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The polymorphic commensal fungus *Candida albicans* causes life-threatening disease via bloodstream and intra-abdominal infections in immunocompromised and transplant patients. Although host immune evasion is a common strategy used by successful human fungal pathogens, *C. albicans* provokes recognition by host immune cells less capable of destroying it. To accomplish this, *C. albicans* white cells secrete a low-molecular-weight chemoattractive stimulant(s) of macrophages, a phagocyte that they are able to survive within and eventually escape from. *C. albicans* opaque cells do not secrete this chemoattractive stimulant(s). We report here a physiological mechanism that contributes to the differences in the interaction of *C. albicans* white and opaque cells with macrophages. *E,E*-Farnesol, which is secreted by white cells only, is a potent stimulator of macrophage chemokinesis, whose activity is enhanced by yeast cell wall components and aromatic alcohols. *E,E*-farnesol results in up to an 8.5-fold increase in macrophage migration *in vitro* and promotes a 3-fold increase in the peritoneal infiltration of macrophages *in vivo*. Therefore, modulation of farnesol secretion to stimulate host immune recognition by macrophages may help explain why this commensal is such a successful pathogen.

Candida albicans is a serious fungal pathogen for humans (1). *C. albicans* is a dimorphic fungus and it has long been recognized that the ability to interconvert between the yeast and filamentous forms is essential for its pathogenicity. Thus, when we discovered that *E,E*-farnesol (referred to here as farnesol) is a quorum sensing molecule (QSM) for *C. albicans* which acts by blocking the conversion of yeasts to mycelia (2), we thought that farnesol would be an attractive lead compound in the design of novel antifungal drugs. However, this goal was not realized when we showed that farnesol itself acted as a virulence factor for *C. albicans* in a mouse intravenous infection model (3). These observations presented a dilemma: how can farnesol act as a virulence factor when part of its mode of action is to block the yeast-mycelium morphogenesis, which is necessary for its pathogenicity? We resolve this conundrum here by showing that farnesol is also a potent stimulator of macrophage migration both *in vitro* and *in vivo*.

C. albicans is one of the best studied examples of an opportunistic fungal pathogen. It is a normal member of the human microbiota where it is found as a commensal primarily in the gastrointestinal and genitourinary tracts and on the skin of healthy individuals without causing significant disease. In healthy individuals, *C. albicans* commensals are controlled but tolerated while *C. albicans* pathogens are eliminated (reviewed in reference 4). The presence of innate immune phagocytes, which include both macrophages and neutrophils, is critical for early detection and elimination of *C. albicans* that have made the transition from commensal to an opportunistic pathogen (5–9; reviewed in reference 10). Thus, when normal immune responses are compromised, *C. albicans* can transition easily from a commensal to an opportunistic pathogen.

Another part of the transition from commensalism to pathogenicity is the ability of *C. albicans* strains that are homozygous at the mating type locus to reversibly convert between two distinct yeast phenotypes, the normal yeast morphology (white) and the mating competent elongated cell type (opaque [11–13]). Approximately 3% of natural isolates are homozygous at the mating type locus (11). The white and opaque morphologies of *C. albicans* are also specifically adapted to colonize distinct host niches; the opaque morphology is best adapted to colonize the skin commensally, whereas the white morphology is commonly associated with systemic infections. Critically, these two yeast morphologies also elicit different responses from host phagocytes, which may account for the partitioning of these morphologies during pathogenesis. First, opaque *C. albicans* only stimulates the random movement of polymorphonuclear leukocytes, including neutrophils, whereas the white phase secretes an unknown chemoattractant(s) that stimulates chemotactic migration directly toward the yeast cells (14). Second, in a study comparing the engulfment of white and opaque cells by macrophages, it was found that a mouse macrophage cell line (RAW264.7 cells) and a *Drosophila melanogaster* hemocyte-derived cell line (S2) both engulfed white *C. albicans* much more effectively than they did opaque cells (15). This difference is intriguing because engulfed white cells are able to survive within and then escape from macrophages *in vitro* (16–18). This *in vitro* result is compatible with the importance of macrophages *in vivo* for the early elimination of *C. albicans* (19, 20) because macrophages which have engulfed *C. albicans* secrete immunoregulatory cytokines including interleukin-6 (IL-6), IL-1β, IL-10, and tumor necrosis factor alpha (TNF-α) (21) prior to the

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escape of *C. albicans*. These cytokines presumably promote the development and chemotactic migration of other innate immune cells, including neutrophils, which are more capable of killing *C. albicans* (22). Even though evading host immunity is a common strategy used by pathogenic fungi (23–25), we hypothesize *C. albicans* white cells produce a chemoattractive stimulant(s) that promotes their engulfment by macrophages. This may explain why *C. albicans* is so virulent in immunocompromised individuals, particularly those with neutropenia (1). One likely candidate for a phagocyte chemoattractive stimulant is the QSM farnesol. Farnesol is an appealing candidate because it is produced by white cells but not by opaque cells (26), and it is already a known virulence factor for *C. albicans* (3). Moreover, farnesol was previously found to activate neutrophils and stimulate proinflammatory cytokine production by monocytes, while dampening dendritic cell activity as antigen-presenting cells (21, 22). We show here that the farnesol excreted by *C. albicans* stimulates macrophage migration by promoting chemokinesis rather than chemotaxis.

MATERIALS AND METHODS

C. albicans* strains, media, and growth conditions.** The *C. albicans* wild-type clinical isolates SC5314 (*MTLa**/*MTL*α) and WO-1 (*MTL*α/*MTL*α) were obtained from Alexander Johnson, University of California at San Francisco, and David Soll, University of Iowa, respectively.

Synthetic complete (SC) medium with uridine at 100 μg/ml in place of uracil (27) was used for routine growth and maintenance of the *C. albicans* strains. Resting cells were prepared as described by Kebaara et al. (28), with the following modifications: single colonies were grown in 25 ml of SC broth at 30°C for 24 h to reach stationary phase, washed three times with 1× phosphate-buffered saline (PBS), resuspended in the same buffer, and stored at 4°C overnight before use. Resting cells were kept no longer than 1 week at 4°C.

For gas chromatography-mass spectrometry (GC-MS) analysis, single colonies of *C. albicans* strains SC5314 and WO-1 (both the white and opaque morphologies) were inoculated into 50 ml of GPP medium (29) and grown at 30°C with 225 rpm aeration until early stationary phase, when the supernatants were harvested, immediately extracted, and derivatized. Microscopically, the opaque cultures appeared to have 100% opaque cells at the times of both inoculation and harvesting.

GC-MS measurement of aromatic alcohol concentrations in *C. albicans* cell-free supernatants. The cell-free supernatants were extracted with ethyl acetate and derivatized prior to GC-MS analysis as described previously (2) with the following modifications. Derivatization of the hydroxyl groups on the aromatic alcohols was accomplished by adding 100 μl of BSTFA+TMCS (99:1; Sylon BFT kit; Supelco Analytical, Bellefonte, PA) and incubating the samples for 1 h at 50°C in a water bath. After cooling, the samples were submitted directly for GC-MS.

Cell culture and mice. RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture medium (Dulbecco modified Eagle medium [DMEM] with 10% fetal bovine serum [FBS] and 50 μg of gentamicin/ml) at 37°C in the presence of 5% CO_2. RAW 264.7 cells were passaged in fresh culture medium every 2 to 3 days and seeded into fresh serum-starved culture medium (DMEM with 0.5% FBS and 50 μg of gentamicin/ml) overnight prior to use in chemotaxis experiments. Only cells from passages 5 through 15 were used for chemotaxis experiments.

Female 8- to 12-week-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Female 8- to 12-week-old B10.s mice were offspring of breeder pairs obtained from Michel Brahic (Stanford University). All experiments involving mice were performed in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Our protocol was approved by the University of Nebraska-Medical Center Institutional Animal Care and Use Committee. All animals were housed and used in accordance with institutional guidelines, and all efforts were made to minimize suffering of animals.

Primary mouse PEC preparation. Mouse peritoneal exudate cells (PECs) were elicited by intraperitoneal (i.p.) injection of 2 ml of thioglycolate broth. Four days later, the peritoneal cavities were flushed with 2 ml of DMEM, and the cells obtained were incubated at 10^6 cells/2 ml of DMEM cell culture medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen) and 50 μg of gentamicin/ml (Invitrogen). These cells were used immediately in the mouse PEC chemotaxis assays.

Mouse macrophage chemotaxis assay. Primary C57BL/6 PECs and RAW264.7 cells were cultured as described above. The migration of both cell types was measured using the CytoSelect 96-well cell migration assay kit according to product specifications (Cell Biolabs, San Diego, CA). Briefly, aliquots of 10^6 cells in culture media were placed into the top chamber of a 5-μm-pore-size polycarbonate membrane transwell plate. Chemoattractant solutions were placed in the bottom chamber. Cells were incubated with each chemoattractant solution for 2 h, at which time the migratory cells were harvested, lysed, and measured by the fluorescence of macrophage nuclei normalized to a control of culture medium without macrophages (normalized RFU). Farnesol was added from a 100 mM stock in methanol prepared fresh each use. Stock solutions of zymosan A (25 mg/ml in PBS) and aromatic alcohols (100 mM in methanol) were also diluted into DMEM, and 1% methanol in DMEM was used as a negative control, whereas 50 ng of monocyte chemotactic protein 1 (MCP-1)/ml in DMEM was used as a positive control.

To test the migratory potential of macrophages toward *C. albicans*-secreted QSMs, supernatants were harvested from early stationary-phase *C. albicans* cultures grown in 25 ml of SC or GPP medium at 30°C with 225-rpm aeration. The supernatants were passed twice through a 20-μm-pore-size nitrate cellulose filter to ensure all *C. albicans* cells were removed prior to the addition of 40 to 120 μl of DMEM in the bottom chamber of the transwell plate.

To test whether farnesol stimulates chemotaxis or chemokinesis, the migration of RAW 264.7 cells was measured using the Neuro Probe ChemoTx system according to product specifications using a checkerboard assay (Neuro Probe, Gaithersburg, MD). Briefly, the bottom wells of the NeuroProbe ChemoTx System chamber were filled with ~300 μl of medium containing or lacking farnesol. Then, 40 μl of 10^6 RAW 264.7 cells/ml suspended in culture medium (DMEM + 0.5% FBS) containing or lacking farnesol was placed on top of a 5-μm-pore-size polycarbonate membrane filter. The ChemoTx System was incubated at 37°C and 5% CO_2 for 6 h. After incubation, nonmigratory cells were removed from the filter surface, and adherent cells that migrated through the filter were detached through addition of 2.5 mM EDTA-PBS to the top of the filter and allowed to incubate for 30 min at room temperature, followed by centrifugation of the plate at 1,400 rpm for 5 min. Migratory cells were examined microscopically and five fields of cells (from a total well area of 25.5 mm^2) were counted per well with five replicate wells per treatment.

Mice and *in vivo* chemotaxis assay. As described by Navarathna et al. (3), commercial *E*,*E*-farnesol (Sigma-Aldrich, St. Louis, MO) was diluted in 0.5% (vol/vol) Tween 80 (Sigma-Aldrich) in sterile, nonpyrogenic PBS. Mice were injected i.p. with 1 ml of fresh 20 mM (4.4 mg) *E*,*E*-farnesol dissolved in 0.5% Tween 80, aged thioglycolate broth, or 0.5% Tween 80. Three days later, the peritoneal cavities were flushed with 2 ml of DMEM, and the PECs were counted. To assay for macrophage populations within the peritoneum, the PECs were stained with peridinin chlorophyll protein-labeled anti-CD11c and fluorescein isothiocyanate-labeled anti-CD11b in the presence of Fc Block (2.4G2; all antibodies were purchased from eBiosciences, San Diego, CA). After surface staining, the cells were fixed in 1% paraformaldehyde and analyzed using a Becton Dickinson FACSCalibur, and the data were processed using FlowJo software (Tree Star, San Carlos, CA).

Statistical analysis. A Student two-tailed unpaired *t* test was used to determine the significance of differences between means for transwell chemotaxis experiments with relative fluorescence measurements of migration (*, $P < 0.05$; **, $P < 0.001$). The GLIMMIX procedure in SAS (SAS Institute,

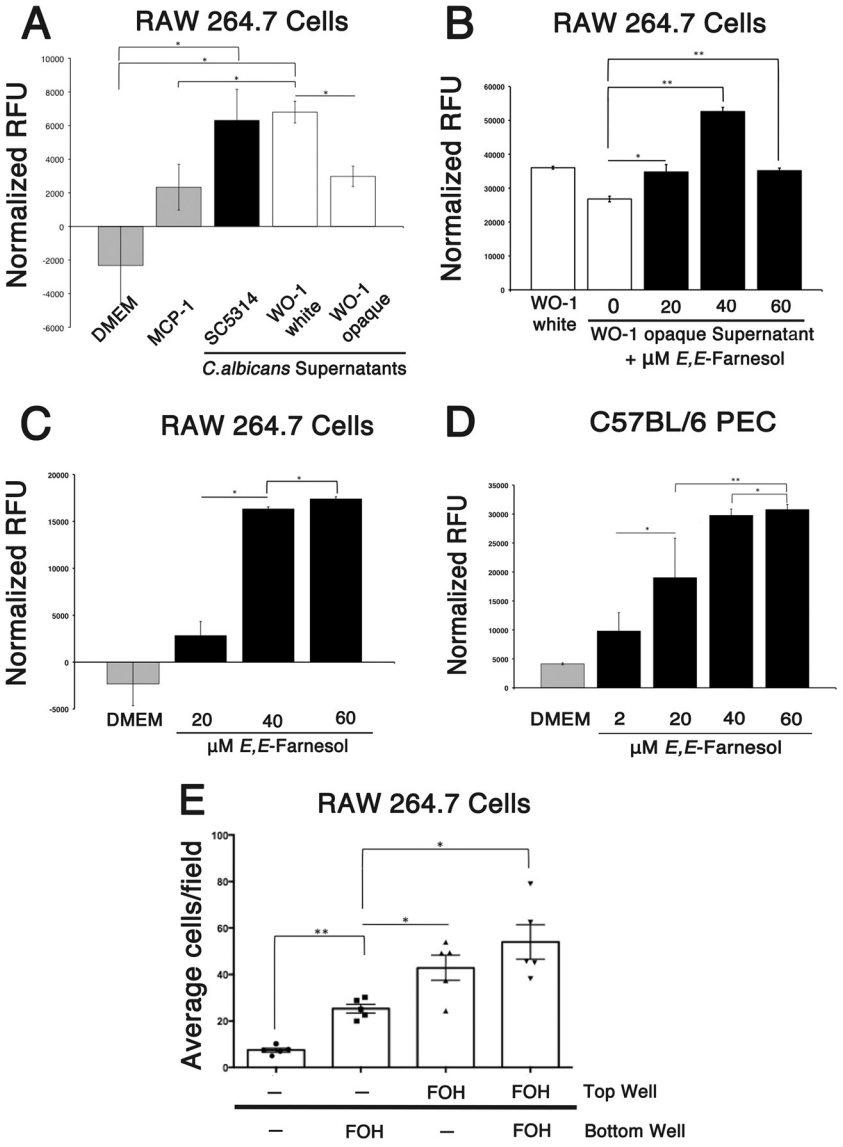

FIG 1 Cell-free supernatants of *C. albicans* and biologically relevant concentrations of *E,E*-farnesol enhance chemokinesis of mouse phagocytes. (A and B) Two-hour migration of mouse macrophage cells toward cell-free supernatants obtained from *C. albicans* growing in the white (SC5314 and WO-1) and opaque (WO-1) morphologies, compared to the negative DMEM control and the MCP-1 positive control (MCP-1, 250 ng/ml). Migration was measured by the fluorescence in the lower well of macrophage nuclei normalized to a DMEM control without macrophages (normalized RFU). Cell-free supernatants were prepared from *C. albicans* cells grown to saturation in synthetic complete medium at 30°C, some of which were supplemented with biologically relevant concentrations of farnesol immediately prior to the chemotaxis assay. (C and D) Biologically relevant concentrations of farnesol stimulate the migration of cells from the mouse cell line RAW 264.7 (C) or primary C57BL/6 PECs (D). (E) Six-hour migration of RAW 264.7 cells toward 20 μM farnesol (FOH) added from a 100 mM stock in methanol prepared fresh for each use. Methanol itself does not stimulate macrophage migration (far-left bar). All graphs show the means ± the standard errors of the mean (SEM) for at least four replicate wells (five replicate wells for panel E) and are representative of three independent experiments. (*, $P < 0.05$; **, $P < 0.001$).

Inc., Cary, NC) was used to determine the treatment least-squares means for transwell chemotaxis experiments with cells/field counts (*, $P < 0.05$; **, $P < 0.001$). The cutoff for statistical significance was set at a P value of <0.05. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) for transwell chemotaxis experiments with relative fluorescence measurements of migration, and using SAS for the transwell chemotaxis experiment with cells/field counts.

RESULTS

Macrophages preferentially migrate toward supernatants of *C. albicans* white cells. White-cell supernatants of *C. albicans*, but not opaque-cell supernatants, stimulate chemotaxis of neutrophils (14). Since macrophages interact differently with opaque cells compared to white cells (15, 20), we hypothesized that, like neutrophils, macrophages might display differential migration patterns toward white and opaque *C. albicans*. We first sought to determine whether *C. albicans* white-cell supernatants preferentially stimulate macrophage migration relative to the opaque-cell supernatants as assayed by a transwell cell migration (Fig. 1A). Briefly, 10^6 serum-starved RAW 264.7 cells were incubated in cell culture medium (i.e., DMEM), while separated from possible che-

moattractants in the respective supernatants by a permeable membrane with a 5-μm-pore-size cutoff. Macrophages attracted to potential chemoattractants distend their pseudopodia and migrate through the membrane toward the chemoattractant. After 2 h, macrophage migration into the lower chamber was quantified by fluorescently staining the macrophage nuclei. Separate cohorts of RAW 264.7 cells were incubated with uninoculated macrophage cell culture medium or with MCP-1 (30, 31) as negative and positive controls, respectively (Fig. 1A). Significantly, supernatants from two *C. albicans* strains, SC5314 and WO-1, stimulated ~3-fold more RAW 264.7 cell migration than did 250 ng of MCP-1/ml. In addition, the supernatants from WO-1 white cells stimulated 2.3-fold-greater migration than the supernatants from WO-1 opaque cells (Fig. 1A). WO-1 opaque-cell supernatants and MCP-1 stimulated similar amounts of RAW 264.7 cell migration. Thus, SC5314 and WO-1 white-cell supernatants contain secreted molecules that stimulate significantly more macrophage migration than supernatants from opaque cells even though these strains differ at their *MTL* locus because SC5314 is heterozygous for the *MTL***a** and *MTL*α alleles, whereas WO-1 is homozygous for the *MTL*α allele (11).

The fungal quorum sensing molecule farnesol stimulates the migration of macrophage *in vitro*. Farnesol is produced naturally by both *C. albicans* SC5314 and WO-1 white cells at concentrations of 2 to 100 μM (32) but not by opaque cells (26), and thus it is a candidate for the white-cell factor that stimulates positive migration of macrophages. We tested the ability of farnesol to promote migration of both RAW 264.7 cells (Fig. 1C) and primary peritoneal exudate cells (PECs) containing 90% inflammatory macrophages (Fig. 1D). Biologically relevant concentrations of farnesol (2 to 60 μM) were sufficient to promote increases in the migration of RAW 264.7 cells and PECs in a dose-dependent manner up to concentrations of farnesol that are toxic for macrophages in cell culture (80 to 100 μM) (Fig. 1C and D and data not shown). Farnesol stimulated up to 8.5- and 5-fold more RAW 264.7 and PEC migration, respectively. Methanol at 0.1%, a vehicle control, did not promote migration of either RAW 264.7 cells or PECs (data not shown). Moreover, the addition of biologically relevant concentrations of farnesol (≥20 μM) added to opaque supernatants was sufficient to stimulate an increase in the migration of RAW 264.7 cells to the same level, as was seen with white-cell supernatants (Fig. 1B), whereas ≤20 μM farnesol did not. Together, these data suggest that farnesol secreted by *C. albicans* white cells promotes the migration of macrophages.

Farnesol stimulates chemokinesis of macrophages *in vitro*. Neutrophils respond to *C. albicans* white-cell supernatants by positive chemotaxis and opaque-cell supernatants by chemokinesis (14). Chemotaxis is directed movement up a chemical concentration gradient, whereas chemokinesis is gradient-independent activation of movement in response to a chemical stimulus. To determine whether the observed induction of migration of RAW 264.7 cells in response to farnesol was due to chemotaxis or chemokinesis, RAW 264.7 cell migration was evaluated in a transwell checkerboard migration assay with 20 μM farnesol in the lower well (−/FOH), the upper well (FOH/−), or both wells (FOH/FOH; Fig. 1E). As before, the RAW 264.7 cells were added to the top well and detected in the bottom well. Consistently, farnesol in the lower well led to macrophage migration from the upper to the lower transwell. Significantly, farnesol added to the upper well also enhanced the migration of macrophages regardless of whether farnesol was present in the lower well (Fig. 1E); a chemical gradient of farnesol was not necessary. These data suggest that farnesol acts as a stimulant for chemokinesis, not chemotaxis.

For neutrophils, Geiger et al. (14) showed that the chemoattractants released by *C. albicans* included at least two components responsible for chemokinesis and chemotaxis. These authors further showed that chemokinesis and chemotaxis were separable and that one of the components necessary for chemotaxis was a peptide of ~1,000 Da because boiling and treatment with proteinase K removed the chemotactic but not the chemokinetic activity. We have now demonstrated, at least for macrophages, that the component responsible for chemokinesis is likely farnesol. Thus, the two activities can be readily distinguished on the basis of the chemical and physical properties of their active components. As we showed when we first identified farnesol as a quorum sensing molecule (2), farnesol (222 Da) is lipid soluble, extractable into ethyl acetate or hexane, stable at 100°C for 30 min, and insensitive to trypsin, pronase, thermolysin, and proteinase K. However, it is still unclear whether cell chemotaxis requires a combination of farnesol and the 1-kDa peptide or whether the peptide by itself is sufficient.

Aromatic alcohols and cell wall components enhance macrophage migration in response to farnesol. In the context of *C. albicans* interactions with the human host, the innate immune system encounters farnesol as part of a complex mixture of immunostimulatory and immunosuppressive signals. *C. albicans* immunomodulatory signals come in the form of cellular debris from dead and dying cells, as well as from excreted metabolic products. Two possible immunomodulatory signals common to both the white and opaque morphologies of *C. albicans* are fungal cell wall carbohydrates and the aromatic alcohols tyrosol, tryptophol, and phenethyl alcohol (21, 33–35). Thus, these immunomodulatory signals were tested for their chemotactic potential both on their own and in combination with farnesol. Zymosan A, a protein-carbohydrate complex prepared from yeast cell walls, stimulates macrophage to express both inflammatory and regulatory cytokines as mediated through a Toll-like receptor 2 (TLR2) and Dectin-1 response (21, 36, 37). We therefore sought to determine whether zymosan A could stimulate mouse macrophage migration on its own or enhance the migration-stimulating activity of farnesol. Zymosan A (25 μg/ml) alone does not stimulate movement of RAW 264.7 cells (Fig. 2A) or primary PECs (Fig. 2B), and it did not enhance macrophage movement due to farnesol (Fig. 2A). These data suggest that even though zymosan A stimulates the expression of both inflammatory and regulatory cytokines, it does not stimulate macrophage chemotaxis or chemokinesis due to farnesol.

Secretion of the aromatic alcohols (tyrosol, phenethyl alcohol, and tryptophol) influences growth, morphogenesis, and biofilm formation by *C. albicans* (reviewed in reference 38), but their role in the innate immune response to *C. albicans* is currently unknown. Biologically relevant concentrations of aromatic alcohols did not stimulate the migration of mouse macrophages (Fig. 2A) or primary PECs (Fig. 2B), a finding that is consistent with the observation that white and opaque cells both secrete significant levels of these aromatic alcohols (Table 1). However, their combinatorial chemotactic potential was evident in that the combination of aromatic alcohols and farnesol stimulated RAW 264.7 cell migration 1.6-fold better than did farnesol alone (Fig. 2A), and the

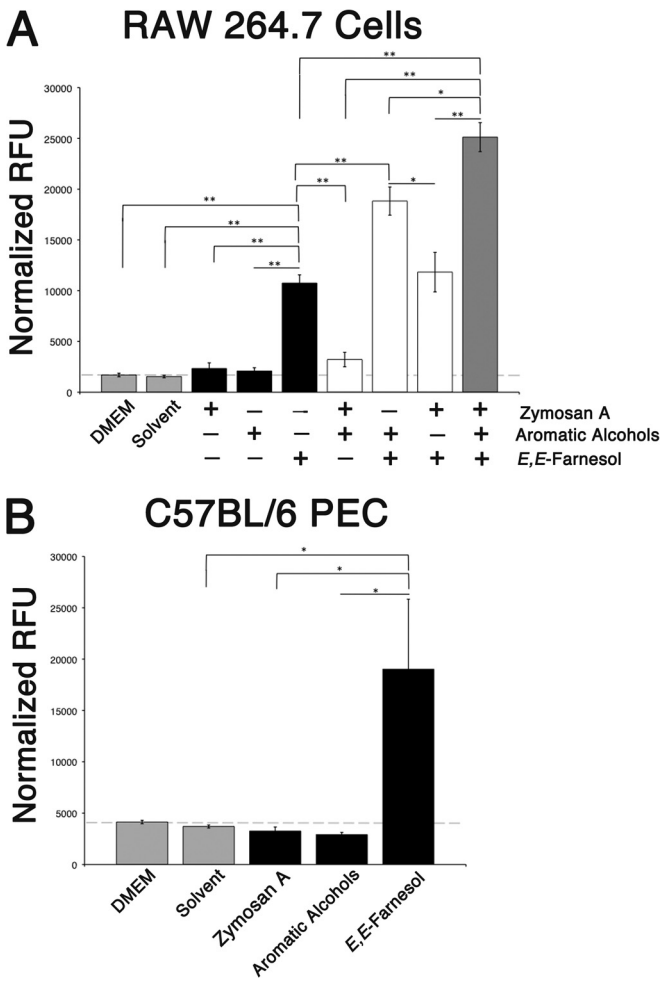

FIG 2 Zymosan A and aromatic alcohols do not stimulate macrophage migration individually, but aromatic alcohols with or without zymosan A enhance farnesol-mediated migration of mouse phagocytes. The results from a 1-h migration of cells from the mouse macrophage cell line, RAW 264.7 (A), or primary C57BL/6 mouse PECs (B) toward 20 μM farnesol, yeast cell wall components (25 μg of zymosan A/ml), and the aromatic alcohols tyrosol, tryptophol, and phenethyl alcohol (1 μM each) as measured by normalized relative fluorescent units (RFU), are shown. Zymosan A (25 mg/ml) in PBS and 100 mM aromatic alcohols in methanol (prepared fresh for each use) were diluted into DMEM. Then, 1% methanol in DMEM was used as a negative control. Although methanol is toxic to macrophages, the macrophages and C57BL/6 PECs remained viable in up to 1% methanol during the time frame of this experiment. Graphs show the means ± the SEM for at least four replicate wells and are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.001$).

combinations of all three signals (zymosan A, aromatic alcohols, and farnesol) were 2.0- and 1.3-fold better than farnesol alone or in combination with the aromatic alcohols, respectively (Fig. 2A). In summary, our results show that zymosan A and the aromatic alcohols do not stimulate the migration of phagocytes on their own, but they do enhance the migration stimulated by farnesol.

Farnesol stimulates the migration of macrophages into the peritoneum *in vivo*. The results thus far demonstrate that farnesol has chemokinetic activity toward macrophages *in vitro*. Therefore, we wondered whether farnesol also elicits macrophage migration *in vivo*. Accordingly, B10.s mice were injected i.p. with 1 ml of 20 mM farnesol in 0.5% Tween 80-PBS and 3 days later PECs were collected and analyzed by flow cytometry (Fig. 3A, middle). This farnesol concentration and delivery vehicle were chosen to replicate our previous work in a mouse model of systemic candidiasis, which showed that farnesol is a virulence factor (3). Separate cohorts of mice were injected with either 1 ml of 0.5% Tween 80-PBS or 2 ml of sterile thioglycolate broth to serve as negative and positive controls, respectively (Fig. 3A, left and right). Macrophages and dendritic cells in the PEC populations were quantified by staining with fluorescent antibodies to CD11b and CD11c (Fig. 3). Mice injected with farnesol had a nearly 3-fold increase in the percentage of PECs that were $CD11b^{hi}$ cells (indicative of macrophages) compared to Tween 80-PBS alone (Fig. 3B). This increase with farnesol was ~1.6-fold greater than that which occurred with the thioglycolate positive control (Fig. 3B). In addition, the absolute number of $CD11b^{hi}$ cells recovered from the PECs of farnesol-treated mice was 60-fold greater than that with Tween 80-PBS alone (Fig. 3C).

In contrast to farnesol stimulating macrophage influx into the peritonea of mice, mice injected with the vehicle control had a 1.5-fold increase in the percentage of $CD11c^{hi}/CD11b^{lo}$ PECs (indicative of dendritic cells) compared to farnesol (Fig. 3D). This decrease in farnesol recruitment of dendritic cells was also seen relative to thioglycolate-injected mice. Thioglycolate-treated mice showed an ~4.5-fold greater percentage of $CD11c^{hi}/CD11b^{lo}$ PECs than the farnesol-treated mice (Fig. 3D) and a nearly 6-fold increase in the absolute number of $CD11c^{hi}/CD11b^{lo}$ cells in the peritoneum compared to farnesol-treated mice (Fig. 3E). Therefore, farnesol is a significant stimulator of macrophage migration, but not dendritic cell migration *in vivo*.

DISCUSSION

In this study, we show that farnesol, which is secreted by *C. albicans* white cells, but not by opaque cells (26), modulates innate immune responses by directly stimulating movement of macrophages thus explaining the greater engulfment of white cells as observed by Lohse and Johnson (15). These results suggest that production of farnesol by *C. albicans* white cells contributes to virulence by stimulating macrophages. In contrast, the lack of farnesol production by opaque-phase cells leads to less macrophage stimulation and attenuated virulence. These results are also consistent with the differences in virulence seen for *C. albicans* cells that produce different levels of farnesol. For example, *C. albicans* pretreated with subinhibitory levels of fluconazole produced up to 12-fold more farnesol and were up to 8.5-fold more lethal (39), while *C. albicans* cells lacking *DPP3* produced 6-fold

TABLE 1 *C. albicans* white- and opaque-phase cells secrete significant concentrations of the aromatic alcohols phenethyl alcohol, tyrosol, and tryptophol

	Mean μg aromatic alcohol/g (dry wt) ± SEM[a]		
C. albicans WO-1 cell phase	Phenethyl alcohol	Tryptophol	Tyrosol
White	2,990 ± 803	439 ± 360	31.5 ± 16.7
Opaque	732 ± 543	198 ± 12.6	2.72 ± 2.14

[a] GC-MS analysis was performed on cell-free supernatants of *C. albicans* WO-1 white- and opaque-phase cells grown at 30°C aerobically in defined GPP medium until early stationary phase. Throughout, phenethyl alcohol, tyrosol, and tryptophol are expressed as micrograms per gram (dry weight) of fungal cells. Data are the averages from triplicate experiments.

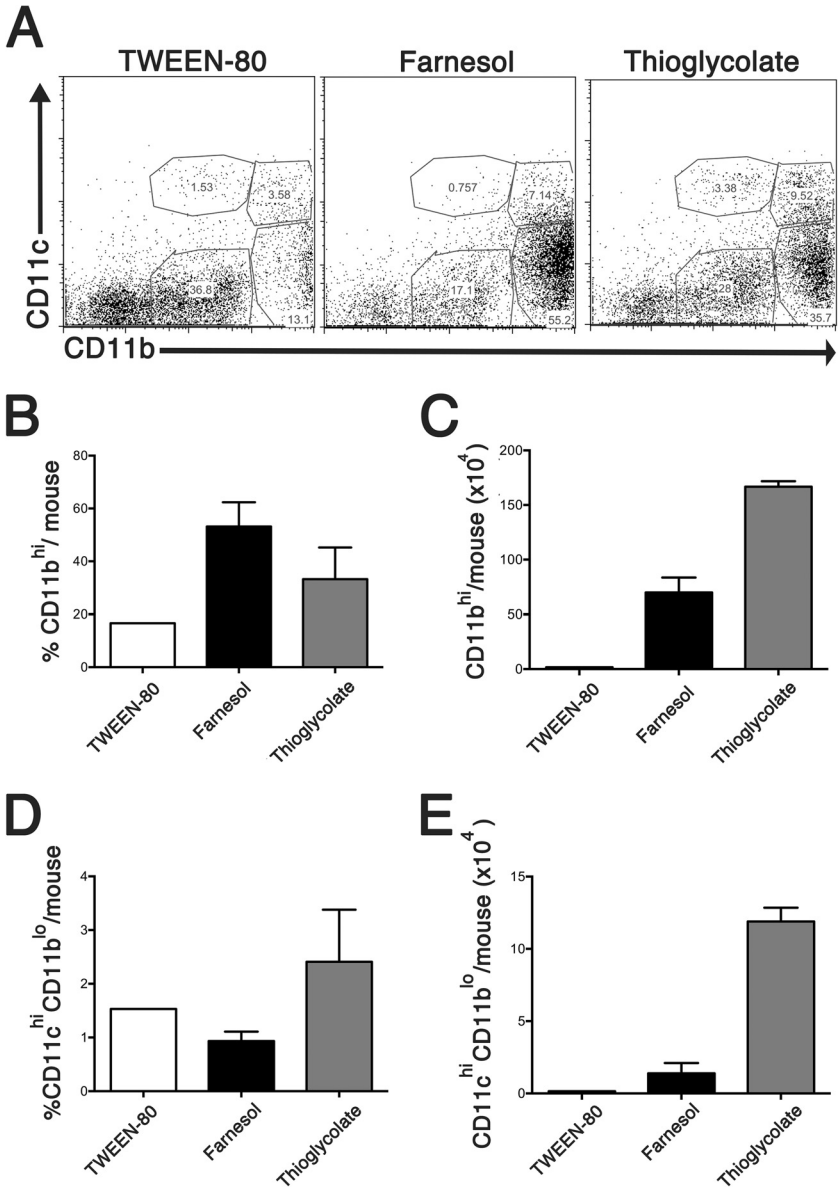

FIG 3 Farnesol promotes the peritoneal infiltration of macrophages *in vivo*. B10.s mice were injected i.p. with 1 ml of 0.5% Tween 80-PBS alone (TWEEN-80) or 1 ml of 20 mM farnesol in 0.5% Tween 80-PBS. Three days later, the PECs were harvested, and the presence of CD11b and CD11c, cell surface markers for macrophages, was quantified by fluorescent antibody staining and flow cytometry. As a positive control, mice were injected i.p. with 2 ml of thioglycolate broth, and the PECs were harvested 4 days later. Representative flow cytometric plots (A), quantification of percent $CD11b^{hi}$ cells in PECs per mouse (B), and quantification of the absolute number of $CD11b^{hi}$ cells per mouse (C) are shown. Similarly, quantification of the percent $CD11c^{hi}/CD11b^{lo}$ cells in PECs per mouse (D) and quantification of the absolute number of $CD11c^{hi}/CD11b^{lo}$ cells per mouse (E) are shown. These results are representative of three independent experiments.

less farnesol and were 4.2-fold less pathogenic (3). Further, *C. albicans* ATCC 10231, a natural strain that produces farnesoic acid, but not farnesol is 10-fold less pathogenic (3). These results are significant because the ineffectiveness of macrophages in killing phagocytosed *C. albicans* helps to explain why farnesol is a virulence factor even though *in vitro* it also prevents yeast-filament morphogenesis, an important virulence trait.

Our observation that farnesol stimulates macrophages to migrate is consistent with work showing that farnesol affects aspects of host immune responses differently (21, 22). Recently, Leonhardt et al. (22) showed that neutrophils, which are more capable of killing *C. albicans* (19, 20), are only weakly stimulated by farnesol at physiologically relevant concentrations (50 to 100 μM). Similarly, monocytes are stimulated by farnesol to produce proinflammatory cytokines (21, 22). In contrast, the differentiation of monocytes into dendritic cells and their subsequent migrational behavior are impaired by farnesol (22). Taken together, these observations suggest in part that farnesol acts as a virulence factor by dampening the adaptive immune response while stimulating the innate immune response. This distinction is important for two reasons. First, we and others have shown that *C. albicans* cells that are phagocytosed by macrophages respond by forming hyphae

that penetrate the macrophage plasma membrane, thus killing the macrophages 3 to 6 h after ingestion and thereby permitting their escape (16–18). Significantly, this escape may occur at a location in the host far distant from where the *C. albicans* was phagocytosed. Second, suppression of the dendritic cell activity with regard to antigen presentation to T cells of the adaptive arm of the immune system suggests that the host can only develop an inefficient adaptive response to *Candida*. Thus, farnesol can modulate the host immune response to favor its survival and dissemination within its host.

We speculate that *C. albicans*, through control of the white-opaque switch, evolved to stimulate macrophages for dissemination through the host. This idea suggests that the first step in the "Trojan horse strategy" for dissemination of some bacterial and viral pathogens (40, 41) is also used by *C. albicans* (19, 42). In terms of the overall balance between host and pathogen, it is highly relevant that prior to their death, macrophages that ingest *C. albicans* secrete immunoregulatory cytokines, including IL-6, IL-1β, IL-10, and TNF-α (21), presumably in order to promote the development and chemotactic migration of other, more capable members of the innate immune system, such as neutrophils (19, 20). This idea is consistent with the importance of macrophages in early elimination of *C. albicans* (43, 44), as well as the predisposition to invasive candidiasis of patients with neutropenia (1). We have not yet determined whether farnesol is a chemoattractive stimulant for neutrophils to the same degree as it is for macrophages.

The roles of phagocytic cells in controlling candidiasis in healthy and immunocompromised individuals are not well understood. However, information about small signaling molecules, such as farnesol, possibly secreted by the gut microbiota may be helpful to tease apart these differences. In immunocompromised hosts secretion of farnesol may promote *C. albicans* dissemination through macrophages as described above. This model could explain in part the high level (70%) of systemic candidiasis in immunodeficient small bowel transplant recipients (45, 46). This scenario is potentially even more profound in patients with neutropenia treated with classes of antifungal drugs that enhance the production of farnesol by *C. albicans* (47–49). In contrast, in immunocompetent hosts, farnesol detection may serve a critical surveillance function for the phagocytes in controlling fungal dissemination. Thus, through the modulation of QSM expression, *C. albicans* has the potential to directly control its fate within the host.

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REFERENCES

1. **Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang CH, Webster KM.** 2009. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin Infect Dis **48:**1695–1703. http://dx.doi.org/10.1086/599039.
2. **Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, Nickerson KW.** 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl Environ Microbiol **67:**2982–2992. http://dx.doi.org/10.1128/AEM.67.7.2982-2992.2001.
3. **Navarathna DH, Hornby JM, Krishnan N, Parkhurst A, Duhamel GE, Nickerson KW.** 2007. Effect of farnesol on a mouse model of systemic candidiasis, determined by use of a *DPP3* knockout mutant of *Candida albicans*. Infect Immun **75:**1609–1618. http://dx.doi.org/10.1128/IAI.01182-06.
4. **Sanchez de Medina F, Ortega-Gonzalez M, Gonzalez-Perez R, Capitan-Canadas F, Martinez-Augustin O.** 2013. Host-microbe interactions: the difficult yet peaceful coexistence of the microbiota and the intestinal mucosa. Br J Nutr **109**(Suppl 2)**:**S12–S20. http://dx.doi.org/10.1017/S0007114512004035.
5. **Cheng SC, Joosten LA, Kullberg BJ, Netea MG.** 2012. Interplay between *Candida albicans* and the mammalian innate host defense. Infect Immun **80:**1304–1313. http://dx.doi.org/10.1128/IAI.06146-11.
6. **Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, Odds FC, Hube B.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. Mol Microbiol **56:**397–415. http://dx.doi.org/10.1111/j.1365-2958.2005.04557.x.
7. **Lilic D.** 2012. Unravelling fungal immunity through primary immune deficiencies. Curr Opin Microbiol **15:**420–426. http://dx.doi.org/10.1016/j.mib.2012.06.003.
8. **Martino P, Girmenia C, Venditti M, Micozzi A, Santilli S, Burgio VL, Mandelli F.** 1989. *Candida* colonization and systemic infection in neutropenic patients: a retrospective study. Cancer **64:**2030–2034.
9. **Urban CF, Reichard U, Brinkmann V, Zychlinsky A.** 2006. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell Microbiol **8:**668–676. http://dx.doi.org/10.1111/j.1462-5822.2005.00659.x.
10. **Seider K, Heyken A, Luttich A, Miramon P, Hube B.** 2010. Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. Curr Opin Microbiol **13:**392–400. http://dx.doi.org/10.1016/j.mib.2010.05.001.
11. **Lockhart SR, Pujol C, Daniels KJ, Miller MG, Johnson AD, Pfaller MA, Soll DR.** 2002. In *Candida albicans*, white-opaque switchers are homozygous for mating type. Genetics **162:**737–745.
12. **Miller MG, Johnson AD.** 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. Cell **110:**293–302. http://dx.doi.org/10.1016/S0092-8674(02)00837-1.
13. **Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR.** 1987. "White-opaque transition": a second high-frequency switching system in *Candida albicans*. J Bacteriol **169:**189–197.
14. **Geiger J, Wessels D, Lockhart SR, Soll DR.** 2004. Release of a potent polymorphonuclear leukocyte chemoattractant is regulated by white-opaque switching in *Candida albicans*. Infect Immun **72:**667–677. http://dx.doi.org/10.1128/IAI.72.2.667-677.2004.
15. **Lohse MB, Johnson AD.** 2008. Differential phagocytosis of white versus opaque *Candida albicans* by *Drosophila* and mouse phagocytes. PLoS One **3:**e1473. http://dx.doi.org/10.1371/journal.pone.0001473.
16. **Ghosh S, Navarathna DH, Roberts DD, Cooper JT, Atkin AL, Petro TM, Nickerson KW.** 2009. Arginine-induced germ tube formation in *Candida albicans* is essential for escape from murine macrophage line RAW 264.7. Infect Immun **77:**1596–1605. http://dx.doi.org/10.1128/IAI.01452-08.
17. **Kolotila MP, Diamond RD.** 1990. Effects of neutrophils and in vitro oxidants on survival and phenotypic switching of *Candida albicans* WO-1. Infect Immun **58:**1174–1179.
18. **Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR.** 1997. Nonfilamentous *Candida albicans* mutants are avirulent. Cell **90:**939–949. http://dx.doi.org/10.1016/S0092-8674(00)80358-X.
19. **Ermert D, Niemiec MJ, Rohm M, Glenthoj A, Borregaard N, Urban CF.** 2013. *Candida albicans* escapes from mouse neutrophils. J Leukoc Biol **94:**223–236. http://dx.doi.org/10.1189/jlb.0213063.
20. **Rudkin FM, Bain JM, Walls C, Lewis LE, Gow NA, Erwig LP.** 2013. Altered dynamics of *Candida albicans* phagocytosis by macrophages and PMNs when both phagocyte subsets are present. mBio **4:**e00810-13. http://dx.doi.org/10.1128/mBio.00810-13.
21. **Ghosh S, Howe N, Volk K, Tati S, Nickerson KW, Petro TM.** 2010.

Candida albicans cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. FEMS Immunol Med Microbiol **60:**63–73. http://dx.doi.org/10.1111/j.1574-695X.2010.00717.x.

22. **Leonhardt I, Spielberg S, Weber M, Albrecht-Eckardt D, Blass M, Claus R, Barz D, Scherlach K, Hertweck C, Loffler J, Hunniger K, Kurzai O.** 2015. The fungal quorum-sensing molecule farnesol activates innate immune cells but suppresses cellular adaptive immunity. mBio **6:**e00143. http://dx.doi.org/10.1128/mBio.00143-15.
23. **Brown GD.** 2011. Innate antifungal immunity: the key role of phagocytes. Annu Rev Immunol **29:**1–21. http://dx.doi.org/10.1146/annurev-immunol-030409-101229.
24. **Collette JR, Lorenz MC.** 2011. Mechanisms of immune evasion in fungal pathogens. Curr Opin Microbiol **14:**668–675. http://dx.doi.org/10.1016/j.mib.2011.09.007.
25. **Jimenez-Lopez C, Lorenz MC.** 2013. Fungal immune evasion in a model host-pathogen interaction: *Candida albicans* versus macrophages. PLoS Pathog **9:**e1003741. http://dx.doi.org/10.1371/journal.ppat.1003741.
26. **Dumitru R, Navarathna DH, Semighini CP, Elowsky CG, Dumitru RV, Dignard D, Whiteway M, Atkin AL, Nickerson KW.** 2007. *In vivo* and *in vitro* anaerobic mating in *Candida albicans*. Eukaryot Cell **6:**465–472. http://dx.doi.org/10.1128/EC.00316-06.
27. **Sherman F.** 2002. Getting started with yeast. Methods Enzymol **350:**3–41. http://dx.doi.org/10.1016/S0076-6879(02)50954-X.
28. **Kebaara BW, Langford ML, Navarathna DH, Dumitru R, Nickerson KW, Atkin AL.** 2008. *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous-growth induction. Eukaryot Cell **7:**980–987. http://dx.doi.org/10.1128/EC.00357-07.
29. **Kulkarni RK, Nickerson KW.** 1981. Nutritional control of dimorphism in *Ceratocystis ulmi.* Exp Mycol **5:**148–154. http://dx.doi.org/10.1016/0147-5975(81)90015-3.
30. **Carr MW, Roth SJ, Luther E, Rose SS, Springer TA.** 1994. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. Proc Natl Acad Sci U S A **91:**3652–3656. http://dx.doi.org/10.1073/pnas.91.9.3652.
31. **Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, Rollins BJ.** 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. J Exp Med **187:**601–608. http://dx.doi.org/10.1084/jem.187.4.601.
32. **Weber K, Sohr R, Schulz B, Fleischhacker M, Ruhnke M.** 2008. Secretion of *E,E*-farnesol and biofilm formation in eight different *Candida* species. Antimicrob Agents Chemother **52:**1859–1861. http://dx.doi.org/10.1128/AAC.01646-07.
33. **Chen H, Fujita M, Feng Q, Clardy J, Fink GR.** 2004. Tyrosol is a quorum-sensing molecule in *Candida albicans*. Proc Natl Acad Sci U S A **101:**5048–5052. http://dx.doi.org/10.1073/pnas.0401416101.
34. **Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, Kasprowicz DJ, Kellar K, Pare J, van Dyke T, Ziegler S, Unutmaz D, Pulendran B.** 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. J Clin Invest **116:**916–928. http://dx.doi.org/10.1172/JCI27203.
35. **Ghosh S, Kebaara BW, Atkin AL, Nickerson KW.** 2008. Regulation of aromatic alcohol production in *Candida albicans*. Appl Environ Microbiol **74:**7211–7218. http://dx.doi.org/10.1128/AEM.01614-08.
36. **Brown GD, Gordon S.** 2001. Immune recognition: a new receptor for beta-glucans. Nature **413:**36–37. http://dx.doi.org/10.1038/35092620, http://dx.doi.org/10.1038/35092617.
37. **Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg BJ, Wijmenga C, Joosten LA, Xavier RJ, van der Meer JW, Stunnenberg HG, Netea MG.** 2012. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe **12:**223–232. http://dx.doi.org/10.1016/j.chom.2012.06.006.
38. **Albuquerque P, Casadevall A.** 2012. Quorum sensing in fungi: a review. Med Mycol **50:**337–345. http://dx.doi.org/10.3109/13693786.2011.652201.
39. **Navarathna DH, Hornby JM, Hoerrmann N, Parkhurst AM, Duhamel GE, Nickerson KW.** 2005. Enhanced pathogenicity of *Candida albicans* pretreated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis. J Antimicrob Chemother **56:**1156–1159. http://dx.doi.org/10.1093/jac/dki383.
40. **Garzoni C, Kelley WL.** 2011. Return of the Trojan horse: intracellular phenotype switching and immune evasion by *Staphylococcus aureus*. EMBO Mol Med **3:**115–117. http://dx.doi.org/10.1002/emmm.201100123.
41. **Sorgeloos F, Jha BK, Silverman RH, Michiels T.** 2013. Evasion of antiviral innate immunity by Theiler's virus L* protein through direct inhibition of RNase L. PLoS Pathog **9:**e1003474. http://dx.doi.org/10.1371/journal.ppat.1003474.
42. **Sasse C, Hasenberg M, Weyler M, Gunzer M, Morschhauser J.** 2013. White-opaque switching of *Candida albicans* allows immune evasion in an environment-dependent fashion. Eukaryot Cell **12:**50–58. http://dx.doi.org/10.1128/EC.00266-12.
43. **Lionakis MS, Swamydas M, Fischer BG, Plantinga TS, Johnson MD, Jaeger M, Green NM, Masedunskas A, Weigert R, Mikelis C, Wan W, Lee CC, Lim JK, Rivollier A, Yang JC, Laird GM, Wheeler RT, Alexander BD, Perfect JR, Gao JL, Kullberg BJ, Netea MG, Murphy PM.** 2013. CX3CR1-dependent renal macrophage survival promotes *Candida* control and host survival. J Clin Invest **123:**5035–5051. http://dx.doi.org/10.1172/JCI71307.
44. **Qian Q, Jutila MA, Van Rooijen N, Cutler JE.** 1994. Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. J Immunol **152:**5000–5008.
45. **Florescu DF, Islam KM, Grant W, Mercer DF, Langnas A, Botha J, Nielsen B, Kalil AC.** 2010. Incidence and outcome of fungal infections in pediatric small bowel transplant recipients. Transpl Infect Dis **12:**497–504. http://dx.doi.org/10.1111/j.1399-3062.2010.00542.x.
46. **Florescu DF, Qiu F, Mercer DF, Langnas AN, Shafer LR, Kalil AC.** 2012. Risk factors for systemic *Candida* infections in pediatric small bowel transplant recipients. Pediatr Infect Dis J **31:**120–123. http://dx.doi.org/10.1097/INF.0b013e31823a66d5.
47. **Hornby JM, Kebaara BW, Nickerson KW.** 2003. Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. Antimicrob Agents Chemother **47:**2366–2369. http://dx.doi.org/10.1128/AAC.47.7.2366-2369.2003.
48. **Hornby JM, Nickerson KW.** 2004. Enhanced production of farnesol by *Candida albicans* treated with four azoles. Antimicrob Agents Chemother **48:**2305–2307. http://dx.doi.org/10.1128/AAC.48.6.2305-2307.2004.
49. **Zhang L, Chang W, Sun B, Groh M, Speicher A, Lou H.** 2011. Bisbibenzyls, a new type of antifungal agent, inhibit morphogenesis switch and biofilm formation through upregulation of *DPP3* in *Candida albicans*. PLoS One **6:**e28953. http://dx.doi.org/10.1371/journal.pone.0028953.