Current Research in Microbial Sciences 7 (2024) 100246

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Common virulence factors between *Histoplasma* and *Paracoccidioides*: Recognition of Hsp60 and Enolase by CR3 and plasmin receptors in host cells

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ARTICLE INFO

Keywords:
Dimorphic fungi
Histoplasma capsulatum
Paracoccidioides spp.
Hsp60
Enolase
CR3 pathway
Plamin receptor
Innate immune response

ABSTRACT

Over the last two decades, the incidence of Invasive Fungal Infections (IFIs) globally has risen, posing a considerable challenge despite available antifungal therapies. Addressing this, the World Health Organization (WHO) prioritized research on specific fungi, notably *Histoplasma* spp. and *Paracoccidioides* spp. These dimorphic fungi have a mycelial life cycle in soil and a yeast phase associated with tissues of mammalian hosts. Inhalation of conidia and mycelial fragments initiates the infection, crucially transforming into the yeast form within the host, influenced by factors like temperature, host immunity, and hormonal status. Survival and multiplication within alveolar macrophages are crucial for disease progression, where innate immune responses play a pivotal role in overcoming physical barriers. The transition to pathogenic yeast, triggered by increased temperature, involves yeast phase-specific gene expression, closely linked to infection establishment and pathogenicity. Cell adhesion mechanisms during host-pathogen interactions are intricately linked to fungal virulence, which is critical for tissue colonization and disease development. Yeast replication within macrophages leads to their rupture, aiding pathogen dissemination. Immune cells, especially macrophages, dendritic cells, and neutrophils, are key players during infection control, with macrophages crucial for defense, tissue integrity, and pathogen elimination. Recognition of common virulence molecules such as heat- shock protein-60 (Hsp60) and enolase by pattern recognition receptors (PRRs), mainly via the complement receptor 3 (CR3) and plasmin receptor pathways, respectively, could be pivotal in host-pathogen interactions for *Histoplasma* spp. and *Paracoccidioides* spp., influencing adhesion, phagocytosis, and inflammatory regulation. This review provides a comprehensive overview of the dynamic of these two IFIs between host and pathogen. Further research into these fungi's virulence factors promises insights into pathogenic mechanisms, potentially guiding the development of effective treatment strategies.

1. Introduction

The incidence of Invasive Fungal Infections (IFIs) has witnessed a substantial surge globally over the past two decades, paralleled by a growing population at risk of developing these fungal infections (Bongomin et al., 2017; Limper et al., 2017; Pemán et al., 2020; Sifuentes-Osornio et al., 2012). Denning and Bromley (2015) estimated that around 1.2 billion individuals annually fall prey to fungal diseases, with 1.5 to 2 million cases succumbing to mortality, surpassing the death toll attributed to malaria and tuberculosis (Denning and Bromley, 2015).

Moreover, despite the current antifungal treatments available, fungal diseases continue to pose a significant public health challenge globally, resulting in an annual economic burden exceeding USD 11.5 billion in the United States alone in 2019 (Benedict et al., 2022). In this context, the dimorphic fungi *Histoplasma* spp. and *Paracoccidioides* spp. have recently been designated as fungi of high and medium priority, respectively, by the World Health Organization (WHO) to guide research efforts and policy interventions to strengthen the global response to fungal

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https://doi.org/10.1016/j.crmicr.2024.100246

Available online 8 June 2024

infections and antifungal resistance (WHO, 2023).

Dimorphic fungi, including genera such as *Histoplasma, Paracoccidioides, Blastomyces, Coccidioides, Sporothrix, Talaromyces*, and *Emergomyces* (Souza and Taborda, 2021), share the unique capability of undergoing morphological transformation in response to temperature changes when transitioning from the environment to the host (Gauthier, 2015). Furthermore, *Histoplasma* and *Paracoccidioides* spp. belongs to the Onygenales order within the Ajellomycetaceae family. This family includes other pathogenic dimorphic fungi, such as *Blastomyces, Emergomyces*, and *Emmonsia*, which share similar characteristics related to host-pathogen interactions (Muñoz et al., 2015).

Histoplasma spp. and *Paracoccidioides* spp. are facultative intracellular pathogens known to initiate respiratory infections in mammals, with the potential to progress to systemic and disseminated ones. The process of fungal acquisition begins when the host inhales aerosolized conidia and hyphae fragments, which subsequently transform into a pathogenic yeast form in response to an increase in temperature from approximately 22 to 37°C (Aristizabal et al., 1998; Edwards et al., 2013; Newman et al., 1990). The severity of the disease is contingent upon the host immune system's capacity to control the pathogen's spread, with innate immune responses playing a crucial role (Shen and Rappleye, 2020).

The fungal cell wall represents the initial point of interaction with the immune system and is one of the principal pathogen-associated molecular patterns (PAMPs) that can be recognized by their counterpart pattern recognition receptors (PRRs) on the host cells' surface. Notably, the fungal cell wall is crucial in safeguarding the fungus against exogenous stressors and constitutes one of the most significant virulence factors (Gow et al., 2017). Other common virulence factors shared by *Histoplasma* spp. and *P. brasiliensis* include α−1,3-glucan and β−1, 3-glucan cell wall components, as well as adhesins that are upregulated during infection and biofilm formation, such as the heat shock proteins (Hsp) and the moonlight proteins like 14–3–3, Gapdh, and enolase, among others (Valdez et al., 2022).

The ability of these fungi to form biofilms raises a health concern, as infections caused by biofilm-forming microorganisms are difficult to control, requiring high drug concentrations that can lead to various adverse effects, potentially worsening the patient's prognosis (Vertes et al., 2012). Biofilms are sessile communities of microorganisms in which cells adhere to a biotic or abiotic substrate and are surrounded by an extracellular matrix (ECM) composed of polymeric substances that differ from free-living (planktonic) cells in terms of growth, gene transcription, and protein translation (Costerton et al., 1995; Donlan and Costerton, 2002; Martinez and Casadevall, 2015). They may be associated with disease recurrence after medication withdrawal, as this ECM hinders drug diffusion and immune system activity (Ferreira and Borges, 2009), challenging the treatment of biofilm-related infections.

Extracellular polymeric substances also play a pivotal role in cell-cell cohesion, enabling cellular aggregation within biofilms by creating an initial adhesion region that promotes cell aggregation and subsequent colonization. The continuous production of polymeric substances facilitates the biofilm's three-dimensional (3D) expansion, creating a favorable microenvironment for cluster development into colonies (Karygianni et al., 2020).

Furthermore, cells derived from biofilms, known as "Persisters," are inherently much more resistant to antifungals and have been demonstrated in fungi such as *Saccharomyces cerevisiae* and *Candida albicans* (Bojsen et al., 2016; LaFleur et al., 2006; Wuyts et al., 2018). In the case of *Paracoccidioides*, it has been reported its capacity to form biofilms, either as a single species or in mixed interactions with other bacterial agents (Sardi et al., 2015; Medina-Alarcón et al., 2021; Oliveira et al., 2020). Although biofilms of *Histoplasma* spp. have not been reported in clinical practice, we believe they represent a resistance mechanism with multiple underexplored virulence factors during the pathogen-host interaction. Since it has been demonstrated that *Histoplasma* biofilms formed *in vitro* require higher doses of antifungals for elimination (Brilhante et al., 2023; Vaso et al., 2022).

This review will highlight the common molecules and activating signaling pathways shared by *Histoplasma* and *Paracoccidioides* during host-pathogen interactions.

2. Histoplasmosis and paracoccidioidomycosis epidemiology

Histoplasmosis accounts for a significant portion of IFIs and ranks as the fourth most common fungal disease in terms of annual cases, affecting approximately 600,000 individuals every year (Bongomin et al., 2017; Goughenour and Rappleye, 2017). Globally, *Histoplasma* spp. is the most common species affecting humans among the dimorphic fungi. This mycosis is caused by the species of the thermal dimorphic fungus *Histoplasma* spp., among which are *H. capsulatum sensu stricto, H. mississippiense, H. ohiense, H. suramericanum*, and *H. africanum* (Sepúlveda et al., 2017). Histoplasmosis has been reported in both endemic and non-endemic areas and is a significant disease in the Americas, with notable occurrences in Mexico, Panama, Colombia, Guatemala, Honduras, Brazil, Argentina, and the United States (Azar et al., 2020; Bahr et al., 2016; Kasuga et al., 2003; Sifuentes-Osornio et al., 2012; M. de M. Teixeira et al., 2016). In Brazil, histoplasmosis outbreaks have been documented in various regions, affecting 19 out of 26 Brazilian states (Almeida et al., 2019). More recently, Moreira et al. (2022) reported the presence of *Histoplasma* spp. in Antarctica for the first time (Moreira et al., 2022).

Paracoccidioidomycosis (PCM) is geographically localized in the Americas, affecting over 4000 people annually, although these numbers do not accurately reflect the reality due to the lack of compulsory disease reporting in most countries (Bongomin et al., 2017). It is considered an endemic disease in Latin America, with the highest cases reported in Brazil, Argentina, Venezuela, and Peru. The significant genetic variability found among strains isolated from each country has led to the classification of different monophyletic species: *P. brasiliensis* (isolated in Argentina, Antarctica, Brazil, Peru, and Venezuela), *P. lutzii* (Brazil and Ecuador), *P. americana* (Brazil and Venezuela), *P. restrepiensis* (Colombia), and *P. venezuelensis* (Venezuela) (Matute et al., 2006; M. M. Teixeira et al., 2014; Turissini et al., 2017).

Paracoccidioides spp. is considered a primary pathogen due to their ability to cause disease in immunocompetent individuals, unlike opportunistic fungal pathogens that require host immune system deficiencies to establish infections, as seen in immunocompromised individuals (Garfoot and Rappleye, 2016; Teixeira et al., 2014). In the case of *Histoplasma* spp., this pathogen could act as a primary or an opportunistic agent; thus, this fungus affects mainly immunocompromised hosts, especially HIV-infected patients with CD4 T cells less than 150 per μL, or could affect individuals who have inhaled a high burden load of the infectious particles independent of their immune status. Because of these capabilities, the diseases exhibit various clinical manifestations depending on the previous host immune system and virulence strain, varying from asymptomatic to symptomatic clinical manifestations (Wheat et al., 2016).

3. Histoplasmosis and paracoccidioidomycosis pathogenesis

In their natural habitat, *Histoplasma* spp. and *Paracoccidioides* spp. predominantly exist in their mycelial form, thriving in nitrogen-rich soils and environments rich in animal excrement, respectively. These fungi thrive within a temperature range of 25 to 28 °C and are characterized by the presence of macro and microconidia for *Histoplasma* spp. and conidia for *Paracoccidioides* spp., as well as septate hyphae. However, during infection, these fungi are typically acquired through the inhalation of conidia or mycelial fragments. Following inhalation, a crucial transformation occurs as the fungal transition into the yeast form. This transition is of utmost importance for the survival of the fungi within the host. It is facilitated by a series of environmental adaptations that are influenced by factors such as temperature, the host's immune

system defenses, and hormonal influences in the case of *Paracoccidioides* spp. (Cleare et al., 2017; Hahn et al., 2022; Loose et al., 1983; Shankar et al., 2011; Wheat et al., 2016).

During their journey from the upper airways to the lungs, the mycelial forms of these fungi experience a temperature rise, reaching 37 °C, which triggers their transformation into yeast cells. Additionally, these fungi encounter and must overcome various physical barriers along their path, including nasal and pharyngeal mucus, mucociliary clearance, and initial host defense mechanisms. Within the alveoli, conidia morphotype or transformed yeast cells confront the initial host defense mechanisms that significantly impact their ability to survive and establish the infection (Elansari et al., 2016; Filler and Sheppard, 2006; Rizzi et al., 2006). Moreover, these yeast cells are engulfed and phagocytosed by resident alveolar macrophages. The progression of the disease hinges on the yeast cells' ability to survive and multiply within these phagocytic cells, making the host-pathogen interaction a highly intricate phenomenon and a critical determinant of the disease's course. In this context, the innate immune response plays a pivotal role in influencing disease progression (Cleare et al., 2017; Cohen et al., 2022; Guimarães, de Cerqueira, et al., 2011; Hahn et al., 2022; Loose et al., 1983; Mittal et al., 2019; N. de S. Pitangui et al., 2021; Shankar et al., 2011).

The thermally dimorphism is a process that requires the expression of yeast phase-specific genes. This process is intricately linked to establishing infection and developing pathogenicity, as isolates incapable of undergoing morphological transitions exhibit reduced virulence (Maresca and Kobayashi, 2000; Nemecek et al., 2006).

Furthermore, cell adhesion is another virulence mechanism implied during host-pathogen interactions in both fungi (Cleare et al., 2017). Adherence to host cells is the first step in tissue colonization, as replication of the yeast form within macrophages leads to macrophage rupture, facilitating pathogen release and dissemination (Antonello et al., 2011; Cohen et al., 2022; Deepe and Gibbons, 2009; Isaac et al., 2015; Kroetz and Deepe, 2012).

Macrophages are the primary immune cells involved in the defense of *Histoplasma* spp. and *Paracoccidioides* spp. infection, but the fungi can also be detected in dendritic cells (DCs) and neutrophils within 1 to 7 days after exposure (Deepe et al., 2008; Giusiano, 2021; Gonzalez and Hernandez, 2016). The phagocytosis of pathogenic fungi by these immune cells is a critical component of the host's defense mechanism. Macrophages, in particular, are noteworthy due to their diverse phenotypes and functions. They contribute to tissue integrity by presenting antigens during the innate immune response, phagocytosing and eliminating pathogens and apoptotic neutrophils, and playing a crucial role in resolving inflammation and tissue healing in pathological processes (Erwig and Gow, 2016).

If these fungi can overcome the physical barriers, they will be phagocytosed in the alveoli. The physical barrier is provided by tight and adherent junctions, desmosomes, and gap junctions among cells in the airway epithelium, which also express a variety of PRRs during infection that recognize different PAMPs and damage-associated molecular patterns (DAMPs). These transmembrane receptors are primarily categorized into C-type lectin receptors (CLRs), toll-like receptors (TLRs), Complement Receptors (CRs), NOD-like receptors (NLRs), and cytoplasmic proteins known as retinoic acid-inducible gene-I-like receptors (RLRs) (Invernizzi et al., 2020; Medzhitov, 2007).

Once *Histoplasma* spp. and *Paracoccidioides* spp. enter the host cell, and it must withstand an inhospitable intracellular environment. Within the phagocytic vacuole, they must endure challenges such as ROS, nitric oxide (NO), phagosomal acidification, and lysosomal fusion, all while acquiring essential nutrients for its survival (Chaves et al., 2021; Garfoot and Rappleye, 2016).

In addition, upon macrophage activation by IFN-γ, secreted by CD4+ *T* cells during the adaptive immune response, macrophages can increase their microbicidal activity, aiding in the fungi elimination by inhibiting intracellular replication (Brummer et al., 1991). As a survival mechanism, fungi can use differential gene expression within macrophages and exploit them to spread into the bloodstream, reaching other organs and tissues, including the potential to cross the blood-brain barrier (De Oliveira et al., 2021; Leopold Wager et al., 2016).

4. Virulence factors of *Histoplasma* spp

The transition from the mycelial to the yeast phase is a critical step in *Histoplasma* spp. infection and disease development, a process that involves changes in glucan composition from β-(1,3)-glucan to α-(1,3)-glucan. Regarding *Histoplasma* spp., this process begins with host-pathogen contact and can take hours (between 24 and 96 h) to complete, during which the fungus expresses various genes, including DRK1 (dimorphism regulating kinase), which is associated with CBP1 (calcium-binding protein) and AGS1 (α-(1,3)-glucan synthase) genes. When DRK1 is silenced, CBP1 and AGS1 are suppressed, leading to a compromised transition. It has also been demonstrated that sulfhydryl-blocking agents can inhibit the transition from mycelial to yeast phase, preventing infection (Nemecek et al., 2006; Rappleye et al., 2004). In addition to AGS1, other genes are involved in the synthesis of α-(1,3)-glucan, such as AMY1, which is responsible for translating the protein α-(1,4)-amylase, and UGP1, which is responsible for generating UTP-glucose-1-phosphate uridyltransferase that produces UDP-glucose monomers. Together, these genes are involved in forming α-(1,4) and α-(1,6) linked glucans, and their silencing may lead to a reduction in α-(1,3)-glucan synthesis (Marion et al., 2006).

Besides the thermal dimorphism and changes in glucan synthesis, several genes are implicated in other virulence mechanisms of *Histoplasma*; these fungal genes include the CBP1, previously described and which is involved in additional mechanisms different from dimorphism, and Yeast Phase-Specific (YPS3). The CBP1 gene serves as a virulence factor that is secreted by fungal cells during the intracellular growth of the yeast morphotype within macrophages; this protein can bind calcium, an essential micronutrient vital for the survival of *Histoplasma* spp. inside the phagosome. Cbp1 has been shown to localize within the macrophage cytosol during *Histoplasma* spp. infection. On the other hand, the Yps3 protein is detected both as a component of the fungal cell wall and as a secreted molecule (Bohse and Woods, 2005; Isaac et al., 2015).

Recent findings have revealed that Cbp1 forms a complex with Yps-3, which in turn targets the cytosol to trigger host cell lysis (Azimova et al., 2022). The cell lysis process is initiated when the Cbp1/Yps-3 complex activates the Integrated Stress Response (ISR), culminating in the activation of the caspase-1 pathway. This activation ultimately leads to the maturation of IL-1β and IL-18. Within *Histoplasma* spp. cells, caspase-1 plays a regulatory role in fungal proliferation and triggers the activation of ISR tolerance genes (Azimova et al., 2022; Bertheloot et al., 2021; Place et al., 2022). Consequently, this process is likely related to macrophage pyroptosis during the cellular stress response.

The ISR pathway plays a crucial role in regulating cellular homeostasis and promoting cell survival in response to various forms of stress through apoptosis and pyroptosis processes. It can also trigger cell death during infections caused by pathogens (Place et al., 2022). This pathway is activated during macrophage infection by planktonic cells of *Histoplasma* spp., facilitated by Cbp1. It involves an increase in eIF2α phosphorylation, the induction of the C/EBP Homologous Protein (CHOP) transcription factor, and the presence of the pseudokinase Tribbles 3 (TRIB3), both were demonstrated as necessary for macrophage death during *in vitro* infection (English et al., 2017).

Recently, our research group has been observed that during infection of human macrophages-like THP-I cell line with *Histoplasma* spp., a significant increase in the expression of the Putative Thiol-Specific Antioxidant Protein (Tsa1) (manuscript under preparation); this protein is among the most encountered thiol-specific proteins in yeast and serves multiple functions in stress protection, including safeguarding against oxidative stress (Weids and Grant, 2014).

Despite the current understanding of *Histoplasma*'s ability to tolerate oxidative stress during infection, further research is required to fully elucidate the mechanisms by which the fungus thrives within the intracellular environment. So far, it has been established that catalases (CatB and CatP) and extracellular superoxide dismutase (Sod3) play a significant role in the antioxidant system of *Histoplasma* spp. (Holbrook et al., 2013; Johnson et al., 2002). CatB, also known as Antigen M, is a glycoprotein with a molecular mass of approximately 90 kDa; this glycoprotein possesses species-specific protein as well as glycosidic epitopes, which facilitate species identification and interaction with human antibodies. Conversely, CatP has a molecular mass of approximately 57 kDa and is characterized as a monofunctional peroxisomal catalase, akin to a previously described CatP in *P. brasiliensis*. Furthermore, the extracellular Sod3 plays a pivotal role in protecting the fungi against particles generated by immune cells (Holbrook et al., 2013; Johnson et al., 2002; Mihu and Nosanchuk, 2012; Place et al., 2022; Weids and Grant, 2014). These antioxidant proteins are considered virulence factors within *Histoplasma*'s defense system.

Histoplasma's ability to form biofilms *in vitro*, first demonstrated by (Pitangui et al., 2012), has revolutionized the concepts of virulence and raised several questions regarding using these structures during infection. In this regard, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) is described as a glycolytic pathway protein and is detected on the fungal cell surface (Barbosa et al., 2006). In a study conducted by our group, transcriptomic analysis showed that the GAPDH gene had a fold change of 482.3 for biofilms formed by the EH-315 strain of *Histoplasma* spp., indicating a higher expression than in the planktonic form (manuscript under preparation).Subsequently, Fregonezi and colleagues demonstrated that Gapdh acts as an adhesin in *Histoplasma* biofilms. Notably, blocking Gapdh led to reduced biofilm formation and decreased its robustness and biomass (Fregonezi et al., 2020). Besides these described genes in *Histoplasma* spp., recent publications have shown that biofilm formation could represent an important virulence mechanism in *Histoplasma* spp.(Gonçalves et al., 2020; Pitangui et al., 2016, 2021; Pitangui et al., 2012).

Pioneer studies of our group confirm *Histoplasma*'s ability to form biofilms *in vitro*, and comparative proteomic and transcriptomic analysis between planktonic and derived-biofilm yeasts has been done. This research revealed a differential expression in biofilms, with various upregulated transcripts related to cell wall-associated hydrolase, membrane proteins, histidine decarboxylase, recombinases, transcriptional regulation family related to *Histoplasma* spp. morphology, signal peptides, and GAPDH, among others (manuscript under preparation). These findings raised several questions regarding the differences in the pathogen-host interaction between yeast and biofilms during human macrophage infection (THP-1 macrophage-like). The differential expression of various miRNAs was observed in biofilm infection. Interactome analysis showed that the most affected metabolic pathways in macrophages were related to the regulation of fungus-host cell adhesion (receptor-ECM adhesion, focal adhesion), polysaccharide biosynthesis (glycosaminoglycans and N-glycans), regulation of cell structure and motility (actin cytoskeleton), protein degradation (ubiquitin-mediated proteolysis), response to proinflammatory stimuli (MAPK signaling), regulation of cellular homeostasis (apoptosis and gap junctions), amino acid metabolism (lysine degradation), and functional cellular effects of proliferation, differentiation, and cell migration (Pitangui et al., 2021). The dysregulation of several metabolic pathways in host cells due to *Histoplasma* spp. biofilm infection demonstrates that the fungus modulates miRNAs, affecting host cell functions and rendering macrophages more susceptible to biofilm infection.

In counterpart, the participation of genes associated with the protection of macrophages against infection has been reported, with their functions encompassing various cellular processes. These genes include those associated with the regulator complex, which plays a role in nutrient stress sensing, glycosylation enzymes, protein degradation machinery, mitochondrial respiration genes, solute transporters, and the endoplasmic reticulum membrane complex, which aids in the folding of transmembrane proteins with multiple membrane-spanning regions. The most effective protective group of genes, consisting of Gnb2, Pdcl, AP-1 subunits, AP-2 subunits, and Arrb2, primarily regulate G-protein coupled receptor (GPCR) signaling and receptor trafficking upon GPCR engagement. One of these genes encodes the GPCR C3a receptor 1 (C3ar1/C3aR), which is essential in the eukaryotic endocytosis process (Cohen et al., 2022; Irannejad and von Zastrow, 2014).

Heat-shock proteins play a pivotal role in conferring tolerance to high temperatures during the infective process, and they are categorized as 60 kDa, 70 kDa, 90 kDa (chaperones), and 100 kDa (catalytic activity) families (Cleare et al., 2017). For *Histoplasma* spp., the Hsp family was initially described as exhibiting increased expression during the transition from mycelial to yeast morphotype (Shearer et al., 1987). Presently, HSP60 stands as the most extensively studied member within the Hsp family, showing a correlation with heightened expression during the infective process. This study renders the protein crucial for pathogenesis and heat stress tolerance (Guimarães, Nakayasu, et al., 2011). Recent investigations have elucidated the role of HSP60 in biofilm formation. Blocking its activity with a monoclonal antibody (mAb 7B6) reduced *Histoplasma*'s biofilm metabolic activity and biomass *in vitro*. Moreover, this intervention increased the survival of *Galleria mellonella* larvae during infection (Fregonezi et al., 2020). Hsp60 binding to Complement Receptor 3 (CR3) (Cohen et al., 2022; Habich et al., 2006), an interaction that will be further discussed.

The literature lacks sufficient data on other proteins crucial for *Histoplasma* spp. pathogenesis, such as 14–3–3 and enolase. However, it is known that during histoplasmosis, the marker CD42b/GP1b expressed on the fungal organism's surface serves as a 14–3–3 ligand for other microorganisms. The heightened expression of this marker could potentially serve as a disease indicator (Ku et al., 2018). For enolase, proteomic analysis during infection has demonstrated increased expression in *Histoplasma* spp. yeast, providing insights into its significance during pathogenesis (Holbrook et al., 2011). Further research demonstrating the importance of 14–3–3 and enolase for *Histoplasma* spp. pathogenesis is still necessary.

Recent studies have identified Ryp transcription factors (Ryp1, Ryp2, Ryp3, and Ryp4) as essential for *Histoplasma* dimorphism. These factors are crucial for the yeast-phase transition, as mutants lacking any one of the Ryp family members are unable to transition to the yeast phase at 37 °C. Ryp4, a zinc-associated transcription factor, has been shown to be a target of the other Ryp factors. This interaction forms a regulatory network that sustains a positive feedback loop at 37 °C, thereby promoting yeast-phase growth (Nguyen and Sil, 2008; Sil, 2019; Webster and Sil, 2008).

On the other hand, the APSES protein family, known for its role as transcription factors involved in gene expression during fungal development, has homologs in Ascomycetes, including Mbp1, Stu1, Swi6, and Xbp1. Their significance during *Histoplasma* infection and other crucial cellular mechanisms has not been demonstrated, consequently no correlation with virulence has been established. Nonetheless, they represent a highly conserved protein family within fungal species, warranting further investigation to elucidate their contributions to *Histoplasma* biology (Longo et al., 2018).

Finally, the cAMP signaling pathway is well-documented for its influence on *Histoplasma* dimorphism, being crucial for the yeast-phase transition (Medoff et al., 1981). cAMP is involved in the regulation of the delta9-desaturase gene, which is expressed during both the mycelial and yeast phases. Studies have identified a stress-responsive *cis* element (STRE) that is responsive to the cAMP and have demonstrated that this element is active in *Histoplasma* (Medoff et al., 1981).

5. Virulence factors of *Paracoccidioides* spp

Regarding *Paracoccidioides* spp. virulence factors, several genes, and proteins that play a crucial role in the yeast transition of this fungal

pathogen have been reported; among these are also temperature tolerance genes, which are essential for the dimorphic switch. During the mycelial phase of *Paracoccidioides* spp., the cell wall comprises β−1,3-glucan, β−1,6-glucan, and chitin. The transition to the yeast phase, characterized by a prevalence of α−1,3-glucan, necessitates changes in gene expression, triggering morphological alterations (Felipe et al., 2005). The protein Drk1, which has also been identified in *Histoplasma* spp. (Nemecek et al., 2006) and *Blastomyces dermatitidis* (Lawry et al., 2017), is indispensable for dimorphism. DRK1 acts by negatively modulating the synthesis of chitin and β-glucans, thereby contributing to their masking and promoting the pathogenicity of *P. brasiliensis* (Navarro et al., 2021).

The heat-shock proteins (Hsp) family is essential to dimorphic fungi (Cleare et al., 2017). Thus, the increase in Hsp70 mRNA level of transcripts and protein in *Paracoccidioides* spp. was also demonstrated as correlated to its dimorphism (Cleare et al., 2017; da Silva et al., 1999). The Hsp90 is notably involved in the dimorphic transformation of *P. brasiliensis*; this Hsp90 exhibits a significant overexpression in the presence of an oxidative environment. Moreover, this chaperone molecule can form a complex with calcineurin, thus contributing to the stabilization of this critical regulatory enzyme. Hsp90 competes with calmodulin for binding to the calcium/calmodulin docking site, indicating its involvement in calcium-dependent signaling pathways (Imai and Yahara, 2000; Matos et al., 2013). Nonetheless, these interactions appear to interfere with *Paracoccidioides* spp. cell differentiation, and intriguingly, when estradiol is present, the HSP family proteins show down-regulation. This observation suggests that hormones, particularly estradiol, exert a notable influence during the process of conferring tolerance to high temperatures (Nicola et al., 2008).

Moreover, studies have demonstrated the influence of estradiol on the dimorphism of *P. brasiliensis*, specifically in the inhibition of mycelium-to-yeast transition. However, the transition from yeast to mycelium is not affected by estradiol (Restrepo et al., 1984). The dimorphism of *Paracoccidioides* is influenced by the gene expression of Hsp90 and Hsp70 pathways in response to estradiol exposure. This modulation occurs through the downregulation of gene signaling related to dimorphism (Shankar et al., 2011).

For successful colonization of the host, *Paracoccidioides* spp. yeast must possess the ability to adhere to the host cell surface. This adhesive process involves recognizing various components within the host ECM proteins, including elastin, laminin, fibronectin, collagens, glycosaminoglycans, proteoglycans, and others. Numerous research studies have investigated the fungal interactions with these ECM components, revealing that the successful disease development and severity of the pathology varies depending on the specific ECM ligand involved. This aspect implies that the selection of an ECM ligand can indeed influence the outcome of the disease and its severity in *Paracoccidioides* spp. infections. (de Oliveira et al., 2015; Gonzalez et al., 2008; Mendes-Giannini et al., 2006).

In addition, other moonlighting proteins, such as Gapdh, 14–3–3, and enolase (Karkowska-Kuleta and Kozik, 2014), which participate in multiple cellular processes, have also been identified as being involved in the adhesion to host cells. Furthermore, proteins like gp43, the hydrolase PbHAD32, 1,6-bisphosphate aldolase (ALD), and malate synthase enzyme (PbMLS) from *Paracoccidioides* spp. also have been demonstrated to play a role in host cell adhesion and pathogenesis (de Oliveira et al., 2015).

The Gapdh protein in *Paracoccidioides* is notably overexpressed during the dimorphic transition and has been detected in both the cell wall and extracellular vesicles. It plays an essential role in cell adhesion since this protein has the capability to bind to host ECM components such as collagen, fibronectin, and laminin (Barbosa et al., 2006; Longo et al., 2014). Furthermore, the enolase protein can interact with plasminogen to promote invasion (Ghosh and Jacobs-Lorena, 2011).

Among the moonlighting proteins, our research group has characterized the 14–3–3 protein as a critical virulence factor. Silencing this protein has been demonstrated to enhance the survival of pneumocytes significantly. This finding strongly implies that the improved survival is a result of reduced interaction between the fungus and the host plasma membrane (Marcos et al., 2016, 2019); this reduction in interaction appears to be mediated by the 14–3–3 protein through modulation of the TLR signaling pathway (Jannuzzi et al., 2019; Marcos et al., 2016). Specifically, it binds to TLR2, TLR3, TLR4, TLR7/8, and TLR9, initiating the production of proinflammatory cytokines such as IL-6, TNFα, and IFN-β. Notably, the binding of 14–3–3 to TLR3 has recently been proposed as an evasion mechanism; thus, this binding dampens the proinflammatory response, inhibits nitric oxide (NO) production, and suppresses the activation of IFNγ/CD8/T cells and IL-17/CD8/T cells, along with cytotoxic functions, including the downregulation of granzyme B and perforin (Jannuzzi et al., 2019).

On the other hand, the capacity of *Paracoccidioides* spp. to form biofilms, either as a single species (Sardi et al., 2015) or in mixed interactions with other bacterial agents (Medina-Alarcón et al., 2021; L. T. Oliveira et al., 2020), presents a significant interest in the pathogenic mechanisms of this fungus (Cattana et al., 2017). The characterization of *P. brasiliensis* biofilms was initially documented by Sardi and colleagues (Sardi et al., 2015). This research also unveiled the overexpression of genes encoding GP43, enolase, GAPDH, and aspartyl proteinase in biofilms compared to planktonic cells. These results demonstrated the enhancement of adhesins as virulence factors in biofilms, constituting a virulence mechanism.

Further studies involving transcriptomic analysis of *Paracoccidioides* are necessary to identify differentially expressed genes in biofilms. This research can provide valuable insights into the molecular mechanisms underlying biofilm formation and the specific genes that play a crucial role in this process.

6. Host - *Histoplasma* and - *Paracoccidioides* interaction mechanisms: the role of C-type lectin receptors (CLRs)

PRRs detect the presence of microorganisms through transmembrane or intracytoplasmic domains found in various cell types (Shirjang et al., 2017). Among PRRs, CLRs constitute one of the best families of transmembrane PRRs studied so far. This family encompassing Dectin-1, Dectin-2, MINCLE, DC-SIGN, and Mannose receptor (MR), predominantly expressed in innate immune cells; these molecules are known as carbohydrate-binding receptors. Notably, Dectin-1 plays an important role in the specific recognition of β-glucans inherent to the fungal cell wall (Osorio and Reis e Sousa, 2011).

Due to its dimorphism, Dectin-1 receptors are not considered important in *Histoplasma* spp. and *Paracoccidioides* spp. recognition, specifically in their pathogenic morphotypes, the yeast cells (Heinsbroek et al., 2005). The changes in glucan biosynthesis that the fungi undergoes during the transition to the yeast phase results in a reduction in the production of β-(1,3)-glucan, which is responsible for leukocyte recruitment and the regulation of inflammatory mediators like leukotrienes and proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α). Simultaneously, there is an increase in the synthesis of α-(1,3)-glucan, which is crucial for pulmonary colonization (Marion et al., 2006).

On the other hand, MR is a transmembrane receptor involved in different processes, mainly pathogen recognition, cell migration, and homeostatic clearance (Gazi et al., 2011). Like Dectin-1, MR was shown to not interfere in *Histoplasma* spp. recognition, suggesting that this fungus lack interactions involving mannan-type PAMPs (Bullock and Wright, 1987; Garfoot and Rappleye, 2016). For *Paracoccidioides* spp., the MR (CD206) in host monocytes binds to the gp43 protein, subsequently initiating mitogen protein and nuclear factor kB (NFkB) activation. This cascade regulates the adaptive immune response by producing cytokines and chemokines (Nakaira-Takahagi et al., 2011). This protein also contains a mannose chain as a ligand, activating this host receptor during fungal adhesion. Notably, gp43 also functions as a

virulence factor since its binding to MR and TLR receptors triggers the inactivation of macrophage functions, including the suppression of reactive oxygen species (ROS) production (Flavia Popi et al., 2002).

7. Host - *Histoplasma* and -*Paracoccidioides* interaction mechanisms: the role of TLRs

TLRs are evolutionarily conserved membrane receptors with characteristics and structures extensively described, constituted as one of the most important protein families among PRRs, and are responsible for recognizing PAMPs through their leucine-rich domains (LRRs). This binding triggers signaling cascades that effectively eliminate microorganisms (Crespo-Lessmann et al., 2010; Mogensen, 2009).

In recent years, several authors have emphasized the significance of TLRs in recognizing fungal pathogens (Bourgeois and Kuchler, 2012; Le et al., 2023; Netea et al., 2004; Reddy et al., 2022). Thus, it has been elucidated the capacity of *Histoplasma*'s Yps3 protein to interact with TLR2 receptors, thereby inducing NF-kB activation (Aravalli et al., 2008). Additionally, recent research utilizing murine hematopoietic stem cells has revealed the internalization of *Histoplasma* spp. yeast via TLR2, TLR4, and Dectin-1 pathways, leading to heightened expression levels of IL-1β, IL-6, IL-10, IL-17, TNF-α, and TGF-β, as well as immune mediators such as Arg-1 and iNOS (Rodríguez-Echeverri et al., 2022).

Additional TLR receptors, notably TLR7 and TLR9, have been shown to play a critical role in the defense mechanism of CD103+ dendritic cells against *Histoplasma* spp. yeast. This defense mechanism operates through the production of type I interferon (IFN-I) and IFNγ, which are essential for the recruitment and activation of CD4+ *T* cells and defense against *Histoplasma* spp. (Van Prooyen et al., 2016).

Concerning *Paracoccidioides* spp., Loures et al. (2009) described the TLR2 activation process during *P. brasiliensis* infection, revealing that TLR2 interacts with *P. brasiliensis*, resulting in increased adhesion/ingestion of the fungus and subsequent activation of neutrophils, considered the first line of defense in the innate immune response. Similarly, other studies have reported an upregulation of TLR2 and TLR4 expression in macrophages against *Paracoccidioides* infection; this interaction is mediated by the adapter molecule MyD88, inducing the activation of these cells and an increase in the production of proinflammatory cytokines such as IL-6, IL-1β, and IL-8 (Burger, 2021).

On the other hand, the negative impact of *Paracoccidioides* interaction through the TLR3 receptor has also been demonstrated. T-CD8 lymphocytes exhibit improved cytotoxic activity in TLR3 knock-out mice compared to wild-type mice. Similarly, $TLR3^{-/-}$ macrophages display a more significant fungicidal effect and increased NO production than $TLR3^{+/+}$ control mice. Additionally, TLR9 has been shown to recognize the yeast form of *P. brasiliensis* and its DNA (Patin et al., 2019). Experimental immunization models in mice have indicated that $TLR9^{-/-}$ mice produce more antibodies, specifically IgG1 and IgG3, than normal mice; this finding underscores a protective effect mediated by recognizing the fungus through TLR9 (Morais et al., 2016; Patin et al., 2019).

Some studies have demonstrated TLRs' role in the interaction of specific adhesins like 14–3–3 in *P. brasiliensis*. Marcos et al. (2016) showed that the 14–3–3 protein present in *P. brasiliensis* plays an important role in the fungus's virulence, as it is associated with adherence to host cells and the subsequent establishment of the disease (Andreotti et al., 2005; Marcos et al., 2016). Additionally, other studies have demonstrated that the 14–3–3 protein plays a significant role in activating innate immunity via TLR4 signaling. It enhances the production of proinflammatory cytokines such as IL-6 and TNFα while negatively regulating TLR2-dependent activation and NFκβ activity. Furthermore, it modulates the production of IL-10 and the chemokine RANTES through signal transduction dependent on the TRIF/TRAM pathway (Schuster et al., 2011).

Finally, the 14–3–3 protein emerges as a key player in the pathogenesis of *P. brasiliensis*, exerting its influence through multiple mechanisms. Studies have elucidated its pivotal role in mediating the interaction of *P. brasiliensis* adhesins with host cells, thereby contributing to the establishment of infection, moreover, its involvement in innate immunity.

8. Host - *Histoplasma* and - *Paracoccidioides* interaction mechanisms: the role of CR3 and plasmin receptor

The CR3 is a heterodimer of transmembrane glycoproteins α (CD11b) and β (CD18); while the α chain is unique to CR3, the β subunit is shared by LFA1, CR4, and αdβ2, all belonging to the β2 integrin family of leukocyte receptors. CR3 is primarily expressed in macrophages, monocytes, granulocytes, and NK cells, playing a pivotal role in cell-cell and cell-matrix interactions. It enables leukocyte adhesion to the endothelium and facilitates the movement of leukocytes through endothelial intercellular junctions. CR3 is also involved in phagocytosis and cell death via oxidative burst and regulates inflammation homeostasis by mediating the apoptosis of extravasated neutrophils (Dunkelberger and Song, 2010; Sarma and Ward, 2011).

Dynamic regulation of CR3 function allows for rapid switching of receptor adhesion to some of its ligands. Such modifications in CR3 occur following the activation of other cell surface molecules like selectins, chemotactic receptors, and cytokine receptors. These receptors transmit signals into the cell, leading to conformational changes in CR3, converting it into an active adhesive form. CR3 can bind to specific ligands in its active form, initiating a cascade of outside-in signaling events. Recent data suggest that CR3 can also transmit signals from proteins linked to glycosylphosphatidylinositol (GPI), such as CD14, FcγRIIIB, and urokinase plasminogen activator receptor (uPAR). Also, GPI-anchored proteins, lacking a transmembrane domain, may capture the ligand while floating in the lipid bilayer of the membrane and transmit inflammatory signals through co-associated CR3 molecules (Dunkelberger and Song, 2010; Sarma and Ward, 2011).

The CR3 pathway is the main common point of host-pathogen interaction between *Histoplasma* spp. and *Paracoccidioides* spp. (Cohen et al., 2022; de Oliveira et al., 2021); and is known for low cytokine production response to infection (Aderem and Underhill, 1999).

The phagocytosis of *Histoplasma* spp. is not reliant on the recognition of β-glucans by Dectin-1. As a result, fungal recognition and phagocytosis primarily occur through β2-integrin receptors, with CR3 (CD11/CD18) being the most extensively studied. Additionally, LFA-1 (CD11a/CD18) and CR4 (CD11c/CD18) play significant roles during macrophage infection and invasion (Edwards et al., 2013).

For *Histoplasma* spp., the binding to the CR3 receptor occurs through Hsp present in the fungus cell wall, which acts as adhesins. The activation and production of 60 kDa (Hsp60) occur due to the increase in temperature. These surface molecules mediate the recognition of the microorganism by the host's immune system, directly affecting the macrophage phagocytosis mechanism, as they act as ligands for the CR3 receptor (CD11/CD18). Moreover, Hsp60 is an essential protein in the cellular adhesion process. The Hsp60/CR3 interaction leads to rapid phagocytosis/invasion without significant phagocyte activation, resulting in an ineffective immune response (Mihu and Nosanchuk, 2012). Blocking CR3 receptors does not directly impact *Histoplasma* spp. phagocytosis (Cohen et al., 2022; Long et al., 2003), suggesting that other interaction pathways may be involved in fungal phagocytosis.

Indeed, recent research has shed light on the significance of the Itgb2 (CD18) gene from macrophages as a crucial recognition mechanism for *Histoplasma* spp. This gene encodes the β-subunit of CR3, whereas the Fermt3 gene facilitates integrin activation (Cohen et al., 2022). Integrins, a family of heterodimeric transmembrane cell adhesion molecules, also serve as signaling receptors. They play pivotal roles in numerous biological processes, such as metazoan development, immunity, hemostasis, wound healing, and the regulation of cell survival, proliferation, and differentiation (Tan, 2012).

In *Paracoccidioides* spp., Jimenez and colleagues first highlighted the importance of activating the CR3 signaling pathway (Jiménez et al.,

2006). Currently, the complete understanding of the activation route in *Paracoccidioides* spp. remains incomplete. However, recent studies have shown that when *P. brasiliensis* is phagocytosed by macrophages with low levels of CD18, the fungus survives at a lower rate after 48 h of infection. This reduced survival is associated with lower levels of nitrate and the production of high levels of IgG1, resulting in an ineffective immune response. As a result, it has been suggested that β2 integrins play a significant role in the survival of *Paracoccidioides* spp. inside macrophages. Once phagocytosed, the macrophage may serve as a protective environment for *P. brasiliensis*, allowing the fungus to evade immune defenses and persist within the host (de Oliveira et al., 2021).

Conversely, the Plasmin Receptor is a ligand for plasminogen and is pivotal in the fibrinolytic system. However, it contributes to pathogenic processes during inflammation, cancer, and thrombosis (Godier and Hunt, 2013). In the context of fungal infections, proteomics analysis has revealed the overexpression of *Histoplasma*'s enolase during macrophage infection. This observation led the authors to hypothesize about the binding of enolase to the Plasmin Receptor, analogous to what has been observed in infections caused by *Pneumocystis* and *Leishmania* (Holbrook et al., 2011). However, further investigations are still necessary to confirm this hypothesis.

Nogueira et al. (2010) demonstrated the binding of enolase to the Plasmin Receptor by utilizing a mutant strain of *P. brasiliensis* expressing recombinant enolase. This binding facilitated the conversion of plasminogen into plasmin, consequently enhancing adherence to epithelial cells and increasing tissue invasion rates in mice. Similar results were observed in *Plasmodium*, where enolase binding to the Plasmin receptor increased pathogen invasion (Ghosh and Jacobs-Lorena, 2011).

While the recognition of Hsp60 in *Paracoccidioides* spp. by the CR3 receptor remains undetermined, it has been evidenced that the Hsp60 in *H. capsulatum* is indeed recognized by this receptor (Long et al., 2003). Given the considerable identity shared between both proteins, it could be hypothesized that a common receptor might recognize this shared ligand, thereby activating analogous pathways within host cells, as represented in Fig. 1. Similarly, enolase binding to the Plasmin Receptor in *P. brasiliensis* and *Cryptococcus* spp. has been documented (Nogueira et al., 2010b; Stie et al., 2009), albeit unconfirmed for *H. capsulatum*. Nevertheless, given the high similarities between the enolase proteins of *H. capsulatum* and *P. brasiliensis*, it is hypothesized that both fungi share mechanisms involving CR3 and Plasmin Receptor interactions as virulence factors and evasion strategies .

The binding of fungal Hsp60 to the CR3 receptor triggers activation of the actin cytoskeleton, fostering phagocytosis. Subsequently, within the phagolysosome, the macrophage induces the expression of IL-1β and IL-18 genes, stimulating autocrine activity, thereby amplifying macrophage activation to increase NO production. This response, mediated by CR3 activation, produces a dampened immune response. The heightened NO production within the phagolysosome inhibits lymphocyte activation, regarded as an evasion mechanism employed by these pathogens. Through a similar mechanism, enolase binding to the Plasmin Receptor facilitates rapid adhesion to the host cell, enabling fungal invasion (Fig. 1). This process prompts alterations in gene expression, the specific consequences of which remain unknown.

9. Conclusions and future directions

Identifying common pathways among dimorphic fungi during infection is crucial in developing new drug targets for treating these diseases. This study elucidated shared host-pathogen interaction mechanisms between *H. capsulatum* and *Paracoccidioides* spp., identifying enolase and Hsp60 as highly homologous proteins. These proteins bind to CR3 and Plasmin receptors, resulting in a dampened immune response, potentially serving as an evasion strategy employed by these fungi.

Future investigations are imperative to challenge this hypothesis and provide further evidence of common host-pathogen interaction mechanisms shared by dimorphic fungi. Such research endeavors will contribute to a deeper understanding of fungal pathogenesis and aid in developing novel therapeutic strategies targeting these shared mechanisms.

Funding

This research was funded by Fundação de Amparo à Pesquisa do

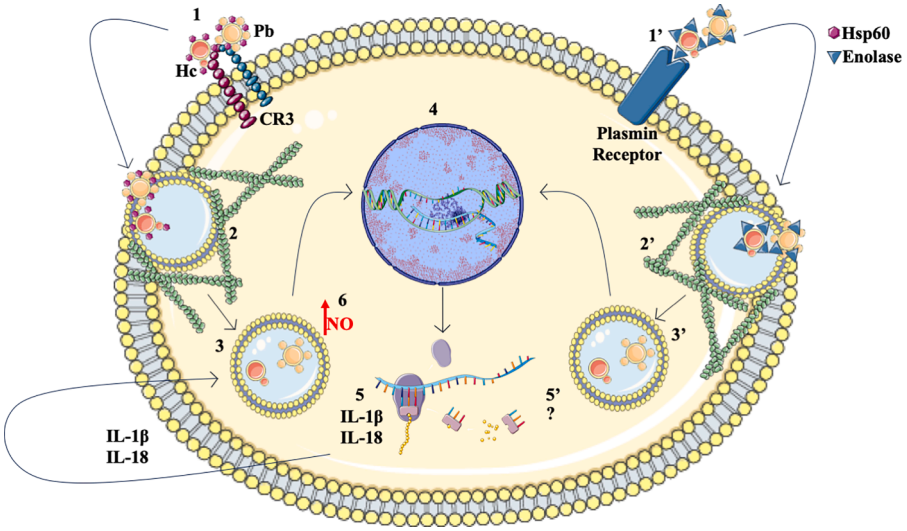

Fig. 1. Common host-pathogen interaction mechanisms of *Histoplasma* spp. and *P. brasiliensis*: Complement Receptor 3 (CR3) and Plasmin Receptor activation appear to share the same activation pathway. 1 to 6 represents the binding of Hsp60 to CR3, while 1′ to 5′ represents the binding of enolase to Plasmin Receptor. 1. Hsp60 binding to CR3 of the host cell; 2. Activation of the cytoskeleton and phagocytosis; 3. Fungi internalization into the phagolysosome; 4. Activation of transcription factor; 5. Production of pro-inflammatory cytokines IL-1β and IL-18 generation an autocrine response in the macrophage; 6. Increase of Nitric Oxid (NO) inside the phagolysosome triggering a dumped adaptative immune response. 1′. Enolase binding to Plasmin Receptor of the host cell; 2′. Activation of the cytoskeleton and phagocytosis; 3′. Fungi internalization into the phagolysosome; 4′. Activation of transcription factor; 5′. Unknow consequences.
Source: Own source. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

Estado de São Paulo (FAPESP) [grant 2022/15826-0 REGULAR]; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) [finance code 001, 88887.600612/2021-00 (SMS), 88887.839588/2023-00 (SMS), 88887.839360/2023-00 (CRE)].

Disclosure

The authors declare no conflicts of interest.

Authors' contributions and potential conflicts of interest

Samanta de Matos Silva, Carolina Rodriguez Echeverri, and Angel Gonzalez conceived the idea and wrote the first draft of the manuscript; Maria José Soares Mendes-Giannini, Ana Marisa Fusco-Almeida provided critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Samanta de Matos Silva reports financial support was provided by São Paulo State University. Carolina Rodriguez Echeverri reports financial support was provided by São Paulo State University. Ana Marisa Fusco Almeida reports financial support was provided by State of Sao Paulo Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

We are grateful to Julio Cesar Jaramillo Alzate and Julian Camilo Arango Rincon from the Universidad de Antioquia (UdeA) for their invaluable assistance with insights into immunology.

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