The Pathogen Candida albicans Hijacks Pyroptosis for Escape from Macrophages

Nathalie Uwamahoro,a Jiyoti Verma-Gaur,a Hsin-Hui Shen,b Yue Qu,a,b Rowena Lewis,c,d Jingxiong Lu,e Keith Bambery,f Seth L. Masters,c,d James E. Vince,c,d Thomas Naderer,a Ana Travena

Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australiaa; Department of Microbiology, Monash University, Clayton, Victoria, Australiaa; Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australiab; Department of Medical Biology, the University of Melbourne, Parkville, Victoria, Australiac; Department of Chemical Engineering, Monash University, Clayton, Victoria, Australiadc; The Australian Synchrotron, Melbourne, Victoria, Australiade;e The Pathogen Candida albicans causes macrophage death and escapes, but the molecular mechanisms remained unknown. Here we used live-cell imaging to monitor the interaction of C. albicans with macrophages and show that C. albicans kills macrophages in two temporally and mechanistically distinct phases. Early upon phagocytosis, C. albicans triggers pyroptosis, a proinflammatory macrophage death. Pyroptosis is controlled by the developmental yeast-to-hypha transition of Candida. When pyroptosis is inactivated, wild-type C. albicans hyphae cause significantly less macrophage killing for up to 8 h postphagocytosis. After the first 8 h, a second macrophage-killing phase is initiated. This second phase depends on robust hyphal formation but is mechanistically distinct from pyroptosis. The transcriptional regulator Mediator is necessary for morphogenesis of C. albicans in macrophages and the establishment of the wild-type surface architecture of hyphae that together mediate activation of macrophage cell death. Our data suggest that the defects of the Mediator mutants in causing macrophage death are caused, at least in part, by reduced activation of pyroptosis. A Mediator mutant that forms hyphae of apparently wild-type morphology but is defective in triggering early macrophage death shows a breakdown of cell surface architecture and reduced exposed 1,3-β-glucan in hyphae. Our report shows how Candida uses host and pathogen pathways for macrophage killing. The current model of mechanical piercing of macrophages by C. albicans hyphae should be revised to include activation of pyroptosis by hyphae as an important mechanism mediating macrophage cell death upon C. albicans infection.

ABSTRACT The fungal pathogen Candida albicans causes macrophage death and escapes, but the molecular mechanisms remained unknown. Here we used live-cell imaging to monitor the interaction of C. albicans with macrophages and show that C. albicans kills macrophages in two temporally and mechanistically distinct phases. Early upon phagocytosis, C. albicans triggers pyroptosis, a proinflammatory macrophage death. Pyroptosis is controlled by the developmental yeast-to-hypha transition of Candida. When pyroptosis is inactivated, wild-type C. albicans hyphae cause significantly less macrophage killing for up to 8 h postphagocytosis. After the first 8 h, a second macrophage-killing phase is initiated. This second phase depends on robust hyphal formation but is mechanistically distinct from pyroptosis. The transcriptional regulator Mediator is necessary for morphogenesis of C. albicans in macrophages and the establishment of the wild-type surface architecture of hyphae that together mediate activation of macrophage cell death. Our data suggest that the defects of the Mediator mutants in causing macrophage death are caused, at least in part, by reduced activation of pyroptosis. A Mediator mutant that forms hyphae of apparently wild-type morphology but is defective in triggering early macrophage death shows a breakdown of cell surface architecture and reduced exposed 1,3-β-glucan in hyphae. Our report shows how Candida uses host and pathogen pathways for macrophage killing. The current model of mechanical piercing of macrophages by C. albicans hyphae should be revised to include activation of pyroptosis by hyphae as an important mechanism mediating macrophage cell death upon C. albicans infection.

Candida albicans is a human commensal but is also an important human pathogen responsible for more than 400,000 cases of invasive disease per year, from which the mortality is high (1). A key virulence attribute for this organism is the ability to undergo developmental transitions that result in morphological plasticity. The budding yeast state is associated commensalism, while the developmental transition to hyphal growth is generally related to disease (2). Hyphae are linked to the ability of C. albicans to evade phagocytic digestion by macrophages (3, 4). Signals within the phagocytic environment trigger the developmental transition to hyphae, resulting in the escape of hyphae at the expense of the host cell (3). Generally, yeast-form cells fail to cause damage and to escape from macrophages (4, 5). The current model is that the highly polarized growth of hyphae enables physical destruction of the macrophage by piercing of the fungal filaments through the macrophage plasma membrane (3). Challenging this model are findings that dissociate the ability of C. albicans to grow as hyphae from the ability to escape from macrophages (5, 6).

In addition to the morphological and size differences, a main distinguishing feature of yeast and hyphal cells is the structure of the cell wall (7–9). The C. albicans cell wall is made of glucose polymers 1,3 and 1,6-β-glucans, chitin, and a range of mannosylated proteins that decorate the cell surface. The differential expression and exposure of cell wall components are thought to be a...
major factor in how immune cells discriminate yeast from invasive hyphal forms (reviewed in reference 7). For example, differences between yeast and hyphae in β-glucan exposure have been proposed to lead to differential engagements with the cell surface pathogen recognition receptor (PRR) dectin-1 (10). Dectin-1 triggers proinflammatory interleukin-1β (IL-1β) expression via Syk kinase signaling, and activation of a cytoplasmic inflammasome that contains NLRP3, ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) and caspase-1 results in cleavage of pro-IL-1β to its bioactive form (11–14). Other pathogen recognition receptors also contribute to this pathway (12–14). Intracellular hyphae, but not yeast forms, induce caspase-1-dependent IL-1β secretion, although it remains unknown how the NLRP3/ASC inflammasome is activated under these conditions (12, 15). Intriguingly, dectin-1 signaling in some C. albicans isolates has been linked to a noncanonical inflammasome in which caspase-8, rather than caspase-1, was proposed to cleave and thereby activate IL-1β (14). That and other studies (16) suggest that C. albicans can adjust the composition of its cell wall during the course of infection to modulate innate immune responses. Indeed, a recent study suggested that factors additional to hyphal morphology lead to production of IL-1β (6).

Inflammasomes that induce IL-1β secretion can also trigger programmed cell death. In the case of caspase-1 activation, macrophages undergo a proinflammatory form of cell death termed pyroptosis. Other programmed cell death pathways, such as the canonical apoptosis and ordered necrosis, which depends on receptor-interacting kinases Rip1 and Rip3 (reviewed in reference 17), have also been shown to protect against viral and bacterial infections by either eliminating the replicative niche of the pathogens or exposing them to the immune system (18, 19). However, the timing of these pathways may be critical, as some microbial pathogens, including Salmonella, induce caspase-1- and Rip3-dependent cell death to trigger escape from macrophages and dissemination from the site of infection (20–22). Cell death pathways have mostly been studied in the context of bacterial and viral infections, and there is only limited evidence indicating whether they play a role in fungal disease (23, 24).

Here we show that C. albicans kills macrophages by inducing pyroptotic programmed cell death at early times post-infection (the first 8 h under our experimental conditions). Hyphal morphogenesis is important for induction of pyroptosis, and our data suggest that proper hyphal cell surface architecture mediates early macrophage killing and fungal escape. Pyroptosis-independent macrophage killing by Candida also occurs, particularly at later stages post-phagocytosis, and this requires robust hyphal morphogenesis. Activation of pyroptosis in response to Candida might serve to augment proinflammatory responses, but C. albicans might in addition hijack activation of this programmed cell death pathway to escape from macrophages and thus evade the innate immune response. These two scenarios are not mutually exclusive and offer an explanation for the paradoxical role of hyphal forms in C. albicans pathogenesis, whereby hyphae are both the virulent form of the pathogen and the form that triggers host immune responses.

RESULTS

C. albicans kills macrophages by triggering pyroptosis. To understand the mechanism by which C. albicans kills macrophages, we devised a time-lapse microscopy assay whereby C. albicans is incubated with macrophages in the presence of the membrane-impermeable dye propidium iodide (PI). This allowed detailed determination of macrophage cell death rates as percentages of PI-positive cells over time (images were taken every 15 min over 21 to 24 h). C. albicans was co-cultivated with bone marrow-derived macrophages (BMDMs) for 1 h (at a multiplicity of infection [MOI] of 1 macrophage to 6 Candida cells), followed by washing of the nonphagocytosed cells and monitoring of macrophage cell death. Thus, the assay monitors the consequences of the interactions between phagocytosed (intracellular) Candida cells and macrophages. A detailed description of the assay is provided in Materials and Methods in the supplemental material. In agreement with other studies (25), BMDM cell death rates were about 20% to 30% within 6 h post-infection with C. albicans (Fig. 1A). During this time, C. albicans formed extended filaments that were clearly extruding from host cells (Fig. 1D; see also Video S1 in the supplemental material). At later times, C. albicans induced a second phase of macrophage killing, which lasted up to 21 h post-infection and resulted in a complete collapse of the host cell culture (Fig. 1A; see also Video S1). Both phases were dependent on live C. albicans, as heat-killed cells failed to induce any death, despite almost wild-type infection rates (Fig. 1A; for rates of infection by heat-killed cells, see Fig. 2A).

In contrast to BMDMs, the RAW 264.7 macrophage-like cell line was resistant to C. albicans killing in the first 8 to 9 h post-infection (Fig. 1B). Filamentation (the appearance of hyphal filaments and germ tubes) in RAW 264.7 cells was similar to what we observed in BMDMs (Fig. 1C; also compare Videos S1 and S2 in the supplemental material). The levels of phagocytosis of Candida cells were also similar between BMDMs and RAW 264.7 macrophages (Fig. 2A). Hyphae eventually escaped from RAW 264.7 cells, followed by rapid killing of the entire host culture within the next 7 to 8 h (Fig. 1B). Therefore, efficient killing of macrophages by Candida hyphae in phase 1 might require a host factor that is inactive in RAW 264.7 macrophages.

RAW 264.7 macrophages lack the inflammasome component ASC, which is required for caspase-1 activation (26), and could thus be defective in activation of pyroptosis. To probe directly for the role of pyroptosis in C. albicans-mediated killing of macrophages, we utilized BMDMs derived from casp1−/− casp11−/− mutant mice (27). As shown in Fig. 1E and F, casp1−/− casp11−/− BMDMs were more resistant to killing by C. albicans within the first 8 to 10 h. Phagocytosis of C. albicans by casp1−/− casp11−/− BMDM was similar to that seen with wild-type BMDMs, and fungal hyphae formed normally (Fig. 1C and 2A), suggesting that lower rates of macrophage cell death are not caused by lower uptake or changes to the morphogenesis of Candida in the mutant BMDMs. Instead, these data show that C. albicans triggers pyroptotic macrophage death during the first phase post-infection. The second phase of macrophage killing by C. albicans hyphae was not defective in casp1−/− casp11−/− BMDMs, as a rapid macrophage-killing phase was seen starting at 10 to 12 h (Fig. 1D and 1E; see also Video S3 in the supplemental material). We note that, even in casp1−/− casp11−/− BMDMs, some macrophage cell death was observed early upon infection (Fig. 1E and F), indicating that C. albicans utilizes mechanisms additional to pyroptosis to cause macrophage death. However, we found no evidence of activation of caspase 3 by C. albicans early post-infection (Fig. 1G), suggesting that the canonical apoptotic pathway was not triggered in phase 1 under our experimental conditions.
FIG 1  C. albicans triggers pyroptotic macrophage cell death. (A) Wild-type (WT) C. albicans was incubated with wild-type BMDMs at MOI 1:6 (macrophage: Candida), and macrophage cell death was monitored over time. Shown are averages and standard errors of the means (SEM) of the results from two independent biological experiments. HKWT, heat-killed wild-type C. albicans cells (yeast morphology). (B) Experiments were performed as described for panel A except that the RAW 264.7 macrophage cell line was used. Averages and SEM are shown (n = 2). (C) Yeast and filamentous forms were counted from images from the live-cell microscopy experiments described for panels B and E at 30 min after the 1-h coincubation. A total of 100 phagocytosed Candida cells were counted for each of the independent biological experiments and classified as yeast, germ tubes, or hyphae. Values shown are means ± SEM (n = 3 for the RAW 264.7 cells and n = 2 for BMDMs). (D) Images corresponding to selected time points (h) from the live-cell microscopy of wild-type C. albicans infecting wild-type or casp1−/− casp11−/− BMDMs. (E) Wild-type C. albicans was incubated with wild-type or casp1−/− casp11−/− BMDMs. Averages and SEM of the results of 4 independent experiments are shown. These data and the data in the graph in panel F are the same as those determined in the wild-type Candida control experiments represented in Fig. 3. They are shown here separately for clarity of the results. (F) Graphs show means and SEM for percentages of macrophage cell death at selected time points from the curves shown in panel E. **, P < 0.01; *, P < 0.05. Representative live-cell microscopy movies from the macrophage-killing experiments represented in this figure are shown in Videos S1 to S3 in the supplemental material. (G) BMDMs were infected with live or heat-killed wild-type (HKWT) Candida at MOI 1:6 (macrophage:Candida) or treated with cycloheximide (CHX; 50 μg/ml) for 3 h, and the generation of cleaved caspase 3 was detected by immune blotting. Loading was visualized by Ponceau staining. Cycloheximide treatment served as a positive control.
Mediator as a new regulator of *C. albicans*-macrophage interactions. We have previously shown that the subunits of the Mediator complex, a central transcriptional regulator, control morphogenesis and cell wall integrity in *C. albicans* (28). The mutant deleted for the Mediator MED31 subunit infected BMDMs similarly to wild-type *C. albicans* (Fig. 2A), but was delayed in filamentation and was primarily in yeast form at 3 h post-phagocytosis (Fig. 2B and C). Filamentous structures were starting to form at later time points, and filaments were visible at 4 to 5 h post-infection (see Video S4 in the supplemental material). This finding is in agreement with our previous data determined *in vitro* and in the worm infection model that showed that the *med31Δ*Δ mutant is impaired in filamentation (28). Consistent with the morphogenesis defect, the *med31Δ*Δ mutant was severely impaired in early escape from macrophages (as judged by microscopy using calcofluor white staining of externalized hyphae) (Fig. 2B and D) and remained associated with the late phagosomal marker Lamp1 for prolonged times (Fig. 2E; images are shown in >Fig. 51 in the supplemental material) at the 2-h time point (following the 1-h coincubation). Three independent experiments were performed, and at least 50 *Candida* cells were counted in each. Averages and SEM are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
shown in Fig. S1). The med31Δ/Δ mutant is impaired for fitness in vitro (28), but was able to survive long-term in BMDMs, although it failed to multiply efficiently at 13.5 h post-infection (see Fig. S2). We note that, for the data determined at 13.5 h post-infection, a number of technical differences between phagocytosed and escaped Candida cells that were replicating in the media) Consistent with fewer hyphae escaping, the med31Δ/Δ mutant induced low levels of macrophage cell death within 8 to 10 h post-infection (Fig. 3A). Notably, this mutant consistently induced higher macrophage cell death rates than heat-killed wild-type yeast cells (see graph in Fig. 3A). After 18 h, macrophage cell death rates increased to about 30%, and by 24 h, the med31Δ/Δ mutant caused an average macrophage cell death rate of 62.5% (Fig. 3A and data not shown). The increased ability of the mutant to cause macrophage death at later time points was most likely due to the eventual formation of filaments. Complementation of the med31Δ/Δ mutant with the plasmid containing the MED31 gene restored macrophage death to wild-type levels (Fig. 3A). Therefore, while hyphal formation is important to induce macrophage death, factors additional to hyphal morphology are important for efficient killing and escape from macrophages, particularly early following phagocytosis. Both Mediator mutants were less virulent in the mouse tail vein systemic candidiasis model (Fig. S2).

We next combined the wild type and the Mediator mutants of Candida albicans, infecting wild-type or casp1Δ/Δ casp11Δ/Δ mutant BMDMs, all assayed together to allow direct comparisons of the effects of host and pathogen mutations. The wild-type C. albicans results are presented separately in >Fig. 1 for clarity of the Results section. For simplicity, the results from wild-type BMDMs and those from casp1 Δ/Δ casp11 Δ/Δ BMDMs are presented in separate graphs. The experiments were performed 3 independent times (for the med31Δ/Δ mutant) or 4 independent times (for the srb9Δ/Δ mutant). The means and SEM for percentages of dead macrophages are shown. Graphs are for means and SEM for individual time points with statistical significance. All numerical results are presented separately in Fig. S2 in the supplemental material. P values are indicated as follows: *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001. Videos of mutant Candida are shown in Videos S4 and S5.
C. albicans with wild-type and casp1Δ−/− casp11Δ−/− BMDMs to address whether the reduced ability of Mediator mutants to cause macrophage cell death was due to defective activation of pyroptosis. If this were the case, then we would expect that the difference in macrophage killing between wild-type and mutant C. albicans would be abrogated in the absence of pyroptosis in casp1Δ−/− casp11Δ−/− BMDMs. As shown in Fig. 3B, in casp1Δ−/− casp11Δ−/− BMDMs there was no statistically significant difference between wild-type C. albicans and the Mediator mutants in the ability to cause macrophage cell death in phase 1 (P values of <0.05 were considered to be significant; for all P values see Fig. S3). This result supports the notion that the Mediator mutants are defective in triggering pyroptosis. That there was no significant difference between wild-type hyphae and the morphogenesis-impaired med31Δ/Δ mutant in causing death of casp1Δ−/− casp11Δ−/− BMDMs early postphagocytosis suggests that an important function of hyphal structures in causing early macrophage death is due to their requirement for a phase 1 (first 8 h following uptake (Fig. 1). Caspase-1 and caspase-11 in C. albicans were able to induce more macrophage death than the Mediator mutants in casp1Δ−/− casp11Δ−/− BMDMs (Fig. 3B), although this difference was smaller than in wild-type BMDMs (compare Fig. 3A to B). Also, all live strains (the wild type and both mutants) were inducing substantially more macrophage death than heat-killed yeast cells not only in wild-type BMDMs but also in the absence of pyroptosis in casp1Δ−/− casp11Δ−/− BMDMs (Fig. 3). These results suggest that while pyroptosis is an important function of hyphal structures in causing early macrophage cell death, functions of hyphal additional determinants, besides filamentous morphology, which contribute to macrophage killing by C. albicans. Atomic force microscopy (AFM) showed that the srb9Δ/Δ mutant hyphae displayed a breakdown of cell surface architecture; the surface of mutant hyphae appeared smoother than that of wild-type filaments as shown in surface topography images (Fig. 5). In addition, force mapping demonstrated that wild-type hyphae contain areas of high adhesion forces, which were absent on srb9Δ/Δ hyphae (Fig. 5). The complemented srb9Δ/Δ + SRB9 strain had an intermediate phenotype (Fig. 5). We have previously found that the srb9Δ/Δ mutant displays lower levels of some hypha-specific cell wall genes in vitro (28). However, in macrophages, the expression of the hyphal cell wall adhesins did not depend on Srb9 (Fig. 6A). Instead, srb9Δ/Δ hyphae displayed reduced exposed 1,3 β-glucan levels compared to the wild type which appeared as punctate staining by confocal microscopy (Fig. 6B). Flow cytometry confirmed this result (Fig. 6C and E). Yeast forms of srb9Δ/Δ did not display reduced 1,3 β-glucan exposure (in contrast, 1,3 β-glucan exposure was slightly higher in the mutant in some experiments; Fig. 6D and F). Taken together, these results show that Srb9 regulates morphology-dependent cell surface exposure of 1,3 β-glucan but also the overall cell wall architecture.

**DISCUSSION**

The interaction of C. albicans with macrophages has most commonly been studied by sampling at defined time points where the events that occur before, after, or between the selected time points are missed (5, 6, 29). To dissect this process in greater detail, we followed C. albicans-macrophage intracellular interactions in real time using live-cell imaging. With our new assay, we show that macrophage killing by C. albicans occurs in two distinct phases: phase 1 (first 6 to 8 h) and phase 2 (8 to 10 h to 18 to 24 h post-phagocytosis). Both phases depend on the presence of wild-type hyphae but are distinguished by the requirement for activation of host responses by C. albicans. Phase 1 requires the activity of the pyroptotic caspases, caspase-1 and caspase-11. Wild-type C. albicans hyphae cause 40% to 50% less macrophage cell death in casp1Δ−/− casp11Δ−/− BMDMs than in wild-type BMDMs in the first 8 h following uptake (Fig. 1). Caspase-1 and caspase-11 induce pyroptosis and are not known to cause any other form of programmed cell death. Therefore, these results show that C. albicans hyphae trigger pyroptotic macrophage cell death in phase 1.
That wild-type *C. albicans* filaments fail to induce normal death rates in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs and also in RAW macrophages, where almost no host cell death is observed for the first 9 h, suggests that mechanical piercing by hyphae, which is currently considered to be a major contributor to macrophage killing (3–5), is not among the main mechanisms of early host cell death upon phagocytosis. Caspase-1 and caspase-11 can independently induce pyroptosis (18, 27). However, caspase-11 is primarily activated by Gram-negative bacteria and LPS (30, 31), suggesting that caspase-1 is the main pyroptotic caspase activated by *C. albicans* hyphae. Our conclusions are supported by a report published while the present manuscript was under review that showed that *C. albicans* hyphae induce macrophage pyroptosis that depends on caspase-1 and the inflammasome subunits NLRP3 and ASC (32). Consistent with our data, the same study showed that pyropoptosis is the predominant mechanism of macrophage cell death when fungal cell numbers are low, such as early upon phagocytosis. It has to be noted that mechanisms additional to pyroptosis also operate in phase 1. First, wild-type *C. albicans* induces more macrophage cell death than heat-killed cells in the absence of pyroptosis in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs. Second, wild-type filaments caused higher cell death rates than the morphogenesis-impaired *med31<sup>Δ/Δ</sup>* mutant not only in wild-type BMDMs, but also in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs. Moreover, phase 2 of killing requires wild-type hyphae, but the mechanism is distinct from pyroptosis, as this phase occurs normally in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs. The additional macrophage cell death mechanism in phase 1, as well as the phase 2 death that occurs when *C. albicans* hyphae are abundant, could depend on mechanical destruction of macrophages by hyphae. Alternatively, another host cell death pathway could be triggered. *C. albicans* has been shown to induce apoptosis in peritoneal macrophages (33) and in the J774 macrophage-like cell line (34). However, we found no activation of apoptotic caspase 3 in BMDMs early upon phagocytosis (Fig. 1), and our results are supported by recent experiments using the RAW 267.4 cell line (23). Moreover, a study of macrophage-*Candida* interactions *in vivo* in kidneys of mice found that there is no activated caspase 3 in wild-type macrophages at day 6 following infection with *Candida* (35). Recently, extracellular *C. albicans* have been shown to activate a caspase-8-containing inflammasome in dendritic cells (14), but a previous study using RAW 267.4 macrophages showed no or minimal activation of caspase 8 and caspase 9 in response to *C. albicans* infection and the authors concluded that apoptosis does not play a major role in macrophage cell death induced by *C. albicans* (36). In addition to apoptosis, another possibility is that *C. albicans* triggers the caspase-independent programmed form of necrosis termed necroptosis (37). Ongoing studies in our laboratories are focused on elucidating the mechanistic features of pyroptosis-independent macrophage cell death caused by *C. albicans*.

We have shown that several aspects of macrophage-*C. albicans* interactions, including morphogenesis, hyphal architecture, and virulence factors of the cell wall, are controlled by the transcriptional regulator Mediator. We have also shown that Mediator subunits Med31 and Srb9 are necessary for wild-type virulence in the mouse systemic model. Med31, which is in the so-called Middle domain of the core Mediator complex, impacts on hyphal morphogenesis (28). In contrast, the Srb9 subunit from the kinase domain does not appear to have an impact on morphogenesis (28). It has to be noted that mechanisms additional to pyroptosis also operate in phase 1. First, wild-type *C. albicans* filaments fail to induce normal death rates in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs and also in RAW macrophages, where almost no host cell death is observed for the first 9 h, suggests that mechanical piercing by hyphae, which is currently considered to be a major contributor to macrophage killing (3–5), is not among the main mechanisms of early host cell death upon phagocytosis. Caspase-1 and caspase-11 can independently induce pyroptosis (18, 27). However, caspase-11 is primarily activated by Gram-negative bacteria and LPS (30, 31), suggesting that caspase-1 is the main pyroptotic caspase activated by *C. albicans* hyphae. Our conclusions are supported by a report published while the present manuscript was under review that showed that *C. albicans* hyphae induce macrophage pyroptosis that depends on caspase-1 and the inflammasome subunits NLRP3 and ASC (32). Consistent with our data, the same study showed that pyropoptosis is the predominant mechanism of macrophage cell death when fungal cell numbers are low, such as early upon phagocytosis. It has to be noted that mechanisms additional to pyroptosis also operate in phase 1. First, wild-type *C. albicans* induces more macrophage cell death than heat-killed cells in the absence of pyroptosis in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs. Second, wild-type filaments caused higher cell death rates than the morphogenesis-impaired *med31<sup>Δ/Δ</sup>* mutant not only in wild-type BMDMs, but also in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs. Moreover, phase 2 of killing requires wild-type hyphae, but the mechanism is distinct from pyroptosis, as this phase occurs normally in *casp1<sup>−/−</sup>* *casp11<sup>−/−</sup>* BMDMs. The additional macrophage cell death mechanism in phase 1, as well as the phase 2 death that occurs when *C. albicans* hyphae are abundant, could depend on mechanical destruction of macrophages by hyphae. Alternatively, another host cell death pathway could be triggered. *C. albicans* has been shown to induce apoptosis in peritoneal macrophages (33) and in the J774 macrophage-like cell line (34). However, we found no activation of apoptotic caspase 3 in BMDMs early upon phagocytosis (Fig. 1), and our results are supported by recent experiments using the RAW 267.4 cell line (23). Moreover, a study of macrophage-*Candida* interactions *in vivo* in kidneys of mice found that there is no activated caspase 3 in wild-type macrophages at day 6 following infection with *Candida* (35). Recently, extracellular *C. albicans* have been shown to activate a caspase-8-containing inflammasome in dendritic cells (14), but a previous study using RAW 267.4 macrophages showed no or minimal activation of caspase 8 and caspase 9 in response to *C. albicans* infection and the authors concluded that apoptosis does not play a major role in macrophage cell death induced by *C. albicans* (36). In addition to apoptosis, another possibility is that *C. albicans* triggers the caspase-independent programmed form of necrosis termed necroptosis (37). Ongoing studies in our laboratories are focused on elucidating the mechanistic features of pyroptosis-independent macrophage cell death caused by *C. albicans*.
phal filaments has been proposed as a trigger for activation of caspase-1 inflammasomes (38). Second, it is possible that 1,3 \( \beta \)-glucan is sensed directly by host receptors. Increased exposure of 1,3 \( \beta \)-glucan on the cell surface of \textit{C. albicans} induces higher levels of IL-1 \( \beta \) (12), and the \( \beta \)-glucan preparation curdlan can activate caspase-1/NLRP3/Asc-containing inflammasomes (39, 40). It is also possible that the \textit{srb9}\( \Delta \)/\( \Delta \) mutant displays changes to other components of the cell wall that impact on the activation of immune responses. Also, compromised hyphal cell wall structure impacts mechanical features of the hyphae that could mediate the ability to cause macrophage cell death.

In conclusion, our data show that the interplay between developmental transitions and survival strategies of \textit{C. albicans} and the activation of host immune pathways is more sophisticated than previously appreciated. It is currently not clear what the consequence of \textit{Candida}-triggered pyroptosis is for disease. Caspase-1 is known to protect against \textit{C. albicans} infections (11, 13, 41), and it is thus possible that pyroptosis has a protective role by increasing inflammatory responses, as is the case for bacterial pathogens. While mice deficient in caspase-1 and the IL-1 receptor are highly susceptible to disseminated candidiasis, \textit{casp1}\( /-\)/\textit{casp11}\( /-\) mice show normal fungal burdens during the first few days in kidneys in the systemic infection model, and at the site of infection, on tongues, in the oral model (11, 41). The PRR dectin-1 is required for activation of caspase-1 by \textit{Candida} in response to some fungal strains (14), and it will be interesting to determine
how a possible role for dectin-1 in pyroptosis contributes to the roles of this PRR in disease caused by C. albicans (16, 42, 43). We suggest that pyroptosis might promote evasion of the innate immune response by C. albicans by providing an escape route for the pathogen (as shown, for example, by reduced escape of \( \text{svr9}_\Delta/\Delta \) mutant hyphae). In other words, the same molecular event—activation of caspase-1 by fungal hyphae—can cause both protective immunity and fungal escape. The outcome of infection likely depends on a balance between these paradoxical consequences of the interactions between Candida hyphae and the innate immune response.

**MATERIALS AND METHODS**

Detailed experimental procedures are provided in the supplemental material.

**Ethics statement.** Animal experiments were performed in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals and approved by the Monash University Animal Ethics Committee (approval number S0BS/M/2010/49) or under conditions approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

**Statistical analysis.** For statistical analysis, the unpaired, two-tailed Student’s t-test was performed using GraphPad Prism software, and \( p \)-values of \(<0.05\) were considered to be significant. For the animal infection model (see Fig. S2 in the supplemental material), statistical analysis was performed with MiniTab version 16 statistical software. Differences in survival rates were estimated with the nonparametric Kaplan-Meier method using the log-rank test and survival curves plotted. Means of organ burdens were compared using one-way analysis of variance (ANOVA).

**Yeast strains and growth conditions.** The C. albicans strains are derivatives of BWP17 and described in reference 28. The strains were propagated at 30°C in yeast extract-peptone-dextrose (YPD) media with the addition of 80 \( \mu \)g/ml uridine. All experiments involving hyphal growth were performed in either RPMI or Spider media at 37°C.

**Microscopy and quantification of 1,3-β-glucan exposure by flow cytometry.** The phagocytosis data in Fig. 2A (percentage of infected macrophages and number of Candida cells/100 macrophages) were determined using the images from the live-cell microscopy experiments presented in Fig. 1B and 3 at 30 min after the 1 h coincubation period. The MOI was 1 macrophage to 6 Candida. The cell morphology of wild-type Candida in the various macrophages in Fig. 1C was determined from the live-cell microscopy experiments as described for phagocytosis above. For determining cell morphology, escape and phagolysosome association of the C. albicans wild type or the Mediator mutants (Fig. 2B to E), BMDMs were infected at a MOI of 1:2 (macrophage:Candida), followed by 1 h coincubation and washing. For monitoring escape, fungal cells were stained with calcifiouor white (5 \( \mu \)g/ml, 10 min). Immunofluorescence experiments with the glucan antibody and for monitoring association with the phagosomal marker Lamp1 are described in detail in the experimental procedures section of the supplemental material.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00003-14/-/DCSupplemental.

Text S1, PDF file, 0.2 MB.

Figure S1, TIF file, 1 MB.

Figure S2, TIF file, 2.1 MB.

Figure S3, TIF file, 0.1 MB.

Figure S4, TIF file, 0.6 MB.

Video S1, AVI file, 20.5 MB.

Video S2, AVI file, 13.4 MB.

Video S3, AVI file, 20.6 MB.

Video S4, AVI file, 13.8 MB.

March/April 2014 Volume 5 Issue 2 e00003-14
ACKNOWLEDGMENTS

We thank Trevor Lithgow and Jamie Rossjohn for comments on the manuscritp and acknowledge the technical support from the Monash University Microfilingimaging facility. We further thank Gilu Abraham for technical assistance.

A.T., T.N., J.E.V., and S.L.M. are supported by grants from the Australian National Health and Medical Research Council (NH&MRC). Y.Q. and H.-H.S. are Australian Research Council (ARC) SuperScience Fellows. J.E.V. is an NH&MRC Career Development Fellow.

REFERENCES


