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***Histoplasma Capsulatum*: Mechanisms for Pathogenesis**

Jamie Mittal,

Department of Medicine (Infectious Diseases), Montefiore Medical Center, Bronx, NY, USA

Maria G. Ponce,

Department of Medicine (Infectious Diseases), Montefiore Medical Center, Bronx, NY, USA

Inessa Gendlina,

Department of Medicine (Infectious Diseases), Albert Einstein College of Medicine, Bronx, NY, USA

Joshua D. Nosanchuk

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA

Abstract

Histoplasmosis, caused by the dimorphic environmental fungus *Histoplasma capsulatum*, is a major mycosis on the global stage. Acquisition of the fungus by mammalian hosts can be clinically silent or it can lead to life-threatening systemic disease, which can occur in immunologically intact or deficient hosts, albeit severe disease is more likely in the setting of compromised cellular immunity. *H. capsulatum* yeast cells are highly adapted to the mammalian host as they can effectively survive within intracellular niches in select phagocytic cells. Understanding the biological response by both the host and *H. capsulatum* will facilitate improved approaches to prevent and/or modify disease. This review presents our current understanding of the major pathogenic mechanisms involved in histoplasmosis.

1 Introduction

Histoplasma capsulatum is an environmental dimorphic fungus (Wheat et al. 2007). Human infection occurs after the fungus (in the form of microconidia or hyphal fragments) is inhaled, travels through the respiratory system, and reaches the alveoli. In the alveoli, it transforms into a yeast form, a process that can occur both inside or outside of phagocytes (Deepe et al. 2008). *H. capsulatum* infections are primarily acquired, and there is no person-to-person transmission with the rare exception of organ transplantation (Lenhart et al. 2004). Once *Histoplasma* enters the host, it must evade immune-mediated and intracellular defenses, and find a favorable niche for growth and reproduction, which may include dissemination and the development of a state of latency within granulomas. Host phagocytes play a central role in the pathogenesis of histoplasmosis, as they are the vehicles for dissemination, spreading initially to the lymph nodes and later to multiple organs (Guimarães et al. 2006). The pathogen's ability to evade inflammatory responses and the

intensity of the host immune response determine the severity of symptoms and clinical presentation, and whether a state of latency develops with the potential for reactivation (Huffnagle and Noverr 2008; Casadevall and Pirofski 2003). The processes involved for this pathogen to survive and cause host damage are discussed here, and they highlight the complexity of the interactions between the host and pathogen. An understanding of pathogenesis provides insight into the clinical manifestations, reasons for reactivation, and future therapeutic targets.

2 History

Histoplasma was first described in 1906 by Dr. Samuel Taylor Darling. His first report was on the autopsy findings of a 27-year-old carpenter from Martinique who was working in the Panama Canal (Darling 1906). He likened his findings to what had been observed in protozoan infections. Dr. Darling coined the name *H. capsulatum* when he saw the invasion of histocyte-like cells with these encapsulated organisms. Histoplasmosis was later referred to as Darling's Disease. Subsequently, in 1912, the correct description of the organism was determined by pathologist Henrique da Rocha-Lima who recognized it as a fungus (Baum and Schwarz 1957). Since then, *Histoplasma* taxonomically has been divided into three groups based on geographic distribution and clinical manifestations: var. *capsulatum* which is the most common worldwide, var. *duboisii* found in Africa, and var. *farciminosum* known to be a horse pathogen. To refine phylogenetic classifications, Kasuga et al. performed phylogenetic analyses on 137 individual isolates representing the three original classifications of *Histoplasma*. Using DNA sequence variation, eight clades were genetically identified with seven of them representing isolated groups. The clades are called: North American class 1, North American class 2, Latin American group A, Latin American group B, Australian, Netherlands, Eurasian, and the African clade. Overall, seven of these clades represent isolated groups except for the Eurasian clade which originated from the Latin American group A clade. Isolates classified in one of the three original categories were intermixed and were found to be present in multiple phylogenetic clades thus making the original nomenclature obsolete (Kasuga et al. 2003). Kasuga's clade classification is used today to identify *Histoplasma* fungus; however, new information on cryptic speciation may lead to further refinements of *H. capsulatum* classifications (Sepúlveda et al. 2017).

3 Epidemiology

As evident from the clade grouping, *H. capsulatum* is distributed throughout the world, including Asia, Africa, Australia, North, Central, and South America (Chakrabarti and Slavin 2011; Loulergue et al. 2007; McLeod et al. 2011; Colombo et al. 2011). In North America, a higher incidence has been reported in the Ohio and Mississippi River Valleys via identification of a high frequency of positive skin testing (Manos et al. 1956). In South America, there is a predominance of disease in Brazil, Ecuador, Venezuela, Paraguay, Uruguay, and Argentina (Guimarães et al. 2006). A possible cause for this difference in overall distribution may be associated with features of the soil in these areas and climate differences (Wheat et al. 2007). Histoplasmosis is the most prevalent endemic fungal infection in the US. It is estimated that 50 million people have latent infection, and 500,000 new infections estimated annually (Nosanchuk and Gacser 2008; Retallack and Woods 1999;

Baddley et al. 2011). Despite wide distribution, there is still a significant underdiagnosis and hence underreporting of this disease in the US. This was evident in a multistate epidemiological surveillance analysis of histoplasmosis performed in US from 2011–2014 (Armstrong et al. 2018). It was noted that over a 3-year period, only 3409 histoplasmosis patients were diagnosed in 12 states including: Alabama, Arkansas, Delaware, Illinois, Indiana, Kentucky, Michigan, Minnesota, Mississippi, Nebraska, Pennsylvania, and Wisconsin. Of those identified infected individuals, most patients were asymptomatic; although, patients who presented with symptoms had a significant risk of mortality as 7% of hospitalized patients with histoplasmosis died (Armstrong et al. 2018). Given its prevalence and potential for causing severe disease, further study and a deeper understanding of pathogenesis are crucial in development and implementation of diagnostic and treatment strategies, with goals of improving patient outcomes and increasing disease awareness.

4 Clinical Manifestations

There is a broad range of presentations for histoplasmosis. Disease signs and symptoms are mediated by the host immune status with immunosuppressed patients being at higher risk for more severe disease. Other factors that play a role in the severity of the disease include the virulence of the fungal strain and the amount of inhaled inoculum (Knox and Hage 2010). Most often individuals will be asymptomatic after the acquisition of the fungus, but 1% will present with symptoms associated with the infection (Kauffman 2007). Symptomatic clinical presentations include acute pulmonary disease, disseminated disease, and chronic pulmonary histoplasmosis. Additional forms of disease can occur (Wheat et al. 2016).

4.1 Acute Histoplasmosis

Symptomatic patients usually present within one to 3 weeks from exposure. They can progress to develop fever, chills, dry cough, chest pain, myalgias, and headaches, often described as a flu-like syndrome. There are no specific imaging findings, but often mediastinal lymphadenopathy and infiltrates can be seen (Nadel et al. 2005). Acute disease can manifest as pneumonia, less frequently as pericarditis or with rheumatologic syndromes including arthritis, arthralgias and erythema nodosum, and mediastinal fibrosis particularly in patients with HLA-A2 in whom fibrosis is associated with abnormal host inflammatory response (Kataria et al. 1981; Wheat et al. 1983; Rosenthal et al. 1983; Davis et al. 2001; Peebles et al. 2000). Other possible manifestations of the acute histoplasmosis are broncholithiasis and pulmonary nodules, occasionally leading to compression syndromes due to their size (Arrigoni et al. 1971; Goodwin and Snell 1969). Nodules can also represent the residual changes of acute disease, and can be seen in radiographs as single or multiple calcified and noncalcified lesions, often described as “coin” lesions that can mimic and cause concern for malignancy (Galetta et al. 2007).

4.2 Disseminated Histoplasmosis

Disseminated disease most often occurs in individuals exposed to *H. capsulatum* who are immunosuppressed, but a large inoculum infection can result in severe disease in ~29% of individuals without immunological disorders (Larrabee et al. 1978). The risk for dissemination is particularly increased in patients with HIV/AIDs, particularly with a CD4

count less than 200 cells per μL , but chronic steroid use and immunosuppression secondary to malignancy are additional risk factors for disease (Kauffman 2007; Knox and Hage 2010; Histoplasmosis Risk & Prevention; Nacher et al. 2014). Symptoms of disseminated disease include fever, weight loss, and respiratory complaints. Clinical findings of lymphadenopathy, hepatomegaly, and splenomegaly, together with bone marrow toxicity on laboratory evaluation are associated with disseminated disease. Skin and mucosal lesions can also manifest. Disseminated disease can involve multiple other organs including endovascular, central nervous system, gastrointestinal and suprarenal glands (Wheat et al. 2016). Since symptoms are nonspecific, even disseminated disease can at times evade recognition, and if not treated, can progress to chronic or latent disease.

4.3 Chronic Pulmonary Histoplasmosis

Chronic pulmonary disease typically occurs in older individuals with previously injured lung tissues (Goodwin et al. 1976). It may follow acute pulmonary infection or develop secondary to persistent, slowly progressive disease, typically a complication of the acute disease. With acute disease, there is a progression of disease from pulmonary infiltrates to fibrosis and finally cavitation (Wheat et al. 1984). Apical bullae can also be part of chronic disease. Patients present with symptoms of fever, weight loss, cough, and dyspnea (Kauffman 2007). Bronchopleural fistulas are a serious complication of chronic disease (Goodwin et al. 1976). Due to the presence of cavitary lesions in chronic pulmonary histoplasmosis, it can be confused with tuberculosis or other mycotic infections and coinfections are possible (Wheat et al. 2016).

5 Diagnosis and Treatment

Although an in-depth discussion on diagnosis and treatment is beyond the scope of the current work, it merits a brief overview. There are currently multiple diagnostic modalities that include antigen detection, serology, pathology, and culture that when combined can achieve high sensitivity and specificity (Azar and Hage 2017). Culture of *Histoplasma* is the gold standard for diagnosis but obtaining adequate tissue and fluid samples for culture can be difficult. Considering fungal growth patterns, it can take more than 2 weeks to observe mycelial growth, which can significantly delay diagnosis. Positive cultures are more likely to be found in disseminated disease due to the presumed higher fungal burden in the host. When histopathology is available, finding yeast cells in tissue is consistent with *Histoplasma* and supports the diagnosis. Another important diagnostic method is antigen detection. Antigen testing (i.e. detection of *Histoplasma* polysaccharide by antibodies) can be done on any body fluids and tissues depending on the suspected site of infection but is most often tested in the urine (Azar and Hage 2017; Nadel et al. 2005; Assi et al. 2011). Additionally, urine and other body fluid antigen levels can be helpful in monitoring treatment response. Serology is more useful for chronic and subacute disease. Various techniques include immunodiffusion, complement fixation enzyme immunoassay, and radioimmunoassay. Finally, molecular methods are not yet FDA-approved, but these approaches can nevertheless be helpful due to high specificity and faster processing time (Azar and Hage 2017).

The decision to treat is based on the severity of the disease as well as the host immune status. Treatment recommendations are largely based on the 2007 update by the Infectious Disease Society of America (Wheat et al. 2007) and the 2011 American Thoracic Society statement (Limpert et al. 2011). When the disease is classified as mild acute pulmonary histoplasmosis there is no indication for treatment, unless symptoms are present for more than 4 weeks or if the patient is immunocompromised, in which cases itraconazole is recommended. If the disease is classified as moderate, then the treatment of choice is itraconazole, which should be initiated regardless of the duration of symptoms. Other possible therapeutic options include voriconazole and posaconazole. For patients identified as having moderately severe to severe acute pulmonary disease, the first line of treatment is liposomal amphotericin B, followed by itraconazole. The use of steroids is recommended at initiation of therapy if a patient is hypoxemic or presents with respiratory distress. Unfortunately, if host immunosuppression persists then the medication will need to be given lifelong. When treating disease with itraconazole, serum drug level monitoring should be performed 2 weeks after therapy is started and every 3–6 months while receiving treatment (Wheat et al. 2007).

Despite having a wide geographic distribution, histoplasmosis continues to be under-recognized. There is a range of disease states discussed above and clinical severity determined to a large extent by the immune status of the host. Additionally, as new immunotherapies are developed, the number of people at risk for disease is expanding. To enrich our understanding of disease epidemiology, treatment development and prevention modalities further, we will further focus on the pathogenesis of this disease.

6 Pathogenesis

6.1 Introduction

H. capsulatum is a member of the family Ascomycetes (Kwon-Chung et al. 1974). It has a mold or mycelial form and a yeast morphology (Nosanchuk and Gacser 2008). The mycelial phase is primarily present in the environment, in nitrogen enriched soil which is the case when it is contaminated with bird or bat droppings. Fungal hyphae measure from 1.25 to 2 µm in diameter, and occasionally, hyphal forms can be seen during human infection, particularly in the setting of endocarditis (Hutton et al. 1985; Svirbely et al. 1985). There are two types of conidia in the mycelial form; macroconidia ranging in size from 8 to 15 µm in diameter, and microconidia ranging in size from 2 to 5 µm in diameter; it is the latter that can effectively be inhaled and travel as far as the host alveoli (Fig. 1). The mycelial-to-yeast phase transition in *H. capsulatum* is primarily induced by changes in temperature. The yeast form is ovoid, measuring from 2 to 5 µm in diameter. The yeast cells reproduce by polar budding giving them their characteristic narrow budding base and the appearance of a bridge between mother and daughter cells (Nadel et al. 2005). The yeast phase predominates in host tissues with an optimal growth rate at 37 °C (Maresca and Kobayashi 1989).

The yeast is the pathogenic form of *H. capsulatum* causing acute disease, or it can become latent, able to potentially reemerge in the setting of immunosuppression. However, *H. capsulatum* has many obstacles to overcome to cause host injury. It must bypass mucosal barriers during acute inoculation, evade host immune cell responses, and find its niche

within host macrophages. Table 1 is a summary of the host-pathogen responses to various components of the immune response. When *H. capsulatum* successfully enters macrophages, it can effectively avoid host effector responses and multiply. Within these cells, the fungus can disseminate throughout the host organs. These processes highlight the complexity of the interaction of the host and *Histoplasma* and the interconnected nature of the host response.

6.2 Transformation to the Pathogen

H. capsulatum pathogenesis begins immediately upon contact with the host. Upon entry into the host and the shift to mammalian body temperature, the mycelial form ceases to be metabolically active. Thereafter, “shunt pathways” mediated by cysteine and sulfhydryl compounds are thought to induce morphogenesis to the yeast form (Sacco et al. 1983). This transition process usually takes from hours to days (Sacco et al. 1983). The transition can be inhibited by sulfhydryl blocking agents, which lock the fungus in the mycelial phase. Significantly, using this blocking system, treated *H. capsulatum* is unable to cause disease in well-established animal models (Medoff et al. 1987), which highlights the necessity of the transformation into the yeast form for virulence.

More recent studies have evaluated genes involved in the transition process. Nemecek et al. evaluated *DRK1* (dimorphism regulating kinase). Silencing of this gene reduced *H. capsulatum* virulence and suppressed additional factors important to pathogenesis such as *CBP1* and *AGS1*, which were no longer expressed (Nemecek et al. 2006). Nguyen et al. demonstrated that *RYPI* (required for yeast phase growth), which is part of the WOPR family of genes, was similarly required for effective morphogenesis and virulence (Nguyen and Sil 2008). Webster et al. identified *RYP2* and *RYP3* that are part of the Velvet family of genes, as necessary for morphogenesis (Webster and Sil 2008). While all the functions of each of these factors have not been elucidated, Beyhan et al. attempted to clarify the interactions between Ryp1, Ryp2, and Ryp3, and identified another transcription factor Ryp4 involved in the transformation process (Beyhan et al. 2013). Using whole genome transcriptional profiling, they observed that 96% of yeast phase transcripts were dependent on the expression of these transcription factor genes, which were found to associate and interact with upstream regions and impact expression of genes that controlled not only transition but also virulence and therefore pathogenesis. Further studies are needed to identify additional factors involved in controlling transformation into the yeast form as well as characterizing possible pathways for interaction. Understanding these processes may lead to the identification of potential therapeutic targets in the future. However, the transformation from the environmental mycelia morphology to the yeast form is only the beginning of the process of successfully infecting the host.

6.3 Mucosal Barriers

At the onset of infection, aerosolized microconidia enter host airways, and the most common types of diseases described are sinus and lung infections. Regarding the former disease state, it is unclear if this is just a part of dissemination or the result of the nares being the entryway of the pathogen (Elansari et al. 2016; Rizzi et al. 2006; Symptoms of Histoplasmosis). In pulmonary and disseminated disease, *Histoplasma* microconidia bypass initial innate defenses, such as nasal and pharyngeal mucus, mucociliary clearance, and initial antibody

defenses, likely due to their small size and the negative pressure present with inhalation. As *Histoplasma* spores travel down the respiratory tract, they undergo phase transition to yeast. In the alveoli, yeast encounter the first of the host defenses that impact their survival.

6.3.1 Surfactant Proteins—During acute infection or initial inoculation, microconidia and newly transitioned yeast forms that successfully pass the filtering systems of the upper airways and reach the alveoli, encounter pulmonary surfactant proteins. Surfactant is a complex fluid that is composed mostly of phospholipids and four proteins (SP-A, SP-B, SP-C, and SP-D), which have different biological functions. SP-A and SP-D are part of the collectin family as they contain a collagen-like region that is part of the structure of the C-type lectin domain (McCormack et al. 2003). These hydrophilic surfactant components play a role in lung immunity (Han and Mallampalli 2015; Carreto-Binaghi et al. 2016). SP-A and SP-D bind viruses, bacteria, fungi, and parasites through a carbohydrate recognition domain (CRD), opsonizing the pathogens to enhance phagocytosis and clearance by neutrophils and macrophages (Nayak et al. 2012; Carreto-Binaghi et al. 2016; van de Wetering et al. 2004).

However, this is not the primary way in which surfactant impacts the survival of *Histoplasma*. Surfactant proteins have inherent fungicidal properties. McCormack et al. showed that *Histoplasma* yeast cells grown in the presence of SP-A and SP-D are greatly inhibited (McCormack et al. 2003). This decrease in viability was associated with a calcium-dependent, surfactant protein-mediated increase in permeability of *Histoplasma* cells. The complete mechanism leading to increased permeability of the yeast cells is not fully understood. The authors proposed that calcium binding leads to conformational shifts in CRD which expose hydrophobic proteins that disrupt the yeast cell wall. Also, SP-A deficient mice were more susceptible to infection compared to wild type. Interestingly, there was a minimal decrease in clearance of the pathogen in these mice, and the authors proposed that this may be due to the continued presence of SP-D or rapid phagocytosis of *Histoplasma* by macrophages. The growth of the pathogen within macrophages was uninhibited by the presence of SP-A and SP-D (McCormack et al. 2003; Carreto-Binaghi et al. 2016). Hence, the primary role of surfactants is to impede further entry of the pathogen into host tissues and cells.

6.4 Developing a Niche Within Host Macrophages

Once they reach alveoli, *H. capsulatum* yeast encounter cells of innate immune system. While shown to interact with various cell types, as part of the innate immune response, yeast cells establish their niche within alveolar macrophages. *Histoplasma* enters the macrophage via phagocytosis and is mediated by complement receptors as this does not require opsonization of the pathogen. This is important as the lungs are a site poor in serum opsonins (Le Cabec et al. 2002). Utilization of this mechanism of phagocytosis is beneficial to the pathogen as it does not trigger additional fungicidal pathways, which aids in the organism's ability to persist within a cell (Long et al. 2003). Additionally, when initially entering the cell, the pathogen must further alter its cell wall to avoid recognition by other macrophage receptors (Garfoot and Rappleye 2016; Garfoot et al. 2016, 2017). Once ingested once in the cell cytoplasm, *H. capsulatum* is contained within a phagocytic vacuole (Long et al. 2003). To persist within this vacuole, it must evade further protective responses

to an invading organism. The ways that it alters normal function include inactivating phagosomal reactive nitrogen and oxygen species, prevention of phagosomal acidification, and potentially lysosomal fusion. Lastly, while *Histoplasma* is contained within the macrophage, it must be able to create and transport its own nutrients. The pathogen, if successful, can continue to grow and divide in this host cell, and eventually will travel to the nucleus to induce host cell apoptosis so that it can infect other cells and disseminate (Pitangui et al. 2015).

6.4.1 Entry into the Macrophage

Complement Receptor-Mediated Phagocytosis: The first step in the interaction between the yeast form and the macrophage is ingestion via complement receptor-mediated phagocytosis. The main receptors on the surface of alveolar macrophages are LFA-1 (CD11a/CD18), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Bullock and Wright 1987). Each of these receptors has a unique β subunit and a common α subunit. When the CD18 subunit of these receptors is blocked, 50–90% of *Histoplasma* binding is impeded (Garfoot and Rappleye 2016; Newman et al. 1990).

The receptor–ligand protein on *Histoplasma*'s surface that is primarily involved with internalization is a heat shock protein (Hsp). Hsp are regulators of protein folding and are upregulated during times of stress (Cleare et al. 2017; Long et al. 2003; Guimarães et al. 2011b). These proteins are distinguished and named for their molecular weight and each plays a different role in fungal pathogenesis. For instance, Hsp70 is upregulated during the morphogenic shift from the environmental filamentous form to the pathogenic yeast phase (Cleare et al. 2017; Leach and Cowen 2013). Hsp60 exists on the cell wall of *Histoplasma* yeast forms and it is the ligand of the CR3 receptor on host macrophages. Interestingly, it is unclear what promotes its expression specifically on the yeast form cell wall (Long et al. 2003).

In a study by Guimarães et al., other functions of Hsp60 within *Histoplasma* were evaluated (Guimarães et al. 2011b). Hsp60 interacted with 126 unique fungal proteins and the number of interactions increased with temperature increase, suggesting that this protein has a wide breadth of cellular functions (Guimarães et al. 2011b). Significantly, it is crucial in the initial steps leading to persistence in the host macrophages. This is evident in a study by Gomez et al., where vaccination with recombinant Hsp60 effectively protected mice challenged with a lethal inoculum of *Histoplasma* (Gomez et al. 1995). A later study by Guimarães et al., had a similar finding using passive immunity with monoclonal antibodies to Hsp60. In their inoculated mice, they observed that certain Hsp60-binding antibodies prolonged survival, decreased fungal burden and organ damage, and increased Th1-type cytokine levels (IL-2, IL-12, TNF α) (Guimarães et al. 2009).

The interaction between Hsp60 and CR3 may be the reason that *Histoplasma* can enter the macrophage without triggering additional inflammatory cascades (Long et al. 2003). However, the interaction of these proteins is not an isolated event in terms of successful entry into the macrophage. The organism has additional changes it will need to undergo to further evade the host immune response.

Alterations in the Cell Wall: Macrophages have additional cell wall receptors that can recognize fungal-pathogen-associated molecular patterns (PAMPs) to trigger an attack on an invading pathogen. One such receptor is Dectin-1. This receptor recognizes β -glucan which comprises a large part of the *Histoplasma* cell wall structure. This interaction of the glucan and Dectin-1 triggers a pro-inflammatory response from the macrophage. The primary mechanism that *H. capsulatum* utilizes to evade Dectin-1 is the production of α -linked glucans that surround and conceal β -glucans (Garfoot and Rappleye 2016; Garfoot et al. 2016).

These α -glucans are synthesized by α -(1,3)-glucan synthase (Ags1). Rappleye et al. demonstrated that reduction or loss of α -glucans results in a significant reduction in virulence (Rappleye et al. 2004). Additional genes are involved in the process of α -glucan synthesis. Marion et al. identified an α -(1,4)-amylase (Amy1) and the gene responsible for producing this protein, *AMY1* (Marion et al. 2006). As a part of the α -amylase family of enzymes, Amy1 may generate α -(1,4) linked oligosaccharides that are then used by Ags1 to generate α -(1,3) linked glucans, or it may be responsible for transglycosylation of the final product. Another gene identified in this study was *UGPI*. This gene generates a UTP-glucose-1-phosphate uridylyltransferase that produces UDP-glucose monomers. These are used by Ags1 to generate α -(1,4) and α -(1,6) linked glucans. With silencing of *UGP*, there was a decrease in substrate and therefore a loss of α -(1,3)-glucan synthesis (Marion et al. 2006).

Strains that have significant quantities of α -(1,3)-glucan as part of their cells walls are called chemotype 2 strains. Some strains of *Histoplasma* lack α -glucan production as an evasive mechanism yet these strains show no difference in their virulence and are called chemotype 1 strains. This is true of the Panamanian G186A and North American isolate G217B (Edwards et al. 2011). In a study by Edwards et al., genome analysis of the promoter region of the Ags1 gene in α -glucan-deficient strains showed a large interrupting sequence that demonstrated a decrease in gene product expression in vitro (Edwards et al. 2011). However, *AGS1* mRNA was detected upon lung infection in mice. The authors proceeded to generate mutant yeast forms that lacked *AGS1* and no defects were seen in their ability to infect the lung and disseminate. These strains were also used in the study described above by Rappleye et al. Those authors found a decrease in virulence with the loss of *AGS1*, and they argue that α -(1,3)-glucan is still part of the cell wall of these strains but it may be modified and undetectable by standard methods (Rappleye et al. 2004). Both studies also propose that chemotype 1 strains may utilize unique mechanisms, other than the manipulation of their cell wall structure, to evade interaction with Dectin-1 (Edwards et al. 2011; Rappleye et al. 2004).

It is possible that secreted glucanases may be the unique or predominately utilized mechanism of certain strains to evade additional interactions with macrophage receptors. The glucanase Eng1 was studied by Garfoot et al., using strains G186A and G217B (Garfoot et al. 2016). When *ENG1* expression was silenced, the growth of the organism itself was not impacted. However, in vivo Eng1 deficiency led to reduced infectivity and increased pro-inflammatory marker production. Hence, Eng1 is a secreted glucanase that reduces the amount of β -glucan exposed to the macrophages (Garfoot et al. 2016).

Exg8 is another studied glucanase whose target is also cell wall β -glucan. Exg8, like Eng1, is only produced by the pathogenic yeast form of *Histoplasma*. A second study by Garfoot et al., using strains G186A and G217B, found that the loss of Exg8 led to a modest attenuation in contrast to Eng1 loss (Garfoot et al. 2017). Also, Exg8 did not significantly impair yeast cell interaction with Dectin-1. Exg8 in this study is characterized as an exo-glucanase and Eng1 is an endo-glucanase. It was proposed then that the structure of cellular β -glucans is not simple chains with exposed terminal ends but loops; hence, the endo-glucanase Eng1 is of greater importance in evasion of Dectin-1. These glucanases maintain the virulence of strains that lack α -glucan production, but they do not alter the receptor–ligand binding ultimately leading to internalization by the macrophage, i.e., CR3 binding to Hsp60 (Garfoot et al. 2017).

6.4.2 Challenges to Overcome Once Within the Macrophage—The processes discussed thus far have focused on the initial entry and phagocytosis by macrophages. Once inside, *Histoplasma* survives within a phagocytic vacuole in the cytoplasm. To persist and grow in this compartment, it needs to overcome additional challenges. Most of the fungus' actions are defensive in nature. It must inactivate reactive oxygen and nitrogen species released into the vacuole, prevent phagosome acidification and lysosomal fusion, and produce and transport needed nutrients to overcome nutritional immunity of the macrophage.

Inactivating Reactive Oxygen Species: Ingestion of the fungus by macrophages triggers an oxidative burst, which is a surge in the production of reactive oxygen species by the phagosome membrane associated complex, NADPH oxidase (Missall et al. 2004). This enzyme reduces oxygen to superoxide; this is secreted into the phagosome and can cause considerable damage to the microbe within it. This attack occurs extracellularly to the organism as superoxide is charged and does not cross the cell membrane of the pathogen (Garfoot and Rappleye 2016; Youseff et al. 2012).

Superoxide is not the only chemical that is potentially fungicidal within the phagosome. It can be broken down into hydrogen peroxide or it can combine with nitric oxide, and both can cause damage to microbes (Missall et al. 2004). Hydrogen peroxide can also be further broken down into hydroxyl radicals. Hydrogen peroxide, unlike superoxide, can pass through the cell membrane of an organism since it carries a neutral charge, and has the potential to cause damage both extra-and intracellularly (Youseff et al. 2012). Overall, the susceptibility of a fungus to generated reactive oxygen species is organism dependent (Missall et al. 2004). For instance, *Histoplasma* yeast cells endure concentrations of reactive oxygen species that would kill other yeast, like *Candida* (Youseff et al. 2012). *Histoplasma* as an intracellular pathogen needs to be able to combat both extracellular and intracellular free radicals while in the phagosome. These survival mechanisms are crucial to the virulence of the organism; hence, production of certain enzymes only occurs within the pathogenic yeast form (Garfoot and Rappleye 2016; Youseff et al. 2012).

Eissenberg et al. suggested that *H. capsulatum* may not induce an oxidative burst in all macrophages it invades (Garfoot and Rappleye 2016; Eissenberg and Goldman 1987). The mechanism of this early finding has never been fully elucidated. One explanation is that

murine peritoneal macrophages were used, which do not behave the same as human macrophages (Eissenberg and Goldman 1987; Youseff et al. 2012). In a 2012 study by Youseff et al., using functional assays on murine intraperitoneal macrophages, further characterized the process of respiratory burst by macrophages infected with *Histoplasma* (Youseff et al. 2012). They first observed that the yeast forms infected inactivated macrophages. They then created a strain that lacked superoxide dismutase (Sod3), which is needed for clearance of free radicals by the fungus within the phagosome. Both the wild-type and mutant strains infected inactivated macrophages, but the survival of the mutant strain was decreased by a small but significant amount. This finding indicates that even in the resting state, macrophages continue to produce reactive oxygen species. The authors also found that a *Histoplasma* superoxide dismutase decreased free radical levels to baseline within 10–15 min of exposure (Youseff et al. 2012). Additionally, they observed that activation with certain cytokines, like IFN- γ and TNF α that are released by CD4 cells, resulted in a further enhancement in the production of reactive oxygen species by macrophages (Youseff et al. 2012). This highlights the connections and cooperation between the innate and adaptive immune response that are discussed in further detail below.

Hydrogen peroxide is produced extracellularly by the phagosome and as a by-product of superoxide production. To combat hydrogen peroxide, *H. capsulatum* produces 3 catalases, CatA, CatB, and CatP (Johnson et al. 2002). CatA is produced by the mycelial form of certain strains and production is inducible in the presence of hydrogen peroxide (Holbrook et al. 2013; Guimarães et al. 2008). CatB and CatP are constantly produced by the yeast form. They differ in that CatB exerts its role extracellularly while CatP works intracellularly, but both function to reduce hydrogen peroxide into water and oxygen to prevent damage to the microbe (Guimarães et al. 2008; Holbrook et al. 2013). In a study by Holbrook et al., the authors evaluated the relative importance of each catalase by studying the impact of their removal in generated mutant strains compared to a wild type (Holbrook et al. 2013). Loss of CatB had no significant impact on virulence in vivo and only minor effect on survival in culture. The proposed explanation is that superoxide may be more abundant and play a more significant role as a fungicidal agent. Mutants that lacked both Sod3 and CatB were evaluated, and there was no additive effect in decreased survival and virulence in vitro and in vivo with the additional loss of CatB. The conclusion made was that CatB and CatP alone are not sufficient to protect the yeast form from damage by generated free radicals, and superoxide is the major fungicidal agent in the phagosome. However, the loss of both catalases in a strain while maintaining Sod3 function was evaluated, and their loss resulted in a minor reduction in virulence. Loss of CatP or CatB alone did not impact virulence to the same degree as seen when both enzymes are silenced. The authors asserted here that there is a redundancy in the function of these enzymes, and it is likely that this is a result of the nature of their target molecule. Hydrogen peroxide carries no charge, and so unlike superoxide, it can pass freely through the pathogen's membrane. Unfortunately, wherever it travels it is flanked by a catalase on either side (Holbrook et al. 2013; Garfoot and Rappleye 2016).

The pathogen, if successful in neutralizing these reactive oxygen species, is another step closer to claiming its niche where it can persist, grow within, and utilize it to disseminate throughout the host.

Inactivating Reactive Nitrogen Species: As mentioned, superoxide can also combine with nitric oxide, which generates reactive nitrogen species that the organism must also contend with (Garfoot and Rappleye 2016; Chao et al. 2008; Lane et al. 1994; Nakamura et al. 1994; Nittler et al. 2005). Activated macrophages utilize the enzyme nitrogen oxide synthase to produce nitric oxide. Activation of this enzyme is induced by the initial infection and by IFN- γ stimulation of macrophages (Garfoot and Rappleye 2016; Lane et al. 1994; Nakamura et al. 1994). Nitric oxide, once generated and transformed into a reactive nitrogen species, can cause DNA and membrane damage, inhibit cell replication, and can inactivate crucial cellular enzymes (Nittler et al. 2005). Reactive nitrogen species in response to infection with *Histoplasma* leads to a fungistatic, not fungicidal, result (Nittler et al. 2005; Garfoot and Rappleye 2016).

Nittler et al. utilized functional genomics to identify a set of genes that were induced in response to reactive nitrogen species in *Histoplasma* infection. They identified 153 gene transcripts that were upregulated with infection, but their function remained unclear, and no core group of genes could be identified (Nittler et al. 2005). One gene found was *NORI*, and this was noted to have high sequence homology with other nitric oxide reductases seen in other fungal and bacterial pathogens that facilitate the conversion of nitric oxide to nitrous oxide, which is no longer toxic to the cell (Nittler et al. 2005). Chao et al. were able to confirm with cell culture, mass spectroscopy, and nitrous oxide detection that Nor1 reduced reactive nitrogen species into less toxic substances in the infected macrophage (Chao et al. 2008). It was also found to have constitutive expression in the mycelial form and inducible expression in the yeast form of *Histoplasma* (Nittler et al. 2005; Chao et al. 2008).

It is evident that *H. capsulatum* has defense mechanisms in place to evade both reactive oxygen and nitrogen species. But these are not the only dangers to the pathogen when it exists intracellularly and within a phagocytic vacuole.

Prevention of Phagosome Acidification and Lysosomal Fusion: As a phagosome matures it creates an acidic pH internally to enhance microbicidal activities against encompassed microbes (Isaac et al. 2013). Vacuolar ATPases (V-ATPase) are pumps on the membrane that are used to bring protons to the inside of the phagosome. Subsequently, the phagosome fuses with a lysosome that contains multiple hydrolases that work best at this generated low pH. These enzymes then further attack an invading microbe. Therefore, for the microbe to persist, it must find a way to manipulate its environment within the macrophage to create a more basic pH and potentially prevent lysosomal fusion.

Strasser et al. found that inhibition of macrophage V-ATPase had no effect on *H. capsulatum* survival, and there was no change in internal pH (Strasser et al. 1999). They also found that there was diminished phagosome-lysosomal fusion. The authors go on to assert that V-ATPase was not required for the acidification of phagosomes containing *Histoplasma*. It was thought also that *Histoplasma* itself contains a pH sensing ability for it to respond to changes in its environment (Strasser et al. 1999). Isaac et al. utilized genetic screening of insertional mutants of *Histoplasma* to identify mutants that were unable to lyse host macrophages (Isaac et al. 2013). They identified HMG CoA lyase (*HCL 1*) as being required for growth within the macrophage and later lysis of the cell particularly in glucose-deficient and leucine-rich

environments. Indirectly, the authors found that this enzyme was also important to maintaining phagolysosomal pH. In mutants lacking this enzyme, there was an accumulation of acidic species when leucine was utilized as the primary energy source while grown in unbuffered media. Hc11 mutants were able to persist in macrophages, but their growth was significantly restricted compared to wild-type strains (Isaac et al. 2013). Unfortunately, in vivo studies with mice models did not show a difference in virulence of the mutant strains compared to the wild type. This would also suggest that there are additional pathways that require alteration to inhibit pathogenesis, and further studies are needed to explore that (Isaac et al. 2013; Garfoot and Rappleye 2016).

Newman et al. further investigated the role of pH in *H. capsulatum* survival in human macrophages (Newman et al. 2005). They found that intraphagosomal pH was about 6.5 when viable yeast forms were present, but there was no change in pH noted using heat-killed cells, fixed yeast cells or nonpathogenic yeast known to cause acidification when digested by macrophages. This would indicate that an acidic pH may in fact not be needed for killing. The authors asserted that additional studies are needed to further explore this relationship out; particularly, if there is a target or signal pathway needed to initiate changes in pH that aid in clearance of *Histoplasma* (Newman et al. 2006).

The prevention of lysosomal fusion may be another method utilized by *Histoplasma* as part of its pathogenesis. P338D1 mice and J774.2 cell line models have normal fusion of these intracellular compartments (Strasser et al. 1999; Taylor et al. 1989; Eissenberg et al. 1988). However, the RAW 264.7 cell line and human macrophages show a decrease in phagosome-lysosome fusion (Newman et al. 2006). It remains unclear why this is the case. Based on the above studies by Newman and Strasser, an acidic pH does not seem to limit nor is it necessary for lysosomal hydrolase function (Newman et al. 2006; Strasser et al. 1999). Additional study in this area would be of value not only to understand how the organism may impact this interaction but for the potential of therapeutic interventions that could increase clearance of *H. capsulatum*.

Nutrient, Essential Metal, and Nucleic Acid Acquisition: At this point, if the pathogen defends itself from reactive oxygen and nitrogen species, prevents lysosomal fusion and maintains a basic pH, as alluded to earlier, there are still certain nutrients it will need to acquire that impact its growth and survival. The phagosome is generally nutrient-poor (Garfoot and Rappleye 2016). This mechanism of limiting nucleic acids, iron, and vitamins has also been termed nutritional immunity (Woods 2016; Garfoot and Rappleye 2016). It is a dynamic process and one that the macrophage possesses to defend itself. Therefore, the pathogen needs to be able to utilize what is present or produce its own nutrients to meet its metabolic needs (Garfoot and Rappleye 2016; Woods 2016).

Iron: *Histoplasma* must find a way to deal with iron deficiency in its environment, so it can continue to proliferate while in the phagosome. Newman et al. investigated the role of intracellular iron and its impact on yeast cell growth (Newman et al. 1994). Initial cultures of the pathogen with iron chelators suppressed growth in a concentration-dependent pattern, and the effect was reversed with the supplementation of iron. Chloroquine had a similar effect on growth, but it did so by raising endocytic pH and induced human macrophages to

kill yeast cells. Its effects were reversed using iron supplementation that was soluble at a basic pH. Chloroquine's effects were both time- and dose-dependent and impacted fungal burden and dissemination. This study highlighted the importance of iron to *Histoplasma* survival and begs the question of potential therapeutic options (Newman et al. 1994).

Within the host, iron is bound and transported into a cell by surface transferrin molecules. Once in the cell, the iron is free or can be bound to ferritin. This binding to ferritin is upregulated in the setting of infection. Lane et al. found that IFN- γ released by T cells during infection caused a decrease in cell surface transferrin receptors (Lane et al. 1991). This suggests that cytokine stimulation also plays a role in limiting iron availability within the macrophage. Intracellular unbound iron is a potential source of this essential metal for *Histoplasma*. When iron is lacking in its surroundings, the yeast cells secrete hydroxamates that function as siderophores or iron chelators (Howard et al. 2000; Garfoot and Rappleye 2016). Howard et al. observed that *H. capsulatum*-derived siderophores are detected 4 days after inoculation of the media. Expression can be suppressed with increasing concentrations of iron in the environment. Also, the authors found that there is not just one siderophore but 5 different ones (Howard et al. 2000). Hwang et al. later identified the gene *SIDI*, which produces the enzymes that catalyze the first step in siderophore production. Strains deficient in this gene showed significant depression in growth (Hwang et al. 2008).

In addition to the chelating activity of siderophores, Timmerman et al. observed the utilization of enzymatic reductants by *Histoplasma* to reduce iron and thus allowed for its uptake (Timmerman and Woods 1999). The expression of these enzymes was upregulated when the pathogen was grown in iron-deficient conditions. These included secreted extracellular glutathione-dependent ferric reductase, extracellular non-proteinaceous ferric reductants, and cell surface ferric reducing agents. Subsequently, they evaluated the relationship between reductases, siderophores, and discussed pH changes leading to the release of iron from transferrin. It was initially believed that an acidic pH allows an organism to gather essential metals while within the macrophage (Isaac et al. 2013; Strasser et al. 1999). Iron is bound to the transferrin receptors, and when the pH falls from 7.0 to 6.0 there is a 50% dissociation of iron and even more when the pH falls below 6.0. Timmerman et al. noted the function of extracellular glutathione-dependent ferric reductase as well as chelation by siderophores despite a pH of 7, and they questioned the true impact of pH changes on iron acquisition. Regardless, they proposed a model for iron acquisition that included this process. The postulate that siderophores, ferric reductants, and changes in pH can function separately to pull iron off of molecules like transferrin and transport it into the cell. Additionally, iron bound to siderophores and iron released by transferrin with pH changes can act as the substrate for ferric reductants and, once reduced, can also be transported into the cell (Timmerman and Woods 2001). The process of iron acquisition is crucial to survival for the pathogen, however, the exact mechanism is complex and there is more to be learned about the genes and enzymes that are involved (Winters et al. 2008). Additionally, other metals, like zinc, are implicated in pathogenesis, but their role and interaction with the pathogen require further study (Garfoot and Rappleye 2016; Dade et al. 2016).

Nucleic Acids: *Histoplasma* can overcome an iron deficient environment; similarly, it must be able to adapt to the nucleic acid deficient environment of the macrophage. To understand how *Histoplasma* build their DNA, studies have focused on developing auxotrophs that are incapable of their own nucleic acid production. Nucleic acid auxotrophs were first derived by Woods et al. (Woods et al. 1998; Woods 2016). The *URA5* gene when interrupted, in a more recent study by Rappleye et al., created uracil auxotrophs that had a decrease in growth unless supplemented with uracil in cell culture (Woods et al. 1998; Rappleye et al. 2004). Additionally, in this study, adenine auxotrophs were created using gene disruption of *ADE2*, which showed a decrease in growth in cell culture (Woods 2016; Rappleye et al. 2004). Supplementation of a pyrimidine was required for continued growth. However, if the pathway was further damaged, such that its synthetic function is compromised, even with supplementation, there is no growth or proliferation of the pathogen (Garfoot and Rappleye 2016; Rappleye et al. 2004; Woods et al. 1998). So, it would seem then that *Histoplasma* is capable of independent production and potentially transport of nucleic acids when needed.

Vitamins: *Histoplasma* can synthesize its own essential vitamins while within the macrophage (Garfoot and Rappleye 2016; Garfoot et al. 2014). Garfoot et al. identified vitamin synthesis pathways within the genome, and then using a medium lacking in nutrients they were able to demonstrate that *Histoplasma* can produce all essential vitamins except thiamine (Garfoot et al. 2014). They proposed that the pathogen may gather this vitamin from the host and have scavenging mechanisms to aid in this process. Additionally, they studied the impact of riboflavin, pantothenate and biotin synthesis on growth and proliferation of the pathogen. They disrupted *RIB2*, *PAN6*, and *BIO2*. *RIB2* mutants persisted in the lungs but they did not replicate in vivo, and a similar decrease in virulence was seen when *PAN6* was disrupted. *RIB2* mutants had only partial restoration of growth with supplementation in the cell media. *BIO2* gene disruption did not impact the virulence of the organism, and it is proposed that there is availability within the host to make up for this induced dysfunction. The authors asserted that information from this study explains more about the vitamins that are available in the phagosome and thus suggests potential therapeutic targets (Garfoot et al. 2014).

6.4.3 Inducing Apoptosis and Dissemination—*H. capsulatum*, by utilizing the previously discussed interactions with the host macrophage, can thrive in its intracellular niche. However, for infection to be propagated in the host and disseminate to other organs in the body, the pathogen needs to induce apoptosis of or otherwise leave the macrophage and infect subsequent phagocytes. This process will also potentially activate components of the adaptive immune response to allow for further control by the host and taking residence within granulomas. Pitangui et al. described the movement of *H. capsulatum* yeast cells within the macrophage. They found that yeast aggregate 5 h after infection around the cell nucleus and this leads to DNA damage and cell death (Pitangui et al. 2015). Deepe et al. further defined how apoptosis occurs and what cytokines are involved in this process. It is particularly intricate, and they describe the utilization of extrinsic pathways that are mediated by and lead to an increased expression of TNF- α and activation of caspases 1 and 3. Additionally, there is an increase in IL-10 production, which is counterintuitive as this is a cytokine that inhibits apoptosis in neighboring cells. The authors propose that these two

cytokines produce a net effect that benefits the pathogen. There is the initial induction of apoptosis, and with the stimulation of neighboring cells with IL-10, the yeast can go on to infect phagocytes that will accommodate their survival (Deepe and Buesing 2012). The question that arises from this is whether *Histoplasma* triggers cell death, or if this is entirely a passive process that occurs when the fungal burden is too high within the macrophage.

Calcium Binding Protein (Cbp1) is specifically produced by *H. capsulatum* yeast cells, and it is involved in promoting cell growth in calcium limited settings (Batanghari et al. 1998). However, it also plays a role in cell death and proliferation (Sebghati et al. 2000). Isaac et al. evaluated the mechanisms used by Cbp1 when involved with cell death (Isaac et al. 2015). The authors screened 14,000 insertional mutants to find those that grew at high levels intracellularly but would not lyse cells, and three such mutants were identified, all lacking *CBP1* expression. Even with sufficient growth without *CBP1*, there was no cell lysis, indicating that this process was actively induced, as opposed to a passive cell death by the pathogen. Using whole genome sequencing, *CBP1* was found to be required for induction of stress-responsive genes that modulate cell death as well as activate caspases 3 and 7 (Isaac et al. 2015). This is further confirmed in a recent study by English et al. which suggests that there is an integrated stress response regulated by Cbp1 that induces the expression of other proapoptotic genes like *CHOP* and *TRIB3*; however, the entire mechanism by which Cbp1 induces cell death is not known and is an area of further research and potential drug targeting (English et al. 2017).

6.5 Host Cellular Immune Response

Although the macrophage is a principal player in the host-pathogen interaction, *H. capsulatum* can interact with a variety of cells before it reaches its niche within the macrophage (Deepe et al. 2008). Deepe et al. demonstrated that the yeast cells preferentially invade different phagocytic cell subpopulations at different times after initial infection. They demonstrated that the yeast cells were present in neutrophils, dendritic cells (DCs) and macrophages, from days 1 to 7 after inoculation; however, DCs contained proportionately more yeast cells by day 1, with this shifting towards neutrophils and then macrophages on subsequent days (Deepe et al. 2008). DCs, neutrophils, and natural killer cells can effectively kill *H. capsulatum* yeast cells and, thus, their host-pathogen interaction is distinct from that described so far for macrophages.

6.5.1 Dendritic Cells—DCs first encounter *H. capsulatum* in the alveoli, and they serve as a link to subsequent host defenses. DCs precursors originate in the bone marrow and mature into more specialized cells that are found in the skin, and most solid organs including the lungs, specifically in airway epithelium, parenchyma, submucosa, alveolar septal wall, and alveolar surfaces (Thind et al. 2015; Sertl et al. 1986; Holt and Schon-Hegrad 1987). These cells are the primary antigen presenting cells of the innate immune system, and serve as a connection to the adaptive immune response as they interact with T cells, once they leave the tissues and travel to the lymph nodes (Clark and Kupper 2005). Immature DCs engulf entire organisms via receptor or non-receptor mediated phagocytosis at the tissue level. These cells process the pathogen and mature to then become antigen presenting cells (Thind et al. 2015). Also, once activated, DCs release cytokines like IL-12 and TNF α to

recruit other DC, additional phagocyte populations, stimulate the adaptive immune response, and aid in granuloma formation (Zhou et al. 2001).

DCs efficiently engage *H. capsulatum*. Gildea et al. observed that after 6 h of incubation 75% of DCs in culture had ingested at least one yeast cell (Gildea et al. 2001). DCs binding of *Histoplasma* occurs via a fibronectin receptor on the DC surface called very late antigen-5 (VLA-5) (Gildea et al. 2001). This was an unexpected finding as there are higher levels of CD18 on the cell's surface. The authors initially hypothesized that CD18 would be utilized as was the case with macrophages. They also observed that DCs inhibited the growth of and killed phagocytized yeast cells unlike what had been observed in macrophages. This is similar to what was observed when microconidia are ingested by DCs as their transformation into yeast cells was inhibited (Newman et al. 2011). The authors proposed that the difference in the receptor interactions of these cells with *Histoplasma* may impact the intracellular survival of the pathogen; although, the mechanism for the preferential receptor binding is unknown (Gildea et al. 2001). Subsequently, Gomez et al. identified the ligand for VLA-5 on the surface of *H. capsulatum* yeast cells, which turned out to be a 20-kDa protein called cyclophilin A (Gomez et al. 2008).

Once phagocytosis occurs, DCs can be both fungistatic and fungicidal. Human DCs phagolysosomal fusion occurs unimpeded (Gildea et al. 2005). Once inside this vesicle, there is either restriction of growth or killing of the organism, which largely occurs through hydrolases. Nitric oxide and oxygen free radicals do not seem to play a role in DC fungicidal activity, as in macrophages (Thind et al. 2015; Gildea et al. 2005). Despite the effective protection provided by the DCs, pathogens that are not phagocytized will encounter additional host defenses that can still impact their survival.

6.5.2 Neutrophils—In addition to early interactions with DCs after acquisition of *H. capsulatum*, the fungus also encounters neutrophils (Deepe et al. 2008). These cells rapidly arrive at the site of infection to engage the pathogen as part of the innate immune response (Thind et al. 2015). Human neutrophils are fungistatic against *H. capsulatum*. Antimicrobial proteins in neutrophils are contained in azurophilic granules (Newman et al. 1993). Within these granules, there are two families of proteins known as defensins and serprocidins, and two additional proteins with unique structures: lysozyme and bactericidal-permeability-increasing protein (BPI). Their interactions were rigorously evaluated by Newman et al. (Newman et al. 2000). Defensins (HNP-1, HNP-2, and HNP-3) are derived from 29 to 30 amino acids, differing only in a single N-terminal amino acid. Each of these when incubated with yeast cells showed concentration-dependent inhibition of growth. Of the three, HNP-2 had the greatest inhibitory activity. Their effect was also noted to be additive (Newman et al. 2000).

Serprocidins are a group of four proteins. Cathepsin G is one of these proteins, it is a neutral protease of molecular mass 29–31 kDa, and it is the only member of this family that inhibits growth of *Histoplasma*. Alone, this protein has inhibitory activity, and its effects are additive when associated with defensins (Newman et al. 2000). BPI is a protein within the granule with significant fungistatic ability toward *H. capsulatum*. It has a molecular mass of 50–60 kDa, characterized by a lysine-rich amino-terminal and carboxy-terminal regions (Gray et al.

1989). This protein inhibits the growth of yeast cells in a concentration-dependent manner and it has an additive effect when combined with either defensins or cathepsin G (Newman et al. 2000).

The entire mechanism of fungistasis by these proteins has not been fully elucidated (Newman et al. 1993, 2000). Newman et al. have described the discrepancy noted in *Histoplasma* endemic areas where many individuals who presumably have been exposed to the fungi at some point had negative skin tests when exposed to histoplasmin. It is hypothesized that the initial response from neutrophils may be sufficient to clear the organism from the host without the need or time for activation of the adaptive immune response, more likely in the setting of a small inoculum (Newman et al. 1993).

Both neutrophils and DC directly impact survival of *Histoplasma*. DCs through cytokine release will impact later control of the infection as well. Natural killer cells are also part of the initial host defenses, and they are connected to this process as they are recruited by DCs and macrophages.

6.5.3 Natural Killer Cells—In a study of intranasally infected mice, Cain et al. examined the inflammatory reactions and cytokine responses with active disease progression (Cain and Deepe 1998). Increased levels of IL-12 were observed by day 3 of infection followed by the increased expression of IL-2 and IFN- γ starting on day 5 till day 10. All of these declined as of day 14. It was observed that myeloid cells had increases in their expression by day 5, and by day 7 they peak with the additional presence of natural killer cells. It is by day 10 that T cells and B cells start to predominate among the inflammatory cell types at sites of disease. This highlights the involvement of natural killer cells in response to infection, and a potential connection between the initial myeloid cell response via stimulating cytokines.

Cohen et al. attempted to further define the connections between myeloid cells and the natural killer cells response to infection with various fungal pathogens (Cohen et al. 2011). Natural killer cell responses were reduced in the presence of *H. capsulatum* yeast cells and associated DCs were unable to produce IL-12. Additionally, when *Histoplasma* cells were grown with DCs lacking Dectin-1 on the cell surface, there was also a reduced natural killer cell response. The authors propose a general mechanism for natural killer cells' function during a systemic fungal infection. They postulate that antigen presenting cells, like DC or macrophages, produce IL-12 in response to fungal cell wall components leading to natural killer cell activation. Also, their data showed that natural killer cells enhance the production of IL-12 from antigen presenting cells as a positive feedback loop (Cohen et al. 2011).

Natural killer cells, akin to DCs, are directly cytotoxic to the target pathogen; however, this mechanism is not fully understood regarding infection with *Histoplasma* (Tewari and Von Behren 2000). Natural killer cells, also like DCs, continue to link the innate and adaptive host response to this pathogen. Activated natural killer cells produce IFN- γ (Zhou et al. 2001), which leads to activation of CD8 and CD4 T cells for further control of the infection and potentially granuloma formation.

6.6 Adaptive Immune Response, Granuloma Formation and Reactivation

The pathogen at this point has now bypassed the mucosal level obstacles, cellular host defenses and is manipulating the macrophage to further disseminate. But as alluded to earlier there is a complex network of cytokine production and the adaptive immune responses that are the host's last line of defense. The adaptive immune response once effectively activated can either clear the organism or lead to granuloma formation. If the latter occurs, there is the potential for reactivation of the organism. Reactivation is a response to impaired immunity (Allen and Deepe 2006). This can occur in the setting iatrogenic immunosuppression with the use of immunomodulating therapy that impacts cytokine function, in conditions like HIV/AIDS where there is a loss of T cell function, and with additional systemic conditions that impact the immune response (Heninger et al. 2006). Ultimately, the pathogen, though potentially controlled after acquisition, seems to never truly be eliminated by host defenses.

The adaptive immune response and granuloma formation is complex and not completely understood, but murine models have been developed to examine this process further (Allen and Deepe 2006; Heninger et al. 2006). As mentioned earlier DC act as antigen presenting cells in lymphoid tissue and activate T cells; therefore, they are a link between the innate and adaptive immune response. Natural killer cells and macrophages serve to connect these responses via cytokine production leading to cell activation. Heninger et al. used a murine model to further characterize granuloma formation (Heninger et al. 2006). On day 5, after infection, macrophages are present at the tissue, and the tissue has a vasculitic appearance that may indicate extravasation of immune cells. Granuloma formation occurred in the liver by day 7. The liver granulomas formed were noted to grow and reach their maximum size by day 10. IL-10 and TGF- β were elevated early in granuloma formation, with the latter coming from infected macrophages. The liver granulomas decreased in size after day 10 as immune stimulation waned, but pulmonary granulomas did not diminish in size. Hence, the organ itself has an impact on granuloma characteristics and control responses to the pathogen (Heninger et al. 2006).

Notably, 70% of the liver granuloma was made up of macrophages and there was an abundance of IFN- γ . Subsequently, these macrophages produced TNF- α . DCs and neutrophils were present to a lesser extent as were CD4 and CD8 T Cells. Early on there were more CD4 cells, but their ratio equalized as time progressed. Additionally, there was a low level of B cells present within the granulomas, which may be a potential link to the formation of protective antibodies. Moreover, there was a diversity of T and B cells present, indicating a diversity in recruitment processes as opposed to single cell type entry, multiplication and granuloma formation (Heninger et al. 2006).

Allen et al. further elucidated the importance of T and B cells in the granuloma, and their role in the control of disease (Allen and Deepe 2006). The authors intranasally infected mice and then depleted them of CD4 and CD8 T cells 42 days later. The mice developed persistent disease when both cell types were eliminated. Also, latently infected mice subjected to B cell and CD4 cell depletion resulted in disease reactivation. These findings demonstrate that there is cooperation between CD8 T cells and B cells, and that this potentially explains why the depletion of either CD4 or CD8 T cells alone was insufficient to

permit reactivation. The role of B cells in the immune response is complicated and has yet to be fully defined. Tristão et al. found that *Histoplasma* cell-free antigens (CFAg) are present during murine infection, and antibodies were generated against these antigens. These antigens competed with actual fungal surface antigens in antibody formation and recognition. So, as with other components of the immune response, this pathogen may have escape mechanisms against antibodies as well (Tristão et al. 2012). However, disease modifying monoclonal antibodies (mAb) have been described and include antibodies to cell surface displayed histone 2B (Nosanchuk et al. 2003; Shi et al. 2008), M antigen (Guimarães et al. 2008; Nosanchuk et al. 2012), and heat shock protein 60 (Guimarães et al. 2009, 2011a, b). However, there are antibodies that do not improve disease outcomes, such as a mAb to H1C (Lopes et al. 2010), and some mAb can enhance disease, such as an IgG2b isotype to heat shock 60 (Guimarães et al. 2009). Hence, the role of antibody therapy in histoplasmosis is complex and requires further investigation.

The Heninger study found that IFN- γ within these granulomas was produced mostly by CD4 and CD8 T cells (Heninger et al. 2006). As discussed, this cytokine activates macrophages to induce killing via reactive oxygen and nitrogen species. The macrophage will also produce TNF- α . To stress the importance of these cytokines in the control of disease, there have been studies and case series that evaluate the loss of these cytokines and how that impacts disease progression and reactivation. Clemons et al. treated rodent models with IFN antibodies and developed *IFN* knockout mice, and compared these to controls. Depletion of the cytokine and gene disruption both resulted in a loss of resistance to lethal infection and early mortality in the mice (Clemons et al. 2000). In a subsequent study by Clemons et al., IFN was evaluated as an adjuvant therapy to amphotericin B in infected mice, and the combination of the cytokine with amphotericin was superior to drug therapy alone (Clemons et al. 2001). This experimental work is supported by a case report from Zerbe et al., where a patient with an inherited IFN- γ receptor deficiency developed recurrent disseminated histoplasmosis (Zerbe and Holland 2005).

TNF- α is also a key regulator of disease (Deepe 2005). It is clinically relevant as seen in the study by Lee et al. where the authors reviewed post-licensure adverse effects of TNF inhibitors and found 10 cases of reactivation of *Histoplasma* after treatment with these agents (Lee et al. 2002). In mouse models, TNF depletion or inhibition leads to higher mortality in both primary and secondary infection (Allendoerfer and Deepe 1998; Deepe 2005). Granulomas continue to form within the tissues with an increase in inflammatory changes seen specifically in the lungs, likely due to an increase in fungal burden (Allendoerfer and Deepe 1998). Additionally, in primary infection, without TNF- α , there is a decrease in nitric oxide production and this may lead to poor clearance of the organism (Deepe 2007). Unexpectedly, no change to IFN levels has been observed in this setting, which indicates independent production, and a possible codependent relationship with TNF- α as both are required for clearance of the organism (Allendoerfer and Deepe 1998; Deepe 2005). In secondary infection, without TNF- α , there is an upregulation of IL-4 and IL-10 which diminish protective immunity. In fact, blockade of both cytokines in TNF- α depleted mice improved survival (Allendoerfer and Deepe 1998; Deepe 2007). More recent studies are evaluating the T cells populations recruited when TNF- α is missing and how this may impact potential therapeutic interventions (Deepe and Gibbons 2008; Kroetz and Deepe

2012). Ultimately, further study is required to understand the relationships between this cytokine and the various immune cell responses in mice, and eventually expansion to human models to see if these same changes exist.

It is evident that the interactions of the adaptive and innate immune response, eventual granuloma formation and potential reactivation are each complex and interconnected connected processes. However, further characterization and definitions of the elements involved are beneficial as this can lead to therapeutic targets against disease. An example of this is evaluated in a study by Lazar-Molnar et al., as they examined the receptors involved in apoptosis (Lázár-Molnár et al. 2008). Programmed cell death-1 receptor (PD-1) is an immune inhibitory receptor that is part of the CD28:B7 family. It is expressed on activated T cells, B cells, and myeloid cells. PD-1 receptor and ligand binding inhibit cytokine production in vitro. In PD-1-deficient mice, there is protection from *Histoplasma capsulatum* infection. In wild-type mice, blockade of the PD-1 pathway generated increased survival by 70%. This is a potential pathway to be manipulated as a future therapeutic target (Lázár-Molnár et al. 2008).

6.7 New Pathogenic Mechanisms

Extracellular Vesicles—Extracellular vesicles are lipid bilayered structures that contain lipids, phospholipids, polysaccharides, nucleic acid, proteins, and other compounds. Extracellular vesicles have been described in all biological kingdoms (Zamith-Miranda et al. 2018). In fungi, extracellular vesicles have been shown to transport diverse compounds that include factors associated with virulence (Joffe et al. 2016; Rodrigues et al. 2008). *H. capsulatum* yeast cells produce extracellular vesicles that carry virulence factors such as heat shock protein 60, catalases, laccases, and phosphatases (Albuquerque et al. 2008; Matos Baltazar et al. 2016). Fungal extracellular vesicles can modulate host–pathogen interactions (Zamith-Miranda et al. 2018; Vargas et al. 2015); hence, *H. capsulatum* extracellular vesicles are postulated to impact pathogenesis. This is supported by the fact that the contents of *H. capsulatum* vesicles are recognized by immune human sera (Albuquerque et al. 2008). Additionally, binding of mAb to *H. capsulatum* heat shock protein 60 to yeast cells induces a change in extracellular cargo loading and the characteristics of released vesicles are distinct from those isolated from untreated yeast cells (Matos Baltazar et al. 2016), which suggests that this process is dynamic and there is an interplay between the fungus and the host immune system. Targeting processes associated with the loading and release of extracellular vesicles is a promising potential approach to modifying the virulence of the fungus.

7 Concluding Remarks

H. capsulatum is the most prevalent endemic fungus worldwide. Nevertheless, histoplasmosis remains under-recognized and it is thus under-reported. Disease caused by *H. capsulatum* has a wide range of presentations and varying degrees of severity of clinical manifestations that impact treatment decisions. The process of infection and damage to the host is complex, and involves a dynamic interaction between host defense mechanisms, both at the mucosal and cellular level, and the pathogen's evasive and reactive responses. If the pathogen is successful, it can cause acute, local, or disseminated disease, or develop a latent

state within tissue granulomas with the potential for reactivation. Further study and understanding of the pathogenesis of *Histoplasma* are needed. New knowledge is essential as it can open the door for therapeutic interventions that can positively impact clinical outcomes.

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Fig. 1.
A depiction of the acquisition process of mycelial *Histoplasma* from the environment to the lung with the transformation to the yeast form upon deposition in alveoli

Table 1

Summary of the immune system components, their response to an invading pathogen, and *Histoplasma*'s protective mechanisms

	Host mechanisms	Pathogen response	References^a
Host entry	None	Phase transition from mycelial to yeast form	(Sacco et al. 1983; Medoff et al. 1987; Nemecek et al. 2006)
Innate defense	Mucociliary clearance, mucus production	None needed	(Elansari et al. 2016; Rizzi et al. 2006; Symptoms of Histoplasmosis)
Surfactant	Pathogen opsonization Alters <i>Histoplasma</i> yeast permeability—Surfactant proteins A and D (SP-A and SP-D)	Rapid entry into macrophage—intracellular localization shields from the action of pulmonary surfactants	(McCormack et al. 2003; Han and Mallampalli 2015; Carreto-Binaghi et al. 2016)
Macrophages	Detects fungal cell components and initiate pro-inflammatory response Attacks phagocytized pathogen with reactive oxygen and nitrogen species Creates an acidic environment in the phagosome which allows for activation of hydrolases upon lysosomal fusion Creates nutritionally deficient environment Attract other immune cells	Avoids immune response on entry Inactivates reactive oxygen and nitrogen species Prevents acidification and lysosomal fusion Produces its own nutrients or has mechanisms for transport to obtain them from the environment Induce apoptosis to further disseminate	(Long et al. 2003; Guimarães et al. 2011b; Garfoot et al. 2017; Missall et al. 2004; Newman et al. 1994)
Dendritic cells	Cytotoxic to the pathogen Acts as an antigen presenting cell and secretes cytokines to further propagate the immune response	No active defense to this cell	(Clark and Kupper 2005; Zhou et al. 2001; Gildea et al. 2001)
Neutrophils	Traps the yeast forms intracellularly in a fungistatic response using enzymes contained within azurophilic granules to impede growth	No active defense to this cell	(Newman et al. 1993, 2000; Gray et al. 1989)
Natural killer cells	Cytotoxic to the pathogen Acts as an antigen presenting cells and secretes cytokines to further propagate the immune response	No active defense to this cell	(Cain and Deepe 1998; Cohen et al. 2011; Tewari and Von Behren 2000)
Adaptive immunity	Cytotoxic to pathogen Further propagate the immune response to eventually trap the pathogen within a granuloma	Utilizes macrophages as a sanctuary Reactivates with changes to the host's immune status	(Allen and Deepe 2006; Heninger et al. 2006; Tristão et al. 2012; Shi et al. 2008; Nosanchuk et al. 2003)

^aSample references provided, please see text for full list of references and details on each topic