Cutting Edge: *Candida albicans* Hyphae Formation Triggers Activation of the Nlrp3 Inflammasome

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The proinflammatory cytokine IL-1β plays an important role in antifungal immunity; however, the mechanisms by which fungal pathogens trigger IL-1β secretion are unclear. In this study we show that infection with *Candida albicans* is sensed by the Nlrp3 inflammasome, resulting in the subsequent release of IL-1β. The ability of *C. albicans* to switch from a unicellular yeast form into a filamentous form is essential for activation of the Nlrp3 inflammasome, as *C. albicans* mutants incapable of forming hyphae were defective in their ability to induce macrophage IL-1β secretion. Nlrp3-deficient mice also demonstrated increased susceptibility to infection with *C. albicans*, which is consistent with a key role for Nlrp3 in innate immune responses to the pathogen *C. albicans*. The Journal of Immunology, 2009, 183: 3578–3581.

*Candida albicans* is a fungal pathogen that can cause severe opportunistic infections in immunocompromised patients. *C. albicans* can switch from a unicellular yeast form into two distinct filamentous multicellular forms, pseudohyphae and hyphae. The ability to switch between yeast and filamentous forms is believed to be important for *Candida* virulence (1). In addition, *C. albicans* dimorphism results in a differential interaction with dendritic cells, skewing Th cell responses both in vitro and in vivo (2).

Signaling through the IL-1R has been shown to be important for host defenses against *C. albicans* (3). Consistent with this, IL-1α- and IL-1β-deficient mice have increased mortality rates and candidal burdens compared with wild-type (WT) mice challenged i.v. with *C. albicans* (4). Nlrp3 (also known as Nalp3 and cryopyrin), a member of the NLR (nucleotide-binding domain leucine-rich repeat containing) family, along with ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and caspase-1, forms a multiprotein complex called the Nlrp3 inflammasome (5). The Nlrp3 inflammasome can activate caspase-1 in response to a number of diverse stimuli, resulting in the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 (5). Nlrp3 plays an important protective role against a number of pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, and the influenza virus (6–9); however, whether and how fungi can activate the inflammasome remains unknown.

In this study we show that *C. albicans* can induce activation of caspase-1 and secretion of IL-1β through activation of the Nlrp3 inflammasome. We also demonstrate that the ability of *C. albicans* to switch from a unicellular yeast form into a filamentous form is essential for activation of the Nlrp3 inflammasome. Finally, we show that the Nlrp3 inflammasome has a profound influence on in vivo control of the fungal pathogen *C. albicans*.

**Materials and Methods**

**Mice**

The generation of Nlrp3-, ASC-, caspase-1-, and Nlrc4-deficient mice has been described previously (10, 11). All protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Iowa (Iowa City, IA).

**Candida strains and mutants**

The *C. albicans* clinical isolates FC20, FC16, FC10, FC5 (12), SC5314 WT and nonfilamentous double mutant *egf1Δ/AKG1ΔΔ* (1), WO-1 (13), and ATCC UC820; *Candida bruntii* clinical isolates P31, 932638 (14), and ATCC 6258; *Candida tropicalis* clinical isolates T14, T362, and T5 (15); and *C. glabrata* clinical isolates 932474, 1480, and 932273 (16) were used in this study. For in vivo and in vitro experiments, 1 ml of a 24-h culture was diluted in 30 ml of YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose) and grown for 4 h at 30°C. For hyphal induction, 1 ml of a 24-h culture was diluted in 30 ml of YPD supplemented with 20% FCS and incubated at 37°C for 3–4 h. Heat-killed *C. albicans* were obtained by incubation for 90 min at 65°C. UV-killed *C. albicans* were obtained by exposure to 4 × 100 millijoules (UV Stratalinker 2400; Stratagene).

**In vitro stimulation of macrophages**

Bone marrow-derived macrophages (BMM) were generated as previously described (14). BMM that were either unstimulated or primed with 50 ng/ml LPS for 3–4 h were infected with *Candida* at a multiplicity of infection (MOI) of one macrophage (M4) to 10 yeast cells for 6 h or for the indicated times and concentrations. Ab pairs for the IL-1β ELISA were from R&D Systems. Western blotting was performed as previously described (14). Cytochalasin D, cytochalasin B, cathespin B inhibitor CA-074-Me, and the cathespin L inhibitor V were obtained from Calbiochem and added 10 min (for cytochalasin) and 30 min (for cathespin inhibitors) before the addition of *C. albicans*.
C. albicans induces IL-1β secretion from LPS-primed Mφ. A, BMM from WT mice were either primed with 50 ng/ml LPS or left untreated. Mφ were infected with the C. albicans clinical isolate FC20 or ATCC strain UC820, and culture supernatants were collected 6 h later; IL-1β was measured by ELISA. B, LPS-primed Mφ were infected with C. albicans strain FC20 at the indicated MOI for 6 h; culture supernatants were collected and IL-1β release was measured by ELISA. C and D, LPS-primed Mφ were infected with the indicated C. albicans strains (C), and live, heat-killed (HK), or UV-killed FC20 and zymosan (100 μg/ml) (D). IL-1β release into culture supernatants 6 h after challenge was measured by ELISA. Determinations were performed in triplicate and presented as the mean ± SEM. Results are representative of two (C and D) and three (A and B) separate experiments.

In vivo infection with C. albicans

Nlrc4-deficient mice (n = 4) and WT mice (n = 4) were infected i.v. with 5 × 10^6 CFU of C. albicans strain FC20. To assess organ colonization, the kidney, liver, and spleen were harvested 6 days postinfection, and dilutions of homogenized organs were plated onto YPD plates and counted.

Results and Discussion

C. albicans induces macrophage IL-1β secretion in vitro

To determine whether C. albicans could directly induce the secretion of IL-1β, we infected WT Mφ with two different strains of C. albicans (Fig. 1A). Infection of unprimed Mφ with C. albicans failed to induce the secretion of IL-1β. However, infection of LPS-primed Mφ resulted in a dose-dependent secretion of IL-1β (Fig. 1, A and B). Maximal IL-1β secretion was reached 4 h after infection (supplemental Fig. 1A). To ensure that the ability of C. albicans to induce IL-1β secretion from LPS-primed Mφ was not strain specific, we tested multiple clinical isolates of C. albicans from different genetic clades (12). All C. albicans strains tested were capable of inducing IL-1β secretion from LPS-primed WT Mφ (Fig. 1C), although the UC820 strain was less potent compared with the clinical isolates at the same MOI. Heat-killed or UV-inactivated C. albicans and zymosan failed to induce IL-1β secretion from LPS-primed Mφ (Fig. 1D), suggesting that C. albicans-mediated activation of Mφ for IL-1β secretion is an active process on the part of Candida and is independent of the sole stimulation of major surface receptors for C. albicans such as Dectin-1 or TLR2.

C. albicans induces IL-1β secretion in an Nlrc4-dependent manner

We used Mφ deficient in specific components of the Nlrc4 inflammasome to test whether they were required for C. albicans-induced IL-1β secretion. LPS-primed Nlrc4−/−, ASC−/−, and caspase-1-deficient Mφ displayed a marked defect in their ability to secrete IL-1β in response to C. albicans (Fig. 2, A and B). In contrast, Mφ deficient in Nlrc4, which is important for Mφ caspase-1 activation in response to infection with type III or type IV secretion system-carrying bacteria (5), had an intact response to infection with C. albicans and were capable of secreting IL-1β (Fig. 2A). C. albicans infection of LPS-primed WT Mφ resulted in the activation of caspase-1 as detected by Western blotting with the appearance of the p10 cleavage product (Fig. 2B). We did not observe caspase-1 activation in response to C. albicans in either Nlrc4−/− or ASC−/−, LPS-primed Mφ (Fig. 2B). Despite the marked defect in caspase-1 activation and IL-1β secretion observed in Nlrc4-deficient Mφ, Nlrc4 deficiency did not alter the cytotoxic effects of C. albicans on the Mφ (supplemental Fig. 1A and B). These data identify the Nlrc4 inflammasome as required for caspase-1-mediated IL-1β secretion in response to infection with C. albicans.

To assess the relevance of these findings in vivo, fungal burdens in organs of WT and Nlrc4−/− mice after i.v. infection with C. albicans were determined (Fig. 2C). Fungal burdens in the kidney, spleen, and liver were higher (10–100-fold) in Nlrc4-deficient mice compared with WT mice. Hence, Nlrc4 is important in the innate immune defenses required to control replication of C. albicans in vivo.
C. albicans-induced IL-1β secretion requires internalization and cathepsin B activity

To understand how *C. albicans* might activate the Nlrp3 inflammasome, we tested whether the endocytic ability of Mφ was required for *C. albicans*-induced IL-1β production. Inhibiting actin polymerization with cytochalasin D or B reduced IL-1β production from Mφ infected with *C. albicans* (supplemental Fig. 2A), suggesting that phagocytosis of *C. albicans* by Mφ is needed to activate the Nlrp3 inflammasome for the resultant processing and secretion of IL-1β. Neither cytochalasin D or B reduced IL-1β production in response to stimulation with ATP, which uses the P2X7 receptor to activate the Nlrp3 inflammasome, confirming that macrophages were still viable and capable of secreting Nlrp3-dependent IL-1β (supplemental Fig. 2A).

Using the cathepsin B inhibitor CA-074-Me, but not the cathepsin L inhibitor V, we observed inhibition in the secretion of IL-1β following infection with *C. albicans* (supplemental Fig. 2B). This result suggests that, similar to activation of the inflammasome by silica, amyloid-β, and influenza, (5, 8, 17), *C. albicans* activation of the Nlrp3 inflammasome may be linked to lysosomal damage. A recent study by Gross and colleagues (18) showed that cathepsin B was dispensable for *C. albicans*-induced IL-1β secretion in dendritic cells. Further studies to examine the differences in the requirement for cathepsin B between dendritic cells and Mφ are needed. *C. albicans*-induced secretion of IL-1β was, however, independent of endogenous ATP release, because Mφ deficient in P2X7 receptors were still capable of secreting IL-1β (supplemental Fig. 2C). Taken together, these data suggest that *C. albicans*-induced activation of the Nlrp3 inflammasome shares similar pathways used by other danger-associated molecular patterns and pathogens that are capable of activating the Nlrp3 inflammasome.

The ability for *C. albicans* to form hyphae is essential for their efficient activation of the Nlrp3 inflammasome

As the switch to a filamentous form is crucial for *C. albicans* to avoid killing by providing a means to evade the phagosome (1), we hypothesized that membrane disruption by hyphae or pseudohyphae may be necessary for inflammasome activation. Interestingly, IL-1β secretion by LPS-primed Mφ exposed to hyphae was markedly lower than that by LPS-primed Mφ challenged with the yeast form (Fig. 3A). Studies examining the interaction between *C. albicans* and Mφ have shown that 1 h following the ingestion of yeast by the Mφ, *C. albicans* start to form germ tubes that eventually develop into hyphae (1). We therefore tested whether the transition to a hyphal form was important for triggering IL-1β secretion as opposed to the Mφ interaction with the hyphae themselves. We studied the ability of the *C. albicans* efg1ΔΔcph1ΔΔ double mutant, locked in the yeast phase and therefore deficient in both pseudohyphae and hyphae (1), to induce IL-1β secretion by LPS-primed Mφ. The *C. albicans* efg1ΔΔcph1ΔΔ double mutant failed to induce IL-1β secretion compared with its parental WT strain even at high MOI values (Fig. 3B). Infection of LPS-primed Mφ with the WT parental strain resulted in the activation of caspase-1 as detected by the appearance of p10 by Western blotting at MOI values of 1:10 and 1:20 (Fig. 3C), whereas no caspase-1 activation was observed in response to the
C. glabrata cph1/H9004 (data not shown). Infection of WT mice with the efg1Δ/cph1Δ/Δ double mutant in vivo also resulted in significantly less serum IL-1β compared with mice infected with WT C. albicans (Fig. 3D). To further examine the role of the C. albicans mcyc2 gene in Nlrp3 inflammasome activation, we studied the ability of phenotypic switching to modulate IL-1β secretion. Phenotypic switching is a spontaneous and reversible mechanism affecting the cellular morphology, colony shape, and cell physiology of most C. albicans strains. The WO-1 strain switching system consists of a reversible transition between a white domed-shape colony “white phase” and a larger flat gray colony “opaque phase” (13). More importantly for our study, the WO-1 opaque phenotype multiplies solely as a unicellular cell (yeast form), whereas the white phenotype retains the ability to form hyphae and pseudohyphae (13). Consistent with our findings that the efg1Δ/cph1Δ/Δ double mutant fails to induce Nlrp3 inflammasome activation, the WO-1 opaque phenotype, which does not produce hyphae and pseudohyphae, had a markedly diminished ability to induce the secretion of Mφ IL-1β (Fig. 3E).

Because hyphae and pseudohyphae are two different filamentous forms with distinct properties, we next evaluated whether inflammasome activation was strictly dependent on the bud-hyphae transition or whether pseudohyphae could also mediate this pathway. We tested the ability of C. tropicalis and C. krusei (which form pseudohyphae but not hyphae) and C. glabrata (which produces rare pseudohyphae under specific conditions) (19) but not hyphae) to induce IL-1β secretion from LPS-primed Mφ. Infection of LPS-primed Mφ with C. tropicalis and C. krusei, but not C. glabrata, resulted in IL-1β secretion (Fig. 3F). Although C. glabrata was still capable of inducing the secretion of TNF-α from LPS-primed Mφ (data not shown), there may be additional species differences, other than hyphae production, between C. glabrata and C. albicans that may account for the lack of induction of IL-1β secretion. Together, these data demonstrate that candidal activation of the inflammasome is dependent upon the ability of Candida to form filaments, although not necessarily the filaments themselves. This further underscores the role of phenotypic plasticity in C. albicans modulation of the host innate immune response.

In conclusion, we demonstrated a key role for the Nlrp3 inflammasome in host defense against C. albicans. A recent study by Gross and colleagues is consistent with our finding that Nlrp3 is important in antiscidental host defense (18). Their findings and our results highlight the differences in the mechanisms of inflammasome activation in different myceloid cells. In this study, we show that C. albicans activation of the Nlrp3 inflammasome in Mφ requires a two-step activation process, with the first signal consisting of a TLR ligand. In contrast, dendritic cells appear to obtain both signal 1 and signal 2 from the yeast to the filamentous phase is a crucial component for signal 2 in Nlrp3 inflammasome activation. Consistent with this difference in the requirement of a separate signal 1 by Mφ compared with dendritic cells, Goodridge et al. demonstrated that Dectin-1-CARD9 failed to activate NF-κB and trigger TNF-α production in resting Mφ (20). Taken together, these findings have intriguing implications for the role of the Nlrp3 inflammasome in the pathogenesis of fungal diseases, and stimulation of the Nlrp3 inflammasome may provide a possible therapeutic adjunct for the treatment of candidal infections.

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Disclosures
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