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Dueling in the lung: How *Cryptococcus* spores race the host for survival

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Summary

Many human fungal pathogens infect people when they are inhaled as spores. Despite the serious impact of fungal spores on human health, little is known about their basic properties or how they interact with the host. This is particularly true for *Cryptococcus neoformans*, a human fungal pathogen that causes more than 600,000 deaths annually. Spores of *C. neoformans* have not been well characterized previously because of technical challenges in isolating them; however, recent advances in spore isolation have lead to the first direct analyses of spores. Novel insights into the spore-host interaction, specifically how spores interact with alveolar macrophages, have provided a new model of cryptococcosis that could have broad implications for human fungal pathogenesis.

Introduction

Sporulation is a strategy used by many organisms, including bacteria, fungi, protozoa, algae, and ferns to survive conditions that are too harsh to sustain vegetative growth. Survival is generally facilitated by developing specialized cells (spores) with physical properties that confer resistance to environmental assault. Many organisms also produce spores on specialized structures that are adapted for efficient dispersal via wind or water currents [1]. Through these adaptations, sporulation is an effective mechanism to either persist until local conditions improve or disperse to new environments conducive for growth.

For pathogenic microbes, favorable growth conditions are often found in a mammalian host, resulting in serious consequences for human health. For example, spores of protozoan parasites, such as the oocytes of *Cryptosporidium* sp., can be found in untreated or fecal waste-contaminated water and have been estimated to cause >50% of water-borne parasitic disease worldwide, including major outbreaks in the United States [2]. Spores of bacterial pathogens, such as those produced by *Bacillus anthracis*, are extremely resistant to physical and chemical insult, making *B. anthracis* a potentially devastating biological weapon [3]. In fungi, spores are thought to be the infectious particles of many fungal pathogens. This has been shown rigorously for a number of plant fungal pathogens, such as the wheat rusts,

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Puccinia sp., which disperse globally on an annual basis and cause damage to food crops totaling ~3 billion dollars per year [1,4].

Among human fungal pathogens, spores are presumed infectious particles for many organisms. The infection-causing potential of spores from human fungal pathogens is exemplified by *Coccidioides immitis*, as few as 10 spores can establish disease and cause fatal disease [5]. Because these highly infectious spores are adapted for wind dispersal, *C. immitis* spores, similar to spores from *Bacillus anthracis*, have been postulated to be serious threats as biological weapons [6]. Despite the demonstrated capacity of spores from human fungal pathogens to infect mammalian hosts, the specific roles that spores play in establishing disease are less clear.

Many human fungal pathogens are free-living in the environment and infect humans via a respiratory route. For example, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Penicillium marneffei*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii* all grow filamentously in the environment and produce conidia (asexual spores) that are dispersed easily by wind currents [7,8]. Conidia are presumed infectious particles because they are small relative to filamentous hyphal cells, thus increasing their chances of being deposited in the alveoli of the lung and causing an infection [9]. Despite the high likelihood that airborne conidia are natural infectious particles for these human fungal pathogens, relatively little is known about the molecular properties of conidia or how they interact with the host.

Another human fungal pathogen for which there is limited information with respect to spores is *Cryptococcus neoformans*. *C. neoformans* is distributed worldwide and causes meningoencephalitis, primarily in immunocompromised individuals, resulting in approximately one million cases and 600,000 deaths annually [10]. *C. neoformans* is a free-living budding yeast that resides in the environment. The natural route of infection has been inferred from a preponderance of clinical evidence, which indicates that fungal cells (likely yeast or spores) are inhaled by a human host, causing an asymptomatic infection within the lung. In immunocompromised individuals, *C. neoformans* can disseminate from the lung to other tissues, including the central nervous system [11]. The resulting disease is uniformly fatal without treatment [12].

C. neoformans has been found in association with trees, soil, and bird droppings, but sources of spores in the environment have not been identified [13]. In the laboratory, spores can be produced via two distinct pathways: sexual development and monokaryotic fruiting. Sexual development occurs when haploid yeast cells of opposite mating types (**a** and α) encounter one another under appropriate environmental conditions and fuse. The resulting binucleate **a**+α cell initiates a new developmental program and grows filamentously off of the substrate. In response to unknown signals, the terminal hyphal cells produce fruiting structures, called basidia, in which nuclear fusion and meiosis occur [14]. The resulting products are replicated and packaged into spores that are budded onto the basidium surface to produce long chains of spores [15]. The other sporulation pathway, known as monokaryotic fruiting, occurs when α cells in nutrient limiting conditions produce hyphae, basidia, and spore chains in the absence of **a** cells [16]. In nature, sexual development and monokaryotic fruiting have not been observed directly; however, population genetics studies show that recombination occurs in some regions of the world, and in other areas both clinical and environmental samples show a strong bias in favor of the α mating type [17–19]. These findings support the hypothesis that spores exist in nature.

However, because sexual and fruiting structures have yet to be observed outside the laboratory, it has been proposed that the infectious propagules in *C. neoformans* infections

are desiccated yeast [20]. Desiccated yeast in the environment are approximately 1–5 μm in size, making them small enough to lodge in the alveoli of the lung [21,22]. One downside to this proposal is that desiccated yeast are not particularly robust. The yeast tend to die over time, making them less appealing as infectious agents [23]. On the other hand, spores are quite robust and fall into the 1–2 μm range, ideal for alveolar deposition [24]. Because both desiccated yeast and spores are small enough to establish infection within the lower airway, the *C. neoformans* system provides an excellent opportunity to compare the abilities of spores and yeast to cause disease. Ultimately, differences between spore-mediated and yeast-mediated disease in animal models may inform the identities of natural infectious particles.

A direct comparison of the properties of spores and yeast has never been conducted because, until recently, it has been impossible to isolate spores away from yeast and filamentous cells in large numbers. Breakthroughs in density gradient centrifugation now provide a reliable method for isolating large numbers of pure spores [24]. Direct analyses of the physiological, biochemical, and virulence properties of isolated spores implicate *C. neoformans* spores as infectious particles.

C. neoformans spores are adapted to withstand external stress

One key feature of microbial spores, and an important difference between spores and vegetative cells, is that spores are adapted to withstand harsh conditions. *C. neoformans* spores are more resistant than yeast to oxidative stress, high temperatures, chemical insult, and desiccation [24]. Because *C. neoformans* spores are produced on basidia that grow away from the substrate, and spores have been shown to be aerosolized by air currents generated under laboratory conditions, it is likely that spores are aerially dispersed in nature [25]. It is therefore important that spores be adapted to withstand desiccating conditions that they might encounter during aerial dispersal.

Broad stress resistance is a feature of many spores, and in a number of fungi, such as *Saccharomyces cerevisiae*, it has been linked to a thick spore coat [26]. The spore coat acts as a barrier preventing water from leaving spores and keeping deadly compounds out of the spore, which protects the contents from damage [27,28]. One major difference between *C. neoformans* spores and yeast is a thick spore coat, which is distinct from the yeast cell wall (Fig. 1) [24]. This coat likely contributes to the broad stress resistance of *C. neoformans* spores. By protecting the spore from damage, the coat could provide an advantage for spores in surviving dispersal conditions and thus increase their chances of encountering a susceptible host.

C. neoformans spores cause disease in a mouse model of cryptococcosis

If spores act as infectious particles in nature, one would anticipate that they could cause disease in an animal model. Decades of studying the virulence properties of *C. neoformans* yeast have provided a reliable murine model of cryptococcosis. In this model, yeast are inoculated via an intranasal route leading to an infection within the lungs. The yeast then proliferate and are capable of disseminating to the brain, which appears to mimic the proposed natural route of infection and dissemination in humans [29]. When mice in this model are infected with spores, they also develop fatal cryptococcosis [30–32]. An inoculum of as few as 500 spores is lethal in this model, indicating that *C. neoformans* spores are effective infectious particles [25,32].

In addition, when mice are infected with similar numbers of either spores or yeast, the mice become moribund at the same time (~30 days post infection). Despite this identical survival time, the fungal burden within the lung of spore-infected mice 6 days post infection is 10-

fold lower than the fungal burden of yeast-infected mice [32]. Through an unknown mechanism, *C. neoformans* spores are able to overcome significantly lower numbers early during disease progression and cause death at the same time as yeast. These data suggest a potentially significant difference between infections caused by spores and infections caused by yeast.

Thus far, the infectious nature of *C. neoformans* spores has been explored using established type strains that have been selected for laboratory use because they are highly virulent in the mouse model of infection. Yeast from many clinical and environmental isolates harbor varying abilities to cause morbidity in the mouse model [33]. It is possible that many strains that are not particularly virulent as yeast may produce spores that are better suited to cause disease. Additional studies of spores from a diverse population of strains may facilitate the development of a sensitized system for studying the natural route of infection. This system would be ideal for studying the differences between spores and yeast in establishing infection within the host and provide insights into how spores may be better adapted to cause disease.

Spore and yeast interactions with the host are distinct

One striking difference between *C. neoformans* spores and yeast is their interactions with macrophages in culture. A long-standing observation is that *C. neoformans* yeast are not phagocytosed by macrophages in culture in the absence of opsonization [34]. Spores on the other hand are readily phagocytosed by both cultured murine macrophages and primary alveolar macrophages in the absence of opsonins [25,32]. Once inside the macrophages, spores germinate into yeast and grow. These yeast can withstand the reactive oxygen and nitrogen intermediates (ROI and RNI, respectively) produced during the macrophage killing response, proliferate, and escape [35].

This scenario is similar to the one in *H. capsulatum*, in which conidia are readily phagocytosed by alveolar macrophages, and can germinate into yeast that are resistant to the anti-microbial activities of the innate immune system [36]. In this case, phagocytosis of *H. capsulatum* conidia provides a means of entry for the fungus into host macrophages, where rapid germination produces yeast that are resistant to the anti-microbial activities of host immune cells, thus initiating an intracellular parasitic lifestyle.

This proposed mechanism is similar to what is observed for many intracellular bacterial pathogens and is known as the Trojan Horse Model. Using alveolar macrophages as Trojan horses, intracellular pathogens are ferried out of the alveoli and into the bloodstream, thus facilitating dissemination [37]. The mechanisms of dissemination of *C. neoformans* from the lung to the central nervous system are not known. It is possible that the phagocytosis of *C. neoformans* spores provides a means for the fungus to leave the lung and disseminate to the brain or remain dormant until conditions in the host support dissemination.

Spore survival is a race between germination and activation

Although spores can be phagocytosed and grow inside macrophages in culture, this is possible only when the macrophages are not activated [32]. In culture, macrophages are not subject to the natural activation responses of the host, but this response can be mimicked by exposing macrophages to lipopolysaccharide (LPS) and interferon-γ (IFN-γ) [38]. When macrophages in culture are activated with LPS and IFN-γ prior to exposure to *C. neoformans* spores, the spores are phagocytosed and then killed rapidly in a manner that is dependent on ROI and RNI produced by the macrophages [32]. In contrast, opsonized yeast that are phagocytosed by activated macrophages are resistant to macrophage killing mechanisms. Because dormant spores are more resistant to environmental stress than yeast,

one might predict that spores would also be more resistant to macrophage killing. However, given the sensitivity of spores to ROI and RNI, it appears that germination is a period of vulnerability when *C. neoformans* is susceptible to the innate immune response.

Because germinating spores are susceptible to the anti-microbial activities of macrophages, it is paradoxical that spores cause disease as efficiently as yeast in mice. One possible explanation for this finding invokes a kinetic model, which balances competition between spore germination and macrophage activation. In this scenario, although the majority of spores are killed and cleared by activated macrophages, a small number of spores germinate before macrophage activation responses can kill them. These spores (now yeast) can grow in the protected environment of the macrophage and use macrophages as Trojan horses to disseminate (Fig. 2). Because yeast may not be phagocytosed as efficiently as spores in vivo, dissemination in spore-infected mice may be more efficient, leading to the same time-to-disease with fewer persisting cells.

This model highlights the importance of the kinetics of spore germination and macrophage activation for determining whether *C. neoformans* causes disease. As a result, the kinetics of macrophage activation within a host could be an important factor in understanding susceptibility to cryptococcosis. Perhaps a rapid immune response from immunocompetent individuals is sufficient to kill and clear the vast majority of germinating spores; as a result, *C. neoformans* spores are unable to persist within this host. However, individuals with immune system defects may not mount a sufficiently early immune response to attack *C. neoformans* spores during the window of germination. In particular, people infected with HIV show dysfunctional cytokine signaling due to viral infection of CD4+ T lymphocytes and alveolar macrophages that can decrease or delay the innate immune response within the lung [39]. This delayed response could allow spores to germinate into yeast and proliferate, leading to disseminated disease.

Similarly, germination kinetics could be a critical factor in determining the fate of spores when phagocytosed. Perhaps, in immunocompetent people, *C. neoformans* spore germination is not as rapid as macrophage activation, and that is why this seemingly ubiquitous human fungal pathogen is capable of causing disease only in immunocompromised individuals. Conversely, it is possible that the spores of *Cryptococcus gattii*, a closely related fungus that causes disease in immunocompetent people, can germinate rapidly enough to escape macrophage killing and allow this fungus to establish infection even in the presence of a rapid immune response in healthy individuals.

Conclusions

Recent advances in spore isolation have led to the first direct analyses of the properties of *C. neoformans* spores and how these properties contribute to the virulence of this human fungal pathogen. *C. neoformans* spores are more stress-resistant than yeast, indicating a possible advantage during dispersal. Furthermore, the differences between the spore and yeast surfaces lead to different interactions with host immune cells, likely resulting in a fundamentally different disease process within the host.

Further analysis of the properties of *C. neoformans* spores will help elucidate the role of *C. neoformans* spores in the natural process of infection. In particular, uncovering the differences between spores produced by sexual development and spores produced by monokaryotic fruiting promises to provide valuable insights into the natural life cycle of *C. neoformans* and the extent to which each type of spore contributes to disease in humans. Ultimately, further understanding *Cryptococcus* spores will also help shed light on common

mechanisms of fungal pathogenesis that will likely have broad implications for current virulence models and guide future attempts to develop novel therapeutic strategies.

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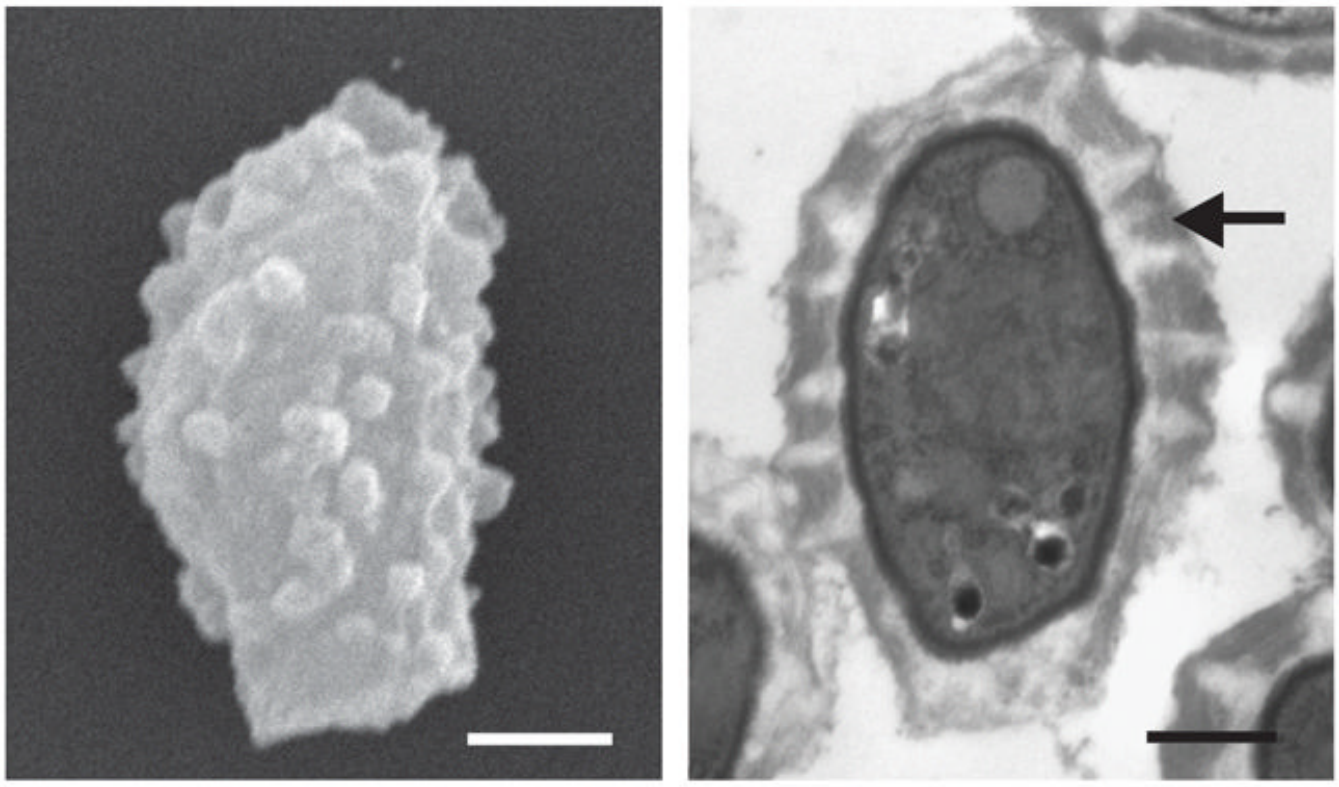

Figure 1. *C. neoformans* spores are covered by a thick spore coat

The left panel is a scanning electron micrograph of a *C. neoformans* spore, showing the characteristic polar morphology with a stalk-like structure on the bottom and a crenulated surface (Bar = 500 nm). The right panel shows a transmission electron micrograph displaying a cross section of a *C. neoformans* spore. The arrow indicates the thick spore coat, which is a heterogeneous structure containing striations of varying electron density (Bar 500 nm).

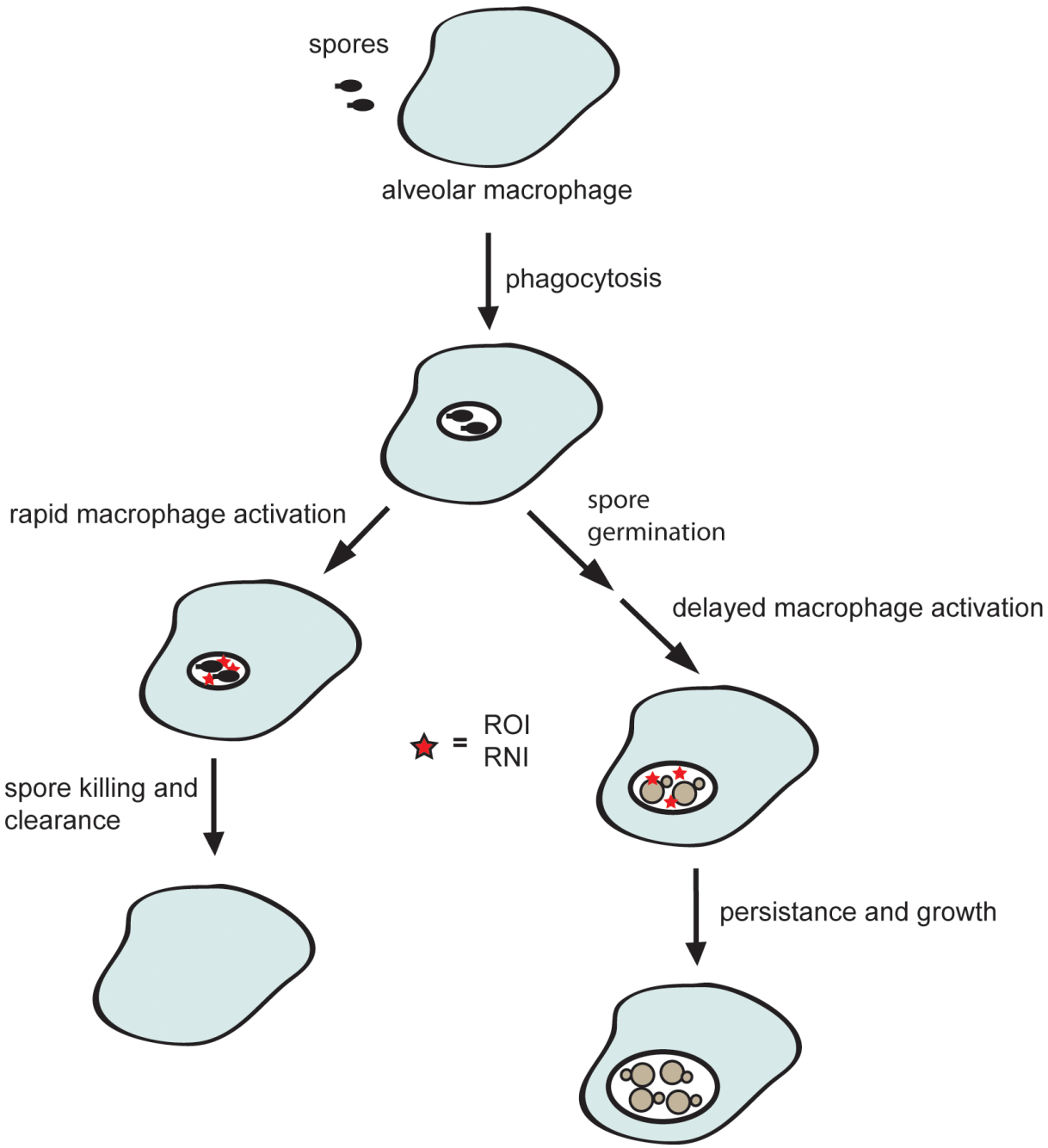

Figure 2. The kinetic model of *C. neoformans* spore-mediated infections

Inhaled spores are phagocytosed by alveolar macrophages. Rapid activation of the macrophage leads to the production of ROI and RNI (stars) before the spores have completed germination, thus killing and clearing the spores. Delayed activation of the macrophages allows the spores to germinate into yeast, which are capable of withstanding the ROI and RNI. These yeast then persist and grow within the macrophage.