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Suman Ghosh
University of Nebraska - Lincoln

Nina Howe
University of Nebraska Medical Center

Katie Volk
University of Nebraska Medical Center

Swetha Tati
University of Nebraska - Lincoln

Kenneth W. Nickerson
University of Nebraska - Lincoln, knickerson1@unl.edu

See next page for additional authors

RESEARCH ARTICLE

Candida albicans cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line

Suman Ghosh[1], Nina Howe[2], Katie Volk[2], Swetha Tati[1], Kenneth W. Nickerson[1], Thomas M. Petro[3]

[1]School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE, USA; [2]Department of Dental Hygiene, University of Nebraska Medical Center, Lincoln, NE, USA; and [3]Department of Oral Biology, University of Nebraska Medical Center, Lincoln, NE, USA

Correspondence: Thomas M. Petro, Department of Oral Biology, University of Nebraska Medical Center, Lincoln, NE 68583-0740, USA. Tel.: +1 402 472 1327; fax: +1 402 472 2551; e-mail: tpetro@unmc.edu

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Abstract

Candida albicans causes candidiasis, secretes farnesol, and switches from yeast to hyphae to escape from macrophages after phagocytosis. However, before escape, macrophages may respond to *C. albicans*' pathogen-associated molecular patterns (PAMPs) through toll-like receptor 2 (TLR2) and dectin-1 receptors by expressing cytokines involved in adaptive immunity, inflammation, and immune regulation. Therefore, macrophages and the RAW264.7 macrophage line were challenged with *C. albicans* preparations of live wild-type cells, heat-killed cells, a live mutant defective in hyphae formation, a live mutant producing less farnesol, or an isolate producing farnesoic acid instead of farnesol. Interleukin-6 (IL-6), and IL-1β, IL-10, and tumor necrosis factor-α (TNF-α) expression were evaluated by ELISA and/or qRT-PCR within 6 h after challenge. All viable strains producing farnesol, regardless of hyphae phenotype, induced IL-6, IL-1β, IL-10, and TNF-α. To determine which components of *C. albicans* induced IL-6, RAW264.7 cells were incubated with farnesol, farnesoic acid, with or without zymosan, a yeast cell wall preparation that contains PAMPs recognized by TLR2 and dectin-1. The highest expression of IL-6, TLR2, and dectin-1 occurred when RAW264.7 cells were stimulated with zymosan and farnesol together. Our results suggest that the rapid expression of cytokines from macrophages challenged with *C. albicans* is due to cell-wall PAMPs combined with farnesol.

Introduction

Candida albicans is a commensal fungus that colonizes the human oral cavity and intestine. It is polymorphic in that it converts between yeast, hyphal, and pseudohyphal forms (Berman, 2006). Individuals with healthy immune systems limit *Candida* growth at mucosal sites. In contrast, a compromised immune system often leads to mucocandidiasis, oral thrush, or systemic candidiasis, with significant mortality (Klein *et al.*, 1984; Launay *et al.*, 1998; Pfaller & Diekema, 2007). Key virulence factors leading to mucosal or systemic candidiasis include the following: (1) cell morphogenesis, i.e. yeast to hyphae switching; (2) phenotypic switching, for example white to opaque switching; (3) epithelial adhesion; (4) extracellular enzymes, for example phospholipase B and aspartyl proteases; and (5) production of farnesol (Navarathna *et al.*, 2005, 2007a, b). Farnesol was first identified as a quorum-sensing molecule (QSM) in that it blocked the yeast to hyphae conversion by *C. albicans* (Hornby *et al.*, 2001). Later, we showed that farnesol also acted as a virulence factor (Navarathna *et al.*, 2005, 2007a, b). At that time, we created a knockout mutation in *DPP3*, the gene encoding one of the two phosphatases that converts farnesyl pyrophosphate to farnesol. This mutant KWN2 (*dpp3*/*dpp3*), which had only one of the phosphatases, produced six times less farnesol and was *c.* 4.2 times less pathogenic to mice than its parent (Navarathna *et al.*, 2007a b). We also created the *DPP3* reconstituted strain (KWN4) such that it regained the mutated farnesyl pyrophosphatases, expressed both farnesyl pyrophosphatases, and as a result regained complete farnesol production and pathogenicity (Navarathna *et al.*, 2007a).

Since that time, we have been interested in farnesol's mode of action as a virulence factor. This mode of action

appears to be distinct from farnesol's mode of action as a QSM, i.e. blocking hyphal development. As a step toward deciphering farnesol's mode of action, we showed that blood from mice pretreated with farnesol had significantly reduced levels of both the critical Th1 cytokine interferon-γ (IFN-γ) and the Th1-inducing cytokine interleukin-12 (IL-12), accompanied by elevated Th2 cytokine IL-5 (Navarathna *et al.*, 2007a, b).

In addition to farnesol production, *C. albicans* conversion from yeast to hyphal forms is also a virulence factor. We have shown that following ingestion by macrophages, wild-type *C. albicans* turns on arginine biosynthesis, metabolizes that arginine via arginase (*CAR1*) and urea amidolyase (*DUR1,2*) to activate the yeast to hyphae switch, and escapes from the macrophage (Ghosh *et al.*, 2009). Wild-type *C. albicans* escaped within 4–6 h, whereas both arginine auxotrophs and *dur1,2*/*dur1,2* mutants were unable to form hyphae or escape (Ghosh *et al.*, 2009). A critical unanswered question concerns the extent to which macrophages are able to produce cytokines before they are killed.

Macrophages are known to produce cytokines that promote inflammation, others that direct $CD4^+$ T cell differentiation to a phenotype that promotes effective immunity to *C. albicans*, and others that regulate inflammation and adaptive immunity. Macrophage IL-1β and tumor necrosis factor-α (TNF-α) promote inflammation by increasing chemokine production and the expression of endothelial cell adhesion molecules. Macrophage IL-12 directs T cell differentiation to the Th1 subset that produces IFN-γ, which in turn activates macrophages (Nathan *et al.*, 1983). We have shown previously that farnesol decreases the expression of IL-12 from macrophages (Navarathna *et al.*, 2007b). Alternately, macrophage IL-6 is pivotally involved in the development of the Th17 (Bettelli *et al.*, 2006) subset, which produces IL-17 that is required for resistance against mucosal (Conti *et al.*, 2009) and systemic candidiasis (Huang *et al.*, 2004). The production of IL-17 leads to the accumulation of neutrophils (Huang *et al.*, 2004), which effectively phagocytize and kill both yeast and hyphal versions of *C. albicans* (Rubin-Bejerano *et al.*, 2003). However, IL-6 is also involved in systemic inflammation and may play a role in pathogenesis when produced in excessive amounts (Cuzzocrea *et al.*, 1999). Therefore, *C. albicans* induction of IL-6 from macrophages plays a role in the development of both innate and adaptive immunity, as well as in its systemic virulence. However, it is still unclear whether macrophages that have phagocytized *C. albicans* express IL-6 before their death.

To mediate cytokine responses to *C. albicans*, monocytes, macrophages, and dendritic cells express 11 leucine-rich toll-like receptors (TLRs) and c-type lectins such as dectin-1, which recognize distinctive pathogen-associated molecular patterns (PAMPs) (Hume *et al.*, 2001; Jones *et al.*, 2001; Netea *et al.*, 2008). Cell surface TLR2 responds to *C. albicans* whole cells, zymosan (an *Saccharomyces cerevisiae* cell-wall-derived preparation that contains β-glucans as a major component, and also mannan and chitin), as well as to phospholipomannans of *C. albicans* (Jouault *et al.*, 2003), while TLR4 responds to *C. albicans* α-mannans (Tada *et al.*, 2002). Indeed, TLR2 is essential for resistance against systemic infection against *C. albicans* (Villamon *et al.*, 2004). However, TLR2 is not alone in its response to zymosan and *C. albicans* cells as dectin-1 binds to β-glucan (Brown *et al.*, 2002) and associates with TLR2 to provide a collaborative response to *C. albicans* (Goodridge *et al.*, 2007). Therefore, TLR2 and dectin-1 are key macrophage cell surface molecules for responses to *C. albicans*.

The present paper takes a further step in elucidating farnesol's mode of action. These experiments are carried out with the same *C. albicans*/murine macrophage system that we used to show the importance of arginine biosynthesis by *C. albicans* (Ghosh *et al.*, 2009). We have adopted a genetic and molecular approach to examine whether postphagocytic hyphae formation and/or farnesol production influence the ability of macrophages to express IL-6 and other cytokines. In this report, we used five strains of *C. albicans* that are defective in hyphae formation or farnesol production to determine the impact of these fungal virulence factors on the postphagocytic induction of IL-6, as well as IL-1β, IL-10, and TNF-α. We show that following phagocytosis of *C. albicans* by macrophages, the expression of IL-6, IL-1β, TNF-α, and IL-10 was rapidly induced regardless of whether those cells were capable of hyphae formation or maximal farnesol production. Using farnesol, farnesoic acid, and zymosan, alone or in combination, we found that farnesol and zymosan act synergistically to induce IL-6 from macrophages.

Materials and methods

Strains, media, growth conditions, and chemicals

The *C. albicans* wild-type SC5314, which can form germ tubes and produce farnesol, and strain SN152 (*URA3/ura3*::*imm434 his1/his1 arg4/arg4 leu2/leu2 IRO1/iro1*::*imm436*) (Noble & Johnson, 2005), which is defective in germ tube formation (GTF), but can induce farnesol, were obtained from Dr Alexander Johnson, University of California at San Francisco. *Candida albicans* 10231, which is a clinical isolate that forms germ tubes, but produces farnesoic acid instead of farnesol, was obtained from the American Type Culture Collection (Rockville, MD). The construction of KWN2 (*dpp3*::*C.d.HIS1*/*dpp3*::*C.m.LEU2*, *his1/his1, leu2/leu2, arg4/arg4*), which produces low farnesol, and KWN4 (*dpp3*::*DPP3*/*dpp3*::*DPP3*, *his1/his1, leu2/leu2, arg4/arg4*), which produces normal farnesol, from the parental strain SN152 was described previously (Navarathna *et al.*, 2007a, b). Thus, SN152 and KWN4 do not produce germ tubes, but do produce farnesol,

while KWN2 does not produce germ tubes and produces low farnesol. Otherwise, SN152, KWN2, and the reconstructed KWN4 are isogenic strains.

Candida albicans strains were grown and maintained in YPD medium (10 g of yeast extract, 5 g of peptone and 20 g of glucose per liter) at 30 °C, while auxotrophic mutants were grown in YPD supplemented with 40 μg mL^{-1} of required amino acid. Resting-phase cells were grown overnight in YPD at 30 °C, washed three times with 50 mM potassium phosphate buffer, and stored in the same buffer. These cells were used for co-culture with murine macrophage RAW264.7 line. Heat-killed (HK) cells were prepared by heating the resting-phase cells at 60 °C for 2 h. This temperature regime was chosen to ensure that the *C. albicans* cells were still intact particles, suitable for phagocytosis. Cell death was confirmed by spreading the HK cells on YPD plates; there was no growth following incubation at 30 °C for 24 h (data not shown). The murine RAW264.7 macrophage-like cell line was grown in a complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) plus 50 μg mL^{-1} gentamicin at 37 °C in the presence of 5% CO_2.

The *trans*, *trans*-farnesol was purchased from Sigma-Aldrich Chemicals, St. Louis, MO, (E, E)farnesoic acid from Echelon Biosciences Inc., Salt Lake City, UT, and zymosan from InVivogen, San Diego, CA. Farnesol and farnesoic acid were dissolved in methanol while zymosan was suspended in ethanol to prepare stock solutions before use.

Preparation of splenic macrophages and challenge with *C. albicans*

Spleens from C57Bl/6 mice were extracted under aseptic conditions and cells were gently dispersed using 90 mesh screens into cold cell culture media (CCM) that contained DMEM (Sigma-Aldrich Chemicals), 50 μg mL^{-1} gentamicin, and 10% FBS. The cell suspensions were centrifuged at 500 ***g*** at 4 °C for 10 min. Erythrocytes were lysed with Gey's reagent for 5 min, and then the remaining cells were washed twice with CCM. The viability and concentration of mononuclear cells were determined using trypan blue and a hemacytometer. Adherent splenic macrophages were obtained by adjusting mononuclear cells to 10^7 per 2 mL of CCM in six-well culture dishes and incubating overnight at 37 °C in 5% CO_2/air. Nonadherent cells were removed from by adding 37 °C-warmed CCM, swirling, and then aspirating away suspended cells. In 2 mL of CCM, adherent splenic macrophages were unstimulated or stimulated with 10^6 viable SN152, KWN2, or KWN4 *C. albicans* cells, based on optimal stimulation determined from preliminary assays. After 6 h of stimulation, the supernatants were collected.

Challenge of RAW264.7 cells with *C. albicans* or their components

We, and many others, have shown that the RAW264.7 cell line expresses similar levels of cytokines compared with splenic macrophages in response to several pattern-recognition receptor (PRR) agonists (Kollet *et al.*, 2001; Al-Salleeh & Petro, 2008). Therefore, RAW264.7 cells were stimulated with *C. albicans*, zymosan, or farnesol. One day before the experiment, RAW264.7 cells that reached confluence in culture media were collected, washed, and counted using a hemacytometer. Cells (10^5) per well were plated in culture media in 12-well plates and grown overnight in 5% CO_2 at 37 °C to allow adherence to the surface. On day 0, the nonadherent cells were removed from the plates by aspiration and a fresh prewarmed complete culture medium was added. Resting-phase *C. albicans* cells were washed with phosphate-buffered saline (PBS) and their concentrations were measured using a Spectronic 20 spectrophotometer. Cells (4×10^5) were added to each well (4 : 1 *C. albicans* : macrophage ratio based on optimal stimulation from preliminary assays), and the plates were incubated for 1, 2, or 3 h in 5% CO_2 at 37 °C. For the challenge of RAW264.7 cells with various *C. albicans* components, cells were treated with: (a) 5 μM of farnesol; (b) 250 μM of farnesoic acid; (c) 25 μg mL^{-1} of zymosan; (d) 5 μM of farnesol and 25 μg mL^{-1} of zymosan or in one experiment 0.1–20 μM of farnesol and 25 μg mL^{-1} of zymosan; (e) 250 μM of farnesoic acid and 25 μg mL^{-1} of zymosan; (f) 5 μM of farnesol and 4×10^5 HK SC5314 cells; or (g) 250 μM of farnesoic acid and 4×10^5 HK SC5314 cells. After 1, 2, and 3 h, the supernatants were removed for enzyme-linked immunosorbent assay (ELISA), 400 μL of lysing buffer (5Prime PerfectPure RNA Cell and Tissue, RNA isolation kit) was added to the cells, and the sample was transferred into tubes and frozen at − 80 °C.

RNA isolation, cDNA, and reverse transcriptase (RT)-PCR

RNA was extracted using the PerfectPure RNA isolation kit of 5Prime Inc. (Gaithersburg, MD) according to the manufacturer's instructions. cDNAs were prepared from 0.3 μg of RNA. Quantitative real-time RT-PCR (qRT-PCR) was carried out in the presence of 1 mM dNTPs, 2 μM $MgCl_2$, 2 U Taq DNA polymerase, 1 μM of each primer, and 1 μL of cDNA in 25 μL. For IL-6, IL-1β, TNF-α, IL-10, dectin-1, TLR2, and GAPDH, qRT-PCR was performed using the Platinum-SYBR Green I-UDG-quantitative PCR SuperMix (Invitrogen) and the following primers: IL-6, 5′-ATGAAGTTCCTCTCTG CAAGAGACT-3′/5′-CACTAGGTTTGCCGAGTAGATCTC-3′ (638 bp); IL-1β, 5′-AACTGTTCCTGAACTCAACTGT-3′/5′-GAGATTTGAAGCTGGATGCTCT-3′ (150 bp); TNF-α, 5′-CA TGATCCGCGACGTGGAACTG-3′/5′-AGAGGGAGGCCATT TGGGAACT-3′ (195 bp); IL-10, 5′-ATGCAGGACTTTAAGG GTTACTTGGGTG-3′/5′-ATTTCGGAGAGAG GTACAAACG

AGGTTT-3′ (455 bp); dectin-1, 5′-AGAGAATCTGGATGAAG ATGGA-3′/5′-ACCACAAAGCACAGGATTCCTA-3′ (150 bp); TLR2, 5′-TCTAAAGTCGATCCGCGACAT-3′/5′-TACCCAGC TCGCTCACTACGT-3′ (344 bp); and GAPDH, 5′-TTGTCAG CAATGCATCCTGCAC-3′/5′-ACAGCTTTCCAGAGGGGCC ATC-3′ (149 bp). qRT-PCR reactions were run on an ABI Prism 7000 thermal cycler in which 1 μL of cDNA was incubated at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Cycle thresholds (C_t) were normalized to C_t for GAPDH for each cDNA and expressed by fold increase using the formula: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta$ = (the difference between the C_t value obtained from cytokine/PRR cDNA of stimulated samples and the C_t value obtained from GAPDH cDNA of stimulated samples) − (the difference between the average C_t of cytokine/PRR of unstimulated samples and the average C_t value obtained from GAPDH cDNA of unstimulated samples).

ELISA

Mouse IL-6 was quantified by coating 96-well assay plates with 2 μg mL^{-1} purified unconjugated anti-IL-6 monoclonal antibody (clone MP5-20F3) in bicarbonate buffer (pH 9.6) at 4 °C overnight. After two washes with PBS/0.05% Tween 20 (Sigma-Aldrich Chemicals), the plate was blocked with PBS/10% FBS. For the standard curve, serial dilutions of recombinant IL-6 (Pharmingen) were added to wells coated with respective anti-IL-6. The plate was incubated at room temperature for 2 h. After washing two times with PBS/Tween 20, the plate was incubated with 2 μg mL^{-1} biotinylated anti-IL-6 (clone MP5-32C11) in PBS/10% FBS at room temperature for 45 min. After six washes with PBS/Tween 20, the plates were incubated with dilute (1 : 1000) streptavidin peroxidase (Sigma-Aldrich Chemicals) at room temperature for 30 min. The plates were washed five times with PBS/Tween 20 and incubated with 3, 3′, 5, 5′ tetramethylbenzidine substrate/hydrogen peroxide solution and readings were taken at $OD_{450\,nm}$ wavelength with a reference of $OD_{570\,nm}$ using an ELISA spectrophotometric plate reader.

Statistical analysis

Cytokine expression measured by PCR and cytokine production measured by ELISA was analyzed using Student's *t*-test to determine the significance of differences; *P*-values of ≤ 0.05 were considered significant.

Results

Yeast–hyphae dimorphism plays no role in the induction of IL-6 by *C. albicans*

Because *C. albicans* escapes from macrophage following phagocytosis, there are few reports describing macrophage cytokine expression following *C. albicans* challenge. We decided to measure IL-6 expression by macrophages challenged with *C. albicans* and identify the cellular components in *C. albicans* that might be responsible for inducing IL-6. Splenic macrophages were challenged with live *C. albicans* SN152 (normal farnesol), KWN2 (low farnesol), and KWN4 (restored farnesol production) for 6 h and IL-6 production was measured using ELISA. This time frame was chosen because in this system wild-type *C. albicans* cells form germ tubes and escape 4–6 h after ingestion (Ghosh *et al.*, 2009). Figure 1a shows that splenic macrophages rapidly (within 6 h) produced significantly more (2–3.5 times over the background) IL-6 in response to the challenge with *C. albicans* than unchallenged splenic macrophages. Similar results were obtained with strain SN152 and the two mutants of SN152 that produce altered levels of farnesol (Fig. 1a).

We have seen that the RAW264.7 macrophage cell line in response to various TLR agonists expresses cytokines at levels equivalent to primary splenic macrophages (Kollet *et al.*, 2001; Al-Salleeh & Petro, 2008). Therefore, the response of RAW264.7 cells to five strains of *C. albicans* was explored by measuring IL-6 mRNA, by qRT-PCR, for 3 h after challenge (Fig. 1b). Wild-type *C. albicans* SC5314 rapidly induced the macrophage expression of IL-6. Note that the IL-6 scale is logarithmic and thus the expression of IL-6 mRNA increased almost 1000-fold by 3 h. This indication of enhanced IL-6 gene expression was confirmed by measurement of the actual IL-6 protein from RAW264.7 cells. The supernatants of RAW264.7 cells phagocytizing *C. albicans* SC5314 were evaluated by ELISA for secreted IL-6. At 6 h, RAW264.7 cells that had phagocytized SC5314 secreted significantly more (8.7 times) IL-6 compared with those that had not (13.07 ± 1.42 vs. 1.5 ± 0.21 pg mL^{-1}, $P = 0.001$).

Like SC5314, *C. albicans* SN152, KWN2, and KWN4, which are all arginine auxotrophs and thus defective in GTF and escape from macrophages (Ghosh *et al.*, 2009), induced ~1000-fold increases in IL-6 expression by RAW264.7 cells (Fig. 1b). Interestingly, strain 10231, which undergoes GTF, but generates farnesoic acid instead of farnesol (Oh *et al.*, 2001; Hornby & Nickerson, 2004) (*vide infra*), failed to induce IL-6 expression from RAW264.7 cells (Fig. 1b). Likewise, IL-6 expression in response to HK SC5314, which cannot produce farnesol *de novo*, was substantially less than that for live SC5314 (Fig. 1b). Similarly, SC5314, SN152, KWN2, and KWN4 induced RAW264.7 cells to produce roughly equivalent levels of IL-1β, IL-10, and TNF-α within 3 h (Fig. 2). In contrast to IL-6 expression, *C. albicans* that underwent GTF were unable to express significant levels of IL-23 and transforming growth factor-β (TGF-β), two other cytokines that contribute to Th17 development, within 3 h (data not shown), while mutant *C. albicans* that are defective in GTF induced low, but detectable IL-23 and TGF-β mRNA within 3 h (data not shown).

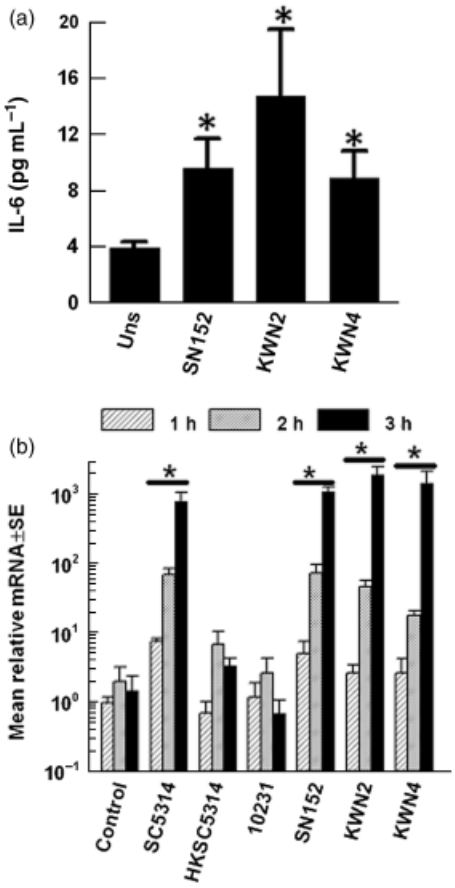

Fig. 1. Splenic macrophages (a) and RAW264.7 cells (b) express IL-6 in response to challenge by *Candida albicans*. (a) IL-6 protein production by splenic macrophages after 6 h or (b) real-time qRT-PCR of mRNA expression in RAW264.7 cells after 1, 2, and 3 h. A total of 1×10^7 splenic mononuclear cells or 1×10^5 RAW264.7 cells in culture media were seeded overnight in CCM. Adherent splenic macrophages were given PBS (Uns) or challenged with 1×10^6 SN152, KWN2, or KWN4 cells, while RAW264.7 cells were control or challenged with 4×10^5 cells of SC5314 (wild type), HK SC5314 (HKSC5314), 10231 (wild-type clinical isolate) that secretes farnesoic acid instead of farnesol, SN152 (*URA3/ura3*::*imm434 his1/his1 arg4/arg4 leu2/leu2 IRO1/iro1*::*imm436*) – defective in GTF inside macrophages, but secretes farnesol similar to the wild type; or KWN2 (*dpp3*::*C.d.HIS1/dpp3*::*C.m.LEU2*, *his1/his1, leu2/leu2, arg4/arg4*) – defective in GTF inside macrophages and secretes six times less farnesol compared with the wild type; or KWN4 (*dpp3*::*DPP3/dpp3*::*DPP3*, *his1/his1, leu2/leu2, arg4/arg4*) – defective in GTF inside macrophages and secrete two times more farnesol compared with the wild type; dissolved in PBS for 1, 2, and 3 h. (a) Bar graphs represent mean IL-6 ± SEM ($n = 5$) as measured by ELISA and (b) Bar graphs represent the mean relative induction of IL-6 mRNA ± SEM ($n = 3$) as measured by qRT-PCR of three independent experiments normalized to GAPDH expression; *Mean is significantly different from the control, $P < 0.05$.

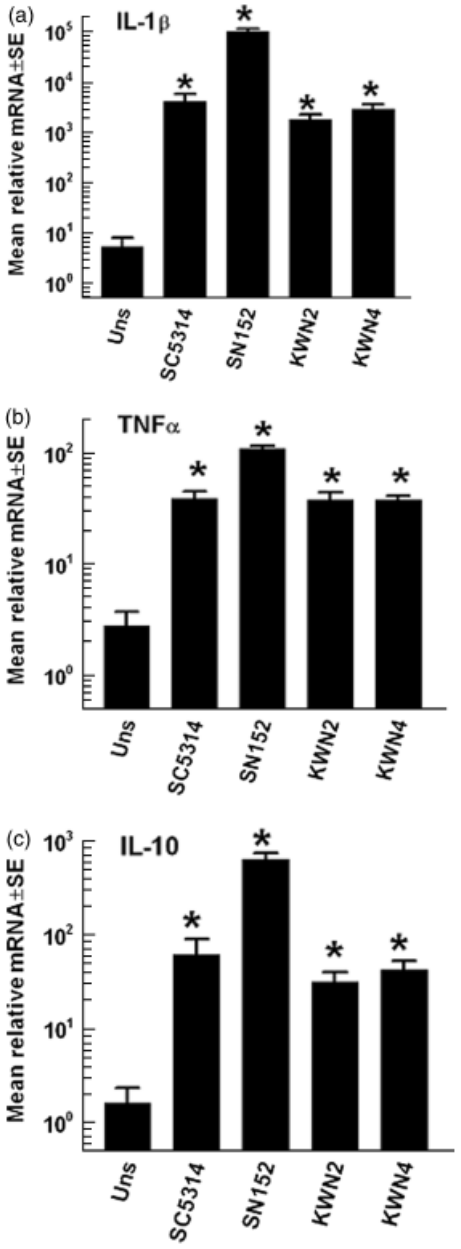

Fig. 2. RAW264.7 cells express inflammatory cytokines IL-1β (a), TNF-α (b), and immunoregulatory cytokine IL-10 (c) in response to a challenge by *Candida albicans*. Real-time qRT-PCR of IL-1β (a), TNF-α (b), and IL-10 (c) mRNA in RAW264.7 cells after 3 h. A total of 1×10^5 RAW264.7 cells in culture media were given PBS (Uns) or challenged with 4×10^5 (MOI 4 : 1) cells of SC5314 (wild type), SN152, KWN2, or KWN4 in PBS for 3 h. Bar graphs represent the mean relative induction ± SEM ($n = 4$) normalized to GAPDH; *Mean is significantly different from the control, $P < 0.05$.

Farnesol stimulates the expression of IL-6 from macrophages

Four strains of *C. albicans* were chosen to determine whether the level of farnesol production influenced cytokine production, either positively or negatively. Strain 10231 is a wild-type strain, capable of GTF and escape from macrophages (Ghosh *et al.*, 2009), but it is unusual because it produces farnesoic acid (Oh *et al.*, 2001) rather than farnesol

(Hornby & Nickerson, 2004). KWN2 is a *dpp3*/*dpp3* mutant that produces only 15% as much farnesol as its parent SN152 due to a defect in one of the pathways for the conversion of farnesyl pyrophosphate to farnesol (Navarathna *et al.*, 2007a, b). Both *DPP3* and farnesol production were reconstituted in KWN4 (Navarathna *et al.*, 2007a, b). Thus, strains SN152, KWN2, and KWN4 are isogenic and they are all derived from SC5314. Figure 1b shows that strains SN152, KWN2, and KWN4 all increased IL-6 expression *c.* 1000-fold, just like the wild-type clinical isolate SC5314. In contrast, strain 10231 and HK SC5314 elicited only background levels of IL-6 expression (Fig. 1b).

Farnesol and zymosan synergistically induce IL-6 expression

The results so far suggest that farnesol, perhaps in conjunction with another component of *C. albicans* cells, induces IL-6 expression by macrophages. Zymosan, a yeast cell wall preparation containing β-glucan, is a likely candidate for a fungal component that could synergize with farnesol to induce cytokines from macrophages. β-1,3-Glucan, a major cell wall component of zymosan, as well as *S. cerevisiae* and *C. albicans* cell walls, is known to stimulate cytokine expression by binding to dectin-1 (Ozinsky *et al.*, 2000; Brown *et al.*, 2002, 2003), a receptor that collaborates with TLR2 (Sato *et al.*, 2003) in stimulating synergistic signaling pathways. Accordingly, we next examined which combinations of zymosan with farnesol or farnesoic acid induced high levels of IL-6 expression by the RAW264.7 cell line (Fig. 3). Zymosan, farnesol, or farnesoic acid by themselves failed to stimulate the expression of IL-6 (Fig. 3). Interestingly, farnesol or farnesoic acid significantly decreased the background expression of IL-6 from RAW264.7 cells (Fig. 3). However, the combination of zymosan and farnesol induced significant expression of IL-6. This combination of two cellular components yielded levels of IL-6 mRNA expression similar to those induced by whole cells of SC5314, SN152, KWN2, or KWN4 (Fig. 1). The combination of farnesoic acid and zymosan, to some extent, also stimulated IL-6 expression, even though strain 10231 failed to do so. It is likely that strain 10231 only excretes very low levels of farnesoic acid in the first 3 h after phagocytosis. As we noted above, the 15% farnesol produced by KWN2 is apparently sufficient for the induction of IL-6 expression from macrophages. To confirm this notion, we stimulated RAW264.7 cells with zymosan plus varying farnesol concentrations. The data indicated that zymosan and farnesol at 1/50th of the original concentration induced as much IL-6 expression from RAW264.7 cells as zymosan plus the original concentration of farnesol or zymosan plus four times more farnesol (data not shown). Therefore, KWN2 produces enough farnesol for the synergistic induction of IL-6 expression from the RAW264.7 line.

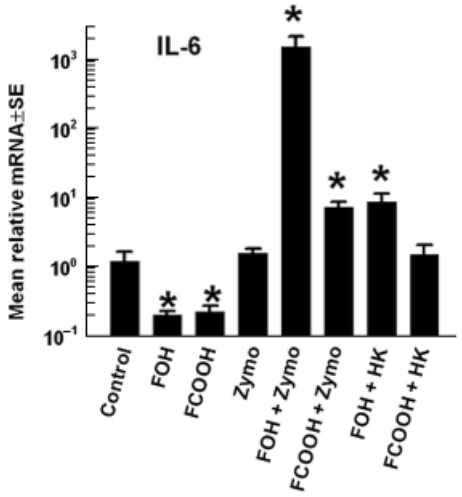

Fig. 3. RAW264.7 cytokine expression in response to the challenge by yeast cell components. Real-time qRT-PCR of IL-6 in RAW264.7 cells. A total of 1×10^5 RAW264.7 cells were incubated with 5 μL of methanol (control); farnesol (5 μM); farnesoic acid (250 μM); zymosan (25 μg mL^{-1}); farnesol (5 μM) and zymosan (25 μg mL^{-1}) together; farnesoic acid (250 μM) and zymosan (25 μg mL^{-1}) together; HK SC5314 (4×10^5) and farnesol (5 μM) together; or HK SC5314 (4×10^5) and farnesoic acid (250 μM) together for 3 h. Farnesol and farnesoic acid were dissolved in 5 μL of methanol. Bar graphs represent mean ± SEM ($n = 3$) of three independent experiments normalized to GAPDH; *Mean is significantly different from the control, $P < 0.05$. FOH, farnesol; FCOOH, farnesoic acid; Zymo, zymosan; HK, heat-killed SC5314 cells.

The combination of farnesol and zymosan stimulates the expression of dectin-1

We and others have shown that the RAW264.7 cells constitutively express TLR2 (Al-Salleeh & Petro, 2007), but low levels of dectin-1 (Taylor *et al.*, 2002; Brown *et al.*, 2003). However, the expressions of both TLR2 and dectin-1 can be stimulated further when macrophages encounter microorganisms. We hypothesize that the synergism of zymosan and farnesol is associated with the increased expression of TLR2 and/or dectin-1 induced by zymosan or farnesol. Therefore, RAW264.7 cell expression of TLR2 and dectin-1 was determined by qRT-PCR following stimulation with zymosan, farnesol, farnesoic acid, or combinations of farnesol plus zymosan or farnesoic acid plus zymosan. The expression of TLR2 was significantly stimulated by zymosan alone, while farnesol plus zymosan did not increase this expression further (Fig. 4a). In contrast, farnesoic acid did not increase the expression of TLR2, either with or without zymosan. In contrast to TLR2 expression, the expression of dectin-1 was significantly enhanced only with the combination of zymosan and farnesol (Fig. 4b). Zymosan alone, farnesol alone, or farnesoic acid alone or in combination with zymosan failed to stimulate the expression of dectin-1 further in RAW264.7 cells. Therefore, the synergism of zymosan and farnesol in

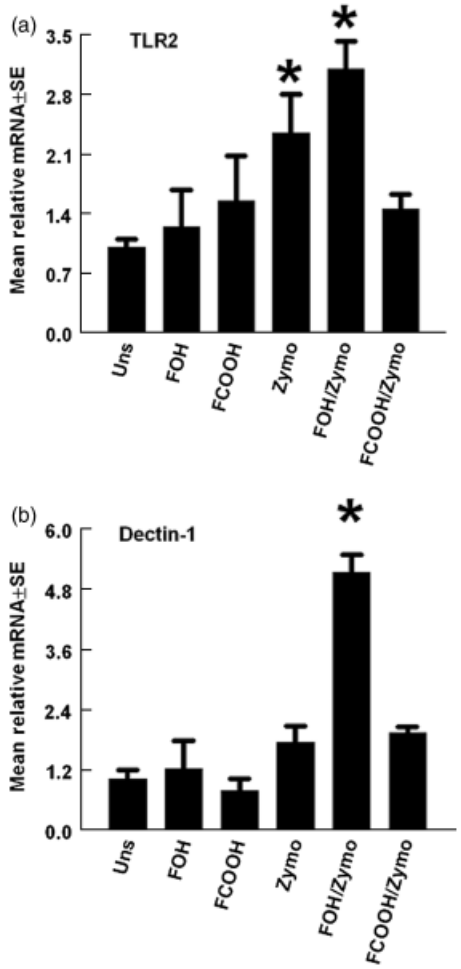

Fig. 4. RAW264.7 TLR2 and dectin-1 expression in response to the challenge by yeast cell components. Real-time qRT-PCR of TLR2 (a) and dectin-1 (b) in RAW264.7 cells. A total of 1×10^5 RAW264.7 cells were incubated with 5 μL of methanol (Uns); farnesol (5 μM); farnesoic acid (250 μM); zymosan (25 μg mL^{-1}); farnesol (5 μM) and zymosan (25 μg mL^{-1}) together; and farnesoic acid (250 μM) and zymosan (25 μg mL^{-1}) together for 3 h. Bar graphs represent mean ± SEM ($n = 4$) normalized to GAPDH; *Mean is significantly different from the control, $P < 0.05$. FOH, farnesol; FCOOH, farnesoic acid; Zymo, zymosan.

the induction of IL-6 expression is related to increased expression of dectin-1.

Discussion

We showed previously (Ghosh *et al.*, 2009) that those macrophages that have phagocytized *C. albicans* die within 4–6 h because the fungal pathogen converts to hyphae inside the phagosome, pierces the phagosomic and cytoplasmic membranes, and escapes. Here, we show that splenic macrophages produce and the RAW264.7 murine macrophage cell line expresses critical cytokines within 3–6 h after phagocytosis of the fungus *C. albicans*. Furthermore, macrophages that phagocytized hyphal-forming *C. albicans* (SC5314) expressed nearly as much cytokines as macrophages that phagocytized mutants of *C. albicans* (SN152) that do not form hyphae. Thus, GTF and hyphal growth are not necessary for the expression of IL-6 or the other macrophage cytokines within 3 h after challenge with *C. albicans*. These results show that macrophages that have phagocytized *C. albicans* can, before they are killed by escaping *C. albicans*, express substantial amounts of IL-6 (Bettelli *et al.*, 2006; Korn *et al.*, 2008), IL-1β and TNF-α for inflammation (Tonnesen, 1989), and IL-10 for immune regulation (Edwards *et al.*, 2006). Furthermore, whether or not the *C. albicans* undergo GTF after phagocytosis does not affect the expression of these cytokines within 3 h. Therefore, *C. albicans* escape from macrophages does not alter the early kinetics of cytokine expression. This rapid time frame for the expression of these cytokines is essential for macrophages' participation in the development of inflammation, adaptive immunity, and immune regulation. Therefore, macrophages that phagocytize *C. albicans*, despite their inability to kill this fungal pathogen, can potentially transmit cytokine signals that contribute toward the development of effective antifungal adaptive immunity, inflammation, and immune regulation. Among the cytokines expressed by macrophages in response to *C. albicans* is IL-6, which, in conjunction with TGF-β and IL-23, is essential for the induction of Th17 (Bettelli *et al.*, 2006; Ivanov *et al.*, 2007). Th17 in turn secretes IL-17 and IL-22 (Liang *et al.*, 2006), which play pivotal roles in anti-*Candida* immunity (Huang *et al.*, 2004) through the induction of defensins from epithelial cells (Liang *et al.*, 2006), some of which are candidacidal. In addition, IL-17 is critical to the accumulation of neutrophils at the site of *Candida* infection (Liang *et al.*, 2007). We also show here that macrophages that phagocytize *C. albicans* also express IL-1β and TNF-α, two cytokines that are also critical for the inflammatory response leading to neutrophil accumulation at infected sites (Tonnesen, 1989). Neutrophils play the pivotal role in anti-*Candida* immunity because, unlike macrophages, they are capable of phagocytizing and killing *C. albicans* (Rubin-Bejerano *et al.*, 2003). In addition to inflammatory cytokines, macrophages that phagocytize *C. albicans* also express the cytokine IL-10 within 3 h. IL-10 is important in control of the inflammation (Mosser & Edwards, 2008) induced by *C. albicans* infection (Del Sero *et al.*, 1999). However, IL-10 may significantly impair resistance against systemic infection with *C. albicans* that leads to infection in the kidney (Vazquez-Torres *et al.*, 1999). Therefore, phagocytosis of *C. albicans* by macrophages, which cannot effectively kill the fungus, stimulates the expression of cytokines from macrophages that contribute to inflammation and immune regulation.

Of interest is the observation that in contrast to live *C. albicans*, HK *C. albicans* failed to induce IL-6 expression

within 3 h. Gow *et al.* (2007) showed that HK *C. albicans*, which exposes more β-glucan than live *C. albicans*, does induce IL-6 production from macrophages. However, their measurement of induced IL-6 production by HK *C. albicans* occurred at 24–48 h after exposure to macrophages. Therefore, significant early induction of IL-6 production from macrophages by HK *C. albicans* does not occur.

It is also clear from this report that two components of viable *C. albicans* are responsible for stimulating these cytokines from macrophages. One of the components is farnesol, a QSM and virulence factor excreted by *C. albicans* (Hornby *et al.*, 2001), and the other is zymosan, which contains yeast cell wall components such as β-1,3-glucan, chitin, and mannans. It is known that zymosan stimulates macrophages through TLR2 acting in collaboration with dectin-1 (Goodridge *et al.*, 2007) or TLR6 (Hajjar *et al.*, 2001). TLR2 is also known to dimerize with TLR1 (Hajjar *et al.*, 2001) and galectin-3 (Jouault *et al.*, 2006). However, dectin-1 has been shown to be critical to antifungal immunity (Taylor *et al.*, 2007; Robinson *et al.*, 2009), and dectin-1 and TLR2 synergistically induce the expression of cytokines from macrophages (Ferwerda *et al.*, 2008). Nevertheless, future experiments will need to sort out which of the dimer partners of TLR2 and which cell wall components of *C. albicans* participate in the heightened expression of the aforementioned cytokines from macrophage following phagocytosis. Previous reports, measuring cytokines at 24 and 48 h, have shown that zymosan (Dillon *et al.*, 2006) and farnesol (Decanis *et al.*, 2009), acting separately, can induce the expression of IL-6. The present report shows that farnesol alone can decrease the background expression of IL-6 from macrophages, while zymosan and farnesol, acting together, can induce the expression of IL-6 within 3 h. The results presented here are clinically relevant because the innate immune response to zymosan also induces aseptic shock, multiorgan failure, and death in the host (Cuzzocrea *et al.*, 1997), probably due to excess production of zymosan-induced IL-6 (Cuzzocrea *et al.*, 1999). However, here, we show that farnesol and zymosan, acting together within 3 h, induce greater expression of IL-6 from macrophages than either of those two factors alone. Therefore, the combination of zymosan and farnesol may induce macrophages to generate IL-6, one of the cytokines necessary for the development of Th17, a T cell subset essential for immunity to *C. albicans* (Huang *et al.*, 2004; Conti *et al.*, 2009). This combination also induces cytokines such as TNF-α, which, along with IL-6, are involved in shock and death following systemic infection.

In addition to IL-6, IL-23 and TGF-β also contribute to Th17 development. However, macrophages that phagocytize *C. albicans* that produced hyphae were unable to express significant levels of IL-23 and TGF-β within 3 h (data not shown). In contrast, macrophages that phagocytize mutant *C. albicans* that do not produce hyphae express low, but detectable IL-23 and TGF-β mRNA within 3 h (data not shown). Further studies will be needed to understand the receptor and signaling system activated for the macrophage response to farnesol. We suggest that farnesol enhances the effect of cell wall PAMPs of *C. albicans* on macrophage cytokine expression. It should be noted that while IL-6 is important in murine induction of Th17, IL-1β is the corresponding macrophage cytokine for the development of Th17 in humans (Acosta-Rodriguez *et al.*, 2007). Here, we show in a murine macrophage cell line that *C. albicans* induces IL-1β 1278-fold within 3 h after phagocytosis. These results suggest that human macrophages phagocytizing *C. albicans* may also express the additional Th17-inducing cytokine, IL-1β.

In general, HK *C. albicans* induced far less early expression of cytokines compared with the live wild-type *C. albicans* cells. HK cells, which are likely to be phagocytized by macrophages, are inactive metabolically and therefore cannot produce farnesol or hyphae. In contrast, live *C. albicans*, which are phagocytized by macrophages, switch their morphology from yeast to hyphae, produce farnesol, kill the macrophage, and escape. Neither *C. albicans* 10 231 nor HK SC5314 caused increased IL-6 expression (Fig. 1a). Although strain 10 231 is of a different genetic background, it is a useful strain for comparison because it is the only known isolate of *C. albicans* that does not secrete farnesol. Therefore, farnesol, but not farnesoic acid, likely plays a role in IL-6 expression by macrophages. Presumably, the 15% farnesol produced by KWN2 (Navarathna *et al.*, 2007a, b) is sufficient for whatever role farnesol plays in the macrophage because the IL-6 expression levels by SN152, KWN2, and KWN4 were equivalent (Fig. 1a and b). Therefore, active farnesol production by *C. albicans*, in combination with cell wall components after phagocytosis, is likely necessary for high IL-6 expression from macrophages, whereas farnesol alone appears to decrease the expression of IL-6 from macrophages. Similarly, hyphae formation in the phagosome is not required, but does not prevent cytokine expression because equivalent levels of IL-6 expression were stimulated by the wild-type SC5314 and the three arginine auxotrophs, which do not produce hyphae.

Our long-term goal is to discern the role of farnesol in pathogenicity and virulence. In doing so, we must reconcile four quite different modes of action for farnesol. Farnesol was first discovered as a QSM for *C. albicans* (Hornby *et al.*, 2001). That is, *in vitro C. albicans* produced and excreted farnesol, and when the farnesol concentration exceeded a threshold level, it prevented the yeast to hypha switch. Next, in the present paper, we found that farnesol, together with cell wall components, acts as a signal to elicit the production of cytokines from macrophages. This suggests that farnesol is a costimulator of PAMPs within the cell wall. However,

whole animal studies showed that farnesol acts instead as a virulence factor. *Candida albicans* mutants that produced sixfold less farnesol were *c*. fourfold less virulent (Navarathna *et al*., 2007a) and wild-type *C. albicans* that had been treated with sublethal levels of fluconazole, thus producing 8–12 times more farnesol (Hornby & Nickerson, 2004; Navarathna *et al*., 2005, 2007a), were *c*. sixfold more virulent (Navarathna *et al*., 2005, 2007a). Our data in Fig. 3 indicate that farnesol alone without costimulation with cell wall components could decrease IL-6 expression, thus fulfilling its role as a virulence factor. It is also possible that the high level of inflammatory cytokines induced by farnesol in synergism with cell wall components *in vitro* is lethal during systemic candidiasis. Additionally, we found that farnesol significantly reduced the mouse serum levels of IFN-γ and IL-12 during systemic candidiasis (Navarathna *et al*., 2007b), which could prove to enhance virulence because both of these cytokines are needed for optimum immunity to *C. albicans*. Further study will be needed to connect these observations. For now, farnesol's exact mode of action as a virulence factor or a costimulator of PAMPs remains elusive.

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