glycosphingolipid of eukaryotic cells and it is expressed in almost all fungal species (Barreto-Bergter et al., 2004). In *C. neoformans*, glucosylceramide has been shown to be cell wall associated and concentrates at specific sites during cell division (Rodrigues et al., 2000). Additionally, antibodies to glucosylceramide have been shown to inhibit *C. neoformans* and *F. pedrosoi* growth and budding (Rodrigues et al., 2000; Nimrichter et al., 2004), block mycelium formation in the plant pathogen *Colletotrichum gloeosporioides* (Da Silva et al., 2004), interfere with filamentation in *P. boydii*, and inhibit germ-tube formation in *C. albicans* (Pinto et al., 2002). CMHs components have been identified in neutral lipids extracted from both filamentous and yeast Hc (Toledo et al., 2001a). Indirect immunofluorescence using an IgG2a monoclonal antibody (mAb) to glucosylceramide, termed MEST-2, labeled the surface of Hc yeast suggesting a cell wall/membrane localization of this molecule (Toledo et al., 2001b). In Hc, the significance of this molecule has not been elucidated. However, the glycosylinositol phosphorylceramides found in the mycelia and yeast phases of Hc (Barr and Lester, 1984; Barr et al., 1984) appears to be required for fungal survival (Dickson and Lester, 1999).

EXTRACELLULAR VESICLES

Macromolecules need to be transferred from the intracellular to the extracellular space through the rigid, complex, and dense cell wall environment.

Recently, Hc extracellular vesicles composed by a lipid bilayer have been described and appear to be secreted through and are present at least transiently in the cell wall (Albuquerque et al., 2008). Using transmission electron microscopy, biochemistry, proteomics, and lipidomics analysis, we and other investigators have described the vesicular transport in fungal pathogens (Yoneda and Doering, 2006; Rodrigues et al., 2007, 2008a; Albuquerque et al., 2008; Vallejo et al., 2011), including *C. neoformans*, *C. albicans*, *C. parapsilosis*, *Sporothrix schenckii*, *Saccharomyces cerevisiae*, *P. brasiliensis*, and Hc. The cellular origin of the extracellular vesicles remains unknown but there is evidence for the participation of different pathways of cellular traffic in vesicle biogenesis (Oliveira et al., 2010). Remarkably, morphological and biochemical features indicate that they are similar to mammalian exosomes (Rodrigues et al., 2008b; Casadevall et al., 2009).

Compositional analysis of Hc vesicles have revealed a very diverse pool of molecules. The lipid content included common components of biological membranes that can be involved in immune response, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Gilbreath et al., 1986; Aramaki, 2000; Hoffmann et al., 2005; Albuquerque et al., 2008). In addition, 206 proteins were identified in Hc vesicles with a broad range of functions, such chaperones (Hsp30, Hsp70, and Hsp60), superoxide dismutase, catalase B, signal transduction proteins, vesicle formation, cell wall and cytoskeleton regulation, cell growth, and sugar, lipid, and amino acid metabolism (Albuquerque et al., 2008).

Hence, some proteins found in Hc vesicles are pathogenic determinants and/or are involved in host–pathogen interactions, so, hypothetically, the concentration of these molecules could provide an efficient release mechanism of virulence factors into host tissues and during infection could directly mediate host cell damage (Rodrigues et al., 2008b; Casadevall et al., 2009; Oliveira et al., 2010). In conclusion, Hc extracellular vesicle may function as "virulence bags," since virulence factors are concentrated within them and these molecules can modulate the host–pathogen interaction (Rodrigues et al., 2008b; Casadevall et al., 2009; Oliveira et al., 2010). In accord with this hypothesis, proteins extracted from Hc vesicles reacted with immune sera from patients with histoplasmosis, showing that vesicles can modulate the immune response (Albuquerque et al., 2008).

CELL WALL PROTEINS

The dynamic nature of the cell wall of Hc includes the changing composition of several proteins that participate in sensing the environment, modifying host–pathogen interactions, and defending the fungus against oxidative stress.

Heat shock proteins

Heat shock proteins (Hsps) are among the most evolutionary highly conserved proteins across all species (Lindquist, 1986). Hsps are essential for maintaining cellular functions, playing crucial roles in protein folding/unfolding, preventing aggregation of nascent polypeptides and toxicity by facilitating protein folding, directing assembly and disassembly of protein complexes, coordinating translocation/sorting of newly synthesized proteins into correct intracellular target compartments, degradation of aged/damaged proteins via the proteasome, regulating cell cycle and signaling, and also protecting cells against apoptosis (Li and Srivastava, 2004; Saibil, 2008). As a key component of the heat shock response, Hsp expression is markedly upregulated when a cell is exposed to challenging conditions (e.g., high temperature,

oxidative stress, radiation, inflammation, exposure to toxins, starvation, hypoxia, nitrogen deficiency, or water deprivation; Wu, 1995).

Hsp60

A Hsp of 60 kDa (Hsp60) has been identified on the surface of Hc, as well as within the cell and within vesicles. Hsp60 can be visualized as clusters on the cell wall by immunogold electron microscopy (Long et al., 2003). It appears to be the major ligand that mediates attachment of Hc to macrophage/monocyte CR3 integrin (CD11b/CD18; Long et al., 2003), resulting in phagocytosis (Long et al., 2003; Habich et al., 2006). Interestingly, a study of seven different Hsp60 species from microbes and mammals revealed that all proteins bound the CR3 receptor, although using different binding sites, and all elicited an inflammatory response in mouse macrophages (Habich et al., 2003). This implies the existence of distinct receptor structures responsible for Hsp60 binding and for Hsp60-induced release of pro-inflammatory mediators. In addition, the interaction of Hsp60 with immune cells exhibits immunoregulatory properties, controlling innate, and adaptive immune reactions (Habich and Burkart, 2007). Besides the interaction of Hsp60 from Hc with CR3 receptor complex on the cell surface of macrophages (Long et al., 2003), this protein could be interacting with other critical macrophages surface proteins, regulating the effector functions of these cells, or even exerting other important chaperonin-like functions that modify the pathogenesis of Hc (Guimaraes et al., 2011c).

Hsp60 from Hc is also an immunogenic molecule and has been described as a potential target for passive immunization therapy (Guimaraes et al., 2009, 2011a). Vaccination with Hc Hsp60 induces protection in a lethal murine infection model. The depletion of CD4+ cells during vaccination completely abolishes this protective effect (Gomez et al., 1995; Deepe and Gibbons, 2002). However, studies in the expressive phase of vaccination show that the elimination of CD4(+) or CD8(+) cells does not significantly modify fungal recovery from organs of infected animals or survival from a lethal challenge. Passive immunization with antibodies to Hsp60 offered protection against Hc, as mice treated with IgG1 or IgG2a mAbs to Hsp60 have significantly prolonged survival, with a reduction in the pulmonary and splenic CFUs after a week and a decrease up to 2.5 logs of yeast numbers in the lungs at 2 weeks (Guimaraes et al., 2009, 2011b).

Hsp60 expression levels are strain and temperature dependent, with an expression peak between 34 and 37°C (Shearer et al., 1987). The mechanism by which the heat shock response to environmental stressors occurs has not been fully elucidated. However, some evidence suggests that an increase in damaged or abnormal proteins activate Hsps (Santoro, 2000). Our group has shown that Hsp60 levels increase in response to temperature stress in both cytoplasm and cell wall subcellular fractions (Guimaraes et al., 2011c). However, Hsp60 cell wall levels was not significantly changed during heat shock, suggesting that in the conditions tested Hsp60 had a constitutive and regulatory function in the cell, orchestrating traffic of proteins to the cell surface where it is present at levels close to saturation, independent of overall expression in the cell.

The capacity of the Hsp60 to interact and work as a carrier molecule suggests innumerous regulatory functions of these proteins. Differential interactions have been dissected in both cytoplasmic and cell wall, and we identified common and unique interactions within each subcellular compartment (Guimaraes et al., 2011c). The interactome reveals that Hc Hsp60 engages nuclear chaperones, small chaperones, and Hsp90 families. Temperature increases interactions between Hsp60 and Hsp70 in the cell wall. Furthermore, cell wall Hsp60 more broadly interacts with enzymes related to carbohydrate metabolism, suggesting a trafficking function of Hsp60 related to enhanced energy acquisition under stress conditions. Additionally, Hsp60 apparently contributes with cell wall changes that allow the pathogen to survive under stress conditions (Shaner et al., 2008). Hence, this protein participates as a key regulator of diverse cellular processes, including amino acid, protein, lipid, and carbohydrate metabolism, cell signaling, replication, and expression of virulence associated proteins.

Hsp70

Hsp70 is a putative chaperone secreted by Hc to the extracellular milieu, probably within vesicles (Albuquerque et al., 2008), but also found on the cellular surface of the fungus (Gomez et al., 1992, 1997; Lopes et al., 2010). Little is known about the function of this protein in Hc. Recombinant Hc Hsp70 elicits a cutaneous delayed-type hypersensitive response in mice. However, vaccination with Hsp70 did not confer protection against Hc infection (Allendoerfer et al., 1996). Hc Hsps, such as Hsp70 and Hsp82 (an Hsp recently associated with virulence, albeit by unknown mechanisms (Edwards et al., 2011), display a similar expression pattern to Hsp60 (Caruso et al., 1987; Minchiotti et al., 1992). Hsp70 is highly expressed by the fungus when undergoing transition from mycelium-to-yeast (Kamei et al., 1992) and it is also strain and temperature dependent, having its peak of expression at 37°C (Shearer et al., 1987). Hsp70 synthesis increases soon after heat shock (Lambowitz et al., 1983; Shearer et al., 1987) and we demonstrated more interactions between Hsp70 and Hsp60 at elevated temperatures. Thus, Hc Hsp70 interacts with Hsp60, in various cellular compartments, and might communicate with other intracellular chaperones, composing a heat shock regulon complex.

M antigen

The M antigen is the major diagnostic antigen of Hc as elicits intense humoral and cellular immune responses (Hamilton et al., 1990; Deepe, 1994; Deepe and Durose, 1995; Hamilton, 1998; Zancopé-Oliveira et al., 1999; Guimaraes et al., 2006). Antibodies to M antigen appear soon after infection and, importantly, antibodies to M antigen can indicate prior exposure, acute disease or a chronic progressive disease. The M precipitin reaction can persist for up to 3 years after disease resolution and people who have never had contact with Hc can become reactive after skin testing with histoplasmin (Klite, 1965; Kaufman, 1992).

Based on its amino acid sequence and cross reactivity of mAbs raised against the M antigen with other fungal catalases, the M protein has been characterized as a B catalase (Hamilton et al., 1990; Zancopé-Oliveira et al., 1999). The M antigen has been detected in cell-free extracts and in solution after permeabilizing Hc yeast,

suggesting that the M antigen is a secreted enzyme (Howard, 1983). Hc expresses two other catalases, CatP, and CatA, both located intracellularly, and all of these proteins are involved in detoxification of reactive oxygen species generated by fungal metabolism and respiration (Johnson et al., 2002).

Our group sought to further characterize the M antigen and confirm its catalase activity. Initially, we constructed a 3-D rendering by homology modeling, and found structures and domains that closely resembled characterized catalases. Specific mAbs against recombinant M antigen labeled the yeast cell surface of Hc and provided a single band in immunoblots using cell wall/membrane preparations. Hence, these mAbs further confirmed the cell surface location of the M antigen. Additionally, we demonstrated that the majority of catalase activity was concentrated in fungal surface preparations. The localization of the M antigen to the cell surface makes the M antigen the most important catalase for detoxification of host derived peroxides, protecting the fungus against oxidative stress, and also makes this protein accessible to host immune cells and antibody (Guimaraes et al., 2008). However, its importance during establishment of infection and as a vaccine candidate awaits characterization.

H antigen

The H antigen has been described as a secreted component of histoplasmin, the most important immunodiagnostic reagent of Hc obtained from culture supernatant of the filamentous culture of the fungus (Ehrhard and Pine, 1972a,b; Zancope-Oliveira et al., 1994; Guimaraes et al., 2006). This antigen consistently reacts with sera from histoplasmosis patient, and antibodies to the H antigen may be detected 1–2 years after the resolution of acute disease. Antibodies to the H antigen usually disappear more quickly than the anti-M antibodies (Davies, 1986). The H precipitin rarely is detected after HMIN skin testing.

Although this protein has been extensively studied as an immunodiagnostic reagent for more than 50 years, its biological function has not been precisely elucidated. The deduced amino acid sequence of the H gene displays homology to secreted fungal β-glucosidases and has a molecular weight from 108 to 120 kDa. However, recombinant protein expressed in a prokaryotic host (Fisher et al., 1999) did not demonstrate β-glucosidase enzymatic activity, probably due to incorrect folding and altered protein structure. Subsequently, expression in the native organism has resulted in production of a full-size, glycosylated H antigen with functional β-glucosidase activity (Fisher et al., 1999). Potential biological activities for this enzyme include nutrient acquisition (by the breakdown of environmental cellulosic or carbohydrate substrates to acquire glucose; Woodward and Wiseman, 1982) or modulation of cell wall architecture (Deepe and Durose, 1995; Fisher et al., 1999; Fisher and Woods, 2000; by the breakdown of cell wall polymers and carbohydrates; Kruse and Cole, 1992; Akiyama et al., 1998).

Recombinant H antigen can stimulate splenocytes from mice immunized with viable yeast or with antigen suspended in adjuvant (Deepe and Durose, 1995; Deepe and Gibbons, 2001). However, despite stimulating a cell-mediated immune response, vaccination with the antigen was not able to protect against either a lethal or a sub-lethal intravenous inoculum of yeast (Deepe and Durose, 1995). Interestingly, vaccination with H antigen protected mice in a pulmonary histoplasmosis model (Deepe and Gibbons, 2001). The protective immunization was associated with production of IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), and interleukin-10 (IL-10) by splenocytes and in parallel, there was major expansion of $CD4^+$ or $CD8^+$ cells in spleens of mice (Deepe and Gibbons, 2001).

Histone 2B

Histones are proteins commonly associated with DNA and have high conserved structure and functions. Although they are classically located in the nucleus, cell surface histones have been described in both eukaryotic and prokaryotic organisms. Histones have been described in the plasma membrane of leukocytes (Rekvig et al., 1987), T cells (functioning as proteoglycan receptors; Ojcius et al., 1991; Khan et al., 1998), B cells (Mecheri et al., 1993), and a human lung carcinoma cell line (as an adhesin to the extracellular matrix; Bilozur and Biswas, 1990). A histone 2B of *Mycobacterium leprae* can be located on the bacterial cell surface and this expressed protein facilitates invasion of Schwann cells (Shimoji et al., 1999; De Melo Marques et al., 2000) by binding laminin on peripheral nerves (Pessolani et al., 1993; De Melo Marques et al., 2000). Similarly *Mycobacterium smegmatis* binds to laminin on human pneumocytes and macrophages through a histone-like protein located on its surface (Pethe et al., 2001). We have described a histone 2B-like protein of 17-kDa antigen expressed on the surface of Hc, and it is speculated that the protein is important in cell–cell signaling and modulation of the immune response by the fungus (Nosanchuk et al., 2003).

Administration of mAbs that bind histone 2B-like protein on the surface of Hc has been shown to reduce fungal burden, decrease pulmonary inflammation, and prolong survival in a murine infection model (Nosanchuk, 2005). The protective response mediated by these mAbs was associated with enhanced levels of IL-4, IL-6, and IFN-γ in the lungs of infected mice. The mAbs increased phagocytosis of yeast by J774.16 cells through a CR3-dependent process and uptake of the opsonized yeast was associated with yeast growth inhibition and killing (Nosanchuk et al., 2003; Nosanchuk, 2005). The altered intracellular fate of the opsonized Hc yeast was characterized by significantly enhanced macrophage phagosome activation and maturation and also a reduced ability of the organism to regulate the phagosomal pH. Opsonization also increased antigen processing and reduced negative PD-1/PDL-1 co-stimulation in macrophages, resulting in more-efficient T-cell activation (Shi et al., 2008).

YPS3

YPS3 is both resident in the cell wall and released in significant quantities into the culture medium during growth of Hc. This protein is expressed only by the pathogenic yeast phase *in vitro* or infecting tissues (Bohse and Woods, 2005). The *yps3* locus is present in all the strains of Hc, but protein production seems to be limited to the North American restriction fragment length polymorphism class 2/NAm 2 group of strains (Bohse and Woods, 2007b). These strains have the highest virulence among all the

Hc isolates and the expression of Yps3 is likely associated with increased virulence (Bohse and Woods, 2005). The Yps3 protein in the NAm 2 strains has an average length of 137 amino acids. However, intragenic hypervariable region of tandem repeats in the *yps3* gene might result in different size fluctuation or protein isoforms among Hc strains (Bohse and Woods, 2007a). Another important feature of this protein is the presence of an N-terminal secretion signal sequence (Bohse and Woods, 2005). As this protein is secreted by the yeast, it binds to the polysaccharide chitin and becomes exposed on the cell wall.

Intriguingly, silencing of the *YPS3* transcript did not result in any detectable phenotypic differences *in vitro* (Bohse and Woods, 2007b). Silenced yeast displayed normal growth at 37°C and similar virulence to wild-type yeasts in co-cultures with a RAW 264.7 murine macrophage-like cell line. However, in an *in vivo* murine infection model, silenced yeast caused significantly less disease than wild-type yeast. Reductions in fungal burden were particularly evident in phagocyte-rich tissues, such spleen and liver. Hence, YPS3 is clearly linked to Hc virulence *in vivo*.

MELANIN

Many pathogenic fungi produce the enigmatic polymer pigment melanin in their cell wall (Nosanchuk and Casadevall, 2006). Hc is also able to synthesize melanin, on both conidia and yeast (Nosanchuk et al., 2002). Melanins are negatively charged, hydrophobic pigments of high molecular weight, formed by the oxidative polymerization of exogenous phenolic and/or indolic compounds. Since Hc conidia synthesize melanin in the absence of exogenous phenolic substrate, it is probable that conidia are melanized in the environment (Nosanchuk et al., 2002). Thus, melanization may protect the conidia from environmental insults, such as damage by solar UV radiation, extreme temperatures and chemical (heavy metals and oxidizing agents Nosanchuk et al., 2002) and also predation in the environment, against free radicals, defensins, and other toxic responses within a host. Melanization reduces Hc susceptibility to host defense mechanisms and antifungal drugs (Van Duin et al., 2002; Taborda et al., 2008). Moreover, melanization of Hc protects the fungus against chemotherapy with amphotericin B or echinocandins (Van Duin et al., 2002; Gomez and Nosanchuk, 2003). Hence, Hc cell wall melanin protects the fungus from a myriad of insults and the polymeric nature of the pigment enhances its structural strength. Moreover, yeast melanization appears to play important roles in virulence and pathogenicity.

CONCLUSION

The fungal cell wall is fundamentally the structure that interacts with environmental and host milieus. A current model for the Hc cell wall is presented on the **Figure 2**. It provides structural support, varying from flexible to a rigid structure, and protects the cell against environmental stressors and host anti-microbial effectors mechanisms. Many fungal antigens are conserved among phylogenetically related species and many that are associated with virulence can be found on their cell walls. Hence, common targets could be used to induce protection against different fungal species described (Casadevall and Pirofski, 2007). Although approaches to developing protective responses vary from vaccination with cells or recombinant antigens to passive protection with mAbs, coordinating knowledge across the fungal pathogens may lead to new, more effective means for combating potentially lethal mycoses. In this survey of Hc surface structures, we demonstrate the function of diverse antigens in regulating Hc biology and discuss the current knowledge base from which we can build upon in future investigations.

REFERENCES

Ajello, L. (1971). Coccidioidomycosis and histoplasmosis. A review of their epidemiology and geographical distribution. *Mycopathol. Mycol. Appl.* 45, 221–230.

Akiyama, T., Kaku, H., and Shibuya, N. (1998). A cell wall-bound beta-glucosidase from germinated rice: purification and properties. *Phytochemistry* 48, 49–54.

Albuquerque, P. C., Nakayasu, E. S., Rodrigues, M. L., Frases, S., Casadevall, A., Zancope-Oliveira, R. M., Almeida, I. C., and Nosanchuk, J. D. (2008). Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. *Cell. Microbiol.* 10, 1695–1710.

Allendoerfer, R., Biovin, G. P., and Deepe, G. S. Jr. (1997). Modulation of immune responses in murine pulmonary histoplasmosis. *J. Infect. Dis.* 175, 905–914.

Allendoerfer, R., Maresca, B., and Deepe, G. S. Jr. (1996). Cellular immune responses to recombinant heat shock protein 70 from *Histoplasma capsulatum*. *Infect. Immun.* 64, 4123–4128.

Alteras, I. (1966). First Romanian isolation of *Histoplasma capsulatum* from the soil. *Dermatol. Int.* 5, 69–71.

Anjos, A. R., Calvi, S. A., Ferracini, R., Peracoli, M. T., Silva, C. L., and Soares, A. M. (2002). Role of *Paracoccidioides brasiliensis* cell wall fraction containing beta-glucan in tumor necrosis factor-alpha production by human monocytes: correlation with fungicidal activity. *Med. Mycol.* 40, 377–382.

Aramaki, Y. (2000). Liposomes as immunomodulator – inhibitory effect of liposomes on NO production from macrophages. *Biol. Pharm. Bull.* 23, 1267–1274.

Azuma, I., Kanetsuna, F., Tanaka, Y., Yamamura, Y., and Carbonell, L. M. (1974). Chemical and immunological properties of galactomannans obtained from *Histoplasma duboisii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and Blastomyces dermatitidis. *Mycopathol. Mycol. Appl.* 54, 111–125.

Barr, K., Laine, R. A., and Lester, R. L. (1984). Carbohydrate structures of three novel phosphoinositol-containing sphingolipids from the yeast *Histoplasma capsulatum*. *Biochemistry* 23, 5589–5596.

Barr, K., and Lester, R. L. (1984). Occurrence of novel antigenic phosphoinositol-containing sphingolipids in the pathogenic yeast *Histoplasma capsulatum*. *Biochemistry* 23, 5581–5588.

Barreto-Bergter, E., Pinto, M. R., and Rodrigues, M. L. (2004). Structure and biological functions of fungal cerebrosides. *An. Acad. Bras. Cienc.* 76, 67–84.

Berliner, M. D. (1973). *Histoplasma capsulatum*: vital staining for the differentiation of the albino and brown phenotypes in vitro. *Sabouraudia* 11, 271–273.

Bernard, M., and Latge, J. P. (2001). Aspergillus fumigatus cell wall: composition and biosynthesis. *Med. Mycol.* 39 (Suppl. 1), 9–17.

Bilozur, M. E., and Biswas, C. (1990). Identification and characterization of heparan sulfate-binding proteins from human lung carcinoma cells. *J. Biol. Chem.* 265, 19697–19703.

Bittencourt, V. C., Figueiredo, R. T., Da Silva, R. B., Mourao-Sa, D. S., Fernandez, P. L., Sassaki, G. L., Mulloy, B., Bozza, M. T., and Barreto-Bergter, E. (2006). An alpha-glucan of *Pseudallescheria boydii* is involved in fungal phagocytosis and Toll-like receptor activation. *J. Biol. Chem.* 281, 22614–22623.

Bohse, M. L., and Woods, J. P. (2005). Surface localization of the Yps3p protein of *Histoplasma capsulatum*. *Eukaryot. Cell* 4, 685–693.

Bohse, M. L., and Woods, J. P. (2007a). Expression and interstrain variability of the YPS3 gene of *Histoplasma capsulatum*. *Eukaryot. Cell* 6, 609–615.

Bohse, M. L., and Woods, J. P. (2007b). RNA interference-mediated silencing of the YPS3 gene of *Histoplasma capsulatum* reveals virulence defects. *Infect. Immun.* 75, 2811–2817.

Borelli, D. (1970). Prevalence of systemic mycosis in Latin America. *Proc. Int. Symp. Mycoses Scient. Publ.* 205.

Bradsher, R. W. (1996). Histoplasmosis and blastomycosis. *Clin. Infect. Dis.* 22(Suppl. 2), S102–S111.

Bromuro, C., Romano, M., Chiani, P., Berti, F., Tontini, M., Proietti, D., Mori, E., Torosantucci, A., Costantino, P., Rappuoli, R., and Cassone, A. (2010). Beta-glucan-CRM197 conjugates as candidates antifungal vaccines. *Vaccine* 28, 2615–2623.

Brown, G. D. (2006). Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* 6, 33–43.

Bulawa, C. E., Miller, D. W., Henry, L. K., and Becker, J. M. (1995). Attenuated virulence of chitin-deficient mutants of *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10570–10574.

Cambi, A., Gijzen, K., De Vries, J. M., Torensma, R., Joosten, B., Adema, G. J., Netea, M. G., Kullberg, B. J., Romani, L., and Figdor, C. G. (2003). The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur. J. Immunol.* 33, 532–538.

Cano, M. V., and Hajjeh, R. A. (2001). The epidemiology of histoplasmosis: a review. *Semin. Respir. Infect.* 16, 109–118.

Caruso, M., Sacco, M., Medoff, G., and Maresca, B. (1987). Heat shock 70 gene is differentially expressed in *Histoplasma capsulatum* strains with different levels of thermotolerance and pathogenicity. *Mol. Microbiol.* 1, 151–158.

Casadevall, A., Nosanchuk, J. D., Williamson, P., and Rodrigues, M. L. (2009). Vesicular transport across the fungal cell wall. *Trends Microbiol.* 17, 158–162.

Casadevall, A., and Pirofski, L. A. (2007). Antibody-mediated protection through cross-reactivity introduces a fungal heresy into immunological dogma. *Infect. Immun.* 75, 5074–5078.

Cash, H. L., Whitham, C. V., and Hooper, L. V. (2006). Refolding, purification, and characterization of human and murine RegIII proteins expressed in *Escherichia coli*. *Protein Expr. Purif.* 48, 151–159.

Couto, M. A., Liu, L., Lehrer, R. I., and Ganz, T. (1994). Inhibition of intracellular *Histoplasma capsulatum* replication by murine macrophages that produce human defensin. *Infect. Immun.* 62, 2375–2378.

Cox, R. A., and Best, G. K. (1972). Cell wall composition of two strains of *Blastomyces dermatitidis* exhibiting differences in virulence for mice. *Infect. Immun.* 5 449–453.

Csillag, A., and Wermer, T. (1956). Histoplasmosis. *Orv. Hetil.* 97, 964–967.

Da Silva, A. F., Rodrigues, M. L., Farias, S. E., Almeida, I. C., Pinto, M. R., and Barreto-Bergter, E. (2004). Glucosylceramides in *Colletotrichum gloeosporioides* are involved in the differentiation of conidia into mycelial cells. *FEBS Lett.* 561, 137–143.

Davies, S. F. (1986). Serodiagnosis of histoplasmosis. *Semin. Respir. Infect.* 1, 9–15.

Davies, S. F., Khan, M., and Sarosi, G. A. (1978). Disseminated histoplasmosis in immunologically suppressed patients. Occurrence in a nonendemic area. *Am. J. Med.* 64, 94–100.

Davis, T. E. Jr., Domer, J. E., and Li, Y. T. (1977). Cell wall studies of *Histoplasma capsulatum* and *Blastomyces dermatitidis* using autologous and heterologous enzymes. *Infect. Immun.* 15, 978–987.

De Melo Marques, M. A., Mahapatra, S., Nandan, D., Dick, T., Sarno, E. N., Brennan, P. J., and Vidal Pessolani, M. C. (2000). Bacterial and host-derived cationic proteins bind alpha2-laminins and enhance *Mycobacterium leprae* attachment to human Schwann cells. *Microbes Infect.* 2, 1407–1417.

Deepe, G. S. Jr. (2005). Modulation of infection with Histoplasma capsulatum by inhibition of tumor necrosis factor-alpha activity. *Clin. Infect. Dis.* 41(Suppl. 3), S204–S207.

Deepe, G. S. Jr., and Durose, G. G. (1995). Immunobiological activity of recombinant H antigen from *Histoplasma capsulatum*. *Infect. Immun.* 63, 3151–3157.

Deepe, G. S. Jr., and Gibbons, R. (2001). Protective efficacy of H antigen from *Histoplasma capsulatum* in a murine model of pulmonary histoplasmosis. *Infect. Immun.* 69, 3128–3134.

Deepe, G. S. Jr., Gibbons, R. S., and Smulian, A. G. (2008). *Histoplasma capsulatum* manifests preferential invasion of phagocytic subpopulations in murine lungs. *J. Leukoc. Biol.* 84, 669–678.

Deepe, G. S. Jr., Smelt, S., and Louie, J. S. (2005). Tumor necrosis factor inhibition and opportunistic infections. *Clin. Infect. Dis.* 41(Suppl. 3), S187–S188.

Deepe, G. S. J., and Gibbons, R. S. (2002). Cellular and molecular regulation of vaccination with heat shock protein 60 from *Histoplasma capsulatum*. *Infect. Immun.* 70, 3759–3767.

Deepe, G. S. Jr. (1994). The immune response to *Histoplasma capsulatum*: unearthing its secrets. *J. Lab. Clin. Med.* 123, 201–205.

Dennehy, K. M., and Brown, G. D. (2007). The role of the beta-glucan receptor Dectin-1 in control of fungal infection. *J. Leukoc. Biol.* 82, 253–258.

Dickson, R. C., and Lester, R. L. (1999). Yeast sphingolipids. *Biochem. Biophys. Acta* 1426, 347–357.

Disalvo, A. F., Bigler, W. J., Ajello, L., Johnson, J. E., and Palmer, J. (1970). Bat and soil studies for sources of histoplasmosis in Florida. *Public. Health. Rep.* 85, 1063–1069.

Domer, J. E. (1971). Monosaccharide and chitin content of cell walls of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. *J. Bacteriol.* 107, 870–877.

Domer, J. E., Hamilton, J. G., and Harkin, J. C. (1967). Comparative study of the cell walls of the yeastlike and mycelial phases of *Histoplasma capsulatum*. *J. Bacteriol.* 94, 466–474.

Duarte-Escalante, E., Zenteno, E., and Taylor, M. L. (2003). Interaction of *Histoplasma capsulatum* yeasts with galactosylated surface molecules of murine macrophages. *Arch. Med. Res.* 34, 176–183.

Edwards, J. A., Zemska, O., and Rappleye, C. A. (2011). Discovery of a Role for Hsp82 in *Histoplasma* virulence through a quantitative screen for macrophage lethality. *Infect. Immun.* 79, 3348–3357.

Edwards, M. R., Hazen, E. L., and Edwards, G. A. (1960). The micromorphology of the tuberculate spores of *Histoplasma capsulatum*. *Can. J. Microbiol.* 6, 65–70.

Ehrhard, H. B., and Pine, L. (1972a). Factors influencing the production of H and M antigens by *Histoplasma capsulatum*: development and evaluation of a shake culture. *Appl. Microbiol.* 23, 236–249.

Ehrhard, H. B., and Pine, L. (1972b). Factors influencing the production of H and M antigens by *Histoplasma capsulatum*: effect of physical factors and composition of medium. *Appl. Microbiol.* 23, 250–261.

Eissenberg, L. G., and Goldman, W. E. (1987). *Histoplasma capsulatum* fails to trigger release of superoxide from macrophages. *Infect. Immun.* 55, 29–34.

Eissenberg, L. G., and Goldman, W. E. (1991). *Histoplasma* variation and adaptive strategies for parasitism: new perspectives on histoplasmosis. *Clin. Microbiol. Rev.* 4, 411–421.

Eissenberg, L. G., Moser, S. A., and Goldman, W. E. (1997). Alterations to the cell wall of *Histoplasma capsulatum* yeasts during infection of macrophages or epithelial cells. *J. Infect. Dis.* 175, 1538–1544.

Eissenberg, L. G., Poirier, S., and Goldman, W. E. (1996). Phenotypic variation and persistence of *Histoplasma capsulatum* yeasts in host cells. *Infect. Immun.* 64, 5310–5314.

Emmons, C. W. (1950). Histoplasmosis: animal reservoirs and other sources in nature of the pathogenic fungus *Histoplasma capsulatum*. *Am. J. Public Health* 40, 436–440.

Emmons, C. W. (1956a). Histoplasmosis in animals. *Public Health Monogr.* 70, 272–273.

Emmons, C. W. (1956b). Isolation of *Histoplasma capsulatum* from soil. *Public Health Monogr.* 70, 237–239.

Emmons, C. W., Klite, P. D., Baer, G. M., and Hill, W. B. Jr. (1966). Isolation of *Histoplasma capsulatum* from bats in the United States. *Am. J. Epidemiol.* 84, 103–109.

Figueiredo, F., Alves, L. M., and Silva, C. L. (1993). Tumour necrosis factor production in vivo and in vitro in response to *Paracoccidioides brasiliensis* and the cell wall fractions thereof. *Clin. Exp. Immunol.* 93, 189–194.

Fisher, K. L., Deepe, G. S. Jr., and Woods, J. P. (1999). *Histoplasma capsulatum* strain variation in both H antigen production and beta-glucosidase activity and overexpression of HAG1 from a telomeric linear plasmid. *Infect. Immun.* 67, 3312–3316.

Fisher, K. L., and Woods, J. P. (2000). Determination of beta-glucosidase enzymatic function of the *Histoplasma capsulatum* H antigen using a native expression system. *Gene* 247, 191–197.

Garrison, R. G., and Boyd, K. S. (1977). The fine structure of microconidial germination and vegetative cells of *Histoplasma capsulatum*. *Ann. Microbiol. (Paris)* 128, 135–149.

Gilbreath, M. J., Hoover, D. L., Alving, C. R., Swartz, G. M. Jr., and Meltzer, M. S. (1986). Inhibition of lymphokine-induced macrophage microbicidal activity against *Leishmania* major by liposomes: characterization of the physicochemical requirements for liposome inhibition. *J. Immunol.* 137, 1681–1687.

Gomez, B. L., Figueroa, J. I., Hamilton, A. J., Ortiz, B. L., Robledo, M. A., Restrepo, A., and Hay, R. J. (1997). Development of a novel antigen detection test for histoplasmosis. *J. Clin. Microbiol.* 35, 2618–2622.

Gomez, B. L., and Nosanchuk, J. D. (2003). Melanin and fungi. *Curr. Opin. Infect. Dis.* 16, 91–96.

Gomez, F. J., Allendoerfer, R., and Deepe, G. S. Jr. (1995). Vaccination with recombinant heat-shock protein 60 from *Histoplasma capsulatum*. *Infect. Immun.* 63, 2587–2595.

Gomez, F. J., Gomez, A. M., and Deepe, G. S. Jr. (1992). An 80-kilodalton antigen from *Histoplasma capsulatum* that has homology to heat shock protein 70 induces cell-mediated immune responses and protection in mice. *Infect. Immun.* 60, 2565–2571.

Goodwin, R. A. Jr., and Des Prez, R. M. (1978). State of the art: histoplasmosis. *Am. Rev. Respir. Dis.* 117, 929–956.

Goodwin, R. A., Loyd, J. E., and Des Prez, R. M. (1981). Histoplasmosis in normal hosts. *Medicine (Baltimore)* 60, 231–266.

Gorocica, P., Taylor, M. L., Alvarado-Vasquez, N., Perez-Torres, A., Lascurain, R., and Zenteno, E. (2009). The interaction between *Histoplasma capsulatum* cell wall carbohydrates and host components: relevance in the immunomodulatory role of histoplasmosis. *Mem. Inst. Oswaldo Cruz* 104, 492–496.

Guimaraes, A. J., Frases, S., Gomez, F. J., Zancope-Oliveira, R. M., and Nosanchuk, J. D. (2009). Monoclonal antibodies to heat shock protein 60 alter the pathogenesis of *Histoplasma capsulatum*. *Infect. Immun.* 77, 1357–1367.

Guimaraes, A. J., Hamilton, A. J., De, M. G. H. L., Nosanchuk, J. D., and Zancope-Oliveira, R. M. (2008). Biological function and molecular mapping of M antigen in yeast phase of *Histoplasma capsulatum*. *PLoS ONE* 3, e3449. doi:10.1371/journal.pone.0003449.g001

Guimaraes, A. J., Frases, S., Pontes, B., De Cerqueira, M. D., Rodrigues, M. L., Viana, N. B., Nimrichter, L., and Nosanchuk, J. D. (2011a). Agglutination of *Histoplasma capsulatum* by IgG monoclonal antibodies against Hsp60 impacts macrophage effector functions. *Infect. Immun.* 79, 918–927.

Guimaraes, A. J., Martinez, L. R., and Nosanchuk, J. D. (2011b). Passive administration of monoclonal antibodies against *H. capsulatum* and other fungal pathogens. *J. Vis. Exp.* doi: 10.3791/2532.

Guimaraes, A. J., Nakayasu, E. S., Sobreira, T. J., Cordero, R. J., Nimrichter, L., Almeida, I. C., and Nosanchuk, J. D. (2011c). *Histoplasma capsulatum* heat-shock 60 orchestrates the adaptation of the fungus to temperature stress. *PLoS ONE* 6, e14660. doi:10.1371/journal.pone.0014660

Guimaraes, A. J., Nosanchuk, J. D., and Zancope-Oliveira, R. M. (2006). Diagnosis of Histoplasmosis. *Braz. J. Microbiol.* 37, 1–13.

Habich, C., and Burkart, V. (2007). Heat shock protein 60: regulatory role on innate immune cells. *Cell. Mol. Life Sci.* 64, 742–751.

Habich, C., Kempe, K., Gomez, F. J., Lillicrap, M., Gaston, H., Van Der Zee, R., Kolb, H., and Burkart, V. (2006). Heat shock protein 60: identification of specific epitopes for binding to primary macrophages. *FEBS Lett.* 580, 115–120.

Habich, C., Kempe, K., Van Der Zee, R., Burkart, V., and Kolb, H. (2003). Different heat shock protein 60 species share pro-inflammatory activity but not binding sites on macrophages. *FEBS Lett.* 533, 105–109.

Hamilton, A. J. (1998). Serodiagnosis of histoplasmosis, paracoccidioidomycosis and penicilliosis marneffei; current status and future trends. *Med. Mycol.* 36, 351–364.

Hamilton, A. J., Bartholomew, M. A., Figueroa, J., Fenelon, L. E., and Hay, R. J. (1990). Evidence that the M antigen of *Histoplasma capsulatum* var. *capsulatum* is a catalase which exhibits cross-reactivity with other dimorphic fungi. *J. Med. Vet. Mycol.* 28, 479–485.

Hay, R. J. (1992). Laboratory techniques in the investigation of fungal infections. *Genitourin. Med.* 68, 409–412.

Hoffmann, P. R., Kench, J. A., Vondracek, A., Kruk, E., Daleke, D. L., Jordan, M., Marrack, P., Henson, P. M., and Fadok, V. A. (2005). Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. *J. Immunol.* 174, 1393–1404.

Hogan, L. H., and Klein, B. S. (1994). Altered expression of surface alpha-1,3-glucan in genetically related strains of *Blastomyces dermatitidis* that differ in virulence. *Infect. Immun.* 62, 3543–3546.

Howard, D. H. (1983). Studies on the catalase of *Histoplasma capsulatum*. *Infect. Immun.* 39, 1161–1166.

Isbister, J., Elliott, M., and Nogrady, S. (1976). Histoplasmosis: an outbreak occurring among young men who visited one cave. *Med. J. Aust.* 2, 243–248.

Johnson, C. H., Klotz, M. G., York, J. L., Kruft, V., and Mcewen, J. E. (2002). Redundancy, phylogeny and differential expression of *Histoplasma capsulatum* catalases. *Microbiology* 148, 1129–1142.

Kamei, K., Brummer, E., Clemons, K. V., and Stevens, D. A. (1992). Induction of stress protein synthesis in *Histoplasma capsulatum* by heat, low pH and hydrogen peroxide. *J. Med. Vet. Mycol.* 30, 385–393.

Kanetsuna, F. (1981). Ultrastructural studies on the dimorphism of *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis* and *Histoplasma capsulatum*. *Sabouraudia* 19, 275–286.

Kanetsuna, F., Carbonell, L. M., Azuma, I., and Yamamura, Y. (1972). Biochemical studies on the thermal dimorphism of *Paracoccidioides brasiliensis*. *J. Bacteriol.* 110, 208–218.

Kanetsuna, F., Carbonell, L. M., Gil, F., and Azuma, I. (1974). Chemical and ultrastructural studies on the cell walls of the yeastlike and mycelial forms of *Histoplasma capsulatum*. *Mycopathol. Mycol. Appl.* 54, 1–13.

Kataoka, K., Muta, T., Yamazaki, S., and Takeshige, K. (2002). Activation of macrophages by linear (1right-arrow3)-beta-D-glucans. Impliations for the recognition of fungi by innate immunity. *J. Biol. Chem.* 277, 36825–36831.

Kauffman, C. A. (2007). Histoplasmosis: a clinical and laboratory update. *Clin. Microbiol. Rev.* 20, 115–132.

Kaufman, L. (1992). Laboratory methods for the diagnosis and confirmation of systemic mycoses. *Clin. Infect. Dis.* 14(Suppl. 1), S23–S29.

Khan, I. U., Wallin, R., Gupta, R. S., and Kammer, G. M. (1998). Protein kinase A-catalyzed phosphorylation of heat shock protein 60 chaperone regulates its attachment to histone 2B in the T lymphocyte plasma membrane. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10425–10430.

Klimpel, K. R., and Goldman, W. E. (1988). Cell walls from avirulent variants of *Histoplasma capsulatum* lack alpha-(1,3)-glucan. *Infect. Immun.* 56, 2997–3000.

Klite, P. D. (1965). The interpretation of agar-gel precipitin reactions in histoplasmosis. *J. Lab. Clin. Med.* 66, 770–787.

Koppel, E. A., Van Gisbergen, K. P., Geijtenbeek, T. B., and Van Kooyk, Y. (2005). Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cell. Microbiol.* 7, 157–165.

Kruse, D., and Cole, G. T. (1992). A seroreactive 120-kilodalton beta-1,3-glucanase of *Coccidioides immitis* which may participate in spherule morphogenesis. *Infect. Immun.* 60, 4350–4363.

Kugler, S., Schurtz Sebghati, T., Groppe Eissenberg, L., and Goldman, W. E. (2000). Phenotypic variation and intracellular parasitism by *Histoplasma capsulatum*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8794–8798.

Kwon-Chung, K. J. (1972). Sexual stage of *Histoplasma capsulatum*. *Science* 175, 326.

Kwon-Chung, K. J. (1975). Perfect state (*Emmonsiella capsulata*) of the fungus causing large-form African histoplasmosis. *Mycologia* 67, 980–990.

Kwon-Chung, K. J., and Bennett, J. E. (1992). *Medical Mycology*. Philadelphia: Lea & Febiger.

Lambowitz, A. M., Kobayashi, G. S., Painter, A., and Medoff, G. (1983). Possible relationship of morphogenesis in pathogenic fungus, *Histoplasma capsulatum*, to heat shock response. *Nature* 303, 806–808.

Li, Z., and Srivastava, P. (2004). Heat-shock proteins. *Curr. Protoc. Immunol.* Appendix 1, Appendix 1T.

Limaye, A. P., Connolly, P. A., Sagar, M., Fritsche, T. R., Cookson, B. T., Wheat, L. J., and Stamm, W. E. (2000). Transmission of *Histoplasma capsulatum* by organ transplantation. *N. Engl. J. Med.* 343, 1163–1166.

Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* 55, 1151–1191.

Lipke, P. N., and Ovalle, R. (1998). Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.* 180, 3735–3740.

Londero, A. T., and Ramos, C. D. (1978). The status of histoplasmosis in Brazil. *Mycopathologia* 64, 153–156.

Long, K. H., Gomez, F. J., Morris, R. E., and Newman, S. L. (2003). Identification of heat shock protein 60 as the ligand on *Histoplasma capsulatum* that mediates binding to CD18 receptors on human macrophages. *J. Immunol.* 170, 487–494.

Lopes, L. C., Guimaraes, A. J., De Cerqueira, M. D., Gomez, B. L., and Nosanchuk, J. D. (2010). A *Histoplasma capsulatum*-specific IgG1 isotype monoclonal antibody, H1C, to a 70-kilodalton cell surface protein is not protective in murine histoplasmosis. *Clin. Vaccine Immunol.* 17, 1155–1158.

Macwilliam, I. C. (1970). The structure, synthesis and functions of the yeast cell wall – a review. *J. Inst. Brew.* 76, 524–535.

Maldonado, G., Gorocica, P., Agundis, C., Perez, A., Molina, J., and Zenteno, E. (1998). Inhibition of phagocytic activity by the *N*-acetyl-D-galactosamine-specific lectin from Amaranthus leucocarpus. *Glycoconj. J.* 15, 615–622.

Maldonado, G., Porras, F., Fernandez, L., Vazquez, L., and Zenteno, E. (1994). Effect of lectins on mouse peritoneal macrophage phagocytic activity. *Immunol. Invest.* 23, 429–436.

Maresca, B., and Kobayashi, G. S. (1989). Dimorphism in *Histoplasma capsulatum*: a model for the study of cell differentiation in pathogenic fungi. *Microbiol. Rev.* 53, 186–209.

Marion, C. L., Rappleye, C. A., Engle, J. T., and Goldman, W. E. (2006). An alpha-(1,4)-amylase is essential for alpha-(1,3)-glucan production and virulence in *Histoplasma capsulatum*. *Mol. Microbiol.* 62, 970–983.

Matsuo, A. L., Tersariol, Ii, Kobata, S. I., Travassos, L. R., Carmona, A. K., and Puccia, R. (2006). Modulation of the exocellular serine-thiol proteinase activity of *Paracoccidioides brasiliensis* by neutral polysaccharides. *Microbes Infect.* 8, 84–91.

Mcmahon, J. P., Wheat, J., Sobel, M. E., Pasula, R., Downing, J. F., and Martin, W. J. II. (1995). Murine laminin binds to *Histoplasma capsulatum*. A possible mechanism of dissemination. *J. Clin. Invest.* 96, 1010–1017.

Mecheri, S., Dannecker, G., Dennig, D., Poncet, P., and Hoffmann, M. K. (1993). Anti-histone autoantibodies react specifically with the B cell surface. *Mol. Immunol.* 30, 549–557.

Medeiros, A. I., Malheiro, A., Jose, P. J., Conroy, D. M., Williams, T. J., and Faccioli, L. H. (2004). Differential release of MIP-1alpha and eotaxin during infection of mice by *Histoplasma capsulatum* or inoculation of beta-glucan. *Inflamm. Res.* 53, 351–354.

Medeiros, A. I., Silva, C. L., Malheiro, A., Maffei, C. M., and Faccioli, L. H. (1999). Leukotrienes are involved in leukocyte recruitment induced by live *Histoplasma capsulatum* or by the beta-glucan present in their cell wall. *Br. J. Pharmacol.* 128, 1529–1537.

Meloan, E. L. (1952). Histoplasmosis. *Miss. Doct.* 29, 256–257.

Mendes-Giannini, M. J., Taylor, M. L., Bouchara, J. B., Burger, E., Calich, V. L., Escalante, E. D., Hanna, S. A., Lenzi, H. L., Machado, M. P., Miyaji, M., Monteiro Da Silva, J. L., Mota, E. M., Restrepo, A., Restrepo, S., Tronchin, G., Vincenzi, L. R., Xidieh, C. F., and Zenteno, E. (2000). Pathogenesis II: fungal responses to host responses: interaction of host cells with fungi. *Med. Mycol.* 38(Suppl. 1), 113–123.

Minchiotti, G., Gargano, S., and Maresca, B. (1992). Molecular cloning and expression of hsp82 gene of the dimorphic pathogenic fungus *Histoplasma capsulatum*. *Biochem. Biophys. Acta* 1131, 103–107.

Mol, P. C., and Wessels, J. G. H. (1987). Linkages between glucosaminoglycan and glucan determine alkali-insolubility of the glucan in walls of Saccharomyces cerevisiae. *FEMS Microbiol. Lett.* 41, 95–99.

Mora-Montes, H. M., Netea, M. G., Ferwerda, G., Lenardon, M. D., Brown, G. D., Mistry, A. R., Kullberg, B. J., O'callaghan, C. A., Sheth, C. C., Odds, F. C., Brown, A. J., Munro, C. A., Gow, N. A., and Deepe, G. S. Jr. (2011). Recognition and blocking of innate immunity cells by *Candida albicans* chitin. *Infect. Immun.* 79, 1961–1970.

Newman, S. L. (2005). Interaction of *Histoplasma capsulatum* with human macrophages, dendritic cells, and neutrophils. *Methods Mol. Med.* 118, 181–191.

Newman, S. L., Lemen, W., and Smulian, A. G. (2011). Dendritic cells restrict the transformation of *Histoplasma capsulatum* conidia into yeasts. *Med. Mycol.* 49, 356–364.

Nimrichter, L., Barreto-Bergter, E., Mendonca-Filho, R. R., Kneipp, L. F., Mazzi, M. T., Salve, P., Farias, S. E., Wait, R., Alviano, C. S., and Rodrigues, M. L. (2004). A monoclonal antibody to glucosylceramide inhibits the growth of Fonsecaea pedrosoi and enhances the antifungal action of mouse macrophages. *Microbes Infect.* 6, 657–665.

Nosanchuk, J. D. (2005). Protective antibodies and endemic dimorphic fungi. *Curr. Mol. Med.* 5, 435–442.

Nosanchuk, J. D., and Casadevall, A. (2006). Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob. Agents Chemother.* 50, 3519–3528.

Nosanchuk, J. D., Gomez, B. L., Youngchim, S., Diez, S., Aisen, P., Zancope-Oliveira, R. M., Restrepo, A., Casadevall, A., and Hamilton, A. J. (2002). *Histoplasma capsulatum* synthesizes melanin-like pigments in vitro and during mammalian infection. *Infect. Immun.* 70, 5124–5131.

Nosanchuk, J. D., Steenbergen, J. N., Shi, L., Deepe, G. S. Jr., and Casadevall, A. (2003). Antibodies to a cell surface histone-like protein protect against *Histoplasma capsulatum*. *J. Clin. Invest.* 112, 1164–1175.

Ojcius, D. M., Muller, S., Hasselkus-Light, C. S., Young, J. D., and Jiang, S. (1991). Plasma membrane-associated proteins with the ability to partially inhibit perforin-mediated lysis. *Immunol. Lett.* 28, 101–108.

Oliveira, D. L., Freire-De-Lima, C. G., Nosanchuk, J. D., Casadevall, A., Rodrigues, M. L., and Nimrichter, L. (2010). Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infect. Immun.* 78, 1601–1609.

Pessolani, M. C., Hunter, S. W., and Brennan, P. J. (1993). Relationship between host histones and armadillo-derived *Mycobacterium leprae*. *Int. J. Lepr. Other Mycobact. Dis.* 61, 381–388.

Pethe, K., Puech, V., Daffe, M., Josenhans, C., Drobecq, H., Locht, C., and Menozzi, F. D. (2001). *Mycobacterium smegmatis* laminin-binding glycoprotein shares epitopes with *Mycobacterium tuberculosis* heparin-binding haemagglutinin. *Mol. Microbiol.* 39, 89–99.

Pietrella, D., Bistoni, G., Corbucci, C., Perito, S., and Vecchiarelli, A. (2006). *Candida albicans* mannoprotein influences the biological function of dendritic cells. *Cell. Microbiol.* 8, 602–612.

Pietrella, D., Lupo, P., Rachini, A., Sandini, S., Ciervo, A., Perito, S., Bistoni, F., and Vecchiarelli, A. (2008). A *Candida albicans* mannoprotein deprived of its mannan moiety is efficiently taken up and processed by human dendritic cells and induces T-cell activation without stimulating proinflammatory cytokine production. *Infect. Immun.* 76, 4359–4367.

Pine, L. (1960). "Morphological and physiological characteristics of *Histoplasma capsulatum*," in *Histoplasmosis* (Springfield, IL: CC Thomas), 40–75.

Pinto, M. R., Rodrigues, M. L., Travassos, L. R., Haido, R. M., Wait, R., and Barreto-Bergter, E. (2002). Characterization of glucosylceramides in *Pseudallescheria boydii* and their involvement in fungal differentiation. *Glycobiology* 12, 251–260.

Rappleye, C. A., Eissenberg, L. G., and Goldman, W. E. (2007). *Histoplasma capsulatum* alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1366–1370.

Rappleye, C. A., Engle, J. T., and Goldman, W. E. (2004). RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. *Mol. Microbiol.* 53, 153–165.

Reiss, E. (1977). Serial enzymatic hydrolysis of cell walls of two serotypes of yeast-form *Histoplasma capsulatum* with alpha(1 leads to 3)-glucanase, beta(1 leads to 3)-glucanase, pronase, and chitinase. *Infect. Immun.* 16, 181–188.

Reiss, E., Miller, S. E., Kaplan, W., and Kaufman, L. (1977). Antigenic, chemical, and structural properties of cell walls of *Histoplasma capsulatum* yeast-form chemotypes 1 and 2 after serial enzymatic hydrolysis. *Infect. Immun.* 16, 690–700.

Reiss, E., Mitchell, W. O., Stone, S. H., and Hasenclever, H. F. (1974). Cellular immune activity of a galactomannan-protein complex from mycelia of *Histoplasma capsulatum*. *Infect. Immun.* 10, 802–809.

Rekvig, O. P., Muller, S., Briand, J. P., Skogen, B., and Van Regenmortel, M. H. (1987). Human antinuclear autoantibodies crossreacting with the plasma membrane and the N-terminal region of histone H2B. *Immunol. Invest.* 16, 535–547.

Rementeria, A., Abaitua, F., Garcia-Tobalina, R., Hernando, F., Ponton, J., and Sevilla, M. J. (1997). Resistance to candidiasis and macrophage activity in chitin-treated mice. *FEMS Immunol. Med. Microbiol.* 19, 223–230.

Rodrigues, M. L., Nakayasu, E. S., Oliveira, D. L., Nimrichter, L., Nosanchuk, J. D., Almeida, I. C., and Casadevall, A. (2008a). Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryot. Cell* 7, 58–67.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Nosanchuk, J. D., and Casadevall, A. (2008b). Vesicular trans-cell wall transport in fungi: a mechanism for the delivery of virulence-associated macromolecules? *Lipid Insights* 2, 27–40.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., and Casadevall, A. (2007). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell* 6, 48–59.

Rodrigues, M. L., Travassos, L. R., Miranda, K. R., Franzen, A. J., Rozental, S., De Souza, W., Alviano, C. S., and Barreto-Bergter, E. (2000). Human antibodies against a purified glucosylceramide from *Cryptococcus neoformans* inhibit cell budding and fungal growth. *Infect. Immun.* 68, 7049–7060.

Ross, G. D. (2002). Role of the lectin domain of Mac-1/CR3 (CD11b/CD18) in regulating intercellular adhesion. *Immunol. Res.* 25, 219–227.

Ruiz-Herrera, J., and Osorio, E. (1974). Isolation and chemical analysis of the cell wall of Morchella sp. *Antonie Van Leeuwenhoek* 40, 57–64.

Saibil, H. R. (2008). Chaperone machines in action. *Curr. Opin. Struct. Biol.* 18, 35–42.

Saliba, A., and Beatty, O. A. (1960). Pulmonary histoplasmosis, importance of diagnostic methods, with report of an early case. *JAMA* 173, 902–904.

Santoro, M. G. (2000). Heat shock factors and the control of the stress response. *Biochem. Pharmacol.* 59, 55–63.

Scheckelhoff, M., and Deepe, G. S. Jr. (2005). A deficiency in gamma interferon or interleukin-10 modulates T-Cell-dependent responses to heat shock protein 60 from *Histoplasma capsulatum*. *Infect. Immun.* 73, 2129–2134.

Schlosser, A., Thomsen, T., Moeller, J. B., Nielsen, O., Tornoe, I., Mollenhauer, J., Moestrup, S. K., and Holmskov, U. (2009). Characterization of FIBCD1 as an acetyl group-binding receptor that binds chitin. *J. Immunol.* 183, 3800–3809.

Serrano-Gomez, D., Leal, J. A., and Corbi, A. L. (2005). DC-SIGN mediates the binding of *Aspergillus fumigatus* and keratinophylic fungi by human dendritic cells. *Immunobiology* 210, 175–183.

Shaner, L., Gibney, P. A., and Morano, K. A. (2008). The Hsp110 protein chaperone Sse1 is required for yeast cell wall integrity and morphogenesis. *Curr. Genet.* 54, 1–11.

Sharon, N. (1984). Carbohydrates as recognition determinants in phagocytosis and in lectin-mediated killing of target cells. *Biol. Cell* 51, 239–245.

Shearer, G. Jr., Birge, C. H., Yuckenberg, P. D., Kobayashi, G. S., and Medoff, G. (1987). Heat-shock proteins induced during the mycelial-to-yeast transitions of strains of *Histoplasma capsulatum*. *J. Gen. Microbiol.* 133, 3375–3382.

Shi, L., Albuquerque, P. C., Lazar-Molnar, E., Wang, X., Santambrogio, L., Gacser, A., and Nosanchuk, J. D. (2008). A monoclonal antibody to *Histoplasma capsulatum* alters the intracellular fate of the fungus in murine macrophages. *Eukaryot. Cell* 7, 1109–1117.

Shimoji, Y., Ng, V., Matsumura, K., Fischetti, V. A., and Rambukkana, A. (1999). A 21-kDa surface protein of *Mycobacterium leprae* binds peripheral nerve laminin-2 and mediates Schwann cell invasion. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9857–9862.

Singh, R. S., Bhari, R., Rana, V., and Tiwary, A. K. (2011). Immunomodulatory and therapeutic potential of a mycelial lectin from *Aspergillus nidulans*. *Appl. Biochem. Biotechnol.* 165, 624–638.

Smith, C. D. (1971a). "Nutritional factors that are required for the growth and sporulation of *Histoplasma capsulatum*," in *Histoplasmosis. Proceedings of the Second National Conference*, eds L. Ajello, E. W. Chick, and M. L. Furcolow (Springfield, IL: C Thomas), 64–70.

Smith, C. D. (1971b). "The role of birds in the ecology of *Histoplasma capsulatum*," in *Histoplasmosis. Proceedings of the Second National Conference*, eds L. Ajello, E. W. Chick, and M. L. Furcolow (Springfield, IL: C Thomas), 140–148.

Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., Kolls, J. K., and Brown, G. D. (2005). The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog.* 1, e42. doi:10.1371/journal.ppat.0010042

Strasser, J. E., Newman, S. L., Ciraolo, G. M., Morris, R. E., Howell, M. L., and Dean, G. E. (1999). Regulation of the macrophage vacuolar ATPase and phagosome-lysosome fusion by *Histoplasma capsulatum*. *J. Immunol.* 162, 6148–6154.

Taborda, C. P., Da Silva, M. B., Nosanchuk, J. D., and Travassos, L. R. (2008). Melanin as a virulence factor of *Paracoccidioides brasiliensis* and other dimorphic pathogenic fungi: a minireview. *Mycopathologia* 165, 331–339.

Taylor, M. L., Duarte-Escalante, E., Perez, A., Zenteno, E., and Toriello, C. (2004). *Histoplasma capsulatum* yeast cells attach and agglutinate human erythrocytes. *Med. Mycol.* 42, 287–292.

Taylor, M. L., Duarte-Escalante, E., Reyes-Montes, M. R., Elizondo, N., Maldonado, G., and Zenteno, E. (1998). Interaction of murine macrophage-membrane proteins with components of the pathogenic fungus *Histoplasma capsulatum*. *Clin. Exp. Immunol.* 113, 423–428.

Toledo, M. S., Levery, S. B., Suzuki, E., Straus, A. H., and Takahashi, H. K. (2001a). Characterization of cerebrosides from the thermally dimorphic mycopathogen *Histoplasma capsulatum*: expression of 2-hydroxy fatty *N*-acyl (E)-Delta(3)-unsaturation correlates with the yeast-mycelium phase transition. *Glycobiology* 11, 113–124.

Toledo, M. S., Suzuki, E., Levery, S. B., Straus, A. H., and Takahashi, H. K. (2001b). Characterization of monoclonal antibody MEST-2 specific to glucosylceramide of fungi and plants. *Glycobiology* 11, 105–112.

Torosantucci, A., Bromuro, C., Chiani, P., De Bernardis, F., Berti, F., Galli, C., Norelli, F., Bellucci, C., Polonelli, L., Costantino, P., Rappuoli, R., and Cassone, A. (2005). A novel glyco-conjugate vaccine against fungal pathogens. *J. Exp. Med.* 202, 597–606.

Vallejo, M. C., Matsuo, A. L., Ganiko, L., Medeiros, L. C., Miranda, K., Silva, L. S., Freymuller-Haapalainen, E., Sinigaglia-Coimbra, R., Almeida, I. C., and Puccia, R. (2011). The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic alpha-Galactosyl epitopes. *Eukaryot. Cell* 10, 343–351.

Van Duin, D., Casadevall, A., and Nosanchuk, J. D. (2002). Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* reduces their susceptibilities to amphotericin B and caspofungin. *Antimicrob. Agents Chemother.* 46, 3394–3400.

Wheat, J. (1994). Histoplasmosis: recognition and treatment. *Clin. Infect. Dis.* 19(Suppl. 1), S19–S27.

Wheat, J. (1996). Histoplasmosis in the acquired immunodeficiency syndrome. *Curr. Top. Med. Mycol.* 7, 7–18.

Wheat, J. (1997). Histoplasmosis. Experience during outbreaks in Indianapolis and review of the literature. *Medicine (Baltimore)* 76, 339–354.

Wheat, L. J. (2001). Laboratory diagnosis of histoplasmosis: update 2000. *Semin. Respir. Infect.* 16, 131–140.

Wheat, L. J., Connolly-Stringfield, P. A., and Baker, R. L. (1990). Disseminated histoplasmosis in the acquired immune deficiency syndrome: clinical findings, diagnosis and treatment, and review of the literature. *Medicine (Baltimore)* 69, 361–374.

Wheat, L. J., Freifeld, A. G., Kleiman, M. B., Baddley, J. W., Mckinsey, D. S., Loyd, J. E., and Kauffman, C. A. (2007). Clinical practice guidelines for the management of patients with histoplasmosis: 2007 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* 45, 807–825.

Wheat, L. J., and Kauffman, C. A. (2003). Histoplasmosis. *Infect. Dis. Clin. North Am.* 17, 1–19, vii.

Woodward, J., and Wiseman, A. (1982). Fungal and other beta-glucosidases – their properties and applications. *Enzyme Microb. Technol.* 4, 73–79.

Wu, C. (1995). Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell Dev. Biol.* 11, 441–469.

Yoneda, A., and Doering, T. L. (2006). A eukaryotic capsular polysaccharide is synthesized intracellularly and secreted via exocytosis. *Mol. Biol. Cell* 17, 5131–5140.

Zancope-Oliveira, R. M., Bragg, S. L., Reiss, E., and Peralta, J. M. (1994). Immunochemical analysis of the H and M glycoproteins from *Histoplasma capsulatum*. *Clin. Diagn. Lab. Immunol.* 1, 563–568.

Zancopé-Oliveira, R. M., Reiss, E., Lott, T. J., Mayer, L. W., and Deepe, G. S. Jr. (1999). Molecular cloning, characterization, and expression of the M antigen of *Histoplasma capsulatum*. *Infect. Immun.* 67, 1947–1953.

Zancope-Oliveira, R. M., Silva Tavares, P. M. E., and De Medeiros Muniz, M. (2005). Genetic diversity of *Histoplasma capsulatum* strains in Brazil. *FEMS Immunol. Med. Microbiol.* 45, 443–449.

Zeidberg, L. D., Ajello, L., Dillon, A., and Runyon, L. C. (1952). Isolation of *Histoplasma capsulatum* from soil. *Am. J. Public Health* 42, 930–935.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 August 2011; paper pending published: 16 September 2011; accepted: 25 October 2011; published online: 18 November 2011.

Citation: Guimarães AJ, de Cerqueira MD and Nosanchuk JD (2011) Surface architecture of Histoplasma capsulatum. Front. Microbio. ***2****:225. doi: 10.3389/fmicb.2011.00225*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 20 December 2011
doi: 10.3389/fmicb.2011.00257

The *Paracoccidioides* cell wall: past and present layers toward understanding interaction with the host

Rosana Puccia*, Milene Carmes Vallejo, Alisson Leonardo Matsuo and Larissa Valle Guilhen Longo

Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo – Escola Paulista de Medicina, São Paulo, Brazil

Edited by:
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Carol Munro, University of Aberdeen, UK
Jean Paul Latge, Institut Pasteur, France
Walter Magliani, University of Parma, Italy

****Correspondence:***
Rosana Puccia, Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo – Escola Paulista de Medicina, Rua Botucatu, 862, oitavo andar, 04023-062 São Paulo, SP, Brazil.
e-mail: rpuccia@unifesp.br; ropuccia@gmail.com

The cell wall of pathogenic fungi plays import roles in the interaction with the host, so that its composition and structure may determine the course of infection. Here we present an overview of the current and past knowledge on the cell wall constituents of *Paracoccidioides brasiliensis* and *P. lutzii*. These are temperature-dependent dimorphic fungi that cause paracoccidioidomycosis, a systemic granulomatous, and debilitating disease. Focus is given on cell wall carbohydrate and protein contents, their immune-stimulatory features, adhesion properties, drug target characteristics, and morphological phase specificity. We offer a journey toward the future understanding of the dynamic nature of the cell wall and of the changes that may occur when the fungus infects the human host.

Keywords: cell wall, *Paracoccidioides brasiliensis*, *P. lutzii*, polysaccharides, adhesins, enzymes, host interaction

The 60s and 70s were prolific decades in the study of the morphological and biochemical aspects of the *Paracoccidioides brasiliensis* cell wall (San Blas and San Blas, 1984, 1994). This fungus belongs to a group of pathogens whose morphology changes according to the temperature of growth: a mycelial phase develops under mild environmental temperatures up to 26°C, while multi-budding yeasts develop at temperatures near 37°C. The infectious particles are supposedly inhaled conidia produced by environmental mycelia (Bustamante-Simon et al., 1985; San Blas, 1986; Samsonoff et al., 1991); transition to yeasts in the lungs is mandatory for the establishment of paracoccidioidomycosis (McEwen et al., 1987), which is a granulomatous mycosis prevalent in Latin America (San Blas et al., 2002). Currently, *Paracoccidioides* isolates from Brazilian central–western areas are considered a new species – *P. lutzii* ("Pb01-like"; Teixeira et al., 2009), whereas *P. brasiliensis* represents a complex of three phylogenetic groups (Matute et al., 2006). There is free access to complete genomic information for isolates Pb18, Pb3, and Pb01 at the Dimorphic Fungal Database of the Broad Institute site (http://www.broadinstitute.org/annotation/genome/dimorph_collab/MultiHome.html), and genome comparative analyses have been reported (Desjardins et al., 2011). Transcriptome data have long been available for Pb18 and Pb01 and differentially expressed genes have been detected (Felipe et al., 2003, 2005; Goldman et al., 2003; Andrade et al., 2005; Nunes et al., 2005; Bailão et al., 2006; Borges et al., 2011). Yet, genetic manipulation in *Paracoccidioides* is only starting (Sturme et al., 2011), thus the association between translated proteins and the cell wall has generally been inferred by comparison with other fungal systems.

The relationship among *P. brasiliensis* cell wall, dimorphism, and virulence has intrigued pioneer researchers (San Blas, 1982). They used sophisticated high resolution microscopy and biochemical techniques available at the time to compare the surface structure of the yeast and budding forms (Carbonell, 1967), hyphae (Carbonell and Rodriguez, 1968), transition phases (Carbonell, 1969), isolated cell walls (Carbonell et al., 1970), and to identify the main cell wall components (Kanetsuna et al., 1969, 1972; Kanetsuna and Carbonell, 1970). They studied a Venezuelan fungal isolate whose cell wall was thicker in yeasts (0.2–0.6 μm) than in hyphae (80–150 nm). Both showed a wide translucent and homogeneous layer that lied over the plasma membrane. In yeasts, an electron dense outer layer decorated with fibrils covered this inner layer. Conidia cell walls (150 to 300-nm wide) were studied only later: they showed homogeneous appearance of medium electron density, being surrounded by a thick coat of long electron dense microfibrils (Edwards et al., 1991).

Carefully isolated yeast cell wall preparations were composed of around 81% carbohydrates (38% hexoses; 43% amino sugars), 10% amino acids, and 11% lipids; for mycelia, these percentages were, respectively, 51% (38% hexoses; 13% amino sugars), 33%, and 8% (Kanetsuna et al., 1972). Isolated cell walls were biochemically fractionated into alkali-insoluble (F1) or alkali-soluble components precipitated (F2) or not (F3) upon neutralization with acetic acid. Striking structural differences were then observed (Kanetsuna et al., 1972): in yeasts, 95% of the glucans were α-linked (F2) and 5% were β-linked (F1), while only β-linked glucans (F1) were found in mycelia. In both anomeric forms, long 1,3-linked chains were prevalent, with few 1,6-linked side chains. Chitin (F1)

was more abundant in yeasts. Freeze-section-shadow techniques (Carbonell et al., 1970) revealed that F3, composed of galactomannan and amino acids, was amorphous. In yeasts, F2 contained only α-1,3-glucans and appeared as thick (100 nm) and short microfibrils that resembled the cell wall outer layer. F1, containing chitin, β-glucan, and amino acids, displayed delicate nets of irregularly disposed thin fibers (20 nm), similar to those seen in the cell wall inner layer. Successive treatments with NaOH, β-1,3-glucanase and pronase damaged the mycelium cell wall, while the round yeast shape was maintained (Kanetsuna, 1981). The importance of randomly orientated chitin microfibrils for rigidity was then stressed. The round yeast shape was also kept in a mutant strain where most α-glucan was replaced by 1,3-mannan, as well as in the parent strain that presented only reduced amounts of α-glucan due to the effect of multiple sub-culturing (San Blas et al., 1976, 1977b). Later analysis of cell wall polysaccharides proved that their composition, however, could vary not only with culture conditions, but also among isolates (San Blas and Vernet, 1977; San Blas and San Blas, 1982; San Blas et al., 1984), and especially in mutants with defects in phase transition (San Blas et al., 1977a; Hallak et al., 1982; San Blas and San Blas, 1992). At that time, these mutants were useful to review the models of dimorphism (Kanetsuna et al., 1972; Kanetsuna, 1981; San Blas and San Blas, 1984, 1994). It has recently been reported that a few single residues of α-1,4-linked glucose may be attached to the linear α-1,3-glucan chains (Sorais et al., 2010).

Although a role for α-glucan in the yeast shape was discarded, its relationship with fungal virulence has long been speculated, especially because decreased virulence and loss of externally arranged α-glucan have been observed to occur concomitantly (San Blas et al., 1976, 1977b; San Blas and Vernet, 1977; Goihman-Yahr et al., 1980; Hallak et al., 1982; Brummer et al., 1990; Kashino et al., 1990). The role of fungal cell wall polysaccharides as pathogen-associated molecular patterns (PAMPs) has been widely explored in *Candida albicans* (Netea et al., 2008). β-glucan is a cell wall PAMP that is mainly recognized by macrophage dectin-1 (Brown et al., 2002) and may interact with TLR2 (toll-like receptor 2) to activate NF-κB signaling (Gantner et al., 2003). The role of external α-glucan to prevent dectin-1 recognition and stimulus of protective immune response has been recently demonstrated in genetically manipulated *Histoplasma capsulatum* (Rappleye et al., 2004, 2007). Dectin-1 activation by wild type *P. brasiliensis* has been suggested (Diniz et al., 2004), as well as cellular immune-stimulatory responses by *P. brasiliensis* F1 cell wall fraction have been demonstrated (Silva and Fazioli, 1985; Alves et al., 1987; Silva et al., 1997, 2011; Anjos et al., 2002; de Pádua Queiroz et al., 2010). Cell wall F1 obtained from avirulent *P. brasiliensis* elicited in mice higher levels of tumor necrosis factor (TNF-α; Figueiredo et al., 1993), leukocyte recruitment, and granuloma formation (Silva and Fazioli, 1985) when compared with F1 from a virulent isolate. The effects correlated with higher β-glucan levels in the cell wall of the avirulent yeasts. Therefore, the amount of both α- and β-glucans might contribute to differential virulence among *Paracoccidioides* isolates.

B-cell activation by *P. brasiliensis* cell wall fractions has also been reported (de Oliveira et al., 1993; Benard et al., 1995; Silva and Silva, 1995; Silva et al., 1997). Complement activation (Crott et al., 1997) and neutrophil chemotaxis (Crott et al., 1993) stimulated by F1 have been observed. It has been demonstrated by immunofluorescence that C3, C3a, C3d, C3g, C4, C5b-9, and factors H and B are present on the cell surface of *P. brasiliensis* yeasts (Munk and Silva, 1992) and that complement 3 and mannose receptors are important for phagocytosis by macrophages (Jiménez et al., 2006). Also, an opsonized cell wall preparation was able to regulate B lymphocyte by complement receptors 1 and 2, which recognize activated C3 and C4 (de Agostino Biella et al., 2006). The results concerning stimulation with whole and fractionated cell wall preparations should be viewed critically, though, taking into consideration the purity of the preparations and the fact that F1 contains chitin, proteins, and β-glucans.

Water soluble galactomannan extracted in *P. brasiliensis* cell wall F3 (Kanetsuna et al., 1972) bears common antibody epitopes among dimorphs (Azuma et al., 1974). The galactomannan backbone has long α-1,6-Man*p* chains, which in hyphae are mostly decorated with α-1,6-Man*p*-α-1,2-Gal*f* ramifications (Ahrazem et al., 2003). In yeasts, these are minor structures, while β-1,6-Gal*f*-α-1,2-Man*p* and α-1,2-Man*p* prevail. Terminal β-galactofuranosyl residues are common fungal epitopes that can evoke cross-reactivity (Puccia and Travassos, 1991; Latge, 2009). Conversely, terminal α-galactofuranose has seldom been described in fungi (San Blas et al., 2005). We have recently characterized α-galactopyranosyl epitopes distributed along the cell wall and inside cytoplasmic vacuoles that were partially mapped in protein *O*-linked sugar chains (Vallejo et al., 2011). They are highly reactive with paracoccidioidomycosis patients' sera; however their role in the interaction with the host remains unknown. On the other hand, non-reducing units of terminal *N*-acetylneuraminic acid linked to sub-terminal β-galactopyranosyl units were observed on the yeast surface forming an anionic layer that possibly protects the fungus against phagocytosis (Soares et al., 1998). The nature of the glycoconjugates bearing these residues has not been described.

Cell wall-related sequences identified in the *Paracoccidioides* transcriptomes (Goldman et al., 2003; Felipe et al., 2005; Tomazett et al., 2005) and genomes (Desjardins et al., 2011) encode enzymes related to carbohydrate synthesis, remodeling, and degradation, as well as cell wall structural or associated molecules. A screening in the Pb01 transcriptome for GPI-anchored sequences (Castro et al., 2005) yielded six expression sequencing tags (ESTs) from possible cell wall structural proteins. A putative GPI-anchored membrane/cell wall β-1,3-glucan elongase capable of mediating fungal cell wall integrity (PbGel3p) was immunolocalized at the yeast surface (Castro et al., 2009).

The structural uniqueness and essentiality of fungal cell wall components indicate that proteins involved in their synthesis/degradation are potential drug targets, e.g., α-1,3-glucan synthase, glucanases [exo-β-1,3, endo-β-1,3(4)], α-1,2- and α-1,6-mannosyltransferases, chitinase, and dolichyl phosphate-D-mannose:protein *O*-D-mannosyltransferase (Desjardins et al., 2011). *Paracoccidioides* has a single β-1,3-glucan synthase gene that is overexpressed in Pb01 yeasts (Tomazett et al., 2010); however, echinocandin inhibitors of β-1,3-glucan synthesis have little effect against the yeast pathogenic phase due to the prevalence of α-glucan (San Blas and Niño-Vega, 2008; Rodríguez-Brito et al., 2010). Thus, a better target would be α-1,3-glucan synthase

and its regulatory molecules including GTPase proteins Rho1, Rho2, Rac1, and Cdc42 (Sorais et al., 2010). Not coincidentally, *P. brasiliensis* mutants knocked down for *CDC42* expression presented altered growth pattern and decreased virulence (Almeida et al., 2009).

Chitin synthases are broader targets for fungi. Genome analysis revealed the existence of seven chitin synthases in *Paracoccidioides*, one of each class, including a functional class III (Barreto et al., 2010), not described previously (Niño-Vega et al., 2000), and Pbrchs6 originally detected in transcriptome studies only in mycelia (Tomazett et al., 2005). Microarray hybridization analysis identified some chitin synthases up-regulated during the mycelium-to-yeast transition, while chitinases and endochitinases were downregulated (Nunes et al., 2005). A chitin deacetylase transcript (*CDA*), which encodes an enzyme that converts chitin to chitosan, was overexpressed in Pb01 yeasts (Felipe et al., 2005).

A number of intracellular proteins, particularly enzymes, have also been localized to the cell wall in *Paracoccidioides* and other fungi (Nombela et al., 2006). Their presumptive roles at the surface might involve essential activities like cell wall biosynthesis and remodeling, microorganism adaptation to different conditions, and also interaction with the host. We reported on a mitochondrial heat-shock chaperone Mdj1 labeled at the *P. brasiliensis* yeast cell wall (Batista et al., 2006, 2007), which is the preferential site in fully formed mycelia (Dourado et al., unpublished). Alkaline phosphatase activity on the cell wall was demonstrated by histochemical techniques in *P. brasiliensis* yeast phase (Campo-Aasen and Albornoz, 1994). A secreted 75-kDa protein with phosphatase activity was identified by confocal microscopy and flow cytometry on the cell surface, preferentially at the budding site of *P. brasiliensis* (Xander et al., 2007). Yeast cells opsonized with anti-75 kDa monoclonal antibodies showed growth inhibition, increasing phagocytic index, and *in vivo* protection against murine paracoccidioidomycosis. Formamidase, which participates in the nitrogen metabolism, was detected by immunoelectron microscopy on the cell wall of Pb01 yeast cells (Borges et al., 2009), as well as a 66-kDa secreted aspartyl protease (PbSAP) that was more abundantly expressed in the yeast phase (Tacco et al., 2009).

Adhesion of pathogens to host components is an important step in microorganism infection and dissemination. The dataset of a recently developed Fungal RV adhesin predictor algorithm (Chaudhuri et al., 2011) identified 27 proteins from *P. lutzii* (Pb01). Among them, only triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (GADPH), two cytoplasmic glycolytic enzymes, have already been identified as cell wall adhesins (Barbosa et al., 2006; Pereira et al., 2007). Both can bind to cell culture pneumocytes and to proteins associated with the extracellular matrix (ECM). Similar results have been observed for glyoxylate pathway malate synthase (da Silva Neto et al., 2009) and for putative α-1,6-mannosidase PbDfg5p (defective for filamentous growth protein), both localizing mainly to budding yeast cell walls (da Silva et al., 2008). PbDfg5p transcripts were preferentially expressed in yeasts, where it was shown to be linked to β-glucan. Enolase is another glycolytic enzyme with fibronectin-binding features and double localization in *P. brasiliensis* (Donofrio et al., 2009; Nogueira et al., 2010). This protein is also capable of activating plasminogen conversion to plasmin, a serine protease that can facilitate pathogen dissemination. Host cell exposure to recombinant enolase had enhanced fungal adhesion, possibly due to an increased exposure of *N*-acetylglucosamine, which might be recognized by paracoccin, a TNF-α stimulatory lectin (Coltri et al., 2006). Paracoccin is a cell wall chitin-binding protein with *N*-acetyl-β-D-glucosaminidase activity that plays a role in fungal growth, supposedly by participating in cell wall remodeling and organization (Ganiko et al., 2007; dos Reis Almeida et al., 2010).

Secreted gp43 is a major diagnostic *P. brasiliensis* antigen (Puccia and Travassos, 1991) that partly resides on the cell wall (Straus et al., 1996). It presents vaccine features due to the presence of T-cell epitopes that stimulate Th-1 protective responses (Taborda et al., 1998; Travassos et al., 2008), but apparently it can also stimulate innate immunity (Nakaira-Takahagi et al., 2011). Gp43 has been appointed as a virulence factor considering its broadly demonstrated adhesive properties to ECM (Vicentini et al., 1994; Gesztesi et al., 1996; Hanna et al., 2000), while an internal peptide (NLGRDAKRHL) was able to compete in binding assays (Mendes-Giannini et al., 2006). A 32-kDa ligand to laminin, fibronectin, and fibrinogen was observed on the surface of yeasts, mycelia, and conidia, which could play a role in the initial attachment of the infectious particles to the lungs (González et al., 2005, 2008). In general, experimental data have positively correlated fungal

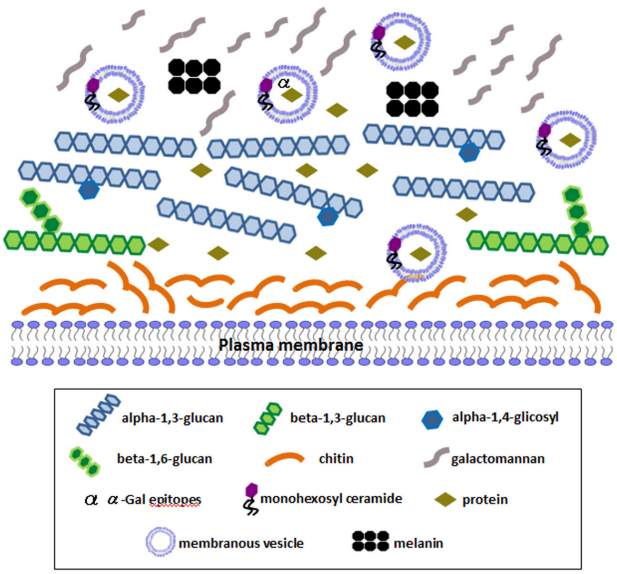


FIGURE 1 | Schematic representation of an updated view of *Paracoccidioides* yeast phase cell wall components. Structural polysaccharides are represented: an outer layer of alpha-1,3-glucan (with eventual single alpha-1,4-linked glucose residues) in higher amounts than beta-1,3-glucan, which lies in internal layers and is decorated with small beta-1,6-glucan side chains. Internal chitin fibers maintain the round shape and galactomannan composes an external fibrillar layer. Melanin is shown on the surface. Extracellular membranous vesicles containing monohexosyl ceramide transport components outside the plasma membrane, including numerous intracellular proteins. A number of intracellular proteins that have been immunolocalized to the cell wall in yeast cells using TEM or confocal microscopy (see text) are represented, as well as α-Gal epitopes. Some of them have also been detected in isolated extracellular vesicles, like enolase, GADPH, formamidase, and triosephosphate isomerase.

adhesion with the degree of virulence among isolates (Hanna et al., 2000), and also with *in vivo* passage (Mendes-Giannini et al., 2006): higher expression of the 30-kDa laminin-binding protein (Andreotti et al., 2005) and enolase (Donofrio et al., 2009) was observed in the latter situation, suggesting once again the induction of cell wall virulence factors within the host or in host-like culture conditions.

Apart from polysaccharides and (glyco)proteins, the negatively charged pigment melanin was found *in vitro* and *in vivo* in the *P. brasiliensis* conidia and yeast cell wall (Gómez et al., 2001), conferring resistance to phagocytosis, antifungal drugs, and antimicrobial oxidants (da Silva et al., 2006, 2009). Data on the composition of cell wall lipids are hard to find, although early works reported up to 80% of lipids in the cell wall F3 fraction of highly subcultured and mutant *P. brasiliensis* strains (San Blas et al., 1976, 1977a). Neutral and acidic glycosphingolipids, specifically glucosylceramide (GlcCer) and glycoinositol phosphoryl-ceramides (GIPCs), have later been fully characterized and found to probably interfere in dimorphism; they bear antigenic oligosaccharide epitopes and present surface localization, possibly residing on the cell wall (Toledo et al., 2001, 2010; Bertini et al., 2007; Takahashi et al., 2009). In *C. neoformans*, a similar GlcCer was localized to the cell wall and correlated with cell wall thickening; anti-GlcCer antibodies were able to inhibit fungal growth and budding (Rodrigues et al., 2000). Later, this component was observed in vesicle membranes that crossed the cell wall toward the extracellular compartment of *C. neoformans* (Rodrigues et al., 2007).

We have recently characterized extracellular membranous vesicles in the yeast phase of *P. brasiliensis* (Vallejo et al., 2011), where a monohexosyl ceramide has also been detected (Vallejo et al., unpublished). They carry antigens recognized by patients' sera, including components containing α-galactopyranosyl epitopes (Vallejo et al., 2011), macrophage stimulatory molecules (Vallejo et al., unpublished), and a large number of proteins recently identified using proteomic approaches (Vallejo et al., submitted). Among them are some of the proteins mentioned above to be localized to the cell wall and also surface proteins mildly extracted from *P. brasiliensis* cells (Longo et al., unpublished). Besides, over 40% of these proteins have orthologs among fungal vesicle proteins (Vallejo et al., submitted; Rodrigues et al., 2007; Albuquerque et al., 2008; Oliveira et al., 2010). In fungi, membranous extracellular vesicles seem to have several possible biogenesis routes; therefore they contain proteins of diverse nature (Casadevall et al., 2009; Oliveira et al., 2010). At least part of vesicle components could remain in the cell wall and be functional. An updated scheme of the *Paracoccidioides* cell wall, yeast phase, is presented in **Figure 1** that considers past and recent findings of cell wall components discussed in this review. It is a future challenge to understand the transport of components outside the plasma membrane using non-conventional pathways, how they interact with structural cell wall molecules and interfere in its biology. These findings will help to update our view of the dynamic nature of this essential organelle and of the changes that may occur when the fungus infects the human host.

ACKNOWLEDGMENTS

The authors receive financial support from Fundação de Amparo à Pesquisa (FAPESP, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

REFERENCES

Ahrazem, O., Prieto, A., San Blas, G., Leal, J. A., Jimenez-Barbero, J., and Bernabé, M. (2003). Structural differences between the alkali-extracted water-soluble cell wall polysaccharides from mycelial and yeast phases of the pathogenic dimorphic fungus *Paracoccidioides brasiliensis*. *Glycobiology* 13, 743–747.

Albuquerque, P. C., Nakayasu, E. S., Rodrigues, M. L., Frases, S., Casadevall, A., Zancope-Oliveira, R. M., Almeida, I. C., and Nosanchuk, J. D. (2008). Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. *Cell. Microbiol.* 10, 1695–1710.

Almeida, A. J., Cunha, C., Carmona, J. A., Sampaio-Marques, B., Carvalho, A., Malavazi, I., Steensma, H. Y., Johnson, D. I., Leão, C., Logarinho, E., Goldman, G. H., Castro, A. G., Ludovico, P., and Rodrigues, F. (2009). Cdc42p controls yeast-cell shape and virulence of *Paracoccidioides brasiliensis*. *Fungal Genet. Biol.* 46, 919–926.

Alves, L. M., Figueiredo, F., Brandão Filho, S. L., Tincani, I., and Silva, C. L. (1987). The role of fractions from *Paracoccidioides brasiliensis* in the genesis of inflammatory response. *Mycopathologia* 97, 3–7.

Andrade, R. V., da Silva, S. P., Torres, F. A., Pocas-Fonseca, M. J., Silva-Pereira, I., Maranhão, A. Q., Campos, E. G., Moraes, L. M., Jesuíno, R. S., Pereira, M., Soares, C. M., Walter, M. E., Carvalho, M. J., Almeida, N. F., Brigido, M. M., and Felipe, M. S. (2005). Overview and perspectives on the transcriptome of *Paracoccidioides brasiliensis*. *Rev. Iberoam. Micol.* 22, 203–212.

Andreotti, P. F., da Silva, J. L. M., Bailão, A. M., Soares, C. M., Benard, G., Soares, C. P., and Mendes-Giannini, M. J. (2005). Isolation and partial characterization of a 30 kDa adhesin from *Paracoccidioides brasiliensis*. *Microbes Infect.* 7, 875–881.

Anjos, A. R., Calvi, S. A., Ferracini, R., Peraçoli, M. T., Silva, C. L., and Soares, A. M. (2002). Role of *Paracoccidioides brasiliensis* cell wall fraction containing beta-glucan in tumor necrosis factor-alpha production by human monocytes: correlation with fungicidal activity. *Med. Mycol.* 40, 377–382.

Azuma, I., Kanetsuna, F., Tanaka, Y., Yamamura, Y., and Carbonell, L. M. (1974). Chemical and immunological properties of galactomannans obtained from *Histoplasma duboisii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*. *Mycopathol. Mycol. Appl.* 54, 111–125.

Bailão, A. M., Schrank, A., Borges, C. L., Dutra, V., Molinari-Madlum, E. E. W. I., Felipe, M. S. S., Mendes-Giannini, M. J. S., Martins, W. S., Pereira, M., and Soares, C. M. A. (2006). Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. *Microbes Infect.* 8, 2686–2697.

Barbosa, M. S., Bao, S. N., Andreotti, P. F., de Faria, F. P., Felipe, M. S., dos Santos, F. L., Mendes-Giannini, M. J., and Soares, C. M. (2006). Glyceraldehyde-3-phosphate dehydrogenase of *Paracoccidioides brasiliensis* is a cell surface protein involved in fungal adhesion to extracellular matrix proteins and interaction with cells. *Infect. Immun.* 74, 382–389.

Barreto, L., Sorais, F., Salazar, V., San Blas, G., and Niño-Vega, G. A. (2010). Expression of *Paracoccidioides brasiliensis* CHS3 in a *Saccharomyces cerevisiae* chs3 null mutant demonstrates its functionality as a chitin synthase gene. *Yeast* 27, 293–300.

Batista, W. L., Barros, T. F., Goldman, G. H., Morais, F. V., and Puccia, R. (2007). Identification of transcription elements in the 5′ intergenic region shared by LON and MDJ1 heat shock genes from the human pathogen *Paracoccidioides brasiliensis*. Evaluation of gene expression. *Fungal Genet. Biol.* 44, 347–356.

Batista, W. L., Matsuo, A. L., Ganiko, L., Barros, T. F., Veiga, T. R., Freymüller, E., and Puccia, R. (2006). The PbMDJ1 gene belongs to a conserved MDJ1/LON locus in thermodimorphic pathogenic fungi and encodes a heat shock protein that localizes to both the mitochondria and cell wall of *Paracoccidioides brasiliensis*. *Eukaryotic Cell* 5, 379–390.

Benard, G., Durandy, A., Assis, C. M., Hong, M. A., Orii, N. M., Sato, M. N., Mendes-Gianini, M. J., Lacaz, C. S., and Duarte, A. J. (1995). Responses of T and B lymphocytes to a *Paracoccidioides brasiliensis* cell wall extract in healthy sensitized and nonsensitized subjects. *Am. J. Trop. Med. Hyg.* 53, 189–194.

Bertini, S., Colombo, A. L., Takahashi, H. K., and Straus, A. H. (2007). Expression of antibodies directed to *Paracoccidioides brasiliensis* glycosphingolipids during the course of paracoccidioidomycosis treatment. *Clin. Vaccine Immunol.* 14, 150–156.

Borges, C. L., Bailão, A. M., Bao, S. N., Pereira, M., Parente, J. A., and Soares, C. M. A. (2011). Genes potentially relevant in the parasitic phase of the fungal pathogen *Paracoccidioides brasiliensis*. *Mycopathologia* 171, 1–9.

Borges, C. L., Parente, J. A., Barbosa, M. S., Santana, J. M., Bao, S. N., de Sousa, M. V., and Soares, C. M. A. (2009). Detection of a homotetrameric structure and protein-protein interactions of *Paracoccidioides brasiliensis* formamidase lead to new functional insights. *FEMS Yeast Res.* 10, 104–113.

Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y., and Gordon, S. (2002). Dectin-1 is a major beta-glucan receptor on macrophages. *J. Exp. Med.* 196, 407–412.

Brummer, E., Restrepo, A., Hanson, L. H., and Stevens, D. A. (1990). Virulence of *Paracoccidiodes brasiliensis*: the influence of in vitro passage and storage. *Mycopathologia* 109, 13–17.

Bustamante-Simon, B., McEwen, J. G., Tabares, A. M., Arango, M., and Restrepo-Moreno, A. (1985). Characteristics of the conidia produced by the mycelial form of *Paracoccidioides brasiliensis*. *Sabouraudia* 23, 407–414.

Campo-Aasen, I., and Albornoz, M. C. (1994). Alkaline phosphatase at the cell wall of the yeast phase of *Paracoccidioides brasiliensis*. *Mycopathologia* 127, 69–71.

Carbonell, L. M. (1967). Cell wall changes during the budding process of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*. *J. Bacteriol.* 94, 213–223.

Carbonell, L. M. (1969). Ultrastructure of dimorphic transformation in *Paracoccidioides brasiliensis*. *J. Bacteriol.* 100, 1076–1082.

Carbonell, L. M., Kanetsuna, F., and Gil, F. (1970). Chemical morphology of glucan and chitin in the cell wall of the yeast phase of *Paracoccidioides brasiliensis*. *J. Bacteriol.* 101, 636–642.

Carbonell, L. M., and Rodriguez, J. (1968). Mycelial phase of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*: an electron microscope study. *J. Bacteriol.* 96, 533–543.

Casadevall, A., Nosanchuk, J. D., Williamson, P., and Rodrigues, M. L. (2009). Vesicular transport across the fungal cell wall. *Trends Microbiol.* 17, 158–162.

Castro, N. S., de Castro, K. P., Orlandi, I., Feitosa, L. S., Rosa e Silva, L. K., Vainstein, M. H., Bao, S. N., Vai, M., and Soares, C. M. (2009). Characterization and functional analysis of the beta-1,3-glucanosyltransferase 3 of the human pathogenic fungus *Paracoccidioides brasiliensis*. *FEMS Yeast Res.* 9, 103–114.

Castro, N. S., Maia, Z. A., Pereira, M., and Soares, C. M. (2005). Screening for glycosylphosphatidylinositol-anchored proteins in the *Paracoccidioides brasiliensis* transcriptome. *Genet. Mol. Res.* 4, 326–345.

Chaudhuri, R., Ansari, F. A., Raghunandanan, M. V., and Ramachandran, S. (2011). FungalRV: adhesin prediction and immunoinformatics portal for human fungal pathogens. *BMC Genomics* 12, 192. doi:10.1186/1471-2164-12-192

Coltri, K. C., Casabona-Fortunato, A. S., Gennari-Cardoso, M. L., Pinzan, C. F., Ruas, L. P., Mariano, V. S., Martinez, R., Rosa, J. C., Panunto-Castelo, A., and Roque-Barreira, M. C. (2006). Paracoccin, a GlcNAc-binding lectin from *Paracoccidioides brasiliensis*, binds to laminin and induces TNF-alpha production by macrophages. *Microbes Infect.* 8, 704–713.

Crott, L. S., Lucisano-Valim, Y. M., Silva, C. L., and Barbosa, J. E. (1997). Interactions of F1 fractions from different strains of *Paracoccidioides brasiliensis* with human complement and with human neutrophils. *Mycopathologia* 140, 19–27.

Crott, L. S., Valim, Y. M., Silva, C. L., and Barbosa, J. E. (1993). The role of the complement system in the neutrophil functions stimulated in vitro by an alkali-insoluble cell wall fraction of *Paracoccidioides brasiliensis*. *J. Med. Vet. Mycol.* 31, 17–27.

da Silva, C. N., Barbosa, M. S., Maia, Z. A., Bao, S. N., Felipe, M. S., Santana, J. M., Mendes-Giannini, M. J. S., Pereira, M., and Soares, C. M. A. (2008). Characterization of *Paracoccidioides brasiliensis* PbDfg5p, a cell-wall protein implicated in filamentous growth. *Yeast* 25, 141–154.

da Silva, M. B., Marques, A. F., Nosanchuk, J. D., Casadevall, A., Travassos, L. R., and Taborda, C. P. (2006). Melanin in the dimorphic fungal pathogen *Paracoccidioides brasiliensis*: effects on phagocytosis, intracellular resistance and drug susceptibility. *Microbes Infect.* 8, 197–205.

da Silva, M. B., Thomaz, L., Marques, A. F., Svidzinski, A. E., Nosanchuk, J. D., Casadevall, A., Travassos, L. R., and Taborda, C. P. (2009). Resistance of melanized yeast cells of *Paracoccidioides brasiliensis* to antimicrobial oxidants and inhibition of phagocytosis using carbohydrates and monoclonal antibody to CD18. *Mem. Inst. Oswaldo Cruz* 104, 644–648.

da Silva Neto, B. R., de Fátima da Silva, J., Mendes-Giannini, M. J., Lenzi, H. L., Almeida Soares, C. M., and Pereira, M. (2009). The malate synthase of *Paracoccidioides brasiliensis* is a linked surface protein that behaves as an anchorless adhesin. *BMC Microbiol.* 9, 272. doi:10.1186/1471-2180-9-272

de Agostino Biella, C., Uecker, M., Silva, M. F., Barbosa, J. E., Silva, C. L., and Crott, L. S. (2006). Investigation of the role of complement and complement receptors in the modulation of B cell activation by a *Paracoccidioides brasiliensis* cell wall fraction. *Clin. Immunol.* 118, 324–331.

de Oliveira, S. L., da Silva, M. F., Soares, A. M., and Silva, C. L. (1993). Cell wall fractions from *Paracoccidioides brasiliensis* induce hypergammaglobulinemia. *Mycopathologia* 121, 1–5.

de Pádua Queiroz, L. Jr., Mattos, M. E. Jr., da Silva, M. F., and Silva, C. L. (2010). TGF-beta and CD23 are involved in nitric oxide production by pulmonary macrophages activated by beta-glucan from *Paracoccidioides brasiliensis*. *Med. Microbiol. Immunol.* 199, 61–69.

Desjardins, C., Champion, M., Holder, J., Muszewsha, A., Goldberg, J., Baptista, A. J., Brígido, M. M., Ferreira, M., Garcia, A., Grynberg, M., Heiman, D., Henn, M., Kodira, C., Leon-Narvaez, H., Longo, L., Ma, L., Malavazi, I., Matsuo, A., Moarais, F., Pereira, M., Brito, S., Sakthikumar, S., Salem-Izacc, S., Sykes, S., Teixeira, M. M., Vallejo, M., Willment, J. A., Young, S., Yandava, C., Zeng, Q., Zucker, J., Felipe, M. S., Goldman, G. H., Haas, B., McEwen, J. G., Nino-Vega, G. A., Puccia, R., San-Blas, G., Soares, C. M., Birren, B., and Cuomo, C. (2011). Comparative genomic analysis of human fungal pathogens causing paracoccidioidomycosis. *PLoS Genet.* 7, e1002345. doi:10.1371/journal.pgen.1002345

Diniz, S. N., Nomizo, R., Cisalpino, P. S., Teixeira, M. M., Brown, G. D., Mantovani, A., Gordon, S., Reis, L. F., and Dias, A. A. (2004). PTX3 function as an opsonin for the dectin-1-dependent internalization of zymosan by macrophages. *J. Leukoc. Biol.* 75, 649–656.

Donofrio, F. C., Calil, A. C., Miranda, E. T., Almeida, A. M., Benard, G., Soares, C. P., Veloso, S. N., Soares, C. M., and Mendes Giannini, M. J. (2009). Enolase from *Paracoccidioides brasiliensis*: isolation and identification as a fibronectin-binding protein. *J. Med. Microbiol.* 58, 706–713.

dos Reis Almeida, F. B., de Oliveira, L. L., Valle de Souza, M, Barreira, M. C., and Hanna, E. S. (2010). Paracoccin from *Paracoccidioides brasiliensis*; purification through affinity with chitin and identification of *N*-acetyl-beta-D-glucosaminidase activity. *Yeast* 27, 67–76.

Edwards, M. R., Salazar, M. E., Samsonoff, W. A., Cano, L. E., Ostrander, G. A., and Restrepo, A. (1991). Electron microscopic study of conidia produced by the mycelium of *Paracoccidioides brasiliensis*. *Mycopathologia* 114, 169–177.

Felipe, M. S., Andrade, R. V., Arraes, F. B., Nicola, A. M., Maranhão, A. Q., Torres, F. A., Silva-Pereira, I., Pocas-Fonseca, M. J., Campos, E. G., Moraes, L. M., Andrade, P. A., Tavares, A. H., Silva, S. S., Kyaw, C. M., Souza, D. P., Pereira, M., Jesuíno, R. S., Andrade, E. V., Parente, J. A., Oliveira, G. S., Barbosa, M. S., Martins, N. F., Fachin, A. L., Cardoso, R. S., Passos, G. A., Almeida, N. F., Walter, M. E., Soares, C. M., Carvalho, M. J., and Brígido, M. M. (2005). Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. *J. Biol. Chem.* 280, 24706–24714.

Felipe, M. S., Andrade, R. V., Petrofeza, S. S., Maranhão, A. Q., Torres, F. A., Albuquerque, P., Arraes, F. B., Arruda, M., Azevedo, M. O., Baptista, A. J., Bataus, L. A., Borges, C. L., Campos, E. G., Cruz, M. R., Daher, B. S., Dantas, A., Ferreira, M. A., Ghil, G. V., Jesuino, R. S., Kyaw, C. M., Leitão, L., Martins, C. R., Moraes, L. M., Neves, E. O., Nicola, A. M., Alves, E. S., Parente, J. A., Pereira, M., Pocas-Fonseca, M. J., Resende, R., Ribeiro, B. M., Saldanha, R. R., Santos, S. C., Silva-Pereira, I., Silva, M. A., Silveira, E., Simões, I. C.,

Soares, R. B., Souza, D. P., Souza, M. T., Andrade, E. V., Xavier, M. A., Veiga, H. P., Venâncio, E. J., Carvalho, M. J., Oliveira, A. G., Inoue, M. K., Almeida, N. F., Walter, M. E., Soares, C. M., and Brígido, M. M. (2003). Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. *Yeast* 20, 263–271.

Figueiredo, F., Alves, L. M., and Silva, C. L. (1993). Tumor necrosis factor production in vivo and in vitro in response to *Paracoccidioides brasiliensis* and the cell wall fractions thereof. *Clin. Exp. Immunol.* 93, 189–194.

Ganiko, L., Puccia, R., Mariano, V. S., Sant'Anna, O. A., Freymüller, E., Roque-Barreira, M. C., and Travassos, L. R. (2007). Paracoccin, an *N*-acetyl-glucosamine-binding lectin of *Paracoccidioides brasiliensis*, is involved in fungal growth. *Microbes Infect.* 9, 695–703.

Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107–1117.

Gesztesi, J. L., Puccia, R., Travassos, L. R., Vicentini, A. P., Moraes, J. Z., Franco, M. F., and Lopes, J. D. (1996). Monoclonal antibodies against the 43,000 Da glycoprotein from *Paracoccidioides brasiliensis* modulate laminin-mediated fungal adhesion to epithelial cells and pathogenesis. *Hybridoma* 15, 415–422.

Goihman-Yahr, M., Essenfeld-Yahr, E., Albornoz, M. C., Yarzábal, L., Gómez, M. H., San Martín, B., Ocanto, A., Gil, F., and Convit, J. (1980). Defect of in vitro digestive ability of polymorphonuclear leukocytes in paracoccidioidomycosis. *Infect. Immun.* 28, 557–566.

Goldman, G. H., Marques, E. R., Duarte Ribeiro, D. C., Souza Bernardes, L. A., Quiapin, A. C., Vitorelli, P. M., Savoldi, M., Semighini, C. P., Oliveira, R. C., Nunes, L. R., Travassos, L. R., Puccia, R., Batista, W. L., Ferreira, L. E., Moreira, J. C., Bogossian, A. P., Tekaia, F., Nobrega, M. P., Nobrega, F. G., and Goldman, M. H. (2003). Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. *Eukaryotic Cell* 2, 34–48.

Gómez, B. L., Nosanchuk, J. D., Díez, S., Youngchim, S., Aisen, P., Cano, L. E., Restrepo, A., Casadevall, A., and Hamilton, A. J. (2001). Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis* in vitro and during infection. *Infect. Immun.* 69, 5760–5767.

González, A., Caro, E., Muñoz, C., Restrepo, A., Hamilton, A. J., and Cano, L. E. (2008). *Paracoccidioides brasiliensis* conidia recognize fibronectin and fibrinogen which subsequently participate in adherence to human type II alveolar cells: involvement of a specific adhesin. *Microb. Pathog.* 44, 389–401.

González, A., Gómez, B. L., Diez, S., Hernández, O., Restrepo, A., Hamilton, A. J., and Cano, L. E. (2005). Purification and partial characterization of a *Paracoccidioides brasiliensis* protein with capacity to bind to extracellular matrix proteins. *Infect. Immun.* 73, 2486–2495.

Hallak, J., San Blas, F., and San Blas, G. (1982). Isolation and wall analysis of dimorphic mutants of *Paracoccidioides brasiliensis*. *Sabouraudia* 20, 51–62.

Hanna, S. A., Monteiro da Silva, J. L., and Giannini, M. J. (2000). Adherence and intracellular parasitism of *Paracoccidioides brasiliensis* in Vero cells. *Microbes Infect.* 2, 877–884.

Jiménez, M. P., Restrepo, A., Radzioch, D., Cano, L. E., and García, L. F. (2006). Importance of complement 3 and mannose receptors in phagocytosis of *Paracoccidioides brasiliensis* conidia by Nramp1 congenic macrophages lines. *FEMS Immunol. Med. Microbiol.* 47, 56–66.

Kanetsuna, F. (1981). Ultrastructural studies on the dimorphism of *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis* and *Histoplasma capsulatum*. *Sabouraudia* 19, 275–286.

Kanetsuna, F., and Carbonell, L. M. (1970). Cell wall glucans of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J. Bacteriol.* 101, 675–680.

Kanetsuna, F., Carbonell, L. M., Azuma, I., and Yamamura, Y. (1972). Biochemical studies on the thermal dimorphism of *Paracoccidioides brasiliensis*. *J. Bacteriol.* 110, 208–218.

Kanetsuna, F., Carbonell, L. M., Moreno, R. E., and Rodriguez, J. (1969). Cell wall composition of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J. Bacteriol.* 97, 1036–1041.

Kashino, S. S., Singer-Vermes, L. M., Calich, V. L., and Burger, E. (1990). Alterations in the pathogenicity of one *Paracoccidioides brasiliensis* isolate do not correlative with its in vitro growth. *Mycopathologia* 111, 173–180.

Latge, J. P. (2009). Galactofuranose containing molecules in *Aspergillus fumigatus*. *Med. Mycol.* 47, S104–S109.

Matute, D. R., McEwen, J. G., Puccia, R., Montes, B. A., San Blas, G., Bagagli, E., Rauscher, J. T., Restrepo, A., Morais, F., Niño-Vega, G., and Taylor, J. W. (2006). Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. *Mol. Biol. Evol.* 23, 65–73.

McEwen, J. G., Bedoya, V., Patiño, M. M., Salazar, M. E., and Restrepo, A. (1987). Experimental murine paracoccidiodomycosis induced by the inhalation of conidia. *J. Med. Vet. Mycol.* 25, 165–175.

Mendes-Giannini, M. J., Andreotti, P. F., Vincenzi, L. R., da Silva, J. L., Lenzi, H. L., Benard, G., Zancopé-Oliveira, R., Matos Guedes, H. L., and Soares, C. P. (2006). Binding of extracellular matrix proteins to *Paracoccidioides brasiliensis*. *Microbes Infect.* 8, 1550–1559.

Munk, M. E., and Silva, W. D. (1992). Activation of human complement system *Paracoccidioides brasiliensis* and its deposition on the yeast form cell surface. *J. Med. Vet. Mycol.* 30, 481–484.

Nakaira-Takahagi, E., Golim, M. A., Bannwart, C. F., Puccia, R., and Peraçoli, M. T. (2011). Interactions between TLR2, TLR4, and mannose receptors with gp43 from *Paracoccidioides brasiliensis* induce cytokine production by human monocytes. *Med. Mycol.* 49, 694–703.

Netea, M. G., Brown, G. D., Kullberg, B. J., and Gow, N. A. (2008). An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Microbiol.* 6, 67–78.

Niño-Vega, G. A., Munro, C. A., San Blas, G., Gooday, G. W., and Gow, N. A. (2000). Differential expression of chitin synthase genes during temperature-induced dimorphic transitions in *Paracoccidioides brasiliensis*. *Med. Mycol.* 38, 31–39.

Nogueira, S. V., Fonseca, F. L., Rodrigues, M. L., Mundodi, V., Abi-Chacra, E. A., Winters, M. S., Alderete, J. F., and Soares, C. M. (2010). *Paracoccidioides brasiliensis* enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells. *Infect. Immun.* 78, 4040–4050.

Nombela, C., Gil, C., and Chaffin, W. L. (2006). Non-conventional protein secretion in yeast. *Trends Microbiol.* 14, 15–21.

Nunes, L. R., Oliveira, R. C, Leite, D. B., Silva, V. S., Marques, E. R., Ferreira, M. E. S., Ribeiro, D. C., Bernardes, L. A. S., Goldman, M. H., Puccia, R., Travassos, L. R., Batista, W. L., Nóbrega, M. P., Nóbrega, F. G., Yang, D. Y., Pereira, C. A. B., and Goldman, G. H. (2005). Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. *Eukaryotic Cell* 4, 2115–2128.

Oliveira, D. L., Nakayasu, E. S., Joffe, L. S., Guimarães, A. J., Sobreira, T. J., Nosanchuk, J. D., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., and Rodrigues, M. L. (2010). Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. *PLoS ONE* 5, e11113. doi:10.1371/journal.pone.0011113

Pereira, L. A., Báo, S. N., Barbosa, M. S., Silva, J. L., Felipe, M. S., Santana, J. M., Mendes-Giannini, M. J., and Soares, C. M. (2007). Analysis of the *Paracoccidioides brasiliensis* triosephosphate isomerase suggests the potential for adhesin function. *FEMS Yeast Res.* 7, 1381–1388.

Puccia, R., and Travassos, L. R. (1991). 43-kilodalton glycoprotein from *Paracoccidioides brasiliensis*: immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. *J. Clin. Microbiol.* 29, 1610–1615.

Rappleye, C. A., Eissenberg, L. G., and Goldman, W. E. (2007). *Histoplasma capsulatum* alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1366–1370.

Rappleye, C. A., Engle, J. T., and Goldman, W. E. (2004). RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. *Mol. Microbiol.* 53, 153–165.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., and Casadevall, A. (2007). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryotic Cell* 6, 48–59.

Rodrigues, M. L., Travassos, L. R., Miranda, K. R., Franzen, A. J., Rozental, S., Souza, W., Alviano, C. S., and Barreto-Bergter, E. (2000). Human antibodies against a purified glucosylceramide from *Cryptococcus neoformans* inhibit cell budding and fungal growth. *Infect. Immun.* 68, 7049–7060.

Rodríguez-Brito, S., Niño-Vega, G., and San Blas, G. (2010). Caspofungin affects growth of *Paracoccidioides brasiliensis* in both morphological phases. *Antimicrob. Agents Chemother.* 54, 5391–5394.

Samsonoff, W. A., Salazar, M. E., McKee, M. L., Restrepo, A., Cano, L. E., and Edwards, M. R. (1991). Scanning electron microscopy of the conidia produced by the mycelial form of *Paracoccidioides brasiliensis*. *Mycopathologia* 114, 9–15.

San Blas, F. (1986). Ultrastructure of spore formation in *Paracoccidioides brasiliensis*. *J. Med. Vet. Mycol.* 24, 203–210.

San Blas, F., and San Blas, G. (1992). Mutants of *Paracoccidioides brasiliensis* strain IVIC Pb9 affected in dimorphism. *J. Med. Vet. Mycol.* 30, 51–60.

San Blas, F., San Blas, G., and Cova, L. J. (1976). A morphological mutant of *Paracoccidioides brasiliensis* strain IVIC Pb9. Isolation and wall characterization. *J. Gen. Microbiol.* 93, 209–218.

San Blas, G. (1982). The cell wall of fungal human pathogens: its possible role in host-parasite relationships. *Mycopathologia* 79, 159–184.

San Blas, G., and Niño-Vega, G. (2008). *Paracoccidioides brasiliensis*: chemical and molecular tools for research on cell walls, antifungals, diagnosis, taxonomy. *Mycopathologia* 165, 183–195.

San Blas, G., Niño-Vega, G., and Iturriaga, T. (2002). *Paracoccidioides brasiliensis* and paracoccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. *Med. Mycol.* 40, 225–242.

San Blas, G., Prieto, A., Bernabé, M., Ahrazem, O., Moreno, B., and Leal, J. A. (2005). Alpha-galf-1,6-alpha-mannopyranoside side chains in *Paracoccidioides brasiliensis* cell wall are shared by members of the Onygenales, but not by galactomannans of other fungal genera. *Med. Mycol.* 43, 153–159.

San Blas, G., and San Blas, F. (1982). Variability of cell wall composition in *Paracoccidioides brasiliensis*: a study of two strains. *Sabouraudia* 20, 31–40.

San Blas, G., and San Blas, F. (1984). Molecular aspects of fungal dimorphism. *Crit. Rev. Microbiol.* 11, 101–127.

San Blas, G., and San Blas, F. (1994). "Biochemistry of *Paracoccidioides brasiliensis* dimorphism," in *Paracoccidioidomycosis*, eds M. Franco, C. S. Lacaz, A. Restrepo-Moreno, and G. Del Negro (Boca Raton: CRC Press), 49–66.

San Blas, G., San Blas, F., Ordaz, D., Centeno, S., and Albornoz, M. C. (1984). Chemical changes in cell wall structure of five strains of *Paracoccidioides brasiliensis*. *Sabouraudia* 22, 255–257.

San Blas, G., San Blas, F., Ormaechea, E., and Serrano, L. E. (1977a). Cell wall analysis of an adenine-requiring mutant of the yeast-like form of *Paracoccidioides brasiliensis* strain IVIC Pb9. *Sabouraudia* 15, 297–303.

San Blas, G., San Blas, F., and Serrano, L. E. (1977b). Host-parasite relationships in the yeastlike form of *Paracoccidioides brasiliensis* strain IVIC Pb9. *Infect. Immun.* 15, 343–346.

San Blas, G., and Vernet, D. (1977). Induction of the synthesis of cell wall alpha-1,3-glucan in the yeastlike form of *Paracoccidioides brasiliensis* strain IVIC Pb9 by fetal calf serum. *Infect. Immun.* 15, 897–902.

Silva, C. L., and Fazioli, R. A. (1985). A *Paracoccidioides brasiliensis* polysaccharide having granuloma-inducing, toxic and macrophage-stimulating activity. *J. Gen. Microbiol.* 131, 1497–1501.

Silva, M. F., Bocca, A. L., Ferracini, R. Jr., Figueiredo, F., and Silva, C. L. (1997). Cellular requirements for immunomodulatory effects caused by cell wall components of *Paracoccidioides brasiliensis* on antibody production. *Clin. Exp. Immunol.* 109, 261–271.

Silva, M. F., Napimoga, M. H., Rodrigues, D. B., Pereira, S. A., and Silva, C. L. (2011). Phenotypic and functional characterization of pulmonary macrophages subpopulations after intratracheal injection of *Paracoccidioides brasiliensis* cell wall components. *Immunobiology* 216, 821–831.

Silva, M. F., and Silva, C. L. (1995). The role of somatic structure of the fungus *Paracoccidioides brasiliensis* upon B cell activation in experimental paracoccidioidomycosis. *Clin. Exp. Immunol.* 101, 321–327.

Soares, R. M., Costa e Silva-Filho, F., Rozental, S., Angluster, J., Souza, W., Alviano, C. S., and Travassos, L. R. (1998). Anionogenic groups and surface sialoglycoconjugate structures of yeast forms of the human pathogen *Paracoccidioides brasiliensis*. *Microbiology* 144, 309–314.

Sorais, F., Barreto, L., Leal, J. A., Bernabé, M., San Blas, G., and Niño-Vega, G. A. (2010). Cell wall glucan synthases and GTPases in *Paracoccidioides brasiliensis*. *Med. Mycol.* 48, 35–47.

Straus, A. H., Freymüller, E., Travassos, L. R., and Takahashi, H. K. (1996). Immunochemical and subcellular localization of the 43 kDa glycoprotein antigen of *Paracoccidioides brasiliensis* with monoclonal antibodies. *J. Med. Vet. Mycol.* 34, 181–186.

Sturme, H., Puccia, R., Goldman, G. H., and Rodrigues, F. (2011). Molecular biology of the dimorphic fungi *Paracoccidioides* spp. *Fungal Biol. Rev.* 25, 89–97.

Taborda, C. P., Juliano, M. A., Puccia, R., Franco, M., and Travassos, L. R. (1998). Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice. *Infect. Immun.* 66, 786–793.

Tacco, B. A., Parente, J. A., Barbosa, M. S., Báo, S. N., Góes, T. D., Pereira, M., and Soares, C. M. (2009). Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*. *Med. Mycol.* 47, 845–854.

Takahashi, H. K., Toledo, M. S., Suzuki, E., Tagliari, L., and Straus, A. H. (2009). Current relevance of fungal and trypanosomatid glycolipids and sphingolipids: studies defining structures conspicuously absent in mammals. *An. Acad. Bras. Cienc.* 81, 477–488.

Teixeira, M. M., Theodoro, R. C., Carvalho, M. J., Fernandes, L., Paes, H. C., Hahn, R. C., Mendoza, L., Bagagli, E., San Blas, G., and Felipe, M. S. (2009). Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus. *Mol. Phylogenet. Evol.* 52, 273–283.

Toledo, M. S., Suzuki, E., Levery, S. B., Straus, A. H., and Takahashi, H. K. (2001). Characterization of monoclonal antibody MEST-2 specific to glucosylceramide of fungi and plants. *Glycobiology* 11, 105–112.

Toledo, M. S., Tagliari, L., Suzuki, E., Silva, C. M., Straus, A. H., and Takahashi, H. K. (2010). Effect of anti-glycosphingolipid monoclonal antibodies in pathogenic fungal growth and differentiation. Characterization of monoclonal antibody MEST-3 directed to Manp-alpha1, 3-Manp alpha1,2 IPC. *BMC Microbiol.* 10, 47. doi:10.1186/1471-2180-10-47

Tomazett, P. K., Cruz, A. H., Bonfim, S. M., Soares, C. M., and Pereira, M. (2005). The cell wall of *Paracoccidioides brasiliensis*: insights from its transcriptome. *Genet. Mol. Res.* 4, 309–325.

Tomazett, P. K., Félix, C. R., Lenzi, H. L., Faria, F. P., Soares, C. M., and Pereira, M. (2010). 1,3-beta-D-Glucan synthase of *Paracoccidioides brasiliensis*: recombinant protein, expression and cytolocalization in the yeast and mycelium phases. *Fungal Biol.* 114, 809–816.

Travassos, L. R., Rodrigues, E. G., Iwai, L. K., and Taborda, C. P. (2008). Attempts at a peptide vaccine against paracoccidioidomycosis, adjuvant to chemotherapy. *Mycopathologia* 165, 341–352.

Vallejo, M. C., Matsuo, A. L., Ganiko, L., Medeiros, L. C., Miranda, K., Silva, L. S., Freymüller-Haapalainen, E., Sinigaglia-Coimbra, R., Almeida, I. C., and Puccia, R. (2011). The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic alpha-Galactosyl epitopes. *Eukaryotic Cell* 10, 343–351.

Vicentini, A. P., Gesztesi, J. L., Franco, M. F., Souza, W., Moraes, J. Z., Travassos, L. R., and Lopes, J. D. (1994). Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. *Infect. Immun.* 62, 1465–1469.

Xander, P., Vigna, A. F., Feitosa, L. S., Pugliese, L., Bailão, A. M., Soares, C. M., Mortara, R. A., Mariano, M., and Lopes, J. D. (2007). A surface 75-kDa protein with acid phosphatase activity recognized by monoclonal antibodies that inhibit *Paracoccidioides brasiliensis* growth. *Microbes Infect.* 9, 1484–1492.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 September 2011; paper pending published: 13 October 2011; accepted: 02 December 2011; published online: 20 December 2011.

*Citation: Puccia R, Vallejo MC, Matsuo AL and Longo LVG (2011) The Paracoccidioides cell wall: past and present layers toward understanding interaction with the host. Front. Microbio. **2**:257. doi: 10.3389/fmicb.2011.00257*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 21 December 2011
doi: 10.3389/fmicb.2011.00243

Sporothrix schenckii cell wall peptidorhamnomannans

Leila M. Lopes-Bezerra*

Laboratório de Micologia Celular e Proteômica, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

Edited by:
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Slawomir Milewski, Gdansk University of Technology, Poland
Everardo López-Romero, Universidad de Guanajuato, Mexico

***Correspondence:**
Leila M. Lopes-Bezerra, Laboratório de Micologia Celular e Proteômica/IBRAG, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier 524 PHLC sl. 501D, CEP 20550-013 Rio de Janeiro, Brazil. e-mail: leila@uerj.br; leila.lopes_bezerra@pq.cnpq.br

This mini-review article is dedicated to clarifying certain important biochemical aspects of *Sporothrix schenckii* cell wall peptidorhamnomannans. Cell wall components involved in the host interaction such as antigens as well as a gp70 adhesin are important molecules present on the surface of the yeast parasitic phase. Other structural glycoconjugates present on the fungus cell surface are also described here. Knowledge of the fine structure of carbohydrate epitopes expressed on the surface in both morphological phases of *S. schenckii* permitted the development of non-invasive immunochemical methods to diagnose human and feline sporotrichosis.

Keywords: *Sporothrix schenckii*, cell wall, antigens, gp70, peptidorhamnomannan

GENERAL ASPECTS OF *S. SCHENCKII*

Sporotrichosis is a subcutaneous mycosis caused by the dimorphic fungus *Sporothrix schenckii*. This disease has several clinical forms and, until now, no clear scientific evidence can explain the occurrence of cutaneous disseminated and/or extracutaneous forms of sporotrichosis in immunocompetent patients (Lima Barros et al., 2003; Schubach et al., 2008). These clinical observations indicate that besides the immunological background of the host, other factors associated with the pathogen can impair the clinical manifestations of this disease, such as virulence factors (i.e., adhesins) and antigenic molecules. Some of these factors of *S. schenckii* will be briefly discussed in this review article, focusing on the fungus surface.

Another important feature that should be highlighted is the recent description of several genotypes indicating that *S. schenckii* is a complex of cryptic species, denominated the *S. schenckii* complex (Marimon et al., 2006, 2007). Within this complex, at least five species are considered of interest due to their pathogenic potential (Arrillaga-Moncrieff et al., 2009). Among these, *S. globosa* have already been associated with human cases (Oliveira et al., 2010) and *S. brasiliensis* with feline sporotrichosis (Lopes-Bezerra L. M., unpublished results) in Brazil. The genome project of two pathogenic species, *S. schenckii* and *S. brasiliensis*, are currently under development (Felipe M. S. and Lopes-Bezerra L. M., personal communication) and will bring new insights regarding virulence factors and other relevant biological aspects of these two pathogenic species, which can be further associated with noteworthy clinical and epidemiological aspects of sporotrichosis.

Subcutaneous fungal infections are usually chronic conditions. They often begin after the skin has been pierced following an injury of some kind, since this allows infectious fungi to penetrate the skin and establish themselves inside the host. Eventually, a subcutaneous infection like sporotrichosis can evolve to severe systemic or disseminated forms. In this rather complicated puzzle, the first step for a pathogen to succeed is its capacity to adhere to and colonize host tissues. The binding of pathogen-associated molecular patterns (PAMPs) as cell wall sugar polymers and proteins, to pattern recognition receptors (PRRs) on innate immune cells triggers the activation of the immune system (Bourgeois et al., 2010; Latgé, 2010). Additionally, adhesion molecules expressed on the fungus cell surface can mediate their interaction with host cells and extracellular matrix components (Tronchin et al., 2008).

One of the well known cell wall components of *S. schenckii* is the peptidorhamnomannan. This glycopeptide or glycoconjugate is a complex of molecules with a wide range of molecular weights that are hard to purify as individual components (Lima and Lopes-Bezerra, 1997). This cell wall peptidorhamnomannan fraction (CWPR) can be recognized by IgG antibodies present in patient sera (Lloyd and Bitoon, 1971; Penha and Lopes-Bezerra, 2000) and by host cell receptors and matricial proteins (Lima et al., 2001; Figueiredo et al., 2004). Another interesting cell wall component, also present in the fungus culture filtrate, is a 70-kDa antigen (Lima and Lopes-Bezerra, 1997; Nascimento and Almeida, 2005). Furthermore, several groups have reported evidence of the role of gp70 as an important adhesin that can mediate fungus adhesion to host tissues and to matricial proteins of the basal lamina (López-Romero et al., 2011).

CELL WALL GLYCOCONJUGATES

Sporothrix schenckii has two morphological phases: a mycelial saprophytic form and a parasitic yeast-like form. Alkali-soluble and -insoluble glucans were detected in both morphological phases of this fungus. Alkali-soluble glucans of the yeast form of *S. schenckii* are linked by β(1,3), β(1,6), and β(1,4) bonds at 44, 28, and 28%, respectively. Insoluble glucans contain 66, 29, and 5%, respectively, of β(1,3), β(1,6), and β(1,4) bonds. No variations in β-glucan composition were correlated with the fungus morphological transition (Previato et al., 1979). Furthermore, there

is no evidence in the literature concerning the presence of an α-glucan on the fungus surface, similar to that described for other dimorphic fungi.

The cell wall peptidorhamnomannan is composed of 33.5% rhamnose, 57% mannose, and 14.2% protein and was characterized in the yeast-phase of *S. schenckii* (Lloyd and Bitoon, 1971). In addition to rhamnose and mannose, polysaccharides containing galactose have also been identified on the surface of this fungus. A similar component containing rhamnose and mannose was detected in a fraction isolated from the culture filtrate of *S. schenckii* (Ishizaki, 1970). This culture filtrate glycopeptide was also shown to be antigenic and its antigenicity varied according to the rhamnose:mannose molar ratio, which is influenced by the culture conditions (Takata and Ishizaki, 1983).

Another intriguing finding was the observation that *S. schenckii* peptidorhamnomannans reacted with the carbohydrate-binding protein Concanavalin A (lectin ConA), but the rhamnomannans extracted by hot alkali treatment was non-reactive (Travassos et al., 1977). These findings did not confirm the preliminary structural evidence indicating the presence of mannose residues at non-reducing ends of the carbohydrate moiety of peptidorhamnomannan (Lloyd and Bitoon, 1971). Further biochemical studies verified that ConA reactivity was associated with the presence of *O*-glycosidically linked chains in the CWPR structure (Lopes-Alves et al., 1992). The *O*-linked oligosaccharides had a common internal core of α-D-Man $1 \rightarrow 2$ α-D-Man (illustrated in **Figure 1**) and the 2-*O* substituted mannose residue was identified as the lectin binding domain present on the fungus cell surface (Lopes-Alves et al., 1992). Immunocytochemical analysis of antigens in the outermost layer of yeast cells of *S. schenckii* revealed labeling of these antigens by polyclonal antibodies colocalized with regions reactive with ConA (Castillo et al., 1990).

The peptidorhamnomannan fraction described by Lloyd and Bitoon (1971) reacted with sera from patients with sporotrichosis. The main carbohydrate epitopes identified in the *N*- and *O*-glycosidically linked chains of CWPR are illustrated in **Figures 1A,B**. In addition, an enriched glycopeptide cell wall fraction inhibited the adhesion of the yeast-phase of *S. schenckii* to extracellular matrix proteins, suggesting the presence of adhesins on the surface of this pathogen (Lima et al., 2001, 2004). In addition to rhamnose and mannose, polysaccharides containing galactose have been identified on the surface of this fungus, suggesting the presence of a cell wall galactomannan (Mendonça et al., 1976; Mendonça-Previato et al., 1980). A peptidorhamnogalactan has previously been isolated from yeast-like cells (Nakamura, 1976). Glucuronic acid residues have been described in an acidic fraction of rhamnomannans from *S. schenckii* (Gorin et al., 1977). In addition, the *O*-glycosidic chains showed important antigen determinants involving α-D-glucuronic acid residues mono- and disubstituted by terminal non-reducing rhamnose residues (**Figure 1A**; Lopes-Alves et al., 1994).

Interestingly, a structurally similar cell surface peptidorhamnomannan was described in the pathogenic fungus *Pseudallescheria boydii* and, this peptidopolysaccharide also presented *O*-linked chains (Pinto et al., 2005). Similarly to *S. schenckii*, the rhamnose containing structures appear to be immunodominant epitopes.

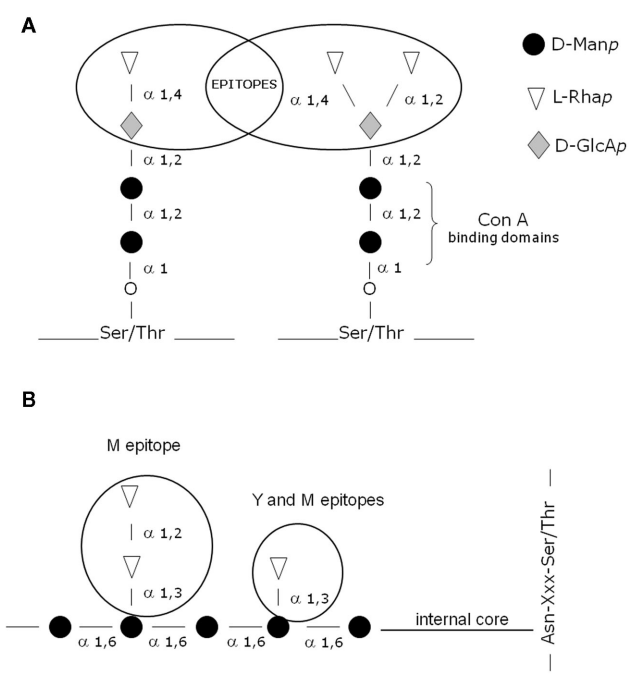


FIGURE 1 | (A,B) Schematic representation showing the general structure of the main antigenic epitopes and ConA-binding sites described on the cell wall peptidorhamonomannan of both the yeast (Y) and mycelial (M) phases of *S. schenckii*.

S. SCHENCKII ANTIGENS

Antigens isolated from both the culture filtrate and the cell wall have been used for intradermal tests in guinea pigs and humans (Nielsen, 1968). Sporotrichin tests, however, have yielded false-positive results in individuals with no history of sporotrichosis (Schneidau et al., 1964). Furthermore, antigens isolated from the culture filtrate are highly heterogeneous (Takata and Ishizaki, 1983).

Since the cell wall is the surface structure of the fungal cell and therefore mediates the relation with the host, it represents an important source of antigens. Several studies have been conducted with the objective of isolating and identifying antigens on the cell wall of *S. schenckii*. The evidence that the *O*-linked tetra- and pentasaccharides carry important antigenic epitopes (**Table 1**), α-L-Rha*p* $1 \rightarrow 2$ α-D-GLcA*p* and α-L-Rha*p* $1 \rightarrow 4$

Table 1 | Maximum inhibition (%) of *S. schenckii* peptidorhamnomannan immunoprecipitation with its derived *O*-linked oligosaccharides (at 1 μmol).

Oligosaccharide	% Inhibition
Trisaccharide	28
Tetrasaccharide	63
Pentasaccharide	82
Tri + pentasaccharide	95

Adapted from Lopes-Alves et al. (1994).

α-D-GlcA*p* (**Figure 1**), which had not been described in other species, suggested that they could be used as potential markers for serodiagnosis (Lopes-Alves et al., 1994). Using the recognition and binding capacity of these CWPR *O*-glycosidic chains to the lectin ConA, Lima and Lopes-Bezerra (1997) purified a cell wall ConA-binding fraction, called *Ss*CBF (*S. schenckii* ConA-*b*inding *f*raction). In ELISA assays using human sera (gold standard), this antigenic fraction was recognized by IgG antibodies present in the sera of patients with all clinical forms of sporotrichosis (Bernardes-Engemann et al., 2005) and this recognition was specifically blocked by the purified *O*-linked tetra- and pentasaccharides (Penha and Lopes-Bezerra, 2000). This serological test has also been useful for therapeutic follow-up of patients (Bernardes-Engemann et al., 2005). Another serological ELISA test was proposed in the literature (Almeida-Paes et al., 2007), based on a culture filtrate antigenic preparation. The ELISA tests described in the current literature showed values of specificity and sensibility ranging from 80 to 95%. Another recent study confirmed that the *Ss*CBF antigen could also be useful for the serodiagnosis of feline sporotrichosis (Fernandes et al., 2011).

In conclusion, non-invasive diagnostic tools are available to promptly diagnose not only the classic lymphocutaneous or fixed cutaneous forms of sporotrichosis, but also the disseminated and atypical forms of this disease or unresolved cases that are negative according to the routine mycological test.

S. SCHENCKII gp70

In the cell wall *Ss*CBF antigenic fraction three main antigens with approximate molecular weights of 84 (gp84), 70 (gp70), and 58 kDa which reacted with a rabbit anti-*S. schenckii* serum were identified (Lima and Lopes-Bezerra, 1997). 2D-immunoblotting with anti-*S. schenckii* antibodies had shown that a 70-kDa glycoprotein was the major antigen detected in the cell wall of the mycelial and the yeast-like phases of *S. schenckii* and, a 60-kDa antigenic glycoprotein was present only on the surface of yeast-like cells (Ruiz-Baca et al., 2011).

Further studies had shown that sera from mice infected with *S. schenckii* had IgG1 and IgG3 antibodies reacting with a gp70 antigenic component present in the fungus culture filtrate (Nascimento and Almeida, 2005). A monoclonal antibody was raised against the secreted gp70 antigen, mAb P6E7. This monoclonal antibody had a protective effect *in vivo* by passive immunization of mice infected with *S. schenckii* (Nascimento et al., 2008). Besides, the secreted gp70 antigen was also shown to be expressed on the cell wall of the yeast-like phase of several clinical isolates of *S. schenckii* (Teixeira et al., 2009). This antigenic glycoprotein seems to have other important functions. There is strong evidence for its role as an adhesin which mediates *S. schenckii* interaction with the extracellular matrix protein fibronectin and also, adhesion of this fungus to the dermis of mouse tails (Ruiz-Baca et al., 2009; Teixeira et al., 2009). The co-localization on the yeast cell surface of a fibronectin adhesin and the mAb P6E7 antibody was confirmed by confocal microscopy, suggesting that the gp70 exoantigen and the cell wall protein with apparent MW of 70 kDa are similar components (Teixeira et al., 2009).

The purified gp70 exhibited a pI of 4.1 and about 5.7% of its molecular mass was contributed by *N*-linked glycans with no evidence for *O*-linked oligosaccharides (Ruiz-Baca et al., 2009).

ACKNOWLEDGMENTS

Leila M. Lopes-Bezerra is a research fellow of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ).

REFERENCES

Almeida-Paes, R., Pimenta, M. A, Pizzini, C. V., Monteiro, P. C., Peralta, J. M., Nosanchuk, J. D., and Zancopé-Oliveira, R. M. (2007). Use of mycelial-phase *Sporothrix schenckii* exoantigens in an enzyme-linked immunosorbent assay for diagnosis of sporotrichosis by antibody detection. *Clin. Vaccine Immunol.* 14, 244–249.

Arrillaga-Moncrieff, I., Capilla, J., Mayayo, E., Marimon, R., Mariné, M., Gené, J., Cano, J., and Guarro, J. (2009). Different virulence levels of the species of *Sporothrix* in a murine model. *Clin. Microbiol. Infect.* 15, 651–655.

Bernardes-Engemann, A. R., Costa, R. C., Miguens, B. R., Penha, C. V., Neves, E., Pereira, B. A., Dias, C. M., Mattos, M., Gutierrez, M. C., Schubach, A., Oliveira Neto, M. P., Lazéra, M., and Lopes-Bezerra, L. M. (2005). Development of an enzyme-linked immunosorbent assay for the serodiagnosis of several clinical forms of sporotrichosis. *Med. Mycol.* 43, 487–493.

Bourgeois, C., Majer, O., Frohner, I. E., Tierney, L., and Kuchler, K. (2010). Fungal attacks on mammalian hosts: pathogen elimination requires sensing and testing. *Curr. Opin. Microbiol.* 13, 401–408.

Castillo, M. C., Tapia, F. J., and Arciniegas, E. (1990). Ultrastructural localization of specific surface antigens in the dimorphic fungus *Sporothrix schenckii*. *J. Med. Vet. Mycol.* 28, 91–94.

Fernandes, G. F., Lopes-Bezerra, L. M., Bernardes-Engemann, A. R., Schubach, T. M., Dias, M. A., Pereira, S. A., and Camargo, Z. P. (2011). Serodiagnosis of sporotrichosis infection in cats by enzyme-linked immunosorbent assay using a specific antigen, SsCBF, and crude exoantigens. *Vet. Microbiol.* 147, 445–449.

Figueiredo, C. C., Lima, O. C., Carvalho, L., Lopes-Bezerra, L. M., and Morandi, V. (2004). The in vitro interaction of *Sporothrix schenckii* with human endothelial cells is modulated by cytokines and involves endothelial surface molecules. *Microb. Pathog.* 36, 177–188.

Gorin, P. A. J., Haskins, R. H., Travassos, L. R., and Mendonça-Previato, L. (1977). Further studies on the rhamnomannans and acidic rhamnomannans of *Sporothrix schenckii* and *Ceratocystis stenoceras*. *Carbohydr. Res.* 55, 21–33.

Ishizaki, H. (1970). Some antigenic substances from culture filtrate of *Sporothrix schenckii*. *Jpn. J. Dermatol. B* 80, 16–23.

Latgé, J. P. (2010). Tasting the fungal cell wall. *Cell. Microbiol.* 12, 863–872.

Lima, O. C., Bouchara, J. P., Renier, G., Marot-Leblond, A., Chabasse, D., and Lopes-Bezerra, L. M. (2004). Immunofluorescence and flow cytometry analysis of fibronectin and laminin binding to *Sporothrix schenckii* yeast cells and conidia. *Microb. Pathog.* 37, 131–140.

Lima, O. C., Figueiredo, C. C., Previato, J. O., Mendonça-Previato, L., Morandi, V., and Lopes-Bezerra, L. M. (2001). Involvement of fungal cell wall components in adhesion of *Sporothrix schenckii* to human fibronectin. *Infect. Immun.* 69, 6874–6880.

Lima, O. C., and Lopes-Bezerra, L. M. (1997). Identification of a concanavalin A-binding antigen of the cell surface of Sporothrix schenckii. *J. Med. Vet. Mycol.* 35, 167–172.

Lima Barros, M. B, de Oliveira Schubach, A., Galhardo, M. C., Schubach, T. M., dos Reis, R. S., Conceição, M. J., and do Valle, A. C. (2003). Sporotrichosis with widespread cutaneous lesions: report of 24 cases related to transmission by domestic cats in Rio de Janeiro, Brazil. *Int. J. Dermatol.* 42, 677–681.

Lloyd, K. O., and Bitoon, M. A. (1971). Isolation and purification of a peptido-rhamnomannan from the yeast form of *Sporothrix schenckii*. Structural and immunochemical studies. *J. Immunol.* 107, 663–671.

Lopes-Alves, L. M., Mendonça-Previato, L., Fournet, D., Degant, P., and Previato, J. O. (1992). O-glycosidically linked oligosaccharides from peptidorhamnomannans of *Sporothrix schenckii*. *Glycoconj. J.* 9, 75–81.

Lopes-Alves, L. M., Travassos, L. R., Previato, J. O., and Mendonça-Previato, L. (1994). Novel antigenic determinants from peptidorhamnomannans of *Sporothrix schenckii*. *Glycobiology* 4, 281–288.

López-Romero, E., Reyes-Montes Mdel, R., Pérez-Torres, A., Ruiz-Baca, E., Villagómez-Castro, J. C., Mora-Montes, H. M., Flores-Carreón, A., and Toriello, C. (2011). *Sporothrix schenckii* complex and sporotrichosis, an emerging health problem. *Future Microbiol.* 6, 85–102.

Marimon, R., Cano, J., Gené, J., Sutton, D. A., Kawasaki, M., and Guarro, J. (2007). *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. *J. Clin. Microbiol.* 45, 3198–3206.

Marimon, R., Gené, J., Cano, J., Trilles, L., Dos Santos Lazéra, M., and Guarro, J. (2006). Molecular phylogeny of *Sporothrix schenckii*. *J. Clin. Microbiol.* 44, 3251–3256.

Mendonça, L., Gorin, P. A. J., Lloyd, K. O., and Travassos, L. R. (1976). Polymorphism of *Sporothrix schenckii* surface polysaccharides as a function of morphological differentiation. *Biochemistry* 15, 2423–2431.

Mendonça-Previato, L., Gorin, P. A. J., and Travassos, L. R. (1980). Galactose-containing polysaccharides from the human pathogen to *Sporothrix schenckii*. *Infect. Immun.* 29, 934–939.

Nakamura, Y. (1976). Purification and isolation of a biologically active peptide-rhamnogalactan from to *Sporothrix schenckii*. *J. Dermatol.* 3, 25–29.

Nascimento, R. C., and Almeida, S. R. (2005). Humoral immune response against soluble and fractionate antigens in experimental sporotrichosis. *FEMS Immunol. Med. Microbiol.* 43, 241–247.

Nascimento, R. C., Espíndola, N. M., Castro, R. A., Teixeira, P. A., Loureiro y Penha, C. V., Lopes-Bezerra, L. M., and Almeida, S. R. (2008). Passive immunization with monoclonal antibody against a 70-kDa putative adhesin of *Sporothrix schenckii* induces protection in murine sporotrichosis. *Eur. J. Immunol.* 38, 3080–3089.

Nielsen, H. S. Jr. (1968). Biological properties of skin test antigens of yeast form *Sporotrichum schenckii*. *J. Infect. Dis.* 118, 173–180.

Oliveira, M. M., Almeida-Paes, R., Medeiros Muniz, M., Lima Barros, M. B., Galhardo, M. C., and Zancope-Oliveira, R. M. (2010). Sporotrichosis caused by *Sporothrix globosa* in Rio de Janeiro, Brazil: case report. *Mycopathologia* 169, 359–363.

Penha, C. V., and Lopes-Bezerra, L. M. (2000). Concanavalin A-binding cell wall antigens of *Sporothrix schenckii*: a serological study. *Med. Mycol.* 38, 1–7.

Pinto, M. R., Gorin, P. A., Wait, R., Mulloy, B., and Barreto-Bergter, E. (2005). Structures of the O-linked oligosaccharides of a complex glycoconjugate from *Pseudallescheria boydii*. *Glycobiology* 15, 895–904.

Previato, J. O., Gorin, P. A. J., Haskins, R. H., and Travassos, L. R. (1979). Soluble and insoluble glucans from different cell types of *Sporothrix schenckii*. *Exp. Mycol.* 3, 92–105.

Ruiz-Baca, E., Mora-Montes, H. M., López-Romero, E., Toriello, C., Mojica-Marín, V., and Urtiz-Estrada, N. (2011). 2D-immunoblotting analysis of *Sporothrix schenckii* cell wall. *Mem. Inst. Oswaldo Cruz* 106, 248–250.

Ruiz-Baca, E., Toriello, C., Perez-Torres, A., Sabanero-Lopez, M., Villagomez-Castro, J. C., and Lopez-Romero, E. (2009). Isolation and some properties of a glycoprotein of 70 kDa (Gp70) from the cell wall of *Sporothrix schenckii* involved in fungal adherence to dermal extracellular matrix. *Med. Mycol.* 47, 185–196.

Schneidau, J. D., Lamar, L. M., and Hairston, M. A. (1964). Cutaneous hypersensitivity to sporotrichin in Louisiana. *JAMA* 188, 371–373.

Schubach, A., Barros, M. B., and Wanke, B. (2008). Epidemic sporotrichosis. *Curr. Opin. Infect. Dis.* 21, 129–133.

Takata, M., and Ishizaki, H. (1983). Correlations among culture times, sugar composition and biological activities of *Sporothrix schenckii* antigens. *Mycopathologia* 84, 31–39.

Teixeira, P. A., Castro, R. A., Nascimento, R. C., Tronchin, G., Perez-Torres, A, Lazéra, M., Almeida, S. R., Bouchara, J. P., Loureiro y Penha, C. V., and Lopes-Bezerra, L. M. (2009). Cell surface expression of adhesins for fibronectin correlates with virulence in *Sporothrix schenckii*. *Microbiology* 155, 3730–3738.

Travassos, L. R., Souza, W., Mendonça-Previato, L., and Lloyd, K. O. (1977). Location and biochemical nature pf surface components reacting with concanavalin A in different cell types of *Sporothrix schenckii*. *Exp. Mycol.* 1, 293–305.

Tronchin, G., Pihet, M., Lopes-Bezerra, L. M., and Bouchara, J. P. (2008). Adherence mechanisms in human pathogenic fungi. *Med. Mycol.* 46, 749–772.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 September 2011; paper pending published: 13 October 2011; accepted: 22 November 2011; published online: 21 December 2011.

*Citation: Lopes-Bezerra LM (2011) Sporothrix schenckii cell wall peptidorhamnomannans. Front. Microbio. **2**:243. doi: 10.3389/fmicb.2011.00243*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 06 September 2011
doi: 10.3389/fmicb.2011.00181

Multiple disguises for the same party: the concepts of morphogenesis and phenotypic variations in *Cryptococcus neoformans*†

Oscar Zaragoza*

Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain

Edited by:
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Andrew Alspaugh, Duke University School of Medicine, USA
Kirsten Nielsen, University of Minnesota, USA

***Correspondence:**
Oscar Zaragoza, Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo, Km2, Majadahonda, 28220 Madrid, Spain.
e-mail: ozaragoza@isciii.es

Although morphological transitions (such as hyphae and pseudohyphae formation) are a common feature among fungi, the encapsulated pathogenic yeast *Cryptococcus neoformans* is found during infection as blastoconidia. However, this fungus exhibits striking variations in cellular structure and size, which have important consequences during infection. This review will summarize the main aspects related with phenotypic and morphological variations in *C. neoformans*, which can be divided in three classes. Two of them are related to changes in the capsule, while the third one involves changes in the whole cell. The three morphological and phenotypic variations in *C. neoformans* can be classified as: (1) changes in capsule structure, (2) changes in capsule size, and (3) changes in the total size of the cell, which can be achieved by the formation of cryptococcal giant/titan cells or microforms. These changes have profound consequences on the interaction with the host, involving survival, phagocytosis escape and immune evasion and dissemination. This article will summarize the main features of these changes, and highlight their importance during the interaction with the host and how they contribute to the development of the disease.

Keywords: *Cryptococcus neoformans*, morphogenesis, capsule enlargement, giant cells, micro-cells, antigenic variations

INTRODUCTION

Adaptation of pathogenic fungi to the host environment is key to understanding the diseases caused by these microorganisms. During infection, adaptive responses are triggered to evade the immune response and survive in the host. Most of these responses are regulated by signaling pathways, which induce adaptation to the host nutritional environment, pH and osmotic pressure, and also provide resistance to free radicals and antimicrobial molecules. In addition, pathogenic fungi frequently change their cellular morphology. For example, it is well known how different yeasts induce pseudohyphae and hyphae during infection. These cellular forms have been well characterized in *Candida albicans*, and are produced by other *Candida* sp (Kurtzman and Fell, 1997; Whiteway and Bachewich, 2007). Filamentous fungi, such as *Aspergillus* spp also induce the formation of filaments after the germination of the spores during infection. Other fungi exhibit dimorphism regulated by the temperature, as happens with *Histoplasma capsulatum* and *Blastomyces dermatitidis* (Kane, 1984; Maresca and Kobayashi, 1989). These changes are important for the course of infection, and provide the fungus a mechanism to reach new nutritional environments, evade the host immune response, and disseminate through the organism.

The pathogenic fungus *Cryptococcus neoformans* represents a unique example of eukaryotic virulence. Its incidence increased significantly at the end of the twentieth century associated with the AIDS pandemic (Casadevall and Perfect, 1998). Recent studies identified that *C. neoformans* also accounts for 10% of fungal infections in transplant recipients (Singh and Forrest, 2009; Hosseini-Moghaddam and Husain, 2010). Although its incidence in countries where the highly active antiretroviral therapy (HAART) is available has decreased, its associated mortality is still high (Dromer et al., 2007). The incidence in developing countries remains unacceptable, since it has been estimated that *C. neoformans* causes more than 650,000 deaths per year in these countries (Park et al., 2009).

Cryptococcus neoformans possesses a complex polysaccharide capsule that surrounds the cell body. The capsule is the main virulence factor of the yeast because it exerts a large number of deleterious effects on the host (see review in Zaragoza et al., 2009). Among others, it confers resistance to stress conditions, such as dehydration, free radicals, and antimicrobial compounds (Aksenov et al., 1973; Zaragoza et al., 2008). The capsule can interfere with the host immune response at multiple levels. For example, it has antiphagocytic properties, inhibits Ab production, depletes complement, inhibits leukocyte migration, and induces apoptosis in macrophages and T cells (Murphy and Cozad, 1972; Kozel et al., 1977; Macher et al., 1978; Lipovsky et al., 2000; Vecchiarelli, 2000; Ellerbroek et al., 2002; Monari et al., 2006). In addition, *C. neoformans* expresses other virulence factors during infection, such as cell wall melanin, proteases, and phospholipases (see reviews in Casadevall and Perfect, 1998; Nosanchuk and Casadevall, 2003; Heitman et al., 2011).

†I dedicate this article to my two sons, Javier and David, for filling up my days with joy and happiness.

Cryptococcus neoformans has traditionally been considered a yeast that does not exhibit filamentous growth or dimorphism, except during the mating process (for reviews, see Heitman et al., 2011; Kozubowski and Heitman, 2011). Although some pseudohyphal forms have occasionally been described during infection (Neilson et al., 1978; Williamson et al., 1996; Gazzoni et al., 2010), this is a rare phenomenon, and it is believed that *C. neoformans* does not undergo morphological changes in the host. While this concept is true, many articles demonstrate that *C. neoformans* displays a complex morphogenetic program which results in the appearance of multiple phenotypic forms. In fact, the final result of these variations is the production of multiple types of yeast cells that may differ in their recognition by the immune system.

In this review, I discuss the concepts of phenotypic and morphologic variations in *C. neoformans* with the purpose of illustrating the complexity of different forms that this pathogen exhibits *in vivo* and how they contribute to the development of the disease. These changes are of three types: changes in capsule structure, changes in capsule size, and changes in total cell size. Some of these different variations are illustrated in **Figure 1**. I describe the main characteristics of these variations, and also highlight their importance in infection.

CHANGES IN CAPSULE STRUCTURE

The capsule of *C. neoformans* is mainly composed of polysaccharide. The major component is glucuronoxylomannan (GXM), which comprises around 90–95% of the total mass of the capsule (Cherniak et al., 1980). The other component has been classically known as galactoxylomannan (GalXM; Cherniak et al., 1982), which accounts for 5–10% of the capsular polysaccharide. Both components have high molecular weights, around 10^6 Da for GXM and 10^5 Da for GalXM (Cherniak et al., 1982; McFadden et al., 2006b). The structure of these polysaccharides has been largely studied and revised (see reviews in Doering, 2000; Bose et al., 2003; Janbon, 2004; Zaragoza et al., 2009; Janbon and Doering, 2011). Briefly, GXM is composed of a chain of mannose residues, with substitutions of xylose and glucuronic acid. GalXM was previously believed to be composed of a chain of galactose with substitutions of xylose and mannose. Recently, it was found that it also contains residues of glucuronic acid (Heiss et al., 2009), so the name of glucuronoxylomannangalactan (GXMGal) has been suggested for this polysaccharide. GXM is highly 6-O-acetylated (Cherniak et al., 1988a; Cherniak and Sundstrom, 1994; Janbon et al., 2001; McFadden et al., 2006b). In addition to polysaccharides, the capsule consists of a small proportion of mannoproteins (MPs; Levitz and Specht, 2006). While GXM localizes throughout the whole capsule, GXMGal and MPs seem to localize in regions close to the cell wall (De Jesus et al., 2010). More recently, other components have been suggested to be present in the capsule too, such as sialic acid and chitin-like structures (Rodrigues et al., 2008; Gahrs et al., 2009), but their role in capsule architecture remains unknown.

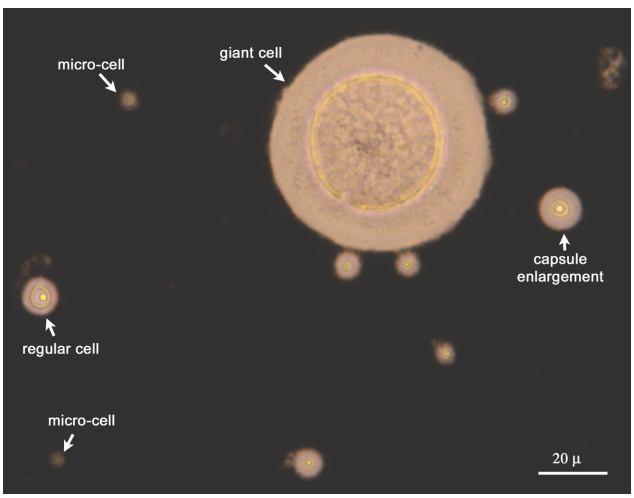


FIGURE 1 | Heterogeneity of fungal population extracted from the lungs of infected mice. The yeast cells isolated from the lungs of infected mice were isolated and suspended in India Ink to visualize the capsule. As shown, multiple forms of *C. neoformans* (regular cells, cells with enlarged capsule, fungal giant/titan cells, and microforms) are present during infection.

The steps involved in capsule synthesis and fibers assembly are poorly understood. A few capsular genes necessary to produce the capsule (known as *CAP*) have been described (Chang and Kwon-Chung, 1994, 1998, 1999; Chang et al., 1995), but their exact biochemical function is not fully characterized. More recently, more genes have been identified by massive analysis of a *C. neoformans* mutant library (Liu et al., 2008), but the interplay of these, and how they regulate capsule synthesis remains to be elucidated. Furthermore, it has been shown that secretion of vesicles loaded with capsular polysaccharide is an important process in capsule synthesis (Rodrigues et al., 2007).

Although the main components of the capsule are characterized, how the polysaccharides are spatially organized is still unknown. The polysaccharide is organized in fibers that can self-associate through non-covalent interactions (Pierini and Doering, 2001; McFadden et al., 2006b). In the case of GXM, seven different basic structures have been described (Cherniak et al., 1998; Nimrichter et al., 2007), but how these structures are organized, and repeated is still unknown. Recent findings indicate that the capsular fibers are highly branched (Cordero et al., 2011). A clear conclusion from the basic analysis of the capsule is that multiple structures, repetitions, and ramifications are possible in the same GXM or GXMGal molecule (see reviews in McFadden et al., 2006a; Rodrigues et al., 2011; and in Rodrigues et al. in this same issue). As a consequence, *C. neoformans* has classically been classified in five different serotypes (A, B, C, D, and AD hybrid), based on the different antigenic properties of the capsular polysaccharide. The use of structural and analytical techniques, such as NMR or mass spectrometry has demonstrated that the capsular variation occurs at inter- and intra-strain levels (Cherniak et al., 1988a,b; McFadden et al., 2006b). The use of monoclonal antibodies that bind to the capsule has been extremely useful to understand the variability and complexity of this structure (Dromer et al., 1987; Eckert and Kozel, 1987; Todaro-Luck et al., 1989; van de Moer et al., 1990; Casadevall et al., 1992, 1994; Pirofski et al., 1995). Most of these mAbs have different affinities and specificities for different epitopes in the capsule, which indicates that the capsule

has a complex and heterogeneous structure. Moreover, the epitope distribution for some of these Abs is not homogeneous through the capsule (Maxson et al., 2007b).

The use of mAbs has been of particular interest to show, not only the variability of the capsule between strains, but also how this structure changes in the same strain depending on the environment. McFadden et al. (2007) demonstrated that the binding of two different antibodies to the capsule changed when the fungal strain was grown in different media. Consistent with these findings, the capsule exhibits a high heterogeneity in the expression of epitopes, which depends on cell age, growth conditions, and serotype (Garcia-Hermoso et al., 2004; Gates-Hollingsworth and Kozel, 2009). The relationship between cell age and capsule structure is supported by the fact that the capsule of the buds is structurally different from the capsule of the mother cells (Pierini and Doering, 2001; Zaragoza et al., 2006; Gates-Hollingsworth and Kozel, 2009). Taken together, these findings clearly illustrate how the capsule is a dynamic structure that undergoes important rearrangements.

Changes of the capsule structure also occur *in vivo*, which suggest that these variations have consequences on the interaction with the host. A seminal article by Charlier et al. (2005) elegantly demonstrates how the capsule undergoes significant rearrangements during infection. The authors use two mAbs which present different *in vitro* serotype specificity (E1 mAb, which binds to serotype A, and CRND-8, which binds to serotype D). When animals are challenged with a serotype A strain, the fungus bound E1 mAb at early times of infection. After several days of infection, the binding specificity changed, and the fungal cells preferentially bound the CRND-8 mAb. This evolution of the capsule structure seems to be organ specific (Garcia-Hermoso et al., 2004). Moreover, there is evidence that similar capsular rearrangements occur during human cryptococcal meningitis (Cherniak et al., 1995), where structural changes in the capsule of isolates from patients with recurrent cryptococcosis have been described. The molecular basis underlying the variability in capsule structures are not yet known.

The capsule can also significantly change the polysaccharide density. Many reports indicate that the density of the capsule varies according to its spatial distribution, being denser at the inner locations close to the cell wall (Pierini and Doering, 2001; Gates et al., 2004; Zaragoza et al., 2006; Maxson et al., 2007b). In addition, the density can significantly increase with cellular age and independently of capsule size (Maxson et al., 2007b). This change is associated with a higher resistance of the capsule to factors that induce its separation from the cell, such as γ-radiation, which suggests that increase in density is associated with a higher crosslinking of the polysaccharide fibers (Maxson et al., 2007b). Increase in polysaccharide density also occurs during infection (Gates et al., 2004).

IMPORTANCE OF CAPSULE STRUCTURE CHANGES DURING THE INTERACTION WITH THE HOST

The fact that capsule structure can change depending on the environmental conditions has profound consequences for the interaction with the host, since this structure is the first barrier recognized by the immune cells. An example of this situation is provided by the opsonic effect of some antibodies that do not bind to Fc receptors in the macrophages, such as IgM or Fab fragments, and that in consequence, are not opsonic by themselves. In these cases, the binding of the antibody produces a structural change in the capsule that exposes polysaccharide epitopes that can bind to CD18 and induce phagocytosis through the complement receptors (Netski and Kozel, 2002; Taborda and Casadevall, 2002). Conversely, changes in capsule structure alter the recognition of Abs. For example, changes in the acetylation can dramatically influence the binding of Abs to the capsule (Todaro-Luck et al., 1989; Kozel et al., 2003). Capsular differences between serotypes also influence the binding pattern of the same Ab (which can be annular or punctuate, depending on the serotype), and this correlates with opsonic efficiency and protection during infection (Cleare and Casadevall, 1998; Mukherjee et al., 1998). These findings suggest that if a specific adaptive response elicits certain Abs during infection, the ability to change the capsular structure will yield fungal cells which will not be recognized by those antibodies.

Changes in capsular structures occur during the cross of the brain–blood barrier, suggesting that capsular variations are required for dissemination and organ colonization (Garcia-Hermoso et al., 2004; Charlier et al., 2005).

In addition to Ab recognition, capsule structure affects the deposition of other molecules of the immune system. For example, the capsule is a potent inducer of the alternative pathway of complement activation, and it has been shown that differences in capsular structure influence the rate of complement deposition on the capsule. While all the serotypes accumulated the same amount of C3 molecules, the kinetics of the process was much faster when strains from serotypes A and D were used compared to strains from serotypes B and C (Young and Kozel, 1993).

The change in capsule density is another factor that determines some aspects of the interaction with the host. In particular, capsule density influences the penetration of different molecules of the immune system, such as complement or Abs, in the capsule, and affects the biological activity of these molecules (Zaragoza et al., 2003b; Gates and Kozel, 2006; Zaragoza and Casadevall, 2006). More recently, it has been demonstrated that a high degree of branching and viscosity of the capsule enhances some properties of the polysaccharide that are important during the interaction with the host, such as complement activation and phagocytosis avoidance, and the ability to act as a protective agent against free radicals (Cordero et al., 2011).

Another phenomenon that highlights the importance of capsule structure during infection is phenotypic switching. In *C. neoformans*, two different types of colony morphologies, smooth and mucoid, have been described (Goldman et al., 1998) that present differences in capsule viscosity (McFadden et al., 2007). These phenotypic variations are involved in virulence, since mucoid isolates are hypervirulent (Goldman et al., 1998; Guerrero et al., 2010).

Changes in capsular structure can influence not only the interaction between the yeast cells and the immune system, but can also affect the diagnosis of the infection. The most widely diagnostic tool used for cryptococcosis is based on serological tests which use reactive sera against the capsular polysaccharide which circulates in the serum. However, this circulating polysaccharide can change its structure over time, resulting in lack of reactivity, and false negative diagnoses (McFadden et al., 2004).

CHANGES IN CAPSULE SIZE

A striking feature of the capsule is that it can undergo significant changes in its size according to the environmental conditions. This finding was first described in the 1950s (Littman, 1958), and in this work, a medium which produced capsule growth was described. More factors that induce capsule enlargement have been described, such as CO_2, iron limitation, mammalian serum, low nutrient concentration at moderate basic pH and mannitol (Anna, 1979; Granger et al., 1985; Vartivarian et al., 1993; Zaragoza et al., 2003a; Zaragoza and Casadevall, 2004; Guimaraes et al., 2010). More importantly, while the capsule of *C. neoformans* is small in the environment and in regular laboratory conditions, it significantly increases in size after a few hours post-infection (Feldmesser et al., 2001).

Capsule enlargement represents a significant change for the cell. In cells with large capsule, this structure accounts for approximately 95% of the total volume of the cell (Maxson et al., 2007a). Capsule enlargement is achieved by addition of new polysaccharide to the old capsule (Maxson et al., 2007b; Frases et al., 2009). An estimation of the mass of the capsule has been made, and it has been found that in media where the capsule enlarges, the total mass of the polysaccharide increases by a 20% in only a few hours. (Maxson et al., 2007a). These changes indicate that capsule enlargement is a dramatic change for the cell, and suggest that it is a high energy cost process. Structural studies have shown that the new polysaccharide fibers are different from the ones that are previously attached to the cell (Frases et al., 2008). Capsule growth is a regulated process, and does not occur indefinitely. After enlargement, the capsule size reaches a limit, which is directly proportional to the size of the cell body, delimited by the cell wall (Zaragoza et al., 2006).

IMPORTANCE OF CAPSULE GROWTH DURING THE INTERACTION WITH THE HOST

Capsule growth occurs during the first hours after being inhaled (Feldmesser et al., 2001), and is considered an "early" response of *C. neoformans* during the interaction with the host. Different studies have shown that this process confers advantages to the pathogen. By increasing the size of the capsule, the pathogen increases its total size, which may impair phagocytosis. However, phagocytosis of *C. neoformans* can occur in the presence of opsonins, such as proteins of the complement (C) system or antibodies. In the case of C-mediated phagocytosis, capsule enlargement plays a prominent role in determining the degree of internalization of the fungus. The localization of C3 protein, which is the main opsonin of the complement system involved in *C. neoformans* uptake, depends on the size of the capsule. In cells with small capsule, C3 binds at the edge of the capsule, a location which is accessible for the complement receptors (Zaragoza et al., 2003b), and phagocytosis occurs. However, when the capsule enlarges, C3 proteins bind deep in the capsule, several microns away from the edge, and is not accessible to the receptors (Zaragoza et al., 2003b). In this way, capsule enlargement promotes phagocytosis avoidance even in the presence of complement proteins.

Another role of capsule enlargement has been described during intracellular pathogenesis of *C. neoformans*. This fungal pathogen can evade killing by phagocytes once it has been internalized, and is considered a facultative intracellular pathogen (Diamond and Bennett, 1973; Levitz et al., 1999; Tucker and Casadevall, 2002). The mechanism of intracellular survival is still unknown. *C. neoformans* does not inhibit phagosome maturation or lysosome fusion and acidification (Levitz et al., 1999), which suggests that *C. neoformans* expresses some phenotypic traits that allow killing escape and survival. In this sense, capsule enlargement confers resistance to stress factors from the phago-lysosome, such as free radicals and antimicrobial peptides (Zaragoza et al., 2008). It is believed that capsule enlargement protects against free radicals by acting as a buffering structure. In cells with large capsule, free radicals would mainly exert their action on the capsular polysaccharide since it constitutes more than 90% of the volume of the cell (Maxson et al., 2007a). Since the capsule is not required for the normal growth of the yeast, alterations in the polysaccharide fibers by free radicals attack would result in increased survival compared to cells with small capsule. This idea is supported by the finding that capsule enlargement is in fact induced by macrophage factors (Ma et al., 2006; Chrisman et al., 2011).

CHANGES IN THE TOTAL SIZE OF THE CELL

Changes in the total cell size of *C. neoformans* occur during infection. Although *C. neoformans* is mainly found as rounded yeast cells in the host, the size of the blastoconidia found in the tissues (in particular, lungs) undergoes enormous variations. The regular cell size of *C. neoformans* cells *in vitro* ranges from 5 to 7 μm. During infection, sizes from 1 to 100 μm are found (see **Figure 1** and Cruickshank et al., 1973; Feldmesser et al., 2001). Hence, *C. neoformans* can produce both microforms and macro cells in the host. These variations have occasionally been described in the literature, but it has not been until recently that these types of cells have raised the interest of the scientific community. These phenomena are extremely interesting and suggest multiple consequences during the interaction with the host.

The best characterized phenomenon is the ability to form cells of a tremendous size in the lungs of infected mice. This transition is observed after several days of infection, so it could be considered as a "late" morphological response of the pathogen. Recently, two independent articles have characterized these cells in detail (Okagaki et al., 2010; Zaragoza et al., 2010). These articles defined them as "giant" or "titan" cells. So I will refer to them as fungal or cryptococcal "giant/titan cells." Using different approaches, these two groups show that fungal giant/titan cells are reproducibly found during infection, but their proportion is highly variable. During infection in murine models, where disseminated disease is accompanied by a strong inflammatory response, the proportion of fungal giant/titan cells is very low, around 5–10% of the total fungal burden. Okagaki et al. (2010) find that this proportion significantly increases when co-infections with MATa and MATα mating types are performed, indicating that the pheromone signaling pathway plays an important role in the development of giant/titan cells. On the other hand, the proportion of cryptococcal giant/titan cells also increases when the fungal burden in the lungs is low and the mice develop chronic asymptomatic infection (Zaragoza et al., 2010). In addition, cAMP signaling pathway is required for cryptococcal giant/titan cell formation (Zaragoza et al., 2010). In agreement, other elements involved in the cAMP signaling pathway have been

recently described to affect giant/titan cell formation, such as Ste3a and Gpr5 (G-protein coupled receptors), and the transcription factor Rim101 (Okagaki et al., 2011). Moreover, analysis of a collection of gene deletion mutants have identified other proteins involved in cellular enlargement, like G1 cyclins, Rho-GTPases, and GTPases-activating proteins, suggesting that titan formation requires the interplay of different pathways in the cell (Okagaki et al., 2011). Ras1 and MAPK signaling pathway do not seem to participate in giant/titan cell induction (Zaragoza et al., 2010; Okagaki et al., 2011). Fungal giant/titan cells are polyploid (Okagaki et al., 2010; Zaragoza et al., 2010), suggesting that the formation of these cells is achieved by endoreduplication, a well known process that yields cells of enormous size. In addition to an increase in the DNA content, fungal giant/titan cells present other phenotypic differences compared to regular cells. The capsule of titan cells has a higher polysaccharide density and presents different antigenic properties compared to cells grown *in vitro* (Zaragoza et al., 2010). In addition, the cell wall is significantly thicker, reaching a width of 2–3 μm (Zaragoza et al., 2010). Titan cells present peculiar intracellular features, such as the presence of a fragmented vacuole. Cryptococcal giant/titan cell formation is achieved not only by an increase in the capsule size, but also in the cell body size, which in some cases could reach a diameter of 100 μm (Okagaki et al., 2010).

Besides the formation of titan cells, *C. neoformans* can also form micro-cells, of a size lower than 1 μm (Feldmesser et al., 2001). These cells have not been studied in detail, but they are a common feature of the *C. neoformans* infection. The fact that *C. neoformans* can exhibit giant/titan and micro-cells during infection suggests that this pathogen has developed a complex morphogenetic program that allows adaptation to the host environment.

IMPORTANCE OF CHANGES IN TOTAL CELL SIZE DURING THE INTERACTION WITH THE HOST

The presence of cryptococcal giant/titan cells during infection represents a multilevel problem for the immune system. These cells present a higher resistance to oxidative agents (Okagaki et al., 2010; Zaragoza et al., 2010). In addition, titan cells avoid phagocytosis, although macrophages seem to recognize and bind to these fungal cellular forms (see **Figure 2** and Okagaki et al., 2010; Zaragoza et al., 2010). These two features confirm that cryptococcal giant/titan cells can evade the host immune system. However, the role of these cells in the pathogenesis of the yeast is still unclear. Co-infection with MATa and MATα cells produced an increase in the proportion of fungal giant/titan cells in the lung, and this correlated with decreased dissemination of the fungus to the brain (Okagaki et al., 2010), suggesting that their large size impairs their ability to exit the lungs and cross biological barriers. During chronic infection, where a low number of yeasts are found in the lungs and there is no inflammation, the proportion of fungal giant cells was around 70–90% (Zaragoza et al., 2010). These observations suggest that, in fact, fungal giant/titan cells are not involved in the development of a disseminated disease. Instead, they provide fungal resistant forms that can persist in the host for long time periods. However, recent findings demonstrate that fungal giant/titan cells are virulent in the non conventional host *Galleria mellonella* (Garcia-Rodas et al., 2011), suggesting that this type of cells can also contribute to the development of the disease in certain situations, most probably by producing a progeny of cells of regular size.

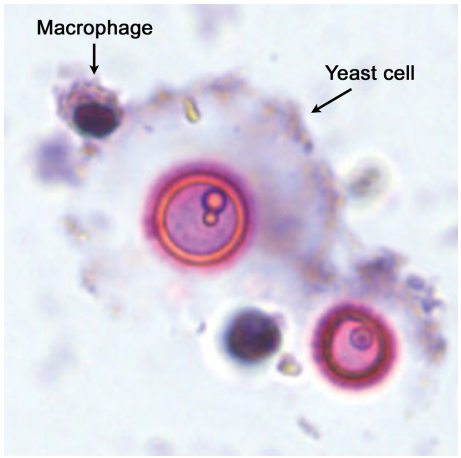


FIGURE 2 | Interaction between macrophages and cryptococcal giant/titan cells. Fungal giant/titan cells obtained from infected mice were co-incubated *in vitro* with RAW264.7 macrophage-like cells. As shown in the figure, the macrophages can recognize and bind to the fungal giant/titan cells, but they cannot internalize the fungal cells due to their enormous size.

Cryptococcal giant/titan cells have been also described in other model hosts. Recently, it has been found that incubation of *C. neoformans* in ameba extracts results in capsule enlargement (Chrisman et al., 2011), and occasionally, also in the appearance of titan cells (Chrisman et al., 2011). Furthermore, unpublished results from our group indicate that during infection in the non-conventional host *Galleria mellonella*, *C. neoformans* also forms giant/titan cells (Garcia-Rodas et al., 2011). These findings indicate that gigantism is a general feature elicited by *C. neoformans* that occurs during infection in different types of hosts, which highlights the idea that this morphological transition plays an important role in fungal survival and persistence in the host.

Cryptococcus neoformans can also produce cells of a smaller size, known as micro-cells. Although their role in virulence has not been described, their presence during infection raises challenging questions. For example, it is tempting to hypothesize that due to their reduced size, these cells could have a particular ability to disseminate and cross biological barriers, such as endothelia and the brain–blood barrier, and in consequence, contribute to the development of cryptococcal meningitis.

FINAL REMARKS ABOUT THE ROLE OF MORPHOLOGICAL AND PHENOTYPIC VARIATIONS IN DIFFERENT PHASES OF THE INFECTION AND DISEASE CAUSED BY *CRYPTOCOCCUS NEOFORMANS*

As it has been described in this review, *C. neoformans* can produce a large number of phenotypic forms by inducing changes in the structure and/or size of the capsule, and in the total size of the cell. These changes have profound effects during the interaction with the host. They contribute to immune evasion, adaptation to the host environment, dissemination, and long term survival

during infection. These phenotypic and morphological variations constitute mechanisms that contribute to the cryptococcal infection, in addition to the well established role of the cryptococcal exopolysaccharide as a virulence factor (see review in Zaragoza et al., 2009). The molecular mechanisms involved in the regulation of these changes, and their contribution to the cryptococcal pathogenesis remain unknown, but it is reasonable to hypothesize that they participate in different states of the cryptococcal disease. After the first contact of the yeast with the host, capsule enlargement (an "early" response of the fungus) contributes to phagocytosis escape and intracellular survival, which allows the yeast to evade the first line of defense elicited by the host. In immunocompetent hosts, the infection is controlled, but the fungal cells are not completely cleared, and it is believed that *C. neoformans* can develop a latent state (Dromer et al., 2011). In these conditions, the production of fungal giant/titan cells (a "late" fungal response) could be a key factor to ensure long term survival. During the most typical clinical manifestation, which is dissemination and meningitis, morphological changes also contribute to the disease. First, the ability to produce capsular rearrangements contributes to the avoidance of recognition by immune cells and antibodies. In addition, capsular rearrangements seem to contribute to yeast dissemination and cross of biological barriers. The capacity to produce micro-cells could also facilitate the dissemination of the fungus and the consequent invasion of the brain. Finally, the large number of different yeast forms that could be found during infection could also play a role in another well known disease caused by *C. neoformans*, which is the immune reconstitution inflammatory syndrome (IRIS) also known as immune restoration disease (IRD). This is a disease developed by some HIV patients who recover their immune system after the initiation of the HAART. In these conditions, if an infection is encountered by the immune system, an exaggerated inflammatory response is elicited, which develops an acute disease. The pathogens which are associated with this disorder are *Mycobacterium tuberculosis* and *C. neoformans* (French, 2009), most probably because their incidence is particularly significant among HIV patients. In the case of *C. neoformans*, I hypothesize that in situations of immune recovery, the presence of multiple variants of cryptococcal cells, with different capsule structures and cell sizes may elicit multiple immune responses simultaneously, which can contribute to the appearance of the IRIS.

FUTURE PERSPECTIVES

In conclusion, although *C. neoformans* has not been classically considered a fungus able to undergo morphological changes, it can in fact elicit multiple cellular types which contribute to the survival of the yeast *in vivo*. Although some effects of cryptococcal morphogenesis have been described, many important questions still need to be addressed. These transitions have been described *in vitro* and in murine models, but their presence during human infection has not been fully demonstrated. Clinical and histopathological studies are needed to confirm the occurrence of capsular variations and the formation of micro- and giant/titan cells in humans. In addition, the cellular mechanisms that regulate the variations in structure and size of the capsule and the formation of fungal giant/titan and micro-cells are unknown, and future studies are required to unveil these molecular processes. For this purpose, it is necessary to better characterize the genes involved in capsule synthesis, and in particular, the interplay between them. The finding that vesicle secretion is involved in capsule synthesis suggests that exocytosis processes might be involved in capsular rearrangements, and that induction or inhibition of extracellular transport could result in variations of capsule structure. Concerning the changes in total cell size, the formation of cryptococcal giant/titan and micro-cells implies that *C. neoformans* can induce alterations in their cell cycle, to undergo endoreduplication (to produce gigantic cells), or to reduce the length of the cell cycle (to produce micro-cells). The idea that cell cycle regulation depends on host factors is a challenging hypothesis that deserves special interest in future studies. To conclude, the full characterization of morphological changes in *C. neoformans*, and in particular, the elucidation of the molecular mechanisms involved in these processes will also contribute to the design of new therapeutical strategies.

ACKNOWLEDGMENTS

I warmly thank my colleague and friend Helene Eisenman for her careful critical reading of the manuscript and for her helpful suggestions. Oscar Zaragoza is funded by grant SAF2008-03761 from the Spanish Ministry of Science and Innovation.

REFERENCES

Aksenov, S. I., Babyeva, I. P., and Golubev, V. I. (1973). On the mechanism of adaptation of microorganisms to conditions of extreme low humidity. *Life Sci. Space Res.* 11, 55–61.

Anna, E. J. (1979). Rapid in vitro capsule production by cryptococci. *Am. J. Med. Technol.* 45, 585–588.

Bose, I., Reese, A. J., Ory, J. J., Janbon, G., and Doering, T. L. (2003). A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryot. Cell* 2, 655–663.

Casadevall, A., Deshaw, M., Fan, M., Dromer, F., Kozel, T. R., and Pirofski, L. A. (1994). Molecular and idiotypic analysis of antibodies to *Cryptococcus neoformans* glucuronoxylomannan. *Infect. Immun.* 62, 3864–3872.

Casadevall, A., Mukherjee, J., Devi, S. J., Schneerson, R., Robbins, J. B., and Scharff, M. D. (1992). Antibodies elicited by a *Cryptococcus neoformans*-tetanus toxoid conjugate vaccine have the same specificity as those elicited in infection. *J. Infect. Dis.* 165, 1086–1093.

Casadevall, A., and Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, DC: ASM Press.

Chang, Y. C., and Kwon-Chung, K. J. (1994). Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol. Cell. Biol.* 14, 4912–4919.

Chang, Y. C., and Kwon-Chung, K. J. (1998). Isolation of the third capsule-associated gene, CAP60, required for virulence in *Cryptococcus neoformans*. *Infect. Immun.* 66, 2230–2236.

Chang, Y. C., and Kwon-Chung, K. J. (1999). Isolation, characterization, and localization of a capsule-associated gene, CAP10, of *Cryptococcus neoformans*. *J. Bacteriol.* 181, 5636–5643.

Chang, Y. C., Wickes, B. L., and Kwon-Chung, K. J. (1995). Further analysis of the *CAP59* locus of *Cryptococcus neoformans*: structure defined by forced expression and description of a new ribosomal protein-encoding gene. *Gene* 167, 179–183.

Charlier, C., Chretien, F., Baudrimont, M., Mordelet, E., Lortholary, O., and Dromer, F. (2005). Capsule structure changes associated with *Cryptococcus neoformans* crossing of the blood-brain barrier. *Am. J. Pathol.* 166, 421–432.

Cherniak, R., Jones, R. G., and Reiss, E. (1988a). Structure determination of *Cryptococcus neoformans* serotype A-variant glucuronoxylomannan by 13C-n.m.r. spectroscopy. *Carbohydr. Res.* 172, 113–138.

Cherniak, R., Jones, R. G., and Slodki, M. E. (1988b). Type-specific polysaccharides of *Cryptococcus neoformans*. n.m.r.-spectral

study of a glucuronomannan chemically derived from a *Tremella mesenterica* exopolysaccharide. *Carbohydr. Res.* 182, 227–239.

Cherniak, R., Morris, L. C., Belay, T., Spitzer, E. D., and Casadevall, A. (1995). Variation in the structure of glucuronoxylomannan in isolates from patients with recurrent cryptococcal meningitis. *Infect. Immun.* 63, 1899–1905.

Cherniak, R., Reiss, E., Slodki, M. E., Plattner, R. D., and Blumer, S. O. (1980). Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. *Mol. Immunol.* 17, 1025–1032.

Cherniak, R., Reiss, E., and Turner, S. (1982). A galactoxylomannan antigen of *Cryptococcus neoformans* serotype A. *Carbohydr. Res.* 103, 239–250.

Cherniak, R., and Sundstrom, J. B. (1994). Polysaccharide antigens of the capsule of *Cryptococcus neoformans. Infect. Immun.* 62, 1507–1512.

Cherniak, R., Valafar, H., Morris, L. C., and Valafar, F. (1998). *Cryptococcus neoformans* chemotyping by quantitative analysis of 1H nuclear magnetic resonance spectra of glucuronoxylomannans with a computer-simulated artificial neural network. *Clin. Diagn. Lab. Immunol.* 5, 146–159.

Chrisman, C. J., Albuquerque, P., Guimaraes, A. J., Nieves, E., and Casadevall, A. (2011). Phospholipids trigger *Cryptococcus neoformans* capsular enlargement during interactions with amoebae and macrophages. *PLoS Pathog.* 7, e1002047. doi: 10.1371/journal.ppat.1002047

Cleare, W., and Casadevall, A. (1998). The different binding patterns of two immunoglobulin M monoclonal antibodies to *Cryptococcus neoformans* serotype A and D strains correlate with serotype classification and differences in functional assays. *Clin. Diagn. Lab. Immunol.* 5, 125–129.

Cordero, R. J., Frases, S., Guimaraes, A. J., Rivera, J., and Casadevall, A. (2011). Evidence for branching in cryptococcal capsular polysaccharides and consequences on its biological activity. *Mol. Microbiol.* 79, 1101–1117.

Cruickshank, J. G., Cavill, R., and Jelbert, M. (1973). *Cryptococcus neoformans* of unusual morphology. *Appl. Microbiol.* 25, 309–312.

De Jesus, M. D., Nicola, A. M., Chow, S. K., Lee, I. R., Nong, S., Specht, C. A., Levitz, S. M., and Casadevall, A. (2010). Glucuronoxylomannan, galactoxylomannan, and mannoprotein occupy spatially separate and discrete regions in the capsule of *Cryptococcus neoformans. Virulence* 1, 500–508.

Diamond, R. D., and Bennett, J. E. (1973). Growth of *Cryptococcus neoformans* within human macrophages in vitro. *Infect. Immun.* 7, 231–236.

Doering, T. L. (2000). How does *Cryptococcus* get its coat? *Trends Microbiol.* 8, 547–553.

Dromer, F., Casadevall, A., Perfect, J. R., and Sorrell, T. C. (2011). "*Cryptococcus neoformans*: latency and disease," in *Cryptococcus. From Human Pathogen to Model Yeast*, eds J. Heitman, T. R. Kozel, K. J. Kwon-Chung, J. R. Perfect, and A. Casadevall (Washington, DC: ASM Press), 431–439.

Dromer, F., Charreire, J., Contrepois, A., Carbon, C., and Yeni, P. (1987). Protection of mice against experimental cryptococcosis by anti-*Cryptococcus neoformans* monoclonal antibody. *Infect. Immun.* 55, 749–752.

Dromer, F., Mathoulin-Pelissier, S., Launay, O., and Lortholary, O. (2007). Determinants of disease presentation and outcome during cryptococcosis: the CryptoA/D study. *PLoS Med.* 4, e21. doi: 10.1371/journal.pmed.0040021

Eckert, T. F., and Kozel, T. R. (1987). Production and characterization of monoclonal antibodies specific for *Cryptococcus neoformans* capsular polysaccharide. *Infect. Immun.* 55, 1895–1899.

Ellerbroek, P. M., Hoepelman, A. I., Wolbers, F., Zwaginga, J. J., and Coenjaerts, F. E. (2002). Cryptococcal glucuronoxylomannan inhibits adhesion of neutrophils to stimulated endothelium in vitro by affecting both neutrophils and endothelial cells. *Infect. Immun.* 70, 4762–4771.

Feldmesser, M., Kress, Y., and Casadevall, A. (2001). Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. *Microbiology* 147, 2355–2365.

Frases, S., Nimrichter, L., Viana, N. B., Nakouzi, A., and Casadevall, A. (2008). *Cryptococcus neoformans* capsular polysaccharide and exopolysaccharide fractions manifest physical, chemical, and antigenic differences. *Eukaryot. Cell* 7, 319–327.

Frases, S., Pontes, B., Nimrichter, L., Viana, N. B., Rodrigues, M. L., and Casadevall, A. (2009). Capsule of *Cryptococcus neoformans* grows by enlargement of polysaccharide molecules. *Proc. Natl. Acad. Sci. U.S.A.* 106, 1228–1233.

French, M. A. (2009). HIV/AIDS: immune reconstitution inflammatory syndrome: a reappraisal. *Clin. Infect. Dis.* 48, 101–107.

Gahrs, W., Tigyi, Z., Emody, L., and Makovitzky, J. (2009). Polarization optical analysis of the surface structures of various fungi. *Acta Histochem.* 111, 308–315.

Garcia-Hermoso, D., Dromer, F., and Janbon, G. (2004). *Cryptococcus neoformans* capsule structure evolution in vitro and during murine infection. *Infect. Immun.* 72, 3359–3365.

Garcia-Rodas, R., Casadevall, A., Rodriguez-Tudela, J. L., Cuenca-Estrella, M., and Zaragoza, O. (2011). *Cryptococcus neoformans* capsular enlargement and cellular gigantism during *Galleria mellonella* infection. *PLoS ONE* (in press).

Gates, M. A., and Kozel, T. R. (2006). Differential localization of complement component 3 within the capsular matrix of *Cryptococcus neoformans. Infect. Immun.* 74, 3096–3106.

Gates, M. A., Thorkildson, P., and Kozel, T. R. (2004). Molecular architecture of the *Cryptococcus neoformans* capsule. *Mol. Microbiol.* 52, 13–24.

Gates-Hollingsworth, M. A., and Kozel, T. R. (2009). Phenotypic heterogeneity in expression of epitopes in the *Cryptococcus neoformans* capsule. *Mol. Microbiol.* 74, 126–138.

Gazzoni, A. F., Oliveira Fde, M., Salles, E. F., Mayayo, E., Guarro, J., Capilla, J., and Severo, L. C. (2010). Unusual morphologies of *Cryptococcus* spp. in tissue specimens: report of 10 cases. *Rev. Inst. Med. Trop. Sao Paulo* 52, 145–149.

Goldman, D. L., Fries, B. C., Franzot, S. P., Montella, L., and Casadevall, A. (1998). Phenotypic switching in the human pathogenic fungus *Cryptococcus neoformans* is associated with changes in virulence and pulmonary inflammatory response in rodents. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14967–14972.

Granger, D. L., Perfect, J. R., and Durack, D. T. (1985). Virulence of *Cryptococcus neoformans.* Regulation of capsule synthesis by carbon dioxide. *J. Clin. Invest.* 76, 508–516.

Guerrero, A., Jain, N., Wang, X., and Fries, B. C. (2010). *Cryptococcus neoformans* variants generated by phenotypic switching differ in virulence through effects on macrophage activation. *Infect. Immun.* 78, 1049–1057.

Guimaraes, A. J., Frases, S., Cordero, R. J., Nimrichter, L., Casadevall, A., and Nosanchuk, J. D. (2010). *Cryptococcus neoformans* responds to mannitol by increasing capsule size in vitro and in vivo. *Cell. Microbiol.* 12, 740–753.

Heiss, C., Klutts, J. S., Wang, Z., Doering, T. L., and Azadi, P. (2009). The structure of *Cryptococcus neoformans* galactoxylomannan contains beta-D-glucuronic acid. *Carbohydr. Res.* 344, 915–920.

Heitman, J., Kozel, T. R., Kwon-Chung, K. J., Perfect, J. R., and Casadevall, A. (2011). *Cryptococcus. From Human Pathogen to Model Yeast.* Washington, DC: ASM Press.

Hosseini-Moghaddam, S. M., and Husain, S. (2010). Fungi and molds following lung transplantation. *Semin. Respir. Crit. Care Med.* 31, 222–233.

Janbon, G. (2004). *Cryptococcus neoformans* capsule biosynthesis and regulation. *FEMS Yeast Res.* 4, 765–771.

Janbon, G., and Doering, T. L. (2011). "Biosynthesis and genetics of the *Cryptococcus* capsule," in *Cryptococcus. From human Pathogen to Model Yeast*, eds J. Heitman, T. R. Kozel, K. J. Kwon-Chung, J. R. Perfect, and A. Casadevall (Washington, DC: ASM Press), 27–41.

Janbon, G., Himmelreich, U., Moyrand, F., Improvisi, L., and Dromer, F. (2001). Cas1p is a membrane protein necessary for the O-acetylation of the *Cryptococcus neoformans* capsular polysaccharide. *Mol. Microbiol.* 42, 453–467.

Kane, J. (1984). Conversion of *Blastomyces dermatitidis* to the yeast form at 37 degrees C and 26 degrees C. *J. Clin. Microbiol.* 20, 594–596.

Kozel, T. R., Gulley, W. F., and Cazin, J. Jr. (1977). Immune response to *Cryptococcus neoformans* soluble polysaccharide: immunological unresponsiveness. *Infect. Immun.* 18, 701–707.

Kozel, T. R., Levitz, S. M., Dromer, F., Gates, M. A., Thorkildson, P., and Janbon, G. (2003). Antigenic and biological characteristics of mutant strains of *Cryptococcus neoformans* lacking capsular O acetylation or xylosyl side chains. *Infect. Immun.* 71, 2868–2875.

Kozubowski, L., and Heitman, J. (2011). Profiling a killer, the development of *Cryptococcus neoformans. FEMS Microbiol. Rev.* doi: 10.1111/j.1574-6976.2011.00286.x. [Epub ahead of print].

Kurtzman, C. P., and Fell, J. W. (1997). *The Yeasts: A Taxonomical Study*. Amsterdam: Elsevier.

Levitz, S. M., Nong, S. H., Seetoo, K. F., Harrison, T. S., Speizer, R. A., and Simons, E. R. (1999). *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. *Infect. Immun.* 67, 885–890.

Levitz, S. M., and Specht, C. A. (2006). The molecular basis for the immunogenicity of *Cryptococcus neoformans* mannoproteins. *FEMS Yeast Res.* 6, 513–524.

Lipovsky, M. M., Tsenova, L., Coenjaerts, F. E., Kaplan, G., Cherniak, R., and Hoepelman, A. I. (2000). Cryptococcal glucuronoxylomannan delays translocation of leukocytes across the blood-brain barrier in an animal model of acute bacterial meningitis. *J. Neuroimmunol.* 111, 10–14.

Littman, M. (1958). Capsule synthesis by *Cryptococcus neoformans. Trans. N. Y. Acad. Sci.* 20, 623–648.

Liu, O. W., Chun, C. D., Chow, E. D., Chen, C., Madhani, H. D., and Noble, S. M. (2008). Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans. Cell* 135, 174–188.

Ma, H., Croudace, J. E., Lammas, D. A., and May, R. C. (2006). Expulsion of live pathogenic yeast by macrophages. *Curr. Biol.* 16, 2156–2160.

Macher, A. M., Bennett, J. E., Gadek, J. E., and Frank, M. M. (1978). Complement depletion in cryptococcal sepsis. *J. Immunol.* 120, 1686–1690.

Maresca, B., and Kobayashi, G. S. (1989). Dimorphism in *Histoplasma capsulatum*: a model for the study of cell differentiation in pathogenic fungi. *Microbiol. Rev.* 53, 186–209.

Maxson, M. E., Cook, E., Casadevall, A., and Zaragoza, O. (2007a). The volume and hydration of the *Cryptococcus neoformans* polysaccharide capsule. *Fungal Genet. Biol.* 44, 180–186.

Maxson, M. E., Dadachova, E., Casadevall, A., and Zaragoza, O. (2007b). Radial mass density, charge, and epitope distribution in the *Cryptococcus neoformans* capsule. *Eukaryot. Cell* 6, 95–109.

McFadden, D., Zaragoza, O., and Casadevall, A. (2006a). The capsular dynamics of *Cryptococcus neoformans. Trends Microbiol.* 14, 497–505.

McFadden, D. C., De Jesus, M., and Casadevall, A. (2006b). The physical properties of the capsular polysaccharides from *Cryptococcus neoformans* suggest features for capsule construction. *J. Biol. Chem.* 281, 1868–1875.

McFadden, D. C., Fries, B. C., Wang, F., and Casadevall, A. (2007). Capsule structural heterogeneity and antigenic variation in *Cryptococcus neoformans. Eukaryot. Cell* 6, 1464–1473.

McFadden, D. C., Zaragoza, O., and Casadevall, A. (2004). Immunoreactivity of cryptococcal antigen is not stable under prolonged incubations in human serum. *J. Clin. Microbiol.* 42, 2786–2788.

Monari, C., Bistoni, F., and Vecchiarelli, A. (2006). Glucuronoxylomannan exhibits potent immunosuppressive properties. *FEMS Yeast Res.* 6, 537–542.

Mukherjee, J., Kozel, T. R., and Casadevall, A. (1998). Monoclonal antibodies reveal additional epitopes of serotype D *Cryptococcus neoformans* capsular glucuronoxylomannan that elicit protective antibodies. *J. Immunol.* 161, 3557–3568.

Murphy, J. W., and Cozad, G. C. (1972). Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. *Infect. Immun.* 5, 896–901.

Neilson, J. B., Ivey, M. H., and Bulmer, G. S. (1978). *Cryptococcus neoformans*: pseudohyphal forms surviving culture with *Acanthamoeba polyphaga. Infect. Immun.* 20, 262–266.

Netski, D., and Kozel, T. R. (2002). Fc-dependent and Fc-independent opsonization of *Cryptococcus neoformans* by anticapsular monoclonal antibodies: importance of epitope specificity. *Infect. Immun.* 70, 2812–2819.

Nimrichter, L., Frases, S., Cinelli, L. P., Viana, N. B., Nakouzi, A., Travassos, L. R., Casadevall, A., and Rodrigues, M. L. (2007). Self-aggregation of *Cryptococcus neoformans* capsular glucuronoxylomannan is dependent on divalent cations. *Eukaryot. Cell* 6, 1400–1410.

Nosanchuk, J. D., and Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. *Cell. Microbiol.* 5, 203–223.

Okagaki, L. H., Strain, A. K., Nielsen, J. N., Charlier, C., Baltes, N. J., Chretien, F., Heitman, J., Dromer, F., and Nielsen, K. (2010). Cryptococcal cell morphology affects host cell interactions and pathogenicity. *PLoS Pathog.* 6, e1000953. doi: 10.1371/journal.ppat.1000953

Okagaki, L. H., Wang, Y., Ballou, E. R., O'meara, T. R., Bahn, Y. S., Alspaugh, J. A., Xue, C., and Nielsen, K. (2011). Cryptococcal titan cell formation is regulated by G-protein signaling in response to multiple stimuli. *Eukaryot. Cell.* doi: 10.1128/EC.05179-11. [Epub ahead of print].

Park, B. I., Wannemuehler, K. A., Marston, B. J., Govender, N., Pappas, P. G., and Chiller, T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* 23, 525–530.

Pierini, L. M., and Doering, T. L. (2001). Spatial and temporal sequence of capsule construction in *Cryptococcus neoformans. Mol. Microbiol.* 41, 105–115.

Pirofski, L., Lui, R., Deshaw, M., Kressel, A. B., and Zhong, Z. (1995). Analysis of human monoclonal antibodies elicited by vaccination with a *Cryptococcus neoformans* glucuronoxylomannan capsular polysaccharide vaccine. *Infect. Immun.* 63, 3005–3014.

Rodrigues, M. L., Alvarez, M., Fonseca, F. L., and Casadevall, A. (2008). Binding of the wheat germ lectin to *Cryptococcus neoformans* suggests an association of chitinlike structures with yeast budding and capsular glucuronoxylomannan. *Eukaryot. Cell* 7, 602–609.

Rodrigues, M. L., Casadevall, A., and Zaragoza, O. (2011). "The architecture and antigenic composition of the polysaccharide capsule," in *Cryptococcus. From Human Pathogen to Model Yeast*, eds J. Heitman, T. R. Kozel, K. J. Kwon-Chung, J. R. Perfect, and A. Casadevall (Washington, DC: ASM Press), 43–54.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., and Casadevall, A. (2007). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell* 6, 48–59.

Singh, N., and Forrest, G. (2009). Cryptococcosis in solid organ transplant recipients. *Am. J. Transplant.* 9(Suppl. 4), S192–S198.

Taborda, C. P., and Casadevall, A. (2002). CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are involved in complement-independent antibody-mediated phagocytosis of *Cryptococcus neoformans. Immunity* 16, 791–802.

Todaro-Luck, F., Reiss, E., Cherniak, R., and Kaufman, L. (1989). Characterization of *Cryptococcus neoformans* capsular glucuronoxylomannan polysaccharide with monoclonal antibodies. *Infect. Immun.* 57, 3882–3887.

Tucker, S. C., and Casadevall, A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3165–3170.

van de Moer, A., Salhi, S. L., Cherniak, R., Pau, B., Garrigues, M. L., and Bastide, J. M. (1990). An anti-*Cryptococcus neoformans* monoclonal antibody directed against galactoxylomannan. *Res. Immunol.* 141, 33–42.

Vartivarian, S. E., Anaissie, E. J., Cowart, R. E., Sprigg, H. A., Tingler, M. J., and Jacobson, E. S. (1993). Regulation of cryptococcal capsular polysaccharide by iron. *J. Infect. Dis.* 167, 186–190.

Vecchiarelli, A. (2000). Immunoregulation by capsular components of *Cryptococcus neoformans. Med. Mycol.* 38, 407–417.

Whiteway, M., and Bachewich, C. (2007). Morphogenesis in *Candida albicans. Annu. Rev. Microbiol.* 61, 529–553.

Williamson, J. D., Silverman, J. F., Mallak, C. T., and Christie, J. D. (1996). Atypical cytomorphologic appearance of *Cryptococcus neoformans*: a report of five cases. *Acta Cytol.* 40, 363–370.

Young, B. J., and Kozel, T. R. (1993). Effects of strain variation, serotype, and structural modification on kinetics for activation and binding of C3 to *Cryptococcus neoformans. Infect. Immun.* 61, 2966–2972.

Zaragoza, O., and Casadevall, A. (2004). Experimental modulation of capsule size in *Cryptococcus neoformans. Biol. Proced. Online* 6, 10–15.

Zaragoza, O., and Casadevall, A. (2006). Monoclonal antibodies can affect complement deposition on the capsule of the pathogenic fungus *Cryptococcus neoformans* by both classical pathway activation and steric hindrance. *Cell. Microbiol.* 8, 1862–1876.

Zaragoza, O., Chrisman, C. J., Castelli, M. V., Frases, S., Cuenca-Estrella, M., Rodriguez-Tudela, J. L., and Casadevall, A. (2008). Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. *Cell. Microbiol.* 10, 2043–2057.

Zaragoza, O., Fries, B. C., and Casadevall, A. (2003a). Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO(2). *Infect. Immun.* 71, 6155–6164.

Zaragoza, O., Taborda, C. P., and Casadevall, A. (2003b). The efficacy of complement-mediated phagocytosis of *Cryptococcus neoformans* is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions. *Eur. J. Immnunol.* 33, 1957–1967.

Zaragoza, O., Garcia-Rodas, R., Nosanchuk, J. D., Cuenca-Estrella, M., Rodriguez-Tudela, J. L., and Casadevall, A. (2010). Fungal cell gigantism during mammalian infection. *PLoS Pathog.* 6, e1000945. doi: 10.1371/journal.ppat.1000945

Zaragoza, O., Rodrigues, M. L., De Jesus, M., Frases, S., Dadachova, E., and Casadevall, A. (2009). The capsule of the fungal pathogen *Cryptococcus neoformans*. *Adv. Appl. Microbiol.* 68, 133–216.

Zaragoza, O., Telzak, A., Bryan, R. A., Dadachova, E., and Casadevall, A. (2006). The polysaccharide capsule of the pathogenic fungus *Cryptococcus neoformans* enlarges by distal growth and is rearranged during budding. *Mol. Microbiol.* 59, 67–83.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 July 2011; accepted: 17 August 2011; published online: 06 September 2011.

Citation: Zaragoza O (2011) Multiple disguises for the same party: the concepts of morphogenesis and phenotypic variations in Cryptococcus neoformans. Front. Microbio. ***2****:181. doi: 10.3389/fmicb.2011.00181*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 15 August 2011
doi: 10.3389/fmicb.2011.00171

Fungal polysaccharides: biological activity beyond the usual structural properties

Marcio L. Rodrigues[1]*, Leonardo Nimrichter[1], Radames J. B. Cordero[2] and Arturo Casadevall[2,3]

[1] *Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[2] *Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA*
[3] *Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA*

Edited by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine, USA

Reviewed by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine, USA
Oscar Zaragoza, Instituto de Salud Carlos III, Spain

****Correspondence:***
Marcio L. Rodrigues, Avenida Carlos Chagas Filho 373, CCS, Bloco I. Instituto de Microbiologia, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21941-902, Brazil.
e-mail: marcio@micro.ufrj.br

Studies on structure and function of polysaccharides in biological systems classically involve sequence and compositional analyses, anomeric configuration, type of glycosidic linkage, and presence of substituents. Recent studies, however, indicates that other structural parameters, so far little explored, can directly influence the biological activity of microbial polysaccharides. Among these parameters, we highlight the molecular dimensions of *Cryptococcus neoformans* polysaccharides, which appear to be inversely correlated with their immunobiological activity. These recent observations raise new concepts about the structure and function of polysaccharides, which stimulates the design of new experimental approaches and suggests previously unknown applications.

Keywords: polysaccharides, fungal pathogens, immunity, structure and function of polysaccharides, *Cryptococcus neoformans*

Polysaccharides are cellular structural components found in the three domains of life. In microorganisms, several studies have shown that polysaccharides play crucial roles in the architecture of the cell envelope (Roberts, 1996; Nimrichter et al., 2005). In both prokaryotic and eukaryotic microbial pathogens, polysaccharides are major cell wall components (Roberts, 1996; Nimrichter et al., 2005). This surface distribution is in agreement with the fact that polysaccharide molecules directly influence host–pathogen interactions (Moxon and Kroll, 1990; Smith, 1990; Levitz, 2004; Zaragoza et al., 2009).

Polysaccharides consist of polymeric structures composed of at least ten monosaccharides sequentially connected by glycosidic bonds (Bertozzi and Rabuka, 2009; Mulloy et al., 2009; Stanley and Cummings, 2009). These structures may be linear or branched, a characteristic that is observed when a monosaccharide constituent of a polysaccharide is involved in more than two glycosidic bonds. Polysaccharides can be classified as homopolymers, a term used to indicate a polymer composed of identical monosaccharides, or heteropolymers, a term used for classification of polysaccharides composed of two or more types of monosaccharides. Classic approaches for the determination of polysaccharide structure include chromatographic methods in association with spectrometric and spectroscopic techniques. These approaches allow determination of sequence and composition, anomeric configuration, type of glycosidic linkage, and presence of substituents (Mulloy et al., 2009).

Capsular polysaccharides were amongst the earliest microbial virulence determinants described in the literature, as demonstrated in the classic Griffith's experiment [review in (Smith, 1990)]. This pioneering study established a direct relationship between the presence of polysaccharide capsules in bacterial pathogens and protection against host defenses. Subsequently, over several decades, the association between microbial virulence and capsular polysaccharides has been consolidated (Moxon and Kroll, 1990; Monari et al., 2006; Vecchiarelli, 2007; Zaragoza et al., 2009), although it is clear that in some cases, these structures work in favor of the host (Mazmanian and Kasper, 2006; Pletz et al., 2008;Kumar et al., 2009). Consequently, microbial polysaccharides may work in favor of the pathogen or induce immune responses that promote infection control, depending on their chemistry and/or structural aspects.

Classically, polysaccharide antigens have been considered poor inducers of cellular immunity. In fact, polysaccharide molecules are considered T-cell independent antigens that are more efficient activators of antibody production than of cell-mediated immune responses (Weintraub, 2003). In the last decade, however, a number of studies have demonstrated the role of polysaccharides in the activation of innate immunological mechanisms [reviewed in (Raetz and Whitfield, 2002; Kumar et al., 2009)]. In addition, the conjugation of polysaccharides to protein structures can generate hybrid molecules of increased immunogenicity. In fact, vaccines containing polysaccharides against several prokaryotic pathogens have proven successful and are commercially available (Pichichero, 2005; Pletz et al., 2008).

Polysaccharides are essential for pathogenic mechanisms and for the immune response during fungal infections (Roeder et al., 2004). Unlike mammalian cells, fungi have a cell wall, a complex compartment mainly composed of polysaccharides (Nimrichter et al., 2005). Glucans, chitin, and mannans (polymers consisting of repeating units of, respectively, glucose, *N*-acetylglucosamine, and mannose) are particularly abundant in the fungal cell wall. Depending on their structural particularities, cell wall

polysaccharides function as regulators of virulence or activators of innate immunity (Roeder et al., 2004; Zaragoza et al., 2009). Well characterized immunoactive polysaccharides produced by fungi include α- and β-glucans (Brown et al., 2003; Hohl et al., 2005; Bittencourt et al., 2006; Rappleye et al., 2007; Wheeler et al., 2008; van de Veerdonk et al., 2009; Chai et al., 2011) and complex mannans (Leitao et al., 2003; Cambi et al., 2008; van de Veerdonk et al., 2009). *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Cryptococcus neoformans,* and *C. gattii* are examples of fungal pathogens in which the immune functions of polysaccharides are known in great detail (Monari et al., 2006; Rappleye et al., 2007; Cambi et al., 2008; Chai et al., 2011).

Cryptococcus neoformans and *C. gattii* are the etiologic agents of cryptococcosis, a disease that presumably begins in the lung. In immunocompromised individuals, *C. neoformans* can disseminate to the central nervous system and other organs (Bicanic and Harrison, 2004). *C. gattii*, on the other hand, can cause disseminated disease in immunocompetent individuals (Byrnes et al., 2011). It is estimated that about one million new cases of cryptococcosis occur annually, with mortality rates that can reach 60% (Park et al., 2009). In Brazil, cryptococcosis is the fungal disease with highest mortality rates among HIV-positive individuals (Prado et al., 2009). *C. neoformans* and *C. gattii* have polysaccharide capsules surrounding the cell body. Bacterial capsules are common in bacterial pathogens, but relatively rare and still poorly defined among eukaryotic organisms.

In *C. neoformans* and *C. gattii*, the capsule is mainly composed of the polysaccharides glucuronoxylomannan (GXM) and galactoxylomannan (GalXM; Zaragoza et al., 2009; Rodrigues et al., 2011). An alteration in the GalXM nomenclature to glucuronoxylomannogalactan (GXMGal) has been recently proposed based on structural aspects and monosaccharide composition (Heiss et al., 2009). The principal capsular polysaccharide component in both species is GXM, which consists of a polymer composed of an α1,3 linked mannosyl backbone with β1,2 and β1,4 xylosyl and glucuronyl substitutions (for details, see Zaragoza et al., 2009). Structural variation resulting from differences in composition, substitution, and conformation results in different serological properties. These GXM properties divide *C. neoformans* strains into four main serotypes: A and D, produced by *C. neoformans*, and B and C, produced by *C. gattii* [review in (Zaragoza et al., 2009; Rodrigues et al., 2011)]. Hybrid serotypes have been also characterized in different regions (Xu et al., 2002). GXM is believed to be synthesized intracellularly, possibly in close association to lipid structures (Rodrigues et al., 2007; Oliveira et al., 2009). For construction of the capsule, yeast cells secrete polysaccharides into the extracellular environment by mechanisms that involve the release of vesicles for subsequent polysaccharide incorporation into the cell surface, for distal capsular enlargement (Zaragoza et al., 2006; Rodrigues et al., 2007).

It is assumed that synthesis of GXM and its release to the extracellular space are crucial for the immunopathogenesis of cryptococcosis. In general, GXM is deleterious to the immune system (Monari et al., 2006), although several reports indicate that this polysaccharide is a potent activator of the complement system and of the innate immunity [review in (Zaragoza et al., 2009)]. Fractions of extracellular GXM released into the medium were recently shown to differ in structure and function from capsular polysaccharide extracts (Frases et al., 2008). These observations suggested that *C. neoformans* and *C. gattii* cells produce highly diverse populations of GXM that show structural peculiarities with possible implications on their biological roles. In fact, various studies have established that the production of polysaccharides by *C. neoformans* includes GXM molecules with highly variable dimensions, which interact through several mechanisms to form the capsular network (Nimrichter et al., 2007; Frases et al., 2009). These studies raised an important question: do polysaccharide samples of identical composition but variable size and degree of polymerization present distinct biological functions?

The hypothesis raised above is supported by observations that have recently become available in the literature. Chitin, a linear polysaccharide found in fungi, crustaceous, insects, and parasites, is a water insoluble polymer composed of units of β1,4-linked *N*-acetylglucosamine. As described by Da Silva et al. (2008, 2009), fractions of chitin with high molecular dimensions are immunologically inert. Polysaccharide samples with reduced dimensions, however, were associated with the effective stimulation of innate immunity and production of pro- and anti-inflammatory cytokines. These observations have established a clear precedent in the literature indicating that polysaccharide samples of identical composition but with varying dimensions may have different functions.

The observation described above and the fact that capsular structures of *Cryptococcus* species are composed of GXM molecules of various sizes (Mcfadden et al., 2006; Frases et al., 2009) support the hypothesis that variation in these structures could translate into different biological effects. In fact, Fonseca et al. (2010) recently established a parallel between the functions of GXM fractions and their dimensions. Through the use of an experimental model that included tests of the activation of cellular responses resulting in nitric oxide production by phagocytes and activation of Toll-like receptors in epithelial cells, it was observed that GXM samples isolated from *C. gattii* (serotype B) with monosaccharide compositions that were similar to other polysaccharide fractions produced by *C. neoformans* (serotypes A and D) and even by other strains of *C. gattii* (serotype C) generated very distinct cellular responses. Measurements of polysaccharide diameter by dynamic light scattering (for details about the technique, see Frases et al., this issue) revealed that the increased capacity of *C. gattii* GXM to induce cellular responses was correlated with a reduced molecular diameter (Fonseca et al., 2010). These results led to the conclusion that serotype B GXM samples with reduced dimensions have greater immunobiological potential, as demonstrated for chitin (Da Silva et al., 2008, 2009; Lee et al., 2008). It remains unknown, however, whether this concept would be pertinent to other GXM-producing species, including members of the *Trichosporon* genus (Fonseca et al., 2009).

The findings discussed above imply new concepts about the structure and function of fungal polysaccharides. Besides structural aspects traditionally studied, such as sequence analysis, compositional determination, anomeric configuration, type of glycosidic linkage type, and presence of substituents, it becomes

clear that other structural parameters, including molecular diameter and degree of polymerization, must be considered for functional studies. In fact, the conclusion that polysaccharide functions are influenced by these additional parameters was further supported by Cordero et al. (2011). Using static and dynamic light scattering, viscosity analysis, and high-resolution microscopy of *C. neoformans* polysaccharides, this study demonstrated that spatial conformation (branching) influences phagocytosis, nitric oxide production by macrophage-like cells, and susceptibility to reactive oxygen species, serology, and clearance during infection. These previously unexplored parameters can generate new insights, for example, on immunogenicity assays of polysaccharides, as well as their use in therapy or prevention of diseases. Studies in this area are still embryonic and, clearly, much remains to be discovered. For conceptual validation, future studies must include evaluation of the relationship between immunoactivity and molecular dimensions of polysaccharides synthesized by other organisms, as well as of fungal glycans other than GXM and chitin.

ACKNOWLEDGMENTS

Marcio L. Rodrigues and Leonardo Nimrichter are supported by grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP,Brazil), and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil). Arturo Casadevall is supported by NIH grants AI033142, AI033774, AI052733, and HL059842.

REFERENCES

Bertozzi, C. R., and Rabuka, D. (2009). Structural basis of glycan diversity, in *Essentials of Glycobiology*, Chap. 2, 2nd Edn. Eds A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, and M. E. Etzler, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Bicanic, T., and Harrison, T. S. (2004). *Cryptococcal meningitis. Br. Med. Bull.* 72, 99–118.

Bittencourt, V. C., Figueiredo, R. T., Da Silva, R. B., Mourao-Sa, D. S., Fernandez, P. L., Sassaki, G. L., Mulloy, B., Bozza, M. T., and Barreto-Bergter, E. (2006). An alpha-glucan of *Pseudallescheria boydii* is involved in fungal phagocytosis and toll-like receptor activation. *J. Biol. Chem.* 281, 22614–22623.

Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S., and Gordon, S. (2003). Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119–1124.

Byrnes, E. J. III, Bartlett, K. H., Perfect, J. R., and Heitman, J. (2011). *Cryptococcus gattii*: an emerging fungal pathogen infecting humans and animals. *Microbes Infect.* doi: 10.1016/j.micinf.2011.05.009. [Epub ahead of print].

Cambi, A., Netea, M. G., Mora-Montes, H. M., Gow, N. A., Hato, S. V., Lowman, D. W., Kullberg, B. J., Torensma, R., Williams, D. L., and Figdor, C. G. (2008). Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. *J. Biol. Chem.* 283, 20590–20599.

Chai, L. Y., Vonk, A. G., Kullberg, B. J., Verweij, P. E., Verschueren, I., Van Der Meer, J. W., Joosten, L. A., Latge, J. P., and Netea, M. G. (2011). *Aspergillus fumigatus* cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes Infect.* 13, 151–159.

Cordero, R. J., Frases, S., Guimaraes, A. J., Rivera, J., and Casadevall, A. (2011). Evidence for branching in cryptococcal capsular polysaccharides and consequences on its biological activity. *Mol. Microbiol.* 79, 1101–1117.

Da Silva, C. A., Chalouni, C., Williams, A., Hartl, D., Lee, C. G., and Elias, J. A. (2009). Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J. Immunol.* 182, 3573–3582.

Da Silva, C. A., Hartl, D., Liu, W., Lee, C. G., and Elias, J. A. (2008). TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. *J. Immunol.* 181, 4279–4286.

Fonseca, F. L., Frases, S., Casadevall, A., Fischman-Gompertz, O., Nimrichter, L., and Rodrigues, M. L. (2009). Structural and functional properties of the *Trichosporon asahii* glucuronoxylomannan. *Fungal Genet. Biol.* 46, 496–505.

Fonseca, F. L., Nohara, L. L., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., and Rodrigues, M. L. (2010). Immunomodulatory effects of serotype B glucuronoxylomannan from *Cryptococcus gattii* correlate with polysaccharide diameter. *Infect. Immun.* 78, 3861–3870.

Frases, S., Nimrichter, L., Viana, N. B., Nakouzi, A., and Casadevall, A. (2008). *Cryptococcus neoformans* capsular polysaccharide and exopolysaccharide fractions manifest physical, chemical, and antigenic differences. *Eukaryot. Cell* 7, 319–327.

Frases, S., Pontes, B., Nimrichter, L., Viana, N. B., Rodrigues, M. L., and Casadevall, A. (2009). Capsule of *Cryptococcus neoformans* grows by enlargement of polysaccharide molecules. *Proc. Natl. Acad. Sci. U.S.A.* 106, 1228–1233.

Heiss, C., Klutts, J. S., Wang, Z., Doering, T. L., and Azadi, P. (2009). The structure of *Cryptococcus neoformans* galactoxylomannan contains beta-D-glucuronic acid. *Carbohydr. Res.* 344, 915–920.

Hohl, T. M., Van Epps, H. L., Rivera, A., Morgan, L. A., Chen, P. L., Feldmesser, M., and Pamer, E. G. (2005). *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog.* 1, e30. doi: 10.1371/journal.ppat.0010030

Kumar, H., Kawai, T., and Akira, S. (2009). Toll-like receptors and innate immunity. Biochem. Biophys. *Res. Commun.* 388, 621–625.

Lee, C. G., Da Silva, C. A., Lee, J. Y., Hartl, D., and Elias, J. A. (2008). Chitin regulation of immune responses: an old molecule with new roles. *Curr. Opin. Immunol.* 20, 684–689.

Leitao, E. A., Bittencourt, V. C., Haido, R. M., Valente, A. P., Peter-Katalinic, J., Letzel, M., De Souza, L. M., and Barreto-Bergter, E. (2003). Beta-galactofuranose-containing O-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* contain immunodominant epitopes. *Glycobiology* 13, 681–692.

Levitz, S. M. (2004). Interactions of toll-like receptors with fungi. *Microbes Infect.* 6, 1351–1355.

Mazmanian, S. K., and Kasper, D. L. (2006). The love-hate relationship between bacterial polysaccharides and the host immune system. *Nat. Rev. Immunol.* 6, 849–858.

Mcfadden, D. C., De Jesus, M., and Casadevall, A. (2006). The physical properties of the capsular polysaccharides from *Cryptococcus neoformans* suggest features for capsule construction. *J. Biol. Chem.* 281, 1868–1875.

Monari, C., Bistoni, F., and Vecchiarelli, A. (2006). Glucuronoxylomannan exhibits potent immunosuppressive properties. *FEMS Yeast Res.* 6, 537–542.

Moxon, E. R., and Kroll, J. S. (1990). The role of bacterial polysaccharide capsules as virulence factors. *Curr. Top. Microbiol. Immunol.* 150, 65–85.

Mulloy, B., Hart, G. W., and Stanley, P. (2009). Structural analysis of glycans, in *Essentials of Glycobiology*, Chap. 47, 2nd Edn. Eds A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, and M. E. Etzler, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Nimrichter, L., Frases, S., Cinelli, L. P., Viana, N. B., Nakouzi, A., Travassos, L. R., Casadevall, A., and Rodrigues, M. L. (2007). Self-aggregation of *Cryptococcus neoformans* capsular glucuronoxylomannan is dependent on divalent cations. *Eukaryot. Cell* 6, 1400–1410.

Nimrichter, L., Rodrigues, M. L., Rodrigues, E. G., and Travassos, L. R. (2005). The multitude of targets for the immune system and drug therapy in the fungal cell wall. *Microbes Infect.* 7, 789–798.

Oliveira, D. L., Nimrichter, L., Miranda, K., Frases, S., Faull, K. F., Casadevall, A., and Rodrigues, M. L. (2009). *Cryptococcus neoformans* cryoultramicrotomy and vesicle fractionation reveals an intimate association between membrane lipids and glucuronoxylomannan. *Fungal Genet. Biol.* 46, 956–963.

Park, B. J., Wannemuehler, K. A., Marston, B. J., Govender, N., Pappas, P. G., and Chiller, T. M. (2009). Estimation of the current global burden of *Cryptococcal meningitis* among persons living with HIV/AIDS. *AIDS* 23, 525–530.

Pichichero, M. E. (2005). Meningococcal conjugate vaccines. *Expert Opin. Biol. Ther.* 5, 1475–1489.

Pletz, M. W., Maus, U., Krug, N., Welte, T., and Lode, H. (2008). Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. *Int. J. Antimicrob. Agents* 32, 199–206.

Prado, M., Da Silva, M. B., Laurenti, R., Travassos, L. R., and Taborda, C. P. (2009). Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. *Mem. Inst. Oswaldo Cruz* 104, 513–521.

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700.

Rappleye, C. A., Eissenberg, L. G., and Goldman, W. E. (2007). Histoplasma capsulatum alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1366–1370.

Roberts, I. S. (1996). The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu. Rev. Microbiol.* 50, 285–315.

Rodrigues, M. L., Casadevall, A., and Zaragoza, O. (2011). "The architecture and antigenic composition of the polysaccharide capsule," in *Cryptococcus: From Human Pathogen to Model Yeast*, eds T. K. Joseph Heitman, J. Kwon-Chung, J. Perfect and A. Casadevall (Washington, DC: American Society for Microbiology), 43–54.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., and Casadevall, A. (2007). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell* 6, 48–59.

Roeder, A., Kirschning, C. J., Rupec, R. A., Schaller, M., Weindl, G., and Korting, H. C. (2004). Toll-like receptors as key mediators in innate antifungal immunity. *Med. Mycol.* 42, 485–498.

Smith, H. (1990). Pathogenicity and the microbe in vivo. The 1989 Fred Griffith Review Lecture. *J. Gen. Microbiol.* 136, 377–393.

Stanley, P., and Cummings, R. D. (2009). Structures common to different glycans, in *Essentials of Glycobiology,* Chap. 13, 2nd Edn. Eds A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, and M. E. Etzler, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

van de Veerdonk, F. L., Marijnissen, R. J., Kullberg, B. J., Koenen, H. J., Cheng, S. C., Joosten, I., Van Den Berg, W. B., Williams, D. L., Van Der Meer, J. W., Joosten, L. A., and Netea, M. G. (2009). The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. *Cell Host Microbe* 5, 329–340.

Vecchiarelli, A. (2007). Fungal capsular polysaccharide and T-cell suppression: the hidden nature of poor immunogenicity. *Crit. Rev. Immunol.* 27, 547–557.

Weintraub, A. (2003). Immunology of bacterial polysaccharide antigens. *Carbohydr. Res.* 338, 2539–2547.

Wheeler, R. T., Kombe, D., Agarwala, S. D., and Fink, G. R. (2008). Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. *PLoS Pathog.* 4, e1000227. doi: 10.1371/journal.ppat.1000227

Xu, J., Luo, G., Vilgalys, R. J., Brandt, M. E., and Mitchell, T. G. (2002). Multiple origins of hybrid strains of *Cryptococcus neoformans* with serotype AD. *Microbiology* 148, 203–212.

Zaragoza, O., Rodrigues, M. L., De Jesus, M., Frases, S., Dadachova, E., and Casadevall, A. (2009). The capsule of the fungal pathogen *Cryptococcus neoformans*. *Adv. Appl. Microbiol.* 68, 133–216.

Zaragoza, O., Telzak, A., Bryan, R. A., Dadachova, E., and Casadevall, A. (2006). The polysaccharide capsule of the pathogenic fungus *Cryptococcus neoformans* enlarges by distal growth and is rearranged during budding. *Mol. Microbiol.* 59, 67–83.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 July 2011; accepted: 28 July 2011; published online: 15 August 2011.
Citation: Rodrigues ML, Nimrichter L, Cordero RJB and Casadevall A (2011) Fungal polysaccharides: biological activity beyond the usual structural properties. Front. Microbio. ***2****:171. doi: 10.3389/fmicb.2011.00171*
This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 10 October 2011
doi: 10.3389/fmicb.2011.00207

Biophysical methods for the study of microbial surfaces

Susana Frases[1,2]*, Nathan B. Viana[3] and Arturo Casadevall[4]

[1] *Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Laboratório de Biologia, Instituto Nacional de Metrologia, Normalização e Qualidade Industrial, Rio de Janeiro, Brazil*
[3] *Laboratório de Pinças Ópticas–Coordenação de Programas de Estudos Avançados, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[4] *Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA*

Edited by:
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Tom Coenye, University of Ghent, Belgium
Floyd Layton Wormley, The University of Texas at San Antonio, USA

****Correspondence:***
Susana Frases, Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Ilha do fundão, Rio de Janeiro, Brazil.
e-mail: susanafrases@biof.ufrj.br

The challenge in studying the surface architecture of different microbial pathogens is to integrate the most current biochemical, spectroscopic, microscopic, and processing techniques. Individually these methods have insufficient sensitivity to reveal complex structures, such as branched, large, viscous polymers with a high structure hydration, size, and complexity. However, when used in combination biophysical techniques are our primary source of information for understanding polydisperse molecules and complex microbial surfaces. Biophysical methods seek to explain biological function in terms of the molecular structures and properties of specific molecules. The sizes of the molecules found in microbial surfaces vary greatly from small fatty acids and sugars to macromolecules like proteins, polysaccharides, and pigments, such as melanin. These molecules, which comprise the building blocks of living organisms, assemble into cells, tissues, and whole organisms by forming complex individual structures with dimensions from 10 to 10,000 nm and larger. Biophysics is directed to determining the structure of specific biological molecules and of the larger structures into which they assemble. Some of this effort involves developing new methods, adapting old methods and building new instruments for viewing these structures. The description of biophysical properties in an experimental model where, properties such as flexibility, hydrodynamic characteristics, and size can be precisely determined is of great relevance to study the affinity of the surfaces with biologically active and inert substrates and the interaction with host molecules. Furthermore, this knowledge could establish the abilities of different molecules and their structures to differentially activate cellular responses. Recent studies in the fungal pathogen *Cryptococcus neoformans* have demonstrated that the physical properties of its unique polysaccharide capsule correlate with the biological functions associated with the intact capsule and the components comprising the capsule. In this review, we describe the application of biophysical techniques to study and characterize this highly hydrated and fragile fungal surface structure.

Keywords: *Cryptococcus* spp., polysaccharides, optical tweezers, light scattering, zeta potential

INTRODUCTION

The study of microbial surfaces is critically important because they contain and display components associated with virulence, such as adhesins, and surface structures are often major targets for immune responses. Despite their importance, microbial surfaces are difficult to study because they are often heterogeneous and compositionally complex. Traditional techniques for studying microbial surfaces include electron microscopy and hydrophobicity, both of which can provide important insights into surface structure and composition. In recent years a variety of biophysical techniques have been developed that can be applied to better understand the nature of microbial surfaces. In this essay, we review several techniques that have been applied to study the surface of the pathogenic fungus *Cryptococcus neoformans*. The importance of this fungus is underscored by the recent report indicating that globally *C. neoformans* is responsible for approximately one million severe infections and ∼600,000 deaths annually (Park et al., 2011). This organism is unusual because it is surrounded by a polysaccharide capsule and has the distinction of being the only encapsulated human pathogenic fungus (Casadevall and Perfect, 1998). Hence, the distal surface of *C. neoformans* is essentially that of a gel composed of polysaccharides. Until physical techniques were applied, the polysaccharide surface of *C. neoformans* resisted characterization because it was highly vulnerable to any method used to study. The capsule is composed largely of water and electron microscopy methods that require drying inevitably lead to damage and the likely formation of artifactual fibrillar structures of uncertain relevance to natural capsular structure. In this regard, the more recent biophysical methods applied to this problem studied the capsule and capsular components in hydrated form and avoided concerns resulting from drying. The individual various biophysical techniques each provided important insights that when combined have yielded a new view of capsular architecture.

BIOPHYSICAL TECHNIQUES AND APPLICATIONS

OPTICAL TWEEZERS

Optical tweezers or optical trap (OT) is a micromanipulation tool that is now widely employed to study mechanical properties in cellular and molecular force scales. It was developed by Arthur Ashkin and his group at the Bell labs (USA) in the early 1970s (Ashkin, 1997). They were initially working on an optical atom trapping experiment and, after encountering certain difficulties in this project, they decided to examine particles in the micron and sub-micron diameter range. The forces involved in the interaction of light with micrometric non-absorbent particles are in the piconewton (10^{-12} N) range. This feature makes OT an appropriate tool to investigate mechanical properties in cellular and molecular scales.

During the first years of development, the attempts to optically trap a particle were performed with counter propagating laser beans, similar to that used in a cooling atom experiment (Ashkin, 1997). In 1987, after placing a Tobacco Mosaic Virus sample in the microscope to test for contamination, they observed that they could isolate and trap contaminant bacteria for many hours by using the laser to illuminate a high numerical aperture lens (Ashkin and Dziedzic, 1987). This result introduced a configuration that by its simplicity became the most used setup for optical tweezers experiments and led to its main application area, biology or mechanical response of biologically related materials.

Although the development of OT started in the 1970s it was only recently that a first principle theory of OT was developed. To date, this theory has been tested by our group and, even though it is not complete, the results indicate confidence in the force measurements performed (Viana et al., 2006, 2007).

Basic optical tweezers applications

Figure 1 shows the basic schematic representation of a simple optical tweezers setup. A laser emitting light with a wavelength in the infrared spectrum region is used to avoid water absorption of light. The laser is directed to the high numerical aperture microscope objective (MO) lens using a dichroic mirror (DM) of the EPI illumination system of the microscope. After passing by the objective the interaction of the laser light with micrometric dielectric non-absorbent particles in the water sample (WS) produces two forces: the radiation pressure (due to the reflection of the light at the particle in the geometric optics limit) and the gradient force (due to the refraction of the light at the particle in the geometric optics limit). When the gradient force is bigger than the radiation pressure, the particle remains trapped near the focus of the lens. The sample is illuminated by the microscope illumination (MI) system and conveniently positioned by step motors (XY Motor) in the microscope plane of observation. Digitized images of the samples are obtained using a charged coupled device (CCD) camera connected to a computer.

The OT is commonly used to manipulate micrometric objects or as a force sensor in the piconewton range. Recently we developed methodologies using these two features of the OT to measure the viscosity of polysaccharide water solutions in order to study the interaction of yeast cells and to examine the mechanical properties of the capsule.

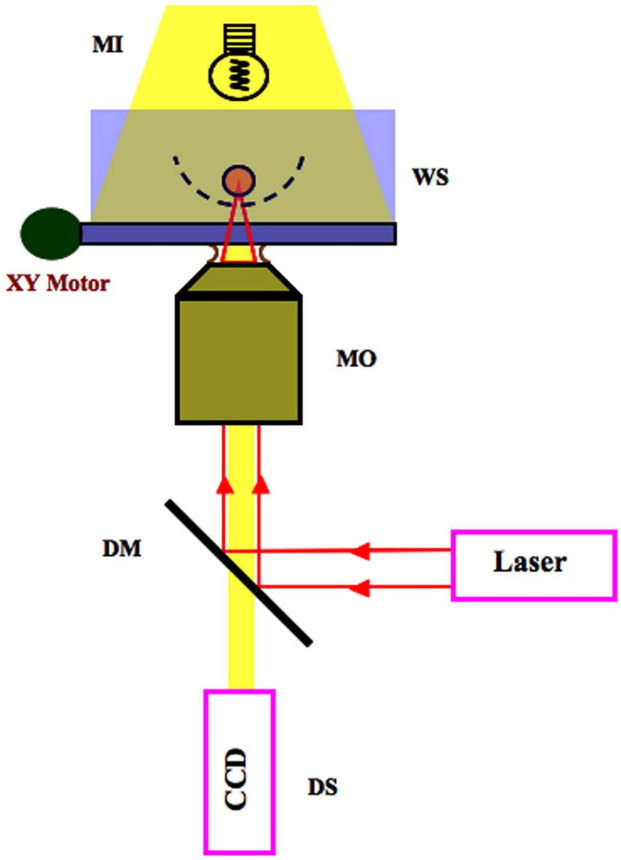


FIGURE 1 | Schematic representation of an optical tweezers setup.

The OT as a micromanipulation tool

To use the OT as a micromanipulation tool it is usually not necessary to know the force acting on the manipulated object. The microscope positioning system is employed to move the entire sample and to put the objects under the action of the focalized laser light. In this way the object to be trapped can be chosen and positioned as desired by the investigator. Different applications that employ OT in this way are: measurements of viscosity of capsule and algae polysaccharide water solutions, measurement of the Young modulus of the *C. neoformans* capsule and calculation of adhesion time of particles.

LIGHT-SCATTERING TECHNIQUES

When electromagnetic radiation passes through a given system, the electric field radiation induces a polarization of the molecules that oscillates in time with the same frequency of radiation. The molecules become secondary sources of radiation, and therefore, radiate apart from the incident energy beam. This radiation is called scattered radiation. The frequency changes, the angular distribution, polarization, and intensity of scattered radiation depend on the size, shape, and molecular interactions within the irradiated material. Therefore, from the characteristics of scattered radiation of a given system, it is possible to obtain information on the molecular structure and dynamics of the scattering medium. The most

common radiation used in scattering experiments is visible light (laser). The choice of the type of radiation is based on the length scale of interest and interaction of radiation with matter.

Static light scattering

In static light scattering (SLS) the angular dependency of the time-mean-intensity of laser light scattered by the particles is measured. The course of the scattered intensity as a function of the detector angle depends on size and structure of the particles. By extrapolating light-scattering measurements made at different angles and concentrations to zero concentration and zero angle we can obtain estimates of the average molecular weight, radius of gyration, and second virial constant without any assumptions.

Dynamic light scattering

When a laser beam is focused on a large number of particles in solution the light scattered from each particle can be measured by detectors. Since small particles are moving around randomly in the liquid, undergoing diffusive Brownian motion, the distance that the scattered waves travel to a detector varies as a function of time. Electromagnetic waves, like water and sound waves, exhibit interference effects. Scattered waves can interfere constructively or destructively depending on the distances traveled to the detector. The result is an average intensity with superimposed fluctuations. The decay times of the fluctuations are related to the diffusion constants and, therefore, the sizes of the particles. Small particles moving rapidly cause faster decaying fluctuations than large particles moving slowly. The decay times of these fluctuations may be determined either in the frequency domain (using a spectrum analyzer) or in the time domain (using a correlator). The correlator generally offers the most efficient means for this type of measurement. Correlation is defined as the average of the products of the two quantities. From this correlation, effective diameter and polydispersity can be measured. The fluctuating signal, originated from the random motion of particles in a liquid phase and the associated alterations in the intensity of the scattered light over time.

Zeta potential

Particles dispersed in a liquid often have a charge on the surface. If an electric field is applied in the liquid then these charged particles will move toward either the positive or the negative pole of the applied field. The direction they select is a clear indication of the sign of the charge they carry. The velocity with which they move is proportional to the magnitude of the charge. Zeta potential is calculated from this information. Units of zeta potential are millivolts.

CHARACTERIZATION OF ADHESION TIME BETWEEN MONOCLONAL ANTIBODIES AND *HISTOPLASMA CAPSULATUM* SURFACE PROTEINS

This approach was used to characterize the interaction of *Histoplasma capsulatum* yeast cells in the presence of monoclonal antibodies (mAbs) to an surface expressed heat shock protein of 60 kDa (Hsp60; Guimaraes et al., 2011). The mAbs induced different degrees of yeast cell agglutination that correlated with differences in efficacy in a histoplasmosis model of infection.

CHARACTERIZATION OF *C. NEOFORMANS* POLYSACCHARIDE

The human pathogenic fungus *C. neoformans* has a large polysaccharide (PS) capsule that enlarges during infection and releases copious amounts of PS (exo-PS) into cultures and infected tissues (Casadevall and Perfect, 1998). The capsular PS is a major virulence factor that can elicit protective antibody responses (Casadevall and Perfect, 1998). The capsule is essential for virulence, but the mechanism for capsular growth is unknown. During infection, cryptococcal PS is secreted resulting in deleterious immunological effects such as antibody unresponsiveness (Murphy and Cozad, 1972; Kozel et al., 1977), inhibition of leukocyte migration (Dong and Murphy, 1995), depletion of complement, cytokine production (Vecchiarelli et al., 1995), interference with antigen presentation, and prevention of phagocytosis by macrophages (Kozel and Gotschlich, 1982). Changes in capsular antigenic structure and size have been described and these alterations are associated with the capacity of *C. neoformans* yeast to invade the central nervous system, correlating structural variability with yeast cells crossing the blood–brain barrier. The PS of *C. neoformans* is the target for experimental PS conjugate vaccines and passive antibody therapies (Pirofski, 2001; Larsen et al., 2005).

The PS that is recovered from culture supernatants has historically provided an ample and convenient source of material for structural and immunological studies. Classical techniques to study polysaccharides, such as serology, did not discriminate structural features of the exo-PS and capsular PS However, a comparison of exo-PS made by two isolation techniques with capsular PS stripped from cells with gamma radiation or dimethyl sulfoxide revealed significant differences in glycosyl composition, mass, size, charge, viscosity, circular-dichroism spectra, and reactivity with mAbs when analyzed by light-scattering techniques (Frases et al., 2008). These tools permitted working with these PS in solution in its native state. The results of these studies strongly suggest that exo-PS and capsular PS are structurally different and this observation has important implications for current views of the relationship between capsular PS and exo-PS for the generation of PS preparations suitable for immunological studies, and for the formulation of PS-based vaccines for the prevention of cryptococcosis (Frases et al., 2008).

In addition, an analysis of capsular PS from cells with small and large capsules by dynamic LS revealed a linear correlation between PS effective diameter and microscopic capsular diameter (Frases et al., 2009). This result implied that capsule growth was achieved by the addition of molecules with larger length, such that some molecules can span the entire diameter of the capsule. Measurement of polystyrene bead penetration of *C. neoformans* capsules by using OT techniques revealed that the outer regions were penetrable, but not the inner regions. These results provided a mechanism for capsular enlargement based on the axial lengthening of PS molecules and suggested the first model for the architecture of a eukaryotic microbial capsule (Frases et al., 2009). In addition, zeta potential experiments demonstrated that aggregation and interaction of PS molecules could be produced by divalent cations which could be one of the capsule formation mediators (Nimrichter et al., 2007).

After determining the basis for the ultra structure of PS capsule, the biophysical techniques allow us to determine the effects

of carbon sources on the capsule and exo-PS production. For example, growth of *C. neoformans* cells in mannitol significantly increased capsular volume compared with cultivation in glucose (Guimaraes et al., 2010). The fiber lengths and glycosyl composition of capsular polysaccharide from yeast grown in mannitol was structurally different from that of yeast grown in glucose demonstrated by dynamic light scattering (DLS). Combining physical and biological data we demonstrated the capacity of the carbohydrate source and concentration to modify the expression of a major virulence factor of *C. neoformans*. These findings may impact the clinical management of cryptococcosis (Guimaraes et al., 2010).

The capsule of *C. neoformans* is composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc), and deacetylated glucosamine units play key roles as capsule constituents (Rodrigues et al., 2008). GlcNAc is the monomeric unit of chitin and chitooligomers, which participate in the connection of capsular polysaccharides to the cryptococcal cell wall. Chromatographic and dynamic light-scattering analyses demonstrated that glucuronoxylomannan (GXM), the major cryptococcal capsular component, interacts with chitin and chitooligomers. Interestingly, chitooligomers can form soluble complexes with GXM and interfere with capsular assembly, as manifested by aberrant capsules with defective connections with the cell wall and no reactivity with a mAb to GXM (Fonseca et al., 2009a).

By combining static and DLS, viscosity analysis, and high-resolution microscopy we tried to correlate our biophysical findings with biological properties. Analysis of the dependence of capsular PS molecular mass and the radius of gyration provided strong evidence against a simple linear PS configuration. Shape factors calculated from light-scattering measurements in solution revealed values consistent with polymer branching. Furthermore, viscosity measurements by OT provided complementary evidence for structural branching that interfere in complement mediated phagocytosis and inhibit nitric oxide (NO) production (Cordero et al., 2011).

In parallel, analysis of several structural parameters of GXM samples from *C. neoformans* (serotypes A and D) and *Cryptococcus gattii* (serotypes B and C) was done in an effort to correlate these variables with the production of NO by phagocytes and the activation of TLRs (Yauch et al., 2004). TLR-mediated responses were most effectively activated by a PS fraction from a *C. gattii* serotype B GXM. This serotype B polysaccharide, which was also highly efficient at eliciting the production of NO by macrophages, was similar to the other GXM samples in monosaccharide composition, zeta potential, and electrophoretic mobility. However, immunofluorescence staining using four different mAbs and dynamic light-scattering analysis revealed that the serotype B GXM showed differences in serological reactivity and had the smallest effective diameter among the GXM samples analyzed in this study. Fractionation of additional serotype B GXMs, followed by exposure of these fractions to macrophages, revealed a correlation between NO production and reduced effective diameters. Our results demonstrate a great functional diversity in GXM samples from different isolates and establish their abilities to differentially activate cellular responses. We propose that serological properties as well as physical chemical parameters, such as the diameter of polysaccharide molecules extracted from DLS, may potentially influence the inflammatory response against *Cryptococcus* spp. and may contribute to the differences in granulomatous inflammation between cryptococcal species (Fonseca et al., 2010).

The insights learned during the surface characterization of *C. neoformans* capsule using light-scattering techniques, zeta potential, and OT have been applied to other organisms. Description of structural features of *Trichosporon asahii* surface was done using these methodology. The virulence attributes of *T. asahii* are virtually unknown, despite its growing relevance as a causative agent of superficial and invasive diseases in humans (Gross and Kan, 2008). Although GXM is a well described virulence factor of pathogenic species in the *Cryptococcus* genus, it is also produced by species of the *Trichosporon* genus. Both polysaccharides share antigenic determinants, but unlike cryptococcal GXM, relatively little work has been done on trichosporal GXMs. Analyses of structural and functional aspects of GXM produced by *T. asahii* and its comparison with the properties of the cryptococcal polysaccharide have revealed that GXM from both species share antigenic reactivity, but PS from *T. asahii* has a smaller effective diameter and is less negatively charged. These results established that despite similarities in cell wall anchoring, antigenic and antiphagocytic properties, trichosporal and cryptococcal GXMs manifest major structural differences that may directly affect polysaccharide assembly at the fungal surface (Fonseca et al., 2009b).

CONCLUSION

In summary, biophysical techniques provide powerful means to study certain aspects of microbial surfaces. The application of OT and light scattering has provided new insights into the structure of the *C. neoformans* capsule and capsular polysaccharide. However, unlike techniques such as crystallography, NMR, and biochemistry that can provide definitive structural information, biophysical approaches often provide a measurement that must be integrated with other information to generate testable models. Hence, biophysical studies constitute a highly intellectual approach to the problem of microbial surfaces whereby information from many different sources must be used to construct a coherent synthesis.

ACKNOWLEDGMENTS

We thank Joshua Nosanchuk for discussions and assistance with various aspects of this work.

REFERENCES

Ashkin, A. (1997). Optical trapping and manipulation of neutral particles using lasers. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4853–4860.

Ashkin, A., and Dziedzic, J. M. (1987). Optical trapping and manipulation of viruses and bacteria. *Science* 235, 1517–1520.

Casadevall, A., and Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, DC: ASM Press.

Cordero, R. J., Frases, S., Guimaraes, A. J., Rivera, J., and Casadevall, A. (2011). Evidence for branching in cryptococcal capsular polysaccharides and consequences on its biological activity. *Mol. Microbiol.* 79, 1101–1117.

Dong, Z. M., and Murphy, J. W. (1995). Effects of the two varieties of *Cryptococcus neoformans* cells and culture filtrate antigens on neutrophil locomotion. *Infect. Immun.* 63, 2632–2644.

Fonseca, F. L., Nimrichter, L., Cordero, R. J., Frases, S., Rodrigues, J., Goldman, D. L., Andruszkiewicz, R., Milewski, S., Travassos, L. R., Casadevall, A., and Rodrigues, M. L. (2009a). Role for chitin and

chitooligomers in the capsular architecture of *Cryptococcus neoformans*. *Eukaryot. Cell* 8, 1543–1553.

Fonseca, F. L., Frases, S., Casadevall, A., Fischman-Gompertz, O., Nimrichter, L., and Rodrigues, M. L. (2009b). Structural and functional properties of the *Trichosporon asahii* glucuronoxylomannan. *Fungal Genet. Biol.* 46, 496–505.

Fonseca, F. L., Nohara, L. L., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., and Rodrigues, M. L. (2010). Immunomodulatory effects of serotype B glucuronoxylomannan from *Cryptococcus gattii* correlate with polysaccharide diameter. *Infect. Immun.* 78, 3861–3870.

Frases, S., Nimrichter, L., Viana, N. B., Nakouzi, A., and Casadevall, A. (2008). *Cryptococcus neoformans* capsular polysaccharide and exopolysaccharide fractions manifest physical, chemical, and antigenic differences. *Eukaryot. Cell* 7, 319–327.

Frases, S., Pontes, B., Nimrichter, L., Viana, N. B., Rodrigues, M. L., and Casadevall, A. (2009). Capsule of *Cryptococcus neoformans* grows by enlargement of polysaccharide molecules. *Proc. Natl. Acad. Sci. U.S.A.* 106, 1228–1233.

Gross, J. W., and Kan, V. L. (2008). *Trichosporon asahii* infection in an advanced AIDS patient and literature review. *AIDS* 22, 793–795.

Guimaraes, A. J., Frases, S., Cordero, R. J., Nimrichter, L., Casadevall, A., and Nosanchuk, J. D. (2010). *Cryptococcus* neoformans responds to mannitol by increasing capsule size in vitro and in vivo. *Cell. Microbiol.* 12, 740–753.

Guimaraes, A. J., Frases, S., Pontes, B., de Cerqueira, M. D., Rodrigues, M. L., Viana, N. B., Nimrichter, L., and Nosanchuk, J. D. (2011). Agglutination of *Histoplasma capsulatum* by IgG monoclonal antibodies against Hsp60 impacts macrophage effector functions. *Infect. Immun.* 79, 918–927.

Kozel, T. R., and Gotschlich, E. C. (1982). The capsule of *cryptococcus neoformans* passively inhibits phagocytosis of the yeast by macrophages. *J. Immunol.* 129, 1675–1680.

Kozel, T. R., Gulley, W. F., and Cazin, J. Jr. (1977). Immune response to *Cryptococcus neoformans* soluble polysaccharide: immunological unresponsiveness. *Infect. Immun.* 18, 701–707.

Larsen, R. A., Pappas, P. G., Perfect, J., Aberg, J. A., Casadevall, A., Cloud, G. A., James, R., Filler, S., and Dismukes, W. E. (2005). Phase I evaluation of the safety and pharmacokinetics of murine-derived anticryptococcal antibody 18B7 in subjects with treated cryptococcal meningitis. *Antimicrob. Agents Chemother.* 49, 952–958.

Murphy, J. W., and Cozad, G. C. (1972). Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. *Infect. Immun.* 5, 896–901.

Nimrichter, L., Frases, S., Cinelli, L. P., Viana, N. B., Nakouzi, A., Travassos, L. R., Casadevall, A., and Rodrigues, M. L. (2007). Self-aggregation of *Cryptococcus neoformans* capsular glucuronoxylomannan is dependent on divalent cations. *Eukaryot. Cell* 6, 1400–1410.

Park, S. Y., Love, T. M., Nelson, J., Thurston, S. W., Perelson, A. S., and Lee, H. Y. (2011). Designing a genome-based HIV incidence assay with high sensitivity and specificity. *AIDS*. PMID: 21716075. [Epub ahead of print].

Pirofski, L. A. (2001). Polysaccharides, mimotopes and vaccines for fungal and encapsulated pathogens. *Trends Microbiol.* 9, 445–451.

Rodrigues, M. L., Alvarez, M., Fonseca, F. L., and Casadevall, A. (2008). Binding of the wheat germ lectin to *Cryptococcus neoformans* suggests an association of chitin-like structures with yeast budding and capsular glucuronoxylomannan. *Eukaryot. Cell.* 7, 602–609.

Vecchiarelli, A., Retini, C., Pietrella, D., Monari, C., Tascini, C., Beccari, T., and Kozel, T. R. (1995). Downregulation by cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1 beta secretion from human monocytes. *Infect. Immun.* 63, 2919–2923.

Viana, N. B., Rocha, M. S., Mesquita, O. N., Mazolli, A., and Maia Neto, P. A. (2006). Characterization of objective transmittance for optical tweezers. *Appl. Opt.* 45, 4263–4269.

Viana, N. B., Rocha, M. S., Mesquita, O. N., Mazolli, A., Maia Neto, P. A., and Nussenzveig, H. M. (2007). Towards absolute calibration of optical tweezers. *Phys. Rev. E. Stat. Nonlin. Soft Matter Phys.* 75(Pt 1), 021914.

Yauch, L. E., Mansour, M. K., Shoham, S., Rottman, J. B., and Levitz, S. M. (2004). Involvement of CD14, toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen *Cryptococcus neoformans* in vivo. *Infect. Immun.* 72, 5373–5382.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 August 2011; accepted: 17 September 2011; published online: 10 October 2011.

Citation: Frases S, Viana NB and Casadevall A (2011) Biophysical methods for the study of microbial surfaces. Front. Microbio. ***2****:207. doi: 10.3389/fmicb.2011.00207*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 05 December 2011
doi: 10.3389/fmicb.2011.00239

Structural analysis of fungal cerebrosides

Eliana Barreto-Bergter[1]*, Guilherme L. Sassaki[2] and Lauro M. de Souza[2]

[1] *Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Departamento de Bioquímica e Biologia Celular, Universidade Federal do Paraná, Curitiba, Brazil*

Edited by:
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Karin Thevissen, Catholic University of Leuven, Belgium
Steven Bruce Levery, University of Copenhagen, Denmark

***Correspondence:**
Eliana Barreto-Bergter, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, Bloco I, 21941-902 Rio de Janeiro, Brazil.
e-mail: eliana.bergter@micro.ufrj.br

Of the ceramide monohexosides (CMHs), gluco- and galactosyl-ceramides are the main neutral glycosphingolipids expressed in fungal cells. Their structural determination is greatly dependent on the use of mass spectrometric techniques, including fast atom bombardment-mass spectrometry, electrospray ionization, and energy collision-induced dissociation mass spectrometry. Nuclear magnetic resonance has also been used successfully. Such a combination of techniques, combined with classical analytical separation, such as high-performance thin layer chromatography and column chromatography, has led to the structural elucidation of a great number of fungal CMHs. The structure of fungal CMH is conserved among fungal species and consists of a glucose or galactose residue attached to a ceramide moiety containing 9-methyl-4,8-sphingadienine with an amidic linkage to hydroxylated fatty acids, most commonly having 16 or 18 carbon atoms and unsaturation between C-3 and C-4. Along with their unique structural characteristics, fungal CMHs have a peculiar subcellular distribution and striking biological properties. Fungal cerebrosides were also characterized as antigenic molecules directly or indirectly involved in cell growth or differentiation in *Schizophyllum commune*, *Cryptococcus neoformans*, *Pseudallescheria boydii*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Colletotrichum gloeosporioides*. Besides classical techniques for cerebroside (CMH) analysis, we now describe new approaches, combining conventional thin layer chromatography and mass spectrometry, as well as emerging technologies for subcellular localization and distribution of glycosphingolipids by secondary ion mass spectrometry and imaging matrix-assisted laser desorption ionization time-of-flight.

Keywords: cerebrosides, pathogenic fungi, structural characterization, mass spectrometry, NMR spectroscopy

INTRODUCTION

Cerebrosides are commonly called monohexosylceramides or ceramide monohexosides (CMH). They are neutral glycosphingolipids that usually contain glucose or galactose, with β-glycosidic linkages to the primary alcohol of an *N*-acyl sphingoid base (ceramide). Cerebrosides can be found in plants, fungi, and animals, although clear differences in the structure of the ceramide backbone of these organisms are observed. Galactosylceramide is present only in fungi and animals. In contrast, glucosylceramide is the unique glycosphingolipid which plants, fungi, and animals have in common. CMHs have been widely detected in fungal cells (reviewed by Warnecke and Heinz, 2003; Barreto-Bergter et al., 2004). The current literature indicates that cerebrosides seem to be present in almost all fungal species studied so far, with *Saccharomyces cerevisiae* representing a well-known exception together with *Candida glabrata* (Tavares et al., 2008). Gluco- and galactosyl-ceramides are the main neutral glycosphingolipids expressed in fungal pathogens (**Figure 1**). The long-chain base (LCB) 9-methyl-4,8-sphingadienine is present in almost all pathogenic fungi studied (Levery et al., 2000; Barreto-Bergter et al., 2004). Structural modifications of the ceramide moiety in these CMHs include different sites of unsaturation as well as varying lengths of fatty acid residues. LCB was first described in monohexosylceramides from *Aspergillus oryzae* (Fujino and Ohnishi, 1976) and was subsequently isolated from *Schizophyllum commune* (Kawai and Ikeda, 1985), from the plant pathogen *Fusicoccum amygdale* (Ballio et al., 1979), and the edible fungi *Clitocybe geotropa* and *Clitocybe nebularis* (Fogedal et al., 1986). CMHs were further characterized in lipid extracts from the fungal species *Alternaria raphani* (Wang et al., 2009), *Aspergillus fumigatus* (Boas et al., 1994; Toledo et al., 1999), *Aspergillus nidulans* (Levery et al., 2002), *Aspergillus niger* (Levery et al., 2000; Levery, 2005), *Aspergillus versicolor* (Boas et al., 1994), *Acremonium chrysogenum* (Sakaki et al., 2001), *Amanita muscaria* (Weiss and Stiller, 1972), *Candida albicans* (Matsubara et al., 1987), *Candida deformans* (Mineki et al., 1994), *Candida utilis* (Wagner and Zofcsik, 1966), *Colletotrichum gloeosporioides* (da Silva et al., 2004), *Cryptococcus neoformans* (Rodrigues et al., 2000), *Fonsecaea pedrosoi* (Nimrichter et al., 2004), *Fusarium graminearum* (Zaüner et al., 2008), *Fusarium solani* (Duarte et al., 1998), *Ganoderma lucidum* (Mizushina et al., 1998), *Hansenula anomala* (Ng and Laneelle, 1977), *Histoplasma capsulatum* (Toledo et al., 2001), *Hypsizygus marmoreus* (Sawabe et al., 1994), *Kluyveromyces waltii* (Takakuwa et al., 2002), *Kluyveromyces thermotolerans* (Takakuwa et al., 2002), *Kluyveromyces lactis* (Takakuwa et al., 2002), *Lentinus edodes* (Kawai, 1989), *Magnaporthe grisea* (Koga et al., 1998; Umemura et al., 2000; Maciel et al., 2002), *Mortierella alpina* (Batrakov et al., 2002), *Metrilium senile* (Karlsson et al., 1979), *Neurospora crassa* (Lester et al., 1974; Park et al., 2005), *Paracoccidioides brasilensis* (Takahashi et al., 1996), *Penicillium chrysogenum*

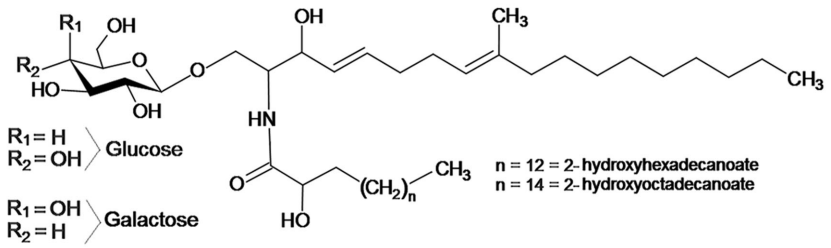


FIGURE 1 | Typical fungal cerebroside structure, containing a C19 sphingoid base with a C-9 methyl group, and two double bonds (Δ4, Δ8).

(Peng et al., 2011), *Pichia pastoris* (Sakaki et al., 2001), *Polyporus ellisii* (Gao et al., 2001), *Polyporus squamosus* (Arigi et al., 2007), *Pseudallescheria boydii* (Pinto et al., 2002), *Rhynchosporium secalis* (Sakaki et al., 2001), *Saccharomyces kluyveri* (Takakuwa et al., 2002), *Sordaria macrospore* (Sakaki et al., 2001), *Sporothrix schenckii* (Toledo et al., 2000), *Termitomyces albuminosus* (Qi et al., 2001), *Zygosaccharomyces cidri*, and *Zygosaccharomyces fermentati* (Takakuwa et al., 2002).

Whereas many studies have focused on the functional roles of glycosphingolipids in mammals, relatively little is known about the structures of glycosphingolipids of pathogenic organisms, and how such pathogen-derived glycosphingolipids influence immune functions of their hosts.

CMHs have been characterized in fungal cells as bioactive molecules with several distinct roles. Fungal cerebrosides induce cell differentiation in *S. commune*, and the 8E-double bond and the methyl group at C-9 in the sphingoid base is essential for this differentiation (Kawai and Ikeda, 1982). Monohexosylceramides isolated from the rice pathogen *M. grisea* are active elicitors of the hypersensitive response in rice (Koga et al., 1998; Umemura et al., 2000).

The presence of CMH as a structural component of the fungal cell wall was clearly demonstrated by electron microscopy of yeast cells of *C. neoformans* labeled with immunogold antibodies (Rodrigues et al., 2000) and by immunofluorescence of mycelium cells of *C. gloeosporioides*, using a monoclonal antibody to GlcCer (da Silva et al., 2004). Glucosylceramides from *P. boydii*, accumulated on the surface of mycelia and pseudo-hyphal forms, were recognized by antibodies from rabbits immunized with *P. boydii* whole cells (Pinto et al., 2002).

A monoclonal antibody to the glucosylceramide synthesized by *P. brasiliensis* was produced and reacted with *A. fumigatus* conidiophore. This finding supported the idea that CMHs are preferentially accumulated on surface sites related to fungal growth, but it also suggested that they are involved in the differentiation process (Toledo et al., 2001).

Using polyclonal antibodies or Mabs anti-monohexosylcera mides, our group showed that CMHs were associated with fungal growth (Rodrigues et al., 2000; Nimrichter et al., 2005) and morphological transitions in *C. neoformans*, *P. boydii*, *C. albicans*, *A. fumigatus*, *C. gloeosporioides*, and *S. apiospermum* (reviewed by Barreto-Bergter et al., 2004).

We now describe methods to extract neutral glycosphingolipids (GSLs) from fungal cells, techniques to separate them by thin-layer chromatography (TLC) or high-performance thin layer chromatography (HPTLC) and, finally, strategies to structurally characterize the glycan and ceramide moieties of the GSLs.

ISOLATION AND PURIFICATION

The methodology described herein follows the steps of purification routinely used in our laboratory for CMH extraction and purification (Boas et al., 1994; Duarte et al., 1998, Pinto et al., 2002; da Silva et al., 2004), although different methods are available in the current literature for isolation and purification of CMHs (reviewed by Barreto-Bergter et al., 2004).

Fungal cells are successively extracted with chloroform–methanol (2:1 and 1:2 v/v). Extracts are combined, dried, and the crude lipid extract is partitioned according to (Folch et al., 1957). The lipids recovered from the Folch lower layer are fractionated on a silica gel column eluted with chloroform, acetone, and then methanol. The acetone and methanol fractions containing CMHs are further purified on a silica gel column, which are sequentially eluted with chloroform–methanol with increasing concentrations of methanol (95:5, 9:1, 8:2, and 1:1 v/v), and finally with 100% methanol. Fractions eluted with chloroform–methanol (9:1 and 8:2 v/v) are further purified by chromatography on Iatrobeads RS 2060 (Macherey and Nagel, Düren, Germany), using the same elution system, to provide a purified glycosphingolipid fraction. A typical example of the purification of cerebrosides is shown in **Figure 2**.

ANALYTICAL TECHNIQUES TO STUDY CEREBROSIDES

HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography is a popular and convenient technique for separation and identification of CMH, and is the first step in analysis of glycosphingolipids, requiring only small amounts (3–4 nM) of material (Scandroglio et al., 2009). This technique does not allow determination of chemical structures, but can give preliminary information on their structures based on chromatographic mobility, in comparison with standards (Wells and Dittmer, 1965; Scandroglio et al., 2009) and the reaction with specific staining reagents (Svennerholm, 1956; Scandroglio et al., 2009). Cerebrosides are analyzed on HPTLC plates developed with $CHCl_3$–MeOH–H_2O (65:25:4 v/v) or $CHCl_3$–MeOH–2M NH_4OH (40:10:1 v/v/v; Pinto et al., 2002; Rollin-Pinheiro et al., unpublished) and visualized through iodine vapor, which is absorbed by both saturated and unsaturated lipids (Kates, 1986), and by spraying the plates with orcinol–sulfuric acid, in which case cerebrosides give violet spots (Svennerholm, 1956).

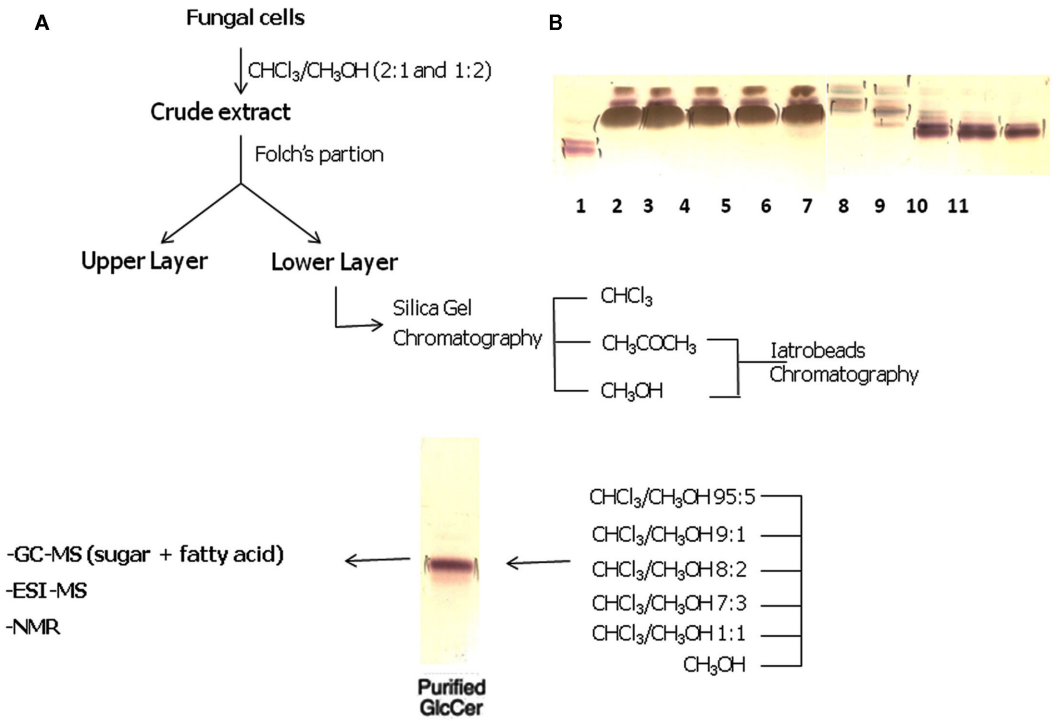


FIGURE 2 | (A) Overview of the strategy used for purification of CMHs from fungal cells. **(B)** High-performance thin-layer chromatography of fungal neutral glycosphingolipids isolated by silica gel column chromatography. Lane 1: cerebroside from bovine brain. Lanes 2–6: Chloroform–methanol (95:5 v/v) fractions. Lanes 7–8: Chloroform–methanol (90:10 v/v) fractions. Lanes 9–11: Chloroform–methanol (80:20 v/v) fractions. Solvent system: chloroform/methanol ½ M NH_4OH (40:10:1 v/v/v). Detection: iodine and orcinol–sulfuric acid reagents.

MASS SPECTROMETRY APPLIED TO FUNGAL LIPID ANALYSIS

Mass spectrometry (MS) is a powerful tool for the analysis of lipids. However, in the early days of its development, the intact lipids could not be directly analyzed by MS, since the available ionization sources produced ions by accelerating electrons electron ionization (EI), which should provide the molecules in a gaseous phase, to transfer their energy on ionization. This was the limiting factor for its application, since the molecules must have a sufficient vapor pressure to enter into the gaseous phase of the mass spectrometer's ion source. Briefly, MS with EI was coupled to gas chromatography (GC–MS) and lipids could be analyzed. However, in order to become analyzable, CMHs should be converted to their constituent moieties (sugar, fatty acids, LCB), submitted to appropriate chemical derivatization and then identified by GC–MS.

To overcome the requirements for volatilization, the glycosphingolipids were methanolyzed and their components analyzed as fatty acid methyl esters (FAMEs) (Duarte et al., 1998).

The monosaccharide components of glycosphingolipids were analyzed by GC–MS, by conversion to alditol acetate derivatives after being liberated by acid hydrolysis (Sawardeker et al., 1965). Long-chain bases were released (LCB, or sphingoid base) from CMH by methanolysis and analyzed by GC–MS as the TMS derivatives after treatment with *bis*-(trimethylsilyl) -trifluoroacetamide/pyridine (Zanetta et al., 1999; Sweeley, 1963; Karlsson and Holm, 1965). A simple derivatization method established by Sassaki et al. (2008) can also be employed for GC–MS identification of monosaccharides and LCB, present in glycosphingolipids.

The highly energetic electrons (70 eV) in EI produce radical ions, but a secondary effect associated with EI is an intense production of fragment-ions, due to the extensive covalent-linkage breakdown promoted by the absorbed energy by the molecules.

In order to overcome the tendency of fragmentation during the ionization process, other ion sources have been developed, called soft ionization methods.

SOFT IONIZATION TECHNIQUES FOR THE DETERMINATION OF THE STRUCTURE OF INTACT LIPIDS

The soft ionization technologies allow ionization and transferring non-volatile and thermolabile molecules to the gaseous phase without extensive production of fragments, impossible with EI. This type of source became available from 1980s with the introduction of fast atom bombardment-mass spectrometry (FAB-MS), followed by electrospray ionization mass spectrometry (ESI-MS), and matrix-assisted laser desorption ionization (MALDI). Another important characteristic is that these techniques allow changing the ion polarity, since it is possible to produce ions via cation or anion interactions, such as protonation, sodiation, lithiation or deprotonation, chlorination, and so on. Nowadays, variations of these techniques are also found, such as nano-ESI-MS, photospray ionization, as well as those called ambient MS, which include direct analysis in real time-mass spectrometry (DART-MS) and desorption electrospray (DESI),

suitable for analysis of analytes deposited on a surface, such as with HPTLC.

The ions obtained from intact molecules provide information on their molecular weight, as well as fragmentation being an important tool for structural analysis. The MS instrumentation allow separation of a specific ion and subjecting it to a fragmentation process, as occurring in collision-induced dissociation (CID) or collision-activated dissociation (CAD). This type of analysis is usually referred to as tandem-MS and the spectrometers operating in tandem mode consist of conjugated analyzers, such as triple quadrupole (TQ or QQQ), quadrupole-time of flight (Q-TOF) or the ion trap, and Fourier-transform ion cyclotron resonance (FT-ICR) analyzers, which allow the re-fragmentation of product ions (fragment-ions from MS/MS) usually referred to as MS^n.

FAST ATOM BOMBARDMENT-MASS SPECTROMETRY

Fast atom bombardment was first introduced by (Barber et al., 1981a,b; Morris et al., 1981). The sample is dissolved in a non-volatile liquid matrix (e.g., glycerol, thioglycerol, triethanolamine), and the mixture is bombarded by a beam of accelerated atoms (typically argon or xenon). Non-volatile, polar, and thermolabile molecules, such as lipids, could be ionized directly analyzed (Murphy et al., 1982). FAB-MS is considered to be a relatively soft ionization technique, since it produces primary the molecular ion (quasi- or pseudo-molecular ions), although numerous fragments ions are typically generated.

Since FAB-MS allowed the analysis of a broad range of intact molecules, it rapidly became one of the most commonly used techniques for structural characterization of lipids, including a wide variety of fungal cerebrosides, using native and peracetylated samples (reviewed by Levery, 2005). The structure of *A. fumigatus* CMH was elucidated, using FAB-MS in the positive mode (Boas et al., 1994). Spectra were obtained using peracetylated samples, in the presence and the absence of sodium acetate, and the main CMH from *A. fumigatus* was identified as 2′-*N*-hydroxyoctadecenoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine. The presence of an additional ceramide containing 2-hydroxy-octadecanoic acid was reported by (Toledo et al., 1999), employing ESI-MS and ESI-MS/CID.

Using peracetylated samples with positive- and negative-ion FAB-MS, other cerebrosides were characterized by our group from *A. versicolor*, *C. neoformans*, *F.pedrosoi*, *F. solani*, *Fusarium* sp., and *M. grisea* (reviewed by Barreto-Bergter et al., 2004). FAB-MS analysis of native and peracetylated samples indicated that the glycolipid from *P. boydii* consisted of two components that differ in their fatty acid compositions. In a negative-ion spectrum, deprotonated molecules appeared at *m/z* 726 and 754, consistent with monohexosylceramides containing hydroxyl-hexadecanoic and hydroxyl-octadecanoic acids, and C19 sphingadienine. On peracetylation with acetic anhydride/pyridine, $[M + Na]^+$ ions were at *m/z* 1030 and 1002, indicating addition of six acetyl groups to the mass of the underivatized glycolipids, consistent with hydroxyl acid-containing monohexosylceramides (**Figure 3**).

Over the past decades, FAB-MS/MS has been used to characterize sphingolipids, since it offers many advantages for structural elucidation and has high-energy CID capabilities (Domon and Costello, 1988; Pittenauer and Allmaier, 2009a,b; Ann and Adams, 1992; Ann and Adams, 1993). Evidence for the location of double bonds in the long-chain base was obtained by collisional activation of the sphingadienine fragments at *m/z* 276. Cleavages of the alkyl chain indicated the presence of a 4,8-sphingadienine structure, with a methyl substituent on C-9. An identical fragment ion

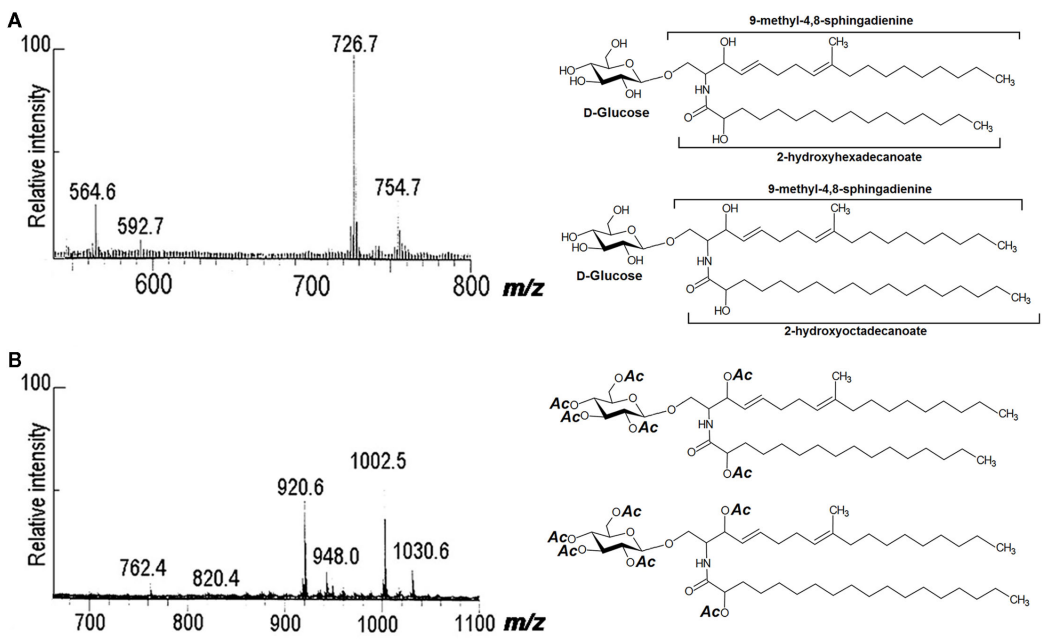


FIGURE 3 | Fast atom bombardment-mass spectrometry spectra of CMHs from *P. boydii*. **(A)** Negative-ion spectrum $[M - H]^-$ of native CMHs, and **(B)** positive-ion spectrum $[M + Na]^+$ of peracetylated CMHs.

spectrum was obtained from collision activation of *m/z* 276 in the FAB spectrum of the CMH from *F. solani* (Duarte et al., 1998) and *A. fumigatus* (Boas et al., 1994).

ELECTROSPRAY IONIZATION MASS SPECTROMETRY

In the mid 1980s, two soft ionization techniques were developed, ESI by John Fenn (Yamashita and Fenn, 1984) and MALDI (Karas et al., 1987). These techniques allowed direct analysis by MS of a great number of lipids.

Electrospray ionization mass spectrometry is an atmospheric pressure ionization technique, in which the sample can be dissolved in common solvents, such as methanol, acetonitrile, water, or in mixtures. The sample passes through a capillary tube, to which an intense electrical field is applied creating a spray of charged particles which are dried with the aid of a nitrogen flow and the ionized analytes are transferred to mass analyzers (Yamashita and Fenn, 1984).

Matrix assisted laser desorption ionization is a desorption ionization (DI) method which deserves special attention because, besides its extraordinary sensitivity, it is efficient in producing intact molecular ions of large biological compounds. The MALDI matrix compounds are usually organic acids that have strong electronic absorption in the region of the applied laser wavelength.

Ions obtained by the soft ionization processes usually appear as protonated or deprotonated species, depending on their chemical characteristics. However, it is also common to find ions produced by the combination of the analyte with different cations or anions, such as Na^+ or K^+ and Cl^-, which are referred to as adducts. These ions are commonly present in the solvent and could aid the ionization of several polar molecules, such as neutral glycolipids. On the other hand, small amounts of specific adducts (e.g., Li^+) can be added to the solvent, to increase ion production.

Ions obtained from intact molecules contain information on their molecular weight, although further information is necessary to define their chemical structures. Accordingly, the majority of mass spectrometers allow the selection of a specific ion and its fragmentation. This type of analysis is usually referred to as tandem-MS (MS/MS). Spectrometers capable of performing tandem-MS consist of conjugated analyzers, such as TQ or QQQ, Q-TOF or the ion trap analyzer.

Positive-ion mode ESI-MS employing quadrupole instruments has been used extensively for fungal cerebroside analysis (Toledo et al., 1999; Levery et al., 2000; Toledo et al., 2000; da Silva et al., 2004; Nimrichter et al., 2005). A GlcCer-enriched fraction of *C. gloeosporioides* was analyzed in the positive ion ESI-MS mode $[M + H]^+$. Two major ion species appeared at *m/z* 754 and 756, as well as their corresponding fragments at *m/z* 736 and 738, generated by the loss of elements of water (**Figure 4A**). ESI-MS/MS has also been applied and major fragment-ions at *m/z* 594 and 592 were obtained, and assigned to 2′-*N*-hydroxyoctadecanoyl-9-methyl-4,8-sphingadienine and 2′-*N*-hydroxyoctadecenoyl-9-methyl-4,8-sphingadienine respectively (**Figures 4B,C**) (da Silva et al., 2004).

As neutral molecules, CMHs can be analyzed by MS with the aid of adducts, the most common one being Na^+. However, the use of other adducts could reveal more details from the structures, by evidencing fragments which could be missed. The use of Li^+, as LiCl, or LiI, to aid cationization of neutral molecules containing carbohydrates has proven to be useful, improving ion detection and fragmentation provided by collision-induced dissociation-mass spectrometry (CID-MS), using less energies than other common alkali cations (i.e., Na^+ and K^+). ESI-MS/CID-MS of lithium adducts was applied for analysis of cerebrosides from *F. pedrosoi* (Nimrichter et al., 2005), *A. niger*, *C. albicans*, *Cryptococcus* spp., and yeast and mycelium forms of *P. brasiliensis* (Levery et al., 2000). More recently, ESI-MS in the positive ionization mode of lithiated

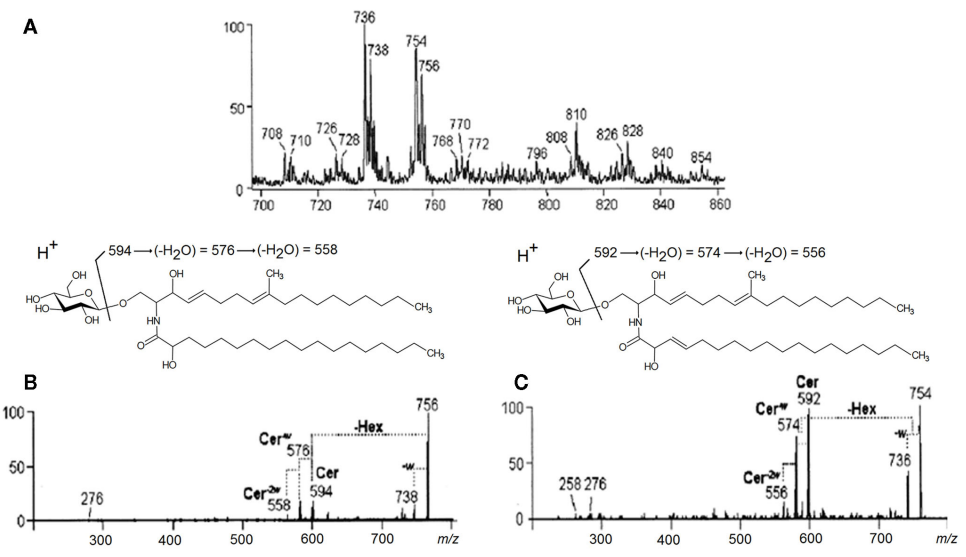


FIGURE 4 | Positive ESI-MS $[M + H]^+$ analysis of the GlcCer species of *C. gloeosporioides*. (A) MS^1 spectrum **(B)** and **(C)** ESI-MS2 of the ion species *m/z* 756 and 754, observed in **(A)**. Cer, ceramide; Hex, hexose; w, water.

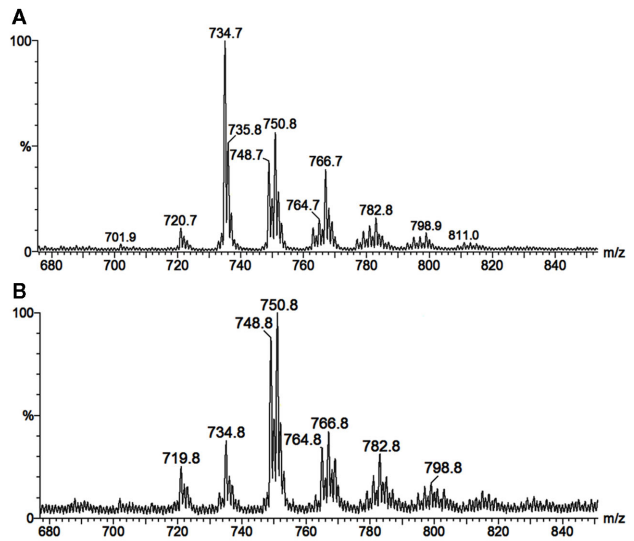


FIGURE 5 | Positive ESI-MS $[M + Li]^+$ of ceramide monohexosides from the opportunistic human pathogen *Scedosporium apiospermum* (A), and the saprophytic *Cladosporium resinae* (B).

adducts has been used by our group for cerebroside analysis of *S. apiospermum*, an opportunistic human pathogen that belongs to the *P. boydii*/*Scedosporium* complex (Rollin-Pinheiro et al., unpublished). Ions with ranging from *m/z* 700 to 800 were detected, with two major ion species appearing at *m/z* 734 $[M + Li]^+$ and 750 $[M + Li]^+$ (**Figure 5A**). Although in a different ratio, similar results were found in the analysis of glycosphingolipids from *Cladosporium resinae* (**Figure 5B**), a saprophytic species commonly found as a contaminant in fuel oil storage tanks (Lindley and Heyderman, 1985; Seifert et al., 2007). CID-MS analysis confirmed their similarities and revealed that the differences were located in the LCB moieties (**Figures 6A–D**).

An ESI-MS examination of lipid extracts from *F. pedrosoi* (Nimrichter et al., 2005), another human pathogen, also showed the presence of an ion at *m/z* 750. This was interpreted as resulting from a ceramide with a LCB containing one more hydroxyl group. This hypothesis was supported by the analysis of the permethylated CMH derivative, showing a molecular, monolithiated ion at *m/z* 862, consistent with addition of eight methyl groups. Fragmentation of permethylated CMH suggested that the additional hydroxyl group is linked to C-4 or C-5 of its long-chain base (Nimrichter et al., 2005). However, this LCB conserves other characteristics of fungal LCBs, such as the presence of a double

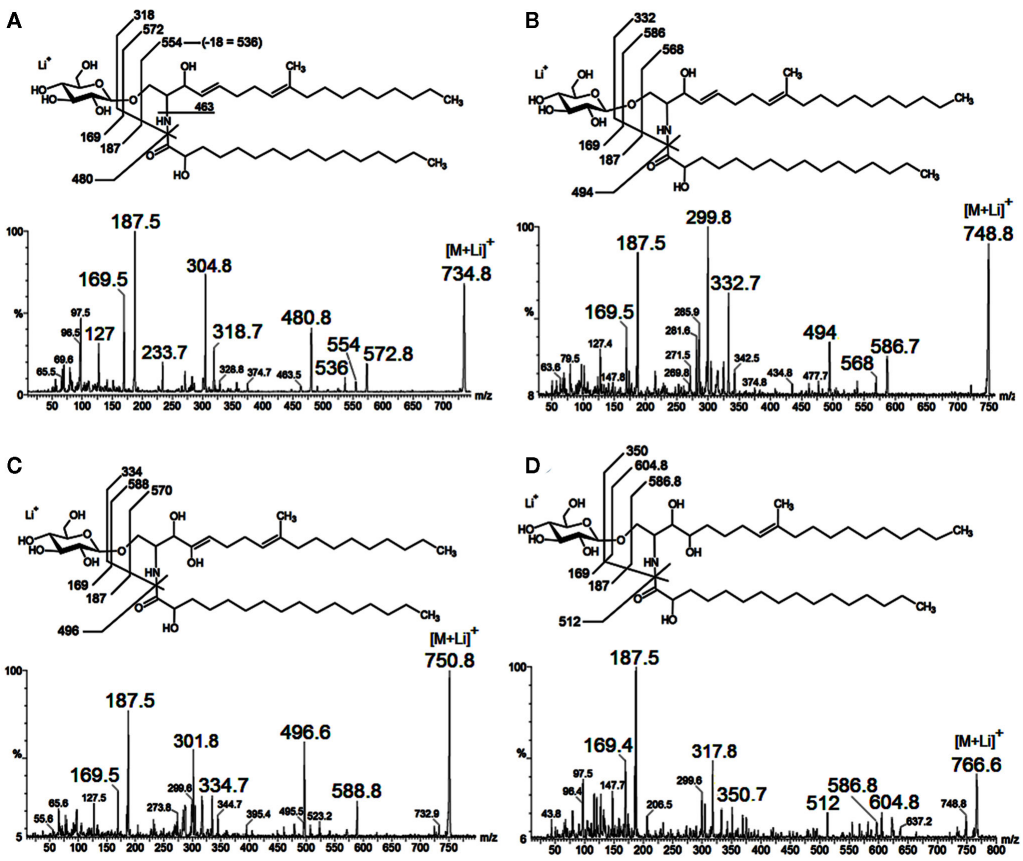


FIGURE 6 | Characteristic tandem-MS profiles obtained from lithiated CMHs from *Scedosporium apiospermum* (A,C) and *Cladosporium resinae* (B,D). The fragmentation indicated differences in the LCB moieties and the putative structures were depicted for each CMH species.

bond (C8 to C-9) and a CH_3 group in C9, distinguishing it from phytosphingosine.

NMR SPECTROSCOPY

Nuclear magnetic resonance (NMR) spectroscopy is a non-destructive method capable of furnishing information concerning the primary structure of cerebrosides, such as monosaccharide composition, anomeric configuration, and size of the sugar rings. The assignments of all ceramide signals could also be obtained without interference of the sugar moiety.

Fungal cerebrosides have been characterized by 1D, 2-D-^{1}H-NMR, and ^{13}C-NMR spectroscopy as native (underivatized) or peracetylated samples.

Native glycosphingolipid fractions isolated from *Paracoccidioides brasiliensis* (Toledo et al., 1999), *Aspergillus fumigatus* (Toledo et al., 1999), *Sporothrix schenckii* (Toledo et al., 2000), *Histoplasma capsulatum* (Toledo et al., 2001), dissolved in DMSO–d6/2% D2O have been extensively analyzed by a series of 1- and 2-D homonuclear and heteronuclear NMR experiments (1H-1H TOCSY, NOESY, ^{1}H-^{13}C HSQC, HMBC). According to the authors, this solvent presents superior solubility characteristics as compared with CDCl3/CD3OD mixtures and chemical shift reproducibility for cerebrosides without the need for derivatization. Using these techniques, a native glycosphingolipid fraction from *Fusarium oxysporum* dissolved in $CDCl_3$–CD_3OD was recently characterized (**Figure 7**).

^{1}H and ^{13}C-NMR spectra for several fungal glucosylceramides have been previously recorded as per-*O*-acetylated compounds in CDCl3 (Fogedal et al., 1986; Boas et al., 1994; da Silva et al., 1997; Duarte et al., 1998) and more recently in *M. alpina* (Batrakov et al., 2002) and *F. graminearum* (Zaüner et al., 2008).

In the ^{1}H-NMR spectrum of the peracetylated GlcCer of *A. fumigatus*, protons corresponding to a β-glucopyranosyl unit were recognized by their characteristic chemical shifts (Boas et al., 1994). The spectrum displayed one sharp doublet at δ 4.47 ppm with a coupling constant $J_{1,2} = 7.9$ Hz due to anomeric nuclei. The remaining protons appearing in the spectrum were in agreement with the β-glucopyranose structure. The structure of the long-chain base was deduced from one- and two-dimensional NMR spectra. The proton to proton COSY spectrum enabled the connectivity of the long-chain base protons to be traced between H-1 and H-11 and those of the fatty acid from H-2 to H-5. The chemical shifts of 5.30 and 5.50 ppm, assigned to the long-chain base H-3 and the fatty acid H-2, respectively, were typical of protons with acetylated OH-groups neighboring the olefinic bonds. For H-4 and H-5 of the long-chain base, we found a coupling constant of 15.3 Hz and for the protons H-3 and H-4 of the fatty acid 14.3 Hz, respectively, typical of *trans*-double bonds. An additional

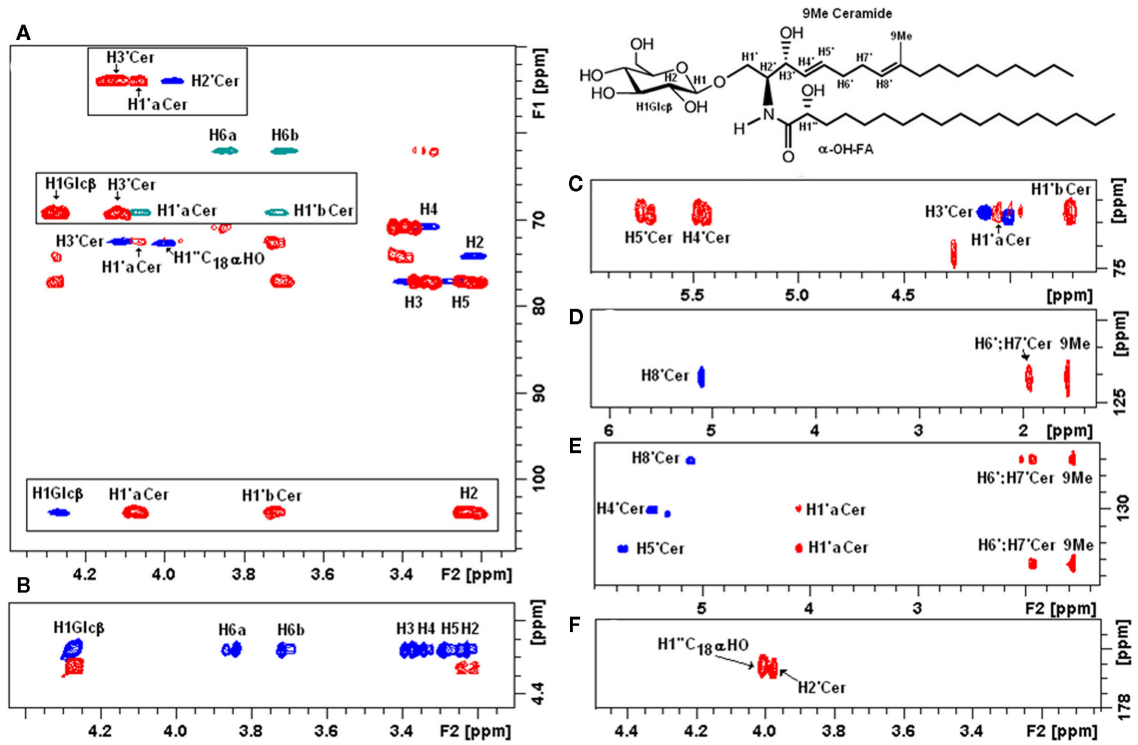


FIGURE 7 | Two-dimensional NMR fingerprints of *F. oxysporum* CMH observed by homonuclear and heteronuclear experiments. (A) Overlaid representation of fingerprint assignments of partial edited HSQC and HMBC spectra; positive phase (blue) correspond to CH and CH3 carbons, negative phase (green) correspond to CH2 carbons, in (red) HMBC cross peaks; (boxes) represent glycosyl-ceramide linkage zones fingerprints. **(B)** Partial TOCSY (blue) and COZY (red) spectra from the anomeric region showing the main cross peaks of the carbohydrate ring, observation of the total ^{1}H–^{11}H axial correlations and reach the monosaccharide composition. **(C–E)** HSQC and HMBC slices of unsaturated carbons in ceramide moiety. **(E,F)** Fingerprint slices of the quaternary carbons (C-9) and amide linkage at 136.4 and 176.5 ppm, respectively.

upfield resonance for the cerebrosides was that of the sphingadienine 9-methyl group which was a singlet at ∼1.545 ppm. However, the chain lengths of the fatty acid and the sphingosine moieties of GlcCer could not be determined by NMR analysis, in contrast with mass spectrometric methods (Levery, 2005).

In summary, a series of CMHs were isolated from pathogenic fungi and their structures elucidated using a combination of HPTLC, FAB-MS, ESI-MS spectrometry, and NMR. Their principal structures are shown in **Figure 8**.

TLC OVERLAY TECHNIQUES

The TLC overlay technique was introduced by Magnani et al. (1980) and consists of resolution of the glycosphingolipid on a TLC plate, with detection of the CMH *in situ* using specific ligands as antibodies, bacterial toxins, carbohydrate-specific viruses, and bacteria. Crude lipid extracts containing glycosphingolipids as minor components could also be analyzed, avoiding time consuming purification procedures. It is a highly sensitive technique that only requires nanogram quantities of CMH (Meisen et al., 2011).

HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY IMMUNOSTAINING

Using anti-glucosylceramide antibodies, CMH could be identified in cell wall lipid extracts of *C. neoformans* separated by HPTLC (Rodrigues et al., 2000). Using an IgG 2a monoclonal antibody anti-glucosylceramide (MEST-2), Toledo et al. (2001) showed that this Mab reacts with glucosylceramide from yeast and mycelium forms of *P. brasiliensis*, *H. capsulatum*, *S. schenckii*, hyphae of *A. fumigatus*, and yeast forms of *C. albicans*, *C. neoformans*, *C. laurentii*, and *C. albidus*. The same results were observed using solid-phase radioimmunoassay (RIA). Studies on the specificity of MEST-2 showed that the epitope required for binding of this Mab with glucosylceramide would involve the β-D-Glc residue and the 2-hydroxyl group of the fatty acid from the ceramide portion of the GlcCer (Toledo et al., 2001).

NEW APPLICATIONS IN THE MASS SPECTROMETRIC ANALYSIS OF CEREBROSIDES

THIN-LAYER CHROMATOGRAPHY–IONIZATION ORTHOGONAL TIME-OF-FLIGHT MASS SPECTROMETRY–MASS SPECTROMETRY

With the recent development of ambient MS, the lipids from different sources have been analyzed by HPTLC, and then subjected to MS using desorption techniques, such as DESI and MALDI. The coupling of complementary techniques as TLC and MS could open new avenues for knowledge of specific structural information using trace amounts of fungal glycosphingolipids.

Using the TLC overlay assay, combined with infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry (IR-MALDI–O-TOF-MS), Müsken et al. (2010) carried out the structural characterization of globo-series neutral GSLs (microbial GSL-receptor), recognized by P-fimbriated *Escherichia coli*, the agent of upper urinary tract infections in humans. The method could be applicable for any GSL-binding non-pathogenic and/or pathogenic *E. coli* strain, or other microbes such as *Helicobacter pylori* or viruses. A detailed survey of methodological aspects of this technique is available in the comprehensive review by Meisen et al. (2011).

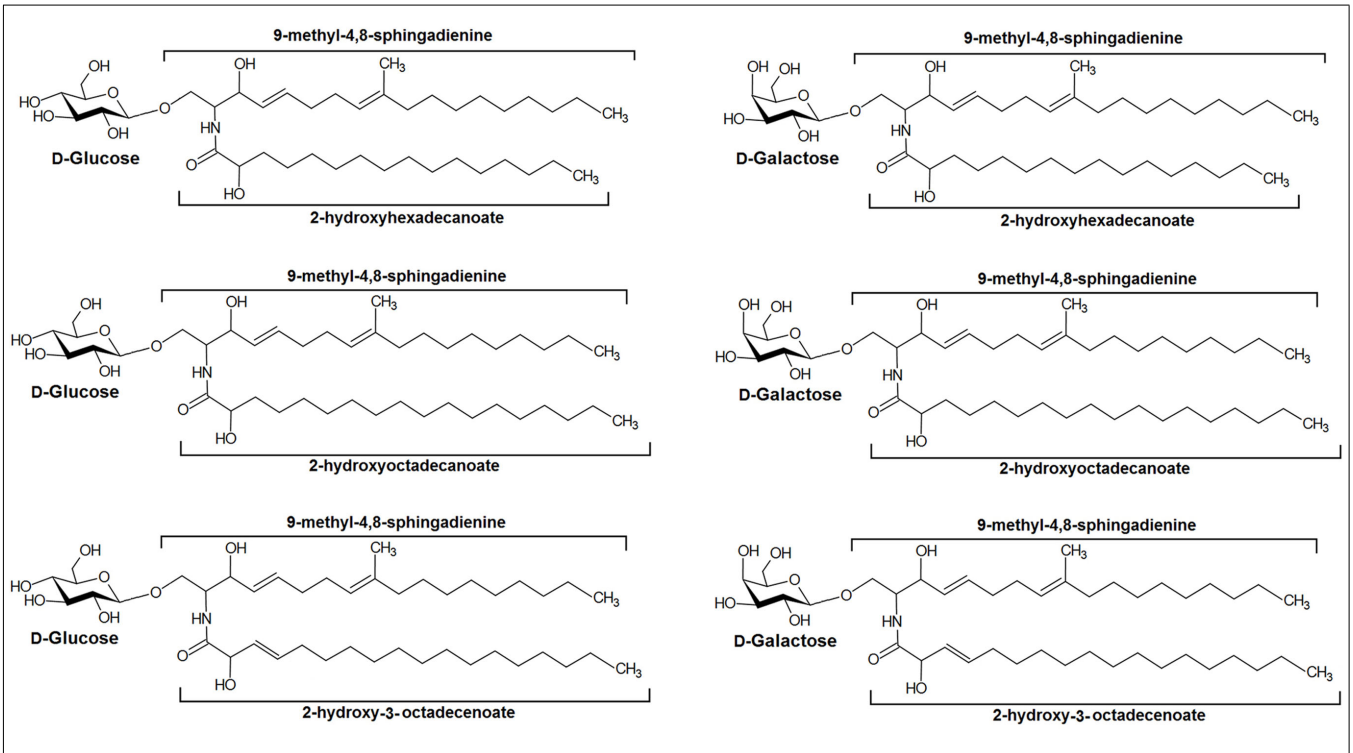


FIGURE 8 | Main structures of cerebrosides isolated from pathogenic fungi.

This new methodology was successfully applied to the identification of cancer-associated GSLs in several types of human tumors (Müthing and Distler, 2010).

IMAGING MASS SPECTROMETRY

Current techniques used to identify and quantify glycosphingolipids require a prior extraction and their removal from biological material (cells, tissues) resulting in a loss of information.

Direct tissue analysis and imaging MS are emerging technologies that can help in answering questions raised by current analytical techniques. The subcellular localization and distribution of glycosphingolipids are now beginning to be probed directly using secondary ion mass spectrometry (SIMS) and MALDI techniques (reviewed by Haynes et al., 2009).

In MALDI–MS, the surface of a sample is coated with a matrix which serves to absorb the laser beam, and then volatilize and ionize the sample. For SIMS, the sample is directly desorbed by a primary ion bombardment. A simultaneous acquisition of a large number of biological compounds in mixtures, with the excellent sensitivity obtained by ToF mass analyzer, are the advantages of these techniques, when compared with others using chemical imaging (reviewed by Touboul et al., 2011).

CONCLUSION

This review summarizes classical analytical techniques used for the identification and structural determination of fungal cerebrosides. MS has emerged as a powerful technique for the analysis of cerebrosides, including analytical variations as FAB/MS, ESI-MS, and low energy ESI-MS/CID-MS. ^{1}H and ^{13}C-NMR have also been successfully used in cerebroside structural analysis. The combination of these techniques is usually sufficient for a complete structural elucidation. A detailed structural information of fungal cerebrosides is required in order to elucidate the biological role of these molecules. Also discussed here are some emerging techniques as TLC–IR-MALDI–MS and imaging MS in the analysis of glycosphingolipids.

ACKNOWLEDGMENTS

The authors thank Prof. P. A. J. Gorin for critical reading of this manuscript. This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), PRONEX, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

Ann, Q., and Adams, J. (1992). Structure determination of ceramides and neutral glycosphingolipids by collisional activation of $[M+Li]^+$ ions. *J. Am. Soc. Mass Spectrom.* 3, 260–263.

Ann, Q., and Adams, J. (1993). Structure specific collision induced fragmentations of ceramides cationized with alkali metal ions. *Anal. Chem.* 65, 7–13.

Arigi, E., Singh, S., Kahlili, A. H., Winter, H. C., Goldstein, I. J., and Levery, S. B. (2007). Characterization of neutral and acidic glycosphingolipids from the lectin-producing mushroom, Polyporus squamosus. *Glycobiology* 17, 754–766.

Ballio, A., Casinovi, C. G., Framondino, M., Marino, G., Nota, G., and Santurbano, B. (1979). A new cerebroside from Fusicoccum amygdali Del. *Biochem. Biophys. Acta* 573, 51–60.

Barber, M., Bordoli, R. S., Garner, G. V., Gordon, D. B., Sedgwick, R. D., Tetler, L. W., and Tyler, A. N. (1981a). Fast-atom-bombardment mass spectra of enkephalins. *Biochem. J.* 197, 401–404.

Barber, M., Bordoli, R. S., Sedgwick, R. D., Tyler, A. N., and Bycroft, B. W. (1981b). Fast atom bombardment mass spectrometry of bleomycin A2 and B2 and their metal complexes. *Biochem. Biophys. Res. Commun.* 101, 632–638.

Barreto-Bergter, E., Pinto, M. R., and Rodrigues, M. L. (2004). Structure and biological functions of fungal cerebrosides. *An. Acad. Bras. Cienc.* 76, 67–84.

Batrakov, S. G., Konova, I. V., Sheichenko, V. I., Esipov, S. E., Galanina, L. A., and Istratova, L. N. (2002). Unusual fatty acid composition of cerebrosides from the filamentous soil fungus *Mortierella alpina*. *Chem. Phys. Lipids* 117, 45–51.

Boas, M. H., Egge, H., Pohlentz, G., Hartmann, R., and Bergter, E. B. (1994). Structural determination of N-2′-hydroxyoctadecenoyl-1-O-beta-D-glucopyranosyl-9-methyl-4,8-sphingadienine from species of *Aspergillus*. *Chem. Phys. Lipids* 70, 11–19.

da Silva, A. F., Rodrigues, M. L., Farias, S. E., Almeida, I. C., Pinto, M. R., and Barreto-Bergter, E. (2004). Glucosylceramides in *Colletotrichum gloeosporioides* are involved in the differentiation of conidia into mycelial cells. *FEBS Lett.* 561, 137–143.

da Silva, M. C. F., Vieira, R. P., Hartmann, R., Mulloy, B., and Barreto-Bergter, E. (1997). The structures of polysaccharides and glycolipids of *Aspergillus fumigatus* grown in the presence of human serum. *Mycopathologia* 137, 17–25.

Domon, B., and Costello, C. E. (1988). Structure elucidation of glycosphingolipids and gangliosides using high-performance tandem mass spectrometry. *Biochemistry* 27, 1534–1543.

Duarte, R. S., Polycarpo, C. R., Wait, R., Hartmann, R., and Bergter, E. B. (1998). Structural characterization of neutral glycosphingolipids from *Fusarium* species. *Biochim. Biophys. Acta* 1390, 186–196.

Fogedal, M., Mickos, H., and Norberg, T(1986). Isolation of of N-2′-hydroxydecanoyl-1-O-β-D-glucopyranosyl-9-methyl-4,8-D-erythro-sphingadienine from fruiting bodies of two Basidiomycetes fungi. *Glycoconj. J.* 3, 233–237.

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509.

Fujino, Y., and Ohnishi, M. (1976). Structure of cerebroside in *Aspergillus oryzae*. *Biochim. Biophys. Acta* 486, 161–171.

Gao, J. M., Hu, L., Dong, Z. J., and Liu, J. K. (2001). New glycosphingolipid containing an unusual sphingoid base from the basidiomycete Polyporus ellisii. *Lipids* 36, 521–527.

Haynes, C. A., Allegood, J. C., Park, H., and Sullards, M. C. (2009). Sphingolipidomics: methods for the comprehensive analysis of sphingolipids. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 877, 2696–2708.

Karas, M., Bachmann, D., Bahr, U., and Hillenkamp, F. (1987). Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Process.* 78, 53e68.

Karlsson, K. A., and Holm, G. A. (1965). Studies on sphingosines. 9. C19-sphingosines, hitherto unknown sphingosines. *Acta Chem. Scand.* 19, 2423–2425.

Karlsson, K. A., Leffler, H., and Samuelsson, B. E. (1979). Characterization of cerebroside (monoglycosylceramide) from the sea anemone, *Metridium senile*. Identification of the major long-chain base as an unusual dienic base with a methyl branch at a double bond. *Biochim. Biophys. Acta* 574, 79–93.

Kates, M. (1986). "Separation of lipid mixtures," in *Techniques of Lipidology: Isolation, Analysis and Identification of Lipids*, eds R. H. Burdon and P. H. van Knippenberg (Amsterdam: Elsevier), 186–278.

Kawai, G. (1989). Molecular species of cerebrosides in fruiting bodies of *Lentinus edodes* and their biological activity. *Biochim. Biophys. Acta* 1001, 185–190.

Kawai, G., and Ikeda, Y. (1982). Fruiting-inducing activity of cerebrosides observed with Schizophlyllum commune. *Biochim. Biophys. Acta* 719, 612–618.

Kawai, G., and Ikeda, Y. (1985). Structure of biologically active and inactive cerebrosides prepared from *Schizophyllum commune*. *J. Lipid Res.* 26, 338–343.

Koga, J., Yamauchi, T., Shimura, M, Ogawa, N, Oshima, K., Umemura, K., Kikuchi, M., and Ogasawara, N. (1998). Cerebrosides A and C, sphingolipid elicitors of hypersensitive cell death and phytoalexin accumulation in rice plants. *J. Biol. Chem.* 273, 31985–31991.

Lester, R. L., Smith, S. W., Wells, G. B., Rees, D. C., and Angus, W. W. (1974). The isolation and partial characterization of two novel sphingolipids from *Neurospora crassa*: di(inositolphosphoryl)ceramide and [(gal)3glu]ceramide. *J. Biol. Chem.* 249, 3388–3394.

Levery, S. B. (2005). Glycosphingolipid structural analysis and glycosphingolipidomics. *Meth. Enzymol.* 405, 300–369.

Levery, S. B., Momany, M., Lindsey, R., Toledo, M. S., Shayman, J. A., Fuller, M., Brooks, K., Doong, R. L., Straus, A. H., and Takahashi, H. K. (2002). Disruption of the glucosylceramide biosynthetic pathway in *Aspergillus nidulans* and *Aspergillus fumigatus* by inhibitors of UDP-Glc: ceramide glucosyltransferase strongly affects spore germination, cell cycle, and hyphal growth. *FEBS Lett.* 525, 59–64.

Levery, S. B., Toledo, M. S., Doong, R. L., Straus, A. H., and Takahashi, H. K. (2000). Comparative analysis of ceramide structural modification found in fungal cerebrosides by electrospray tandem mass spectrometry with low energy collision-induced dissociation of Li^+ adduct ions. *Rapid Commun. Mass Spectrom.* 14, 551–563.

Lindley, N. D., and Heyderman, M. T. (1985). Alkane utilization by *Cladosporium resinae*: the importance of extended lag phases when assessing substrate optima. *FEMS Microbiol. Ecol.* 31, 307–310.

Maciel, D. M., Rodrigues, M. L., Wait, R., Villas Boas, M. H., Tischer, C. A., and Barreto-Bergter, E. (2002). Glycosphingolipids from Magnaporthe grisea cells: expression of a ceramide dihexoside presenting phytosphingosine as the long-chain base. *Arch. Biochem. Biophys.* 405, 205–213.

Magnani, J. L., Smith, D. F., and Ginsburg, V. (1980). Detection of gangliosides that bind cholera toxin: direct binding of 125I-labeled toxin to thin-layer chromatograms. *Anal. Biochem.* 109, 399–402.

Matsubara, T., Hayashi, A., Banno, Y., Morita, T., and Nozawa, Y. (1987). Cerebroside of the dimorphic human pathogen, *Candida albicans. Chem. Phys. Lipids* 43, 1–12.

Meisen, I., Mormann, M., and Müthing, J. (2011). Thin-layer chromatography, overlay technique and mass spectrometry: a versatile triad advancing glycosphingolipidomics. *Biochim. Biophys. Acta* 1811, 875–896.

Mineki, S., Iida, M., and Tsutsumi, T. (1994). A new cerebroside of the n-alkane-assimilating yeast *Candida deformans. J. Ferment. Bioeng.* 78, 327–330.

Mizushina, Y., Hanashima, L., Yamaguchi, T., Takemura, M., Sugawara, F., Saneyoshi, M., Matsukage, A., Yoshida, S., and Sakaguchi, K. (1998). A mushroom fruiting body-inducing substance inhibits activities of replicative DNA polymerases. *Biochem. Biophys. Res. Commun.* 249, 17–22.

Morris, H. R., Panico, M., Barber, M., Bordoli, R. S., Sedgwick, R. D., and Tyler, A. (1981). Fast atom bombardment: a new mass spectrometric method for peptide sequence analysis. *Biochem. Biophys. Res. Commun.* 101, 623–631.

Murphy, R. C., Mathews, W. R., Rokach, J., and Fenselau, C. (1982). Comparison of biological-derived and synthetic leukotriene C4 by fast atom bombardment mass spectrometry. *Prostaglandins* 23, 201–206.

Müsken, A., Souady, J., Dreisewerd, K., Zhang, W., Distler, U., Peter-Katalinic, J., Miller-Podraza, H., Karch, H., and Müthing, J. (2010). Application of thin-layer chromatography/infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry to structural analysis of bacteria-binding glycosphingolipids selected by affinity detection. *Rapid Commun. Mass Spectrom.* 24, 1032–1038.

Müthing, J., and Distler, U. (2010). Advances on the compositional analysis of glycosphingolipids combining thin-layer chromatography with mass spectrometry. *Mass Spectrom. Rev.* 29, 425–479.

Ng, K. H., and Laneelle, M. A. (1977). Lipids of the yeast *Hansenula anomala. Biochimie* 59, 97–104.

Nimrichter, L., Barreto-Bergter, E., Mendonca-Filho, R. R., Kneipp, L. F., Mazzi, M. T., Salve, P., Farias, S. E., Wait, R., Alviano, C. S., and Rodrigues, M. L. (2004). A monoclonal antibody to glucosylceramide inhibits the growth of *Fonsecaea pedrosoi* and enhances the antifungal action of mouse macrophages. *Microbes Infect.* 6, 657–665.

Nimrichter, L., Cerqueira, M. D., Leitao, E. A., Miranda, K., Nakayasu, E. S., Almeida, S. R., Almeida, I. C.,Alviano, C. S., Barreto-Bergter,E,, and Rodrigues, M. L. (2005). Structure, cellular distribution, antigenicity, and biological functions of *Fonsecaea pedrosoi* ceramide monohexosides. *Infect. Immun.* 73, 7860–7868.

Park, C., Bennion, B., François, I. E. J. A., Ferket, K. K. A., Cammue, B. P. A., Thevissen, K., and Levery, S. B. (2005). Neutral glycolipids of the filamentous fungus *Neurospora crassa*: altered expression in plant defensin-resistant mutants. *J. Lipid Res.* 46, 759–768.

Peng, X., Wang, Y., Sun, K., Liu, P., Yin, X., and Zhu, W. (2011). Cerebrosides and 2-pyridone alkaloids from the halotolerant fungus *Penicillium chrysogenum* grown in a hypersaline medium. *J. Nat. Prod.* 74, 1298–1302.

Pinto, M. R., Rodrigues, M. L., Travassos, L. R., Haido, R. M., Wait, R., and Barreto-Bergter, E. (2002). Characterization of glucosylceramides in *Pseudallescheria boydii* and their involvement in fungal differentiation. *Glycobiology* 12, 251–260.

Pittenauer, E., and Allmaier, G. (2009a). High-energy collision induced dissociation of biomolecules: MALDI-TOF/RTOF mass spectrometry in comparison to tandem sector mass spectrometry. *Comb. Chem. High Throughput Screen.* 12, 137–155.

Pittenauer, E., and Allmaier, G. (2009b). The renaissance of high-energy CID for structural elucidation of complex lipids: MALDI-TOF/RTOF-MS of alkali cationized triacylglycerols. *J. Am. Soc. Mass Spectrom.* 20, 1037–1047.

Qi, J., Ojika, M., and Sakagami, Y. (2001). Neuritogenic cerebrosides from an edible Chinese mushroom. Part 2: structures of two additional termitomycesphins and activity enhancement of an inactive cerebroside by hydroxylation. *Bioorg. Med. Chem.* 9, 2171–2177.

Rodrigues, M. L., Travassos, L. R., Miranda, K. R., Franzen, A. J., Rozental, S., de Souza, W., Alviano, C. S., and Barreto-Bergter, E. (2000). Human antibodies against a purified glucosylceramide from *Cryptococcus neoformans* inhibit cell budding and fungal growth. *Infect. Immun.* 68, 7049–7060.

Sakaki, T., Zahringer, U., Warnecke, D. C., Fahl, A., Knogge, W., and Heinz, E. (2001). Sterol glycosides and cerebrosides accumulate in Pichia pastoris, *Rhynchosporium secalis* and other fungi under normal conditions or under heat shock and ethanol stress. *Yeast* 18, 679–695.

Sassaki, G. L., Souza, L. M., Serrato, R. V., Cipriani, T. R., Gorin, P. A., and Iacomini, M. (2008). Application of acetate derivatives for gas chromatography-mass spectrometry: novel approaches on carbohydrates, lipids and amino acids analysis. *J. Chromatogr. A* 1208, 215–222.

Sawabe, A., Morita, M., Okamoto, T., and Ouchi, S. (1994). The location of double bonds in a cerebroside from edible fungi (mushroom) estimated by B/E linked scan fast atom bombardment mass spectrometry. *Biol. Mass Spectrom.* 23, 660–664.

Sawabi, A., Morita, M., Okamoto, T., and Ouchi, S. (1996). Fast atom bombardment mass spectrometry and linked scan analyses at constant B/E in the structural characterization of new polyisoprenepolyols isolated from an edible mushroom (*Hypsizygus marmoreus*). *J. Mass. Spectrom.* 31, 921–925.

Sawardeker, J,S., Slonecker, J, H., and Jeanes, A. (1965). Quantitative determination of monosaccharides as alditol acetates by gas chromatography. *Anal. Chem.* 37, 1602–1604.

Scandroglio, F., Loberto, N., Valsecchi, M., Chigorno, V., Prinetti, A., and Sonnino, S. (2009).Thin layer chromatography of gangliosides. *Glycoconj. J* 26, 961–973.

Seifert, K. A., Hughes, S. J., Boulay, H., and Louis-Seize, G. (2007). Taxonomy, nomenclature and phylogeny of three Cladosporium-like hyphomycetes, *Sorocybe resinae*, *Seifertia azaleae* and the *Hormoconis anamorph* of *Amorphotheca resinae. Stud. Mycol.* 58, 235–245.

Svennerholm, L. (1956). The quantitative estimation of cerebrosides in nervous tissue. *J. Neurochem.* 1, 42–53.

Sweeley, C. C. (1963). Purification and partial characterization of sphingomyelin from human plasma. *J. Lipid Res.* 4, 402–406.

Takahashi, H. K., Levery, S. B., Toledo, M. S., Suzuki, E., Salyan, M. E., Hakomori, S., and Straus, A. H. (1996). Isolation and possible composition of glucosylceramides from *Paracoccidioides brasiliensis. Braz. J. Med. Biol. Res.* 29, 1441–1444.

Takakuwa, N., Kinoshita, M., Oda, Y., and Ohnishi, M. (2002). Existence of cerebroside in *Saccharomyces kluyveri* and its related species. *FEMS Yeast Res.* 2, 533–538.

Tavares, P. M., Thevissen, K., Cammue, B. P., Francois, I. E., Barreto-Bergter, E., Taborda, C. P., Marques, A. F., Rodrigues, M. L., and Nimrichter, L. (2008). In vitro activity of the antifungal plant defensin RsAFP2 against *Candida* isolates and its in vivo efficacy in prophylactic murine models of candidiasis. *Antimicrob. Agents Chemother.* 52, 4522–4525.

Toledo, M. S., Levery, S. B., Straus, A. H., Suzuki, E., Momany, M., Glushka, J., Moulton, J. M., and Takahashi, H. K. (1999). Characterization of sphingolipids from mycopathogens: factors correlating with expression of 2-hydroxy fatty acyl (E)-Delta 3-unsaturation in cerebrosides of *Paracoccidioides brasiliensis* and *Aspergillus fumigatus*. *Biochemistry* 38, 7294–7306.

Toledo, M. S., Levery, S. B., Straus, A. H., and Takahashi, H. K. (2000). Dimorphic expression of cerebrosides in the mycopathogen *Sporothrix schenckii*. *J. Lipid Res.* 41, 797–806.

Toledo, M. S., Levery, S. B., Suzuki, E., Straus, A. H., and Takahashi, H. K. (2001). Characterization of cerebrosides from the thermally dimorphic mycopathogen *Histoplasma capsulatum*: expression of 2-hydroxy fatty N-acyl (E)-Delta(3)-unsaturation correlates with the yeast-mycelium phase transition. *Glycobiology* 11, 113–124.

Touboul, D., Brunelle, A., and Laprévote, O. (2011). Mass spectrometry imaging: towards a lipid microscope. *Biochimie* 93, 113-119

Umemura, K., Ogawa, N., Yamauchi, T., Iwata, M., Shimura, M., and Koga, J. (2000). Cerebroside elicitors found in diverse phytopathogens activate defense responses in rice plants. *Plant Cell Physiol.* 41, 676–683.

Wagner, H., and Zofcsik, W. (1966). On new sphingolipids of yeast. *Biochem. Z.* 344, 314–316.

Wang, W., Wang, Y., Tao, H., Peng, X., Liu, P., and Zhu, W. (2009). Cerebrosides of the halotolerant fungus *Alternaria raphani* isolated from a sea salt field. *J. Nat. Prod.* 72, 1695–1698.

Warnecke, D., and Heinz, E. (2003). Recently discovered functions of glucosylceramides in plants and fungi. *Cell. Mol. Life Sci.* 60, 919–941.

Weiss, B., and Stiller, R. L. (1972). Sphingolipids of mushrooms. *Biochemistry* 11, 4552–4557.

Wells, M. A., and Dittmer, J. C. (1965). A preparative method for the isolation of brain cerebrosides, sulfatide and sphingomyelin. *J. Chromatogr.* 18, 503–511.

Yamashita, M., and Fenn, J. B. (1984). Electrospray ion source. Another variation on the free-jet theme. *J. Phys. Chem.* 88, 4451–4459.

Zanetta, J. P., Timmerman, P., and Leroy, Y. (1999). Determination of constituents of sulphated proteoglycans using a methanolysis procedure and gas chromatography/mass spectrometry of heptafluorobutyrate derivatives. *Glycoconj. J.* 16, 617–627.

Zaüner, S., Zähringer, U., Lindner, B., Warnecke, D., and Sperling, P. (2008). Identification and functional characterization of the 2-hydroxy fatty N-acyl-(3(E)-desaturase from *Fusarium graminearum*. *J. Biol. Chem.* 283, 36734–36742.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 September 2011; accepted: 13 November 2011; published online: 05 December 2011.

Citation: Barreto-Bergter E, Sassaki GL and de Souza LM (2011) Structural analysis of fungal cerebrosides. Front. Microbio. ***2****:239. doi: 10.3389/fmicb.2011.00239*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 20 October 2011
doi: 10.3389/fmicb.2011.00212

Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials

Leonardo Nimrichter* and Marcio L. Rodrigues

Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Edited by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine, USA

Reviewed by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine, USA
Attila Gacser, University of Szeged, Hungary

****Correspondence:***
Leonardo Nimrichter, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciencias da Saude, Bloco E, sub-solo. 21941590 Rio de Janeiro, Brazil.
e-mail: nimrichter@micro.ufrj.br

The first work reporting synthesis of glucosylceramide (cerebrin, GlcCer) by yeasts was published in 1930. During approximately 70 years members of this class of glycosphingolipids (GSL) were considered merely structural components of plasma membrane in fungi. However, in the last decade GlcCer was reported to be involved with fungal growth, differentiation, virulence, immunogenicity, and lipid raft architecture in at least two human pathogens. Fungal GlcCer are structurally distinct from their mammalian counterparts and enriched at the cell wall, which makes this molecule an effective target for antifungal activity of specific ligands (peptides and antibodies to GlcCer). Therefore, GSL are promising targets for new drugs to combat fungal diseases. This review discusses the most recent information on biosynthesis and role of GlcCer in fungal pathogens.

Keywords: glucosylceramide, fungal pathogens, antifungal targets

INTRODUCTION

The family of glycosphingolipids (GSL) combines a diversity of molecules consisting of at least one saccharide unit covalently linked to a ceramide (Schnaar et al., 2009). The biosynthesis of GSL is complex and involves a repertoire of enzymes that must be carefully expressed and distributed over endoplasmic reticulum (ER) and Golgi in all eukaryotic cells (Halter et al., 2007; Daniotti and Iglesias-Bartolome, 2011). Due to their structural properties and primary cellular location, GSL were initially described as architectural components of cell membranes, straightly connected with fluidity and stability (Feinstein et al., 1975; Tinker et al., 1976; Aaronson and Martin, 1983; Campanella, 1992). Recent finding, however, clearly demonstrate that their role goes beyond the former concepts, since these molecules are major components of specialized membrane domains called lipid rafts (Bagnat et al., 2000; Hakomori, 2003, 2008; Wachtler and Balasubramanian, 2006). In addition, GSL have been characterized as relevant structures in events of cell–cell interaction, cell signaling and protein sorting (Bagnat et al., 2000; Bagnat and Simons, 2002; Nimrichter et al., 2008; Lopez and Schnaar, 2009; Schnaar et al., 2009; Staubach and Hanisch, 2011).

In fungal organisms two types of GSL have been reported. Inositol phosphorylceramides (IPC) are used to make complex fungal GSL and have been extensively studied in the yeast model *Saccharomyces cerevisiae* (Sugimoto et al., 2004; Dickson et al., 2006; Dickson, 2008). They consist of a ceramide usually containing phytosphingosine as long chain base, associated with a very long fatty acid ($C_{24:1}$–$C_{26:1}$). A unit of inositol is covalently linked to the ceramide and the preformed molecule, which is not found in mammalian cells, is the substrate for mannosyltransferases for the synthesis of mannosylinositol phosphorylceramide (MIPC) and mannosyldiinositol phosphorylceramide [$M(IP)_2C$]. The second major class of GSL synthesized by fungal cells is the monohexosylceramide (CMH, glycosylceramide, cerebroside, or cerebrin). The sugar moiety covalently linked to a distinct ceramide is usually glucose (GlcCer) or, to a minor extent, galactose (GalCer; Boas et al., 1994; Takahashi et al., 1996; Levery et al., 2000, 2002; Rodrigues et al., 2000; Toledo et al., 2000; Pinto et al., 2002; Barreto-Bergter et al., 2004; da Silva et al., 2004; Nimrichter et al., 2004, 2005a). GlcCer and GalCer are not synthesized by *S. cerevisiae* but are part of the lipid arsenal of classical fungal pathogens, excepting *Candida glabrata* (Saito et al., 2006; Tavares et al., 2008).

A very conserved structure was described as the major GlcCer in fungal pathogens, the *N*-2′-hydroxy(hexa/octa)de(ca/ce)noyl-1-beta-D-glucopyranosyl-9-methyl-4,8-sphingadienine. GlcCer distribution is not limited to fungal membranes, since large amounts of this GSL have been also found at cell wall (Rodrigues et al., 2000; Nimrichter et al., 2005a; Rhome et al., 2011). Although GlcCer have been identified as fungal components decades ago, knowledge about their functions during fungal growth/dimorphism, lipid raft formation, and correlation with virulence has been recently reported. In this review we discuss the most recent findings that characterize the functions of GlcCer and its biosynthetic steps as potential targets for new antifungal drugs. The putative correlation between GlcCer, lipid rafts and secretion and the potential role of this GSL as virulence regulators are also reviewed.

THE UNCOVERED PATHWAY FOR FUNGAL GlcCer SYNTHESIS

Sphingoid bases of fungal GlcCer are structurally distinct from mannosylated IPCs and from their counterparts in animal cells. They have a methyl group at C-9 and an extra unsaturation at C-8, forming the typical fungal base mentioned above (for review please see Barreto-Bergter et al., 2004). However, initial steps of ceramide biosynthesis are the same independent of the cell type studied.

Ceramide synthesis always begins with condensation of palmitoyl-CoA and serine, followed by sphinganine formation through the activity of a 3-ketosphinganine reductase (Barreto-Bergter et al., 2004; Li et al., 2006; Schnaar et al., 2009; Gault et al., 2010). The product, sphinganine, is then *N*-acylated to generate ceramide. Two distinct pools of ceramide operate as building blocks for major fungal GSL (CMH and IPCs). They differ according to the hydroxylation level of long chain base (dihydroxy or trihydroxy) and the extension of hydroxylated fatty acids ($C_{16:1}$–$C_{18:1}$ or $C_{24:1}$–$C_{26:1}$)(Barreto-Bergter et al., 2004; Dickson et al., 2006; Takakuwa et al., 2008; Rittenour et al., 2011; Ternes et al., 2011). All enzymes involved in fungal ceramide synthesis operate specifically according to their substrate leading to precise production of GlcCer or MIPCs. So far, the only exceptions appear to be *Magnaporthe grisea* and *Neurospora crassa*, discussed later on (Lester et al., 1974; Maciel et al., 2002). It implies that preformed ceramide must be considerably modified before GlcCer or IPC synthesis. This section is focused on fungal GlcCer biosynthesis. Approaches utilized for structural characterization of these molecules are discussed in detail by Barreto-Bergter et al. (this issue).

Synthesis of IPC and GlcCer must include a branching point, since the enzymes required for this process make part of common and specific biosynthetic pathways. A very elegant study published by Ternes et al. (2011) contributed significantly to the understanding of GlcCer biosynthesis. Briefly, these authors demonstrated that in *Pichia pastoris*, similarly to *S. cerevisiae*, *Lag*1 and *Lac*1 appear to be the genes responsible for coding the enzymes that make trihydroxy ceramides carrying very long chain fatty acids (Ternes et al., 2011). On the other hand, *BarA* (*b*iocontrol *a*gent *r*esistance) is related to synthesis of dihydroxy ceramides bearing hydroxylated C_{16}–C_{18} fatty acids in *P. pastoris*. *BarA* was originally identified by a screen assay performed to isolate mutants from *A. nidulans* resistant to the polyketide HSAF, a *h*eat-*s*table *a*ntifungal *f*actor isolated from the biocontrol agent *Lysobacter enzymogenes* (Zhang and Yuen, 1999). In *A. nidulans*, exposure to HSAF leads to a rapid loss of the formin SepA at growing hyphal tips and disturbs the polarized growth (Li et al., 2006). Phylogenetic analysis indicated that BarAp belongs to a distinct clade of ceramide synthases when compared to Lag1p and Lac1p. Accordingly, *A. nidulans* lacking BarAp do not synthesize GlcCer (Li et al., 2006). In *Kluyveromyces lactis*, overexpression of *Lac*1 increased GlcCer synthesis while gene disruption blocked this pathway, leading to IPC accumulation (Takakuwa et al., 2008). Disruption of *Lag*1 appears to be lethal in *K. lactis*. Orthologous expression of *Lac*1 from *K. lactis* in *S. cerevisiae* resulted in IPC with $C_{18:1}$ fatty acids, which are presumably exclusive to GlcCer. Sequence analysis suggests that *Lac*1 of *K. lactis* belongs to the same clade as *BarA* (Rittenour et al., 2011). These data indicated that when *BarA* is absent in fungi that express GlcCer, *Lac*1 is the gene involved with GlcCer production.

The biosynthetic steps occurring right after ceramide hydroxylation at C-2 of fatty acid are Δ4-desaturation, Δ8-desaturation, C-9-methylation, and then glucose addition by ceramide glucosyl ceramide-synthetase (GCS; Ternes et al., 2002, 2011; Michaelson et al., 2009). Membrane-bound desaturases are part of a superfamily defined by the histidine boxes H*X*3–4H, H*X*2–3HH, and (H/Q)*X*2–3HH that usually require NAD(P)H and O_2 as cofactors (Sperling et al., 1995; Zauner et al., 2008). Searching proteins with histidine box sequence motifs a family of Δ4-desaturase was discovered. *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, and *Candida albicans* Δ4-desaturases were expressed in *S. cerevisiae* and biochemically characterized (Ternes et al., 2002). Sphing-4-enine and 4-hydroxysphinganine were characterized as products in all situations, indicating a bifunctional activity (Δ4-desaturase and C14-hydroxylase). This enzymatic step appears to be essential, given that *P. pastoris* mutant lacking Δ4-desaturase is not able to make GlcCer (Ternes et al., 2011). Insertion of a double bond between C-8 and C-9 of long chain base, the step catalyzed by a Δ8-desaturase, does not appear to be a requirement for GlcCer. *P. pastoris* lacking the enzyme is still able to build regular amounts of GlcCer.

Transference of the C-9-methyl group to the sphingoid base is the last step of ceramide synthesis used to construct fungal GlcCer. The enzyme responsible for this step belongs to the superfamily of *S*-adenosylmethionine-(SAM)-dependent methyltransferases, appears to be membrane-bound and requires a Δ4,8-desaturated ceramide as substrate (Ternes et al., 2006). As observed for Δ8-desaturase, absence of C-9 methyltransferase in *P. pastoris* (coding the enzyme Ppmt1) does not impair GlcCer synthesis, resulting in the exclusive formation of methylated ceramide. In *C. albicans*, strains lacking GlcCer, or C-9 methyltransferase were also viable, although a deficient hyphal growth was observed when compared to the wild type strain (Oura and Kajiwara, 2010). Two genes encoding putative C-9 methyltransferase were found in *Fusarium graminearum*, FgMT1, and FgMT2 (Ramamoorthy et al., 2009). Both genes complemented the lack of C-9 methyltransferase activity in *P. pastoris* mutant Ppmt1. FgMT1 disruption did not change the ceramide methylation and mutants grew as wild type cells. On the other hand, disruption of FgMT2 reduced the enzymatic product in approximately 35%, decreased growth ratio, altered shape, and size of conidia, reduced mycelial formation and virulence to *A. thaliana* leaves and wheat. Double-knockouts were not viable, suggesting that C-9 methyltransferase is essential to fungal growth in *F. graminearum*.

Glucose transfer to ceramide occurs at Golgi stacks in animal cells through a CGS (Halter et al., 2007). A fraction of newly synthesized GlcCer returns to ER and is transported to Golgi lumen to synthesize LacCer and other complex GSL. Using a second pathway, intact GlcCer reaches the cell surface through a non-vesicular pathway. In yeast, ceramide is also transported to Golgi through vesicular and non-vesicular routes (Funato and Riezman, 2001) where complex GSL will be synthesized. It is not clear whether *N*-2′-hydroxy(hexa/octa)de(ca/ce)noyl-1-beta-D-glucopyranosyl-9-methyl-4,8-sphingadienine follows a similar pathway.

The complexity of fungal GSL biosynthesis is supported by studies on the phytopathogens *F. graminearum* and *M. grisea*. *F. graminearum* is a causative agent of head blight, a plant disease that results in crop loss or grain unsuitability for animal consumption due to mycotoxin production (Xu and Nicholson, 2009). As observed for *A. nidulans*, *Bar*1 mutants of *F. graminearum* do not synthesize GlcCer and are resistant to HSAF (Rittenour et al., 2011). GlcCer was also isolated from lipid extracts of the rice blast agent *M. grisea* (Maciel et al.,

2002). The typical fungal GlcCer structure was characterized in this model, consisting of *N*-2′-hydroxy(octa/hexa)decanoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine. The *M. grisea* crude extract showed an extra orcinol positive TLC band migrating above GlcCer, indicative of a dihexosylceramide (CDH). The purified molecule was identified as lactosylceramide (LacCer). Unusually, the lipid moiety of LacCer consisted of a phytosphingosine covalently linked to a $C_{24:2}$ fatty acid. A tetrahexosylceramide has been also reported in *N. crassa* (Lester et al., 1974). By using gas and thin layer chromatography the identified components were Gal_3Glc, sphinganine, and a $C_{24:1}$ fatty acid.

These results establish that GSL biosynthesis is conserved in fungal cells; however, it cannot be summarized by a simple pathway. It seems important to consider that different species must be investigated individually. Reconstituted strains on GSL pathways also should be carefully investigated, since proper enzyme location usually cannot be assured, which may result in unusual products.

CORRELATION BETWEEN FUNGAL GlcCer, LIPID RAFTS, AND SECRETION MECHANISMS

To our knowledge, the mechanisms displayed by fungal organisms to traffic GlcCer intracellularly have never been investigated. Fungal GlcCer destiny includes plasma membrane and cell wall (Rodrigues et al., 2000; Nimrichter et al., 2005a), as well as the extracellular space (Rodrigues et al., 2007a, 2008a,b; Nosanchuk et al., 2008).

As mentioned previously, along with sterol and GPI-anchored proteins, GSL are components of lipid domains or lipid rafts (Hakomori, 2003, 2008). These lipid platforms are found in different cell types, including non-pathogenic and pathogenic fungi, where they participate in polarization and compartmentalization steps of the plasma membrane (Bagnat et al., 2000; Bagnat and Simons, 2002; Martin and Konopka, 2004; Siafakas et al., 2006; Wachtler and Balasubramanian, 2006). In the *C. albicans* model, the ability of fungal cells to switch from yeast to hyphae is considered a crucial step for host cell colonization (Mitchell, 1998). Such morphological changes are dependent on a polarized growth mediated by cytoskeleton (Whiteway and Bachewich, 2007). The first correlation between lipid rafts and polarization in *C. albicans* was suggested by Martin and Konopka (2004). They observed accumulation of sterols at the growing tip of hyphal cells and at sites of septum formation in mature hyphae. In addition, treatment with myriocin, an inhibitor of serine palmitoyltransferase, decreases filipin staining, and reduces Pma1p recruitment to lipid rafts. Pma1p is a plasma membrane proton pump that is considered a raft marker in *C. albicans* and *S. cerevisiae* (Bagnat et al., 2001; Insenser et al., 2006). Along with Pma1p, heat shock proteins (HSP70, HSP90) and molecules required for sterol metabolism, energy production, and polysaccharide synthesis were compartmentalized in lipid domains in *C. albicans* (Insenser et al., 2006). A connection between polarized growth, lipid rafts, and GlcCer was also observed in *F. graminearum* and *A. nidulans* lacking Bar1p and GlcCer as well. In *F. graminearum*, germ tube formation occurs regularly in the absence of Bar1p, but hyphal tip organization is considerably altered. Although sterols accumulate in hyphal tips of a wild type strain, Δ*bar*1 mutants lack this distribution. Similarly, no filipin staining was visualized at hyphal tips of *A. nidulans* lacking Bar1p. These mutants do not display regular Spitzenkorper when stained with FM4-64, a fluorescent lipid marker. Polarization defects were confirmed by the altered tropomyosin distribution. Together, these data suggested a requirement of GlcCer and intact lipid rafts for hyphal growth and polarization of fungal cells.

Lipid rafts were also isolated from the opportunistic pathogen *C. neoformans*. GlcCer, phospholipase B1 (PLB1), and Cu/Zn superoxide dismutase (SOD1), molecules involved with fungal virulence, were characterized as raft components in this fungus (Siafakas et al., 2006). It was hypothesized that membranes of *C. neoformans* contain lipid domains that concentrate some virulence factors at fungal surface. Secretion of virulence factors could be associated to their aggregation to lipid rafts.

A few years ago, we discussed aspects of cell wall that, at that point, were considered unusual (Nimrichter et al., 2005b). We then raised the possibility that compact vesicles capable of crossing polar environments, such as the fungal cell wall, could be used by fungal organisms for the extracellular release of macromolecules. Lipid rafts would be likely necessary to increase vesicle stability. In fact, we recently demonstrated that *C. neoformans* produces extracellular vesicles containing GlcCer, sterol, and SOD1 (Rodrigues et al., 2007a, 2008a). Trans-cell wall traffic of GlcCer-containing vesicles could in fact explain the high amounts of GlcCer that are found at the cell wall of fungal pathogens (Rodrigues et al., 2000; Nimrichter et al., 2005a). Further studies revealed that extracellular vesicles are produced by different fungal organisms, possibly representing a conserved mechanism of trans-cell wall transport (Nosanchuk et al., 2008; Oliveira et al., 2010a,b). A number of proteins can reach the extracellular environment through these vesicles, including other lipid raft components, such as Pma1p, HSP70, and HSP90 (Albuquerque et al., 2008; Rodrigues et al., 2008a). The putative correlation between extracellular vesicle formation and fungal lipid rafts is then suggested.

Molecular exportation to the extracellular space through vesicles is not limited to proteins. The polysaccharide glucuronoxylomannan (GXM), a major capsule component of *C. neoformans,* follows a vesicle association pathway to leave the cells, in a process that apparently requires a close association with GlcCer (Rodrigues et al., 2007a; Oliveira et al., 2009). These data indicate that GlcCer regulates/participates on polysaccharide and protein sorting in fungal cells. Such processes involve compartmentalization and changes in membrane shape. This attribute could be correlated with another property of some complex GSL, the capacity to control membrane curvature and vesicle formation (Sonnino et al., 2007). Recently, a correlation between GlcCer and Golgi morphology was observed in plant cells (Melser et al., 2010). Treatment of *A. thaliana* with the inhibitor of GlcCer synthesis PDMP disturbed transport of soluble and membrane-associated secretory proteins to plant cell surface. This phenotype was accompanied by remarkable morphological changes and protein retention in the Golgi of treated cells. Reduction on Golgi cisternal thickness and stack width together with an increase in the number of vesicular structures surrounding this organelle was visualized after treatment with drugs that inhibit ceramide glycosylation.

As depicted above, pharmacological drugs that inhibit GSL synthesis are important tools to investigate the role of GSL and lipid

rafts physiological events in animal cells (Tifft and Proia, 2000). Their activity against fungi is controversial, as discussed below.

Treatment of *Aspergillus* species with D-threo-1-phenyl-2-palmitoyl-3-pyrrolidino propanol (P4) and D-threo-3P,4P-ethylenedioxy-P4 (EDO-P4), inhibitors of ceramide glycosylation, was followed by a decrease on GlcCer expression and defects in spore germination, cell cycle, and hyphal growth (Levery et al., 2002). Hillig et al. (2005) extended these findings by evaluating the activity of GSL inhibitors on *P. pastoris* expressing heterologous GCS. The activity of these enzymes was measured in cell-free membrane fractions in the presence of EDO-P4, a GCS inhibitor. Although the human enzyme was strongly inhibited by this drug, it showed no effect over fungal GCS (for instance, *C. albicans*). These results, however, must be carefully interpreted, given the lack of information in the literature on the properties of GCS activity from other fungal species. However, we could not exclude the possibility that other fungal species are sensitive to derivatives of these inhibitors.

GlcCer AND FUNGAL VIRULENCE IN MURINE MODELS

As discussed above it is unquestionable that GlcCer is a key molecule during physiological events in fungal organisms. However, mutants lacking GCS are usually viable. These mutant strains have been used to evaluate the role of GlcCer during infection. In this section we will debate the capacity of fungal GlcCer to regulate virulence in animal models of mycoses.

The first report showing that GlcCer could influence the capacity of a fungal organism to cause disease was established by Rittershaus et al. (2006). *C. neoformans* lacking GlcCer synthesis was generated after GCS disruption and used in murine models of cryptococcosis. Although the capacity to produce key virulence factors of *C. neoformans* was intact in the mutant, this strain was avirulent when inhaled by CBA/J mice. Dissemination to the brain was not observed even after 90 days of infection. When injected intravenously using the same mouse background, yeasts of *C. neoformans* reached different tissues, including the brain, with a similar outcome observed for WT strain. Histopathological sections confirmed these data and also showed yeast cells confined to granulomas in the lung of mice after intranasal infection. These granulomas were necessary to control fungal growth since brain dissemination was observed after inhalation of GCS mutants by T- and NK-cell-immunodeficient mice, where granuloma formation is impaired (Kechichian et al., 2007). A refined mechanism of yeast cell cycle transition correlated with pH and CO_2 concentration was suggested. For establishment of cryptococcosis, GlcCer appears to be required for fungal growth in the blood and alveolar spaces of the lung, which, in contrast to the acidic intracellular environment in macrophages, have a neutral pH. Corroborating with these data T- and NK-cell-immunodeficient mice depleted of alveolar macrophages had decreased dissemination to the brain and prolonged survival. The hypothesis raised by Rittershaus et al. (2006) is also in agreement with experiments published by Saito et al. (2006). These authors reported a relationship between GlcCer synthesis and the capacity of fungal cells to grow under neutral or alkaline pH.

Results from a meticulous work developed by Noble et al. (2010) using the model *C. albicans* reinforce the role of GlcCer as a virulence regulator. These authors disrupted 674 genes in *C. albicans* using auxotrophic markers with no effect on virulence. Mutants were then screened for three major phenotypes: (i) pathogenicity in mouse model of candidiasis, (ii) morphological switching (yeast to hypha conversion), and (iii) cell proliferation *in vitro*. The experiments *in vivo* disclosed 115 mutants with impaired pathogenicity. Among these only 15 mutants displayed impairment to differentiate and grow as hypha. In addition, in the group of 115 mutants with impaired pathogenicity, 89 exhibited growth rates that were close to the WT strain. Therefore, delay in fungal growth did not correlate with capacity to cause disease in mice. The authors then analyzed the mutants with impaired capacity to colonize mice but efficient in proliferating and in promoting morphological changes. Strikingly, four mutants were involved with synthesis of GlcCer.

Previous studies by Oura and Kajiwara (2010) have shown that mutants of *C. albicans* lacking C-9 methyltransferase, GCS, and Δ8-saturase have growth rates that are similar to those observed in WT cells. Delayed morphological changes were visualized when these mutants were cultivated in spider medium, but hypha formation was observed in all situations. Contrasting with *C. neoformans* mutants lacking GlcCer, mutants of *C. albicans* isolated by Noble and colleagues were able to grow under neutral and alkaline pH similar to WT. Thus, a distinct mechanism of virulence regulation is dependent on GlcCer in *C. albicans*. The authors identified GlcCer as the first molecule specifically required to govern virulence in *C. albicans* (Noble et al., 2010).

FUNGAL GlcCer AS TARGETS TO NEW ANTIFUNGAL DRUGS

Together with other groups we have demonstrated the use of anti-GlcCer antibodies to prevent fungal differentiation and growth of fungal pathogens. For instance, these antibodies blocks germ tube formation in *C. albicans, Colletotrichum gloeosporioides*, and *Pseudallescheria boydii* (Pinto et al., 2002; da Silva et al., 2004). Furthermore, anti-GlcCer antibodies were able to protect mice in a lethal infection by *C. neoformans* (Rodrigues et al., 2007b). The mechanism of action for these antibodies is still unknown. The enrichment of GlcCer at budding sites in *C. neoformans*, suggested that the antibodies could interfere with the cell wall biosynthesis and organization as previously described for the lectin WGA (Ciopraga et al., 1999). Alternatively, these antibodies could associate to GlcCer on lipid rafts, impairing cleavage of GPI-anchored proteins, essential to cell wall assembly. Cross linking of components found on lipid rafts by antibodies or ligands could also initiate a cell signaling response as observed in mammalian cells (Landry and Xavier, 2006; Marta et al., 2008; Bingaman et al., 2010). In this context, the only information available was the prominent cellular damage, with organelle destruction, membrane retraction, and increased vacuolization observed in yeasts from *C. neoformans* treated with anti-GlcCer (Nimrichter et al., 2005b). Some TEM images suggested an apoptotic mechanism, but no biochemical evidences were characterized.

A set of experiments published by Thevissen et al. (2004) strongly suggested that targeting fungal GlcCer could in fact initiate a cell signaling response in fungal organisms (Thevissen et al., 2004; Aerts et al., 2007). These authors demonstrated that a peptide isolated from radish seeds and named RsAFP2 was able to

specifically bind to fungal GlcCer (Thevissen et al., 2004). The peptide displayed a potent microbicidal activity in micromolar concentrations against fungal species that express GlcCer. The exact region of GlcCer where the peptide binds is not known, but apparently C-9 methylation at ceramide is not required, since mutants where this enzyme was impaired remained susceptible to the peptide (Ramamoorthy et al., 2009). Binding of peptide was followed by ROS production and fungal death (Aerts et al., 2007). The peptide activity was impaired by anti-oxidative molecules, confirming that fungal death was caused by an oxidative activity. Further studies by our group demonstrated a prophylactic activity for RsAFP2 in a murine model of candidiasis (Tavares et al., 2008). The peptide was biologically active against distinct *Candida* species and its potency was proportional to the amount of GlcCer expressed by the *Candida* strain tested.

Although the use of current GCS inhibitors is still questionable, a new alternative to decrease fungal GlcCer content in pathogenic fungi was recently proposed by Rhome et al. (2011): the administration of cerezyme (Cz) to mice lethally infected with *C. neoformans*. Cz is a human recombinant enzyme that removes glucose from GlcCer. It has been used as an alternative treatment for patients with Gaucher's, disease where hydrolysis of GlcCer is impaired and the GSL accumulates in lysosomes (Charrow, 2009). This enzyme was proven to hydrolyze fungal GlcCer and reduce its content in *C. neoformans in vitro* and *in vivo*. Cz-induced GlcCer decrease was associated with reduction of fungal membrane stability. In a murine model of cryptococcosis Cz was also able to increase mice survival (Rhome et al., 2011).

CONCLUSION AND PERSPECTIVES

For over half a century, GlcCer was classified as a structural membrane component of eukaryotic cells. In the last decade, however, a number of studies have uncovered sophisticated functions for GlcCer in eukaryotes. In fungi, it is now clear that knowledge on functions related to virulence, growth, and morphological transitions can be connected to specific structural features and particular biosynthetic steps to validate GlcCer as a potential target to development of new antifungal drugs. A major challenge, however, is to identify these drugs. The literature suggests several inhibitors of fungal GlcCer synthesis as candidates. So far, none of them have appeared to be sufficiently specific to allow use in human patients. In parallel, GlcCer ligands are apparently efficient and selective antifungal agents where in fact, peptides and monoclonal antibodies were already administered in murine models with encouraging results. Other candidates, including the antifungal factor HSAF and the enzyme Cz (which is already approved for use in humans), are also promising GlcCer-binding candidates. We need now more details on how antibodies, peptides, and enzymes interfering with the structure of GlcCer alter the biology of fungal cells, as well as how they impact human/animal physiology. Considering the critical side effects usually observed during antifungal therapy, the low efficacy of currently approved antifungal drugs and the emerging resistance of fungal cells against antifungals, we believe GlcCer is a very promising target for the activity of new compounds with potential to be used in human and animal individuals with severe mycoses.

ACKNOWLEDGMENTS

Leonardo Nimrichter and Marcio L. Rodrigues were supported by grants from Conselho Nacional de Desenvolvimento Tecnológico (CNPq, Brazil), CAPES, and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil).

REFERENCES

Aaronson, L. R., and Martin, C. E. (1983). Temperature-induced modifications of glycosphingolipids in plasma membranes of *Neurospora crassa*. *Biochim. Biophys. Acta* 735, 252–258.

Aerts, A. M., Francois, I. E., Meert, E. M., Li, Q. T., Cammue, B. P., and Thevissen, K. (2007). The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. *J. Mol. Microbiol. Biotechnol.* 13, 243–247.

Albuquerque, P. C., Nakayasu, E. S., Rodrigues, M. L., Frases, S., Casadevall, A., Zancope-Oliveira, R. M., Almeida, I. C., and Nosanchuk, J. D. (2008). Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. *Cell. Microbiol.* 10, 1695–1710.

Bagnat, M., Chang, A., and Simons, K. (2001). Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. *Mol. Biol. Cell* 12, 4129–4138.

Bagnat, M., Keranen, S., Shevchenko, A., and Simons, K. (2000). Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3254–3259.

Bagnat, M., and Simons, K. (2002). Lipid rafts in protein sorting and cell polarity in budding yeast *Saccharomyces cerevisiae*. *Biol. Chem.* 383, 1475–1480.

Barreto-Bergter, E., Pinto, M. R., and Rodrigues, M. L. (2004). Structure and biological functions of fungal cerebrosides. *An. Acad. Bras. Cienc.* 76, 67–84.

Bingaman, M. G., Basu, G. D., Golding, T. C., Chong, S. K., Lassen, A. J., Kindt, T. J., and Lipinski, C. A. (2010). The autophilic anti-CD20 antibody DXL625 displays enhanced potency due to lipid raft-dependent induction of apoptosis. *Anticancer Drugs* 21, 532–542.

Boas, M. H., Egge, H., Pohlentz, G., Hartmann, R., and Bergter, E. B. (1994). Structural determination of N-2′-hydroxyoctadecanoyl-1-O-beta-D-glucopyranosyl-9-methyl-4, 8-sphingadienine from species of *Aspergillus*. *Chem. Phys. Lipids* 70, 11–19.

Campanella, R. (1992). Membrane lipids modifications in human gliomas of different degree of malignancy. *J. Neurosurg. Sci.* 36, 11–25.

Charrow, J. (2009). Enzyme replacement therapy for Gaucher disease. *Expert Opin. Biol. Ther.* 9, 121–131.

Ciopraga, J., Gozia, O., Tudor, R., Brezuica, L., and Doyle, R. J. (1999). *Fusarium* sp. growth inhibition by wheat germ agglutinin. *Biochim. Biophys. Acta* 1428, 424–432.

da Silva, A. F., Rodrigues, M. L., Farias, S. E., Almeida, I. C., Pinto, M. R., and Barreto-Bergter, E. (2004). Glucosylceramides in *Colletotrichum gloeosporioides* are involved in the differentiation of conidia into mycelial cells. *FEBS Lett.* 561, 137–143.

Daniotti, J. L., and Iglesias-Bartolome, R. (2011). Metabolic pathways and intracellular trafficking of gangliosides. *IUBMB Life* 63, 513–520.

Dickson, R. C. (2008). Thematic review series: sphingolipids. New insights into sphingolipid metabolism and function in budding yeast. *J. Lipid Res.* 49, 909–921.

Dickson, R. C., Sumanasekera, C., and Lester, R. L. (2006). Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*. *Prog. Lipid Res.* 45, 447–465.

Feinstein, M. B., Fernandez, S. M., and Sha'afi, R. I. (1975). Fluidity of natural membranes and phosphatidylserine and ganglioside dispersions. Effect of local anesthetics, cholesterol and protein. *Biochim. Biophys. Acta* 413, 354–370.

Funato, K., and Riezman, H. (2001). Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast. *J. Cell Biol.* 155, 949–959.

Gault, C. R., Obeid, L. M., and Hannun, Y. A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. *Adv. Exp. Med. Biol.* 688, 1–23.

Hakomori, S. (2003). Structure, organization, and function of glycosphingolipids in membrane. *Curr. Opin. Hematol.* 10, 16–24.

Hakomori, S. I. (2008). Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. *Biochim. Biophys. Acta* 1780, 325–346.

Halter, D., Neumann, S., Van Dijk, S. M., Wolthoorn, J., De Maziere, A. M., Vieira, O. V., Mattjus, P., Klumperman, J., Van Meer, G., and Sprong, H. (2007). Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. *J. Cell Biol.* 179, 101–115.

Hillig, I., Warnecke, D., and Heinz, E. (2005). An inhibitor of glucosylceramide synthase inhibits the human enzyme, but not enzymes from other organisms. *Biosci. Biotechnol. Biochem.* 69, 1782–1785.

Insenser, M., Nombela, C., Molero, G., and Gil, C. (2006). Proteomic analysis of detergent-resistant membranes from *Candida albicans*. *Proteomics* 6(Suppl. 1), S74–S81.

Kechichian, T. B., Shea, J., and Del Poeta, M. (2007). Depletion of alveolar macrophages decreases the dissemination of a glucosylceramide-deficient mutant of *Cryptococcus neoformans* in immunodeficient mice. *Infect. Immun.* 75, 4792–4798.

Landry, A., and Xavier, R. (2006). Isolation and analysis of lipid rafts in cell-cell interactions. *Methods Mol. Biol.* 341, 251–282.

Lester, R. L., Smith, S. W., Wells, G. B., Rees, D. C., and Angus, W. W. (1974). The isolation and partial characterization of two novel sphingolipids from *Neurospora crassa*: di(inositolphosphoryl)ceramide and ((gal)3glu)ceramide. *J. Biol. Chem.* 249, 3388–3394.

Levery, S. B., Momany, M., Lindsey, R., Toledo, M. S., Shayman, J. A., Fuller, M., Brooks, K., Doong, R. L., Straus, A. H., and Takahashi, H. K. (2002). Disruption of the glucosylceramide biosynthetic pathway in *Aspergillus nidulans* and *Aspergillus fumigatus* by inhibitors of UDP-Glc:ceramide glucosyltransferase strongly affects spore germination, cell cycle, and hyphal growth. *FEBS Lett.* 525, 59–64.

Levery, S. B., Toledo, M. S., Doong, R. L., Straus, A. H., and Takahashi, H. K. (2000). Comparative analysis of ceramide structural modification found in fungal cerebrosides by electrospray tandem mass spectrometry with low energy collision-induced dissociation of Li+ adduct ions. *Rapid Commun. Mass Spectrom.* 14, 551–563.

Li, S., Du, L., Yuen, G., and Harris, S. D. (2006). Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. *Mol. Biol. Cell* 17, 1218–1227.

Lopez, P. H., and Schnaar, R. L. (2009). Gangliosides in cell recognition and membrane protein regulation. *Curr. Opin. Struct. Biol.* 19, 549–557.

Maciel, D. M., Rodrigues, M. L., Wait, R., Villas Boas, M. H., Tischer, C. A., and Barreto-Bergter, E. (2002). Glycosphingolipids from *Magnaporthe grisea* cells: expression of a ceramide dihexoside presenting phytosphingosine as the long-chain base. *Arch. Biochem. Biophys.* 405, 205–213.

Marta, C. B., Bansal, R., and Pfeiffer, S. E. (2008). Microglial Fc receptors mediate physiological changes resulting from antibody cross-linking of myelin oligodendrocyte glycoprotein. *J. Neuroimmunol.* 196, 35–40.

Martin, S. W., and Konopka, J. B. (2004). Lipid raft polarization contributes to hyphal growth in *Candida albicans*. *Eukaryot. Cell* 3, 675–684.

Melser, S., Batailler, B., Peypelut, M., Poujol, C., Bellec, Y., Wattelet-Boyer, V., Maneta-Peyret, L., Faure, J. D., and Moreau, P. (2010). Glucosylceramide biosynthesis is involved in Golgi morphology and protein secretion in plant cells. *Traffic* 11, 479–490.

Michaelson, L. V., Zauner, S., Markham, J. E., Haslam, R. P., Desikan, R., Mugford, S., Albrecht, S., Warnecke, D., Sperling, P., Heinz, E., and Napier, J. A. (2009). Functional characterization of a higher plant sphingolipid Delta4-desaturase: defining the role of sphingosine and sphingosine-1-phosphate in *Arabidopsis*. *Plant Physiol.* 149, 487–498.

Mitchell, A. P. (1998). Dimorphism and virulence in *Candida albicans*. *Curr. Opin. Microbiol.* 1, 687–692.

Nimrichter, L., Barreto-Bergter, E., Mendonca-Filho, R. R., Kneipp, L. F., Mazzi, M. T., Salve, P., Farias, S. E., Wait, R., Alviano, C. S., and Rodrigues, M. L. (2004). A monoclonal antibody to glucosylceramide inhibits the growth of *Fonsecaea pedrosoi* and enhances the antifungal action of mouse macrophages. *Microbes Infect.* 6, 657–665.

Nimrichter, L., Burdick, M. M., Aoki, K., Laroy, W., Fierro, M. A., Hudson, S. A., Von Seggern, C. E., Cotter, R. J., Bochner, B. S., Tiemeyer, M., Konstantopoulos, K., and Schnaar, R. L. (2008). E-selectin receptors on human leukocytes. *Blood* 112, 3744–3752.

Nimrichter, L., Cerqueira, M. D., Leitao, E. A., Miranda, K., Nakayasu, E. S., Almeida, S. R., Almeida, I. C., Alviano, C. S., Barreto-Bergter, E., and Rodrigues, M. L. (2005a). Structure, cellular distribution, antigenicity, and biological functions of *Fonsecaea pedrosoi* ceramide monohexosides. *Infect. Immun.* 73, 7860–7868.

Nimrichter, L., Rodrigues, M. L., Rodrigues, E. G., and Travassos, L. R. (2005b). The multitude of targets for the immune system and drug therapy in the fungal cell wall. *Microbes Infect.* 7, 789–798.

Noble, S. M., French, S., Kohn, L. A., Chen, V., and Johnson, A. D. (2010). Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nat. Genet.* 42, 590–598.

Nosanchuk, J. D., Nimrichter, L., Casadevall, A., and Rodrigues, M. L. (2008). A role for vesicular transport of macromolecules across cell walls in fungal pathogenesis. *Commun. Integr. Biol.* 1, 37–39.

Oliveira, D. L., Nakayasu, E. S., Joffe, L. S., Guimaraes, A. J., Sobreira, T. J., Nosanchuk, J. D., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., and Rodrigues, M. L. (2010a). Biogenesis of extracellular vesicles in yeast: many questions with few answers. *Commun. Integr. Biol.* 3, 533–535.

Oliveira, D. L., Nakayasu, E. S., Joffe, L. S., Guimaraes, A. J., Sobreira, T. J., Nosanchuk, J. D., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., and Rodrigues, M. L. (2010b). Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. *PLoS ONE* 5, e11113. doi:10.1371/journal.pone.0011113

Oliveira, D. L., Nimrichter, L., Miranda, K., Frases, S., Faull, K. F., Casadevall, A., and Rodrigues, M. L. (2009). *Cryptococcus neoformans* cryoultramicrotomy and vesicle fractionation reveals an intimate association between membrane lipids and glucuronoxylomannan. *Fungal Genet. Biol.* 46, 956–963.

Oura, T., and Kajiwara, S. (2010). *Candida albicans* sphingolipid C9-methyltransferase is involved in hyphal elongation. *Microbiology* 156, 1234–1243.

Pinto, M. R., Rodrigues, M. L., Travassos, L. R., Haido, R. M., Wait, R., and Barreto-Bergter, E. (2002). Characterization of glucosylceramides in *Pseudallescheria boydii* and their involvement in fungal differentiation. *Glycobiology* 12, 251–260.

Ramamoorthy, V., Cahoon, E. B., Thokala, M., Kaur, J., Li, J., and Shah, D. M. (2009). Sphingolipid C-9 methyltransferases are important for growth and virulence but not for sensitivity to antifungal plant defensins in *Fusarium graminearum*. *Eukaryot. Cell* 8, 217–229.

Rhome, R., Singh, A., Kechichian, T., Drago, M., Morace, G., Luberto, C., and Del Poeta, M. (2011). Surface localization of glucosylceramide during *Cryptococcus neoformans* infection allows targeting as a potential antifungal. *PLoS ONE* 6, e15572. doi:10.1371/journal.pone.0015572

Rittenour, W. R., Chen, M., Cahoon, E. B., and Harris, S. D. (2011). Control of glucosylceramide production and morphogenesis by the Bar1 ceramide synthase in *Fusarium graminearum*. *PLoS One* 6, e19385. doi:10.1371/journal.pone.0019385

Rittershaus, P. C., Kechichian, T. B., Allegood, J. C., Merrill, A. H. Jr., Hennig, M., Luberto, C., and Del Poeta, M. (2006). Glucosylceramide synthase is an essential regulator of pathogenicity of *Cryptococcus neoformans*. *J. Clin. Invest.* 116, 1651–1659.

Rodrigues, M. L., Nakayasu, E. S., Oliveira, D. L., Nimrichter, L., Nosanchuk, J. D., Almeida, I. C., and Casadevall, A. (2008a). Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryot. Cell* 7, 58–67.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Nosanchuk, J. D., and Casadevall, A. (2008b). Vesicular trans-cell wall transport in fungi: a mechanism for the delivery of virulence-associated macromolecules? *Lipid Insights* 2, 27–40.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., and Casadevall, A. (2007a). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell* 6, 48–59.

Rodrigues, M. L., Shi, L., Barreto-Bergter, E., Nimrichter, L., Farias, S. E., Rodrigues, E. G., Travassos, L. R., and Nosanchuk, J. D. (2007b). Monoclonal antibody to fungal glucosylceramide protects mice against lethal *Cryptococcus neoformans* infection. *Clin. Vaccine Immunol.* 14, 1372–1376.

Rodrigues, M. L., Travassos, L. R., Miranda, K. R., Franzen, A. J., Rozental, S., De Souza, W., Alviano, C. S., and Barreto-Bergter, E. (2000). Human antibodies against a purified glucosylceramide from *Cryptococcus neoformans* inhibit cell budding and fungal growth. *Infect. Immun.* 68, 7049–7060.

Saito, K., Takakuwa, N., Ohnishi, M., and Oda, Y. (2006). Presence of glucosylceramide in yeast and its relation to alkali tolerance of yeast. *Appl. Microbiol. Biotechnol.* 71, 515–521.

Schnaar, R. L., Suzuki, A., and Stanley, P. (2009). "Glycosphingolipids," in *Essentials of Glycobiology,* Chap. 10, 2nd Edn. Eds A.Varki, R. D.Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G.W. Hart, and M. E. Etzler (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Siafakas, A. R., Wright, L. C., Sorrell, T. C., and Djordjevic, J. T. (2006). Lipid rafts in *Cryptococcus neoformans* concentrate the virulence determinants phospholipase B1 and Cu/Zn superoxide dismutase. *Eukaryot. Cell* 5, 488–498.

Sonnino, S., Mauri, L., Chigorno, V., and Prinetti, A. (2007). Gangliosides as components of lipid membrane domains. *Glycobiology* 17, 1R–13R.

Sperling, P., Schmidt, H., and Heinz, E. (1995). A cytochrome-b5-containing fusion protein similar to plant acyl lipid desaturases. *Eur. J. Biochem.* 232, 798–805.

Staubach, S., and Hanisch, F. G. (2011). Lipid rafts signaling and sorting platforms of cells and their roles in cancer. *Expert Rev. Proteomics* 8, 263–277.

Sugimoto, Y., Sakoh, H., and Yamada, K. (2004). IPC synthase as a useful target for antifungal drugs. *Curr. Drug Targets Infect. Disord.* 4, 311–322.

Takahashi, H. K., Levery, S. B., Toledo, M. S., Suzuki, E., Salyan, M. E., Hakomori, S., and Straus, A. H. (1996). Isolation and possible composition of glucosylceramides from *Paracoccidioides brasiliensis. Braz. J. Med. Biol. Res.* 29, 1441–1444.

Takakuwa, N., Ohnishi, M., and Oda, Y. (2008). Significance of the KlLAC1 gene in glucosylceramide production by *Kluyveromyces lactis. FEMS Yeast Res.* 8, 839–845.

Tavares, P. M., Thevissen, K., Cammue, B. P., Francois, I. E., Barreto-Bergter, E., Taborda, C. P., Marques, A. F., Rodrigues, M. L., and Nimrichter, L. (2008). In vitro activity of the antifungal plant defensin RsAFP2 against *Candida* isolates and its in vivo efficacy in prophylactic murine models of candidiasis. *Antimicrob. Agents Chemother.* 52, 4522–4525.

Ternes, P., Franke, S., Zahringer, U., Sperling, P., and Heinz, E. (2002). Identification and characterization of a sphingolipid delta 4-desaturase family. *J. Biol. Chem.* 277, 25512–25518.

Ternes, P., Sperling, P., Albrecht, S., Franke, S., Cregg, J. M., Warnecke, D., and Heinz, E. (2006). Identification of fungal sphingolipid C9-methyltransferases by phylogenetic profiling. *J. Biol. Chem.* 281, 5582–5592.

Ternes, P., Wobbe, T., Schwarz, M., Albrecht, S., Feussner, K., Riezman, I., Cregg, J. M., Heinz, E., Riezman, H., Feussner, I., and Warnecke, D. (2011). Two pathways of sphingolipid biosynthesis are separated in the yeast *Pichia pastoris. J. Biol. Chem.* 286, 11401–11414.

Thevissen, K., Warnecke, D. C., Francois, I. E., Leipelt, M., Heinz, E., Ott, C., Zahringer, U., Thomma, B. P., Ferket, K. K., and Cammue, B. P. (2004). Defensins from insects and plants interact with fungal glucosylceramides. *J. Biol. Chem.* 279, 3900–3905.

Tifft, C. J., and Proia, R. L. (2000). Stemming the tide: glycosphingolipid synthesis inhibitors as therapy for storage diseases. *Glycobiology* 10, 1249–1258.

Tinker, D. O., Pinteric, L., Hsia, J. C., and Rand, R. P. (1976). Perturbation of lecithin bilayer structure by globoside. *Can. J. Biochem.* 54, 209–218.

Toledo, M. S., Levery, S. B., Straus, A. H., and Takahashi, H. K. (2000). Dimorphic expression of cerebrosides in the mycopathogen *Sporothrix schenckii. J. Lipid Res.* 41, 797–806.

Wachtler, V., and Balasubramanian, M. K. (2006). Yeast lipid rafts? – an emerging view. *Trends Cell Biol.* 16, 1–4.

Whiteway, M., and Bachewich, C. (2007). Morphogenesis in *Candida albicans. Annu. Rev. Microbiol.* 61, 529–553.

Xu, X., and Nicholson, P. (2009). Community ecology of fungal pathogens causing wheat head blight. *Annu. Rev. Phytopathol.* 47, 83–103.

Zauner, S., Zahringer, U., Lindner, B., Warnecke, D., and Sperling, P. (2008). Identification and functional characterization of the 2-hydroxy fatty N-acyl-Delta3(E)-desaturase from *Fusarium graminearum. J. Biol. Chem.* 283, 36734–36742.

Zhang, Z., and Yuen, G. Y. (1999). Biological control of *Bipolaris sorokiniana* on tall fescue by *Stenotrophomonas maltophilia* strain C3. *Phytopathology* 89, 817–822.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 September 2011; accepted: 30 September 2011; published online: 20 October 2011.

*Citation: Nimrichter L and Rodrigues ML (2011) Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials. Front. Microbio. **2**:212. doi: 10.3389/fmicb.2011.00212*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 19 December 2011
doi: 10.3389/fmicb.2011.00248

Fungal surface and innate immune recognition of filamentous fungi

Rodrigo T. Figueiredo[1]*, Leticia A. M. Carneiro[2] and Marcelo T. Bozza[2]*

[1] *Instituto de Ciências Biomédicas/Pólo de Xerém, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Edited by:
Marcio Rodrigues, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Dario S. Zamboni, Universidade de São Paulo, Brazil
Erin McClelland, The Commonwealth Medical College, USA

***Correspondence:**
Rodrigo T. Figueiredo, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, CCS Bloco F 014, Avenida Carlos Chagas Filho, 373 Cidade Universitária, Rio de Janeiro, RJ, 21941-902, Brazil.
e-mail: rotifi@icb.ufrj.br;
Marcelo T. Bozza, Departamento de Imunologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, CCS Bloco D 036, Avenida Carlos Chagas Filho, 373 Cidade Universitária, Rio de Janeiro, RJ, 21941-902 Brazil.
e-mail: mbozza@micro.ufrj.br

The innate immune system performs specific detection of molecules from infectious agents through pattern recognition receptors. This recognition triggers inflammatory responses and activation of microbicidal mechanisms by leukocytes. Infections caused by filamentous fungi have increased in incidence and represent an important cause of mortality and morbidity especially in individuals with immunosuppression. This review will discuss the innate immune recognition of filamentous fungi molecules and its importance to infection control and disease.

Keywords: fungi, PRRs, innate immunity, inflammation, signaling, macrophages, cytokines

INTRODUCTION

Fungal infections are an important cause of morbidity and mortality. Infections caused by filamentous fungi have increased in incidence, especially in immunosuppressed individuals (Singh, 2001; Marr et al., 2002; Camilos et al., 2006; Enoch et al., 2006; Segal and Walsh, 2006). Risk factors are generally associated with immunosuppression and include therapy with corticoids or myeloablative drugs, neutropenia, and solid organ transplants (Marr et al., 2002; Morgan et al., 2005; Camilos et al., 2006). Sporadic cases of infection in immunocompetent individuals have also been described, particularly in situations where traumatic inoculation occurs, like the use of catheters, surgeries, or wounds (Pasqualotto and Denning, 2006; Meersseman et al., 2008; Murray et al., 2008).

Pathogenic fungi are variable in their biology, and can also include commensal microorganisms such as *Candida albicans*, and opportunist filamentous fungi like *Aspergillus fumigatus*, *Scedosporium* spp., and *Fusarium* spp.

Filamentous fungi are saprophytic and widely distributed in the environment. These fungi present multiseptated forms, the hyphae, and forms of dispersion referred to as conidia. Conidia are extremely small and are responsible for the establishment of the infection once they reach deeper tissues, such as the lung alveoli or wounds. In the absence of adequate removal by macrophages and/or killing mediated by neutrophils, conidia germinate giving rise to hyphae that promote tissue invasion and injury (Groll and Walsh, 2001).

The pathogenesis of filamentous fungi is related to their ubiquitous presence in the environment, their high capacity of dispersion and small size of the conidia, which contribute to the access of these structures to host tissues, like the respiratory tract. Filamentous fungi are able to survive and grow in a wide range of temperatures, varying from 25 to 37°C or even higher temperatures which, in association with their low nutritional requirements, permits host tissue colonization in a context of deficiencies in microbicidal mechanisms of leukocytes and mobilization of the inflammatory response (Groll and Walsh, 2001; Latgé, 2001).

Molecules from infectious agents are recognized by the innate immune system triggering an inflammatory response that is fundamental to the initial control of infection. This early response

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; BIR, baculovirus inhibitor of apoptosis protein repeat domain; CARD, caspase activation and recruitment domain; C/EBP, CCAAT/enhancer biding protein; CLRs, C-type receptors; CRD, carbohydrate recognition domain; DC-SIGN, dendritic cell-specific ICAM3 grabbing non-integrin; GMX, glucuronoxylomannans; IL-1, interleukin-1; ITAM, immunoreceptor tyrosine activation based motifs; LPS, lipopolysaccharides; LRR, leucine-rich repeat; MAP kinases, mitogen-activated protein kinases; MDP, muramyl dipeptide; mincle, macrophage-inducible C-type lectin; MR, mannose receptor; NACHT, Naip, *C*IITA, *H*ET-E (plant het product involved in vegetative incompatibility) and *T*P-1 (telomerase-associated protein 1); NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor-κB; NLR, Nod-like receptors; Nod, nucleotide oligomerization domain; PRRs, pattern recognition receptors; PYD, pyrin; ROS, reactive oxygen species; TLRs, Toll like receptors; TNF-α, tumor necrosis factor-α.

dictates the activation of antigen specific lymphocytes, the hallmark of the adaptive immune response. Each microorganism is composed of a peculiar set of molecules and ways to interact with the host. The innate immune system also recognizes molecules from damaged host cells and tissues, promoting unique immune/inflammatory responses.

The specific recognition of conserved microbial molecules from infectious agents is performed by pattern recognition receptors (PRRs; Akira et al., 2006; Medzhitov, 2007). Activation of these receptors triggers intracellular signaling pathways resulting in the production of cytokines, lipid mediators, and reactive oxygen/nitrogen species. This activation of the immune system is instrumental for pathogen killing but is also critically involved in tissue damage (Nathan, 2002).

Toll like receptors (TLRs) and the C-type lectin receptors (CLRs) are the best defined receptors involved in fungal recognition. They couple pathogen detection to signaling cascades that promote induction of pro-inflammatory mediators, phagocytosis, and induction of adaptive immunity (Van de Veerdonk et al., 2008).

Toll like receptors form a group of receptors that share homology with *Drosophila* Toll receptor. Toll receptor was initially demonstrated to be involved in immunity to fungal infections in *Drosophila* through sensing of the protein Spätzle that is produced by proteolysis during the infection caused by *A. fumigatus* (Lemaitre et al., 1996). Characterization of the *Drosophila* Toll receptor opened the way for identification of mammalian homologs designated TLRs which have been implicated in detection of microbial products and activation of innate immunity to pathogens (Medzhitov, 2007).

TLR4 detects bacterial lipopolysaccharides (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999). TLR2 forms heterodimers with TLR1 or TLR6, recognizing diacylated (TLR2/TLR6) and triacylated bacterial lipoproteins (TLR2/TLR1; Takeuchi et al., 2000, 2001, 2002), lipoteichoic acid (TLR2/TLR6; Schwandner et al., 1999; Schröder et al., 2003; Travassos et al., 2004), lipoarabinomannans (TLR2/TLR1; Means et al., 1999; Sandor et al., 2003; Tapping and Tobias, 2003), and GPI anchors of protozoans (Campos et al., 2001; Krishnegowda et al., 2005; Debierre-Grockiego et al., 2007). TLR5 recognizes bacterial flagellin (Hayashi et al., 2001). TLR3, TLR7, TLR8, and TLR9 are present in endosomes and are involved in recognition of internalized nucleic acids (Kawai and Akira, 2011). TLR3 senses double-stranded RNA produced during viral infections (Alexopoulou et al., 2001). TLR7/8 is responsible for detection of single stranded RNA enriched in GU-containing sequences and nucleotides analogs (Hemmi et al., 2002; Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). TLR9 recognizes DNA sequences containing non-methylated CpG (Hemmi et al., 2000).

C-type lectin receptors are transmembrane proteins involved in pathogen recognition by means of carbohydrate detection. This group of receptors includes molecules involved in fungal recognition such as Dectin-1 (CLEC7A), Dectin-2 (CLEC4n), dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN), Macrophage-inducible C-type lectin (Mincle, CLEC4e), and mannose receptor (MR; Willment and Brown, 2008). Dectin-1, Mincle, and Dectin-2 recognize fungal products, and signal through immunoreceptor tyrosine based motifs (ITAM), leading to activation of transcriptional factors such as NFAT and NF-κB and induction of cytokines (Kerrigan and Brown, 2010). Furthermore, some CLRs have been implicated in internalization of pathogens, such as Dectin-1, DC-SIGN, and MR. Dectin-1 recognizes fungal β-1,3-glucans (Brown and Gordon, 2001; Brown et al., 2003), Dectin-2 is involved in cellular responses to mannans expressed on fungal surfaces (Saijo et al., 2010). MR binds to polysaccharides containing mannose and fucose residues and is involved in recognition of many fungal pathogens (Kerrigan and Brown, 2009). Mincle senses mycobacterial glycolipid, trehalose dimycolate, and fungal molecules still uncharacterized, like *C. albicans* (Miyake et al., 2010). DC-SIGN is involved in recognition of several pathogens and has been implicated in internalization and cellular infection by microorganisms like *Mycobacterium tuberculosis*, HIV, Dengue virus, and fungal pathogens (van Kooyk and Geijtenbeek, 2003).

This review will discuss the receptors involved in innate immune recognition of opportunist filamentous fungi and the fungal surface molecules recognized by these receptors.

FUNGAL SURFACES AND MOLECULAR PATTERNS

Fungal pathogens have a thick cell wall that is essential for their survival by providing protection against environmental stress and allowing interaction with host cells and tissues. The distinct composition of the fungal surface in relation to the membrane of mammalian cells offers a wide array of molecular patterns that are important targets for recognition by the immune system and impact the relationship between fungal pathogens and their hosts (Latgé, 2010).

Zymosan, a particulate material obtained from the *Saccharomyces cerevisiae* cell wall, has been extensively used as a model for investigation of the mechanisms of innate immune recognition of fungal pathogens. Zymosan is composed mainly of polysaccharides that include chitin, mannans, and β-glucans, in addition to fatty acids and proteins (Di Carlo and Fiore, 1958). Zymosan is promptly phagocytosed by macrophages and is a potent inducer of pro-inflammatory cytokine release and reactive oxygen species (ROS) production by macrophages and neutrophils (Murata et al., 1987; Brown et al., 2003; Gantner et al., 2003).

Candida albicans is a well-studied fungal pathogen and its surface has been characterized in detail. The *C. albicans* cell wall is composed of an external coat of mannans and mannoproteins that covers a deeper layer composed of β-glucans and chitin (Chaffin et al., 1998; Masuoka, 2004). The mannans found in the *C. albicans* surface are polymers of mannose linked by α-1,4, α-1,6, and α-1,2 bonds. The *C. albicans* cell wall also presents β-1,2 oligomannosides joined to α-1,6 linked chains by phosphodiester bonds. The surface linked proteins are heavily glycosylated with long chains of mannans that are components of glycoproteins or glycolipids present in the surface. Internally, the *C. albicans* cell wall is filled with a tough cross-linked layer of chitin and β-glucan with glycolipids and glycoproteins anchored by glycosylphosphatidylinositol groups. The core of the internal layer of the *C. albicans* cell wall is formed by chains of β-glucans joined through β-1,3 and β-1,6 linkages. Chitin is a polymer of units of *N*-acetyl-D-glucosamine linked by β-1,4 bonds that is covalently linked to the

net of β-glucans, thus conferring extensive cross-linking to the β-glucan chains (Chaffin et al., 1998; Masuoka, 2004).

Filamentous fungi like *A. fumigatus* present a distinct composition in their surfaces in comparison with *C. albicans*. The cell wall of *A. fumigatus* has an external coat covered with α-1,3/α-1,4-glucans and galactomannans (Fontaine et al., 2000). In contrast with yeasts, *A. fumigatus* presents peptidogalactomannans containing oligomers of galactofurans linked to units of α-1,2 mannoses and these polysaccharides are antigens released during infection (Leitão et al., 2003). Curiously, these mannans are generally bound to glucans without a peptidic core (Fontaine et al., 2000). As in yeasts, the *A. fumigatus* cell wall is constituted by a rigid insoluble layer of β-1,3-glucans that is branched by β-1,6 linkages, but in the case of *A. fumigatus*, β-1,6 glucans are not present and β-1,3/1,4 linear chains are part of the cell wall (Fontaine et al., 2000). Chitin, galactomannans, and β-1,3/1,4-glucans are covalently bound to the mesh of β-1,3-glucans that forms the core of the cell wall (Fontaine et al., 2000). GPI-anchored glycoproteins and polysaccharides are also present on the *A. fumigatus* membrane (Fontaine et al., 2003; Costachel et al., 2005).

Aspergillus fumigatus resting conidia present a hydrophobic layer formed by the protein RodA. This layer masks β-glucans and uncharacterized TLR activators, making resting conidia unable to induce cytokine release by macrophages. During germination, the rodlet layer of RodA is degraded and molecules that are recognized by PRRs in macrophages and dendritic cells are then exposed and promote cytokine production and expression of co-stimulatory molecules. Similarly, other environmental non-pathogenic fungi also express a layer of hydrophobins and their resting conidia are also unable to induce dendritic cell activation, while chemical removal of hydrophobins permits dendritic cell activation by the resting conidia. Thus, the outermost hydrophobic layer of many mold fungi prevents an undesired inflammatory response to resting conidia in what seems to be an evolutionary interplay between environmental fungi and mammals. Thus the only morphological stages that represent a real threat for host tissue invasion, like germ tubes, swollen conidia, and hyphae, would be recognized by the immune system (Aimanianda et al., 2009).

TOLL LIKE RECEPTORS AND RECOGNITION OF PATHOGENIC FUNGI

TLR2 and TLR4 have been implicated in fungal recognition (Van de Veerdonk et al., 2008). TLR2/TLR6 heterodimers are responsible for activation of NF-κB and cytokine induction by zymosan in macrophages (Underhill et al., 1999; Ozinsky et al., 2000). TLR2 and TLR4 are involved in innate immune recognition of *C. albicans* and *A. fumigatus* (Van de Veerdonk et al., 2008).

Mice with non-functional TLR4 (C3H/HeJ) present an increased fungal load in the kidneys and deficiencies in neutrophil recruitment upon *C. albicans* challenge when compared to TLR4 responsive mice (C3H/HeN; Netea et al., 2002). TLR4 is required for the production of neutrophil chemoattractants MIP-2/CXCL2 and KC/CXCL1 by mouse macrophages stimulated with heat-killed *C. albicans* blastoconidia, while TLR2 is required for TNF-α and IL-1 release by human peripheral blood mononuclear cells (Netea et al., 2002). *Tlr2*$^{-/-}$ mice are more susceptible to *C. albicans* infection showing a reduction in neutrophil mobilization during infection. TLR2 is also required for MIP-2/CXCL2 and TNF-α release by murine macrophages in response to *C. albicans* yeasts and hyphae (Villamón et al., 2004). Bellocchio et al. (2004) found that, in a model of gastric colonization by *C. albicans*, *Tlr4*$^{-/-}$, and *Tlr2*$^{-/-}$ mice display increased fungal loads. These authors observed a reduced TNF-α release by *Tlr4*$^{-/-}$ macrophages, but not *Tlr2*$^{-/-}$ macrophages, stimulated with *C. albicans* yeasts or hyphae. On the other hand, these authors did not observe any increase in the susceptibility of *Tlr4*$^{-/-}$ or *Tlr2*$^{-/-}$ mice to systemic *C. albicans* infection in regards to lethality and fungal load. Even though TLR2 and TLR4 have been clearly demonstrated to recognize *C. albicans*, discrepancies among different studies probably reflect heterogeneity of experimental settings. Additionally, the differences in the experimental models of infection are most likely due to the different routes of infections used in the study.

TLR2 and TLR4 are also involved in *A. fumigatus* recognition. TLR2 is required for TNF-α release by macrophages in response to *A. fumigatus* resting conidia and hyphae (Mambula et al., 2002). In contrast, TLR4 deficiency does not impact TNF-α induction by *A. fumigatus* in macrophages. Similar results were obtained with HEK cells overexpressing TLR4 and TLR2, in which TLR2 expression promoted NF-κB activation in response to *A. fumigatus*, while TLR4 expression failed to do so (Mambula et al., 2002). Meier et al. (2003) observed that *A. fumigatus* induces NF-κB activation in macrophages and overexpression of either TLR2 or TLR4 in HEK cells leads to NF-κB activation upon stimulation with *A. fumigatus* conidia and hyphae (Meier et al., 2003). Furthermore, TLR4 but not TLR2 deficiency impaired cytokine release by murine peritoneal macrophages stimulated with *A. fumigatus* conidia and hyphae (Meier et al., 2003). Neutrophil recruitment upon *A. fumigatus* challenge is reduced in C3H/HeJ mice, and although a residual cytokine release and neutrophil recruitment were still observed, C3H/HeJ/*Tlr2*$^{-/-}$ mice show absence of the cytokine release by macrophages and a greater reduction in neutrophil mobilization *in vivo*, implying that both receptors are involved in *A. fumigatus* recognition with TLR2 single deficiency being compensated by TLR4 signaling (Meier et al., 2003). Human polymorphisms in TLR4, TLR1, and TLR6 have been associated with susceptibility to *A. fumigatus* infections in recipients of hematopoietic transplants, indicating that these receptors are involved in immunity to *A. fumigatus* (Kesh et al., 2005; Bochud et al., 2008). Latter works demonstrated that innate immune recognition of *A. fumigatus* requires germination of conidia. Resting conidia are unable to induce cytokine release by macrophages, but upon germination swollen conidia and germ tubes are recognized by TLR2 and Dectin-1, thus triggering cytokine release and ROS production by macrophages (Hohl et al., 2005; Steele et al., 2005; Gersuk et al., 2006).

Toll like receptors are also involved in the innate recognition of *Pseudallescheria boydii*, an important pathogenic filamentous fungus. TLR4 is required for cytokine production by macrophages in response to the stimulation with *P. boydii* conidia, while TLR2 seems to be dispensable. In contrast, induction of TNF-α by *P. boydii* hyphae is independent of either TLR2 or TLR4 mediated recognition (Figueiredo et al., 2010).

Recognition of zymosan with subsequent cytokine production by macrophages requires cooperation between TLR2 and Dectin-1 (Brown et al., 2003; Gantner et al., 2003). The capacity to activate TLR2 is abolished by hot alkali treatment or oxidation of zymosan, while Dectin-1 signaling is not impaired (Gantner et al., 2003; Saijo et al., 2007). Macrophage activation by β-glucans is independent of TLR signaling, but completely dependent on Dectin-1 triggering (Rogers et al., 2005; Yoshitomi et al., 2005; Saijo et al., 2007). These results indicate that different molecules are involved in zymosan recognition by macrophages, with β-glucans inducing Dectin-1 activation and unknown molecules promoting TLR2 triggering.

Candida albicans phospholipomannans induce NF-κB activation and TNF-α release in murine macrophages by a mechanism dependent on TLR2 activation that also requires TLR4 and TLR6 signaling for full-blown activation (Jouault et al., 2003). However, it is uncertain if other pathogenic fungi express phospholipomannans, in particular filamentous fungi. A phospholipomannan of *C. albicans* was structurally characterized and shown to be composed of β-1,2 oligomannosides linked to a phosphoinositol ring that, in turn, is linked to a C18/C20 phytosphingosine and a ramified C24/C25/C26 fatty acid (Trinel et al., 2002). However, the structural requirements involved in TLR activation by phospholipomannans are still unknown and direct interaction with TLR2, TLR4, or TLR6 was not analyzed.

Glucuronoxylomannans (GXM) are central components of the capsule present in the fungal pathogens *Cryptococcus neoformans* and *C. gattii*. GXMs consist of polyanionic polysaccharides composed of α-1,3 linked mannans, *O*-acetylated in some mannosyl residues with substitutions of β-1,2 glucuronyl and β-1,2/β-1,4 xylosyl units (Rodrigues et al., 2011). GXM is recognized by TLR4 (Shoham et al., 2001), TLR2/TLR1, and TLR2/TLR6 (Fonseca et al., 2010). Activation of TLR2/TLR1 and TLR2/TLR6 is associated with a smaller size of GXM particles (Fonseca et al., 2010). Interaction of GXM with TLR4, TLR2/TLR1, or TLR2/TLR6 promotes NF-κB activation (Shoham et al., 2001; Fonseca et al., 2010), and expression of co-stimulatory molecules by macrophages (Monari et al., 2005). However, in contrast to prototypical TLR agonists, GXM does not induce TNF-α production or activation of MAP kinases by macrophages (Shoham et al., 2001; Monari et al., 2005). Nevertheless, other studies have demonstrated the induction of cytokines by human neutrophils and monocytes stimulated with GXM (Retini et al., 1996; Vecchiarelli et al., 1996; Delfino et al., 1997). Curiously, human monocytes, like macrophages, do not produce the pro-inflammatory cytokines TNF-α and IL-1β when stimulated with GXM (Retini et al., 1996; Shoham et al., 2001; Monari et al., 2005). Although TLR2 and TLR4 are clearly involved in recognition of GXM particles, the structural determinants involved are still uncharacterized. In fact, GXMs are highly heterogeneous molecules and it is possible that GXM recognition could trigger activation of surface receptors other than TLRs that promote immunomodulatory effects distinct from those activated by TLR classical ligands. Furthermore the heterogenicity of GXM composition could contribute to the differences in the induction of cytokines, observed in different works. The absence of induction of TNF-α by GXMs in mononuclear phagocytes, in contrast to neutrophils, might contribute to immune evasion of pathogenic *Cryptococcus* spp., since macrophages carry intracellular *Cryptococcus* yeasts during infection.

Mannans isolated from *C. albicans* and *Saccharomyces* spp. are inducers of cytokine release in macrophages through TLR4 recognition (Tada et al., 2002). Interestingly, TLR4 seems to cooperate with the MR. TLR4 recognizes O-linked mannans, while N-linked mannans are recognized by MR (Netea et al., 2006). TLR4 is also required for macrophage activation induced by *P. boydii* conidia, and removal of mannans results in a great reduction in TNF-α production by macrophages, indicating that mannans play an important role in the innate immune recognition of *P. boydii*. Rhamnomannans isolated from *P. boydii* are inducers of cytokine production in macrophages through TLR4 activation. Interestingly, mannans isolated from *P. boydii* present terminal non-reducing units of rhamnose and chemical removal of these units abolish cytokine induction, implying a role for these terminal residues in recognition of *P. boydii* derived mannans (Figueiredo et al., 2010).

P. boydii derived α-glucans induce cytokine production by macrophages and dendritic cells, by means of recognition mediated by TLR2 and CD14 (Bittencourt et al., 2006). Polysaccharides extracted from the medicinal fungi *Ganoderma lucidum* and *Cordyceps sinensis* are potent immunomodulators and have antitumoral activity (Hsu et al., 2009). The ability of these polysaccharides to induce cytokine production and B cell activation is dependent on TLR2 and TLR4 activation. Binding assays using recombinant chimeric TLR4-Fc and TLR2-Fc demonstrated direct binding to polysaccharides derived from *G. lucidum*. Furthermore, the binding of TLR4 and TLR2 to these polysaccharides was inhibited by soluble mannans, indicating that TLR4 and TLR2 competitively bind both mannans and polysaccharides (Hsu et al., 2009). It remains undefined how TLR4 and TLR2 detect fungal molecules. Fungal polysaccharides such as mannans, α-glucans, and GXMs are structurally distinct from the prototypical agonists of TLR4 and TLR2. It is possible that fungal structures, particularly complex polysaccharides or fragments of cell wall, could present uncharacterized glycolipids anchored to their structures in such a way that these compounds could act as true "agonists" of TLR4 and/or TLR2. To shed light into these questions, sensitive analytical techniques, including nuclear magnetic resonance and mass spectrometry, could prove to be useful. In addition, complementary approaches like genetic models of deficiency in the synthesis of specific molecules of fungal pathogens and selective inactivation of investigated molecules (enzymatic treatments, selective chemical reactions), in order to exclude contamination with bacterial ligands or even the contribution of minor components of fungal preparations. Direct binding of bacterial lipid A and lipopeptides have been described for TLR4 and TLR2, respectively. In addition, crystallographic analysis of lipid A/TLR4/MD2 complex and lipopeptides to TLR1/TLR2 or TLR6/TLR2 complexes have also been obtained, indicating physical interaction between these ligands and their receptor complexes (Jin et al., 2007; Kang et al., 2009; Park et al., 2009). Thus it will be important to establish the structural basis for TLR4 and TLR2 interaction with fungal molecules, which would help to understand how these receptors are able to detect such distinct structures like LPS (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999) and lipoproteins

(Takeuchi et al., 2000, 2001, 2002), as well as endogenous mammalian molecules such as carboxy alkyl pyrroles, hyaluronic acid, heme, HMGB1, and heparan sulfate (Brunn et al., 2005; Figueiredo et al., 2007; Taylor et al., 2007b; West et al., 2010; Yang et al., 2010).

Although TLR2 and TLR4 are involved in *A. fumigatus* recognition, the molecules involved in activation of these receptors were not characterized. The *A. fumigatus* cell wall presents α-glucans and mannans (Latgé, 2010) and, as it was found for *P. boydii*, these molecules could be targets of recognition mediated by TLR2 and TLR4, respectively. Thus, it will be important to perform the isolation and characterization of highly purified cell wall components of *A. fumigatus* and other filamentous fungi, and screening of such molecules for activation of selective PRRs including TLR4 and TLR2.

CD14 CONTRIBUTES TO TLR RECOGNITION OF FUNGAL MOLECULES

CD14 was initially characterized as a receptor involved in LPS binding to cell membrane and LPS-induced cellular responses. CD14 is a glycosylphosphatidylinositol-anchored protein that lacks an intracellular signaling domain (Wright et al., 1990). CD14 was implied in cellular responses to several microbial molecules, such as lipoproteins (Schröder et al., 2004) and lipoteichoic acid (Schröder et al., 2003). Although unable to trigger cellular signaling, CD14 promotes loading of TLR4/MD2 and TLR2 with their ligands, a mechanism that contributes to detection of these soluble molecules in small amounts (Wright et al., 1990; Schröder et al., 2004).

CD14 is involved in the recognition of filamentous fungi. CD14 overexpression in human cell lines results in increased NF-κB activation and TNF-α production in response to *A. fumigatus*. These results, however, are in contrast with others showing no difference in TNF-α production by $Cd14^{-/-}$ and wild-type macrophages stimulated with *A. fumigatus*, indicating that in the case of murine macrophages CD14 is dispensable for *A. fumigatus* recognition (Mambula et al., 2002). Activation of human monocytes by *A. fumigatus* hyphae is dependent on recognition mediated by CD14 and TLR4, since blocking these receptors with monoclonal antibodies results in reduction of TNF-α production (Wang et al., 2001). Recognition of *P. boydii* conidia requires CD14, as demonstrated by reduction in TNF-α release by $Cd14^{-/-}$ macrophages when compared with wild-type cells (Figueiredo et al., 2010). The α-glucans isolated from *P. boydii* also require CD14 for cytokine induction by murine macrophages (Bittencourt et al., 2006). Mannans derived from *C. albicans* and *S. cerevisiae* induce macrophage activation with induction of pro-inflammatory cytokines in a mechanism that is also dependent on CD14 recognition (Tada et al., 2002). *C. neoformans* derived GXM induces macrophage and epithelial cell activation by a mechanism requiring CD14 recognition. Furthermore CD14 is involved in GXM internalization by macrophages (Barbosa et al., 2007).

The mechanism by which CD14 promotes leukocyte activation in response to fungal molecules is still largely unknown. As observed with bacterial activators of TLR2 and TLR4, CD14 is probably involved in the transfer of fungal molecules to TLR2 or TLR4. Studies using isolated fungal molecules and measurements of their binding to CD14 and TLR2/TLR4 and cellular activation would be valuable in defining the role of CD14 in fungal recognition.

ROLE OF C-TYPE LECTIN RECEPTORS ON FUNGI RECOGNITION

Dectin-1 is a type II transmembrane protein with an extracellular CLR domain and was identified by means of a search in a cDNA library obtained from RAW264.7 cells for genes able to confer β-glucan binding (Brown and Gordon, 2001). Dectin-1 binds and promotes phagocytosis of particles containing β1,3-glucans (Brown and Gordon, 2001; Brown et al., 2002). Dectin-1 couples detection of β-glucans to induction of phagocytosis, since internalization, but not binding of zymosan, requires a functional ITAM motif in its intracellular tail (Herre et al., 2004). Dectin-1 presents an intracellular domain containing an hemi-ITAM motif that is phosphorylated upon clustering of Dectin-1. The hemi-ITAM present in the cytoplasmic tail of Dectin-1 recruits Syk kinase and results in its activation, an upstream event in the Dectin-1 signaling pathway (Rogers et al., 2005). Activation of Dectin-1 induces NF-κB and NFAT activation and the production of cytokines in response to purified β-glucans, by a signaling pathway requiring activation of Syk and the adaptor CARD9 (Rogers et al., 2005; Gross et al., 2006; Goodridge et al., 2007; Hara et al., 2007). As opposed to TLRs that recognize soluble ligands, Dectin-1 signaling requires its clustering by aggregates of β-glucans in order to promote Syk activation and exclusion of tyrosine phosphatases CD45 and CD48 (Goodridge et al., 2011). Dectin-1 is absolutely required for macrophage recognition of β-glucans, since macrophages obtained from Dectin-1 deficient mice are unable to produce cytokines in response to β-glucans (Saijo et al., 2007; Taylor et al., 2007a).

Dectin-1 cooperates with TLR2 in cell activation induced by zymosan, amplifying NF-κB activation and cytokine production by macrophages, and inducing generation of ROS (Brown et al., 2003; Gantner et al., 2003). Zymosan submitted to hot alkali treatment or oxidation with NaClO is unable to induce TLR2 signaling but it still promotes Dectin-1 activation, indicating that Dectin-1 is able to induce macrophage activation independently of signaling mediated by TLR2, although cooperation between these receptors is required for cytokine production (Gantner et al., 2003; Saijo et al., 2007).

Phagocytosis of *C. albicans* yeasts requires recognition of β-glucans by Dectin-1. Interestingly, β-glucans are exposed on the *C. albicans* surface during budding of yeasts, while hyphae have a low expression of β-glucans on the cell surface (Gantner et al., 2005). Dectin-1 is also involved in innate immune recognition of *A. fumigatus*. During germination, exposure of β-glucans on surfaces of *A. fumigatus* conidia promotes recognition mediated by Dectin-1 (Gersuk et al., 2006; Luther et al., 2007). The pattern of expression of β-glucans seems to influence the phagocytosis of *A. fumigatus* conidia, since resting conidia show a reduced internalization by macrophages when compared to swollen conidia that have exposed β-glucans on the surface as a consequence of the degradation of the hydrophobic layer during germination (Hohl et al., 2005; Steele et al., 2005; Gersuk et al., 2006). Phagocytosis of *A. fumigatus* resting conidia obtained from a *pksP* deficient strain is increased, in comparison to the wild-type resting conidia.

Resting conidia from *pksP* mutants are deficient in the synthesis of melanin and show higher exposure of β-glucans on their surface, thus implying exposure of β-glucans as a regulator of the phagocytosis of *A. fumigatus* conidia (Luther et al., 2007).

Dectin-1 activation also triggers generation of ROS upon recognition of fungal pathogens such as *C. albicans* yeasts, zymosan, and *A. fumigatus* germ tubes (Gantner et al., 2003, 2005; Gersuk et al., 2006). Thus, Dectin-1 integrates fungal phagocytosis and production of inflammatory mediators through recognition of β-1,3-glucans exposed on the fungal surface.

Dectin-1 (also known as CLEC7A) is involved in innate immune recognition of *C. albicans* (Gantner et al., 2005; Saijo et al., 2007; Taylor et al., 2007a), *Pneumocystis carinii* (Steele et al., 2003), and *A. fumigatus* (Hohl et al., 2005; Steele et al., 2005; Gersuk et al., 2006). However, discrepant results have been reported in experimental models of infection with *C. albicans* that demonstrated either a higher susceptibility of *Clec7a*$^{-/-}$ mice to infection or no role for Dectin-1 (Saijo et al., 2007; Taylor et al., 2007a). On the other hand, Dectin-1 is absolutely necessary for the immune response to *A. fumigatus*. *Clec7a*$^{-/-}$ mice succumb to pulmonary infection due to increased growth of *A. fumigatus* hyphae. This increased susceptibility is associated with an impairment in microbicidal activity and generation of ROS by neutrophils, as well a reduction in neutrophil recruitment to the lungs. *Clec7a*$^{-/-}$ mice present a reduced production of pro-inflammatory cytokines during the infection (Werner et al., 2009). It is interesting that *A. fumigatus*, an opportunist pathogen, causes lethal infections in *Clec7a*$^{-/-}$ mice in the absence of immunosuppressive treatments, a phenotype that is not observed in TLR2, TLR4, or MyD88 knockout mice, indicating a critical role for Dectin-1 in innate immunity to *A. fumigatus* (Dubourdeau et al., 2006; Bretz et al., 2008).

Dectin-2 (also known as CLEC4N) was initially identified by the screening of a cDNA library obtained from dendritic cells (Ariizumi et al., 2000). Dectin-2 recognizes hyphae of *C. albicans*, *Trichophyton rubrum*, and *Microsporum audouinii* (Sato et al., 2006). Although Dectin-2 lacks intracellular signaling motifs, its association with FcRγ chain, which contains ITAM motifs, leads to the activation of Syk kinase and recruitment of CARD9 (Sato et al., 2006; Saijo et al., 2010). Activation of the Dectin-2 signaling pathway results in activation of NF-κB thus promoting the induction of transcription of pro-inflammatory cytokines (Bi et al., 2010; Saijo et al., 2010).

A soluble recombinant version of Dectin-2 carbohydrate recognition domain (CRD) binds mannans and fungal surfaces by a mechanism dependent on Ca^{+2}, and blocked by mannose or fucose monosaccharides (McGreal et al., 2006). Glycan array analysis reveals that Dectin-2 presents selectivity to structures with a high content of mannosyl residues (McGreal et al., 2006). *Clec4n*$^{-/-}$ macrophages have impaired cytokine production and fail to activate Syk, MAP kinases, and NF-κB in response to *C. albicans* α-mannans or cell wall preparations. *Clec4n*$^{-/-}$ mice are susceptible to *C. albicans* infection presenting higher fungal loads in kidneys than wild-type mice. *Clec4n*$^{-/-}$ macrophages also show impaired production of cytokines in response to *C. albicans* yeasts or hyphae. Furthermore, *Clec4n*$^{-/-}$ mice show a deficient Th17 polarization whose induction with consequent production of IL17A is essential to immunity and survival during infection (Saijo et al., 2010).

The role of Dectin-2 in detection of filamentous fungi has not been investigated. Since α-mannans constitute the core of polysaccharides in many surface associated glycoconjugates from filamentous fungi, Dectin-2 could be a receptor involved in innate immune recognition of fungi like *A. fumigatus* and other mold fungi.

Mincle (also known as CLEC4E) was identified as a downstream gene of C/EBPβ, a member of the family of CCAAT/enhancer binding protein (C/EBP) of transcription factors, by screening of a subtraction library in wild-type and *Cebpb*$^{-/-}$ macrophages (Matsumoto et al., 1999). Mincle is a type II transmembrane receptor of the group of CLRs (Miyake et al., 2010). Similarly to Dectin-2, Mincle engages signaling by recruitment of FcRγ chain, which leads to NFAT activation (Yamasaki et al., 2008).

Soluble recombinant Mincle binds to *C. albicans* and *S. cerevisiae* (Bugarcic et al., 2008; Wells et al., 2008). In experimental models of *C. albicans* infection, *Clec4e*$^{-/-}$ mice present higher fungal loads in kidneys than *Clec4e*$^{+/+}$ mice. *Clec4e*$^{-/-}$ macrophages show impaired production of TNF-α in response to *C. albicans* yeasts (Wells et al., 2008). Mincle also recognizes *Malassezia* spp., a commensal yeast associated with dermatitis and infections in newborns. Mincle binds to *Malassezia* spp. and this involves a domain for recognition of mannoses. In a microarray for glyconjugates, Mincle was demonstrated to bind to α-mannosyl residues but not mannans, indicating recognition of terminal α-mannoses (Yamasaki et al., 2009). *Clec4e*$^{-/-}$ macrophages present impaired production of cytokines in response to *M. pachydermatis*. Furthermore *Clec4e*$^{-/-}$ mice show impaired production of cytokines and leukocyte recruitment during infection by *M. pachydermatis* (Yamasaki et al., 2009).

The role of Mincle in the response to filamentous fungi is unclear. Yamasaki et al. (2009) failed to observe Mincle mediated responses to *Aspergillus* spp. or *Scedosporium apiospermum* when Mincle expressing cells carrying a NFAT reporter system were stimulated with these fungi (Yamasaki et al., 2009). However, the use of Mincle overexpression systems could fail to mimic the responses of cells that naturally express it, like macrophages or dendritic cells. For example, *C. albicans* has been demonstrated to require Mincle for macrophage activation, although in the NFAT reporter system employed by Yamasaki et al. (2009) *C. albicans* strains were not able to induce NFAT activation (Wells et al., 2008). Fungal molecules responsible for Mincle activation are still unknown. Although Mincle has been demonstrated to bind α-mannosyl residues (Yamasaki et al., 2009), it remains to be established if α-mannosyl residues expressed on fungal molecules are the natural ligands of Mincle.

Dendritic cell-specific ICAM3 grabbing non-integrin is a type II transmembrane protein that contains a C-type lectin extracellular portion. DC-SIGN was initially characterized as a protein able to bind to HIV-1 glycoprotein, gp120. Subsequent studies demonstrated a role for DC-SIGN in interaction of dendritic cells and macrophages with several pathogens such as *M. tuberculosis*, Dengue virus, HIV, and Ebola virus (van Kooyk and Geijtenbeek, 2003).

Dendritic cell-specific ICAM3 grabbing non-integrin is involved in *C. albicans* recognition by mediating *C. albicans* binding to dendritic cells. Furthermore, DC-SIGN colocalizes with *C. albicans*-containing phagosomes during its internalization by

dendritic cells (Cambi et al., 2003). DC-SIGN mediates binding and internalization of *A. fumigatus* conidia in macrophages and dendritic cells. (Serrano-Gómez et al., 2004). Recognition of *C. albicans* and *A. fumigatus* by DC-SIGN is blocked by soluble mannans, suggesting that these are the ligands for DC-SIGN on fungal surfaces (Cambi et al., 2003; Serrano-Gómez et al., 2004).

The role of DC-SIGN in microbial phagocytosis is uncertain. It is not clear whether DC-SIGN mediates only pathogen binding or also signals microbial internalization. Mutations in two adjacent leucines in a conserved intracellular motif of DC-SIGN result in a preserved binding to particles containing DC-SIGN ligands, although internalization of these particles is impaired, thus indicating a role for DC-SIGN in the activation of phagocytosis (Azad et al., 2008). However, it remains to be defined whether DC-SIGN is able to promote ingestion of pathogens in professional phagocytes, such as dendritic cells and macrophages, or if it is a receptor that mediates microbial binding to phagocytes for internalization mediated by phagocytic receptors.

Chimeric DC-SIGN-Fc protein binds to mannans, α-mannosyl, and α-fucosyl residues but not to sialylated Lewis carbohydrates (Lee et al., 2011). Thus, it seems that DC-SIGN is involved in the recognition of a large spectrum of pathogens, including fungal pathogens, by means of detection of mannose/fucose containing glycoconjugates. In contrast to other PRRs, like Dectin-1 and TLRs, DC-SIGN has been described to inhibit production of pro-inflammatory cytokines, particularly IL-12p70, while promoting IL-10 production, which has been interpreted as a mechanism for immune evasion of pathogens that express DC-SIGN ligands (van Kooyk and Geijtenbeek, 2003). However it remains to be established if fungal pathogens target DC-SIGN in order to evade immune responses during infections.

Mannose receptor is a type I transmembrane protein containing an extracellular cysteine rich domain, a fibronectin type II repeat, eight C-type lectin domains, and a stalk portion connecting the extracellular domain to a short intracellular region. MR recognizes mannose, fucose, or *N*-acetylglucosamine residues present in glycoconjugates (Ezekowitz et al., 1990; Lee et al., 2011). MR is a receptor for binding and internalization of several pathogens, including *M. tuberculosis*, *Leishmania* spp., and fungi such as *C. albicans* and *P. carinii* (Kerrigan and Brown, 2009).

Mannose receptor was the first phagocytic receptor described that was able to mediate fungal internalization through recognition of fungal derived molecules, in the absence of opsonins (Ezekowitz et al., 1990). MR expression confers phagocytic capacity to *P. carinii*, yeasts and zymosan in non-phagocytic cells, and the ingestion of these particles is inhibited by soluble mannans (Ezekowitz et al., 1990). The role of MR in phagocytosis is not only due to particle binding, but involves signaling by its intracellular portion, since chimeric proteins containing the extracellular portion of FcγRI and transmembrane and intracellular regions of MR are able to promote internalization of IgG-opsonized erythrocytes, thus indicating that intracellular motifs of MR are able to couple detection of particles to activation of phagocytic machinery (Kruskal et al., 1992). MR has been described as a receptor mediating internalization of *C. neoformans* and *P. carinii* by dendritic cells and macrophages (Ezekowitz et al., 1990; Syme et al., 2002; Zhang et al., 2005).

Cytokine production by macrophages in response to *C. albicans* N-linked mannans is mediated by MR (Netea et al., 2006). Internalization and activation of dendritic cells by *C. albicans* requires recognitions of N-linked mannans, by mechanisms involving recognition by DC-SIGN and MR (Cambi et al., 2008). *C. neoformans* mannoproteins promote dendritic cell activation thorough MR detection (Tachado et al., 2007). MR mediates internalization of *P. carinii* and IL-8/CXCL8 release by alveolar macrophages in cooperation with TLR2 signaling, during *P. carinii* recognition (Tachado et al., 2007). Although MR has been identified as a receptor involved in the recognition of fungal pathogens, experimental models of infection do not show increased susceptibility to *C. albicans* or *P. carinii* infection in *Mrc*1$^{-/-}$ mice. Still, *Mrc*1$^{-/-}$ mice show increased leukocyte recruitment and pulmonary pathology during infection by *P. carinii* (Lee et al., 2003; Swain et al., 2003). *C. neoformans* infection results in increased mortality and higher fungal loads in *Mrc*1$^{-/-}$ mice (Dan et al., 2008). In summary, MR seems to be dispensable for immunity to some fungal pathogens, possibly due to the recognition of mannans by other PRRs, like Dectin-2 and TLR4 (Netea et al., 2006; Saijo et al., 2010). The role of MR in recognition of mold fungi is unknown, but since mannans are present on the external layer of these fungi, MR must be involved in immune recognition of these pathogens.

The growing knowledge on CLR biology has made clear the involvement of these receptors in fungal recognition. The ability of Dectin-1, Dectin-2, Mincle, DC-SIGN, and MR to recognize carbohydrates makes these proteins important sensors for fungi since these are the major components of fungal surfaces. CLRs integrate fungal detection to cellular events like phagocytosis, microbicidal activity, and cytokine induction. In addition, they also cooperate with other classes of PRRs such as TLRs. Thus, a better understanding of the role of CLRs in the recognition of fungal pathogens should provide an important contribution to the comprehension of the immunopathogenesis of fungal infections.

ACTIVATION OF CYTOSOLIC NOD-LIKE RECEPTORS BY FUNGAL INFECTION

Soon after the discovery of the TLRs, it became clear that, despite their key role in microbial detection, TLRs could not account for full host protection. The apparent inability of TLRs to sense intracellular pathogens led to the concept that other PRRs could be involved in cytosolic microbial detection. Bioinformatic analyses have identified the existence of more than 20 Nod-like receptors (NLR) in the human genome. Due to the lack of signal peptides and transmembrane domains, NLRs are thought to localize in the cytosol, where they would continuously screen this compartment for the presence of microbes. A typical NLR protein displays (i) a C-terminal leucine-rich repeat (LRR) domain that is possibly involved in recognition of conserved microbial patterns or other ligands; (ii) a centrally located NACHT domain that mediates self-oligomerization and is essential for activation of the NLRs, and (iii) a N-terminal effector domain, which is responsible for protein–protein interaction with adapter molecules that result in signal transduction. Based on the nature of their N-terminal domain, the NLRs have been divided into subfamilies: the Nods (Nod1 and Nod2) and NLRC4 possess a caspase recruitment domain (CARD), the NLRPs (NLRP1–14) display a

pyrin domain (PYD), and Naip presents a baculovirus inhibitor of apoptosis protein repeat domain (BIR). Like the TLRs, the NLRs sense characteristic microbial products and possibly "danger signals" but to date only few have known ligands (Carneiro et al., 2008).

Nod1 and Nod2 have been characterized as bacterial peptidoglycan sensors. A putative role for Nod2 in response to *A. fumigatus* conidia was suggested based on the observations that, in a mouse model of intranasal inoculation of *A. fumigatus* conidia, Nod2 protein expression was upregulated in the lungs and that stimulation with muramyl dipeptide (MDP) increased the secretion of TNF-α induced by *A. fumigatus* conidia (Zhang et al., 2008). On the other hand, no role for Nod2 was observed in *Candida* infection – neither did the prevalence of Nod2 polymorphisms increase in *Candida* infections nor was the pattern of cytokines produced in response to *Candida* infection altered in individuals with Nod2 polymorphisms (Van der Graaf et al., 2006).

The NLRs involved in inflammasome assembly, on the other hand, have been implicated in the innate immune response to a variety of fungi, including *C. albicans*, *A. fumigatus*, and *S. cerevisiae*. By definition, inflammasomes are multi-protein complexes comprising the intracellular adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD, also known as TMS1) and a "sensor NLR" that function as molecular scaffolds for the activation of caspase-1. Several inflammasomes have been characterized and each one is named upon the unique NLR protein that forms the molecular platform (NLRP1 inflammasome, NLRP3 inflammasome, and the NLRC4 inflammasome; Davis et al., 2011). In particular, the NLRP3 inflammasome has been proposed to play an important role in antifungal host defense.

It has been shown that large particulate β1,3-glucans, including curdlan, glucan from baker's yeast, paramylon, and zymosan, activate the transcription of pro-IL-1β through a Dectin-1-dependent pathway in human macrophages (Kankkunen et al., 2010). The Dectin-1/Syk pathway is essential for β-glucan-induced IL-1β mRNA expression (Kankkunen et al., 2010). Additionally, β-glucans activate the NLRP3 inflammasome resulting in IL-1β processing and secretion. Interestingly, β-glucan appears to be able to provide the double-hit necessary for IL-1β secretion, i.e., induce transcription of pro-IL-1β and promote IL-1β processing and secretion by inducing inflammasome formation. This unique ability seems to be related to its capacity to trigger the Dectin-1/Syk pathway as well as inducing ROS formation and potassium efflux (Kankkunen et al., 2010).

The first reports to demonstrate a role for the inflammasomes during fungal infection described the stimulation of the NLRP3 inflammasome in the innate immune response to *C. albicans*. Infection of macrophages and dendritic cells with *C. albicans* results in activation of the NLRP3 inflammasome with the resultant activation of caspase-1 and processing and secretion of IL-1β. The known fungal PRRs TLR2 and Dectin-1 regulate IL-1β gene transcription, whereas the NLRP3 inflammasome controls caspase-1 mediated cleavage of pro-IL-1β. Together, these three PRRs are essential for defense against dissemination of mucosal infection and mortality *in vivo* (Gross et al., 2009; Hise et al., 2009).

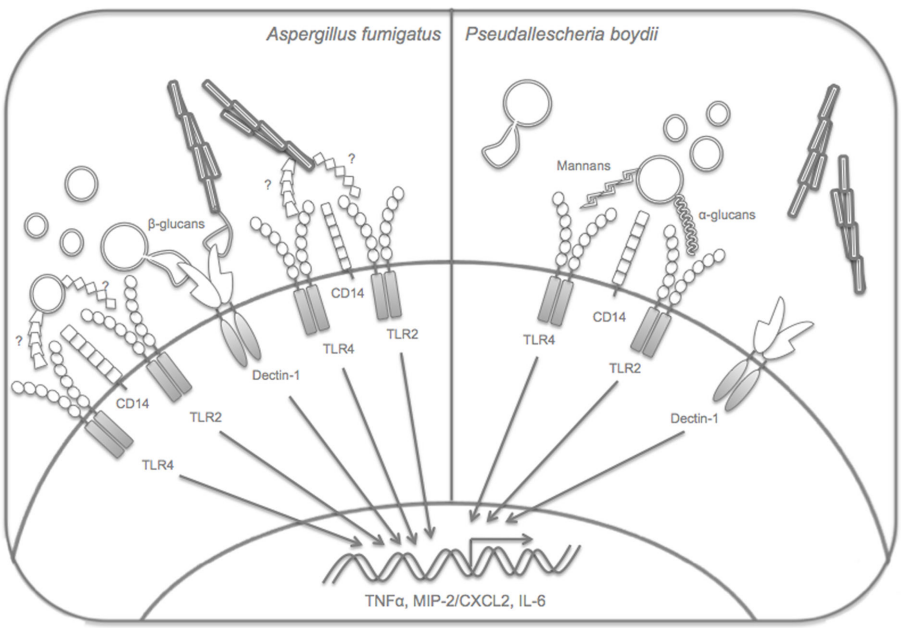


FIGURE 1 | TLR2, TLR4, Dectin-1, and CD14 are involved in innate immune recognition of *A. fumigatus* developmental stages. *A. fumigatus* resting conidia do not induce cytokine production by macrophages and dendritic cells, but during the process of germination, swollen conidia, germ tubes, and hyphae expose β-glucans, agonists of Dectin-1, and uncharacterized ligands for TLR2, TLR4 which induce signaling pathways that culminate with cytokine release by innate immune cells. Recognition of *P. boydii* involves recognition of α-glucans and rhamnomannans by TLR2 and TLR4, respectively. *P. boydii* conidia require CD14 and TLR4 activation for the induction of macrophage activation, while activation of TLR2 or TLR4 is dispensable for macrophage recognition by *P. boydii* hyphae. The role of Dectin-1 in *P. boydii* recognition is still unknown.

As mentioned above, Syk, operating downstream of the Dectin-1/CARD9 axis controls transcription of several cytokine genes, upon fungal recognition, including pro-IL-1β. Interestingly, inhibition of Syk not only results in inhibition of pro-IL-1β transcription, as expected, but also affects the NLRP3 inflammasome activation after cell stimulation with *C. albicans*. These results suggest that the ability of *C. albicans* to induce both pro-IL-1β transcription and secretion rely on the activity of Syk. Whereas Syk signaling for pro-IL-1β transcription selectively depends on CARD9, the activation of the NLRP3 inflammasome involves production of ROS and potassium efflux. These two events are a common theme to many NLRP3 activators but the inhibition of Syk selectively abrogates inflammasome activation by *C. albicans* and Dectin-1 and not other known NLRP3 activators (Gross et al., 2009; Kumar et al., 2009; Kankkunen et al., 2010).

In addition to its role as a regulator of inflammation and innate immunity, IL-1β also exerts its effects by controlling Th1 and Th17 adaptive responses. A recent study demonstrated a crucial role of the inflammasome components ASC and caspase-1 in regulating antifungal Th1/Th17 protective responses and preventing increased fungal overgrowth and disseminated candidiasis. The increased susceptibility of ASC- and caspase-1-deficient mice to *C. albicans* infection is in line with the reports mentioned above reporting increased susceptibility to candidiasis of NLRP3- and IL-1β-deficient mice (Van de Veerdonk et al., 2011).The relevance of the NLRP3 inflammasome in response to *C. albicans* infection is illustrated by experiments showing hyper-susceptibility of NLRP3-deficient mice. NLRP3-deficient mice show diminished concentrations of IL-1β in the sera, reduced survival, and higher fungal burdens in kidney, spleen, liver, and lung following infection (Gross et al., 2009; Hise et al., 2009; Van de Veerdonk et al., 2011). In a mucosal model of *C. albicans* challenge, inflammasome-deficient mice show susceptibility to infection with higher fungal loads and lethality, demonstrating that NLRP3, ASC, and caspase-1 mediate *C. albicans*-induced IL-1β responses and antifungal defense *in vivo* (Hise et al., 2009).

Aspergillus fumigatus induces secretion of IL-1β by the human monocyte/macrophage cell line THP1 (Saïd-Sadler et al., 2010). Of note, the morphological stage of these organisms seems to be critical for their ability to induce inflammasome activation providing further evidence for a differential regulation of immune responses based on the morphological forms of fungi (Netea et al., 2003). *A. fumigatus* hyphae, but not conidia, induce inflammasome activation. As described for β-glucans and *C. albicans*, the ability of *A. fumigatus* hyphae to activate the NLRP3 inflammasome in a human monocyte cell line is dependent on potassium efflux, ROS generation, and Syk mediated signaling pathways (Saïd-Sadler et al., 2010). Thus, NLRs and inflammasomes have been shown to be important modulators of the innate immune response to a number of medically relevant fungi.

CONCLUDING REMARKS

Innate immune recognition of filamentous fungi is still poorly understood. Even in the case of more studied pathogens such as *A. fumigatus*, the molecules involved in the triggering of PRRs are largely unknown. **Figure 1** outlines the molecules and receptors that have been implicated in recognition of the filamentous fungi *A. fumigatus* and *P. boydii* by macrophages. **Table 1** lists the PRRs that have been described in the recognition of fungal molecules.

Comprehension of innate immune recognition of fungal molecules is relevant to the understanding of the pathogenesis of fungal infections. Characterization of the fungal molecules responsible for the activation of PRRs and their roles in fungal recognition could uncover new possibilities to immunotherapy in fungal infections, and help establish effectors mechanisms in immunosuppressed individuals. Alternatively, these molecules could also be used as antigens or adjuvants to induce prophylactic immune responses to fungal pathogens (Xin et al., 2008). On the other hand, activation of innate immune receptors by fungal molecules could be involved in immunopathogenesis by promoting increased inflammation and tissue injury. Thus modulation of innate immune receptor activation during fungal infections could

Table 1 | Pattern recognition receptors involved in detection of fungal molecules.

Receptor	Fungal molecules	Fungal species
TLR4	O-linked mannans	*C. albicans*
	Mannans	*Saccharomyces* spp.
	Rhamnomannans	*P. boydii*
	Phospholipomannans	*C. albicans*
	?	*A. fumigatus*
TLR2/TLR6	Phospholipomannans	*C. albicans*
	Glucuronoxylomannans	*C. neoformans*
TLR2/TLR1	Glucuronoxylomannans	*C. neoformans*
TLR2	α1,4-glucans	*P. boydii*
	?	*A. fumigatus*
MR	N-linked mannans	*C. albicans*
	Mannans	*P. carinii*
	Mannoproteins	*C. neoformans*
DC-SIGN	Galactomannans	*A. fumigatus*
	Mannans	*C. albicans*
Dectin-1	β1,3-glucans	*A. fumigatus*
		C. albicans
		Saccharomyces spp.
Dectin-2	α-mannans	*C. albicans*
Mincle	Polysaccharides containing	*C. albicans*
	α-mannosyl residues?	*Malassezia* spp.
CD14	Mannans	*Saccharomyces* spp.
	?	*A. fumigatus*
	α1,4-glucans	*P. boydii*
NLRP3	β1,3-glucans	*C. albicans*
inflammasome		*S. cerevisiae*
		A. fumigatus

The table summarizes PRRs that have been identified in recognition of fungal molecules, but in many cases the molecules responsible for activation are unknown. Activation of the NLRP3 inflammasome by β-glucans and C. albicans is dependent on Dectin-1. In this sense NLRP3 does not recognize fungal molecules directly but instead it senses intracellular signals generated by activation of PRRs, like Dectin-1. The question mark indicates that the fungal molecules recognized by the indicated receptor are unknown.

be useful in order to increase the immune response or in some cases prevent immunopathology.

ACKNOWLEDGMENTS

Rodrigo T. Figueiredo was supported by grants of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ). Marcelo T. Bozza was supported by grants of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ), PRONEX. Letícia A. M. Carneiro was supported by Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ) and by the Antonio Luis Vianna Program of the PR2-UFRJ.

REFERENCES

Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., Clavaud, C., Paris, S., Brakhage, A. A., Kaveri, S. V., Romani, L., and Latgé, J. P. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124, 783–801.

Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413, 732–738.

Ariizumi, K., Shen, G. L., Shikano, S., Ritter, R. III, Zukas, P., Edelbaum, D., Morita, A., and Takashima, A. (2000). Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J. Biol. Chem.* 275, 11957–11963.

Azad, A. K., Torrelles, J. B., and Schlesinger, L. S. (2008). Mutation in the DC-SIGN cytoplasmic triacidic cluster motif markedly attenuates receptor activity for phagocytosis and endocytosis of mannose-containing ligands by human myeloid cells. *J. Leukoc. Biol.* 84, 1594–1603.

Barbosa, F. M., Fonseca, F. L., Figueiredo, R. T., Bozza, M. T., Casadevall, A., Nimrichter, L., and Rodrigues, M. L. (2007). Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production. *Clin. Vaccine Immunol.* 14, 94–98.

Bellocchio, S., Montagnoli, C., Bozza, S., Gaziano, R., Rossi, G., Mambula, S. S., Vecchi, A., Mantovani, A., Levitz, S. M., and Romani, L. (2004). The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J. Immunol.* 172, 3059–3069.

Bi, L., Gojestani, S., Wu, W., Hsu, Y. M., Zhu, J., Ariizumi, K., and Lin, X. (2010). CARD9 mediates dectin-2-induced IkappaBalpha kinase ubiquitination leading to activation of NF-kappaB in response to stimulation by the hyphal form of *Candida albicans*. *J. Biol. Chem.* 285, 25969–25977.

Bittencourt, V. C., Figueiredo, R. T., da Silva, R. B., Mourão-Sá, D. S., Fernandez, P. L., Sassaki, G. L., Mulloy, B., Bozza, M. T., and Barreto-Bergter, E. (2006). An alpha-glucan of *Pseudallescheria boydii* is involved in fungal phagocytosis and Toll-like receptor activation. *J. Biol. Chem.* 281, 22614–22623.

Bochud, P. Y., Chien, J. W., Marr, K. A., Leisenring, W. M., Upton, A., Janer, M., Rodrigues, S. D., Li, S., Hansen, J. A., Zhao, L. P., Aderem, A., and Boeckh, M. (2008). Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. *N. Engl. J. Med.* 359, 1766–1777.

Bretz, C., Gersuk, G., Knoblaugh, S., Chaudhary, N., Randolph-Habecker, J., Hackman, R. C., Staab, J., and Marr, K. A. (2008). MyD88 signaling contributes to early pulmonary responses to *Aspergillus fumigatus*. *Infect. Immun.* 76, 952–958.

Brown, G. D., and Gordon, S. (2001). A new receptor for β-glucans. *Nature* 413, 36–37.

Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S., Gordon, S. (2003). Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119–1124.

Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y., Gordon, S. (2002) Dectin-1 is a major beta-glucan receptor on macrophages. *J. Exp. Med.* 196, 407–412.

Brunn, G. J., Bungum, M. K., Johnson, G. B., and Platt, J. L. (2005). Conditional signaling by Toll-like receptor 4. *FASEB J.* 19, 872–874.

Bugarcic, A., Hitchens, K., Beckhouse, A. G., Wells, C. A., Ashman, R. B., and Blanchard, H. (2008). Human and mouse macrophage-inducible C-type lectin (mincle) bind *Candida albicans*. *Glycobiology* 18, 679–685.

Cambi, A., Gijzen, K., de Vries, J. M., Torensma, R., Joosten, B., Adema, G. J., Netea, M. G., Kullberg, B. J., Romani, L., and Figdor, C. G. (2003). The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur. J. Immunol.* 33, 532–538.

Cambi, A., Netea, M. G., Mora-Montes, H. M., Gow, N. A., Hato, S. V., Lowman, D. W., Kullberg, B. J., Torensma, R., Williams, D. L., and Figdor, C. G. (2008). Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. *J. Biol. Chem.* 283, 20590–20599.

Camilos, G., Luna, M., Lewism, R. E., Bodey, G. P., Chemaly, R., Tarrand, J. J., Safdar, A., Raad, I. I., and Kontoyiannis, D. P. (2006). Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003). *Haematologica* 91, 986–989.

Campos, M. A., Almeida, I. C., Takeuchi, O., Akira, S., Valente, E. P., Procópio, D. O., Travassos, L. R., Smith, J. A., Golenbock, D. T., and Gazzinelli, R. T. (2001). Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J. Immunol.* 167, 416–423.

Carneiro, L. A., Magalhaes, J. G., Tattoli, I., Philpott, D. J., and Travassos, L. H. (2008). Nod-like proteins in inflammation and disease. *J. Pathol.* 214, 136–148.

Chaffin, W. L., López-Ribot, J. L., Casanova, M., Gozalbo, D., and Martínez, J. P. (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol. Mol. Biol. Rev.* 62, 130–180.

Costachel, C., Coddeville, B., Latgé, J. P., and Fontaine, T. (2005). Glycosylphosphatidylinositol-anchored fungal polysaccharide in *Aspergillus fumigatus*. *J. Biol. Chem.* 280, 39835–39842.

Dan, J. M., Kelly, R. M., Lee, C. K., and Levitz, S. M. (2008). Role of the mannose receptor in a murine model of Cryptococcus neoformans infection. *Infec. Immun.* 76, 2362–2367.

Davis, B. K., Wen, H., and Ting, J. P. (2011). The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu. Rev. Immunol.* 29, 707–735.

Debierre-Grockiego, F., Campos, M. A., Azzouz, N., Schmidt, J., Bieker, U., Resende, M. G., Mansur, D. S., Weingart, R., Schmidt, R. R., Golenbock, D. T., Gazzinelli, R. T., and Schwarz, R. T. J. (2007). Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from *Toxoplasma gondii*. *J. Immunol.* 179, 1129–1137.

Delfino, D., Cianci, L., Lupis, E., Celeste, A., Petrelli, M. L., Curró, F., Cusumano, V., and Teti, G. (1997). Interleukin-6 production by human monocytes stimulated with *Cryptococcus neoformans* components. *Infect. Immun.* 65, 2454–2456.

Di Carlo, F. J., and Fiore, J. V. (1958). On the composition of zymosan. *Science* 127, 756–757.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303, 1529–1531.

Dubourdeau, M., Athman, R., Balloy, V., Huerre, M., Chignard, M., Philpott, D. J., Latgé, J. P., and Ibrahim-Granet, O. (2006). *Aspergillus fumigatus* induces innate immune responses in alveolar macrophages through the MAPK pathway independently of TLR2 and TLR4. *J. Immunol.* 177, 3994–4001.

Enoch, D. A., Ludlam, H. A., and Brown, N. M. (2006). Invasive fungal infections: a review of epidemiology and management options. *J. Med. Microbiol.* 55, 809–818.

Ezekowitz, R. A., Sastry, K., Bailly, P., Warner, A. (1990) Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. *J. Exp. Med.* 172, 1785–1794.

Figueiredo, R. T., Fernandez, P. L., Dutra, F. F., González, Y., Lopes, L. C., Bittencourt, V. C., Sassaki, G. L., Barreto-Bergter, E., and Bozza, M. T. (2010). TLR4 recognizes *Pseudallescheria boydii* conidia and purified rhamnomannans. *J. Biol. Chem.* 285, 40714–40723.

Figueiredo, R. T., Fernandez, P. L., Mourão-Sá, D. S., Porto, B. N., Dutra, F. F., Alves, L. S., Oliveira, M. F., Oliveira, P. L., Graça-Souza, A. V., and Bozza, M. T. (2007). Characterization of heme as activator of Toll-like receptor 4. *J. Biol. Chem.* 282, 20221–20229.

Fonseca, F. L., Nohara, L. L., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., and Rodrigues, M. L. (2010). Immunomodulatory effects of serotype B glucuronoxylomannan from *Cryptococcus gattii* correlate with polysaccharide diameter. *Infect. Immun.* 78, 3861–3870.

Fontaine, T., Magnin, T., Melhert, A., Lamont, D., Latgé, J. P., and Ferguson, M. A. (2003). Structures of the glycosylphosphatidylinositol membrane anchors from *Aspergillus fumigatus* membrane proteins. *Glycobiology* 13, 169–177.

Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., Vorgias, C. E., Diaquin, M., and Latgé, J. P. (2000). Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *J. Biol. Chem.* 275, 41528.

Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 107–117.

Gantner, B. N., Simmons, R. M., and Underhill, D. M. (2005). Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. *EMBO J.* 24, 1277–1286.

Gersuk, G. M., Underhill, D. M., Zhu, L., and Marr, K. A. (2006). Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. *J. Immunol.* 176, 3717–3724.

Goodridge, H. S., Reyes, C. N., Becker, C. A., Katsumoto, T. R., Ma, J., Wolf, A. J., Bose, N., Chan, A. S., Magee, A. S., Danielson, M. E., Weiss, A., Vasilakos, J. P., and Underhill, D. M. (2011). Activation of the innate immune receptor dectin-1 upon formation of a phagocytic synapse. *Nature* 472, 471–475.

Goodridge, H. S., Simmons, R. M., and Underhill, D. M. (2007). Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J. Immunol.* 178, 3107–3115.

Groll, A. H., and Walsh, T. J. (2001). Uncommon opportunistic fungi: new nosocomial threats. *Clin. Microbiol. Infect.* 7, 8–24.

Gross, O., Gewies, A., Finger, K., Schäfer, M., Sparwasser, T., Peschel, C., Förster, I., and Ruland, J. (2006). Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442, 651–656.

Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschläger, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V., Mocsai, A., Tschopp, J., and Ruland, J. (2009). Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459, 433–437.

Hara, H., Ishihara, C., Takeuchi, A., Imanishi, T., Xue, L., Morris, S. W., Inui, M., Takai, T., Shibuya, A., Saijo, S., Iwakura, Y., Ohno, N., Koseki, H., Yoshida, H., Penninger, J. M., and Saito, T. (2007). The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat. Immunol.* 8, 619–629.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099–1103.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 303, 1526–1529.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3, 196–200.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745.

Herre, J., Marshall, A. S., Caron, E., Edwards, A. D., Williams, D. L., Schweighoffer, E., Tybulewicz, V., Reis e Sousa, C., Gordon, S., and Brown, G. D. (2004). Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104, 4038–4045.

Hise, A. G., Tomalka, J., Ganesan, S., Patel, K., Hall, B. A., Brown, G. D., and Fitzgerald, K. A. (2009). An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. *Cell Host Microbe* 5, 487–497.

Hohl, T. M., Van Epps, H. L., Rivera, A., Morgan, L. A., Chen, P. L., Feldmesser, M., and Pamer, E. G. (2005). *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog.* 1, e30. doi:10.1371/journal.ppat.0010030

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162, 3749–3752.

Hsu, T. L., Cheng, S. C., Yang, W. B., Chin, S. W., Chen, B. H., Huang, M. T., Hsieh, S. L., and Wong, C. H. (2009). Profiling carbohydrate-receptor interaction with recombinant innate immunity receptor-Fc fusion proteins. *J. Biol. Chem.* 284, 34479–34489.

Jin, M. S., Kim, S. E., Heo, J. Y., Lee, M. E., Kim, H. M., Paik, S. G., Lee, H., and Lee, J. O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 130, 1071–1082.

Jouault, T., Ibata-Ombetta, S., Takeuchi, O., Trinel, P. A., Sacchetti, P., Lefebvre, P., Akira, S., and Poulain, D. (2003). *Candida albicans* phospholipomannan is sensed through Toll-like receptors. *J. Infect. Dis.* 188, 165–172.

Kang, J. Y., Nan, X., Jin, M. S., Youn, S. J., Ryu, Y. H., Mah, S., Han, S. H., Lee, H., Paik, S. G., and Lee, J. O. (2009). Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* 31, 873–884.

Kankkunen, P., Teirilä, L., Rintahaka, J., Alenius, H., Wolff, H., and Matikainen, S. (2010). (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J. Immunol.* 184, 6335–6342.

Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34, 637–650.

Kerrigan, A. M., and Brown, G. D. (2009). C-type lectins and phagocytosis. *Immunobiology* 214, 562–575.

Kerrigan, A. M., and Brown, G. D. (2010). Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. *Immunol. Rev.* 234, 335–352.

Kesh, S., Mensah, N. Y., Peterlongo, P., Jaffe, D., Hsu, K., Van Den Brink, M., O'reilly, R., Pamer, E., Satagopan, J., and Papanicolaou, G. A. (2005). TLR1 and TLR6 polymorphisms are associated with susceptibility to invasive aspergillosis after allogeneic stem cell transplantation. *Ann. N. Y. Acad. Sci.* 1062, 95–103.

Krishnegowda, G., Hajjar, A. M., Zhu, J., Douglass, E. J., Uematsu, S., Akira, S., Woods, A. S., and Gowda, D. C. (2005). Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J. Biol. Chem.* 280, 8606–8016.

Kruskal, B. A., Sastry, K., Warner, A. B., Mathieu, C. E., and Ezekowitz, R. A. (1992). Phagocytic chimeric receptors require both transmembrane and cytoplasmic domains from the mannose receptor. *J. Exp. Med.* 176, 1673–1680.

Kumar, H., Kumagai, Y., Tsuchida, T., Koenig, P. A., Satoh, T., Guo, Z., Jang, M. H., Saitoh, T., Akira, S., and Kawai, T. (2009). Involvement of the NLRP3 inflammasome in innate and humoral adaptive immune responses to fungal β-glucan. *J. Immunol.* 183, 8061–8067.

Latgé, J. (2001). The pathobiology of *Aspergillus fumigatus*. *Trends Microbiol.* 9, 382–389.

Latgé, J. (2010). Tasting the fungal cell wall. *Cell. Microbiol.* 12, 863–872.

Lee, R. T., Hsu, T. L., Huang, S. K., Hsieh, S. L., Wong, C. H., and Lee, Y. C. (2011). Survey of immune-related, mannose/fucose-binding C-type lectin receptors reveals widely divergent sugar-binding specificities. *Glycobiology* 21, 512–520.

Lee, S. J., Zheng, N. Y., Clavijo, M., and Nussenzweig, M. C. (2003). Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infect. Immun.* 71, 437–445.

Leitão, E. A., Bittencourt, V. C., Haido, R. M., Valente, A. P., Peter-Katalinic, J., Letzel, M., de Souza, L. M., and Barreto-Bergter, E. (2003). Beta-galactofuranose-containing O-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* contain immunodominant epitopes. *Glycobiology* 13, 681–692.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86, 973–983.

Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A., and Flavell, R. A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. U.S.A.* 101, 5598–5603.

Luther, K., Torosantucci, A., Brakhage, A. A., Heesemann, J., and Ebel, F. (2007). Phagocytosis of *Aspergillus fumigatus* conidia by murine macrophages involves recognition by the dectin-1 beta-glucan receptor and Toll-like receptor 2. *Cell. Microbiol.* 9, 368–381.

Mambula, S. S., Sau, K., Henneke, P., Golenbock, D. T., and Levitz, S. M. (2002). Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus. J. Biol. Chem.* 277, 39320–39326.

Marr, K. A., Carter, R. A., Crippa, F., Wald, A., and Corey, L. (2002). Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. *Clin. Infect. Dis.* 34, 909–917.

Masuoka, J. (2004). Surface glycans of *Candida albicans* and other pathogenic fungi: physiological roles, clinical uses, and experimental changes. *Clin. Microbiol. Rev.* 17, 281–310.

Matsumoto, M., Tanaka, T., Kaisho, T., Sanjo, H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Akira, S. (1999). A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. *J. Immunol.* 163, 5039–5048.

McGreal, E. P., Rosas, M., Brown, G. D., Zamze, S., Wong, S. Y., Gordon, S., Martinez-Pomares, L., and Taylor, P. R. (2006). The carbohydrate-recognition domain of dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology* 16, 422–430.

Means, T. K., Lien, E., Yoshimura, A., Wang, S., Golenbock, D. T., and Fenton, M. J. (1999). The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J. Immunol.* 163, 6748–6755.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature* 449, 819–826.

Meersseman, W., Lagrou, K., Maertens, J., Wilmer, A., Hermans, G., Vanderschueren, S., Spriet, I., Verbeken, E., and Van Wijngaerden, E. (2008) Galactomannan in bronchoalveolar lavage fluid: a tool for diagnosing aspergillosis in intensive care unit patients. *Am. J. Respir. Crit. Care Med.* 177, 27–34.

Meier, A., Kirschning, C. J., Nikolaus, T., Wagner, H., Heesemann, J., and Ebel, F. (2003). Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. *Cell. Microbiol.* 5, 561–570.

Miyake, Y., Ishikawa, E., Ishikawa, T., and Yamasaki, S. (2010). Self and nonself recognition through C-type lectin receptor, mincle. *Self Nonself* 1, 310–313.

Monari, C., Bistoni, F., Casadevall, A., Pericolini, E., Pietrella, D., Kozel, T. R., and Vecchiarelli, A. (2005). Glucuronoxylomannan, a microbial compound, regulates expression of costimulatory molecules and production of cytokines in macrophages. *J. Infect. Dis.* 191, 127–137.

Morgan, J., Wannemuehler, K. A., Marr, K. A., Hadley, S., Kontoyiannis, D. P., Walsh, T. J., Fridkin, S. K., Pappas, P. G., and Warnock, D. W. (2005) Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. *Med. Mycol.* 43(Suppl. 1), S49–S58.

Murata, T., Sullivan, J. A., Sawyer, D. W., and Mandell, G. L. (1987). Influence of type and opsonization of ingested particle on intracellular free calcium distribution and superoxide production by human neutrophils. *Infect. Immun.* 55, 1784–1791.

Murray, C. K., Loo, F. L., Hospenthal, D. R., Cancio, L. C., Jones, J. A., Kim, S. H., Holcomb, J. B., Wade, C. E., and Wolf, S. E. (2008). Incidence of systemic fungal infection and related mortality following severe burns. *Burns* 34, 1108–1112.

Nathan, C. (2002). Points of control in inflammation. *Nature* 420, 846–852.

Netea, M. G., Gow, N. A., Munro, C. A., Bates, S., Collins, C., Ferwerda, G., Hobson, R. P., Bertram, G., Hughes, H. B., Jansen, T., Jacobs, L., Buurman, E. T., Gijzen, K., Williams, D. L., Torensma, R., McKinnon, A., MacCallum, D. M., Odds, F. C., Van der Meer, J. W., Brown, A. J., and Kullberg, B. J. (2006). Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* 116, 1642–1650.

Netea, M. G., Van Der Graaf, C. A., Vonk, A. G., Verschueren, I., Van Der Meer, J. W., and Kullberg, B. J. (2002). The role of Toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J. Infect. Dis.* 185, 1483–1489.

Netea, M. G., Warris, A., Van der Meer, J. W., Fenton, M. J., Verver-Janssen, T. J., Jacobs, L. E., Andresen, T., Verweij, P. E., and Kullberg, B. J. (2003). *Aspergillus fumigatus* evades immune recognition during germination through loss of Toll-like receptor-4-mediated signal transduction. *J. Infect. Dis.* 188, 320–326.

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13766–13771.

Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., and Lee, J. O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458, 1191–1195.

Pasqualotto, A. C., and Denning, D. W. (2006). Post-operative aspergillosis. *Clin. Microbiol. Infect.* 12, 1060–1076.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085–2088.

Qureshi, S. T., Larivière, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.* 189, 615–625.

Retini, C., Vecchiarelli, A., Monari, C., Tascini, C., Bistoni, F., and Kozel, T. R. (1996). Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. *Infect. Immun.* 64, 2897–2903.

Rodrigues, M. L., Nimrichter, L., Cordero, R. J., and Casadevall, A. (2011). Fungal polysaccharides: biological activity beyond the usual structural properties. *Front. Microbiol.* 2:171. doi:10.3389/fmicb.2011.00171

Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., Williams, D. L., Gordon, S., Tybulewicz, V. L., Brown, G. D., and Reis e Sousa, C. (2005). Syk-dependent cytokine induction by dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22, 507–517.

Saïd-Sadler, N., Padilla, E., Langsley, G., and Ojcius, D. M. (2010). *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS ONE* 5, e10008. doi:10.1371/journal.pone.0010008

Saijo, S., Fujikado, N., Furuta, T., Chung, S. H., Kotaki, H., Seki, K., Sudo, K., Akira, S., Adachi, Y., Ohno, N., Kinjo, T., Nakamura, K., Kawakami, K., and Iwakura, Y. (2007). Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans. Nat. Immunol.* 8, 39–46.

Saijo, S., Ikeda, S., Yamabe, K., Kakuta, S., Ishigame, H., Akitsu, A., Fujikado, N., Kusaka, T., Kubo, S., Chung, S. H., Komatsu, R., Miura, N., Adachi, Y., Ohno, N., Shibuya, K., Yamamoto, N., Kawakami, K., Yamasaki, S., Saito, T., Akira, S., and Iwakura, Y. (2010). Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans. Immunity* 32, 681–691.

Sandor, F., Latz, E., Re, F., Mandell, L., Repik, G., Golenbock, D. T., Espevik, T., Kurt-Jones, E. A., and Finberg, R. W. (2003). Importance of extra- and intracellular domains of TLR1 and TLR2 in NFkappa B signaling. *J. Cell Biol.* 162, 1099–1110.

Sato, K., Yang, X. L., Yudate, T., Chung, J. S., Wu, J., Luby-Phelps, K., Kimberly, R. P., Underhill, D., Cruz, P. D. Jr., and Ariizumi, K. (2006). Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J. Biol. Chem.* 281, 38854–38866.

Schröder, N. W., Heine, H., Alexander, C., Manukyan, M., Eckert, J., Hamann, L., Göbel, U. B., and Schumann, R. R. (2004). Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. *J. Immunol.* 173, 2683–2691.

Schröder, N. W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U. B., Weber, J. R., and Schumann, R. R. (2003) Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* 278, 15587–15594.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999). Peptidoglycan, and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* 274, 17406–17409.

Segal, B. H., and Walsh, T. J. (2006). Current approaches to diagnosis and treatment of invasive aspergillosis. *Am. J. Respir. Crit. Care Med.* 173, 707–717.

Serrano-Gómez, D., Domínguez-Soto, A., Ancochea, J., Jimenez-Heffernan, J. A., Leal, J. A., and Corbí, A. L. (2004). Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin mediates binding and internalization of *Aspergillus fumigatus* conidia by dendritic cells and macrophages. *J. Immunol.* 173, 5635–5643.

Shoham, S., Huang, C., Chen, J. M., Golenbock, D. T., and Levitz, S. M. (2001). Toll-like receptor 4 mediates intracellular signaling without TNF-alpha release in response to *Cryptococcus neoformans* polysaccharide capsule. *J. Immunol.* 166, 4620–4626.

Singh, N. (2001). Trends in the epidemiology of opportunistic fungal infections: predisposing factors and the impact of antimicrobial use practices. *Clin. Infect. Dis.* 33, 1692–1696.

Steele, C., Marrero, L., Swain, S., Harmsen, A. G., Zheng, M., Brown, G. D., Gordon, S., Shellito, J. E., and Kolls, J. K. (2003). Alveolar macrophage-mediated killing of *Pneumocystis carinii* f. spp. muris involves molecular recognition by the dectin-1 beta-glucan receptor. *J. Exp. Med.* 198, 1677–1688.

Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., Kolls, J. K., and Brown, G. D. (2005). The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus. PLoS Pathog.* 1, e42. doi:10.1371/journal.ppat.0010042

Swain, S. D., Lee, S. J., Nussenzweig, M. C., and Harmsen, A. G. (2003). Absence of the macrophage mannose receptor in mice does not increase susceptibility to *Pneumocystis carinii* infection in vivo. *Infect. Immun.* 71, 6213–6221.

Syme, R. M., Spurrell, J. C., Amankwah, E. K., Green, F. H., and Mody, C. H. (2002). Primary dendritic cells phagocytose *Cryptococcus neoformans* via mannose receptors and Fcgamma receptor II for presentation to T lymphocytes. *Infect. Immun.* 70, 5972–5981.

Tachado, S. D., Zhang, J., Zhu, J., Patel, N., Cushion, M., and Koziel, H. (2007). Pneumocystis-mediated IL-8 release by macrophages requires coexpression of mannose receptors and TLR2. *J. Leukoc. Biol.* 81, 205–211.

Tada, H., Nemoto, E., Shimauchi, H., Watanabe, T., Mikami, T., Matsumoto, T., Ohno, N., Tamura, H., Shibata, K., Akashi, S., Miyake, K., Sugawara, S., Takada, H. (2002). *Saccharomyces cerevisiae*, and *Candida albicans*-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14, and Toll-like receptor 4-dependent manner. *Microbiol. Immunol.* 46, 503–512.

Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Mühlradt, P. F., and Akira, S. (2000). Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* 164, 554–557.

Takeuchi, O., Kawai, T., Mühlradt, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., Takeda, K., and Akira, S. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13, 933–940.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R. L., and Akira, S. (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* 169, 10–14.

Tapping, R. I., and Tobias, P. S. (2003). Mycobacterial lipoarabinomannan mediates physical interactions between TLR1, and TLR2 to induce signaling. *J. Endotoxin Res.* 9, 264–268.

Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., Haynes, K., Steele, C., Botto, M., Gordon, S., and Brown, G. D. (2007a). Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31–38.

Taylor, K. R., Yamasaki, K., Radek, K. A., Di Nardo, A., Goodarzi, H., Golenbock, D., Beutler, B., and Gallo, R. L. (2007b). Recognition of hyaluronan released in sterile injury involves a unique receptor complex dependent on Toll-like receptor 4, CD44, and MD-2. *J. Biol. Chem.* 282, 18265–18275.

Travassos, L. H., Girardin, S. E., Philpott, D. J., Blanot, D., Nahori, M. A., Werts, C., and Boneca, I. G. (2004). Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep.* 5, 1000–1006.

Trinel, P. A., Maes, E., Zanetta, J. P., Delplace, F., Coddeville, B., Jouault, T., Strecker, G., and Poulain, D. (2002). *Candida albicans* phospholipomannan, a new member of the fungal mannose inositol phosphoceramide family. *J. Biol. Chem.* 277, 37260–37271.

Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M., and Aderem, A. (1999). The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 401, 811–815.

Van de Veerdonk, F. L., Joosten, L. A., Shaw, P. J., Smeekens, S. P., Malireddi, R. K., van der Meer, B. J., Netea, M. G., and Kanneganti, T. D. (2011). The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. *Eur. J. Immunol.* 41, 2260–2268.

Van de Veerdonk, F. L., Kullberg, B. J., van der Meer, J. W., Gow, N. A., and Netea, M. G. (2008). Host-microbe interactions: innate pattern recognition of fungal pathogens. *Curr. Opin. Microbiol.* 11, 305–312.

Van der Graaf, C. A., Netea, M. G., Franke, B., Girardin, S. E., van der Meer, J. W., and Kullberg, B. J. (2006). Nucleotide oligomerization domain 2 (Nod2) is not involved in the pattern recognition of *Candida albicans. Clin. Vaccine Immunol.* 13, 423–425.

van Kooyk, Y., and Geijtenbeek, T. B. (2003). DC-SIGN: escape mechanism for pathogens. *Nat. Rev. Immunol.* 3, 697–709.

Vecchiarelli, A., Retini, C., Monari, C., Tascini, C., Bistoni, F., and Kozel, T. R. (1996). Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. *Infect. Immun.* 64, 2846–2849.

Villamón, E., Gozalbo, D., Roig, P., O'Connor, J. E., Fradelizi, D., and Gil, M. L. (2004). Toll-like receptor-2 is essential in murine defenses against *Candida albicans* infections. *Microbes Infect.* 6, 1–7.

Wang, J. E., Warris, A., Ellingsen, E. A., Jørgensen, P. F., Flo, T. H., Espevik, T., Solberg, R., Verweij, P. E., and Aasen, A. O. (2001). Involvement of CD14, and Toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae. *Infect. Immun.* 69, 2402–2406.

Wells, C. A., Salvage-Jones, J. A., Li, X., Hitchens, K., Butcher, S., Murray, R. Z., Beckhouse, A. G., Lo, Y. L., Manzanero, S., Cobbold, C., Schroder, K., Ma, B., Orr, S., Stewart, L., Lebus, D., Sobieszczuk, P., Hume, D. A., Stow, J., Blanchard, H., and Ashman, R. B. (2008). The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans. J. Immunol.* 180, 7404–7413.

Werner, J. L., Metz, A. E., Horn, D., Schoeb, T. R., Hewitt, M. M., Schwiebert, L. M., Faro-Trindade, I., Brown, G. D., and Steele, C. (2009). Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus. J. Immunol.* 182, 4938–4946.

West, X. Z., Malinin, N. L., Merkulova, A. A., Tischenko, M., Kerr, B. A., Borden, E. C., Podrez, E. A., Salomon, R. G., and Byzova, T. V. (2010). Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. *Nature* 467, 972–976.

Willment, J. A., and Brown, G. D. (2008). C-type lectin receptors in antifungal immunity. *Trends Microbiol.* 16, 27–32.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249, 1431–1433.

Xin, H., Dziadek, S., Bundle, D. R., and Cutler, J. E. (2008). Synthetic glycopeptide vaccines combining beta-mannan and peptide epitopes induce protection against candidiasis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13526–13531.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., and Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* 9, 1179–1188.

Yamasaki, S., Matsumoto, M., Takeuchi, O., Matsuzawa, T., Ishikawa, E., Sakuma, M., Tateno, H., Uno, J., Hirabayashi, J., Mikami, Y., Takeda, K., Akira, S., and Saito, T. (2009). C-type lectin mincle is an activating receptor for pathogenic fungus, *Malassezia. Proc. Natl. Acad. Sci. U.S.A.* 106, 1897–1902.

Yang, H., Hreggvidsdottir, H. S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., Rosas-Ballina, M., Al-Abed, Y., Akira, S., Bierhaus, A., Erlandsson-Harris, H., Andersson, U., and Tracey, K. J. (2010). A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11942–11947.

Yoshitomi, H., Sakaguchi, N., Kobayashi, K., Brown, G. D., Tagami, T., Sakihama, T., Hirota, K., Tanaka, S., Nomura, T., Miki, I., Gordon, S., Akira, S., Nakamura, T., and Sakaguchi, S. (2005) A role for fungal {beta}-glucans and their receptor dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201, 949–960.

Zhang, H. J., Qu, J. M., Shao, C. Z., Zhang, J., He, L. X., and Yuan, Z. H. (2008). *Aspergillus fumigatus* conidia upregulates NOD2 protein expression both in vitro and in vivo. *Acta Pharmacol. Sin.* 29, 1202–1208.

Zhang, J., Zhu, J., Bu, X., Cushion, M., Kinane, T. B., Avraham, H., and Koziel, H. (2005). Cdc42 and RhoB activation are required for mannose receptor-mediated phagocytosis by human alveolar macrophages. *Mol. Biol. Cell* 16, 824–834.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 September 2011; accepted: 23 November 2011; published online: 19 December 2011.

Citation: Figueiredo RT, Carneiro LAM and Bozza MT (2011) Fungal surface and innate immune recognition of filamentous fungi. Front. Microbio. ***2****:248. doi: 10.3389/fmicb.2011.00248*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.