The Pathogen Candida albicans Hijacks Pyroptosis for Escape from Macrophages

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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.

IMPORTANCE Upon phagocytosis by macrophages, Candida albicans can transition to the hyphal form, which causes macrophage death and enables fungal escape. The current model is that the highly polarized growth of hyphae results in macrophage piercing. This model is challenged by recent reports of C. albicans mutants that form hyphae of wild-type morphology but are defective in killing macrophages. We show that C. albicans causes macrophage cell death by at least two mechanisms. Phase 1 killing (first 6 to 8 h) depends on the activation of the pyroptotic programmed host cell death by fungal hyphae. Phase 2 (up to 24 h) is rapid and depends on robust hyphal formation but is independent of pyroptosis. Our data provide a new model for how the interplay between fungal morphogenesis and activation of a host cell death pathway mediates macrophage killing by C. albicans hyphae.

Candida albicans is a human commensal but 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 with 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 mannose-lated proteins that decorate the cell surface. The differential expression and exposure of cell wall components are thought to be a


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major factor in how immune cells discriminate yeast from inva-
dative hyphal forms (reviewed in reference 7). For example, dif-
fferences between yeast and hyphae in β-glucan exposure have been
proposed to lead to different engagements with the cell surface
pathogen recognition receptor (PRR) dectin-1 (10). Dectin-1 trig-
gers proinflammatory interleukin-1β (IL-1β) expression via Syk
kinase signaling, and activation of a cytoplasmic inflammasome
that contains NLRP3, ASC (apoptosis-associated speck-like pro-
tein 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 un-
known 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 inflam-
masome 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
in 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, mac-
rophages 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 patho-
gens 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 dis-
semination from the site of infection (20–22). Cell death pathways
have mostly been studied in the context of bacterial and viral in-
fecions, 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-
phagocytosis (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-
dependent macrophage killing by Candida also occurs, particu-
larly 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 mutu-
ally 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 un-
derstand 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-cultured with bone marrow-
derived macrophages (BMDMs) for 1 h (at a multiplicity of infec-
tion [MOI] of 1 macrophage to 6 Candida cells), followed by
washing of the nonphagocytosed cells and monitoring of macro-
phage 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 agree-
ment 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 sec-
ond phase of macrophage killing, which lasted up to 21 h post-
infection and resulted in a complete collapse of the host cell cul-
ture (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 in-
fection 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 fil-
aments 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 mac-
rophages (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 macro-
phages, 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 fun-
gal hyphae formed normally (Fig. 1C and 2A), suggesting that
lower rates of macrophage cell death are not caused by lower up-
take or changes to the morphogenesis of Candida in the mutant
BMDMs. Instead, these data show that C. albicans triggers pyro-
ptotic 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), suggest-
ing 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 of 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 med31ΔΔ mutant is impaired in filamentation (28). Consistent with the morphogenesis defect, the med31ΔΔ 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).
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. S2D; we note that, for the data determined at 13.5 h in our assay, we do not differentiate between phagocytosed and escaped Candida cells that were replicating in the media). Consistent with the morphogenesis and macrophage escape defects, 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).

The C. albicans mutant lacking the SRB9 subunit from the kinase domain of Mediator infected and formed filaments in BMDMs similarly to wild-type C. albicans (Fig. 2A to C; see also Video S5 in the supplemental material). The srb9Δ/Δ mutant survived and multiplied normally during the infection period (Fig. S2D). Surprisingly, although it formed hyphal filaments, the srb9Δ/Δ mutant was deficient in early escape from macrophages (Fig. 2C) and showed increased association with Lamp1-positive compartments (Fig. 2D, E). Consistent with fewer hyphal escaping, loss of SRB9 resulted in reduced macrophage killing in the first 4 h post-infection compared to wild-type C. albicans results (Fig. 3A). For example, at 3 h, the mutant caused approximately 40% less macrophage cell death than the wild type (mutant/wild-type ratio, 0.63 ± 0.098 standard deviation [SD]). The srb9Δ/Δ mutant killed at the same rate as wild-type C. albicans in the second phase of cell death, and the kinetics of macrophage killing was restored to wild-type levels after re-expression of SRB9 (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...
would be abrogated in the absence of pyroptosis in sis. If this were the case, then we would expect that the difference macrophage cell death was due to defective activation of pyroptosis.

address whether the reduced ability of Mediator mutants to cause secretion induced by Mediator mutant BMDMs (Fig. 4; see also Fig. S4). In BMDMs early postphagocytosis suggests that an important function of the hyphal cell wall adhesins did not depend on Srb9 (Fig. 6A). wall genes mediate phenotype (Fig. 5). We have previously found that the srb9 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, srb9 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 srb9 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 morphogenesis-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.
FIG 5  
srb9Δ/Δ mutant hyphae display a breakdown of cell surface architecture. AFM (atomic force microscopy) was performed on hyphae grown in vitro under conditions that mimic those of the macrophage experiments (RPMI media, 37°C). Deflection images of hyphal tips from wild-type and srb9Δ/Δ mutant hyphae are presented on the left and force measurements on the right. The regions in which force measurements were done were squares of the following sizes: 1.7 μm-by-1.7 μm for the wild type, 1.2 μm-by-1.2 μm for srb9Δ/Δ, and 1.5 μm-by-1 μm for the complemented strain). The adhesion forces, extracted from force-distance curves, were measured in an 8-by-8 matrix for the wild-type and mutant strains, or a 7-by-5 matrix for the complemented strain, as shown in the figure (the unit of adhesion force is nN). The measurements are color coded from gray (low intensity) to red (high intensity). Multiple hyphae were measured for each of the strains and gave equivalent results. The scale bar is 1 μm.

That wild-type C. albicans filaments fail to induce normal death rates in casp1−/− casp11−/− 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 pyrop-
phal filaments has been proposed as a trigger for activation of caspase-1 inflammasomes (38). Second, it is possible that 1,3-β-glucan is sensed directly by host receptors. Increased exposure of 1,3-β-glucan on the cell surface of \textit{C. albicans} induces higher levels of IL-1β (12), and the β-glucan preparation curdlan can activate caspase-1/NLRP3/Asc-containing inflammasomes (39, 40). It is also possible that the \textit{srb9ΔΔ} 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−/−, 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 *srb9ΔΔ* 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 SOBS/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 Unistat 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 WBP17 and described in reference 28. The strains were propagated at 30°C in yeast extract-peptone-dextrose (YPD) media with the addition of 80 μg/ml uridine. All experiments involving hyphal growth were performed in either RPMI or Spider media at 37°C.

**Animal infections and isolation of bone marrow-derived macrophages.** Bone marrow from 6- to 8-week-old C57Bl/6 wild-type or *casp1<sup>−/−</sup>/casp11<sup>−/−</sup>* mice was isolated and macrophages were differentiated essentially as described in reference 44. For assaying virulence, 6- to 8-week-old BALB/c mice were infected using the mouse tail vein systemic infection method. Animal infections and isolation of bone marrow-derived macrophages, *C. albicans* strains, and other methods are described in detail in the experimental procedures section of the supplemental material.

**Live-cell imaging and quantification of macrophage killing by *C. albicans*.** Macrophages were challenged with *C. albicans* at a MOI of 1:6 (macrophage:*Candida*). After 1 h of coincubation, nonphagocytosed fungal cells were removed by washing with phosphate-buffered saline (PBS). PI was added to the wells for monitoring macrophage cell death. Experiments were performed on a Leica AF6000 LX live-cell imaging system with an inverted, fully motorized microscope driven by Leica Advanced Suite Application software. Time-lapse images were acquired with bright-field and TxRed filters every 15 min for up to 24 h using a 20×/0.8-A objective. Fluorescent (PI) images were converted into binary images with ImageJ software using the same signal threshold for all samples, and total PI signal was measured for each of the images. The time point where maximum macrophage death occurred was determined by assessing the time-lapse images and maximum percentage of dead macrophages calculated by manually counting PI-positive and total macrophages in fluorescent (PI) and bright-field images, respectively. This was used to calculate percentage death at earlier time points from the same sample based on total PI signal. Calculations were done using Microsoft Excel and data analyzed with GraphPad Prism software. Representative movies were made by merging the bright and fluorescent fields, and images were compressed and brightness and contrast adjusted evenly for the entire movie for ease of viewing using ImageJ. For methods to determine *C. albicans* survival in macrophages (see Fig. S2), please see the procedures described in the supplemental material.

**Quantification of IL-1β production and caspase-3 activation.** For IL-1β production experiments, macrophages were pretreated with LPS (50 ng/ml for 3.5 h) and infected with *C. albicans* wild-type or Mediator mutant strains at a MOI of 1:6 (macrophages: *Candida*). IL-1β levels were determined from supernatants at 2 and 3 h after the 1-h coincubation period using enzyme-linked immunosorbent assay (ELISA), as described in reference 44. Cleaved caspase-3 was detected in whole-cell extracts after coincubation of BMDMs with wild-type *C. albicans* or heat-killed yeast cells, or treatment with cycloheximide for 3 h, by probing with an antibody that recognizes cleaved caspase-3 (Asp175; Cell Signaling).

**Gene expression analysis.** Analysis of adhesin gene expression was performed after 3 h of coincubation of *C. albicans* and wild-type BMDMs, using a 1:6 multiplicity of infection (macrophages:*Candida*). The levels of the adhesins were determined by quantitative PCR as described in the experimental procedures section of the supplemental material.

**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. 1E 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 calcein white (5 μg/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. Imaging was done on an Olympus IX81 or a Nikon C1 confocal microscope. Representative images were selected and cropped and brightness and contrast adjusted in CorelDRAW even for the entire image. For Fig. 6, fluorescent images of β-glucan in wild-type and mutant hyphae were taken with the same exposure and camera settings, and contrast and brightness adjusted evenly only for the bright-field images using CorelDRAW (the fluorescent images were not altered). Confocal stack images were used to construct three-dimensional (3D) images of representative hyphal cells, using ImageJ software with the 3D project function. AFM was performed on hypha grown in vitro in RPMI media at 37°C. AFM measurements were performed at room temperature under dry conditions using the NanoWizard II AFM system at the Melbourne Centre for Nanofabrication. AFM contact-mode images were obtained using Si3N4 cantilevers (MSNL-10; Bruker, Santa Barbara, CA). Reflection images were simultaneously acquired and analyzed with IPK data software (IPK Instruments AG, Germany). Force-distance measurements were collected on single *C. albicans* hyphae, and several hyphae/strain were measured. Wild-type *C. albicans* and the *srb9ΔΔ* mutant were assayed on several separate occasions, while the complemented *srb9ΔΔ*::SR29 strain was analyzed on one occasion (5 independent hyphae).

**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.

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