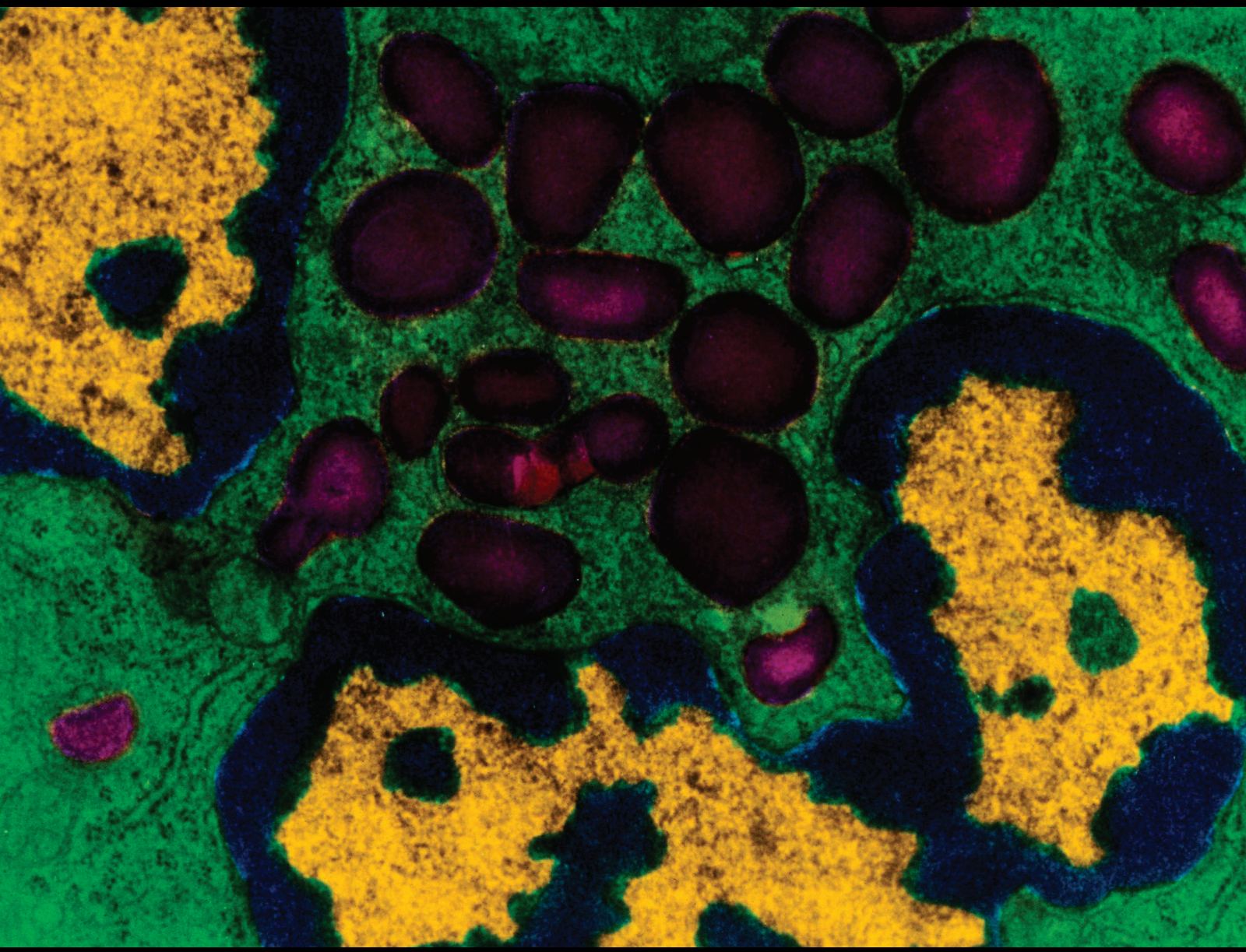
Mediators of Inflammation

Intracellular Eukaryotic Pathogens' Virulence Attributes and Their Interplay with Host Immune Defenses

Lead Guest Editor: Ildinete Silva-Pereira
Guest Editors: Anamélia L. Bocca, Joshua D. Nosanchuk, and Célia M. A. Soares



Intracellular Eukaryotic Pathogens' Virulence Attributes and Their Interplay with Host Immune Defenses

Mediators of Inflammation

Intracellular Eukaryotic Pathogens' Virulence Attributes and Their Interplay with Host Immune Defenses

Lead Guest Editor: Ildinete Silva-Pereira
Guest Editors: Anamélia L. Bocca, Joshua D. Nosanchuk, and Célia M. A. Soares

Editorial Board

Contents

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 1264974, 2 pages
https://doi.org/10.1155/2017/1264974

Editorial

Intracellular Eukaryotic Pathogens' Virulence Attributes and Their Interplay with Host Immune Defenses

Anamélia Lorenzetti Bocca,[1] Célia Maria de Almeida Soares,[2] Joshua D. Nosanchuk,[3] and Ildinete Silva-Pereira[4]

[1]*Cell Biology Department of Biology Institute, Laboratory of Applied Immunology, University of Brasília, 70910-900 Brasília, DF, Brazil*
[2]*Laboratory of Biochemistry and Molecular Biology of the Interaction between Host-Pathogenic Fungi, Biology Institute, University of Goiás, 74001-970 Goiânia, GO, Brazil*
[3]*Departments of Medicine and Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[4]*Cell Biology Department of Biology Institute, Laboratory of Molecular Biology of Pathogenic Fungi, University of Brasília, 70910-900 Brasília, DF, Brazil*

Correspondence should be addressed to Ildinete Silva-Pereira; xocolau@unb.br

Received 2 July 2017; Accepted 2 July 2017; Published 5 November 2017

The incidence of life-threatening infections by intracellular eukaryotic pathogens has risen sharply as a result of modern medical care that diminishes immunity in patients, such as invasive catheters, chemotherapy, and steroids, as well as the increased incidence of immunosuppressive diseases, such as those due to the human immunodeficiency virus (HIV). For example, *Cryptococcus neoformans* is responsible for between ~180,000 and 600,000 deaths annually, primarily in sub-Saharan Africa and principally in individuals with HIV [1, 2]. Thus, there is an urgent need for new and deeper insights into the pathobiology of these diverse intracellular invaders' mechanisms for virulence including their strategies to evade or subvert host immune defenses.

Host-pathogen interactions are complex, dynamic, and multifactorial processes. In order to survive and proliferate within the host, eukaryotic pathogens must be able to sense different host microenvironment signals and regulate transcription and translation reprogramming resulting in metabolic adaptations, alterations in cellular morphology, and adjustments and remodeling of their surface envelope (cell wall/plasmatic membrane), among other processes. For example, signals derived through the binding of the fungal cell wall by antibody can result in alterations of gene activation [3] or protein loading in released extracellular vesicles [4]. Osmotic changes can lead to dramatic alterations in protein regulation, such as in *Paracoccidioides lutzii* [5]. In this special issue, areas that are discussed include the dynamics of phase variation in response to stressors, regulation of enzyme secretion, and considerations of metabolic routes as drug targets. In this special issue, E. Camacho and G. A. Niño-Vega detail virulence factors that facilitate the survival of *Paracoccidioides* spp. The pathway to the identification and development of new antifungal drugs through studies on antifungal resistance and metabolism is thoroughly addressed in an article by J. A. Parente-Rocha et al.

On the other hand, effective host responses require the ability of the host to recognize and respond to the pathogen employing several mechanisms to eradicate and/or control the pathogen through the activation of an efficient immune response. The host defense mechanisms include harnessing the functions of macrophages, dendritic cells, T cells, B cells, Th1, Th2 & Th17 responses, antibody, and complement as well as the engagement of such cells through recognition receptors such as TLRs, Dectin-1,

complement, mannose & other lectin receptors, scavenger receptors, IL-1 receptor, E-cadherein, EGFR-HER2, Gp96, CD14, CD44, and CDw17. For example, dectin-1 is required for the upregulation of miR155 in macrophages challenged with *Candida albicans* [6] and NLRP3 inflammasome activation by *Paracoccidioides brasiliensis* is linked to a protective response against this pathogen [7]. This special edition will examine cellular and humoral systems in responding to intracellular eukaryotic pathogens. Additionally, issues on how vaccination (both with pathogen products or primed cells, such as dendritic cell) can alter the host-pathogen dynamic will be explored. The interplay between the host and pathogen will be highlighted by a focus on the ability of microbes to undergo morphogenesis as a means to escape immune surveillance. For example, the topic of fungal dimorphism and virulence will be carefully detailed at the molecular level by G. M. Gauthier.

Understanding of the interplay between intracellular eukaryotic pathogens and host cells requires dissection at the levels of both pathogen and host. Dynamic ongoing shifts in responses within both the invader cells and the host cells dictate the outcome of the interaction, to the benefit or detriment of each party. The overall complexity of the processes occurring in such struggles is daunting, yet major insights into the pathobiology of these diseases have been achieved. With this special issue, we have provided a platform that presents significant findings that offer insights into host-pathogen interactions.

Anamélia Lorenzetti Bocca
Célia Maria de Almeida Soares
Joshua D. Nosanchuk
Ildinete Silva-Pereira

References

[1] B. J. Park, K. A. Wannemuehler, B. J. Marston, N. Govender, P. G. Pappas, and T. M. Chiller, "Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS," *AIDS*, vol. 23, no. 4, pp. 525–530, 2009.

[2] R. Rajasingham, R. M. Smith, B. J. Park et al., "Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis," *The Lancet Infectious Diseases*, vol. 17, no. 8, pp. 873–881, 2017.

[3] E. E. McClelland, A. M. Nicola, R. Prados-Rosales, and A. Casadevall, "Ab binding alters gene expression in *Cryptococcus neoformans* and directly modulates fungal metabolism," *The Journal of Clinical Investigation*, vol. 120, no. 4, pp. 1355–1361, 2010.

[4] L. Matos Baltazar, E. S. Nakayasu, T. J. Sobreira et al., "Antibody binding alters the characteristics and contents of extracellular vesicles released by *Histoplasma capsulatum*," *mSphere*, vol. 1, no. 2, 2016.

[5] L. N. Rodrigues, A. Brito Wde, A. F. Parente et al., "Osmotic stress adaptation of *Paracoccidioides lutzii*, Pb01, monitored by proteomics," *Fungal Genetics and Biology*, vol. 95, pp. 13–23, 2016.

[6] D. P. Agustinho, M. A. de Oliveira, A. H. Tavares et al., "Dectin-1 is required for miR155 upregulation in murine macrophages in response to *Candida albicans*," *Virulence*, vol. 8, no. 1, pp. 41–52, 2017.

[7] A. H. Tavares, K. G. Magalhães, R. D. Almeida, R. Correa, P. H. Burgel, and A. L. Bocca, "NLRP3 inflammasome activation by *Paracoccidioides brasiliensis*," *PLoS Neglected Tropical Diseases*, vol. 7, no. 12, article e2595, 2013.

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 7375818, 16 pages
https://doi.org/10.1155/2017/7375818

Research Article

The Lymphotoxin β Receptor Is Essential for Upregulation of IFN-Induced Guanylate-Binding Proteins and Survival after *Toxoplasma gondii* Infection

Kristina Behnke,[1,2] Ursula R. Sorg,[1] Helmut E. Gabbert,[3] and Klaus Pfeffer[1]

[1]*Institute of Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany*
[2]*Molecular Medicine II, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany*
[3]*Institute of Pathology, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany*

Correspondence should be addressed to Klaus Pfeffer; klaus.pfeffer@hhu.de

Received 14 February 2017; Revised 23 May 2017; Accepted 7 June 2017; Published 6 August 2017

Academic Editor: Célia M. A. Soares

Lymphotoxin β receptor (LTβR) signaling plays an important role in efficient initiation of host responses to a variety of pathogens, encompassing viruses, bacteria, and protozoans via induction of the type I interferon response. The present study reveals that after *Toxoplasma gondii* infection, LTβR$^{-/-}$ mice show a substantially reduced survival rate when compared to wild-type mice. LTβR$^{-/-}$ mice exhibit an increased parasite load and a more pronounced organ pathology. Also, a delayed increase of serum IL-12p40 and a failure of the protective IFNγ response in LTβR$^{-/-}$ mice were observed. Serum NO levels in LTβR$^{-/-}$ animals rose later and were markedly decreased compared to wild-type animals. At the transcriptional level, LTβR$^{-/-}$ animals exhibited a deregulated expression profile of several cytokines known to play a role in activation of innate immunity in *T. gondii* infection. Importantly, expression of the IFNγ-regulated murine guanylate-binding protein (mGBP) genes was virtually absent in the lungs of LTβR$^{-/-}$ mice. This demonstrates clearly that the LTβR is essential for the induction of a type II IFN-mediated immune response against *T. gondii*. The pronounced inability to effectively upregulate host defense effector molecules such as GBPs explains the high mortality rates of LTβR$^{-/-}$ animals after *T. gondii* infection.

1. Introduction

Core members of the tumor necrosis factor (TNF)/TNF receptor (TNFR) superfamily such as TNF and lymphotoxin (LT) β and their receptors TNFRp55 and LTβR are important mediators of innate immune responses and are considered to be essential for controlling pathogens [1–6]. It has been demonstrated that LTα, TNF, and TNFRp55 but not TNFRp75 are vital for host defense against the intracellular parasite *Toxoplasma gondii* [2, 7, 8]. Although the LTβR has been shown to play an important role in the defense against *Listeria monocytogenes* and *Mycobacterium tuberculosis* [5] as well as CMV [9], it is still unclear, however, whether signaling via the LTβR also contributes to an effective host response to *T. gondii*. *T. gondii*, a member of the phylum Apicomplexa, is an obligate intracellular parasite [7, 10]. Definitive hosts in which sexual reproduction occurs are felids. Due to low host specificity, *T. gondii* is able to infect most warm blooded mammals and prevalence in humans is estimated 30–70% throughout the world [11, 12]. In immune competent hosts, *T. gondii* infection elicits a protective immune response that may initially, in the acute phase, cause mild flu-like symptoms which then resolve [13]. As specific host immune mechanisms set in, *T. gondii* forms tissue cysts (stage conversion), in humans and mice preferably in brain and muscle tissue, and transition into the symptomless, chronic form of toxoplasmosis is effected, in which cysts persist lifelong [14]. In immune incompetent hosts, primary *T. gondii* infection may have severe and sometimes lethal consequences such as pneumonia or encephalitis [13, 15]. Furthermore, existing, chronic *T. gondii* infection may be reactivated in immunocompromised hosts such as AIDS patients or recipients of immunosuppressive drugs with similar

Table 1: Primer and probe sequences for RT-PCR.

Target	Primers	Probe
β-Actin	5′TGACAGGATGCAGAAGGAGA 3′CGCTCAGGAGGAGCAATG	[a]106
mGBP1	5′CAGACTCCTGGAAAGGGACTC 3′CTTGGACCTGGAACATTCACTGAC	[a]41
mGBP2	5′TGAGTACCTGGAACATTCACTGAC 3′AGTCGCGGCTCATTAAAGC	[a]17
mGBP3	5′GGCTGAGGACTGTCCCTGT 3′CATGGTCCACTCGGAAGC	[a]21
mBGP4	5′GCCAAGATCAAGACCCTCAG 3′CCACGTAGGTTGTCACCAGA	[a]48
mGBP5	5′TCACTGAAGCTGAAGCAAGG 3′GCGTCAAAAACAAAGCATTTC	[a]48
mGBP6	5′ATATTTCAACATTTTTTGTTCCTTGT 3′GAAATGGGAGAAAAAATAAATGAAGC	FAM-AGTCATGTTCAATCTTCTCCCTCTTGTCC-DB
mGBP7	5′GCAGAGAATCCGGTGCAG 3′TTTCCACTAGGCACACAGGA	[a]93
mGBP8	5′AAGAAGCTGAAGGAACAAAAGGC 3′GAAATGGGAGAAAAAATAAATGAAGC	FAM-TGTTTCAGTTGCTGTATCTCTCCGTCCA-TMR
mGBP9	5′TTCCAAAACTTTCTCCAGTCACAGTA 3′GGCACGCTCCTCTGCAA	FAM-CCAGCAGTGAGGGCTCTATCTGCCT-TMR
GTPBP1	5′GGTGCAGAGCAAAGATGATG 3′ATCTGGAATATCGGGCACAT	[a]75
IL-4	5′CATCGGCATTTTGAACGAG 3′CGAGCTCACTCTCTGTGGTG	[a]2
IL-12p40	5′GATTCAGACTCCAGGGGACA 3′TGGTTAGCTTCTGAGGACACATC	[a]27
iNOS	5′CTTTGCCACGGACGAGAC 3′TCATTGTACTCTGAGGGCTGAC	[a]13
LTα	5′TCCCTCAGAAGCACTTGACC 3′GAGTTCTGCTTGCTGGGGTA	[a]62
LTβ	5′CCTGGTGACCCTGTTGTTG 3′TGCTCCTGAGCCAATGATCT	[a]76
IFNβ	5′CAGGCAACCTTTAAGCATCAG 3′CCTTTGACCTTTCAAATGCAG	[a]95

[a]Numbers identify probes obtained from the Roche Universal ProbeLibrary (Roche).

repercussions [16, 17]. In addition, primary infection during pregnancy may, via placental transmission of the parasite, lead to fetal pathology, including irreversible neurological defects and, in the worst case, termination of pregnancy [13, 18, 19]. It has been demonstrated that innate immune responses are vital for the efficient control of *T. gondii* [20–22]. Although *T. gondii* lacks classical viral and bacterial pathogen-associated molecular patterns, unique protozoan-associated molecules such as GPI-anchors and profilin are recognized via toll like receptors (TLRs) [23–25]. TLR2 and TLR4-mediated signaling induces secretion of IL-12 and TNF by macrophages, and TLR11 or TLR12-mediated signaling induces secretion of IL-12 by CD8α^+ dendritic cells (DC) [22]. IL-12 in turn induces secretion of IFNγ by NK cells [26, 27]. Besides being required for the induction of T cell responses, IFNγ mediates various innate effector mechanisms such as induction of IDO and production of reactive oxygen species and NO in *T. gondii* infection [28–31]. Another important effect of IFNγ is the induction of IFNy-inducible genes such as immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) [32–34]. It has been demonstrated in mouse models that murine (m)GBPs, a family of 65 kDa guanylate-binding proteins, play an important role in host defense against intracellular pathogens such as *T. gondii* [35–37] and *Neospora caninum* [38]. mGBPs are highly induced via IFNγ after infection and are localized in intracellular vesicle-like structures. mGBP1, mGBP2, mGBP3, mGBP6, mGBP7, and mGBP9 relocate to the parasitophorous vacuole of *T. gondii* after entry of the pathogen into the cell [35]. The importance of mGBPs for the efficient control of *T. gondii* is underscored by findings that mice deficient for mGBP2 or showing a deletion of a cluster of mGBPs (1, 2, 3, 5, and 7) are more susceptible to *T. gondii* infection [35–37, 39]. The present study demonstrates that LTβR-deficient mice likewise show dramatically reduced survival after *T. gondii* infection,

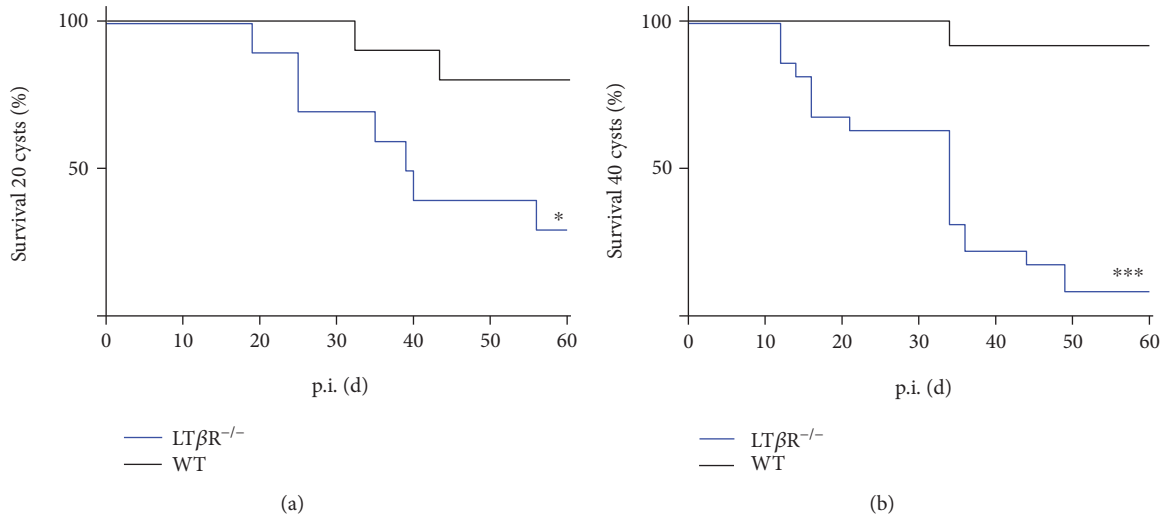


Figure 1: LTβR$^{-/-}$ animals show significantly reduced survival after infection with *T. gondii* (ME 49) cysts compared to WT animals. WT and LTβR$^{-/-}$ animals were infected i.p. with (a) 20 cysts (WT: $n = 10$, LTβR$^{-/-}$: $n = 10$) or (b) 40 cysts (WT: $n = 12$, LTβR$^{-/-}$: $n = 22$) of *T. gondii* (ME49) freshly isolated from the brains of CD1 mice. $^{*}p < 0.05$, $^{***}p < 0.001$.

most probably due to an inability to induce appropriate IFNγ responses and a marked failure to adequately upregulate mGBPs.

2. Materials and Methods

2.1. Animals. This study was carried out in strict accordance with the German Animal Welfare Act. The protocol was approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Permit number 84-02.04.2011.A394). All efforts were made to minimize suffering of laboratory animals. LTβR$^{-/-}$ mice were generated as described previously [40] and had been backcrossed for at least 10 generations onto a C57BL/6 background. Wild-type (WT) littermates were used as controls. Mice were housed under specified pathogen-free conditions in the animal facility of the Heinrich Heine University of Düsseldorf and were between 10 and 12 weeks of age at the time of infection. *T. gondii* strain ME49 was used for all experiments and maintained in the CD1 mouse strain purchased from Charles River Breeding Laboratories.

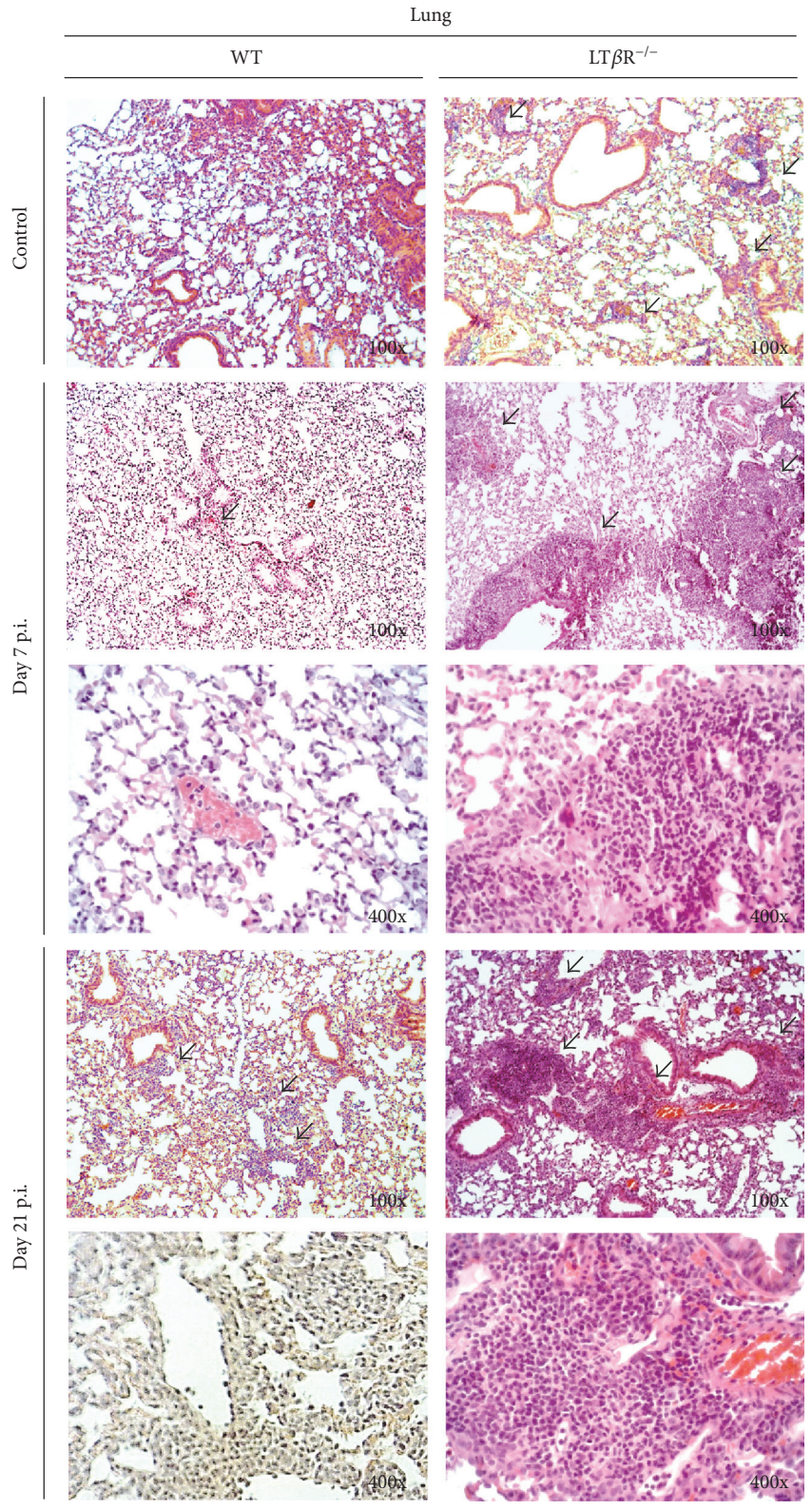
2.2. T. gondii Infection. ME49 cysts were isolated from CD1 mice 6 weeks after infection as described previously [41]. Briefly, the murine cerebrum was homogenized by passaging through successively thinner cannulas. A first centrifugation step (5 min, 60 ×g, 22°C) removed cell debris. The pellet was then resuspended in PBS (Invitrogen, Karlsruhe, Germany), and an underlayer of Ficoll Paque™ Plus (GE Healthcare, Munich, Germany) was added before centrifugation (500 ×g, 25 min, 22°C, without brakes). The pelleted cysts were counted and resuspended in the appropriate amount of PBS. Infections were carried out by intraperitoneally injecting either 20 or 40 cysts (as indicated) of *T. gondii* ME49 in a volume of 0.2 mL PBS.

2.3. Blood and Tissue Processing. Mice were anaesthetized with 100 mg/kg Ketamin and 10 mg/kg Xylazine (both *Vétoquinol GmbH, Ravensburg, Germany*) and bled via the *vena cava inferior* on the days post infection (p.i.) as indicated. Serum was obtained by coagulating the blood (30 min at room temperature) and collecting the serum after two centrifugation steps (10 min, 8000 ×g). The brain, lung, liver, and spleen were removed, rinsed in PBS, and weighed. To determine cell numbers, spleens were collected, digested with collagenase D (Sigma-Aldrich, Taufkirchen, Germany) for 30 min in DMEM/10% FCS, and passed through a 40 μm cell strainer (BD Biosciences, Heidelberg) before lysis of red blood cells with Erylysis buffer (Morphisto, Frankfurt am Main, Germany).

2.4. Histology. Formalin-fixed and paraffin-embedded tissue blocks of the isolated organs were collected; 1 μm sections were cut, transferred onto glass slides, and stained with a standard hematoxylin/eosin protocol.

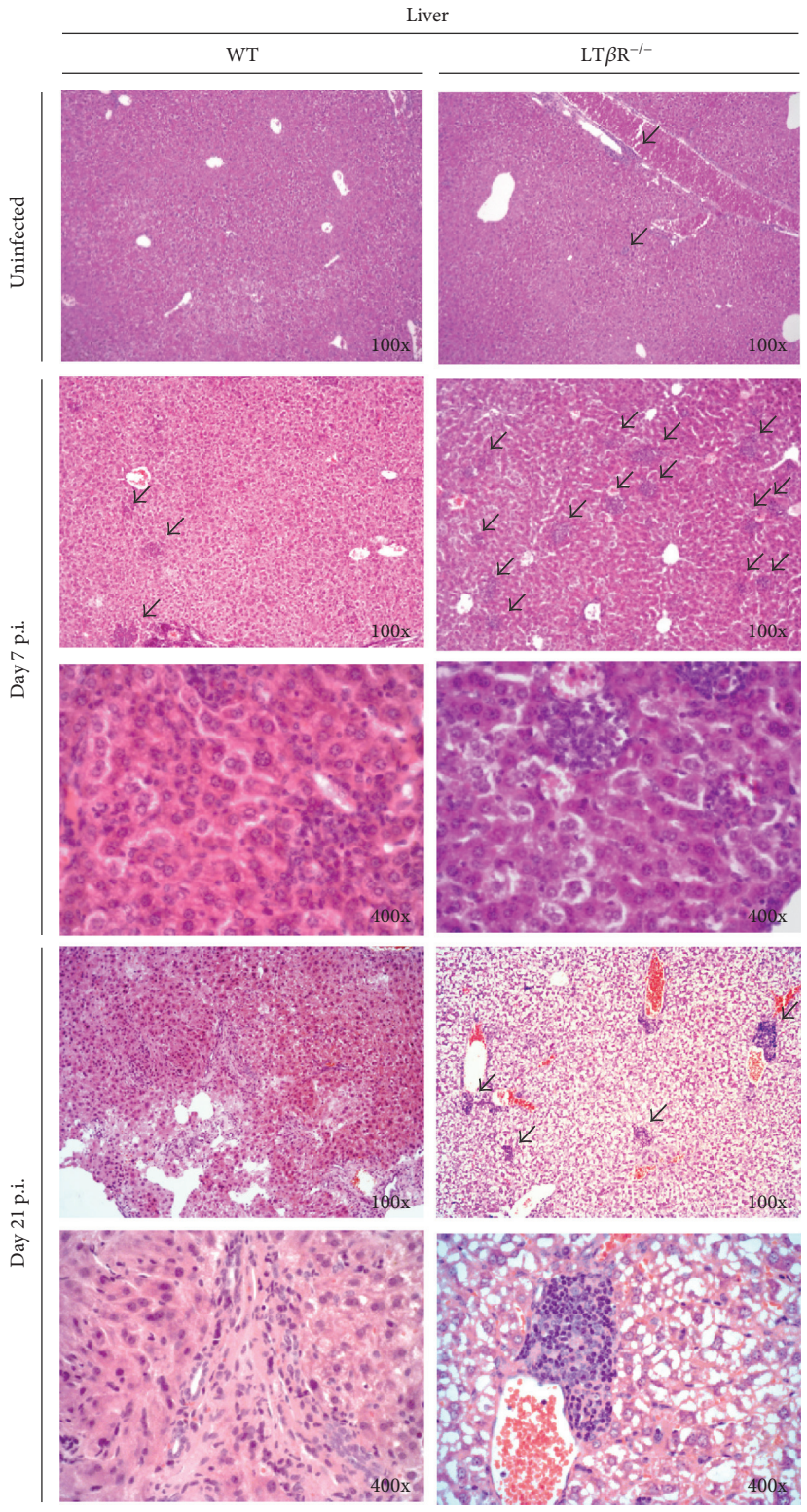
2.5. Serum Biochemistry and Cytokine Quantification. Serum was tested for concentrations of aspartate transaminase (AST), bilirubin, and lactate dehydrogenase (LDH) using the automated biochemical analyzer Spotchem EZ SP-4430 (Arkray, Amstelveen, Netherlands) and the Spotchem EZ Reagent Strip Liver-1 (Arkray). Commercially available ELISA kits were used to quantify serum TNF, IL-4, IFNγ (R&D Systems, Minneapolis, MN), and IL-12p40 (BioSciences, Heidelberg, Germany) levels. NO concentrations were analyzed using the Total Nitric Oxide and Nitrate/Nitrite Kit from R&D Systems.

2.6. Quantitative RT-PCR. Total RNA from single cell suspensions of lung tissue was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using 3 μg of total RNA with Moloney murine leukemia virus



(a)

Figure 2: Continued.



(b)

Figure 2: LTβR$^{-/-}$ animals show more and larger inflammatory areas in the (a) lung and (b) liver 7 and 21 days after infection with *T. gondii* (ME49) cysts compared to WT animals. The lung and liver were isolated from uninfected control mice 7 and 21 days after i.p. infection with 40 *T. gondii* (ME49) cysts and fixed in formalin. Tissues were embedded in paraffin, 10 μm sections were generated, and HE staining was performed. Original magnification as indicated. 3 animals were analyzed for each time point, and a representative section from one organ is shown in each case. Arrows indicate small, dense lymphocyte infiltrates that are considered part of the basal LTβR$^{-/-}$ phenotype. Arrowheads indicate inflammatory infiltrates seen in infected animals.

Table 2: Inflammatory infiltrates in the lung and liver.

Organ	Genotype	Days p.i. 0	3	5	7	12	14	21	30	36
Lung	WT	–	–	–	+	++	–	(+)	–	–
	[a]LTβR$^{-/-}$	–	+++	+++	+++	+++	+++	+++	+++	++
Liver	WT	–	–	–	++	++	–	+	–	–
	[a]LTβR$^{-/-}$	–	–	++	++	+++	+	–	–	–

The number of inflammatory infiltrates per visual field were scored in HE-stained sections, at least 10 visual fields were evaluated per slide. No infiltrates: –; 1–3 infiltrates: (+); 4–8 infiltrates: +; 9–12 infiltrates: ++; 13–18 infiltrates: +++. [a]Infiltrates considered to be part of the basal LTβR$^{-/-}$ phenotype were not included in the scoring.

Table 3: Cyst count in the liver and lung.

Organ	Genotype	Days p.i. 0	3	5	7	12	14	21	30	36
Lung	WT	—	—	—	—	—	2	—	—	—
	LTβR$^{-/-}$	—	—	0.5	1	2	1	—	—	—
Liver	WT	—	—	—	3	2.5	—	—	—	—
	LTβR$^{-/-}$	—	—	—	2.5	2	6	—	—	—
Brain	WT	—	—	—	—	—	0.5	1	1.5	3
	LTβR$^{-/-}$	—	—	—	—	—	2.5	2.5	10.5	16.5

Organ sections from 3 animals per time point were evaluated, except on day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were evaluated. The number of cysts per organ section was counted.

reverse transcriptase and oligo (dT) primer (both Invitrogen Life Technologies). RT-PCR (40 cycles) was performed in triplicate. Primer and probe sequences (listed in Table 1) were synthesized by Metabion (Martinsried, Germany) and based on the conventional TaqMan Probe finder software (TIB MOLBIOL, Berlin, Germany) for mGBP6, mGBP8, and mGBP9 and the Universal ProbeLibrary (Roche, Mannheim, Germany) for all other genes. The PCR primer sets used spanned at least one intron to avoid detection of genomic DNA. Results are expressed relative to expression in uninfected WT mice and normalized to β-actin ($2^{-\Delta\Delta CT}$).

2.7. Statistical Analysis. Quantifiable data are expressed as means ± SD. Statistical analysis was performed using the GraphPad Prism 5.01 software for Student's *t*-test.

3. Results

3.1. LTβR$^{-/-}$ Mice Show Increased Susceptibility to Infection with T. gondii (ME49). It has been demonstrated that the LTβR plays a role in controlling infections with intracellular pathogens such as *M. tuberculosis* and *L. monocytogenes* [5]. To determine whether the LTβR is also required to contain infections with *T. gondii*, LTβR$^{-/-}$ mice were infected with 20 or 40 cysts of the ME49 strain of *T. gondii* (Figure 1). Initially, mice were challenged with 20 cysts (*i.p.*) and significantly decreased survival could be observed (Figure 1(a)). Interestingly, LTβR$^{-/-}$ mice survived the acute phase of infection and only started succumbing to the infection in the early chronic phase on day 19 with an overall survival of 30%. WT littermates started dying considerably later (day 32) and showed an overall survival rate of 80%. After infection with 40 cysts of *T. gondii* ME49, LTβR$^{-/-}$ mice started to succumb to infection by day 12 and overall survival was 9.1%. In contrast, WT mice did not show earlier onset of death (day 34) and an overall survival rate of 90% (Figure 1(b)). These data clearly indicate that the LTβR plays a major role in surviving *T. gondii* infections.

3.2. LTβR$^{-/-}$ Mice Show Marked Exacerbation of Organ Pathology. To analyze tissue pathology, formalin-fixed, paraffin-embedded, and HE-stained tissue sections (10 μm) from the lung and liver were assayed for inflammatory infiltrates (Figure 2). It is important to note that in uninfected/untreated LTβR$^{-/-}$ animals, lymphocyte infiltrates have been described in the kidneys, lungs, liver, pancreas, submandibular glands, mesenterium, cortex of the suprarenal glands, and fatty tissue of the mediastinum [40] and could accordingly be observed in the lungs (Figure 2(a)) of uninfected LTβR$^{-/-}$animals. In addition to these small infiltrates, LTβR$^{-/-}$ lungs showed large inflammatory infiltrates on days 7 and 21 after infection. In contrast, only very few such inflammatory infiltrates could be found in the lungs of WT littermates on days 7 and 21 and they tended to be considerably smaller and less dense (Figure 2(a)). Similarly, the livers of uninfected LTβR$^{-/-}$ mice were characterized by small lymphocyte infiltrates which could not be found in WT livers (Figure 2(b)). On day 7 p.i., the LTβR$^{-/-}$ livers show a marked increase of infiltrates, whereas in the livers of WT mice, the number of inflammatory infiltrates is much lower. By day 21, the LTβR$^{-/-}$ livers still showed considerable number of inflammatory infiltrates, while these have disappeared from the livers of WT mice. These findings are quantified and summarized in Table 2, showing that in the lungs of WT animals, inflammatory infiltrates could mainly be observed on days 7 and 12. In contrast, these infiltrates are much more persistent in LTβR$^{-/-}$ mice: they were observed from day 3 through day 36 in the lungs. Findings were similar in the livers: infiltrates were detected in WT animals mainly on days 7 and 12, while they could be observed in LTβR$^{-/-}$ animals from day 5 through day 14. Thus, organ pathology was much more pronounced and persisted for a longer period of time in LTβR$^{-/-}$ compared to WT animals.

3.3. LTβR$^{-/-}$ Animals Have Higher and More Persistent Cyst Count. To determine whether LTβR$^{-/-}$ mice showed differences in the progression into and through the chronic phase of *T. gondii* infection, bradyzoite containing

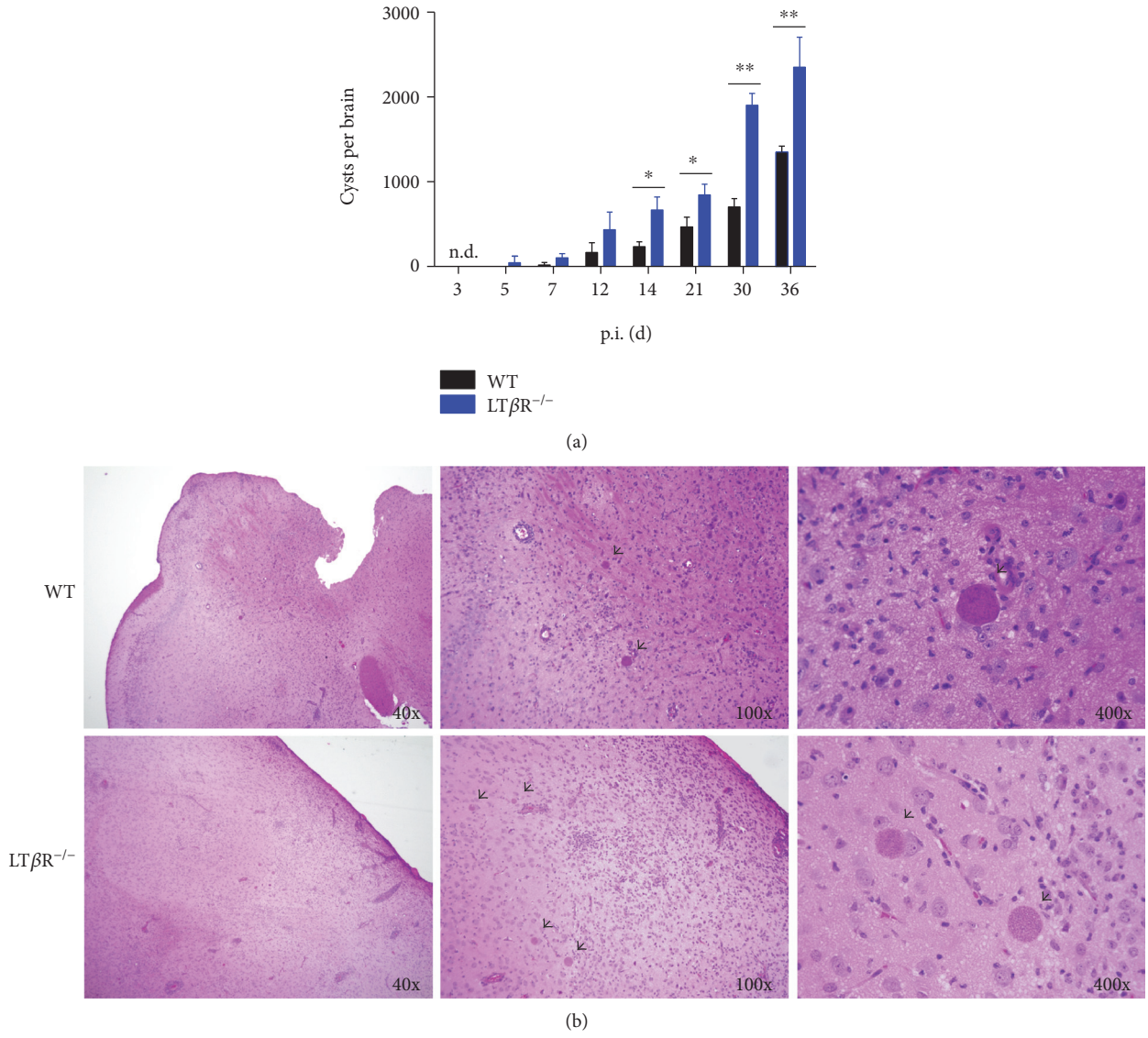


Figure 3: Analysis of parasite burden in the brain of WT and LTβR$^{-/-}$ animals. Animals were infected i.p. with 40 cysts of *T. gondii* (ME49), sacrificed on the days indicated, and the brains were prepared. One hemisphere was used for isolation of cysts, which were isolated by mincing the tissue with a scalpel and then passing it through consecutively higher gauge cannulas, followed by two centrifugation steps to first remove pelleted cells and tissue debris and then pellet the cysts. One half of the second hemisphere was used to generate HE stains from paraffin sections after formalin fixing of tissue. (a) Cysts per brain were calculated by multiplying cyst number counted in one hemisphere by two ($n = 3$ in all cases, except day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were analyzed). (b) Cysts (arrows) in HE-stained brain sections 60 days after i.p. infection with *T. gondii* (ME49) are shown. One representative section of brain tissue from one of three animals is shown. Original magnifications as indicated. $^{*}p < 0.05$, $^{**}p < 0.01$.

cysts were counted in HE sections of liver, lung, and brain (Table 3). Cysts first appeared in the lungs of LTβR$^{-/-}$ mice on day 5 and could be observed on days 7, 12, and 14. In contrast, in the lungs of WT mice cysts could only be found on day 14. While cysts appeared in the liver in both genotypes on day 7 and persisted only slightly longer in LTβR$^{-/-}$ animals compared to WT animals (days 14 and 12, respectively), the number of cysts was elevated in the LTβR$^{-/-}$ mice. Differences in cyst counts were most obvious in the brain. While cysts appeared at the same time after infection (day 14), actual numbers were much higher in LTβR$^{-/-}$ animals than in WT animals (13–18 versus 2–5, respectively, on day 36). The increased presence of cysts in the brain of LTβR$^{-/-}$ mice was confirmed by isolating and counting cysts from the brains (Figure 3(a)). Formalin-fixed, paraffin-embedded, and HE-stained tissue sections also showed an increased presence of cysts in brains of LTβR$^{-/-}$ mice (Figure 3(b)). While disease progression (entry into the acute phase and progression into the chronic phase) apparently occurred within a similar time frame in both genotypes, LTβR$^{-/-}$ animals were less able to contain reproduction of the parasites, leading to a more pronounced tissue pathology, higher cyst numbers, and longer persistence of cysts.

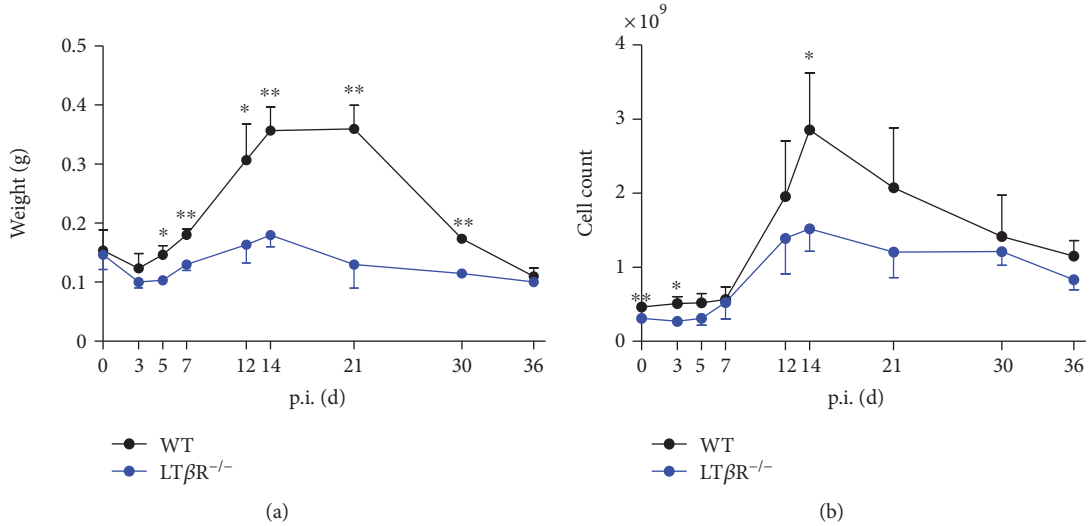


Figure 4: Splenomegaly is observed only in WT but not in LTβR$^{-/-}$ animals after infection with *T. gondii* (ME49). Mice were infected with 40 cysts and sacrificed on the days indicated. Controls were uninfected animals. (a) Spleens were isolated and weighed. (b) Cell numbers were determined by mincing and homogenizing the spleen, passing the obtained cell supension through a 40 μm cell strainer and counting live cells ($n = 3$ in all cases except day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were analyzed). $^{*}p < 0.05$, $^{**}p < 0.01$.

3.4. LTβR$^{-/-}$ Mice Do Not Show Splenic Enlargement and Increase in Splenic Cell Count after Infection with T. gondii. To assess the inflammatory response in LTβR$^{-/-}$ mice after *T. gondii* infection, spleen weight was analyzed. In WT mice, a roughly twofold increase of spleen weight during acute infection could be found which returned to preinfection levels by day 36. In contrast, in LTβR$^{-/-}$ mice, spleen weight increased only marginally during acute infection and returned to physiological levels by day 21 (Figure 4(a)). Splenic cell counts peaked on day 14 both in WT and LTβR$^{-/-}$ animals, but were significantly lower in the latter (Figure 4(b)).

3.5. LTβR$^{-/-}$ Mice Show Minor Alterations in Various Tissue Injury Parameters. Alanine transaminase (ALT) levels were measured to determine liver stress after *T. gondii* infection (Figure 5(a)). In WT animals, ALT levels rose quickly until day 7 p.i., then gradually dropped to preinfection levels by day 60 p.i. ALT levels of LTβR$^{-/-}$ animals progressed in a similar manner, except for a marked but not significant transient increase on day 14. On day 60, ALT levels were significantly higher in LTβR$^{-/-}$ compared to WT animals. Bilirubin is also considered to indicate liver damage. Interestingly, after infection with *T. gondii*, bilirubin levels did not markedly change early during infection (Figure 5(b)), although levels were slightly but significantly increased in LTβR$^{-/-}$ animals on day 5 p.i. Later in infection (days 21 and 30), an increase in bilirubin levels could be observed in both genotypes. On day 60, LTβR$^{-/-}$ animals again show a significant increase in bilirubin compared to WT animals. Since increased LDH is an indicator of cell destruction, LDH levels were determined. Only a slight increase in LDH levels was measured in WT animals throughout the course of infection, with the exceptions of day 7 and day 30 p.i., when a moderate increase occurred. LDH levels of LTβR$^{-/-}$ animals tended to be higher, with a significant increase on days 14, 21, and 60 (Figure 5(c)).

3.6. LTβR$^{-/-}$ Mice Show Lacking or Delayed Cytokine Responses after Infection with T. gondii. Secretion of IL-12 by macrophages and DC is one of the initial steps in the innate immune response to *T. gondii* and induces release of IFNγ by NK and T cells [7]. Compared to LTβR$^{-/-}$ animals, WT animals were observed to have significantly increased levels of serum IL-12p40 by day 5, whereas LTβR$^{-/-}$ animals exhibited this increase two days later (Figure 6(a)). Interestingly, although slightly higher amounts of IFNγ could be found in LTβR$^{-/-}$ compared to WT animals before infection, these amounts did not increase after infection, as seen in WT animals, where levels rose about 4-fold (Figure 6(b)). Despite this marked increase, the difference was not significant, probably due to the high variance found in LTβR$^{-/-}$ animals. TNF is another cytokine that is secreted by macrophages early in infection [7]. While WT animals showed a marked increase of TNF already on day 7 p.i., LTβR$^{-/-}$ animals initially exhibited significantly lower TNF levels which reached WT levels only on day 14 p.i. (Figure 7(a)). As NO produced by macophages is considered to be an important microbicidal mechanism in the innate immune response to *T. gondii* [42], total NO in serum of WT and LTβR$^{-/-}$ mice was analyzed. Figure 7(b) reveals a strong and transient increase of serum NO in WT on day 7 p.i. For the remainder of the observation period, serum NO levels remain moderately elevated in WT animals. In contrast, LTβR$^{-/-}$ animals showed a delayed and reduced increase of serum NO levels on day 12 p.i. and an additional similar peak on day 30 p.i. that could not be detected in WT animals.

3.7. Differential Expression of Genes Involved in Early Innate Immune Response to T. gondii. Expression levels of IL-12p40,

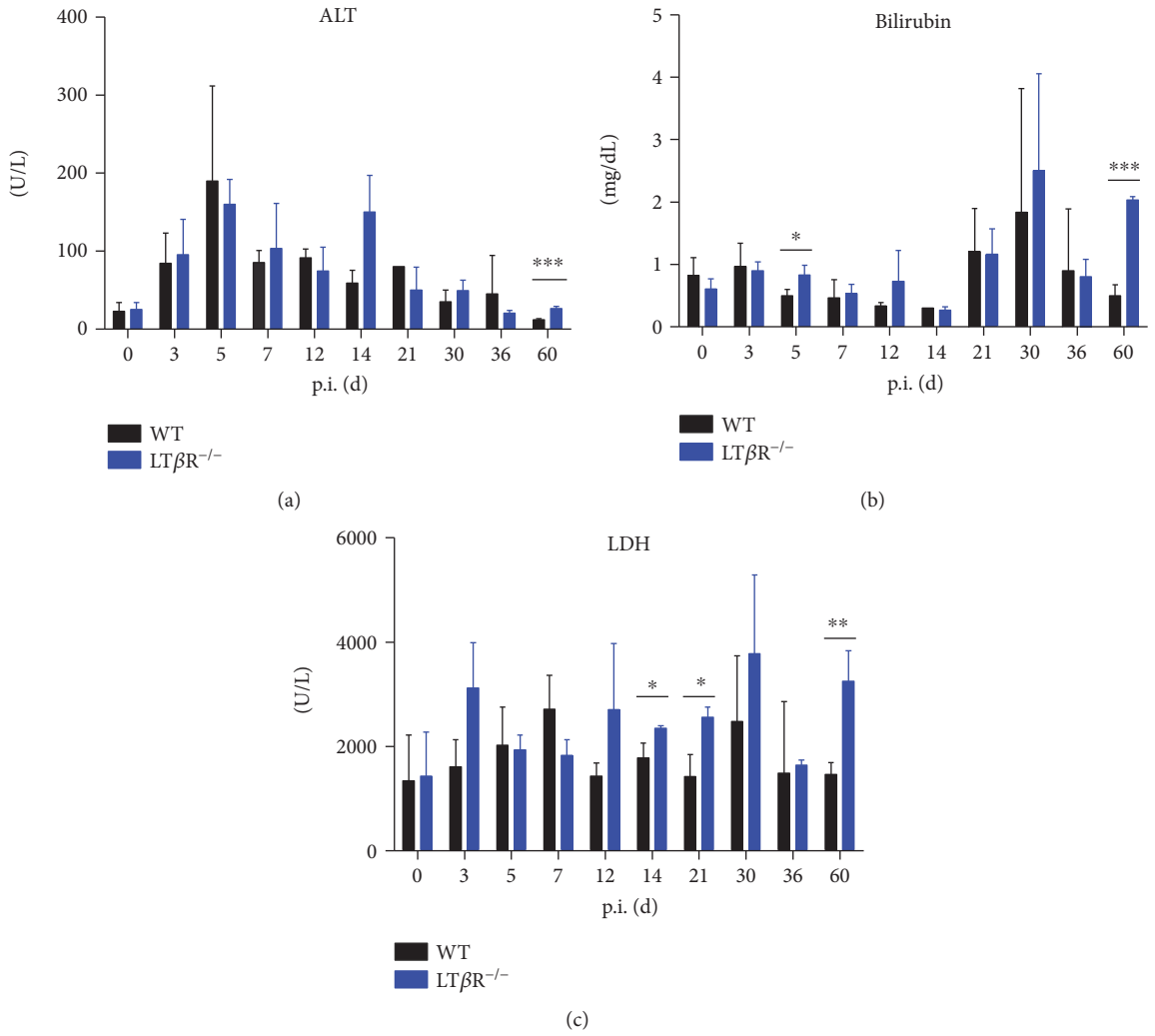


Figure 5: Serum parameters in WT and LTβR$^{-/-}$ animals. Mice were infected with 40 cysts of *T. gondii* (ME49) and sacrificed on the days indicated. Controls were uninfected animals. Serum was obtained by accessing the vena cava inferior, bleeding the animals, and removing cells by centrifugation after allowing a suitable time for clotting. Analysis was performed on a Spotchem 4430. (a) ALT, (b) bilirubin, and (c) LDH ($n = 3$ in all cases except day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were analyzed). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

IFNγ, GTP-binding protein 1 (GTPBP1), IL-4, IFNβ, LTα, and LTβ in the lungs of WT and LTβR$^{-/-}$ animals after *T. gondii* infection were compared. Expression levels for IL-12p40 decreased in WT animals by day 7 p.i, whereas LTβR$^{-/-}$ animals showed much lower expression levels compared to WT animals before infection, but a transient increase in IL-12p40 expression on day 14 p.i. (Figure 8(a)). 7 days after infection with *T. gondii*, IFNγ expression levels increased dramatically in WT animals, returned to normal by day 12, and showed only a mild increase during the further course of infection (Figure 8(b)). In contrast, in LTβR$^{-/-}$ animals, IFNγ levels did not increase until day 14, but then reached levels comparable to WT animals. Also, IFNγ levels remained high at least up to day 40 p.i. and only returned to slightly higher than normal levels by day 60. On the other hand, expression of induced nitric oxide synthase (iNOS) was much lower in LTβR$^{-/-}$ animals compared to WT animals before infection and did not increase markedly after infection (Figure 8(c)). In WT animals, iNOS expression decreased after infection and remained at low levels at least until day 60 p.i. Expression of GTPBP1 increased transiently but markedly in WT animals on day 12 p.i and then remained at slightly elevated levels (Figure 8(d)). LTβR$^{-/-}$ animals did not exhibit such a distinct increase p.i.; GTPBP1 expression levels were only moderately increased during the course of infection. WT animals showed only a slight (around 2-fold) and transient increase of IL-4 expression 7 days p.i. (Figure 8(e)). Of note, IL-4 expression in LTβR$^{-/-}$ animals was increased more than 10-fold before infection when compared to WT animals and this expression decreased markedly early after infection (days 7 and 12), followed by a distinct but transient increase on day 14 p.i. IFNβ expression levels in WT animals showed a 20-fold increase on day 12 p.i. (Figure 8(f)). Then levels dropped again, but rose about 70-fold between days 30 and 60 levels. In LTβR$^{-/-}$ animals, INFβ levels remained low until day

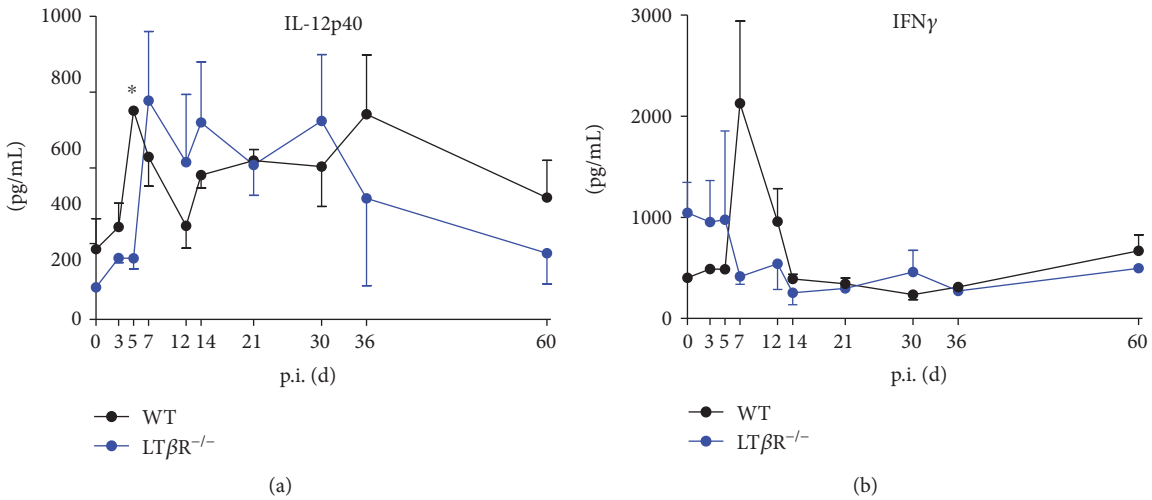


Figure 6: Cytokine production is disturbed in LTβR$^{-/-}$ animals. 50 μL of murine serum was collected from uninfected and infected WT and LTβR$^{-/-}$ animals (*T. gondii* (ME49), 40 cysts) on the days indicated. (a) IL-12p4 and (b) IFNγ amounts were determined by ELISA. ($n = 3$ in all cases except day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were analyzed). $^{*}p < 0.05$.

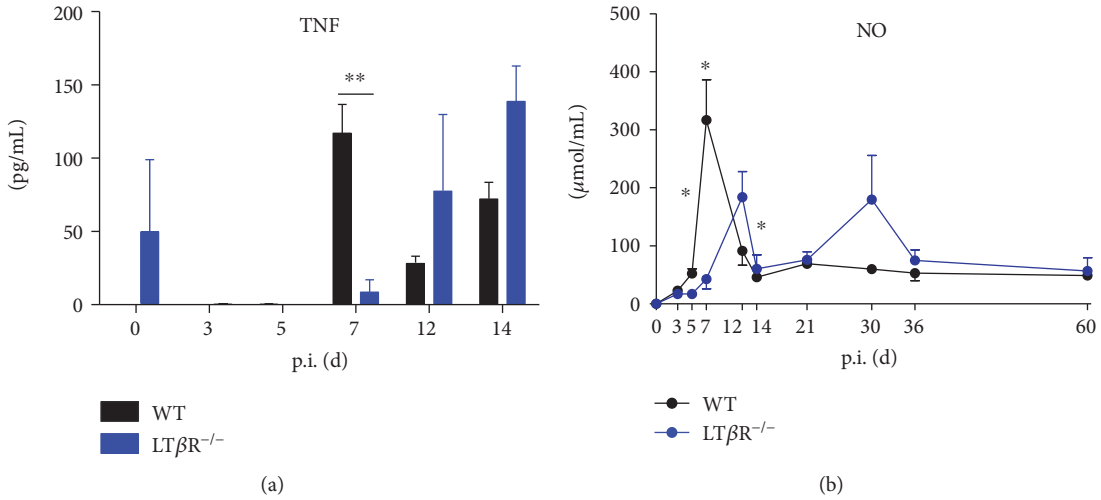


Figure 7: Compared to WT animals, LTβR$^{-/-}$ animals show delayed increase of TNFα in the serum in the acute phase of infection with *T. gondii*. 50 μL of murine serum was collected from uninfected and infected WT and LTβR$^{-/-}$ animals, TNFα levels were determined by ELISA (a), and nitric oxide levels were determined by colorimetric detection of nitrite after conversion of nitrate to nitrite (b). ($n = 3$ in all cases except d 0 (both genotypes) and d 14 (LTβR$^{-/-}$), where only 2 animals were analyzed). $^{*}p < 0.05$, $^{**}p < 0.01$.

12, but steeply increased on day 14 (60-fold), remained at this level until day 30, but then dropped to normal titers again by day 60. Expression patterns of LTα and LTβ were similar (Figures 8(g) and 8(h)): expression in WT animals exhibited a distinct peak on day 12 (approximately 8-fold for LTα and approximately 80-fold for LTβ), whereas expression in LTβR$^{-/-}$ animals was only moderately increased.

3.8. IFNγ-Induced Expression of mGBPs Is Strikingly Reduced in LTβR$^{-/-}$ Animals. mGBPs play an important role in the immune defense against *T. gondii* and are prominent IFNγ-induced genes [35]. Analysis of mGBP expression in the lung after *T. gondii* infection revealed a consistent picture (Figure 9). Generally, mGBP expression before infection tended to be lower in LTβR$^{-/-}$ animals. Early after infection, expression of most mGBPs was increased transiently, but markedly in WT animals. Exceptions were mGBP1 (Figure 9(a)), where a second increase of expression could be observed later in infection and mGBP7 (Figure 9(g)) where no increase of expression levels could be observed. In contrast, the expression of mGBPs in LTβR$^{-/-}$ animals either remained more or less at levels before infection (mGBP2, mGBP4, mGBP5, mGBP6, and mGBP9) or the increase was much lower (mGBP3 and mGBP8) or lower and delayed (mGBP1) when compared to WT animals. Similar to WT animals, no expression of mGBP7 could be observed in LTβR$^{-/-}$ animals. Analysis of spleen tissue showed a similar

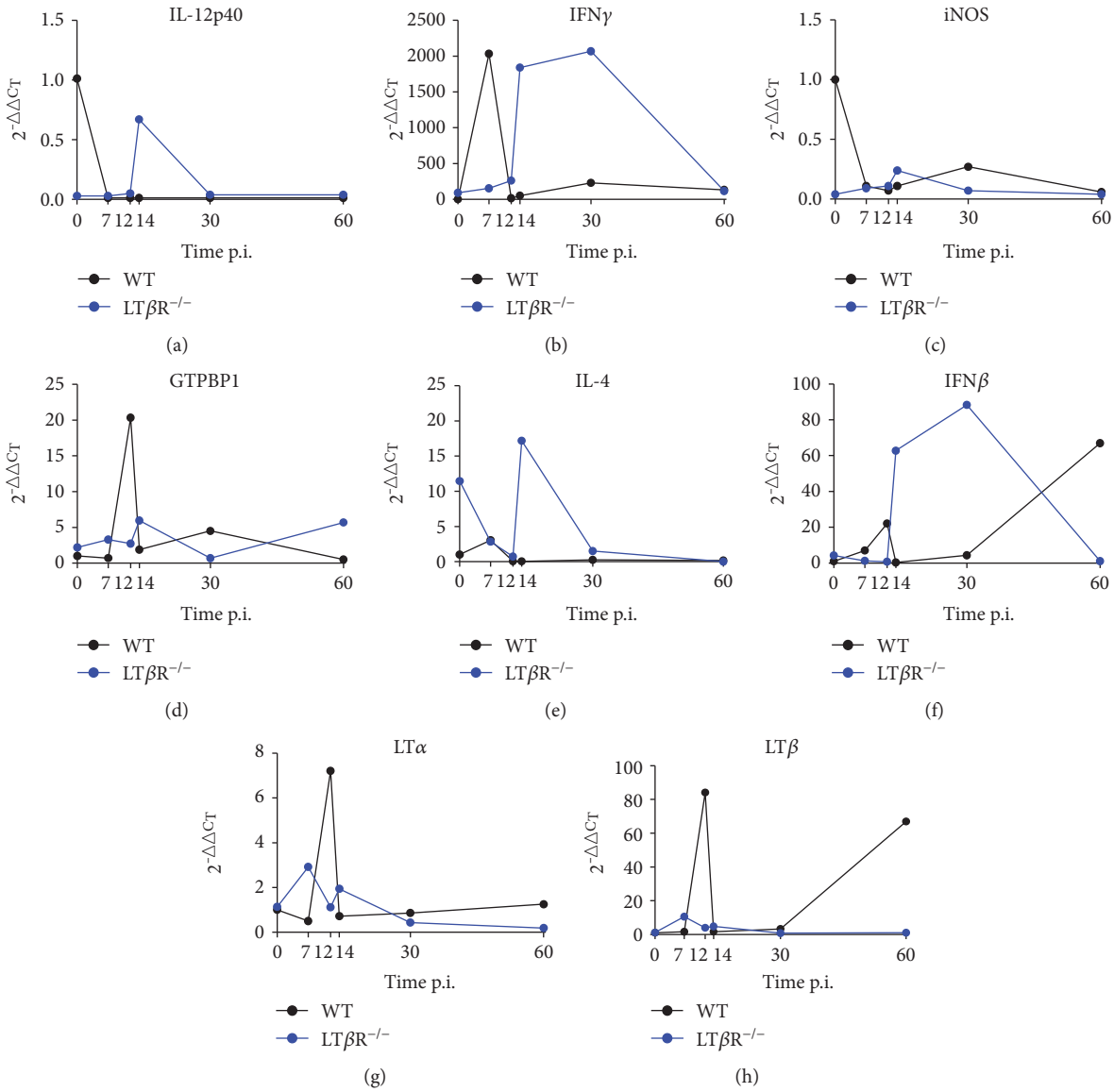


Figure 8: LTβR$^{-/-}$ animals show differential expression of immune relevant genes in the lung in comparison to WT animals after infection with *T. gondii* (ME49). Mice were sacrificed, RNA was isolated from the lungs from uninfected and infected WT and LTβR$^{-/-}$ animals on the days indicated, and expression levels were determined via quantitative RT-PCR. (a) IL-12p40, (b) IFNγ, (c) iNOS, (d) GTPBP1, (e) IL-4, (f) IFNβ, (g) LTα, and (h) LTβ. ($n = 3$ in all cases except day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were analyzed).

absence of mGBP expression in LTβR$^{-/-}$ animals compared to WT animals after *T. gondii* infection (data not shown). Taken together, these results strongly suggest that LTβR-initiated upregulation of immune relevant genes, most notably mGBPs, is essential for the survival of *T. gondii* infection.

4. Discussion

To date, there has been no evidence for a role of the LTβR in the immune defense to *T. gondii*. The present study clearly demonstrates substantially reduced overall survival of *T. gondii* infection in LTβR$^{-/-}$ mice which begins to succumb to the infection around day 12. Around 50% of the LTβR-deficient animals survive the acute phase of the *T. gondii* infection and are able to progress into the chronic phase of the disease before survival rates drop again. LTβR$^{-/-}$ mice fail to induce IFNγ, and mGBPs are subsequently not upregulated, leading to a breakdown of the antitoxoplasma immune response. These results point towards a major role for the LTβR in an efficient immune response to *T. gondii* and are in accordance with other studies suggesting that the LTβR acts as an important immune regulator, not only in bacterial infection models for listeriosis or tuberculosis [5, 43, 44] but also in intracellular parasite infection models for malaria [45, 46] or leishmaniasis [47–50]. The role of the LTβR in these disease models is quite diverse. In infection models with *L. monocytogenes* and *M. tuberculosis*, LTβR$^{-/-}$ mice not only show a delayed/abrogated activation of the innate immune response [5, 44] but also an absence of specific T cell responses [43]. In cutaneous leishmaniasis, the presence of peripheral lymph nodes (LN)

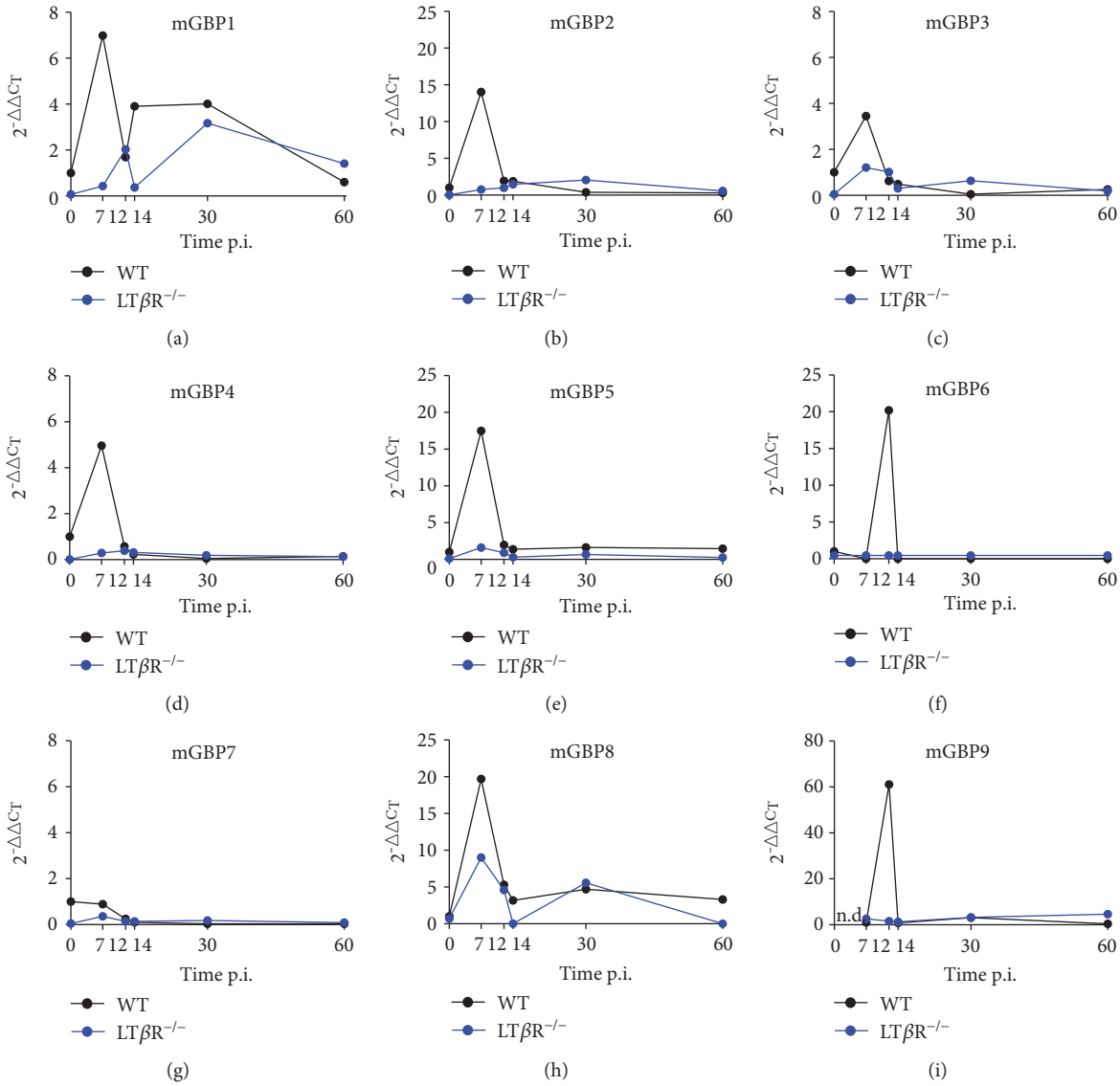


Figure 9: LTβR$^{-/-}$ animals show abrogated or delayed expression of mGBP genes in comparison to WT animals after infection with *T. gondii* (ME49). Mice were sacrificed, RNA was isolated from lungs from uninfected and infected WT and LTβR$^{-/-}$ animals on the days indicated, and expression levels were determined via quantitative RT-PCR. (a) mGBP1, (b) mGBP2, (c) mGBP3, (d) mGBP4, (e) mGBP5, (f) mGBP6, (g) mGBP7, (h) mGBP8, and (i) mGBP9 ($n = 3$ in all cases except day 30 and day 36 from LTβR$^{-/-}$ animals, where only 2 animals were analyzed).

is essential for driving a T_H1 response and the absence of all LN in LTβR$^{-/-}$ mice leads to a marked susceptibility to the disease [48], whereas in visceral leishmaniasis, signaling through the LTβR is protective via promoting DC development and maturation [47]. The current model is that the immune response to *T. gondii* is initiated by activation of DCs via TLR11/12 MyD88 interaction after recognition of the protozoan profilin-like protein [51]. Downstream signaling via the canonical NFκB pathway then leads to secretion of IL-12 by DCs which in turn induces NK cells to release IFNγ. Since LTβR signaling occurs via the classic and the alternative NFκB signaling pathway, it might be envisaged that LTβR$^{-/-}$ animals show delay in IL-12p40 secretion. Interestingly, Xu et al. [49] have demonstrated that blocking of LTβR signaling via HVEM-Ig or LTβR-Ig leads to defective IL12p40 production and increased susceptibility to *Leishmania major infection*. It can be speculated therefore that cooperation of LTβR and TNFRp55 signaling pathways is required for an efficient immune response to *T. gondii*. Since LT$\alpha_1\beta_2^{-/-}$ mice do not succumb to *L. major* infection, LIGHT seems to be the relevant LTβR ligand in this case. Therefore, the susceptibility of TNFRp55$^{-/-}$, LIGHT$^{-/-}$, and functional LTβR/TNFRp55 doubly deficient mice to *T. gondii* is being studied to evaluate to what extent either pathway and which ligands are required for an efficient immune response. Furthermore, imperfect DC differentiation might be responsible for a diminished IL-12 production (see below) in LTβR$^{-/-}$ mice [52]. Interestingly, expression of the LTβR is essential for the development of experimental cerebral malaria (ECM) after infection with *Plasmodium berghei* ANKA and

prolongs survival in LTβR$^{-/-}$-deficient mice due to their inability to generate an effective (CD8$^+$) T cell response, which is responsible for ECM pathophysiology [53, 54]. These findings are explained by the role that LTβR signaling plays in the development and homeostasis of the secondary lymphoid organs [40], its essential role in optimizing DC maturation and function, in supporting CD4 T cell maturation, and its ability to polarize T cells [52, 55]. IFN type I and type II have been shown to be important for survival of viral and nonviral infections [31, 56]. In the defense against MCMV, LTβR signaling has been demonstrated to initiate the type I IFN response [57, 58]. In listeria and mycobacteria infections, LTβR signaling has been shown to induce IFN type I and type II responses [5, 22, 44, 49, 59]. In toxoplasmosis, recognition of parasitic profilin via toll like receptors 11 and 12 is one of the major signals triggering IL-12 production in DC which in turn induces IFNγ production by NK cells [22, 60–62]. Here, in *T. gondii* infected LTβR$^{-/-}$ mice, a delayed increase of serum IL-12p40 and a failure to upregulate serum IFNγ levels could be demonstrated. IFNγ signaling is essential for an efficient antitoxoplasma immune response since neither IFN$\gamma^{-/-}$ nor IFNγR$^{-/-}$ mice are able to efficiently contain *T. gondii* infections and die early during the acute phase [62, 63]. IFNγ triggers several antiparasitic mechanisms including the induction of iNOS which leads to elevated levels of microbicidal NO and the induction of mGBP expression, both of which play an important role in the host defense against *T. gondii* [22, 35, 36, 64, 65]. LTβR$^{-/-}$ mice show a delayed increase of serum NO levels. Compared to WT mice, induction of mGBPs was virtually absent. Recently, members of the mGBP family have been shown to be important for survival after *T. gondii* infection [35–37, 39]. Interestingly, mGBPs are IFNγ and, to a lesser degree, IFN type I responsive genes [35]. Most mGBP proteins are rapidly recruited to the *T. gondii* parasitophorous vacuole in *T. gondii*-infected cells, and expression of at least mGBP2 is required for efficient elimination of the parasite [36, 39]. The marked failure of mGBP family member induction in LTβR$^{-/-}$ mice therefore provides an explanation for the high mortality observed. In addition, WT mice exhibit splenomegaly due to increased cell numbers in the spleen. In contrast, spleen weights and cell numbers increase to a significantly lesser degree in LTβR$^{-/-}$ mice. It has been described previously that LTα/β-LTβR signaling is activated in *T. gondii*-infected WT mice and may, at least in part, be responsible for modulating spleen architecture and organization via chemokine modulation [66]. It has been shown that in LTβR$^{-/-}$ mice, peripheral lymphoid organs, Peyer's patches, and gut-associated lymphoid tissue are absent [40]. Furthermore, dendritic cell (DC) maturation is impaired in these animals [52, 67, 68]. To address the question whether the susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection is due to the lack of adequate priming of immature T cells by DC, further studies are required, for example, using bone marrow chimera models [69]. In addition, since LTβR$^{-/-}$ animals also lack B cell follicles in the spleen [40, 70], it will be interesting to see whether these mice are able to mount a *T. gondii*-specific antibody response and develop an antigen-specific T cell response. The failure to mount an effective specific T and B cell response against *T. gondii* and the possible inability to drive the parasite into its chronic stage and/or to prevent reactivation of chronic toxoplasmosis might explain the higher parasite numbers observed in the brains of LTβR$^{-/-}$ animals and concurs with the increased parasitemia described in LTβR$^{-/-}$ animals in the ECM model by other groups [53, 54]. Taken together, this underscores the importance of LTβR signaling in innate as well as adaptive immunity. We therefore speculate that LTβR signaling is necessary for either driving *T. gondii* infection into the chronic stage or maintaining this chronic stage, and further analysis of the role of the LTβR in this context may lead to a better understanding of the mechanisms of *T. gondii* stage conversion.

5. Conclusions

These data demonstrate that beyond being responsible for the development of secondary lymphatic organs, which provide the environment required to mount an efficient adaptive immune response, LTβR signaling modulates these responses which are important for establishing and maintaining chronic toxoplasmosis and the LTβR is necessary, via inducing an IFN type II response, for initiating innate effector mechanisms essential for containing acute *T. gondii* infection.

Abbreviations

AST: Aspartate transaminase
DC: Dendritic cell
ECM: Experimental cerebral malaria
GBP: Guanylate-binding protein
GTPBP1: GTP-binding protein 1
iNOS: Induced nitric oxide synthase
IRG: Immunity-related gene 1
LDH: Lactate dehydrogenase
LN: Lymph node
LT: Lymphotoxin
LTβR: Lymphotoxin beta receptor
mGBP: Murine guanylate-binding protein
p.i.: Post infection
TNFR: TNF receptor
WT: Wild type.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Kristina Behnke and Ursula R. Sorg contributed equally to this work.

Acknowledgments

The authors thank Nicole Küpper, Julia Hartmann, and Iris Niepel for expert technical assistance. This work was supported by the German Research Council (CRC 974 and RTG 1045) and the Jürgen Manchot Foundation.

References

[1] T. Hehlgans and K. Pfeffer, "The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games," *Immunology*, vol. 115, no. 1, pp. 1–20, 2005.

[2] D. Schluter, L. Y. Kwok, S. Lütjen et al., "Both lymphotoxin-alpha and TNF are crucial for control of Toxoplasma gondii in the central nervous system," *Journal of Immunology*, vol. 170, no. 12, pp. 6172–6182, 2003.

[3] R. Endres, A. Luz, H. Schulze et al., "Listeriosis in p47(phox−/−) and TRp55−/− mice: protection despite absence of ROI and susceptibility despite presence of RNI," *Immunity*, vol. 7, no. 3, pp. 419–432, 1997.

[4] K. Pfeffer, T. Matsuyama, T. M. Kündig et al., "Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection," *Cell*, vol. 73, no. 3, pp. 457–467, 1993.

[5] S. Ehlers, C. Hölscher, S. Scheu et al., "The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens mycobacterium tuberculosis and *Listeria monocytogenes*," *Journal of Immunology*, vol. 170, no. 10, pp. 5210–5218, 2003.

[6] R. M. Locksley, N. Killeen, and M. J. Lenardo, "The TNF and TNF receptor superfamilies: integrating mammalian biology," *Cell*, vol. 104, no. 4, pp. 487–501, 2001.

[7] C. A. Hunter and L. D. Sibley, "Modulation of innate immunity by Toxoplasma gondii virulence effectors," *Nature Reviews. Microbiology*, vol. 10, no. 11, pp. 766–778, 2012.

[8] M. Deckert-Schluter, H. Bluethmann, A. Rang, H. Hof, and D. Schlüter, "Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis," *Journal of Immunology*, vol. 160, no. 7, pp. 3427–3436, 1998.

[9] T. A. Banks, S. Rickert, and C. F. Ware, "Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus," *Immunologic Research*, vol. 34, no. 3, pp. 243–254, 2006.

[10] L. E. Kemp, M. Yamamoto, and D. Soldati-Favre, "Subversion of host cellular functions by the apicomplexan parasites," *FEMS Microbiology Reviews*, vol. 37, no. 4, pp. 607–631, 2013.

[11] A. M. Tenter, A. R. Heckeroth, and L. M. Weiss, "Toxoplasma gondii: from animals to humans," *International Journal for Parasitology*, vol. 30, no. 12-13, pp. 1217–1258, 2000.

[12] J. P. Dubey, "Toxoplasmosis," *The Veterinary Clinics of North America. Small Animal Practice*, vol. 17, no. 6, pp. 1389–1404, 1987.

[13] D. H. M. Joynson and T. G. Wreghitt, *Toxoplasmosis: A Comprehensive Clinical Guide*, Cambridge University Press, Cambridge, UK, 2001.

[14] C. D. Dupont, D. A. Christian, and C. A. Hunter, "Immune response and immunopathology during toxoplasmosis," *Seminars in Immunopathology*, vol. 34, no. 6, pp. 793–813, 2012.

[15] J. G. Montoya and O. Liesenfeld, "Toxoplasmosis," *Lancet*, vol. 363, no. 9425, pp. 1965–1976, 2004.

[16] G. Saadatnia and M. Golkar, "A review on human toxoplasmosis," *Scandinavian Journal of Infectious Diseases*, vol. 44, no. 11, pp. 805–814, 2012.

[17] Y. Suzuki, S. Y. Wong, F. C. Grumet et al., "Evidence for genetic regulation of susceptibility to toxoplasmic encephalitis in AIDS patients," *The Journal of Infectious Diseases*, vol. 173, no. 1, pp. 265–268, 1996.

[18] R. McLeod, K. M. Boyer, D. Lee et al., "Prematurity and severity are associated with Toxoplasma gondii alleles (NCCCTS, 1981-2009)," *Clinical Infectious Diseases*, vol. 54, no. 11, pp. 1595–1605, 2012.

[19] A. C. Lepage, D. Buzoni-Gatel, D. T. Bout, and L. H. Kasper, "Gut-derived intraepithelial lymphocytes induce long term immunity against Toxoplasma gondii," *Journal of Immunology*, vol. 161, no. 9, pp. 4902–4908, 1998.

[20] O. Liesenfeld, "Immune responses to Toxoplasma gondii in the gut," *Immunobiology*, vol. 201, no. 2, pp. 229–239, 1999.

[21] E. Y. Denkers and R. T. Gazzinelli, "Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection," *Clinical Microbiology Reviews*, vol. 11, no. 4, pp. 569–588, 1998.

[22] F. Yarovinsky, "Innate immunity to toxoplasma gondii infection," *Nature Reviews Immunology*, vol. 14, no. 2, pp. 109–121, 2014.

[23] F. Plattner, F. Yarovinsky, S. Romero et al., "Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response," *Cell Host & Microbe*, vol. 3, no. 2, pp. 77–87, 2008.

[24] F. Debierre-Grockiego, M. A. Campos, N. Azzouz et al., "Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii," *Journal of Immunology*, vol. 179, no. 2, pp. 1129–1137, 2007.

[25] F. Yarovinsky, D. Zhang, J. F. Andersen et al., "TLR11 activation of dendritic cells by a protozoan profilin-like protein," *Science*, vol. 308, no. 5728, pp. 1626–1629, 2005.

[26] I. A. Khan, T. Matsuura, and L. H. Kasper, "Interleukin-12 enhances murine survival against acute toxoplasmosis," *Infection and Immunity*, vol. 62, no. 5, pp. 1639–1642, 1994.

[27] R. T. Gazzinelli, S. Hieny, T. A. Wynn, S. Wolf, and A. Sher, "Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 13, pp. 6115–6119, 1993.

[28] K. Heseler, K. Spekker, S. K. Schmidt, M. K. CR, and W. Däubener, "Antimicrobial and immunoregulatory effects mediated by human lung cells: role of IFN-gamma-induced tryptophan degradation," *FEMS Immunology and Medical Microbiology*, vol. 52, no. 2, pp. 273–281, 2008.

[29] E. R. Pfefferkorn, "Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 3, pp. 908–912, 1984.

[30] T. M. Scharton-Kersten, G. Yap, J. Magram, and A. Sher, "Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii," *The Journal of Experimental Medicine*, vol. 185, no. 7, pp. 1261–1273, 1997.

[31] C. Bogdan, J. Mattner, and U. Schleicher, "The role of type I interferons in non-viral infections," *Immunological Reviews*, vol. 202, pp. 33–48, 2004.

[32] J. C. Howard, J. P. Hunn, and T. Steinfeldt, "The IRG protein-based resistance mechanism in mice and its relation to virulence in Toxoplasma gondii," *Current Opinion in Microbiology*, vol. 14, no. 4, pp. 414–421, 2011.

[33] G. A. Taylor, C. G. Feng, and A. Sher, "Control of IFN-gamma-mediated host resistance to intracellular pathogens

by immunity-related GTPases (p47 GTPases)," *Microbes and Infection*, vol. 9, no. 14-15, pp. 1644–1651, 2007.

[34] G. A. Taylor, C. G. Feng, and A. Sher, "p47 GTPases: regulators of immunity to intracellular pathogens," *Nature Reviews Immunology*, vol. 4, no. 2, pp. 100–109, 2004.

[35] D. Degrandi, C. Konermann, C. Beuter-Gunia et al., "Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense," *Journal of Immunology*, vol. 179, no. 11, pp. 7729–7740, 2007.

[36] D. Degrandi, E. Kravets, C. Konermann et al., "Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 1, pp. 294–299, 2013.

[37] E. Kravets, D. Degrandi, S. Weidtkamp-Peters et al., "The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular localization and recruitment to the parasitophorous vacuole of Toxoplasma gondii," *The Journal of Biological Chemistry*, vol. 287, no. 33, pp. 27452–27466, 2012.

[38] K. Spekker, M. Leineweber, D. Degrandi et al., "Antimicrobial effects of murine mesenchymal stromal cells directed against Toxoplasma gondii and Neospora caninum: role of immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs)," *Medical Microbiology and Immunology*, vol. 202, no. 3, pp. 197–206, 2013.

[39] M. Yamamoto, M. Okuyama, J. S. Ma et al., "A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii," *Immunity*, vol. 37, no. 2, pp. 302–313, 2012.

[40] A. Futterer, K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer, "The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues," *Immunity*, vol. 9, no. 1, pp. 59–70, 1998.

[41] G. Reichmann, W. Walker, E. N. Villegas et al., "The CD40/CD40 ligand interaction is required for resistance to toxoplasmic encephalitis," *Infection and Immunity*, vol. 68, no. 3, pp. 1312–1318, 2000.

[42] L. B. Adams, J. B. Hibbs Jr., R. R. Taintor, and J. L. Krahenbuhl, "Microbiostatic effect of murine-activated macrophages for Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine," *Journal of Immunology*, vol. 144, no. 7, pp. 2725–2729, 1990.

[43] M. Kursar, N. Jänner, K. Pfeffer, V. Brinkmann, S. H. Kaufmann, and H. W. Mittrücker, "Requirement of secondary lymphoid tissues for the induction of primary and secondary T cell responses against *Listeria monocytogenes*," *European Journal of Immunology*, vol. 38, no. 1, pp. 127–138, 2008.

[44] S. Kutsch, D. Degrandi, and K. Pfeffer, "Immediate lymphotoxin beta receptor-mediated transcriptional response in host defense against *L. monocytogenes*," *Immunobiology*, vol. 213, no. 3-4, pp. 353–366, 2008.

[45] J. Krucken, J. V. Braun, M. A. Dkhil, A. Grunwald, and F. Wunderlich, "Deletion of LTbetaR augments male susceptibility to Plasmodium chabaudi," *Parasite Immunology*, vol. 27, no. 6, pp. 205–212, 2005.

[46] L. M. Randall and C. R. Engwerda, "TNF family members and malaria: old observations, new insights and future directions," *Experimental Parasitology*, vol. 126, no. 3, pp. 326–331, 2010.

[47] A. C. Stanley, F. de Labastida Rivera, A. Haque et al., "Critical roles for LIGHT and its receptors in generating T cell-mediated immunity during Leishmania donovani infection," *PLoS Pathogens*, vol. 7, no. 10, article e1002279, 2011.

[48] J. M. Ehrchen, J. Roth, K. Roebrock et al., "The absence of cutaneous lymph nodes results in a Th2 response and increased susceptibility to Leishmania major infection in mice," *Infection and Immunity*, vol. 76, no. 9, pp. 4241–4250, 2008.

[49] G. Xu, D. Liu, I. Okwor et al., "LIGHT is critical for IL-12 production by dendritic cells, optimal CD4+ Th1 cell response, and resistance to Leishmania major," *Journal of Immunology*, vol. 179, no. 10, pp. 6901–6909, 2007.

[50] S. de Kossodo, G. E. Grau, T. Daneva et al., "Tumor necrosis factor alpha is involved in mouse growth and lymphoid tissue development," *The Journal of Experimental Medicine*, vol. 176, no. 5, pp. 1259–1264, 1992.

[51] B. Hou, A. Benson, L. Kuzmich, D. F. AL, and F. Yarovinsky, "Critical coordination of innate immune defense against Toxoplasma gondii by dendritic cells responding via their toll-like receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 1, pp. 278–283, 2011.

[52] Y. G. Wang, K. D. Kim, J. Wang, P. Yu, and Y. X. Fu, "Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion," *Journal of Immunology*, vol. 175, no. 10, pp. 6997–7002, 2005.

[53] D. Togbe, P. L. de Sousa, M. Fauconnier et al., "Both functional LTbeta receptor and TNF receptor 2 are required for the development of experimental cerebral malaria," *PLoS One*, vol. 3, no. 7, article e2608, 2008.

[54] L. M. Randall, F. H. Amante, Y. Zhou et al., "Cutting edge: selective blockade of LIGHT-lymphotoxin beta receptor signaling protects mice from experimental cerebral malaria caused by Plasmodium berghei ANKA," *Journal of Immunology*, vol. 181, no. 11, pp. 7458–7462, 2008.

[55] V. Upadhyay and Y. X. Fu, "Lymphotoxin signalling in immune homeostasis and the control of microorganisms," *Nature Reviews Immunology*, vol. 13, no. 4, pp. 270–279, 2013.

[56] H. Hengel, U. H. Koszinowski, and K. K. Conzelmann, "Viruses know it all: new insights into IFN networks," *Trends in Immunology*, vol. 26, no. 7, pp. 396–401, 2005.

[57] K. Schneider, A. Loewendorf, C. De Trez et al., "Lymphotoxin-mediated crosstalk between B cells and splenic stroma promotes the initial type I interferon response to cytomegalovirus," *Cell Host & Microbe*, vol. 3, no. 2, pp. 67–76, 2008.

[58] T. A. Banks, S. Rickert, C. A. Benedict et al., "A lymphotoxin-IFN-beta axis essential for lymphocyte survival revealed during cytomegalovirus infection," *Journal of Immunology*, vol. 174, no. 11, pp. 7217–7225, 2005.

[59] J. L. Gommerman, J. L. Browning, and C. F. Ware, "The lymphotoxin network: orchestrating a type I interferon response to optimize adaptive immunity," *Cytokine & Growth Factor Reviews*, vol. 25, no. 2, pp. 139–145, 2014.

[60] R. Pifer and F. Yarovinsky, "Innate responses to Toxoplasma gondii in mice and humans," *Trends in Parasitology*, vol. 27, no. 9, pp. 388–393, 2011.

[61] F. Yarovinsky, S. Hieny, and A. Sher, "Recognition of Toxoplasma gondii by TLR11 prevents parasite-induced immunopathology," *Journal of Immunology*, vol. 181, no. 12, pp. 8478–8484, 2008.

[62] T. M. Scharton-Kersten, T. A. Wynn, E. Y. Denkers et al., "In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to

control acute infection," *Journal of Immunology*, vol. 157, no. 9, pp. 4045–4054, 1996.

[63] M. Deckert-Schluter, A. Rang, D. Weiner et al., "Interferon-gamma receptor-deficiency renders mice highly susceptible to toxoplasmosis by decreased macrophage activation," *Laboratory Investigation: A Journal of Technical Methods and Pathology*, vol. 75, no. 6, pp. 827–841, 1996.

[64] D. Schluter, M. Deckert-Schlüter, E. Lorenz, T. Meyer, M. Röllinghoff, and C. Bogdan, "Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in Toxoplasma gondii-susceptible C57BL/6 mice but does not reactivate the latent disease in T. gondii-resistant BALB/c mice," *Journal of Immunology*, vol. 162, no. 6, pp. 3512–3518, 1999.

[65] J. MacMicking, Q. W. Xie, and C. Nathan, "Nitric oxide and macrophage function," *Annual Review of Immunology*, vol. 15, pp. 323–350, 1997.

[66] A. Glatman Zaretsky, J. S. Silver, M. Siwicki, A. Durham, C. F. Ware, and C. A. Hunter, "Infection with Toxoplasma gondii alters lymphotoxin expression associated with changes in splenic architecture," *Infection and Immunity*, vol. 80, no. 10, pp. 3602–3610, 2012.

[67] C. De Trez, "Lymphotoxin-beta receptor expression and its related signaling pathways govern dendritic cell homeostasis and function," *Immunobiology*, vol. 217, no. 12, pp. 1250–1258, 2012.

[68] K. Kabashima, T. A. Banks, K. M. Ansel, T. T. Lu, C. F. Ware, and J. G. Cyster, "Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells," *Immunity*, vol. 22, no. 4, pp. 439–450, 2005.

[69] K. Abe, F. O. Yarovinsky, T. Murakami et al., "Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo," *Blood*, vol. 101, no. 4, pp. 1477–1483, 2003.

[70] R. Endres, M. B. Alimzhanov, T. Plitz et al., "Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells," *The Journal of Experimental Medicine*, vol. 189, no. 1, pp. 159–168, 1999.

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 8952878, 10 pages
https://doi.org/10.1155/2017/8952878

Research Article

Relationship between the Antifungal Susceptibility Profile and the Production of Virulence-Related Hydrolytic Enzymes in Brazilian Clinical Strains of *Candida glabrata*

Maria Helena Galdino Figueiredo-Carvalho,[1] Lívia de Souza Ramos,[2] Leonardo Silva Barbedo,[1] Jean Carlos Almeida de Oliveira,[1] André Luis Souza dos Santos,[2] Rodrigo Almeida-Paes,[1] and Rosely Maria Zancopé-Oliveira[1]

[1]*Laboratório de Micologia, Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil*
[2]*Laboratório de Investigação de Peptidases, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Rosely Maria Zancopé-Oliveira; rosely.zancope@ini.fiocruz.br

Received 16 November 2016; Revised 6 March 2017; Accepted 16 April 2017; Published 26 July 2017

Academic Editor: Ildinete Silva-Pereira

Candida glabrata is a facultative intracellular opportunistic fungal pathogen in human infections. Several virulence-associated attributes are involved in its pathogenesis, host-pathogen interactions, modulation of host immune defenses, and regulation of antifungal drug resistance. This study evaluated the in vitro antifungal susceptibility profile to five antifungal agents, the production of seven hydrolytic enzymes related to virulence, and the relationship between these phenotypes in 91 clinical strains of *C. glabrata*. All *C. glabrata* strains were susceptible to flucytosine. However, some of these strains showed resistance to amphotericin B (9.9%), fluconazole (15.4%), itraconazole (5.5%), or micafungin (15.4%). Overall, *C. glabrata* strains were good producers of catalase, aspartic protease, esterase, phytase, and hemolysin. However, caseinase and phospholipase in vitro activities were not detected. Statistically significant correlations were identified between micafungin minimum inhibitory concentration (MIC) and esterase production, between fluconazole and micafungin MIC and hemolytic activity, and between amphotericin B MIC and phytase production. These results contribute to clarify some of the *C. glabrata* mechanisms of pathogenicity. Moreover, the association between some virulence attributes and the regulation of antifungal resistance encourage the development of new therapeutic strategies involving virulence mechanisms as potential targets for effective antifungal drug development for the treatment of *C. glabrata* infections.

1. Introduction

Candida glabrata is a facultative intracellular opportunistic fungal pathogen, with the ability to survive and replicate in several cell types, such as osteoblasts [1], neutrophils [2], and macrophages [3]. This yeast can be isolated from different areas of the human body such as mouth, gastrointestinal tract, and vaginal mucosa, without causing disease in most individuals [4]. Nevertheless, due to the increased use of immunosuppressive drugs and the advent of AIDS, the frequency of *C. glabrata* infections has significantly increased worldwide in the last years [5–8].

In the last decade, two new species phenotypically related to *C. glabrata* have been described in the literature: *Candida nivariensis* and *Candida bracarensis*. These three species are phenotypically indistinguishable, but genetically heterogeneous [9, 10]. It is necessary to periodically monitor the *C. glabrata* species complex in order to determine the frequency of these clinically relevant *Candida* species, their geographical distribution, their virulence attributes, and their propensity to harbor antifungal resistance mechanisms [11, 12].

The therapeutic and prophylactic use of azole antifungals administered for prolonged periods to invasive candidiasis treatment, especially in immunocompromised patients, has

contributed to the increase phenomenon of resistance in *C. glabrata* [5, 7, 13]. The echinocandins have emerged as preferred agents for most episodes of candidemia and invasive candidiasis according to the recent guideline for the management of candidiasis [14]. Nevertheless, echinocandin resistance is increasing in *C. glabrata* [15], including among fluconazole-resistant isolates [5, 15, 16].

The pathogenicity of *Candida* spp. is facilitated by expression on several virulence-associated factors, especially the adherence to host cells, the ability to form biofilms, the resistance to hydrogen peroxide and derivatives, and the capacity to produce and secrete hydrolytic enzymes, particularly proteases, phospholipases, and hemolysins [17, 18]. In comparison with *C. albicans,* there are fewer studies about the potential virulence attributes produced by *C. glabrata*.

The present study aimed to evaluate the in vitro antifungal susceptibility profile, the production of hydrolytic enzymes, and the relationship between these phenotypes in a collection of *C. glabrata* clinical strains isolated from Brazilian hospitals.

2. Materials and Methods

2.1. Fungal Strains. A total of 91 yeast strains, collected between 1998 and 2015 in two tertiary hospitals located in Rio de Janeiro, Brazil, and preliminarily identified by the API 20C AUX (bioMérieux, France) as *C. glabrata*, were included in this study. Strains were isolated from several clinical specimens, such as gastric aspirate ($n = 1$); renal abscess secretion ($n = 1$); pleural fluid ($n = 1$); secretion of surgical drain ($n = 1$); secretion of postoperative wound ($n = 1$); ascitic fluid ($n = 2$); abdominal secretion ($n = 3$); peritoneal fluid ($n = 4$); sputum ($n = 4$); venous catheter ($n = 4$); bronchoalveolar lavage ($n = 5$); vaginal secretion ($n = 7$); feces ($n = 9$); tracheal secretion ($n = 10$); urine ($n = 13$); and blood ($n = 25$). Before the experiments, these clinical strains were recovered from storage (−20°C) and grown on Sabouraud Dextrose Agar and CHROMagar *Candida* medium (both at 37°C for 48 h) in order to evaluate their viability and purity, respectively. The phenotypic confirmation of the species after storage was achieved by a biochemical analysis with the Vitek 2 system (bioMérieux, Marcy-L'Etoile, France) using the YST card according to the manufacturer's guidelines. In addition, *C. glabrata* ATCC 2001 type strain was included as a control strain in all experiments.

2.2. Molecular Identification. Yeast cells obtained from pure colonies were recovered from Sabouraud Dextrose Agar and used for DNA extraction with the Gentra® Puregene® Yeast and G+ Bacteria Kit (Qiagen®). The strains were identified by sequencing the ITS1-5.8S-ITS2 region of the rDNA as previously described [9], using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Sequences were edited using the Sequencher™ version 4.9 and compared by BLAST with sequences available from the NCBI/GenBank database.

2.3. Antifungal Susceptibility Testing. In vitro antifungal susceptibility testing was performed according to the recommendations proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 protocol [19]. Amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), micafungin (MCF), and 5-flucytosine (5-FC) (Sigma-Aldrich Chemical Corporation, St. Louis, MO, USA) were tested. Briefly, RPMI 1640 medium with L-glutamine and without bicarbonate (Gibco BRL, Life Technologies, Woerden, The Netherlands), buffered with 0.165 M 3-*N*-morpholinepropanesulfonic acid (MOPS), pH 7.0, was used for the broth microdilution test. Two-fold dilutions of the drugs were performed and distributed in 96-well flat bottom plates in concentrations ranging from 64–0.125 μg/mL for FLC and 5-FC, 8–0.015 μg/mL for AMB and ITC, or 4–0.008 μg/mL for MCF. The fungal inoculum was prepared from a 24 h Sabouraud Dextrose Agar culture incubated at 35°C; the cells were harvested in RPMI medium and diluted to about $1–5 \times 10^3$ cells/mL. The plates were incubated at 35°C for 24 h. The minimal inhibitory concentrations (MIC) of the drugs were determined according to the CLSI M27-A3 recommendations [19]; and the MIC values for AMB, ITC, and 5-FC were interpreted following the CLSI M27-S3 protocol; and the MIC values for FLC and MCF were interpreted according to the CLSI M27-S4 protocol [20, 21]. MICs were validated after a second experiment performed under the same conditions with the same MIC value verified for each strain.

2.4. Production of Hydrolytic Enzymes. The production of hydrolytic enzymes was carried out in agar plate assays as described previously by Price et al. [22]. Briefly, the aspartic protease activity was determined using 1.17% yeast carbon base medium supplemented with 0.2% bovine serum albumin according to Rüchel et al. [23]. Caseinase activity was assessed using Sabouraud Dextrose Agar provided with 1% casein as previously described by Ziccardi et al. [24]. The determination of phospholipase activity was performed using the egg yolk agar plate method (2% glucose, 1% peptone, 0.5% yeast extract, 4% NaCl, 0.074% $CaCl_2$, 1.5% agar, then, 2% of fresh egg yolk was added to the medium) as previously described by Price et al. [22]. The esterase production was assayed using the Tween agar plate (0.5% yeast extract, 1% peptone, 0.01% $CaCl_2$, 1.5% agar, and 0.1% Tween 80, pH 7.0) according to Aktas et al. [25]. Phytase activity was evaluated using the calcium phytate agar (1% glucose, 0.05% $(NH_4)_2SO_4$, 0.02% KCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.2% calcium phytate, 0.05% yeast extract, 0.0005% $MnSO_4$, 0.0005% $FeSO_4$, and 1.5% agar, pH 7.0) according to Tsang [26]. The hemolytic activity was evaluated in a commercial blood agar plate assay (Plast Labor, Brazil). To determine enzymatic activities, aliquots (10 μl) of 48 h old cultured fungal cells (10^7 cells) were spotted on the surface of each agar medium and incubated at 37°C for up to 7 days. The colony diameter (a) and the diameter of the colony plus the precipitation zone (b) were measured by a graduated ruler, and the enzymatic activities were expressed as *Pz* value (a/b) as previously described [22]. The *Pz* value was scored into four categories: *Pz* of 1.0 indicated no enzymatic activity; *Pz* between 0.999 and 0.700 indicated weak producers; *Pz* between 0.699 and 0.400 corresponded to good producers; and *Pz* lower than 0.399 meant excellent producers [22].

Determination of catalase activity was performed using a semiquantitative assay with slight modifications according to Metchock et al. [27]. In brief, screw-cap tubes containing Sabouraud Dextrose Agar medium were inoculated with 200 μL of a suspension of *C. glabrata* cells corresponding to the 0.5 McFarland standard and incubated at 37°C for 48 h. After this incubation, 1 mL of a freshly prepared 1 : 1 mixture of 10% Tween 80 and 30% hydrogen peroxide was added to the cultures. The column bubble was measured in millimeters after 5 min at room temperature. Uninoculated medium was used as a negative control. A column of bubbles of <45 mm was classified as low catalase producers, while a column bubble of >45 mm was classified as high catalase producers [27].

Since media and conditions may play a key role in the gene expression of the enzymes studied, all enzymatic tests were performed with culture media prepared from a single bottle and tested using the same equipment. Moreover, all the tests for the determination of production of hydrolytic enzymes were performed in duplicate, and results of enzymatic activities are presented as mean ± standard deviation (SD).

2.5. Statistical Analysis. The statistical analyses were performed with the GraphPad Prism 5 computer software®. The correlation between MIC values and the enzymatic activity was performed using the Spearman's rank correlation, since the variables do not meet the bivariate normal distribution assumption. The strength of the relationship between paired data was interpreted through the Spearman's correlation coefficient (r_s) analysis, where the closer r_s is to ±1, the stronger the relationship. Additionally, strains were grouped according to their susceptibility profile (susceptible-dose dependent/resistant for FLC; susceptible/nonsusceptible for other drugs), and the median value of the enzymatic activity of each group was compared using the Mann–Whitney *U* test. *P* values of 0.05 or less were considered to be statistically significant in all tests.

3. Results

3.1. Phenotypic and Molecular Identification of Fungal Strains. All the 91 clinical yeast strains produced colonies with a coppery pigment and smooth texture on chromogenic CHROMagar *Candida* medium, and contamination or mixed colonies were not detected. According to the biochemical analysis by the Vitek 2 system, these strains were identified as *C. glabrata* with an average probability of 98%.

Moreover, all the 91 yeast strains were identified through sequencing of ITS1-5.8S-ITS2 region of the rDNA. These clinical strains showed 99-100% similarity when compared to the *C. glabrata* AY939793 sequence deposited in the GenBank database, thus confirming their identity as *C. glabrata*. No *C. nivariensis* or *C. bracarensis* was found in this study. The obtained sequences to ITS1-5.8S-ITS2 region of the clinical strains were deposited in GenBank under the accession numbers KX450781-KX450814, KX450816-KX450833, KX450835-KX450861, and KX450863-KX450874.

3.2. Susceptibility of C. glabrata against Five Antifungal Drugs. Concerning the antifungal susceptibility profile (Table 1), all the 91 clinical strains of *C. glabrata* were susceptible to 5-FC. However, some of these strains showed resistance to AMB, FLC, ITC, or MCF. In brief, nine *C. glabrata* strains (9.9%) were likely to be resistant to AMB as follows: five strains exhibited MIC of 2 μg/mL, one strain presented MIC of 4 μg/mL, and three strains exhibited MIC of 8 μg/ml to this polyene agent. FLC was the azole with the highest number of resistant strains (MIC ≥ 64 μg/mL). A total of 14 strains (15.4%) were resistant to FLC, whereas five (5.5%) presented resistance to ITC. Fourteen strains of *C. glabrata* (15.4%) exhibited MIC > 0.12 μg/mL to MCF.

The *C. glabrata* ATCC 2001 type strain was classified as susceptible-dose dependent to FLC (MIC of 8 μg/mL) and susceptible to AMB, ITC, MCF, and 5-FC (MIC of 0.12, 0.06, 0.06, and 0.12 μg/mL, resp.).

Eleven of the 91 strains tested (12.1%) were classified as resistant to at least two antifungal drugs. Table 2 summarizes the resistance profile of the Brazilian tested clinical strains of *C. glabrata*.

Association between resistance and the clinical origin of strains or year of isolation was not detected for any of the tested antifungal drugs ($P > 0.05$).

3.3. Production of Hydrolytic Enzymes. In this set of experiments, the in vitro abilities of the *C. glabrata* to produce proteases (aspartic protease and caseinase), phospholipase, esterase, phytase, hemolysin, and catalase were evaluated. Phospholipase and caseinase activities were not detected under the employed experimental conditions for any of the tested strains. Eighty-seven strains of *C. glabrata* (95.6%) were able to produce aspartic protease (*Pz* ranging from 0.100 to 0.583), while four strains (4.4%) showed no enzymatic activity for this hydrolytic enzyme ($Pz = 1.0$). The clinical strains of *C. glabrata* producing aspartic protease were classified as follows: 30 clinical strains (33.0%) were considered excellent producers (*Pz* ranging from 0.100 to 0.395), and 57 clinical strains (62.6%) were classified as good producers (*Pz* ranging from 0.400 to 0.583).

Esterase was detected in 51 *C. glabrata* strains (56.0%), being one strain (1.1%) classified as excellent esterase producer (*Pz* mean = 0.393 ± 0.050), 48 strains (52.7%) were considered good producers (*Pz* ranging from 0.414 to 0.667), and two strains (2.2%) were considered weak producers (*Pz* ranging from 0.762 to 0.800).

Regarding the phytase production, all the strains were positive (*Pz* ranging from 0.114 to 0.762), in which 10 strains (11.0%) were considered excellent producers (*Pz* ranging from 0.114 to 0.380), 80 strains (87.9%) were classified as good producers (*Pz* ranging from 0.400 to 0.692), and one strain (1.1%) was considered weak phytase producer (*Pz* mean = 0.762 ± 0.050).

Hemolytic activity was observed in 90 *C. glabrata* strains (98.9%), being one strain (1.1%) considered excellent producer of hemolysins (*Pz* mean = 0.385 ± 0.000), 82 strains (90.1%) classified as good producers (*Pz* ranging from 0.409 to 0.688), and seven strains (7.7%) were considered weak producers (*Pz* ranging from 0.722 to 0.795).

Table 1: In vitro antifungal susceptibility profile in 91 Brazilian clinical strains of *Candida glabrata*.

Antifungals	MIC (μg/mL)			CLSI interpretation Number of strains (%)		
	Range	MIC_{50}/MIC_{90}	GM	S	SDD or I	R
Amphotericin B[1]	0.06–8	0.5/2	0.61	82 (90.1)	—	9 (9.9)
Fluconazole[2]	0.5–≥64	16/64	11.23	—	77 (84.6)	14 (15.4)
Itraconazole[1]	0.016–4	0.25/0.5	0.22	31 (34.1)	55 (60.4)	5 (5.5)
Micafungin[2]	0.016–1	0.06/0.25	0.08	51 (56.0)	26 (28.6)	14 (15.4)
5-Flucytosine[1]	0.12	0.12/0.12	0.12	91 (100.0)	—	—

MIC: minimal inhibitory concentration; CLSI: clinical and laboratory standards institute; GM: geometric mean; S: susceptible; SDD: susceptible-dose dependent; I: intermediary; R: resistant. [1]Breakpoints established by M27-S3 protocol [20]. In sum, strains with amphotericin B MIC > 1 μg/mL are likely to be resistant to this drug; itraconazole MIC ≤ 0.125 μg/mL are likely to be susceptible, 0.25 ≤ MIC ≤ 0.5 μg/mL are likely to be intermediary, and MIC ≥ 1 μg/mL are likely to be resistant to this drug; 5-flucytosine MIC ≤ 4 μg/mL are likely to be susceptible, 8 ≤ MIC ≤ 16 μg/mL are likely to be intermediary, and MIC ≥ 32 μg/mL are likely to be resistant to this drug. [2]Breakpoints established by M27-S4 protocol [21]. In sum, *C. glabrata* strains with fluconazole MIC ≤ 32 μg/mL are likely to be susceptible-dose dependent and MIC ≥ 64 μg/mL are likely to be resistant to this drug; *C. glabrata* strains with micafungin MIC ≤ 0.06 μg/mL are likely to be susceptible, MIC = 0.12 μg/mL are likely to be intermediary, and MIC ≥ 0.25 μg/mL are likely to be resistant to this drug.

Table 2: Resistance to at least two antifungal drugs in Brazilian clinical strains of *Candida glabrata*.

Antifungals	Number of strains (%)	Clinical specimen (number of strains)
AMB and FLC	5 (5.5)	Bronchoalveolar lavage (1), pleural fluid (1), blood (2), vaginal secretion (1)
AMB, FLC, and MCF	1 (1.1)	Feces (1)
FLC and ITC	2 (2.2)	Sputum (1), urine (1)
FLC and MCF	2 (2.2)	Blood (1), vaginal secretion (1)
FLC, ITC, and MCF	1 (1.1)	Vaginal secretion (1)

AMB: amphotericin B; FLC: fluconazole; ITC: itraconazole; MCF: micafungin.

The *C. glabrata* ATCC 2001 type strain was considered an excellent aspartic protease producer (*Pz* mean = 0.357 ± 0.034) and a weak producer of phytase (*Pz* mean = 0.714 ± 0.000). Caseinase, phospholipase, esterase, and hemolytic activities were not detected under the employed experimental conditions for this strain.

The activity of catalase was detected in all *C. glabrata* strains studied, including *C. glabrata* ATCC 2001 type strain. All the strains produced bubbles almost immediately after hydrogen peroxide hydrolysis, and these strains were classified as high catalase producers.

The profile of hydrolytic enzymes related to virulence of the strains was not related to the clinical origin of the strains nor the year of strain isolation ($P > 0.05$).

3.4. Relationship between Antifungal Susceptibility Profile and Virulence Attributes. Spearman's correlation revealed significant associations between phytase production and AMB MIC, hemolysin production and FLC MIC, esterase production and MCF MIC, and hemolysin production and MCF MIC (Table 3). According to the r_s analysis, phyatse *Pz* and AMB MIC, hemolysin *Pz* and FLC MIC, hemolysin *Pz* and MFC MIC have a negative monotonic correlation, whereas esterase *Pz* and MCF MIC are positively monotonically correlated. Moreover, the strength of all negatively correlated variables was classified as weak, and the esterase/MCF correlation was classified as moderate.

Regarding the enzymatic activities of strains grouped according to their susceptibility profile, differences in the median production value of all studied hydrolytic enzymes were not detected in strains of *C. glabrata* with different susceptibilities to AMB ($P > 0.05$). However, statistically significant differences on the median esterase *Pz* values were noticed between strains with different MCF susceptibility profiles and also on the median *Pz* values for hemolytic activity between strains with different FLC, ITC, and MCF susceptibility profiles (Figure 1).

4. Discussion

Phenotypic methods are not able to discriminate among *C. glabrata*, *C. nivariensis*, and *C. bracarensis* [9, 10]. Therefore, as suggested by others authors [9, 28], a molecular method based on sequencing of ITS1-5.8S-ITS2 region of the rDNA was employed to conclude the identification of the clinical strains analyzed in this study. *C. glabrata* was the sole species found. These results are in agreement with the previous studies [12, 29], showing the high prevalence of *C. glabrata* taken into consideration the *C. glabrata* species complex. The correct identification of yeast species causing invasive mycoses is fundamental to ensure proper management of the patient and specific, early, and effective antifungal therapy [9, 30, 31].

Among the antifungal agents used in the management of candidiasis, we can highlight the amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole, echinocandins, and 5-flucytosine [14]. Unfortunately, only FLC and the echinocandins have clinical breakpoints described by the CLSI to *C. glabrata* [21]. Although no

Table 3: Correlation between production of five potential fungal virulence-related enzymes and minimum inhibitory concentrations of four different antifungal drugs in 91 Brazilian clinical strains of *Candida glabrata*.

Antifungals	Hydrolytic enzymes, P^* (r_s)				
	Aspartic protease	Esterase	Phytase	Hemolysin	Catalase
Amphotericin B	0.7409 (0.04)	0.3865 (0.09)	**0.0353 (−0.22)**	0.1771 (−0.14)	0.2567 (0.12)
Fluconazole	0.7230 (−0.04)	0.1493 (0.15)	0.0910 (−0.18)	**0.0040 (−0.30)**	0.4128 (−0.09)
Itraconazole	0.6495 (0.05)	0.4667 (0.08)	0.3749 (0.09)	0.1418 (−0.16)	0.8592 (−0.02)
Micafungin	0.0559 (−0.20)	**<0.0001 (0.40)**	0.3768 (−0.09)	**0.0034 (−0.30)**	0.0922 (−0.18)

*P values of 0.05 or less (in bold) were considered statistically significant.

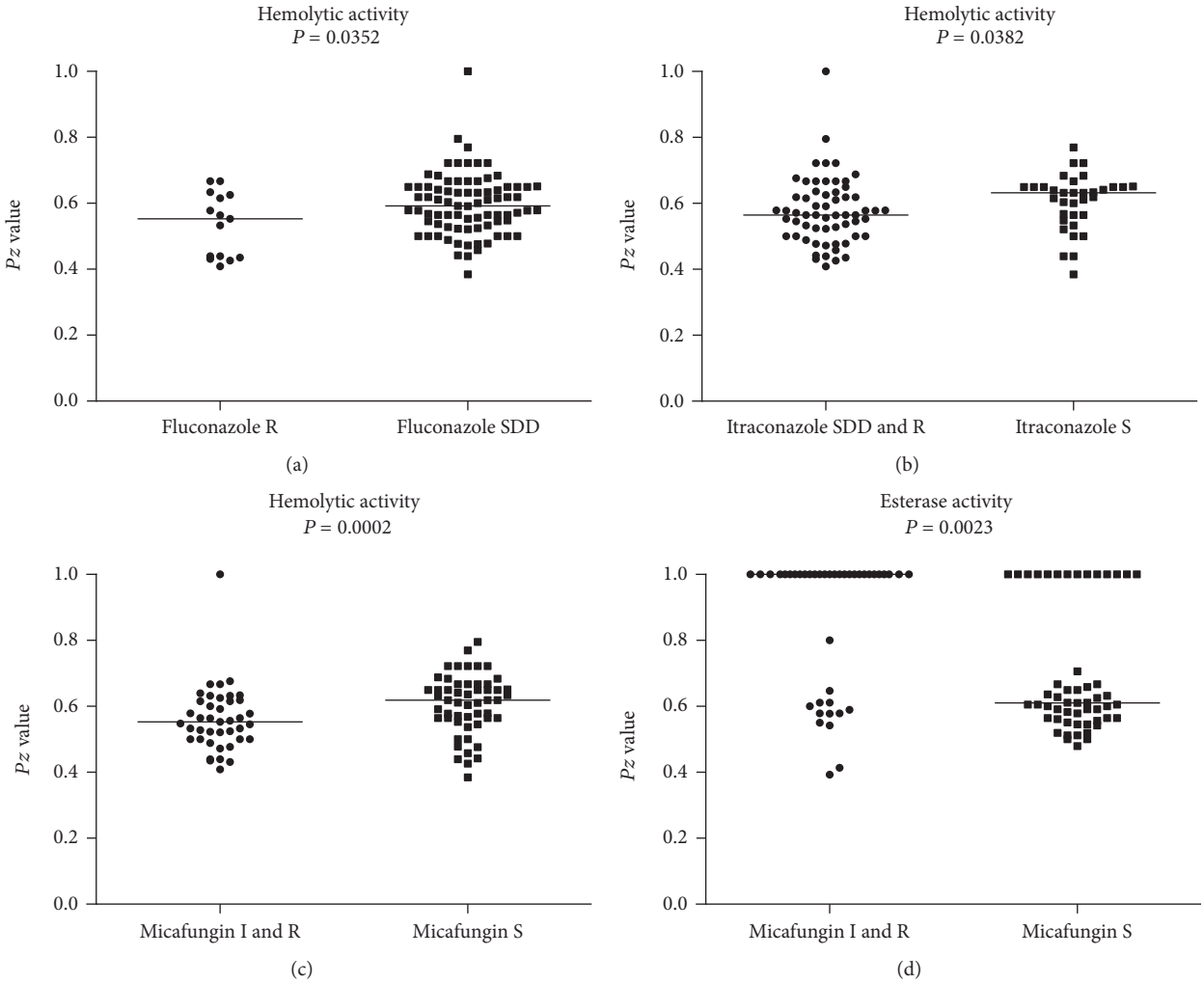


Figure 1: Differential expression of hydrolytic enzymes by 91 *Candida glabrata* strains with different susceptibility profiles against antifungal drugs: (a) hemolytic activity of strains regarding fluconazole susceptibility; (b) hemolytic activity of strains regarding itraconazole susceptibility; (c) hemolytic activity of strains regarding micafungin susceptibility; and (d) esterase activity of strains regarding micafungin susceptibility. Straight lines in each group represent the median for that group. In all the cases, differences between medians of groups with different susceptibility profiles were statistically significant ($P < 0.05$).

clinical breakpoints for AMB have been suggested, the CLSI document indicates that MIC values for this antifungal drug higher than 1 μg/mL are suggestive of resistance [19], the reason for the inclusion of this drug in our analysis. Clinical breakpoints for caspofungin and *C. glabrata* have been described. However, some studies have pointed that the broth microdilution testing is not suitable for caspofungin MIC determination, since unexplained interlaboratory differences are very common for this drug [32–34], and therefore caspofungin was not included in this study. Instead, MCF was chosen to check whether virulence attributes regulate echinocandins' resistance, since this drug does not raise

the same problems observed during MIC determination of caspofungin [34].

In this study, the majority of *C. glabrata* strains presented a MIC ≤ 1 μg/mL to AMB. Fluconazole and micafungin resistance were noted among some *C. glabrata* strains. Similar results were observed in a Portuguese multicenter survey [35] and in a global study developed during the 2014 SENTRY antifungal surveillance program. [36]. However, previous studies developed in Peru [37] and Brazil [38], with a small number of strains ($N = 8$ and 15 isolates, resp.) did not find *C. glabrata* strains with AMB MIC > 1 μg/mL.

According to the clinical breakpoints for *C. glabrata*, it was observed that the frequency of resistant strains was higher to FLC and MCF. Moreover, some *C. glabrata* strains were resistant to both FLC and MCF. Similar results were found in other studies showing that fluconazole-resistant *C. glabrata* isolates were resistant to one or more echinocandins [5, 15]. Echinocandins' resistance appears to be associated with prior exposure to these drugs as well as the presence of *FKS* mutations [15, 16], while azole's resistance can be the result of an alteration of the lanosterol 14α-demethylase target enzyme by either overexpression or mutations in its encoding gene *ERG11* [39], or overexpression of efflux pumps mediated by the activation of expression of ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters [40–42].

In this study, flucytosine demonstrates the greatest in vitro antifungal activity against *C. glabrata* clinical strains. However, in vivo, this drug is usually given in combination with another antifungal agent due to a high rate emergence of resistance during monotherapy for candidiasis [14].

In addition to the CLSI method employed in this study, the only other international standard method for antifungal susceptibility testing of yeasts is that published by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [43]. Pfaller et al. [44] compared these two standardized methods for 10 antifungal agents, including amphotericin B, fluconazole, itraconazole, micafungin, and flucytosine against a collection of clinical isolates of *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. The results indicate that the CLSI and EUCAST methods produce similar results for antifungal susceptibility testing against the five most common species of *Candida*, indicating that their use should not result in resistance profiles different enough to affect direct treatment decisions.

In *Candida* species, extracellular hydrolytic enzymes facilitate the nutrition, adherence, colonization, penetration of tissues or cells, invasion, dissemination, and escape from host immune responses [18, 45]. Moreover, secretion of hydrolytic enzymes has the ability to regulate *Candida* spp. antifungal drug resistance [46].

Aspartic proteases are enzymes with high proteolytic activity and stability at acid pH [47]. These enzymes control several steps in innate immune evasion, and they degrade proteins related to immunological defense such as antibodies, complement, and citokines, allowing the fungus to escape from the first line of host defenses [48]. Moreover, a study developed by Silva et al. [46] suggests that naturally resistant *Candida* spp. or isolates that have developed resistance after prolonged exposure to drugs may present an increase in the secretion pattern and proteolytic activity of secreted aspartic proteases (SAP), but more studies are needed to elucidate its relation. In our study, most strains of *C. glabrata* were classified as good aspartic protease producers. However, *C. glabrata* does not possess classical *SAP* genes in its genome [46, 49]. Probably the enzymatic degradation of albumin verified herein may be due to the production of yapsins. The yapsins (YPS) are a family of five nonsecreted glycosylphosphatidyinositol-linked aspartic proteases that have a well-known role in cell wall integrity and increase the capacity of the fungus to survive inside human macrophages [50]. A study developed by Swoboda-Kopeć et al. [51] confirmed the prevalence of three genes (*YPS2*, *YPS4*, and *YPS6*) in the majority of *C. glabrata* strains isolated from clinical specimens.

Casein is a mixture of phosphoproteins that can be hydrolyzed by a series of enzymes collectively called caseinases. These enzymes belong most likely to the metallo and serine protease families [52]. Caseinase activity was not detected under the employed experimental conditions for any of the tested *C. glabrata* strains. However, these results were discordant from those found by Abbes et al. [53] who reported caseinase activity in 16 *C. glabrata* isolates. Secretion of caseinase has also been observed in *Candida parapsilosis* sensu stricto [24], *Candida haemulonii* species complex [54], and *Yarrowia lipolytica* [53]. Pärnänen et al. [55] identified a serine protease in *C. glabrata* linked to the fungal cell wall, but its role in virulence of *C. glabrata* remains uncertain.

Phospholipases and esterases are extracellular lipolytic enzymes involved in virulence of *Candida* spp. [24, 54]. Their possible functions include digestion of lipids for nutrient acquisition, adhesion to cells and tissues of the host, synergistic interactions with other enzymes, nonspecific hydrolysis, initiation of inflammatory processes by affecting cells of the immune system, and self-defense [56]. In this work, none of the *C. glabrata* strains had detectable levels of phospholipase. Udayalaxmi et al. [57] also did not find phospholipase activity in 14 *C. glabrata* clinical strains isolated from the genitourinary tract. A study from Brazil detected phospholipase activity by the agar plate methodology only in one *C. glabrata* strain isolated from the nasolacrimal duct outlet of a horse [58], thus confirming the low phospholipase production in *C. glabrata*, especially those isolated from human clinical specimens. In a survey among *Candida* vaginal isolates from Egypt, phospholipase activity was observed in a small number of *C. glabrata* strains. This same study also detected the phospholipase *PB2* gene in a few strains studied. On the other hand, the incidence of the phospholipase *PB1* gene in the *Candida* population studied was high, ranging from 87.5% to 95%, depending on the patient history for diabetes [59].

Esterase production was the virulence-related phenotype with more variation among the strains of this study. In a study from Iran with eight *C. glabrata* strains isolated from the oral mucosa, the esterase production showed less variation than the present work, with most strains classified as

esterase producers [60]. On the other hand, a study from Turkey revealed that only one from 14 *C. glabrata* strains isolated from bloodstream infection was considered positive in the esterase agar assay. These data suggest that esterase production in *C. glabrata* may be highly heterogeneous according to the source of the clinical material or the geographic region from which the strains were isolated. A major production of esterase was observed in MCF susceptible *C. glabrata* strains. Enzymes with the ability to degrade chitin are also classified as esterases [61], and high chitin levels are associated with a resistance to caspofungin in some *Candida* species [62]. We are unaware to what extent the esterase agar plate assay employed in this study can also detect chitin desacetylases or if the expression of genes for all esterase families has the same regulation in *C. glabrata* strains. The Spearman's correlation analysis revealed that as MCF MIC increases, esterase production does not increase, which could be the reflex of a higher chitin content in the cell walls of resistant strains due to a lower chitin degradation. Further studies are under way to check this hypothesis.

Phytase is a phosphohydrolase that cleaves phytate-releasing inorganic phosphate and inositol, two essential nutrients for all living cells [63]. In this study, phytase activity was detected in all *C. glabrata* strains. Similar results have been reported in different *Candida* spp., including *C. glabrata* [26], *Candida parapsilosis* species complex [24, 64], and *Candida haemulonii* species complex [54]. In *Candida* spp., the maintenance of a supply of inositol and phosphate mediated by phytase seems to be especially important for pathogen survival and persistence in the host [26]. It was observed that as AMB MIC increases, phytase production does not decrease in the *C. glabrata* strains of our study. To the best of our knowledge, there are no reports of a correlation between phytase production and AMB MIC. Although we were not able to find differences between median phytase *Pz* values among susceptible and resistant AMB strains, the *P* value obtained by the Mann–Whitney test was low ($P = 0.07$), and the difference observed between the two statistic tests may be explained by the low number of AMB-resistant strains in the studied population.

Iron uptake is one of the fundamental requirements for pathogenic fungi to survive and grow into their hosts. Therefore, their survival depends on specialized mechanisms in order to adapt to the restrictions of micronutrients during pathogenesis. In general, fungi have to lyse red blood cells to assimilate the iron associated with hemoglobin [65]. Only one of the *C. glabrata* strains of this study was unable to produce hemolysins, results that agree with previous publications [66–68], reflecting the importance of this virulence factor for this yeast. In fact, iron uptake mechanisms have been demonstrated as necessary for virulence in *C. glabrata* [69]. Iron uptake is also involved in resistance of *Cryptococcus neoformans* [70] and *Candida* species [71] to FLC. During FLC resistance acquirement by a *C. glabrata* strain exposed to crescent concentrations of this azole, an enhancement of hemolytic activity associated with an overexpression of the hemolysin gene was also observed [72]. Therefore, we would expect that azole-resistant strains would express more hemolysins. Since low numbers of FLC and ITC cross-resistance were observed in our study, we could speculate that the different iron-dependent mechanisms regulate resistance to the different azoles. Surprisingly, it was also noticed that expressions of hemolysins were higher in *C. glabrata* strains resistant to MCF. A synergistic effect between MCF and deferasirox, an iron chelator, has been described for *Pythium insidiosum*, suggesting that iron enhances resistance to this echinocandin [73]. Our results support that a similar mechanism occurs in *C. glabrata*. In sum, iron uptake is associated not only to azole resistance in *C. glabrata*, but also to the resistance to echinocandin drugs, such as MCF. These results encourage the development of new therapeutic strategies involving iron depletion, already described for *C. albicans* [74], for the treatment of invasive *C. glabrata* infections.

Catalase was expressed by all tested strains. However, no correlation was observed between the activity of this enzyme and the antifungal susceptibility of these clinical isolates. *C. glabrata* possesses both enzymatic and glutathione mechanisms to resist to the oxidative stress induced by the host immune defenses [75], and our results reinforce the importance of enzymatic mechanisms to maintain redox homeostasis in clinical *C. glabrata* strains.

5. Conclusions

These findings contribute to a better understanding of the *C. glabrata* pathogenesis, showing that aspartic protease, esterase, phytase, hemolysin, and catalase are present in strains from clinical origin. Moreover, the association between expression of some virulence factors with the antifungal resistance to polyenes, azoles, and echinocandins encourages the development of new therapeutic synergistic strategies involving virulence mechanisms such as hydrolytic enzymes as potential targets against drug resistance in *C. glabrata* infections.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

The authors are thankful to CAPES-PROEX, for funding the publication of this work, to Alessandra Leal da Silva Chaves, for providing some strains included in this study, and to Marilia Martins Nishikawa, for providing the *C. glabrata* ATCC 2001 type strain. Automated sequencing was performed using the DNA sequencing platform (ABI-3730; Applied Biosystems) (PDTIS/Fiocruz-Rio de Janeiro, Brazil). Rodrigo Almeida-Paes and Rosely Maria Zancopé-Oliveira are supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant nos. 305487/2015-9 and 304976/2013-0).

References

[1] A. R. Muñoz-Duarte, N. S. Castrejón-Jiménez, S. L. Baltierra-Uribe et al., "*Candida glabrata* survives and replicates in human osteoblasts," *Pathogens and Disease*, vol. 74, no. 4, article ftw030, 2016.

[2] F. Essig, K. Ünniger, S. Dietrich, M. T. Figge, and O. Kurzai, "Human neutrophils dump *Candida glabrata* after intracellular killing," *Fungal Genetics and Biology*, vol. 84, pp. 37–40, 2015.

[3] L. Kasper, K. Seider, and B. Hube, "Intracellular survival of *Candida glabrata* in macrophages: immune evasion and persistence," *FEMS Yeast Research*, vol. 15, no. 5, article fov042, 2015.

[4] P. L. Fidel Jr., J. A. Vazquez, and J. D. Sobel, "*Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *Candida albicans*," *Clinical Microbiology Reviews*, vol. 12, no. 1, pp. 80–96, 1999.

[5] M. A. Pfaller, G. J. Moet, S. A. Messer, R. N. Jones, and M. Castanheira, "Geographic variations in species distribution and echinocandin and azole antifungal resistance rates among *Candida* bloodstream infection isolates: report from the SENTRY antimicrobial surveillance program (2008 to 2009)," *Journal of Clinical Microbiology*, vol. 49, no. 1, pp. 396–399, 2011.

[6] D. Diekema, S. Arbefeville, L. Boyken, J. Kroeger, and M. Pfaller, "The changing epidemiology of healthcare-associated candidemia over three decades," *Diagnostic Microbiology and Infectious Disease*, vol. 73, no. 1, pp. 45–48, 2012.

[7] A. L. Colombo, M. Garnica, L. F. Aranha Camargo et al., "*Candida glabrata*: an emerging pathogen in Brazilian tertiary care hospitals," *Medical Mycology*, vol. 51, no. 1, pp. 38–44, 2013.

[8] M. T. Montagna, G. Lovero, E. Borghi et al., "Candidemia in intensive care unit: a nationwide prospective observational survey (GISIA-3 study) and review of the European literature from 2000 through 2013," *European Review for Medical and Pharmacological Sciences*, vol. 18, no. 5, pp. 661–674, 2014.

[9] J. Alcoba-Flórez, S. Méndez-Alvarez, J. Cano, J. Guarro, E. Pérez-Roth, and M. Pilar Arévalodel, "Phenotypic and molecular characterization of *Candida nivariensis* sp. nov., a possible new opportunistic fungus," *Journal of Clinical Microbiology*, vol. 43, no. 8, pp. 4107–4111, 2005.

[10] A. Correia, P. Sampaio, S. James, and C. Pais, "*Candida bracarensis* sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, Part 1, pp. 313–317, 2006.

[11] M. Cuenca-Estrella, A. Gomez-Lopez, G. Isla et al., "Prevalence of *Candida bracarensis* and *Candida nivariensis* in a Spanish collection of yeasts: comparison of results from a reference centre and from a population-based surveillance study of candidemia," *Medical Mycology*, vol. 49, no. 5, pp. 525–529, 2011.

[12] M. C. Esposto, A. Prigitano, O. Romeo et al., "Looking for *Candida nivariensis* among a large Italian collection of *C. glabrata* isolates results of the FIMUA working group," *Mycoses*, vol. 56, no. 3, pp. 395-396, 2013.

[13] M. Nucci, F. Queiroz-Telles, T. Alvarado-Matute et al., "Epidemiology of candidemia in Latin America: a laboratory-based survey," *PloS One*, vol. 8, no. 1, article e59373, 2013.

[14] P. G. Pappas, C. A. Kauffman, D. R. Andes et al., "Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America," *Clinical Infectious Diseases*, vol. 62, no. 4, pp. e1–e50, 2016.

[15] B. D. Alexander, M. D. Johnson, C. D. Pfeiffer et al., "Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of *FKS* mutations and elevated minimum inhibitory concentrations," *Clinical Infectious Diseases*, vol. 56, no. 12, pp. 1724–1732, 2013.

[16] R. K. Shields, M. H. Nguyen, E. G. Press et al., "The presence of an *FKS* mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to *Candida glabrata*," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 9, pp. 4862–4869, 2012.

[17] S. Silva, M. Negri, M. Henriques, R. Oliveira, D. W. Williams, and J. Azeredo, "*Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance," *FEMS Microbiology Reviews*, vol. 36, no. 2, pp. 288–305, 2012.

[18] J. C. Sardi, L. Scorzoni, T. Bernardi, A. M. Fusco-Almeida, and M. J. Mendes Giannini, "*Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options," *Journal of Medical Microbiology*, vol. 62, Part 1, pp. 10–24, 2013.

[19] Clinical and Laboratory Standards Institute (CLSI), *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard-Third Edition. CLSI Document M-27A3*, CLSI, Wayne, PA, 2008.

[20] Clinical and Laboratory Standards Institute (CLSI), *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Third Informational Supplement. CLSI Document M27-S3*, CLSI, Wayne, PA, 2008.

[21] Clinical and Laboratory Standards Institute (CLSI), *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Fourth Informational Supplement. CLSI Document M27-S4*, CLSI, Wayne, PA, 2012.

[22] M. F. Price, I. D. Wilhinson, and L. O. Gentry, "Plate method for detection of phospholipase activity in *Candida albicans*," *Sabouraudia*, vol. 20, no. 1, pp. 7–14, 1982.

[23] R. Rüchel, R. Tegeler, and M. Trost, "A comparison of secretory proteinases from different strains of *Candida albicans*," *Sabouraudia*, vol. 20, no. 3, pp. 233–244, 1982.

[24] M. Ziccardi, L. O. Souza, R. M. Gandra et al., "*Candida parapsilosis* (sensu lato) isolated from hospitals located in the southeast of Brazil: species distribution, antifungal susceptibility and virulence attributes," *International Journal of Medical Microbiology*, vol. 305, no. 8, pp. 848–859, 2015.

[25] E. Aktas, N. Yigit, and A. Ayyildiz, "Esterase activity in various *Candida* species," *The Journal of International Medical Research*, vol. 30, no. 3, pp. 322–324, 2002.

[26] P. W. Tsang, "Differential phytate utilization in *Candida* species," *Mycophatologia*, vol. 172, no. 6, pp. 473–479, 2011.

[27] B. G. Metchock, F. S. Nolte, and R. J. Wallace Jr., "Mycobacterium," in *Manual of Clinical Microbiology*, P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover and R. H. Yolken, Eds., pp. 399–437, ASM Press, Washington, DC, USA, 1999.

[28] A. Enache-Angoulvant, J. Guitard, F. Grenouillet et al., "Rapid discrimination between *Candida glabrata, Candida nivariensis* and *Candida bracarensis* by use of a singleplex PCR," *Journal of Clinical Microbiology*, vol. 49, no. 9, pp. 3375–3379, 2011.

[29] J. Pemán, E. Cantón, G. Quindós et al., "Epidemiology, species distribution and in vitro antifungal susceptibility of fungaemia in a Spanish multicentre prospective survey," *Journal of Antimicrobial Chemotherapy*, vol. 67, pp. 1181–1187, 2012.

[30] S. A. Meyer and D. Yarrow, "*Candida glabrata*," in *The Yeasts: A Taxonomia Study*, C. P. Kurtzman, J. W. Fell and T. Boekhout, Eds., vol. 2, pp. 1097-1098, Elsevier, Amsterdam, 2011.

[31] A. Chowdhary, H. S. Randhawa, Z. U. Khan et al., "First isolations in India of *Candida nivariensis,* a globally emerging opportunistic pathogen," *Medical Mycology*, vol. 48, no. 2, pp. 416–420, 2010.

[32] M. C. Arendrup and D. S. Perlin, "Echinocandin resistance: an emerging clinical problem?" *Current Opinion in Infectious Diseases*, vol. 27, no. 6, pp. 484–492, 2014.

[33] R. Ben-Ami, Y. Hilerowicz, A. Novikov, and M. Giladi, "The impact of new epidemiological cutoff values on *Candida glabrata* resistance rates and concordance between testing methods," *Diagnostic Microbiology and Infectious Diseases*, vol. 79, no. 2, pp. 209–213, 2014.

[34] D. S. Perlin, "Echinocandin resistance, susceptibility testing and prophylaxis: implications for patient management," *Drugs*, vol. 74, no. 14, pp. 1573–1585, 2014.

[35] I. Faria-Ramos, J. Neves-Maia, E. Ricardo et al., "Species distribution and in vitro antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey," *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 33, pp. 2241–2247, 2014.

[36] M. A. Pfaller, S. A. Messer, P. R. Rhomberg, and M. Castanheira, "Activity of a long-acting echinocandin (CD101) and seven comparator antifungal agents tested against a global collection of contemporary invasive fungal isolates in the SENTRY 2014 antifungal surveillance program," *Antimicrobial Agents and Chemotherapy*, vol. 61, no. 3, pp. e02045–e02016, 2017.

[37] B. Bustamante, M. A. Martins, L. X. Bonfietti et al., "Species distribution and antifungal susceptibility profile of *Candida* isolates from bloodstream infections in Lima, Peru," *Journal of Medical Microbiology*, vol. 63, pp. 855–860, 2014.

[38] E. R. Santos, C. F. Dal Forno, M. G. Hernandez et al., "Susceptibility of *Candida* spp. isolated from blood cultures as evaluated using the M-27ª3 and new M-27-S4 aproved breakpoints," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 56, no. 6, pp. 477–482, 2014.

[39] K. W. Henry, J. T. Nickels, and T. D. Edlind, "Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors," *Antimicrobiol Agents and Chemotherapy*, vol. 44, no. 10, pp. 2693–2700, 2000.

[40] D. Sanglard, K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille, "Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters," *Antimicrobiol Agents and Chemotherapy*, vol. 39, no. 11, pp. 2378–2386, 1995.

[41] K. H. Chen, T. Miyazaki, H. F. Tsai, and J. E. Bennett, "The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in *Candida glabrata*," *Gene*, vol. 386, no. 1-2, pp. 63–72, 2007.

[42] S. Paul and W. S. Moye-Rowley, "Multidrug resistance in fungi: regulation of transporter-enconding gene expression," *Frontiers in Physiology*, vol. 5, article 143, 2014.

[43] M. C. Arendrup, M. Cuenca-Estrella, C. Lass-Florl, W. W. Hope, and EUCAST-AFST, "EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST)," *Clinical Microbiology and Infection*, vol. 18, pp. E246-E247, 2012.

[44] M. A. Pfaller, M. Castanheira, S. A. Messer, P. R. Rhomberg, and R. N. Jones, "Comparison of EUCAST and CLSI broth microdilution methods for the susceptibility testing of 10 systemically active antifungal agents when tested against *Candida* spp," *Diagnostic Microbiology and Infectious Disease*, vol. 79, pp. 198–204, 2014.

[45] R. Ells, W. Kilian, A. Hugo, J. Albertyn, J. L. Kock, and C. H. Pohl, "Virulence of South African *Candida albicans* strains isolated from different clinical samples," *Medical Mycology*, vol. 52, no. 3, pp. 246–253, 2014.

[46] N. C. Silva, J. M. Nery, and A. L. Dias, "Aspartic proteinases of *Candida* spp.: role in pathogenicity and antifungal resistance," *Mycoses*, vol. 57, no. 1, pp. 1–11, 2014.

[47] M. Rao, A. Tanksale, M. Gratge, and V. Deshpande, "Molecular biotechnological aspects of microbial proteases," *Microbiology and Molecular Biology Reviews*, vol. 62, no. 3, pp. 597–635, 1998.

[48] M. Staniszewska, B. Małgorzata, and O. Zbigniew, "Contribution of aspartic proteases in *Candida* virulence. Protease inhibitors against *Candida* infections," *Current Protein & Peptide Science*, vol. 17, 2016.

[49] B. Parra-Ortega, H. Cruz-Torres, L. Villa-Tanaca, and C. Hernández-Rodríquez, "Phylogeny and evolution of the aspartyl protease family from clinically relevant *Candida* species," *Memórias do Instituto Oswaldo Cruz*, vol. 104, no. 3, pp. 505–512, 2009.

[50] D. J. Krysan, E. L. Ting, C. Abeijon, L. Kroos, and R. S. Fuller, "Yapins are a family of aspartyl proteases required for cell wall integrity in *Saccharomyces cerevisiae*," *Eukaryotic Cell*, vol. 4, no. 8, pp. 1364–1374, 2005.

[51] E. Swoboda-Kopeć, M. Sikora, M. Golas, K. Piskorska, D. Gozdowski, and I. Netsvyetayeva, "*Candida nivariensis* in comparison to different phenotypes of *Candida glabrata*," *Mycoses*, vol. 57, no. 12, pp. 747–753, 2014.

[52] M. F. Clincke, E. Guedon, F. T. Yens, V. Ogier, and J. L. Goergen, "Characterization of metalloprotease and serine protease activities in batch CHO cell cultures: control of human recombinant IFN-γ proteolysis by addition of iron citrate," *BMC Proceedings*, vol. 5, Supplement 8, p. 115, 2011.

[53] S. Abbes, I. Amouri, H. Trabelsi et al., "Analysis of virulence factors and in vivo biofilm-forming capacity of *Yarrowia lipolytica* isolated from patients with fungemia," *Medical Mycology*, vol. 55, no. 4, pp. 193–202, 2017.

[54] L. S. Ramos, M. H. Branquinha, and A. L. Santos, "Different classes of hydrolytic enzymes produced by multidrug-resistant yeasts comprising the *Candida haemulonii* complex," *Medical Mycology*, vol. 55, no. 2, pp. 228–232, 2017.

[55] P. Pärnänen, J. H. Meurman, and P. Nikula-Ijäs, "A novel *Candida glabrata* cell wall associated serine protease," *Biochemical and Biophysical Research Communications*, vol. 457, no. 4, pp. 676–680, 2015.

[56] M. Schaller, C. Borelli, H. C. Korting, and B. Hube, "Hydrolitic enzymes as virulence factors of *Candida albicans*," *Mycoses*, vol. 48, no. 6, pp. 365–377, 2005.

[57] J. Udayalaxmi, S. Jacob, and D. D'Souza, "Comparison between virulence factors of *Candida albicans* and non-*albicans* species of *Candida* isolated from genitourinary tract," *Journal of Clinical and Diagnostic Research*, vol. 8, no. 11, pp. 15–17, 2014.

[58] R. S. Brilhante, P. V. Bittencourt, D. S. Castelo-Branco et al., "Trends in antifungal susceptibility and virulence of *Candida* spp. from the nasolacrimal duct of horses," *Medical Mycology*, vol. 54, no. 2, pp. 147–154, 2016.

[59] R. H. Bassyouni, A. A. Wegdan, A. Abdelmoneim, W. Said, and F. AboElnaga, "Phospholipase and aspartyl proteinase activities of *Candida* species causing vulvovaginal candidiasis in patients with type 2 diabetes mellitus," *Journal of Microbiology and Biotechnology*, vol. 25, no. 10, pp. 1734–1741, 2015.

[60] M. Fatahinia, F. Poormohamadi, and A. Zarei Mahmoudabadi, "Comparative study of esterase and hemolytic activities in clinically important *Candida* species, isolated from oral cavity of diabetic and non-diabetic individuals," *Jundishapur Journal of Microbiology*, vol. 8, no. 3, article e20893, 2015.

[61] F. Caufrier, A. Martinou, C. Dupont, and V. Bouriotis, "Carbohydrate esterase family 4 enzymes: substrate specificity," *Carbohydrate Research*, vol. 338, no. 7, pp. 687–692, 2003.

[62] L. A. Walker, N. A. Gow, and C. A. Munro, "Elevated chitin content reduces the susceptibility of *Candida* species to caspofungin," *Antimicrobiol Agents and Chemotherapy*, vol. 57, no. 1, pp. 146–154, 2013.

[63] X. G. Lei and J. M. Porres, "Phytase enzymology, applications, and biotechnology," *Biotechnology Letters*, vol. 25, no. 21, pp. 1787–1794, 2003.

[64] É. A. Abi-chacra, L. O. Souza, L. P. Cruz et al., "Phenotypical properties associated with virulence from clinical isolates belonging to the *Candida parapsilosis* complex," *FEMS Yeast Research*, vol. 13, no. 8, pp. 831–848, 2013.

[65] A. Crawford and D. Wilson, "Essential metals at the host-pathogen interface: nutritional immunity and micronutrient assimilation by human fungal pathogens," *FEMS Yeast Research*, vol. 15, no. 7, 2015.

[66] V. K. Chin, K. J. Foong, A. Maha et al., "*Candida albicans* Isolates from a Malaysian hospital exhibit more potent phospholipase and haemolysin activities than non-*albicans* *Candida* isolates," *Tropical Biomedicine*, vol. 30, no. 4, pp. 654–662, 2013.

[67] R. D. Rossoni, J. O. Barbosa, S. F. Vilela, A. O. Jorge, and J. C. Junqueira, "Comparison of the hemolytic activity between *C. albicans* and non-*albicans* *Candida* species," *Brazilian Oral Research*, vol. 27, no. 6, pp. 484–489, 2013.

[68] C. J. Seneviratne, S. S. Wong, K. Y. Yuen et al., "Antifungal susceptibility and virulence attributes of bloodstream isolates of *Candida* from Hong Kong and Finland," *Mycopathologia*, vol. 172, no. 5, pp. 389–395, 2011.

[69] V. K. Srivastava, K. J. Suneetha, and R. Kaur, "A systematic analysis reveals an essential role for high-affinity iron uptake system, haemolysin and CFEM domain-containing protein in iron homoeostasis and virulence in *Candida glabrata*," *The Biochemical Journal*, vol. 463, no. 1, pp. 103–114, 2014.

[70] J. Kim, Y. J. Cho, E. Do et al., "A defect in iron uptake enhances the susceptibility of *Cryptococcus neoformans* to azole antifungal drugs," *Fungal Genetics and Biology*, vol. 49, no. 11, pp. 955–966, 2012.

[71] T. Prasad, A. Chandra, C. K. Mukhopadhyay, and R. Prasad, "Unexpected link between iron and drug resistance of *Candida* spp.: iron depletion enhances membrane fluidity and drug diffusion, leading to drug-susceptible cells," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 11, pp. 3597–3606, 2006.

[72] Y. H. Samaranayake, B. P. Cheung, J. Y. Yau, K. W. Yeung, and L. P. Samaranayake, "Genotypic, phenotypic, and proteomic characterization of *Candida glabrata* during sequential fluconazole exposure," *Journal of Investigative and Clinical Dentistry*, vol. 2, no. 2, pp. 117–127, 2011.

[73] R. A. Zanette, F. P. Jesus, M. B. Pilotto et al., "Micafungin alone and in combination therapy with deferasirox against *Pythium insidiosum*," *Journal de Mycologie Medicale*, vol. 25, no. 1, pp. 91–94, 2015.

[74] T. Kobayashi, H. Kakeya, T. Miyazaki et al., "Synergistic antifungal effect of lactoferrin with azole antifungals against *Candida albicans* and a proposal for a new treatment method for invasive candidiasis," *Japanese Journal of Infectious Diseases*, vol. 64, no. 4, pp. 292–296, 2011.

[75] M. Briones-Martin-Del-Campo, E. Orta-Zavalza, J. Juarez-Cepeda et al., "The oxidative stress response of the opportunistic fungal pathogen *Candida glabrata*," *Revista Iberoamericana de Micología*, vol. 31, no. 1, pp. 67–71, 2014.

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 9870679, 16 pages
https://doi.org/10.1155/2017/9870679

Review Article

Antifungal Resistance, Metabolic Routes as Drug Targets, and New Antifungal Agents: An Overview about Endemic Dimorphic Fungi

Juliana Alves Parente-Rocha,[1] Alexandre Melo Bailão,[1] André Correa Amaral,[2] Carlos Pelleschi Taborda,[3] Juliano Domiraci Paccez,[1] Clayton Luiz Borges,[1] and Maristela Pereira[1]

[1]*Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[2]*Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[3]*Departamento de Microbiologia do Instituto de Ciências Biomédicas e Laboratório de Micologia Médica LIM53/IMTSP, Universidade de São Paulo, São Paulo, SP, Brazil*

Correspondence should be addressed to Maristela Pereira; maristelaufg@gmail.com

Received 2 February 2017; Revised 28 April 2017; Accepted 23 May 2017; Published 13 June 2017

Academic Editor: Teresa Zelante

Diseases caused by fungi can occur in healthy people, but immunocompromised patients are the major risk group for invasive fungal infections. Cases of fungal resistance and the difficulty of treatment make fungal infections a public health problem. This review explores mechanisms used by fungi to promote fungal resistance, such as the mutation or overexpression of drug targets, efflux and degradation systems, and pleiotropic drug responses. Alternative novel drug targets have been investigated; these include metabolic routes used by fungi during infection, such as trehalose and amino acid metabolism and mitochondrial proteins. An overview of new antifungal agents, including nanostructured antifungals, as well as of repositioning approaches is discussed. Studies focusing on the development of vaccines against antifungal diseases have increased in recent years, as these strategies can be applied in combination with antifungal therapy to prevent posttreatment sequelae. Studies focused on the development of a pan-fungal vaccine and antifungal drugs can improve the treatment of immunocompromised patients and reduce treatment costs.

1. Introduction

Endemic mycoses as well as some other mycoses include self-limiting cutaneous, subcutaneous, systemic, and disseminated infections. People living in areas of endemic mycosis caused by dimorphic fungi are exposed to and can acquire fungal infections [1–3]. Fungal infections are responsible for morbidity and mortality which are accompanied by high costs to the health system. Antifungal chemotherapy is generally required to treat fungal infections. Despite its modest efficacy against fungal cells and the frequent relapse observed, it is currently the best treatment option. Unfortunately, treatment lasts several months, and the derivation of treatment may last for up to 2 years with sulfonamides, polyenes, azoles, or echinocandins (reviewed in [4]).

Increasing mortality rates caused by fungal diseases, often with the appearance of antifungal-resistant lineages, motivates a scientific race to discover new drug targets. Fungal infection can occur in patients who are immunosuppressed due to organ transplantation, intensive care unit hospitalization, cancer, HIV, surgery, or leukemia as well as in patients who use antibiotics able to modify human microbiota. The number of immunocompromised patients has increased in recent decades and unfortunately continues to grow. Cases of fungal resistance have accompanied this growth [5]. Due to the immunological conditions of patients,

the microorganisms grow in an environment where the current therapeutic arsenal is not efficient because the natural immune system is essential to assist in combating infection [6]. The overall inefficiency and high toxicity of current antifungal therapies are also noteworthy [5]. In this context, it is very important to develop a new class of broad-spectrum antifungal agents to circumvent these problems.

2. Antifungal Resistance Mechanisms

From a clinical perspective, drug resistance occurs when the appropriate drug therapy is not effective, causing persistence/progression of an infection [7]. However, the molecular mechanisms that lead to antifungal resistance are complex. Fungal cells must commonly adapt to the presence of toxic drugs; the primary molecular survival strategies include (1) mutation of drug targets that reduces its affinity for the drug, (2) overexpression of the targeted protein by modification of the promoter region of the gene, (3) expression of an efflux system, (4) degradation of the drugs, and (5) pleiotropic drug responses [8]. The similarity of the biology of the eukaryotic host and fungal pathogens has led to a drug development focused on the specific characteristics of fungal cells. For example, the fungal cell wall is a specific antifungal drug target. In addition, ergosterol is present in fungal membranes and can be used as a drug target, because it is distinct from the cholesterol present in mammalian cells. Commercial antifungal drugs that act on the cell wall or on ergosterol include azoles, echinocandins, and polyenes.

2.1. Fungal Resistance to Azoles. In the last 25 years, several azole drugs (including imidazoles and triazoles) have been used against fungal diseases. These drugs target lanosterol demethylase (cytochrome P450), which has evolved to participate in ergosterol biosynthesis. This enzyme is encoded by the *ERG*11 gene; its inactivation prevents ergosterol production, leading to the accumulation of methylated sterols such as the toxic 14-α-methyl-ergosta-8-ene-3,6-diol in the fungal cell [9]. The resistance mechanisms to these drugs are complex. The mutations in the genes *CYP*51A and *CYP*51B (homologous to *ERG*11) are described as reducing the interaction of the drug with the genetic product. The substitutions G54, P216, F219, M220, and G448 have been described as conferring azole resistance [10]. The efflux of azoles has also been reported and is performed by an ATP-binding cassette (ABC) transporter. In *Saccharomyces cerevisiae*, *PDR5* and *PDR15* transporters are overexpressed in azole-resistant strains and are described as conferring azole resistance through efflux [11]. In the last ten years, azole resistance caused by environmental exposure has been studied extensively. This resistance is likely the result of a TR motif (a tandem repeat motif with at least 34-base TR) in the promoter region of *CYP*51A and amino acid mutation, which is well characterized in *Aspergillus fumigatus*, that leads to overexpression of the drug protein target. It is established that this resistance mechanism was developed after exposure of fungal cells to azoles [12]. Recently, the TR34 motif was found associated with the sterol regulatory element-binding protein (SREBP) SrbA and the CCAAT-binding complex (CBC), which is related to repression of sterol synthesis [13]. In this case, the azole tolerance is the result of CBC repression that increases sterol synthesis. In *Cryptococcus neoformans*, the exposure of infected mice to a low dose of fluconazole increases the minimum inhibitory concentration (MIC) of colonies recovered from animals and increases the melanization and capsule size, which are classical virulence factors in this fungus, showing that previous exposure to antifungals leads to different resistance mechanisms in different fungal species [14]. The degradation of azoles is not well characterized, but studies performed with *A. fumigatus* correlate the mitochondrial complex I with metabolism of azoles, causing drug resistances, as mutants of this complex lost azole resistance [15]. Multidrug resistance (MDR) can also lead to azole resistance and includes the phenotype achieved by interactions between transcriptional activators, mediator complexes, and efflux pumps acting as a network in response to several molecules. Azole resistance caused by MDR is described in *S. cerevisiae* and includes the expression of genes regulated by two homologous transcriptional factors *PDR*1 and *PDR*3 [16]. Azole resistance related to the MDR efflux system is also reported in the economically important plant pathogen *Zymoseptoria tritici*, because overexpression of the MgMSF1 transporter was detected in azole-resistant strains [17].

The fluconazole resistance was reported in the dimorphic fungus *Histoplasma capsulatum*, causing treatment failure in HIV patients. In this case, itraconazole was more effective for treatment [18].

2.2. Fungal Resistance to Echinocandins. The echinocandins (lipopeptide molecules) are one of few new classes of antifungals to reach the clinic in the last decade. This class includes caspofungin, micafungin, and anidulafungin. These lipopeptides act on fungal cell walls by specific inhibition of 1,3-β-D-glucan synthase, which is responsible for the biosynthesis of β-1,3-glucan, the key fungal cell wall component [19]. The enzyme consists of three FKS subunits, called FKS1, FKS2, and FKS3 [20]. Resistance is often acquired during therapy by modifying amino acid residues in the FKS1 and FKS2 subunits of β-1,3-glucan synthase. [21–23]. The function of the FKS3 subunit remains unclear [23–25].

The mutations in FKS1 and FKS2 that code for catalytic subunit genes can be amino acid substitutions that increase MIC levels accompanied by a dramatic reduction in glucan synthase activity [26–28]. For example, substitutions related to the FKS1 gene in residues Phe641, Pro649, and Arg1361 (*C. albicans* homolog) have been described [29, 30]. For the FKS2 subunit, the substitutions Ser641 and Ser645 strongly reduce enzyme activity, causing a pronounced resistance phenotype [20, 31]. The FKS1 and FKS2 amino acid residue substitutions can vary depending on fungal species but also cause echinocandin resistance [32–34].

β-1,3-glucan synthase echinocandin inhibition leads to cell wall defects, which in turn cause cellular stress, and several genes are expressed to adapt to this stress condition. The signaling pathway driven by protein kinase C (PKC) alters the biosynthesis of various carbohydrates related to the cell wall, HOG (high-osmolarity glycerol) [35] and chitin

biosynthesis [36, 37]; higher chitin levels are correlated with echinocandin tolerance [38–40]. Altogether, these salvaging pathway phenomena can help the fungus in adaptive cell wall remodeling that enables the cells to survive, even with greater echinocandin doses.

Dimorphic fungi have a natural resistance to echinocandins during the pathogenic phase, but the resistance mechanisms of the β-glucan synthase inhibitors are currently unknown [41].

2.3. Fungal Resistance to Polyenes. Polyenes are fungicidal and act on the membrane. These molecules are natural fermentation products of *Streptomyces*, and the most used polyene drug is amphotericin B (used in systemic mycoses). For many years, it was believed that polyenes (amphipathic drugs) bind strongly to ergosterol, destroying the proton gradient and allowing leakage of ions [42]. Today, it is known that drug-lipid complexes extract ergosterol from phospholipid in the membrane, depleting ergosterol in the cell [43]. Amphotericin B is refractory to the development of resistance, despite its use for 50 years in clinical treatment. In *Candida tropicalis*, low ergosterol content in the membrane is associated with reduced susceptibility to amphotericin B [44]. Because amphotericin B increases the level of reactive oxygen species (ROS) in fungal cells [45], amphotericin B-resistant *C. tropicalis* produces less ROS and alters mitochondrial activity [46]. In *Aspergillus terreus*, the oxidative damage caused by amphotericin B is more important in causing cell injury than membrane permeation [47].

3. Metabolic Routes as Antifungal Drug Targets

In recent decades, the ability of researchers to identify and validate antifungal targets has significantly improved due to improvements in genetic tools for manipulation of fungal pathogens, which lead to a wave of data arising from omics studies and the standardization of animal models for fungal infection. This research progress has begun to allow rational drug design, which creates more efficient antifungal effects with high specific toxicity [48]. In the pipeline for novel investigation of new molecular targets, the following major concerns should be taken into account: (1) the fungicidal target must be essential for fungal survival throughout the infectious process; (2) as mammalian and fungi share basic eukaryotic characteristics, the target or inhibitor must present highly selective toxicity to provide a favorable therapeutic-toxic ratio; and (3) widespread targets among the fungal pathogens must be economically attractive. In addition, the new drug must be safe for use in fragile patients with fungal infections.

3.1. Trehalose Metabolism Enzymes as Targets. In the search for new antifungal drugs, the trehalose [α-D-glucopyranosyl-(1 $\rightarrow$ 1)-α-D-glucopyranoside] biosynthetic pathway arises as a potential target. Trehalose is a simple nonreducing disaccharide containing two glucose units and plays critical roles in general stress adaptation in fungi, as well as energy reserves in certain fungi that might be used for ATP production under certain stresses [49–51]. Trehalose can interact with proteins and phospholipids to protect cell structures such as the plasma membrane from degradation, along with denaturation of intracellular proteins. This cell stress protectant has been shown to be important in fungal adaptation to mammalian body temperatures. In addition, studies with human pathogens suggest that trehalose is a reactive oxygen species scavenger and may protect against host oxidative burst [48]. During central nervous system infection, *C. neoformans* expresses high amounts of trehalose synthase 1 gene (*tps1*). Consistent with this result, cryptococcomas accumulates considerable quantities of this disaccharide. Moreover, functional studies with *C. neoformans* and *C. gattii tps1* mutants revealed that this gene is important for growth at 37°C. However, deletion of *tps1* has a fungicidal effect in host tissues. Thus, such evidence strongly indicates that trehalose counteracts host-imposed stresses other than temperature. This hypothesis was validated when cryptococcal Δ*tps1* was found to be severely attenuated in nematode and zebrafish models, in which temperatures fell below mammalian temperatures [52, 53]. TPS2 is a very specific phosphatase with no characteristics that suggest it as a promiscuous phosphate enzyme target. The trehalose phosphate synthase 2 is essential to keep *C. neoformans* growing at human body temperature; furthermore, its absence substantially impairs fungal virulence. This enzyme is a broad-spectrum target, as strains of *C. albicans* and *A. fumigatus* lacking its activity also exhibit decreased virulence. Trehalases degrade trehalose in glucose via a fuel-providing mechanism in fungi [54]. Although neutral trehalase genes do not have an impact on *C. albicans* and *C. neoformans*, an acidic enzyme influences *C. albicans* pathogenesis [55]. Validamycin A, a trehalase inhibitor, has a limited effect as an antifungal on *C. albicans* infection [56]. Thus, trehalases are still not potential targets due to the presence of multiple enzymes and different impacts on fungal cells. There is no data indicating them as a broad-spectrum target. Future efforts are encouraged to explore the impact of trehalose metabolism on fungal biology and virulence.

3.2. Amino Acid Metabolism-Related Targets. Amino acid biosynthetic routes have been shown as druggable targets, because during infection, fungi are exposed to nutritional stresses that require amino acid metabolism and present fungus-specific enzymes/processes. Evidence shows that efficient responses to amino acid starvation and requirements are important for fungal pathogenicity; the absence of CpcA, a transcriptional activator in amino acid starvation, and AreA, a nitrogen metabolic repressor activated when preferable nitrogen sources are not available, impairs *A. fumigatus* virulence in a murine model of pulmonary aspergillosis [57, 58]. Growing knowledge suggests that biosynthetic pathways have purposes beyond nutritional requirements; additional functions may exists that could be even more important for pathogenicity [59]. During fungal infection, the microorganism encounters specific niches with variable amounts of amino acids/nitrogen sources. Consequently, different infection models or routes may interfere in the essentiality of a specific amino acid biosynthesis in infection. *A. fumigatus* that lacks the HscA gene (homocitrate synthase) required for lysine biosynthesis

revealed that spores, but not hyphae, need free lysine to grow, while hyphae use proteases to harvest lysine. Infection experiments additionally demonstrated that in inhalation and disseminated models, mutants presented avirulence and full virulence, respectively, indicating differential availability of lysine in these host niches [60]. More congruent niche-specific phenotypes were observed in isoleucine/valine auxotrophic *A. fumigatus* strain, which lacks dihydroxy-acid dehydratase (*ilv3A*); Δ*ilv3A* cells were avirulent in systemic infection but only slightly attenuated in pulmonary infection. Interestingly, the depletion of the paralogue *ilv3B* resulted in fungal avirulence in any infection model [61]. However, some biosynthetic pathways rise as niche-independent routes that are essential for fungal pathogenicity. The *A. fumigatus* HisB (imidazoleglycerol phosphates dehydrogenase) null mutant is unable to grow in blood agar and hydrolyzed BSA as a nitrogen source in vitro; thus, it is unable to establish infection in the lungs and bloodstream, which suggests that those niches are unable to provide sufficient amounts of histidine to support fungal growth.

Some amino acid biosynthetic genes are essential for fungi. For instance, *A. fumigatus* mutants that exhibit auxotrophy for the three aromatic amino acids are impossible to obtain. In addition, the *AroM* (dehydroquinate hydrolyase) mutant could not be generated because of its essentiality. As a result, a conditional promoter strategy driving the expression of A*roB* (chorismate synthase) was used to obtain aromatic amino acid auxotroph strain, which was unable to grow in medium containing the three aromatic amino acids and displayed attenuated virulence in pulmonary and systemic murine infection models. This fact could be explained by the accumulation of toxic chorismatic acid, a mitochondrial function inhibitor [62]. Tryptophan biosynthetic genes of *C. neoformans* seem to be essential for this fungus; however, exogenous supplementation with tryptophan, in nitrogen-depressed conditions (using proline as a nitrogen source), partially restored growth [63]. Conditional threonine mutants of *C. neoformans* were more susceptible to growth at human body temperature, but growth was partially rescued when threonine dipeptides were offered [64]. Although biosynthesis of amino acids has been explored in *A. fumigatus* and *C. neoformans*, knowledge of those pathways in dimorphic fungi such as *Blastomyces dermatitidis*, *H. capsulatum*, *Penicillium marneffei*, and *Paracoccidioides* is still poor. Based on our current knowledge, amino acid biosynthetic routes are suitable targets for antifungal development, because they are essential for fungal pathogens and have enzymes not found in humans. However, some aspects must be considered in targeting amino acid pathways: auxotrophy for certain amino acids can be restored by uptake of exogenous molecules from proteolytic products of fungal proteases and the niche-specific requirement varies dramatically [59]. Thus, the generation of conditional expressing mutants appears to be the best strategy to analyze in vivo amino acid acquisition, when null mutants are not obtainable.

3.3. Mitochondrial Proteins as Antifungal Targets. Comparison of mitochondrial genomes across databases resulted in evidence that some mitochondrial protein complexes may present species-specific proteins. Further, analysis of fungal mitochondrial proteins identified both conserved and fungus-specific molecules, among them were potential targets for the development of antifungal therapies. However, functional assays will be required to determine their role in pathogenesis. Current data from functional studies show a correlation of defects in mitochondrial function with virulence attenuation in *C. albicans*. The Ras1-Cyr-PKA signaling pathway controls fungal virulence and filamentation. The optimal functioning of this pathway requires a high cell energy status. The RAS pathway interacts with complexes I and IV but not with complex II or alternative oxidase [65]. Nuo1 and Nuo2 are NADH:ubiquinone oxidoreductase proteins that were identified as nonmammalian complex I proteins and are broadly conserved among fungi. The mutants for these molecules presented low ATP synthesis and respiration, defects in complex I assembly, and avirulence in a mouse model, which confirm these two proteins as interesting targets for antifungal development [66, 67]. Mitochondrial biogenesis includes the SAM (sorting and assembly machinery) and ERMES (ER-mitochondria encounter structure) protein complexes, which are crucial for the import of proteins into the intermembrane space and then to the matrix with joint action of the membrane transporters. The disruption of Sam57 or Mmm1, belonging to SAM and ERMES complexes, respectively, resulted in avirulent *C. albicans* strains [68, 69]. Another fungal-specific protein from the ERMES system is the GTPase Fzo. The lack of this protein is related to increased hydrogen peroxide and azole susceptibility, probably due to the energy-dependent drug efflux pumps [66]. Some studies have identified fission/fusion mitochondrially related genes in the pathogen *A. fumigatus*. The fusion genes *Mgm1*, *Ugo1*, and *Fzo1* play roles in fungal viability and virulence in a galleria model. In contrast, fission mutants showed impaired sporulation and are not essential for virulence [70]. A recent investigation identified a broad-spectrum antifungal candidate with activity against *Candida*, *Aspergillus*, and *Cryptococcus*, namely, ilicicolin. This natural polyketide inhibits the cytochrome Bc1 reductase of complex III and has no effect on the mammalian enzyme [71–73]. Thus, several fungal-specific mitochondrial proteins are promising because inhibition of these genes/proteins abrogates fungal pathogenicity.

3.4. Alternative Carbon Source Pathways. Carbon sources may vary and fungal nutritional requirement differs throughout the course and site of infection. In addition to the abovementioned amino acid-related attenuation of virulence, nonmammalian carbon metabolizing pathways have been described as important pieces of the fungal virulence arsenal. Transcription profile studies indicate that *C. albicans* downregulates glycolytic genes and upregulates glyoxylate cycle genes, which facilitate the assimilation of two carbon compounds with generation of anaplerotic oxaloacetate to gluconeogenesis. Further studies showed that yeasts use the glyoxylate cycle and gluconeogenesis when inside the phagosome, while the glycolysis supports survival in tissues [74–76]. However, other pathogenic fungi are not strictly dependent on the glyoxylate cycle for successful colonization;

A. fumigatus strains tested for the impact of isocitrate lyase (ICL) resulted in no virulence attenuation [77]. Similar results for ICL mutants were observed in *C. neoformans* [78]. Differently, *A. fumigatus* relies more on the methylcitrate cycle (MCC) for survival in mammalian hosts [79]. Experimental infections with a methylcitrate synthase mutant showed that efficient degradation of propionyl-CoA is required for pathogenicity in a murine model of disease and, furthermore, the fungal cells were cleared from host tissues [80]. In this respect, transcriptional and proteomic analyses support the hypothesis that dimorphic fungi may rely on the MCC to adapt to carbon sources in host niches. The mRNA and protein of MCC-specific genes accumulate in infection-like conditions and during mycelium-to-yeast differentiation [81–85]. Additionally, *B. dermatitidis* cells recovered from mouse lungs show increased levels of mRNAs encoding MCC genes, indicating that fungal cells are exposed to propionyl-CoA-generating compounds [86].

3.5. Vitamin Synthesis, Ergosterol Metabolism, and Cell Wall as Targets for the Development of New Antifungals. Proteins involved in de novo vitamin biosynthesis are also suitable targets for antimycotic therapies. Unlike *C. albicans* and *A. fumigatus*, *H. capsulatum* is strongly dependent on phagocytes. This fungus is able to survive and grow inside phagosomes of the host's immune cells. *H. capsulatum* strains with interrupted biosynthesis of panthothenate and riboflavin were unable to proliferate in macrophage phagosomes and revealed attenuated virulence in vivo [87]. Thus, as these routes compose the set of metabolic adaptation to host conditions and are absent in humans, their components are strong candidates for antifungal therapy.

The metabolic processes of ergosterol biosynthesis, membrane stability and maintenance, cell wall remodeling, and folate synthesis are long-established drug targets. Although the existing antifungal agents used to inhibit the abovementioned targets exhibit toxicity for patients and resistant strains are often identified, these pathways are still considered to be in the pipeline for antifungal drug development, because they are absent in mammals and important for virulence. Polyoxins are peptide-derived compounds from *Streptomyces* that inhibit chitin synthesis and consequently are a promising therapy option. Drug repositioning and rational design strategies are used to elaborate new fungicidal compounds. The azole class of antifungals is a great option to manage mycoses; nonetheless, drugs that stick to heme-binding site of *Erg11* also target host cytochromes, resulting in liver toxicity. Recently, the compound VT-1161, a new azole, was rationally designed to have low affinity to mammalian proteins and high efficiency against *C. albicans* and *Coccidioides* [88, 89]. This promising antifungal has recently entered clinical trials [90]. Repositioning of anticancer drugs rendered the identification of AR-12, an antimicrobial compound that is highly potent against fungi. AR-12 inhibits acetyl-CoA synthetase, an essential fungal enzyme [90], and is effective against a broad collection within the kingdom of fungi, including the dimorphic clade [91].

4. Strategies to Develop New Antifungal Agents

Fungal infections represent an important medical issue particularly for immunocompromised patients, such as those with organ transplants or suffering from cancer and HIV infection, for whom fungal infections are frequently life-threatening [92–96]. The rate of these fungal infections has increased significantly in recent decades; pathogenic fungi are responsible for approximately 1.5 million cases of infection per year [92–96]. Despite these alarming statistics, the impact of fungal infections on human health has generally been neglected [97].

Although drugs with antifungal properties are available, they are limited when compared to antibacterial drugs. While the discovery of drugs based on polyenes, azoles, and allylamines may represent significant advances in the field of antifungal agent research, several challenges common to other pathogenic organisms such as side effects, narrow spectrum of activity, and the development of drug-resistant fungi must be overcome [98, 99]. The majority of antifungal drugs, except for amphotericin B, have a fungistatic effect [100]. However, the use of amphotericin B is restricted due to its side effects [100, 101]. Moreover, fungi are eukaryotic parasites that colonize a eukaryotic host, and the narrow range of physiologic differences between the host and colonizer may represent hurdles to developing safe and wide-ranging antifungal agents.

Several strategies may be employed to develop drugs; one of the foremost requirements for drug development is identification of relevant cellular targets to test these therapies. In this regard, identification and development of novel antifungal agents that have minimal toxicity to the host, as well as identification of fungal-specific molecular targets, are essential. Notably, distinct strategies can be applied to develop new antifungal agents to circumvent fungal resistance or promote a better quality of life for patients affected by fungal infections. One of the most employed methods for the development of new therapeuticals involves identifying bioactive compounds present in plants, animals, and microorganisms [99, 102–106]. In addition, a recent approach applies bioinformatics analysis to search in genomic databases for peptide sequences that have the physical-chemical characteristics of the antifungal drugs [107–109]. This may employ the use of several methodologies, including global approaches such as transcriptomic and proteomic methods that may contribute to the understanding of the mechanism of action of these new drugs [110, 111]. The next sections describe different approaches that could lead to the development of new antifungal compounds.

4.1. Identification of Bioactive Compounds. Natural compounds are widely used to identify antifungal molecules, and several natural compounds from various classes such as essential oils [112], lignan (reviewed in [113]), and curcumin (reviewed in [114]) have been studied.

Screening of libraries of synthetic small molecules or natural products is one of the most employed methods to identify drugs [99, 102, 103] and represents the vast majority of the available antibiotics for clinical use [99, 115–117].

Studies performed by Shi and colleagues characterized two acetophenone derivatives, 2-hydroxy-4,6-dimethoxyacetophenone and 2,4-dihydroxy-5-methylacetophenon, as plant antifungal agents [118]. These compounds were isolated from *Melicope borbonica* and *Polyporus picipes*, respectively, and were demonstrated to be effective in *Cytospora* sp., *Glomerella cingulata*, *Pyricularia oryzae*, *Botrytis cinerea*, and *Alternaria solani* [118]. Therefore, this strategy may be employed to screen for antifungal agents against human pathogenic fungi.

In fact, the antifungal agents, echinocandins and polyenes, were discovered through screening of natural products [119–121], and recently, Hein and colleagues reported the isolation of psoriasin from lesional psoriasis scale extracts. They identified this molecule as a potent fungicidal protein active against *Trichophyton rubrum* and *A. fumigatus*. The proposed mechanism of action is chelation of free intracellular zinc, which leads to fungal apoptosis [122]. It is important to note that biofilms are important in clinical settings mainly because they are associated with high drug resistance [123–126]. Recently, Seleem et al. demonstrated the effect of lichochalcone-A, a natural compound found in licorice roots of *Glycyrrhiza* species, on biofilms produced by *C. albicans* [127]. In this work, the authors showed that lichochalcone-A reduces *C. albicans* biofilm and decreases the proteolytic enzymatic activities of proteinases and phospholipases secreted by this fungus. Most importantly, mice treated with lichochalcone-A exhibited a significant reduction in fungal load five days postinfection, suggesting that this compound may be a candidate for a preclinical trial [127].

Despite the promise of these strategies, a problem encountered in the use of these molecules, which is in the majority of peptides, is their poor stability when applied in vivo. Peptides belonging to the most bioactive compounds were explored as antifungal agents. They can be obtained as metabolites of plants, insects, animals, and microorganisms [104, 105, 107] as the first-line arsenal to combat infections. These molecules are attracted to act against fungi because of a conformational structure that generally interacts with the fungal membrane causing an imbalance in the cell.

In general, the ability of antimicrobial peptides (AMPs) to destabilize microbial membranes is due to the conformation of these molecules that are approximately 50 amino acid residues long, with an overall residual positive charge and hydrophobic residues that provide an amphiphilic three-dimensional structure [107, 128]. Unlike most classic antifungal agents, their mechanism of action involves interruption of ergosterol biosynthesis. AMPs directly disrupt membrane integrity, culminating in cell death. Experimental data suggest that the mechanism of action of peptide-like lactoferrin B (Lfcin B) on biological membranes (using liposomes as a membrane model) is the formation of pores that results in loss of cell components [129].

Two cathelicidins (a LL-37 linear alpha-helical cationic peptide identified in the human vaginal tract and the bovine ortholog BMAP-28 peptide) were evaluated in vitro against *Candida* spp. isolated from patients. The mechanism of action for cathelicidins is to target the cell membrane, which causes its disruption and release of the intracellular components [125, 130]. They showed equal or better activity against biofilms formed by *Candida albicans* SC5314 compared to the antifungal agents, miconazole and amphotericin B (AMB). It was also observed that pH influenced the bioactivity of these peptides [125].

Oguro and colleagues demonstrated that a defensin (which is approximately 50 amino acid residues long and is frequently rich in cysteine) from *Brassica juncea* is capable of causing permeabilization and production of reactive oxygen species (ROS) in *C. albicans* by binding to sphingolipid glucosylceramide [131]. Generally, these molecules interact with membranes, inducing oxidative damage by an excessive increase in ROS [132].

The histatins are another group of amphipathic AMPs with helical structure that is important for their antifungal activity. Histatin 5 is found in human saliva and is toxic to fungal cells but causes low toxicity to human cells. It binds to fungal cell wall proteins, causing the release of intracellular ATP-activating P2X receptors in membranes forming ion channels [49, 50]. Because this molecule is capable of preventing biofilm formation caused by *C. albicans* [133], its hybridization with other molecules [134] has been used to improve the effect of these AMPs, which could possibly be applied in formulations to treat oropharyngeal candidiasis.

Despite the antimicrobial potential of these classes of biomolecules, they may be unstable when in biological media. For example, when exposed to pH values, which are not ideal for a structural conformation that presents antifungal activity, they may no longer be efficient. In addition, the histatins are amphipathic AMPs with helical structure that is important for their antifungal activity. Microorganisms can induce the production of metabolites by the host affecting AMP activity [135]. *C. albicans* can degrade LL-37 peptide by expressing secreted aspartic protease (SAP) family [136].

While these AMPs identified in natural sources are good candidates to be used in the development of new antifungal drugs, they must be chemically synthesized with various changes in their structure to improve their activity. López-Abarrategui et al. [106] presented a good example of changes in cationicity and hydrophobicity properties and Boman index in a peptide extracted from a mollusk. In this study, a more than three-fold increase in AMP activity against *C. albicans* was observed, compared to that in the wild-type peptide [106].

Based on the properties described for AMP, computational programs have been developed to identify AMP-similar sequences by exploring the genomic databases of sequenced organisms. Amaral et al. reported the identification of four peptide sequences, two in the human genome and two in the *Paracoccidioides brasiliensis* transcriptome, which are potential candidates as antifungal agents [107].

However, peptides often do not present the expected activity in vivo even when they present good in vitro results for several reasons, including enzymatic degradation or peptide structure destabilization [137]. A possible alternative to solve these issues is by incorporating these molecules into nanostructured drug delivery systems [138]. Such systems are prepared using nanotechnological approaches that allow rational delivery of bioactive compounds, such as AMP.

4.2. Nanostructured Antifungals. According to some studies, nanotechnology is capable of improving antifungal activity for both conventional drugs [139] and bioactive molecules [140]. These improvements may be attributed to nanoscale properties that allow biomolecule protection against biodegradation. Association of AMP with a nanoparticle-based drug delivery system can increase its delivery to the site of action.

d'Angelo et al. developed a nanostructured system for the delivery of colistin, a cationic AMP within poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles engineered with mucoadhesive chitosan [137]. In addition to allowing the protection of peptides against degradation, this approach also permits more effective targeting to the site of action.

Nanotechnology has also been applied to exploit the antimicrobial properties of certain nanomaterials such as silver nanoparticles, which are naturally effective against microorganisms by destabilizing their membrane through electrostatic interactions [138]. However, these nanoparticles also exhibit high toxicity to mammalian cells, which can compromise their use. One way to avoid or reduce this undesirable effect is by incorporating AMP onto the surface of these nanoparticles, decreasing the toxicity against erythrocytes [141]. This combination increased AMP efficiency and further increased its stability compared to the AMP without complexation with nanoparticles. Silver nanoparticles coated with a peptide had diminished cytotoxicity against erythrocytes [141].

Nanotechnology properties are also used to enhance the activity of classic antifungals, especially when the aim is to reduce their unwanted toxic effects, such as in amphotericin B (AMB), which is considered one of the front lines in antifungal therapy [139, 142]. A classic nanostructured formulation for AMB is AmBisome®, a liposomal formulation that minimizes unwanted toxic effects of this potential drug.

Amaral et al. developed a nanoformulation containing AMB within PLGA and functionalized with dimercaptosuccinic acid (DMSA), which presents a tropism to the lungs. The formulation showed no genotoxicity and its therapeutic effect was better than that observed in vivo for AMB deoxycholate in a chronic paracoccidioidomycosis murine model [139]. The authors suggested that the effect might be due to DMSA, which drives the entire nanostructure toward the lungs, guiding the drug to the site of action.

Xu and colleagues also tested another polymeric formulation for AMB using α-butyl-cyanoacrylate in experimental meningitis caused by *C. neoformans* [143]. In this study, the AMB in brain tissue was detected 0.5 hours after injection into animals and a maximum AMB concentration was detected within 3 hours when compared to that in the animals treated with conventional AMB. In addition, animals with cryptococcal meningitis treated with the formulation showed a high survival rate [143]. This nanoformulation was first developed by the same research group that demonstrated the potential of nanoparticles containing AMB coated with polysorbate 80 to deliver this drug across blood brain barrier when compared to the conventional formulation for AMB [144]. Thus, nanoformulations could be used to deliver toxic drugs, such as AMB, in cases of antimicrobial resistance to less toxic drugs, such as azoles, used mainly by immunocompromised patients.

4.3. Drug Repositioning. Although the approaches described above are important for antifungal drug development, it is important to emphasize that the process of drug discovery and development requires 10–17 years on average and the success rate is lower than 10%. A repositioning approach is based on previous research and development of conventional medications that have already been tested in humans in terms of toxicology and pharmacology [145–149]. In this context, drug-repositioning screening may be a valid and affordable alternative method to obtain antifungal drugs. In fact, this strategy has been employed to expedite identification of new therapeutic applications and has been successfully used in therapy of parasitic diseases [150] and several types of cancer [151, 152].

Sun and colleagues, using high-throughput assays, screened approved drugs to identify potent effects against *Exserohilum rostratum* infections [153]. They identified bithionol (antiparasitic drug), tacrolimus (immunosuppressive agent), and floxuridine (antimetabolite) as anti-*E. rostratum* agents, confirming that drug repositioning can be performed [153].

Another approach using repositioning drugs as antifungal agents has been performed. In addition to identifying a drug that has an antifungal effect individually, researchers are working to identify compounds that may synergize with available antifungal drugs. An outstanding example of this approach was performed by Robbins et al. [154]. In this work, a library of compounds was evaluated in combination with subinhibitory concentrations of known antifungals such as amphotericin B, fluconazole, terbinafine, caspofungin, benomyl, and cyprodinil. Remarkably, this approach led to the identification of a synergistic effect with clofazimine (an antimycobacterial drug not reported as antifungal) with caspofungin and posaconazole, indicating that this may represent a potential therapeutic approach against diverse fungal pathogens.

5. Vaccine Development against Fungal Diseases

The immune response against microorganisms such as fungi consists of two major systems: the innate and adaptive immune responses. The adaptive immune response is constituted by cellular and humoral immune responses. The innate response is the first pathway for fungal detection. Immune cells recognize fungal pathogen-associated molecular patterns (PAMP) through pattern recognition receptors (PRR) present in host cells. After invasion by pathogens and recognition, cells are recruited and stimulated to the infection site, such as monocytes, neutrophils, macrophages, natural killer cells, and dendritic cells, which develop an important role to link and stimulate the adaptive immune response (reviewed in [155, 156]).

When activated, those cells secrete soluble molecules that participate in effector mechanisms for fungal clearance. Among these molecules, there are complement proteins,

antimicrobial peptides, costimulatory molecules, chemokines, and cytokines. Phagocytic cells are sufficient to trigger all effector mechanisms. Phagocytosis initiates a signaling pathway, and this microenvironment determines the cytokine pattern and antimicrobial functions that will be developed. Both innate and adaptive immune responses will work together. Furthermore, the effective mechanism to adaptive immune response is based on innate response (reviewed in [155, 156]).

There are different classes of cytokines as proinflammatory (IL-1, IL-6, IL-17, IFN-γ, and TNF-α), anti-inflammatory (IL-4, IL-13, and TGF-β), and immune regulatory (IL-10). When proinflammatory cytokines are prevalent, cellular immunity mediated by $CD4^+$ T cells (Th1 and Th17) is stimulated, whereas when these cytokines are downregulated, humoral immunity takes place ($CD4^+$ T cells—Th2, or regulatory T cells (Treg)). Both types of cytokines stimulate antibody production, fungal killing, transcription factors such as NF-κB. It is widely accepted that induction of Th1-/Th17-type cellular response is crucial for the defense against fungal infection. Th2 humoral response is usually considered not protector, since it stimulates antibody switching to nonopsonizing isotypes. The isotype IgG2a is considered the most protective antibody for fungal infection because it induces a cross-talking with effector molecules of cellular response. Cytotoxic T cells ($CD8^+$ T cells) are the major producers of IFN-γ, TNF-α, and IL-2. These cells naturally occur in the host response to fungal pathogens in the lungs. In conclusion, the interaction of fungal derivatives with immune cells depends on the nature of compounds for yeasts/filamentous and is developed by the complex microenvironment (reviewed in [155, 156]).

The use of vaccine strategies that can be applied together with drug therapy could reduce treatment times, reestablish a protective immune response, and prevent post-treatment sequelae [157]. In the past decade, researchers have been investigating vaccine development against the major human and veterinary fungal pathogens. Most studies are focused on protection against the pathogenic fungi *Aspergillus* spp. [158], *Candida* spp. [159], *B. dermatitidis* [160], *Cryptococcus* spp. [161], *Coccidioides* spp., *Histoplasma capsulatum* [162], *Microsporum canis* [163], *Paracoccidioides* spp. [164], *Pneumocystis jirovecii* [165], *Sporothrix* spp. [166], and *Talaromyces* (*Penicillium*) *marneffei* [167, 168], which cause cutaneous, subcutaneous, and systemic mycoses in all regions of the world and account for millions of new infections per year. The development of safe and efficacious vaccines against fungi is still a challenge due to our lack of knowledge about immunity against fungal infection. However, the use of animal models is helping us to better understand the interaction between the immune system and fungi [169].

There is no doubt that $CD4^+$ T cells play a major role in mediating resistance against fungal infection in immunocompetent and immunosuppressed patients [170]. The mechanism of resistance conferred by $CD4^+$ T cells occurs through secretion of T-helper 1 (Th1) or Th17 cytokines such as IFN-γ, TNF-α, GM-CSF, and IL-17A, which activate various cell populations including neutrophils, macrophages, and dendritic cells. Natural killer cells and natural killer T cells also play an important role in fungal control by modulating immune response through the production of proinflammatory cytokines such as IFN-γ and TNF-α [171–174]. The function of B cells and production of protective antibodies were once controversial. However, today, it is known that the protective function of antibodies depends on various factors [175–177]. Although $CD4^+$ T cells play a major role in fungal control, mediation of protective immunity by $CD8^+$ T cells against fungal infection has been documented. The studies involving $CD8^+$ T cells as vaccine strategies gain special importance in patients with impaired $CD4^+$ T cells as occurring under HIV infection [169, 178].

Protective immunity mediated by $CD8^+$ T cells has been documented in different fungal infections such as aspergillosis, histoplasmosis, cryptococcosis, blastomycosis, paracoccidioidomycosis, pnemocystosis, and mucosal candidiasis. Usually, antifungal $CD8^+$ T cells are elicited by cross-presentation of fungal peptides by MHC class I. Antifungal immunity in the absence of the $CD4^+$ T cell can be mediated by cytotoxic type I $CD8^+$ T cells, which secrete IFN-γ, TNF-α, and GM-CSF, as well as by the cytotoxic factors perforin, granulysin, and granzyme K or IL-17/IFN-γ double-producing $CD8^+$ T cells [169]. Nanjappa et al. showed that even in the absence of $CD4^+$ T cells, vaccinated mice with 105-106 yeast of attenuated *B. dermatitidis* induced IL-17 producing $CD8^+$ T cell, which conferred resistance against *B. dermatitidis* infection [179]. Although there is no licensed vaccine for humans, the results of experimental models are promising and can indicate candidates for clinical trials. Below, we will discuss some vaccine models in development against endemic fungal infections.

5.1. P. brasiliensis and P. lutzii. A main candidate for a vaccine against *P. brasiliensis* is the P10 peptide, whose sequence is QTLIAIHTLAIRYAN. $CD4^+$ T cell modulation with a significant increase in IL-12 and IFN-γ and a decrease in IL-4 and IL-10 was observed in mice immunized with P10 alone or in association with antifungal drugs [180]. Another antigenic protein from *P. brasiliensis*, rPb27, showed protective results in an experimental model using mice [181]. The immunization of BALB/c mice with radioattenuated yeast cells of *P. brasiliensis* promoted long-lasting protection against an infective yeast form [182]. The passive transfer of monoclonal antibodies against the 43 kDa glycoprotein gp43 [183] or gp70 [184] from *P. brasiliensis* was shown to be protective against experimental infection of *P. brasiliensis*. Monoclonal antibodies generated against the heat shock protein 60 from *H. capsulatum* also interact with *P. lutzii* yeast cells and enhance phagocytosis by macrophage cells. The passive transfer of 7B6 and 4E12 mAbs against Hsp60 was protective and reduced the fungal burden in the lungs of BALB/c mice intratracheally infected with *P. lutzii* [175].

5.2. H. capsulatum. Vaccination with recombinant HSP60 from *H. capsulatum* is able to elicit protection mediated by $CD4^+$ T cells and induces IFN-γ production [185]. Immunization with apoptotic phagocytes containing heat-killed *H. capsulatum* efficiently activated $CD8^+$ T cells, whose

contribution was equal to that of $CD4^+$ T cells in protecting against *Histoplasma* challenge [186]. Treatment of mice with a monoclonal antibody against HSP60 before infection reduced the fungal burden in the lungs [175].

5.3. Coccidioides posadasii and C. immitis. The first vaccine tested against endemic mycosis using a killed spherule vaccine failed during a phase 3 clinical trial [169]. A decapeptide agonist of the biologically active C-terminal region of the human complement component C5a, termed EP67, was conjugated with lysine residues on the surface of live arthroconidia. The use of the EP67 vaccine induced phagocytosis and antigen presentation. BALB/c mice immunized with conjugated EP67 increased survival and reduced inflammatory pathology and fungal burden. This protection was mediated by augmenting the T-helper 1 (Th1) and Th17 responses [187, 188]. Whole glucan particles prepared from *Saccharomyces cerevisiae* conjugated with BSA induced significant protection in CD-1 mice [189]. A recombinant Ag2/PRA106 + CSA chimeric fusion protein vaccine in ISS/Montanide adjuvant administered intramuscularly showed promising results in adult female cynomolgus macaques challenged with *C. posadasii* [190]. Synthetic peptides corresponding to the five selected epitopes from fungal aspartyl protease, alpha-mannosidase, and phospholipidase B were incorporated into the vaccine with synthesized CpG ODN in incomplete Freund's adjuvant. Mice vaccinated and challenged intranasally with *C. posadasii* showed reduced fungal burden and infiltration of activated T-helper 1 (Th1), Th2, and Th17 cells, as well as elevated IFN-γ and IL-17 [2].

5.4. Sporothrix brasiliensis and S. schenckii. The passive transfer of monoclonal antibody P6E7 (which recognizes a 70 kDa glycoprotein—gp70—an important factor of virulence) reduced the fungal burden of mice infected with some but not all *Sporothrix* isolates tested. Protection was detected during the early stages and relapse in the final period of infection (21 days) [191].

Serum from mice immunized with a 44 kDa (peptide hydrolase) and 47 kDa (enolase) *Sporothrix schenckii* cell wall protein (predicted to be an adhesein) in aluminum hydroxide was used in a passive transfer to mice systemically infected with *S. schenckii* and resulted in protection. In vitro assays with yeast cells opsonized with serum from immunized animals increased phagocytosis and inhibited the adhesion of the fungi to the fibroblasts [192].

5.5. B. dermatitidis. Virulence-attenuated yeast cells of *B. dermatitidis* (strain 55) were injected subcutaneously into C57Bl/6 mice. Animals were challenged by intratracheal infection with wild-type *B. dermatitidis* 26199 yeasts. Vaccine-induced protection requires dectin-2 to promote differentiation of activated T-helper 1 (Th1) and Th17 cells [160]. A genetically engineered live-attenuated strain of *B. dermatitidis* lacking the major virulence factor BAD-1 was shown to be protective against lethal experimental infection in mice [193]; vaccine immunity in endemic dimorphic fungal infection is primarily mediated by T-helper 1 (Th1) and Th17 cells, but not by antibodies as observed in the experimental model using mice [194]. The use of BAD-1-deficient *B. dermatitidis* has also been investigated as a pan-fungal vaccine against endemic mycosis in North America (*C. posadasii*, *H. capsulatum*, and *B. dermatitidis*). This vaccine induced Th17 and was sufficient to protect against all fungi tested. The protection was mediated by Th17 cells, which recruited and activated neutrophils and macrophages to the alveolar space [162].

5.6. Aspergillus spp. and Candida spp. There are no licensed vaccines against *Aspergillus*. The literature described four *Aspergillus* vaccine categories: pan-fungal, crude, subunit, and therapeutic. All candidates for *Aspergillus* vaccines are being tested in an experimental mice model. A pan-fungal vaccine has advantages when compared with other categories of vaccines (reviewed in [195]). A conjugated β-1,3-D-glucan to diphtheria toxin has been shown to be immunogenic, and antibodies were protective as an immunoprophylactic vaccine against systemic and vaginal *Candida albicans* and *A. fumigatus* [196]. On the other hand, subcutaneous mice vaccination with heat-killed *Saccharomyces cerevisiae* yeast protected against *Aspergillus*, *Coccidioides*, or *Candida* challenge [158]. Using transgenic $CD4^+$ T cells, a sequence of amino acids was identified within chaperone calnexin that is conserved in phylum Ascomycota. Vaccine-conjugated recombinant calnexin protein or calnexin peptide with different adjuvants induced resistance to lethal challenge against *B. dermatitidis*, *H. capsulatum*, and *C. posadasii*. Although this vaccine has not been tested against *Aspergillus* and *Candida*, proteomic studies found potential [197]. The other categories, such as live or killed *A. fumigatus* (crude); recombinant proteins Asp f3, Gel1, Asp f9 (Crf1), Asp f16, and Pep1(subunit); or adoptive transfer of *Aspergillus*-specific $CD4^+$ T cells (therapeutic), showed to be potential candidates for a human vaccine [195]. Due to the importance of both *Candida* spp. and *Aspergillus* spp. infections, there are several vaccine formulations in development. The most common protocols include the following: live-attenuated *C. albicans* strain, purified protein (Sap2p and Als3p), HSP90p, Hyr1p (glycosylphosphatidylinositol- (GPI-) anchored mannoprotein on the cell wall), cell wall extract (β-mercaptoethanol extract), glycoconjugated vaccines (polysaccharide as a carrier), β-mannan and peptide conjugates, and β-glucan conjugate with MF59 adjuvant. Although most experiments reported protection in murine models, there is no knowledge yet if these vaccines will be effective for human protection [198].

6. Concluding Remarks

It is possible that fungal infection will continue to rise as a result of increased numbers of immunocompromised patients. The development of new tools to treat patients with fungal infection is a priority. In addition to the discovery of new antifungal drugs, vaccines are an important alternative to be used alone or in combination with antifungal drugs. Researchers and the pharmaceutical industry are currently investing in the development of a pan-fungal vaccine to reach the largest number of patients.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] C. P. Taborda, M. E. Urán, J. D. Nosanchuk, and L. R. Travassos, "Paracoccidioidomycosis: challenges in the development of a vaccine against an endemic mycosis in the Americas," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 57 Supplement 1, no. 1, pp. 21–24, 2015.

[2] B. J. Hurtgen, C. Y. Hung, G. R. Ostroff, S. M. Levitz, and G. T. Cole, "Construction and evaluation of a novel recombinant T cell epitope-based vaccine against coccidioidomycosis," *Infection and Immunity*, vol. 80, no. 11, pp. 3960–3974, 2012.

[3] N. P. Medici and M. D. Poeta, "New insights on the development of fungal vaccines: from immunity to recent challenges," *Memorias do Instituto Oswaldo Cruz*, vol. 110, no. 8, pp. 966–973, 2015.

[4] O. Mayorga, J. E. Muñoz, N. Lincopan et al., "The role of adjuvants in therapeutic protection against paracoccidioidomycosis after immunization with the P10 peptide," *Frontiers in Microbiology*, vol. 3, pp. 1–6, 2012.

[5] R. Rajendran, L. Sherry, C. J. Nile et al., "Biofilm formation is a risk factor for mortality in patients with *Candida albicans* bloodstream infection-Scotland, 2012-2013," *Clinical Microbiology and Infection*, vol. 22, no. 1, pp. 87–93, 2016.

[6] M. Staniszewska, M. Bondaryk, M. Wieczorek, E. Estrada-Mata, H. M. Mora-Montes, and Z. Ochal, "Antifungal effect of novel 2-bromo-2-chloro-2-(4-chlorophenylsulfonyl)-1-phenylethanone against Candida strains," *Frontiers in Microbiology*, vol. 7, p. 1309, 2016.

[7] T. C. White, K. A. Marr, and R. A. Bowden, "Clinical, cellular, and molecular factors that contribute to antifungal drug resistance," *Clinical Microbiology Reviews*, vol. 11, no. 2, pp. 382–402, 1998.

[8] M. A. Pfaller, "Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment," *The American Journal of Medicine*, vol. 125, 1 Supplement, pp. S3–13, 2012.

[9] F. Abe and T. Hiraki, "Mechanistic role of ergosterol in membrane rigidity and cycloheximide resistance in *Saccharomyces cerevisiae*," *Biochimica et Biophysica Acta*, vol. 1788, no. 3, pp. 743–752, 2009.

[10] D. Hagiwara, A. Watanabe, K. Kamei, and G. H. Goldman, "Epidemiological and genomic landscape of azole resistance mechanisms in Aspergillus fungi," *Frontiers in Microbiology*, vol. 7, p. 1382, 2016.

[11] S. Paul and W. S. Moye-Rowley, "Multidrug resistance in fungi: regulation of transporter-encoding gene expression," *Frontiers in Physiology*, vol. 5, p. 143, 2014.

[12] E. Snelders, A. Karawajczyk, R. J. Verhoeven et al., "The structure-function relationship of the *Aspergillus fumigatus* cyp51A L98H conversion by site-directed mutagenesis: the mechanism of L98H azole resistance," *Fungal Genetics and Biology*, vol. 48, no. 11, pp. 1062–1070, 2011.

[13] F. Gsaller, P. Hortschansky, T. Furukawa et al., "Sterol biosynthesis and azole tolerance is governed by the opposing actions of SrbA and the CCAAT binding complex," *PLoS Pathogens*, vol. 12, no. 7, article e1005775, 2016.

[14] A. C. Fontes, D. Bretas Oliveira, J. R. Santos et al., "A subdose of fluconazole alters the virulence of *Cryptococcus gattii* during murine cryptococcosis and modulates type I interferon expression," *Medical Mycology*, vol. 55, no. 2, pp. 203–212, 2017.

[15] M. Bromley, A. Johns, E. Davies et al., "Mitochondrial complex I is a global regulator of secondary metabolism, virulence and azole sensitivity in fungi," *PloS One*, vol. 11, no. 7, article e0158724, 2016.

[16] P. Yibmantasiri, P. W. Bircham, D. R. Maass, D. S. Bellows, and P. H. Atkinson, "Networks of genes modulating the pleiotropic drug response in *Saccharomyces cerevisiae*," *Molecular BioSystems*, vol. 10, no. 1, pp. 128–137, 2014.

[17] S. Omrane, H. Sghyer, C. Audeon et al., "Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates," *Environmental Microbiology*, vol. 17, no. 8, pp. 2805–2823, 2015.

[18] L. J. Wheat, P. Connolly, M. Smedema, E. Brizendine, R. Hafner, and AIDS Clinical Trials Group and the Mycoses Study Group of the National Institute of Allergy and Infectious Diseases, "Emergence of resistance to fluconazole as a cause of failure during treatment of histoplasmosis in patients with acquired immunodeficiency disease syndrome," *Clinical Infectious Diseases*, vol. 33, no. 11, pp. 1910–1913, 2001.

[19] D. W. Denning, "Echinocandin antifungal drugs," *Lancet*, vol. 362, no. 9390, pp. 1142–1151, 2003.

[20] S. K. Katiyar, A. Alastruey-Izquierdo, K. R. Healey, M. E. Johnson, D. S. Perlin, and T. D. Edlind, "Fks1 and Fks2 are functionally redundant but differentially regulated in *Candida glabrata*: implications for echinocandin resistance," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 12, pp. 6304–6309, 2012.

[21] G. R. Thompson 3rd, N. P. Wiederhold, A. C. Vallor, N. C. Villareal, J. S. Lewis 2nd, and T. F. Patterson, "Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection," *Antimicrobial Agents and Chemotherapy*, vol. 52, no. 10, pp. 3783–3785, 2008.

[22] C. Garnaud, F. Botterel, N. Sertour et al., "Next-generation sequencing offers new insights into the resistance of *Candida* spp. to echinocandins and azoles," *The Journal of Antimicrobial Chemotherapy*, vol. 70, no. 9, pp. 2556–2565, 2015.

[23] D. S. Perlin, "Current perspectives on echinocandin class drugs," *Future Microbiology*, vol. 6, no. 4, pp. 441–457, 2011.

[24] G. J. Dijkgraaf, M. Abe, Y. Ohya, and H. Bussey, "Mutations in Fks1p affect the cell wall content of beta-1,3- and beta-1,6-glucan in *Saccharomyces cerevisiae*," *Yeast*, vol. 19, no. 8, pp. 671–690, 2002.

[25] S. Ishihara, A. Hirata, S. Nogami, A. Beauvais, J. P. Latge, and Y. Ohya, "Homologous subunits of 1,3-beta-glucan synthase are important for spore wall assembly in *Saccharomyces cerevisiae*," *Eukaryotic Cell*, vol. 6, no. 2, pp. 143–156, 2007.

[26] R. K. Shields, M. H. Nguyen, E. G. Press et al., "Rate of FKS mutations among consecutive Candida isolates causing bloodstream infection," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 12, pp. 7465–7470, 2015.

[27] C. D. Pham, N. Iqbal, C. B. Bolden et al., "Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 8, pp. 4690–4696, 2014.

[28] G. Garcia-Effron, S. Park, and D. S. Perlin, "Correlating echinocandin MIC and kinetic inhibition of fks1 mutant glucan

synthases for *Candida albicans*: implications for interpretive breakpoints," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 1, pp. 112–122, 2009.

[29] S. K. Katiyar and T. D. Edlind, "Role for Fks1 in the intrinsic echinocandin resistance of *Fusarium solani* as evidenced by hybrid expression in *Saccharomyces cerevisiae*," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 5, pp. 1772–1778, 2009.

[30] S. Katiyar, M. Pfaller, and T. Edlind, "*Candida albicans* and Candida glabrata clinical isolates exhibiting reduced echinocandin susceptibility," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 8, pp. 2892–2894, 2006.

[31] G. Garcia-Effron, S. Lee, S. Park, J. D. Cleary, and D. S. Perlin, "Effect of Candida glabrata FKS1 and FKS2 mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 9, pp. 3690–9, 2009.

[32] A. Fekkar, I. Meyer, J. Y. Brossas et al., "Rapid emergence of echinocandin resistance during *Candida kefyr* fungemia treatment with caspofungin," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 5, pp. 2380–2382, 2013.

[33] R. H. Jensen, H. K. Johansen, and M. C. Arendrup, "Stepwise development of a homozygous S80P substitution in Fks1p, conferring echinocandin resistance in *Candida tropicalis*," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 1, pp. 614–617, 2013.

[34] T. Pasquale, J. R. Tomada, M. Ghannoun, J. Dipersio, and H. Bonilla, "Emergence of *Candida tropicalis* resistant to caspofungin," *The Journal of Antimicrobial Chemotherapy*, vol. 61, no. 1, p. 219, 2008.

[35] R. Garcia, J. Botet, J. M. Rodriguez-Pena et al., "Genomic profiling of fungal cell wall-interfering compounds: identification of a common gene signature," *BMC Genomics*, vol. 16, p. 683, 2015.

[36] D. S. Perlin, "Mechanisms of echinocandin antifungal drug resistance," *Annals of the new York Academy of Sciences*, vol. 1354, pp. 1–11, 2015.

[37] R. E. Gardiner, P. Souteropoulos, S. Park, and D. S. Perlin, "Characterization of *Aspergillus fumigatus* mutants with reduced susceptibility to caspofungin," *Medical Mycology*, vol. 43, Supplemenr 1, pp. S299–S305, 2005.

[38] L. A. Walker, N. A. Gow, and C. A. Munro, "Elevated chitin content reduces the susceptibility of *Candida* species to caspofungin," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 1, pp. 146–154, 2013.

[39] L. A. Walker, C. A. Munro, I. de Bruijn, M. D. Lenardon, A. McKinnon, and N. A. Gow, "Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins," *PLoS Pathogens*, vol. 4, no. 4, article e1000040, 2008.

[40] K. Ueno, Y. Namiki, H. Mitani, M. Yamaguchi, and H. Chibana, "Differential cell wall remodeling of two chitin synthase deletants Deltachs3A and Deltachs3B in the pathogenic yeast *Candida glabrata*," *FEMS Yeast Research*, vol. 11, no. 5, pp. 398–407, 2011.

[41] K. D. Goughenour and C. A. Rappleye, "Antifungal therapeutics for dimorphic fungal pathogens," *Virulence*, vol. 8, no. 2, pp. 211–221, 2017.

[42] W. I. Gruszecki, M. Gagos, M. Herec, and P. Kernen, "Organization of antibiotic amphotericin B in model lipid membranes. A mini review," *Cellular & Molecular Biology Letters*, vol. 8, no. 1, pp. 161–170, 2003.

[43] T. M. Anderson, M. C. Clay, A. G. Cioffi et al., "Amphotericin forms an extramembranous and fungicidal sterol sponge," *Nature Chemical Biology*, vol. 10, no. 5, pp. 400–406, 2014.

[44] A. Forastiero, A. C. Mesa-Arango, A. Alastruey-Izquierdo et al., "*Candida tropicalis* antifungal cross-resistance is related to different azole target (Erg11p) modifications," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 10, pp. 4769–4781, 2013.

[45] A. C. Mesa-Arango, N. Trevijano-Contador, E. Román et al., "The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug," *Antimicrobial Agents and Chemotherapy*, vol. 58, pp. 6627–6638, 2014.

[46] B. M. Vincent, A. K. Lancaster, R. Scherz-Shouval, L. Whitesell, and S. Lindquist, "Fitness trade-offs restrict the evolution of resistance to amphotericin B," *PLoS Biology*, vol. 11, no. 10, article e1001692, 2013.

[47] G. Blum, C. Hortnagl, E. Jukic et al., "New insight into amphotericin B resistance in *Aspergillus terreus*," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 4, pp. 1583–1588, 2013.

[48] J. R. Perfect, J. L. Tenor, Y. Miao, and R. G. Brennan, "Trehalose pathway as an antifungal target," *Virulence*, vol. 8, no. 2, pp. 143–149, 2017.

[49] S. H. Lillie and J. R. Pringle, "Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation," *Journal of Bacteriology*, vol. 143, no. 3, pp. 1384–1394, 1980.

[50] B. Elliott, R. S. Haltiwanger, and B. Futcher, "Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*," *Genetics*, vol. 144, no. 3, pp. 923–933, 1996.

[51] M. A. Singer and S. Lindquist, "Thermotolerance in *Saccharomyces cerevisiae*: the yin and yang of trehalose," *Trends in Biotechnology*, vol. 16, no. 11, pp. 460–468, 1998.

[52] P. Ngamskulrungroj, U. Himmelreich, J. A. Breger et al., "The trehalose synthesis pathway is an integral part of the virulence composite for *Cryptococcus gattii*," *Infection and Immunity*, vol. 77, no. 10, pp. 4584–4596, 2009.

[53] E. W. Petzold, U. Himmelreich, E. Mylonakis et al., "Characterization and regulation of the trehalose synthesis pathway and its importance in the pathogenicity of *Cryptococcus neoformans*," *Infection and Immunity*, vol. 74, no. 10, pp. 5877–5887, 2006.

[54] R. Sanchez-Fresneda, P. Gonzalez-Parraga, O. Esteban, L. Laforet, E. Valentín, and J. C. Argüelles, "On the biochemical classification of yeast trehalases: *Candida albicans* contains two enzymes with mixed features of neutral and acid trehalase activities," *Biochemical and Biophysical Research Communications*, vol. 383, no. 1, pp. 98–102, 2009.

[55] Y. Pedreno, P. Gonzalez-Parraga, M. Martinez-Esparza, R. Sentandreu, E. Valentín, and J. C. Argüelles, "Disruption of the *Candida albicans* ATC1 gene encoding a cell-linked acid trehalase decreases hypha formation and infectivity without affecting resistance to oxidative stress," *Microbiology*, vol. 153, no. Pt 5, pp. 1372–1381, 2007.

[56] J. P. Guirao-Abad, R. Sanchez-Fresneda, E. Valentin, M. Martínez-Esparza, and J. C. Argüelles, "Analysis of validamycin as a potential antifungal compound against *Candida albicans*," *International Microbiology*, vol. 16, no. 4, pp. 217–225, 2013.

[57] M. Hensel, H. N. Arst Jr., A. Aufauvre-Brown, and D. W. Holden, "The role of the *Aspergillus fumigatus* areA gene in invasive pulmonary aspergillosis," *Molecular & General Genetics*, vol. 258, no. 5, pp. 553–557, 1998.

[58] S. Krappmann, E. M. Bignell, U. Reichard, T. Rogers, K. Haynes, and G. H. Braus, "The *Aspergillus fumigatus* transcriptional activator CpcA contributes significantly to the virulence of this fungal pathogen," *Molecular Microbiology*, vol. 52, no. 3, pp. 785–799, 2004.

[59] J. Amich and E. Bignell, "Amino acid biosynthetic routes as drug targets for pulmonary fungal pathogens: what is known and why do we need to know more?" *Current Opinion in Microbiology*, vol. 32, pp. 151–158, 2016.

[60] F. Schobel, I. D. Jacobsen, and M. Brock, "Evaluation of lysine biosynthesis as an antifungal drug target: biochemical characterization of *Aspergillus fumigatus* homocitrate synthase and virulence studies," *Eukaryotic Cell*, vol. 9, no. 6, pp. 878–893, 2010.

[61] J. D. Oliver, S. J. Kaye, D. Tuckwell et al., "The *Aspergillus fumigatus* dihydroxyacid dehydratase Ilv3A/IlvC is required for full virulence," *PloS One*, vol. 7, no. 9, article e43559, 2012.

[62] A. Sasse, S. N. Hamer, J. Amich, J. Binder, and S. Krappmann, "Mutant characterization and in vivo conditional repression identify aromatic amino acid biosynthesis to be essential for *Aspergillus fumigatus* virulence," *Virulence*, vol. 7, no. 1, pp. 56–62, 2016.

[63] J. M. Kingsbury and J. H. McCusker, "Threonine biosynthetic genes are essential in *Cryptococcus neoformans*," *Microbiology*, vol. 154, no. Pt 9, pp. 2767–2775, 2008.

[64] W. Fan, P. R. Kraus, M. J. Boily, and J. Heitman, "*Cryptococcus neoformans* gene expression during murine macrophage infection," *Eukaryotic Cell*, vol. 4, no. 8, pp. 1420–1433, 2005.

[65] N. Grahl, E. G. Demers, A. K. Lindsay et al., "Mitochondrial activity and Cyr1 are key regulators of Ras1 activation of *C. albicans* virulence pathways," *PLoS Pathogens*, vol. 11, no. 8, article e1005133, 2015.

[66] D. Li, X. She, and R. Calderone, "Functional diversity of complex I subunits in *Candida albicans* mitochondria," *Current Genetics*, vol. 62, no. 1, pp. 87–95, 2016.

[67] X. She, K. Khamooshi, Y. Gao et al., "Fungal-specific subunits of the *Candida albicans* mitochondrial complex I drive diverse cell functions including cell wall synthesis," *Cellular Microbiology*, vol. 17, no. 9, pp. 1350–1364, 2015.

[68] R. Calderone, D. Li, and A. Traven, "System-level impact of mitochondria on fungal virulence: to metabolism and beyond," *FEMS Yeast Research*, vol. 15, no. 4, p. fov027, 2015.

[69] J. M. Becker, S. J. Kauffman, M. Hauser et al., "Pathway analysis of *Candida albicans* survival and virulence determinants in a murine infection model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 51, pp. 22044–22049, 2010.

[70] M. Neubauer, Z. Zhu, M. Penka, C. Helmschrott, N. Wagener, and J. Wagener, "Mitochondrial dynamics in the pathogenic mold *Aspergillus fumigatus*: therapeutic and evolutionary implications," *Molecular Microbiology*, vol. 98, no. 5, pp. 930–945, 2015.

[71] S. B. Singh, W. Liu, X. Li et al., "Structure-activity relationship of cytochrome bc1 reductase inhibitor broad spectrum antifungal ilicicolin H," *Bioorganic & Medicinal Chemistry Letters*, vol. 23, no. 10, pp. 3018–3022, 2013.

[72] E. B. Gutierrez-Cirlos, T. Merbitz-Zahradnik, and B. L. Trumpower, "Inhibition of the yeast cytochrome bc1 complex by ilicicolin H, a novel inhibitor that acts at the Qn site of the bc1 complex," *The Journal of Biological Chemistry*, vol. 279, no. 10, pp. 8708–8714, 2004.

[73] R. Covian and B. L. Trumpower, "Ilicicolin inhibition and binding at center N of the dimeric cytochrome bc1 complex reveal electron transfer and regulatory interactions between monomers," *The Journal of Biological Chemistry*, vol. 284, no. 13, pp. 8614–8620, 2009.

[74] M. C. Lorenz, J. A. Bender, and G. R. Fink, "Transcriptional response of *Candida albicans* upon internalization by macrophages," *Eukaryotic Cell*, vol. 3, no. 5, pp. 1076–1087, 2004.

[75] C. Fradin, P. De Groot, D. MacCallum et al., "Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood," *Molecular Microbiology*, vol. 56, no. 2, pp. 397–415, 2005.

[76] C. J. Barelle, C. L. Priest, D. M. Maccallum, N. A. Gow, F. C. Odds, and A. J. Brown, "Niche-specific regulation of central metabolic pathways in a fungal pathogen," *Cellular Microbiology*, vol. 8, no. 6, pp. 961–971, 2006.

[77] F. Schobel, O. Ibrahim-Granet, P. Ave, J. P. Latgé, A. A. Brakhage, and M. Brock, "*Aspergillus fumigatus* does not require fatty acid metabolism via isocitrate lyase for development of invasive aspergillosis," *Infection and Immunity*, vol. 75, no. 3, pp. 1237–1244, 2007.

[78] T. H. Rude, D. L. Toffaletti, G. M. Cox, and J. R. Perfect, "Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*," *Infection and Immunity*, vol. 70, no. 10, pp. 5684–5694, 2002.

[79] M. Brock, "Fungal metabolism in host niches," *Current Opinion in Microbiology*, vol. 12, no. 4, pp. 371–376, 2009.

[80] O. Ibrahim-Granet, M. Dubourdeau, J. P. Latge et al., "Methylcitrate synthase from *Aspergillus fumigatus* is essential for manifestation of invasive aspergillosis," *Cellular Microbiology*, vol. 10, no. 1, pp. 134–148, 2008.

[81] A. M. Bailão, A. Schrank, C. L. Borges et al., "Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis," *Microbes and Infection*, vol. 8, no. 12-13, pp. 2686–2697, 2006.

[82] M. Costa, C. L. Borges, A. M. Bailao et al., "Transcriptome profiling of *Paracoccidioides brasiliensis* yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction," *Microbiology*, vol. 153, no. Pt 12, pp. 4194–4207, 2007.

[83] A. F. Parente, P. E. Naves, L. L. Pigosso et al., "The response of *Paracoccidioides* spp. to nitrosative stress," *Microbes and Infection*, vol. 17, no. 8, pp. 575–585, 2015.

[84] K. P. Bastos, A. M. Bailao, C. L. Borges et al., "The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process," *BMC Microbiology*, vol. 7, p. 29, 2007.

[85] T. C. Rezende, C. L. Borges, A. D. Magalhaes et al., "A quantitative view of the morphological phases of *Paracoccidioides brasiliensis* using proteomics," *Journal of Proteomics*, vol. 75, no. 2, pp. 572–587, 2011.

[86] J. F. Munoz, G. M. Gauthier, C. A. Desjardins et al., "The dynamic genome and transcriptome of the human fungal

pathogen *Blastomyces* and close relative *Emmonsia*," *PLoS Genetics*, vol. 11, no. 10, article e1005493, 2015.

[87] A. L. Garfoot, O. Zemska, and C. A. Rappleye, "*Histoplasma capsulatum* depends on de novo vitamin biosynthesis for intraphagosomal proliferation," *Infection and Immunity*, vol. 82, no. 1, pp. 393–404, 2014.

[88] W. J. Hoekstra, E. P. Garvey, W. R. Moore, S. W. Rafferty, C. M. Yates, and R. J. Schotzinger, "Design and optimization of highly-selective fungal CYP51 inhibitors," *Bioorganic & Medicinal Chemistry Letters*, vol. 24, no. 15, pp. 3455–3458, 2014.

[89] L. F. Shubitz, H. T. Trinh, J. N. Galgiani et al., "Evaluation of VT-1161 for treatment of coccidioidomycosis in murine infection models," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 12, pp. 7249–7254, 2015.

[90] A. G. Warrilow, C. M. Hull, J. E. Parker et al., "The clinical candidate VT-1161 is a highly potent inhibitor of *Candida albicans* CYP51 but fails to bind the human enzyme," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 12, pp. 7121–7127, 2014.

[91] K. Koselny, J. Green, L. DiDone et al., "The celecoxib derivative AR-12 has broad-spectrum antifungal activity in vitro and improves the activity of fluconazole in a murine model of cryptococcosis," *Antimicrobial Agents and Chemotherapy*, vol. 60, no. 12, pp. 7115–7127, 2016.

[92] G. D. Brown, D. W. Denning, N. A. Gow, S. M. Levitz, M. G. Netea, and T. C. White, "Hidden killers: human fungal infections," *Science Translational Medicine*, vol. 4, no. 165, p. 165rv13, 2012.

[93] C. d'Enfert, "Hidden killers: persistence of opportunistic fungal pathogens in the human host," *Current Opinion in Microbiology*, vol. 12, no. 4, pp. 358–364, 2009.

[94] Z. Erjavec and P. E. Verweij, "Recent progress in the diagnosis of fungal infections in the immunocompromised host," *Drug Resistance Updates*, vol. 5, no. 1, pp. 3–10, 2002.

[95] C. Y. Low and C. Rotstein, "Emerging fungal infections in immunocompromised patients," *F1000 MedicineReports*, vol. 3, p. 14, 2011.

[96] B. Wanke, M.o. S. Lazéra, and M. Nucci, "Fungal infections in the immunocompromised host," *Memórias do Instituto Oswaldo Cruz*, vol. 95 Supplement 1, pp. 153–158, 2000.

[97] D. Armstrong-James, G. Meintjes, and G. D. Brown, "A neglected epidemic: fungal infections in HIV/AIDS," *Trends in Microbiology*, vol. 22, no. 3, pp. 120–127, 2014.

[98] J. Jampilek, "How can we bolster the antifungal drug discovery pipeline?" *Future Medicinal Chemistry*, vol. 8, no. 12, pp. 1393–1397, 2016.

[99] T. Roemer and D. J. Krysan, "Antifungal drug development: challenges, unmet clinical needs, and new approaches," *Cold Spring Harbor Perspectives in Medicine*, vol. 4, no. 5, 2014.

[100] J. W. Rippon, *Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes*, Saunders, Philadelphia, 1988.

[101] R. Laniado-Laborín and M. N. Cabrales-Vargas, "Amphotericin B: side effects and toxicity," *Revista Iberoamericana de Micología*, vol. 26, no. 4, pp. 223–227, 2009.

[102] K. M. Pianalto and J. A. Alspaugh, "New horizons in antifungal therapy," *Journal of Fungi*, vol. 2, 2016.

[103] N. Osherov and D. P. Kontoyiannis, "The anti-Aspergillus drug pipeline: is the glass half full or empty?" *Medical Mycology*, vol. 55, no. 1, pp. 118–124, 2017.

[104] N. Tene, E. Bonnafe, F. Berger et al., "Biochemical and biophysical combined study of bicarinalin, an ant venom antimicrobial peptide," *Peptides*, vol. 79, pp. 103–113, 2016.

[105] A. R. da Silva, J. B. de Andrade Neto, C. R. da Silva et al., "Berberine antifungal activity in fluconazole-resistant pathogenic yeasts: action mechanism evaluated by flow cytometry and biofilm growth inhibition in Candida spp," *Antimicrobial Agents and Chemotherapy*, vol. 60, no. 6, pp. 3551–3557, 2016.

[106] C. López-Abarrategui, C. McBeth, S. M. Mandal et al., "Cm-p5: an antifungal hydrophilic peptide derived from the coastal mollusk *Cenchritis muricatus* (Gastropoda: Littorinidae)," *The FASEB Journal*, vol. 29, pp. 3315–3325, 2016.

[107] A. C. Amaral, O. N. Silva, N. C. Mundim et al., "Predicting antimicrobial peptides from eukaryotic genomes: in silico strategies to develop antibiotics," *Peptides*, vol. 37, no. 2, pp. 301–308, 2012.

[108] A. K. Abadio, E. S. Kioshima, V. Leroux, N. F. Martins, B. Maigret, and M. S. Felipe, "Identification of new antifungal compounds targeting thioredoxin reductase of *Paracoccidioides* genus," *PloS One*, vol. 10, no. 11, article e0142926, 2015.

[109] X. Cao, Y. Xu, Y. Cao et al., "Design, synthesis, and structure-activity relationship studies of novel thienopyrrolidone derivatives with strong antifungal activity against Aspergillus fumigates," *European Journal of Medicinal Chemistry*, vol. 102, pp. 471–476, 2015.

[110] R. S. Prado, A. M. Bailão, L. C. Silva et al., "Proteomic profile response of Paracoccidioides lutzii to the antifungal argentilactone," *Frontiers in Microbiology*, vol. 6, p. 616, 2015.

[111] F. S. Araújo, L. M. Coelho, L.o. C. Silva et al., "Effects of argentilactone on the transcriptional profile, cell wall and oxidative stress of Paracoccidioides spp.," *PLoS Neglected Tropical Diseases*, vol. 10, no. 1, article e0004309, 2016.

[112] L. G. De Toledo, M. A. Ramos, L. Spósito et al., "Essential oil of *Cymbopogon nardus* (L.) Rendle: a strategy to combat fungal infections caused by Candida species," *International Journal of Molecular Sciences*, vol. 17, no. 8, 2016.

[113] S. Hemmati and H. Seradj, "Justicidin B: a promising bioactive lignan," *Molecules*, vol. 21, no. 7, 2016.

[114] S. Z. Moghadamtousi, H. A. Kadir, P. Hassandarvish, H. Tajik, S. Abubakar, and K. Zandi, "A review on antibacterial, antiviral, and antifungal activity of curcumin," *BioMed Research International*, vol. 2014, Article ID 186864, 12 pages, 2014.

[115] T. Roemer, J. Davies, G. Giaever, and C. Nislow, "Bugs, drugs and chemical genomics," *Nature Chemical Biology*, vol. 8, no. 1, pp. 46–56, 2011.

[116] T. Roemer, D. Xu, S. B. Singh et al., "Confronting the challenges of natural product-based antifungal discovery," *Chemistry & Biology*, vol. 18, no. 2, pp. 148–164, 2011.

[117] T. Roemer and C. Boone, "Systems-level antimicrobial drug and drug synergy discovery," *Nature Chemical Biology*, vol. 9, no. 4, pp. 222–231, 2013.

[118] W. Shi, W. J. Dan, J. J. Tang et al., "Natural products as sources of new fungicides (III): antifungal activity of 2,4-dihydroxy-5-methylacetophenone derivatives," *Bioorganic & Medicinal Chemistry Letters*, vol. 26, no. 9, pp. 2156–2158, 2016.

[119] D. W. Denning, "Echinocandins and pneumocandins—a new antifungal class with a novel mode of action," *The*

Journal of Antimicrobial Chemotherapy, vol. 40, no. 5, pp. 611–614, 1997.

[120] D. W. Denning, "Echinocandins: a new class of antifungal," *The Journal of Antimicrobial Chemotherapy*, vol. 49, no. 6, pp. 889–891, 2002.

[121] J. M. Hamilton-Miller, "Chemistry and biology of the polyene macrolide antibiotics," *Bacteriological Reviews*, vol. 37, no. 2, pp. 166–196, 1973.

[122] K. Z. Hein, H. Takahashi, T. Tsumori et al., "Disulphide-reduced psoriasin is a human apoptosis-inducing broad-spectrum fungicide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 42, pp. 13039–13044, 2015.

[123] J. Chandra, P. K. Mukherjee, S. D. Leidich et al., "Antifungal resistance of candidal biofilms formed on denture acrylic in vitro," *Journal of Dental Research*, vol. 80, no. 3, pp. 903–908, 2001.

[124] L. J. Douglas, "Medical importance of biofilms in Candida infections," *Revista Iberoamericana de Micología*, vol. 19, no. 3, pp. 139–143, 2002.

[125] M. Scarsini, L. Tomasinsig, A. Arzese, F. D'Este, D. Oro, and B. Skerlavaj, "Antifungal activity of cathelicidin peptides against planktonic and biofilm cultures of Candida species isolated from vaginal infections," *Peptides*, vol. 71, pp. 211–221, 2015.

[126] D. Seleem, E. Chen, B. Benso, V. Pardi, and R. M. Murata, "In vitro evaluation of antifungal activity of monolaurin against *Candida albicans* biofilms," *PeerJ*, vol. 4, article e2148, 2016.

[127] D. Seleem, B. Benso, J. Noguti, V. Pardi, and R. M. Murata, "In vitro and in vivo antifungal activity of lichochalcone-A against *Candida albicans* biofilms," *PloS One*, vol. 11, no. 6, article e0157188, 2016.

[128] R. E. Hancock and H. G. Sahl, "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies," *Nature Biotechnology*, vol. 24, no. 12, pp. 1551–1557, 2006.

[129] M. Moniruzzaman, J. M. Alam, H. Dohra, and M. Yamazaki, "Antimicrobial peptide lactoferricin B-induced rapid leakage of internal contents from single giant unilamellar vesicles," *Biochemistry*, vol. 54, no. 38, pp. 5802–5814, 2015.

[130] M. F. Burton and P. G. Steel, "The chemistry and biology of LL-37," *Natural Product Reports*, vol. 26, no. 12, pp. 1572–1584, 2009.

[131] Y. Oguro, H. Yamazaki, M. Takagi, and H. Takaku, "Antifungal activity of plant defensin AFP1 in *Brassica juncea* involves the recognition of the methyl residue in glucosylceramide of target pathogen *Candida albicans*," *Current Genetics*, vol. 60, no. 2, pp. 89–97, 2014.

[132] B. M. Hayes, M. R. Bleackley, J. L. Wiltshire, M. A. Anderson, A. Traven, and N. L. van der Weerden, "Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against *Candida albicans*," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 8, pp. 3667–3675, 2013.

[133] E. B. Moffa, M. C. Mussi, Y. Xiao et al., "Histatin 5 inhibits adhesion of *C. albicans* to reconstructed human oral epithelium," *Frontiers in Microbiology*, vol. 6, p. 885, 2015.

[134] J. Han, M. A. Jyoti, H. Y. Song, and W. S. Jang, "Antifungal activity and action mechanism of histatin 5-halocidin hybrid peptides against Candida ssp," *PloS One*, vol. 11, no. 2, article e0150196, 2016.

[135] W. Baranska-Rybak, A. Sonesson, R. Nowicki, and A. Schmidtchen, "Glycosaminoglycans inhibit the antibacterial activity of LL-37 in biological fluids," *The Journal of Antimicrobial Chemotherapy*, vol. 57, no. 2, pp. 260–265, 2006.

[136] M. Rapala-Kozik, O. Bochenska, M. Zawrotniak et al., "Inactivation of the antifungal and immunomodulatory properties of human cathelicidin LL-37 by aspartic proteases produced by the pathogenic yeast *Candida albicans*," *Infection and Immunity*, vol. 83, no. 6, pp. 2518–2530, 2015.

[137] I. d'Angelo, B. Casciaro, A. Miro, F. Quaglia, M. L. Mangoni, and F. Ungaro, "Overcoming barriers in *Pseudomonas aeruginosa* lung infections: engineered nanoparticles for local delivery of a cationic antimicrobial peptide," *Colloids and Surfaces. B, Biointerfaces*, vol. 135, pp. 717–725, 2015.

[138] I. Pal, V. P. Brahmkhatri, S. Bera et al., "Enhanced stability and activity of an antimicrobial peptide in conjugation with silver nanoparticle," *Journal of Colloid and Interface Science*, vol. 483, pp. 385–393, 2016.

[139] A. C. Amaral, A. L. Bocca, A. M. Ribeiro et al., "Amphotericin B in poly(lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) nanoparticles against paracoccidioidomycosis," *The Journal of Antimicrobial Chemotherapy*, vol. 63, no. 3, pp. 526–533, 2009.

[140] A. M. Piras, G. Maisetta, S. Sandreschi et al., "Chitosan nanoparticles loaded with the antimicrobial peptide temporin B exert a long-term antibacterial activity in vitro against clinical isolates of *Staphylococcus epidermidis*," *Frontiers in Microbiology*, vol. 6, p. 372, 2015.

[141] L. Liu, J. Yang, J. Xie et al., "The potent antimicrobial properties of cell penetrating peptide-conjugated silver nanoparticles with excellent selectivity for gram-positive bacteria over erythrocytes," *Nanoscale*, vol. 5, no. 9, pp. 3834–3840, 2013.

[142] A. C. Souza, A. L. Nascimento, N. M. de Vasconcelos et al., "Activity and in vivo tracking of amphotericin B loaded PLGA nanoparticles," *European Journal of Medicinal Chemistry*, vol. 95, pp. 267–276, 2015.

[143] N. Xu, J. Gu, Y. Zhu, H. Wen, Q. Ren, and J. Chen, "Efficacy of intravenous amphotericin B-polybutylcyanoacrylate nanoparticles against cryptococcal meningitis in mice," *International Journal of Nanomedicine*, vol. 6, pp. 905–913, 2011.

[144] T. Ren, N. Xu, C. Cao et al., "Preparation and therapeutic efficacy of polysorbate-80-coated amphotericin B/PLA-b-PEG nanoparticles," *Journal of Biomaterials Science. Polymer Edition*, vol. 20, no. 10, pp. 1369–1380, 2009.

[145] R. Calderone, N. Sun, F. Gay-Andrieu et al., "Antifungal drug discovery: the process and outcomes," *Future Microbiology*, vol. 9, no. 6, pp. 791–805, 2014.

[146] A. Butts and D. J. Krysan, "Antifungal drug discovery: something old and something new," *PLoS Pathogens*, vol. 8, no. 9, article e1002870, 2012.

[147] T. T. Ashburn and K. B. Thor, "Drug repositioning: identifying and developing new uses for existing drugs," *Nature Reviews. Drug Discovery*, vol. 3, no. 8, pp. 673–683, 2004.

[148] A. Katragkou, E. Roilides, and T. J. Walsh, "Can repurposing of existing drugs provide more effective therapies for invasive fungal infections?" *Expert Opinion on Pharmacotherapy*, vol. 17, no. 9, pp. 1179–1182, 2016.

[149] R. Huang, N. Southall, Y. Wang et al., "The NCGC pharmaceutical collection: a comprehensive resource of clinically approved drugs enabling repurposing and chemical

genomics," *Science Translational Medicine*, vol. 3, no. 80, p. 80ps16, 2011.

[150] C. Z. Chen, L. Kulakova, N. Southall et al., "High-throughput *Giardia lamblia* viability assay using bioluminescent ATP content measurements," *Antimicrobial Agents and Chemotherapy*, vol. 55, no. 2, pp. 667–675, 2011.

[151] M. Shen, Y. Zhang, N. Saba, C. P. Austin, A. Wiestner, and D. S. Auld, "Identification of therapeutic candidates for chronic lymphocytic leukemia from a library of approved drugs," *PloS One*, vol. 8, no. 9, article e75252, 2013.

[152] L. F. Zerbini, M. K. Bhasin, J. F. De Vasconcellos et al., "Computational repositioning and preclinical validation of pentamidine for renal cell cancer," *Molecular Cancer Therapeutics*, vol. 13, no. 7, pp. 1929–1941, 2014.

[153] W. Sun, Y. D. Park, J. A. Sugui et al., "Rapid identification of antifungal compounds against Exserohilum rostratum using high throughput drug repurposing screens," *PLoS One*, vol. 8, no. 8,article e70506, 2013.

[154] N. Robbins, M. Spitzer, T. Yu et al., "An antifungal combination matrix identifies a rich pool of adjuvant molecules that enhance drug activity against diverse fungal pathogens," *Cell Reports*, vol. 13, no. 7, pp. 1481–1492, 2015.

[155] J. A. Roussey, M. A. Olszewski, and J. J. Osterholzer, "Immunoregulation in fungal diseases," *Microorganisms*, vol. 4, no. 4, 2016.

[156] K. Chen and J. K. Kolls, "T cell-mediated host immune defenses in the lung," *Annual Review of Immunology*, vol. 31, pp. 605–633, 2013.

[157] L. R. Travassos, E. G. Rodrigues, L. K. Iwai, and C. P. Taborda, "Attempts at a peptide vaccine against paracoccidioidomycosis, adjuvant to chemotherapy," *Mycopathologia*, vol. 165, no. 4-5, pp. 341–352, 2008.

[158] D. A. Stevens, K. V. Clemons, and M. Liu, "Developing a vaccine against aspergillosis," *Medical Mycology*, vol. 49 Supplement 1, pp. S170–S176, 2011.

[159] D. Pietrella, A. Rachini, A. Torosantucci et al., "A beta-glucan-conjugate vaccine and anti-beta-glucan antibodies are effective against murine vaginal candidiasis as assessed by a novel in vivo imaging technique," *Vaccine*, vol. 28, no. 7, pp. 1717–1725, 2010.

[160] H. Wang, M. Li, T. Lerksuthirat, B. Klein, and M. Wüthrich, "The C-type lectin receptor MCL mediates vaccine-induced immunity against infection with Blastomyces dermatitidis," *Infection and Immunity*, vol. 84, no. 3, pp. 635–642, 2016.

[161] A. K. Chaturvedi, R. S. Hameed, K. L. Wozniak et al., "Vaccine-mediated immune responses to experimental pulmonary Cryptococcus gattii infection in mice," *PLoS One*, vol. 9, no. 8, 2014.

[162] M. Wüthrich, B. Gern, C. Y. Hung et al., "Vaccine-induced protection against 3 systemic mycoses endemic to North America requires Th17 cells in mice," *Journal of Clinical Investigation*, vol. 121, no. 2, pp. 554–568, 2011.

[163] N. Antoine and B. Mignon, "Assessment of immunogenicity and protective efficacy of Microsporum canis secreted components coupled to monophosphoryl lipid-A adjuvant in a vaccine study using guinea pigs," *Veterinary Microbiology*, vol. 175, no. 2–4, pp. 304–311, 2015.

[164] A. C. Amaral, A. F. Marques, J. E. Muñoz et al., "Poly(lactic acid-glycolic acid) nanoparticles markedly improve immunological protection provided by peptide P10 against murine paracoccidioidomycosis," *British Journal of Pharmacology*, vol. 159, no. 5, pp. 1126–1132, 2010.

[165] D. R. Samuelson, N. M. de la Rua, T. P. Charles et al., "Oral immunization of mice with live Pneumocystis murina protects against Pneumocystis pneumonia," *The Journal of Immunology*, vol. 196, no. 6, pp. 2655–2665, 2016.

[166] J. R. F. De Almeida, G. H. Kaihami, G. P. Jannuzzi, and S. R. de Almeida, "Therapeutic vaccine using a monoclonal antibody against a 70-kDa glycoprotein in mice infected with highly virulent *Sporothrix schenckii* and Sporothrix brasiliensis," *Medical Mycology*, vol. 53, no. 1, pp. 42–50, 2014.

[167] L. P. Wong, P. C. Y. Woo, A. Y. Y. Wu, and K. Y. Yuen, "DNA immunization using a secreted cell wall antigen Mp1p is protective against Penicillium marneffei infection," *Vaccine*, vol. 20, no. 23-24, pp. 2878–2886, 2002.

[168] Y. Feng, S. Guo, T. Jiang et al., "Active immunization against Pneumocystis carinii with p55-v3 DNA vaccine in rats," *Canadian Journal of Microbiology*, vol. 57, no. 5, pp. 375–381, 2011.

[169] S. G. Nanjappa and B. S. Klein, "Vaccine immunity against fungal infections," *Current Opinion in Immunology*, vol. 28, no. 1, pp. 27–33, 2014.

[170] L. R. Travassos, C. P. Taborda, and A. L. Colombo, "Treatment options for paracoccidioidomycosis and new strategies investigated," *Expert Review of Anti-Infective Therapy*, vol. 6, no. 2, pp. 251–262, 2008.

[171] V. G. Batista, L. Moreira-Teixeira, M. C. Leite-de-Moraes, and G. Benard, "Analysis of invariant natural killer T cells in human paracoccidioidomycosis," *Mycopathologia*, vol. 172, no. 5, pp. 357–363, 2011.

[172] D. I. Godfrey, S. Stankovic, and A. G. Baxter, "Raising the NKT cell family," *Nature Immunology*, vol. 11, no. 3, pp. 197–206, 2010.

[173] V. L. G. Calich, T. A. Da Costa, M. Felonato et al., "Innate immunity to Paracoccidioides brasiliensis infection," pp. 223–236, 2008.

[174] L. N. Longhi, R. M. da Silva, M. C. Fornazim et al., "Phenotypic and functional characterization of NK cells in human immune response against the dimorphic fungus Paracoccidioides brasiliensis," *Journal of Immunology*, vol. 189, no. 2, pp. 935–945, 2012.

[175] L. Thomaz, J. D. Nosanchuk, D. C. P. Rossi, L. R. Travassos, and C. P. Taborda, "Monoclonal antibodies to heat shock protein 60 induce a protective immune response against experimental Paracoccidioides lutzii," *Microbes and Infection*, vol. 16, no. 9, pp. 788–795, 2014.

[176] C. P. Taborda, J. Rivera, O. Zaragoza, and A. Casadevall, "More is not necessarily better: prozone-like effects in passive immunization with IgG," *Journal of Immunology (Baltimore, Md. : 1950)*, vol. 170, no. 7, pp. 3621–3630, 2003.

[177] C. P. Taborda and A. Casadevall, "CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are involved in complement-independent antibody-mediated phagocytosis of *Cryptococcus neoformans*," *Immunity*, vol. 16, no. 6, pp. 791–802, 2002.

[178] S. Bernardino, A. Pina, M. Felonato et al., "TNF-alpha and CD8+ T cells mediate the beneficial effects of nitric oxide synthase-2 deficiency in pulmonary paracoccidioidomycosis," *PLoS Neglected Tropical Diseases*, vol. 7, no. 8, article e2325, 2013.

[179] S. G. Nanjappa, E. Heninger, M. Wuthrich, D. J. Gasper, and B. S. Klein, "Tc17 cells mediate vaccine immunity against

lethal fungal pneumonia in immune deficient hosts lacking CD4+ T cells," *PLoS Pathogens*, vol. 8, no. 7, article e1002771, 2012.

[180] A. F. Marques, M. B. da Silva, M. A. P. Juliano, J. E. Munhõz, L. R. Travassos, and C. P. Taborda, "Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of Paracoccidioides brasiliensis," *Microbes and Infection*, vol. 10, no. 12-13, pp. 1251–1258, 2008.

[181] V. C. Fernandes, E. M. N. Martins, J. N. Boeloni, J. B. Coitinho, R. Serakides, and A. M. Goes, "Additive effect of rPb27 immunization and chemotherapy in experimental paracoccidioidomycosis," *PLoS One*, vol. 6, no. 3, 2011.

[182] E. M. do Nascimento Martins, B. S. Reis, V. C. Fernandes, M. M. Costa, A. M. Goes, and A. S. de Andrade, "Immunization with radioattenuated yeast cells of Paracoccidioides brasiliensis induces a long lasting protection in BALB/c mice," *Vaccine*, vol. 25, no. 46, pp. 7893–7899, 2007.

[183] R. Buissa-Filho, R. Puccia, A. F. Marques et al., "The monoclonal antibody against the major diagnostic antigen of Paracoccidioides brasiliensis mediates immune protection in infected BALB/c mice challenged intratracheally with the fungus," *Infection and Immunity*, vol. 76, no. 7, pp. 3321–3328, 2008.

[184] D. De Mattos Grosso, S. R. De Almeida, M. Mariano, and J. D. Lopes, "Characterization of gp70 and anti-gp70 monoclonal antibodies in Paracoccidioides brasiliensis pathogenesis," *Infection and Immunity*, vol. 71, no. 11, pp. 6534–6542, 2003.

[185] G. S. Deepe Jr, R. Gibbons, G. D. Brunner, and F. J. Gomez, "A protective domain of heat-shock protein 60 from Histoplasma capsulatum," *The Journal of Infectious Diseases*, vol. 174, no. 4, pp. 828–834, 1996.

[186] S. H. Hsieh, J. S. Lin, J. H. Huang et al., "Immunization with apoptotic phagocytes containing *Histoplasma capsulatum* activates functional CD8(+) T cells to protect against histoplasmosis," *Infection and Immunity*, vol. 79, no. 11, pp. 4493–4502, 2011.

[187] G. T. Cole, C. Y. Hung, S. D. Sanderson et al., "Novel strategies to enhance vaccine immunity against coccidioidomycosis," *PLoS Pathogens*, vol. 9, no. 12, pp. 1–4, 2013.

[188] C. Y. Hung, B. J. Hurtgen, M. Bellecourt, S. D. Sanderson, E. L. Morgan, and G. T. Cole, "An agonist of human complement fragment C5a enhances vaccine immunity against Coccidioides infection," *Vaccine*, vol. 30, no. 31, pp. 4681–4690, 2012.

[189] K. V. Clemons, M. A. Antonysamy, M. E. Danielson et al., "Whole glucan particles as a vaccine against systemic coccidioidomycosis," *Journal of Medical Microbiology*, vol. 64, no. 10, pp. 1237–1243, 2015.

[190] S. M. Johnson, N. W. Lerche, D. Pappagianis, J. L. Yee, J. N. Galgiani, and R. F. Hector, "Safety, antigenicity, and efficacy of a recombinant coccidioidomycosis vaccine in cynomolgus macaques (Macaca fascicularis)," *Annals of the new York Academy of Sciences*, vol. 1111, pp. 290–300, 2007.

[191] J. R. Almeida, G. H. Kaihami, G. P. Jannuzzi, and S. R. de Almeida, "Therapeutic vaccine using a monoclonal antibody against a 70-kDa glycoprotein in mice infected with highly virulent *Sporothrix schenckii* and *Sporothrix brasiliensis*," *Medical Mycology*, vol. 53, no. 1, pp. 42–50, 2015.

[192] D. L. Portuondo, A. Batista-Duharte, L. S. Ferreira et al., "A cell wall protein-based vaccine candidate induce protective immune response against *Sporothrix schenckii* infection," *Immunobiology*, vol. 221, no. 2, pp. 300–309, 2016.

[193] M. Wüthrich, T. Krajaejun, V. Shearn-Bochsler et al., "Safety, tolerability, and immunogenicity of a recombinant, genetically engineered, live-attenuated vaccine against canine blastomycosis," *Clinical and Vaccine Immunology*, vol. 18, no. 5, pp. 783–789, 2011.

[194] M. Wüthrich, G. S. Deepe, and B. Klein, "Adaptive immunity to fungi," *Annual Review of Immunology*, vol. 30, no. 1, pp. 115–148, 2012.

[195] S. M. Levitz, "*Aspergillus* vaccines: hardly worth studying or worthy of hard study?" *Medical Mycology*, vol. 55, no. 1, pp. 103–108, 2017.

[196] A. Torosantucci, C. Bromuro, P. Chiani et al., "A novel glycoconjugate vaccine against fungal pathogens," *The Journal of Experimental Medicine*, vol. 202, no. 5, pp. 597–606, 2005.

[197] M. Wuthrich, T. T. Brandhorst, T. D. Sullivan et al., "Calnexin induces expansion of antigen-specific CD4(+) T cells that confer immunity to fungal ascomycetes via conserved epitopes," *Cell Host & Microbe*, vol. 17, no. 4, pp. 452–465, 2015.

[198] X. J. Wang, X. Sui, L. Yan, Y. Wang, Y. B. Cao, and Y. Y. Jiang, "Vaccines in the treatment of invasive candidiasis," *Virulence*, vol. 6, no. 4, pp. 309–315, 2015.

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 8491383, 8 pages
https://doi.org/10.1155/2017/8491383

Review Article

Fungal Dimorphism and Virulence: Molecular Mechanisms for Temperature Adaptation, Immune Evasion, and In Vivo Survival

Gregory M. Gauthier

Department of Medicine, Division of Infectious Diseases, University of Wisconsin School of Medicine & Public Health, Madison, WI, USA

Correspondence should be addressed to Gregory M. Gauthier; gmg@medicine.wisc.edu

Received 18 November 2016; Accepted 12 April 2017; Published 23 May 2017

Academic Editor: Anamélia L. Bocca

The thermally dimorphic fungi are a unique group of fungi within the Ascomycota phylum that respond to shifts in temperature by converting between hyphae (22–25°C) and yeast (37°C). This morphologic switch, known as the phase transition, defines the biology and lifestyle of these fungi. The conversion to yeast within healthy and immunocompromised mammalian hosts is essential for virulence. In the yeast phase, the thermally dimorphic fungi upregulate genes involved with subverting host immune defenses. This review highlights the molecular mechanisms governing the phase transition and recent advances in how the phase transition promotes infection.

1. Introduction

The ability for fungi to switch between different morphologic forms is widespread throughout the fungal kingdom and is a fundamental part of their biology. A small subset of fungi within the Ascomycota phylum is considered dimorphic, which refers to capacity to convert between two specific morphologic forms, yeast and hyphae. These fungi are capable of infecting mammals, plants, and insects, and can be subdivided into thermal and nonthermal dimorphic fungi [1]. Thermally dimorphic fungi infect humans and other mammals such as dogs, cats, armadillos, and rodents (Table 1) [2–8]. The thermally dimorphic fungi are unique among fungal pathogens because they can infect humans with normal and impaired immune defenses. This includes the etiologic agents for blastomycosis, histoplasmosis, coccidioidomycosis, paracoccidioidomycosis, and sporotrichosis. In contrast, penicilliosis and emmonsiosis occur in persons with long-standing HIV infection that has progressed to AIDS ($CD4^+$ T lymphocytes ≤ 200 cells/mm^3) or have impaired cell-mediated immunity for other reasons (e.g., solid organ transplant) [9–11]. Nonthermal dimorphic fungi can also cause human infection (e.g., *Malassezia furfur*) [12] but are more typically phytopathogenic or entomopathogenic. For example, *Ophiostoma novo-ulmi*, the etiologic agent of Dutch elm disease, has destroyed millions of elm trees in Europe and United States [13]. The "zombie ant" fungus, *Ophiocordyceps unilateralis*, secretes metabolites to alter the behavior of infected ants [14]. This review will focus on how the morphologic switch between hyphae and yeast contributes to virulence with an emphasis on thermally dimorphic fungi relevant to human health.

2. The Phase Transition

The reversible morphologic transition between hyphae and yeast, which is known as the phase transition, is fundamental feature of the biology and lifestyle of the dimorphic fungi [1]. In the soil (22–25°C), these fungi grow as septate hyphae that produce conidia. Disruption of soil by human activities such as construction or natural disasters can aerosolize conidia and hyphal fragments. When inhaled into the warm lungs of a mammalian host (37°C), these infectious propagules convert into pathogenic yeast (or spherules for *Coccidioides*) to cause pneumonia [1]. Once infection is established in the

Table 1: Thermally dimorphic fungi pathogenic to humans and mammals.

Fungus	Clinical Disease
Blastomyces dermatitidis and *gilchristii*	Blastomycosis
Histoplasma capsulatum	Histoplasmosis
Coccidioides immitis and *posadasii*	Coccidioidomycosis
Paracoccidioides brasiliensis and *lutzii*	Paracoccidioidomycosis
Sporothrix schenckii	Sporotrichosis
Talaromyces marneffei	Penicilliosis
Emmonsia spp.	Emmonsiosis
Lacazia loboi	Lacaziosis

lungs, the yeast (or spherules) can disseminate to other organs such as the skin, bone, or brain.

Although temperature is the predominate stimulus that influences the phase transition—hyphae at 22–25°C and yeast at 37°C, additional stimuli that impact the dimorphic switch include carbon dioxide (CO_2) tension, exogenous cysteine, and estradiol. Elevated CO_2 tension (5% CO_2) is required for the arthroconidia of *Coccidioides* spp. to germinate into spherules at 37°C and for optional growth of *Histoplasma capsulatum* yeast [15, 16]. In the human lung, CO_2 tension is approximately 150-fold higher than ambient air, which provides an optimal amount of CO_2 for phase transition [17]. In response to an upshift in temperature, mitochondrial respiration ceases in *Histoplasma, Blastomyces,* and *Paracoccidioides* [18, 19]. To reactivate respiration and complete the morphologic switch to yeast, the uptake of exogenous cysteine is required [18, 19]. The production of 17β-estradiol by humans influences the morphologic shift and growth of *Coccidioides* and *Paracoccidioides*, which in turn, modulates the severity of infection in women. In the presence of 17β-estradiol, the growth of *Coccidioides* spherules at 37°C is accelerated, which may explain the increased risk for disseminated coccidioidomycosis in pregnant women [20, 21]. Moreover, in vitro analysis has demonstrated that *Coccidioides* spherules exhibit saturable binding of 17β-estradiol [21]. In contrast to *Coccidioides*, the morphologic switch from hyphae or conidia to yeast in *Paracoccidioides* is blocked by 17β-estradiol [22, 23]. In a murine model of pulmonary infection, the conversion of conidia to yeast is impaired in female, but not in male mice [24]. In humans, the incidence of paracoccidioidomycosis is 11–30-fold higher in adult males than in adult females despite similar frequency of *Paracoccidioides* exposure. Prior to puberty, the male-to-female ratio is 1 : 1 [25].

These observations have prompted investigation to the mechanisms by which estradiol and gender influence fungal development and the host response. Gene expression microarray analysis of *P. brasiliensis* strain Pb01 demonstrated that impaired conversion to yeast at 37°C in the presence of 17β-estradiol reduced the transcription of genes involved with cell signaling (small GTPase RhoA, palmitoyltransferase), heat shock (*HSP40, HSP70,* and *HSP90*), chitin synthesis (chitin synthase), and glucan remodeling (β-1,3-glucan synthase, α-1,3-glucan synthase) [25]. When stimulated with paracoccin, a lectin-binding protein with chitinase activity, female mice exhibit a stronger Th1 cytokine response with increased production of tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and interleukin 12 (IL-12), along with increased macrophage fungicidal activity when compared to male mice [26]. Following oophorectomy and treatment with testosterone, the cytokine response shifted from Th1 to Th2 in female mice. Castration of male mice coupled with estradiol therapy favored a Th1 cytokine response instead of a Th2 cytokine response [26]. Collectively, these findings highlight the importance of sex steroid hormones and gender on fungal development and host susceptibility.

3. Yeast-Phase Virulence Factors and Subversion of Host Immune Defenses

Once inhaled into the lungs, conidia are ingested by macrophages, where they germinate into yeast (or spherules for *Coccidioides*) and replicate. *Histoplasma capsulatum*, *Coccidioides immitis and posadasii*, *Sporothrix schenckii*, *Paracoccidioides brasiliensis* and *lutzii*, and *Talaromyces marneffei* replicate inside and outside of innate immune cells [27–31]. Traditionally, *Blastomyces* spp. were thought to be exclusively extracellular; however, recent research demonstrates that *B. dermatitidis* conidia ingested by macrophages survive and convert to yeast [32].

During the phase transition, the thermally dimorphic fungi upregulate yeast-phase specific genes including *Blastomyces adhesion-1* (*BAD-1*), calcium-binding protein-1 (*CBP1*), yeast-phase specific-3 (*YPS3*), and spherule outer wall glycoprotein (*SOWgp*) to actively subvert host immune defenses. *B. dermatitidis* and *B. gilchristii* express BAD1 (formerly WI-1), a 120 kDA secreted, multifunctional protein serves as an adhesion and immune evasin [33–38]. Secreted BAD1 binds back to the yeast cell surface via interactions with chitin and also remains soluble in the extracellular milieu [33–35]. Cell surface-bound BAD1 binds yeast to host cells via complement receptors (CR3, CD14) and heparan sulfate to promote yeast cell adhesion to host cells. BAD-1 bound to the yeast cell surface inhibits production of TNF-α by macrophages and neutrophils in a transforming growth factor-β (TGF-β) dependent manner [33, 36–38]. In contrast, soluble BAD-1 blocks TNF-α production independent of TGF-β [36]. TNF-α is a critical cytokine for proper host defense against the dimorphic fungi. Neutralization of TNF-α in a murine model of infection results in progressive pulmonary blastomycosis [37]. Moreover, in 2008, the Food and Drug Administration (FDA) issued a warning of increased risk for histoplasmosis, blastomycosis, and coccidioidomycosis for persons on TNF-α inhibitors for treatment of autoimmune disorders (e.g., rheumatoid arthritis and Crohn's disease) [39]. In addition to affecting TNF-α production, BAD1 also impairs the adaptive immune response by inhibiting the activation of CD4+ T lymphocytes, which decreases IL-17 and INF-γ production [33]. The adhesion and immunomodulatory activities of BAD1 are essential for *Blastomyces* pathogenesis. Deletion of *BAD1* renders *Blastomyces* yeast avirulent in murine model of pulmonary infection [40]. In addition to BAD1, *Blastomyces dermatitidis*

secretes a dipeptidyl-peptidase IVA (DppIVA) to modulate host immunity. DppIV is a serine protease that cleaves GM-CSF, a potent cytokine that activates macrophages and neutrophils to kill fungi [41]. Silencing DppIVA by RNA interference (RNAi) reduces survival of *B. dermatitidis* yeast cocultured with GM-CSF-activated macrophages and neutrophils [41]. Moreover, DppIVA-RNAi strains have attenuated virulence during pulmonary infection [41]. In contrast to *B. dermatitidis*, *H. capsulatum* DppIVA is not detected extracellularly and does not contribute to virulence [42, 43].

Analogous to BAD1, *Coccidioides* SOWgp is localized to the spherule cell surface and an important virulence factor. SOWgp facilitates binding of spherules to host extracellular matrix (ECM) proteins including laminin, fibronectin, and collagen [44]. Deletion of SOWgp (*SOWgp*Δ) in *Coccidioides* impairs spherule adherence to ECM proteins and results in attenuated virulence in a murine model of pulmonary infection [44].

In *H. capsulatum*, *CBP1* is a secreted virulence factor that promotes intracellular replication of yeast [45, 46]. CBP1 binds calcium, exists as homodimer, is resistant to protease degradation, and is structurally related to a group of membrane lipid-binding proteins known as saposins [47, 48]. CBP1 secreted by intracellular *H. capsulatum* yeast induces macrophage apoptosis and lysis by inducing transcription of host cell caspases, transcription factors (*NUPR1*/p8, *TRB3*), and genes involved with endoplasmic reticulum (ER) stress [46]. Thus, macrophage lysis is an active process directed by the fungus and not due to high intracellular fungal burden. Similar to *BAD1*, *CBP1* is an essential virulence factor. *CBP1* null mutants (*CBP1*Δ) are unable to induce macrophage apoptosis and are avirulent in murine model of pulmonary infection [45, 46]. In addition to *CBP1*, *H. capsulatum* secretes *YPS3*, which binds back to chitin in the yeast cell wall and facilitates extrapulmonary dissemination to the liver and spleen [49].

During the morphologic switch from hyphae to yeast or conidia to yeast, dimorphic fungi undergo extensive remodeling of the cell wall including glucan composition. Reorganization of glucan content has the potential to impede recognition of pathogen-associated molecular patterns (PAMPs) by host immune cells. During the morphologic switch, the amount of β-(1,3)-glucan in the cell wall of *Blastomyces* and *Paracoccidioides* declines from ≈40% in hyphae to ≈5% in yeast [50, 51]. The reduction of β-(1,3)-glucan in the yeast cell wall may limit its recognition by dectin-1 on innate immune cells and mannose-binding lectins [17, 52]. In contrast, *H. capsulatum* does not reduce β-(1,3)-glucan in yeast cells, but rather it uses α-(1,3)-glucan as a "shield" to block dectin-1 recognition of β-(1,3)-glucan [53]. Thus, the dimorphic fungi utilize multiple strategies including secreted virulence factors and modification of the yeast cell wall to subvert host immune defenses to establish infection including in persons with intact immune systems.

The ability of thermally dimorphic fungi to subvert host immune defenses is not 100% effective. The host can mount an immune response to halt the progression of infection. Epidemiologic studies have demonstrated that ≈50% of persons exposed to *Blastomyces* spp. develop symptomatic infection, whereas ≈50% have asymptomatic or subclinical infection [54, 55]. Similarly, inhalation of *Histoplasma capsulatum*, *Coccidioides* spp., and *Paracoccidioides* spp. results in symptomatic infection in <10%, 33–50%, and <5% of healthy persons, respectively [56–58]. Intact innate and adaptive immune defenses along with the ability to "wall-off" yeast in granulomas are critical for host defense against infection. Following the conversion of conidia to yeast, dendritic cells and macrophages interact with and engulf yeast cells. Gene expression analysis of dendritic cells that have phagocytozed *P. brasiliensis* yeast demonstrated upregulation of transcripts involved with generating a protective immune response including TNF-α, IL-12, and chemokines (CCL22, CCL27, and CXCL10) [59]. In addition, the dectin-1 receptor was upregulated, which induces phagocytosis, generation of reactive oxygen species, and proinflammatory cytokines and chemokines in response to binding β-(1,3)-glucan [59, 60]. Chemokines promote leukocyte migration to sites of infection [59]. Similarly, macrophages infected with *P. brasiliensis* also induce a proinflammatory response with upregulation of TNF-α, chemokines (CCL21, CCL22, CXCL4, CXC11, and CXCL14), and kinases (IRAK2) [61]. These findings highlight the ability of the immune defenses to limit the impact of fungal virulence factors.

4. Regulation of the Phase Transition

The transition from hyphae or conidia to yeast at 37°C is essential for virulence. The discovery of a hybrid histidine kinase encoded by *DRK1* in *Blastomyces* and *Histoplasma* provided the first genetic proof that the morphologic switch to yeast is directly linked to virulence [62]. *DRK1* null (*DRK1*Δ), insertional mutants, and RNA interference-(RNAi-) silenced strains grow as hyphae at 37°C instead of yeast, fail to upregulate yeast-phase specific virulence factors such as *BAD1* and *CBP1*, and are avirulent in a murine model of infection [62]. The function of *DRK1* is conserved among the thermally dimorphic fungi. In *T. marneffei*, *DRKA* (a *DRK1* homolog) is critical for the conversion of conidia to yeast in macrophages [63]. In *Sporothrix, Paracoccidioides*, and *T. marneffei*, the transcript abundance of *DRK1* is higher in yeast (37°C) than in hyphae (25°C) [63–65]. *DRK1* is predicted to function as part of the high-osmolarity glycerol (HOG) signaling cascade, which facilitates adaptation to osmotic, oxidative, and temperature stresses [17]. Accordingly, *DRK1* transcription is also upregulated in response to osmotic stress in *Paracoccidioides* and *T. marneffei* [63, 65]. In addition to facilitating adaptation to temperature and osmotic stress, *DRK1* also influences the integrity of the cell wall [62, 63].

Regulation of the morphologic shift is complex and not limited to *DRK1*. The transcription factors encoded by *RYP1-4* (required for yeast phase) also govern the phase transition and regulate a set of yeast-phase specific genes involved in virulence at 37°C. These transcription factors are upregulated at 37°C and are conserved among dimorphic and filamentous fungi [66–68]. *RYP1* is a homolog of the

master regulator *WOR1* in *C. albicans*, whereas *RYP2* and *RYP3* are part of the velvet complex, *VosA* and *VelB,* respectively. *RYP4* is a $Zn(II)_2Cys_6$ zinc binuclear cluster domain protein that is homologous to *A. nidulans FacB*; however, it does not appear to be involved acetate utilization [68]. These transcription factors form an integrated network in which they directly bind and regulate a common set of core genes including those important for virulence such as *CBP1* and *YPS3* [68]. Silencing *RYP1-4* transcription results in cells that fail to properly undergo the phase transition and grow as hyphae at 37°C [66–68].

The morphologic switch in the opposite direction, yeast to hyphae, is also important for pathogenesis. Growth as hyphae promotes survival in the environment, generation of conidia to facilitate transmission to new hosts, and genetic diversity through mating [1]. *B. dermatitidis SREB* and *H. capsulatum SRE1* encode a GATA transcription factor that governs the transition to hyphae following a drop in temperature from 37°C to 22–25°C [69–71]. *SREB* null mutants (*SREB*Δ) and *SRE1*-RNAi strains fail to complete the conversion to hyphae [69–71]. The role of this GATA transcription factor on temperature adaptation is conserved in other fungi. A homolog of *SREB* and *SRE1* in *C. neoformans*, *CIR1*, is essential for thermotolerance at 37°C [72]. In *B. dermatitidis*, the defect in the morphologic switch corresponds to a decrease in the biosynthesis of neutral lipids (ergosterol, triacylglycerol) and lipid droplets [70]. Supplementation with exogenous saturated fatty acids (palmitic acid, 16 : 0, and stearic acid, 18 : 0) partially corrected the defects in morphogenesis and lipid droplet formation [70]. This suggests that neutral lipid metabolism has to potentially influence the phase transition to hyphae at ambient temperature. *SREB* and *SRE1* also act as negative regulators of genes involved with siderophore biosynthesis and iron uptake; however, this role appears to be independent of the phase transition [69, 70]. In *H. capsulatum*, deletion of *VMA1*, which encodes a vacuolar ATPase involved with intracellular iron homeostasis, results in cells that fail to convert to hyphae at 25°C. This indicates the potential for iron metabolism not regulated by *SREB* to affect the temperature-dependent morphologic switch [73]. In *T. marneffei*, conversion to hyphae and maintenance of filamentous morphology at 25°C is governed by transcription factors encoded by *HGRA* and *TUPA*, respectively [74, 75]. In addition to transcriptional regulators, N-acetylglucosamine (GlcNAc) accelerates the conversion from yeast to hyphae in *B. dermatitidis* and *H. capsulatum* via *NGT1* and *NGT2* transmembrane transporters [76].

5. In Vivo Transcriptional Profiling

The use of forward genetic strategies such as insertional mutagenesis has substantively advanced the field of medical mycology as related to the thermally dimorphic fungi. This has led to the discovery of novel genes and gene networks that regulate the phase transition (e.g., *DRK1*, *RYP1-3*, and *SREB*). In the age of genome-wide association studies, an untapped reservoir for uncovering novel genes or gene networks in the dimorphic fungi is transcriptional profiling of yeast during infection. To identify genes important for pathogenicity, in vivo transcription profiling was performed for *Blastomyces dermatitidis* strain 26199 using a murine model of pulmonary infection [77, 78]. A novel, 2-step technique was developed to efficiently separate *B. dermatitidis* yeast from murine lung tissue to obtain high-quality RNA for RNA-sequencing (RNA-Seq) [77]. To identify *B. dermatitidis* genes with altered transcription independent of temperature or other conditions, the transcriptional profile of yeast isolated from mouse lungs was compared to yeast cocultured with macrophages at 37°C, yeast grown in vitro without bone marrow-derived macrophages at 37°C, and hyphae at 22°C using K-means cluster analysis [78]. This analysis identified 72 genes that were upregulated in vivo >2-fold and independent of temperature, macrophage cocultivation, and media conditions. A subset of these genes included those that encode proteins secreted into the extracellular milieu, metal cation uptake and transport, and amino acid metabolism [78].

Genes involved with zinc acquisition are upregulated by *B. dermatitidis* yeast during pulmonary infection. This includes a zincophore (*PRA1*/*ZPS1*), high-affinity zinc transporter (*ZRT1*), and low affinity zinc transporter (*ZRT2*) [78]. In *Candida albicans*, *PRA1* is secreted in the extracellular environment to bind zinc and deliver it to the fungus via its interaction with *ZRT1* at the cell surface [79]. In *C. albicans*, *Aspergillus fumigatus*, and *Ustilago maydis*, *PRA1* and *ZRT* are coregulated and syntenic. Although *PRA1* and *ZRT1* appear to be coregulated in *Blastomyces*, these genes are not syntenic. Surprisingly, *PRA1* is not well conserved among the dimorphic fungi and is absent in the genomes of *H. capsulatum, Paracoccidioides* spp., and *Emmonsia*; however, homologs are present in *Coccidioides*. In *C. albicans, PRA1* is postulated to impact pathogenesis. Deletion of *PRA1* results in mutants that have reduced ability to lyse endothelial cells under zinc-deplete conditions [79]. The impact of *PRA1* during in vivo infection has not yet been investigated.

In addition to upregulating zinc-scavenging mechanisms in vivo, *B. dermatitidis* increases the transcription of *NIC1*, which encodes a nickel transporter [78]. Nickel is required for the proper function of urease, an enzyme that catalyzes the conversion of urea to ammonia and CO_2. Urea is found in mammalian tissues as a product of purine nucleotide catabolism [80]. In *Coccidioides*, urease is released from spherules during replication and damages tissue through production of ammonia, which alkalinizes the microenvironment [81]. Deletion of the urease gene (*URE*Δ) in *C. posadasii* results in attenuated virulence in murine model of pulmonary infection. At sites of pulmonary infection, *URE*Δ cells are unable to catabolize urea in lung tissue and fail to lower the pH (tissue pH 7.2 for *URE*Δ versus pH 7.7 for wild type). Moreover, mice infected with the null mutant exhibited a more organized immune response with well-formed granulomas encasing *URE*Δ cells [81]. In *Cryptococcus neoformans*, *NIC1* and *URE1* contribute to invasion of the brain. Deletion of either gene results in decreased ability for *NIC1*Δ and *URE1*Δ yeast cells to penetrate the central nervous system [82]. *URE1* also contributes to the pathogenesis of *Cryptococcus gattii*, which primarily causes pulmonary infection

without an increased predilection for CNS invasion in animal models [83, 84]. *C. gattii URE1*Δ have attenuated virulence during pulmonary infection, reduced capacity to disseminate to the bloodstream, and impaired intracellular replication within macrophages [83].

During pulmonary infection, *B. dermatitidis* upregulates dioxygenases involved in the catabolism of amino acids [78]. This includes 4-hydroxyphenylpyruvate dioxygenase (4-HPPD, *HpdA*), homogentisate 1,2-dioxygenase (*HmgA*), indoleamine 2,3-dioxygenase (*IDO*), and cysteine dioxygenase (*CDG*). *HpdA* and *HmgA* are conserved among the dimorphic fungi and are localized on a gene cluster [85]. Although the precise role for *HpdA* and *HmgA* is not known in *B. dermatitidis*, research on *T. marneffei* has illuminated how these genes involved with tyrosine catabolism influence pathogenesis. *HpdA* and *HmgA* null mutants are hypersensitive to oxidative stress and have impaired spore germination to yeast in murine and human macrophages [85]. Inhibition of 4-HPPD activity appears to be important for the temperature-dependent morphologic shift. Chemical inhibition of 4-HPPD by NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1, 3-dione) in *T. marneffei* and *P. brasiliensis* blocks the conversion of conidia or hyphae to yeast following an increase in temperature from 25°C to 37°C [85, 86].

The role of fungal *IDO* on tryptophan degradation is poorly understood; however, tumor cells upregulate *IDO* to degrade tryptophan in the microenvironment to evade host immune cells [87]. Pulmonary infection with *H. capsulatum* and *P. brasiliensis* induces host IDO, which reduces fungal growth, inhibits Th17 T lymphocyte differentiation, and limits excessive tissue inflammation [88, 89].

In addition to cysteine dioxygenase (*CDG*), *B. dermatitidis* upregulates cysteine synthase A (*CSA*) and a sulfite efflux pump (*SSU1*) during pulmonary infection [78]. *CSA* encodes an enzyme involved with the biosynthesis of L-cysteine from acetyl-L-serine. *CDG* breaks down L-cysteine to L-cysteine sulfonic acid which can be further catabolized to pyruvate and sulfite. The accumulation sulfite is potentially toxic to cells and is secreted via an efflux pump encoded by *SSU1*. In *C. albicans*, deletion of *CDG1* and *SSU1* impairs hyphal development in the presence of cysteine and *CDG1*Δ, but not *SSU1*Δ, and attenuates virulence during murine infection [90]. In dermatophytes such as *Arthroderma benhamiae*, the catabolism of cysteine to sulfite by *CDO1* followed by efflux of sulfite into the extracellular environment by *SSU1* is postulated to promote breakdown of keratin to facilitate fungal growth [91]. *A. benhamiae CDO1* and *SSU1* null mutants have impaired ability to grow on keratin-rich substrates such as hair and nails [91]. On the basis of these data, there is potential that the breakdown of cysteine and sulfite secretion could promote the growth of *Blastomyces* yeast in skin, which is abundant in keratin and the most common site for extrapulmonary dissemination.

6. Conclusions

The thermally dimorphic fungi are a unique group of ascomycetes that are capable of infecting persons with intact and impaired immune defenses. Their ability to adapt to core body temperature (37°C) and transition to yeast morphology is essential for virulence. The morphologic switch to yeast is associated with the upregulation of specific virulence factors that promote adhesion to host tissues, growth in and lysis of macrophages, blunt proper cytokine responses, and impair cell-mediated immunity. The regulation of the reversible transition between hyphae and yeast requires these fungi to adapt and respond to numerous stimuli including temperature, CO_2 tension, and sex hormones. In vivo transcriptional profiling has begun to uncover previously unrecognized genes important for propagation and virulence in the mammalian host.

Conflicts of Interest

The author declares that he has no conflicts of interest.

References

[1] G. M. Gauthier, "Dimorphism in fungal pathogens of mammals, plants, and insects," *PLoS Pathogens*, vol. 11, no. 12, article e1004608, 2015.

[2] G. A. Sarosi, M. R. Eckman, S. F. Davies, and W. K. Laskey, "Canine blastomycosis as a harbinger of human disease," *Annals of Internal Medicine*, vol. 91, no. 5, pp. 733–755, 1979.

[3] J. L. Anderson, J. L. Dieckman, K. D. Reed, and J. K. Meece, "Canine blastomycosis in Wisconsin: a survey of small-animal veterinary practices," *Medical Mycology*, vol. 52, no. 7, pp. 774–779, 2014.

[4] C. Brömel and J. E. Sykes, "Histoplasmosis in dogs and cats," *Clinical Techniques in Small Animal Practice*, vol. 20, no. 4, pp. 227–232, 2005.

[5] A. Graupmann-Kuzma, B. A. Valentine, L. F. Shubitz, S. M. Dial, B. Watrous, and S. J. Tornquist, "Coccidioidomycosis in dogs and cats: a review," *Journal of the American Animal Hospital Association*, vol. 44, no. 5, pp. 226–235, 2008.

[6] R. S. Reis, R. Almeida-Paes, M. Mde Muniz et al., "Molecular characterization of *Sporothrix schenckii* isolates from humans and cats involved in the sporotrichosis epidemic in Rio de Janeiro, Brazil," *Memórias do Instituto Oswaldo Cruz* vol. 104, no. 5, pp. 769–774, 2009.

[7] T. D. Arantes, R. C. Theodoro, M. Mde Teixeria, M. Sde Bosco, and E. Bagagli, "Environmental mapping of *Paracoccidioides* spp. in Brazil reveals new clues into genetic diversity, biogeography and wild host association," *PLoS Neglected Tropical Diseases*, vol. 10, no. 4, article e0004606, 2016.

[8] C. Cao, L. Liang, W. Wang et al., "Common reservoirs for *Penicillium marneffei* infection in humans and rodents, China," *Emerging Infectious Diseases*, vol. 17, no. 2, pp. 209–214, 2011.

[9] C. Kenyon, K. Boorchis, C. Corcoran et al., "A dimorphic fungus causing disseminated infection in South Africa," *New England Journal of Medicine*, vol. 369, no. 15, pp. 1416–1424, 2013.

[10] I. S. Schwartz, N. P. Govender, C. Corcoran et al., "Clinical characteristics, diagnosis, management, and outcomes of disseminated emmonsiosis: a retrospective case series," *Clinical Infectious Diseases*, vol. 61, no. 6, pp. 1004–1012, 2015.

[11] T. Le, M. Wolbers, N. H. Chi et al., "Epidemiology, seasonality, and predictors of outcome of AIDS-associated *Penicillium*

marneffei infection in Ho Chi Minh City, Viet Nam," *Clinical Infectious Diseases*, vol. 52, no. 7, pp. 945–952, 2011.

[12] S. Youngchim, J. D. Nosanchuk, S. Pornsuwan, S. Kajiwara, and N. Vanittanakom, "The role of L-DOPA on melanization and mycelial production in *Malassezia furfur*," *PLoS One*, vol. 8, no. 6, article e63764, 2013.

[13] M. Nigg, J. Laroche, C. R. Landry, and L. Bernier, "RNAseq analysis highlights specific transcriptome signatures of yeast and mycelial growth phases of Dutch elm disease fungus *Ophiostoma novo-ulmi*," *G3*, vol. 5, no. 11, pp. 2487–2495, 2015.

[14] H. C. Evans, S. L. Elliot, and D. P. Hughes, "*Ophiocordyceps unilateralis*: a keystone species for unraveling ecosystem functioning and biodiversity of fungi in tropical forests?" *Communicative & Integrative Biology*, vol. 4, no. 5, pp. 598–602, 2011.

[15] S. A. Klotz, D. J. Drutz, M. Huppert, S. H. Sun, and P. L. DeMarsh, "The critical role of CO_2 in the morphogenesis of *Coccidioides immitis* in cell-free subcutaneous chambers," *Journal of Infectious Diseases*, vol. 150, no. 1, pp. 127–134, 1984.

[16] L. Pine, "Studies on the growth of *Histoplasma capsulatum*. I. Growth of the yeast phase in liquid media," *Journal of Bacteriology*, vol. 68, no. 6, pp. 671–679, 1954.

[17] G. M. Gauthier and B. S. Klein, "Insights into fungal morphogenesis and immune evasion: fungal conidia, when situated in mammalian lungs, may switch from mold to pathogenic yeasts or spore-forming spherules," *Microbe*, vol. 3, no. 8, pp. 416–423, 2008.

[18] B. Maresca, A. M. Lambowitz, V. B. Kumar, G. A. Grant, G. S. Kobayashi, and G. Medof, "Role of cysteine in regulating morphogenesis and mitochondrial activity in the dimorphic fungus *Histoplasma capsulatum*," *Proceeding of the National Academy of Sciences, USA*, vol. 78, no. 7, pp. 4596–4600, 1981.

[19] G. Medoff, A. Painter, and G. S. Kobayashi, "Mycelial-to-yeast-phase transitions of the dimorphic fungi *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*," *Journal of Bacteriology*, vol. 169, no. 9, pp. 4055–4060, 1987.

[20] D. J. Drutz, M. Huppert, S. H. Sun, and W. L. McGuire, "Human sex hormones stimulate the growth and maturation of *Coccidioides immitis*," *Infection and Immunity*, vol. 32, no. 2, pp. 897–907, 1981.

[21] B. L. Powell, D. J. Drutz, M. Huppert, and S. H. Sun, "Relationship of progesterone- and estradiol-binding proteins in *Coccidioides immitis* to coccidioidal dissemination in pregnancy," *Infection and Immunity*, vol. 40, no. 2, pp. 478–485, 1983.

[22] A. Restrepo, M. E. Salazar, L. E. Cano, E. P. Stover, D. Feldman, and D. A. Stevens, "Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis," *Infection and Immunity*, vol. 46, no. 2, pp. 346–353, 1984.

[23] M. E. Salazar, A. Restrepo, and D. A. Stevens, "Inhibition by estrogens of conidium-to-yeast conversion in the fungus *Paracoccidioides brasiliensis*," *Infection and Immunity*, vol. 56, no. 3, pp. 711–713, 1988.

[24] B. H. Aristizábal, K. V. Clemons, A. M. Cock, A. Restrepo, and D. A. Stevens, "Experimental *Paracoccidioides brasiliensis* infection in mice: influence of the hormonal status of the host on tissue responses," *Medical Mycology*, vol. 40, no. 2, pp. 169–178, 2002.

[25] J. Shankar, T. D. Wu, K. V. Clemons, J. P. Monteiro, L. F. Mirels, and D. A. Stevens, "Influence of 17β-estradiol on gene expression of *Paracoccidioides* during mycelia-to-yeast transition," *PLoS One*, vol. 6, no. 12, article e28402, 2011.

[26] C. F. Pinzan, L. P. Ruas, A. S. Casabona-Fortunato, F. C. Carvalho, and M.-C. Roque-Barreira, "Immunologic basis for the gender differences in murine *Paracoccidioides brasiliensis* infection," *PLoS One*, vol. 5, no. 5, article e10757, 2010.

[27] D. O. Inglis, M. Voorhies, D. R. Hocking Murray, and A. Sil, "Comparative transcriptomics of infectious spores from the fungal pathogen *Histoplasma capsulatum* reveals a core set of transcripts that specify infectious and pathogenic states," *Eukaryotic Cell*, vol. 12, no. 6, pp. 828–852, 2013.

[28] L. Beaman, E. Benjamini, and D. Pappagianis, "Role of lymphocytes in macrophage-induced killing of *Coccidioides immitis* in vitro," *Infection and Immunity*, vol. 34, no. 2, pp. 347–353, 1981.

[29] P. Mdel Jiménez, A. Restrepo, D. Radzioch, L. E. Cano, and L. F. Garcia, "Importance of complement 3 and mannose receptors in phagocytosis of *Paracoccidioides brasiliensis* conidia by Nramp1 congenic macrophage cell lines," *FEMS Immunology and Medical Microbiology*, vol. 47, no. 1, pp. 56–66, 2006.

[30] S. Guzman-Beltran, A. Perez-Torres, C. Coronel-Cruz, and H. Torres-Guerrero, "Phagocytic receptors on macrophages distinguish between different *Sporothrix schenckii* morphotypes," *Microbes and Infection*, vol. 14, no. 12, pp. 1093–1101, 2012.

[31] K. J. Boyce and A. Andrianopoulos, "Morphogenetic circuity regulating growth and development in the dimorphic pathogen *Penicillium marneffei*," *Eukaryotic Cell*, vol. 12, no. 2, pp. 154–160, 2013.

[32] A. K. Sterkel, R. Mettelman, M. Wüthrich, and B. S. Klein, "The unappreciated intracellular lifestyle of *Blastomyces dermatitidis*," *Journal of Immunology*, vol. 914, no. 4, pp. 1796–1805, 2015.

[33] T. T. Brandhorst, R. Roy, M. Wüthrich et al., "Structure and function of a fungal adhesion that binds heparin and mimics thrombospondin-1 by blocking T cell activation and effector function," *PLoS Pathogens*, vol. 9, no. 7, article e1003464, 2013.

[34] A. Beaussart, T. Brandhorst, Y. F. Dufrêne, and B. S. Klein, "*Blastomyces* virulence adhesion-1 protein binding to glycosaminoglycans is enhanced by protein disulfide isomerase," *MBio*, vol. 6, no. 5, pp. e014303–e014315, 2015.

[35] T. Brandhorst, M. Wüthrich, B. Finkel-Jimenez, and B. S. Klein, "A C-terminal EGF-like domain governs BAD1 localization to the yeast cell surface and fungal adherence to phagocytes, but is dispensable in immune modulation and pathogenicity of *Blastomyces dermatitidis*," *Molecular Microbiology*, vol. 48, no. 1, pp. 53–65, 2003.

[36] B. Finkel-Jimenez, M. Wüthrich, and B. S. Klein, "BAD1, an essential virulence factor of *Blastomyces dermatitidis*, suppresses host TNF-alpha production through TGF-beta-dependent and –independent mechanisms," *Journal of Immunology*, vol. 168, no. 11, pp. 5746–5755, 2002.

[37] B. Finkel-Jimenez, M. Wüthrich, T. T. Brandhorst, and B. S. Klein, "The WI-1 adhesin blocks phagocyte TNF-alpha production, imparting pathogenicity on *Blastomyces dermatitidis*," *Journal of Immunology*, vol. 166, no. 4, pp. 2665–2673, 2001.

[38] T. T. Brandhorst, M. Wüthrich, B. Finkel-Jimenez, T. Warner, and B. S. Klein, "Exploiting type 3 complement receptor for TNF-alpha suppression, immune evasion, and progressive

pulmonary infection," *Journal of Immunology*, vol. 173, no. 12, pp. 7444–7453, 2004.

[39] FDA Alert, *Tumor Necrosis Factor-Alpha Blockers (TNF Blockers), Cimzia (Certolizumab Pegol), Enbrel (Etanercept), Humira (Adalimumab), and Remicade (Infliximab)*, 2008.

[40] T. T. Brandhorst, M. Wüthrich, T. Warner, and B. S. Klein, "Targeted gene disruption reveals an adhesion indispensable for pathogenicity of *Blastomyces dermatitidis*," *Journal of Experimental Medicine*, vol. 189, no. 8, pp. 1207–1216, 1999.

[41] A. K. Sterkel, J. L. Lorenzini, J. S. Fites et al., "Fungal mimicry of a mammalian aminopeptidase disables innate immunity and promotes pathogenicity," *Cell Host and Microbe*, vol. 19, no. 3, pp. 361–374, 2016.

[42] K. G. Cooper, R. Zarnowski, and J. P. Woods, "*Histoplasma capsulatum* encodes a dipeptidyl peptidase active against the mammalian immunoregulatory peptide, substance P," *PLoS One*, vol. 4, no. 4, article e5281, 2009.

[43] K. G. Cooper and J. P. Woods, "Secreted dipeptidyl peptidase IV activity in the dimorphic fungal pathogen *Histoplasma capsulatum*," *Infection and Immunity*, vol. 77, no. 6, pp. 2447–2454, 2009.

[44] C. Y. Hung, J. J. Yu, K. R. Seshan, U. Reichard, and G. T. Cole, "A parasite phase-specific adhesion of *Coccidioides immitis* contributes to the virulence of this respiratory fungal pathogen," *Infection and Immunity*, vol. 70, no. 7, pp. 3443–3456, 2002.

[45] T. S. Sebghati, J. T. Engle, and W. E. Goldman, "Intracellular parasitism by *Histoplasma capsulatum*: fungal virulence and calcium dependence," *Science*, vol. 290, no. 4595, pp. 1368–1372, 2000.

[46] D. T. Isaac, C. A. Berkes, B. C. English et al., "Macrophage cell death and transcriptional response are actively triggered by the fungal virulence factor Cbp1 during *H. capsulatum* infection," *Molecular Microbiology*, vol. 98, no. 5, pp. 910–929, 2015.

[47] M. R. Beck, G. T. DeKoster, D. M. Hambly, M. L. Gross, D. P. Cistola, and W. E. Goldman, "Structural features responsible for the biological stability of *Histoplasma's* virulence factor CBP," *Biochemistry*, vol. 47, no. 15, pp. 4427–4438, 2008.

[48] M. R. Beck, G. T. DeKoster, D. P. Cistola, and W. E. Goldman, "NMR structure of fungal virulence factor reveals structural homology with mammalian saposin," *Molecular Microbiology*, vol. 72 no. 2, pp. 344–353, 2009.

[49] M. L. Bohse and J. P. Woods, "RNA interference-mediated silencing of the YPS3 gene of *Histoplasma capsulatum* reveals virulence defects," *Infection and Immunity*, vol. 75, no. 6, pp. 2811–2817, 2007.

[50] F. Kanetsuna and L. M. Carbonell, "Cell wall composition of the yeastlike and mycelial forms of *Blastomyces dermatitidis*," *Journal of Bacteriology*, vol. 106, no. 3, pp. 946–948, 1971.

[51] F. Kanetsuna, L. M. Carbonell, R. E. Moreno, and J. Rodriguez, "Cell wall composition of the yeast and mycelial forms of *Paracoccidioides brasiliensis*," *Journal of Bacteriology*, vol. 97, no. 3, pp. 1036–1041, 1969.

[52] A. Koneti, M. J. Linke, E. Brummer, and D. A. Stevens, "Evasion of innate immune responses: evidence for mannose binding lectin inhibition of tumor necrosis factor alpha production by macrophages in response to *Blastomyces dermatitidis*," *Infection and Immunity*, vol. 76, no. 3, pp. 994–1002, 2008.

[53] C. A. Rappleye, L. G. Eissenberg, and W. E. Goldman, "*Histoplasma capsulatum* alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor," *Proceedings of the National Academy of Sciences, USA*, vol. 104, no. 4, pp. 1366–1370, 2007.

[54] B. S. Klein, J. M. Vergeront, R. J. Weeks et al., "Isolation of *Blastomyces dermatitidis* from soil associated with a large outbreak of blastomycosis in Wisconsin," *New England Journal of Medicine*, vol. 314, no. 9, pp. 529–534, 1986.

[55] B. S. Klein, J. M. Vergeront, A. F. DiSalvo, L. Kaufman, and J. P. Davis, "Two outbreaks of blastomycosis along rivers in Wisconsin. Isolation of *Blastomyces dermatitidis* from river-bank soil and evidence of its transmission along waterways," *American Review of Respiratory Diseases*, vol. 136, no. 6, pp. 1333–1338, 1987.

[56] G. S. Deepe, "Chapter 265–"*Histoplasma capsulatum* (histoplasmosis)"," in *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, J. E. Bennett, R. Dolin and M. J. Blaser, Eds., pp. 2949–2962, Elsevier Saunders, Philadelphia, PA, USA, 2015.

[57] J. N. Galgiani, "Chapter 267–"Coccidioidomycosis (*Coccidioides* species)"," in *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, J. E. Bennett, R. Dolin and M. J. Blaser, Eds., pp. 2974–2984, Elsevier Saunders, Philadelphia, PA, USA, 2015.

[58] J. A. Parente, C. L. Borges, and M. Pereira, "*Paracoccidioides* mechanisms of pathogenesis and virulence," in *Human Pathogenic Fungi Molecular Biology and Pathogenic Mechanisms*, D. J. Sullivan and G. P. Moran, Eds., pp. 317–338, Caister Academic Press, Norfolk, UK, 2014.

[59] A. H. Tavares, L. S. Derengowski, K. S. Ferreira et al., "Murine dendritic cells transcriptional modulation upon *Paracoccidioides brasiliensis* infection," *PLoS Neglected Tropical Diseases*, vol. 6, no. 1, article e1459, 2012.

[60] R. A. Drummond and G. D. Brown, "The role of Dectin-1 in the host defense against fungal infections," *Current Opinion in Microbiology* vol. 14, no. 4, pp. 392–399, 2011.

[61] S. S. Silva, A. H. F. P. Tavares, D. G. Passos-Silva et al., "Transcriptional response of murine macrophages upon infection with opsonized *Paracoccidioides brasiliensis* yeast cells," *Microbes and Infection*, vol. 10, no. 1, pp. 12–20, 2008.

[62] J. C. Nemecek, M. Wüthrich, and B. S. Klein, "Global control of dimorphism and virulence in fungi," *Science*, vol. 312, no. 5773, pp. 583–588, 2016.

[63] K. J. Boyce, L. Schreider, L. Kirszenblat, and A. Andrianopoulos, "The two-component histidine kinases DrkA and SlnA are required for *in vivo* growth in the human pathogen *Penicillium marneffei*," *Molecular Microbiology*, vol. 82, no. 5, pp. 1164–1184, 2011.

[64] B. Hou, Z. Zhang, F. Zheng, and X. Liu, "Molecular cloning, characterization, and differential expression of *DRK1* in *Sporothrix schenckii*," *International Journal of Molecular Medicine*, vol. 31, no. 1, pp. 99–104, 2013.

[65] A. F. Chaves, M. V. Navarro, D. G. Castilho, J. C. Calado, P. M. Conceição, and W. L. Batista, "A conserved dimorphism-regulating histidine kinase controls the dimorphic switching in *Paracoccidioides brasiliensis*," *FEMS Yeast Research*, vol. 16, no. 5, article fow047, 2016.

[66] N. Q. Nguyen and A. Sil, "Temperature-induced switch to the pathogenic yeast form of *Histoplasma capsulatum* requires Ryp1, a conserved transcriptional regulator," *Proceeding of the National Academy of Sciences, USA*, vol. 105, no. 12, pp. 4880–4885, 2008.

[67] R. H. Webster and A. Sil, "Conserved factors Ryp2 and Ryp3 control cell morphology and infectious spore formation in the fungal pathogen *Histoplasma capsulatum*," *Proceeding of the National Academy of Sciences, USA*, vol. 105, no. 39, pp. 14573–14578, 2008.

[68] S. Beyham, M. Gutierrez, M. Voorhies, and A. Sil, "A temperature-responsive network links cell shape and virulence traits in a primary fungal pathogen," *PLoS Biology*, vol. 11, no. 7, article e1001614, 2013.

[69] G. M. Gauthier, T. D. Sullivan, S. S. Gallardo et al., "SREB, a GATA transcription factor that directs disparate fates in *Blastomyces dermatitidis* including morphogenesis and siderophore biosynthesis," *PLoS Pathogens*, vol. 6, no. 4, article e1000846, 2010.

[70] A. J. Marty, A. T. Broman, R. Zarnowski et al., "Fungal morphology, iron homeostasis, and lipid metabolism regulated by a GATA transcription factor in *Blastomyces dermatitidis*," *PLoS Pathogens*, vol. 11, no. 6, article e1004959, 2015.

[71] L. H. Hwang, E. Seth, S. A. Gilmore, and A. Sil, "SRE1 regulates iron-dependent and –independent pathways in the fungal pathogen *Histoplasma capsulatum*," *Eukaryotic Cell*, vol. 11, no. 1, pp. 16–25, 2012.

[72] W. H. Jung, A. Sham, R. White, and J. W. Kronstad, "Iron regulation of the major virulence factors in the AIDS-associated pathogen *Cryptococcus neoformans*," *PLoS Biology*, vol. 4, no. 12, article e410, 2006.

[73] J. Hilty, A. G. Smulian, and S. L. Newman, "The *Histoplasma capsulatum* vacuolar ATPase is required for iron homeostasis, intracellular replication in macrophages, and virulence in a murine model of histoplasmosis," *Molecular Microbiology*, vol. 70, no. 1, pp. 127–139, 2008.

[74] H. E. Bugeja, M. J. Hynes, and A. Andrianopoulos, "HgrA is necessary and sufficient to drive hyphal growth in the dimorphic pathogen *Penicillium marneffei*," *Molecular Microbiology*, vol. 88, no. 5, pp. 998–1014, 2013.

[75] R. B. Todd, J. R. Greenhalgh, M. J. Hynes, and A. Adrianopoulos, "TupA, the *Penicillium marneffei* Tup1p homologue, represses both yeast and spore development," *Molecular Microbiology*, vol. 48, no. 1, pp. 85–94, 2003.

[76] S. A. Gilmore, S. Naseem, J. B. Konopka, and A. Sil, "N-acetylglucosamine (GlcNAc) triggers a rapid, temperature-responsive morphogenetic program in thermally dimorphic fungi," *PLoS Genetics*, vol. 9, no. 9, article e1003799, 2013.

[77] A. J. Marty, M. Wüthrich, J. C. Carmen et al., "Isolation of *Blastomyces dermatitidis* yeast from lung tissue during murine infection for *in vivo* transcriptional profiling," *Fungal Genetics and Biology*, vol. 56, pp. 1–8, 2013.

[78] J. F. Muñoz, G. M. Gauthier, C. A. Desjardins et al., "The dynamic genome and transcriptome of the human fungal pathogen *Blastomyces* and close relative *Emmonsia*," *PLoS Genetics*, vol. 11, no. 10, article e1005493, 2015.

[79] F. Citiulo, I. D. Jacobsen, P. Miramón et al., "*Candida albicans* scavenges host zinc via Pra1 during endothelial invasion," *PLoS Pathogens*, vol. 8, no. 6, article e1002777, 2012.

[80] H. Z. Wise, C. Y. Hung, E. Whiston, J. W. Taylor, and G. T. Cole, "Extracellular ammonia at sites of pulmonary infection with *Coccidioides posadasii* contributes to severity of the respiratory disease," *Microbial Pathogenesis*, vol. 59, no. 60, pp. 19–28, 2013.

[81] F. Mirbod-Donovan, R. Schaller, C. Y. Hung, J. Xue, U. Reichard, and G. T. Cole, "Urease produced by *Coccidioides posadasii* contributes to the virulence of this respiratory pathogen," *Infection and Immunity*, vol. 74, no. 1, pp. 504–515, 2006.

[82] A. Singh, R. J. Panting, A. Varma et al., "Factors required for activation of urease as a virulence determinant in *Cryptococcus neoformans*," *MBio*, vol. 7, no. 3, pp. e00220–e00213, 2013.

[83] V. Feder, L. Kmetzsch, C. C. Staats et al., "*Cryptococcus gattii* urease as a virulence factor and the relevance of enzymatic activity in cryptococcosis pathogenesis," *The FEBS Journal*, vol. 282, no. 8, pp. 1406–1418, 2015.

[84] S. C. Chen, W. Meyer, and T. C. Sorrell, "*Cryptococcus gattii* infections," *Clinical Microbiology Reviews*, vol. 4, no. 27, pp. 980–1024, 2014.

[85] K. J. Boyce, A. McLauchlan, L. Schreider, and A. Andrianopoulos, "Intracellular growth is dependent on tyrosine catabolism in the dimorphic fungal pathogen *Penicillium marneffei*," *PLoS Pathogens*, vol. 11, no. 3, article e1004790, 2015.

[86] L. R. Nunes, R. Costa de Oliveira, and D.B. Leite DB, et al., "Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition," *Eukaryotic Cell*, vol. 4, no. 11, pp. 2115–2128, 2005.

[87] C. Uyttenhove, L. Pilotte, I. Théate et al., "Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase," *Nature Medicine*, vol. 9, no. 10, pp. 1269–1274, 2003.

[88] C. A. Hage, D. J. Horan, M. Durkin et al., "*Histoplasma capsulatum* preferentially induces IDO in the lung," *Medical Mycology*, vol. 51, no. 3, pp. 270–279, 2013.

[89] E. F. Araújo, F. V. Loures, S. B. Bazan et al., "Indoleamine 2,3-dioxygenase controls fungal loads and immunity in Paracoccidioidomycosis but is more important to susceptible than resistant hosts," *PLoS Neglected Tropical Diseases*, vol. 8, no. 11, article e3330, 2014.

[90] F. Hennicke, M. Grumbt, U. Lermann et al., "Factors supporting cysteine tolerance and sulfite production in *Candida albicans*," *Eukaryotic Cell*, vol. 12, no. 4, pp. 604–613, 2013.

[91] M. Grumbt, M. Monod, T. Yamada, C. Hertweck, J. Kunert, and P. Staib, "Keratin degradation by dermatophytes relies on cysteine dioxygenase and a sulfite efflux pump," *Journal of Investigative Dermatology*, vol. 133, no. 6, pp. 1550–1555, 2013.

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 5313691, 19 pages
https://doi.org/10.1155/2017/5313691

Review Article

Paracoccidioides Spp.: Virulence Factors and Immune-Evasion Strategies

Emma Camacho[1] and Gustavo A. Niño-Vega[2]

[1]*Department of Molecular Microbiology and Immunobiology, Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA*
[2]*Departamento de Biología, División de Ciencias Naturales y Exactas, Universidad de Guanajuato, Guanajuato, GTO, Mexico*

Correspondence should be addressed to Gustavo A. Niño-Vega; gustavo.nino@ugto.mx

Received 17 November 2016; Revised 1 February 2017; Accepted 21 February 2017; Published 2 May 2017

Academic Editor: Joshua D. Nosanchuk

Paracoccidioides spp. are dimorphic fungal pathogens responsible for one of the most relevant systemic mycoses in Latin America, paracoccidioidomycosis (PCM). Their exact ecological niche remains unknown; however, they have been isolated from soil samples and armadillos (*Dasypus novemcinctus*), which have been proposed as animal reservoir for these fungi. Human infection occurs by inhalation of conidia or mycelia fragments and is mostly associated with immunocompetent hosts inhabiting and/or working in endemic rural areas. In this review focusing on the pathogen perspective, we will discuss some of the microbial attributes and molecular mechanisms that enable *Paracoccidioides* spp. to tolerate, adapt, and ultimately avoid the host immune response, establishing infection.

1. Introduction

Paracoccidioides spp. are causative agents of paracoccidioidomycosis (PCM), a human systemic mycosis endemic to Latin America and one of the most prevalent deep mycoses of the region. PCM can go from an acute/subacute clinical type to a chronic progressive disease [1, 2]. Brazil accounts for over 80% of all reported cases, followed by Venezuela, Colombia, Ecuador, Bolivia, and Argentina [1].

To date, the genus *Paracoccidioides* have been reported as constituted by two species: *Paracoccidioides lutzii*, compose of a single monophyletic population so far found in Central West of Brazil and present in Ecuador [3–5], and *Paracoccidioides brasiliensis*, which comprises a complex of at least four cryptic species, namely, S1 (present in Southeast and Central West of Brazil, as well as Argentina), PS2 (found in Southeast Brazil and Venezuela), PS3 (restricted to Colombia), and PS4 (only found in Venezuela) [5–8]. Both species are thermodimorphic, growing as yeast-like multibudding cells, both in cultures at 37°C and in infected tissues, and as mycelium at temperatures of 20–23°C, which has been regarded as its environmental morphotype.

PCM is acquired by inhalation of conidia [9], and all *Paracoccidioides* species can cause both acute/subacute and chronic diseases although some differential clinical features have been observed in patients infected with either *P. lutzii* or the *P. brasiliensis* species complex [8, 10]. Indeed, infections reported in endemic areas of *P. lutzii* frequently present lymphatic-abdominal clinic manifestation, which are not reported in areas endemic for the *P. brasiliensis* species complex [10]. Also, sera recovered from patients infected with *P. lutzii* are not recognized by *P. brasiliensis* antigens and conversely [8, 11–13].

The interaction between *Paracoccidioides* spp. and its extracellular environment, either in their free-living stages or inside the host, has driven the molecular evolution of these fungi, particularly in the microbial components involved in virulence [14]. However, virulence is not an independent microbial property because it cannot be defined independently from a host. Virulence is the outcome of the interaction

between a host and a microbe, whereas the host aims to effectively control the pathogen causing little or nontissue damage. Thusly, in the context of the "damage-response framework," a virulence factor is a microbial component that can damage a susceptible host [15]. Furthermore, the successful microbial clearance after a microbial invasion into a mammalian host relies on the host cellular immunity, mediated by the cells of the innate and adaptive systems. An initial response involves dendritic cells' and macrophages' recognition and presentation of fungal antigens (e.g., chitin, β-glucans, and mannans), to T-lymphocytes (reviewed by [16]). Those fungal antigens are known as pathogen-associated molecular patterns (PAMPs), which are recognized by the cells of the innate immune system through receptors, namely, pattern recognition receptors (PRRs), such as Toll-like receptors (TLR), nucleotide-binding oligomerization domain- (NOD-) like proteins, and C-type lectin receptors (CLRs) (reviewed by [17]). During a later stage, an effective T-cell response must lead to the generation of Th1 cytokines, such as tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ), resulting in a classic activation of macrophages to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) that kill fungi or inhibit their growth [16, 18].

Colonization and invasion of the host is based on a myriad of fungal components and strategies to bypass host defense mechanisms. Indeed, microbial attributes that confer *Paracoccidioides* spp. the potential to become pathogens are intimately related to escape strategies to avoid clearance and bypass host defense mechanisms. Identification of genes related to fungal virulence factors has occurred mainly using molecular tools to genetically manipulate these organisms. Functional analyses in the genus *Paracoccidioides* are still hindered by the highly complex task of achieving viable and stable mutants. In this review, we discuss some of the tools and strategies developed by *Paracoccidioides* spp. to efficiently evade/manipulate the host immune response, occasionally based on studies performed in other endemic dimorphic fungi (*Blastomyces dermatitidis*, *Histoplasma capsulatum*), where there is deeper understanding of the molecular mechanisms associated with key microbial components.

2. Adaptation

2.1. Morphogenesis. In *Paracoccidioides* spp., once conidia or hyphal fragments are inhaled into the lung alveoli, the morphological switch to multibudding yeast cells is a requirement for the disease to be established [19]. Therefore, the mechanisms involved in this morphological change are potential targets for the development of antifungal drugs against these dimorphic fungi. One of those mechanisms studied in *Paracoccidioides* spp. is the synthesis of polyamines, a metabolic process that has been related to the dimorphic change of some fungi [20]. These are micromolecules required for cellular growth and differentiation in eukaryotic systems and originated by the decarboxylation of ornithine by ornithine decarboxylase (ODC), which gives rise to putrescine, the first polyamine in the metabolic pathway. In *P. brasiliensis*, high levels of ODC activity are induced at the onset of the budding process during the yeast growth and during the mycelium-to-yeast transition in vitro [21, 22]. Also, the dimorphic transition can be repressed by the addition of the ODC inhibitor 1,4-diamino-2-butanone (DAB) [22].

In other fungi, at least three signaling pathways that induce dimorphic switching and yeast growth at 37°C have been identified: (a) the two-component signaling, (b) heterotrimeric G protein and Ras signaling, and (c) calcium signaling (reviewed by [23]). The two-component signaling system is regulated through DRK1 (dimorphism-regulating histidine kinase 1). Reports in *B. dermatitidis* and *H. capsulatum* showed that *DRK1* mutants are avirulent in a murine model of infection. These mutant strains fail to convert to the pathogenic yeast form and grow as mycelia at 37°C [24]. In *Paracoccidioides* spp., an ortholog *DKR1* is highly expressed in the virulence phase and is fundamental in the mycelia-to-yeast transition [25, 26]. Ras GTPases are shown to control multiple processes including cAMP signaling, morphogenesis, differentiation, cell cycle progression, and fungal pathogenic gene expression [23]. Evidences that a heterotrimeric G protein and the Ras signaling pathway influence dimorphic switching in *Paracoccidioides* spp. were shown by Nunes et al. [27] and Fernandes et al. [28]. α and β subunits of heterotrimeric G proteins are induced during the mycelium-to-yeast switch, and farnesyltransferase inhibitors (which disrupt Ras protein function by avoiding its correct membrane association) promote yeast-to-mycelium transition, respectively. Thermal dimorphism in *P. brasiliensis* is also found to be closely associated with the calcium signaling pathway through the heat shock protein 90 (HSP90), which binds and stabilizes calcineurin, thus controlling the cell differentiation [29]. *Pbhsp90* is a single-copy gene that reaches a 25-fold relative induction at one hour after mycelium-to-yeast transition, indicative of its participation upon a thermo-dependent response. Its expression was also found to be strongly induced under oxidative stress. Treatment with geldanamycin and radicicol, specific HSP90 inhibitors that affect the protein's ATPase activity, was shown lethal to the yeast cell in a dose-responsive manner, enforcing the potential of HSP90 as a target for novel antifungal therapies [30]. Further work using antisense technology demonstrated that PbHsp90 function is essential to *Paracoccidioides* physiology [31]. PbHSP90 plays a relevant role not only upon oxidative injury but also during growth in acid environment, which correlated with yeast cell viability 3 h postinteraction with activated macrophages, indicating that this protein increases the fungus' capability to adapt to the host.

On the other hand, several studies have shown that estrogens, specifically 17β-estradiol (E_2), impair *P. brasiliensis* morphological transformation of the mycelial to the yeast form, which may explain the strong gender differences among adult population [32–35]. The exact mechanism involved in such modulation remains unclear; however, further analysis of this phenomenon using microarray technology revealed a correlation between estradiol, cell wall remodeling, energy metabolism, and cell signaling during the mycelium-to-yeast transition [36]. This study showed that as a response to

Table 1: Relative content of the main polysaccharides present in the yeast cell wall of different strains of *Paracoccidioides brasiliensis*. Strains belonging to at least three different cryptic species were grown at 37°C on RPMI 1640 (Gibco) liquid medium, buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) to pH 7.0 for 4 days.

Morphological phase	Polysaccharide	Polysaccharide content per *P. brasiliensis* strain (cryptic species)				
		Pb73 (PS3)	Pb300 (PS4)	Pb377 (PS4)	Pb444 (PS4)	Pb381 (S1)
M	α-(1,3)-Glucan	1.06 ± 0.5	tr	tr	tr	7.0 ± 0.3
	β-(1,3)-Glucan	31.4 ± 0.4	25.4 ± 0.1	27.7 ± 0.2	20.2 ± 1.3	22.2 ± 1.1
	Chitin	13.2 ± 0.7	17.3 ± 0.4	12.6 ± 0.6	8.6 ± 0.2	13.5 ± 0.5
Y	α-(1,3)-Glucan	22.4 ± 0.9	23.7 ± 0.2	23.8 ± 0.4	24.1 ± 0.8	32.6 ± 1.0
	β-(1,3)-Glucan	10.6 ± 0.6	6.8 ± 0.5	3.9 ± 0.2	8.6 ± 0.4	6.3 ± 0.3
	Chitin	35.1 ± 1.3	31.4 ± 0.6	18.0 ± 0.2	26.6 ± 0.8	23.5 ± 0.8

tr stands for traces.

overcome the presence of E_2, the fungus delays or alters normal cellular responses triggered by high temperature, thus affecting subsequent morphological changes that compromised fungal adaptation and pathogenesis.

Simultaneously to the thermotolerance dimorphism, pioneer studies analyzing expressed sequence tags (ESTs) of cDNA libraries from *Paracoccidioides* spp. allowed to identify differently expressed genes during the mycelium-to-yeast transition and various host-interaction conditions, thus revealing the genus *Paracoccidioides*' specific metabolic adaptations intimately related to its environment [27, 37–40]. Moreover, initial proteomic approaches performed in the members of the *Paracoccidioides* spp. [41–43] characterized proteins expressed at their morphological phases and upon interaction with macrophages, reinforcing the complex multifaceted response mount by these fungi to facilitate their survival within the host and even modulate macrophages. As might be expected during the dimorphic transition [41], these authors showed preferential expression of proteins involved in the metabolism of amino acids, nitrogen, signal transduction, and several heat shock/stress-related proteins, including HSP88, HSP90, and isoforms of HSP70, consistent with the previous transcriptional analysis [27]. Notably, the enzymes transaldolase and transketolase are induced during the mycelium-to-yeast transition, indicating an upregulation of the pentose phosphate pathway, linked to the production of intermediates (fructose 6P and glyceraldehyde 3P) and recycling of NADP+ to NADPH, which are subsequently used by the yeast cell to produce ATP under anaerobic conditions. Altogether, these transcriptional and proteomic analyses set a starting point for integrative approaches on infection mimicking conditions to gain better knowledge about the interplay between the expression of microbial components, focusing on adapting/tolerating a harsh environment, and the host immune system (mainly macrophages), which produces profuse ROS and RNS activating antimicrobial activities to kill the fungal pathogen.

It is known that in the lungs, inhaled microorganisms are quickly phagocytized by macrophages supported by neutrophils and dendritic cells. Particularly, macrophages are considered a glucose- and amino acid-depleted environment; thereby, *Paracoccidioides* spp. have evolved defense mechanisms to survive under nutrient deprivation. Lima et al. [44] determined *P. lutzii* response in the absence of glucose performing a high-resolution transcriptomics and proteomic approach on cultured yeast cells and recovered yeast cells after macrophage internalization. The transcriptome analysis showed that under carbon starvation stress (6 h of carbon starvation), abundance of specific transporters such as those for copper, hexoses, and monosaccharides was augmented, indicating that carbohydrate, amino acid, and metal uptake processes are required for survival. Additionally, the ability to respond to oxidative stress was also demonstrated under carbon deprivation, since cellular responses against ROS such as superoxide dismutase, catalase, and cytochrome c peroxidase were elevated. In agreement with the transcriptome analysis, the proteomic response to carbon starvation involved an increase of proteins associated with metabolism (amino acid degradation, ß-oxidation, and ethanol production) and reduction of those related to core cellular processes (fatty acid biosynthesis). This study demonstrated how carbon-starved yeast cells modulate their metabolism by induction or repression of cellular activities. Overall data presented by these authors reveals that *P. lutzii* undergoes a global metabolic switch towards gluconeogenesis and ethanol production supported by precursors (acetyl-CoA, pyruvate, oxaloacetate, and succinate) from ß-oxidation, tricarboxylic acid (TCA), and glyoxylate cycles as a mechanism to adapt to carbon-starving conditions and survive in the hostile environment during macrophage infection.

Also, *P. brasiliensis* overcome the cell-mediated immune system by regulating morphogenesis. This can be achieved by a fungal Rho GTPase, Cdc42, which is involve in controlling actin-mediated polarized growth and supports the large size of the yeast cell and its multibudding state, a morphology that inhibits phagocytosis [45]. Indeed, RNAi *cdc42* strains are more efficiently phagocytosed by macrophages and display decreased pathogenicity [45].

2.2. Changes in Cell Wall Polysaccharide Composition. *Paracoccidioides* spp. are characterized by a distinctive structure and chemical differentiation in its cell wall components per the morphological phase in which it stands at a given moment. While the mycelial phase cell wall has β-1,3-glucan as the main neutral glucose polymer, the multibudding yeast-like phase reduces this polysaccharide to a minimum and

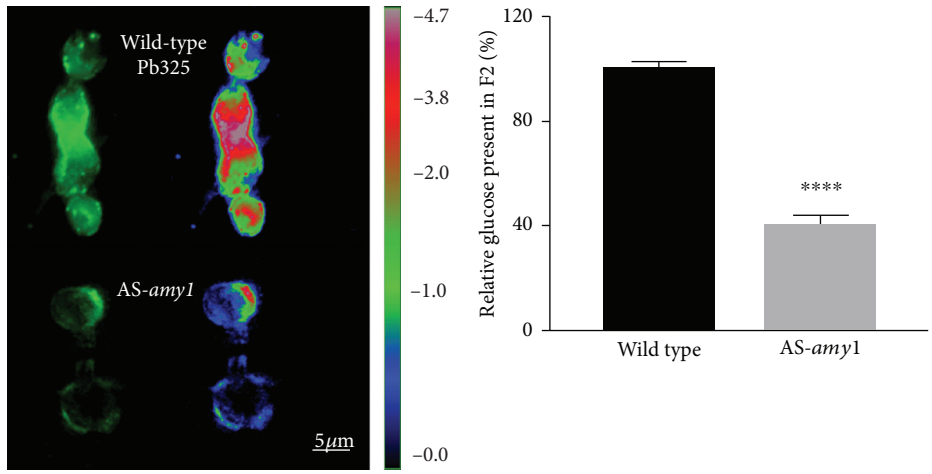


Figure 1: Silencing of Pb*AMY1* reduces α-(1,3)-glucan of *P. brasiliensis* yeast cell. (a) Semiqualitative estimation of α-(1,3)-glucan on *Paracoccidioides* yeast cells by immunofluorescence. Pseudocolor mask for saturation (ImageJ). (b) Quantification of α-(1,3)-glucan in *Paracoccidioides* yeast cells by anthrone assay. $^{****}P < 0.00001$, Welch's test.

substitutes it by α-1,3-glucan (Table 1; [46]), a change that has been correlated with pathogenicity, since spontaneous loss of the polysaccharide correlated with decreased virulence [47]. This initial observation relating α-(1,3)-glucan as a fungal virulence factor was demonstrated 30 years later in *H. capsulatum* [48]. The presence of α-(1,3)-glucan in the outermost layer of the cell wall of *H. capsulatum* yeast masks β-(1,3)-glucan, an immunogenic component of fungal cell walls, avoiding its recognition from pattern recognition receptors (PRR) found on host phagocytic cells [48]. Disturbance of the α-(1,3)-glucan synthesis by depletion of the *H. capsulatum* α-(1,4)-amylase (*AMY1*) transcript, which is involved in priming the oligosaccharide synthesis, reduces cell wall α-(1,3)-glucan content and fungal virulence [49, 50]. Preliminary data from immunofluorescence and biochemical studies after silencing *P. brasiliensis AMY1* (Pb*AMY1*) showed 60% reduction of the α-(1,3)-glucan content on AS-*amy1* yeast cell wall, indicating that indeed PbAMY1p plays a relevant role in the *Paracoccidioides* spp. α-(1,3)-glucan synthesis (Figure 1). In yeast cells, the drastic reduction of the immunogenic polysaccharide β-(1,3)-glucan in its cell wall, and its substitution by α-(1,3)-glucan as an outermost layer when compared to the mycelial phase [51], might be an evolutive feature, hampering the recognition of the yeast cell by the phagocytic cells of the host, as in *H. capsulatum* and, therefore, acting as a protective shield against host defense.

It has been reported for *P. brasiliensis* that the relative content of cell wall polysaccharide is not a constant when different strains are compared and could vary not only with culture conditions but also among isolates [52–54]. However, common features still stand: in the mycelial phase, β-1,3-glucan is present as the mayor structural polysaccharide in different strains, regardless of the phylogenetic group to which each strain belongs, while in the yeast form, α-(1,3)-glucan is present as the mayor neutral polysaccharide and chitin as the mayor structural polysaccharide (Table 1).

Table 2: Relative content of the main polysaccharides present in the yeast cell wall of *Paracoccidioides brasiliensis* strain Pb73 yeast cells, grown on different culture media for 4 days at 37°C. HS, horse serum.

Cell wall polysaccharide content	*P. brasiliensis* strain Pb73, yeast phase Grown on		
	RPMI	YPD	YPD + 5%HS
α-(1,3)-Glucan	22.4 ± 0.9	17.91 ± 0.17	32.52 ± 1.05
β-(1,3)-Glucan	10.6 ± 0.6	5.83 ± 0.28	5.14 ± 0.07
Chitin	35.1 ± 1.3	15.75 ± 0.27	12.87 ± 0.32

These features also apply to a single strain growing on different culture media (Table 2).

Furthermore, it is also well known that long periods of successive subculturing of *Paracoccidioides* spp. lead to attenuation or loss of virulence due to compositional changes of the cell wall [55, 56], which can be re-establish after passage in animals [47], or epithelial culture cells [57] or by supplementing culture media with growth factors such as fetal calf serum [52]. Nevertheless, a biochemical study of *P. brasiliensis* and *P. lutzii* cell wall composition in the presence of horse serum showed interesting differences among them (Table 3). *P. lutzii* showed no increase in its α-(1,3)-glucan content after growth in the presence of horse serum while *P. brasiliensis* did, reinforcing the role of molecular evolution in microbial attributes associated with virulence of these two organisms.

Cell wall turnover during infection after morphological switching is a survival strategy used by dimorphic fungi to avoid recognition by the PRRs of the host phagocytic cells. Dectin-1, a PRR present on the surface of host phagocytic cells, recognizes fungal cell wall β-(1,3)-glucan and triggers phagocytosis, respiratory burst, and release of cytokines such as TNF-α, IL-12, and other interleukins. The spatial arrangement of the yeast cell wall α-(1,3)-glucan in *Paracoccidioides*

Table 3: Biochemical study of *P. brasiliensis* and *P. lutzii* cell wall composition in the presence of horse serum. Yeast cells were grown on YPD or YPD supplemented with 5% horse serum for 4 days at 37°C. HS, horse serum.

Cell wall polysaccharides content	*P. brasiliensis* strain Pb73, yeast phase Grown on		*P. lutzii* strain Pb01, yeast phase Grown on	
	YPD	YPD+ 5%HS	YPD	YPD + 5%HS
Chitin	15.7 ± 0.3	12.8 ± 0.3	21.1 ± 0.7	21.4 ± 1.0
α-(1,3)-Glucan	17.9 ± 0.2	32.5 ± 1.1	25.7 ± 0.4	25.2 ± 0.3
β-(1,3)-Glucan	5.8 ± 0.3	5.1 ± 0.1	3.8 ± 0.3	2.3 ± 0.3

spp. and *H. capsulatum*, present as an outermost layer, covering the immune stimulatory PAMP β-(1,3)-glucan, could actively interfere with these events (Figure 1(a)). A molecular study made for *H. capsulatum* revealed that silencing macrophage Dectin-1 gene expression suppressed the production of proinflammatory TNF-α by phagocytes, suggesting that α-(1,3)-glucan effectively shields β-(1,3)-glucan from innate immune recognition by the Dectin-1 receptor [48, 52] (Figure 2(a)). Additionally, a recent study in α-glucan-containing *Histoplasma* strains showed that yeast cells of this organism secrete an endo-β-(1,3)-glucanase, Eng1, which plays a role in fine scale hydrolysis of cell wall β-glucans [58]. Eng1 acts trimming β-glucan segments exposed on the fungal cell surface further minimizing potential Dectin-1 recognition, decreasing production of proinflammatory cytokines by phagocytes thereby enhancing *Histoplasma* ability to escape detection by host phagocytes. Interestingly, two endoglucanases associated with *P. brasiliensis* extracellular proteome have been reported [59]; however, none shows homology to *Histoplasma* Eng1.

3. Adhesion and Invasion

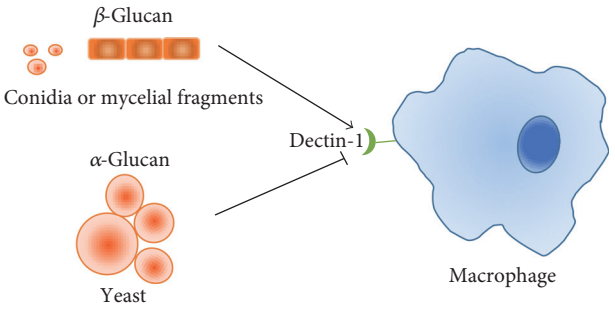
As the infection process advances through the respiratory pathway, *Paracoccidioides* spp. are required to cross tissue planes aiming their intracellular persistence within the host; therefore, the fungus initially invades normally non-phagocytic host cells such as epithelial cells and endothelial cells inducing their own uptake and causing host cell apoptosis [60–62].

3.1. Adhesins. At this stage, *Paracoccidioides* spp. surface proteins known as adhesins play a critical role in the establishing of the infection by interacting with the host cells to promote successful colonization and/or dissemination of the fungi into the host organism [63]. Adhesins mediate fungal cell binding to host extracellular matrix (ECM) components (mainly fibronectin, laminin, fibrinogen, type I and IV collagen, and plasminogen) as well as to epithelial lung cells [64]. Differences in adhesion capacity to Vero cells [65], pneumocytes, and ECM components [66] have been observed for *Paracoccidioides* spp., which might also be attributable to changes in the cell wall composition [67]. Several studies have allowed to identify a diverse number of adhesins in *Paracoccidioides* spp., which are involved in the interaction with host cells and in the in vitro biofilm formation, revealing this fungus a high level of adaptability to a new environment (reviewed by [68]) [63, 69, 70].

A surface glycoprotein of 43 kDa, the first adhesin described in *P. brasiliensis* known as gp43, showed adhesion to laminin and fibronectin [71, 72]. It was the first adhesin to be reported as enhancer of pathogenesis in this fungus. Gp43 inhibits both phagocytosis and fungal intracellular killing [73], may induce protection depending on the route of infection [74], and strongly induces in vitro granuloma-like formation by B-1 cells and macrophages [75]. Downregulation of Pb*GP43* correlated with reduced fungal burden in the lungs of the infected BALB/c mice [76]. Gp43 is likely to be found within vesicles [59] and also happens to be the predominant antigen used for immune detection of *P. brasiliensis* [2]. In the case of *P. lutzii*, a gp43 ortholog, named Plp43, shares only few epitopes in common; therefore, gp43 should not be used in the diagnosis of PCM patients infected with *P. lutzii* [77]. PbHad32p, a 32 kDa protein member of the hydrolase family, able to bind to laminin, fibronectin, and fibrinogen, has been shown to be important in the initial attachment of the infectious particles to the lungs [78–81]. Once into the host, *Paracoccidioides* spp. infective propagules switch to yeast cells, which manage to ease their invasion into pulmonary epithelial cells and keratinocytes by altering the host cell cytoskeleton structure, a process that is promoted by gp43, which acts as an adherence receptor in the internalization of the yeast into the host cell (Figure 2(b)). When into the pulmonary epithelial cell, the fungus induces cytokeratin degradation and apoptosis of the host cells [61, 67].

A phospholipase B (PLB), involved in the early fungus-macrophage interaction, has been reported crucial during the invasion of the host by *Paracoccidioides* spp. and suggested to possibly modulate the innate immune response [82]. Many other potential adhesins, previously described as upregulated genes in yeast cells derived from models of infection, have been uncovered by a comparative transcriptome analysis of annotated ESTs during in vitro adherence assays to type I collagen and fibronectin, including C-5 sterol desaturase, cap20 protein, high-affinity copper transporter, hexokinase, and transketolase [37, 39, 83].

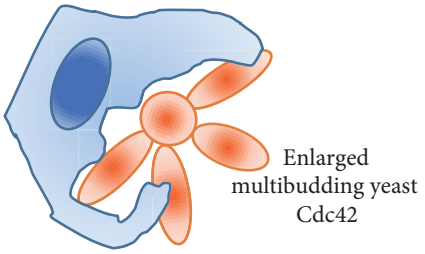
Another set of surface adhesins well characterized as moonlighting proteins in *Paracoccidioides* spp. includes enolase (ENO), fructose 1,6 bisphosphate aldolase (FBA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase (TPI), malate synthase (MLS), isocitrate lyase (ICL), and aconitase (ACO) (reviewed by [69]). These are multifunctional proteins that can perform



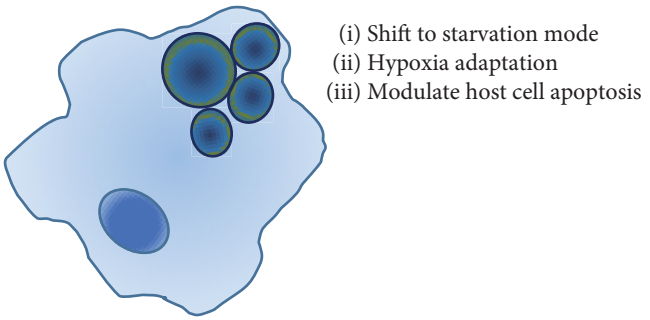
(a) Shielding of stimulatory PAMPs

(1) Promote invasion

(2) Phagocytosis inhibition

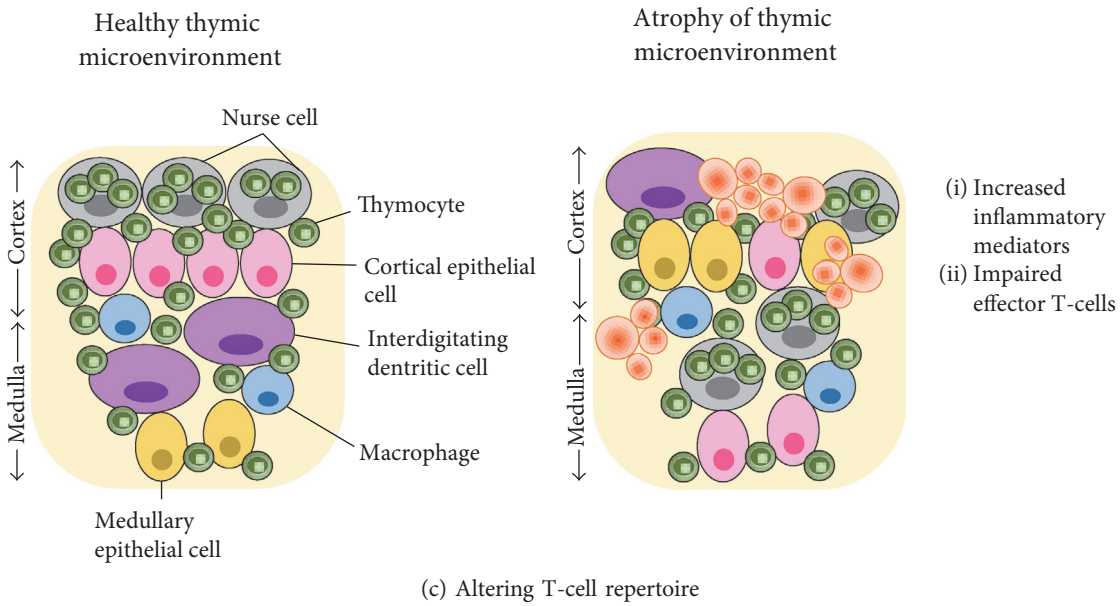


(3) Overcome stress conditions



(b) Intracellular survival

Figure 2: Continued.



(c) Altering T-cell repertoire

Figure 2: Diagram of proposed *Paracoccidioides* spp. immune-evasion mechanisms. (a) Shielding of stimulatory PAMPs. The cell wall beta-glucan present in the fungus saprophytic forms (conidia and mycelia) is recognized by the macrophage Dectin-1 receptor; however, pathogenic yeast cell α-(1,3)-glucan masks β-(1,3)-glucan, avoiding its recognition. (b) Intracellular survival. *Paracoccidiodes* spp. use several strategies to overcome the host harsh environment, among them are the following: (1) promoting invasion to the pulmonary epithelial cells by altering their cytoskeleton structure, a process assisted by gp43; (2) avoiding phagocytosis by displaying an enlarged multibudding morphology, boosted by Cdc42 expression, which physically impairs engulfment by macrophages; and (3) adapting to the host environment. The phagocytosed fungus shifts its metabolism to tolerate macrophage stress conditions and even modulate host apoptosis enabling fungal killing. (c) Altering T-cell repertoire. During acute fungal infections, yeast cells invade the thymus altering its epithelial cells' spatial arrangement crucial for T-cell differentiation and pathogen-specific immune response.

several additional functions, besides their role in chemical metabolic reactions. Most likely, moonlighting proteins act as enzymes constitutively expressed at low levels, but when performing moonlighting functions, they are expressed at high levels [84].

Paracoccidioides spp. ENO, FBA, GAPDH, and TPI are glycolysis enzymes that have been detected in the fungus surface as well as in the vesicle proteome [59], in addition to their conventional cytoplasmic localization. ENO is a 54 kDa protein that binds to laminin, fibronectin, plasminogen, and type I and IV collagen [85, 86]. PbEno expression reported a 10-fold increment on yeast cells derived from the lungs, livers, and spleen of mice after 7 days. Heterologous expression of *Paracoccidioides* enolase (r*Pb*Eno) allowed to evaluate its role in the infection of host cells, suggesting that r*Pb*Eno promoted an increase in the association (adhesion/invasion) of *Paracoccidioides* spp. with host cells in ex vivo models of infection [87]. PbEno's ability to bind plasminogen seems to favor the yeast cell attachment and internalization to host tissues by modifying the surface of host cells (degradation of fibronectin), therefore playing a key role in the establishment of PCM [83, 87]. The enolase plasminogen-binding ability and its role in the degradation of host tissues and ECM components also have been related to the invasion process in *Plasmodium* parasites and other pathogens [88]. A proteomic analysis of *P. lutzii* secretome allowed the identification of fifteen plasminogen-binding proteins, among them is FBA [89]. FBA's ability to bind plasminogen increased fibrinolytic capacity of the fungus, as demonstrated in the fibrin degradation assay. Its participation in the host-pathogen interaction was also evaluated using recombinant protein or anti-FBA antibody in which reduction of adherence/internalization by macrophages was demonstrated [89]. The GADPH binds to laminin, fibronectin, and type I collagen. Its expression is increased during the mycelium-to-yeast transition and parasitic yeast phase; thus, it seems to be involved at the early stages of the fungal infection promoting adhesion to host tissues. In vitro assays treating *Paracoccidioides* spp. yeast cells with polyclonal anti-GAPDH antibody or pneumocytes with the recombinant protein demonstrated reduced interaction between the host and fungus [90, 91]. TPI was initially described as a fungal antigen able to react with the sera of PCM patients [92]. Further characterization and production of an antirecombinant TPI (r*Pb*TPI) polyclonal antibody showed TPI role as an adhesin, which binds preferentially to laminin, and it is involved in the initial fungal adherence and invasion [93].

MLS and ICL are key enzymes of the glyoxylate cycle required for fungal virulence [94]. In *Paracoccidioides* spp., their transcript levels are induced during the mycelium-to-yeast transition and the yeast cell [25, 95], particularly during nutritional stress conditions. MLS is upregulated in yeasts during phagocytosis by macrophages [96], while ICL during the fungus-macrophage interaction upon carbon starvation [44], suggesting their relevance for infection. MLS also

participates in the allantoin degradation pathway, which allows the cells to use purine as a nitrogen source [97]. PbMLS also showed differential accumulation and reactivity on *Paracoccidioides* spp. surface and cytoplasm of budding cells, respectively, and not in the mother cell, indicating that this enzyme is metabolically relevant and mainly synthesized by young cells. The recombinant protein demonstrated ability to recognize fibronectin and type I and IV collagen, as well as pulmonary epithelial cells, implying PbMLS involvement in the interaction of the fungus with host components [98]. ICL binds fibronectin, type IV collagen, and epithelial cells; it is also secreted to the fungal surface [99], supporting the protein relevance during the host-pathogen interaction. Notably, *Pb*ICL is regulated by carbon sources, and its inhibition by argentilactone, a natural drug previously used in the experimental treatment of cutaneous leishmaniasis, can affect cell growth and differentiation [100].

ACO is involved in energy generation catalyzing the isomerization of citrate to isocitrate in both the TCA cycle and the glyoxylate cycle. *Paracoccidioides* spp. ACO (*Pb*ACO) is a 80 kDa protein found in the extracellular fluid, preferentially expressed in yeast cells associated with cell wall, mitochondria, cytosol, and peroxisomes. *Pb*ACO protein levels in yeast cells were induced when fungal growth used potassium acetate or ethanol as carbon sources and in the presence of high-iron concentrations, indicating a potential role in iron metabolism [101].

Furthermore, a 30 kDa adhesin also identified as a 14-3-3 glycoprotein might also be considered a moonlighting protein in *Paracoccidioides* spp. [102]. Initial studies of *P. brasiliensis* 14-3-3 protein showed that it preferentially binds to laminin and presented evidence that adhesion capacity could be related to virulence [57]. 14-3-3 is localized in both the cytoplasm and the cell wall [59]; however, its concentration on the cell wall largely increased during infection, stressing that 14-3-3 plays an essential role in the host-pathogen interaction [103]. Functional analysis of *Pb14-3-3* in *Saccharomyces cerevisiae* partially complemented Bmh1p and Bmh2p proteins supporting the role as an adhesin and demonstrating reduced susceptibility to fluconazole in *S. cerevisiae* transformants [104]. This study shows that Pb14-3-3 might be involved in the ergosterol biosynthesis revealing a potential new drug target. Recent work silencing *Pb14-3-3* distinctly altered the yeast morphology and hampered the morphological switching without affecting cell vitality or viability [105]. Additionally, these authors demonstrated that binding of the *Pb14-3-3* mutant to laminin and fibrinogen was reduced compared to that of the control, which correlated with a significant reduction of the virulence phenotype in the invertebrate infection model *Galleria mellonella*. This study established multifaceted roles of *Pb14-3-3* in morphology, attachment/infection to host components, and virulence, therefore supporting the previous report that suggested 14-3-3 as interesting therapeutic target for the treatment of PCM. Further intracellular survival and dissemination of *Paracoccidioides* spp. is accomplished by modulating programmed cell death of macrophages and epithelial cells, through expression of caspase-2, caspase-3, and caspase-8, strongly influenced by the 30 kDa 14-3-3 and gp43 adhesins [106, 107].

4. Defenses to Host Environment Stressors

Upon successful invasion of the mammalian host and reaching an intracellular niche, *Paracoccidioides* spp. require to overcome environmental stressors, persist intracellularly, and manipulate the progression of disease in the host. Other microbial determinants of *Paracoccidioides* spp. that play a relevant role in their pathogenesis are the following.

4.1. Melanin. Melanin pigments are remarkable substances present in all biological kingdoms, which have been associated with myriad functions based upon their unique physicochemical properties (reviewed by [108]) [109]. Melanins are polymers of phenolic and/or indolic compounds, negatively charged, hydrophobic in nature, and with high molecular weight and unknown structure [110]. Most fungi, bacteria, and helminths synthesize melanin via the polyketide synthase pathway or catalyze it by phenoloxidases (reviewed by [111]). Particularly in the field of fungal pathogenesis, their role in virulence has been well established (reviewed by [112–115]). In *Paracoccidioides* spp., melanin characterization was first described by Gomez et al. [116]. These authors revealed *P. brasiliensis*' ability to produce melanin when recovering dark particles that retained the size and shape of conidia or yeast, after enzymatic digestion and harsh acid treatment. Interestingly, melanized conidia were obtained after growing mycelia on water agar, while melanized yeasts were observed during growth in minimal media supplemented with L-3,4-dihydroxyphenylalanine (L-DOPA) and also recovered from infected mouse tissue, indicating the fungus capacity to synthesize melanin in the absence or presence of L-DOPA. Data also demonstrated that melanized yeast cells, either grown in vitro or recovered from infected tissue, were reactive to melanin-binding monoclonal antibodies (MAbs) isolated from *Cryptococcus neoformans* [117], showing consistency with in vivo melanization. Analysis by electron spin resonance (ESR) spectroscopy of *Paracoccidioides* spp. melanin recovered from yeast cells demonstrated a strong signal characteristic of a stable free-radical population, a key criterion in defining a melanin. Moreover, melanin synthesis by yeast cells was supported by the presence of laccase activity in cytoplasmic extracts. Additionally, upregulation of genes related to melanin synthesis such as tyrosinase and aromatic L-amino acid decarboxylase was shown in infected mice [37].

Fungal melanin distribution varies among species, for example, *Candida albicans* melanin can be found in the outer part of the cell wall and/or clustered on the cell wall surface [118], while in *C. neoformans*, melanin is first detectable close to the plasmatic membrane and fills throughout the cell wall over time [113]. Using transmission electron microscopy, *Paracoccidioides* spp. melanin was shown as electron-dense granules distributed on the yeast cell surface as well as in the cytoplasm [119]. Latest studies about cryptococcal melanin revealed that this polymer is composed of granular particles with an average size of 75 nm in diameter [120, 121].

Moreover, melanin synthesis takes place within laccase-containing vesicles known as fungal melanosomes [122], which might interact with cell wall components such as chitin to facilitate melanin deposition within the cell wall [123–126].

MAbs against *Paracoccidioides* melanin have been generated [127]. This study reported that the melanin-binding MAbs (IgG and IgM) successfully labeled conidia from mycelial cultures grown in water and yeasts grown in the presence of L-DOPA, as well as condium-infected mouse lung tissue. Melanin production during PCM was demonstrated by the detection of IgG Abs in serum specimens from patients; however, sera from patients with different mycoses displayed cross-reactivities against a wide spectrum of fungal melanin types, which supports the hypothesis that melanin may represent a "common" or immunological target for pathogenic fungi [128]. Antibodies to fungal melanin have provided protection against *C. neoformans* [129] and *Fonsecaea pedrosoi* [130].

Initial studies concerning melanin capacity to protect *P. brasiliensis* yeast cells from the host immune system reported that mannan can partially inhibit phagocytosis and that melanized cells were more resistant than nonmelanized cells to fungicidal and fungistatic effects of macrophages; however, increased macrophage uptake of opsonized yeast cell was documented when adding complement and/or antibody against melanin [119]. Further analyses on this area investigated the effect of *P. brasiliensis* melanized yeast cells on antimicrobial oxidants and phagocytosis using carbohydrates and monoclonal antibody to CD18 [131]. This study showed significant reduction in the phagocytosis of melanized yeast cells by macrophages, previously treated with mannan or laminarin; moreover, phagocytosis was virtually abolished when phagocytic cells were treated with mannan and *N*-acetylglucosamine in the presence of anti-CD18 antibodies, suggesting that macrophage internalization of melanized yeasts requires multiple receptors. In vitro analyses demonstrated that melanized cells were less susceptible to chemically generated nitric oxide, oxygen-derived oxidants, chloride-free sodium hypochlorite, and to killing by hydrogen peroxide than nonmelanized cells. These data correlated with an infection in a murine model, which resulted in increased fungal burden in the lungs by melanized yeast compared to nonmelanized cells, most likely attributable to reduced internalization by phagocytic cells and enhanced resistance to intracellular death. Therefore, melanin promotes fungal virulence by inhibiting phagocytosis and neutralizing oxidative radicals generated in the host effector cells.

Furthermore, in search for an alternative treatment to PCM skin lesions and oral mucosa, the influence of melanin produced by sixteen isolates of the *Paracoccidioides* spp. complex on the effects of treatment with antimicrobial photodynamic inhibition (aPI) and antifungal drugs was evaluated [132]. These authors demonstrated that aPI can reduce the viability of *Paracoccidioides* spp.; however, melanized yeast cells were more resistant than nonmelanized cells, which was attributable to lower levels of ROS and RNS due to melanin interference with the absorbance peak of toluidine blue. In addition, MIC data showed that melanized yeast cells were less susceptible to amphotericin and itraconazole, while in the previous study, da Silva et al. [119] found no differences between melanized and nonmelanized yeast cells. Nevertheless, studies from da Silva et al., using an antifungal killing assay for melanized yeast cells of *Paracoccidioides* spp., revealed increased resistance to antifungal drugs mainly amphotericin B and less pronounced with ketoconazole, fluconazole, itraconazole, and sulfamethoxazole, which could be thought to be attributable to reduced cell wall permeability or that melanin quenched free radicals released by cell membrane damaged by drugs [133].

Interestingly, studies by Baltazar et al. [132] showed that melanin can interact with amphotericin, itraconazole, and toluidine blue, consequently changing their antifungal activities. Other authors have demonstrated that melanin binds amphotericin and not itraconazole by analyzing the elemental composition of C:N:O after incubation of these drugs with melanin [134], suggesting that melanin alters the drug composition; however, Baltazar et al. reported that melanin might physically block itraconazole entrance to the yeast thus reducing its activity, while decrease in the antifungal activity of amphotericin is due to the alteration of the drug structure that reduces its affinity for ergosterol. Altogether, these data confirm that melanization contributes to virulence by acting as a ROS scavenger and through binding to antifungal drugs, thereby altering their activities [119, 131, 132].

4.2. Extracellular Vesicles. Fungal extracellular vesicles (EVs) resembling mammalian exosomes have been reported (reviewed by [135–139]). So far it is known that EVs, using a noncannonical pathway of secretion, are able to cross the cell wall and transport molecules that play a role in nutrient acquisition, cell defense, and even modulation of the host immune defense; however, many questions about their biogenesis, mechanisms through which EV transverse the cell wall and reach the extracellular space, and how they modulate host interactions remain to be elucidated. Nevertheless, the compositional analysis of such EVs present in the fungal pathogens *C. neoformans*, *H. capsulatum*, *C. albicans*, *Candida parapsilosis*, and *Sporothrix schenckii* suggests that they might act as "virulence bags" [140]. In fact, it is reported that in *C. neoformans*, glucoronoxylomannan (GXM), the major capsular polysaccharide, is transported within vesicles to the extracellular space where it is released and reincorporated into the cell surface as an alternative pathway for capsule growth [141]. These extracellular compartments composed of lipid bilayers have the potential to regulate key pathogenic steps during fungal infections. Particularly in the genus *Paracoccidioides*, a pioneer study characterized EVs isolated from culture supernatants of *P. brasiliensis* yeast cells cultivated in defined media [42]. This study demonstrated that the fungus EVs carry antigenic components bearing highly immunogenic α-galactopyranosyl (α-Gal) epitopes, which were found both at the vesicle surface and at the lumen. Both PCM and chagasic anti-α-Gal IgG reacted intensely with EVs, in contrast with the slight reaction evoked by natural anti-α-Gal antibodies, thereby suggesting that in *Paracoccidioides* spp., there is a high variety of nonreducing terminal α-linked galactopyranosyl

epitopes that may resemble those found in *Trypanosoma cruzi* mucins.

Furthermore, a unique proteomic analysis of EVs and vesicle-free released proteins from *Paracoccidioides* spp. pathogenic yeast phase provided a comparative analysis with other pathogenic fungi EV proteomes [59]. This study identified 205 and 260 proteins in vesicle and vesicle-free preparations, respectively. According to their sequences, almost 70% of them were predicted secretory, mostly involved in nonclasical secretory pathways. The comparative analysis of *Paracoccidioides* EV proteins with orthologs present in vesicles from *C. neoformans*, *H. capsulatum*, and *Saccharomyces cerevisiae* revealed that 63% of the *Paracoccidioides* vesicle-associated sequences had orthologs in other fungal extracellular vesicles, and among them, 72 were common to *Paracoccidioides* spp. in at least two other species, while 26 were identified in all four species analyzed. Some of these proteins might have clear roles during infection, for instance, superoxide dismutase, mitochondrial peroxiredoxin, and thioredoxin, which are involved in the ROS homeostasis and promote fungal intracellular survival. Interestingly, this analysis also revealed that the composition of the secretome is strongly affected by the growth conditions, suggesting that adaptation and survival to certain environments are closely associated with the profile of released proteins. Overall, it was reinforced with this study that EV cargo is complex, and it might involve proteins with diverse physiological functions from signaling to cell division to response to stress.

Concerning the complexity of fungal EVs, da Silva et al. [142] demonstrated that mannose and *N*-acetylglucosamine residues are found in *Paracoccidioides* EV surface, which are recognized by the innate immune system receptors DC-SIGN and DC-SIGNR, but not Dectin-1 or Dectin-2. Moreover, the influence of EVs produced by *P. brasiliensis* yeast cells on the host immune cells was evaluated [143]. These authors showed that incubation during 48 h of EVs and murine peritoneal macrophages induced the release of proinflammatory mediators such as NO, IL-12p40, IL-12p70, IL-6, TNF-α, IL-1α, and IL-1ß in a dose-dependent manner. Similarly, it was shown that EVs promote a proinflammatory profile in murine macrophage J774A.1 cells. Additionally, it was demonstrated with this study that EVs favor the development of macrophages towards the "classical" M1 activation phenotype, and even more, *Paracoccidioides* EVs can stimulate macrophage switching from an M2 towards an M1 phenotype. Remarkably, EV-stimulated macrophages, during 24 h, exhibited a higher fungicidal activity than those macrophages activated with IFN-γ, which was evident by the lower recovery of yeast CFU from lysed macrophages. Therefore, this study suggests that EV component from *Paracoccidioides* spp. can modulate the host immune response and affect the interplay of fungus-host immune cells.

4.2.1. How Paracoccidioides Spp. Overcome Host Environmental Stressors? Macrophage oxidative burst is characterized by increased oxygen uptake and ROS production that along the release of hydrolytic enzymes and toxic metabolites inside the phagolysosome intend to kill fungal pathogens. Nitrosative molecules (such as nitric oxide), produced mainly by INF-γ-activated macrophages, are fungicidal to *Paracoccidioides* spp. [144, 145]. *Paracoccidioides* complex initiates a metabolic switch to tolerate the macrophages' carbon-depleted environment, particularly by activating the pentose phosphate pathway, which additionally provides a defensive mechanism to the yeast cells against sulfhydryl groups and oxygen radicals from the host by maintaining glutathione in a reduced state [146]. Moreover, high-throughput transcriptional and proteomic analysis studies in *Paracoccidioides* spp. revealed that upon macrophage phagocytosis [147, 148], mimicking oxidative stress by exposure of yeast cells to H_2O_2 [149], or inducing nitrosative stress to yeast cells by incubation with S-nitrosoglutathione (GSNO) [150], which produces RNS, the fungus can cope with oxidative and nitrosative stress. In response to H_2O_2, *Paracoccidioides* spp. present a prominent activation of antioxidant enzymes (catalases, cytochrome c peroxidase, thioredoxin, and superoxide dismutases) and induce a metabolic shift to the pentose phosphate pathway, characterized by increased NADPH production in the cytoplasm as an electron source for glutathione peroxidase system, in order to restore the cellular redox potential [149]. This data correlates with the upregulation of transcripts of genes encoding peroxisomal catalase and Mn superoxide dismutase in yeast cells infecting macrophages associated with glucose and amino acid limitation [147]. Other studies evaluated the role of an alternative respiratory chain (AOX) in *Paracoccidioides* spp. during host-pathogen interaction [151, 152], which has been shown to be involved in the control of ROS and other oxidative molecules [153, 154]. Through generation of a knockdown strain PbAOX-aRNA, these authors demonstrated reduced fungal viability during infection of alveolar macrophages, particularly during the morphological transition, thus decreased fungal burden in the lungs of infected mice and increased survival rate. These data support that PbAOX is essential during the establishment of the fungal infection, possibly by assisting redox balancing during cell growth and the morphological switch of *Paracoccidioides* spp.

Understanding *Paracoccidioides* yeast cell behavior to nitrosative stress was achieved by identifying genes and proteins that might contribute to this response; this study demonstrated reduced levels of enzymes related to aerobic respiration (specifically cytochromes, succinate dehydrogenase, and ATP synthases), indicative of reduced activity of the mitochondrial electron transport chain [150]. In the presence of GSNO, these authors also reported alterations in lipids and branched chain amino acid metabolism and noticed increased expression of the enzymes cytochrome C peroxidase (CCP) and superoxide dismutase (SOD), which have been involved in *Paracoccidioides* spp. oxidative stress response [149]. Consequently, the overlapping role of CCP and SOD in both stress responses was confirmed by knockdown approaches [148, 150, 155]. *Paracoccidioides* spp. *ccp*-aRNA strains are more sensitive to RNS [150] and mitochondrial-generated ROS stress [148], suggesting that CCP avoids cell damage caused by nitrosative and oxidative stress. Additionally, these authors reported that CCP

silencing promoted a reduction in the number of recovered fungi in macrophages and in an animal model, thereby CCP can be considered a virulence factor since it is relevant for the establishment of the infection by *Paracoccidioides* spp. [148]. Another study has reported *Paracoccidioides* spp. ability to reduce nitric oxide (NO) levels by secreting the adhesin gp43, which prevents the release of NO from macrophages and stimulates the release of IL-10, hence reducing the iNOS expression and its enzymatic activity [73].

Concerning SOD role in the response to oxidative stress, Tamayo et al. [155] identified and characterized six isoforms encoded in the *P. brasiliensis* genome, among which Pb*SOD1* and Pb*SOD3* expressions were increased during the morphological switching to the pathogenic yeast phase, as well as under treatment with oxidative agents and during interaction with phagocytic cells (PMNs and alveolar macrophages). Interestingly, as shown by these authors, silencing of Pb*SOD1* and Pb*SOD3* genes has no detrimental effect on yeast cells' growth rate; however, both knockdown strains were similarly susceptible to H_2O_2- and menadione-induced oxidative stress, while PbSod3p was required for virulence. This study propose a well-coordinated response to oxidative stress in *Paracoccidioides* spp., in which intracellular Sods (mostly Sod1p) defense against endogenous-produced ROS while Sod3p, supported by its extracellular activity and cell surface localization, assists in combating the superoxide radicals generated during the host-pathogen interaction.

Furthermore, Tamayo et al. [156] recently identified and characterized the three members of the catalase (CAT) gene family in different fungal strains of *Paracoccidioides* spp., covering each phylogenetic lineage, as well as in other Onygenales. This study revealed that *Coccidioides* and dermatophyte genomes do not encode the extracellular catalase CATB, suggesting that Onygenales may have evolved different mechanisms to counteract oxidative stress via catalases. Moreover, in correlation with the SOD study [155], yeast cells from *P. brasiliensis* showed higher expression of *CATP* than those of *P. lutzii*. Having a similar experimental strategy as in the SOD analysis, these authors demonstrated that PbCATA and PbCATB play a major role in endogenous ROS homeostasis in yeast cells, whereas PbCATP is mainly triggered in the presence of exogenous ROS and the reduced expression of this isoform negatively affected fungal virulence in a mouse model. The data shows that *Paracoccidioides* spp. rely on CAT isoforms to control ROS homeostasis along the different stages of the infectious process to promote fungal survival and virulence [155].

Iron and zinc are essential micronutrients in fungi, due to their participation as cofactors in many biological processes inside the cell. Therefore, host cells impede intracellular microbial proliferation by restraining access to iron through its sequestration by high-affinity iron-binding proteins, such as transferrin and ferritin. Particularly, under iron starvation conditions, a proteomic analysis of *Paracoccidioides* spp. yeast cells grown in media supplemented with the iron chelator bathophenanthrolinedisulfonate (BPS) showed potential repression of the TCA cycle which is mediated by enzymes containing Fe/S clusters, reduced expression of enzymes involved in the glyoxylate pathway and methylcitrate cycle, downregulation of the electron transport chain, and decreased in oxidative phosphorylation thus lowering ATP production. Overall, these data revealed that in response to iron deprivation, the fungus adjusts their energy metabolism to iron-independent pathways by increasing glycolytic activity thus compensating for the decrease of aerobic pathways [157]. Furthermore, in *Paracoccidioides* spp., it has been shown that host hemoglobin and siderophore production and transport are iron sources for the fungus [158, 159]. Hemoglobin uptake is mediated by the hemoglobin fungal receptor ortholog, Rbt5 [158]. A *rbt5* knockdown strain of *Paracoccidioides* spp. showed a lower survival rate inside macrophages and lower fungal burden in vivo in a mouse model of infection, a result which suggests Rbt5 as a virulence factor and a possible way to overcome low levels of iron by a highly effective iron uptake by the fungus [158]. Another way to accumulate intracellular iron, described in *Paracoccidioides* spp., is a nontraditional reductive iron assimilation (RIA) pathway, involving iron reduction and zinc-regulated transporter homologs (Zrt1 and Zrt2), able to transport zinc and iron inside the fungal cell [160, 161]. This would suggest that under stress conditions, like those found inside the macrophage, those *Paracoccidioides* spp. yeast cells successfully phagocyted by macrophages, could shift to a starvation mode, and activate highly effective iron and zinc uptake pathways, in order to persist inside the microenvironmental conditions of phagocytic cells.

It is established that in inflamed tissues, oxygen supply is limited by the high volume of host phagocytic cells or the microbe itself at blood vessels. *Paracoccidioides* spp. must tolerate and overcome stress conditions caused by low oxygen levels. Characterization of *P. lutzii* hypoxia response by a proteomic approach revealed differential protein expression for 134 and 154 proteins at 12 and 24 hours under hypoxy conditions when compared to the control [162]. At 12 hours under hypoxia, 50% of the proteins showing differential expression were increased, while the same percentage was decreased when compared to control cells, while at 24 hours under hypoxia, around 66% of proteins showing differential expression were increased, while around 33% were decreased. An evaluation of mitochondrial activity showed a lower activity at the first 12 hours under hypoxia, and restoration of activity at 24 hours, in agreement with the proteomic results, showing the potential for adaptation of this fungus under low oxygen levels [162]. The same work revealed that *P. lutzii* contains homologs of SrbA, a sterol regulatory element binding protein (SREBP) and key regulator of hypoxia adaptation in fungi [162]. Functional complementation of an *Aspergillus fumigatus srbA* null mutant by the *Paracoccidioides srbA* (*PbsrbA*) gene restored the null mutant hyphal growth under hypoxia, which suggests that *PbsrbA* may promote adaptation to hypoxic microenvironments. Furthermore, this study also showed that *Paracoccidioides* SrbA is likely involved in azole drug resistance responses. Perhaps this resistance could be achieved by regulating brassicasterol biosynthesis, which is found in *Paracoccidioides* spp. yeast cells' cytoplasmic membranes, instead of ergosterol [163], compensating the effects on membrane fluidity due to low oxygen levels.

Table 4: *Paracoccidioides* spp. genes shown by functional molecular studies using antisense technology to be involved in virulence and/or immune-evasion strategies from the host.

Gene	Encodes	Biological role	References
Pb*CDC42*	Rho GTPase	(i) Coordination of cell growth/morphogenesis of yeast cells, promoting an enhanced ability to evade the host immune system.	[45]
Pb*HAD32*	Hydrolase	(i) Adhesin involved in initial attachment of the infectious particles to the lungs.	[80, 81]
Pb*AOX*	Oxidase part of the electron transport chain in mitochondria	(i) Essential during the establishment of the fungal infection, possibly by assisting redox balancing during cell growth and the morphological switch.	[151, 152, 154]
Pb*HSP90*	Molecular chaperone	(i) Binds and stabilizes calcineurin thus controlling the cell differentiation. (ii) Essential upon thermo-dependent response and oxidative injury promoting fungal adaptation to the host.	[29–31]
Pb*GP43*	Cell-surface component	(i) Adhesin that inhibits the phagocytic and fungicidal capacity of macrophages, through binding to mannose receptors and inducing IL-18 production. (ii) Ability to reduce nitric oxide levels. (iii) Adherence receptor in the internalization of the yeast into the host cell altering its cytoskeleton structure. (iv) Modulation of host cells apoptosis.	[73, 76, 106, 107]
Pb*P27*	Protein mainly localized in cytoplasm and cell wall of yeast cells	(i) Involved in the yeast cellular morphological and glucose metabolism. (ii) Possible role in promoting latency in the host.	[173]
Pb*Rbt5*	Surface glycosylphosphatidylinositol-(GPI-) anchored protein	(i) Hemoglobin uptake as an iron source for intracellular survival. (ii) Potential virulence factor.	[158]
Pb*CCP*	Cytochrome c oxidase	(i) Avoids cell damage caused by nitrosative and oxidative stress. (ii) Promote fungal survival within macrophages. (iii) Potential virulence factor.	[148, 150]
Pb*SOD1*	Cytosolic superoxide dismutase	(i) Defense against endogenous-produced ROS.	[155]
Pb*SOD3*	Extracellular superoxide dismutase	(i) Pronounced extracellular activity involved in combating superoxide radicals generated during the host-pathogen interaction. (ii) Potential virulence factor.	[155]
Pb*14-3-3*	30 kDa protein	(i) Adhesin able to bind laminin. (ii) Critical role in attachment/infection to host components and fungal virulence. (iii) Involved in the morphological switching, ergosterol biosynthesis, and modulating apoptosis of host phagocytic and epithelial cells.	[105–107]
Pb*SCONC*	Member of the Toll-like receptor family encoding a negative regulator of the inorganic sulfur assimilation pathway	(i) Dimorphism regulator by modulating the inorganic sulfur metabolism and influencing virulence. (ii) Novel virulence determinant.	[174]
Pb*CATA* Pb*CATB*	Catalases	(i) Major role in endogenous ROS homeostasis in *Paracoccidioides* cells.	[156]
Pb*CATP*	Catalases	(i) Mainly triggered in the presence of exogenous ROS and highly relevant for fungal virulence.	[156]

5. Dissemination

On the last stage of the infectious process, the ability to establish the fungal infection in distant niches through biofilm formation represents a critical virulence factor in *Paracoccidioides* spp. A recent report showed that *P. brasiliensis* can colonize surfaces and form biofilms in its yeast phase [70]. The fungus biofilm consisted of a dense network of yeast cells characterized by the expression of genes encoding adhesins (gp43, GAPDH) and hydrolytic enzymes

(aspartyl proteinase), consistent with the established steps of adhesion, invasion, and tissue destruction also reported for *C. albicans* biofilms [164]. Biofilm formation by the fungus might be a critical factor in the persistence of the fungal infection, since it could hinder the action of antifungal drugs and may contribute to a chronic state of the disease. Additionally, gp43 inhibits the phagocytic and fungicidal capacity of macrophages, through binding to mannose receptors and inducing IL-18 production [73, 165].

Particularly, a serine-thiol extracellular proteinase (PbST) with hydrolytic activity at 37°C has been reported in the pathogenic yeast phase of *P. brasiliensis* [166, 167], in line with its transcript upregulation [38]. This serine proteinase is involved in the cleavage of the main components of the basal membrane in vitro, including laminin, fibronectin, collagen type IV, and proteoglycans, suggesting a potential role for fungal tissue invasion and dissemination. In a *P. brasiliensis* vesicle proteome study, a subtilase-type proteinase psp3 (PADG_07422) was identified [59]. Its identity could be PbST, since it showed a free cysteine residue in its sequence; however, further experimental evidence is still required.

Further escape of *Paracoccidioides* spp. from the immune system is done by altering T-cell repertoire. Differentiation and maturation of T-cells occurs in the thymus, thus integrity of the thymic microenvironment is crucial for the maturation of thymocytes. Experimental data in a murine model of acute paracoccidioidomycosis shows that infection with *Paracoccidioides* yeast cells promotes thymus atrophy as a consequence of epithelial cell spatial disarrangement and increased gene expression of inflammatory mediators [168, 169]. These results suggest that a decreased differentiation of pathogen-specific T-cells leads to host immunosuppression, favoring *Paracoccidioides* spp. ability to thrive and multiply in the thymus microenvironment (Figure 2(c)).

6. Conclusions

Recent molecular evolutionary studies have shown differences in the ecoepidemiology of *Paracoccidioides* spp. [8, 170], suggesting diversifying mechanisms of pathogenicity and intracellular survival across these species that could also be explained by the complex and stochastic adaptation process of evolving within two particular ecological niches, the soil and live tissues of animal hosts. Likewise, Pigosso et al. [171] have demonstrated that the genus *Paracoccidioides* have important differences in their metabolic profiles, which must play a critical role during the host-pathogen interaction at the onset of the infection. However, while there is room for mammalian virulence adaptation in *Paracoccidioides* spp., it is important to always have in mind that virulence is a microbial property exclusively expressed in a susceptible host, and the outcome of this interaction is dependent on both players [172]. PCM is mostly related to low-income male workers on rural endemic areas of Central and South America, which are often related to rural poverty and malnutrition.

Over the last years, using antisense RNA technology, significant progress has been made to enhance our understanding of *Paracoccidioides* spp. host-pathogen interaction, pathogen resistance, and fungal virulence. In Table 4, we summarized genes shown by functional molecular studies using antisense technology to be involved in virulence and/or immune-evasion strategies from the host. In the near future, with the emergence of CRISPR technology and full access to diverse databanks (complete genomes, transcriptome, proteomic, metabolomics, lipidomics, etc.), we will gain more knowledge on the virulence processes that eventually should translate into patient's benefits.

Conflicts of Interest

The authors declare no competing interests regarding the publication of this paper.

Acknowledgments

The authors are very grateful to Sabrina Rodriguez for her biochemical studies on cell wall polysaccharide composition (Mycology Laboratory, Instituto Venezolano de Investigaciones Cientificas, Venezuela) and to Victor Salazar (Microscopy Service, Instituto Venezolano de Investigaciones Cientificas, Venezuela) for his support on performing the immunofluorescence studies.

References

[1] A. Restrepo, A. Tobón, and L. E. Cano, "Paracoccidioides brasiliensis," in *Principles and Practice of Infectious Diseases*, J. E. Bennett, R. Dolin and M. J. Blaser, Eds., pp. 2995–3002, Elsevier, Philadelphia, PA, 2015.

[2] R. Buccheri, Z. Khoury, L. C. Barata, and G. Benard, "Incubation period and early natural history events of the acute form of paracoccidioidomycosis: lessons from patients with a single Paracoccidioides spp. exposure," *Mycopathologia*, vol. 181, no. 5-6, pp. 435–439, 2016.

[3] L. L. Carrero, G. Nino-Vega, M. M. Teixeira et al., "New Paracoccidioides brasiliensis isolate reveals unexpected genomic variability in this human pathogen," *Fungal Genetics and Biology*, vol. 45, no. 5, pp. 605–612, 2008.

[4] M. M. Teixeira, R. C. Theodoro, M. J. de Carvalho et al., "Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus," *Molecular Phylogenetics and Evolution*, vol. 52, no. 2, pp. 273–283, 2009.

[5] M. Teixeira Mde, R. C. Theodoro, F. F. Oliveira et al., "Paracoccidioides lutzii sp. nov.: biological and clinical implications," *Medical Mycology*, vol. 52, no. 2, pp. 19–28, 2014.

[6] D. R. Matute, J. G. McEwen, R. Puccia et al., "Cryptic speciation and recombination in the fungus Paracoccidioides brasiliensis as revealed by gene genealogies," *Molecular Biology and Evolution*, vol. 23, no. 1, pp. 65–73, 2006.

[7] C. Salazar-Salgado, L. R. Jones, A. Restrepo, and J. G. McEwen, "The human fungal pathogen Paracoccidioides brasiliensis (Onygenales: Ajellomycetaceae) is a complex of two species: phylogenetic evidence from five mitochondrial markers," *Cladistics*, vol. 26, no. 6, pp. 613–624, 2010.

[8] M. M. Teixeira, R. C. Theodoro, G. Nino-Vega, E. Bagagli, and M. S. Felipe, "Paracoccidioides species complex: ecology, phylogeny, sexual reproduction, and virulence," *PLoS Pathogens*, vol. 10, no. 10, article e1004397, 2014.

[9] B. Bustamente-Simon, J. G. McEwen, A. M. Tabares, M. Arango, and A. Restrepo-Moreno, "Characteristics of the conidia produced by the mycelial form of *Paracoccidioides brasiliensis*," *Sabouraudia*, vol. 23, no. 6, pp. 407–414, 1985.

[10] G. C. Machado, D. V. Moris, T. D. Arantes et al., "Cryptic species of Paracoccidioides brasiliensis: impact on paracoccidioidomycosis immunodiagnosis," *Memórias do Instituto Oswaldo Cruz*, vol. 108, no. 5, pp. 637–643, 2013.

[11] J. J. Batista, Z. P. de Camargo, G. F. Fernandes, A. P. Vicentini, C. J. Fontes, and R. C. Hahn, "Is the geographical origin of a *Paracoccidioides brasiliensis* isolate important for antigen production for regional diagnosis of paracoccidioidomycosis?" *Mycoses*, vol. 53, no. 2, pp. 176–180, 2010.

[12] G. Gegembauer, L. M. Araujo, E. F. Pereira et al., "Serology of paracoccidioidomycosis due to Paracoccidioides lutzii," *PLoS Neglected Tropical Diseases*, vol. 8, no. 7, article e2986, 2014.

[13] P. Queiroz Júnior Lde, Z. P. de Camargo, T. Tadano et al., "Serological and antigenic profiles of clinical isolates of Paracoccidioides spp. from Central Western Brazil," *Mycoses*, vol. 57, no. 8, pp. 466–472, 2014.

[14] D. R. Matute, L. M. Quesada-Ocampo, J. T. Rauscher, and J. G. McEwen, "Evidence for positive selection in putative virulence factors within the Paracoccidioides brasiliensis species complex," *PLoS Neglected Tropical Diseases*, vol. 2, no. 9, article e296, 2008.

[15] A. Casadevall and L. A. Pirofski, "Virulence factors and their mechanisms of action: the view from a damage-response framework," *Journal of Water and Health*, vol. 7, Supplement 1, pp. S2–S18, 2009.

[16] S. K. Thind, C. P. Taborda, and J. D. Nosanchuk, "Dendritic cell interactions with Histoplasma and Paracoccidioides," *Virulence*, vol. 6, no. 5, pp. 424–432, 2015.

[17] V. L. Calich, A. Pina, M. Felonato, S. Bernardino, T. A. Costa, and F. V. Loures, "Toll-like receptors and fungal infections: the role of TLR2, TLR4 and MyD88 in paracoccidioidomycosis," *FEMS Immunology and Medical Microbiology*, vol. 53, no. 1, pp. 1–7, 2008.

[18] G. Gauthier and B. S. Klein, "Insights into fungal morphogenesis and immune evasion: fungal conidia, when situated in mammalian lungs, may switch from mold to pathogenic yeasts or spore-forming spherules," *Microbe Wash DC*, vol. 3, no. 9, pp. 416–423, 2008.

[19] J. G. McEwen, V. Bedoya, M. M. Patino, M. E. Salazar, and A. Restrepo, "Experimental murine paracoccidiodomycosis induced by the inhalation of conidia," *Journal of Medical and Veterinary Mycology*, vol. 25, no. 3, pp. 165–175, 1987.

[20] F. Sorais, G. Nino-Vega, and G. San-Blas, "El peculiar mecanismo de degradación de la ornitina descarboxilasa fúngica," *Revista Iberoamericana de Micología*, vol. 20, no. 1, pp. 1–5, 2003.

[21] G. San-Blas, F. Sorais, F. San-Blas, and J. Ruiz-Herrera, "Ornithine decarboxylase in Paracoccidioides brasiliensis," *Archives of Microbiology*, vol. 165, no. 5, pp. 311–316, 1996.

[22] G. San-Blas, F. San Blas, F. Sorais, B. Moreno, and J. Ruiz-Herrera, "Polyamines in growth and dimorphism of *Paracoccidioides brasiliensis*," *Archives of Microbiology*, vol. 166, no. 6, pp. 411–413, 1997.

[23] K. J. Boyce and A. Andrianopoulos, "Fungal dimorphism: the switch from hyphae to yeast is a specialized morphogenetic adaptation allowing colonization of a host," *FEMS Microbiology Reviews*, vol. 39, no. 6, pp. 797–811, 2015.

[24] J. C. Nemecek, M. Wuthrich, and B. S. Klein, "Global control of dimorphism and virulence in fungi," *Science*, vol. 312, no. 5773, pp. 583–588, 2006.

[25] K. P. Bastos, A. M. Bailao, C. L. Borges et al., "The transcriptome analysis of early morphogenesis in Paracoccidioides brasiliensis mycelium reveals novel and induced genes potentially associated to the dimorphic process," *BMC Microbiology*, vol. 7, no. 1, p. 29, 2007.

[26] A. F. Chaves, M. V. Navarro, D. G. Castilho, J. C. Calado, P. M. Conceicao, and W. L. Batista, "A conserved dimorphism-regulating histidine kinase controls the dimorphic switching in Paracoccidioides brasiliensis," *FEMS Yeast Research*, vol. 16, no. 5, 2016.

[27] L. R. Nunes, R. Costa de Oliveira, D. B. Leite et al., "Transcriptome analysis of Paracoccidioides brasiliensis cells undergoing mycelium-to-yeast transition," *Eukaryotic Cell*, vol. 4, no. 12, pp. 2115–2128, 2005.

[28] L. Fernandes, H. C. Paes, A. H. Tavares et al., "Transcriptional profile of ras1 and ras2 and the potential role of farnesylation in the dimorphism of the human pathogen Paracoccidioides brasiliensis," *FEMS Yeast Research*, vol. 8, no. 2, pp. 300–310, 2008.

[29] T. G. Matos, F. V. Morais, and C. B. Campos, "Hsp90 regulates Paracoccidioides brasiliensis proliferation and ROS levels under thermal stress and cooperates with calcineurin to control yeast to mycelium dimorphism," *Medical Mycology*, vol. 51, no. 4, pp. 413–421, 2013.

[30] A. M. Nicola, R. V. Andrade, A. S. Dantas et al., "The stress responsive and morphologically regulated hsp90 gene from Paracoccidioides brasiliensis is essential to cell viability," *BMC Microbiology*, vol. 8, no. 1, p. 158, 2008.

[31] D. Tamayo, J. F. Munoz, I. Torres et al., "Involvement of the 90 kDa heat shock protein during adaptation of Paracoccidioides brasiliensis to different environmental conditions," *Fungal Genetics and Biology*, vol. 51, pp. 34–41, 2013.

[32] A. Restrepo, M. E. Salazar, L. E. Cano, E. P. Stover, D. Feldman, and D. A. Stevens, "Estrogens inhibit mycelium-to-yeast transformation in the fungus Paracoccidioides brasiliensis: implications for resistance of females to paracoccidioidomycosis," *Infection and Immunity*, vol. 46, no. 2, pp. 346–353, 1984.

[33] M. E. Salazar, A. Restrepo, and D. A. Stevens, "Inhibition by estrogens of conidium-to-yeast conversion in the fungus Paracoccidioides brasiliensis," *Infection and Immunity*, vol. 56, no. 3, pp. 711–713, 1988.

[34] B. H. Aristizabal, K. V. Clemons, D. A. Stevens, and A. Restrepo, "Morphological transition of Paracoccidioides brasiliensis conidia to yeast cells: in vivo inhibition in females," *Infection and Immunity*, vol. 66, no. 11, pp. 5587–5591, 1998.

[35] C. F. Pinzan, L. P. Ruas, A. S. Casabona-Fortunato, F. C. Carvalho, and M. C. Roque-Barreira, "Immunological basis for the gender differences in murine Paracoccidioides brasiliensis infection," *PLoS One*, vol. 5, no. 5, article e10757, 2010.

[36] J. Shankar, T. D. Wu, K. V. Clemons, J. P. Monteiro, L. F. Mirels, and D. A. Stevens, "Influence of 17beta-estradiol on gene expression of Paracoccidioides during mycelia-to-yeast transition," *PLoS One*, vol. 6, no. 12, article e28402, 2011.

[37] A. M. Bailao, A. Schrank, C. L. Borges et al., "Differential gene expression by Paracoccidioides brasiliensis in host interaction conditions: representational difference analysis

identifies candidate genes associated with fungal pathogenesis," *Microbes and Infection*, vol. 8, no. 12-13, pp. 2686–2697, 2006.

[38] A. M. Bailao, A. Shrank, C. L. Borges et al., "The transcriptional profile of Paracoccidioides brasiliensis yeast cells is influenced by human plasma," *FEMS Immunology and Medical Microbiology*, vol. 51, no. 1, pp. 43–57, 2007.

[39] M. Costa, C. L. Borges, A. M. Bailao et al., "Transcriptome profiling of Paracoccidioides brasiliensis yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction," *Microbiology*, vol. 153, Part 12, pp. 4194–4207, 2007.

[40] M. Pereira, A. M. Bailao, J. A. Parente, C. L. Borges, S. M. Salem-Izacc, and C. M. Soares, "Preferential transcription of Paracoccidioides brasiliensis genes: host niche and time-dependent expression," *Memórias do Instituto Oswaldo Cruz*, vol. 104, no. 3, pp. 486–491, 2009.

[41] T. C. Rezende, C. L. Borges, A. D. Magalhaes et al., "A quantitative view of the morphological phases of Paracoccidioides brasiliensis using proteomics," *Journal of Proteomics*, vol. 75, no. 2, pp. 572–587, 2011.

[42] M. C. Vallejo, A. L. Matsuo, L. Ganiko et al., "The pathogenic fungus Paracoccidioides brasiliensis exports extracellular vesicles containing highly immunogenic alpha-galactosyl epitopes," *Eukaryotic Cell*, vol. 10, no. 3, pp. 343–351, 2011.

[43] S. S. Weber, A. F. Parente, C. L. Borges, J. A. Parente, A. M. Bailao, and C. M. de Almeida Soares, "Analysis of the secretomes of Paracoccidioides mycelia and yeast cells," *PLoS One*, vol. 7, no. 12, article e52470, 2012.

[44] S. Lima Pde, L. Casaletti, A. M. Bailao, A. T. de Vasconcelos, R. Fernandes Gda, and C. M. Soares, "Transcriptional and proteomic responses to carbon starvation in Paracoccidioides," *PLoS Neglected Tropical Diseases*, vol. 8, no. 5, article e2855, 2014.

[45] A. J. Almeida, C. Cunha, J. A. Carmona et al., "Cdc42p controls yeast-cell shape and virulence of Paracoccidioides brasiliensis," *Fungal Genetics and Biology*, vol. 46, no. 12, pp. 919–926, 2009.

[46] F. Kanetsuna, L. M. Carbonell, R. E. Moreno, and J. Rodriguez, "Cell wall composition of the yeast and mycelial forms of Paracoccidioides brasiliensis," *Journal of Bacteriology*, vol. 97, no. 3, pp. 1036–1041, 1969.

[47] G. San-Blas, F. San-Blas, and L. E. Serrano, "Host-parasite relationships in the yeastlike form of Paracoccidioides brasiliensis strain IVIC Pb9," *Infection and Immunity*, vol. 15, no. 2, pp. 343–346, 1977.

[48] C. A. Rappleye, L. G. Eissenberg, and W. E. Goldman, "Histoplasma capsulatum alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 4, pp. 1366–1370, 2007.

[49] C. L. Marion, C. A. Rappleye, J. T. Engle, and W. E. Goldman, "An alpha-(1,4)-amylase is essential for alpha-(1,3)-glucan production and virulence in Histoplasma capsulatum," *Molecular Microbiology*, vol. 62, no. 4, pp. 970–983, 2006.

[50] E. Camacho, V. E. Sepulveda, W. E. Goldman, G. San-Blas, and G. A. Nino-Vega, "Expression of Paracoccidioides brasiliensis AMY1 in a Histoplasma capsulatum amy1 mutant, relates an alpha-(1,4)-amylase to cell wall alpha-(1,3)-glucan synthesis," *PLoS One*, vol. 7, no. 11, article e50201, 2012.

[51] L. M. Carbonell, F. Kanetsuna, and F. Gil, "Chemical morphology of glucan and chitin in the cell wall of the yeast phase of Paracoccidioides brasiliensis," *Journal of Bacteriology*, vol. 101, no. 2, pp. 636–642, 1970.

[52] G. San-Blas and D. Vernet, "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," *Infection and Immunity*, vol. 15, no. 3, pp. 897–902, 1977.

[53] G. San Blas and F. San Blas, "Variability of the cell wall composition in Paracoccidioides brasiliensis: a study of two strains," *Sabouraudia*, vol. 20, no. 1, pp. 31–40, 1982.

[54] G. San Blas, F. San Blas, D. Ordaz, S. Centeno, and M. C. Albornoz, "Chemical changes in cell wall structure of five strains of Paracoccidioides brasiliensis," *Sabouraudia*, vol. 22, no. 3, pp. 255–257, 1984.

[55] F. Kanetsuna, L. M. Carbonell, I. Azuma, and Y. Yamamura, "Biochemical studies on the thermal dimorphism of Paracoccidioides brasiliensis," *Journal of Bacteriology*, vol. 110, no. 1, pp. 208–218, 1972.

[56] E. Brummer, A. Restrepo, L. H. Hanson, and D. A. Stevens, "Virulence of Paracoccidiodes brasiliensis: the influence of in vitro passage and storage," *Mycopathologia*, vol. 109, no. 1, pp. 13–17, 1990.

[57] P. F. Andreotti, J. L. Monteiro da Silva, A. M. Bailao et al., "Isolation and partial characterization of a 30 kDa adhesin from Paracoccidioides brasiliensis," *Microbes and Infection*, vol. 7, no. 5-6, pp. 875–881, 2005.

[58] A. L. Garfoot, Q. Shen, M. Wuthrich, B. S. Klein, and C. A. Rappleye, "The Eng1 beta-glucanase enhances Histoplasma virulence by reducing beta-glucan exposure," *MBio*, vol. 7, no. 2, pp. e01388–e01315, 2016.

[59] M. C. Vallejo, E. S. Nakayasu, A. L. Matsuo et al., "Vesicle and vesicle-free extracellular proteome of Paracoccidioides brasiliensis: comparative analysis with other pathogenic fungi," *Journal of Proteome Research*, vol. 11, no. 3, pp. 1676–1685, 2012.

[60] P. C. Souto, V. N. Brito, J. Gameiro, M. A. da Cruz-Hofling, and L. Verinaud, "Programmed cell death in thymus during experimental paracoccidioidomycosis," *Medical Microbiology and Immunology*, vol. 192, no. 4, pp. 225–229, 2003.

[61] M. J. Mendes-Giannini, S. A. Hanna, J. L. da Silva et al., "Invasion of epithelial mammalian cells by Paracoccidioides brasiliensis leads to cytoskeletal rearrangement and apoptosis of the host cell," *Microbes and Infection*, vol. 6, no. 10, pp. 882–891, 2004.

[62] S. G. Filler and D. C. Sheppard, "Fungal invasion of normally non-phagocytic host cells," *PLoS Pathogens*, vol. 2, no. 12, article e129, 2006.

[63] M. J. Mendes-Giannini, M. L. Taylor, J. B. Bouchara et al., "Pathogenesis II: fungal responses to host responses: interaction of host cells with fungi," *Medical Mycology*, vol. 38, Supplement 1, pp. 113–123, 2000.

[64] M. J. Mendes-Giannini, P. F. Andreotti, L. R. Vincenzi et al., "Binding of extracellular matrix proteins to Paracoccidioides brasiliensis," *Microbes and Infection*, vol. 8, no. 6, pp. 1550–1559, 2006.

[65] S. A. Hanna, J. L. Monteiro da Silva, and M. J. Giannini, "Adherence and intracellular parasitism of Paracoccidioides brasiliensis in Vero cells," *Microbes and Infection*, vol. 2, no. 8, pp. 877–884, 2000.

[66] H. C. de Oliveira, J. F. da Silva, L. Scorzoni et al., "Importance of adhesins in virulence of Paracoccidioides spp.," *Frontiers in Microbiology*, vol. 6, p. 303, 2015.

[67] R. Puccia, M. C. Vallejo, A. L. Matsuo, and L. V. Longo, "The paracoccidioides cell wall: past and present layers toward understanding interaction with the host," *Frontiers in Microbiology*, vol. 2, p. 257, 2011.

[68] H. C. de Oliveira, P. A. Assato, C. M. Marcos et al., "Paracoccidioides-host interaction: an overview on recent advances in the paracoccidioidomycosis," *Frontiers in Microbiology*, vol. 6, p. 1319, 2015.

[69] C. M. Marcos, H. C. de Oliveira, J. F. da Silva, P. A. Assato, A. M. Fusco-Almeida, and M. J. Mendes-Giannini, "The multifaceted roles of metabolic enzymes in the Paracoccidioides species complex," *Frontiers in Microbiology*, vol. 5, p. 719, 2014.

[70] C. Sardi Jde, S. Pitangui Nde, A. R. Voltan et al., "In vitro Paracoccidioides brasiliensis biofilm and gene expression of adhesins and hydrolytic enzymes," *Virulence*, vol. 6, no. 6, pp. 642–651, 2015.

[71] J. D. Lopes, M. C. Moura-Campos, A. P. Vicentini, J. L. Gesztesi, W. de-Souza, and Z. P. Camargo, "Characterization of glycoprotein gp43, the major laminin-binding protein of Paracoccidioides brasiliensis," *Brazilian Journal of Medical and Biological Research*, vol. 27, no. 9, pp. 2309–2313, 1994.

[72] A. P. Vicentini, J. L. Gesztesi, M. F. Franco et al., "Binding of Paracoccidioides brasiliensis to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis," *Infection and Immunity*, vol. 62, no. 4, pp. 1465–1469, 1994.

[73] A. F. Flavia Popi, J. D. Lopes, and M. Mariano, "GP43 from Paracoccidioides brasiliensis inhibits macrophage functions. An evasion mechanism of the fungus," *Cellular Immunology*, vol. 218, no. 1-2, pp. 87–94, 2002.

[74] V. L. Calich and S. S. Kashino, "Cytokines produced by susceptible and resistant mice in the course of Paracoccidioides brasiliensis infection," *Brazilian Journal of Medical and Biological Research*, vol. 31, no. 5, pp. 615–623, 1998.

[75] A. F. Vigna, S. R. Almeida, P. Xander, E. Freymuller, M. Mariano, and J. D. Lopes, "Granuloma formation in vitro requires B-1 cells and is modulated by Paracoccidioides brasiliensis gp43 antigen," *Microbes and Infection*, vol. 8, no. 3, pp. 589–597, 2006.

[76] I. Torres, O. Hernandez, D. Tamayo et al., "Inhibition of PbGP43 expression may suggest that gp43 is a virulence factor in Paracoccidioides brasiliensis," *PloS One*, vol. 8, no. 7, article e68434, 2013.

[77] N. P. Leitao, M. C. Vallejo, P. M. Conceicao, Z. P. Camargo, R. Hahn, and R. Puccia, "Paracoccidioides lutzii Plp43 is an active glucanase with partial antigenic identity with *P. brasiliensis* gp43," *PLoS Neglected Tropical Diseases*, vol. 8, no. 8, article e3111, 2014.

[78] A. Gonzalez, B. L. Gomez, S. Diez et al., "Purification and partial characterization of a Paracoccidioides brasiliensis protein with capacity to bind to extracellular matrix proteins," *Infection and Immunity*, vol. 73, no. 4, pp. 2486–2495, 2005.

[79] A. Gonzalez, E. Caro, C. Munoz, A. Restrepo, A. J. Hamilton, and L. E. Cano, "Paracoccidioides brasiliensis conidia recognize fibronectin and fibrinogen which subsequently participate in adherence to human type II alveolar cells: involvement of a specific adhesin," *Microbial Pathogenesis*, vol. 44, no. 5, pp. 389–401, 2008.

[80] O. Hernandez, A. J. Almeida, A. Gonzalez et al., "A 32-kilodalton hydrolase plays an important role in Paracoccidioides brasiliensis adherence to host cells and influences pathogenicity," *Infection and Immunity*, vol. 78, no. 12, pp. 5280–5286, 2010.

[81] O. Hernandez, A. J. Almeida, D. Tamayo et al., "The hydrolase PbHAD32 participates in the adherence of Paracoccidioides brasiliensis conidia to epithelial lung cells," *Medical Mycology*, vol. 50, no. 5, pp. 533–537, 2012.

[82] D. A. Soares, R. V. de Andrade, S. S. Silva, A. L. Bocca, S. M. Soares Felipe, and S. Petrofeza, "Extracellular Paracoccidioides brasiliensis phospholipase B involvement in alveolar macrophage interaction," *BMC Microbiology*, vol. 10, no. 1, p. 241, 2010.

[83] A. M. Bailao, S. V. Nogueira, S. M. Rondon Caixeta Bonfim et al., "Comparative transcriptome analysis of Paracoccidioides brasiliensis during in vitro adhesion to type I collagen and fibronectin: identification of potential adhesins," *Research in Microbiology*, vol. 163, no. 3, pp. 182–191, 2012.

[84] H. V. Baker, "GCR1 of *Saccharomyces cerevisiae* encodes a DNA binding protein whose binding is abolished by mutations in the CTTCC sequence motif," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 21, pp. 9443–9447, 1991.

[85] F. C. Donofrio, A. C. Calil, E. T. Miranda et al., "Enolase from Paracoccidioides brasiliensis: isolation and identification as a fibronectin-binding protein," *Journal of Medical Microbiology*, vol. 58, no. Pt 6, pp. 706–713, 2009.

[86] C. M. Marcos, J. de Fatima da Silva, H. C. de Oliveira, R. A. Moraes da Silva, M. J. Mendes-Giannini, and A. M. Fusco-Almeida, "Surface-expressed enolase contributes to the adhesion of Paracoccidioides brasiliensis to host cells," *FEMS Yeast Research*, vol. 12, no. 5, pp. 557–570, 2012.

[87] S. V. Nogueira, F. L. Fonseca, M. L. Rodrigues et al., "Paracoccidioides brasiliensis enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells," *Infection and Immunity*, vol. 78, no. 9, pp. 4040–4050, 2010.

[88] A. K. Ghosh and M. Jacobs-Lorena, "Surface-expressed enolases of plasmodium and other pathogens," *Memórias do Instituto Oswaldo Cruz*, vol. 106, Supplement 1, pp. 85–90, 2011.

[89] E. G. Chaves, S. S. Weber, S. N. Bao et al., "Analysis of Paracoccidioides secreted proteins reveals fructose 1,6-bisphosphate aldolase as a plasminogen-binding protein," *BMC Microbiology*, vol. 15, no. 1, p. 53, 2015.

[90] M. S. Barbosa, D. A. Cunha Passos, M. S. Felipe, R. S. Jesuino, M. Pereira, and C. M. de Almeida Soares, "The glyceraldehyde-3-phosphate dehydrogenase homologue is differentially regulated in phases of Paracoccidioides brasiliensis: molecular and phylogenetic analysis," *Fungal Genetics and Biology*, vol. 41, no. 7, pp. 667–675, 2004.

[91] M. S. Barbosa, S. N. Bao, P. F. Andreotti et al., "Glyceraldehyde-3-phosphate dehydrogenase of Paracoccidioides brasiliensis is a cell surface protein involved in fungal adhesion to extracellular matrix proteins and interaction with cells," *Infection and Immunity*, vol. 74, no. 1, pp. 382–389, 2006.

[92] C. A. da Fonseca, R. S. Jesuino, M. S. Felipe, D. A. Cunha, W. A. Brito, and C. M. Soares, "Two-dimensional electrophoresis and characterization of antigens from Paracoccidioides brasiliensis," *Microbes and Infection*, vol. 3, no. 7, pp. 535–542, 2001.

[93] L. A. Pereira, S. N. Bao, M. S. Barbosa et al., "Analysis of the Paracoccidioides brasiliensis triosephosphate isomerase suggests the potential for adhesin function," *FEMS Yeast Research*, vol. 7, no. 8, pp. 1381–1388, 2007.

[94] M. C. Lorenz and G. R. Fink, "The glyoxylate cycle is required for fungal virulence," *Nature*, vol. 412, no. 6842, pp. 83–86, 2001.

[95] M. S. Felipe, R. V. Andrade, F. B. Arraes et al., "Transcriptional profiles of the human pathogenic fungus Paracoccidioides brasiliensis in mycelium and yeast cells," *The Journal of Biological Chemistry*, vol. 280, no. 26, pp. 24706–24714, 2005.

[96] L. S. Derengowski, A. H. Tavares, S. Silva, L. S. Procopio, M. S. Felipe, and I. Silva-Pereira, "Upregulation of glyoxylate cycle genes upon Paracoccidioides brasiliensis internalization by murine macrophages and in vitro nutritional stress condition," *Medical Mycology*, vol. 46, no. 2, pp. 125–134, 2008.

[97] P. F. Zambuzzi-Carvalho, A. H. Cruz, L. K. Santos-Silva, A. M. Goes, C. M. Soares, and M. Pereira, "The malate synthase of Paracoccidioides brasiliensis Pb01 is required in the glyoxylate cycle and in the allantoin degradation pathway," *Medical Mycology*, vol. 47, no. 7, pp. 734–744, 2009.

[98] B. R. da Silva Neto, J. de Fatima da Silva, M. J. Mendes-Giannini, H. L. Lenzi, C. M. de Almeida Soares, and M. Pereira, "The malate synthase of Paracoccidioides brasiliensis is a linked surface protein that behaves as an anchorless adhesin," *BMC Microbiology*, vol. 9, no. 1, p. 272, 2009.

[99] A. H. Cruz, M. Brock, P. F. Zambuzzi-Carvalho et al., "Phosphorylation is the major mechanism regulating isocitrate lyase activity in Paracoccidioides brasiliensis yeast cells," *The FEBS Journal*, vol. 278, no. 13, pp. 2318–2332, 2011.

[100] R. S. Prado, R. J. Alves, C. M. Oliveira et al., "Inhibition of Paracoccidioides lutzii Pb01 isocitrate lyase by the natural compound argentilactone and its semi-synthetic derivatives," *PloS One*, vol. 9, no. 4, article e94832, 2014.

[101] A. Brito Wde, T. C. Rezende, A. F. Parente et al., "Identification, characterization and regulation studies of the aconitase of Paracoccidioides brasiliensis," *Fungal Biology*, vol. 115, no. 8, pp. 697–707, 2011.

[102] N. N. Sluchanko and N. B. Gusev, "Moonlighting chaperone-like activity of the universal regulatory 14-3-3 proteins," *The FEBS Journal*, 2016.

[103] J. F. da Silva, H. C. de Oliveira, C. M. Marcos et al., "Paracoccidoides brasiliensis 30 kDa adhesin: identification as a 14-3-3 protein, cloning and subcellular localization in infection models," *PloS One*, vol. 8, no. 4, article e62533, 2013.

[104] P. A. Assato, J. F. da Silva, H. C. de Oliveira et al., "Functional analysis of Paracoccidioides brasiliensis 14-3-3 adhesin expressed in *Saccharomyces cerevisiae*," *BMC Microbiology*, vol. 15, no. 1, p. 256, 2015.

[105] C. M. Marcos, F. Silva Jde, H. C. Oliveira et al., "Decreased expression of 14-3-3 in Paracoccidioides brasiliensis confirms its involvement in fungal pathogenesis," *Virulence*, vol. 7, no. 2, pp. 72–84, 2016.

[106] M. A. Vericimo, K. M. Franca, A. C. Arnholdt, and T. L. Kipnis, "Increased apoptosis during the early phase of experimental paracoccidioidomycosis as a phenotypic marker of resistance," *Microbes and Infection*, vol. 8, no. 12-13, pp. 2811–2820, 2006.

[107] F. Silva Jde, J. Vicentim, H. C. Oliveira et al., "Influence of the Paracoccidioides brasiliensis 14-3-3 and gp43 proteins on the induction of apoptosis in A549 epithelial cells," *Memórias do Instituto Oswaldo Cruz*, vol. 110, no. 4, pp. 476–484, 2015.

[108] R. J. Cordero and A. Casadevall, "Functions of fungal melanin beyond virulence," *Fungal Biology Reviews*, 2017, In press.

[109] C. Pacelli, R. A. Bryan, S. Onofri, L. Selbmann, I. Shuryak, and E. Dadachova, "Melanin is effective in protecting fast and slow growing fungi from various types of ionizing radiation," *Environmental Microbiology*, vol. 19, no. 4, pp. 1612–1624, 2017.

[110] J. D. Nosanchuk, R. E. Stark, and A. Casadevall, "Fungal melanin: what do we know about structure?" *Frontiers in Microbiology*, vol. 6, p. 1463, 2015.

[111] M. H. Wheeler and A. A. Bell, "Melanins and their importance in pathogenic fungi," in *Current Topics in Medical Mycology*, M. R. McGinnis, Ed., vol. 2, no. 10, pp. 338–387, Springer-Verlag, New York, NY, USA, 1988.

[112] B. L. Gomez and J. D. Nosanchuk, "Melanin and fungi," *Current Opinion in Infectious Diseases*, vol. 16, no. 2, pp. 91–96, 2003.

[113] J. D. Nosanchuk and A. Casadevall, "The contribution of melanin to microbial pathogenesis," *Cellular Microbiology*, vol. 5, no. 4, pp. 203–223, 2003.

[114] J. D. Nosanchuk and A. Casadevall, "Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 11, pp. 3519–3528, 2006.

[115] C. P. Taborda, M. B. da Silva, J. D. Nosanchuk, and L. R. Travassos, "Melanin as a virulence factor of Paracoccidioides brasiliensis and other dimorphic pathogenic fungi: a minireview," *Mycopathologia*, vol. 165, no. 4-5, pp. 331–339, 2008.

[116] B. L. Gomez, J. D. Nosanchuk, S. Diez et al., "Detection of melanin-like pigments in the dimorphic fungal pathogen Paracoccidioides brasiliensis in vitro and during infection," *Infection and Immunity*, vol. 69, no. 9, pp. 5760–5767, 2001.

[117] A. L. Rosas, J. D. Nosanchuk, B. L. Gomez, W. A. Edens, J. M. Henson, and A. Casadevall, "Isolation and serological analyses of fungal melanins," *Journal of Immunological Methods*, vol. 244, no. 1-2, pp. 69–80, 2000.

[118] C. A. Walker, B. L. Gomez, H. M. Mora-Montes et al., "Melanin externalization in *Candida albicans* depends on cell wall chitin structures," *Eukaryotic Cell*, vol. 9, no. 9, pp. 1329–1342, 2010.

[119] M. B. da Silva, A. F. Marques, J. D. Nosanchuk, A. Casadevall, L. R. Travassos, and C. P. Taborda, "Melanin in the dimorphic fungal pathogen Paracoccidioides brasiliensis: effects on phagocytosis, intracellular resistance and drug susceptibility," *Microbes and Infection*, vol. 8, no. 1, pp. 197–205, 2006.

[120] H. C. Eisenman, J. D. Nosanchuk, J. B. Webber, R. J. Emerson, T. A. Camesano, and A. Casadevall, "Microstructure of cell wall-associated melanin in the human pathogenic fungus *Cryptococcus neoformans*," *Biochemistry*, vol. 44, no. 10, pp. 3683–3693, 2005.

[121] H. C. Eisenman and A. Casadevall, "Synthesis and assembly of fungal melanin," *Applied Microbiology and Biotechnology*, vol. 93, no. 3, pp. 931–940, 2012.

[122] H. C. Eisenman, S. Frases, A. M. Nicola, M. L. Rodrigues, and A. Casadevall, "Vesicle-associated melanization in *Cryptococcus neoformans*," *Microbiology*, vol. 155, no. 12, pp. 3860–3867, 2009.

[123] N. Fang, V. Chan, H. Q. Mao, and K. W. Leong, "Interactions of phospholipid bilayer with chitosan: effect of

molecular weight and pH," *Biomacromolecules*, vol. 2, no. 4, pp. 1161–1168, 2001.

[124] A. J. Franzen, M. M. Cunha, E. J. Batista, S. H. Seabra, W. De Souza, and S. Rozental, "Effects of tricyclazole (5-methyl-1,2,4-triazol[3,4] benzothiazole), a specific DHN-melanin inhibitor, on the morphology of Fonsecaea pedrosoi conidia and sclerotic cells," *Microscopy Research and Technique*, vol. 69, no. 9, pp. 729–737, 2006.

[125] J. Zhong, S. Frases, H. Wang, A. Casadevall, and R. E. Stark, "Following fungal melanin biosynthesis with solid-state NMR: biopolymer molecular structures and possible connections to cell-wall polysaccharides," *Biochemistry*, vol. 47, no. 16, pp. 4701–4710, 2008.

[126] S. Chatterjee, R. Prados-Rosales, B. Itin, A. Casadevall, and R. E. Stark, "Solid-state NMR reveals the carbon-based molecular architecture of *Cryptococcus neoformans* fungal eumelanins in the cell wall," *The Journal of Biological Chemistry*, vol. 290, no. 22, pp. 13779–13790, 2015.

[127] M. E. Uran, J. D. Nosanchuk, A. Restrepo, A. J. Hamilton, B. L. Gomez, and L. E. Cano, "Detection of antibodies against Paracoccidioides brasiliensis melanin in in vitro and in vivo studies during infection," *Clinical and Vaccine Immunology*, vol. 18, no. 10, pp. 1680–1688, 2011.

[128] A. Casadevall and L. A. Pirofski, "Antibody-mediated protection through cross-reactivity introduces a fungal heresy into immunological dogma," *Infection and Immunity*, vol. 75, no. 11, pp. 5074–5078, 2007.

[129] A. L. Rosas, J. D. Nosanchuk, and A. Casadevall, "Passive immunization with melanin-binding monoclonal antibodies prolongs survival of mice with lethal *Cryptococcus neoformans* infection," *Infection and Immunity*, vol. 69, no. 5, pp. 3410–3412, 2001.

[130] D. S. Alviano, A. J. Franzen, L. R. Travassos et al., "Melanin from Fonsecaea pedrosoi induces production of human antifungal antibodies and enhances the antimicrobial efficacy of phagocytes," *Infection and Immunity*, vol. 72, no. 1, pp. 229–237, 2004.

[131] M. B. Silva, L. Thomaz, A. F. Marques et al., "Resistance of melanized yeast cells of Paracoccidioides brasiliensis to antimicrobial oxidants and inhibition of phagocytosis using carbohydrates and monoclonal antibody to CD18," *Memórias do Instituto Oswaldo Cruz*, vol. 104, no. 4, pp. 644–648, 2009.

[132] L. M. Baltazar, S. M. Werneck, B. M. Soares et al., "Melanin protects Paracoccidioides brasiliensis from the effects of antimicrobial photodynamic inhibition and antifungal drugs," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 7, pp. 4003–4011, 2015.

[133] Y. Wang and A. Casadevall, "Growth of *Cryptococcus neoformans* in presence of L-dopa decreases its susceptibility to amphotericin B," *Antimicrobial Agents and Chemotherapy*, vol. 38, no. 11, pp. 2648–2650, 1994.

[134] D. van Duin, A. Casadevall, and J. D. Nosanchuk, "Melanization of *Cryptococcus neoformans* and Histoplasma capsulatum reduces their susceptibilities to amphotericin B and caspofungin," *Antimicrobial Agents and Chemotherapy*, vol. 46, no. 11, pp. 3394–3400, 2002.

[135] M. L. Rodrigues, J. D. Nosanchuk, A. Schrank, M. H. Vainstein, A. Casadevall, and L. Nimrichter, "Vesicular transport systems in fungi," *Future Microbiology*, vol. 6, no. 11, pp. 1371–1381, 2011.

[136] D. L. Oliveira, J. Rizzo, L. S. Joffe, R. M. Godinho, and M. L. Rodrigues, "Where do they come from and where do they go: candidates for regulating extracellular vesicle formation in fungi," *International Journal of Molecular Sciences*, vol. 14, no. 5, pp. 9581–9603, 2013.

[137] M. L. Rodrigues, E. S. Nakayasu, I. C. Almeida, and L. Nimrichter, "The impact of proteomics on the understanding of functions and biogenesis of fungal extracellular vesicles," *Journal of Proteomics*, vol. 97, pp. 177–186, 2014.

[138] L. Brown, J. M. Wolf, R. Prados-Rosales, and A. Casadevall, "Through the wall: extracellular vesicles in gram-positive bacteria, mycobacteria and fungi," *Nature Reviews. Microbiology*, vol. 13, no. 10, pp. 620–630, 2015.

[139] L. Nimrichter, M. M. de Souza, M. Del Poeta et al., "Extracellular vesicle-associated transitory cell wall components and their impact on the interaction of fungi with host cells," *Frontiers in Microbiology*, vol. 7, p. 1034, 2016.

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

[141] M. L. Rodrigues, E. S. Nakayasu, D. L. Oliveira et al., "Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence," *Eukaryotic Cell*, vol. 7, no. 1, pp. 58–67, 2008.

[142] R. Peres da Silva, C. Heiss, I. Black et al., "Extracellular vesicles from Paracoccidioides pathogenic species transport polysaccharide and expose ligands for DC-SIGN receptors," *Scientific Reports*, vol. 5, p. 14213, 2015.

[143] T. A. da Silva, M. C. Roque-Barreira, A. Casadevall, and F. Almeida, "Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro," *Scientific Reports*, vol. 6, p. 35867, 2016.

[144] A. L. Bocca, E. E. Hayashi, A. G. Pinheiro et al., "Treatment of Paracoccidioides brasiliensis-infected mice with a nitric oxide inhibitor prevents the failure of cell-mediated immune response," *Journal of Immunology*, vol. 161, no. 6, pp. 3056–3063, 1998.

[145] A. Gonzalez, W. de Gregori, D. Velez, A. Restrepo, and L. E. Cano, "Nitric oxide participation in the fungicidal mechanism of gamma interferon-activated murine macrophages against Paracoccidioides brasiliensis conidia," *Infection and Immunity*, vol. 68, no. 5, pp. 2546–2552, 2000.

[146] B. Rui, T. Shen, H. Zhou et al., "A systematic investigation of *Escherichia coli* central carbon metabolism in response to superoxide stress," *BMC Systems Biology*, vol. 4, p. 122, 2010.

[147] A. H. Tavares, S. S. Silva, A. Dantas et al., "Early transcriptional response of Paracoccidioides brasiliensis upon internalization by murine macrophages," *Microbes and Infection*, vol. 9, no. 5, pp. 583–590, 2007.

[148] J. A. Parente-Rocha, A. F. Parente, L. C. Baeza et al., "Macrophage interaction with Paracoccidioides brasiliensis yeast cells modulates fungal metabolism and generates a response to oxidative stress," *PLoS One*, vol. 10, no. 9, article e0137619, 2015.

[149] G. D. de Arruda, A. M. Bailao, T. C. Vieira Rezende et al., "Response to oxidative stress in Paracoccidioides yeast cells as determined by proteomic analysis," *Microbes and Infection*, vol. 15, no. 5, pp. 347–364, 2013.

[150] A. F. Parente, P. E. Naves, L. L. Pigosso et al., "The response of Paracoccidioides spp. to nitrosative stress," *Microbes and Infection*, vol. 17, no. 8, pp. 575–585, 2015.

[151] O. H. Ruiz, A. Gonzalez, A. J. Almeida et al., "Alternative oxidase mediates pathogen resistance in Paracoccidioides brasiliensis infection," *PLoS Neglected Tropical Diseases*, vol. 5, no. 10, article e1353, 2011.

[152] O. Hernandez, P. Araque, D. Tamayo et al., "Alternative oxidase plays an important role in Paracoccidioides brasiliensis cellular homeostasis and morphological transition," *Medical Mycology*, vol. 53, no. 3, pp. 205–214, 2015.

[153] V. P. Martins, F. M. Soriani, T. Magnani et al., "Mitochondrial function in the yeast form of the pathogenic fungus Paracoccidioides brasiliensis," *Journal of Bioenergetics and Biomembranes*, vol. 40, no. 4, pp. 297–305, 2008.

[154] V. P. Martins, T. M. Dinamarco, F. M. Soriani et al., "Involvement of an alternative oxidase in oxidative stress and mycelium-to-yeast differentiation in Paracoccidioides brasiliensis," *Eukaryotic Cell*, vol. 10, no. 2, pp. 237–248, 2011.

[155] D. Tamayo, J. F. Munoz, A. Lopez et al., "Identification and analysis of the role of superoxide dismutases isoforms in the pathogenesis of Paracoccidioides spp.," *PLoS Neglected Tropical Diseases*, vol. 10, no. 3, article e0004481, 2016.

[156] D. Tamayo, J. F. Munoz, A. J. Almeida et al., "Paracoccidioides spp. catalases and their role in antioxidant defense against host defense responses," *Fungal Genetics and Biology*, vol. 100, pp. 22–32, 2017.

[157] A. F. Parente, A. M. Bailao, C. L. Borges et al., "Proteomic analysis reveals that iron availability alters the metabolic status of the pathogenic fungus Paracoccidioides brasiliensis," *PLoS One*, vol. 6, no. 7, article e22810, 2011.

[158] E. F. Bailao, J. A. Parente, L. L. Pigosso et al., "Hemoglobin uptake by Paracoccidioides spp. is receptor-mediated," *PLoS Neglected Tropical Diseases*, vol. 8, no. 5, article e2856, 2014.

[159] M. G. Silva-Bailao, E. F. Bailao, B. E. Lechner et al., "Hydroxamate production as a high affinity iron acquisition mechanism in Paracoccidioides spp.," *PLoS One*, vol. 9, no. 8, article e105805, 2014.

[160] A. F. Parente, T. C. de Rezende, K. P. de Castro et al., "A proteomic view of the response of Paracoccidioides yeast cells to zinc deprivation," *Fungal Biology*, vol. 117, no. 6, pp. 399–410, 2013.

[161] E. F. Bailao, S. Lima Pde, M. G. Silva-Bailao et al., "Paracoccidioides spp. ferrous and ferric iron assimilation pathways," *Frontiers in Microbiology*, vol. 6, p. 821, 2015.

[162] S. Lima Pde, D. Chung, A. M. Bailao, R. A. Cramer, and C. M. Soares, "Characterization of the Paracoccidioides hypoxia response reveals new insights into pathogenesis mechanisms of this important human pathogenic fungus," *PLoS Neglected Tropical Diseases*, vol. 9, no. 12, article e0004282, 2015.

[163] G. Visbal, A. Alvarez, B. Moreno, and G. San-Blas, "S-Adenosyl-L-methionine inhibitors delta(24)-sterol methyltransferase and delta(24(28))-sterol methylreductase as possible agents against Paracoccidioides brasiliensis," *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 9, pp. 2966–2970, 2003.

[164] G. Ramage, B. Coco, L. Sherry, J. Bagg, and D. F. Lappin, "In vitro *Candida albicans* biofilm induced proteinase activity and SAP8 expression correlates with in vivo denture stomatitis severity," *Mycopathologia*, vol. 174, no. 1, pp. 11–19, 2012.

[165] L. A. Dias-Melicio, R. K. Fernandes, D. R. Rodrigues, M. A. Golim, and A. M. Soares, "Interleukin-18 increases TLR4 and mannose receptor expression and modulates cytokine production in human monocytes," *Mediators of Inflammation*, vol. 2015, Article ID 236839, p. 9, 2015.

[166] R. Puccia, A. K. Carmona, J. L. Gesztesi, L. Juliano, and L. R. Travassos, "Exocellular proteolytic activity of Paracoccidioides brasiliensis: cleavage of components associated with the basement membrane," *Medical Mycology*, vol. 36, no. 5, pp. 345–348, 1998.

[167] R. Puccia, M. A. Juliano, L. Juliano, L. R. Travassos, and A. K. Carmona, "Detection of the basement membrane-degrading proteolytic activity of Paracoccidioides brasiliensis after SDS-PAGE using agarose overlays containing Abz-MKALTLQ-EDDnp," *Brazilian Journal of Medical and Biological Research*, vol. 32, no. 5, pp. 645–649, 1999.

[168] V. N. Brito, P. C. Souto, M. A. Cruz-Hofling, L. C. Ricci, and L. Verinaud, "Thymus invasion and atrophy induced by Paracoccidioides brasiliensis in BALB/c mice," *Medical Mycology*, vol. 41, no. 2, pp. 83–87, 2003.

[169] R. Di Gangi, T. Alves da Costa, R. Thome, G. Peron, E. Burger, and L. Verinaud, "Paracoccidioides brasiliensis infection promotes thymic disarrangement and premature egress of mature lymphocytes expressing prohibitive TCRs," *BMC Infectious Diseases*, vol. 16, p. 209, 2016.

[170] J. F. Munoz, R. A. Farrer, C. A. Desjardins et al., "Genome diversity, recombination, and virulence across the major lineages of Paracoccidioides," *mSphere*, vol. 1, no. 5, article e00213-16, 2016.

[171] L. L. Pigosso, A. F. Parente, A. S. Coelho et al., "Comparative proteomics in the genus Paracoccidioides," *Fungal Genetics and Biology*, vol. 60, pp. 87–100, 2013.

[172] A. Casadevall and L. A. Pirofski, "Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity," *Infection and Immunity*, vol. 67, no. 8, pp. 3703–3713, 1999.

[173] I. Torres, O. Hernandez, D. Tamayo et al., "Paracoccidioides brasiliensis PbP27 gene: knockdown procedures and functional characterization," *FEMS Yeast Research*, vol. 14, no. 2, pp. 270–280, 2014.

[174] J. F. Menino, M. Saraiva, J. Gomes-Rezende et al., "*P. brasiliensis* virulence is affected by SconC, the negative regulator of inorganic sulfur assimilation," *PloS One*, vol. 8, no. 9, article e74725, 2013.