Supplemental Experimental Procedures

Yeast strains and growth conditions

The *C. albicans* wild type (DAY185) and Mediator mutant strains were constructed by standard methods based on PCR and homologous recombination and selected using the *ARG4* and *URA3* auxotrophic markers. Complementation was done using the pDDB78 (*HIS1*) vector. The strains are described in (Uwamahoro et al 2012). For yeast growth, media was YPD (1% yeast extract, 2% peptone, 2% glucose, with the addition of 80 µg/ml uridine) at 30°C. For hyphal growth media was RPMI (complete culture RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and 12 mM pH 7.4 HEPES) or Spider (1% nutrient broth from Oxoid, 1% D-mannitol, 2 g/l K_2HPO_4, 80 µg/ml uridine) at 37°C. RPMI media was used for AFM and microscopy analysis. For FACS analysis of 1,3 ß-glucan exposure, filamentous growth was induced using Spider media because growth in RPMI did not produce consistent results due to technical reasons. For heat killed wild type (HKWT), wild type *Candida* cells were heated for 1 h at 80°C.

Mice strains and preparation of bone-marrow derived macrophages

Mice strains (wild type or *casp1*$^{-/-}$ *casp11*$^{-/-}$ mutants) are on the C57BL/6 background. Animals were sacrificed according to the ethics protocol approved by either the Monash University Animal Ethics committee (SOBS/M/2010/49) or under conditions approved by the Walter and Eliza Hall Institute Animal Ethics Committee. After extraction, the marrow of tibial and femoral bones was flushed out using a 21-gauge syringe and 10 ml of BMDM media (RPMI containing 20% L929 conditioned medium, 15% Fetal Bovine Serum, and penicillin and streptomycin (Sigma) at 100 units/ml and 0.1 mg/ml, respectively), and incubated overnight at 37°C, 5% CO_2 in tissue culture treated flasks. The following day, non-adherent cells (20 ml cell suspension) were diluted in 100 ml fresh BMDM media and incubated for another seven days in untreated Petri dishes. Cells were harvested within 3 days post differentiation by gently scraping them off the plates using a sterile cell scraper (Biologix).

Experiments to monitor fungal morphology, escape and phagocytocis, and Lamp1-association analysis

For assaying fungal escape and morphology (Figure 2B-D), experiments were conducted as described by Fernandez-Arenas et al 2009. Freshly streaked *C. albicans* strains were cultured at 30°C in 2 ml of YPD+ uridine media, while 500,000 cells of primary macrophages were seeded into wells of a 24 well plate containing microscope glass slide coverslips and incubated at 37°C, 5% CO_2 atmosphere overnight. *Candida* and macrophages were co-incubated for 1 h at the multiplicity of infection 1 macrophage: 2 *Candida*, and un-phagocytosed cells washed off with PBS. One milliliter of BMDM medium was added and cells were allowed to co-incubate a further 3 h. For monitoring escape, cells were stained with 5 µg/ml calcoflour white for 10 min, washed 4 times and mounted on glass slides with Dako fluorescent medium (Invitrogen). Imaging was done using an Olympus IX81 microscope with the Olympus cell^{M} software, using the 60x objective with DIC, or the DAPI filter for calcofluor white stained cells. Three independent experiments were performed and at least 200 cells/strain counted in each of

the experiments. For Lamp1 quantification, cells were prepared and stained with calcofluor white as described above, except that cells were allowed to co-incubate for a further 2 h, instead of 3 h. After washes with PBS, cells were fixed with 4% para-formaldehyde for 10 min, and washed thrice with PBS, followed by 10 min incubation in 50 mM ammonium chloride. Cells were washed thrice and permeabilised with 0.1% saponin for 5 min, before adding blocking solution (3% BSA, 0.1% saponin, 1x PBS) for 15 min on ice. 1/800 dilution of anti-Lamp1 antibody (CD107a from BD Pharmagen) was added and allowed to bind overnight at 4°C. The next day, cells were washed twice for 15 min with ice cold PBS, and 1/800 dilution of Alexa-fluor 594 Chicken anti-rat IgA was added. Incubation was overnight in blocking solution. The next day, cells were washed thrice with 50 ml PBS and mounted. Cells were imaged using confocal microscopy on the Nikon C1 Invert Microscope (Monash MicroImaging facility). Three independent experiments were performed and Lamp1 association was counted for at least 50 *Candida* cells for each of the strains in each of the independent experiments. Calcoflour white staining was used to discriminate between internal and external *Candida* during quantification.

For determining phagocytosis in Figure 2A images at the 30 min time point post the 1h co-incubation were used from three independent experiments conducted together for wild type and *casp1*$^{-/-}$*casp11*$^{-/-}$ BMDMs (the experiments are shown in Figures 1E, 3A and 3B). Similarly, for the RAW 264.7 cells, the experiment shown in Figure 1B was used for quantification at the 30 min time point post the 1 h co-incubation (two independent experiments). A total of 200 macrophages were counted for each of the independent biological experiments. The MOI was 1 macrophage to 6 *Candida* cells. Morphology of *Candida* cells within macrophages in Figure 1C was determined from the same experiments as above counting 100 *Candida* cells in each of the biological repeats.

For all experiments testing phagocytosis, fungal morphology, escape and phagolysosome association, Figures show averages from the biological repeats and the standard error of the mean. Statistical analysis was performed using the Graph Pad Prism version 6.00 for Windows, GraphPad Software (La Jolla California USA, www.graphpad.com), using the unpaired, two tailed student t-test. The asterix above error bars indicates *p*-values (*<0.05, **<0.01, ***<0.001 and ****<0.0001).

Quantification of macrophage cell death using time-lapse imaging
Primary macrophages or RAW 264.7 macrophage cell lines (500,000 cells) were seeded in 24 well plates and infected with *C. albicans* (cultured in YPD+uridine at 30°C over night) at a multiplicity of infection of 1:6 (macrophage:*Candida*). After 1 h, macrophages were washed 4 times with 1x PBS to remove all un-phagocytosed yeast cells (as previously described by Fernandez-Arenas et al, 2009). Cells were replenished with 1 ml of RPMI media containing 10 μg/ml propidium iodide (PI) (Invitrogen). Experiments were performed on the Leica AF6000 LX live cell imaging system with an inverted, fully motorized microscope, driven by the Leica Advanced Suite Application software (Monash MicroImaging facility). During the course of the experiment, cells were maintained in a humidified chamber at 37°C and 5% CO_2. Time-lapse images were

acquired with bright field and TxRed filter every 15 min for up to 24 h using a 20x/0.8A objective, typically capturing over 300 cells per well.

Macrophage cell death was determined after conversion of propidium iodide images into binary images using the Fiji ImageJ software (Schindelin et al 2012); the same signal threshold was applied for all samples. The binary images were then used to measure PI signal for the area of each image with the particle analyzer. To determine the percentage of macrophage cell death from the PI signal, we assessed the time-lapse images within each movie to find the time point at which maximum macrophages death was reached. For this time point, the percentage of dead macrophages was determined by counting manually using bright field and PI images and determining PI positive versus total macrophages. This value represented the maximum percentage of macrophage cell death for that movie. For instance, maximum macrophage death rates in wild type *C. albicans* infections occurred ~18-21 h post infection at the MOI used (1 macrophage : 6 *Candida* cells), and reached 100%. In contrast, heat-killed *Candida* cells caused little death, and macrophage cell death for the *Candida med31Δ/Δ* mutant did not reach 100% by the end of the experiment. The values for maximum percentage macrophage death and the respective total PI signal were used to calculate the percentage of dead macrophages at the earlier time points from the same movie based on total PI signal. Calculations were done with Microsoft Excel and data analyzed by the GraphPad Prism Software. Each experiment was performed independently at least 3 times using macrophages derived from different mice and independent *Candida* cultures, except for the RAW 264.7 experiments, which were conducted twice. Graphs show the mean of biological repeats and the standard error of the mean (SEM). Statistical significance was determined using the unpaired Student *t*-test with two-tailed *p*-values (GraphPad Prism Software La Jolla California USA, www.graphpad.com). Asterix above error bars indicate *p*-values (*<0.05, **<0.01, ***<0.001 and ****<0.0001)

Quantification of IL-1β production

Experiments were conducted as described by Joly et al, 2006. Briefly, BMDMs were primed with 50 ng/ml LPS for 3.5 h in 6 well plates. Overnight cultures of *C. albicans* strains were counted and co-incubated with primed BMDMs at a ratio of 1:6 (BMDM:*Candida*) for the time points indicated in Figure 4 and Supplemental Figure 4. In one of the experiments (Figure S3A, comparing wild type and *srb9Δ/Δ* Mediator mutant of *Candida*), after the 1 h of co-incubation non-phagocytosed cells were washed off with PBS. This additional washing step did not cause an observable difference in IL-1β levels. Therefore, relative IL-1β levels for the *srb9Δ/Δ* mutant in Figure 4 were calculated using data from all four experiments. To obtain heat killed fungal cells, wild type *Candida* were heated for 1 h at 80°C before co-incubation with BMDMs. Supernatants were collected and IL-1β quantified by ELISA according to the manufacturer's recommendations (R&D systems).

Western blots for detection of cleaved caspase 3

BMDMs were infected with *C. albicans* wild type or heat killed cells and incubated for 3 h. Alternatively, BMDMs were treated with cycloheximide (CHX, 50µg/ml) for 3 h as a positive control for caspase 3 activation. Macrophages were lysed in reducing SDS-

loading buffer and protein extracts equivalent to $5x10^4$ cells separated on 12% polyacrylamide gels and transferred to nitrocellulose membranes (Amersham). After ponceau S staining, membranes were blocked in 5% skim milk (in PBS with 0.05% Tween-20) and probed with anti-cleaved caspase 3 antibody (Asp175, Cell Signaling), diluted in 5% skim milk overnight. After incubation with secondary antibody, membranes were incubated with ECL-reagents (Promega) and exposed to film (Kodak).

Gene expression analysis

Gene expression was studied following co-incubation of macrophages and *Candida* (1 macrophage : 6 *Candida*) in 100-mm dishes for 3 h. Isolation of *Candida* cells was done by washing the dishes with PBS, followed by addition of 5 ml/plate of Trizol reagent to lyse the macrophages. Fungal cells were collected by centrifugation and washed two times with Trizol to remove most of macrophage DNA and RNA. Cells were frozen in dry-ice and stored at –80°C until use. RNA was extracted using the hot-phenol method and contaminating genomic DNA removed by treatment with DNase I (Ambion). Reverse transcription was performed using Superscript III (Invitrogen). Quantitative PCR was performed using the Fast-Start universal Sybr Green Master (Roche) on an Eppendorf Realplex master cycler, and analysed by absolute quantification. The expression levels of the transcripts were normalized to the level of 18s rRNA. Sequences for qPCR primers used in this study are listed in Table 1 below. Three independent biological experiments were performed, with two technical replicates each. Averages shown in Figure 6 are from the biological repeats and the error bars represent the standard error of the mean (SEM).

Table 1. Primers used in this study	
18s rRNA-forward	GGATTTACTGAAGACTAACTACTG
18s rRNA-reverse	GAACAACAACCGATCCCTAGT
HWP1-forward-	AATCCTCCTCAACCTGATCAGCCTG
HWP1-reverse-	AGCTGGAGTTGTTGGCTTTTCTGGA
ALS1-forward	ACCAATCCAGTTCCAACTGTGGCA
ALS1-reverse	TGGATGCTGATTCATGAGAACCGCT
ALS3-forward	ACTTCCACAGCTGCTTCCACTTCT
ALS3-reverse	TCCACGGAACCGGTTGTTGCT.

Fluorescence microscopy and quantification of 1,3 ß-glucan exposure using flow cytometry

For immunofluorescence labeling of 1,3 β-glucan, overnight YPD grown *C. albicans* strains were washed twice with PBS and transferred into a 24 well tissue culture plate with coverslips at OD^{600}=0.05 in RPMI with 10% serum, followed by incubation for 4 h at 37°C in a humidified atmosphere with 5% CO_2. These conditions mimic the conditions during the macrophage infection experiments. *Candida* bound coverslips were collected after 4 h of filamentation, washed twice with 1 ml PBS, and incubated for 10 min with PBS containing 0.1% BSA. β-glucan staining was done by incubating coverslips with 200 µl monoclonal mouse anti-β-1,3-glucan antibody (Biosupplies Australia, 1 µg/ml in PBS with 0.1 % BSA), at room temperature for 30 min. After washing with PBS to remove unbound primary antibody, cells were incubated with 200 µl AlexaFluor 488-

labelled goat anti-mouse IgG (Invitrogen, 4 μg/ml in PBS with 0.1% BSA). In negative control samples, only the secondary antibody was added (no signal was detected in these negative control experiments, data not shown). After incubation at room temperature for 30 min, cells were washed 3 times with PBS, mounted on slides and observed using the Nicon1 confocal microscope (Monash MicroImaging Facility) as described above.

For flow cytometry, overnight YPD grown *C. albicans* strains were washed twice with PBS and transferred to 37°C into pre-warmed Spider medium at OD^{600}=0.05 and incubated for 3 hours at 37°C. Cells were washed twice with PBS, collected by centrifugation (200 rpm) and re-suspended in 500 μl of monoclonal mouse anti-β-1,3-glucan antibody. After 30 min at room temperature, cells were collected by centrifugation and washed three times with 1 ml PBS, followed by 30 min incubation with AlexaFluor 488-labelled goat anti-mouse IgG as above. After washing, cells were re-suspended in PBS and immediately analyzed by flow cytometry (FACSCalibur, BD Biosciences). For data acquisition, forward and side scatter were detected on linear scales, while AlexaFluor 488 fluorescence was analysed on logarithmic scales. The AlexaFluor 488 fluorescence intensity of at least 10000 gated cells was calculated using FACSDiva 5.0 software (BD Biosciences). The fluorescence graphs were made in Weasel 3.1 (WEHI, Australia). The experiment was performed on three independent occasions.

Atomic force microscopy

Freshly streaked *C. albicans* strains were cultured at 30°C in 2 ml of YPD+ uridine media, while sterile microscope glass slides were immersed in petri dishes containing 15 ml of RPMI media at 37°C in a humidified atmosphere with 5% CO_2. The following day, *C. albicans* cells were washed with sterile water and diluted into the prepared dishes to OD^{600}=0.05 and incubated 37°C in a humidified atmosphere with 5% CO_2. *Candida* bound slides were collected after 4 h of filamentation and washed twice with milli-Q water before imaging. The experiment comparing wild type and *srb9*Δ/Δ mutant hyphae was performed three times, and at least 10 individual hyphae measured for each of the strains across the independent experiments. In one of the experiments, the complemented *srb9*Δ/Δ + *SRB9* strain was also included, and 5 hyphae measured for this strain.

Immediately before measurement, the glass slides were washed 3 times with water and were dried in air. AFM measurements were performed at room temperature using NanoWizard® II AFM system at Melbourne Centre for Nanofabrication. AFM contact mode images were obtained using Si3N4 cantilevers (MSNL-10, Bruker, Santa Barbara, CA). Prior to making measurements, the spring constant was corrected and a scan rate was set at 1 Hz. Deflection images were simultaneously acquired and analyzed with the JPK data software (JPK instruments AG, Germany). Force-distance measurements were collected on a single *C. albicans* cell by approaching the cantilever tip towards the cell, pressing against the cell surface and retracting the tip from the cell. The force-map of wild type and mutant cells consisted of ~64 measurements in a selected region on individual cells (1.7 μm x 1.7 μm for the wild type strain, and a 1.2 μm x 1.2 μm section for the *srb9*Δ/Δstrain). For the complemented strain, a 1.7 μm x 1 μm region was selected with 31 measurements.

Quantification of C. albicans survival in macrophages

Primary bone marrow derived macrophages were seeded in 96 well plates and infected with *C. albicans* (cultured in YPD+uridine at 30°C over night) at a multiplicity of infection of 1:2 (macrophage:*Candida*). After 1 h (T_0), macrophages were washed 4 times with 1x PBS to remove all un-phagocytosed yeast cells (as previously described by Fernandez-Arenas et al, 2009). Cells were replenished with BMDM media and allowed to continue co-incubation for 13.5 h ($T_{13.5}$). Macrophage lysis buffer (150 µl of 50mM Tris pH7.5, 2mM EDTA, 0.1% Triton X-100) was added to the wells, followed by 10 min incubation. Lysed macrophages were transferred into microcentrifuge tubes and wells washed with 1x sterile water using a multichannel pipette until all *Candida* cells were transferred to the microcentrifuge tubes (this was confirmed by microscopic analysis of wells at 20x magnification). Samples were vortexed for 30 seconds and centrifuged at 5000 rpm. Pelleted cells were washed, and diluted before plating on YPD +uridine plates. Plates were incubated at 30°C for 36-48 h and colony forming units (CFUs) counted. Experiments were performed at least 3 independent times with each strain assayed in triplicate wells. Figure S2D indicates the relative ($T_{13.5}/T_0$) increase in CFUs.

Virulence assays in the mouse systemic candidiasis model

The virulence assays were performed essentially as described by Dagley et al, 2011. *C. albicans* cells were grown for 20 h in YPD at 30°C with vigorous shaking (250 rpm) before inoculation into mice. Six to eight week-old (15~20 g) BALB/C female mice (Monash Animal Services, Melbourne, Australia) were randomly allocated to groups of five mice in polycarbonate cages and housed in the Monash Animal Research Platform Holding Facilities. Mice were maintained in heated, thermostatically controlled rooms for the duration of the study. The mice were fed a commercial rodent diet. Tap water was provided in plastic bottles fitted with stainless-steel nipples mounted in rubber corks. The mice were housed for a 7 to 10-days acclimatization period before the initiation of the study.

For the survival study, groups of 10-15 mice were inoculated with each of the *C. albicans* strains (3 x10^5 CFU of *Candida* cells in 100 µl PBS) by intravenous (iv) injection in the lateral tail vein using a 27-gauge needle. The degree of clinical illness was monitored twice a day in the first 4 days and then daily in the next 10 days. In each experiment, mice that had severe clinical signs of illness were euthanized immediately by placing them in a closed chamber filled with CO_2 gas. The ill-health criteria for the survival study were loss of more than 10% of the pre-infection weight plus presentation of a debilitating clinical sign such as ruffled fur, hunched gait, and reduced mobility. For organ burdens, groups of three mice were used for each of the strains respectively. Mice from each group were euthanized on day 1 post infection and processed for tissue collection. Kidneys were harvested, weighted, and homogenized for determination of colony-forming units on YPD agar plates. For kidney histopathology examinations, initial inocula of 1.5 x10^6 CFU of *Candida* cells in 100 µl PBS per mouse was adopted. Kidneys were harvested on day 1 post infection and fixed in 10% neutral buffered formalin (NBF). Selected tissue blocks were placed into plastic cassettes and processed overnight using a routine overnight cycle in a tissue processor. The tissue blocks were then embedded in wax and serially sliced into 5 µm sections. Slides holding the sections were stained with Periodic Acid Schiff

(PAS) stain and examined with an Olympus Provis Ax70 microscope at 400X. At the conclusion of the experiment (Day 14), all remaining mice were euthanized. The experimental protocol was approved by the Monash University Animal Ethics Committee.

Statistical analysis was performed with Minitab version 16 statistical software. Differences in survival rate were estimated with the non-parametric Kaplan–Meier method using log-rank test, and the survival curves were plotted. One-way ANOVA was used to compare means of organ burden between groups.

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