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# The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of *Candida albicans*

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## ABSTRACT

**In the mucosa, the immune pathways discriminating between colonizing and invasive *Candida*, thus inducing tolerance or inflammation, are poorly understood. Th17 responses induced by *Candida albicans* hyphae are central for the activation of mucosal antifungal immunity. An essential step for the discrimination between yeasts and hyphae and induction of Th17 responses is the activation of the inflammasome by *C. albicans* hyphae and the subsequent release of active IL-1β in macrophages. Inflammasome activation in macrophages results from differences in cell-wall architecture between yeasts and hyphae and is partly mediated by the dectin-1/Syk pathway. These results define the dectin-1/inflammasome pathway as the mechanism that enables the host immune system to mount a protective Th17 response and distinguish between colonization and tissue invasion by *C. albicans*. *J. Leukoc. Biol.* 90: 357–366; 2011.**

## Introduction

*Candida albicans* is a common fungal microorganism that colonizes the mucosa and skin. Approximately one-third of individuals is colonized with *C. albicans* at any given time. Despite this, *C. albicans* does not normally invade host tissues unless the mucosal or skin barrier is breached, or the immune system is compromised, leading to mucosal or even disseminated *Candida* infection [1]. Recently, Moyes and colleagues [2] have described the differential pathways of activation of epithelial cells by *C. albicans* yeasts and hyphae that are likely to play an important role in the response to mucosal invasion. However, the mechanism enabling discrimination between colonizing and invading *Candida* cells by the immune system is practically unknown. The presence of colonizing *Candida* on the mucosa does not induce a strong inflammatory reaction, but the immune system is triggered as a consequence of tissue invasion. This implies that mucosal macrophages and/or DCs, which survey the luminal flora and other mucosal surfaces, have evolved tailored signaling-sensing mechanisms that discriminate between colonizing and invading forms of the fungus. However, the nature of these mechanisms, which are crucial for host defense and immune tolerance of the mucosa, has yet to be identified.

The Th17 response has been reported to be crucial for anti-*Candida* host defense, principally resulting in the recruitment of neutrophils [3]. Consequently, IL-17 knockout mice are highly susceptible to disseminated and mucosal candidiasis [4]. It has been also suggested that IL-17 is impaired in patients with mucosal fungal infections [5, 6], reinforcing the role of Th17 for anti-*Candida* host defenses. IL-1β has been shown to be essential for Th17 differentiation [7]. The production of bioactive IL-1β is achieved via enzymatic

Abbreviations: CMC=chronic mucocutaneous candidiasis, HIES=hyper-IgE syndrome, IL-1Ra=IL-1R antagonist, Nlrp3=nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3, $Pam_3Cys_4$=tripalmitoyl-S-glycerol-cysteine, PLL=poly-L-lysine, Syk=spleen tyrosine kinase, UC820=*C. albicans* ATCC MYA-3573, YVAD=Ac-Tyr-Val-Ala-Asp-2,6 dimethylbenzoyloxymethyl-ketone

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cleavage of the procytokine form by active caspase-1, which is strictly regulated by a protein complex called the inflammasome [8, 9]. Among the known inflammasomes, Nlrp3 inflammasome has been suggested to be responsible for anti-*C. albicans* defense [10, 11].

In the present study, we aimed to identify the mechanisms that help macrophages to discriminate between benign colonization and potentially destructive, invasive phases of the host interaction with *C. albicans*. By comparing WT and mutant strains of *C. albicans*, which are unable to germinate and form hyphae, we demonstrate that a vital discriminative factor for inducing protective mucosal responses is the morphological transition from yeast to hyphae. In contrast to yeasts that do not activate the inflammasome, *C. albicans* hyphae were recognized by macrophages and induced inflammasome activation, leading to IL-1$\beta$ production by dectin-1-dependent and -independent pathways. This was followed by Th17 differentiation of naive Th cells with IL-17 and IL-22 production.

## MATERIALS AND METHODS

### Reagents

The irreversible caspase-1 inhibitor YVAD was purchased from Alexis Biochemicals (San Diego, CA, USA), reconstituted in 10 mmol/L DMSO, and subsequently diluted to the desired concentration in medium (RPMI 1640). Syk inhibitor was purchased from Calbiochem (San Diego, CA, USA). In experiments using pharmacological inhibitors, control cells were treated with an equivalent concentration of vehicle (0.01–0.1% DMSO). Synthetic $Pam_3Cys_4$ (TLR2 agonist) was purchased from EMC Microcollections (Germany), and the production of highly purified, particulate $\beta$-glucan has been described elsewhere [12].

### *Candida* strains

UC820 was used in the experiments, unless otherwise indicated. The Δ*efg1*/Δ*cph1* transcription factor double-knockout strain was a kind gift from Dr. Gerald. Fink(Whitehead Institute, Cambridge, MA, USA). CAI-4 strain is the parental strain of the mutants used in this study. The Δ*och1* and Δ*och1* + *OCH1*-complemented strains have been characterized previously [13]. The Δ*hgc1* hyphae-deficient mutant and Δ*hgc1* +*HGC1*-complemented strains were kindly provided by Dr. Yue Wang (Institute of Molecular and Cell Biology, Singapore) [14]. Bwp17, the Δ*eed1* and Δ*eed1* + *EED1*-complemented strains were kindly provided by Dr. Benhard Hube (Hans Knöll Institute, Jena, Germany). *C. albicans* was grown overnight in Sabouraud broth at 25°C, and cells were thereafter harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI 1640; ICN Biomedicals, Aurora, OH, USA). *C. albicans* was killed for 1 h at 100°C or by treatment with 0.04% thimerosal overnight. The killed *C. albicans* cells were washed three times with PBS and resuspended in culture medium for macrophage stimulation and cytokine induction.

### Patients and donors

Peripheral blood was taken from healthy individuals, three patients with CMC, four patients with HIES, and two patients with Y238X dectin-1 mutation with mucocutaneous fungal infection under Institutional Review Board approval of Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands).

### Monocyte-derived macrophages

Separation and stimulation of human monocytes or macrophages were performed as described elsewhere [15]. Human monocytes were cultured in complete RPMI-1640 medium (ICN Biomedicals) supplemented with 100 ng/ml human M-CSF and 10% pooled human serum for 6 days. Cocultures of monocytes or macrophages with lymphocytes were performed for the induction of IL-17 production.

### PBMC and macrophage stimulation

A sample of $5 \times 10^5$ PBMCs/well (or $5\times10^4$ macrophages/well) was seeded in 96-well plates and stimulated with $1 \times 10^5$/ml live or $1 \times 10^6$/ml heat-killed *C. albicans* yeast cells (final volume of 200 $\mu$l/well) in RPMI. Supernatant was collected after 24 h (for TNF, IL-6, and IL-1$\beta$ measurement) or 7 days (for IL-17 and IL-22 measurement).

### Cytokine measurements

IL-6, TNF, IL-1$\beta$, IL-17, and IL-22 concentrations were measured by commercial sandwich ELISA kits (R&D Systems, The Netherlands), according to the manufacturer's instructions. For intracellular IL-1$\beta$ measurement, after collecting of supernatant for extracellular cytokine measurement, 200 $\mu$l fresh RPMI was added to the macrophage. Then, the plate was put into a −80°C freezer, rapidly freezing and thawed at room temperature. This freeze-thaw cycle was repeated three times. Intracellular IL-1$\beta$ was determined by ELISA.

### Intracellular cytokine staining

PBMCs were stimulated for 4–6 h with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and ionomycine (1 mg/ml) (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocols. Cells were first stained using an anti-CD4 APC antibody (BD Biosciences). Subsequently, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and then stained with anti-IFN-$\gamma$ PE (eBioscience, San Diego, CA, USA) and anti-IL-17 FITC (eBioscience). Samples were measured on a FACSCalibur, and data were analyzed using the CellQuest Pro software (BD Biosciences).

### Cell-wall porosity measurement

The porosity of the cell walls of exponential-phase yeast cells grown in RPMI and hyphae grown in 2.5% (v/v) serum at 37°C for 4 h was compared. This assay is based on the ability of polycationic polymers (DEAE-dextran and PLL) to enhance the permeability of the cell membrane, resulting in release of UV-absorbing compounds measured at 260 nm [16].

### Freeze-substitution TEM

*C. albicans* hyphal cells were grown in 20% (v/v) serum with 50 $\mu$g/ml uridine supplementation for 3 h. Samples were collected by centrifugation and immobilized immediately by high-pressure freezing with a Leica EM PACT2 (Leica Microsystems UK, Milton Keynes, UK). After freezing, cells were freeze-substituted in substitution reagent (1% $OsO_4$/0.1% uranyl acetate in acetone) with a Leica EM AFS2. Ultrathin sections were prepared with a Diatome diamond knife on a Leica UC6 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM10 transmission microscope (FEI UK, Cambridge, UK).

### Caspase-1 activity measurement

Caspase-1 activity was determined by a caspase-1 fluorometric assay kit (BioVision, Mountain View, CA, USA), according to the manufacturer's instruction. Briefly, $2 \times 10^6$ macrophages were stimulated with *C. albicans* UC820 or Δ*efg1*/Δ*cph1* strain for 6 h and lysed with cell lysis buffer on ice for 10 min. Then, equal volumes of 2× reaction buffer and YVAD-7-amino-4-trifluoromethylcoumarin were added to the cell lysate within a 96-well plate at 37°C, and the emitted fluorescence was read by FLUOstar Optima microplate (BMG Labtech, Cary, NC, USA) at 90-s intervals for 200 cycles using

400 nm excitation and 505 nm emission filters. The relative activity was calculated compared with recombinant caspase-1 enzymatic activity.

## RESULTS

### The Th17 pathway is compromised in patients with mucosal candidiasis

Our first step was to define the main components of the mucosal antifungal host defense mechanism that were responsible for the elimination of the fungus once invasion occurred. To identify this mechanism, individuals with specific inborn defects of mucosal-antifungal immunity were studied, such as HIES [6] and CMC patients [5]. We first assessed the innate host response triggered in PBMCs of CMC patients by *C. albicans*. Production of monocyte-derived proinflammatory cytokines, such as TNF and IL-6, was normal (**Fig. 1A**). Similar results were found in HIES patients (data not shown). In contrast, T cells isolated from CMC and HIES patients secreted

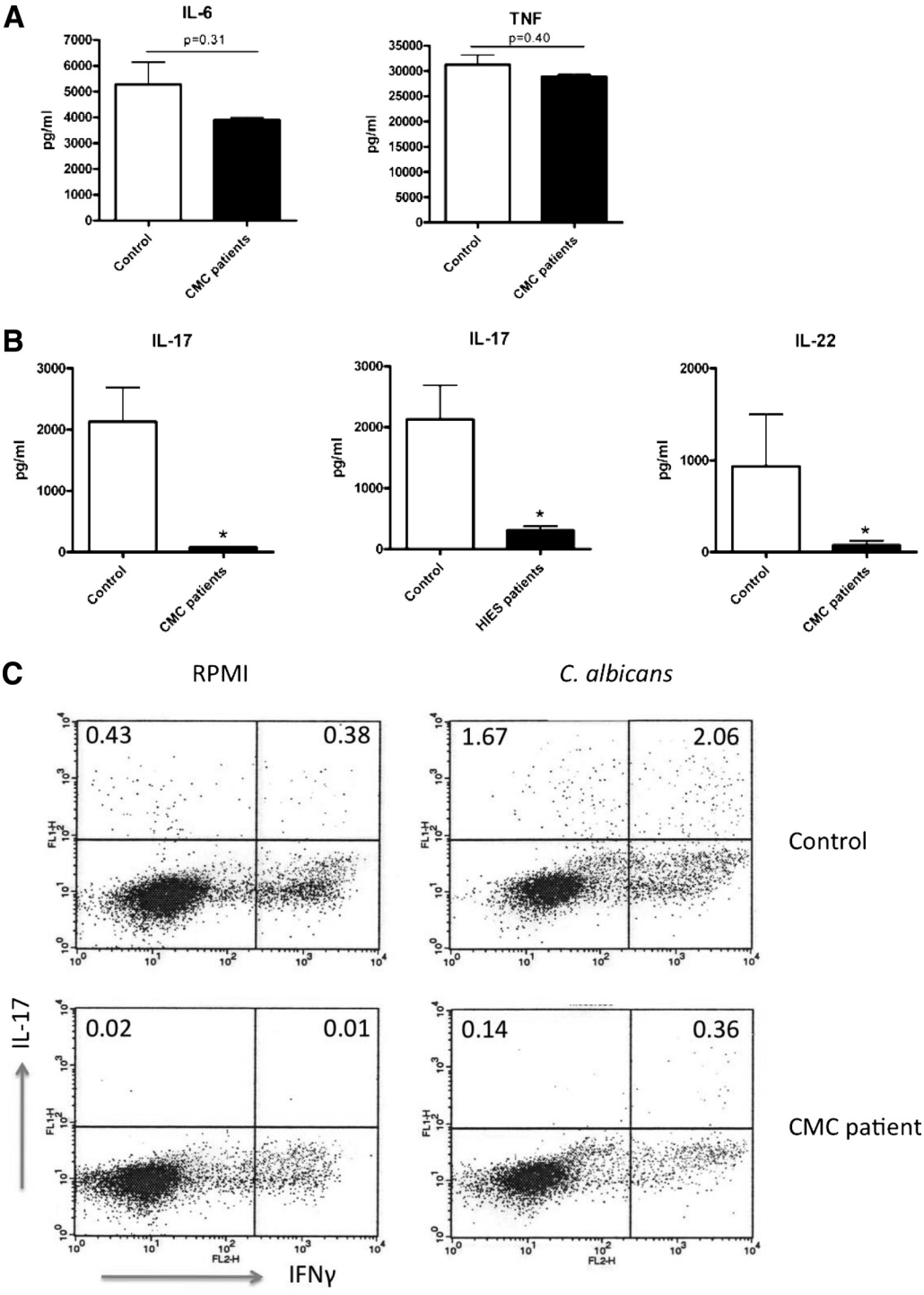


**Figure 1. CMC and HIES patients have defective IL-17 production upon *C. albicans* stimulation.** (A) PBMCs from CMC patients ($n$=3), consisting of a coculture of monocytes and lymphocytes, and healthy controls were stimulated with heat-killed *C. albicans* for 24 h. Supernatant was harvested, and TNF and IL-6 concentration was determined by ELISA ($n \geq 6$, $^*P<0.05$). (B) PBMCs from CMC ($n$=3) and HIES ($n$=4) patients and healthy control were stimulated with heat-killed *C. albicans* for 7 days. Supernatant was harvested, and IL-17 and IL-22 concentration was determined by ELISA. (C) Intracellular cytokine staining of IL-17 and IFN-γ in human PBMCs stimulated for 5 days with RPMI or *C. albicans* and then stimulated for 4 h with PMA and ionomycin. Cells were gated for CD4. Representative data from two separate experiments with a total $n$ = 3 are shown here.

significantly less IL-17 in response to *C. albicans* stimulation than those of healthy controls, as shown by secreted IL-17-, IL-22 (Fig. 1B)-, and IL-17-positive T cells (Fig. 1C). These results are supported by the previous reports that CMC [5] and HIES [6] patients failed to induce protective IL-17 production in response to *C. albicans* stimulation. Th17 responses seem therefore to be one of the main components of the mucosal antifungal defense mechanism.

### *C. albicans* germ-tube formation is critical for stimulation of IL-1β production in macrophages

As the transformation of *Candida* yeasts into hyphae is a crucial step in tissue invasion, we hypothesized that differential recognition of colonizing yeasts and invasive hyphae by macrophages may induce distinct cytokine profiles that may be inductive or not for Th17 responses. IL-1β and IL-6 are the two main cytokines required for the initiation of Th17 responses [7], and IL-23 is important for the proliferation of Th17 cells [17]. To test this hypothesis, we stimulated human macrophages derived from monocytes in culture with a yeast-locked Δ*efg1/*Δ*cph1* transcription factor double-knockout *C. albicans* strain, which is unable to form hyphae [18] (mimicking colonization), and its corresponding WT strain, which forms germ tubes in culture (mimicking tissue invasion). IL-6 stimulation did not differ between germ-tube-positive and germ-tube-negative strains. However, the hyphae-forming WT strain induced strong IL-1β responses, whereas the yeast-locked Δ*efg1/*Δ*cph1* *C. albicans* strain was completely unable to stimulate production of IL-1β (**Fig. 2A**). TNF production induced by the Δ*efg1/*Δ*cph1* strain was also slightly lower, most likely as a result of endogenous, IL-1β-induced TNF. In contrast, live *Candida* strains induced a comparable amount of IL-1β in

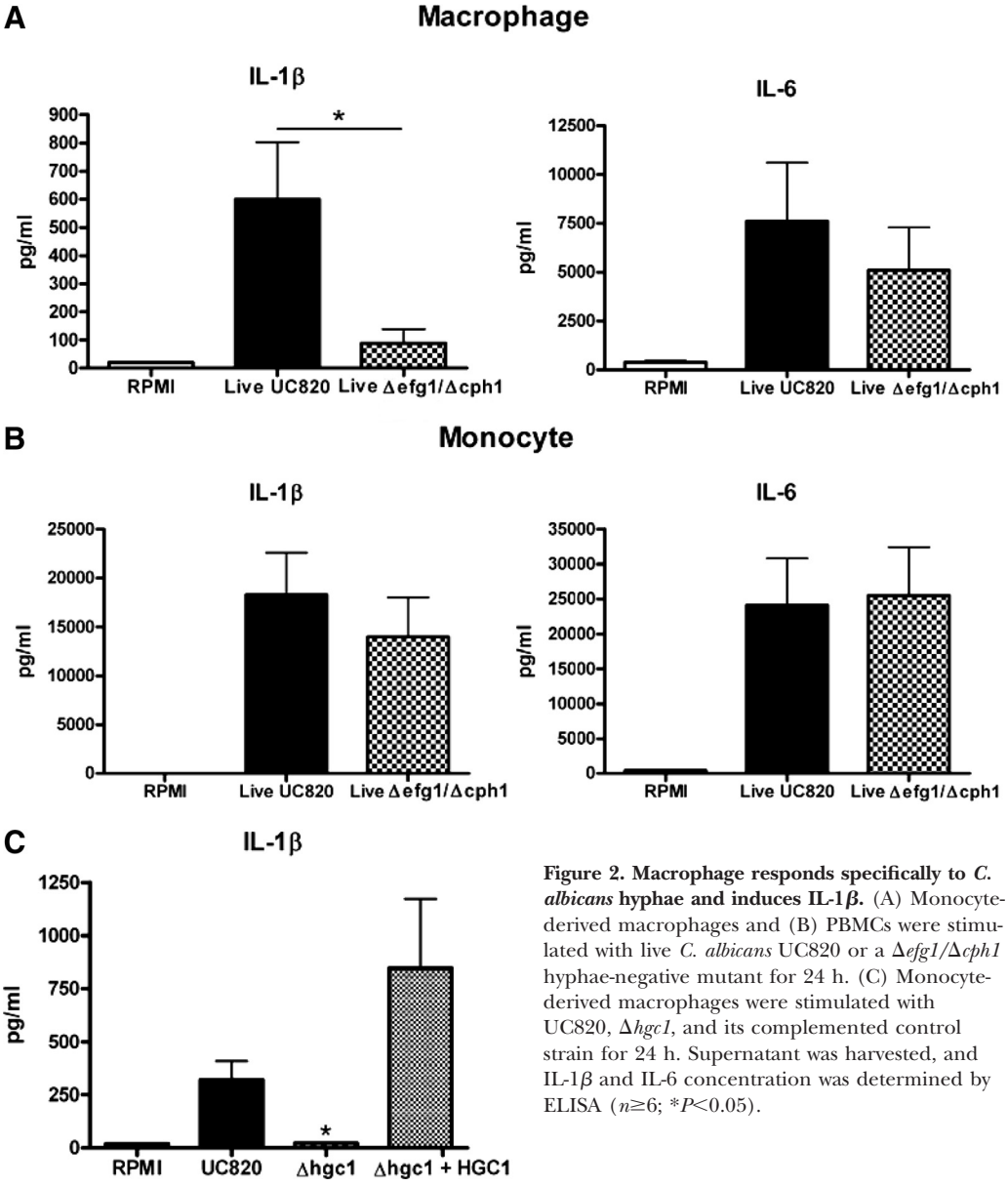


**Figure 2. Macrophage responds specifically to *C. albicans* hyphae and induces IL-1β.** (A) Monocyte-derived macrophages and (B) PBMCs were stimulated with live *C. albicans* UC820 or a Δ*efg1/*Δ*cph1* hyphae-negative mutant for 24 h. (C) Monocyte-derived macrophages were stimulated with UC820, Δ*hgc1*, and its complemented control strain for 24 h. Supernatant was harvested, and IL-1β and IL-6 concentration was determined by ELISA ($n \geq 6$; $^*P < 0.05$).

monocytes (Fig. 2B). To exclude the possibility that the defective IL-1β production was a pleiotropic consequence of the Δ*efg1/*Δ*cph1* genetic background, we also examined the *C. albicans* strain Δ*hgc1* [14], which is also unable to form germ tubes, and the Δ*eed1* mutant, which was unable to sustain hyphal growth [19]. The relevant, complemented control strains were also tested for their capacity to induce production of IL-1β in macrophages. Similar results were observed as with the Δ*efg1/*Δ*cph1*strain (Fig. 2C and Supplemental Fig. 1). These data suggest strongly that induction of IL-1β production delineates the level at which the immune system discriminates the colonization and invasive forms of *C. albicans* and suggest that the capacity to form and sustain hyphal growth is a requirement for a vigorous IL-1β response.

## *C. albicans* hyphae induction of IL-1β secretion is dependent on caspase-1 activity

IL-1β production is regulated at multiple levels: mRNA transcription, translation into inactive pro-IL-1β, and activation of the protein platform called the inflammasome, which results in caspase-1 activation and processing of the procytokine into the mature, active IL-1β [20]. First, we investigated whether this difference in IL-1β induction by yeast and hyphal cells is caused by the differential activation of transcription or pro-IL-1β synthesis. IL-1β mRNA expression did not differ when cells were stimulated with the WT or the Δ*efg1/*Δ*cph1* mutant (**Fig. 3A**), indicating that transcription is equally induced by yeasts and hyphae. Moreover, WT and the Δ*efg1/*Δ*cph1* mutant induced similar concentrations of intracellular pro-IL-1β (Fig.

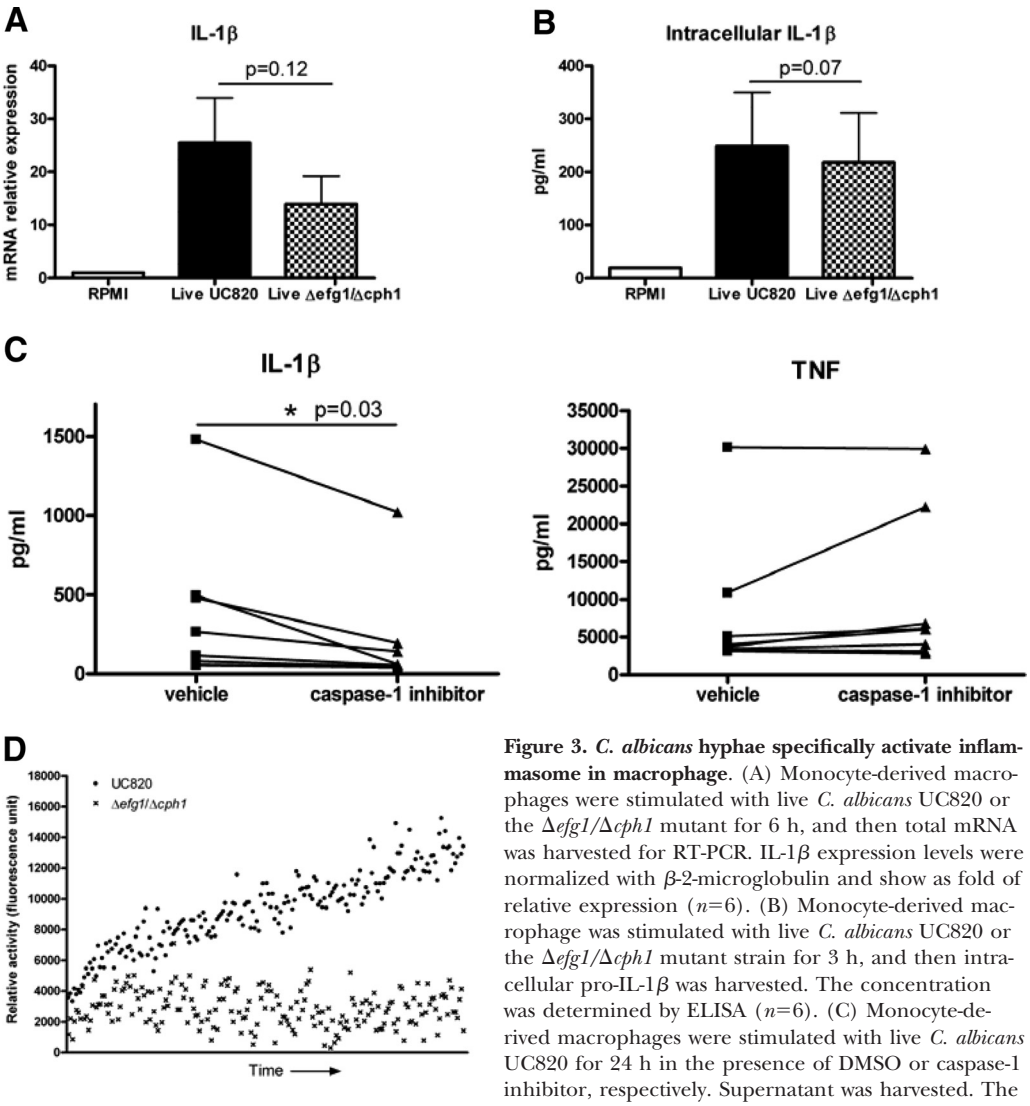


**Figure 3. *C. albicans* hyphae specifically activate inflammasome in macrophage**. (A) Monocyte-derived macrophages were stimulated with live *C. albicans* UC820 or the Δ*efg1/*Δ*cph1* mutant for 6 h, and then total mRNA was harvested for RT-PCR. IL-1β expression levels were normalized with β-2-microglobulin and show as fold of relative expression ($n$=6). (B) Monocyte-derived macrophage was stimulated with live *C. albicans* UC820 or the Δ*efg1/*Δ*cph1* mutant strain for 3 h, and then intracellular pro-IL-1β was harvested. The concentration was determined by ELISA ($n$=6). (C) Monocyte-derived macrophages were stimulated with live *C. albicans* UC820 for 24 h in the presence of DMSO or caspase-1 inhibitor, respectively. Supernatant was harvested. The concentration of IL-1β and TNF was determined by ELISA ($n$=8; *$P$<0.05). (D) Caspase-1 activity was induced by *C. albicans* UC820 but not by the hyphae-deficient Δ*efg1/*Δ*cph1* mutant. Cells were stimulated with the two different *C. albicans* strains for 6 h, and the caspase-1 activity was measured in the cell lysate for a duration of 5 h. Each dot represents the measured value of the fluorescence intensity with the interval of 60 s between each dot. Representative data from two separate experiments were shown here.

3B), indicating that the subsequent translation of pro-IL-1β is not affected. We further investigated whether differential inflammasome activation could be the cause of the difference in IL-1β secretion. Blocking caspase-1 activity with a specific inhibitor led to inhibition of IL-1β secretion induced by WT *C. albicans*, indicating that active caspase-1 is required for IL-1β secretion (Fig. 3C). Monocytes express activated caspase-1 constitutively, and this explains the IL-1β induction by yeasts and hyphae in monocytes. In contrast, macrophages lack a constitutively active caspase-1 [21], providing a target for differential activation by yeast cells and hyphae. Indeed, only the hyphae-competent WT strain, and not the Δ*efg1*/Δ*cph1 C. albicans* strain, was able to activate caspase-1, indicating that hyphae formation is important for macrophage inflammasome activation and IL-1β secretion (Fig. 3D).

## Hyphal PAMPs are crucial in the induction of inflammasome activation

Next, we investigated the recognition mechanism responsible for the capacity of the macrophage to distinguish *C. albicans* hyphae from yeast, leading to IL-1β secretion. Recognition of fungal PAMPs by specific PRRs is responsible for the innate immunity stimulation [22]. We manipulated the profile of PAMPs exposed by the *C. albicans* yeasts and hyphae by heat-

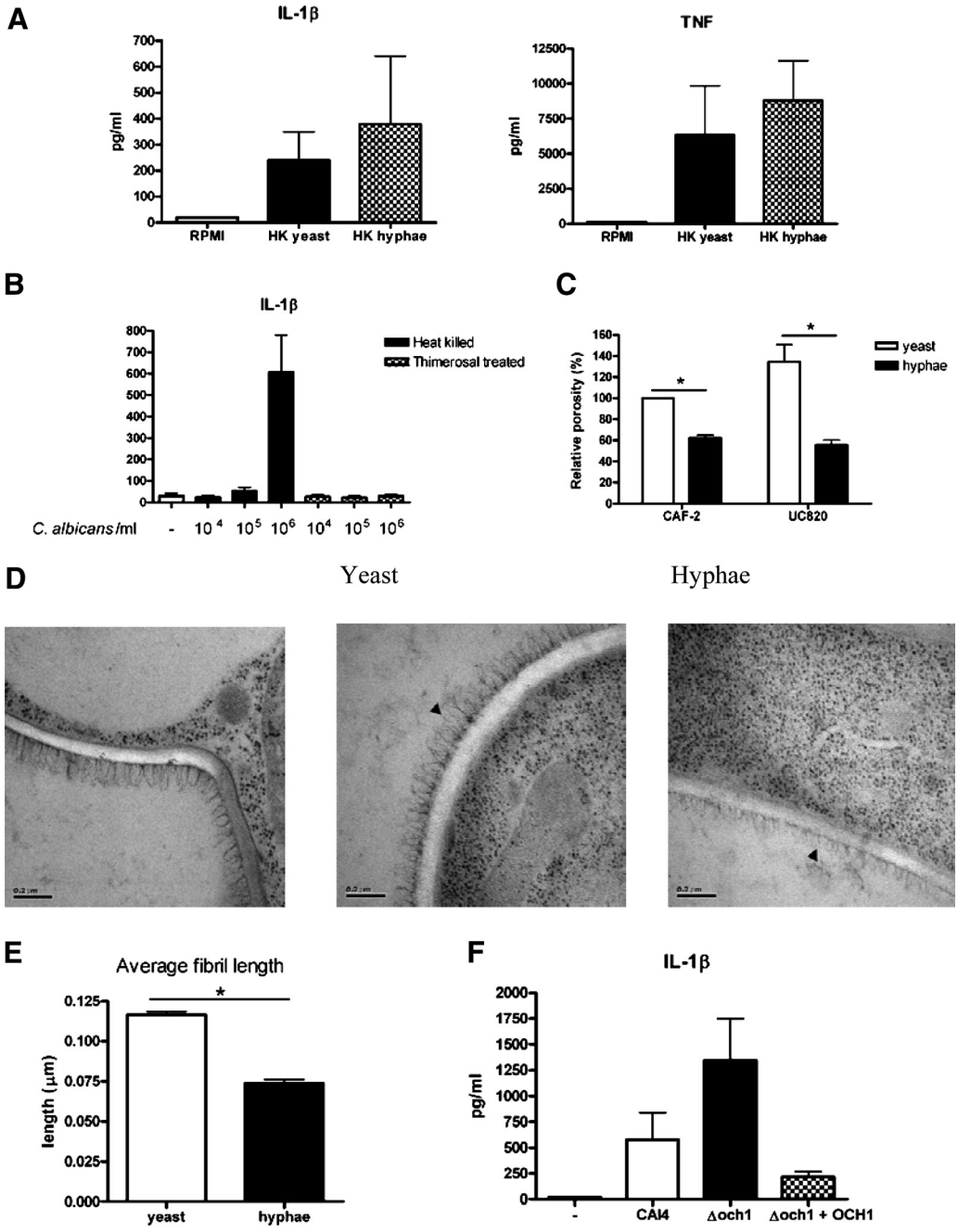


**Figure 4. Exposure of PAMPs situated underneath the mannan layer in hyphae is responsible for IL-1β production.** (A) Monocyte-derived macrophages were stimulated with heat-killed (HK) *C. albicans* yeast or hyphae for 24 h. Supernatant was harvested, and the concentration of IL-1β and TNF was determined by ELISA. (B) Monocyte-derived macrophages were stimulated with heat-killed or thimerosal-treated *C. albicans* yeast for 24 h. Supernatant was harvested, and the concentration of IL-1β and TNF was determined by ELISA. (C) The porosity of two *C. albicans* strains, CAF-2 and UC820, was measured by the relative sensitivity to PLL and DEAE-dextran sensitivity. (D) TEM of yeasts and hyphae (arrowheads indicate mannan fibrils). (E) Average mannan fribril length from 30 randomly selected *C. albicans* yeast and hyphae. (F) Monocyte-derived macrophage was stimulated with heat-killed CAI-4, the Δ*och1 N*-mannan glycosylation mutant, and its complemented control strain, respectively, for 24 h. Supernatant was harvested, and the concentration of IL-1β was determined by ELISA ($n$=6; *$P$<0.05).

killing, which is known to expose inner cell-wall components such as β-glucans and chitin [23]. In contrast, thimerosal fixation kills cells while preserving the native cell-wall architecture. Interestingly, heat-killed yeast induced IL-1β production, similarly to heat-killed hyphae (**Fig. 4A**). In contrast, thimerosal-killed *C. albicans* induced very little IL-1β (Fig. 4B), and heat treatment of thimerosal-killed *C. albicans* restored the IL-1β-inducing ability (not shown). This suggests that recognition of inner cell-wall PAMPs of yeast cells can also lead to IL-1β secretion.

Based on these findings, we hypothesized that the differential ability of *C. albicans* yeast and hyphae to activate the inflammasome was a result of differences in the profile of PAMPs expressed in the yeast and hyphal cell walls and that this difference was most likely to be a result of differences in the components of the inner cell-wall layer that are known to be exposed during invasion of the tissue by *C. albicans* hyphae [24]. Hypothetically, differences in porosity of the cell wall between yeasts and hyphae could facilitate the recognition of the inner hyphal cell-wall layers by PRRs. However, this proved not to be the case, as measurements of porosity of the cell wall demonstrated that hyphal wall porosity was less than that of the yeast cell wall (Fig. 4C). We therefore assessed the capacity of individual cell-wall components to induce IL-1β. Mannoproteins constitute the major component of the outer cell wall of both forms, but the rapid growth of hyphae, which does not allow the complex branching of mannans, is hypothesized to result in significant structural differences in mannans of yeasts and hyphae (Fig. 4D). Indeed, TEM analyses showed that the outer-wall mannan fringe length was longer in mother cell (yeast) than in hyphae (0.116±0.014 μm vs. 0.073±0.013 μm; Fig. 4E). The Δ*och-1 C. albicans* glycosylation mutant lacks most of the branched outer-wall *N*-mannan fibrils [15]. We therefore compared IL-1β production by macrophages stimulated with heat-killed CAI-4 (parental strain) with the Δ*och1* null mutant and its cognate *OCH1*-complemented strain. Interestingly, IL-1β production was significantly greater when macrophages are stimulated with the Δ*och-1 C. albicans* strain compared with the isogenic parental strain and the complemented control strain (Fig. 4F). Therefore, short mannan fibrils, which permit "tasting" of the *C. albicans* PAMPs of the inner cell wall, are associated with induction of IL-1β production by macrophages.

## Dectin-1 is partially responsible for the *C. albicans*-induced IL-1β production

The C-type lectin receptors, dectin-1 and dectin-2, recognize the *C. albicans* β-glucans and α-mannans, respectively, and both signal through Syk kinase [25, 26]. A pharmacological Syk inhibitor partially inhibited *C. albicans*-induced IL-1β secretion in cells from healthy volunteers (**Fig. 5A**), suggesting a role of a Syk-coupled receptor in IL-1β secretion. Macrophages isolated from patients homozygous for the early stop codon mutation Tyr238X in dectin-1, which completely lacks functional dectin-1 expression [27], were exposed to *C. albicans* in the presence or absence of the Syk inhibitor or a caspase-1 inhibitor. Macrophages from dectin-1 Tyr238X pa-

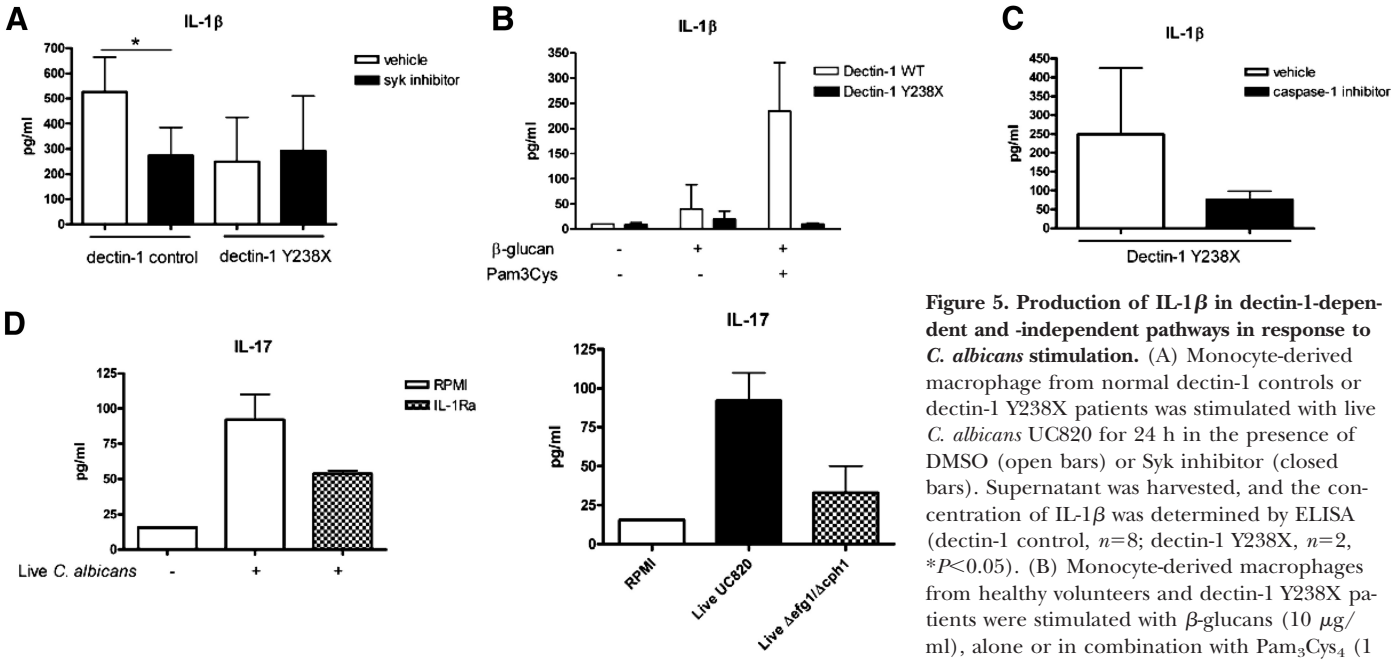


**Figure 5. Production of IL-1β in dectin-1-dependent and -independent pathways in response to *C. albicans* stimulation.** (A) Monocyte-derived macrophage from normal dectin-1 controls or dectin-1 Y238X patients was stimulated with live *C. albicans* UC820 for 24 h in the presence of DMSO (open bars) or Syk inhibitor (closed bars). Supernatant was harvested, and the concentration of IL-1β was determined by ELISA (dectin-1 control, $n=8$; dectin-1 Y238X, $n=2$, $^*P<0.05$). (B) Monocyte-derived macrophages from healthy volunteers and dectin-1 Y238X patients were stimulated with β-glucans (10 μg/ml), alone or in combination with $Pam_3Cys_4$ (1 μg/ml) for 24 h. Supernatant was harvested, and the concentration of IL-1β was determined by ELISA (dectin-1 control, $n=3$; dectin-1 Y238X, $n=2$). (C) Monocyte-derived macrophages from dectin-1 Y238X patients were stimulated with live *C. albicans* UC820 for 24 h in the presence of DMSO and caspase-1 inhibitor. Supernatant was harvested, and the concentration of IL-1β was determined by ELISA. (D) Monocyte-derived macrophages were stimulated with live *C. albicans* UC820 in the presence of autologous lymphocytes, in the presence or absence of IL-1Ra for 7 days. Supernatant was harvested, and the concentration of IL-17 was determined by ELISA. (E) Monocyte-derived macrophages were stimulated with live *C. albicans* UC820 of the Δ*efg1*/Δ*cph1* strain in the presence of autologous lymphocytes, in the presence or absence of IL-1Ra for 7 days. Supernatant was harvested, and the concentration of IL-17 was determined by ELISA.

tients released significantly less IL-1β than macrophages from healthy individuals when challenged with *C. albicans* cells (Fig. 5A). Moreover, unlike the macrophage from healthy individuals, macrophage from dectin-1 Tyr238X patients was not affected by the Syk inhibitor, underlining the putative role of the dectin-1/Syk pathway for induction of IL-1β by macrophages (Fig. 5A). This conclusion was further supported by experiments showing the stimulation of IL-1β by purified β-glucans, which is an entirely dectin-1-dependent mechanism (Fig. 5B). However, at least one additional pathway must be responsible for IL-1β induction in macrophages, as residual IL-1β production was consistently observed in macrophages defective for dectin-1 expression. Recognition of mannans by mannose receptor [15] or dectin-2 [26] was not responsible for this residual production. This was confirmed by small interfering RNA-directed knockdown of expression of these receptors, which failed to show any effect on IL-1β production (not shown). Although chitin is an important candidate for residual IL-1β production, and the chitin-enriched Δ*och1* strain led to increased IL-1β production (Fig. 4F), the unknown nature of the PRR for chitin prevented us from exploring the hypothesis that this residual pathway is related to the recognition of cell-wall chitin. This second, minor pathway of IL-1β stimulation was also shown to be inflammasome-dependent, as a caspase-1 inhibitor also reduced IL-1β production in cells isolated from dectin-1-deficient patients (Fig. 5C).

### IL-1β induced by *C. albicans* hyphae leads to IL-17 production

IL-1β has been demonstrated to be critical for Th17 differentiation in humans [7], and blocking the IL-1RI signaling pathway by IL-1Ra inhibited the induction of IL-17 induced by *C. albicans* (Fig. 5D). We assessed whether the differences in IL-1β induction by yeasts and hyphae also lead to differences in Th17 responses. Indeed, the yeast-locked Δ*efg1*/Δ*cph1 C. albicans* mutant failed to induce IL-17 in macrophages that were cocultured with T cells, whereas hyphae-forming WT *C. albicans* induced Th17 differentiation and IL-17 production (Fig. 5E). These results demonstrate that the induction of IL-1β by the macrophage is critical for Th17 differentiation, and the phenotypic switch between yeasts and hyphae determines the triggering of a Th17 response.

## DISCUSSION

Much attention has been devoted to the understanding of how the immune system defends the host from fungal infection. Th1 and Th17 responses have been shown to have beneficial effects for the host defense against *C. albicans* infection [4, 28]. However, the mechanisms of discrimination between *C. albicans* colonization and invasion are still poorly understood. A recent study has described colonizing yeasts and invading hyphae, causing differential activation of the c-Jun and c-Fos transcription factors in epithelial cells [2]. The discriminatory mechanisms operating at the level of the immune response are unknown. In the present study, we report that antifungal defense depends on mounting an efficient Th17 response. This is the major immune defect in patients with chronic mucosal fungal infections such as CMC and HIES, which share Th17 defects [5, 6]. The protective anti-*C. albicans* Th17 response is initiated when *C. albicans* forms germ tubes, which are recognized by macrophages via a dectin-1/Syk-dependent mechanism. The hyphae-induced, protective Th17 differentiation is dependent on inflammasome activation and IL-1β production.

In the CMC patients, we observed defective production of IL-17 and a partial defective IFN-γ production but no difference in IL-6, TNF, and IL-1β production. The importance of Th17 responses for antifungal host defense is supported by two additional lines of evidence: IL-17 is important for host defense against systemic fungal infection [29, 30], and IL-17 has been shown to be a crucial component of mucosal antifungal defense in a murine experimental model [4]. Th17 responses are initiated by two signals: presentation of a fungal antigen in the context of the activation by costimulatory molecules and the release of an appropriate cytokine milieu by the APCs, such as tissue macrophages [22]. IL-1β production is essential for mounting an effective Th17 response, and this is dependent on the processing of the inactive pro-IL-1β into active IL-1β by the inflammasome and caspase-1 activation.

Efg1 and Cph1 are two important transcription factors that regulate *C. albicans* yeast to hyphae morphogenesis [30]. The Δ*efg1*/Δ*cph1* double-knockout *C. albicans* strain is unable to form hyphae, even when grown in strong hyphae-inducing culture conditions [31]. It has been also shown that Δ*efg1*/Δ*cph1* is avirulent in the mouse model [31], demonstrating that hyphae formation is critical for *C. albicans* invasion. In addition, from the point of view of the clinical histology of mucosal candidiasis patients, germ-tube formation in serum is a much-used clinical diagnostic test. By stimulating macrophage with WT and Δ*efg1*/Δ*cph1 C. albicans* strains, we showed that hyphae development during tissue invasion is crucial for macrophage recognition and IL-1β production via inflammasome activation. Similarly, macrophages stimulated with Δ*hgc1* and Δ*eed1* strains, which are unable to form [14] or to sustain hyphal growth [19], produced significantly lower IL-1β, demonstrating that sustained hyphal development is required for effective inflammasome activation and IL-1β production. This was not a result of alterations in the host transcription of the IL-1β gene but rather, a hyphae-specific induction of inflammasome activation in the macrophage. In contrast, the WT and the yeast-locked *C. albicans* strains induced IL-1β production in blood monocytes, which are cells that are present in the normally sterile bloodstream, where presence of yeast or hyphal cells is indicative of invasion. The production of IL-1β by monocytes stimulated with yeasts and hyphae is a result of the constitutively active caspase-1 in these circulating monocytes [21]. These data indicate that the macrophage is the key cell type recognizing the invading *C. albicans* in tissues. Similar mechanisms could also be present in DCs, which also lack a constitutively active caspase-1 [21].

Reinforcing these observations in human cells, Joly and colleagues [32] showed earlier that hyphal formation triggers Nlrp3 inflammasome activation in murine macrophages. However, there may be intrinsic differences between murine and human macrophages in terms of hyphal recognition and inflammasome

activation. In contrast to human macrophages, in which hyphae induce IL-1β transcription and inflammasome activation, the priming of murine macrophage with LPS is a prerequisite for effective IL-1β production by fungal hyphae. This implies that in mice hyphae, formation triggers Nlrp3 inflammasome activation but not pro-IL-1β translation. As LPS by itself could only induce pro-IL-1β translation in macrophages, no active IL-1β could be released without the activation of inflammasome [21]. Nevertheless, the finding by Joly et al. [32] laid the foundation to explore the role of hyphal germination and inflammasome activation in human macrophages.

We also addressed the question as to why only hyphae, but not yeasts, induced inflammasome activation in human macrophages. An important difference between these fungal cell types was noted in terms of the length of the mannan fibrils in yeasts and hyphae. The length of these fibrils is shorter, and mannans may be less branched on the hyphal cell wall. This could make the inner PAMPs more easily accessible for macrophage PRRs. To test this hypothesis, we stimulated macrophages with the glycosylation defection Δ*och1 C. albicans* strain, which lacks highly branched outer-chain mannans. The Δ*och1 C. albicans* strain induced a stronger stimulation of IL-1β secretion. From these findings, we conclude that although the mannan fibrils may not be responsible for the induction of IL-1β, the absence of a fully matured, branched outer-mannan layer permits the enhanced recognition of inner structures of the *C. albicans* cell wall, which in turn induces IL-1β production.

The loss of branched mannans in the Δ*och1 C. albicans* strain results in the activation of cell-wall biosynthetic pathways, which result in higher relative amounts of β-glucans (88.3% vs. 59.5% in WT) and chitin (1.8% vs. 1.0% in WT) [13]. This suggests that the enhanced recognition of these fungal components by specific PRRs, such as the dectin-1/Syk pathway for the recognition of β-glucans, may be responsible for the augmented production of IL-1β. To test this, we performed a set of experiments using Syk inhibitors and cells isolated from patients with a dectin-1 deficiency [27]. We observed that the dectin-1/Syk pathway was partly responsible for the induction of IL-1β. A possible secondary mechanism could involve the recognition of chitin by an as-yet-unknown receptor. These data are supported by a recent in vitro study implicating the β-glucan recognition pathway by dectin-1 for the activation of the inflammasome [33].

Colonization by *C. albicans* yeasts is tolerated by the host defense system and does not elicit a strong inflammatory reaction. However, during conversion of *C. albicans* yeasts to hyphae and tissue invasion, inflammasome activation leads to caspase-1 activation and IL-1β secretion, which is instrumental in inducting a protective Th17 response (Fig. 5E). The dectin-1/Syk pathway appears therefore to act as a master regulator of the differential recognition of colonizing yeasts versus invading hyphae, mainly through the activation of the IL-1β/Th17 pathway.

The model proposed in this study is based on experiments in human cells, but it is supported by murine studies. Mouse macrophages activate the inflammasome in response to hyphal components [32], whereas mice defective in inflammasome components [10, 11] or IL-17Rs [4] are highly susceptible to systemic or oropharyngeal candidiasis. In addition, a critical role of macrophages in mucosal immunity has been shown in clearing primary and secondary pneumococcal colonization in a Th17-dependent manner [34]. Here, we propose that in addition to their role in controlling bacterial colonization, macrophages discriminate colonization from invasion during *C. albicans* infection.

In conclusion, in this study, we demonstrate that the immune mechanism responsible for the activation of antifungal defense during tissue invasion by *C. albicans* hyphae is the IL-1β-driven Th17 response. The differential induction of Th17 responses by colonizing yeasts (no induction) and invading hyphae (potent induction of Th17) represents one of the main immune mechanisms discriminating between *C. albicans* colonization and invasion states. The mechanisms behind this process are complex: (i) conversion from yeast cells into hyphae is essential for Th17 induction; (ii) inflammasome activation associated with hyphae formation is the discriminating step during production of IL-1β and the subsequent IL-17 induction; (iii) dectin-1/Syk recognition of β-glucans is an essential component of inflammasome activation and IL-1β release (this step is defective in patients with dectin-1 deficiency); and (iv) differences in the mannan architecture in yeast and hyphal cells may result in the recognition of inner cell-wall components such as β-glucans in hyphae. This is supported by the observation that glycosylation-defective *C. albicans* strains induce higher IL-1β production by macrophages.

These findings have important conceptual and practical consequences. First, they describe one of the likely mechanisms responsible for discriminating between benign colonization and harmful invasion by *C. albicans*. The proper discrimination of these two states is crucial for avoiding inappropriate inflammation of colonized mucosal surfaces, while retaining a vigorous inflammatory response to cells that penetrate beyond the most superficial layers of the mucosa. Second, this model of tissue recognition of invasion is likely to be important for other colonizing microorganisms: in case of bacteria, the second signal needed to trigger inflammasome activation may be the release of ATP from dead cells during tissue invasion [21]. Our model suggests that during nonsymptomatic carriage of *C. albicans* in nondiseased individuals, an equilibrium may be established in which commensal carriage of yeast cells is sustained by successful Th17-dependent elimination of opportunistic infiltration of hyphae in the outer mucosal layers. When the normal microbiological or immunological balance at the mucosal surface is perturbed, increases in the numbers of invading hyphae may then represent the trigger for a vigorous inflammatory defense. Future validation of these findings in a mucosal candidiasis experimental model, assessing in vivo the role of the dectin-1/Syk/inflammasome pathway for IL-17 production, will be of great importance to complement the in vitro findings reported here.

Finally, our findings provide important clues about the mechanisms through which tolerance may be lost during inflammatory bowel disease: it is important to note that in Crohn's disease, specific anti-*C. albicans* antibodies have been described [35]; genetic variants in the inflammasome component *NLRP3* predispose to disease [36]; and *C. albicans* exacerbates colitis in mice [37]. This study provides clues about the mechanisms that may have contributed to a defective anti-*C. albicans* response in the intestinal mucosa of these patients and may offer a new therapeutic approach to this disease.

## AUTHORSHIP

S-C.C., N.A.R.G., and M.G.N. designed, performed, analyzed, and wrote the paper. F.L.V. performed experiments from CMC and HIES patients. M.L. performed TEM experiments. M.S., T.P., and S.S. performed dectin-1-deficient patient experiments. L.M. and K.P. performed *C. albicans* cell-wall porosity experiments. L.R., D.C., T-D.K., J.W.M.M., B.J.K., L.A.B.J., N.A.R.G., and M.G.N. anayzed the data and contributed to writing the manuscript.

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