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TROPICAL MYCOSES (L MARTINEZ, SECTION EDITOR)

"Feast-Fit-Fist-Feat": Overview of *Free-living Amoeba* Interactions with Fungi and Virulence as a Foundation for Success in Battle

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Abstract

Purpose of Review Free-living amoebae (FLAs) are ubiquitous and can co-habit similar niches and interact with fungi. Herein, we discuss theories on FLAs and the origin, evolution, and conservation of fungal virulence, proposing the "feast-fit-fist-feat" hypothesis that covers the knowledge on FLA-fungi interactions, and could be extended during evolutionarily host escalation. Overall, by bridling this selective pressure, fungi might return to environment and by serendipity, infect superior hosts. The selected traits might grant the fungus with an enhanced capacity to cause damage, or virulence. The fungal virulence factors that might be expressed during infection to amoeba and that grant a fungal benefit during infection to mammals are discussed. However, how they are induced during infection of FLAs is still an open field. Here we discuss also the "Trojan Horse" role of FLAs and the importance of co-infections and disease outcome.

Recent Findings Herein, we discuss also at the molecular level the early steps on how FLAs are able to attach and internalize fungal pathogens. Upon entrance, amoeba interaction might pose selective pressures, and the result is usually a more virulent phenotype of the fungus. Amoeba is able to modulate several fungal virulence factors, most of them with relative importance for infection to superior or more evolved hosts. This interaction fungi-FLAs makes an attractive model for the application of the "One Health" concept in order to avoid new emerging more virulent fungal species.

Summary Amoeba-fungi interactions are still an open field, with several avenues yet to be explored, which might explain the origin of microbial virulence and innate immunity evolution. Several mechanisms of direct or indirect regulation might be involved.

Keywords Free-living amoeba (FLA) · Acanthamoeba castellanii · Pathogenic fungi · Interactions · Receptors · Virulence factors · And pathogenesis

Introduction

Free-living amoebae (FLAs) are ubiquitous protozoa widely distributed in several ecological niches and that can be found both in natural environments such as soil, air, dust, and fresh and saltwater, as well as man-made devices such as air-conditioning, cooling towers, and medical appliances, e.g., contact lenses, medical valves, and prosthesis [1–8]. FLAs, such

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as *Acanthamoeba*, *Naegleria*, *Balamuthia*, and *Sappinia*, frequently cause superficial infections ranging from ocular to skin keratitis, as well as severe granulomatous encephalitis in humans [1, 9, 10].

The FLAs survive in the environment by feasting on surrounding particles as a consequence of their natural predatory behavior in searching for nutrients, with resulting intracellular signaling and particle engulfment for digestion. FLAs could ingest indigestible polystyrene beads, silica and carbon particles, or many microorganisms such as viruses, bacteria, fungi, and algae. In fact, FLAs predilection for nutrients depends on the environment they are exposed to [9, 11]: *Hartmannella glebae* feed preferably on gram-positive instead of gram-negative bacteria, yeast, algae, or moss, whereas *A. castellanii* is a classic mycophagic amoeba.

Once inside FLAs, microorganisms can be completely digested; however, those surviving predation or so-called amoeba-resistant microorganisms (ARMs) find in their intracellular milieu a propitious locale replication and dissemination. Besides, upon contact with FLAs, these ARMs can be protected against environmental stressors such as unfavorable osmotic conditions, extremes of pH, and biocides, making their dissemination and adaptation more effective [9, 11, 12].

The FLAs thereby have gained attention lately by becoming the main pivots of emerging epidemics, not only for being potential human pathogens, causing from keratitis to blindness and amoebic encephalitis [13, 14] but also for its ecosystemic ability to work as "Trojan Horses," being accidental carriers of different endosymbiont microorganisms and reservoirs to medically important bacteria, viruses, and fungi that can also be harmful to the health of animals [12, 15–17].

The diversity of host-pathogens interactions occurring randomly in the environment appears to offer a broad "training ground" for fitness and best adaptation of microorganisms to the intracellular milieu of a wide variety of hosts.

Possibly, the high FLAs phagocytic activity and the built-in capacity of some pathogens (ARMs) to adapt the intracellular microenvironment of amoebas gave rise to endosymbiosis relationships that contributed to microbial pathogenesis development [18]. Upon amoeba death, pathogens could return to the environment with the acquired fitness to adapt and survive the intracellular lifestyle, thus distinguishing themselves from pathogens that have not gone through the same virulence shaping process, and thereby being able to cause disease in greater evolutionarily complex hosts [9, 19]. Bringing Darwin and John Berger into the host-pathogen evolutionary perspective, "the way a pathogen sees a host is completely affected by what it has previously experienced" [20, 21].

Overall, the whole phenomenon seems to follow a similar pattern, independently on the pathogen or phagocytic amoeba in focus, and could have been repeated through evolution on the escalation, from amoeba to other lower complexity host and finally to higher complexity hosts, such as mammals: (i) FLAs "feast" on the microorganism for nutrient acquisition and survival; (ii) microorganism adapts to and survive in the intracellular milieu of the phagocytic amoeba, becoming "fit"; (iii) fitness acquisition allows infection and the "fist" for survival within a more complex host; and (iv) host damage as the "feat outcome" and returning to environment. By using alliterations, we would like to name it as "feast-fit-fist-feat" or "4F" hypothesis (Fig. 1).

FLAs and the Hypotheses for the Origin of Fungal Virulence

One central question in microbial pathogenesis is directed to the emergence of virulence in pathogenic fungi within a group of organisms mostly found freely in the environment. The fungal saprophytic lifestyle in several ecological niches, with a possibility of a wide range of encounters by serendipity with a myriad of environmental hosts and their nutritional flexibility, raises several hypotheses on the origin of virulence of these facultative pathogens. The "accidental virulence" hypothesis states that the origin and conservation of virulence factors of environmental fungi are not necessarily associated with animal contact, but arose from selective pressures by fortuitous interactions with environmental predators in the soil, including FLAs [12, 22].

The "Amoeboid Predator-Fungal Animal Virulence" hypothesis [12] states that fungal successful virulence strategies to mammals may have emerged mainly to overcome amoeba predation [12, 23]. Association to FLAs may have shaped the fungal pathogenic phenotype by allowing the selection/maintenance of virulence factors needed for survival within these phagocytes and a wide variety of environmental hosts. This, along with the transfer or exchange of genes with endosymbionts, consequently, might also have been an evolutionary trigger for the adaptation of pathogenic fungi to the inside of macrophages [9, 12, 24]. In fact, many similar aspects in the interactions between fungal pathogens and FLAs are similar to other phagocytic mammalian cells; many fungal virulence factors have a so-called "dual-use" role being important for the pathogenicity to many environmental hosts, as well as during mammalian infections [12, 25, 26].

The "Amoeboid Predator-Fungal Animal Virulence" hypothesis [12] can also be universally extended to other microorganisms, such as bacteria. *Legionella pneumophila* and *Pseudomonas aeruginosa* are opportunistic environmental pathogens that can actively escape the phagocytosis of amoeboid cells in the environment and also during infections in mammals [19, 27, 28].

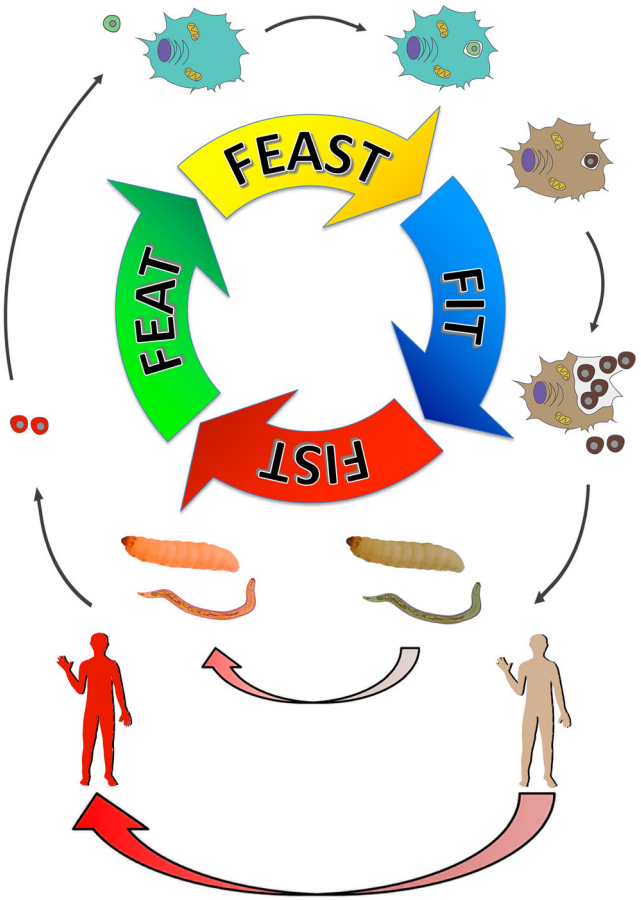

Fig. 1 "Feast-Fit-Fist-Feat" hypothesis: FLAs feast on microorganisms and therefore selecting/maintaining their virulence factors (Fit, according to the previous "training ground hypothesis"). This process could have occurred for the adaptation to more evolutionarily complex hosts upon encounter (Fist) and upon death of the host, the microorganism could have returned to the environment (feat). Then, new interactions with amoeba could have occurred until scalation (cycling) to more evolutionarily complex hosts, such as mammals ("Amoeboid Predator-Fungal Animal Virulence")

FLAs, such as *Acanthamoeba* sp., might act as replicative niche/reservoir for the propagation of fungal pathogens in the environment and a key player for selecting and maintaining fungal virulence factors, which increase their fitness acting as a "training ground" for the endosymbiont ARMs (Fig. 1) [12, 22, 23]. Therefore, new studies on the interactions of FLAs and its surrounding environment, including classes of pathogens that inhabit the same niches as FLAs, would provide great advance into understanding their relationships and importance in the control of microbial populations [9, 12, 16, 17].

Combining all the hypothesis, the "feast-fit-fist-feat" theory would integrate the evolutive participation of the ancient FLAs on virulence selection and maintenance; this process could be repeated as the "training ground" hypothesis and also allows the infection through stochastic events of a more evolutionarily complex host, such as humans, as given specifically by the "Amoeboid Predator-Fungal Animal Virulence."

Amoeba-Fungus Co-Habitation

Saprophytic fungi may eventually encounter and infect several hosts in the environment, including amoeboid, nematode, and insects, categorizing them as nonspecific pathogens [29]. As FLAs are ubiquitous and important in the predatory control of microbial communities [9, 12, 16, 17], many FLAs species, such as *Allovahlkampfia spelaea*, *Vermamoeba* (*Hartmannella*) *vermiformis*, and *Acanthamoeba* spp., share several environmental niches to various fungal species, facilitating possible encounters and interactions between these organisms. Therefore, the dynamics of interaction of FLAs and a wide variety of human pathogens and how they could survive inside amoeboid hosts have not been fully elucidated [12].

Fungal species such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, the thermally dimorphic *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Sporothrix brasiliensis* are saprophytic fungi found in soil, might share similar niches to and likely being able to interact in the environment with *A. castellanii*, recognized in the literature as a classic yeast predator [15, 22, 30]. *C. neoformans* and *H. capsulatum* are commonly isolated from pigeon droppings, where FLAs can also be regularly found [31]; in arid environments, amoebae could interact with *Coccidioides immitis* [22]. In fact, the real frequency of contacts between FLAs and fungi in the environment has not been evaluated, but their co-occurrence is believed to be fairly high [4, 30, 32, 33].

Some fungal species such as *Mallassezia* sp., anthropophilic dermatophytes, and the commensal yeast *Candida albicans*, frequently acquired by contact between individuals using complex mechanisms of interaction, display higher dependence on a host for survival and replication; however, they can be transiently isolated from the environment [34–36]. Although they are less likely to survive for longer periods in the environment and face *A. castellanii*, they could fairly adapt to the mammalian host upon infection and encounter this amoeba during co-infections [35, 37–39].

Amoeba-Fungus Interactions

Several pathogenic fungi are part of the ARMs group, and understanding their relationship with FLAs might provide evidence on how these organisms scaled up in the numbers of possible hosts and evolutionarily acquired the capacity to cause disease in humans [9, 40].

The first observation of a fungus-amoeba association dates from 1930, with the isolation of an amoeba from a culture of *Cryptococcus pararoseus* [41] and posteriorly characterized

as *Acanthamoeba* (*Hartmanella)* sp. [42]. Later, it was demonstrated that besides *C. neoformans* [43], they could phagocytose and feed on *Candida* (*Torulopsis) famata* and *C. parapsilosis* [44].

From the 1970s onward, the interaction between FLAs and *C. neoformans* became the subject of important studies. Among them, Bunting et al. demonstrated that *A. polyphaga* was capable of phagocytosing and killing *C. neoformans*, highlighting its capacity of regulating the fungus population in the environment [45, 46]. However, surviving *C. neoformans* recovered from *A. polyphaga* trophozoites displayed the more resistant pseudohyphae phenotype, despite their hypovirulence in mouse models [45, 47]. Additional studies demonstrated that *A. palestinenses* could co-exist with *C. neoformans* in pigeon droppings, and, by interacting and killing the yeast, could also limit its spread in the wild [46]. *C. neoformans* interactions with *A. castellanii* and *Dictyostelium discoideum* have also been well elucidated in several studies over the years [40, 48, 49]. Therefore, due to association with several fungal species, *A. castellanii* has been recognized as one of the most important mycophagic amoebas and by far the most studied FLA model to address their interactions with fungi [9, 12].

Several findings show that yeasts are more likely detected within *A. castellanii* and probably more ingested than hyphal forms. In fact, fungal dimensions might corroborate to fungal escape by simply engulfment limitation; distinct shapes and cell wall composition might also alter fungal recognition by FLAs and dictate their mycophagic capacity as observed for *Protostelium aurantium* [50].

A. castellanii Fungal Recognition Receptors

A. castellanii interaction capacity to a myriad of microorganisms has been fairly recognized; however, the molecular mechanisms involved in this phenomenon have been unknown for years [9]. To address this subject, our group recently performed a multivariable study to characterize the kinetics of interactions between *A. castellanii* and fungi, including the classical yeasts *C. albicans*, *C. neoformans*, and *S. cerevisiae* and the thermally dimorphic fungi *H. capsulatum*, *P. brasiliensis*, and *S. brasiliensis*, all displaying different cell wall compositions and offering distinct interaction scenarios [15]. The initial evaluation demonstrated that amoeba-fungus association is accumulated as a linear function at early timepoints or small multiplicity of infection (MOI, or also defined as fungi to amoeba (fungi:amoeba) ratio) (Fig. 2). Increasing both variables yielded in enhanced fungus-amoeba interaction rates, despite an overall differential decrease on curve slopes, indicating interaction saturation and the suggestive participation of receptors intermediating *A. castellanii*-yeast associations (Fig. 2). Time-lapse experiments showed vomocytosis (exocytosis; cell extrusion) events for all aforementioned fungi, which started from 15 to 80 min upon interaction. In the same study, it was possible to visualize the free trafficking of amoeba-resistant yeasts between different trophozoites units [15].

The hypothesis of a receptor-mediated fungal attachment and internalization by *A. castellanii* led us to characterize two amoeba surface "universal" receptors that were able to bind and recognize all fungi tested. Both proteins belonged to the superfamily of mannose-affinity lectins identified as mannose-binding protein and mannose-binding protein-1 (MBP and MBP1, respectively, Fig. 2). Inhibitions with soluble mannose further confirmed the high dependence of these MBPs on fungi-amoeba interaction; *C. albicans*, a fungus with a highly mannosylated cell wall, had the mostly impacted interactions.

However, the impact of the recognition through mannose receptors (MR) on fungal survival in the intracellular environment of phagocytic cells and the selection of fungal virulence factors are yet to be confirmed [15].

Overall, MBPs' engagement on fungal mannans and mannoproteins recognition appears to have a fundamental role and to be a conserved mechanism through evolution, as observed also in mammalian phagocytic cells, such as macrophages and Langerhans cells [51–53]. MRs belong to the family of C-type lectin-receptors (CLRs) and consist in one of the main pathogen recognition receptors (PRR) engaged in antifungal immunity [53]. In fact, these MRs are essential for triggering mammalian pro-inflammatory antifungal immune responses such as phagocytosis, oxidative stress, cytokines, and chemokines production by innate immunity effector cell and activation of adaptive immunity via Th17 responses [51–54].

Fungal Fate upon Interaction with FLAs

Upon the predatory activity of FLAs, several fungi appear replicate and develop resistance, and survival strategies, which are similar to those developed against a myriad of hosts [12, 25, 26, 55]. The fungus *C. neoformans* was phagocytosed and able to survive killing by the amoebas *D. discoideum*, and escape from its interior through exocytosis (vomocytosis). A similar phenomenon has been described upon interaction with macrophages [56].

Among the amoeba-resistant fungi described in seminal studies that elucidate fungal survival and adaptation exclusively to the *A. castellanii*, it is possible to highlight the model fungus *S. cerevisiae* [15, 57] and clinically relevant pathogenic fungi such as *Blastomyces dermatitidis* and *H. capsulatum* [15, 49], *S. schenkii* sensu stricto and *S. brasiliensis* [15, 58], *P. brasiliensis* [15, 30], *Aspergillus fumigatus* [59], *C. albicans* [15, 37], *C. auris* [60], *C. neoformans* [15, 40, 61], and *F. solani* [62].

A. castellanii was able to digest and kill *S. cerevisiae*, with 50% of viability decay within 90 min upon interaction [15, 57]. The viability of the yeasts of *H. capsulatum* was

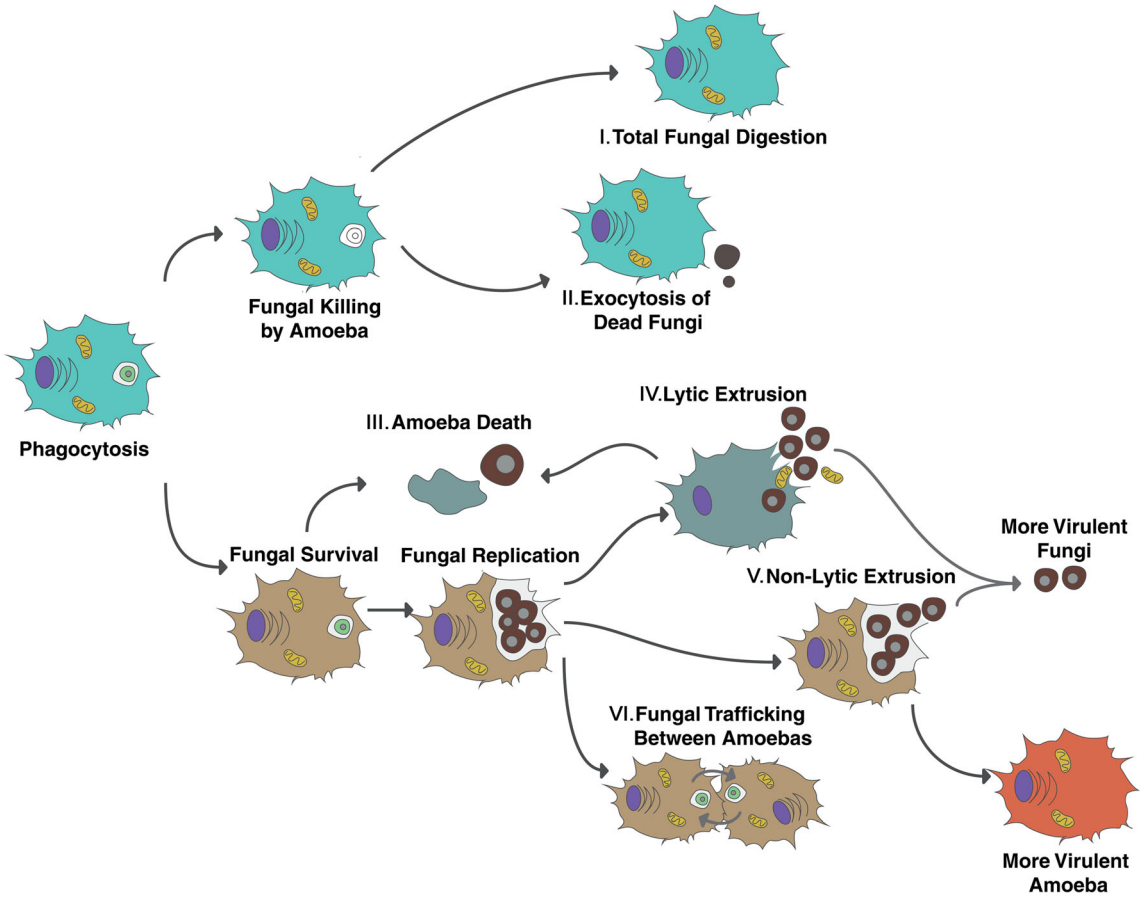

Fig. 2 Illustrative representation of the sequential steps of interaction between *A. castellanii* and fungi. Both time and multiplicity of infection (MOI) contribute to attachment and internalization of fungi by amoeba, which appears to involve at least two mannose surface receptors, a mannose-binding protein (MBP) and mannose-binding protein-1 (MBP-1). As time progress, fungus might develop strategies to survive and replicate within amoeba. Fungal no lytic exocytosis, or vomocytosis, occurs at later time points, which varied according to the fungal species. Upon this process, both fungal and amoeba viability are maintained. Fungi might infect other trophozoites, characterizing as a free traffic among distinct *A. castellanii* units

dramatically decreased (~90%) within 6 h of co-incubation with *A. castellanii* [15]. However, despite its highly predatory behavior, *A. castellanii*-fungus interaction outcome is not always fungal mortality. The viability of *P. brasiliensis* and *S. brasiliensis* yeasts remained unaltered up to 48 h of co-cultivation with *A. castellanii*, whereas *C. albicans* and *C. neoformans* drastically increased in numbers [15]. In fact, it was later demonstrated that phagocytosis of either *Sporothrix schenckii* sensu stricto or *S. brasiliensis* by *A. castellanii* triggered conidial and hyphal growth within 72 h of co-incubation [58]. *A. castellanii* also enhanced the growth of the filamentous fungi *Fusarium oxysporum* and *F. solani*, with conidia germination occurring within the trophozoites [62, 63].

In summary, for the fungus *C. neoformans* and possibly other fungi upon interactions with *A. castellanii*, three main scenarios can take place (Fig. 3): fungi could be killed by amoeba with (i) total digestion of fungal cells or (ii) exocytosis of undigested fungal elements; fungal survival might occur along with (iii) rapid amoeba death, with possible digestion by fungal hydrolases [23], or (iv) fungal replication with mechanical lysis of amoeba (lytic extrusion) by pseudohyphae formation or conidia germination [23, 47, 49, 64]; and survival of both fungi and amoeba with (v) fungal replication and non-lytic extrusion (vomocytosis) [15, 48] and (vi) fungal-trafficking, with exocytosis and entrance to new neighboring amoeba cells.

Fungal survival might be accompanied with the possibility of higher virulence as an outcome (Figs. 3 and 4) [15]. However, these events are strictly dependent on the nutritional conditions that the amoeba is exposed to, the fungal species and even the variability among fungal strains [65].

For *Cryptococcus* sp. resistance to amoeba phagocytosis and killing might be first correlated to the expression of several virulence factors, including capsular polysaccharides and melanin that provide physical barriers to amoeba antifungal responses. Additionally, the resistance to phagocytosis and killing by amoeba has been also linked to the expression of 3-hydroxy- fatty acid by *C. neoformans* protecting the fungus against the membrane permeabilizing amoebapore [66, 67].

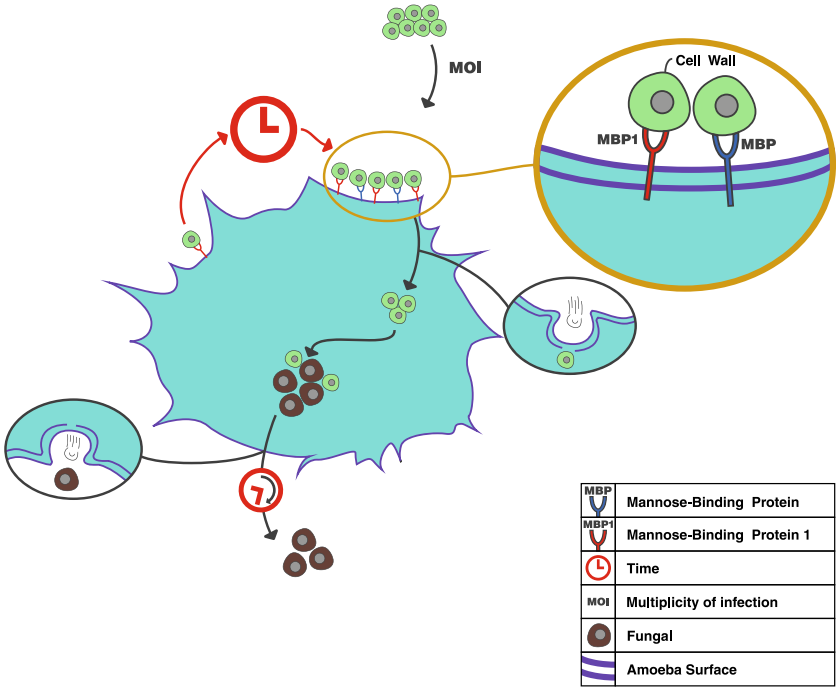

Fig. 3 Schematic representation of known events that may occur after the interactions between *A. castellanii* and pathogenic fungi. The results after the amoeba phagocytosis of the fungi are variable depending on the fungal counterpart. Upon amoeba (cyan) phagocytosis it might be killed, with (i) total digestion of fungal elements or (ii) killed fungus undergoes exocytosis. When the outcome is fungal survival, upon phagocytosis results might be (iii) rapid amoeba death, or fungal replication within amoeba and (iv) lytic extrusion, resulting also in amoeba death. Fungus might escape amoeba milieu by (v) non-lytic extrusion, and fungus might infect other amoebas, configuring free trafficking among them. When the outcome in fungal (brown) and amoeba (orange) survival, both display a more virulent phenotype

Fungal Virulence Factors and FLAs

Although amoebae are capable of killing some fungal species, amoeba-resistant fungi can undergo modifications that affect their virulence (Fig. 4). Therefore, only few studies addressed the emergence or alterations of virulence among FLAs-interacting fungi, to highlight species such as *C. neoformans*, which is by far the most characterized, and *Aspergillus fumigatus*, and how they can contribute for the fungal adaptation to the powerful response of mammals against infections [22, 68, 69].

Thus, against the pressure exerted by amoeboid predators, factors such as metabolic adaptation, dimorphism, and capsule are essential for fungal survival (Fig. 4) [12, 25, 26]. Herein, we discuss, specifically on the prism of individual virulence determinants, how this modulation by FLAs might occur.

Fungal Dimorphism

Fungal dimorphism with the transition to the more resistant filamentous is an important feature for fungal survival. The conversion into hyphal forms provides resistance by physical constraints against the predatory activity of FLAs and might cause mechanical disruption of the trophozoite [12, 70].

In the presence of *A. polyphaga*, yeasts of *C. neoformans* differentiate to pseudohyphae that demonstrate greater phagocytosis resistance and survival, as opposed to yeasts that are promptly internalized and killed by either amoebas or macrophages [45, 47]. Similarly, *C. albicans* differentiate into hyphae in the presence of *D. discoideum* [71].

Upon phagocytosis by trophozoites of *A. castellanii,* the yeasts of the thermally dimorphic fungus *H. capsulatum*, *B. dermatitidis*, *Sporothrix schenckii* sensu stricto, or *S. brasiliensis* can trigger the conversion to hyphal forms at 37 °C, which is usually a temperature permissive for yeast growth [49, 58]; therefore, interactions with FLAs might consist of an alternative to overcome thermic regulation and promote fungal morphological transition [15, 30].

FLAs also induce conidia germination into hyphae in other models: *A. fumigatus* conidia in contact with *V. vermiformis* [64, 72], and *F. oxysporum* and *F. solani* co-incubated with *A. castellanii* demonstrated enhanced conidia germination and growth of filamentous forms [62, 63].

Metabolic Adaptation

Upon interaction, fungal metabolic adaptation to the intracellular milieu of either amoebae or any other phagocyte is crucial for survival. During lung infection in mammalian models, *C. neoformans* overexpresses transcripts related to carbon metabolism (GPA1, PKA1, PKR1, and RAS1) [73–75],

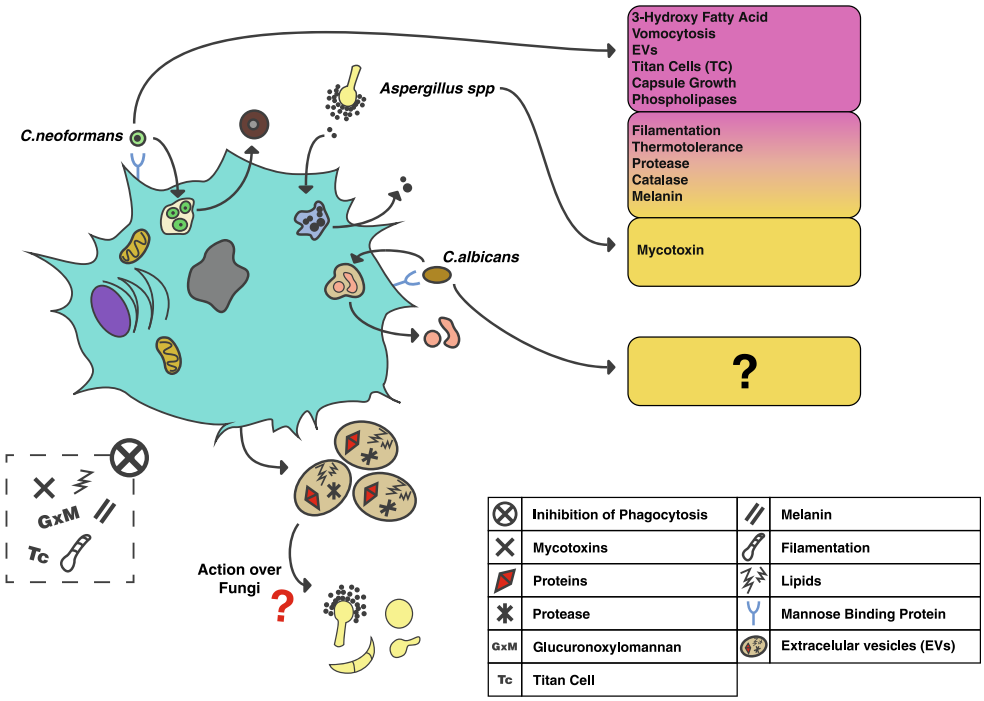

Fig. 4 Summary diagram of the virulence factors of the main pathogenic fungi described in the context of interaction with *A. castellanii*. Important virulence factors are selected exclusively in *C. neoformans* (magenta), after the fungus has passed through the amoeba. *A. fumigatus* undergoes selection of common (magenta to yellow gradient) or exclusive (yellow) virulence attributes to *C. neoformans*. For *C. albicans*, virulence changes upon contact with amoeba are uncertain and yet to be described (question mark). Other fungal virulence factors related to the escape of phagocytosis and metabolic adaptation to amoeboid predators and are represented by symbols (Adapted from [12]). Virulence change might be the result of direct interactions or indirect interactions with its products

transporters for monosaccharides, metals, and acetate and those related to stress responses [76].

Gene expression of *C. neoformans* was compared during in vitro infections of macrophages and amoeba [77]. Among the most differentially expressed genes in both hosts, the ORF CNAG_05662 (named PTP1, Polyol Carrier Protein 1), characterized as a transporter of 5 and 6 carbons sugars, was twofold more expressed in amoeba than macrophages. Recently, Gerstein *et. al.* [78] evaluated the polymorphisms on the sequence type ST93 genomes of *C. neoformans,* identifying 40 potential gene candidates that could impact clinical outcome and host survival. From these, 17 gene deletion strains were tested in murine models and 6 proved to directly influence mouse survival. These included the deletion strain for the same aforementioned gene CNAG_05662, encoding an ITR4 (inositol transporter 4, previously named PTP1), which was overexpressed in clinical isolates and involved in adaptation to inositol-enriched environment, such as the central nervous system (CNS).

Capsule

Among the virulence factors that may have arisen as a result of the interaction with amoeboid predators, the most known is the cryptococcal polysaccharide capsule, which provides phagocytosis resistance in addition to facilitating the escape of the host's immune system [12, 75, 79–81]. Capsular enlargement appears to be a critical conserved cryptococcal sensing response for the general presence of phagocytes. Polar fractions (upper phase) of either *A. castellanii* or macrophages obtained by Folch fractionation induced capsular enlargement and exopolysaccharide release in a wide range of conditions. Mutants lacking phospholipase B (*plb-/-*) were unable to express such phenotype in contact with either viable phagocytes or their respective polar fractions, but promptly reacted to exogenously added glycerophosphocholine (GPC) or glycerophosphoethanolamine (GPE) in vitro. Therefore, the "cryptococcal phagocyte-sensing" mechanism appears to be dependent on the sequential action of fungal proteases, releasing host phospholipids from phospholipids-proteins complex, and PlB enzymatic digestion of host phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) into their polar heads GPC and GPE, respectively, that in turn could rapidly induce capsule enlargement [82].

Titan Cells

C. neoformans has the ability to modulate its size during infection, with the capacity to form abnormally large "titan" blastoconidia (10–100 μm) [74, 81, 83]. Titan cells large sizes, along with reshaping of cell wall contents by increased chitin and decreased glucans, facilitate the escaping and modulation of host immune system recognition, with deleterious anti-inflammatory immune responses [84]. Conditions such as low nutrients, serum supplementation, static incubations, and high CO_2 concentrations can also promote cell enlargement and titan cell formation [85]. Moreover, low cell densities appear to be crucial, as quorum-sensing molecules such as pantothenic acid, peptide Qsp1p, and phospholipids could also regulate titan cells [74]. This phenomenon appears to be a complex cascade of signaling pathways involving positive

and negative regulators [74, 83]. Overall, titan cell formation appears to play a role in virulence for the establishment of pulmonary infections, fungal dissemination, and long-term host persistence [86].

C. neoformans sensing of host cell membrane phospholipids of *A. castellanii* results in capsular enlargement, along with increased dimensions of the fungal cell body, and the formation of "titan-like cells" [82]. Thus, titan cell formation also appears to be a conserved mechanism of physical impairment and escaping phagocytosis triggered by *C. neoformans* against evolutionarily distinct phagocytes, as described in *A. castellanii* [82], hemocytes of *Galleria mellonella* [87] and macrophages [82, 88].

Melanin

Fungal melanin can be defined as highly diverse and structurally complex polyphenolic pigments produced by several fungal species using a multitude of substrates and complex pathways [89, 90]. The DOPA-melanin synthesis by many pathogenic fungi involves the initial hydroxylation of L-tyrosine into L-DOPA, the precursor of many catecholamines, and these are in turn oxidized by the expression and activity of laccase/phenol oxidases into quinones that can polymerize into eumelanin [90]. L-tyrosine can also be deaminated by the action of aminotransferases into 4-hydroxyphenylpyruvate (4-HPP), that is sequentially converted by dioxygenases into homogentisic acid, which in turn undergoes oxidation to benzoquinone acetate that polymerizes into pyomelanin [89–91]. The DHN melanin, commonly expressed in conidia of *Aspergillus* sp., is produced by the linkage of acetyl-CoA molecules through the polyketide synthesis pathway, with the sequential action on synthases/dehydratases/reductases enzymes to form the precursor 1,8-dihydroxynaphthalene, for the further polymerization into DHN-melanin [59],

Upon synthesis in melanosomes, fungal melanin is exported to the cell wall [92] and acts by protecting fungi from environmental harsh conditions such as high temperatures and desiccation, radiation, and oxidative and osmotic stress [89]. In vivo, melanin is a potent immunomodulator, inhibiting the fungal cell wall recognition by phagocytes and also serving as a potent antioxidant, enhancing fungal tolerance to phagosomal oxidative attack and better-overcome killing by macrophages [93].

Fungal melanin also has important contribution to resistance against environmental predators by shielding the fungal recognition by amoeba and nematodes [40]. *A. fumigatus* melanized conidia had lower internalization rates by *D. discoideum* when compared to non-melanized structures. Ingested non-melanized conidia induce rapid phagosomal formation and transient acidification, with subsequent neutralization and conidia exocytosis; in contrast, for melanized conidia, this process was significatively prolonged, and therefore establishing a germination niche inside the amoeba, consequently facilitating fungal survival, conidia aggregation, and amoeba rupture [59]. Therefore, DHN melanin in *Aspergillus* spp. appears to have the capacity of prevention of phagolysosome acidification in amoebas and macrophages, guaranteeing survival within these phagocytes [23, 44, 94].

Despite the evident importance of melanin during infection, whether pathogenic fungi could have the melanin synthesis induced upon interaction with amoeba is still unclear. The expression levels of melanin synthesis-related enzymes have not been pursued in any fungal model; however, this hypothesis is completely plausible, as some precursors for the different melanin synthesis pathway can be found in *A. castellanii* [95]. A phenoloxidase believed to be a laccase was highly expressed during encystation; however, its expression level in trophozoites is unknown. This enzyme was not able to oxidize tyrosine, but its activity on other catecholamines was not determined. Additionally, the expression of other functional phenoloxidases cannot be discarded [96]. *A. castellanii* expresses enzymes involved in the tyrosine metabolism, such as tyrosine aminotransferase [KEGG enzyme entry EC:2.6.1.5] [97] and 4-HPP dioxygenase [EC:1.13.11.27] for the production of homogentisate and other precursors for the pyomelanin synthesis. Despite the known abundance of metabolites such as acetyl-CoA that serve as precursors of DHN-melanin, the genome of *D. discoideum* contains two polyketide synthases that fuse to form the "Steel complex" that catalyzes the formation of 4-methyl-5-pentylbenzene-1,3-diol (MPBD) involved in the induction of spore maturation [98]. Apparently, the expression of the described enzymes is upregulated under stress, with consequent induction of sporulation; therefore, they could also increase the intracellular availability of the fungal melanin synthesis precursors, enhancing fungal resistance.

Interactions with FLAs and Potential Impact on Fungal Thermotolerance

Thermotolerance, or the relative capacity to survive and grow at a given temperature, becomes essential for replication in a host and the establishment of successful infections. Thermotolerance in fungi infecting warm-blooded hosts [99] provides genomic stability and yielding the capacity to grow at temperatures around 37 °C, which is essential to pathogenicity in mammals [55, 100].

Several overlapping signaling pathways seem to regulate the expression of many virulence factors, contributing to adaptation to a multitude of external environmental stressors, such as high temperature, osmotic, UV irradiation, and osmotic and oxidative stress [99, 101, 102]. Capsule enlargement in *C. neoformans* as a response to stress involves the cAMP pathway and Pka1 activation, which in turn activates Nrg1

and Rim101 transcription factor that leads to the induction of expression of genes involved in capsule synthesis [103]. Ssa1, from the Hsp70 family, functions as a stress-related transcriptional co-activator for fungal virulence, regulating the expression of laccase and capsule induction [104]. The HOG pathway negatively regulated capsule induction and laccase expression [105]. In fact, the Hog1 protein might act as a repressor for the Ada1, which encodes a putative DNA-binding, that regulates in turn the expression of capsule and other virulence related attributed to *Cryptococcus* such as thermotolerance, filamentation, and antiphagocytic response [103]. Thus, as these pathways are also involved in sensing and responding to temperature stress, it is reasonable to think that fungi expressing enlarged capsules, melanin, or other virulence factors for the adaptation to stress imposed by the intracellular milieu of FLAs could also upregulated the pathways involved in adaptation to higher temperatures and therefore display a thermotolerant phenotype [55].

Additionally, capsule and melanin induced from the adaptation to FLAs could also have direct implications on fungal resistance to water loss and desiccation induced by high temperatures [106]. Once better adapted to higher temperatures, these fungi could display success in infection to homeothermic hosts, such as mammals [12, 22].

Additional Virulence Factors

Several other virulence factors involved in mammalian fungal pathogenesis have already been described as environmental resistance factors, such as fungal hydrolases, iron acquisition systems, and mannitol [12]. Fungal proteases, phospholipases, lipases, and ureases are pivotal for the acquisition of nutrients and metabolism, and are considered truly virulence factors as they can damage directly the host cells. In *C. neoformans* as mentioned, proteases along with phospholipase (PlB) activities could trigger the "host sensing mechanism" of capsular enlargement, with direct implications on resistance to *A. castellanii* predation, as well macrophage phagocytic activity [40, 82].

Amoeba produce amoebapore, an amoeba-specific effector peptides released in the phagolysosome with membrane-permeabilizing activity against several pathogens [107]; however, hypothetically, fungal proteases could contribute to escaping the action of these molecules and fungal survival [66]. Similarly, fungal proteases also digest C3 proteins, as a complement system evasion mechanism, resulting also in escape to opsonization and phagocytosis by macrophages [108].

Mannitol production is widely known for its accumulation in vivo and direct implications on capsular enlargement during pathogenesis of *C. neoformans* in mammals [109]; however, its importance in the interaction with amoebae remains unclear [12]. Urease is related to nutrient acquisition, and involved in resistance to acidic environments through the synthesis of ammonia and involved in non-lytic exocytosis, as already described for macrophages. Absence of urease seems not to impact yeast survival when co-incubating with *A. castellanii*; however, its importance for intracellular adaptation within amoebas is yet to be determined [12, 110].

The production of toxic secondary metabolites by *A. fumigatus*, such as gliotoxin, could also be an important attribute of fitness, representing yet another important escape mechanism, as it appears to be lethal to *D. discoideum* [13]. Gliotoxin is also characterized by inhibiting phagocytosis of macrophages and functioning of other immune system cells [111]. In fact, these findings indicate that mycotoxin secretion is conserved, with universal antiphagocytic properties [91, 112, 113].

Interactions of the soil saprophytic *Paracoccidioides* spp. fungi with amoeboid hosts are also associated with the selective pressures and maintenance of virulence. Sharing the same environment, *A. castellanii* can internalize *Paracoccidioides* sp. and enhance fungal virulence by increasing the cell wall deposition of polysaccharides, such as α-1,3-glucan. A similar phenotype, which is also observed during filamentous-to-yeast conversion at 37 °C, results in masking of the recognition by PRR on innate phagocytes, favoring *Paracoccidioides* sp. mammalian infections [30].

Exposure of avirulent strains of *H. capsulatum* maintained for a long periods under laboratory cultivation to *A. castellanii* was able to select or induce fungal phenotypes capable of causing persistent infection and increases pulmonary inflammation in murine models upon intranasal infection, when compared to control yeasts kept in culture [80]. Additionally, in a relevant study in this sense, we have observed that yeasts of *H. capsulatum*, *P. brasiliensis*, *S. brasiliensis*, *C. neoformans*, *C. albicans*, and *S. cerevisiae* recovered upon interaction with *A. castellanii* and injected into the Lepidoptera *G. mellonella* model killed larvae more quickly than those that did not pass through the amoeboid intracellular environment [15]. However, changes in the expression of singular virulence factors or on the virulome during passages through *A. castellanii* or other FLAs are yet to be determined.

Amoebas as "Trojan Horses" for Fungi

The clear role of FLAs as serving as a favorable place for protection against adverse environmental conditions makes fungal survival in the environment more successful [9, 15]. Lately, *Acanthamoeba* sp. has received attention for being possible "Trojan horses" for fungi [12, 18, 22], with the possibility to harbor and carry agents to places such as eyes and central nervous system (CNS). Co-infection cases of *A. castellanii* and *Fusarium* sp. have been increasingly reported, mostly linked to the use of contact lenses [114]. Interaction

of both organisms during co-infection might be a complicating factor, since it affects the virulence of both organisms to humans [62], emphasizing the importance of diagnosis for the simultaneous detection [115].

Both *C. neoformans* and *Acanthamoeba* sp. have tropism for the CNS. *C. neoformans* seems to sense neurotransmitter molecules such as epinephrine, DOPA, and norepinephrine, which are important substrates for melanin synthesis [116], dictating yeast resistance and fungal neurotropism [117, 118]. The exact mechanism of *A. castellanii* neurotropism is unknown; however, the simple fact that this protozoa is chemically attracted by expression of receptors to neuromediators cannot be excluded [119]. Although there are no cases of *C. neoformans* and *Acanthamoeba* sp. co-infections in the literature, hypothetically, they could synergistically increase the penetrability of both organisms in the CNS and possibly enhance the synthesis of fungal melanin, which, in parallel, contributes to the suppression of host responses [120]. Both given examples, among many other possibilities, could result in worst prognosis of the mycoses.

Future Trends

Fungal virulence is only expressed in a host. In general, many mammalian models of infection can mimic fungal pathogenicity to humans. As many models are linked to ethical restrictions, alternative infection models could be widely accessed to understand fungal pathogenesis in vivo [121].

Phagocytes appear to have similarities on the way they recognize fungal pathogens. Upon interaction of FLAs and macrophages with fungi, phagocytosis occurs through a "coiling process," with the lateral emission of pseudopods that rotates around the engulfing particle, originating whorl-like structures [19]. Similarities go beyond the morphological, structural, biochemical and motility features, and receptor levels, suggesting a convergent evolution of both organisms [122]. FLAs have become an attractive model to understand the evolution of innate immunity cells [70].

Therefore, and due to the extensive association with several fungal pathogens, FLAs such as *A. castellanii*, *A. polyphaga*, and *D. discoideum* consist of a cheap system with no ethical implications and have been widely used as models for studying host-parasite interactions and characterization of symbionts [49, 71, 123]. Besides, they are easily grown in defined axenic environments, offering the possibility of setting up several controllable experimental variables.

Determining at the molecular level how fungi interact with FLAs may pose a promising strategy for understanding evolutionary relationships and origin of virulence in fungi. Fungal surface recognition by amoeba occurs via mannose receptors; therefore, the participation of other receptors of distinct nature cannot be ruled out and need to be pursued. Additionally, it is extremely necessary to understand the cellular biology of FLAs and comprehend the direct selection mechanisms imposed by the intracellular milieu of these organisms that result in fungal virulence enhancement. In addition, *A. castellanii* is able to synthetize and secrete extracellular vesicles (Fig. 3) of complex composition with several components potentially able to indirectly modulate fungal virulence, from genetic to metabolic level.

The establishment of fungal FLAs-fungi co-infection or "Trojan horse" models is an unexplored field and would essentially allow us to understand whether these symbiotic relationships promote the access of fungal cells to environments easily accessible to amoebae or vice-versa, with a huge impact on pathogens surveillance and public health.

Lastly, the environmental association of FLAs with epidemiologically relevant fungal species and their role as carriers of endosymbionts fungal pathogens direct and indirect impact on human health, making this relationship perfectly suited to the concept of "One Health." Controlling the spread of FLAs species to new environments might prevent new FLAs-fungi encounters, and thereby the emergence of new of more virulent pathogenic fungal species. The continuous evaluation of the interacting and replicative capacity of new or emerging fungal species in commonly found environmental FLAs and their impact on the expression of fungal virulence factors is also of extreme importance [102, 124]. Both approaches could certainly contribute to avoid new potential fungal infections to humans.

Compliance with Ethical Standards

Conflict of Interest Marina da Silva Ferreira, Diego de Souza Gonçalves, Elisa Gonçalves Medeiros, José Mauro Peralta and Allan J. Guimarães declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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