AAC Journals.ASM.org

Macrophage Reporter Cell Assay for Screening Immunopharmacological Activity of Cell Wall-Active Antifungals

Russell E. Lewis,[a]* Guangling Liao,[a] Katherine Young,[b] Cameron Douglas,[b] Dimitrios P. Kontoyiannis[c]

University of Houston College of Pharmacy, Houston, Texas, USA[a]; Merck Research Laboratories, Rahway, New Jersey, USA[b]; The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA[c]

Antifungal exposure can elicit immunological effects that contribute to activity *in vivo*, but this activity is rarely screened *in vitro* in a fashion analogous to MIC testing. We used RAW 264.7 murine macrophages that express a secreted embryonic alkaline phosphatase (SEAP) gene induced by transcriptional activation of NF-κB and activator protein 1 (AP-1) to develop a screen for immunopharmacological activity of cell wall-active antifungal agents. Isolates of *Candida albicans* and *Aspergillus fumigatus* that conditionally express genes involved in cell wall synthesis were also tested with the reporter macrophages. We found that growth of fungi in subinhibitory concentrations of glucan synthesis inhibitors (caspofungin and enfumafungin A) or repression of the β-glucan catalytic subunit of glucan synthase, *FKS1*, increased macrophage NF-κB/AP-1 activation in a dectin-1-dependent manner. This pattern of activation was also transiently observed with repression of chitin synthesis in *C. albicans* or when yeast cells were incubated in low concentrations of the chitin synthesis inhibitor nikkomycin Z.

The host immune response plays a critical role in shaping the efficacy and safety of antifungal therapy *in vivo*. Some antifungals, such as amphotericin B, directly activate immune cells through microbial pattern recognition receptors (PRRs) (toll-like receptor 2 [TLR2] and CD14), causing the release of proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin-6 [IL-6], IL-1Ra, and IL-1β), chemokines (IL-8, monocyte chemoattractant protein 1 [MCP-1], and MIP-1β), nitric oxide, and prostaglandins (1). This direct stimulation of immune cells underlies the frequent and sometimes severe infusion-related reactions (fever, chills, rigors, nausea, and tachypnea) observed in patients during amphotericin B infusions (2). Alternatively, antifungals can also indirectly stimulate host immune cells through their inhibitory or lethal effects in fungi. Exposure of *Candida* or *Aspergillus* species to echinocandin antifungals increases exposure of normally concealed branched glucan polymers, which elicit strong inflammatory responses from macrophages *in vitro* (3, 4) and *in vivo* (5). Although these mechanisms can enhance antifungal drug efficacy, immunopharmacological mechanisms are infrequently considered components of antifungal activity or spectrum *in vivo*.

Similarly, the immunopharmacological activity of antifungals is rarely screened for established or novel antifungal agents in a fashion similar to MIC assays. To that end, we sought to develop a facile assay for screening drug immunopharmacological activity that could complement other phenotypic endpoints used for screening of drug activity or conditional mutant libraries. We also reasoned that an immunopharmacological screen would be useful in the evaluation of resistance in clinical isolates in the research laboratory. For example, certain antifungal resistance mutations may also facilitate immunoevasion strategies in the host even in the absence of antifungal selective pressure, contributing to the persistence of resistant strains *in vivo* (6, 7).

Specifically, the purpose of our study was to develop a simple, cell-based reporter assay suitable for screening indirect immunopharmacological effects of antifungal agents in *Candida albicans* and *Aspergillus fumigatus*. During the development of this assay, we characterized the immunopharmacological profiles of caspofungin; a nonechinocandin glucan synthesis inhibitor, enfumafungin A; and the chitin synthase inhibitor nikkomycin Z over a wide range of clinically achieved concentrations. Additionally, we examined whether strains of *C. albicans* and *A. fumigatus* with conditional mutations in cell wall synthesis genes could be used to phenocopy the immunopharmacological effects of antifungals tested in the assay.

MATERIALS AND METHODS

Antifungal agents. Amphotericin B and tetracycline hydrochloride (Sigma, St. Louis, MO), fluconazole and voriconazole (Pfizer, New York, NY), and caspofungin and enfumafungin A (Merck Research Laboratories, Rahway, NJ) were prepared as stock solutions from analytical-grade powder, as described in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M38-A2 protocols (8, 9). Amphotericin B lipid complex (ABLC; Sigma-Tau, Gaithersburg, MD) was obtained from the manufacturer and diluted in phosphate-buffered saline (PBS) prior to testing.

Test isolates. Table 1 lists the *Candida albicans* ($n = 5$) and *Aspergillus fumigatus* ($n = 3$) isolates used for all experiments (kindly provided by Merck Research Laboratories). *Candida albicans* strains consisted of the parent wild-type (WT) strain, *C. albicans* CAI4, and four strains constructed in the CAI4 background with conditional heterozygous or homozygous loss-of-function mutations in chitin synthase (*CHS3*) or glucan synthase (*FKS1*) under the control of a tetracycline promoter (10). *A. fumigatus* isolates consisted of an *A. fumigatus* CEA17 uracil/uridine auxotroph parent strain and strains with conditional loss-of-function mutations in chitin synthase (*CHS2*) and glucan synthase (*FKS1*) under

Received 7 November 2013 Returned for modification 29 November 2013
Accepted 28 December 2013
Published ahead of print 6 January 2014
Address correspondence to Dimitrios P. Kontoyiannis, dkontoyi@mdanderson.org.
* Present address: Russell E. Lewis, Infectious Diseases Unit, S. Orsola-Malpighi Hospital, Department of Medical Sciences and Surgery, University of Bologna, Bologna, Italy.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02408-13.

doi:10.1128/AAC.02408-13

TABLE 1 Isolates used for testing[b]

Isolate	Description	Reference
Candida albicans CAI4[a]	Wild-type parent strain; MICs (μg/ml): CAS, 0.25; ENF-A, 0.125	10
CAI4-GRACE-*FKS1* (TET-heterozygous)	Tetracycline-repressible glucan synthase	10
CAI4-GRACE-*FKS1* (TET-homozygous)	Tetracycline-repressible glucan synthase	10
CAI4-GRACE-*CHS3* (TET-heterozygous)	Tetracycline-repressible chitin synthase 3	10
CAI4-GRACE-*CHS3* (TET-homozygous)	Tetracycline-repressible chitin synthase 3	10
Aspergillus fumigatus CEA17[a]	Uracil/uridine auxotroph parent strain; MECs (μg/ml): CAS, 0.125; ENF-A, 0.06	11
A. fumigatus CEA17-pn-*FKS1*	Nitrogen-repressible glucan synthase	11
A. fumigatus CEA17-pn-*CHS2*	Nitrogen-repressible chitin synthase 2	11

[a] Although morphological changes were observed with nikkomycin Z exposure, no definitive MIC or MEC endpoint was evident.
[b] Abbreviations: TET, tetracycline; CAS, caspofungin; ENF-A, enfumafungin A.

the control of the p*NiiA* nitrogen-regulated promoter (11). *Candida* MICs were determined according to CLSI M27-A3 (9) methods in RPMI 1640 plus 0.165 M MOPS [3-(*n*-morpholino)propanesulfonic acid], pH 7.0, without tetracycline. Minimal effective plasma concentrations (MECs) for *Aspergillus* were determined using a modification of the CLSI M38-A2 method (8) in *Aspergillus* complete medium (ACM) with uridine/uracil supplementation (12).

Macrophage reporter cells. We used a commercially available reporter macrophage cell line (RAW-Blue cells; InvivoGen, San Diego, CA). Cells were derived from murine RAW 264.7 macrophages with chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct, induced by NF-κB and activator protein 1 (AP-1) transcriptional activation. An NF-κB reporter was selected because of its rapid posttranslational activation in response to activation of microbial pattern recognition receptors (PRRs). The macrophage reporter cells express PRRs, including toll-like receptors (TLRs) and NOD-like, RIG-I-like, and C-type lectin receptors, including the PRR for fungal 1,3-β-glucan, dectin-1 (13). Activation of these PRRs by fungal organisms induces signaling pathways leading to the activation of NF-κB and AP-1 and the subsequent production of SEAP. Concentrations of SEAP are then measured by spectrophotometry to characterize the magnitude of NF-κB and AP-1 transcriptional activation using a colorimetric enzyme assay (Quanti-Blu; InvivoGen).

Macrophages were propagated, banked, and prepared for testing daily using protocols developed by the manufacturer. Cells were grown to 80% confluence in T-25 culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose, heat-inactivated 10% fetal bovine serum, 2 mM L-glutamine, and 200 μg/ml Zeocin antibiotic (InvivoGen) at 37°C in 5% CO_2. To minimize experimental variability, only cells with fewer than 10 passages were used. On each experimental day, cells were harvested by removing growth medium, followed by washing with sterile phosphate-buffered saline (PBS), trypsinization, and resuspension of the cells in fresh DMEM growth medium. Cell viability was confirmed to be >95% by trypan blue counting using a Countess cell imager (Life Technologies, Grand Island, NY). The final test concentration of reporter macrophages used for all experiments was 5×10^5 cells/well.

***Candida albicans* drug analysis.** We used a modification of methods described by Wheeler and Fink (3) for evaluating macrophage response to inactivated *C. albicans*. Yeasts were grown for 18 h in a shaking incubator at 35°C in 50-ml polypropylene tubes containing RPMI growth medium alone or in the presence of increasing serial concentrations (0 to 200 ng/ml) of antifungal agents. Additional replicate tubes were included for higher antifungal concentrations that produced near-fungicidal effects to achieve the required fungal inoculum required for testing. On the morning of the experiment, yeast cells were pelleted by centrifugation (5,000 rpm), washed twice with PBS, and then resuspended in 5 ml of PBS. An aliquot (1 ml) of the suspension was extracted using the Y-PER yeast protein extraction kit (Thermo Scientific, Rockford, IL) and analyzed for protein concentration using a modified Lowry assay (Bio-Rad detergent compatible protein kit; Bio-Rad, Hercules, CA) using bovine serum albumin standards as recommended by the manufacturer. Once the protein concentration was determined, the inoculum was standardized to dispense 20 μg of *C. albicans* protein equivalent into each well of a 96-well, flat-bottom microtiter tray. This protein concentration was roughly equivalent to 5×10^6 *C. albicans* CAI cells per well, confirmed by hemocytometer counts.

Microtiter trays were then subjected to 4 cycles of UV irradiation (120,000 μJ/cm^2) (Stratalinker UV cross-linker 1800; Stratagene, La Jolla, CA) to inactivate cells with shaking between each cycle. Cells were inactivated to reduce the confounding effects of growth in the cell culture medium, which could alter the cell wall surface. Each microplate was centrifuged (3,500 rpm) to pellet cells, and PBS was carefully aspirated using a micropipette, avoiding disruption of the fungal pellet.

Freshly harvested and washed macrophages (5×10^5) were then added (180 μl) to each well of a microplate containing the fungal inoculum (fungal cell/macrophage ratio, 10) and mixed by micropipette. A negative control (endotoxin-free PBS) and a positive control (10 μg zymosan, a TLR2/dectin-1 agonist) were included in each experimental run, as well as macrophages preincubated for 30 min with 10 μg/ml anti-dectin-1 monoclonal antibody (MAb) (InvivoGen) to assess the specific contribution of β-1,3-glucan/dectin-1-mediated activation. After 6 h of incubation at 37°C in 5% CO_2 with intermittent shaking, the microtiter tray was centrifuged to pellet macrophages and fungal material, and 50 μl of the cell culture supernatant and negative and positive controls was transferred for analysis of SEAP concentrations using methods described by the manufacturer (Quanti-Blu assay; InvivoGen). SEAP absorbance was read at an optical density at 655 nm (OD_{655}) on a microplate spectrophotometer at 6, 12, and 24 h. Data reported in this publication are for 24-h readings.

***Candida albicans* conditional mutants.** Isolates with heterozygous or homozygous conditional mutations in *FKS1* and *CHS3* were grown in nonrepressing (RPMI alone) or repressing (RPMI plus 100 μg/ml tetracycline) liquid medium for 18 h in a shaking incubator at 35°C (10). Fungal cells were then collected and processed for testing in a fashion similar to that of antifungal-exposed cells. We also examined how macrophage inflammatory responses changed with different "simulated antifungal doses" by growing the homozygous *FKS1* mutant in different (repressing) tetracycline concentrations.

***Aspergillus fumigatus* drug analysis.** A modification of methods described by Hohl et al. (4) was used to test macrophage responses against drug-exposed *A. fumigatus*. *A. fumigatus* CEA17 conidia (5×10^6) suspended in 200 μl of ACM plus uridine/uracil alone or ACM plus uridine/uracil with antifungal agents (0 to 64 μg/ml) were prepared and dispensed into a 96-well flat-bottom microtiter tray. After 18 h of incubation at 35°C, microplates were centrifuged (3,500 rpm) and medium was carefully aspirated by micropipette using an inverted microscope to leave a hyphal mat on the bottom of each well. Hyphae were then gently washed twice with PBS and subjected to 4 cycles of UV irradiation (120,000 μJ/cm^2) with mixing between each cycle, and the remaining PBS was carefully aspirated. No protein standardization of inoculum was performed, as the antifungal agents had minimal effects on total fungal biomass (<15%) at the tested concentrations (14). Freshly harvested macrophages (5×10^5) or macrophages preincubated with anti-dectin-1 MAb were then added to each well with PBS (negative control) or zymosan (positive control) in a fashion similar to that in *Candida* studies. After 6 h of coincubation, the culture medium was collected and analyzed for SEAP concentrations as previously described.

***Aspergillus fumigatus* conditional mutants.** *A. fumigatus CHS2* and *FKS1* conditional mutants, controlled by the nitrogen-responsive p*NiiA* promoter, were grown overnight (18 h) from 5×10^6 conidia per well in a microplate containing inducing (*Aspergillus* minimal medium [AMM] plus

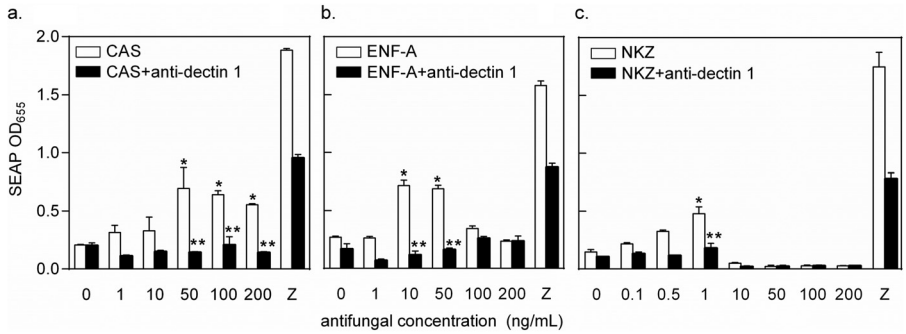

FIG 1 Exposure of *C. albicans* to subinhibitory concentrations of caspofungin, enfumafungin A, or nikkomycin Z increases activation of macrophage NF-κB/AP-1 through dectin-1. (a) Caspofungin (CAS); (b) enfumafungin A (ENF-A); (c) nikkomycin Z (NKZ). Anti-dectin-1 antibody, 10 μl. Z, zymosan, 10 μg/ml (TLR2/dectin-1-positive control). Each experiment was performed in triplicate. *, $P \leq 0.05$ versus control, Kruskal-Wallis test with Dunn's *post hoc* test; **, $P \leq 0.05$ versus non-anti-dectin-1-exposed macrophages.

nitrate) or repressing (AMM plus ammonium tartrate) medium as described by Hu et al. (11). Microplates were then centrifuged to pellet the hyphal mat, washed, and UV inactivated as previously described before addition of macrophages (5×10^5), anti-dectin-1-blocked macrophages, PBS (negative control), or zymosan (positive control). Plates were incubated for 6 h before medium was collected for analysis of SEAP concentrations.

Statistical analysis. Experiments were performed in duplicate on two separate days. Mean SEAP concentrations ± standard deviations were compared by Kruskal-Wallis test, with Dunn's *post hoc* test for individual comparisons. Differences in SEAP concentrations associated with a *P* value of <0.05 were used to define significant differences in macrophage NF-κB/AP-1 activation status.

RESULTS

Macrophage reporter cell assay. A number of test variables were examined during assay development, including fungal cell culture conditions, the optimal target inoculum/macrophage ratio, and the potential for direct macrophage activation of the reagents and antifungals. Among antifungals initially screened for direct immunostimulatory activity, only amphotericin B was found to activate NF-κB/AP-1 transcription at concentrations above 1 μg/ml (see Fig. S1 in the supplemental material). Exposure to caspofungin, enfumafungin A, or nikkomycin Z did not significantly increase macrophage transcriptional activation of NF-κB/AP-1 up to concentrations of 10 μg/ml. The final test conditions described in Materials and Methods produced consistent results that were in agreement with previous work using RAW 264.7 macrophages or primary cell lines (3, 4).

Glucan and chitin synthesis inhibitors enhance macrophage inflammatory responses to *C. albicans*. Macrophages exposed to *C. albicans* CAI4 grown without antifungals elicited marginal, nonsignificant increases in NF-κB/AP-1 macrophage activation over that in the negative controls. However, growth of CAI4 in the presence of subinhibitory (0.125 to 0.5× MIC) concentrations of caspofungin or enfumafungin elicited 2- to 4-fold-higher rates of NF-κB/AP-1 transcriptional activation in macrophages ($P < 0.05$) compared to non-drug-exposed fungi, which were blocked by the addition of anti-dectin-1 MAb (Fig. 1). Notably, macrophage activation diminished with higher drug concentrations (200 ng/ml), particularly for enfumafungin A. Similarly, CAI4 grown in the presence of low (0.1- to 1-ng/ml) but not higher (10- to 500-ng/ml) concentrations of nikkomycin Z induced 2-fold-higher rates ($P < 0.05$) of NF-κB/AP-1 transcriptional activation in macrophages, which was attenuated by anti-dectin-1 MAb, suggesting that chitin synthesis inhibitors may also induce increased surface exposure of β-1,3-glucan in *C. albicans*.

Conditional cell wall mutants of *C. albicans* phenocopy immunopharmacological activity of glucan and chitin synthesis inhibitors. Transcriptional repression of echinocandin (*FKS1*) or chitin synthesis (*CHS3*) drug targets should elicit patterns of dectin-1-dependent macrophage activation similar to those of drug-exposed fungi. Macrophages exposed to homozygous *FKS1* and *CHS3* conditional mutants grown under repressing conditions (100 μg/ml tetracycline) induced patterns of dectin-1-dependent macrophage activation similar to those of cells exposed to glucan synthesis inhibitors (Fig. 2). Similar experiments performed with heterozygous mutants did not

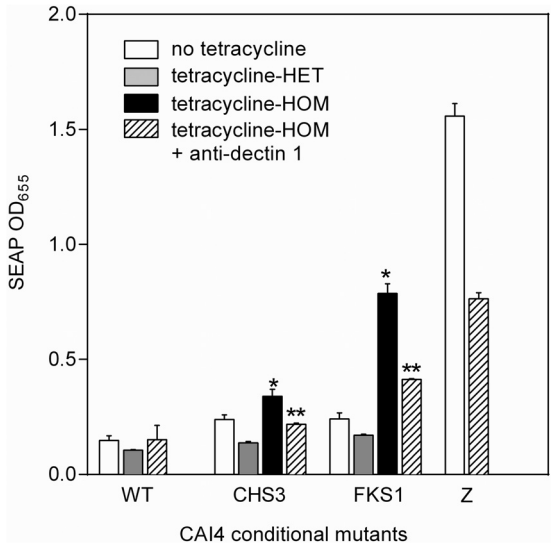

FIG 2 Tetracycline repression of homozygous *CHS3* and *FKS1* in *C. albicans* phenocopies growth in the presence of nikkomycin Z and caspofungin. Tetracycline concentration, 100 μg/ml; anti-dectin-1 antibody, 10 μg/ml; HET, heterozygous conditional mutant; HOM, homozygous conditional mutant; WT, wild type; CHS3, chitin synthase 3; FKS1, β-1,3-glucan synthase subunit; Z, zymosan, 10 μg/ml (TLR2/dectin-1-positive control). Experiments were performed with four replicates. *, $P \leq 0.05$ versus control, Kruskal-Wallis test with Dunn's *post hoc* test; **, $P \leq 0.05$ versus non-anti-dectin-1-exposed macrophages.

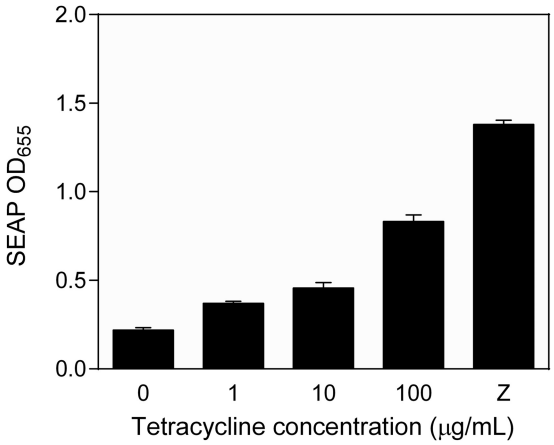

FIG 3 Graded NF-κB/AP-1 transcriptional activation against CAI4-GRACE-*FKS1* grown in increasing concentrations of tetracycline in culture medium. Experiments were performed in four replicates. Z, zymosan, 10 μg/ml (TLR2/dectin-1-positive control).

elicit increased macrophage activation, confirming that the homozygous deletion background was required to phenocopy immunopharmacological effects of exposure to glucan synthesis inhibitors or nikkomycin Z in the assay. These results suggested that the immunopharmacological effects of the antifungals result from direct target inhibition, rather than pleiotropic nonspecific mechanisms on the fungal cells.

The degree of macrophage NF-κB/AP-1 transcriptional activation by homozygous mutants correlated with degree of tetracycline-mediated repression in the medium (Fig. 3). Graded increases in tetracycline proportionally increased NF-κB/AP-1 activation. No evidence of a paradoxical decrease in inflammatory responses (as was observed with higher concentrations in drug experiments) was evident over the range of tested tetracycline concentrations.

Glucan and chitin synthesis inhibitors enhance macrophage inflammatory responses to *A. fumigatus*. *A. fumigatus* hyphae grown in the absence of antifungal agents caused minimal increases in macrophage activation (OD_{655} of <0.2 for SEAP) relative to negative controls. In contrast, hyphae grown in the presence of caspofungin or enfumafungin (≥0.5× MEC) elicited a 4- to 5-fold increase in NF-κB/AP-1 transcriptional activation in macrophages ($P < 0.05$) relative to that of non-drug-exposed fungi (Fig. 4). This activation was blocked by anti-dectin-1 antibodies. Similar to studies with *C. albicans*, the magnitude of dectin-1-dependent macrophage activation decreased with higher caspofungin and enfumafungin A concentrations. In contrast to studies with CAI4, no increase in macrophage transcriptional ac-

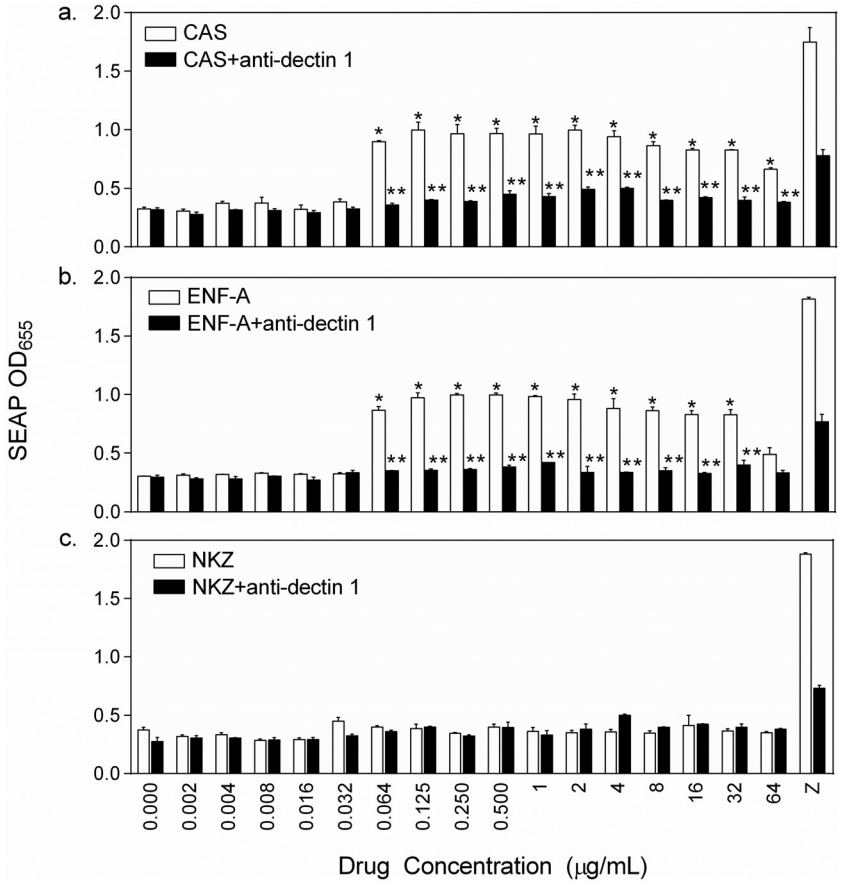

FIG 4 Exposure of *A. fumigatus* to increasing concentrations of caspofungin or enfumafungin A, but not nikkomycin Z, increases activation of macrophage NF-κB/AP-1 through dectin-1. (a) Caspofungin (CAS); (b) enfumafungin A (ENF-A); (c) nikkomycin Z (NKZ). Anti-dectin-1 antibody, 10 μl; Z, zymosan, 10 μg/ml (TLR2/dectin-1-positive control).*, $P \leq 0.05$ versus control, Kruskal-Wallis test with Dunn's *post hoc* test; **, $P \leq 0.05$ versus non-anti-dectin-1-exposed macrophages.

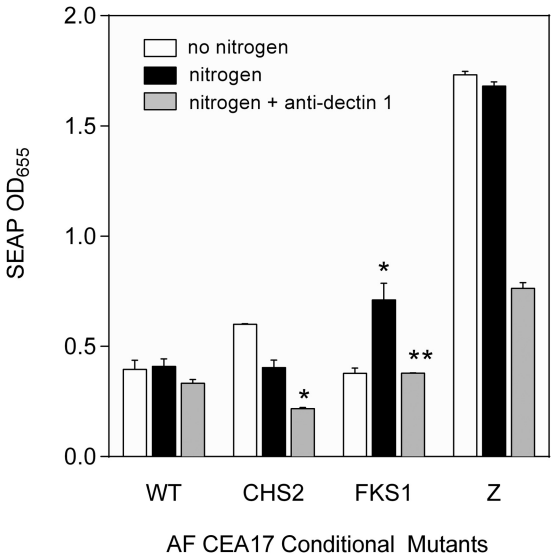

FIG 5 Nitrogen repression of *FKS1* in *A. fumigatus* phenocopies growth in the presence of caspofungin or enfumafungin. Repressing conditions were induced by addition of 10 mM ammonium tartrate to *Aspergillus* minimal medium. Anti-dectin-1 antibody, 10 μg/ml; WT, wild type; CHS2, chitin synthase isoenzyme 2; FKS1, β-1,3-glucan synthase subunit; Z, zymosan, 10 μg/ml (TLR2/dectin-1-positive control). Experiments were performed in four replicates. *, $P \leq 0.05$ versus control, Kruskal-Wallis test with Dunn's *post hoc* test; **, $P \leq 0.05$ versus non-anti-dectin-1-exposed macrophages exposed to isolates grown under repressing conditions.

tivity was observed when *A. fumigatus* CEA17 was grown in the presence of nikkomyzin Z.

The conditional glucan synthase mutant of *A. fumigatus* phenocopies immunopharmacological activity of glucan synthesis inhibitors. Transcriptional repression of *FKS1* caused by ammonium tartrate in growth medium induced patterns of dectin-1-dependent macrophage activation similar to those of hyphae grown in the presence of caspofungin or enfumafungin A (Fig. 5). Similar to the studies with nikkomycin Z-exposed hyphae, conditional repression of *CHS2* was not associated with increased macrophage transcriptional NF-κB/AP-1 activation.

DISCUSSION

Studies in antimicrobial pharmacology typically focus on the ability of the drug to inhibit a specific target and direct phenotypic effects of the target inhibition (15). While these biochemical concepts are central to understanding the intrinsic activity of antifungal agents, direct or indirect interactions with the host immune response also shape the efficacy and safety of the antifungals *in vivo* (16). Indirect immunological effects may be especially important for cell wall-active antifungals, such as the echinocandins. For example, despite their lack of appreciable *in vitro* activity against a number of clinically important molds (Mucorales, *Fusarium*, and *Scedosporium*), echinocandins have been found to be modestly effective *in vivo*, and sometimes highly effective in combination with other agents, in experimental models of mucormycosis (17–19), fusariosis (20), and scedosporiosis (21). Counterintuitively, lower doses of echinocandins were generally more effective than higher doses in these animal infection models. This unusual dose-response pattern may be a signature of indirect immunopharmacological effects *in vivo* and/or limited activation of fungal compensatory responses to echinocandin exposure at lower doses that have been shown (at least with *Candida albicans*) to be associated with diminished activation of host immune cells (22–24).

Given the potential importance of these immunological effects, we sought to develop a simple assay for screening antifungal-mediated modulation of inflammatory responses that could provide information complementary to conventional assays typically used to screen antifungal activity. To this end, we developed a cell-based assay with a commercially available mouse macrophage reporter cell where transcriptional activation of NF-κB/AP-1 induces production of SEAP, an easily measured reporter frequently employed in high-throughput assays.

In agreement with previous studies (3, 13), we found that echinocandin-treated *C. albicans* and *A. fumigatus* increased dectin-1-mediated inflammatory responses in macrophages. This enhanced inflammatory response with caspofungin occurred over a range of drug concentrations that have been observed *in vivo* with 50- to 70-mg doses of caspofungin (100 to 10,000 ng/ml) (25). New findings were that we also observed similar patterns of dectin-1-dependent macrophage activation in *C. albicans* and *A. fumigatus* cells treated with enfumafungin A, a semisynthetic, nonechinocandin glucan synthesis inhibitor synthesized from an antimicrobial peptide produced by the fungal endophyte *Hormonema* species (26). We also found that *C. albicans* treated with low concentrations of nikkomycin Z, a specific inhibitor of chitin synthase (*CHS3*) with relatively poor inhibitory activity against *Candida albicans* (27), elicited a modest but significant increase in dectin-1 macrophage activation in *C. albicans* in a fashion similar to that of glucan synthase inhibitors. It is possible that exposure to higher concentrations of nikkomycin Z induces activation of other chitin synthase isozymes in *Candida* (*CHS2* and *CHS1*), reversing the inhibitor's effects at higher concentrations (27). Macrophage inflammatory responses were largely unchanged in *A. fumigatus* exposed to nikkomycin Z.

Notably, *C. albicans* and *A. fumigatus* isolates with conditional mutations in glucan (*FKS1*) and chitin (*CHS3*) synthase elicited macrophage inflammatory responses in a fashion similar to that of drug treatment when the mutants were grown under repressing conditions. These results suggest that a macrophage reporter cell-based assay could be used as an immunopharmacological screen to complement typical static or fungicidal phenotypic endpoints of candidate drug molecules or libraries of isolates with different mutations affecting cell wall synthesis.

Although the assay is relatively simple and can be performed in any laboratory with cell culture facilities, a couple of limitations should be highlighted. Testing of *Candida* cells was complicated by the dimorphism of the fungus, which elicits different macrophage responses depending on cell morphology and in the presence of fungicidal drugs. Therefore, it was necessary to standardize the inoculum by protein concentration and include replicate tubes to account for lower fungal inoculum at test concentrations near the MIC. Similar protein or biomass standardization approaches would be required for testing fungicidal agents against *Aspergillus*. Culture medium composition (i.e., nutrient-rich versus -poor medium) can also profoundly influence the dynamic and adaptable composition of *Candida* and *Aspergillus* cell walls (28, 29), which also alters macrophage responses. Consequently, test medium needs to be carefully controlled and individualized to the pathogens of interest and the study objective. Finally, although transcriptional activation of NF-κB/AP-1 was simple to monitor

in this assay and often parallels cytokine production, it does not provide information regarding specific immune effector mechanisms such as the generation of reactive oxygen species.

In summary, we describe the development of a simple, cell-based reporter assay suitable for screening indirect immunopharmacological effects of antifungal agents in *Candida albicans* and *Aspergillus fumigatus*. This assay has provided interesting insight into how *FKS1* mutations associated with echinocandin resistance in *A. fumigatus* (30) and *C. albicans* (6) *in vivo* may impact treatment outcome and organism persistence and fitness *in vivo*. Therefore, this assay could be a useful complementary screen in the evaluation of new antifungal agents or mutation libraries or studies of intrinsically resistant or adaptively resistant pathogens *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

This project was funded by Merck & Co., Inc. K.Y. and C.D. are employees of Merck & Co., Inc. D.P.K. acknowledges the Francis King Black Endowment.

REFERENCES

1. **Sau K, Mambula SS, Latz E, Henneke P, Golenbock DT, Levitz SM.** 2003. The antifungal drug amphotericin B promotes inflammatory cytokine release by a toll-like receptor- and CD14-dependent mechanism. J. Biol. Chem. **278:** 37561–37568. http://dx.doi.org/10.1074/jbc.M306137200.
2. **Rogers PD, Jenkins JK, Chapman SW, Ndebele K, Chapman BA, Cleary JD.** 1998. Amphotericin B activation of human genes encoding for cytokines. J. Infect. Dis. **178:**1726–1733. http://dx.doi.org/10.1086/314495.
3. **Wheeler RT, Fink GR.** 2006. A drug-sensitive genetic network masks fungi from the immune system. PLoS Pathog. **2:**e35. http://dx.doi.org/10.1371/journal.ppat.0020035.
4. **Hohl TM, Feldmesser M, Perlin DS, Pamer EG.** 2008. Caspofungin modulates inflammatory responses to *Aspergillus fumigatus* through stage-specific effects on fungal β-glucan exposure. J. Infect. Dis. **198:**176–185. http://dx.doi.org/10.1086/589304.
5. **Wheeler RT, Kombe D, Agarwala SD, Fink GR.** 2008. Dynamic, morphotype-specific *Candida albicans* β-glucan exposure during infection and drug treatment. PLoS Pathog. **4:**e1000227. http://dx.doi.org/10.1371/journal.ppat.1000227.
6. **Ben-Ami R, Garcia-Effron R, Lewis RE, Gamarra S, Leventakos K, Perlin DS, Kontoyiannis DP.** 2011. Fitness and virulence costs of *Candida albicans* FKS1 hot spot mutations associated with echinocandin resistance. J. Infect. Dis. **204:**626–635. http://dx.doi.org/10.1093/infdis/jir351.
7. **Lewis RE, Viale P, Kontoyiannis DP.** 2012. The potential impact of antifungal drug resistance mechanisms on the host immune response to *Candida*. Virulence **3:**368–376. http://dx.doi.org/10.4161/viru.20746.
8. **Clinical and Laboratory Standards Institute.** 2008. Reference method for both dilution susceptibility testing of filamentous fungi, 2nd ed, M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution of antifungal susceptibility testing of yeasts; approved standard, 3rd ed, M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **Roemer TB, Jiang J, Davison J, Ketela T, Veillette K, Breton A, Tandia F, Linteau A, Sillaots S, Marta C, Martel N, Veronneau S, Lemieux S, Kauffman S, Becker J, Storms R, Boone C, Bussey H.** 2003. Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. Mol. Microbiol. **50:**167–181. http://dx.doi.org/10.1046/j.1365-2958.2003.03697.x.
11. **Hu W, Sillaots S, Lemieux S, Davison J, Kauffman S, Breton A, Linteau A, Xin C, Bowman J, Becker J, Jiang B, Roemer T.** 2007. Essential gene identification and drug target prioritization in *Aspergillus fumigatus*. PLoS Pathog. **3:**e24. http://dx.doi.org/10.1371/journal.ppat.0030024.
12. **Cove D.** 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim. Biophys. Acta **113:**51–56. http://dx.doi.org/10.1016/S0926-6593(66)80120-0.
13. **Brown GD.** 2006. Dectin-1: a signaling non-TLR pattern-recognition receptor. Nat. Rev. Immunol. **6:**33–43. http://dx.doi.org/10.1038/nri1745.
14. **Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, Liberator PA, Douglas CM.** 2002. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. Antimicrob. Agents Chemother. **46:**3001–3012. http://dx.doi.org/10.1128/AAC.46.9.3001-3012.2002.
15. **Drusano GL.** 2004. Antimicrobial pharmacodynamics: critical interactions of "bug and drug". Nat. Rev. Microbiol. **2:**289–300. http://dx.doi.org/10.1038/nrmicro862.
16. **Ben-Ami R, Lewis RE, Kontoyiannis DP.** 2008. Immunocompromised hosts: immunopharmacology of modern antifungals. Clin. Infect. Dis. **47:**226–235. http://dx.doi.org/10.1086/589290.
17. **Ibrahim AS, Gebremariam T, Fu Y, Edwards JS, Spellberg B.** 2008. Combination echinocandin-polyene treatment of murine mucormycosis. Antimicrob. Agents Chemother. **52:**1556–1558. http://dx.doi.org/10.1128/AAC.01458-07.
18. **Ibrahim AS, Bowman JC, Avanessian V, Brown K, Spellberg B, Edwards JE, Douglas CM.** 2005. Caspofungin inhibits *Rhizopus oryzae* 1,3-β-D-glucan synthase, lowers burden in brain measured by quantitative PCR, and improves survival at a low but not a high dose during murine disseminated zygomycosis. Antimicrob. Agents Chemother. **49:**721–727. http://dx.doi.org/10.1128/AAC.49.2.721-727.2005.
19. **Lewis RE, Leventakos K, Liao G, Kontoyiannis DP.** 2011. Efficacy of caspofungin in neutropenic and corticosteroid-immunosuppressed murine models of invasive pulmonary mucormycosis. Antimicrob. Agents Chemother. **55:**3584–3587. http://dx.doi.org/10.1128/AAC.01812-10.
20. **Spellberg B, Schwartz J, Fu Y, Avanesian V, Adler-Moore J, Edwards JE, Ibrahim AS.** 2006. Comparison of antifungal treatments for murine fusariosis. J. Antimicrob. Chemother. **58:**973–979. http://dx.doi.org/10.1093/jac/dkl378.
21. **Bocanegra R, Najvar LK, Hernandez S, McCarthy DI, Graybill JR.** 2005. Caspofungin and liposomal amphotericin B therapy of experimental murine scedosporiosis. Antimicrob. Agents Chemother. **49:**5139–5141. http://dx.doi.org/10.1128/AAC.49.12.5139-5141.2005.
22. **Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, Kullberg BJ, O'Callaghan CA, Sheth CC, Odds FC, Brown AJP, Munro CA, Gow NAR.** 2011. Recognition and blocking of innate immunity cells by *Candida albicans* chitin. Infect. Immun. **79:**1961–1970. http://dx.doi.org/10.1128/IAI.01282-10.
23. **Walker LA, Gow NAR, Munro CA.** 2013. Elevated chitin content reduces the susceptibility of *Candida* species to caspofungin. Antimicrob. Agents Chemother. **57:**146–154. http://dx.doi.org/10.1128/AAC.01486-12.
24. **Marakalala MJ, Vautier S, Potrykus J, Walker LA, Shepardson KM, Hopke A, Mora-Montes HM, Kerrigan A, Netea MG, Murray GI, MacCallum DM, Wheeler R, Munro CA, Gow NA, Cramer RA, Brown AJ, Brown GD.** 2013. Differential adaptation of *Candida albicans* in vivo modulates immune recognition by dectin-1. PLoS Pathog. **9:**e1003315. http://dx.doi.org/10.1371/journal.ppat.1003315.
25. **Stone JA, Xu X, Winchell GA, Deutsch PJ, Pearson PG, Migoya EM, Mistry GC, Xi L, Miller A, Sandhu P.** 2004. Disposition of caspofungin: role of distribution in determining pharmacokinetics in plasma. Antimicrob. Agents Chemother. **48:**815–823. http://dx.doi.org/10.1128/AAC.48.3.815-823.2004.
26. **Aly AH, Debbab A, Proksch P.** 2011. Fungal endophytes: unique plant inhabitants with great promises. Appl. Microbiol. Biotechnol. **90:**1829–1845. http://dx.doi.org/10.1007/s00253-011-3270-y.
27. **Gaughran JP, Lai MH, Kirsch DR, Silverman SJ.** 1994. Nikkomycin Z is a specific inhibitor of *Saccharomyces cerevisiae* chitin synthase isozyme Chs3 *in vitro* and *in vivo*. J. Bacteriol. **176:**5857–5860.
28. **Kruppa M, Greene RR, Noss I, Lowman DW, Williams DL.** 2011. *C. albicans* increases cell wall mannoprotein, but not mannan, in response to blood, serum and cultivation at physiological temperature. Glycobiology **21:**1173–1180. http://dx.doi.org/10.1093/glycob/cwr051.
29. **Clavaud C, Beauvais A, Barbin L, Munier-Lehmann H, Latge JP.** 2012. The composition of the culture medium influences the β-1,3-glucan metabolism of *Aspergillus fumigatus* and the antifungal activity of inhibitors of β-1,3-glucan synthesis. Antimicrob. Agents Chemother. **56:**3428–3431. http://dx.doi.org/10.1128/AAC.05661-11.
30. **Lewis RE, Liao G, Hou J, Prince RA, Kontoyiannis DP.** 2011. Comparative in vivo dose-dependent activity of caspofungin and anidulafungin against echinocandin-susceptible and -resistant *Aspergillus fumigatus*. J. Antimicrob. Chemother. **66:**1324–1331. http://dx.doi.org/10.1093/jac/dkr142.