EC
Journals.ASM.org

Candida albicans Triggers NLRP3-Mediated Pyroptosis in Macrophages

Melanie Wellington,[a] Kristy Koselny,[a] Fayyaz S. Sutterwala,[c] Damian J. Krysan[a,b]

Departments of Pediatrics[a] and Microbiology/Immunology,[b] University of Rochester School of Medicine and Dentistry, Rochester, New York, USA; Department of Internal Medicine, University of Iowa Carver College of Medicine, Iowa City, Iowa, USA[c]

Pyroptosis is an inflammasome-mediated programmed cell death pathway triggered in macrophages by a variety of stimuli, including intracellular bacterial pathogens. Activation of pyroptosis leads to the secretion of interleukin-1β (IL-1β) and pore-mediated cell lysis. Although not considered an intracellular pathogen, *Candida albicans* is able to kill and, thereby, escape from macrophages. Here, we show that *C. albicans*-infected bone marrow-derived macrophages (BMDM) and murine J774 macrophages undergo pyroptotic cell death that is suppressed by glycine and pharmacologic inhibition of caspase-1. Infection of BMDM harvested from mice lacking components of the inflammasome revealed that pyroptosis was dependent on caspase-1, ASC, and NLRP3 and independent of NLRC4. In contrast to its role during intracellular bacterial infection, pyroptosis does not restrict *C. albicans* replication. Nonfilamentous *Candida* spp. did not trigger pyroptosis, while *Candida krusei*, which forms pseudohyphae in macrophages, triggered much lower levels than did *C. albicans*. Interestingly, a *Saccharomyces cerevisiae* strain from the filamentous background Σ1278 also triggered low, but significant, levels of pyroptosis. We have found that deletion of the transcription factor *UPC2* decreases pyroptosis but has little effect on filamentation in the macrophage. In addition, a gain-of-function mutant of *UPC2* induces higher levels of pyroptosis than does a matched control strain. Taken together, these data are most consistent with a model in which filamentation is necessary but not sufficient to trigger NLRP3 inflammasome-mediated pyroptosis. This is the first example of a fungal pathogen triggering pyroptosis and indicates that *C. albicans*-mediated macrophage damage is not solely due to hypha-induced physical disruption of cellular integrity.

Candida albicans is a commensal organism that is part of the normal flora of the human gastrointestinal and urogenital tract (1). In immunocompetent individuals, *C. albicans* causes superficial mucosal diseases such as thrush in infants and vulvovaginitis in women. Immunocompromised individuals develop either more severe mucosal disease, as is the case with people living with HIV/AIDS, or invasive infections of the bloodstream and deep organs (2). One major risk factor for the latter manifestation of *C. albicans* infection is neutropenia, or decreased numbers of circulating neutrophils (2). In addition to neutrophils, other phagocytes, such as dendritic cells and macrophages, also play an important role in the immune response to *C. albicans* as well as other *Candida* spp (3). Recent studies have shown that the role of macrophages varies over the course of infection and appears to be most crucial during the early stages of candidiasis (4). In addition, macrophages are more important to the immune response in organs such as the kidneys and less crucial in others (5). Although the importance of macrophages to the immune response to candidiasis is becoming better appreciated, much remains to be learned about the mechanisms of this host-pathogen interaction.

One of the key mechanisms by which macrophages respond to *C. albicans* is through activation of the inflammasome (6, 7, 8, 9). The inflammasomes are a set of multiprotein complexes which participate in the host response to a variety of stimuli, including microbes, endogenous lipids, amyloid, and silica and other particles (10). Because of their extensive roles in both the response to pathogens and inflammatory diseases, the inflammasomes have been the subject of intensive research. A variety of inflammasomes has been characterized, and they are classified by the nucleotide-binding domain leucine-rich repeat receptor (NLR) component of the complex (11). Multiple inflammasomes have been shown to play a role in mediating the macrophage and/or dendritic cell response to *C. albicans* (6, 7, 8, 9, 12). For example, the NLRP3 inflammasome is activated in macrophages (6, 7, 8) while the NLRC4 inflammasome is triggered in mucosal stromal cells (9). These inflammasomes, composed of an NLR, ASC, and caspase-1, mediate the processing of procaspase-1 to its active mature form (11). The best-characterized function of caspase-1 in response to *C. albicans* is to process pro-interleukin-1β (pro-IL-1β) and/or pro-IL-18 into their mature, active forms. Thus, activation of the NLRP3 and/or NLRC4 inflammasomes typically provides the tightly regulated "second signal" required for elaboration of IL-1β in response to pathogens; the "first signal" is activation of pathogen recognition receptors (PRR), which leads to production of pro-IL-1β. Importantly, mice lacking components of the inflammasome are more susceptible to disseminated *C. albicans* infection (6, 7, 8) and less able to limit superficial candidiasis to the mucosa (9).

The *C. albicans* factor(s) that mediates inflammasome activation has not been specifically identified. In response to other stimuli, activation of the NLRP3 inflammasome has been associated with direct physical or chemical damage to the phagolysosome (13). Within macrophages (14), *C. albicans* undergoes a morpho-

Received 17 December 2013 **Accepted** 20 December 2013
Published ahead of print 27 December 2013
Address correspondence to Melanie Wellington, melanie_wellington@urmc.rochester.edu, or Damian J. Krysan, damian_krysan@urmc.rochester.edu.

doi:10.1128/EC.00336-13

genetic transformation from round yeast forms to filaments (hyphae and pseudohyphae). The filaments have long been thought to cause physical disruption of both the phagolysosome and, ultimately, the macrophage, allowing the fungus to escape from the macrophage. Putting these two phenomena together, filamentation-mediated physical disruption of the vacuole provides a reasonable model for the mechanism of inflammasome activation by *C. albicans*. Further supporting this mechanism, multiple laboratories observed that the nonfilamentous *C. albicans* strain lacking Efg1, a master regulator of filamentation, failed to activate capase-1 processing and trigger IL-1β production (6, 7, 8). However, our laboratory has recently shown that *C. albicans* strains lacking the transcription factor Upc2 or Ahr1 form filaments within the macrophage but fail to trigger IL-1β secretion (15), indicating that filamentation alone is not sufficient to trigger inflammasome activation and that additional factors associated with the filamentous form of *C. albicans* are also required.

In our previous work (15), we also observed that the ability of *C. albicans* mutants to cause macrophage lysis correlated with their ability to induce IL-1β production. For example, the filamentation-competent *upc2*Δ/Δ and *ahr1*Δ/Δ mutants were dramatically deficient in the ability to cause macrophage lysis, indicating that the physical presence of filamentous *C. albicans* within the macrophage was not sufficient to cause lysis. To explain these observations, we hypothesized that *C. albicans* may trigger the inflammasome-mediated programmed cell death pathway pyroptosis. Pyroptosis is a caspase-1-dependent process which leads to IL-1β and IL-18 production as well as macrophage cell lysis (16). Pyroptosis is distinguished from apoptosis in that the macrophages lose cell integrity and the process is proinflammatory; it is distinct from simple cell necrosis because it is mediated by the inflammasome and caspase-1. Initially described in macrophages infected with intracellular bacteria such as *Salmonella* and *Legionella* species, pyroptosis appears to facilitate clearance of some pathogens by destroying their replicative niche and activating other components of the immune response (17).

As described here, we demonstrate that *C. albicans* triggers macrophage pyroptosis through the NLRP3 inflammasome. To our knowledge, this represents the first example of pyroptosis being triggered by a fungal or other eukaryotic pathogen. Our observations indicate that a substantial portion of macrophage damage caused by *C. albicans* is due to a programmed cell death pathway and not simply due to the physical disruption of the macrophage by growing hyphae.

MATERIALS AND METHODS

Yeast strains and macrophage cell lines. Unless otherwise indicated, all experiments were performed using *C. albicans* strain SN250 (18). *Candida krusei* ATCC 6258, obtained via the University of Rochester clinical microbiology laboratory, is now listed as *Issatchenkia orientalis* (the teleomorph of *C. krusei*) at the American Type Culture Collection (Manassas, VA). *Candida parapsilosis* RO78-G5 was a clinical isolate obtained from Wendy Watson (19). *Candida glabrata* BG2 (20) was obtained via Constantine Haidaris, University of Rochester Medical Center. *C. albicans* mutants lacking the transcription factor Upc2, Ahr1, Bcr1, Ndt80, or Rca1 are derived from SN152, constructed by Homann et al., and obtained from the Fungal Genetics Stock Center (21). Clinical isolates S1 (*UPC2/UPC2*) and S2 (*UPC2/upc2*^{GOF}) were obtained from David Rogers, University of Tennessee Health Sciences Center (22). *Saccharomyces cerevisiae* strain BY4741 (S288c background) was obtained from Open Biosystems, and HLY335 was a generous gift of Gerry Fink (Whitehead Institute). All yeasts were grown overnight at 30°C in yeast extract-peptone-dextrose medium (YPD) and washed three times in phosphate-buffered saline (PBS) prior to use in macrophage coculture assays. Where indicated, yeasts were heat killed by incubation at 65°C for 90 min or UV inactivated in a Stratalinker chamber with four sequential doses of 0.1 J/cm, as previously described (23). The mouse macrophage-like cell line J774 was obtained from the ATCC (specifically, J774A.1 cells, TIB-67) and maintained as recommended by the supplier.

Mouse strains and animal handling. The Institutional Animal Care and Use Committee at the University of Rochester and the University of Iowa approved all protocols used in this study. BALB/c, C57BL/6, and A/J mice were obtained from the National Cancer Institute (Frederick, MD). The generation of NLRP3 (23)-, ASC (24)-, NLRC4 (25)-, and caspase-1 (26)-deficient mice has been previously described. NLRP3-, ASC-, and NLRC4-deficient mice have been backcrossed onto the C57BL/6 genetic background for 9 generations. Caspase-1-deficient mice have been backcrossed onto the C57BL/6 genetic background for 10 generations; however, these mice have recently been shown to be deficient for functional caspase-11. For clarity, the designation "caspase-1" is used to include both caspase-1 and caspase-11 throughout this work.

Harvest and culture of mouse BMDM. Bone marrow-derived macrophages (BMDM) were prepared by harvesting bone marrow from femurs and tibias of the relevant mouse strain. Bone marrow was then strained, washed, and cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% GlutaMAX, 1% penicillin-streptomycin, 10 mM HEPES, and macrophage colony-stimulating factor (m-CSF) (50 ng/ml; eBioscience, San Diego, CA). Cells were cultured in untreated plastic dishes to allow separation of adherent macrophages from other nonadherent cell types. Cells were fed every 2 to 3 days, and nonadherent cells were removed by gentle washing. Adherent BMDM were removed from the dishes by gentle scraping just prior to use.

Macrophage-yeast coculture. J774A.1 cells or BMDM were counted using a hemocytometer, diluted appropriately, and plated in a 96-well tissue culture dish at a final density of 1e5 cells per well in high-glucose (4.5-g/liter) RPMI medium containing 1% FBS. Unless indicated, macrophages were stimulated with lipopolysaccharide (LPS) (*Escherichia coli* O111:B4; Calbiochem) at a final concentration of 50 ng/ml. Where indicated, macrophages were pretreated by addition of glycine (5 mM) or the caspase-1 inhibitor z-YVAD-fmk (100 μM), diluted in medium. For control samples, macrophages were treated with medium containing the relevant carrier: PBS for LPS and/or glycine or dimethyl sulfoxide (DMSO) for z-YVAD-fmk (final DMSO concentration, 0.1%). For all experiments, the final volume during pretreatment was 100 μl/well. Treated macrophages were incubated for 2 h at 37°C, in a 5% CO_2, humidified environment prior to inoculation. The titer of the inoculum of washed yeast was determined with a hemocytometer, and the inoculum was diluted as needed in PBS and added to macrophages in a volume of 20 μl. Unless otherwise noted, experiments were carried out at a multiplicity of infection (MOI) of 2 yeasts to 1 macrophage. After inoculation, cocultures were incubated for 5 h at 37°C, in a 5% CO_2, humidified environment. At the end of the coculture period, one 40-μl aliquot of the culture supernatant from each well was transferred into an opaque white 96-well plate for analysis of lactate dehydrogenase (LDH) release and a second 40-μl aliquot was transferred into a separate plate containing 160 μl/well of enzyme-linked immunosorbent assay (ELISA) blocking solution. LDH release was measured immediately using the Promega CytoTOX One assay kit (Promega, Madison, WI). Samples for cytokine analysis were frozen at −20°C until use. The IL-1β concentration in the coculture supernatants was determined using a ReadySetGo ELISA kit (eBioscience, San Diego, CA).

Quantification of fungistatic activity of macrophages. The ability of macrophages to kill *C. albicans* or limit its replication was quantified using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] metabolism assay as previously described (23). Briefly, a freshly prepared solution containing XTT (0.5 mg/ml), glucose (4.5 mg/

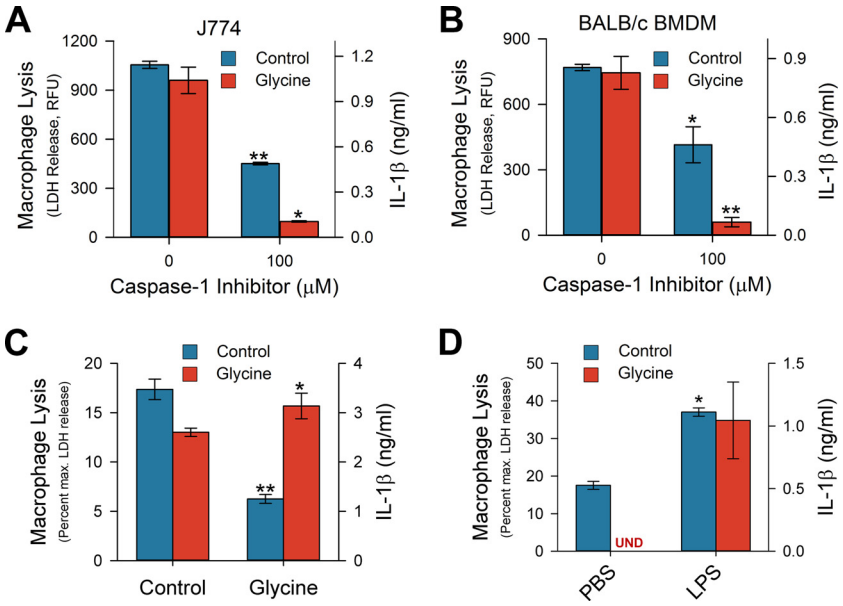

FIG 1 *C. albicans*-induced macrophage lysis has characteristic features of pyroptosis. (A and B) J774 macrophages (A) or bone marrow-derived macrophages (BMDM) from BALB/c mice (B) were treated with the caspase-1 inhibitor z-YVAD-fmk or carrier, prestimulated with LPS for 2 h, and then exposed to *C. albicans* at an MOI of 2:1 for 5 h. LDH (blue) and IL-1β (red) were measured from the supernatant of the coculture. LDH release was measured using a fluorogenic assay; data are presented as mean relative fluorescence units (RFU). (C) LPS-stimulated J774A.1 macrophages were exposed to *C. albicans* in the presence or absence of 5 mM glycine and evaluated as described above. (D) J774 macrophages were prestimulated with LPS (50 ng/ml) or PBS as a control and exposed to *C. albicans* as described above. LDH activity of supernatants (C and D) is expressed as a percentage of the maximum LDH release measured from an identical sample of detergent-treated macrophages. Each experiment was performed at least twice with similar results, and a representative single experiment is shown. Bar heights are the means of least three technical replicates, and error bars indicate standard deviations. Data were analyzed by Student's *t* test: **, $P \leq 0.001$ compared to control; *, $P \leq 0.05$ compared to control. UND, undetectable.

ml), and coenzyme Q (40 μg/ml) in PBS was filter sterilized and kept in the dark until use. After coculture, plates were centrifuged at 250 × *g* for 2 min, the supernatant was aspirated, and ice-cold 0.2% Triton X-100 in water (200 μl) was added to lyse the macrophages. After incubation for 15 min at 37°C, the plates were centrifuged again, all wells were aspirated and washed with an additional 200 μl of 0.2% Triton X-100, and the plates were centrifuged a final time. After washing, the lysis solution was aspirated from each well and replaced with XTT solution (100 μl). The plate was incubated at 37°C for 15 min in the dark, after which absorbance was measured at 450 nm with subtraction of background absorbance at 570 nm.

1,3-β-Glucan immunofluorescence assay. Surface-exposed 1,3-β-glucan was detected by indirect immunofluorescence, as previously described (23). Briefly, yeasts from an overnight culture in YPD were harvested, washed three times with PBS, treated with 3% bovine serum albumin in PBS blocking solution, and incubated for 2 h at 4°C with anti-1,3-β-glucan antibody (Biosupplies Australia Pty. Ltd., Parkville, Australia). Samples were then washed in PBS and treated with 15 μg/ml Texas Red-conjugated goat anti-mouse IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA) for 60 min at 4°C. After secondary antibody treatment, samples were washed with PBS. Fluorescence microscopy was performed using a Nikon ES80 epifluorescence microscope equipped with a CoolSnap charge-coupled device (CCD) camera using NIS-Elements software. Image processing in ImageJ and Adobe PhotoShop was limited to cropping and adjustment of brightness/contrast for ease of viewing. All images were processed identically.

RESULTS

***C. albicans* induces macrophage lysis via triggering NLRP3- and caspase-1-dependent pyroptosis.** As has been well established in the literature, *C. albicans* induces NLRP3/caspase-1-dependent IL-1β production in macrophages (6, 7, 8, 9). In addition, we and others have shown that *C. albicans*-infected macrophages lose cell membrane integrity and undergo lysis (15). To investigate whether caspase-1 activation also contributes to *C. albicans*-induced macrophage lysis via the programmed cell death pathway known as pyroptosis, we tested the effect of the caspase-1 inhibitor z-YVAD-fmk on this process. The murine macrophage cell line J774 was prestimulated with LPS and then cocultured with *C. albicans* strain SC5314 at an MOI of 2:1 for 2 h in the presence or absence of z-YVAD-fmk. As expected, z-YVAD-fmk dramatically reduced IL-1β production (Fig. 1A). Macrophage cell lysis, as determined by standard LDH release cytotoxicity assay (15), was also reduced ~2.5-fold in the presence of the caspase-1 inhibitor, consistent with the notion that caspase-1-dependent pyroptosis plays a role in *C. albicans*-mediated macrophage damage. To confirm the effect of z-YVAD-fmk on macrophage lysis, we repeated the experiments with bone-marrow-derived macrophages (BMDM) isolated from BALB/c mice. As shown in Fig. 1B, the inhibitor reduced LDH by 2-fold relative to untreated cocultures and blocked nearly all IL-1β release, further supporting a role for caspase-1-dependent processes in mediating *C. albicans*-induced macrophage lysis.

Caspase-1-dependent macrophage cell lysis appears to be mediated by pore formation in the plasma membranes (27). Although the mechanism of this process has not been fully characterized, the addition of solutes such as glycine to the culture medium suppresses pyroptotic cell lysis, presumably by blocking the pores. Since caspase-1 remains active, the addition of glycine to the extracellular milieu blocks pyroptotic lysis but does not affect release of IL-1β. We, therefore, infected J774 cells with *C.*

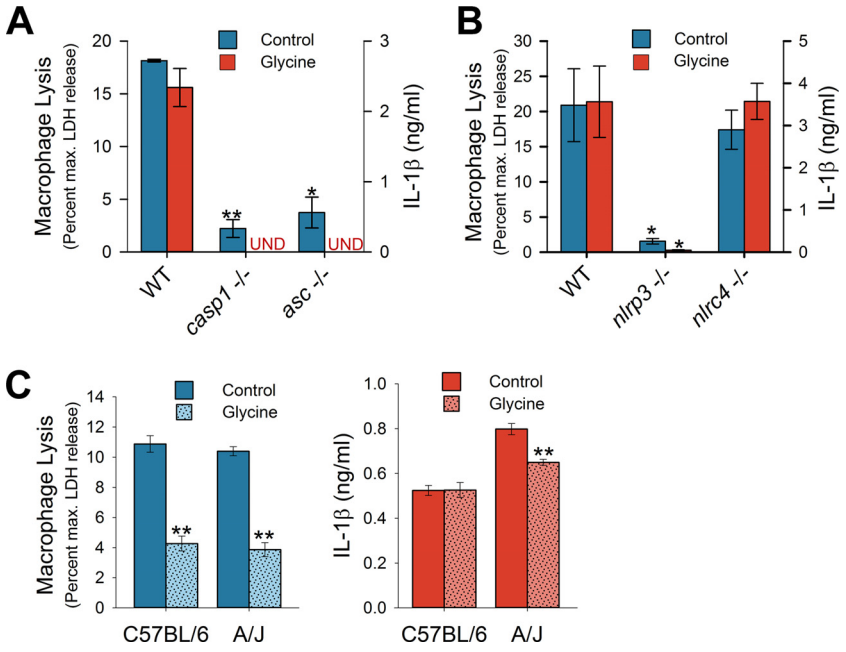

FIG 2 *C. albicans*-induced macrophage lysis is dependent on caspase-1, ASC, and NLRP3. (A and B) Bone-marrow-derived macrophages (BMDM) from C57BL/6 mice (WT) or the indicated knockout strain were prestimulated with LPS and exposed to *C. albicans* at an MOI of 2:1 for 5 h. (C) BMDM from C57BL/6 or A/J mice were treated with 5 mM glycine or PBS as a control, prestimulated with LPS, and exposed to *C. albicans* at an MOI of 2:1 for 5 h. LDH and IL-1β were measured from the supernatant of cocultures after 5 h. LDH results were expressed as a percentage of the maximum LDH release as described in the legend to Fig. 1. Each experiment was performed at least twice with similar results, and a representative single experiment is shown. Bar heights are the means of least three technical replicates, and error bars indicate standard deviations. Data were analyzed by Student's *t* test: **, $P \leq 0.001$ compared to control; *, $P \leq 0.05$ compared to WT in panels A and B and compared to control in panel C. UND, undetectable.

albicans in standard medium and medium supplemented with glycine. As shown in Fig. 1C, addition of glycine decreased *C. albicans*-induced macrophage lysis approximately 2-fold relative to exposure in standard medium. As expected, glycine did not affect *C. albicans*-induced IL-1β production. These data further support a role for pyroptosis in the interaction between *C. albicans* and macrophages.

Inflammasome-mediated, caspase-1-dependent production of IL-1β is a two-step process (11). The first signal functions to initiate transcription and translation of pro-IL-1β. The second signal then leads to assembly of the appropriate inflammasome, activation of caspase-1, and, ultimately, IL-1β processing. Priming macrophages with lipopolysaccharide (LPS) provides the first signal and is typically used in the study of the interaction of *C. albicans* with macrophages. *C. albicans* induces both pro-IL-1β production and inflammasome activation (8); however, the long incubation time necessary for *C. albicans* to trigger robust pro-IL-1β expression complicates the interpretation of experiments using unprimed macrophages because the cultures become overgrown with fungus. Interestingly, prestimulation of macrophages has been shown to promote *Yersinia*-induced pyroptosis relative to that with naive macrophages (28). Therefore, we compared the amount of *C. albicans*-induced lysis that occurs in unstimulated macrophages to that from LPS-stimulated macrophages. As shown in Fig. 1D, LDH release from LPS-stimulated macrophages was 2-fold higher than that observed with naive cells. As expected, naive cells produced no IL-1β while LPS-primed macrophages did so robustly. The increased lysis in LPS-treated cells relative to untreated cells corresponds closely to the amount of lysis that is suppressible by either glycine or z-YVAD-fmk, suggesting that the first signal also promotes *C. albicans*-induced macrophage pyroptosis.

To more definitively characterize the role of the inflammasome in *C. albicans*-induced macrophage lysis, we isolated BMDM from mice lacking caspase-1 (*casp1*$^{-/-}$) and its upstream adaptor protein ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD, *asc*$^{-/-}$) and compared the levels of LDH release/IL-1β production induced in the mutant macrophages to those isolated from wild-type (WT) mice. Consistent with the findings of others (6, 7, 8, 9), *C. albicans*-induced IL-1β production was completely abolished. LDH release was also reduced in *casp1*$^{-/-}$ and *asc*$^{-/-}$ macrophages relative to the wild type (Fig. 2A), confirming that the terminal components of the inflammasome are required for the majority of macrophage cell lysis at the time point examined. To our knowledge, this represents the first example of a fungal pathogen inducing macrophage pyroptosis and provides a previously unrecognized mechanism by which *C. albicans* causes macrophage damage.

The nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs) are the cytosolic receptors that mediate assembly of the various versions of the inflammasome in response to a variety of infectious and noninfectious stimuli (11). As discussed above, previous studies have shown that NLRP3 and NLRC4 contribute to the host response to *C. albicans* (6, 7, 8, 9). NLRP3 and NLRC4 are required for *C. albicans*-induced IL-1β production in macrophages and mucosal stromal cells, respectively. In addition, NLRC4 mediates pyroptosis in response to a number of Gram-negative bacteria (17). To identify which NLR mediates *C. albicans*-induced pyroptosis, we isolated *nlrp3*$^{-/-}$ and *nlrc4*$^{-/-}$ BMDM and exposed them to *C. albicans*. Consistent with the de-

pendence of *C. albicans*-induced IL-1β production on NLRP3 (6, 7, 8, 9), *nlrp3*$^{-/-}$ macrophages underwent essentially no lysis while *nlrc4*$^{-/-}$ macrophages were indistinguishable from the wild type (Fig. 2B). These data firmly establish that *C. albicans* induces NLRP3-dependent pyroptosis in macrophages.

In order to confirm that *C. albicans*-induced macrophage pyroptosis was not limited to a specific strain of mice, we examined the extent of macrophage lysis in BMDM isolated from A/J mice. A/J mice have a variant NAIP5 (NLR family, apoptosis inhibitory protein 5) that fails to stimulate NLRC4, and thus, unlike BMDM from C57BL/6 mice, A/J BMDM that have ingested *Legionella pneumophila* fail to undergo pyroptosis (29). The lack of macrophage pyroptosis appears to fully account for the hypersusceptibility of A/J mice to *Legionella*; C57BL/6 mice, with normal NAIP5, are relatively resistant to *Legionella*. C57BL/6 mice are also relatively resistant to systemic candidiasis, whereas A/J mice are more susceptible (30), which raises the question that susceptibility to both organisms may be related to NAIP5 and/or NLRC4 activation. However, our results with the *nlrc4*$^{-/-}$ BMDM predicted that A/J-derived BMDM would undergo pyroptosis similarly to wild-type C57BL/6 mice. Consistent with that expectation, BMDM from A/J mice undergo lysis and IL-1β production at levels very similar to those derived from C57BL/6 mice in response to *C. albicans* (Fig. 2C). Furthermore, treatment with glycine prevented lysis in both A/J and C57BL/6 macrophages, indicating that macrophage pyroptosis occurs in both types of BMDM. Thus, while NAIP5 may contribute to *C. albicans* resistance *in vivo*, it is unlikely that macrophage pyroptosis plays a role in the susceptibility of A/J mice to candidiasis. In addition, these results indicate that macrophage pyroptosis is not unique to a single strain of mice.

The fungistatic action of macrophages is independent of pyroptosis. One of the apparent functions of pyroptosis is to limit the replication of intracellular bacteria, presumably by destroying the protective niche and exposing the bacteria to other components of the immune system (17). Intracellular bacteria replicate more effectively in macrophages deficient for pyroptosis (17). *C. albicans* is not considered a professional, intracellular pathogen and presumably does not require the phagolysosome to replicate; in addition, macrophages have a modest fungistatic effect on *C. albicans*. Therefore, we investigated the effect of pyroptosis on the fungistatic activity of macrophages toward *C. albicans*. During a 5-h exposure, as in the assays above, the addition of glycine to cultures decreased macrophage lysis by 2.5-fold and yet the *C. albicans* organism burden was essentially identical to that in cultures without supplemental glycine (Fig. 3A and B). Since longer exposure times at our standard MOI of 2:1 resulted in *C. albicans* overgrowth (data not shown), we reduced the MOI to 1:5; this adjustment allowed us to extend the time of *C. albicans* exposure to 24 h. Under these conditions, glycine reduced macrophage lysis 4-fold (Fig. 3C) but had no effect on *C. albicans* replication within the macrophages (Fig. 3D). Thus, while pyroptosis occurs in response to *C. albicans*, it does not enhance the ability of macrophages to clear *C. albicans in vitro*.

***C. albicans*-induced pyroptosis is dependent on multiplicity of infection and duration of coculture.** In the studies described above, macrophages and *C. albicans* were cocultured for 5 h. This time point was chosen because it allowed ample time for *C. albicans* filamentation and for activation of macrophage responses; as noted above, longer exposure times lead to dramatic fungal overgrowth of the coculture at our standard MOI. Macrophages appear to undergo pyroptosis early during infections with intracellular bacteria, while other mechanisms of cell death become important at later time points (31). To determine if a similar shift in mechanism occurs in *C. albicans*-induced macrophage lysis, we compared lysis in BMDM derived from wild-type and *casp1*$^{-/-}$ mice over time (Fig. 4A). At the standard MOI of 2:1, there is a clear difference in the extent of macrophage lysis between *casp1*$^{-/-}$ and wild-type mice up to 9 h of exposure; at 12 and 24 h, macrophage lysis is independent of caspase-1.

One potential explanation for this observation is that the fungal burdens within the cocultures overwhelm the macrophages, leading to extensive, nonpyroptotic lysis. To test this possibility, we carried out time course experiments at different MOIs. In order to easily compare the data from different MOI at the various time points, we determined the ratio of lysis in *casp1*$^{-/-}$ macrophages to that in wild-type macrophages and plotted the data as a percentage of caspase-1-dependent lysis (Fig. 4B). At all MOIs,

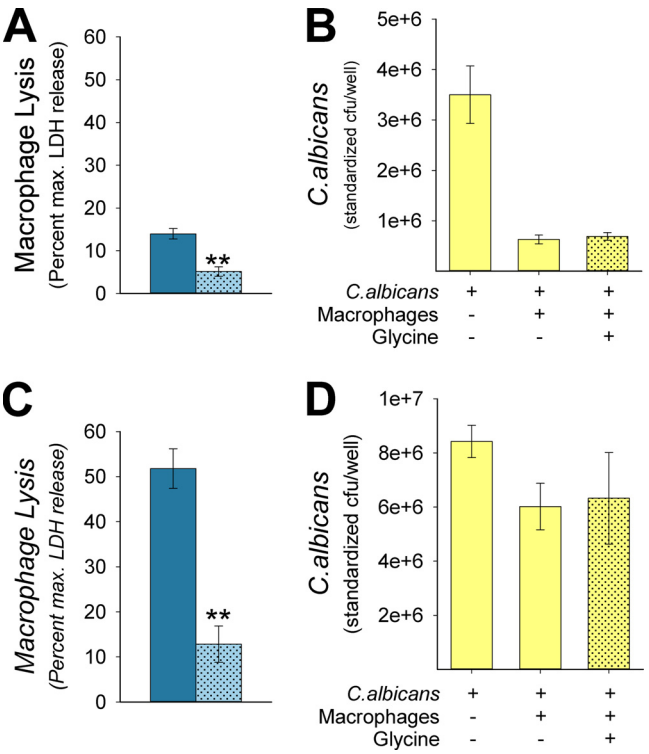

FIG 3 Pyroptotic macrophage lysis does not alter macrophage killing of *C. albicans*. J774 cells were treated with 5 mM glycine or PBS as a control, prestimulated with LPS, and exposed to *C. albicans* at an MOI of 2:1 for 5 h (A) or an MOI of 1:5 for 24 h (C). LDH was measured from the supernatant and is expressed as the percentage of maximum release as described in the legend to Fig. 1. The metabolic activity of *C. albicans* present in the well at the end of the coculture was measured using the XTT assay (B and D). A standard curve from wells containing known numbers of *C. albicans* yeast cells was used to calculate the organism density, expressed as "standardized CFU/well" because the standard curve was generated using yeast, but organisms in experimental wells were predominantly hyphae. Each experiment was performed at least three times, and results of representative experiments are shown. Bar heights represent the mean results of technical replicates ($n = 3$), and error bars indicate standard deviations. Data were analyzed by Student's t test: **, $P \leq 0.001$ compared to control. For panel B, the P value is 0.096 for comparison of wells containing *C. albicans* and macrophages with and without glycine. For panel D, the P value is 0.52 for the same comparison.

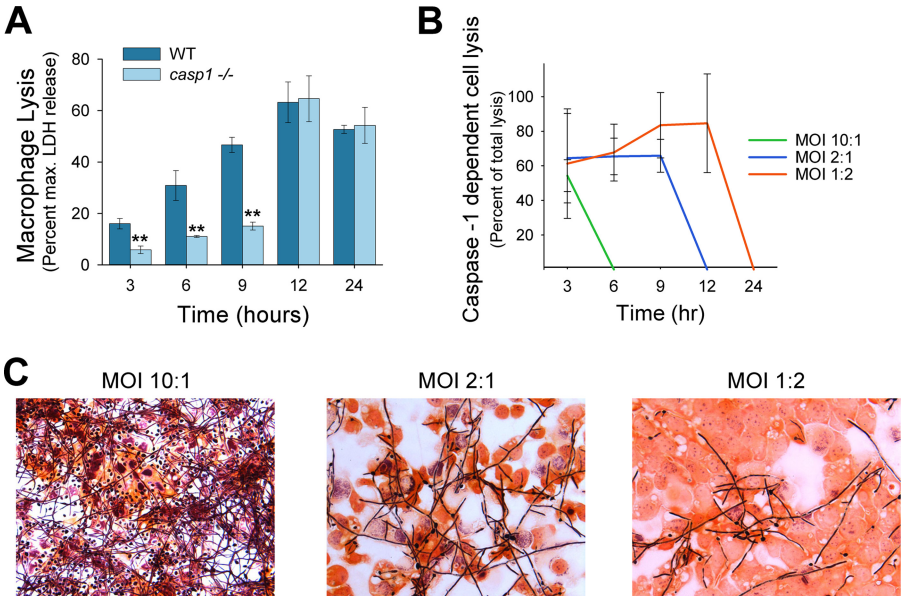

FIG 4 Caspase-1-independent macrophage lysis occurs at later time points and/or higher organism burdens. (A) Bone marrow-derived macrophages (BMDM) from C57BL/6 (WT) or caspase-1-deficient (*casp1*$^{-/-}$) mice were prestimulated with LPS for 2 h and then exposed to *C. albicans* at an MOI of 2:1 for 5 h. LDH was measured from the supernatant and is expressed as the percentage of maximum release as described in the Fig. 1 legend. Each experiment was performed at least three times, and results of representative experiments are shown. Bar heights represent the mean results of technical replicates ($n = 3$), and error bars indicate standard deviations. (B) The percentage of caspase-1-dependent cell lysis was calculated as 100 × [100 − (result for *casp1*$^{-/-}$ BMDM)/(result for WT BMDM)]. Data points represent the means and error bars indicate the propagated errors as calculated from the original standard deviations. Data were analyzed by Student's *t* test: **, $P \leq 0.001$ compared to control. (C) Parallel samples were cocultured in 8-well chamber slides. At the end of the exposure, slides were rinsed once with PBS and once with distilled water, allowed to dry, and then Gram stained. Photomicrographs of representative fields are presented. The experiment was performed twice; results were similar for the two experiments.

caspase-1-dependent lysis was predominant at 3 h. For the highest MOI (10:1), caspase-1-independent lysis was operative by 6 h, whereas for the lowest MOI (1:2), the switch from caspase-1-dependent to -independent lysis did not occur until 24 h. To illustrate the differences in organism burden associated with the MOIs examined, samples of macrophages that were inoculated with MOIs of 10:1, 2:1, and 1:2 were Gram stained after 6 h of coculture; the resulting photomicrographs are shown in Fig. 4C. After 6 h of exposure, pyroptosis remains the predominant mechanism of macrophage lysis in samples inoculated at an MOI of 2:1 or 1:2 despite the formation of extensive hyphae and apparently robust organism burden as revealed by Gram staining. At 6 h, only macrophages that were inoculated with an MOI of 10:1 demonstrate substantial nonpyroptotic lysis. In these samples, the Gram stain shows that the macrophages are almost completely overgrown by a dense network of *C. albicans* hyphae. Thus, these data indicate that pyroptosis occurs at low to moderate levels of organism burden whereas lysis independent of pyroptosis dominates if the initial inoculum is high or if the infection is allowed to proceed sufficiently to allow high levels of organism replication.

Polymorphic yeasts are stronger inducers of pyroptosis than are nonfilamentous yeasts. In order to characterize *C. albicans*-induced pyroptosis in more detail, we asked whether the process required live yeast and if it was dependent upon filamentation. First, we determined if pyroptosis required live organisms. To do so, we exposed J774 cells to live, heat-killed, and UV-inactivated *C. albicans*. Neither heat-killed *C. albicans* yeast nor UV-inactivated yeast triggered detectable macrophage lysis; as expected, heat-killed and UV-inactivated *C. albicans* also induced only low levels of IL-1β production (Fig. 5A). *C. albicans* rapidly undergoes morphogenetic transition to form hyphae and pseudohyphae (32). Thus, one possible explanation for these data is that pyroptosis is associated with filamentous growth of *C. albicans*.

Our previously published data demonstrated that formation of hyphae alone is not sufficient to trigger macrophage lysis or IL-1β production (15). In addition to changes in the physical morphology, hyphae possess a number of cellular characteristics that differ from the yeast form which could trigger inflammasome activation. To further investigate the role of filamentous growth in yeast-induced pyroptosis, we compared the responses of J774A.1 cells to various *Candida* spp. with different abilities to undergo polarized growth (Fig. 5B). Joly et al. had previously shown that *C. krusei* induced IL-1β secretion, although at lower levels than did *C. albicans* (6). In contrast, *C. glabrata* triggered essentially no IL-1β production. Both the *C. albicans* strain SN250, used as the reference strain in this work, and the well-studied *C. albicans* laboratory strain SC5314 readily provoked pyroptosis at MOIs of 2:1 and lower (Fig. 5B and data not shown). At an MOI of 2:1, *C. krusei* induced approximately 4-fold less pyroptosis than did *C. albicans*; at a substantially increased MOI (20:1), *C. krusei* induced slightly more lysis than did *C. albicans* at an MOI of 2:1. Thus, *C. krusei* is capable of triggering pyroptosis, although it is not as strong an inducer as *C. albicans*. Interestingly, the macrophage lysis induced by *C. krusei* was almost completely suppressed by glycine, suggesting that almost all of the cell death occurring in response to *C. krusei* is due to pyroptosis. These data are particularly interesting because *C. krusei* is one of the few non-*albicans* *Candida* spp. that are able to form filaments of any type; *C. krusei* forms pseudohyphae, not true hyphae, in macrophages (33). The ability of *Candida* species to trigger IL-1β production also corre-

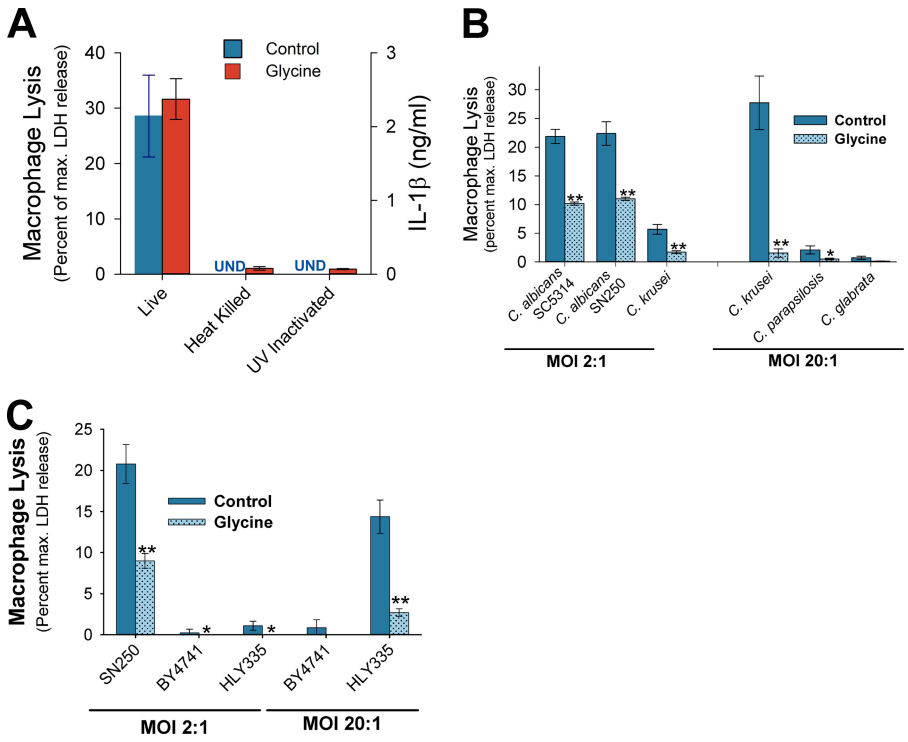

FIG 5 Yeasts that filament are stronger inducers of pyroptosis. (A) J774 macrophages were prestimulated with LPS for 2 h and then exposed to live, heat-killed, or UV-inactivated *C. albicans* at an MOI of 2:1 for 5 h. LDH (blue) and IL-1β (red) were measured from the supernatant of the coculture as described for Fig. 1. (B) LPS-stimulated J774 macrophages were exposed to the indicated *Candida* species at an MOI of 2:1 or 20:1 for 5 h and analyzed for LDH release as described for Fig. 1. (C) LPS-stimulated J774 macrophages were exposed to *C. albicans* (SN250) or *S. cerevisiae* (BY4741 and HLY335) at an MOI of 2:1 or 20:1 for 5 h, and LDH activity of the supernatant was determined as described for Fig. 1. Each experiment was performed at least three times, and results of representative experiments are shown. Bar heights represent the mean results of technical replicates ($n \geq 3$), and error bars indicate standard deviations. Data were analyzed by Student's *t* test: **, $P \leq 0.001$ compared to control; *, $P \leq 0.05$ compared to control. UND, undetectable.

lated with their ability to induce macrophage lysis (data not shown). Thus, although hyphal formation is not sufficient to trigger pyroptosis, the ability of the non-*albicans Candida* species to induce pyroptosis appears to be linked to their ability to form hyphae or pseudohyphae within macrophages.

To explore this further, we also tested the ability of the model yeast *Saccharomyces cerevisiae* to cause pyroptosis. Gross et al. had previously shown that *S. cerevisiae* induced very low levels of IL-1β secretion in mouse bone marrow-derived dendritic cells; however, the specific strain was not identified (8). *S. cerevisiae* strain BY4741, which is derived from the S288C background and is not filamentous, induced very low levels of pyroptosis at both high and low MOIs (Fig. 5C). In contrast, strain HLY335 (34), which is derived from the filamentous Σ1278 background, did not induce pyroptosis well at an MOI of 2:1 but triggered substantially increased pyroptosis at an MOI of 20:1. Consistent with the behavior observed for *C. krusei*, the lysis induced by HLY335 is highly sensitive to glycine suppression, indicating that pyroptosis contributes to the vast majority of the observed macrophage lysis (Fig. 5C). As with the non-*albicans Candida* species, this pattern correlates well with the ability of these strains to undergo polarized, pseudohyphal growth: S288C strains contain mutations that prevent them from forming pseudohyphae (35), whereas Σ1278 strains are competent in polarized growth and are used regularly in the study of pseudohyphal formation (36). Thus, hyphal and pseudohyphal phases of growth provide a much stronger trigger for pyroptosis than does the yeast phase. Taken together with our previous data, these data indicate that while filamentation is not sufficient to trigger pyroptosis, it may be necessary for pyroptosis to occur.

Upc2 regulates the *C. albicans* factor(s) that induces pyroptosis. In our previous work, we identified a set of mutants that trigger altered levels of macrophage lysis and IL-1β production (15); presumably, this is caused by differences in their abilities to trigger pyroptosis. Deletion of the transcription factor Upc2 leads to decreased lysis and IL-1β production in J774 cells. Upc2 has been well studied due to its role in regulation of ergosterol production and, consequently, fluconazole resistance (22). To determine if Upc2 function also regulates the ability of *C. albicans* to induce pyroptosis, we first asked whether the *upc2*Δ/Δ mutant induces lower levels of pyroptosis (Fig. 6A). As observed previously, the *upc2*Δ/Δ mutant induced lower levels of macrophage lysis than did the reference strain. When glycine was present, the levels of lysis triggered by SN250 and the *upc2*Δ/Δ mutant were quite similar, suggesting that the two strains do not differ in their abilities to induce pyroptosis-independent lysis, or, in other words, that the decrease in lysis induced by the *upc2*Δ/Δ mutant relative to the wild type is nearly all attributable to differences in the ability of the mutant to activate pyroptosis.

If Upc2 regulates processes required for *C. albicans* to induce pyroptosis, then we hypothesized that increasing the activity of Upc2 could increase pyroptosis. Gain-of-function alleles of *UPC2* have been isolated in *C. albicans* strains resistant to fluconazole. We obtained a pair of well-characterized *C. albicans* clinical iso-

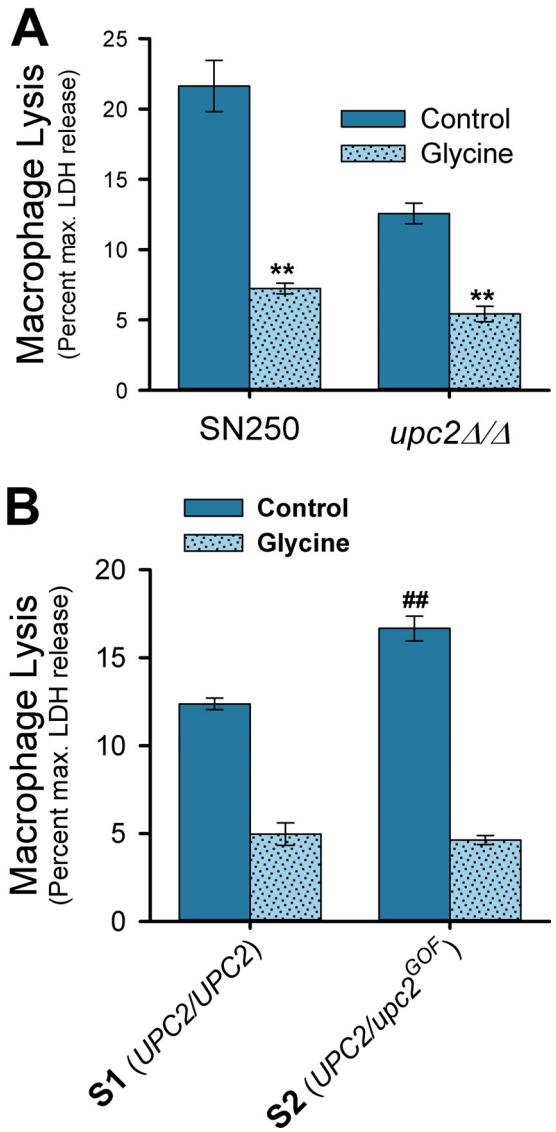

FIG 6 The *C. albicans* transcription factor Upc2 is important for induction of pyroptosis. (A) J774 macrophages were prestimulated with LPS for 2 h and then exposed to WT (SN250) or *upc2*Δ/Δ *C. albicans* at an MOI of 2:1 for 5 h in the presence or absence of 5 mM glycine. LDH was measured from the supernatant of the cocultures as described for Fig. 1. (B) LPS-stimulated J774 macrophages were exposed to *C. albicans* clinical isolate S1 or S2, as in panel A. Each experiment was performed at least two times, and results of representative experiments are shown. Bar heights represent the means of technical replicates ($n \geq 3$), and error bars indicate standard deviations. Data were analyzed by Student's *t* test: **, $P \leq 0.001$ compared to control; ##, $P \leq 0.001$ comparing S1 to S2 in the absence of glycine.

lates in which one isolate has a wild-type allele of *UPC2* while the second, obtained from the same patient but following treatment with fluconazole, has a gain-of-function mutation in one allele of *UPC2* (22). As with the *upc2*Δ/Δ mutant, the two clinical isolates triggered similar levels of lysis in the presence of glycine but the isolates differed in the amount of lysis that could be ameliorated by glycine (Fig. 6B). Thus, the *UPC2/upc2*^{GOF} strain was a stronger inducer of pyroptosis than was its *UPC2/UPC2* counterpart. As expected, the *UPC2/upc2*^{GOF} strain also produced higher levels of IL-1β (data not shown). Thus, there is a direct correlation between the level of Upc2 activity and the ability of *C. albicans* to induce pyroptosis. Although we did not complement the *upc2*Δ/Δ mutant, the converse phenotypes of *upc2*Δ/Δ and *UPC2/upc2*^{GOF} strains in different strain backgrounds strongly indicate that the *UPC2* mutations are responsible for the observed differences in pyroptosis induction.

While most studies of Upc2 function have focused on its role in the regulation of ergosterol production, it may also regulate several genes that are related to the cell wall (37). Several studies have found that recognition of *C. albicans* 1,3-β-glucan by the lectin receptor dectin-1 has a role in triggering pro-IL-1β transcription and possibly in the processing of pro-IL-1β into its mature form (7, 38, 39). Our data with heat-killed organisms, which have increased surface exposure of 1,3-β-glucan compared with that of live organisms (40), suggest that 1,3-β-glucan recognition is not a major trigger of pyroptosis in response to *C. albicans* (Fig. 5A). To verify this, we assayed 1,3-β-glucan exposure in a set of *C. albicans* mutants that we previously identified as having altered abilities to trigger macrophage lysis and IL-1β production (15). Mutants lacking the transcription factor *AHR1* or *UPC2*, which triggered low levels of macrophage lysis, or those lacking *NDT80* or *RCA1*, which triggers normal or high levels of macrophage lysis, respectively (Fig. 7A), had relatively normal surface 1,3-β-glucan exposure compared to that of the wild type (WT) (Fig. 7B). In contrast, the mutant lacking *BCR1*, a transcription factor that regulates genes related to cell wall integrity in *C. albicans* (41), triggered nearly normal levels of macrophage lysis (Fig. 7A) and had variable but substantially increased 1,3-β-glucan exposure (Fig. 7B). Thus, the ability of *Candida* to trigger pyroptosis cannot be explained by a simple increase in surface exposure of 1,3-β-glucan. Rather, our data suggest that the role of Upc2 in induction of pyroptosis is more likely due to its other effects on other aspects of *C. albicans* physiology. Intriguingly, fatty acids (42) and sterols (43) trigger NLRP3 inflammasome activation and Upc2 is a regulator of lipid biosynthesis in *C. albicans* (37, 44); however, additional work will be required to identify the specific Upc2-regulated molecules or processes that modulate the ability of *C. albicans* to trigger the inflammasome.

DISCUSSION

Over the past decade, our understanding of macrophage responses to infection has blossomed. In particular, the identification and characterization of the inflammasome-mediated programmed cell death pathway, pyroptosis, have shed substantial light on the mechanisms through which macrophages respond to intracellular bacteria such as *Salmonella*, *Legionella*, *Yersinia*, and *Listeria* species (10, 11, 13, 17). Here, we have demonstrated for the first time that a fungal pathogen, *C. albicans*, triggers pyroptosis. This discovery represents a paradigm shift in our understanding of *C. albicans*-induced macrophage damage. Previously, *C. albicans*-mediated damage to phagocytes was primarily attributed to its ability to physically disrupt the integrity of the cell by converting from the round yeast form to the filamentous form.

Our work indicates that the mechanism of *C. albicans*-induced macrophage damage is more complex. Specifically, we find that *C. albicans*-induced lysis is primarily mediated by pyroptosis early in the infection and/or when the MOI is low. Under these conditions, the majority of macrophage lysis is dependent on the caspase-1/NLRP3 inflammasome, although pyroptosis-independent lysis also makes a contribution to the damage. Filaments of *C.*

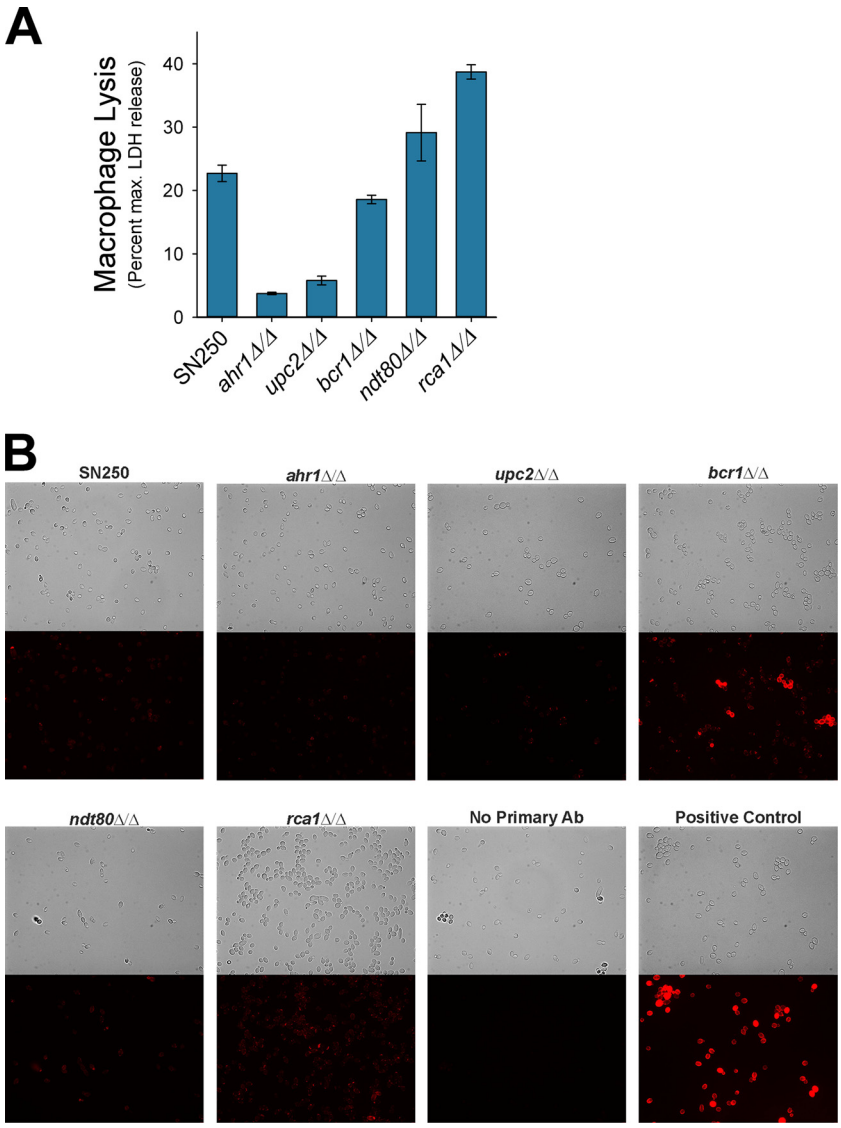

FIG 7 *C. albicans*-induced pyroptosis does not correlate with surface 1,3-β-glucan exposure. (A) Macrophage lysis data are from the work of Wellington et al. (15) and are included in this figure for comparison to panel B. J774 macrophages were prestimulated with LPS for 2 h and then exposed to *C. albicans* WT (SN250) or mutants at an MOI of 2:1 for 5 h. LDH was measured from the supernatant of the cocultures as described for Fig. 1. (B) Photomicrographs of bright-field or Texas Red immunofluorescence of live *C. albicans* WT (SN250) or the indicated mutants incubated with a mouse monoclonal anti-1,3-β-glucan antibody followed by Texas Red-conjugated goat anti-mouse secondary antibody. All images were taken with the same exposure settings and processed only to adjust contrast. All images were processed identically.

albicans are well recognized to break the cell membrane of macrophages and other phagocytes and escape into the tissue or culture medium. These observations led to the reasonable conclusion that the physical disruption of the macrophage was directly linked to cell death. In contrast to this model, the experiments described here indicate that many macrophages have undergone pyroptotic cell lysis well before the *C. albicans* hyphae disrupt the cell membrane and are essentially "dead men walking." Thus, we propose that *C. albicans* destruction of the macrophage results from a combination of NLRP3-mediated pyroptosis, physical destruction of the cell, and potentially other NLRP3-independent mechanisms.

Previously, we identified *C. albicans* mutants that form hyphae normally within macrophages but have a defect in their ability to trigger macrophage lysis and production of IL-1β (15). This strongly suggested that these mutants are deficient in the ability to induce pyroptosis. In the current study, we determined that the *upc2*Δ/Δ mutant is, in fact, deficient in its ability to trigger pyroptosis despite normal filamentation. Thus, the physical formation of hyphae is not sufficient cause for the macrophage to undergo pyroptosis. However, we have demonstrated here that yeast species that form hyphae or pseudohyphae within macrophages are much stronger inducers of pyroptosis than are species that are limited to growth as yeast, suggesting that polarized growth is necessary for pyroptosis. Therefore, we suspect that a physiologic process that is at least partially dependent upon the transcription factor Upc2 and that occurs during polarized growth is the trigger for pyroptosis. The finding that *S. cerevisiae* strain HLY335 is capable of triggering pyroptosis, albeit at a high MOI, opens this

topic to investigation using the robust techniques of *S. cerevisiae* genetic analysis. Our data are most consistent with a model by which filament formation is necessary but not sufficient for *C. albicans*-mediated activation of the NLRP3 inflammasome and pyroptosis.

Gain-of-function alleles of *UPC2* (*UPC2/upc2*^{GOF}) have been identified in clinical isolates that are fluconazole resistant (22). We found that a strain with this mutation triggers increased levels of pyroptosis in macrophages but similar levels of glycine-suppressible lysis, further supporting the notion that Upc2 regulates the expression of genes that play an important role in the activation of the NLRP3 inflammasome and pyroptosis. Recently, Lohberger et al. have shown that *UPC2* gain-of-function mutants have decreased virulence *in vivo* (45). Importantly, the decreased virulence of the *UPC2* gain-of-function mutants is not related to defects in growth or fitness *in vitro*. At this time, the basis for the decreased fitness of the *UPC2* gain-of-function mutant *in vivo* remains unknown; however, our observations suggest that increased pyroptosis could contribute to this phenomenon. Interestingly, Lohberger et al. also found that *UPC2* gain-of-function mutants showed a delay in their ability to form filaments in macrophages (45). Thus, the *UPC2/upc2*^{GOF} mutant has a decreased propensity to filament and an increased ability to trigger pyroptosis and is consistent with our model that pyroptosis is not simply due to filament formation within the phagolysosome. However, we have not yet found a mutant that is unable to filament and yet retains its ability to trigger pyroptosis. Therefore, the available data are most consistent with *C. albicans* filamentation being potentially necessary but clearly not sufficient for pyroptosis.

Although pyroptosis is induced by a variety of infectious and noninfectious stimuli (10, 11, 13), the infectious stimulus which has been most extensively characterized is intracellular bacteria (17). Pyroptosis plays two crucial roles in the immune response to intracellular bacteria. First, cytokine production recruits additional phagocytes to the site of infection, and second, macrophage lysis prevents bacteria adapted to survival in the phagolysosome from replicating within this immunologically protected niche. As a result of this second function, pyroptosis is important for clearance of intracellular bacteria *in vivo* even in the absence of IL-1β/IL-18 production (46). Although *C. albicans* replicates within and escapes from the macrophage phagolysosome (14), it is not generally considered an intracellular pathogen in the same sense as bacteria such as *Salmonella* and *Legionella* or fungi such as *Cryptococcus neoformans* or *Histoplasma capsulatum*. *In vitro*, bacteria that are professional intracellular pathogens replicate more efficiently within macrophages that are unable to undergo pyroptosis than within wild-type macrophages. In contrast, we found that suppressing macrophage pyroptosis with glycine had no effect on *C. albicans* replication *in vitro*, despite a substantial decrease in macrophage lysis. Similarly, Lohberger et al. found that the *UPC2* gain-of-function strains replicated within macrophages *in vitro* similarly to wild-type controls (45). Nevertheless, pyroptosis may play an important role in organism clearance *in vivo*, a possibility partially supported by the fact that the *UPC2* gain-of-function mutant is less fit *in vivo*. Alternatively, pyroptosis could lead to the release of both cytokine and noncytokine mediators of inflammation and facilitate the recruitment of other components of the immune system to the site of infection (47). Thus, the cell lysis that accompanies pyroptosis may serve not to limit replication but to augment the inflammatory response to *C. albicans* infection. Finally, it is also possible that *C. albicans*-mediated pyroptosis represents an immune evasion mechanism. However, this seems less likely since ASC-, caspase-1-, and IL-1β-deficient mice have higher organism burdens in mouse models of candidiasis and since the *UPC2* gain-of-function mutant is less virulent (7, 39). However, additional studies will be required before a detailed understanding of the specific function of pyroptosis in the immune response to *C. albicans* can be established.

Not all pathogenic *Candida* spp. appear to induce pyroptosis. As such, we have uncovered a profound difference in the manners by which these relatively closely related fungal pathogens interact with the innate immune system. The two species of *Candida* that trigger pyroptosis, *C. albicans* and *C. krusei*, both form hyphae or pseudohyphae, and thus, this is an observation that is consistent with filamentation playing a role in the process. Previous studies of inflammasome activation had shown that an unspecified strain of *S. cerevisiae* induced very low levels of IL-1β secretion. We found that *S. cerevisiae* cells from a nonfilamentous background induce essentially no pyroptosis, while a strain from the filamentous Σ1278 background is able to trigger pyroptosis, albeit at low levels. In addition, nearly all of the lysis induced by Σ1278 is due to pyroptosis even though high MOIs are required. Although *S. cerevisiae* is most closely related to *C. glabrata*, a stark difference in their biology is that *S. cerevisiae* is filamentous while *C. glabrata* is not. Consequently, *S. cerevisiae* is more like *C. albicans* and *C. krusei* in its interaction with macrophages. Taken together, these observations indicate that there are significant differences in the ways that macrophages react to filamentous and nonfilamentous pathogenic *Candida* spp.

In summary, we have demonstrated that the human fungal pathogen *C. albicans* provokes the programmed cell death pathway pyroptosis in macrophages, resulting in macrophage lysis and production of IL-1β/IL-18. Thus, we propose a refined model of *C. albicans*-induced macrophage damage. In this model, (i) macrophages encounter and ingest *C. albicans*, and (ii) the phagosome environment triggers both filamentation and an additional factor(s) which together (iii) activate the NLRP3 inflammasome and caspase-1, which (iv) trigger macrophage pyroptosis. Our data indicate that filamentation is necessary but not sufficient to trigger pyroptosis; furthermore, the additional *C. albicans* factor(s) required for pyroptosis is at least partially regulated by Upc2. The elucidation of the function and mechanism of *C. albicans*-mediated pyroptosis will lead to a deeper understanding of the interaction between this important human fungal pathogen and the host.

ACKNOWLEDGMENTS

We thank David Rogers (University of Tennessee Health Sciences Center) for providing *UPC2* gain-of-function mutants.

This work was supported by grants from the National Institute of Allergy and Infectious Diseases, 1R01AI098450 (D.J.K.) and R01 AI087630 (F.S.S.), and from the Strong Children's Research Center at The University of Rochester (M.W.).

REFERENCES

1. **Moran GP, Coleman D, Sullivan D.** 2012. An introduction to the medically important *Candida* species, p 11–25. *In* Calderone R, Clancy CJ (ed), *Candida* and candidiasis, 2nd ed. ASM Press, Washington, DC.
2. **Vazquez JA, Sobel JD.** 2003. Candidiasis, p 143–187. *In* Dismukes WE, Pappas PG, Sobel JD (ed), Clinical mycology. Oxford University Press, Oxford, United Kingdom.
3. **Miramon P, Kasper L, Hube B.** 2013. Thriving in the host: *Candida* spp.

interaction with phagocytic cells. Med. Microbiol. Immunol. **202:**183–195. http://dx.doi.org/10.1007/s00430-013-0288-z.
4. **Ngo LY, Karahara S, Kumaska DK, Knoblaugh SE, Jhingran A, Hohl TM.** 2014. Inflammatory monocytes mediate early and organ-specific innate defense during systemic candidiasis. J. Infect. Dis. **209:**109–119. http://dx.doi.org/10.1093/infdis/jit413.
5. **Lionakis MS, Lim JK, Lee CC, Murphy PM.** 2011. Organ specific immune responses in a mouse model of invasive candidiasis. J. Innate Immun. **3:**180–199. http://dx.doi.org/10.1159/000321157.
6. **Joly S, Ma N, Sadler JJ, Soll DR, Cassel SL, Sutterwala FS.** 2009. Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. J. Immunol. **183:**3578–3581. http://dx.doi.org/10.4049/jimmunol.0901323.
7. **Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, Fitzgerald KA.** 2009. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe **5:**487–497. http://dx.doi.org/10.1016/j.chom.2009.05.002.
8. **Gross O, Poeck H, Bscheider M, Dostert C, Hannesschlager N, Endres S, Hartmann G, Tardivel A, Schweighoffer E, Tybulewicz V, Mocsai A, Tschopp J, Ruland J.** 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature **459:**433–436. http://dx.doi.org/10.1038/nature07965.
9. **Tomalka J, Ganesan S, Azodi E, Patel K, Majmudar P, Hall BA, Fitzgerald KA, Hise AG.** 2011. A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen *Candida albicans*. PLoS Pathog. **7:**e1002379. http://dx.doi.org/10.1371/journal.ppat.1002379.
10. **Franchi L, Munoz-Planillo R, Nunez G.** 2012. Sensing and reacting to microbes through the inflammasomes. Nat. Immunol. **13:**325–332. http://dx.doi.org/10.1038/ni.2231.
11. **Schroder K, Tschopp J.** 2010. The inflammasomes. Cell **140:**821–832. http://dx.doi.org/10.1016/j.cell.2010.01.040.
12. **Joly S, Eisenbarth SC, Olivier AK, Williams A, Kaplan DH, Cassel SL, Flavell RA, Sutterwala FS.** 2012. Cutting edge: Nlrp10 is essential for protective antifungal adaptive immunity against *C. albicans*. J. Immunol. **189:**4713–4717. http://dx.doi.org/10.4049/jimmunol.1201715.
13. **Pedra JHF, Cassel SL, Sutterwala FS.** 2009. Sensing pathogens and danger signals by the inflammasome. Curr. Opin. Immunol. **21:**10–16. http://dx.doi.org/10.1016/j.coi.2009.01.006.
14. **Jimenez-Lopez C, Lorenz MC.** 2013. Fungal immune evasion in a model host-pathogen interaction: *Candida albicans* versus macrophages. PLoS Pathog. **11:**e1003741. http://dx.doi.org/10.1371/journal.ppat.1003741.
15. **Wellington M, Koselny K, Krysan DJ.** 2012. *Candida albicans* morphogenesis is not required for macrophage IL-1β production. mBio **4**(1): e00433–12. http://dx.doi.org/10.1128/mBio.00433-12.
16. **Miao E, Rajan JV, Aderm A.** 2011. Caspase-1-induced pyroptotic cell death. Immunol. Rev. **243:**206–214. http://dx.doi.org/10.1111/j.1600-065X.2011.01044.x.
17. **Chen KW, Schroder K.** 2013. Antimicrobial functions of the inflammasomes. Curr. Opin. Microbiol. **16:**311–318. http://dx.doi.org/10.1016/j.mib.2013.02.004.
18. **Noble SM, French S, Kohn LA, Chen V, Johnson AD.** 2010. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat. Genet. **42:**590–598. http://dx.doi.org/10.1038/ng.605.
19. **Bliss JM, Basavegowda KP, Watson WJ, Sheikh AV, Ryan RM.** 2008. Vertical and horizontal transmission of *Candida albicans* in very low weight infants using DNA fingerprinting techniques. Pediatr. Infect. Dis. J. **27:**231–235. http://dx.doi.org/10.1097/INF.0b013e31815bb69d.
20. **Cormack BP, Falkow S.** 1999. Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen *Candida glabrata*. Genetics **151:**979–987.
21. **Homann OR, Dea J, Noble SM, Johnson AD.** 2009. A phenotypic profile of the *Candida albicans* regulatory network. PLoS Genet. **5:**e1000783. http://dx.doi.org/10.1371/journal.pgen.1000783.
22. **Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhäuser J, Rogers PD.** 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. Eukaryot. Cell **7:**1180–1190. http://dx.doi.org/10.1128/EC.00103-08.
23. **Wellington M, Kolan K, Krysan DJ.** 2009. Live *Candida albicans* suppresses production of reactive oxygen species in phagocytes. Infect. Immun. **77:**405–413. http://dx.doi.org/10.1128/IAI.00860-08.
24. **Sutterwala FS, Ogura Y, Szcsepanik M, Lara-Tejero M, Lichtenberger GS, Grant EP, Bertin J, Coyle AJ, Galan JE, Askenase PW, Flavell RA.** 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity though its regulation of caspase-1. Immunity **24:**317–327. http://dx.doi.org/10.1016/j.immuni.2006.02.004.
25. **Lara-Tejero M, Sutterwala FS, Ogura Y, Gran EP, Bertin J, Coyle AJ, Flavell FA, Galan JE.** 2006. The role of caspase-1 in *Salmonella typhimurium* pathogenesis. J. Exp. Med. **203:**1407–1412. http://dx.doi.org/10.1084/jem.20060206.
26. **Zheng TS, Hunot S, Kuida K, Momoi Srinivasan TA, Nicholson DW, Lazebnik Y, Flavell RA.** 2000. Deficiency in caspage-9 or caspase-3 induces compensatory caspase activation. Nat. Med. **6:**1241–1247. http://dx.doi.org/10.1038/81343.
27. **Fink SL, Cookson BL.** 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cell. Microbiol. **8:**1812–1825. http://dx.doi.org/10.1111/j.1462-5822.2006.00751.x.
28. **Bergsbaken T, Cookson BT.** 2007. Macrophage activation redirects *Yersinia*-infected host cell death from apoptosis to caspase-1-dependent pyroptosis. PLoS Pathog. **3:**e161. http://dx.doi.org/10.1371/journal.ppat.0030161.
29. **Fortier A, Diez E, Gros P.** 2005. Naip5/Birc1e and susceptibility to *Legionella pneumophila*. Trends Microbiol. **13:**328–335. http://dx.doi.org/10.1016/j.tim.2005.05.007.
30. **Tuite A, Elias M, Picard S, Mullick A, Gros P.** 2005. Genetic control of susceptibility to *Candida albicans* in susceptible A/J. and resistant C57BL/6J mice. Genes Immun. **6:**672–682. http://dx.doi.org/10.1038/sj.gene.6364254.
31. **Puri AW, Broz P, Shen A, Monack DM, Bogyo M.** 2012. Caspase-1 activity is required to bypass macrophage apoptosis upon *Salmonella* infection. Nat. Chem. Biol. **8:**745–747. http://dx.doi.org/10.1038/nchembio.1023.
32. **Gow NAR, van de Veerdonk FL, Brown AJP, Netea MG.** 2011. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat. Rev. Microbiol. **10:**112–122. http://dx.doi.org/10.1038/nrmicro2711.
33. **Garcia-Rodas R, Gonzalez-Camacho F, Rodriguez-Tudela JL, Cuenca-Estrella M, Zaragoza O.** 2011. The interaction between *Candida krusei* and murine macrophages results in multiple outcomes, including intracellular survival and escape from killing. Infect. Immun. **79:**2136–2144. http://dx.doi.org/10.1128/IAI.00044-11.
34. **Liu H, Styles CA, Fink GR.** 1993. Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science **262:**1741–1744. http://dx.doi.org/10.1126/science.8259520.
35. **Liu Styles H, Styles CA, Fink GR.** 1996. *Saccharomyces cerevisiae* S288c has a mutation in *FL08*, a gene required for filamentous growth. Genetics **144:**967–978.
36. **Ryan O, Shapiro RS, Kurat CF, Mayhew D, Baryshnikova A, Chin B, Lin ZY, Cox MJ, Vizeacoumar F, Cheung D, Bahr S, Tsui K, Tebbji F, Sellam A, Istel F, Schwarzmüller T, Reynolds TB, Kuchler K, Gifford DK, Whiteway M, Giaever G, Nislow C, Costanzo M, Gingras AC, Mitra RD, Andrews B, Fink GR, Cowen LE, Boone C.** 2012. Global gene deletion analysis exploring yeast filamentous growth. Science **337:**1353–1356. http://dx.doi.org/10.1126/science.1224339.
37. **Flowers SA, Barker KS, Berlow EL, Chadwick SG, Gygax SE, Morschhauser J, Rogers PD.** 2012. Gain-of-function mutations in *UPC2* are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. Eukaryot. Cell **11:**1289–1299. http://dx.doi.org/10.1128/EC.00215-12.
38. **Gringhuis SI, Kaptein TM, Wevers BA, van der Vlist M, Boekhout T, Feijtenbeek TB.** 2012. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1β via a noncanonical caspase-8 inflammasome. Nat. Immunol. **13:**246–254. http://dx.doi.org/10.1038/ni.2222.
39. **van de Veerdonk FL, Joosten LA, Devesa I, Mora-Montes HM, Kanneganti TD, Dinarello CA, van der Meer JW, Gow NAB, Kullberg BJ, Netea MG.** 2009. Bypassing pathogen-induced inflammasome activation for the regulation of interleukin-1beta production by the fungal pathogen *Candida albicans*. J. Infect. Dis. **199:**1087–1096. http://dx.doi.org/10.1086/597274.
40. **Wheeler RT, Kombe D, Agarwala SD, Fink GR.** 2008. Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. PLoS Pathog. **4:**e1000227. http://dx.doi.org/10.1371/journal.ppat.1000227.

41. **Finkel JS, Xu W, Huang D, Hill EM, Desai JV, Wooldford CA, Nett JE, Taff H, Norice CT, Andes DR, Lanni F, Mitchell AP.** 2012. Portrait of *Candida albicans* adherence regulators. PLoS Pathog. **8:**e1002525. http://dx.doi.org/10.1371/journal.ppat.1002525.
42. **Snodgrass RG, Huang S, Choi I-W, Rutledge JC, Hwang DH.** 2013. Inflammasome-mediated secretion of IL-1β in human monocytes through TLR2 activation; modulation by dietary fatty acids. J. Immunol. **191:**4337–4347. http://dx.doi.org/10.4049/jimmunol.1300298.
43. **Youm Y-H, Kanneganti T-D, Bandanmagsar B, Zhu X, Ravussin A, Adijiang A, Owen JS, Thomas MJ, Francis J, Parks JS, Dixit VD.** 2012. The NLRP3 inflammasome promotes age-related thymic demise and immunosenescence. Cell Rep. **1:**56–68. http://dx.doi.org/10.1016/j.celrep.2011.11.005.
44. **Znaidi S, Weber S, Al-Abdin OZ, Bomme P, Saidane S, Drouin S, Lemieux S, De Deken X, Robert F, Raymond M.** 2008. Genomewide location analysis of *Candida albicans* Upc2p, a regulator of sterol metabolism and azole drug resistance. Eukaryot. Cell **7:**836–847. http://dx.doi.org/10.1128/EC.00070-08.
45. **Lohberger A, Coste AT, Sanglard D.** 2014. Distinct roles of *Candida albicans* drug resistance transcription factors *TAC1*, *MRR1*, and *UPC2* in virulence. Eukaryot. Cell **13:**127–142. http://dx.doi.org/10.1128/EC.00245-13.
46. **Miao E, Leaf IA, Treuting PM, Mao DP, Dors M, Sakar A, Warren SE, Wewers MD, Aderem A.** 2010. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. Nat. Immunol. **11:**1136–1142. http://dx.doi.org/10.1038/ni.1960.
47. **Lamkanfi M.** 2011. Emerging inflammasome effector mechanisms. Nat. Rev. Immunol. **11:**213–220. http://dx.doi.org/10.1038/nri2936.