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Exploiting Lipids to Develop Anticryptococcal Vaccines

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Abstract

Purpose of Review *Cryptococcus* spp. are responsible for life-threatening infections in humans causing mortality rates of 70% in developing countries. Antifungal therapy to combat cryptococcosis is based on the combination of amphotericin B, azoles, and 5-flucytosine. However, treatment failure is frequently triggered by antifungal resistance, drug-drug interactions, and toxicity. New alternatives to prevent cryptococcosis are imperative. Here, we discuss the roles of lipids in the immunological control of the disease caused by *Cryptococcus* spp.

Recent Findings Recently, remarkable advances on immunology of fungal infections have been made and a number of studies indicated the potential of vaccine formulations to combat cryptococcosis. New formulations exploiting virulence regulators and genetically modified attenuated strains have been tested. In this context, lipids have emerged as virulence regulators and immunogens to be explored.

Summary Glucosylceramide (GlcCer), sterylglycosides (SGs), and lipid-containing extracellular vesicles have been recently tested in vaccine formulations and their anticryptococcal efficacy was confirmed in vivo. Together, the data discussed here encourage the use of fungal lipids in anticryptococcal vaccinal strategies.

Keywords Cryptococcosis · Immunogenic lipids · Glucosylceramide · Sterylglycosides · Extracellular vesicles · Antifungal vaccine

Introduction

The predominant species of *Cryptococcus* involved with lethal infections in humans are *Cryptococcus neoformans* and *Cryptococcus gattii* [1]. The disease caused by these pathogens is called cryptococcosis, which usually begins after inhalation of infectious propagules that colonize the lung resulting in clinical or sub-clinical symptoms and signs of pneumonia [1, 2]. The lack of ability to control fungal growth or/and to eliminate the fungus in the lung can be followed by its dissemination to other organs and tissues [2–4]. In immunocompromised patients, *C. neoformans* frequently disseminates to the brain, causing meningoencephalitis, which is one of the major causes of death in HIV patients [1, 5]. Global incidence of meningoencephalitis due to *Cryptococcus* spp. was estimated in approximately 200,000 cases per year [6]. Current antifungal therapy to combat cryptococcosis is limited to a combination of drugs that comprise amphotericin B, azoles, and 5-flucytosine (5-FC) [7, 8]. However, treatment is frequently accompanied by pitfalls that cause failure or interruption of drug administration [8, 9]. These drawbacks include primarily adverse side effects and the emergence of resistance [8–10]. Furthermore, anticryptococcal therapeutic protocols are considerably long and very expensive [8]. Drug-drug interactions are frequent and high toxicity is common [8, 9]. Considering that treatment failure during cryptococcosis results in high rates of mortality and morbidity, new strategies for treatment or prevention are imperative. There are

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simply no vaccines in the clinic against any fungal infections. The research and development in this area has been scarce, most likely because it is difficult to envision a protective immunity induced by a fungal vaccine when fungal infections mostly occur in the condition of immunodeficiency. However, recent investigations of new vaccine formulations to combat fungal infections have re-emerged in the last decade, mainly because a better understanding of the antifungal immune response prompts the elaboration of novel vaccination protocols and immunotherapeutic strategies [11–14]. The major focus of this review is to discuss the most recent published work using lipids as tools to induce, and perhaps preserve, a robust host response that would protect the host from cryptococcosis (and perhaps other fungal infections) even in the condition of immunodeficiency.

Antifungal Vaccines

Vaccination has been successfully used to prevent human diseases caused by several infectious agents, mostly bacteria and virus [15–17]. However, after decades of research, only a few vaccine formulations have reached clinical trials to combat fungal infections, and so far, none of them was approved to be used in humans [14, 18]. This scenario can change considerably in the next years, considering the number of promising formulations that have been developed recently [14]. Purified and recombinant antigens have been tested, especially using combinations of proteins and polysaccharides found at the fungal cell wall [14, 19, 20]. In addition, killed organisms and attenuated strains were reported as protective in mice models of invasive fungal infections [21, 22]. Other immunotherapy protocols have also been suggested, including adoptive and antibody-based therapies [23]. In this manuscript, we will limit the discussion to antigen preparations based on the immunological role of fungal lipids.

Very little is known about the use of fungal lipids as immunogens. Nami and colleagues have recently listed and discussed studies where the activity of antifungal vaccines was investigated. Remarkably, out of 61 vaccine prototypes, only one formulation was prepared testing lipids as antigens [14]. Using murine CpG oligodeoxynucleotide or dendritic cells as adjuvants, Bozza and colleagues compared the protective effect of different *Aspergillus fumigatus* antigens, including membrane glycosphingolipids (GSL) glycosylinositolphosphoceramide and the GPI-anchored lipophosphogalactomannan [24]. Although immunization with GSL induced a Th17 response, usually associated with protection, this response was accompanied by a concomitant Th2 activation and no protection was observed for both models.

Fungal Lipids: Structure and Function for Antifungal Targets

Overall, lipids such as fatty acids, phospholipids, triacylglycerol, and sterols are usually associated with membrane architecture, permeability, fluidity control, and signaling [25–28]. More complex lipids, including sphingolipids and its derivatives, are also typical plasma membrane-enriched molecules. However, in recent years, they gained attention as potent immunogenic and regulators of fungal virulence [28, 29•, 30, 31]. Importantly, immunogenicity, pathogenic profile, and membrane physiology are all interconnected. For instance, changes in membrane asymmetry through deletion of Apt1, a flippase responsible for aminophospholipid translocation, not only directly affected the synthesis of phospholipids, glucosylceramide, and ergosterylglycoside but also impaired the synthesis and secretion of glucuronoxylomannan (GXM) by *C. neoformans* and resulted in reduced pathogenic potential [32]. Other fungal lipids were functionally implicated in processes of morphogenesis and pathogenesis. For instance, farnesol is a sesquiterpene produced by *C. albicans* and released extracellularly as a quorum-sensing molecule that inhibited yeast-to-hypha differentiation [33]. The response to farnesol by other species is diverse. Farnesol reverses the deficiency in conidiation that occurs in *Neurospora crassa* after deletion of genes related with *frequence* or *white collar* [34]. In addition, farnesol induces apoptosis in *Aspergillus nidulans* [35] and inhibits *Histoplasma capsulatum*, *C. neoformans*, and *C. gattii* growth in vitro [36, 37].

The lipid structures and their biosynthesis pathways are highly conserved between mammalian and fungal organisms. However, key differences have been identified and particular lipids and enzymes have been explored as targets of antifungal drugs [9]. The broad-spectrum drug amphotericin B (AmB), for instance, was described in the 1950s and until now is the principal drug choice for cryptococcosis treatment [7]. This amphipathic polyene binds to ergosterol in fungal membranes compromising their function, leading to pore formation and consequent fungal death [9]. Additional studies suggested that AmB stimulates the oxidative response of phagocytes, contributing to the anticryptococcal activity [38, 39]. The ergosterol biosynthesis pathway is also a target for azoles [9]. This class of antifungal drugs inhibits the lanosterol 14 alpha-demethylase, reducing ergosterol production and causing accumulation of toxic precursors. As a consequence, the fungal membrane loses stability and vital functions. Other lipid biosynthesis inhibitors (e.g., myriocin, fumonisin, australifungin) displayed potent antifungal activity. However, the use of these molecules in humans was impaired by the toxic effects or due to their limited effect against molds (e.g., aureobasidin) [9, 13]. Recently, Mor and colleagues identified a new class of antifungal molecules derived from hydrazides [40]. These compounds indirectly inhibited the synthesis of fungal

glucosylceramide (GlcCer) and were effective in vitro and in vivo against a variety of fungal pathogens, including *C. neoformans*, *C. albicans*, and *Pneumocystis* and displayed reduced toxicity to mammalian cells. Hydrazide derivatives will be discussed in more detail later in this review. It is clear, in summary, that the search for new drugs that bind to or impair lipid biosynthesis pathway in fungal organisms is highly promising.

Lipid Immunogens Produced by *Cryptococcus*

Initially considered as a structural component of fungal membranes, GlcCer (Fig. 1A) was first characterized as an antigen and a component of *C. neoformans* cell wall by Rodrigues and colleagues in 2000 [30]. A few years later, GlcCer was reported as a pathogenicity regulator in *C. neoformans* [29•]. It was established that the absence of GlcCer (Δ*gcs1* strain, Fig. 1B) in *C. neoformans* affects cell cycle progression and growth in neutral/alkaline pH at physiologically CO_2 concentrations. Mutants lacking GlcCer lost the ability to cause cryptococcosis in mice challenged intranasally with lethal doses of *C. neoformans*. These studies were followed by an extensive investigation on the relationship between structure and function of GlcCer in *C. neoformans* and other fungal pathogens [31]. By knocking out the enzymes required for the GlcCer biosynthesis pathway, Del Poeta's group has demonstrated that changes in GlcCer structure profoundly impact the *C. neoformans* surface, interfering with membrane physical properties and virulence [29•, 41, 42]. For instance, when the *C. neoformans* ability to methylate the sphingosine backbone is blocked through the deletion of the *smt1* gene, yeasts accumulate demethylated sphingosine and demethylated GlcCer (Fig. 1B) [41]. Membrane permeability and rigidity were strongly affected in Δ*smt1* strain, which seemed to impact adaptation to neutral/alkaline environment found in the lung alveoli. The Δ*smt1* phenotype was similar to that observed in the Δ*gcs1* strain, linking a unique GlcCer lipid structure (methylated GlcCer) to a phenotype. Furthermore, virulence of the Δ*smt1* mutant was also reduced when this strain was administered intranasally in mice. Yeasts of the Δ*smt1* strain were trapped in lung granulome with a consequent decrease in dissemination and virulence. Changes on membrane permeability also occurred when the enzyme Sld8, a sphingolipid desaturase, was knocked out in *C. neoformans* (Δ*sld8* strain, Fig. 1B) [42]. Membrane permeability of the Δ*sld8* strain was similar to that of the Δ*smt1* mutant and significantly reduced when compared with the WT strain. In addition, the Δ*sld8* mutant was more sensitive to ionic and non-ionic detergents. Differently from the Δ*gcs1* and Δ*smt1* mutants, the Δ*sld8* strain was able to grow in vitro under acidic or neutral/alkaline pH at 37 °C and 5% CO_2. Yet, a reduced intracellular growth inside macrophages was observed when compared with WT and the reconstituted strain. Despite its ability to grow under different pH levels, the mutant strain lost completely its virulence when administered intranasally in mice.

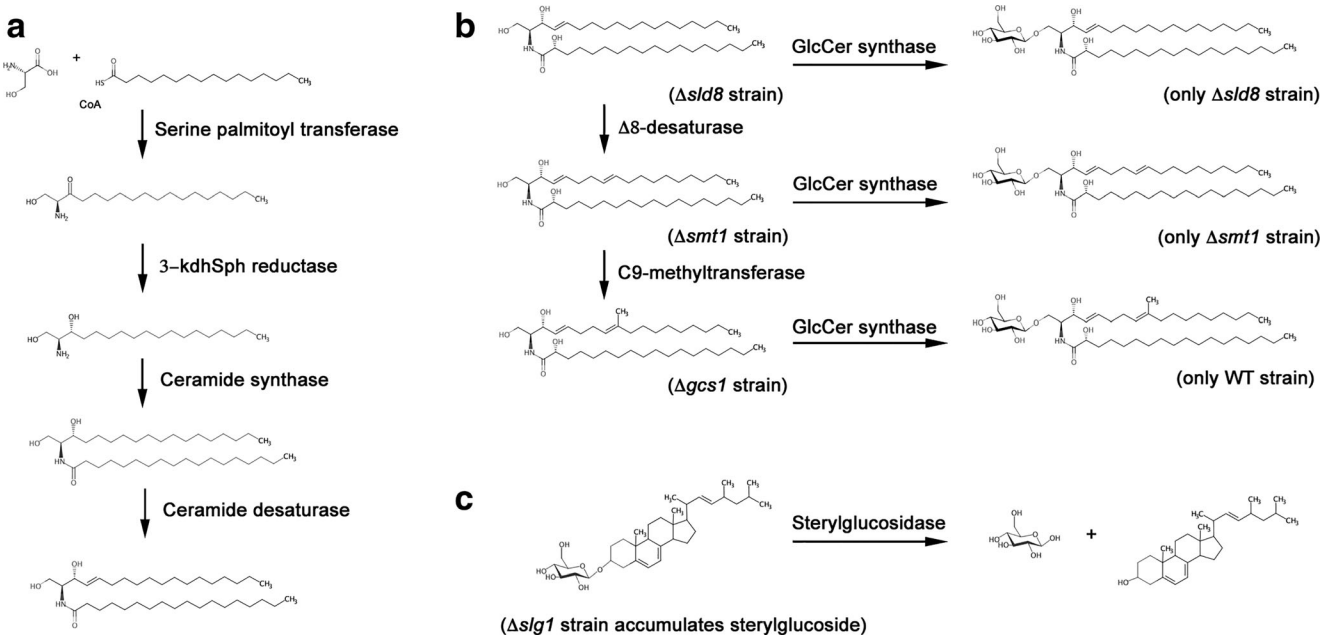

Fig. 1 Structures of GlcCer, GlcCer intermediates, and sterylglucoside produced by *C. neoformans*. **a** Initial steps for *C. neoformans* GlcCer biosynthesis (pathway conserved between humans and fungi). **b** Final steps of GlcCer biosynthesis (specific for fungi and mutant strains discussed here). **c** Sterylglucoside hydrolysis by sterylglucosidase

GlcCer has been also considered a target for new antifungal drugs [9]. Cerezyme, an enzyme that metabolizes GlcCer, promoted deficiencies in *C. neoformans* that were similar to those observed in the Δ*gcs1* strain, including membrane integrity defects, and reduced its ability to grow in physiological CO_2 atmosphere. Treatment with Cerezyme prolonged the survival of intranasally infected mice [43]. More recently, a new class of a synthetic drug able to reduce synthesis of fungal GlcCer was reported by Mor and colleagues [40]. The hydrazide derivatives D0 and BHBM were efficient in vivo against several fungal pathogens, including *C. neoformans*. Both drugs affected fungal cell morphology promoting the accumulation of intracellular vesicles. According to this study, proteins involved with vesicular transport and cell cycle progression were targeted. These studies were followed by the generation of acylhydrazones derivatives with even lower toxicity to mammalian cells and a higher anticryptococcal activity in vitro and in vivo [44].

The results discussed above characterize GlcCer as a virulence regulator that participates intensively during membrane and cell surface organization. In addition, GlcCer is conserved in a list of fungal pathogens [45, 46]. Thus, if immunogenic, GlcCer would be an interesting target for the development of a vaccine formulation. A set of data validates this hypothesis. Almost two decades ago, Rodrigues and colleagues demonstrated that antibodies against GlcCer are produced by patients with cryptococcosis and other fungal infections, including histoplasmosis, aspergillosis, and paracoccidioidomycosis [30]. The anti-GlcCer reactivity was concentrated at the *C. neoformans* cell wall and distinctively enriched at budding sites. Interestingly, localization of GlcCer at the cell surface was considerably higher under neutral pH and high CO_2, conditions regularly found by yeasts of *C. neoformans* during infection [43]. Furthermore, incubation of *C. neoformans* with anti-GlcCer resulted in inhibition of cell budding and fungal growth in vitro [30]. In a subsequent study, passive immunization of mice with anti-GlcCer followed by a lethal inoculum with *C. neoformans* prolonged the survival of A/J mice [47]. Intriguingly, at the first week of infection, the antibody administration did not reduce the CFU number in the lungs. In contrast, anti-GlcCer reduced significantly the inflammatory response when compared with control systems. The mechanisms by which anti-GlcCer controls inflammation are not clear, but it could be related to neutralization of GlcCer-containing extracellular vesicles (EVs). In fact, in the initial investigation showing the localization of GlcCer in *C. neoformans*, an association with vesicles was suggested [30]. This hypothesis was confirmed years later when *C. neoformans* EVs were isolated for the first time [48]. Analysis of lipid composition of *C. neoformans* EVs revealed GlcCer and sterols as their major neutral lipids. Since EVs produced by *C. neoformans* were associated with an increase in blood-brain barrier permeability and inflammatory response, treatment with anti-GlcCer antibodies could have a direct effect in disease control [49]. In fact, monoclonal antibodies can change the protein loading and modulatory activity of EVs released by *Histoplasma capsulatum* [50, 51]. It is possible then that anti-GlcCer impairs EV release or neutralizes the EVs already released inside the host.

The confirmation that GlcCer could indeed be used in a vaccine formulation to prevent infection with *C. neoformans* was published by Mor and colleagues [52]. GlcCer was administered intraperitoneally in different mice strains followed by a lethal dose of *C. neoformans*. Administration of GlcCer induced production of anti-GlcCer antibodies and prevented fungal dissemination. A significant reduction of fungal burden was observed. Interestingly, protection was observed even in the absence of adjuvants. Furthermore, histopathological analysis revealed absence of fungi in the brain and no major abnormalities when the mice were immunized with GlcCer. Additionally, normal structures were visualized in the lung of immunized mice, contrasting with high inflammatory response and massive infiltration by *C. neoformans* yeasts in the control. Importantly, when GlcCer was administered alone, no toxicity or damage was observed. These results confirm that fungal GlcCer can be exploited alone or in combination with other structures in vaccine formulations.

Ergosterol is another classical component of fungal membranes required for a number of physiological events. In *Cryptococcus* species, at least 13 different species of sterol derivatives were characterized, with a predominance of ergosterol [28]. As discussed previously, ergosterol is the major target for the currently antifungal drugs [9]. Besides that, additional functions have been described for this lipid in plants and mammals. In plants, this lipid is considered as a microbe-associated molecular pattern (MAMP) [53]. For instance, ergosterol reduces the activity of H^+ ATPase in sugar beet and induces a rapid and transient increase in H^+ flux, an early marker of elicitor action in plants [54]. Koselny and colleagues [55] recently investigated the role of ergosterol during pyroptosis in macrophages. They demonstrated that ergosterol levels and localization at the fungal cell surface are correlated with the ability of *C. neoformans*, *C. albicans* and *Saccharomyces cerevisiae* to initiate pyroptosis. The participation of ergosterol in this process was confirmed using liposomes. Ergosterol-containing liposomes were able to induce pyroptosis and mediated macrophage lysis. Together, these data suggest that ergosterol could be better explored as components of antifungal vaccines. Importantly, previous attempts to generate anti-ergosterol antibodies have failed. Rabbits were immunized with BSA-conjugated ergosterol and the development of antibodies was monitored. Although high titers of antibodies were observed and binding was inhibited by free ergosterol, the serum was not reactive to the membrane of fungi [56].

In several organisms, including animals, plants, and fungi, sterol derivatives can be modified by glycosyltransferases to

produce sterylglycosides (SGs) (Fig. 1C) [57–59]. Little is known about the functions of SG in animals and fungi, but a number of studies have reported the involvement of SG during plant stress [58]. In addition, the effect of plant SGs in animals has also been investigated and anti-inflammatory, anti-tumoral, immunomodulatory, and neurodegenerative activities were reported [60–62]. Considering that plant SGs are able to stimulate a Th1 response [63], Lee and colleagues investigated whether this glycolipid displayed a protective effect against *C. albicans* in vivo [61]. Pre-treatment of mice with sitosterylglucoside stimulated the production of IFN-γ and IL-2 by splenocytes. During infection, pre-treatment with sitosterylglucoside reduced the fungal burden in the kidney of mice infected intravenously with *C. albicans*. Protection was not observed when the $CD4^+$ T cells were depleted and the adoptive transference with sitosterylglucoside $CD4^+$ T treated cells reversed the resistance, confirming the relevance of Th1 response during protection [61].

Although bacteria lack the enzymatic machinery to produce sterols, SGs can be produced using cholesterol from host cells [58]. For instance, *Helicobacter pylori* extracts cholesterol from epithelial membranes of the host and converts it into SGs. SGs produced by *H. pylori* are able to reduce the bacterial uptake by macrophages [64]. These SGs can be subsequently acylated or phosphorylated in the C6′-primary hydroxy group of glucose [63]. Importantly, acylated SGs are presented by CD1d and recognized by murine and human invariant TCR-bearing NKT cells [65]. *Borrelia burgdorferi* is another bacterial species that glycosylates host cholesterol [66]. Production of cholesteryl 6′-*O*-acyl β-galactoside during Lyme disease resulted in antibody production, demonstrating that acylated SGs are indeed immunogenic [67]. Thus, glycosylation of sterols can modify their functions and improve their ability to activate the immune response, including the development of specific antibodies.

The immunomodulatory activity of SGs in *C. neoformans* was also investigated. Rella and colleagues explored the potential use of fungal SG as a vaccine strategy during cryptococcosis [68]. By deleting the sterylglucosidase (Sgl1) in *C. neoformans*, an attenuated strain with higher SG content was generated and tested as an anticryptococcal vaccine. Remarkably, all mice pre-treated with the attenuated strain Δ*sgl1* survived to a lethal challenge with *C. neoformans* or *C. gattii*. Vaccination using the Δ*sgl1* strain was efficient even in immunosuppressed mice where $CD4^+$ T cells were depleted.

The accumulation of SGs in the *C. neoformans* Δ*sgl1* strain was accompanied by formation of a thicker and highly branched capsule, suggesting that changes in SG metabolism could also impact capsule architecture and/or GXM composition and release [69•]. In fact, protection was lost when GXM was absent in condition of SG accumulation. Since GXM is a potent immunomodulator, changes in its structure could be also attributed to the protection conferred by the Δ*sgl1* strain. This hypothesis has been addressed very recently by using a double knockout strain lacking the abilities to synthesize capsule and to degrade SGs. In summary, *CAP59*, a putative regulator of capsule export, was deleted in the Δ*sgl1* strain [69•]. As expected, the double mutant strain, Δ*sgl1*/Δ*cap59*, accumulated SGs and had no capsule. However, this strain was unable to promote protection when used as an attenuated vaccine formulation. To investigate the mechanism of protection generated by the Δ*sgl1* strain, parameters of the immune response and fungal burden were compared after infection with WT, Δ*sgl1*, Δ*cap59*, and Δ*sgl1*/Δ*cap59* strains. In the first days post infection, a higher number of monocytes, eosinophils, and dendritic cells were found in the lung of mice treated with the Δ*sgl1* strain. The initial cytokine production stimulated by the WT and Δ*sgl1* strains was similar, but the WT infection sustained this response during the infection, suggesting a hyper-inflammatory reaction that could culminate with mice death. The response to the Δ*sgl1*/Δ*cap59* strain was similar to that induced by Δ*cap59* cells and apparently not sufficient to promote protection. The vaccination/infection models showed the presence of yeasts only in systems where WT and Δ*cap59* strains were used. However, only the WT strain was lethal. During the re-challenge with the WT strain, an increase in dendritic cells and $CD4^+$ cells was observed only in mice previously infected with the Δ*sgl1* strain. Also, the inflammatory response of Δ*sgl1*-treated mice displayed the same kinetic, with an initial acute response followed by a decrease in the cytokine production. The results discussed here suggest that an association between higher levels of SG and GXM is required for the protection promoted by the Δ*sgl1* strain.

EVs: a Platform Carrying Immunogenic Lipids of *C. neoformans*

The extracellular release of EVs is a conserved strategy developed by fungal cells to export lipids, proteins, polysaccharides, pigments, and nucleic acids [48, 70–77]. These compartments have been associated with a number of processes in *C. neoformans* and other fungal pathogens, including host cell activation, pathogenesis, virulence transference, and resistance to antifungal drugs [70, 78–82].

Some of the most effective vaccines used to prevent microbial infections are multi-antigenic, especially those using inactivated and attenuated fungal strains [14, 22] [69•]. Organisms with attenuated virulence can induce a stronger response than inactivated cells, but their application is limited since the target population is immunocompromised. However, they represent efficient models to investigate the mechanisms of protection and to inspire the development of safer vaccine formulations. EVs have been successfully explored in vaccine formulations against other diseases, including bacterial

meningitis [83–89]. A major advantage of the use of vaccinal EVs is the use of cell-free preparations containing multiple structures in their native form. As discussed above, the protection given by the Δ*sgl1* strain of *C. neoformans* required a combination of SGs and GXM [69•]. On the basis that EVs from the Δ*sgl1* strain are enriched in SGs and in the immunogenic structures GlcCer and GXM, these compartments were tested in a *Galleria mellonella* model of cryptococcosis. Pre-treatment of *G. mellonella* larvae with EVs from the Δ*sgl1* strain increased the survival of the animals after a lethal challenge with *C. neoformans*. Importantly, EVs from the Δ*cap59* and Δ*sgl1*/Δ*cap59* strains showed no effects, while EVs produced by the WT strain accelerated killing of infected larvae. Protection by EVs was previously reported in a candidiasis model of *G. mellonella* [70]. These data suggested that fungal EVs can be explored as vaccination prototypes. One major limitation of the use of EVs in vaccination models is the low yields of the protocols traditionally used for EV isolation [72]. However, the recent establishment of an improved protocol of isolation of fungal EVs with high yields has the potential to change this scenario (Reis et al., *in press*). Alternatively, one could envision the production of giant synthetic vesicles containing GXM and various lipids, such as the immunogenic SG and GlcCer.

Concluding Remarks and Future Vaccine Formulations for Cryptococcosis

The urgent need of alternatives for prevention of fungal diseases associated with the recent advances in the understanding of the immunological mechanisms controlling fungal infections revitalized the vaccine field in medical mycology. New vaccinal formulations exploiting virulence regulators and genetically modified attenuated strains were described in the last decades. Apart from other promising immunogens, lipids consist of molecular species with highly complex biosynthesis pathways and consequently huge structural diversity. Remarkably, minimal changes in lipid structures or relative concentration can modify completely their functions and impact fungal physiology and virulence. GlcCer, SGs, and lipid-containing EVs have been recently tested in vaccine formulations and their anticryptococcal efficacy was confirmed in vivo. Together, the data discussed here encourage the use of fungal lipids in anticryptococcal vaccinal strategies. One major challenge in the field is to generate vaccines to prevent cryptococcosis in immunosuppressed patients. In addition, considering that cryptococcosis mainly affects neglected populations, it is important that such preparations will be affordable in underdeveloped and developing countries.

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Compliance with Ethical Standards

Conflict of Interest Leonardo Nimrichter and Marcio L. Rodrigues declare no conflict of interest. Dr. Maurizio Del Poeta is the co-founder and Chief Scientific Officer (CSO) of MicroRid Technologies Inc. MLR is currently in leave from a position of associate professor at the Microbiology Institute of UFRJ.

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.

References

Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

1. Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnurm A, et al. Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis. Cold Spring Harb Perspect Med. 2014;4(7):a019760.
2. Esher SK, Zaragoza O, Alspaugh JA. Cryptococcal pathogenic mechanisms: a dangerous trip from the environment to the brain. Mem Inst Oswaldo Cruz. 2018;113(7):e180057.
3. Chen SC, Meyer W, Sorrell TC. Cryptococcus gattii infections. Clin Microbiol Rev. 2014;27(4):980–1024.
4. Skolnik K, Huston S, Mody CH. Cryptococcal lung infections. Clin Chest Med. 2017;38(3):451–64.
5. Limper AH, Adenis A, Le T, Harrison TS. Fungal infections in HIV/AIDS. Lancet Infect Dis. 2017;17(11):e334–43.
6. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, et al. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect Dis. 2017;17(8):873–81.
7. Coelho C, Casadevall A. Cryptococcal therapies and drug targets: the old, the new and the promising. Cell Microbiol. 2016;18(6):792–9.
8. Mourad A, Perfect JR. Present and future therapy of Cryptococcus infections. J Fungi (Basel). 2018;4(3).
9. Rollin-Pinheiro R, Singh A, Barreto-Bergter E, Del Poeta M. Sphingolipids as targets for treatment of fungal infections. Future Med Chem. 2016;8(12):1469–84.

10. Bongomin F, Oladele RO, Gago S, Moore CB, Richardson MD. A systematic review of fluconazole resistance in clinical isolates of Cryptococcus species. Mycoses. 2018;61(5):290–7.
11. Medici NP, Del Poeta M. New insights on the development of fungal vaccines: from immunity to recent challenges. Mem Inst Oswaldo Cruz. 2015;110(8):966–73.
12. Sui X, Yan L, Jiang YY. The vaccines and antibodies associated with Als3p for treatment of Candida albicans infections. Vaccine. 2017;35(43):5786–93.
13. Nami S, Aghebati-Maleki A, Morovati H, Aghebati-Maleki L. Current antifungal drugs and immunotherapeutic approaches as promising strategies to treatment of fungal diseases. Biomed Pharmacother. 2019;110:857–68.
14. Nami S, Mohammadi R, Vakili M, Khezripour K, Mirzaei H, Morovati H. Fungal vaccines, mechanism of actions and immunology: a comprehensive review. Biomed Pharmacother. 2019;109:333–44.
15. Keshavan P, Pellegrini M, Vadivelu-Pechai K, Nissen M. An update of clinical experience with the quadrivalent meningococcal ACWY-CRM conjugate vaccine. Expert Rev Vaccines. 2018;17(10):865–80.
16. Sullivan SG, Price OH, Regan AK. Burden, effectiveness and safety of influenza vaccines in elderly, paediatric and pregnant populations. Ther Adv Vaccines Immunother. 2019;7:2515135519826481.
17. Willis ED, Woodward M, Brown E, Popmihajlov Z, Saddier P, Annunziato PW, et al. Herpes zoster vaccine live: a 10 year review of post-marketing safety experience. Vaccine. 2017;35(52):7231–9.
18. Cassone A. Vulvovaginal Candida albicans infections: pathogenesis, immunity and vaccine prospects. BJOG. 2015;122(6):785–94.
19. Torosantucci A, Chiani P, Bromuro C, De Bernardis F, Palma AS, Liu Y, et al. Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. PLoS One. 2009;4(4):e5392.
20. Portuondo DL, Batista-Duharte A, Ferreira LS, Martinez DT, Polesi MC, Duarte RA, et al. A cell wall protein-based vaccine candidate induce protective immune response against Sporothrix schenckii infection. Immunobiology. 2016;221(2):300–9.
21. Saville SP, Lazzell AL, Chaturvedi AK, Monteagudo C, Lopez-Ribot JL. Efficacy of a genetically engineered Candida albicans tet-NRG1 strain as an experimental live attenuated vaccine against hematogenously disseminated candidiasis. Clin Vaccine Immunol. 2009;16(3):430–2.
22. Wuthrich M, Filutowicz HI, Warner T, Deepe GS Jr, Klein BS. Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to CD8+ T cells: implications for vaccine development in immune-deficient hosts. J Exp Med. 2003;197(11):1405–16.
23. Naran K, Nundalall T, Chetty S, Barth S. Principles of immunotherapy: implications for treatment strategies in cancer and infectious diseases. Front Microbiol. 2018;9:3158.
24. Bozza S, Clavaud C, Giovannini G, Fontaine T, Beauvais A, Sarfati J, et al. Immune sensing of Aspergillus fumigatus proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. J Immunol. 2009;183(4):2407–14.
25. Mansilla MC, Banchio CE, de Mendoza D. Signalling pathways controlling fatty acid desaturation. Subcell Biochem. 2008;49:71–99.
26. Uemura H. Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives. Appl Microbiol Biotechnol. 2012;95(1):1–12.
27. Mishra P, Bolard J, Prasad R. Emerging role of lipids of Candida albicans, a pathogenic dimorphic yeast. Biochim Biophys Acta. 1992;1127(1):1–14.
28. Singh A, MacKenzie A, Girnun G, Del Poeta M. Analysis of sphingolipids, sterols, and phospholipids in human pathogenic Cryptococcus strains. J Lipid Res. 2017;58(10):2017–36.
29.• Rittershaus PC, Kechichian TB, Allegood JC, Merrill AH Jr, Hennig M, Luberto C, et al. Glucosylceramide synthase is an essential regulator of pathogenicity of Cryptococcus neoformans. J Clin Invest. 2006;116(6):1651–9 **This work reports for the first time that GlcCer from *Cryptococcus neoformans* is a pathogenesis regulator and confirms previous publications suggesting that this lipid could be explored as an antifungal target**.
30. Rodrigues ML, Travassos LR, Miranda KR, Franzen AJ, Rozental S, de Souza W, et al. Human antibodies against a purified glucosylceramide from Cryptococcus neoformans inhibit cell budding and fungal growth. Infect Immun. 2000;68(12):7049–60.
31. Rella A, Farnoud AM, Del Poeta M. Plasma membrane lipids and their role in fungal virulence. Prog Lipid Res. 2016;61:63–72.
32. Rizzo J, Colombo AC, Zamith-Miranda D, Silva VKA, Allegood JC, Casadevall A, et al. The putative flippase Apt1 is required for intracellular membrane architecture and biosynthesis of polysaccharide and lipids in Cryptococcus neoformans. Biochim Biophys Acta, Mol Cell Res. 2018;1865(3):532–41.
33. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, et al. Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol. Appl Environ Microbiol. 2001;67(7):2982–92.
34. Granshaw T, Tsukamoto M, Brody S. Circadian rhythms in Neurospora crassa: farnesol or geraniol allow expression of rhythmicity in the otherwise arrhythmic strains frq10, wc-1, and wc-2. J Biol Rhythm. 2003;18(4):287–96.
35. Semighini CP, Hornby JM, Dumitru R, Nickerson KW, Harris SD. Farnesol-induced apoptosis in Aspergillus nidulans reveals a possible mechanism for antagonistic interactions between fungi. Mol Microbiol. 2006;59(3):753–64.
36. Brilhante RS, de Lima RA, Marques FJ, Silva NF, Caetano EP, Castelo-Branco Dde S, et al. Histoplasma capsulatum in planktonic and biofilm forms: in vitro susceptibility to amphotericin B, itraconazole and farnesol. J Med Microbiol. 2015;64(Pt 4):394–9.
37. Cordeiro Rde A, Nogueira GC, Brilhante RS, Teixeira CE, Mourao CI, Castelo-Branco Dde S, et al. Farnesol inhibits in vitro growth of the Cryptococcus neoformans species complex with no significant changes in virulence-related exoenzymes. Vet Microbiol. 2012;159(3–4):375–80.
38. Mozaffarian N, Berman JW, Casadevall A. Enhancement of nitric oxide synthesis by macrophages represents an additional mechanism of action for amphotericin B. Antimicrob Agents Chemother. 1997;41(8):1825–9.
39. Mesa-Arango AC, Trevijano-Contador N, Roman E, Sanchez-Fresneda R, Casas C, Herrero E, et al. The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. Antimicrob Agents Chemother. 2014;58(11):6627–38.
40. Mor V, Rella A, Farnoud AM, Singh A, Munshi M, Bryan A, et al. Identification of a new class of antifungals targeting the synthesis of fungal sphingolipids. MBio. 2015;6(3):e00647.
41. Singh A, Wang H, Silva LC, Na C, Prieto M, Futerman AH, et al. Methylation of glycosylated sphingolipid modulates membrane lipid topography and pathogenicity of Cryptococcus neoformans. Cell Microbiol. 2012;14(4):500–16.
42. Raj S, Nazemidashtarjandi S, Kim J, Joffe L, Zhang X, Singh A, et al. Changes in glucosylceramide structure affect virulence and membrane biophysical properties of Cryptococcus neoformans. Biochim Biophys Acta Biomembr. 2017;1859(11):2224–33.
43. Rhome R, Singh A, Kechichian T, Drago M, Morace G, Luberto C, et al. Surface localization of glucosylceramide during Cryptococcus neoformans infection allows targeting as a potential antifungal. PLoS One. 2011;6(1):e15572.
44. Lazzarini C, Haranahalli K, Rieger R, Ananthula HK, Desai PB, Ashbaugh A, et al. Acylhydrazones as antifungal agents targeting the synthesis of fungal sphingolipids. Antimicrob Agents Chemother. 2018;62(5).
45. Barreto-Bergter E, Pinto MR, Rodrigues ML. Structure and biological functions of fungal cerebrosides. An Acad Bras Cienc. 2004;76(1):67–84.

46. Rhome R, McQuiston T, Kechichian T, Bielawska A, Hennig M, Drago M, et al. Biosynthesis and immunogenicity of glucosylceramide in Cryptococcus neoformans and other human pathogens. Eukaryot Cell. 2007;6(10):1715–26.
47. Rodrigues ML, Shi L, Barreto-Bergter E, Nimrichter L, Farias SE, Rodrigues EG, et al. Monoclonal antibody to fungal glucosylceramide protects mice against lethal Cryptococcus neoformans infection. Clin Vaccine Immunol. 2007;14(10):1372–6.
48. Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O, et al. Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell. 2007;6(1):48–59.
49. Huang SH, Wu CH, Chang YC, Kwon-Chung KJ, Brown RJ, Jong A. Cryptococcus neoformans-derived microvesicles enhance the pathogenesis of fungal brain infection. PLoS One. 2012;7(11):e48570.
50. Baltazar LM, Zamith-Miranda D, Burnet MC, Choi H, Nimrichter L, Nakayasu ES, et al. Concentration-dependent protein loading of extracellular vesicles released by Histoplasma capsulatum after antibody treatment and its modulatory action upon macrophages. Sci Rep. 2018;8(1):8065.
51. Matos Baltazar L, Nakayasu ES, Sobreira TJ, Choi H, Casadevall A, Nimrichter L, et al. Antibody binding alters the characteristics and contents of extracellular vesicles released by Histoplasma capsulatum. mSphere. 2016;1(2).
52. Mor V, Farnoud AM, Singh A, Rella A, Tanno H, Ishii K, et al. Glucosylceramide administration as a vaccination strategy in mouse models of cryptococcosis. PLoS One. 2016;11(4):e0153853.
53. Klemptner RL, Sherwood JS, Tugizimana F, Dubery IA, Piater LA. Ergosterol, an orphan fungal microbe-associated molecular pattern (MAMP). Mol Plant Pathol. 2014;15(7):747–61.
54. Rossard S, Roblin G, Atanassova R. Ergosterol triggers characteristic elicitation steps in Beta vulgaris leaf tissues. J Exp Bot. 2010;61(6):1807–16.
55. Koselny K, Mutlu N, Minard AY, Kumar A, Krysan DJ, Wellington M. A genome-wide screen of deletion mutants in the filamentous Saccharomyces cerevisiae background identifies ergosterol as a direct trigger of macrophage pyroptosis. MBio. 2018;9(4).
56. Tejada-Simon MV, Pestka JJ. Production of polyclonal antibody against ergosterol hemisuccinate using Freund's and Titermax adjuvants. J Food Prot. 1998;61(8):1060–3.
57. Shimamura M. Immunological functions of steryl glycosides. Arch Immunol Ther Exp. 2012;60(5):351–9.
58. Ferrer A, Altabella T, Arro M, Boronat A. Emerging roles for conjugated sterols in plants. Prog Lipid Res. 2017;67:27–37.
59. Grille S, Zaslawski A, Thiele S, Plat J, Warnecke D. The functions of steryl glycosides come to those who wait: recent advances in plants, fungi, bacteria and animals. Prog Lipid Res. 2010;49(3):262–88.
60. Bouic PJ. The role of phytosterols and phytosterolins in immune modulation: a review of the past 10 years. Curr Opin Clin Nutr Metab Care. 2001;4(6):471–5.
61. Lee JH, Lee JY, Park JH, Jung HS, Kim JS, Kang SS, et al. Immunoregulatory activity by daucosterol, a beta-sitosterol glycoside, induces protective Th1 immune response against disseminated candidiasis in mice. Vaccine. 2007;25(19):3834–40.
62. Khabazian I, Bains JS, Williams DE, Cheung J, Wilson JM, Pasqualotto BA, et al. Isolation of various forms of sterol beta-D-glucoside from the seed of Cycas circinalis: neurotoxicity and implications for ALS-parkinsonism dementia complex. J Neurochem. 2002;82(3):516–28.
63. Shimamura M, Hidaka H. Therapeutic potential of cholesteryl O-acyl alpha-glucoside found in Helicobacter pylori. Curr Med Chem. 2012;19(28):4869–74.
64. Wunder C, Churin Y, Winau F, Warnecke D, Vieth M, Lindner B, et al. Cholesterol glucosylation promotes immune evasion by Helicobacter pylori. Nat Med. 2006;12(9):1030–8.
65. Chang YJ, Kim HY, Albacker LA, Lee HH, Baumgarth N, Akira S, et al. Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity. J Clin Invest. 2011;121(1):57–69.
66. Crowley JT, Toledo AM, LaRocca TJ, Coleman JL, London E, Benach JL. Lipid exchange between Borrelia burgdorferi and host cells. PLoS Pathog. 2013;9(1):e1003109.
67. Stubs G, Fingerle V, Wilske B, Gobel UB, Zahringer U, Schumann RR, et al. Acylated cholesteryl galactosides are specific antigens of borrelia causing Lyme disease and frequently induce antibodies in late stages of disease. J Biol Chem. 2009;284(20):13326–34.
68. Rella A, Mor V, Farnoud AM, Singh A, Shamseddine AA, Ivanova E, et al. Role of Sterylglucosidase 1 (Sgl1) on the pathogenicity of Cryptococcus neoformans: potential applications for vaccine development. Front Microbiol. 2015;6:836.
69. • Colombo AC, et al. Cryptococcus neoformans Glucuronoxylomannan and Sterylglucoside are required for host protection in an animal vaccination model. MBio. 2019;(10):e02909-18 **This work confirms the potential use of fungal extracellular vesicles as a vaccination strategy.**
70. Vargas G, Rocha JD, Oliveira DL, Albuquerque PC, Frases S, Santos SS, et al. Compositional and immunobiological analyses of extracellular vesicles released by Candida albicans. Cell Microbiol. 2015;17(3):389–407.
71. Peres da Silva R, Puccia R, Rodrigues ML, Oliveira DL, Joffe LS, Cesar GV, et al. Extracellular vesicle-mediated export of fungal RNA. Sci Rep. 2015;5:7763.
72. Rodrigues ML, Oliveira DL, Vargas G, Girard-Dias W, Franzen AJ, Frases S, et al. Analysis of yeast extracellular vesicles. Methods Mol Biol. 2016;1459:175–90.
73. Zamith-Miranda D, Nimrichter L, Rodrigues ML, Nosanchuk JD. Fungal extracellular vesicles: modulating host-pathogen interactions by both the fungus and the host. Microbes Infect. 2018;20:501–4.
74. Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancope-Oliveira RM, et al. Vesicular transport in Histoplasma capsulatum: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol. 2008;10(8):1695–710.
75. Eisenman HC, Frases S, Nicola AM, Rodrigues ML, Casadevall A. Vesicle-associated melanization in Cryptococcus neoformans. Microbiology. 2009;155(Pt 12:3860–7.
76. Peres da Silva R, Heiss C, Black I, Azadi P, Gerlach JQ, Travassos LR, et al. Extracellular vesicles from Paracoccidioides pathogenic species transport polysaccharide and expose ligands for DC-SIGN receptors. Sci Rep. 2015;5:14213.
77. Rayner S, Bruhn S, Vallhov H, Andersson A, Billmyre RB, Scheynius A. Identification of small RNAs in extracellular vesicles from the commensal yeast Malassezia sympodialis. Sci Rep. 2017;7:39742.
78. Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. Infect Immun. 2010;78(4):1601–9.
79. Bielska E, Sisquella MA, Aldeieg M, Birch C, O'Donoghue EJ, May RC. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen Cryptococcus gattii. Nat Commun. 2018;9(1):1556.
80. Zarnowski R, Sanchez H, Covelli AS, Dominguez E, Jaromin A, Bernhardt J, et al. Candida albicans biofilm-induced vesicles confer drug resistance through matrix biogenesis. PLoS Biol. 2018;16(10):e2006872.
81. da Silva TA, Roque-Barreira MC, Casadevall A, Almeida F. Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro. Sci Rep. 2016;6:35867.
82. Johansson HJ, Vallhov H, Holm T, Gehrmann U, Andersson A, Johansson C, et al. Extracellular nanovesicles released from the

commensal yeast Malassezia sympodialis are enriched in allergens and interact with cells in human skin. Sci Rep. 2018;8(1):9182.
83. Oftung F, Korsvold GE, Aase A, Naess LM. Cellular immune responses in humans induced by two serogroup B meningococcal outer membrane vesicle vaccines given separately and in combination. Clin Vaccine Immunol. 2016;23(4):353–62.
84. Su EL, Snape MD. A combination recombinant protein and outer membrane vesicle vaccine against serogroup B meningococcal disease. Expert Rev Vaccines. 2011;10(5):575–88.
85. Wang S, Gao J, Wang Z. Outer membrane vesicles for vaccination and targeted drug delivery. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2019;11(2):e1523.
86. Shears RK, Bancroft AJ, Hughes GW, Grencis RK, Thornton DJ. Extracellular vesicles induce protective immunity against Trichuris muris. Parasite Immunol. 2018;40(7):e12536.
87. Di Bonito P, Accardi L, Galati L, Ferrantelli F, Federico M. Anticancer vaccine for HPV-associated neoplasms: focus on a therapeutic HPV vaccine based on a novel tumor antigen delivery method using endogenously engineered exosomes. Cancers (Basel). 2019;11(2).
88. Jungbauer A. Exosomes enter vaccine development: strategies meeting global challenges of emerging infections. Biotechnol J. 2018;13(4):e1700749.
89. Bottero D, Gaillard ME, Errea A, Moreno G, Zurita E, Pianciola L, et al. Outer membrane vesicles derived from Bordetella parapertussis as an acellular vaccine against Bordetella parapertussis and Bordetella pertussis infection. Vaccine. 2013;31(45):5262–8.
90.•• Reis FCG, et al. A Novel Protocol for the Isolation of Fungal Extracellular Vesicles Reveals the Participation of a Putative Scramblase in Polysaccharide Export and Capsule Construction in Cryptococcus gattii. mSphere 2019;(4):e00080-19 **A fast and easy protocol for fungal extracellular vesicle isolation is reported in this work.**