Pharmacodynamic and Immunomodulatory Effects of Micafungin on Host Responses against Biofilms of *Candida parapsilosis* in Comparison to Those of *Candida albicans*

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ABSTRACT Micafungin (MFG) demonstrates potent activity against biofilms of *Candida albicans* and *Candida parapsilosis*, the most frequent opportunistic fungal pathogens. Little is known about its immunopharmacologic effect on antibiofilm activity of phagocytic cells following exposure to *Candida* biofilms. In this study, we investigated the effects of MFG on human neutrophil-mediated damage of *C. albicans* and *C. parapsilosis* biofilms by XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] and the potential mechanisms underlying the immunomodulatory MFG activities on cultured monocyte-derived THP-1 cells in response to these biofilms by reverse transcription-PCR and sandwich and multiplex enzyme-linked immunosorbent assay. Preexposure of *C. albicans* to subinhibitory MFG concentrations significantly enhanced neutrophil-mediated biofilm damage, an effect that appears to be species specific since a comparable effect was not observed with drug-pretreated *C. parapsilosis* biofilms. Human THP-1 cells responded to both *Candida* biofilms through Toll-like receptor 2 (TLR2) and TLR4 upregulation, modest TLR6 involvement, and enhanced NLRP3 activation, whereas the signal was relayed to the nucleus via NF- κ B p65 activation. MFG caused 2- to 3-fold lower TLR2 and TLR4 mRNA levels than those caused by either organism. *C. albicans* biofilms induced a robust proinflammatory response, whereas *C. parapsilosis* biofilms either alone or in the presence of MFG caused increased interleukin-1 β (IL-1 β) production, but small amounts of IL-8, IL-23, and tumor necrosis factor alpha. In conclusion, MFG may condition THP-1 cells toward an inflammatory response through TLR2/TLR4 recruitment. Inflammatory signals observed with *C. albicans* biofilms are considerably reduced upon exposure of THP-1 cells to *C. parapsilosis* biofilms, possibly enhancing fungal survival and increasing biofilm pathogenicity.

KEYWORDS micafungin, antibiofilm activity, immunomodulation, *Candida* biofilms

While *Candida albicans* has been the most frequently isolated species in invasive fungal infections, an increased incidence of candidemia due to *Candida parapsilosis* has been observed at the last years (1, 2). Both *Candida* spp. are considered primary causes of morbidity and potential mortality among immunocompromised patients and great threats in neonatal intensive care units in newborns with central lines or parenteral nutrition (3–5). A major virulence factor that contributes to infections

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is the propensity of *Candida* spp. to develop biofilms on vascular or urinary catheters, as well as on other foreign bodies (1).

For the treatment of biofilms, echinocandins demonstrate potent fungicidal activity documented both *in vitro* (6, 7) and *in vivo* (8). At subinhibitory doses, echinocandins have been shown to possess an indirect immunomodulatory effect, as they expose immunogenic cell wall determinants on the surfaces of *Candida* blastoconidia, causing an increased immune response (9, 10). In phagocytes, activation of host responses is achieved through recognition of fungal cell wall structures by immune cell surface receptors on blastoconidia; the signal delivered intracellularly initiates a cascade of events leading to activation and nuclear translocation of NF- κ B, production of cytokines, recruitment of polymorphonuclear leukocytes, and release of reactive oxygen species. NLRP3 inflammasome activation holds also a central role in anti-*Candida* host defense as it is involved in processing of pro-interleukin-1 β (pro-IL-1 β) and IL-1 β secretion, leading to the priming of an inflammatory response (11–13). We hypothesized that subinhibitory concentrations of micafungin (MFG) may alter biofilm formation, enhancing the antifungal efficacy of immune cells and, indirectly, modulating immune responses to the benefit of host defenses in the containment of *Candida* biofilms.

In this study, we (i) assessed whether preexposure of *C. albicans* or *C. parapsilosis* biofilms to subinhibitory MFG concentrations can augment the efficacy of human polymorphonuclear neutrophils (PMN) and (ii) evaluated whether MFG exerts a differential immunomodulatory effect on cultured monocyte-derived THP-1 cells exposed to mature biofilms of *C. albicans* or *C. parapsilosis*. We also followed the molecular events involved from pathogen cell surface recognition to nuclear signal transduction to cytokine protein expression.

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RESULTS

Antifungal effect of PMN against MFG-pretreated *C. albicans* or *C. parapsilosis* biofilms. The median biofilm MICs (MIC₅₀) of MFG against the *C. albicans* and *C. parapsilosis* isolates in this study were determined to be 0.25 mg/liter (range, 0.06 to 0.5 mg/liter) and 4 mg/liter (range, 4 to 8 mg/liter), respectively. Based on these results, we chose to study the antifungal effect of three subinhibitory concentrations of MFG against each organism. Following MFG treatment, both organisms exhibited similar percentage of metabolically active biofilms compared to untreated control (*C. albicans*: 90 to 82%, Fig. 1A; *C. parapsilosis*: 77 to 82%, Fig. 1C). Nevertheless, when PMN were added to drug-pretreated *C. albicans* biofilms, there was a significant augmentation of PMN-induced biofilm damage against the organism exposed to 0.5 $\times$ MIC MFG (0.125 mg/liter) compared to drug-untreated biofilms (81% $\pm$ 1.8% versus 60% $\pm$ 3.5%; P < 0.01, Fig. 1B). In contrast, PMN addition to MFG preexposed *C. parapsilosis* biofilms exhibited similar biofilm damage compared to the damage induced by PMN on intact biofilms (32% $\pm$ 2.3%, 36% $\pm$ 6.4%, and 30% $\pm$ 6.8% versus 35% $\pm$ 3.8%, respectively; P = not significant [ns], Fig. 1D).

TLR2, TLR4, TLR6, DECTIN-1, and NLRP3 mRNA expression in THP-1 cells incubated with *C. albicans* or *C. parapsilosis* biofilms and MFG. While mRNA levels of both Toll-like receptor 2 (TLR2) and TLR4 induced by the incubation of THP-1 cells with biofilms of both *Candida* spp. increased significantly, TLR2 and TLR4 induced by *C. parapsilosis* biofilms were 2- to 3-fold greater than those observed after incubation with *C. albicans* biofilms (Table 1). MFG alone caused increased mRNA expression of TLR2 and TLR4, albeit to a lesser extent than those observed with biofilms of both *Candida* spp. or in response to the combination treatment. While TLR6 was modestly upregulated, NLRP3 mRNA was significantly increased in THP-1 cells exposed to biofilms of both *Candida* species in the presence or absence of MFG (Table 1). DECTIN-1 mRNA

FIG 1 Effect of MFG subinhibitory concentrations on metabolic activity and human PMN-mediated biofilm damage of *C. albicans* and *C. parapsilosis*. (A and C) Percent metabolic activity of *C. albicans* and *C. parapsilosis* drug-pretreated biofilms (black columns) compared to untreated biofilms (striped columns). (B and D) Planktonic cells of *C. albicans* or *C. parapsilosis* were exposed to subinhibitory concentrations of MFG for 24 h. Untreated and drug-pretreated cells were washed and grown for 48 to 72 h for biofilm formation. Untreated (white columns) and pretreated (gray columns) mature biofilms were incubated with PMN at an E:T ratio of 5:1 for an additional period of 24 h. Metabolic activity and biofilm damage were assessed by the XTT assay (see Materials and Methods). Data are presented as means $\pm$ the standard errors (SE; bars) derived from four experiments with each experimental condition being tested in pentaplicate. Comparisons between PMN-mediated damage of drug-pretreated and untreated biofilms (A and C) or between metabolically active drug-pretreated and untreated biofilms (B and D) were performed by ANOVA with Dunnett's test for multiple comparisons. Statistically significant differences from untreated control with P values of ≤ 0.05 are indicated by an asterisk.

expression was not detected in THP-1 cells exposed to either species under any experimental condition.

NF- κ B p65 protein expression in nuclear extracts of THP-1 cells in response to MFG and *C. albicans* or *C. parapsilosis* biofilms. We observed more NF- κ B p65 protein present in nuclear extracts of THP-1 cells exposed to the combination treatment for both *Candida* spp. than in the cells exposed to biofilms alone (*C. albicans*: 321 ± 22 ng/ml versus 279 ± 19 ng/ml, *C. parapsilosis*: 329 ± 31 ng/ml versus 182 ± 52 ng/ml, $P < 0.05$; Fig. 2).

Differential cytokine release from THP-1 cells exposed to *C. albicans* or *C. parapsilosis* biofilms in the presence or absence of MFG. Of the 16 cytokines tested, detectable levels were obtained for IL-1 β , IL-8, IL-23, and tumor necrosis factor alpha (TNF- α). The combination treatment of MFG plus *C. albicans* biofilms (CABF) caused the release of significantly increased levels of IL-1 β (CABF+MFG [0.06, 1, and 4 mg/liter] versus CABF, $P < 0.001$; Fig. 3) and IL-8 (CABF+MFG [0.06 mg/liter] versus CABF, $P < 0.001$; Fig. 3) from THP-1 cells compared to those released by THP-1 cells stimulated with CABF. MFG alone stimulated THP-1 cells to produce significantly reduced amounts of IL-1 β , IL-8, IL-23, and TNF- α compared to those produced by THP-1 cells exposed to

TABLE 1 Fold changes in the expression of TLR2, TLR4, TLR6, and NLRP3 genes involved in immune recognition

Gene and <i>Candida</i> sp.		Fold change ^a in THP-1 cells treated with:						
		Biofilm	MFG			MFG+biofilm		
			0.06 mg/liter	1 mg/liter	4 mg/liter	0.06 mg/liter	1 mg/liter	4 mg/liter
TLR2								
<i>C. albicans</i>	3.0	1.0	1.2	1.3	2.0	1.3	2.4	
<i>C. parapsilosis</i>	6.5	3.4	3.0	3.0	7.5	8.0	7.0	
TLR4								
<i>C. albicans</i>	2.1	1.2	1.4	1.7	2.0	1.0	2.2	
<i>C. parapsilosis</i>	7.0	2.4	2.0	2.0	7.0	5.4	7.0	
TLR6								
<i>C. albicans</i>	0.6*	0.8*	1.0	0.6*	0.7*	0.6*	1.3	
<i>C. parapsilosis</i>	0.7*	0.6*	1.1	0.5*	1.0	1.2	1.4	
NLRP3								
<i>C. albicans</i>	3.0	3.0	2.5	3.1	2.0	3.5	2.2	
<i>C. parapsilosis</i>	3.0	3.0	2.6	2.4	3.3	3.0	3.0	

^aIncreases in gene expression of ≥ 1 -fold were considered significant. *, fold changes of < 1 were considered not significant.

the combination treatment (MFG [1 and 4 mg/liter] versus CABF+MFG [1 and 4 mg/liter] for IL-1 β ; MFG [0.06 mg/liter] versus CABF+MFG [0.06 mg/liter] for IL-8; MFG [4 mg/liter] versus CABF+MFG [4 mg/liter] for IL-23; MFG [0.06, 1, and 4 mg/liter] versus CABF+MFG [0.06, 1, and 4 mg/liter] for TNF- α ; $P < 0.05$, Fig. 3).

In contrast, except for the level of IL-8 which was significantly upregulated in the combined treatment of *C. parapsilosis* biofilms (CPBF) with 0.06 mg/liter of MFG compared to the IL-8 amounts released by THP-1 cells incubated with CPBF ($P < 0.05$, Fig. 4), similar amounts were observed for IL-1 β , IL-8, IL-23, or TNF- α in the supernatants from MFG-treated THP-1 cells or the combination treatment of CPBF plus MFG (Fig. 4; $P = \text{ns}$).

DISCUSSION

Biofilm formation of *C. albicans* and *C. parapsilosis* in catheters or other implanted devices in patients with weakened immune host defenses most often leads to the development of candidemia with serious repercussions for the health of these patients. As early catheter removal may not always improve outcome (14), an effective antibiofilm treatment is of great clinical importance. Echinocandins, despite reports of breakthrough infections, retain their potency against *Candida* species and in situations where the foreign body cannot be removed they constitute potential options for systemic and

FIG 2 NF- κ B p65 protein in nuclear extracts of THP-1 cells is upregulated after stimulation of THP-1 cells with *C. albicans* or *C. parapsilosis* biofilms in the presence or absence of MFG. Untreated THP-1 cells (white column) were incubated with biofilms from each organism at an E:T ratio of 10:1 or with the combination of MFG (0.06 mg/liter) plus *C. albicans* (black column) or *C. parapsilosis* (gray column) biofilms for 4 h. Nuclear NF- κ B p65 protein expressed in ng/ml represent means $\pm$ the SE from three independent experiments. Differences between biofilm-stimulated THP-1 and THP-1 cells incubated with MFG with or without biofilms were tested by ANOVA by Dunnett's posttest for multiple comparisons. Values significantly different ($P < 0.05$) are indicated by an asterisk.

FIG 3 Profiles of IL-1 β , IL-8, IL-23, and TNF- α cytokine levels released from THP-1 cells stimulated with *C. albicans* biofilms and MFG. THP-1 cells alone (white column) were incubated with MFG (0.06, 1, and 4 mg/liter; light gray columns), with *C. albicans* biofilms (black column), or with a combination of biofilms plus MFG (dark gray columns) for 4 h. Protein levels were measured using multiplexed human cytokine Strip wells 16-plex assay. Data are presented as means $\pm$ the SE derived from three independent experiments. Comparisons between biofilm-treated THP-1 cells with MFG alone or with the combined treatment of MFG with or without *C. albicans* biofilms were performed by ANOVA with Dunnett's test for multiple comparisons. Statistically significant differences with *P* values of ≤ 0.05 are indicated by an asterisk.

lock antifungal therapy (15, 16). To our knowledge, no information exists about the immune molecules involved in biofilm recognition and signal transduction for the activation of an immune response. In addition, limited investigations have been performed on the impact of echinocandins on immune cells' antibiofilm activity or the type of immune responses that are elicited by phagocytes against biofilms of *C. albicans* or *C. parapsilosis* (17–22).

Our results demonstrate that preexposure of *C. albicans* to subinhibitory MFG concentrations significantly enhances subsequent PMN-mediated biofilm damage, an observation that appears to be species specific since a comparable effect is not seen with drug-pretreated *C. parapsilosis* biofilms. In addition, our findings on host-fungal interactions show that THP-1 cells respond to *Candida* mature biofilms through TLR2 and TLR4 upregulation, modest TLR6 expression level, and NLRP3 activation, whereas the signal is relayed to the nuclei of THP-1 cells via NF- κ B p65 activation. *C. parapsilosis*

FIG 4 Profiles of IL-1 β , IL-8, IL-23, and TNF- α cytokine levels released from THP-1 cells stimulated with *C. parapsilosis* biofilms and MFG. THP-1 cells alone (white column) were incubated with MFG (0.06, 1, and 4 mg/liter; light gray columns), with *C. parapsilosis* biofilms (black column), or with a combination of biofilms plus MFG (dark gray columns) for 4 h. Protein levels were measured using multiplexed human cytokine strip wells 16-plex assay. Data are presented as means $\pm$ the SE derived from three independent experiments. Comparisons between biofilm-treated THP-1 cells with MFG alone or with the combined treatment of MFG plus *C. parapsilosis* biofilms were performed by ANOVA with Dunnett's test for multiple comparisons. Statistically significant differences with P values of ≤ 0.05 are indicated by an asterisk.

biofilms either alone or in the presence of MFG cause increased IL-1 β production, but small amounts of IL-8, IL-23, and TNF- α . In contrast, *C. albicans* biofilms cause a higher overall innate immune response as larger amounts of IL-8, IL-23, and TNF- α are released by THP-1 cells than those observed in response to *C. parapsilosis* biofilms. MFG tends to enhance cytokine release when combined with *C. albicans* biofilms without reaching significance.

Taking together the results of the pharmacodynamic experiments with PMN and the host-*Candida* biofilm interaction experiments with THP-1 human macrophages, the antifungal action of MFG seems to have a beneficial effect toward innate immune cells in host defense against *C. albicans* biofilms, but a blunt effect against *C. parapsilosis* biofilms: preexposure of *C. albicans* to MFG facilitates the subsequent biofilm killing

FIG 5 Overview of the effects of micafungin to innate immune cells in the defense of *C. albicans* and *C. parapsilosis* biofilms. (A) Preexposure of *C. albicans* to MFG benefits the subsequent biofilm killing capacity of PMN against mature biofilms. The candida modulates THP-1 human macrophages toward a proinflammatory program upon contact of immune cells with biofilm structures that induce IL-1 β , IL-8, IL-23, and TNF- α to be released using mainly TLR2 and TLR4 receptors and NF- κ B/NLRP3 signal transduction molecules. (B) In contrast, the killing capacity of PMN is similar against either intact or MFG-pretreated biofilms of *C. parapsilosis*. THP-1 human macrophages respond to the stimulus of *C. parapsilosis* biofilms with a strong TLR2/TLR4 response. However, such response leads to an increased release of IL-1 β but to moderate amounts of the chemoattracting cytokine IL-8 and very small amounts of IL-23 and TNF- α .

capacity of PMN against mature biofilms. The echinocandin modulates THP-1 cells toward a proinflammatory program upon contact of immune cells with biofilm structures that induce cytokines to be released using mainly TLR2 and TLR4 receptors and NF- κ B/NLRP3 signal transduction molecules (Fig. 5A). On the other hand, the killing capacity of PMN is similar against either intact or MFG-pretreated biofilms of *C. parapsilosis*. THP-1 cells respond to the stimulus of *C. parapsilosis* biofilms with a TLR2/TLR4 upregulation that is stronger than that to *C. albicans*. However, such a response leads to an increased release of IL-1 β and moderate amounts of the chemoattracting cytokine IL-8 mediated through the NLRP3 inflammasome activation and the NF- κ B p65 signal transduction molecule (Fig. 5B).

Treating *C. albicans* blastoconidia with subinhibitory concentrations of MFG or caspofungin not only affects β -glucan biosynthesis but also changes protein compo-

sition that leads to a cell wall configuration that interferes with *Candida* virulence and immunogenicity, making *C. albicans* vulnerable to natural immune cells (23, 24). *C. albicans* and *C. parapsilosis*, two of the most clinically important *Candida* spp., form different biofilm structures: whereas *C. albicans* biofilms are composed of a continuous cell layer with thick matrix, *C. parapsilosis* biofilms form less dense interspersed structures characterized by irregular clusters of blastoconidial aggregates and pseudohyphal cells (25, 26). Nevertheless, *C. parapsilosis* biofilms exhibit variable and relatively more resistant profile to echinocandins than that observed with *C. albicans* biofilms (6–8, 27). As structural analysis is beyond the scope of this study, we can only speculate that drug-pretreated *C. parapsilosis* cells do develop altered cell wall architecture, but the clustered growth phenotype of biofilms maintains an efficient shield that counteracts the antifungal activity of PMN.

Studies on host-*Candida* interactions have revealed that the NLRP3 inflammasome is activated by a diverse array of fungal stimuli and involves several receptors on the surfaces of human macrophages, including DECTIN-1, DECTIN-2, mincle, TLR2, or TLR4, which lead to the production of cytokines through NF- κ B activation (28–30). Reports link IL-1 β production induced by *C. albicans* to the NLRP3 inflammasome, whereas the increased susceptibility of *Nlrp3* knockout mice has shown the protective role of NLRP3 in host defense against disseminated candidiasis (29, 31). However, the role of DECTIN-1 in connection with fungal structures is unclear, as studies have shown that macrophage DECTIN-1 receptors recognize the yeast form of *C. albicans* through β -(1,3)-glucan binding, but not hyphal forms, whereas recognition depends on the accessibility of the ligand on the fungal cell wall (32, 33). Moreover, the degree of exposure and distribution of β -glucan structures in *Candida* biofilms may depend on biofilm maturation or surface attachment, as β -glucan has been shown to be present during early biofilm growth but not in mature biofilms formed in abiotic surfaces (34). In our study we show that mature biofilms of both *Candida* species induce expression of TLR2, TLR4, and NLRP3 receptors with a modest upregulation of TLR6, but DECTIN-1 gene expression is not detected. As the biofilm structure adds an additional layer of protection, the most accessible *Candida* ligands that predominate on the outer cell wall are those recognized by TLRs. TLR2 and TLR4 assisted by TLR6 may cooperatively act to cope with the invading fungus. However, additional experiments are needed to dissect further the connection of DECTIN-1 and biofilm ligand accessibility in relation to biofilm age and MFG exposure, as the antifungal concentrations used in this study do not seem to have a beneficial effect toward “unmasking” β -glucan ligands in the setting of mature biofilms.

IL-1 β , TNF- α , and IL-23 are secreted by macrophages that promote Th1-mediated immunity and provide a profoundly fungicidal microenvironment for the destruction of fungal organisms. However, deregulated secretion of inflammatory cytokines or a shift from an inflammatory to an anti-inflammatory immune response may enhance fungal survival and increase fungal pathogenicity. A recent study on the differences in the proteomic profile between M1-type and M2-type macrophages in response to *C. albicans* has shown that the fungus has the ability, during a 3- to 18-h time window, to cause a switch from a more inflammatory M1 phenotype to a less inflammatory M2 phenotype thus escaping clearance by the innate immune system (35). Chandra et al., using a coculture system for growing *C. albicans* in the presence of human monocytes, observed that biofilms induce monocytes to secrete differential levels of cytokines that could explain the demonstrated immunosuppressive effect of fungal biofilms to the monocyte's phagocytic activity (17). However, anidulafungin, a drug belonging to the same class of echinocandins, was shown to disrupt such an effect and induce a Th1 response by increased TNF- α production after a 22-h exposure of THP-1 cells to *C. albicans* mature biofilms (18). By investigating an earlier time point of an immune response, we support and extend the data presented above demonstrating that MFG increases inflammatory responses in response to *C. albicans* biofilms.

To our knowledge, there are no other studies reporting interactions between *C. parapsilosis* biofilms and human cells. Nevertheless, our results indicate that soluble

factors released by *C. parapsilosis* biofilms might act on macrophages deregulating their release of inflammatory cytokines. Furthermore, MFG seems to have a restricted role in the augmentation of immune responses against *C. parapsilosis* biofilms.

Focusing on the development of new therapeutic strategies to treat biofilm-related infections, it is of the utmost importance to define precisely the factors that regulate differential species-dependent interactions between biofilms and host immune cells, as well as delineate the interplay between biofilms, host immunity, and echinocandins, as this antifungal class still presents the preferred choice to treat several types of candidiasis. However, as drug resistance is associated with long-term prophylactic treatments, poor drug penetration at sites of infection and suboptimal drug exposure especially in immunocompromised patients, the selection of the optimal antimicrobial drug dose, route of administration, and duration of therapy should be considered for a successful therapeutic outcome.

MATERIALS AND METHODS

Clinical isolates and growth conditions. Four *C. albicans* and four *C. parapsilosis* blood isolates recovered from immunocompromised adult patients at the Hippokration Hospital of Thessaloniki were studied, along with two well-documented biofilm-producing strains: *C. albicans* M61 and *C. parapsilosis* strain A71 (25). Species identification of the eight isolates was performed using the Vitek II system or the API ID32C (bioMérieux, Marcy l'Etoile, France). Stocks were maintained at -80°C in yeast-peptone-dextrose broth with 25% glycerol (Oxoid, Cambridge, UK) solution. After strain revival on Sabouraud (Scharlau Chemie, S.A., Barcelona, Spain) agar plates, each isolate was transferred to yeast nitrogen base (Difco Laboratories, Detroit, MI) medium supplemented with 2% glucose and incubated at 37°C overnight on a rocking table. The grown cultures were harvested by centrifugation at 2,000 rpm for 10 min, washed twice with phosphate-buffered saline solution (PBS; 0.02 M phosphate, 0.15 M NaCl [pH 7.2]), and resuspended in RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Antifungal agent. MFG was obtained from Astellas Pharma, Inc. (Tokyo, Japan). The antifungal drug was dissolved in sterile water to a final concentration of 10 mg/ml and maintained as stock solution at -35°C for up to 1 month. An aliquot of antifungal stock was further diluted to 1 and 10 mg/liter in RPMI 1640 and used to prepare concentrations of 0.06, 1, and 4 mg/liter.

Biofilm formation. Mature *Candida* biofilms were formed using 96-well microtiter polystyrene plates as previously described (36). Briefly, aliquots of 100 μl of yeast suspension were inoculated into each well and incubated for 48 h (*C. albicans*) or 72 h (*C. parapsilosis*) at 37°C with shaking. Plates were then centrifuged at 4,000 rpm for 20 min and washed once with PBS to remove unattached cells, and 100 μl of fresh RPMI 1640 was subsequently added. Mature biofilm production was documented by staining the polysaccharide structure of the extracellular matrix of biofilms with 0.1% safranin for 5 min and measuring the optical absorbance at 492 nm with a microplate reader (ChroMate 4300; Inter Bio-Lab, Palm City, FL) (37).

Preparation of PMN. Human polymorphonuclear leukocytes (PMN) were purified from healthy adult volunteers by dextran sedimentation and Ficoll centrifugation. Whole blood was mixed with dextran to a final concentration of 1%, and the red blood cells (RBCs) were allowed to sediment for 20 min. The top layer of the biphasic solution was placed above Ficoll and centrifuged at $400 \times g$ for 20 min. The supernatant was removed, and the remaining RBCs were lysed by hypotonic shock. The PMN were washed with Hanks balanced salt solution (HBSS), resuspended in the same buffer solution, and counted with a hemocytometer. Neutrophil preparations were $>95\%$ pure and $>95\%$ viable as visualized by their ability to exclude the trypan blue dye. PMN were adjusted to $5 \times 10^6/\text{ml}$ and used immediately for the functional assays.

Antifungal treatment of biofilms and PMN exposure. Portions (10^6 blastoconidia/ml) of *C. albicans* or *C. parapsilosis* were grown in RPMI solution (pH 7.2) at 37°C for 24 h in the presence of either 0.03, 0.06, or 0.125 mg/liter MFG or with 0.5, 1, or 2 mg/liter MFG, respectively. After a brief washing step with PBS for drug removal, untreated and MFG-preexposed isolates were grown in fresh RPMI at 37°C for 48 h (*C. albicans*) or 72 h (*C. parapsilosis*) for biofilm formation. Mature biofilms were subsequently incubated with PMN at an effector-to-target ratio (E:T) of 5:1 for an additional period of 24 h at 37°C and 5% CO_2 in an incubator.

Assessment of biofilm damage. The biofilm MIC of MFG and the PMN-induced biofilm damage were assessed by an XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay, as described previously (6). Metabolic activity and biofilm damage were calculated according to the following two formulae: percent metabolic activity = $(X/C) \times 100$, and percent biofilm damage = $[1 - (X/C)] \times 100$, where X is the absorbance of experimental wells and C is the absorbance of control wells. Each experimental condition was tested in pentaplicate. Four independent experiments were performed.

Cell culture treatment. The THP-1 monocytic cell line (ATCC TIB202; American Type Culture Collection, Manassas, VA) was grown in a humidified CO_2 incubator at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (FCM; Gibco BRL/Life Technologies, Ltd., Paisley, Scotland). THP-1 monocytes were differentiated into macrophages with phorbol myristate acetate (PMA) at a final concentration of 100 nM at 37°C with 5% CO_2 for 6 h in a humidified atmosphere. The cells were then washed once with HBSS

TABLE 2 PCR primers used for the amplification of TLR2, TLR4, TLR6, DECTIN1, NLRP3, and ACT1 genes

Primer	Sequence (5'–3')	T_m (°C)	Amplicon size (bp)
TLR2 FRW	TCCTCCAATCAGGCTTCTCTGTCTT	54.8	628
TLR2 REV	CTCGCAGTTCCAAACATTCC		
TLR4 FRW	CACAGACTTGCGGGTTCTAC	54.8	1,341
TLR4 REV	AGGACCGACACACCAATGATG		
TLR6 FRW	GGCCTGCCCATCTGTAAGG	54.8	886
TLR6 REV	ACTCTCAACCCAAGTGCAGT		
DECTIN1 FRW	CGCCTCATTGCTGTAATTTTGG	54.8	463
DECTIN1 REV	ATCCATCCTCCCAGAGCCA		
NLRP3 FRW	CCGCCATGTAACCTGCT	56.9	451
NLRP3 REV	GACAGTGACTCCACCCGAT		
ACT1 FRW	TACAGGAAGTCCCTTGCCAT	54.8	243
ACT1 REV	ACCAAAAGCCTTCATACATCTCA		

without Ca^{2+} and Mg^{2+} (Gibco BRL) and rested in fresh PMA-free FCM media at 37°C overnight. A total of 10^6 cells/ml were then incubated with mature biofilms of *C. albicans* M61 strain or *C. parapsilosis* A71 strain at an E:T ratio of 10:1 either alone or in the presence of 0.06, 1, or 4 mg/liter MFG at 37°C with 5% CO_2 for 4 h. Culture supernatants were collected to measure the released cytokines using multiplex enzyme-linked immunosorbent assay (ELISA), whereas total RNA from THP-1 cells was extracted from each experimental condition to follow the gene expression profile of membrane-bound and intracellular receptors by reverse transcription-PCR (RT-PCR).

Cell viability. The viability of PMN or THP-1 cells was tested using the trypan blue exclusion method by counting stained (dead) versus unstained (alive) cells on a hemocytometer. Cell viability was >95% in all experiments.

Total RNA isolation. Total RNA was extracted from THP-1 cells under each experimental condition according to the protocol for the isolation of RNA using TRIreagent (Bioline, GmbH, Germany). Briefly, cells were lysed by adding 1 ml of TRIreagent, and proteins from each lysate were removed by adding 0.2 ml of chloroform. The aqueous phase was precipitated by mixing with 0.5 ml of cold isopropyl alcohol, followed by a centrifugation step at $12,000 \times g$ for 10 min at 4°C. The RNA pellet was dissolved in diethylpyrocarbonate-treated sterile water and quantified by spectrophotometry (UV101; Pocklington, United Kingdom). The total RNA purity measured at A260/280 was >1.8, whereas the amount of total RNA recovered was in the range of 1 to 2 $\mu\text{g}/\mu\text{l}$. Samples were subsequently stored at -70°C for no more than 1 week prior to downstream processing.

RT-PCR analysis. PCR primers (Table 2) specific for the TLR2, TLR4, TLR6, DECTIN-1, and NLRP3 genes were designed using Oligo 7 primer analysis software. cDNA synthesis and PCR amplification was performed using the SuperScript One-Step RT-PCR System (Invitrogen, Carlsbad, CA). PCR mixtures contained 10 μM concentrations of each primer, $2\times$ reaction mix, 1 μl of superscript RT/platinum *Taq* mix, and 500 ng of total RNA. cDNA was synthesized in one cycle at 55°C for 30 min after a denaturation step at 94°C for 10 min. PCRs were then carried out in 35 cycles of denaturation at 94°C for 15 s, with primer annealing at 54.8°C for 30 s (NLRP3: 56.9°C, 30 s) and extension at 72°C for 1 min. A final 5-min extension at 72°C completed the reaction. PCR products were separated by gel electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. Fold expression analysis between untreated THP-1 cells and drug-treated THP-1 cells exposed to biofilms was calculated by measuring signal intensities using the TotalLab Quant software. The relative intensity of the target amplification product from treated samples over the untreated control expressed the change in folds with respect to the untreated control. Actin mRNA (ACT1) was used as an internal control for gene normalization. Two independent RT-PCR experiments were performed. A >1-fold change was considered significant.

Measurement of NF- κ B p65 activity. To measure NF- κ B p65 activity, THP-1 cells were incubated with either *C. albicans* or *C. parapsilosis* biofilms at a 10:1 E:T ratio in the presence of 0.06 mg/liter MFG for 4 h. The cells were then harvested, the cell lysates and nuclear extracts were prepared according to the manufacturer's instructions (Novus Biologicals, Abingdon, UK), and the nuclear translocation of NF- κ B p65 was measured using an NF- κ B ActivELISA kit. One hundred microliters from each experimental condition was added into appropriate wells in duplicate. NF- κ B p65 was detected colorimetrically by reading the color development at 405 nm in an ELISA plate reader. Three independent experiments were performed.

Measurement of cytokine production. At the end of the 4-h incubation period, the culture supernatants were collected and stored at -30°C until assayed. The levels of 16 cytokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, gamma interferon, TNF- α , and TNF- β) were measured simultaneously using multiplexed human cytokine Strip wells 16-plex assay (Quansys Biosciences, West Logan, UT). Thawed samples were centrifuged at $13,000 \times g$ for 10 min at 4°C immediately prior to testing. The chemiluminescence absorbance intensities were captured by the Q-View imager, and the concentrations of the relevant cytokines were determined for each sample using standards provided by the manufacturer. Data analysis was performed with the Q-View software using log-based regression analysis and a log-log curve fitting model. The overall coefficient of variation for duplicate samples was <20%. Each experimental condition was tested in duplicate. Three independent experiments were performed.

Statistical analysis. The average values of all replicates were used in the data analysis to determine the mean standard error for each condition. The PMN-induced percent biofilm damage of drug-treated biofilms compared to untreated control and differences in protein concentrations between biofilm-treated THP-1 cells with MFG-treated THP-1 cells and MFG+biofilm-treated THP1 cells were tested by analysis of variance (ANOVA) with Dunnett's posttest. Data were analyzed using the Instat 3 biostatistics software (GraphPad, Inc., San Diego, CA). A two-tailed *P* value of <0.05 was considered significant.

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