Caspofungin Modulates Inflammatory Responses to *Aspergillus fumigatus* through Stage-Specific Effects on Fungal *β*-Glucan Exposure

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Abstract

Echinocandins target fungal *β*-1,3 glucan synthesis and are used clinically to treat invasive aspergillosis. Although echinocandins do not completely inhibit in vitro growth of *Aspergillus fumigatus,* they do induce morphological changes in fungal hyphae. Because *β*-1,3 glucans activate host antifungal pathways via the Dectin-1 receptor, we investigated the effect of echinocandins on inflammatory responses to *A. fumigatus.* Caspofungin- or micafungin-treated conidia and germlings induced less secretion of tumor necrosis factor (TNF) and CXCL2 by macrophages than did their untreated counterparts. Diminished secretion of TNF and CXCL2 correlated with diminished *β*-glucan exposure on echinocandin-treated germ tubes. In contrast to treated conidia and germlings, echinocandin-treated hyphae stimulated increased release of TNF and CXCL2 by macrophages and demonstrated intense staining with a *β*-glucan-specific antibody, particularly at hyphal tips. Our experiments demonstrate that echinocandin-induced morphological changes in *A. fumigatus* hyphae are accompanied by increased *β*-glucan exposure, with consequent increases in Dectin-1-mediated inflammatory responses by macrophages.

In 2001, the echinocandin caspofungin was licensed in the United States as a second-line agent for the treatment of invasive aspergillosis (IA), which is a common cause of infection-associated morbidity and mortality among immunocompromised patients [1-3]. IA begins with the inhalation of airborne spores (i.e., conidia) that escape weakened pulmonary host defenses and germinate into tissue-invasive hyphae [4,5]. Caspofungin is a noncompetitive inhibitor of *β*-1,3 glucan synthase, an essential enzyme located on the growing filaments (i.e., hyphae) of *Aspergillus fumigatus* [6], the most common etiologic agent of IA.

Although caspofungin has been found to be effective in animal models of IA [7-10], it does not fully inhibit growth of *A. fumigatus,* even at high concentrations [11,12]. Echinocandins reduce bulk *β*-1,3 glucan levels [13] and induce profound morphological changes that include the formation of swollen germ tubes; short, broad-based hyphae with aberrant branching; and distended, balloon-type cells [11]. The minimum drug concentration associated with these changes is referred to as the "minimum effective concentration" (MEC).

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In contrast, caspofungin induces complete growth inhibition and cell lysis of the dimorphic fungus *Candida albicans* [12]. At sub-MIC concentrations, caspofungin increases *β*-glucan exposure on *C. albicans,* and drug-treated yeast cells trigger enhanced macrophage inflammatory responses [14]. In the case of *A. fumigatus,* caspofungin appears to induce membrane depolarization in a subset of cells located at the tips and branch points of the growing mycelium [15], a process that is sufficient to stunt but not arrest filamentous growth.

Not only is *β*-1,3 glucan an obligate fungal cell wall constituent, it represents a target of the innate immune system. The mammalian receptor Dectin-1 [16,17] binds to *β*-1,3-glucan from *Pneumocystis carinii* [18], *C. albicans* [19], *A. fumigatus* [20-22], and *Coccidioides posadasii* [23]. It also triggers fungicidal responses that include phagocytosis, release of inflammatory mediators, and generation of reactive oxygen intermediates [17].

Although echinocandins increase *β*-glucan exposure on the surface of *C. albicans,* whether this change occurs in such clinically important molds as *A. fumigatus* is not known. Therefore, we treated *A. fumigatus* spores with echinocandins in vitro and measured surface *β*-glucan exposure and macrophage inflammatory responses. TNF and CXCL2, a neutrophil chemoattractant, were chosen as a readout for inflammatory responses, because both have established roles in host defense against *A. fumigatus* [24-27]. During the early stages of conidial swelling and germ tube formation, drug-treated cells elicited weaker inflammatory responses than did control cells. In contrast, drug-treated hyphae induced stronger inflammatory responses than did control cells. In all cases, the magnitude of the inflammatory response reflected changes in surface *β*-glucan exposure. Our findings suggest that echinocandins, by increasing *β*-glucan exposure on the surface of invading *A. fumigatus* hyphae, may enhance antifungal inflammatory responses to hyphae, independent of direct growth inhibition.

MATERIALS AND METHODS

Reagents and antibodies

Chemical and cell culture reagents were purchased from Sigma-Aldrich and Gibco, respectively. Caspofungin (Merck) and micafungin (Astellas) in powder form were obtained from the Memorial Sloan-Kettering Cancer Center pharmacy, dissolved in sterile PBS at 5 mg/mL, and stored at -80°C. The anti-Dectin-1 antibody 2A11 and an IgG isotype control antibody were purchased from AbD Serotec. Monoclonal antibody (MAb) 744 has been described elsewhere [20], and an Alexa Fluor 594-linked anti-mouse IgM antibody was obtained from Invitrogen.

Fungal growth and culture

C. albicans strain SC5314 was grown in yeast extract peptone dextrose overnight at 25°C. Yeast cells were diluted 1:1000 in fresh medium ± 5 ng/mL caspofungin, shaken overnight at 25°C, washed 3 times in PBS, killed by UV irradiation from a Stratalinker UV Crosslinker 1800 (dose, 4×10^5 mJ/cm^2), and added to bone marrow-derived macrophages (BMMϕs) at a ratio of 10:1. The MIC of caspofungin for these conditions was 10 ng/mL.

A. fumigatus strains Af293 and ATCC 90906 were cultured as described elsewhere [20]. For germination reactions, 5×10^4 conidia were seeded in 96-well culture dishes (Invitrogen) in 0.2 mL of RP-10$^+$ (i.e., RPMI 1640, 10% heat-inactivated fetal calf serum [FCS], 5 mmol/L HEPES [pH 7.4], 1.1 mmol/L ʟ-glutamine, 0.5 U/mL penicillin, 0.5 μg/mL gentamicin, 50 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol) containing caspofungin (0-1000 ng/mL) or micafungin (0-1000 ng/mL) and incubated at 37°C for the times indicated. Culture

supernatants were aspirated, and fungal cells were inactivated by UV irradiation, as described above.

Cell culture and ELISA

BMMϕs obtained from 8- to 12-week-old C57BL/6 female mice were prepared as described elsewhere [20], harvested after 5 days in culture, adjusted to 7.5×10^5 cells/mL in RP-10$^+$, and added (in a final volume of 0.2 mL) to empty wells or wells containing UV-inactivated fungal cells in 96-well plates.

For experiments with live *A. fumigatus* conidia, BMMϕs were cultured with 1.5×10^6 conidia in 0.2 mL of RP-10$^+$ with 0-1000 ng/mL caspofungin or micafungin for 18 h at 37°C. For specific experiments, 10 μg/mL anti-Dectin-1 or isotype control antibody was added to BMMϕs 10 min before coculture with fungal cells. For experiments with UV-inactivated fungal cells, the BMMϕ coincubation period was 6 h. Culture supernatants were analyzed using commercially available ELISA kits for the detection of CXCL2 (R & D Systems) and TNF (BD Biosciences) by use of a VersaMax Microplate Reader (Molecular Devices).

Confocal microscopy

A total of 5×10^4 conidia per well was grown on 4-well Lab-Tek glass chamber slides (Nalge Nunc International) in RP-10$^+$ with the indicated drug concentration for 8-24 h at 37°C. Pilot experiments determined that UV inactivation did not alter β-glucan immunoreactivity. Fungal cells were processed for confocal microscopy by use of MAb 744 and Alexa Fluor 594-linked anti-mouse IgM, as described elsewhere [20]. No immunoreactivity was observed in samples stained with a control isotype antibody. The samples were scanned in 0.5- to 1.0-μm intervals (in the *z*-plane), with the use of an upright Leica TCS SP2 AOBS confocal microscopy system equipped with a 63$\times$ Leica HPX PL APO water-immersion objective (numeric aperture 1,2) using the 488-nm and 594-nm laser lines and transmitted light. Composite fluorescence images were assembled from individually scanned optical sections obtained from a 30- to 40-μm interval in the *z*-plane. For all images displayed, the sensitivity of the recording photomultiplier tube was set in the linear range and was identical for drug-treated and untreated cells.

For image analysis of drug-treated and untreated hyphae, cellular autofluorescence was recorded using the 488-nm laser line to visualize the hyphal area (in cross-section) in optical sections measuring 237.5 μm $\times$ 237.5 μm $\times$ 1 μm. Autofluorescence and immunofluorescence recordings underwent image analysis in MetaMorph software (version 7.1.1.0; Molecular Devices). Threshold settings were applied uniformly for all paired data sets. A low-pass filter (2 $\times$ 2 pixels) was applied to autofluorescence stacks to suppress single-pixel noise, and the area above a set threshold was measured in each optical slice and then summed across all slices within a field of view, to obtain a measure of the total hyphal volume within an image stack.

For image processing of immunofluorescence recordings, no filters were applied, and the integrated fluorescence intensity in the red channel above a set threshold was measured for each optical slice and summed across all slices within the same field of view. To associate β-glucan immunoreactivity with fungal biomass for drug-treated and untreated hyphae (after 24 h of incubation), the total integrated fluorescence intensity in the red channel was divided by the summed threshold area delineated by the autofluorescent signal, resulting in a measurement of β-glucan immunoreactivity per hyphal volume. In a single experiment, 4-5 optical sections, each consisting of ~30-40 optical slices, were evaluated for each condition.

RESULTS

No direct elicitation by caspofungin of macrophage TNF production

To determine whether caspofungin directly elicits inflammatory responses, the drug was added to BMMϕ cultures. No difference in the secretion of TNF or CXCL2 by BMMϕ was observed in drug-treated or control samples (figure 1*A*) (not shown), even with concurrent addition of lipopolysaccharide (LPS) or killed, swollen *A. fumigatus* conidia. Thus, caspofungin does not trigger secretion of TNF or CXCL2 by BMMϕ at rest or in conjunction with inflammatory stimuli. In contrast, *C. albicans* yeast cells grown in sub-MIC levels of caspofungin elicited TNF secretion that was ~4-fold higher than that of control cells (figure 1*B*), in accordance with results achieved using a different strain [14].

Caspofungin and the diminishment of inflammatory responses to live A. fumigatus conidia

At rest, *A. fumigatus* conidia trigger minimal inflammatory responses [20]. Conidial swelling represents the first step of germination and is required for macrophages to initiate inflammatory and fungicidal responses [28,29].

To examine the effect of caspofungin on inflammatory responses to conidia, BMMϕs and conidia (derived from *A. fumigatus* strain Af293) were incubated for 18 h in the presence of 0-500 ng/mL caspofungin. Caspofungin diminished inflammatory responses in a dose-dependent manner, with a reduction evident at drug concentrations at or above the MEC, which was determined to be 63 ng/mL for the experimental conditions (figure 2*A*). At a drug concentration of 500 ng/mL, the average percentage ($\pm$SD) of TNF and CXCL2 released into the supernatants by BMMϕs was 49% $\pm$ 4% (range, 46%-54%; $n = 4$ experiments) and 55% $\pm$ 10% (range, 43%-62%; $n = 4$ experiments), respectively, compared with the amount released for untreated samples. Similar results were obtained with conidia derived from *A. fumigatus* strain ATCC 90906 (not shown).

To determine whether exposure of conidia to caspofungin diminishes Dectin-1 signaling by macrophages, the effect of antibody-mediated receptor blockade on inflammatory responses to drug-treated and untreated conidia was examined. MAb 2A11 binds to the extracellular portion of Dectin-1 and blocks signal transduction through its intracellular immunoregulatory tyrosine-based activation motif-like motif [30]. Macrophages isolated from Dectin-1$^{-/-}$ mice do not bind MAb 2A11 [31], indicating that the antibody is specific for Dectin-1 and does not bind to other C-type lectins (e.g., dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin or Dectin-2, receptors with different carbohydrate-binding properties implicated in the recognition of fungal cells) [32-34].

In agreement with previous studies [20-22,31], antibody-blocking experiments demonstrated that Dectin-1 is largely responsible for macrophage production of TNF and CXCL2 in response to conidia (figure 2*B*). The reduced macrophage inflammatory response observed in association with drug-treated conidia reflects the diminished Dectin-1-associated signaling triggered by these cells.

The conidia-macrophage coincubation assay described above measures inflammatory responses to the earliest stage of conidial germination. In caspofungin-treated and drug-free samples, >99% of conidia associated with macrophages at the conclusion of the incubation period did not form germ tubes (figure 2*C*). Rare hyphae that escaped macrophage control were observed in all samples (<1% of cells), consistent with the inability of caspofungin to prevent germination (figure 2*C*, *white arrows*). In sum, these results suggest that caspofungin reduces the inflammatory responses of BMMϕs to live conidia.

Caspofungin and diminishment of inflammatory responses to A. fumigatus *germlings*

To examine the effect of caspofungin on inflammatory responses triggered at later stages of germination, 5×10^4 conidia per well were incubated in the presence or absence of drug for 8 h, to complete the swelling reaction and form short germ tubes. BMMϕs were then added to UV-inactivated fungal cells for 6 h. Similar results were observed with *A. fumigatus* strain Af293 (figure 3) and ATCC 90906 (not shown).

The amounts of TNF and CXCL2 released in BMMϕ culture supernatants decreased in a dose-dependent manner if the caspofungin concentration exceeded the MEC (figure 3*A*). At a drug concentration of 500 ng/mL, the average percentage ($\pm$SD) of TNF and CXCL2 released by BMMϕs was 51% $\pm$ 7% (range, 43%-64%; $n = 7$ experiments) and 61% $\pm$ 8% (range, 53%-74%; $n = 7$ experiments) respectively, compared with the amount released from untreated samples. Inflammatory responses of BMMϕs were 90%-95% attributable to Dectin-1 signaling, and drug treatment reduced Dectin-1-associated production of TNF and CXCL2 (figure 3*B*). Germlings grown in micafungin elicited a similar decrease in TNF and CXCL2 secretion by BMMϕs (figure 3*C*), indicating a general effect of echinocandin drugs.

Caspofungin and enhancement of inflammatory responses to A. fumigatus *hyphae*

To examine inflammatory responses to hyphae, 5×10^4 conidia were grown for 24 h in medium containing 0-500 ng/mL caspofungin, and they were then inactivated by UV exposure. Despite a marked reduction in fungal growth in drug-treated wells (see below), BMMϕ inflammatory responses were enhanced in a dose-dependent manner (figure 4*A*). Fungal hyphae grown in 500 ng/mL caspofungin induced an average ($\pm$SD) of 4.11-fold ($\pm$2.39-fold) more TNF (range, 1.90- to 7.84-fold; $n = 8$ experiments) and 2.90-fold ($\pm$1.40-fold) more CXCL2 (range, 1.53- to 5.41-fold; $n = 8$ experiments) than did hyphae grown in drug-free medium. Enhanced inflammatory responses to drug-treated hyphae were observed with *A. fumigatus* strain ATCC 90906 as well (not shown).

The increase in secretion of TNF and CXCL2 by BMMϕs in caspofungin-treated samples was attributable to increased Dectin-1-associated signaling (figure 4*B*). Enhanced BMMϕ inflammatory responses were observed with micafungin-treated hyphae as well (figure 4*C*). This effect was seen in association with different fungal growth media, both containing serum (RP-10$^+$) and lacking serum (RPMI 1640, 0.165 mol/L 3-(*N*-morpholino) propanesulfonic acid [pH 7.0]), and with conidial inocula that ranged from 5×10^3 to 5×10^4 cells (data not shown).

Hyphae may elaborate factors that reduce the inflammatory responses of BMMϕs in proportion to mycelial growth. Because growth is markedly reduced in echinocandin-treated samples (see below), excluding this explanation for the observed difference in inflammatory response is important. To examine whether macrophage responsiveness diminished when cells were stimulated with control hyphae, rather than with drug-treated hyphae, LPS was added to both experimental conditions. Macrophage inflammatory responses were nearly identical in both cases (figure 4*D*). This finding supports the notion that low-level inflammatory responses to non-drug-treated hyphae and enhanced inflammatory responses to drug-treated hyphae are not associated with changes in macrophage responsiveness to other inflammatory stimuli.

To examine inflammatory responses to *A. fumigatus* during the transition from germlings to hyphae, fungal cells were grown in drug-free medium or in medium containing caspofungin, and, at defined time points between 8 h and 24 h, they were inactivated by UV exposure. Inflammatory responses decreased as fungal cells completed the transition from germlings to hyphae in drug-free medium, despite the increase in fungal mass that occurred during hyphal growth (figure 4*E*). In contrast, fungal cells exposed to caspofungin induced a large increase in the secretion of TNF and CXCL2 by macrophages after 12-18 h of growth in medium

containing drugs (figure 4*E*). Drug-treated hyphae were more inflammatory than drug-treated germlings. In all cases examined, inflammatory responses to drug-treated and control cells were highly associated with Dectin-1 signaling.

Caspofungin and alteration of β-glucan immunoreactivity on germ tubes and hyphae

Fungal cells were examined by confocal microscopy to examine drug-dependent changes in surface *β*-glucan exposure during germ tube formation and hyphal growth. A monoclonal antibody that recognizes *β*-1,3-glucan was used for experiments involving immunofluorescence [20]. Untreated germlings demonstrated strong *β*-glucan immunoreactivity on the remnant of the swollen conidium and diffuse staining along the protruding germ tube (figure 5*A*). In contrast, their drug-treated counterparts lacked *β*-glucan immunoreactivity on the protruding germ tube.

As filaments increased in length (during 10-12 h of incubation in RP-10$^+$), they remained immunoreactive along the entire structure (figure 5*B* and *C*). In drug-treated samples, *β*-glucan immunoreactivity became apparent on the tips of germ tubes after 10 h of growth (figure 5*B*), with marked enhancement at distal segments noted after 2 additional hours of growth (figure 5*C*). At this later point in time, the growth inhibitory effects of caspofungin were apparent.

At 15 h of growth, drug-free samples contained weakly immunoreactive and nonreactive hyphae (figure 5*D*). The morphologically distinct hyphae observed in drug-treated samples displayed intense *β*-glucan immunoreactivity concentrated at peripheral segments (figure 5*D*). Drug-treated hyphae displayed persistent *β*-glucan immunoreactivity after 18 h of growth in caspofungin, predominantly at distal segments but extending to more central areas as well (figure 5*E*). In contrast, control samples were markedly less immunoreactive than drug-treated cells (at the same time point) and less mature hyphae (figure 5*D*). At the final time point examined (at 24 h), drug-treated hyphae displayed far greater *β*-glucan immunoreactivity than did untreated hyphae (figure 6). This finding was noted with different conidial inocula (5×10^3-5×10^4 conidia), fungal growth media, and echinocandin drugs (caspofungin and micafungin) (figure 6) (not shown).

To examine differences in *β*-glucan immunoreactivity between drug-treated and untreated hyphae, the integrated fluorescence intensity of *β*-glucan immunoreactivity was determined for all hyphal filaments within a field of view consisting of ~30 - 40 optical slices (see Materials and Methods for detail). To relate the *β*-glucan fluorescence intensity to the hyphal mass within each field of view, fungal cell autofluorescence was recorded to visualize the hyphal cross-sectional area in each optical slice (and field of view). In 2 experiments, drug-treated hyphae displayed >10-fold greater *β*-glucan immunoreactivity than did untreated hyphae, normalized for hyphal mass (table 1).

DISCUSSION

In the present study, we found that echinocandins shape inflammatory responses to *A. fumigatus* through modulation of surface *β*-glucan content. Surprisingly, this process occurs in a stage-specific manner: drug-treated germlings display diminished surface *β*-glucan immunoreactivity and elicit reduced inflammatory responses, whereas drug-treated hyphae exhibit a dramatic increase in surface *β*-glucan immunoreactivity and trigger enhanced inflammatory responses. The latter result is consistent with the finding that macrophages cooperate with caspofungin to inhibit hyphal growth in an additive manner [35]. The results of the present study support echinocandins increasing hyphal susceptibility to Dectin-1-mediated responses by direct effects on the fungal cell wall. Drug-dependent changes in *β*-glucan exposure and macrophage inflammatory responses occur at drug levels that are relevant for therapy [36].

A second finding is that *A. fumigatus,* under drug-free conditions, limits *β*-glucan exposure during hyphal growth, given the low-level Dectin-1-dependent inflammatory response to hyphae, compared with the response to germlings. *C. albicans* conceals *β*-glucan during yeast cell and pseudohyphal growth beneath a mannoprotein layer [19]. Shielding *β*-glucan or limiting surface exposure may provide a growth or survival advantage within the diverse ecological niches inhabited by these organisms [14,37]. Caspofungin disrupts the normal cell wall architecture of both organisms and exerts similar proinflammatory effects on *C. albicans* yeast cells [14] and *A. fumigatus* hyphae. However, in contrast to their effect on responses to *C. albicans,* echinocandin drugs can decrease inflammatory responses to specific growth stages of *A. fumigatus.*

The *A. fumigatus* Δ*pksP* mutant defective in pigment biosynthesis forms conidia with high levels of surface *β*-glucan [38]. Δ*pksP* conidia display heightened in vitro susceptibility to phagocyte defense mechanisms [39] and reduced virulence in animal models of IA [40]. Although this mutant strain likely harbors other changes that influence disease outcome, it is striking that enhanced *β*-glucan exposure correlates with decreased virulence.

A. fumigatus spores contain minimal amounts of *β*-glucan on their surface [20], and *β*-glucans become exposed during swelling and germ tube formation. When caspofungin exposure occurs, a reduction in de novo synthesis may account for the reduced *β*-glucan surface display on the initial germ tube segment. Alternatively, the initial step of germ tube formation could depend, in part, on preformed stores. Either way, caspofungin appears to prevent the immunoreactive polymer from reaching the fungal surface at the earliest stage of filamentous growth, given the near absence of immunoreactive *β*-glucan on short drug-treated filaments. Compensatory changes in cell wall constituents or assembly may be associated with the reduced inflammatory responses observed with swollen conidia and germ tubes, at the eventual cost of dysmorphic and stunted growth.

Continued inhibition of glucan synthase leads to a paradoxical increase in surface *β*-glucan at the ends of growing filaments. Increased surface accumulation of *β*-glucan may result from secondary changes in the activities of enzymes involved in polymer biosynthesis and turnover (e.g., *β*-1,3-glucanosyltransferases [41] and *β*-1,3-glucanases [42-44]). Because Dectin-1 binds exclusively to *β*-1,3-linked glucose oligomers [45], echinocandin-induced changes in other polysaccharide constituents of the cell wall are unlikely to contribute directly to the Dectin-1-dependent inflammatory responses measured in these studies.

The antifungal properties of echinocandin drugs may not be adequately described by their effects on growth [37,46]. Compounds with direct immune stimulatory properties or those that enhance the immune recognition of target microbes have an increasing role in the treatment of infectious diseases. For example, the Toll-like receptor (TLR) agonist imiquimod is effective against lesions associated with human papillomavirus [47]. A variety of compounds or constituents of antifungal agents have immune modulatory properties that may enhance fungal clearance. Amphotericin B directly stimulates TLR-dependent inflammatory cytokine release in a fungus-independent manner [48]. Recently, empty liposomes were found to have beneficial immune stimulatory effects in a murine model of IA [49]. In contrast, echinocandin drugs are unlikely to cause general activation of inflammatory responses. Their immunomodulatory properties depend on the presence of fungal cells, providing a mechanism by which cell-mediated and humoral responses [50] are focused to sites of hyphal growth. Recognition that caspofungin modulates inflammatory responses to specific growth forms of *A. fumigatus* has potential implications for prophylactic and therapeutic strategies. Because host inflammatory responses make a critical contribution to the clearance of invasive aspergillosis, echinocandin drugs may be most effective for hyphal lesions and at the time of host myeloid cell recovery.

Acknowledgments

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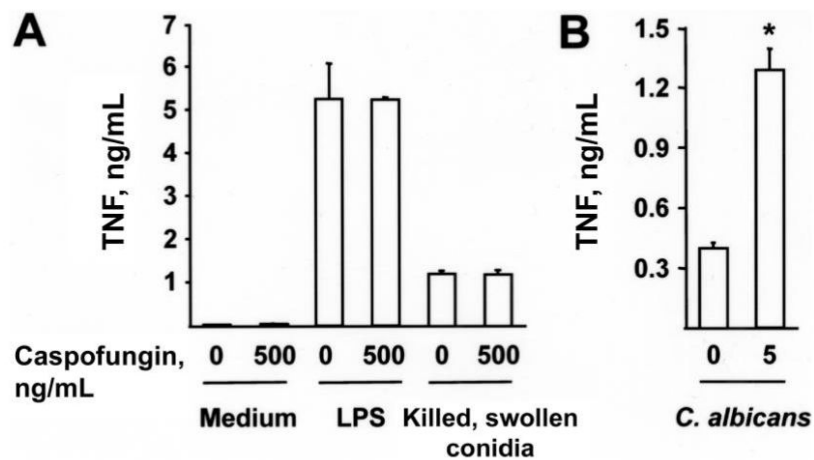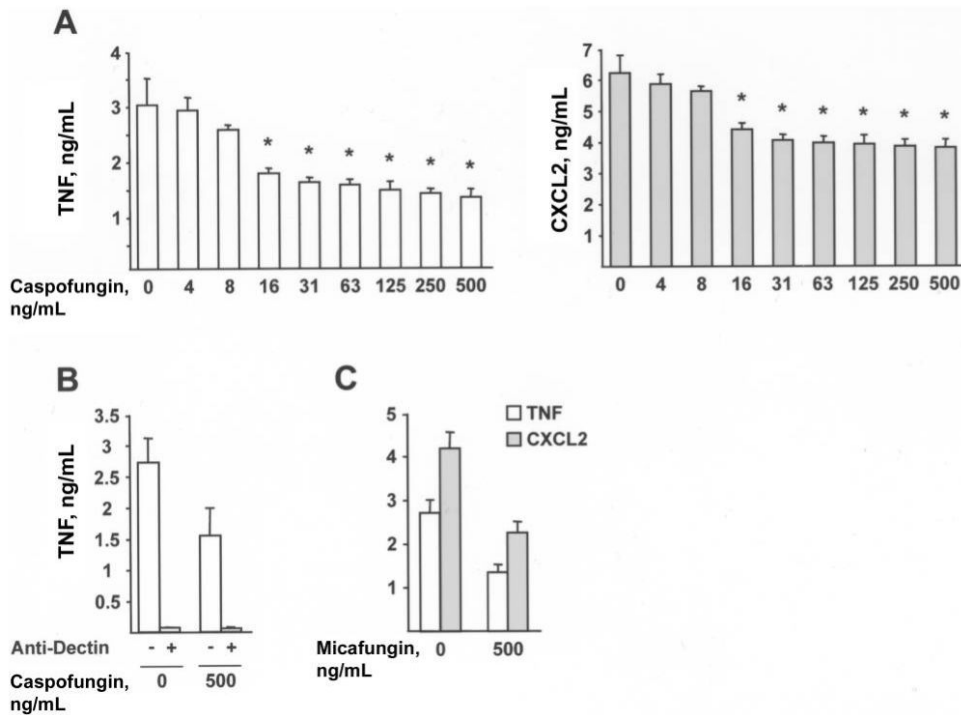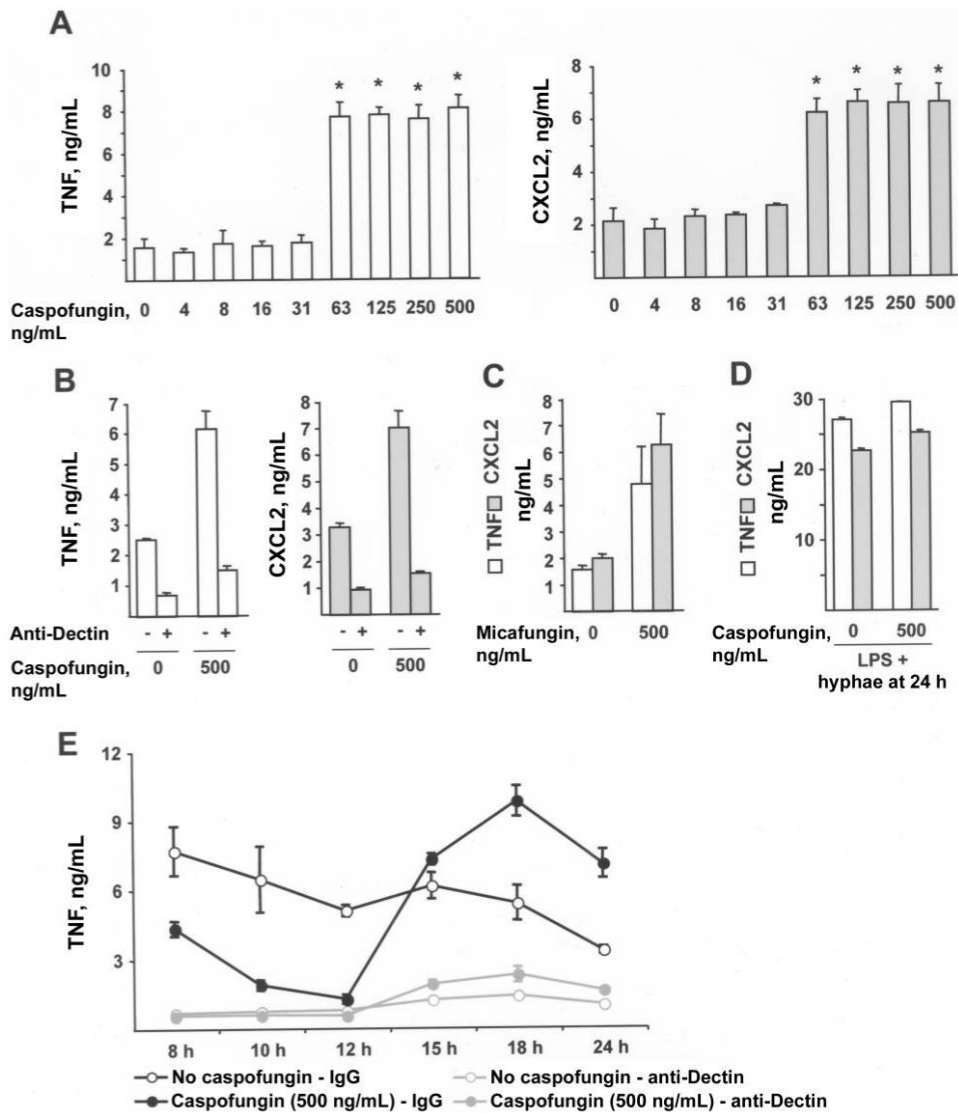
Figure 1.
Caspofungin and a lack of direct inducement of inflammatory responses. *A,* Secretion of tumor necrosis factor (TNF) by 10^5 bone marrow-derived macrophages (BMMϕs) cultured in RP-10$^+$ (see Materials and Methods for details) with 0 or 500 ng/mL caspofungin. Samples contained 100 ng/mL lipopolysaccharide (LPS) or 5×10^5 heat-killed, swollen *Aspergillus fumigatus* conidia, as indicated. *B,* Secretion of TNF by 10^5 BMMϕs incubated with 10^6 UV-inactivated *Candida albicans* yeast cells grown in yeast extract peptone dextrose containing 0 or 5 ng/mL caspofungin. *A* and *B,* Bar graphs denote the average TNF concentration (+SD) in 3 wells per condition. One of 3 representative experiments is shown. *$P < .05$, by 2-tailed *t* test, compared with control condition (i.e., no drug treatment).
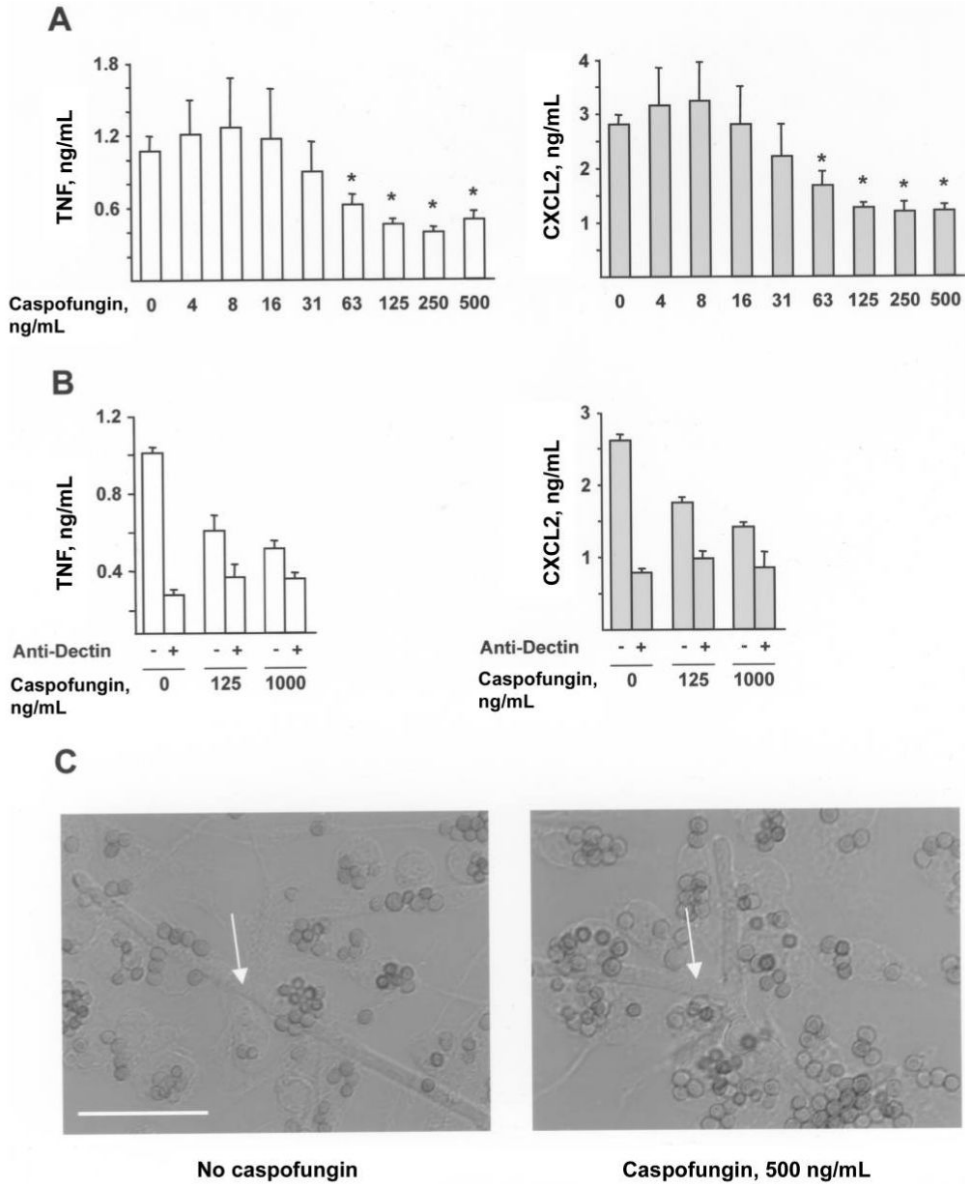
Figure 2.
Caspofungin and reductions in inflammatory responses to conidia. *A* and *B,* Secretion of tumor necrosis factor *(white bars)* or CXCL2 *(gray bars)* by 1.5×10^5 bone marrow-derived macrophages (BMMφs) incubated for 18 h with 7.5×10^5 *Aspergillus fumigatus* conidia *(A)* in RP-10+ (see Materials and Methods for details) with 0 or 500 ng/mL caspofungin or *(B)* under the same conditions, with the addition of 10 *μ*g/mL anti-Dectin (+) or isotype control antibody (-). Bar graphs denote the average TNF or CXCL2 concentration (+SD) in 3-4 wells per condition. One of 4 *(A)* or 3 *(B)* representative experiments is shown. **P* < .05, by 2-tailed *t* test, compared with control condition (i.e., no drug treatment). *C,* Differential interference contrast image of BMMφs and conidia after 18 h in RP-10+ with 0 ng/mL *(left)* or 500 ng/mL *(right)* caspofungin. Arrows denote hyphae. Scale bar, 20 *μ*m.
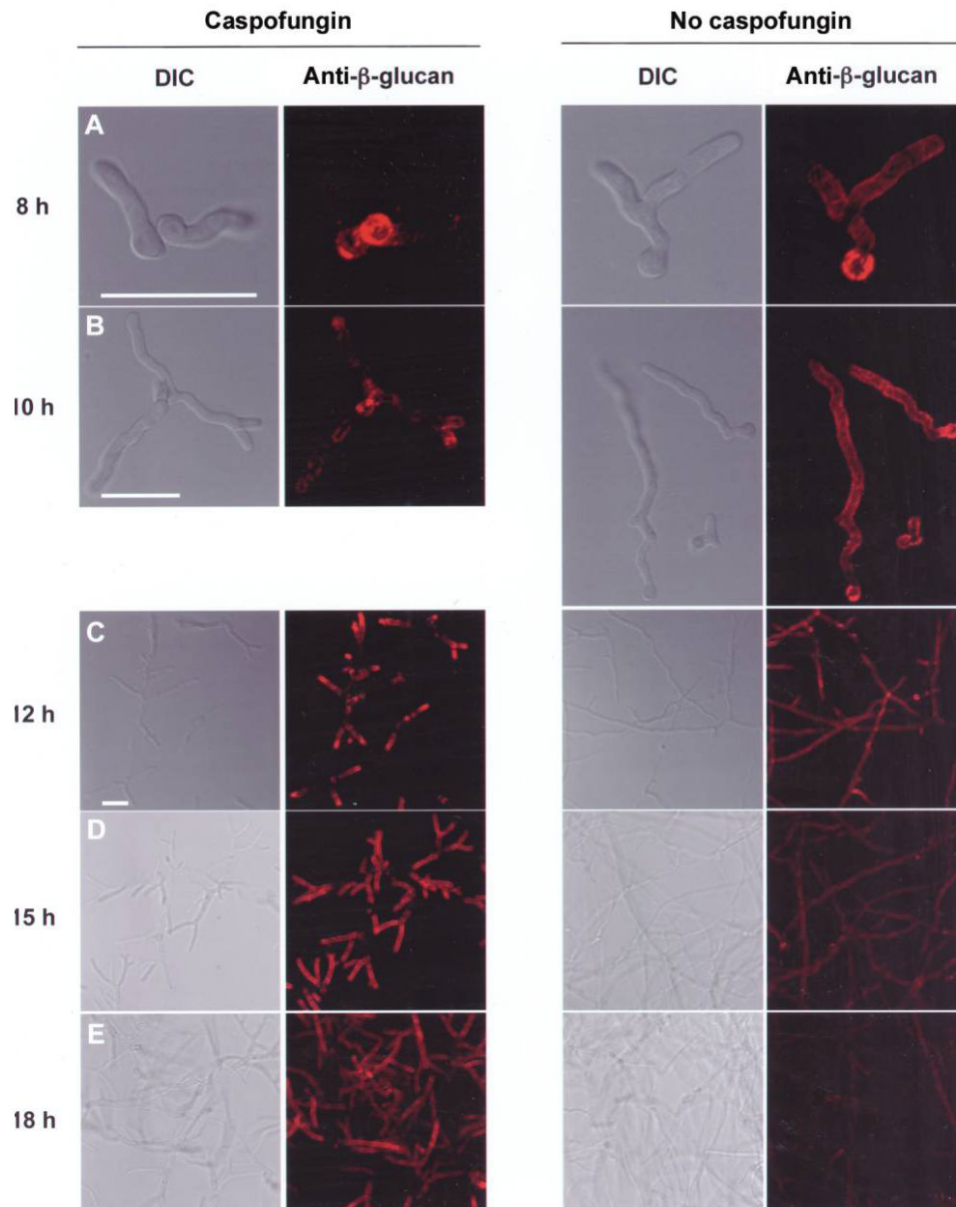
Figure 3.
Caspofungin and decreases in inflammatory responses to germlings. *A-C,* Secretion of tumor necrosis factor (TNF) or CXCL2 by 1.5×10^5 bone marrow-derived macrophages (BMMϕs) incubated with *Aspergillus fumigatus* germlings for 6 h. A total of 5×10^4 conidia were grown in RP-10$^+$ (see Materials and Methods for details) for 8 h at 37°C with 0-500 ng/mL caspofungin *(A, B)* or micafungin *(C)* before UV inactivation and the addition of BMMϕs. *B,* Cocultures contained 10 μg/mL anti-Dectin (+) or 10 μg/mL isotype control antibody (-). Bar graphs denote the average TNF or CXCL2 concentration (+SD) in 3-4 wells per condition. One of 7 *(A)* or 3 *(B, C)* representative experiments is shown. *$P < .05$, by 2-tailed *t* test, compared with control condition (i.e., no drug treatment).
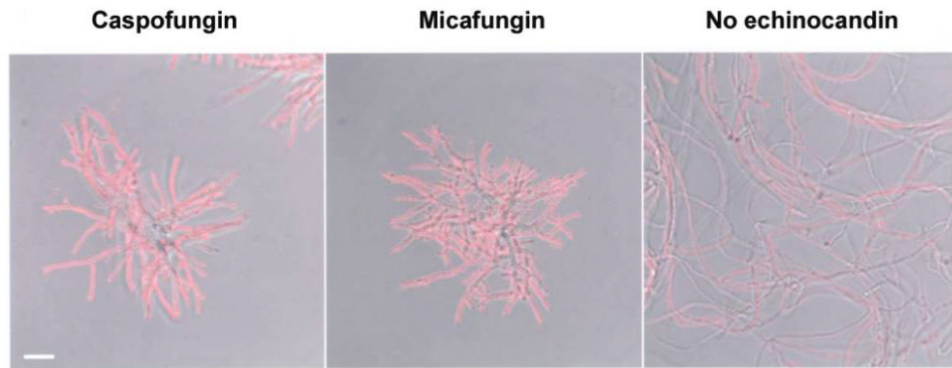
Figure 4.
Caspofungin and enhancement of inflammatory responses to hyphae. *A-D,* Secretion of tumor necrosis factor (TNF) or CXCL2 by 1.5×10^5 bone marrow-derived macrophages (BMMϕs) incubated for 6 h with *Aspergillus fumigatus* hyphae. A total of 5×10^4 conidia were grown in RP-10$^+$ (see Materials and Methods for details) for 24 h at 37°C with 0-500 ng/mL caspofungin *(A, B, D)* or micafungin *(C)* before UV inactivation and the addition of BMMϕs. *B* and *D,* Cocultures contained 10 μg/mL anti-Dectin (+), 10 μg/mL isotype control antibody (-), or 100 ng/mL lipopolysaccharide, as indicated. *E,* TNF secretion by BMMϕs stimulated with UV-inactivated fungal cells grown for the indicated period in RP-10$^+$ *(empty circles)* or in RP-10$^+$ with 500 ng/mL caspofungin *(filled circles).* Coincubations were performed in the presence of anti-Dectin *(red lines)* or control isotype antibody *(black lines),* as described for panel *B. A-E,* Bar graphs denote the average TNF or CXCL2 concentration (+SD) in 3-6 wells per condition. One of either 8 *(A)* or 3 *(B-E)* representative experiments is shown. **P < .05,* by 2-tailed *t* test, compared with control condition (i.e., no drug treatment).

Figure 5.
Caspofungin and modulation of *β*-glucan immunoreactivity in a stage-specific manner. *A-E,*
A total of 5×10^4 conidia were grown in RP-10$^+$ (see Materials and Methods for details) without
drug *(left)* or with 500 ng/mL caspofungin *(right)* for 8-18 h, as indicated; stained with an anti-
β-glucan antibody (monoclonal antibody 744); and examined by confocal microscopy.
Representative epifluorescence and differential interference contrast (DIC) images are shown.
Fluorescence images are a composite of scanned images obtained at 0.5-*μ*m *(A-B)* or 1.0-*μ*m
(C-E) intervals. Scale bar, 20 *μ*m.

Figure 6.
Echinocandins and increases in β-glucan immunoreactivity on hyphae. A total of 5×10^3 conidia were grown in RP-10+ (see Materials and Methods for details) for 24 h with 500 ng/mL caspofungin *(left)* or 500 ng/mL micafungin *(middle)* or without drug *(right)*. Representative fields are shown with composite fluorescence images overlaid on differential interference contrast images. Scale bar, 20 μm.

Table 1

Quantitative analysis of β-glucan immunoreactivity for caspofungin-treated and untreated *Aspergillus fumigatus* hyphae

Experiment	Integrated fluorescence intensity/fungal mass,[a] by hyphae group	
	Caspofungin treated	Untreated
1	21.41[b] ± 8.34	1.83 ± 0.73
2	43.73[b] ± 6.96	2.96 ± 4.67

NOTE. Data are the average ratio (±SD) of β-glucan immunofluorescence intensity normalized to hyphal mass, as calculated from 4-5 fields of view per condition.

[a] Arbitrary units.

[b] $P < .02$, by 2-tailed *t* test, compared with control condition (untreated hyphae).