Role of the innate immune system in host defence against fungal infections

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Abstract. – OBJECTIVE: The objective of this narrative review is to present a summary of current knowledge of host-fungal pathogen interaction focusing on the importance of the innate immune system in host defense against invasive fungal infections. In addition, the emergence of drug resistance in the treatment of invasive fungal infections has also been highlighted.

MATERIALS AND METHODS: A literature review was conducted to identify articles documenting the role of the host innate immune system against fungal pathogen and the emergence of drug resistance in the treatment of invasive fungal infections.

RESULTS: In this review, we provide an update from the most recent studies on the role of the host innate immune system against fungal pathogen and we also highlight the mechanisms that these pathogens use to evade the innate immune system.

CONCLUSIONS: This review highlights the existence of different cellular mechanisms that, following the recognition of fungal PAMPS, induce the production of different sets of defense factors. The development of new diagnostic methods and antifungal drugs along with a better understanding of the host immune response are key approaches to controlling invasive fungal infections.

Key Words:
   Invasive fungal infections, PRRs, PAMPS, Innate immune responses.

Introduction

Since the latter half of the last century, significant medical advances have extended the lives of many people with previously fatal health conditions, such as AIDS and cancer[1]. These improvements have been followed by an increase in the number of immunocompromised patients who, being more susceptible to invasive fungal infections, have greatly increased the incidence of such infections worldwide[2]. Although the fungal kingdom has approximately six million species widely distributed throughout the world, only more than 200 species are known to cause disease in humans[3]. The fungal infections are differentiated, according to the part of the body affected, into superficial, cutaneous, subcutaneous and invasive[4]. Among these, invasive fungal infection (IFI) results in increased morbidity and mortality in healthy and immunocompromised patients, respectively[5,6]. Opportunistic fungi, such as *Candida*, *Aspergillus* and *Cryptococcus* species are the most frequent cause of IFIs worldwide[7,8]. Invasive candidiasis account for approximately 60% of all IFIs and together with other fungal infections (cryptococcal meningitis, pneumocystis pneumonia, disseminated histoplasmosis, and chronic pulmonary aspergillosis) account for nearly 50% of all AIDS-related deaths[9,10]. *Aspergillus* spp. is a ubiquitous saprophytic fungus that can cause pneumonia, invasive infection, and allergic bronchopulmonary especially in asthmatics and cystic fibrosis patients[11,12]. Invasive aspergillosis has a mortality rate of approximately 50% that can reach 80% with untimely diagnosis and/or inadequate treatment in vulnerable populations, such as neutropenic, HIV, bone marrow transplant, or cancer patients[8,13].

Cryptococcosis is caused by the yeasts *Cryptococcus* spp and it is estimated to affect around one million individuals each year[14,15]. This mycosis is associated with significant mortality and morbidity, including severe neurological complications[14-16]. Several factors influence the outcome of IFI, such as the pathogenicity of the fungus, the host immune response, and the site of infection[8,17]. Early diagnosis of fungal infection based on clinical symptoms is also not easy because of nonspecific symptoms[18]. Some fungi cause symptoms that resemble bacterial and/or viral infections, providing unnecessary or harmful

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treatment[4,19]. In addition, because humans come into contact with fungal spores every day, it is often difficult to discriminate between commensal and clinically relevant isolates[20,21]. According to IFI guidelines, a proven IFI requires microbiological analysis of clinical specimens which, through identification of microorganisms, allows the initiation of appropriate antifungal therapy[18,22]. Conventional diagnosis of these infections is time-consuming and lacks sensitivity[23]. Delayed clinical diagnosis and treatment of IFIs may contribute to the persistently high mortality rate in critically ill patients in the ICU[24,25]. In light of the above, for the early diagnosis of IFIs, there is an urgent need to improve the development of rapid diagnostic tests, particularly in immunocompromised patients who are unable to mount a normal immune response to fungal infections[26].

Antifungal drugs currently available for the treatment of IFIs are often limited by dose-limiting toxicities and potentially deleterious drug-drug interaction effects[27]. In addition, abuse and overuse of antifungal drugs in the treatment of fungal diseases can lead to the development of multidrug-resistant pathogens that have made IFIs a serious public health threat especially in immunocompromised individuals[28,29]. A better understanding of the early host immune response against fungal pathogens is considered of primary importance for the development of effective therapeutic approaches against these pathogens[30]. Here, we will primarily discuss host-fungal interaction and the role of innate immune cells in host defense against fungal pathogens. We will also discuss the emergence and spread of drug-resistant pathogens that are a serious threat to global health.

The Role of Pattern Recognition Receptors in Antifungal Immunity

Nearly every cell in the body expresses a wide variety of germline-encoded receptors, called pattern recognition receptors (PRRs), that are capable of detecting structural and conserved motifs from microbes, known as pathogen-associated molecular patterns (PAMPs)[31]. Toll-like receptors (TLRs), C-type lectin like receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs)32 belong to the class of receptors responsible for overseeing sites in the body and initiating innate immunity, which in turn triggers adaptive immune responses[33]. After engagement

of PRRs by individual PAMPs, PRRs activate downstream signaling pathways that induce the transcription of several genes, including nuclear factor (NF-) κB, activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK); the activation of the latter is critical for the production of proinflammatory cytokines, such as interleukin- (IL-) l and tumor necrosis factor- (TNF-) α[33,34] (Figure 1). Over the past decade, there have been rapid advances in the understanding of the biological role of PRRs in antifungal immunity[35]. In particular, some reports[35-37] have investigated the mechanisms underlying fungal recognition and how the PRRs modulate immune responses by initiating various signaling cascades that lead to the production of various cytokines and/or chemokines. Here, we discuss recent findings on the early immune response during host-fungal interaction and the importance of these findings for the development of new strategies directed at mounting a stronger and more effective antifungal immune response.

The Early Host Immune Response During Host-Fungus Interaction

Binding of PRRs to their ligands is necessary to induce the innate immune response that represent the host's first line of defense against pathogens. PRRs are expressed not only in myeloid cells, such as monocytes, macrophages, dendritic cells (DCs) and polymorphonuclear leukocytes (PMNs), but also in nonmyeloid cells such as epithelial cells, endothelial cells, fibroblasts and some lymphoid cells. PRRs are present on the cell membrane, in endosomes or in cytoplasm[38]. The main PRRs families include Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs)[39]. These receptors sense different fungal cell PAMPS such as β-glucans, N- and O-linked mannans, mannoproteins, fungal RNA and unmethylated CpG DNA. The recognition of PAMPs by PRRs leads to the activation of specific intracellular signaling cascades including phagocytosis, respiratory burst, cytokine and chemokine release[33]. The role of the main PRR families in the detection of fungal pathogens is briefly summarized below. Up to now there are ten functional TLRs in human (TLR1–10) and twelve (TLR1–TLR9, TLR11–TLR13) in mice. While TLR1, 2, 4, 5, 6, 10 are expressed on the surface of immune cells and recognize the membrane components of patho-
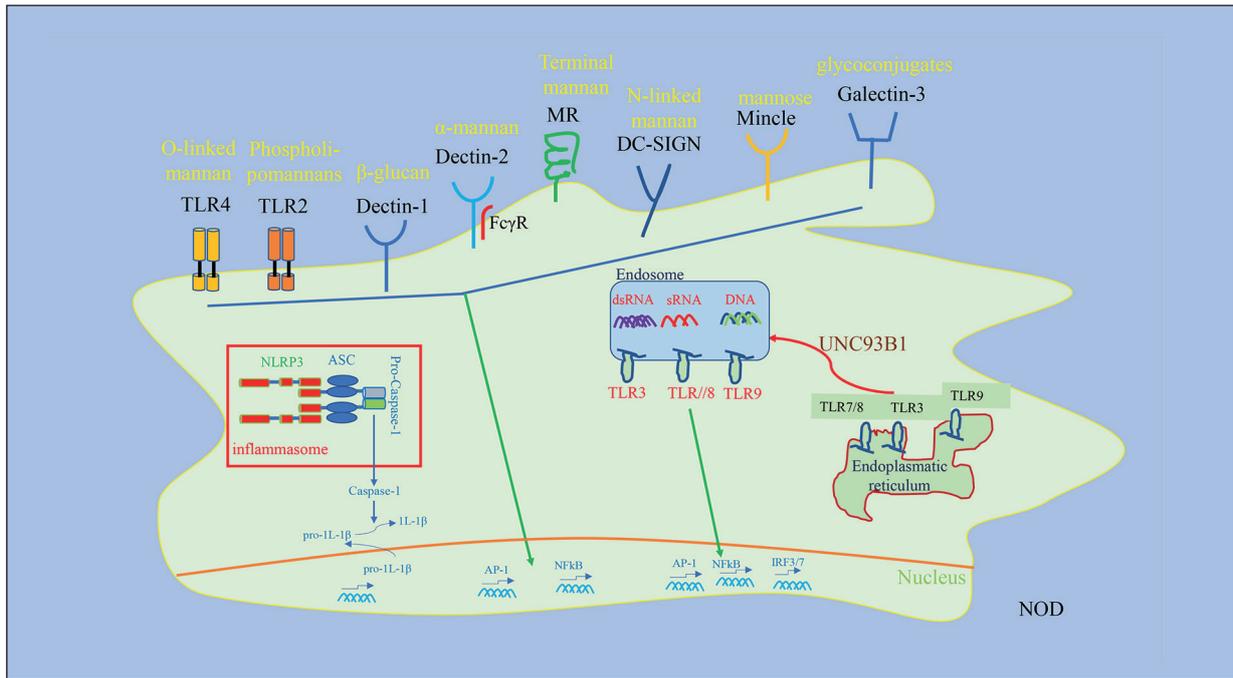
Figure 1. Pattern recognition receptors (PRRs) involved in fungal detection. After engagement of PRRs by individual PAMPs, PRRs modulate immune responses by initiating various signaling cascades that lead to the production of various cytokines and/or chemokines.

genic microorganisms, TLR3, 7, 8, 9 are found intracellularly in endosomes and recognize the nucleic acids of microorganisms[35]. TLR signaling usually includes at least two intracellular pathways, the MyD88-dependent pathway common to all TLRs, except TLR3, and the TRIF-dependent pathway used by TLR3 and 4. Several TLRs are able to initiate innate immune responses following recognition of fungal PAMPs. TLR2 has been shown to be involved in zymosan-induced signaling and this receptor is required for TNF-α production by mouse macrophages in response to *Candida albicans* yeasts or hyphae[34]. TLR4 recognizes O-linked mannans in the cell wall of *C. albicans* and TLR4-deficient mice are more susceptible to invasive candidiasis than WT mice[40]. TLR2, but not TLR4, are required for host defense against *Cryptococcus neoformans*, a pathogenic encapsulated yeast, through the induction of increased cytokines expression[41]. TLR7 plays a crucial role in fungal ssRNA recognition and the lack of TLR7 was associated with increased susceptibility to experimental *C. albicans* infection[38]. In an experimental model of *C. albicans* infection, the recognition of yeast nucleic acids by TLR7 and TLR9 can induce host-protective type-I interferons responses although the suscep-

tibility of TLR9 KO mice to *C. albicans* infection was not significantly different from that of WT mice[8,38]. Recent studies[42] indicate that TLR9 has an important role in the containment and clearance of *Aspergillus* by modulating the innate immune response against swollen fungal conidia. TLR3, another endosomal receptor, has been shown to be important for host defense against experimental pulmonary aspergillosis[43].

C-type lectin-like receptors (CLRs) are a family of transmembrane receptors that play an important role in innate immune defense against fungal pathogens[35]. The family of CLRs are preferentially expressed by myeloid cells and comprises receptors that share similar structures, such as: a carbohydrate recognition domain (CRD) and a transmembrane region[44]. CLRs are capable of recognizing a wide range of ligands among them those mainly involved in antifungal immune responses are: Dectin-1, Dectin-2, Dectin-3, MR, Mincle and DC-SIGN. Dectin-1, Dectin-2, Dectin-3, Mincle and DC-SIGN[44]. Based on their intracellular signaling motifs, it is possible to subdivide CLR receptors into two groups: CLRs with immunoreceptor tyrosine-based activation motifs (ITAMs) and CLRs containing non-ITAMs[45]. To this second group belong the recep-

tors MR and DC-SIGN. Recent studies[45] showed that Dectin-1 specifically recognizes β-1, 3-glucans, whereas Dectin-2 and Dectin-3 specifically recognize α-mannans. Macrophage mannose receptor (MR), macrophage inducible C-type lectin (Mincle), and dendritic cell (DC)-specific ICAM3-grabbing non-integrin (DC-SIGN) would specifically recognize mannose on the surface of the fungus, resulting in the production of TNF-α and IL-10[35]. Dectin-1 transduces signals through the involvement of numerous proteins such as: spleen tyrosine kinase, protein kinase C-delta, and caspase recruitment domain family member 9 (syk/CARD9 pathway). The latter act to induce the transcription of NF-κB and cytokine and chemokine production including TNF-α, IL-10, CXCL2, IL-1β, IL-6 and IL-23 which play an important role in the development of antifungal Th1 and Th17 responses[46]. The interaction of many CLRs with fungal carbohydrate motifs initiates an intracellular signaling cascade that are CARD9-dependent. The fundamental role of this signaling adaptor protein in antifungal defense is evidenced by the fact that CARD9 is required for developing an innate immune response against infection by invasive fungi such as *Candida*, *Aspergillus* and *Cryptococcus* spp[44]. Unlike Dectin-1, Dectin-2 recognizes α-mannans and is therefore able to detect fungal hyphae of different fungal pathogens, including *C. albicans, C. neoformans, A. fumigatus, Paracoccidioides brasiliensis, Histoplasma capsulatum, Microsporum audouinii and Trichophyton rubrum*. Some studies[39,47] have shown that both Dectin-2 and Dectin-3 play a crucial role in $T_H 17$ cell differentiation that has been reported to be important in host defense against *C. albicans* or *Blastomyces dermatitidis.*

Mincle, a sensor for α-mannose and glycolipid trehalose 6′-dimycolate (TDM), is important in host defenses against *Malasezzia spp.,* whereas the role of this receptor in fungal recognition would be ancillary[35,48]. This receptor can induce regulatory responses affecting signaling pathways triggered by TLRs or other CLRs such as Dectin-1. Mincle has been shown to be involved in the recognition of Fonsecaea spp, a pleomorphic fungus responsible for chronic fungal skin infection (chromoblastomycosis)[44].

DC-SIGN is a transmembrane receptor that is able to detect different carbohydrate-based ligands, including mannose and fucose for the induction of successful immune responses against diverse organisms such as HIV-1, *Helicobacter pylori*, *C. albicans* and *A. fumigatus*[44,49,50]. In particular, it has been shown[51] the N-mannosylation and galactomannan are required for the binding, phagocytosis, and immune sensing of, respectively, *C. albicans* or *A. fumigatus* conidia by human DCs.

MR recognizes N-linked mannan and plays an important role in the development of Th17 responses to *C. albicans* although the importance of MR in antifungal immunity has yet to be disclosed since MR is not required for resistance to systemic candidiasis or *Pneumocystis carinii* or *Coccidioides immitis* infection[35,43].

NLRs

NLRs are classified into two main subsets: inflammasome-forming NLRs and non-inflammasome-forming NLRs that are unable to directly activate caspase-1 (i.e., NOD1 and NOD2). Among all NLRs, NLRP3 is a well-studied inflammasome NLR that plays a critical role in the control of disseminated *C. albicans* infections through the activation of a process that leads to caspase-mediated cleavage of pro-IL-1β and pro-IL-18 into functional IL-1β and IL-18[52]. Importantly, both NLRP3 activation and IL-1β production require that the fungus is able of forming hyphae since *C. albicans* incapable of hyphal growth does not induce this response[35]. The activation of the NLRP3 inflammasome has also been shown in response to *Aspergillus* hyphae infection[53]. The role of non-inflammasome forming NLRs, such as NOD1 and NOD2, during fungal infections would be ancillary and should be studied in more detail.

RLRs

Only recently, the role of the RIG-I-like receptors (RLRs), a family of pattern-recognition receptors able to recognize a vast array of RNA viruses[35], has been investigated in anti-fungal immunity[35]. A recent study[54] reports the involvement of the RIG-I-like helicase receptor MDA5 in host innate defenses against *Candida* infection. However, further studies are required to clearly define the role of the RLRs during anti-fungal immunity.

Innate Immune Responses to Fungal Pathogens

Innate immune responses to pathogens, such as bacteria, fungi, and viruses, play a crucial role also in the initiation of pathogen-specific adaptive immunity (Figure 2)[30]. Macrophages are effector
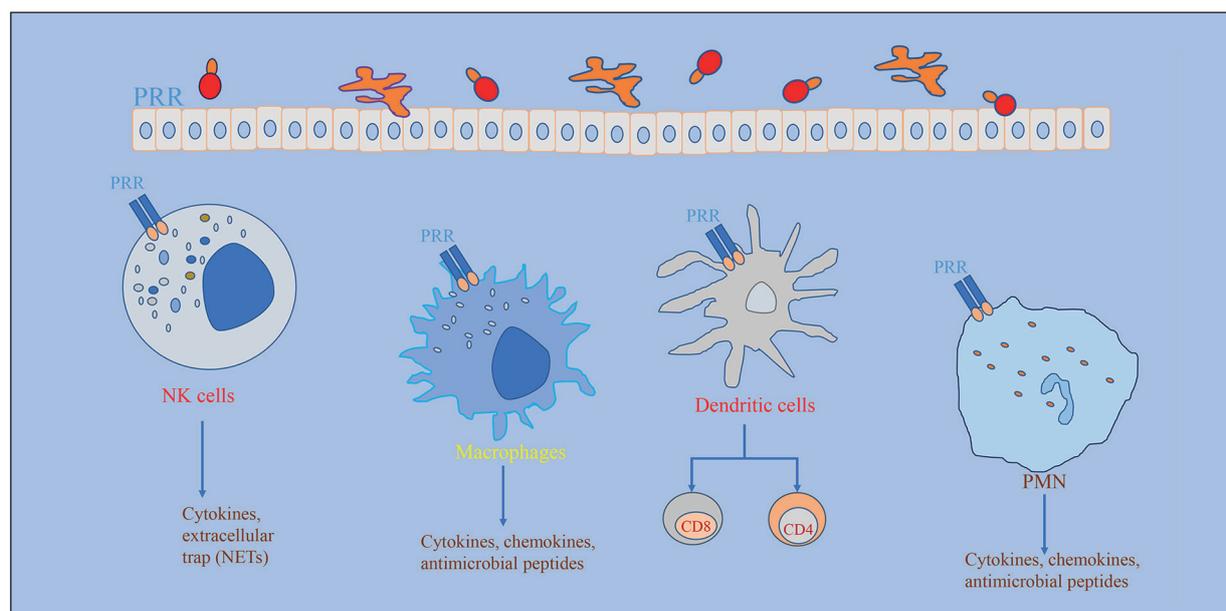
Figure 2. Innate immune response to fungal infections. Innate immune cells are equipped to recognize pathogenic fungi and orchestrate innate immune responses and the activation of adaptive immunity

cells of the innate immune system that link innate and adaptive immunity. During the initial inflammatory phase, the action of macrophages is aimed at eliminating the pathogens and limiting their spread. Macrophages engulf some of the fungal cells by phagocytosis and initiate an acute inflammatory response by producing cytokines and chemokines, which recruit and activate other immune cells to the site of the infection[8]. Recent studies[55] in mice reported that macrophages along with other innate immune cells play a key role in phagocytosis and *Candida* clearance. Further, inflammatory monocytes and macrophages play an important role in host resistance against aspergillosis and candidiasis by inhibiting the conidial germination of these fungi[56]. Recent reports[57] suggest a dual role of monocytes in *Cryptococcus* infection. Specifically, these phagocytes play a beneficial role in the host's innate responses against *C. neoformans*, but they are also detrimental to the host when phagocytosing the fungus protects it from the immune system, thereby promoting its spread and brain invasion.

Dendritic cells (DC) are among the major antigen-presenting cells able to migrate to lymph nodes and prime naïve T cell activation and differentiation towards T-helper (Th) subsets, including Th1, Th2 and Th17 cells which in turn play a major role in protective immune responses against fungal infections[58]. DC can be differen-

tiated into various subsets and, in mice, a DC subset is able to discriminate between yeast and filamentous forms of *C. albicans* and induce different Th cell responses. In humans, two major distinct subsets of DCs, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) have been identified[59]. Evidence[59] suggests that human pDC inhibits the hyphal growth of *A. fumigatus* and the depletion of these cells reduces the survival rate of mice infected with *Aspergillus*. Further studies are needed to clear the exact role of these subsets since DC cells can involve in both protective and pathological T cell phenotypes.

Polymorphonuclear leukocytes (PMLs), also known as granulocytes, are the most abundant leukocytes and include neutrophils, eosinophils, basophils, and mast cells. The neutrophils, which under normal conditions are the most common PMLs, play a central role in initiating the primary immune response to invasive infection caused by some fungal pathogens, such as *Candida* and *Aspergillus* while they play an ancillary role in other infections caused by other fungi, such as *Cryptococcus neoformans*, *Pneumocystis jirovecii* and *Histoplasma capsulatum*[8,60].

Neutrophils employ several strategies to cope with fungal pathogens invading the host, including the production of chemokines, enzymes (myeloperoxidase, NADPH oxidase, and SOD), and antimicrobial peptides. Another distinct antimi-

crobial activity of neutrophils is the production of neutrophil extracellular traps (NETs) involved in the restriction of fungal growth and containment of hyphal forms of fungal pathogens[61].

NK cells constitute ~15% of the cells in the human peripheral blood and they are able to directly kill different microbes including fungi and bacteria. These cells are active against the hyphal forms of *C. albicans* and *A. fumigatus* and have cytotoxic activity against *C. neoformans*, and the dimorphic fungi (*Paracoccidioides braziliensis* and *Coccidioides immitis*)[62]. Moreover, it has been demonstrated that NK are able to regulate the functions of other immune cells by the production of pro-inflammatory cytokines, such as IFN-γ, GM-CSF and RANTES[63]. Recent studies[64] indicate that adoptive transfer of NK cells in immunocompromised patients with IFI could be of great benefit to these individuals.

Antifungal Resistance in Pathogenic Fungi

Microbial resistance refers to microorganisms no longer responding to drugs to which they were previously susceptible[65]. Antifungal drug resistance is a complex phenomenon that depends on different host and microbial factors. Overuse and misuse of antifungal agents are not the only factors responsible for the spread of antifungal resistance[66,67]. Other factors that have contributed significantly to the emergence of antifungal drug resistance include: 1) the inability to sustain the action of the antifungal drug by the immune response of the immunocompromised host, resulting in prolonged use of the drug; 2) the biofilm formation on indwelling catheters makes the microorganisms more resistant to the antifungal drug because the drug cannot reach the site of infection at a sufficient concentration for antimicrobial action; 3) the exposition of some microorganisms to suboptimal levels of drugs contributes to the selection of resistant strains that can proliferate giving rise to the formation of new subclinical reservoirs capable of generating new infections. For more than 40 years, amphotericin B remained the sole treatment option for IFIs[68]. Although very powerful and with a broad-spectrum antifungal activity, its use has been limited by significant renal toxicity. The discovery of new lipid-based formulations of amphotericin B with reduced nephrotoxicity has caused it to remain for much longer for the treatment of IAI. Several reasons have contributed to the reduced development of antifungal drugs and among them, the

most important ones are the reduced incidence in the past of IAI and the eukaryotic structure of the fungal cell that has limited the discovery of antifungal targets[27]. Only a few chemical classes are available for the treatment of IFIs such as polyenes [i.e., amphotericin B (AMB), azoles (i.e., fluconazole, itraconazole), and echinocandins (i.e., caspofungin, micafungin, and anidulafungin] that target different structural features or cellular processes of the fungal pathogen. Azoles block fungal wall synthesis by inhibiting biosynthesis of ergosterol, polyenes bind to ergosterol making pores in the cell membrane while echinocandins act by inhibiting the synthesis of 1,3-β-D-glucan located in the fungal cell wall[67]. The main mechanisms by which fungi exhibit resistance to fungal drugs are: reduced drug uptake, altered drug targets, drug efflux pump activation and drug inactivation. For example, the azole resistance is usually a combination of aforementioned mechanisms which specifically concern: 1) alteration by mutation overexpression of the drug target gene (i.e., *erg11*), reported in *Aspergillus* and *Candida* spp; 2) upregulation of drug efflux pumps mediated by several members of ATP-binding cassette (ABC), reported in *Candida*, *Aspergillus* and *Cryptococcus* spp.; 3) poor drug absorption due to changes in the cell wall or plasma membrane of the fungus[69]. Compared with the first and second-generation of azole drugs, the newest azole released in 2015 are broad-spectrum agents with fewer drug interactions[70]. The echinocandins drugs were introduced in the 2000s for the first time since azoles[71]. They target the fungal cell wall exhibiting low toxicity and synergistic effect in combination therapy; however, they have a limited spectrum, showing fungicidal activity against *Candida* spp, *Aspergillus* spp. and ineffectiveness against fungi of the genus *Mucorales*, *Fusarium*, *Rizpous*, *Scedosporium*, *Trichosporon* and *Cryptococcus* [67]. Moreover, echinocandins being poorly absorbed through the gastrointestinal system are only available as parenteral formulations and consequently they are not recommended for the treatment of mucosal candidiasis[27]. Echinocandin resistance primarily occurs in two highly conserved hot-spots (HS) regions of the *fks* gene-encoding glucan synthase. The frequency of resistance among *Candida* spp. varies among species with an average of about 3%; *C. parapsilosis* and *C. guilliermondii* are the species with the greatest increase in resistance to echinocandins[72]. Exposure to echinocandins activates fungal stress responses that through

activation of protein kinase C (PKC), calcineurin and Hsp90 trigger increased chitin synthesis[73]. This increased amount of chitin contributes not only to the maintenance of cell wall integrity by replacing 1,3-β-D-glucan but also increases the resistance of *Candida spp* to echinocandins[73]. Of particular note is the emergence of a multi-drug resistant *Candida* strain known as *C. auris*, capable of causing nosocomial infections associated with a very high mortality rate. Recent studies indicate that more than 90% of *C. auris* isolates would have extremely high levels of resistance to azoles, and a percentage close to 5% of isolates would be resistant to all three classes of drugs[29]. Antifungal resistance has become a major concern in the treatment of hospitalized patients, particularly those with indwelling medical devices such as stents, prostheses, and catheters[74]. Although much progress has been made in the past decade to identify the mechanisms of resistance to azoles and echinocandins, the factors contributing to their rapid development are only partially known. Identification of these factors is important for the development of new therapeutic strategies to reduce the likelihood of resistance developing during antifungal therapy.

Conclusions

Understanding of how fungi disseminate from the initial infection is essential for correct diagnosis and management. The main barriers against fungal infections are physical barriers such as the skin and mucous surfaces of respiratory, gastrointestinal and urinary tracts. These barriers work in conjunction with cells of innate immune system to prevent colonization and infection. The activation of PRRs in response to PAMPs, such as β-glucan and α-mannan, is crucial for the initiation of innate immune responses. Numerous reports suggest the presence of several cellular mechanisms underlying fungal recognition that lead to the production of different host defense factors. Besides C-type lectin-like receptors (CLRs) other receptors, such as the intracellular receptors that recognize cytoplasmic nucleic acids are crucial in inducing host innate immune defenses against fungal pathogens. One of the best characterized of these mechanisms is the detection of cell-wall structures by receptors located on the host cell surface. This mechanism that leads to the production of inflammatory cytokines, such as TNF-α and IL-23 is independent from TLRs. Conversely, the second best characterized mechanism is dependent from TLR or TRL adaptors and requires the release of fungal nucleic acids that are detected by TLR7 and TLR9 triggering MyD88- and IRF1-dependent responses. Patients with certain immunocompromised or immunosuppressed conditions are unable to mount an effective innate immune response capable of preventing potentially life-threatening infections by fungal pathogens. IFIs continue to be associated with high morbidity and mortality rates due to the spread of drug-resistant strains and lack of rapid diagnostic tools. Rapid and accurate diagnosis of fungal infection is critical to begin effective treatment as soon as possible. In this context, understanding the complex host-pathogen interaction such as the cooperation between CLRs and TLRs in the development of antifungal innate immunity will be critical for the development of effective treatments in the future.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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