Innate Inflammatory Response and Immunopharmacologic Activity of Micafungin, Caspofungin, and Voriconazole against Wild-Type and FKS Mutant Candida glabrata Isolates

Nicholas D. Beyda, a Guangling Liao, a Bradley T. Endres, a Russell E. Lewis, b Kevin W. Garey a

Department of Pharmacy Practice and Translational Research, University of Houston College of Pharmacy, Houston, Texas, USA a; Department of Medical Sciences and Surgery, University of Bologna, Bologna, Italy b

The direct or indirect interactions that antifungals have with the host immune response may play a significant role in defining their activity in vivo. However, the impact that acquired antifungal resistance has on the immunopharmacologic activity of antifungals is not well described. We assessed the immunopharmacologic activity of caspofungin, micafungin, and voriconazole among isolates of Candida glabrata with or without FKS-mediated echinocandin resistance. Clinical bloodstream isolates of C. glabrata from patients who did (n = 5) or did not (n = 3) develop persistent candidemia and who did (n = 2) or did not (n = 11) harbor FKS gene mutations were included. A cell-based assay was used to compare differences in macrophage activation among isolates when grown in the presence or absence of subinhibitory concentrations of caspofungin, micafungin, or voriconazole. In the absence of antifungals, macrophage activation was significantly lower for index C. glabrata isolates obtained from persistent candidemia patients than for those from nonpersistent patients (33% versus 79% increase over negative controls, respectively; P < 0.01). Growth of isolates possessing wild-type FKS genes in subinhibitory concentrations of micafungin and caspofungin, but not voriconazole, significantly increased macrophage inflammatory responses compared to untreated controls (1.25- to 2.75-fold increase, P < 0.01). For isolates harboring the FKS2 hot spot 1 (HS1) S663P mutation, however, a significant increase was observed only with micafungin treatment (1.75-fold increase versus negative control, P < 0.01). Macrophage activation correlated with the level of unmasking of β-glucan in the cell wall. The diminished macrophage inflammatory response to isolates that caused persistent candidemia and differential immunopharmacologic activity of echinocandins among FKS mutants suggest that certain strains of C. glabrata may have a higher propensity for immune evasion and development of antifungal resistance during treatment.

Candida species are a leading cause of bloodstream infections among hospitalized patients and are associated with significant morbidity and mortality. The echinocandin antifungals are recommended as first-line therapy, especially for patients at risk for azole-resistant species such as Candida glabrata (1). In addition to their direct fungicidal or fungistatic activity, the echinocandins can also indirectly stimulate host immune cell inflammatory responses to Candida and Aspergillus (2–5). The echinocandins are cell wall-active agents that work by inhibiting β-glucan synthase (encoded by FKS1 and FKS2 genes), which forms β-1,3-glucan, a key structural component of the fungal cell wall (6). Treatment of Candida albicans and Aspergillus fumigatus with echinocandins has been shown to increase exposure of the normally concealed cell wall β-glucans, which in turn elicits a strong macrophage inflammatory response both in vitro and in vivo (3–5). Although this indirect immunopharmacologic activity may play an important role in shaping their efficacy in vivo, these mechanisms are not often considered to be a component of their antifungal activity. A better understanding of how echinocandins modulate the innate immune response can improve our efforts to optimize the use of these antifungals, especially against C. glabrata, which has a high incidence of antifungal resistance and propensity for immune evasion (7–13). It may also provide insight on how specific antifungal resistance mutations (such as FKS gene mutations conferring resistance to echinocandins) parallel immunevasive strategies and contribute to the persistence of resistant strains in vivo (11, 14).

To date, few studies have evaluated the immunopharmacologic properties of antifungals against C. glabrata. Furthermore, whether acquired echinocandin resistance (due to the development of an FKS gene mutation) impacts the immunopharmacologic activity of antifungals has not been well described. In this study, we used a cell-based reporter assay to profile the innate inflammatory response and immunopharmacologic activity of caspofungin, micafungin, and voriconazole against clinical bloodstream isolates of C. glabrata. Paired strains of echinocandin-susceptible (harboring wild-type FKS genes) and echinocandin-resistant (harboring FKS gene mutations) isolates were tested from patients that developed persistent candidemia to evaluate the impact of acquired resistance on the innate inflammatory response and immunopharmacologic activity of antifungals.
**MATERIALS AND METHODS**

**Test isolates.** Clinical bloodstream isolates of *C. glabrata* obtained from patients admitted to a large, academic teaching hospital in the Texas Medical Center between 2009 and 2013 as part of an institutional review board (IRB)-approved protocol were used in this study. Isolates from patients who did or did not develop persistent candidemia were screened for inclusion. Persistent candidemia was defined as a repeat positive blood culture for candidemia after receiving 5 or more days of echinocandin (micafungin) therapy. Isolates from patients who did not develop persistent candidemia and who had an index wild-type (WT) isolate (the isolate from the first positive blood culture did not harbor an FKS gene mutation conferring echinocandin resistance) were randomly chosen for inclusion. Among patients who did develop persistent candidemia, only those who had an index WT isolate with the same genotype as the persistent WT or persistent FKS mutant isolate were included.

**Antifungal susceptibility testing.** Antifungal susceptibility testing for caspofungin, micafungin, and voriconazole was performed by broth microdilution according to CLSI M27-A3 guidelines using RPMI 1640 + 2% glucose. Endpoints were determined by spectrophotometry after 24 h of incubation at 35°C. The concentration at which turbidity was reduced by 80% compared to the growth control well was defined as the MIC$_{80}$.

**FKS gene sequence analysis.** Genomic DNA was extracted from cells passaged twice on Sabouraud dextrose agar (Hardy Diagnostics, Santa Maria, CA) at 35°C for 24 h using the UltraClean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Hot spot 1 (HS1) and HS2 of FKS1 and FKS2 were amplified by PCR as previously described (15). Standard Sanger sequencing of purified PCR amplicons was performed using a 3730 DNA analyzer (Applied Biosystems, Carlsbad, CA). DNA Dynamo software (Blue Tractor Software, North Wales, United Kingdom) was used to analyze DNA sequences.

**Strain typing.** Genotyping was performed using the DiversiLab Microbial Typing System (bioMérieux) based on repetitive-sequence-based PCR (rep-PCR), which amplifies repetitive, noncoding DNA sequences in *Candida* genomes. For genotyping experiments, DNA was extracted using the MoBio UltraClean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) as recommended by the manufacturer. DNA concentration was measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 35 ng/μL. DNA was then amplified using the commercially available DiversiLab Candida Fingerprinting kit (bioMérieux). Amplification products were separated by microfluidic electrophoresis using a DNA LabChip and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Data were analyzed with Web-based Diversilab software (v3.4) using the Kullback-Leibler coefficient to determine distance matrices and a 3 band differences). Strain typing.

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**Macrophage reporter cells.** We used a commercially available reporter macrophage cell line (RAW-Blue cells; InvivoGen, San Diego, CA). Cells were derived from murine RAW 264.7 macrophages with chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct, induced by NF-κB and activator protein 1 (AP-1) transcriptional activation. An NF-κB reporter was selected because of its rapid posttranslational activation in response to activation of microbial pattern recognition receptors (PRRs). The macrophage reporter cells express PRRs, including Toll-like receptors (TLRs) and NOD-like, RIG-1 like, and C-type lectin receptors, including the PRR for fungal 1,3-β-glucan, dectin-1. Activation of these PRRs by fungal organisms induces signaling pathways leading to the activation of NF-κB and AP-1 and the subsequent production of SEAP. Concentrations of SEAP were then measured by spectrophotometry to characterize the magnitude of NF-κB and AP-1 transcriptional activation using a colorimetric enzyme assay (Quanti-Blu; InvivoGen).

Macrophages were propagated, banked, and prepared for testing daily using protocols developed by the manufacturer. Cells were grown to 80% confluence in T-25 culture flasks in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose, heat-inactivated 10% fetal bovine serum, 2 mM L-glutamine, and 200 μg/ml Zeocin antibiotic (InvivoGen) at 37°C in 5% CO$_2$. To minimize experimental variability, only cells with fewer than 15 passages were used. On each experimental day, cells were harvested by removing growth medium, followed by washing with sterile phosphate-buffered saline (PBS), trypsinization, and resuspension of the cells in fresh DMEM growth medium. Cell viability was confirmed to be >95% by trypan blue counting using a Countess Cell imager (Life Technologies, Grand Island, NY). The final test concentration of reporter macrophages used for all experiments was 5 × 10$^5$ cells/well.

**Candida glabrata drug analysis.** Evaluation of the macrophage response to inactivated *C. glabrata* was performed as previously described by Lewis et al. with some modifications (2). *C. glabrata* isolates were grown for 18 h in a shaking incubator at 35°C in Erlenmeyer flasks containing RPMI growth medium alone or in the presence of 0.25× or 0.5× the MIC$_{90}$ of antifungal agents. On the morning of the experiment, yeast cells were collected using a concentrating pipette with a 0.1 μm polyethersulfone single-use pipette tip and PBS elution fluid (Innovaprep, Drexel, MO). Yeast cells were then pelleted by centrifugation (5,000 rpm) and washed twice with PBS. The inoculum was then counted by a hemocytometer, diluted to a concentration of 5 × 10$^5$ *C. glabrata* cells/ml, and added to the wells of a microtiter tray.

To reduce the confounding effects of growth in the cell culture medium (which could alter the cell well), yeast cells were inactivated by UV by subjecting the microtiter trays to 6 cycles of UV irradiation (120,000 μW/cm$^2$; Stratalinker UV cross-linker 1800; Stratagene, La Jolla, CA). The microtiter plate was then centrifuged (3,500 rpm) to pellet the cells, and PBS supernatant was removed with a micropipette to avoid disruption of the fungal pellet.

Freshly harvested and washed macrophages (5 × 10$^5$) were then added to each well of the microplate containing the fungal inoculum and mixed with a micropipette to create a multiplicity of infection (MOI) of 1:100 (macrophage to fungal cells). Endotoxin-free PBS and Zymosan 10 μg/μL (a potent TLR-2/1,3-glucan elicitor) were used as negative and positive controls, respectively, in each experimental run. After 6 h of coincubation at 37°C in 5% CO$_2$ with intermittent shaking, the microtiter tray was centrifuged, and 50 μL of the cell culture supernatant was carefully removed and transferred to another microtiter plate for analysis of SEAP concentrations according to the manufacturer’s instructions (Quanti-Blu assay; InvivoGen, San Diego, CA). SEAP absorbance was read at an optical density of 655 nm (OD$_{655}$) on a microplate spectrophotometer at 16 h.

**Immunolabeling and relative quantification of cell wall β-1,3-glucan.** *C. glabrata* isolates were grown for 18 h in a shaking incubator at 35°C in RPMI growth medium alone or in the presence of 0.5× the MIC$_{90}$ of antifungal agents as described before. The cells were collected and fixed for 40 min in 4% paraformaldehyde. Following fixation, the cells were washed and then incubated for 30 min in PBS containing 1% bovine serum albumin (BSA). After blocking, the cells were washed and then stained with the anti-β-glucan antibody (Bioспysios, Inc., Australia) diluted 1:200 in PBS for 1 h at 37°C. The cells were washed three times with PBS and then allowed to incubate for 1 h at 37°C in darkness with donkey anti-mouse Alexa Fluor 555-conjugated secondary antibody (Life Technologies), which was diluted 1:100 in PBS. Finally, the cells were washed again, rediluted in PBS, and imaged using a Leica SP8 confocal microscope. All images were processed with FIJI software.

**Statistical analysis.** Experiments were performed in at least triplicate. Mean SEAP concentrations ± standard deviations (SD) were compared by the Kruskal-Wallis test with Dunn’s post hoc test for individual comparisons. Differences in SEAP concentrations associated with a P value of <0.05 were considered to reflect a significant difference in macrophage NF-κB/AP-1 transcription activation.
RESULTS

C. glabrata isolates included in the analysis. A total of 13 clinical bloodstream isolates of C. glabrata obtained from 8 patients were included in this analysis (Fig. 1). Of the 8 patients included, 3 did not develop persistent candidemia and 5 developed persistent candidemia while receiving antifungal treatment. Of the 5 that developed persistent candidemia, the persistent isolates from 2 patients (patients 7 and 8) harbored an FKS gene mutation conferring echinocandin resistance (FKS2 H5663P) while the persistent isolates from 3 patients (patients 4, 5, and 6) did not harbor an FKS gene mutation. None of the index isolates harbored an FKS mutation. Rep-PCR analysis indicated that the same strain was present during the course of infection among patients with persistent candidemia, as no difference in banding or fingerprinting pattern was observed between the index or persistent isolates for each patient (Fig. 1).

Macrophage reporter cell assay. During assay development, SEAP levels were assessed after macrophages were coincubated with antifungal-exposed and -unexposed C. glabrata at MOIs of 1:1, 1:10, and 1:100 (macrophage/fungal cells). Compared to macrophages alone, a significant increase in SEAP levels could be detected in response to antifungal-exposed C. glabrata at MOIs of 1:1, 1:10, and 1:100, and for antifungal-unexposed C. glabrata at 1:100 (see Fig. S1 in the supplemental material). An MOI of 1:100 was chosen as it provided the best dynamic range for capturing changes in macrophage NF-kB/AP-1 transcription induced by antifungal-exposed and -unexposed C. glabrata strains.

Macrophage inflammatory responses to index WT C. glabrata isolates grown in the absence or presence of caspofungin, micafungin, or voriconazole. Macrophage inflammatory responses were compared among index wild-type isolates obtained from patients that did (n = 5) or did not (n = 3) develop persistent candidemia.

Macrophages exposed to index WT isolates grown without antifungals elicited modest but significant increases in NF-kB/AP-1 transcription activation overall compared to negative controls (45% ± 4%, P < 0.001). However, macrophage NF-kB/AP-1 transcription activation was less robust for index WT isolates from patients that developed persistent candidemia (33% ± 2% increase) than for those from patients who did not develop persistent candidemia (79% ± 7% increase; P < 0.05) (Fig. 2).

Growth of index WT isolates in subinhibitory concentrations

<table>
<thead>
<tr>
<th>Isolate</th>
<th>FKS mutation</th>
<th>CAS (µg/ml)</th>
<th>MFG (µg/ml)</th>
<th>VOR (µg/ml)</th>
<th>Prior antifungal exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index</td>
<td>WT</td>
<td>0.25</td>
<td>0.06</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>Persistent</td>
<td>S6663P (FKS2)</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>Micafungin</td>
</tr>
<tr>
<td>Index</td>
<td>WT</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>Fluconazole</td>
</tr>
<tr>
<td>Persistent</td>
<td>WT</td>
<td>0.5</td>
<td>0.12</td>
<td>0.5</td>
<td>Fluconazole + micafungin</td>
</tr>
<tr>
<td>Index</td>
<td>WT</td>
<td>0.25</td>
<td>0.06</td>
<td>0.12</td>
<td>None</td>
</tr>
<tr>
<td>Persistent</td>
<td>S6663P (FKS2)</td>
<td>16</td>
<td>8</td>
<td>0.12</td>
<td>Fluconazole + micafungin</td>
</tr>
<tr>
<td>Index</td>
<td>WT</td>
<td>0.5</td>
<td>0.06</td>
<td>0.12</td>
<td>Fluconazole + micafungin</td>
</tr>
<tr>
<td>Persistent</td>
<td>WT</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>Fluconazole</td>
</tr>
<tr>
<td>Persistent</td>
<td>WT</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>Fluconazole + micafungin</td>
</tr>
<tr>
<td>Index</td>
<td>WT</td>
<td>0.5</td>
<td>0.06</td>
<td>0.5</td>
<td>Fluconazole</td>
</tr>
<tr>
<td>Persistent</td>
<td>WT</td>
<td>0.5</td>
<td>0.06</td>
<td>4</td>
<td>Fluconazole</td>
</tr>
</tbody>
</table>

FIG 1 (Left) Dendrogram of 13 C. glabrata isolates obtained from 8 hospitalized patients with candidemia. (Right) The presence or absence of an FKS mutation, antifungal susceptibility (MIC80) to caspofungin (CAS), micafungin (MFG), and voriconazole (VOR), and the patients’ antifungal exposure history prior to the collection of each isolate are provided.

FIG 2 Macrophage NF-kB/AP-1 activation is significantly lower for index WT C. glabrata isolates from patients who developed persistent candidemia than for those from patients who did not. Each experiment was performed in at least triplicate. *, P < 0.05 versus negative control, Kruskal-Wallis test with Dunn’s post hoc test. **, P < 0.05 between index WT isolates from patients with and without persistent candidemia.
performed in at least triplicate. *, P < 0.05 versus negative control, Kruskal-Wallis test with Dunn’s post hoc test.

(0.25× and 0.5× the MIC) of caspofungin or micafungin elicited 1.25- to 2.75-fold-higher rates of macrophage NF-κB/AP-1 transcriptional activation (P < 0.05) than those of nonantifungal-exposed controls (Fig. 3 and Table 1). No significant difference was observed when isolates were grown in subinhibitory concentrations of voriconazole.

**Macrophage inflammatory responses to persistent WT C. glabrata isolates grown in the absence or presence of caspofungin, micafungin, or voriconazole.** The macrophage inflammatory responses to isogenic paired strains consisting of an index WT and persistent C. glabrata mutants, however, a significant increase in NF-κB/AP-1 transcription was observed only when isolates were grown in subinhibitory concentrations of caspofungin or micafungin elicited similar increases in macrophage NF-κB/AP-1 transcription (1.25- to 1.75-fold increases; P < 0.05) compared to their antifungal-unexposed controls. Growth of the index WT or persistent WT isolates in subinhibitory concentrations of voriconazole did not significantly increase NF-κB/AP-1 transcription compared to their antifungal-unexposed controls.

**Macrophage inflammatory response to persistent FKS mutant C. glabrata in the absence or presence of caspofungin, micafungin, or voriconazole.** Macrophage inflammatory response to isogenic paired strains consisting of an index WT and persistent FKS mutant isolate from two patients were evaluated (Fig. 1). Both persistent FKS mutant isolates harbored the FKS2 HS1 S663P mutation and had significantly elevated MICs to both caspofungin (8 to 16 μg/ml) and micafungin (4 to 8 μg/ml), while the MICs were significantly lower for the index WT isolates (0.25 and 0.06 μg/ml, respectively). When grown without antifungals, no significant difference in macrophage NF-κB/AP1 transcription was observed between the paired strains (Fig. 5). Growth of the index WT isolates in subinhibitory concentrations of caspofungin or micafungin elicited 1.25- to 2.75-fold increases in macrophage NF-κB/AP-1 transcription levels (P < 0.05) compared to unexposed controls. For the persistent FKS mutants, however, a significant increase in NF-κB/AP-1 transcription was observed only when isolates were grown in 0.5× the MIC of micafungin (1.8-fold increase over unexposed controls; P < 0.05) (Fig. 5). No significant change in NF-κB/AP-1 transcription was observed for index WT or persis-

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**TABLE 1** Percent increase in macrophage NF-κB/AP-1 transcription activation by index WT C. glabrata isolates relative to antifungal-unexposed controls after growth in subinhibitory concentrations of caspofungin, micafungin, or voriconazole

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Caspofungin</th>
<th>Micafungin</th>
<th>Voriconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25× MIC</td>
<td>0.5× MIC</td>
<td>0.25× MIC</td>
</tr>
<tr>
<td>1</td>
<td>138 ± 23</td>
<td>96 ± 11</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>177 ± 16</td>
<td>179 ± 26</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>130 ± 5</td>
<td>179 ± 4</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>4</td>
<td>55 ± 13</td>
<td>47 ± 15</td>
<td>6 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>103 ± 3</td>
<td>49 ± 7</td>
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<tr>
<td>6</td>
<td>155 ± 7</td>
<td>125 ± 8</td>
<td>27 ± 4</td>
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<tr>
<td>7</td>
<td>98 ± 9</td>
<td>87 ± 5</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>261 ± 15</td>
<td>215 ± 15</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>Overall</td>
<td>152 ± 14b</td>
<td>133 ± 11b</td>
<td>36 ± 3b</td>
</tr>
</tbody>
</table>

*a* NA, not available: data were excluded due to contamination or insufficient growth.

*b* P < 0.05, Kruskal-Wallis test with Dunn’s post hoc test.
tent FKS mutant isolates grown in subinhibitory concentrations of voriconazole compared to unexposed controls.

**β-glucan unmasking in response to echinocandin treatment.** Echinocandins have been shown to unmask immunogenic β-glucans in the cell wall of *C. albicans* and *A. fumigatus*; however, this effect has not been studied in *C. glabrata* or in isolates with FKS-mediated echinocandin resistance. In order to assess this, we used an indirect immunofluorescence assay with β-1,3-glucan-specific antibodies to examine differences in β-glucan exposure among paired strains of index WT and persistent FKS mutant isolates when grown in the presence or absence of subinhibitory concentrations (0.5× the MIC) of caspofungin or micafungin. Since β-1,3-glucan is localized primarily to the inner layer of the cell wall, relatively little fluorescent signal was observed in both the WT and FKS mutant isolates when grown in the absence of antifungals (Fig. 6). In contrast, growth of the index WT isolates in caspofungin or micafungin led to distinct β-glucan staining at the cell wall, corresponding to a 63% (*P < 0.05*) and 80% (*P < 0.05*) increase in fluorescence, respectively, compared to growth without antifungals. Interestingly, growth of the persistent FKS mutant isolates with caspofungin did not unmask β-glucan, while micafungin caused a significant increase (*P < 0.05*) in β-glucan presence at the cell wall compared to growth without antifungals.

**DISCUSSION**

The outcome of invasive fungal infections involves a complex dynamic interplay between host, pathogen, and antifungal treatment factors (16). Direct or indirect interactions of antifungals with the

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**FIG 4** Similar levels of macrophage NF-κB/AP-1 transcriptional activation are observed between paired strains of index WT and persistent WT *C. glabrata* isolates when grown with or without caspofungin, micafungin, or voriconazole at subinhibitory concentrations (0.25× and 0.5× MIC). Growth in subinhibitory concentrations of caspofungin and micafungin significantly increased macrophage NF-κB/AP-1 transcription relative to unexposed controls. Each experiment was performed in at least triplicate. *, *P < 0.05* versus negative control, Kruskal-Wallis test with Dunn’s *post hoc* test.

**FIG 5** Growth of index WT isolates in subinhibitory concentrations of caspofungin or micafungin elicits significant increases in macrophage NF-κB/AP-1 transcription activation relative to unexposed controls. In contrast, for persistent FKS mutant isolates a significant increase in NF-κB/AP-1 transcription is observed only when grown in 0.5× the MIC of micafungin. Each experiment was performed in at least triplicate. *, *P < 0.05* versus negative control, Kruskal-Wallis test with Dunn’s *post hoc* test.
host immune response have been shown to play a role in shaping their efficacy against fungal pathogens in vitro and in vivo (3, 9, 16, 17). Similarly, acquired gene mutations conferring antifungal resistance and/or adaptive cell wall stress responses may diminish innate immune cell recognition and response, which may promote the survival and proliferation of resistant strains in vivo (11, 14). Given the importance of these immunological effects, we sought to evaluate the immunopharmacologic activity of caspofungin, micafungin, and voriconazole among clinical bloodstream isolates of \textit{C. glabrata} obtained from patients with or without persistent candidemia. Furthermore, we determined the impact of acquired echinocandin resistance on both the innate immune response and the immunopharmacologic activity of micafungin and caspofungin.

In this study, we observed that the macrophage inflammatory response was significantly dampened against index WT \textit{C. glabrata} isolates obtained from patients who developed persistent candidemia compared to the response of those who did not. We also observed macrophage activation levels to be similar between the persistent WT (or persistent \textit{FKS} mutant) and index WT isolate obtained from patients with or without persistent candidemia. Furthermore, we determined the impact of acquired echinocandin resistance on both the innate immune response and the immunopharmacologic activity of micafungin and caspofungin.

In agreement with studies performed with \textit{C. albicans} and \textit{A. fumigatus} using a similar methodology, macrophage inflammatory responses were found to be significantly enhanced when index WT \textit{C. glabrata} isolates were treated with subinhibitory concentrations of caspofungin or micafungin (2, 3, 5). This enhanced inflammatory response occurred consistently for both caspofungin and micafungin at concentrations that have been observed in human plasma after administration of standard doses (0.01 to 10 μg/ml) (18, 19). We also observed that treatment of persistent WT \textit{C. glabrata} isolates with caspofungin or micafungin significantly enhanced macrophage activation, with a magnitude similar to what was observed with the paired index WT strains. In contrast, only micafungin treatment significantly enhanced macrophage activation against the persistent \textit{FKS} mutant isolates, despite both caspofungin and micafungin displaying significant activity against the parent index WT strains. Based on these results, it is possible that continuing micafungin therapy (in addition to another class of antifungals) in a patient who develops an \textit{FKS} mutant isolate...
may be beneficial by indirectly stimulating the host immune response despite retaining minimal fungicidal activity. However, further in vitro and in vivo studies that include isolates with other FKS mutations will be required to test this hypothesis.

Despite including C. glabrata isolates demonstrating both low and high voriconazole MICs, treatment with subinhibitory concentrations of voriconazole did not result in significant macrophage activation for any isolate. In contrast to the echinocandins, voriconazole acts on the cell membrane by inhibiting ergosterol synthesis. While supra-MICs (4× the MIC) of voriconazole have been shown to impact the ultrastructure of C. glabrata, it is possible that subinhibitory concentrations failed to induce a significant change in the fungal cell wall and thus had minimal impact on macrophage detection and inflammatory response in our model (22).

Although the assay and methodology used in this study were robust and relatively simple to perform, several limitations should be acknowledged. First, our multiplicity of infection (MOI) (1 macrophage for 100 fungal cells) is higher than what is typically reported in the literature. In our preliminary studies, we found this MOI to provide the best dynamic range for capturing differences in macrophage NF-κB/AP1 transcription (measured by OD 655) after exposure to C. glabrata grown in the presence and absence of antifungals. The composition of the culture medium (nutrient rich versus poor) can impact ultrastructural characteristics of the fungal cell, which in turn may alter macrophage responses. Lastly, while monitoring the transcriptional activation of NF-κB/AP-1 often correlates directly with cytokine production, it does not provide information regarding specific immune effector mechanisms.

Conclusions. In summary, differences in macrophage inflammatory responses were observed between index WT C. glabrata isolates obtained from patients who did and did not develop persistent candidemia. We also observed a significant increase in macrophage activation when index WT C. glabrata isolates were treated with caspofungin or micafungin but not voriconazole. However, for the persistent FKS mutant isolates, only micafungin treatment led to a significant increase macrophage activation. Activation of macrophage correlated with the level of unmasking β-glucan in the cell wall. Our findings highlight that caspofungin and micafungin have differential patterns of immunopharmacologic activity against C. glabrata, which changes in the setting of specific FKS mutations. Further studies to identify specific changes in the fungal cell wall induced by exposure to echinocandins in isolates with or without FKS gene mutations should be conducted to better understand the interplay between the host, pathogen, and antifungal treatment.

ACKNOWLEDGMENTS

Transparency declarations: N.D.B. has served as a consultant to and received research grants from Astellas and T2 Biosystems Inc.; R.E.L. has served as a consultant for Gilead and Merck; K.W.G. has received research grants from Astellas and T2 Biosystems Inc.; R.E.L. has received research grants from Astellas, Merck, and T2 Biosystems Inc. All other authors have no conflicts of interest to declare.

This work was supported in part by an unrestricted research grant from Astellas Pharma Inc. The study sponsors had no role in the study design or the collection, analysis, or interpretation of the data.

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