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Micafungin Enhances the Human Macrophage Response to *Candida albicans* through β-Glucan Exposure

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ABSTRACT Micafungin belongs to the antifungal family of echinocandins, which act as noncompetitive inhibitors of the fungal cell wall β-1,3-D-glucan synthase. Since *Candida albicans* is the most prevalent pathogenic fungus in humans, we study the involvement of micafungin in the modulation of the inflammatory response developed by human tissue macrophages against *C. albicans*. The MIC for micafungin was 0.016 μg/ml on the *C. albicans* SC5314 standard strain. Micafungin induced a drastic reduction in the number of exponential SC5314 viable cells, with the fungicidal effect being dependent on the cellular metabolic activity. Notably, micafungin also caused a structural remodelling of the cell wall, leading to exposure of the β-glucan and chitin content on the external surface. At the higher doses used (0.05 μg/ml), the antifungal also induced the blowing up of budding yeasts. In addition, preincubation with micafungin before exposure to human tissue macrophages enhanced the secretion of tumor necrosis factor alpha (TNF-α), interleukin-17A (IL-17A), and IL-10 cytokines. Our results strongly suggest that in *C. albicans* treatment with micafungin, in addition to having the expected toxic antifungal effect, it potentiates the immune response, improving the interaction and activation of human macrophages, probably through the unmasking of β-glucans on the cell wall surface.

KEYWORDS *Candida albicans*, β-glucans, antifungal, chitin, cytokine, macrophage, micafungin

Candida albicans remains the most important pathogenic fungus responsible for life-threatening infection in immunocompromised patients. Rapid diagnosis is very important for choosing the correct antifungal treatment and thus reducing mortality (1). However, the structural and metabolic similarities between fungal and host cells limit the number of antifungal targets and lead to a reduction in the selective toxicity of the available antifungal compounds, causing harmful side effects in patients (2). For these reasons, a search for new and more selective antifungal drugs is essential. We also need to improve our knowledge of the mechanisms of action of available compounds and their interaction with host cells.

Macrophages are phagocytic cells of the immune system able to recognize and eliminate the microorganisms that cross the epithelial barrier. These cells play a key role in defense against pathogens because they engulf and destroy pathogens, even in the absence of an adaptive immune response. Macrophages recognize microorganisms through pattern recognition receptors (PRRs) and trigger the innate proinflammatory response, which subsequently enables the development of an adaptive response.

PRRs bind to conserved microbial structures called pathogen-associated molecular patterns (PAMPs). The recognition of yeasts by macrophages is mainly based on the PAMPs present as components of the cell wall, a complex structure composed mainly

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of glycans (3). The outer layers of the cell wall are made up of phosphopeptidomannan, a polymer of *O*- and *N*-linked mannose residues commonly referred to as mannans. In addition, the cell wall contains chitin and two structural polymers of glucose, namely, β-1,3- and β-1,6-linked glucans. These β-glucans lie deep within the wall and are usually masked in live cells but accessible to the cell surface after treatments which remove the mannan, such as by heating, infection, or the supply of drugs (4, 5).

Lectin-like receptors (LRLs) and Toll-like receptors (TLRs), mainly TLR-2 and TLR-4, are host PRRs that recognize sugars on the yeast cell wall, inducing the immune response. Dectin-2 recognizes α-mannans, a kind of *O*-linked mannoproteins, and dectin-3 binds to α-mannans. Macrophage mannose receptor (MR) recognizes fungal *N*-linked mannans. Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) recognizes galactomannan and complex mannoside structures exposed on yeast cell walls. Mincle C-lectin binds to α-mannosyl residues and galectin-3, binds *Candida*-specific β-1,2 mannosides, and finally, dectin-1 confers cellular responsiveness to microbial β-glucans (3, 6). Other receptors participate in the recognition of sugars on the cell wall, like CD23, which binds to α-mannans and β-glucans, while CR3 recognizes β-glucans and CD14 recognizes α-(1,4)-glucans (7).

The interaction between host PRRs and *C. albicans* PAMPs results in intracellular signaling and the secretion of cytokines leading, in most cases, to the proinflammatory response against the yeast that is necessary to acquire resistance to infection, or to an anti-inflammatory response, which allows the yeast to establish itself inside the body (3, 8).

Echinocandins are antifungal compounds with inhibitory activity on β-1,3-D-glucan synthase and, to a lesser extent, β-1,6-D-glucan synthase. The fungal cell wall β-1,3-D-glucan synthase complex is constituted by two subunits, Fks1p and Rho1p. Fks1p (encoded by the genes *FKS1* and *FKS2*) is the active site of the enzyme, responsible for cell wall remodeling, while Rho1p has a regulatory function in the synthesis of β-1,3-D-glucan (9). The echinocandins are noncompetitive inhibitors of β-1,3-D-glucan synthase which specifically target the FKS1 gene product. They induce alterations in the cell wall structure and function, since β-1,3-D-glucan is the major structural polysaccharide of the cell wall (10). Anidulafungin, caspofungin, and micafungin (MF) are members of this family in current clinical use (11).

Several studies have shown that caspofungin is an antifungal drug with an important immunomodulatory role in the response against *Candida albicans*. Subinhibitory doses of this echinocandin are able to unmask cell wall β-glucan, which participates in the production of proinflammatory cytokines released by murine macrophages (12, 13). Micafungin (MF) has been shown to have a fungicidal action like caspofungin, and it exerts a postantifungal effect that kills diverse *Candida* spp., disturbs cell walls of viable organisms (14, 15), reduces adherence to epithelial cells, and enhances the susceptibility to phagocytosis mediated by a murine macrophage cell line (14). Despite the fact that MF and caspofungin belong to the echinocandin family, differences in their pharmacokinetics, pharmacodynamics, adverse effects, and/or drug interactions have been reported (9). In addition, no data are available about the involvement of the alterations caused by MF in *C. albicans* in the immune response against the yeast in a human model. For this reason, our objective was to analyze the effect of MF on the *C. albicans* SC5314 yeast strain and the modulation of the inflammatory response developed by human tissue macrophages as a consequence of MF yeast treatment.

RESULTS

Micafungin reduces metabolic activity and cell viability and has a toxic effect on *C. albicans*. First, the MIC for MF on the SC5314 yeast cell strain was determined using the CLSI protocol (see Materials and Methods). Since the MIC was 0.016 mg/liter, we selected the following concentrations of this echinocandin for the assays performed in this study: 0.005 μg/ml (0.3× the MIC), 0.016 μg/ml (MIC), and 0.05 μg/ml (3× the MIC). After 1 h of treatment at 37°C in yeast extract-peptone-glucose (YPD) medium, MF induced a dose-dependent reduction in cell viability, as determined by the number of

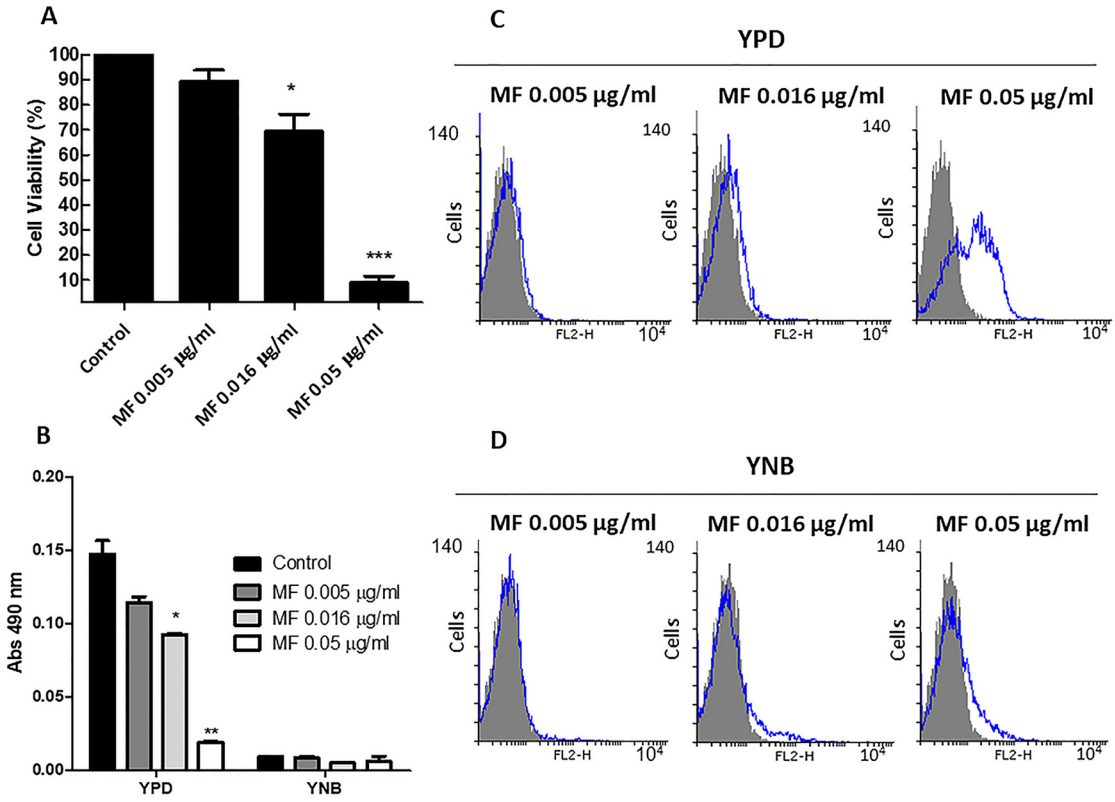

FIG 1 Cell survival, metabolic activity, and level of toxicity in *Candida albicans* upon micafungin treatment is a function of the nutritional status of the medium. SC5314 exponential cell cultures were treated with MF for 1 h at 37°C in YPD or YNB. (A) Viability determined after MF treatment in YPD. (B) Metabolic activity measured by XTT assay in YPD and YNB. Data shown are the results from at least three independent experiments performed in triplicate. (C and D) Toxicity assay performed by PI staining and flow cytometric analysis in cultures treated in YPD (C) or YNB (D). Gray histograms, control cells; blue histograms, MF-treated cells. The data shown are representative of the results from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; statistically significant differences with respect to an untreated control according to Mann-Whitney U test. Abs, absorbance.

CFU (Fig. 1A), with the highest concentration of the antifungal (0.05 μg/ml) causing a reduction in cell viability of 90%. Then, the effect of MF treatment on the metabolic activity of yeasts was studied by a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay. As shown in Fig. 1B, the metabolism was reduced in a dose-dependent manner in response to MF treatment in a rich medium (YPD), especially at the highest concentration. However, when the experiment was performed in a minimal medium (yeast nitrogen base [YNB]), a very low metabolic activity was detected in the control assay or in the MF-treated cells (Fig. 1B).

When the fungicidal effect of MF was examined using the propidium iodide (PI) staining method in identical cultures, a significant degree of cell killing was recorded only after treatment with a 0.05 μg/ml concentration of the echinocandin, while slight or no differences could be observed with 0.016 μg/ml or 0.005 μg/ml, respectively, compared to the control cells in the experiments performed in YPD (Fig. 1C). Time-lapse recordings of yeast cell cultures under these experimental conditions showed the blowing up of budding cells while treating to grow (data not shown). In contrast, when the experiments were performed in YNB, there was no detectable fungicidal effect, even at the highest MF concentration (Fig. 1D).

MF unmasks cell wall β-glucans and increases the chitin content. The effect of MF on *C. albicans* cell wall β-glucan exposure was studied by an indirect immunofluorescence assay with specific monoclonal antibodies against β-glucans and subsequent analysis by flow cytometry (Fig. 2) or by fluorescence microscopy (Fig. 3A). In the flow cytometry assay, a cell population with the same size and complexity was chosen in all cases (Fig. 2A, gate R-1). No fluorescence signal was detected in untreated control cells

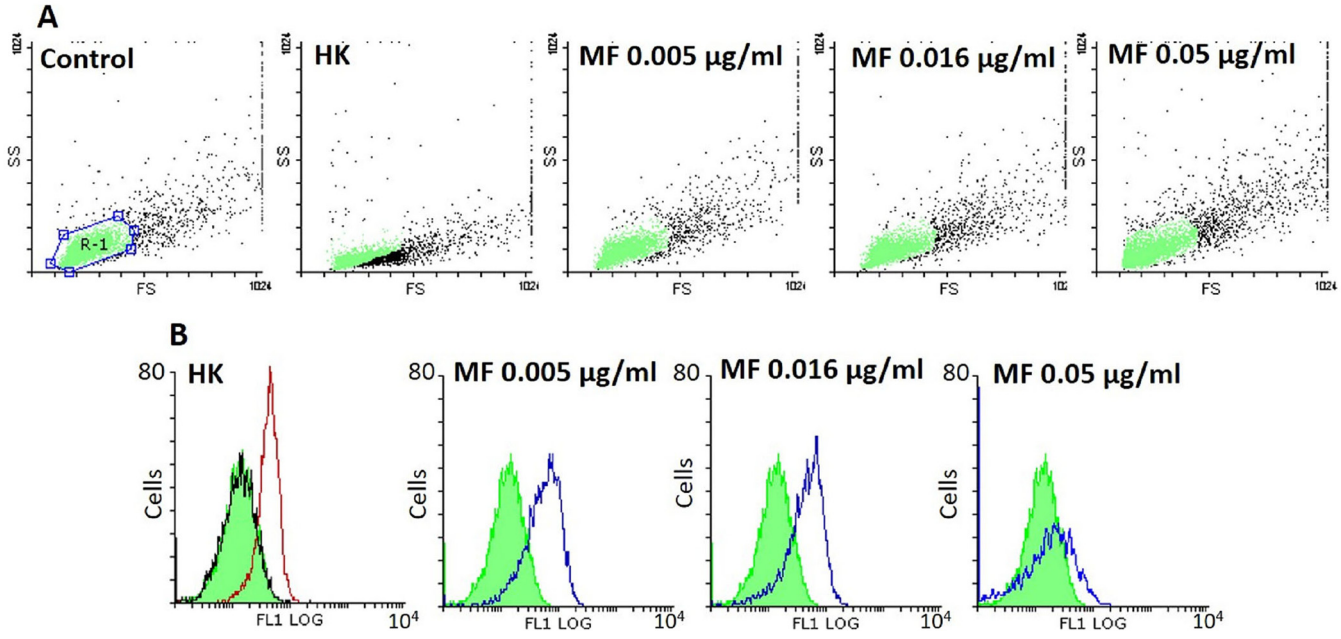

FIG 2 Micafungin induces cell wall β-glucan surface exposure in *C. albicans*. SC5314 exponential cell cultures were treated with MF for 1 h in YPD at 37°C, and surface β-glucan exposure was analyzed by flow cytometry. Healthy yeast cells were used as a negative control and heat-killed cells (HK; 120°C for 15 min) as a positive control. (A) Dot plots represent the cell size (SS) with respect to the cellular complexity (FS). (B) Histograms show the cell number with respect to the green fluorescence intensity (FL1) in the population gated in R-1. Black, negative staining control; green, untreated control cells; red, HK cells; light blue, violet, and dark blue, MF-treated cells at 0.005, 0.16 and 0.05 μ/ml MF, respectively. The data shown are representative of the results from three independent experiments.

since the polysaccharides are located in the inner layer of the cell wall, and therefore, they are not accessible to the specific antibodies used to detect it (Fig. 2B). Conversely, the heat-killing (HK) treatment (120°C for 30 min) induced a partial loss of the components of the cell wall and the exposure of β-1,3-glucans, with the fluorescence staining being clearly detectable (Fig. 2B). In addition, a strong increase in β-glucan exposure on the cell wall surface was recorded when cells were treated with 0.005 and 0.16 μg/ml MF, which are concentrations below and equal to the MIC, respectively (Fig. 2B). The highest doses of MF (0.05 μg/ml) showed only a slight increase in the fluorescence intensity over that in the control cells (Fig. 2B).

The yeast population was analyzed by fluorescence microscopy (Fig. 3A). The results revealed that MF exposed the β-glucans on the external surface, as can be observed by the presence of a sharp fluorescent green signal around the cells. This intense signal was also evident in the surviving cells with the highest concentration of the antifungal (0.05 μg/ml). Surprisingly, daughter cells (arrows) were not stained, probably due to their inability to synthesize the polysaccharide as a consequence of the toxic effect triggered by MF. Furthermore, the chitin content detected by calcofluor white (Fig. 3B) increased in cells treated with MF at a concentration equal to or above the MIC (0.016 μg/ml and 0.05 μg/ml), but no differences with respect to control cells were observed with the lowest MF concentration of 0.005 μg/ml (0.3$\times$ the MIC).

MF treatment of *C. albicans* enhances the macrophage response against the yeast. To evaluate the hypothetical effect of MF application on *C. albicans* in the stimulation of the immune response, peritoneal macrophages were isolated from human donors, and their ability to secrete cytokines in response to *C. albicans* infection was studied (Fig. 4). The release of tumor necrosis factor alpha (TNF-α) (Fig. 4A) and interleukin-10 (IL-10) (Fig. 4B) cytokines after 4 h of infection increased significantly in cell cultures of primary macrophages cocultured with *C. albicans* (1:5 macrophage-to-yeast ratio). MF treatment enhanced the macrophage response to *C. albicans*, as revealed by the increased production of TNF-α (Fig. 4A), IL-10 (Fig. 4B), and IL-17A (Fig. 4C) cytokines after infection with MF-treated yeast compared with the untreated

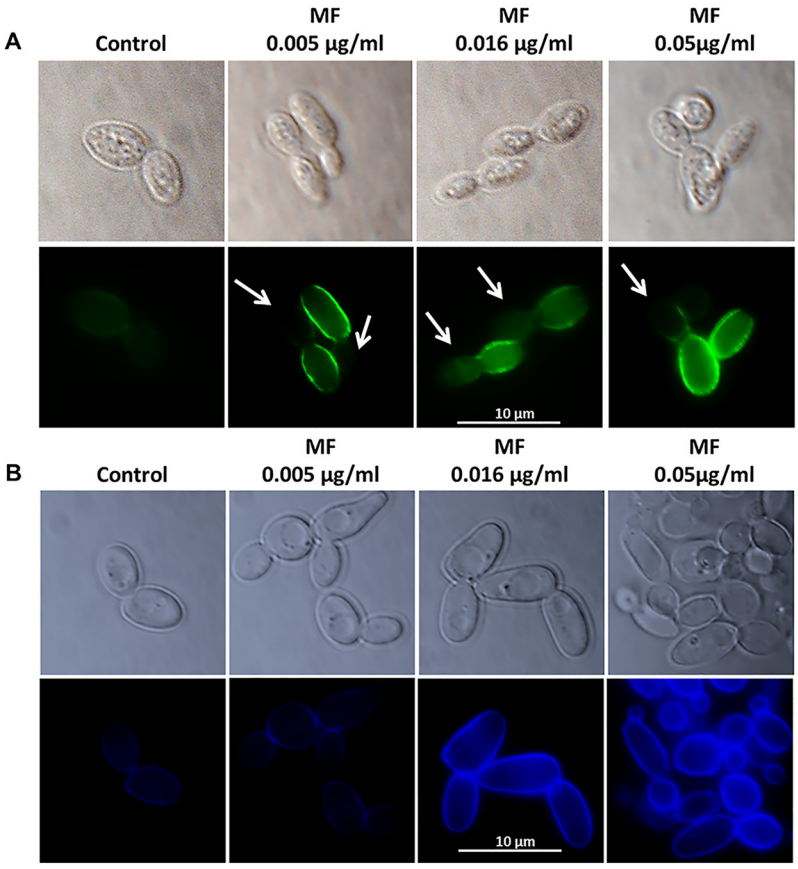

FIG 3 Levels of β-glucans and chitin in the cell wall are increased in response to micafungin treatment in *C. albicans*. SC5314 exponential cells were treated with different concentrations of MF for 1 h at 37°C in YPD. β-Glucan exposure (A) or chitin content (B) was revealed by either specific antibody or calcofluor white staining, respectively, as described in Materials and Methods. Images were taken with a Nikon 90i microscope (objective ×100) by phase contrast (A and B, top images), or with green fluorescence filter (A, bottom images) or under UV light (B, bottom images) with 500-ms exposure times. Arrows signal the daughter cells. The data shown are representative of the results from three independent experiments.

control. The MF-induced immunostimulatory response was dose dependent. Thus, only 0.05 μg/ml caused a significant increase in TNF-α and IL-10 levels (Fig. 4A and B), while the increase in IL-17A could be detected at lower doses of MF (Fig. 4C).

DISCUSSION

The limited number of antifungals used in clinical practice belong to three main families: azoles, polyenes, and echinocandins. However, a growing number of cases of azole resistance have been described in *Candida* species (16). In turn, the resistance to polyenes is very infrequent, but their high nonselective toxicity recommends them as an alternative therapy when the other antifungal compounds are not sufficient (2, 17). Conversely, echinocandin resistance in *Candida* spp. is unusual, except in *C. glabrata*, and important side events are less frequent (10, 11, 18). Therefore, echinocandins seem to be the best option for use in clinical practice, meaning that a study of their mechanism of action and the effects produced on both *Candida* spp. and host cells would be useful to optimize and improve clinical results.

Our data indicate that the MIC for MF in *C. albicans* SC5314 was 0.016 μg/ml, which agrees with the results of a study performed with 4,283 *C. albicans* clinical isolates (19), and it validates the use of the standard SC5314 *C. albicans* strain as a model for this study. The lower MIC dose for MF than for anidulafungin and caspofungin (0.03 μg/ml) highlights the efficiency of this echinocandin for therapeutic candidiasis treatments (19).

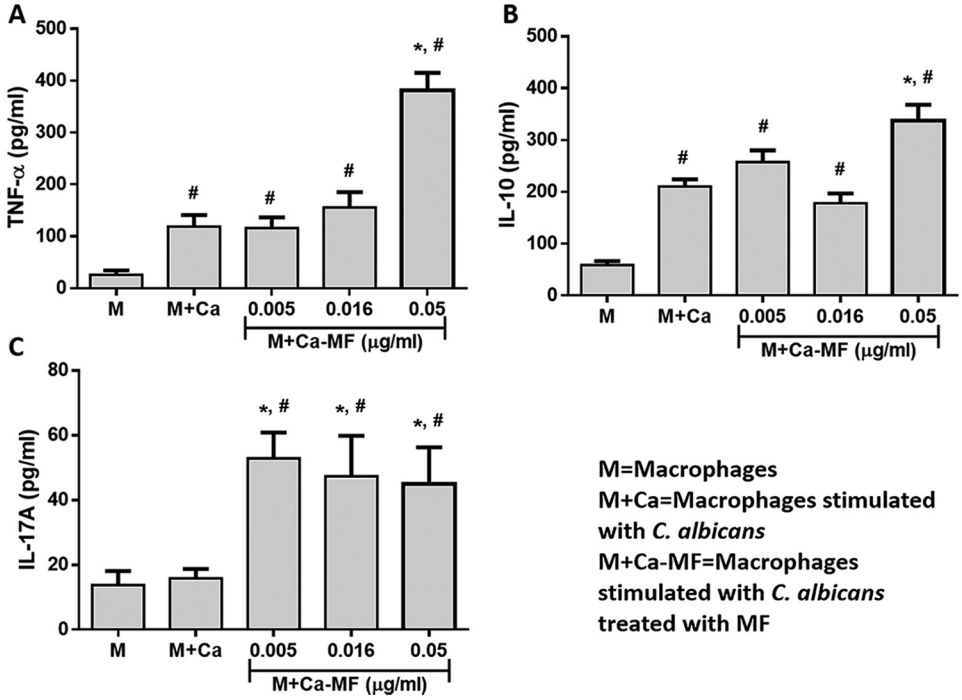

FIG 4 Micafungin treatment of *C. albicans* enhances cytokine production induced in peritoneal macrophages. Exponential cultures of SC5314 strain were exposed to different MF concentrations for 1 h in YPD at 37°C and then washed in PBS. Human peritoneal macrophages (M) were stimulated with untreated (Ca) or MF-treated yeast (Ca-MF), for 4 h at 37°C at a 1:5 ratio of macrophage to yeast. After stimulation, TNF-α (A), IL-10 (B), and IL-17A (C) cytokine concentrations on the cell-free supernatants were determined by ELISA. #, $P < 0.05$ with respect to M; *, $P < 0.05$ with respect to M+Ca, according to a Mann-Whitney U test.

We have demonstrated that MF significantly decreased cell viability at doses equal to and higher than the MIC (0.016 μg/ml and 0.05 μg/ml, respectively) (Fig. 1A). The fungicidal assays performed in YPD medium revealed a strong effect of MF on cell death, which was only evident at the highest concentration (0.05 μg/ml [3$\times$ the MIC]) (Fig. 1C). Time-lapse recordings of yeast cell cultures under these experimental conditions showed that when budding cells started to grow, they blow up probably as a consequence of the loss of cell wall integrity, caused by their inability to synthetize new fungal wall β-glucan (data not shown). The MF effect depends on the metabolic status of the cells, since the biosynthesis of different structural components for the cell wall requires energy, which is partially obtained from mitochondrial activity (20). Thus, yeasts grown in a medium rich in nutrients with an active metabolism were sensitive to the echinocandin effect, in contrast to cells grown in YNB (Fig. 1D). Our results also revealed that MF reduced the *C. albicans* metabolic activity in a dose-dependent way, which could probably represent a mechanism to prevent the echinocandin-mediated cell damage (Fig. 1B).

We and other groups have previously reported that MF treatment induces structural remodelling of the cell wall in *C. albicans*, as evident in electron micrographs (14, 15). Here, we show that the β-glucans located in the inner layer of the cell wall were exposed on the cell surface as consequence of the antifungal treatment (Fig. 2 and 3). As expected, the exposure of β-glucans on the surface was not observed in daughter cells due to their MF-mediated inability to synthesize the polysaccharide (Fig. 3A). These structural alterations were accompanied by an increase in chitin content in cultures treated with MF doses equal to and higher than the MIC (0.016 μg/ml and 0.05 μg/ml, respectively) (Fig. 3B). The same behavior has been observed after exposure to caspofungin, which induced the unmasking of β-glucans (13) and the activation of both the cell wall integrity and the calcineurin signaling pathways, which are involved in chitin synthesis to compensate for the loss of β-glucan content (12, 21).

Studies on host-pathogen interactions in human models are usually performed with immortalized macrophage cell lines or blood monocyte-derived macrophages. Both the phenotype and functional ability of the macrophages obtained display notable differences, depending on the protocol chosen for *in vitro* differentiation, and do not necessarily reflect the physiological situation in the tissues. Thus, despite the difficulties in obtaining samples, the present study used human peritoneal macrophages isolated from healthy donors. These results show for the first time that healthy human tissue macrophages increase the release of TNF-α, IL-17A, and the anti-inflammatory cytokine IL-10 when *C. albicans* is treated with MF (Fig. 4). The recognition of β-glucans by phagocytic cells is mediated by several receptors, including dectin-1, CD11b (part of complement receptor-3, CR3), CD23 (the c-type lectin Fc epsilon RII), and lactosylceramide (7, 22). This induces phagocytic, cytotoxic, and antimicrobial activities and stimulates the production of proinflammatory mediators, cytokines, and chemokines, including IL-8, IL-1β, IL-6, TNF-α, and IL-17, in vertebrates (22, 23). Therefore, fungal β-glucan masking represents a mechanism for immune evasion (8).

Moreover, NOD2, TLR9, and the mannose receptor are essential fungal chitin recognition receptors that induce the secretion of the anti-inflammatory cytokine IL-10 and activate arginase-1 in human macrophages, reducing the nitric oxide production which leads to a decrease in the efficiency of fungal killing (24, 25). Thus, the augmented proinflammatory response mediated by MF-treated *C. albicans* through TNF-α and IL-17 secretion by human macrophages (Fig. 4A to C) can mainly be explained by the β-glucan exposure (Fig. 3A), while the anti-inflammatory response mediated through IL-10 must be a consequence of the appearance of chitin on the yeast cell surface (Fig. 3B and 4C).

Similar immunomodulatory behavior has been reported for MF in other fungal species; for instance, the hyphae of *Aspergillus fumigatus*, but not conidia or germlings, increased β-glucan exposure after MF treatment, with a subsequent increase in TNF and CXCL2 secretion by murine macrophages (26). These results also agree with the increase in TNF-α (13) and IL-17A (12) observed in murine macrophages stimulated with caspofungin-treated *C. albicans* cells, although no significant variation in IL-10 levels was reported under the experimental conditions used (12).

Our results lead us to conclude that MF is a powerful antifungal compound whose effect is dependent on the metabolic activity of cells and which is responsible for cell wall remodelling in *C. albicans* following β-glucan and chitin exposure on the external cell surface. This restructuration facilitates the recognition of *C. albicans* by human tissue macrophages, which increase cytokine production, enhancing the inflammatory response directed at eliminating the fungal infection.

MATERIALS AND METHODS

Yeast strains and culture conditions. The *C. albicans* SC5314 wild-type strain was used throughout this study. Growth was monitored by measuring the optical density (OD) of cultures at 600 nm in a Shimadzu U/V spectrophotometer or by direct cell counting in a Neubauer hemocytometer chamber. Exponential yeast cell cultures (blastoconidia) were grown by shaking at 37°C in YPD medium (2% peptone, 1% yeast extract, and 2% glucose) or in minimal medium YNB (0.7% yeast nitrogen base without ammonium sulfate, supplemented with 0.5% ammonium sulfate and 2% glucose). The cultures were harvested in exponential phase (OD, 0.8 to 1.0) and treated with different concentrations of MF (Astellas Pharma Europe B.V.) during 1 h. MF was prepared in dimethyl sulfoxide (DMSO) and stored at −80°C until use.

MIC determination. The MIC for MF in SC5314 cells was determined according to a Clinical and Laboratory Standards Institute (CLSI) protocol (29). Briefly, 10^6 yeasts were grown in a microtiter plate (96 wells) filled with a 2-fold dilution of MF ranging from 0.001 to 1 μg/ml in RPMI 1640 without glutamine plus 0.2% glucose (buffered at pH 7.0 with 0.164 M morpholinepropanesulfonic acid [MOPS]). The plates were incubated at 37°C for 24 h, and the reading of the MIC was performed by spectrophotometry at 490 nm. The quality control *Candida parapsilosis* ATCC 22019 strain, required by the CLSI procedure, was run in parallel with the expected value for MF (1 μg/ml).

Antifungal sensitivity. SC5314 cells were treated with different concentrations of MF for 1 h at 37°C, and antifungal sensitivity was measured using (i) a viability assay, performed by counting the number of yeast colonies formed on plates; (ii) a metabolic activity assay, performed by evaluating the XTT reduction; and (iii) a fungicidal assay, performed by staining with propidium iodide (PI) to evaluate the level of yeast death.

(i) Viability assay. After each appropriate treatment, yeast samples were diluted in sterile water and plated in triplicate on solid YPD, and the number of CFU per milliliter was determined after incubation for 24 h at 37°C. Between 30 and 300 colonies were counted per plate. The results were referred to an untreated control sample (100% viability).

(ii) Metabolic activity assay. After MF exposure, the yeasts were washed twice with phosphate-buffered saline (PBS), plated in a 96 well-plate, and incubated during 1 h at 37°C with 0.5 mg/ml XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt] (Sigma-Aldrich). Then, the microplates were centrifuged at 1,000 rpm for 1 min, and the supernatants were transferred to another microplate to measure the absorbance at 490 nm in a microtiter plate reader (Asus Jupiter). The amount of accumulated formazan following the XTT conversion is proportional to the metabolic activity of cells.

(iii) Fungicidal assay. After the indicated MF treatments in YPD or YNB, the cells were washed twice with PBS, stained with PI (10 μg/ml), and analyzed by flow cytometry (27).

Yeast cell surface staining. The presence of β-glucans and chitin on the *C. albicans* cell wall was detected by staining with specific antibodies and calcofluor white, respectively.

For chitin detection, the blastoconidia were stained with 10 μg/ml of calcofluor white (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. Then, they were washed twice with PBS and examined with a Nikon 90i microscope. Fluorescence images were taken with a 500-ms exposure time.

Yeast immunostaining was performed as previously described (28). Briefly, 10^6 SC5314 cells were washed with PBS containing 2% fetal calf serum (FCS) and then incubated for 15 min with rabbit polyclonal antibody anti-β-1,3-D-glucan (kindly provided by M. D. Moragues, Universidad del País Vasco, Bilbao, Spain). After washing, cells were incubated for 15 min with secondary fluorescein isothiocyanate (FITC)-labeled secondary antibody (BD-Pharmingen, San Diego, CA, USA). The negative control was performed by adding labeled second antibody at the same concentration. The whole process was carried out at 4°C. After washing, cells were fixed in 0.4% paraformaldehyde and analyzed by flow cytometry.

Human macrophage isolation and culture conditions. Human macrophages were obtained from the peritoneal cavity of healthy women after clinical surgery at the Gynecological Unit of the Hospital Universitario Virgen de la Arrixaca, Murcia, Spain. Cell samples were obtained during exploratory or therapeutic laparoscopies for benign gynecological pathology (simple ovarian cysts or uterine fibroids) or tubal ligation. After opening the abdomen by incision, the peritoneal cavity was instilled with 50 ml PBS that was collected from the rectouterine pouch, or pouch of Douglas, strictly avoiding contamination by blood. Nevertheless, red samples indicative of peripheral blood contamination were excluded. Abdominal surgery continued after this brief lavage procedure. The samples were then maintained at 4°C to avoid cell attachment to plastic. The cells were washed, and 0.2×10^6 white cells were stained with 5 μl of anti-human CD14-FITC antibody (eBioscience, San Diego, CA) 15 min at 4°C, washed in PBS, and analyzed by flow cytometry. The cells were then seeded for panning at 0.2×10^6 macrophages/well in 96-well plates, according to the percentage of $CD14^+$ cells in the sample.

After an overnight incubation at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Gibco Invitrogen, Paisley, UK) containing 10% fetal bovine serum and 1% penicillin-streptomycin (complete culture medium [CCM]), nonadhered cells were washed out with CCM. The purity of the macrophages in cell culture was >95%. Then, macrophages were treated with *C. albicans* SC5314, untreated, or pretreated during 1 h with MF, at a 1/5 cell-to-yeast ratio, and culture supernatants were collected after 4 h for cytokine detection.

The ethics committees (Comité Ético del Hospital Universitario Virgen de la Arrixaca and Comité de Bioética de la Universidad de Murcia) approved the study protocol, and all peritoneal cell donors gave informed written consent to be included in this study.

Flow cytometry. Flow cytometry analysis was performed on three-color fluorescence Epics XL (Beckman Coulter) using the Flowing Software version 2.5.1. Forward scatter (FS) and side scatter (SS) were analyzed on linear scales, while the analysis of green (FL1) or red (FL2) fluorescence intensity was made on logarithmic scales. Analysis gates were set around debris and intact cells on an FS-versus-SS dot plot. The fluorescence histograms corresponding to 5,000 cells were generated using the gated data.

Cytokine detection. Cell culture supernatants from macrophages cultured for 4 h under different conditions were collected, and the concentrations of TNF-α, IL-17A, and IL-10 cytokines were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (eBioscience Ready-set-GO!).

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Statistical differences were analyzed using a Mann-Whitney U test. Every experiment was performed in triplicate and repeated at least three times.

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We declare no conflicts of interest.

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