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The potential impact of antifungal drug resistance mechanisms on the host immune response to Candida

Russell E. Lewis,[1,*] Pierluigi Viale[1] and Dimitrios P. Kontoyiannis[2]

[1]Division of Infectious Diseases; S. Orsola Malpighi Hospital; University of Bologna; Bologna, Italy;
[2]Department of Infectious Diseases; The University of Texas MD Anderson Cancer Center; Houston, TX USA

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A large number of studies have been published over the last two decades examining molecular mechanisms of antifungal resistance in *Candida* species. However, few of these studies have explored how such mechanisms influence the host immune response to this opportunistic pathogen. With recent advances in our understanding of host immunity to Candida, a body of emerging literature has begun to explore how intrinsic and adaptive resistance mechanisms in Candida alter host immune system evasion and detection, which could have important implications for understanding (1) why certain resistance mechanisms and *Candida* species predominate in certain patient populations, (2) the biological context for understanding why high in vitro levels of resistance in may not necessarily correlate with risk of drug failure in vivo and (3) insight into effective immunotherapeutic strategies for combatting Candida resistance. Although this area of research is still in its infancy, two themes are emerging: First, the immunoevasion and intracellular persistence of *C. glabrata* may be a key factor in the capability of this species to persist in the course of multiple antifungal treatments and develop multidrug resistance. Second, changes in the cell wall associated with antifungal resistance often favor evasion for the host immune response.

Introduction

Candida species are capable of a wide spectrum of infections in human hosts, ranging from benign colonization of the skin and mucosal surfaces to invasion of the bloodstream with dissemination to internal organs. The most common risk factors for invasive candidiasis include major surgery, especially involving the abdomen, immunosuppression (e.g., neutropenia, glucocorticoids and immunomodulators) and many supportive care measures used in the critically ill patient such as broad-spectrum antimicrobials, total parenteral nutrition, renal replacement therapies and central venous catheters.[1] The ubiquity of these risk factors explains, in part, the continuing high prevalence of Candida infections in cancer, transplant and ICU patient populations.[2,3] Although the prompt administration of effective systemic antifungal therapy can significantly reduce the morbidity and mortality associated with invasive candidiasis, increasing rates of antifungal resistance, particularly among *C. glabrata,* are threatening to diminish the efficacy of current frontline agents for invasive candidiasis.[4-6]

A multitude of papers have been published over the last two decades examining the molecular mechanisms of virulence and antifungal resistance in *Candida* spp. Few of these studies have explored how antifungal resistance mechanisms alter pathogen recognition by the innate immune system, or conversely how host immunological responses shape the evolution antifungal resistance in vivo. Yet a number of recent studies have begun to explore how the microevolution of antifungal resistance in vivo may be shaped by intact or residual host immune responses. Indeed, the host immune response may act as a "second drug" (if not the primary drug) that allows emergence of a resistant subpopulation that gives rise to a breakthrough infection. An improved understanding of the interplay between resistance mechanisms and the host immune response could broaden our understanding of the antifungal resistance landscape in *Candida* spp and possibly help prioritize drug resistance/pathogen mechanisms that are most likely to emerge in patients. These studies could also aid our understanding why high MICs for some drug-pathogen combinations have limited utility for predicting clinical failure of therapy in patients. In this review, we will examine the emerging data on how antifungal resistance mechanisms alter host immune response to Candida, and project the possible clinical and laboratory implications of these interactions for interpreting susceptibility testing and treating patients with invasive candidiasis.

Overview of Host Immunity to Invasive Candidiasis

Until recently, relatively little was known about how the host immune differentiated benign colonizing yeast forms of Candida from invasive hyphal forms and what triggers were responsible for activation of the inflammatory response. The discovery of Toll-like receptors (TLRs) in the 1990s heralded a revolution in knowledge of innate immunity that revealed a diverse array of receptors and pathways in leukocytes and epithelial cells capable of detecting specific pathogen-associated molecular patterns

*Correspondence to: Russell E. Lewis; Email: russ.e.lewis@gmail.com
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(PAMPs) expressed at various stages of Candida growth.[7-9] Progress since these early discoveries have led to an integrated model for how the host immune response recognizes *Candida albicans* through pathogen recognition receptors (PRRs) and initiates the early inflammatory response as well as adaptive immunity. A number of excellent reviews have been recently published on this topic.[8,10,11] Therefore, the model for host response to Candida is only briefly summarized below.

Morphogenesis and the cell wall. *Candida* species are capable of growth as yeast, pseudohyphal or hyphal forms. When *C. albicans* infect humans and animals, hyphae predominate at the primary site of infection in epithelial layers and tissue, whereas yeast forms found on the epithelial cell surface or merging from penetrating hyphae in surrounding tissue (**Fig. 1A**).[9,10] The capacity to undergo the reversible yeast-hyphal switch has been shown to be an essential virulence trait of *C. albicans*.[11]

The yeast to hyphal transition is also associated with marked changes in the organization of cell wall carbohydrates and proteins.[8] The cell wall of *C. albicans* is organized into two major layers: an outer layer consisting of glycoproteins (*O*- and *N*-linked mannose polymers, mannoproteins) as well as an inner layer containing skeletal polysaccharides (chitin, β-1,3-glucan and β-1,6-glucan).[8] For the outer layer, yeast to hyphal transition causes changes in the type of cell surface mannans that are expressed, as well as the highly regulated production of proteins that play a role in adhesion and invasion of epithelial cells.[8] For the inner layer, the yeast to hyphal transition has been shown to alter the organization and concentration of structural polysaccharides, including a 3- to 5-fold increase in cell wall chitin and decreased surface exposure of immunogenic β-glucans.[8] These changes could be especially important to the host immune response, as the carbohydrates and proteins found in the cell wall represent the major PAMPs used by immune cells for detecting Candida invasion. Hence, the masking of immunostimulatory cell walls glucans and fortification of the hyphal cell wall with less immunogenic chitin could be key immune evasion strategy employed by *Candida* species during early stages of infection.[8]

Host immune response to Candida. The first encounter with the host immune response occurs at the epithelium, where the mucosa possesses a complex system for differentiating harmless colonizing yeast from invasive hyphal forms.[10,12] Consequently, Candida can colonize but do not typically invade through the mucosal or epithelial layers unless they are damaged resulting in mucosal infection, or in the case of immunocompromised patients, could cause invasive disease that spreads via the bloodstream to distal organs. Inflammatory reactions to yeast forms of Candida are limited in healthy hosts by a low fungal load that is held in check by competing bacterial flora, and cellular morphotype of the fungus, which has limited surface exposure of PAMPs such as β-1,3-D-glucan in the yeast form (**Fig. 1B**).[8] As yeast transition to the invasive hyphal morphotype, they exhibit a greater capacity for endocytosis and damage of epithelium, which causes the release of immunogenic cell wall constituents and proinflammatory cytokines and chemokines from the epithelium. Cytokine and chemokine release acts as the initial trigger for attracting monocytes and neutrophils in the circulation, as well as tissue macrophages (**Fig. 1C**). These cells express a repertoire of PAMP receptors including TLRs (i.e., TLR2 and TLR4), which aid in the discrimination of yeast vs. hyphal morphotypes, and C-type lectin receptors (e.g., Dectin-1, Dectin-2, mannose receptor, DC-sign, Mincle and others), which recognize the matrix of glycosylated proteins (mannoproteins) and glucan and chitin polysaccharides that are the major structural elements of the fungal cell wall.[8,10]

The pattern of PRR ligation that occurs when epithelial or immune cells "taste" Candida cells initiates specific and redundant pathways of immune cell activation leading to cytokine production, phagocytosis, and fungal killing (**Fig. 1C**). This activation pattern also shapes the subsequent pattern of T-cell activation. For example, the balance between TLR2 and TLR4 activation by

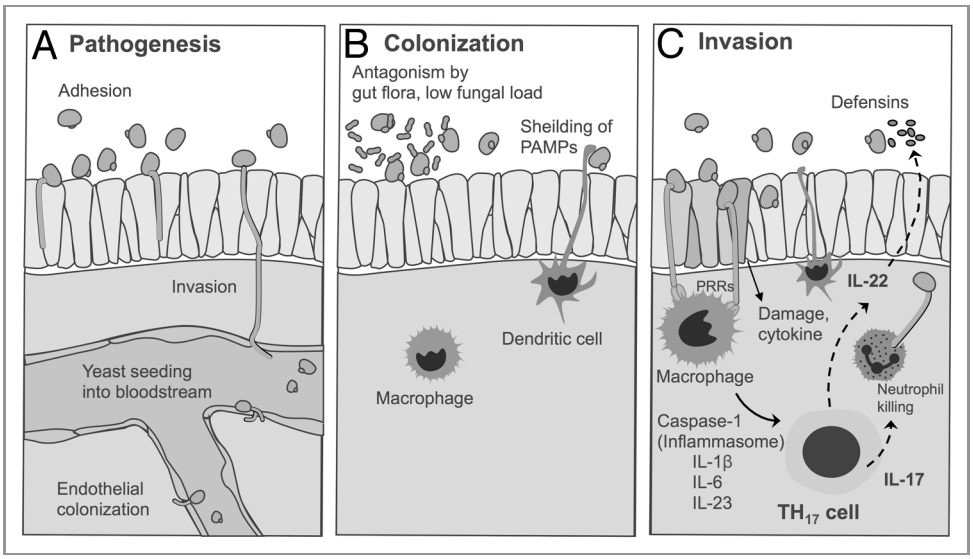

Figure 1. Pathogenesis and host immune response to invasive candidiasis. This figure was recreated in a different format from the review by Gow et al.[8]

Candida cell morphotypes determines the dominant type of T helper cell response.[11] Ligation of TLR4 is more prominent with the PRRs expressed in yeast form of Candida, which induces a pro-inflammatory response resulting in the production TH1-type cytokines such as INF-γ that boost Candida killing.[9] In contrast, hyphal forms of Candida preferentially activate TLR2, which induce a much weaker TH1-response, thus promoting conditions favorable for a TH2 or T-regulatory cell expansion driven by IL-10 that in experimental infections models is associated with reduced Candida clearance.[9,13,14] Hence, the shift from the yeast to hyphal morphotype in Candida may represent a key evasive mechanism employed by the pathogen to down-regulate protective host immune responses.[9]

Similar to epithelial cells, tissue macrophages and dendritic cells monitor the microbial flora on the epithelial surface. The mechanism that allows these cells to discriminate between colonizing yeast and invasive hyphal forms was not well understood until the role of inflammasomes and TH17 cells were elucidated for host response to infection and autoimmunity.[15] Activation of the PRRs that recognize Candida cell wall β-glucan (Dectin-1), phospholipomannans (TLR2) and mannan (macrophage mannose receptor) induce transcription of pro IL-1β, that under colonizing conditions is not processed to its active form because of limited availability of caspase-1.[8,16] However, epithelial damage triggers the activation of the NLRP3 inflammasome in macrophages and dendritic cells, which leads to the activation of caspase-1 and processing of pro IL-1β into the active cytokine form.[15] IL-1β subsequently induces TH17-type response, which includes the production of IL-17, IL-22, neutrophil recruitment for hyphal killing, and boosting of epithelial cell responses through production of defensins (**Fig. 1C**).[15] Interestingly, a recent report by Zelante et al. suggests that *C. albicans* can directly sense IL-17A in human hosts, resulting in downregulation of signal transduction pathways, increased adhesion and filamentous growth as well as enhanced biofilm formation that facilitates resistance to attack by phagocytic cells.[17]

While this model for host immunity to Candida is far from complete, it suggests that the host response is highly adapted and evolved to detect changes in the fungal cell wall and morphology. Growth of fungal cells under varying culture conditions can markedly change cell wall content even if cell morphology is unaltered.[8] Therefore, it seems likely that antifungal therapy in vivo and compensatory resistance mechanisms influencing cell wall composition and organization would similarly alter detection by the host immune cells. The nature of these interactions may have important consequences on the emergence of resistance in vivo and its impact on the host.

Intrinsically Resistant *Candida* Species

Approximately 95% of all invasive Candida infections are caused by five species: *C. albicans, C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*.[18] Among these species, only *C. glabrata* and *C. krusei* are numerically increasing in some geographic areas such as the United States due in part to their intrinsic and acquired resistance to azoles and other commonly used antifungal agents.[18] The prevalence of *C. glabrata* infections is highest among severely ill patients greater than 60 y of age, with one-third of bloodstream infections caused by this species.[19] Importantly, reports of bloodstream infections due to *C. glabrata* resistant to multiple triazoles and echinocandins have increased in recent years.[6] In a review of data from population-based and lab-based surveillance programs in the US, Pfaller and colleagues noted that 9.7% of *C. glabrata* strains were resistant to fluconazole, of which 99% were cross-resistant to voriconazole and 8–9% were also cross-resistant to anidulafungin, caspofungin and micafungin.[6] In contrast, no echinocandin-resistant strains were detected in isolates collected from 2001–2004. These data suggest that while a majority of *C. glabrata* remains susceptible to echinocandin antifungals, the recent increase in MDR strains may represent an ominous trend justifying continued surveillance and antimicrobial susceptibility testing.

The haploid nature of *C. glabrata* genome makes the pathogen particularly well suited for acquiring and expressing MDR resistance traits in the presence of drug pressure.[20,21] However, *C. glabrata* lacks several key virulence factors reported to be essential for virulence in diploid organisms *C. albicans*.[21] For example, *C. glabrata* is the only *Candida* species that does not form pseudohyphae at temperatures above 37°C or secrete hydrolases that have been shown to be essential for tissue invasion and persistence in *C. albicans*.[22] *C. glabrata* also behaves differently from other *Candida* species in the classic mouse intravenous infection challenge.[23] Whereas *C. albicans* and *C. tropicalis* induce severe systemic inflammation and rapidly progressive invasion of kidneys and brain, depending on the mouse strain and inoculum level, inoculation with *C. krusei* and *C. parapsilosis* are not lethal even at high inoculum levels, and are eventually cleared by infected animals. On the other hand, intravenous challenge with even high inoculum of *C. glabrata* is non-lethal in immunocompentent mice, but produces a sustained high fungal burden in animal kidneys that appears to be tolerated by animals with minimal inflammation.[23] Remarkably, *C. glabrata* can be isolated from infected immunocompetent mice over several weeks, without evidence of rapid immune system clearance observed with other *Candida* species.[24] These observations suggest that *C. glabrata* may have a fundamentally greater capacity for immune evasion, which could favor its persistence and emergence as a MDR pathogen in the setting of continuous or repetitive pressure with antifungal agents.

The concept that immune evasion is a key element in *C. glabrata* infection, and by extension the emergence of multidrug resistance, is also supported by studies that have examined interactions of this species with phagocytic cells.[25] *C. glabrata* can survive attack by phagocytes and even replicate inside macrophages after engulfment.[25] Sieder and colleagues recently demonstrated that intracellularly proliferating *C. glabrata* in human macrophages do not elicit the production of reactive oxygen species and only marginally induce production of pro- or anti-inflammatory cytokines.[26] Interestingly, phagosomes containing viable *C. glabrata*, but not heat-killed yeast, failed to recruit cathepsin D and were only weakly acidified. Therefore it appeared

that viable *C. glabrata* was able to subvert normal macrophage phagosome maturation, survive and replicate within these immune cells for considerable periods of time without damaging the host cell or eliciting a proinflammatory immune response.[26] While this interaction could be mutually beneficial during commensal carriage, it would be detrimental for clearance of an invasive infection in a debilitated host during invasive infections.[26]

The exploitation of an intracellular niche as part of an immune evasion and persistence strategy could also favor the development of antifungal resistance in *C. glabrata* and possibly *C. parapsilosis*. In a series of recent studies, Baltch et al. and Bopp et al. examined intracellular yeast killing kinetics of voriconazole and echinocandins (caspofungin and micafungin) in macrophages infected with various *Candida* species.[27-30] In contrast to *C. krusei* where viable CFU counts inside macrophages decreased even in the absence of antifungal exposure, reductions in viable colony forming unit counts of intracellular *C. parapsilosis* and *C. glabrata* required voriconazole or micafungin concentrations that were often 2.5 to 5 times higher than the extracellular MIC.[28] Intracellular anti-candidal activity could be improved if these agents where administered in combination, or in macrophages primed with granulocyte-macrophage colony stimulating factor.

Collectively, these studies provide a plausible hypothesis of how *C. glabrata* may be capable of surviving and persisting in the face of prolonged antifungal therapy to later emerge in a weakened host. Treatment strategies that improve the intracellular activity of antifungals or host immune responses may be a key pathway for reducing antifungal resistance of treating persistent *C. glabrata* fungemia.

Amphotericin B acquired resistance. Amphotericin B exhibits fungicidal effects in *Candida* species by binding to ergosterol in the fungal cell membrane forming pores that cause membrane destabilization and leakage of intracellular contents. Amphotericin B resistance in Candida is generally assumed to be rare, although many broth-based methods used for detecting resistance in *Candida* species may lack the sensitivity to reliably detect resistance in vitro.[4] *Candida* species for which MICs $>$ 1 mg/L are unusual, but at the very least, may require higher doses of amphotericin B for optimal treatment.[4,31] Compared with *C. albicans, C. glabrata* and *C. krusei* are less susceptible to amphotericin B in vitro and display delayed killing kinetics by time-kill studies.[4] *C. lusitaniae* is notorious for developing resistance during amphotericin B therapy, although the species is often susceptible upon initial isolation from the bloodstream.[32] The acquired resistance in this species has been linked to high-frequency phenotypic switching from susceptibility to resistance upon amphotericin B exposure.[33,34] To our knowledge no study has explored differences in host immune responses or virulence for the amphotericin B susceptible vs. resistant phenotypes of *C. lusitaniae*.

The most commonly cited mechanisms of amphotericin B resistance in *Candida* species include alterations in ergosterol biosynthesis leading to a decrease in the amount of ergosterol in the plasma membrane or increased production of catalases reducing drug-associated oxidative damage.[32] However, amphotericin B-resistant strains are rarely isolated from patients suggesting that either the sterol substitutions may be associated with significant fitness costs to infecting isolates in vivo, or possibly enhanced eradication by the host immune response. Indeed, inactivation of enzyme sterol $\Delta^{5,6}$-desaturase (*ERG3*) in *C. albicans*, which results in ergsoterol sterol membrane substitutions and diminished fluconazole and amphotericin B susceptibility, produces *C. albicans* strains locked in the yeast form with attenuated virulence in animal models.[35] A recent report from Vale-Silva and colleagues, however, reported that unlike *C. albicans*, loss-of-function $\Delta^{5,6}$-desaturase (*ERG3*) mutations in *C. glabrata* do not necessarily result in decreased virulence in animal models.[36]

Triazole acquired resistance. Triazoles inhibit 14-α-demethylase, an enzyme responsible for conversion of lanosterol to ergosterol in pathogenic fungi. Inhibition of this rate-limiting step in the ergosterol biosynthesis pathway results in abnormal cell membrane fluidity and function, arresting fungal cell growth. Several resistance mechanisms are commonly associated with triazole resistance in *Candida* species. First, mutations in the gene encoding the drug target *Erg11* alter the drug-binding domain of triazoles, reducing the potency of some, but not necessarily all triazoles.[32,37] Second, high level triazole resistance may result from overexpression of genes involved in the sterol biosynthesis pathway as well as upregulation of two families of efflux pumps, the ATP-binding cassette (ABC) (*Cdr1* and *Cdr2*) and the major facilitator superfamily (MFS) *Mdr1*.[38] Frequently, the co-expression of these resistance mechanisms results in cross-resistance to all triazoles, isolated most frequently in patients with breakthrough *C. glabrata* fungemia.[32] A less common mechanism of resistance in *C. glabrata* involves mitochondrial dysfunction in *C. glabrata*, resulting in the triazole-resistant "petite mutant" growth phenotype in vitro that is resistant to triazoles.[39,40]

Biofilm formation is clearly an important virulence trait and resistance mechanism for chronic or relapsing Candida infections that acts as a physical barrier protecting underlying cells from phagocytes and limiting drug penetration.[41-43] Recent evidence suggest that β-1,3-glucans are a major component of Candida biofilms and may directly bind triazole antifungals such as fluconazole.[41,43] Because drug efflux pumps are upregulated when cells grow in biofilm condition,[44] it is possible that selection of mutants overexpressing efflux mechanisms during drug therapy may favor biofilm-oriented growth and escape from the host immune system.[42,45,46]

Several studies have surveyed the impact of these acquired triazole resistance mechanisms on virulence in *Candida* species (**Table 1**). Graybill and colleagues examined this question in *C. albicans* by testing the virulence of azole-resistant isolates in a mouse model of invasive candidiasis compared with their azole-susceptible susceptible parental isolate.[47] The authors concluded that no direct relationship between fluconazole susceptibility and survival (virulence) was evident. A similar study by Schulz et al. using clonally related *C. albicans* strains from patients with oropharyngeal candidiasis reported that while the fluconazole-susceptible strain was more virulent and exhibited faster growth kinetics and increased biofilm formation, the resistant strain adhered more avidly to epithelial cells facilitating colonization.[48]

Table 1. Acquired Candida resistance mechanisms and potential impact on host pathogen interaction

Resistance mechanism	Drugs affected	Genotypic or phenotypic changes associated with resistance that may alter host immune response
Alterations in the ergosterol biosynthetic pathway (i.e., loss of function in *ERG3)*	Triazoles Amphotericin B	Impaired yeast to hyphal transition; impaired biofilm formation Alterations in membrane cell wall protein localization and function
Alterations in drug target binding (i.e., *ERG11* mutation)	Triazoles	Changes in cell wall mannoproteins, decreased β-1,2-linked mannose residues and side chain structure
Mitochondrial dysfunction	Triazoles	Enhanced cell wall remodeling, increased biofilm formation
Drug efflux (i.e., *CDR1* and *CDR2*), *MDR1*	Triazoles	Possible decreased lysozomal acidification inside macrophages, increased biofilm formation?
Alterations in glucan synthases (i.e., *FKS1* and *FKS2* mutations)	Echinocandins	Impaired yeast to hyphal transition, increased cell wall remodeling; increased cell wall chitin, alterations in biofilm matrix

The impact of antifungal resistance on the host immune response and pathogen virulence may differ, however, for *C. glabrata*. Ferrari and colleagues recently reported that gain of function mutations in the transcriptional regulator CgPDR1—the key modulator of Cdr1 and Cdr2 expression in *C. glabrata*, was associated not only with higher levels of in vitro/in vivo resistance to fluconazole, but also increased virulence and "dominance" of the fungal population in mice even in the absence of drug selection.[49,50] Enhanced in vivo virulence has also been reported in other fluconazole-resistant *C. glabrata* isolates selected in vivo where triazole resistance developed from mitochondrial DNA deficiency independent of gain-of-function mutations in drug efflux pumps.[49] Interestingly, these petite mutants displayed enhanced expression of stress response pathways and cell wall remodeling, similar to that reported after exposure to echinocandin antifungals.[51] Although the specific role of these resistance mechanisms has not yet been fully elucidated with respect to host pathogen interactions, overexpression of drug efflux transporters in other yeast species such as *Cryptococcus neoformans* has been shown to interfere with lysosome acidification in macrophages to increase intracellular fungal survival.[52]

Finally, Takahashi and colleagues reported that acquired triazole and echinocandin resistance in *C. glabrata* was associated with significant changes in the antigenic cell wall mannoprotein structure of *C. glabrata*.[53] Specifically, *C. glabrata* isolates exhibiting resistance to both itraconazole and micafungin contained very low cell concentrations of β-1,2-linked mannose residues relative to susceptible strains. These β-1,2-linked mannose residues have been previously shown to induce TNF-α synthesis through TLR2.[54] Although it was not specifically tested in this study, the authors' findings would suggest that the echinocandin and triazole-resistant *C. glabrata* strains would not elicit as potent response (i.e., TNF-α) release from epithelial or phagocytic cells.[54]

Collectively, these studies suggest that the acquired triazole resistance, particularly in *C. glabrata*, may be associated with significant and possibly advantageous changes in the fungal cell wall and compensatory mechanisms for evading or surviving the initial host immune response. The acquired host immune evasion mechanisms could further facilitate the capability of this species to persist in the setting of antifungal therapy and mutate into multidrug resistant forms.

Echinocandin acquired resistance. Echinocandin antifungals (anidulafungin, caspofungin and micafungin) are among the most widely prescribed antifungals in patients with invasive candidiasis. Despite some pharmacokinetic differences, all three echinocandins act by inhibiting 1,3-β-D-glucan synthase, thereby disrupting glucan biosynthesis in the cell wall.[55] Candida cells exposed to echinocandin concentrations near the MIC have defective cell walls that render the cell susceptible to osmotic lysis. However, even subinhibitory concentrations of echinocandins affect cell wall organization resulting in increased cell surface exposure of immunogenic β-glucans normally hidden by surface mannoproteins, resulting in strong stimulation of immune responses and increases levels of cytokines such as tumor necrosis factor α, interleukin-6 (IL-6), IL-10 and γ-interferon.[56] This β-glucan "unmasking" effect has been shown to be sufficient for fungicidal activity in animals with intact innate immunity.[57] Interestingly, this "unmaking" effect of β-glucan may persist even in some echinocandin-resistant strains, which may be an important limiting factor for the emergence of some resistant subpopulations during treatment.[58,59]

Echinocandin resistance in *Candida* species is most frequently caused by mutations in the genes encoding 1,3-β-D-glucan synthase complex, often in conserved "hot spot" regions of the FKS1 catalytic subunit, although mutations in FKS2 and FKS3 catalytic subunits have also been observed in *C. glabrata*.[60] Mutations in the FKS catalytic subunits alter the kinetics of the glucan synthase enzyme complex resulting in higher inhibitory constant 50% (IC_{50}) and a 50- to several thousand-fold increased kinetic inhibition (ki) for the mutant enzymes for all three echinocandins compared with sensitive wild-type strains.[61] Therefore, mutations in the FKS catalytic sites generally result in cross-resistance to all three echinocandins.[62]

A second pattern of resistance or tolerance to echinocandin fungicidal effects is also sometimes observed when Candida cells are exposed to echinocandin concentrations above the MIC.[63] This paradoxical persistence at higher echinocandin concentrations appears to be mediated through fungal cellular homeostatic cell wall remodeling pathways.[64] Following echinocandin exposure in *Candida albicans*, HOG1, CEK1, PKC MAP kinase and Ca^{2+}-calcineurin signaling pathways are upregulated resulting in increased cellular glucan and chitin synthesis, changes in cellular protein, and increased tolerance to echinocandins

with paradoxical growth at supra-MIC concentrations.[65-67] Interestingly, isolates exposed to paradoxical growth inducing concentrations of glucan synthesis inhibitors such as caspofungin often display reduced ability to activate RAW 264.7 macrophages through Dectin-1 (β-D-glucan) dependent mechanisms (Lewis et al., submitted). Deletion of genes involved in these homeostatic pathways or pharmacologic inhibition (i.e., with calcineurin inhibitors) often reverses paradoxical growth at high echinocandin concentrations and enhances the anti-Candida potency of echinocandins.[68,69] Interestingly, the phenomena of echinocandin paradoxical growth varies between the echinocandin tested and *Candida* species, with paradoxical effects observed most frequently when caspofungin is tested in vitro against in clinical isolates of *C. parapsilosis*, *C. albicans*, *C. dubliniensis*, *C. tropicalis* and occasionally *C. krusei*.[70] Paradoxical growth was not observed when *C. glabrata* is treated with echinocandins. Because paradoxical growth of Candida exposed to high echinocandin concentrations is difficult to detect in vivo,[71-73] some experts have suggested that this phenomena is only an artifact of in vitro testing. Nevertheless, paradoxical growth clearly represents an adaptive response of the fungus to drug and probably influences host immune responses.

Early studies performed by Douglas and Kurtz examining the effects of *FKS1* mutations on echinocandin susceptibility suggested that some mutations associated with high-level echinocandin-resistance may be associated with significant growth defects, impaired yeast to hyphal transition in *C. albicans* and decreased virulence in vivo.[74] Consistent with this observation, a clinical study examining echinocandin-resistant breakthrough *C. tropicalis* infections in leukemic patients found that resistant isolates rarely caused metastatic infection or sepsis.[75] However, generalizations regarding the clinical outcome of echinocandin-resistant strains are difficult, given the heterogeneity and diversity of clinical risk factors of patients in published clinical reports.

Ben-Ami and colleagues recently examined the fitness and virulence costs of *FKS1* hot spot mutations associated with echinocandin-resistance in invertebrate and vertebrate models of invasive candidiasis.[58] Compared with wild-type stains, *C. albicans* strains with homozygous *FKS1* mutations had reduced catalytic activity of glucan synthase, thicker cell walls attributable to increased cell wall chitin and reduced growth rate and capacity for filamentation.[59] *FKS1* mutants were hypovirulent in fly and mouse infection models, including mixed growth competition assays with wild-type strains. In vivo virulence was highly correlated with the cell wall chitin content of the infecting strain. Importantly, *FKS1* mutants with increased cell wall chitin content induced weaker Dectin-1 dependent inflammatory responses when coincubated with RAW264.7 macrophages compared with Wt or *FKS* mutant strains that had minimal increases in cell wall chitin. Data concerning the effect of *FKS1* mutations on biofilm formation capacity in echinocandin-resistant isolates was less conclusive, but several studies have suggested that *FKS1* mutant strains produce biofilm with a less dense matrix but with similar mass as wild-type cells.

These studies were corroborated by investigators from Aberdeen, who reported that *C. albicans* strains harboring *FKS1* mutations often display higher basal production of chitin.[64,76] Animals infected with *C. albicans* cells with elevated cell wall chitin concentrations were similarly resistant to echinocandin treatment.[76] Similar to Ben-Ami et al., the mean survival time of mice infected with high-chitin cells was considerably longer than that of mice infected with normal-chitin cells. Interestingly, compensatory increases in cell wall chitin synthesis were also found to be a good indicator if which stain display paradoxical growth at high echinocandin concentrations.[65] The investigators were able to demonstrate that chitin purified from *Candida albicans* cell wall blocked Dectin-1 mediated recognition of human peripheral blood mononuclear cells and murine macrophages, leading to significant reductions in cytokine production.[76] Hence, chitin may be a signature of "less invasive" form of the pathogen, and consequently does not invoke as vigorous of immune response as β-D-glucan.

Taken as a whole, these studies demonstrated that *C. albicans* remodels its cell wall in response to echinocandin therapy and this adaptation can have a significant impact on pathogen virulence and recognition by host immune cells.[77] These studies may also provide some explanation of why the prevalence of *FKS1* hot spot mutations continues to be low in *C. albicans* and why some patients with bloodstream infection have limited evidence of visceral dissemination/sepsis, or sometimes paradoxically improved clinical response when infected with echinocandin-resistant strains. Nevertheless, other investigators have reported increased virulence in *C. albicans* harboring *FKS1* mutations,[78,79] although immunological responses against these strains were not investigated. The in vivo impact of *FKS1* mutations may be very different in *C. glabrata* vs. *C. albicans*, as suggested in current epidemiological trends of resistance. These questions will provide fascinating avenues for future laboratory, clinical and epidemiological research of invasive candidiasis.

Possible Clinical Implications

Knowledge of how drug resistance mechanisms affect host immune responses is fundamental to understanding the clinical impact of antifungal resistance in opportunistic pathogens such as *C. albicans*. As the key elements in the host immune response to Candida become clearer, an evaluation of how these central elements are affected by drug resistance should be a research priority. These studies could shed light on: (1) the potential for resistance spreading in given patient populations, (2) biological context for understanding why high levels of resistance in vitro may not necessarily correlate with high risk of drug failure in vivo or (3) effective immunotherapeutic strategies for combatting resistance. These studies may also help explain why despite developing resistance mechanisms that may favor the spread to different patient populations, many less common *Candida* species never spread beyond their "classic" host niches.[18]

Host immunity to *Candida* spp is complex by design, as are the pathogen's responses strategies for coexisting with, or escaping the host immune response. This complexity should not prevent the search for patterns of altered host immune responses and pathogen virulence that develop with antifungal resistance

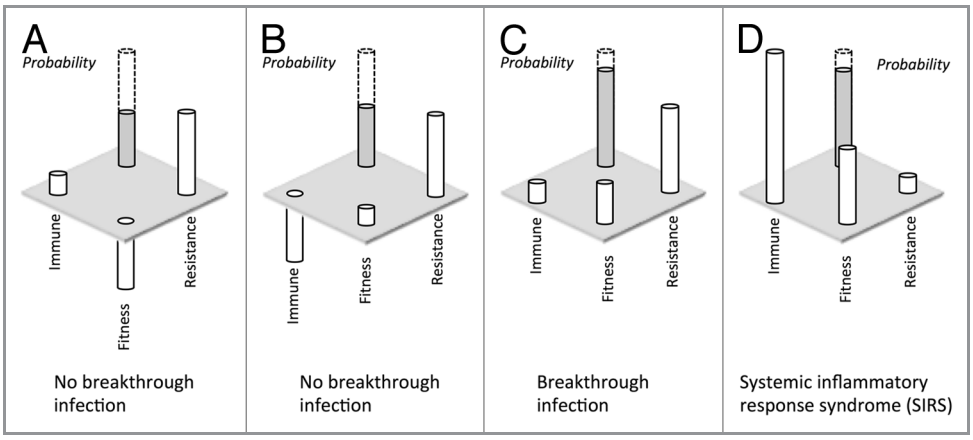

Figure 2. Illustrative hypothetical scenarios for the risk of breakthrough infection with resistant *Candida* species. In (A), expression of a new resistance mechanisms in the presence of antifungal therapy does not alter host immune detection/elimination, but diminishes pathogen fitness. Therefore, the probability of breakthrough infection is low at the current clinical status of the host. In (B), the expression of the resistance mechanism is not associated with a significant fitness cost but does impact immune evasion strategies, therefore the emergence of the resistant subpopulation in held in check by the immune response. In (C), expression of the resistance mechanism is not associated with significant costs in terms of pathogen fitness or host immune evasion, therefore the resistant subpopulation "emerges" in the presence of antifungal therapy. In (D), the isolate does not express resistance mechanisms in the presence of drug, but the high virulence and strong induction of immune responses lead to sepsis (SIRS). The concept for this figure was derived from review by James Anderson.[80]

(**Fig. 2**). Such studies will undoubtedly lead to more questions than answers, but the answers are potentially clinically important and may help solve the even larger mystery of "clinical resistance"—the catch-all term for why patients fail antifungal therapy when the pathogen appears susceptible in the laboratory.

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