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Morphogenesis Is Not Required for *Candida albicans-Staphylococcus aureus* Intra-Abdominal Infection-Mediated Dissemination and Lethal Sepsis

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Intra-abdominal polymicrobial infections cause significant morbidity and mortality. An established experimental mouse model of *Staphylococcus aureus-Candida albicans* intra-abdominal infection results in ~60% mortality within 48 h postinoculation, concomitant with amplified local inflammatory responses, while monomicrobial infections are avirulent. The purpose of this study was to characterize early local and systemic innate responses during coinfection and determine the role of *C. albicans* morphogenesis in lethality, a trait involved in virulence and physical interaction with *S. aureus*. Local and systemic proinflammatory cytokines were significantly elevated during coinfection at early time points (4 to 12 h) compared to those in monoinfection. In contrast, microbial burdens in the organs and peritoneal lavage fluid were similar between mono- and coinfected animals through 24 h, as was peritoneal neutrophil infiltration. After optimizing the model for 100% mortality within 48 h, using 3.5×10^7 *C. albicans* (5× increase), coinfection with *C. albicans* yeast-locked or hypha-locked mutants showed similar mortality, dissemination, and local and systemic inflammation to the isogenic control. However, coinfection with the yeast-locked *C. albicans* mutant given intravenously (i.v.) and *S. aureus* given intraperitoneally (i.p.) failed to induce mortality. These results suggest a unique intra-abdominal interaction between the host and *C. albicans-S. aureus* that results in strong inflammatory responses, dissemination, and lethal sepsis, independent of *C. albicans* morphogenesis.

Intra-abdominal infections (IAIs) are a group of human infections that are often polymicrobial (1, 2) and are caused by invasion and replication of microbes in the abdominal cavity. Severe IAIs result from a variety of insults, including bowel perforation, laparotomy surgery, intestinal hernias, and insertion of medical devices, such as peritoneal catheters (reviewed in reference 3). If IAIs are left untreated or misdiagnosed, microorganisms can migrate into the bloodstream, causing sepsis or systemic inflammatory response syndrome (SIRS) (4–6).

In general, polymicrobial infections are most virulent when fungi are implicated. During polymicrobial IAIs involving fungi, mortality rates escalate between 50 and 75%, compared to 10 to 30% during polymicrobial bacterial infections (7–10). Fungal/bacterial IAIs, specifically those involving *Candida albicans*, have been detected with increasing frequency (11, 12), with *Staphylococcus aureus* coinfections occurring in approximately 15 to 50% of these polymicrobial infections (13–15). As nosocomial and opportunistic pathogens, *C. albicans* and *S. aureus* can be coisolated from a number of anatomical sites and infections (16, 17). Additionally, they are able to form polymicrobial biofilms on a variety of surfaces, including peritoneal catheters, which serve as a source of contamination leading to development of IAIs (17, 18). Moreover, the relationship between *C. albicans* and *S. aureus* is mutually beneficial; in *C. albicans-S. aureus* polymicrobial biofilms, each species displays increased growth, *S. aureus* acquires antimicrobial drug resistance (19), and they modulate one another's proteomic profiles *in vitro* (20). Additionally, during *in vitro* and *in vivo* biofilm growth, *S. aureus* preferentially adheres to *C. albicans* hyphae 30-fold more than yeast (19–21).

Previous studies using an experimental mouse model of IAI involving *C. albicans* and *S. aureus* have demonstrated synergistic lethality, with 40 to 60% mortality by 48 h postinoculation (p.i.) (21), compared to 0% mortality in mice inoculated with either organism alone (21, 22). Coinfection is associated with increased intra- and retroperitoneal tissue microbial burdens, dramatic increases in innate proinflammatory cytokines, prostaglandin E_2 (PGE_2) production, and neutrophil recruitment to the peritoneal cavity at 24 h p.i. However, it is still unclear whether the heightened inflammatory response or enhanced microbial burden and associated virulence factors are the major contributors to mortality in coinfected mice.

The transition from yeast to hyphae is a well-established virulence factor in *C. albicans* infections (reviewed in references 23 and 24). The role of morphogenesis in virulence during systemic infection was demonstrated by intravenous inoculation of a yeast-locked *C. albicans* strain, which resulted in no mortality until hyphal transition was induced *in vivo* (25). Morphogenesis is accompanied by upregulation of hypha-specific genes, including virulence factors, such as adhesins, oxidative stress response genes, and secreted aspartic proteases (SAPs) (reviewed in refer-

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ence 26), that mediate tissue invasion and cellular damage *in vitro* (27–30). Furthermore, the predilection of *S. aureus* to bind to *C. albicans* hyphae suggests that there may be role for this morphotype in pathogenesis during polymicrobial IAIs.

Therefore, the purpose of this study was to characterize the early local and systemic inflammatory events during *C. albicans*-*S. aureus* intra-abdominal infection and determine the role of *Candida* morphogenesis in mediating these events.

MATERIALS AND METHODS

Strains and growth conditions. The methicillin-resistant *S. aureus* strain NRS383 was obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) repository and used in all experiments. NRS383 is positive for toxic shock syndrome toxin (*tst*) and delta-toxin genes. Frozen stocks of NRS383 were kept at −80°C, streaked onto Trypticase soy agar (TSA) (Becton, Dickinson, Sparks, MD), and grown at 37°C. A single colony was transferred to 10 ml of Trypicase soy broth (TSB) (Becton, Dickinson) incubated with shaking at 37°C overnight, diluted 1:100 in fresh TSB, and incubated similarly for 3 h until reaching the log phase. The wild-type *C. albicans* strain used in these experiments was DAY185, a prototrophic control strain that has the *HIS1*, *URA3*, and *ARG4* genes reinserted into strain BWP17, an auxotrophic derivative of strain SC5314 (31). Construction of *C. albicans* morphogenesis mutants was described previously (32–34). Briefly, mutants were constructed using the tetracycline-repressible *tetO* promoter (35), which overexpresses either the hyphal repressor Nrg1p (34) or hypha-specific transcriptional activator Ume6p (36, 37). The resulting mutants are "locked" in the yeast (TNRG1) or hyphal (TUME6) forms in the absence of doxycycline. The isogenic control, TT21, contains the *tetO* promoter with no genes under its control and responds normally to morphogenesis cues (data not shown). Frozen stocks of *Candida* strains were kept at −80°C and streaked onto Sabouraud dextrose agar (SAB) (Becton, Dickinson). TUME6 colonies were plated on SAB containing 20 μg/ml of doxycycline to prevent hyphal formation. A single colony was transferred to 20 ml of yeast extract-peptone-dextrose (YPD) broth and incubated with shaking at 30°C for 18 h. The YPD broth used for culturing TUME6 also contained 20 μg/ml of doxycycline. Prior to inoculation, all organisms were washed 3 times by centrifugation in sterile phosphate-buffered saline (PBS), counted on a hemocytometer, and diluted in sterile PBS to the desired inocula.

Murine model of IAI. All animals were housed and handled according to institutionally recommended guidelines. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the LSU Health Sciences Center. Peritoneal infection was conducted as previously described (21) with some modifications. Briefly, 6-week-old outbred Swiss Webster mice, purchased from Charles Rivers at the National Cancer Institute (NCI), Frederick, MD, were injected intraperitoneally with 0.2 ml of *C. albicans* at various inocula with or without 0.2 ml of 4×10^8 CFU/ml *S. aureus*. Mice intraperitoneally injected with 0.2 ml of saline served as the negative control (referred to as "naïve"). Alternatively, mice were injected intravenously (i.v.) with 0.2 ml of 5×10^6 CFU/ml of the *C. albicans* TNRG1 strain. After inoculation, mice were observed over 5 days for morbidity (hunched posture, inactivity, lethargy, and ruffled fur) and mortality. In addition, separate groups of mice were sacrificed at 4, 8, 12, 18, 24, and 48 h postinoculation and at the end of the 5-day time course. At each time point, several specimens were collected. Whole blood was collected by retro-orbital bleeding, and serum was separated by centrifugation in Microtainer serum separator tubes (BD) at $10{,}000 \times g$ for 2 min. Peritoneal cavities were lavaged by injection of 2 ml of sterile PBS containing EDTA-free protease inhibitor (Roche, Basel, Switzerland) followed by gentle massaging of the peritoneal cavity. Peritoneal lavage fluid was then removed using a pipette inserted into a small incision in the abdominal cavity. Spleens and brains were removed, weighed, and placed into either 1 ml sterile PBS with protease inhibitor or 10% phosphate-buffered formalin for histological analysis. Tissues were mechanically homogenized prior to CFU analysis.

Microbial burden. Microbial burdens in the peritoneal lavage fluid, spleen, and brain were enumerated after serial dilution and plating onto SAB agar plates containing 40 μg/ml gentamicin (Invitrogen, Carlsbad, CA) and 2 μg/ml vancomycin (Sigma Chemicals Co., St. Louis, MO) for *C. albicans*, and TSA containing 2.5 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO) and 40 μg/ml gentamicin for *S. aureus*. SAB plates were incubated at 35°C for 48 h, and TSA plates were incubated for 37°C for 24 h. Spleen and brain CFU were normalized to tissue weights (g).

Cytokine analysis. Concentrations of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and IL-1β in serum and peritoneal lavage supernatant were determined by single-plex enzyme-linked immunosorbent assays (ELISAs) (eBioscience, San Diego, CA). Total protein levels were also measured in peritoneal lavage supernatants using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL), and cytokine data from lavage fluid were expressed as pg cytokine per mg total protein.

Neutrophil analysis. Neutrophils and mononuclear cells were identified and quantified in the peritoneal lavage fluid by differential staining. Peritoneal lavage fluid was centrifuged at $500 \times g$ for 5 min at 4°C and resuspended in 1 ml PBS. Lavage fluid cells were stained with trypan blue and counted on a hemocytometer to determine the total cell number. Cells were then diluted to 1×10^5 cells in 200 μl PBS and cytospun onto Vectabond-treated (Bector Labs, Burlingame, CA) glass slides (5 min at 1,000 rpm). Slides ($n = 1$/animal) were stained with hematoxylin and eosin (H&E) to identify cells with single (mononuclear cells) or trilobed (neutrophils) nuclei. Slides were visualized by light microscopy. Five random fields of mono- and trilobed cells were counted per slide, and the number of neutrophils was averaged per animal and ultimately for each group per time point. Percentages of neutrophils were calculated from the total number of cells in each field and similarly averaged.

Brain histology. Formalin-fixed tissues were sent for histological preparation and were paraffin embedded, sectioned, and stained with hematoxylin-eosin (H&E) or periodic acid-Schiff stain (PAS) (Morphology Imaging Core, Louisiana State University Health Sciences Center [LSUHSC]). Slides were visualized by standard light microscopy.

Statistics. All experiments used groups of 4 to 10 mice and were repeated in duplicate, except where noted. All assays were repeated in duplicate, and the results were averaged. Survival data were analyzed using the Kaplan-Meier test. The Mann-Whitney U test was used to analyze CFU data, while the unpaired Student's *t* test was used to analyze cytokine data and neutrophil infiltration. Significant differences were defined at a confidence level where P is <0.05. All statistical analyses were performed using Prism software (Graph Pad, San Diego, CA).

RESULTS

Polymicrobial infection differentially stimulates inflammation. Using a murine model of IAI, we previously reported that coinfection with 7×10^6 *C. albicans* and 8×10^7 *S. aureus* cells resulted in significantly higher mortality concomitant with significantly elevated levels of organ-associated (kidney/spleen) proinflammatory cytokines and microbial burden compared to either monomicrobial infection (10- to 100-fold) at 24 h p.i. (21). To better understand this synergistic mortality, we conducted a series of kinetic studies evaluating local (peritoneal) and systemic (blood/organs) cytokines and microbial burden in polymicrobial versus monomicrobially infected mice using the same inocula as in the studies previously performed. In the peritoneum, *C. albicans* and *S. aureus* microbial burdens did not differ significantly from 4 to 24 h p.i. between mono- and coinfections (Fig. 1A). By 48 h p.i., only the bacterial burden in *S. aureus* monomicrobially infected mice was reduced. In the spleen, both *C. albicans* and *S. aureus* burdens were high and did not differ significantly between monoinfections and coinfections through 18 h p.i. (Fig. 1B). By 24 h p.i., monoinfected but not coinfected mice began to show a

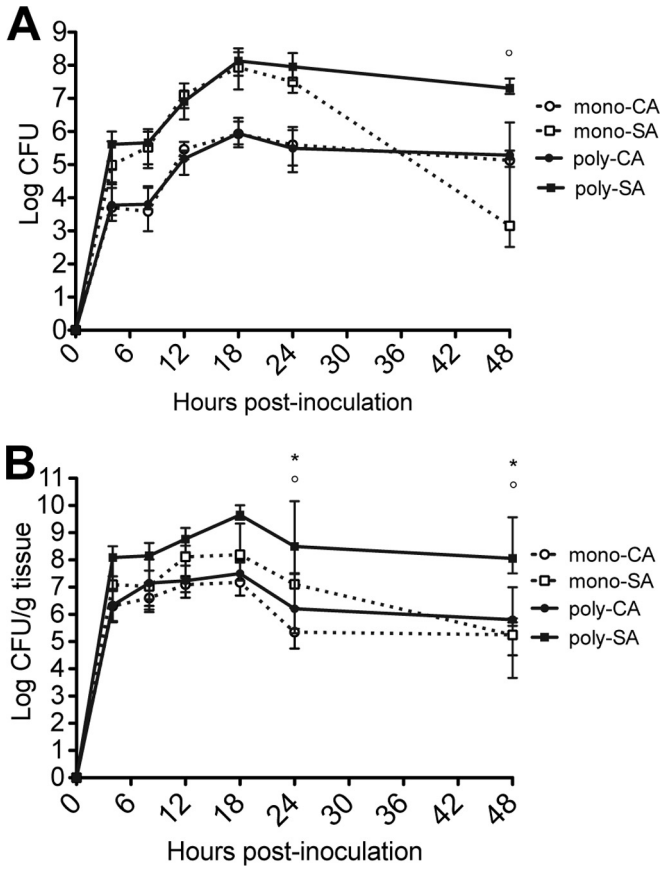

FIG 1 Microbial burden in monomicrobial and polymicrobial peritoneal infection with *C. albicans* and *S. aureus*. Mice ($n = 5$ mice/group) were injected i.p. with 0.2 ml of 3.5×10^7 CFU/ml *C. albicans* alone (7×10^6 CFU), 0.2 ml of 4×10^8 CFU/ml *S. aureus* alone (8×10^7 CFU), or 0.2 ml containing 3.5×10^7 CFU/ml *C. albicans* and 4×10^8 CFU/ml *S. aureus* (8.7×10^7 total organisms). At various time points postinoculation, mice were sacrificed to quantitate *C. albicans* (mono-CA [open circles] versus poly-CA [closed circles]) and *S. aureus* (mono-SA [open squares] versus poly-SA [closed squares]) in peritoneal lavage fluid (A) and spleen (B). Results are expressed as the median CFU ± interquartile range. Shown are cumulative data from two repeat experiments. Mono- and polyinfection groups were analyzed using the Mann-Whitney U test. *, $P < 0.05$ for *C. albicans* versus *C. albicans-S. aureus*; °, $P < 0.05$ for *S. aureus* versus *C. albicans-S. aureus*.

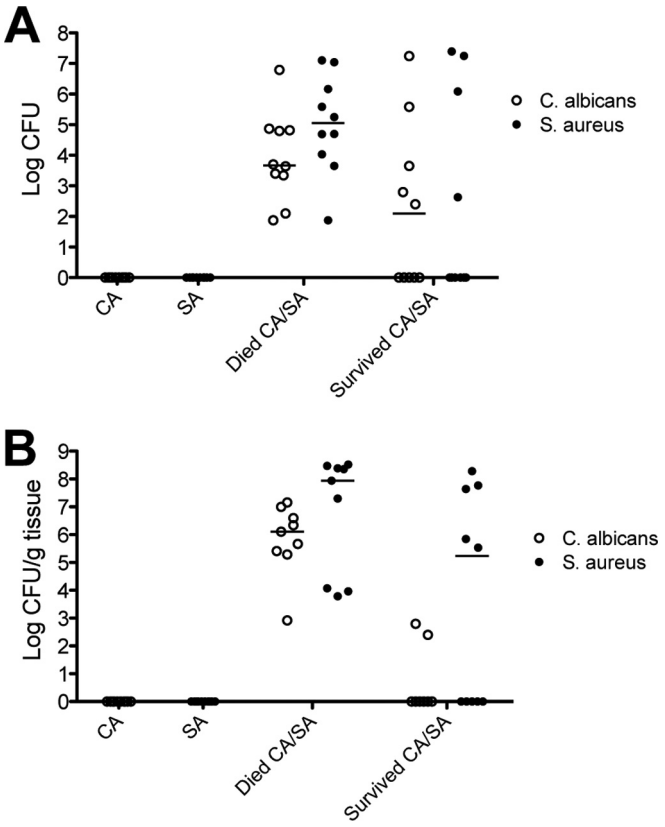

FIG 2 Microbial burden in dead versus surviving coinfected mice. Mice were inoculated i.p. with *C. albicans* (CA) and *S. aureus* (SA) as described in the legend to Fig. 1. Mice ($n = 5$ to 10 mice/group) were sacrificed when moribund or after 5 days postinoculation (p.i.) to assess fungal and bacterial CFU in peritoneal lavage fluid (A) and spleen (B) (*C. albicans*, open circles; *S. aureus*, closed circles). Results are expressed as the median CFU. Shown are cumulative data from three repeat experiments.

reduced microbial burden consistent with our previous data (21). Reduced microbial burden in the kidney, a retroperitoneal organ, was also detected at 24 h p.i. (data not shown), thus indicating similar levels of dissemination.

To determine whether monoinfected mice eventually clear the infection, we analyzed microbial burdens at day 5 p.i. (Fig. 2). The results showed no detectable *C. albicans* or *S. aureus* CFU locally in the peritoneal cavity (Fig. 2A) or the spleen (Fig. 2B). In coinfected mice, recognizing that only ~60% succumb to infection, those that succumbed had high levels of both *C. albicans* and *S. aureus* locally and in organs, whereas the majority of those that survived showed reduced local and organ burdens.

To determine whether coinfection differentially stimulates inflammation despite equivalent microbial burdens, we analyzed the kinetics of inflammatory cytokines produced locally in the peritoneal cavity (Fig. 3). We evaluated the proinflammatory cytokines IL-6, TNF-α, and IL-1β, which have been studied in other IAI models (38). Accordingly, local production of IL-6 peaked at 4 h p.i, with significantly higher levels produced during coinfection through 24 h p.i. compared with either *C. albicans* or *S. aureus* monoinfection (Fig. 3A). TNF-α production peaked between 4 and 12 h p.i., with significantly higher levels in coinfected mice at these time points (Fig. 3B). IL-1β production peaked at 12 h p.i., with dramatic increases in coinfected mice through 24 h p.i. (Fig. 3C).

Based on our previous report showing that neutrophils are recruited to the peritoneal cavity with increasing numbers during coinfection (21), it was possible that the increase in proinflammatory cytokines observed early during coinfection was due to increases in neutrophil recruitment and stimulation. However, when local neutrophil infiltration was analyzed, similar percentages of neutrophils were observed by 4 h p.i. in all infected groups (Fig. 4). The neutrophil levels began to decrease in monoinfected mice by 24 h p.i. and by 48 h in coinfected mice. The absolute numbers of peritoneal neutrophils reflected these data (data not shown).

Polymicrobial infection leads to dissemination and sepsis. The proinflammatory cytokines TNF-α and IL-6, which were elevated locally during coinfection, are also hallmark cytokines involved in sepsis (39, 40). Therefore, we analyzed whether there were also elevated levels of these cytokines systemically. Serum

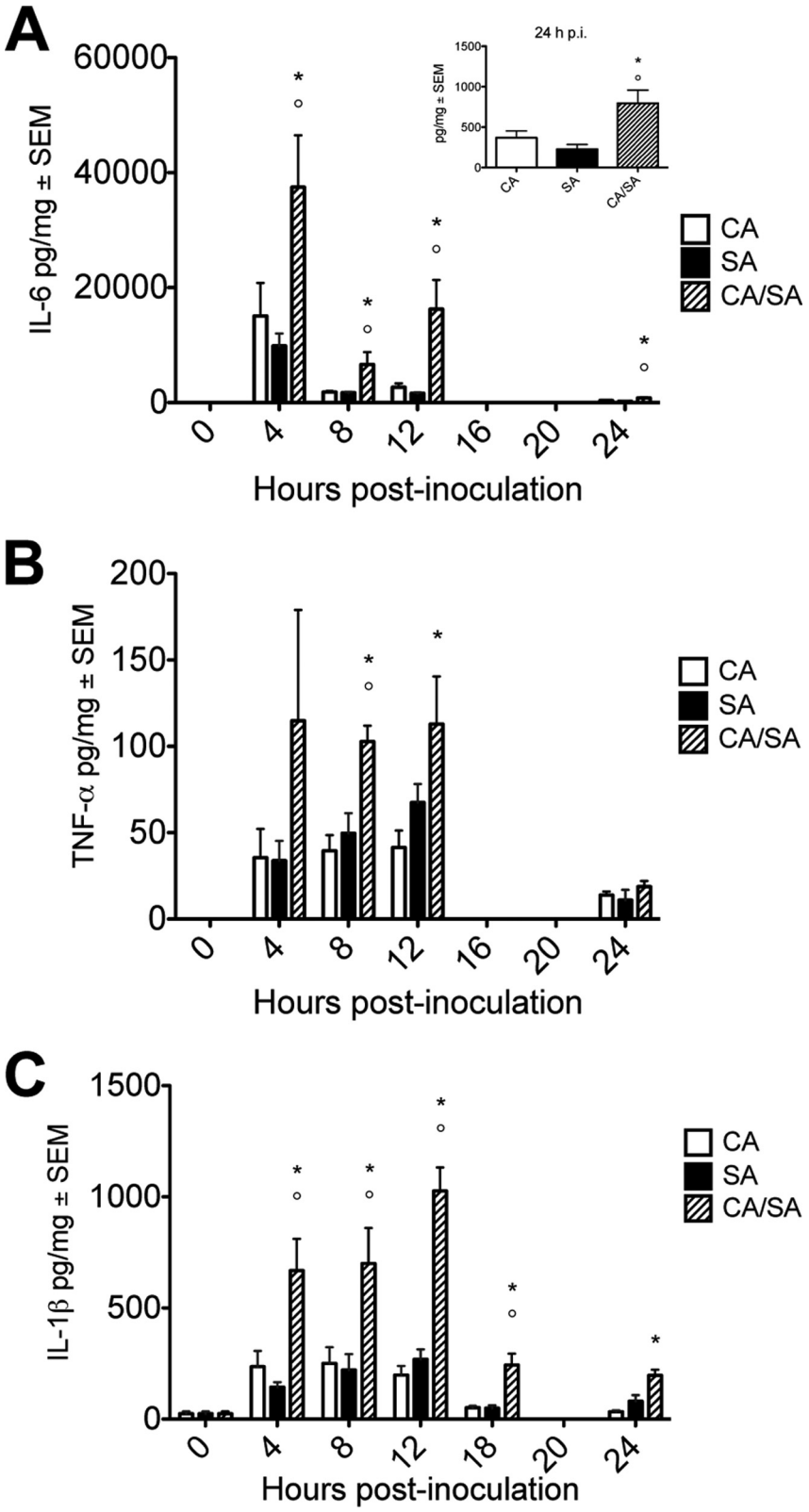

FIG 3 Local proinflammatory cytokines during polymicrobial peritoneal infection with *C. albicans* and *S. aureus*. Mice ($n = 5$ mice/group) were inoculated i.p. with *C. albicans* (CA) and *S. aureus* (SA) as described in the legend to Fig. 1. At various time points postinoculation, mice were sacrificed, and the peritoneal cavity was lavaged. IL-6 (with inset showing 24 h p.i.) (A), TNF-α (B), and IL-1β (C) concentrations were quantified in peritoneal lavage fluid by ELISA from *C. albicans*-infected mice (white bars), *S. aureus*-infected mice (black bars), and coinfected mice (diagonally striped bars). Results are expressed as the mean cytokine level ± standard error of the mean (SEM). Shown are cumulative data from two repeat experiments. Data were analyzed using the unpaired Student's *t* test. *, $P < 0.05$ for *C. albicans* versus *C. albicans-S. aureus*; °, $P < 0.05$ for *S. aureus* versus *C. albicans-S. aureus*.

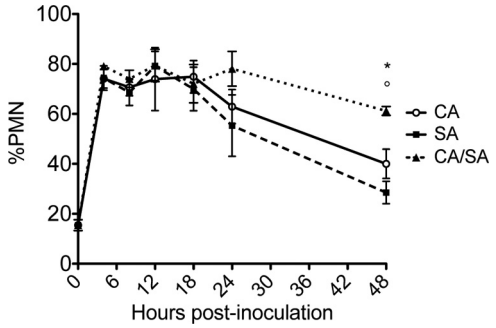

FIG 4 PMN infiltration in monomicrobial and polymicrobial peritoneal infection with *C. albicans* and *S. aureus*. Mice ($n = 5$ mice/group) were inoculated i.p. with *C. albicans* (CA) and *S. aureus* (SA) as described in the legend to Fig. 1. At various time points postinoculation, peritoneal lavage fluid from mice infected with *C. albicans* alone (open circles), *S. aureus* alone (closed squares), or both pathogens (closed triangles) was cytospun and stained with H&E. PMNs and total cells from each infection group were counted in 5 nonadjacent fields, and the percentage of PMNs was calculated. Results are expressed as the mean percentage of PMNs ± SEM. Shown are cumulative data from two repeat experiments. Data were analyzed using the unpaired Student's *t* test. *, $P < 0.05$ for CA versus *C. albicans-S. aureus*; °, $P < 0.05$ for SA versus *C. albicans-S. aureus*.

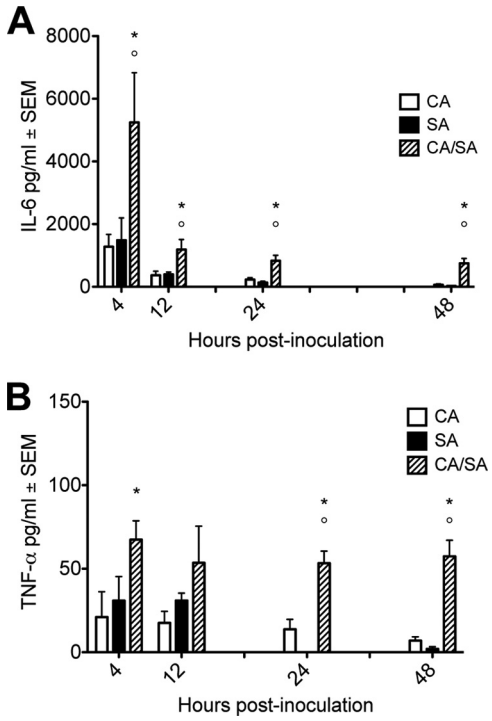

FIG 5 Systemic proinflammatory cytokines in polymicrobial peritoneal infection with *C. albicans* and *S. aureus*. Mice ($n = 5$ mice/group) were inoculated i.p. with *C. albicans* (CA) and *S. aureus* (SA) as described in the legend to Fig. 1. At various time points postinoculation, mice were sacrificed, and serum was analyzed for IL-6 (A) and TNF-α (B) by ELISA. Cytokine concentrations were analyzed in mice infected with *C. albicans* alone (white bars), *S. aureus* alone (black bars), and both pathogens combined (striped bars). Results are expressed as the mean cytokine level ± SEM. Shown are cumulative data from two repeat experiments. Data were analyzed using the unpaired Student's *t* test. *, $P < 0.05$ for *C. albicans* versus *C. albicans-S. aureus*; °, $P < 0.05$ for *S. aureus* versus *C. albicans-S. aureus*.

levels of IL-6 peaked at 4 h p.i. and remained elevated through 48 h, with significantly higher levels produced during coinfection at most time points (Fig. 5A). Serum TNF-α production peaked 4 h p.i. and was significantly elevated in coinfected mice from 4 to 48 h p.i. (Fig. 5B).

To determine the kinetics and the extent of bloodstream dissemination, we examined microbial burden in the brain, a distal target organ of *Candida* and *Staphylococcus* disease. Surprisingly, dissemination to the brain occurred in both mono- and coinfected mice as early as 4 h p.i. (Fig. 6). No differences in dissemination were observed at early time points between mono- and coinfected mice, similar to what was observed in the kidney and spleen. However, by 24 h p.i., significant increases in *S. aureus* CFU were observed in coinfected mice. These increases were not associated with pathology, as brain histology showed no abscesses, signs of encephalitis, meningitis, or increases in immune cell infiltration in monoinfected or coinfected mice (data not shown).

Morphogenesis does not play a role in dissemination and lethal sepsis. Conversion from the yeast to the hyphal form of *C. albicans* has been shown to be required for virulence during bloodstream infection via intravenous inoculation (25). In addition, the hyphal and yeast forms of *C. albicans* elicit different responses from innate immune cells (41–43). These differing stimuli may be contributing factors to the early inflammatory events and synergistic pathology observed during coinfection, especially since inflammatory cytokine production was independent of microbial burden. Furthermore, because *S. aureus* primarily adheres to *C. albicans* hyphae, virulence factors from both organisms may be modulated (20). Therefore, we sought to determine whether the ability of *C. albicans* to form hyphae was required for virulence during intra-abdominal coinfection. Because the model results in only 40 to 60% mortality during coinfection, with survivors eventually clearing the infection, data from early time points include animals that will succumb to infection as well as those that survive. This contributes to inherent variability in results and limits the ability to determine the role of a specific virulence factor during infection. Therefore, we sought to optimize the model by increasing the *Candida* inocula to achieve 100% mortality in coinfected mice by day 5, without affecting morbidity or mortality in the monomicrobially infected animals. Results in Fig. 7 show that increasing the *C. albicans* inocula 5-fold (3.5×10^7 CFU) resulted in 100% mortality by day 3 during coinfection and no mortality in the monoinfected animals. Therefore, this increased *Candida* inoculum was utilized for subsequent studies.

Morphogenesis is controlled by several different transcriptional regulators with overlapping function in *C. albicans* (reviewed in references 44 and 45). Therefore, knockouts in single regulators often do not produce a uniformly strong phenotype *in vivo*. We therefore chose to use tet-regulatable strains that remain locked in either the yeast form (TNRG1) or hyphal form (TUME6) *in vivo* in the absence of doxycycline and the associated parental control strain (TT21) (33). We visually confirmed that the TNRG1 and TUME6 strains were locked in the yeast form or hyphal form, respectively, *in vivo* and that the TT21 wild-type strain formed both yeast and hyphae *in vivo* (data not shown) (33). As expected, no mortality was observed with the wild-type strain TT21 during the monomicrobial infection using the higher inocula. Surprisingly, coinfection with either the yeast-locked *C. albicans* strain TNRG1 or the hypha-locked *C. albicans* strain TUME6 at the higher inocula resulted in virtually 100% mortality

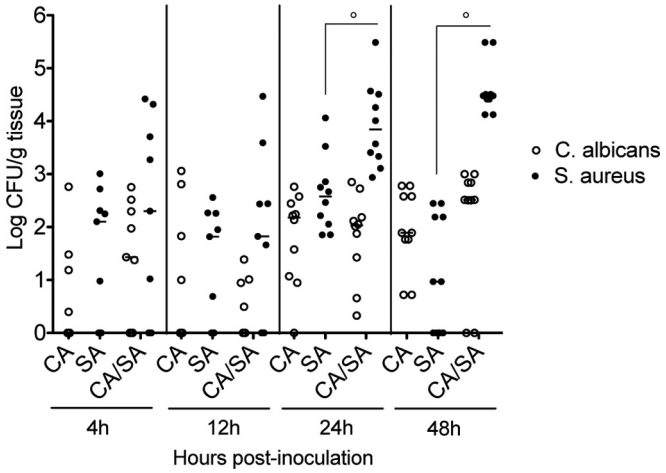

FIG 6 Dissemination of microbial burdens in monomicrobial and polymicrobial peritoneal infections with *C. albicans* and *S. aureus*. Mice ($n = 5$ mice/group) were inoculated i.p. with *C. albicans* (CA) and *S. aureus* (SA) as described in the legend to Fig. 1. At various time points postinoculation, mice were sacrificed, and the microbial burden in the brain was assessed in mice with monomicrobial and polymicrobial infections (*C. albicans*, open circles; *S. aureus*, closed circles). Results are expressed as the median CFU. Shown are cumulative data from two repeat experiments. Data were analyzed using the Mann-Whitney U test. °, $P < 0.05$ for *S. aureus* versus *C. albicans*-*S. aureus*.

(range of 80 to 100% mortality in separate experiments) by day 2 p.i. Most monoinfected mice showed no mortality: the exception was 20% mortality in mice inoculated with TUME6 (Fig. 8A). To confirm that this morphogenesis-independent mortality was due to synergistic effects, mice were inoculated with the yeast-locked TNRG1 or *S. aureus* alone at the total microbial burden, 1.15×10^8 CFU. No mortality was observed with either monomicrobial infection, confirming a synergistic effect in the absence of hyphal formation (data not shown). Regarding dissemination, no differences were observed in brain CFU between wild-type and the yeast-locked or hypha-locked strains during coinfection (Fig. 8B). Locally in the peritoneal cavity, coinfection with TNRG1 or TUME6 resulted in similar levels of proinflammatory cytokines to the wild-type strain (Fig. 9A to C). The lone exception was increased TNF-α in the TUME6-coinfected mice compared to mice infected with the wild-type strain. Serum cytokines showed a similar pattern, with equivalent IL-6, TNF-α, and IL-1β levels during coinfection regardless of the *C. albicans* strain (Fig. 9D to F).

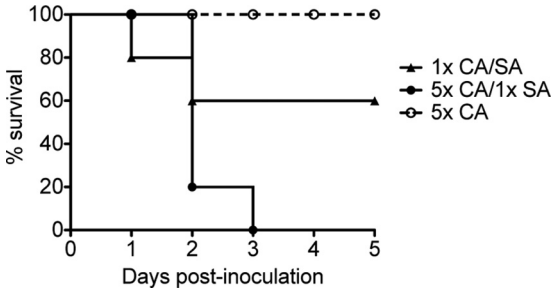

FIG 7 Effects of increased *C. albicans* inocula on mortality during coinfection. Mice ($n = 5$ mice/group) were inoculated i.p. with the standard inocula of 7×10^6 *C. albicans* and 8×10^7 *S. aureus* cells (1× CA/SA [closed triangles]), 3.5×10^7 *C. albicans* and 8×10^7 *S. aureus* cells (5× CA/1× SA [closed circles]), or with *C. albicans* alone (5× CA [open circles]). Mice were monitored for morbidity/mortality through 5 days p.i. Results are from one experiment with all groups inclusive.

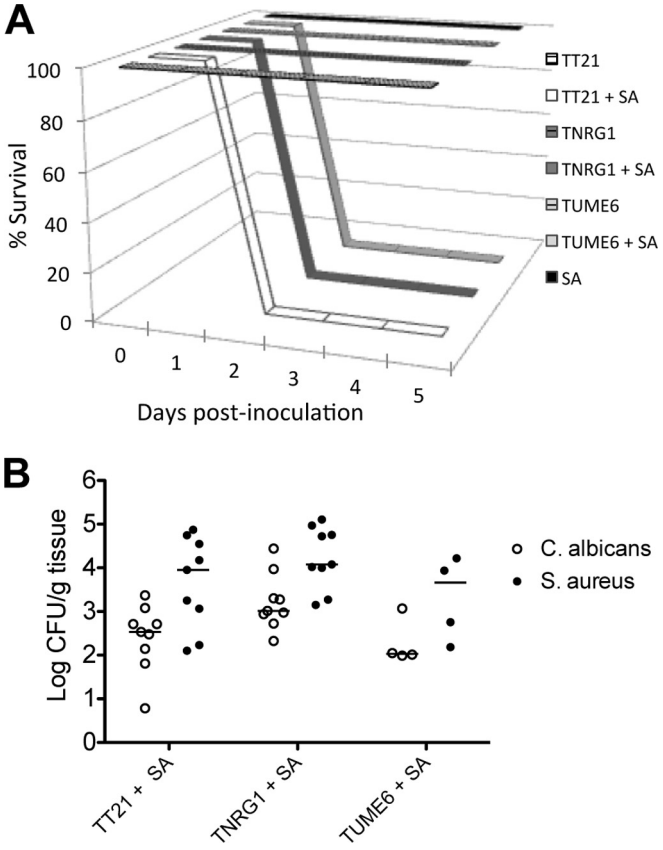

FIG 8 *C. albicans* morphogenesis is not required for dissemination or synergistic mortality. Mice ($n = 4$ or 5 mice/group) were inoculated with *S. aureus* (SA) and either *C. albicans* (CA) strain TNRG1, TUME6, or the isogenic control strain TT21 using 5× inocula as described in the legend to Fig. 7. (A) Survival of mice coinfected with *S. aureus* and TNRG1 using a 5× inoculum (dark gray line), *S. aureus* and TUME6 using a 5× inoculum (light gray line), or *S. aureus* and the isogenic control using a 5× inoculum (white line). Analysis was performed using the Kaplan-Meier test ($P < 0.05$). (B) At 24 h postinoculation, brains from coinfected mice were assessed for fungal and bacterial burden (*C. albicans*, open circles; *S. aureus*, closed circles). Results are expressed as the median CFU. Data shown are cumulative data from two repeat experiments, except for the TUME6 and TUME6-*S. aureus* inoculation, which was performed once. Data were analyzed using the Mann-Whitney U test.

Coorigination of disseminated infection from the abdominal cavity is required to induce mortality. While our results indicate that coinfection with a fungal and bacterial pathogen results in exacerbated stimulation of innate immune responses, leading to sepsis and mortality, it remained unclear whether local versus systemic responses are more important for lethality and whether the presence of both pathogens is required in the same anatomical space. To address this, we asked whether synergistic lethality could occur if the pathogens originated from different routes of inoculation (intravenous [i.v.] versus i.p.). The 50% lethal dose (LD_{50}) for either *C. albicans* or *S. aureus* is much lower for i.v. infection than that for i.p. infection, limiting the ability to compare effects during coinfection in our model of IAI. However, previous studies have demonstrated that mice survive high-dose i.v. inoculations with a *C. albicans* yeast-locked strain similar to TNRG1 (34). This is not due to quick clearance of infection or lack of dissemination,

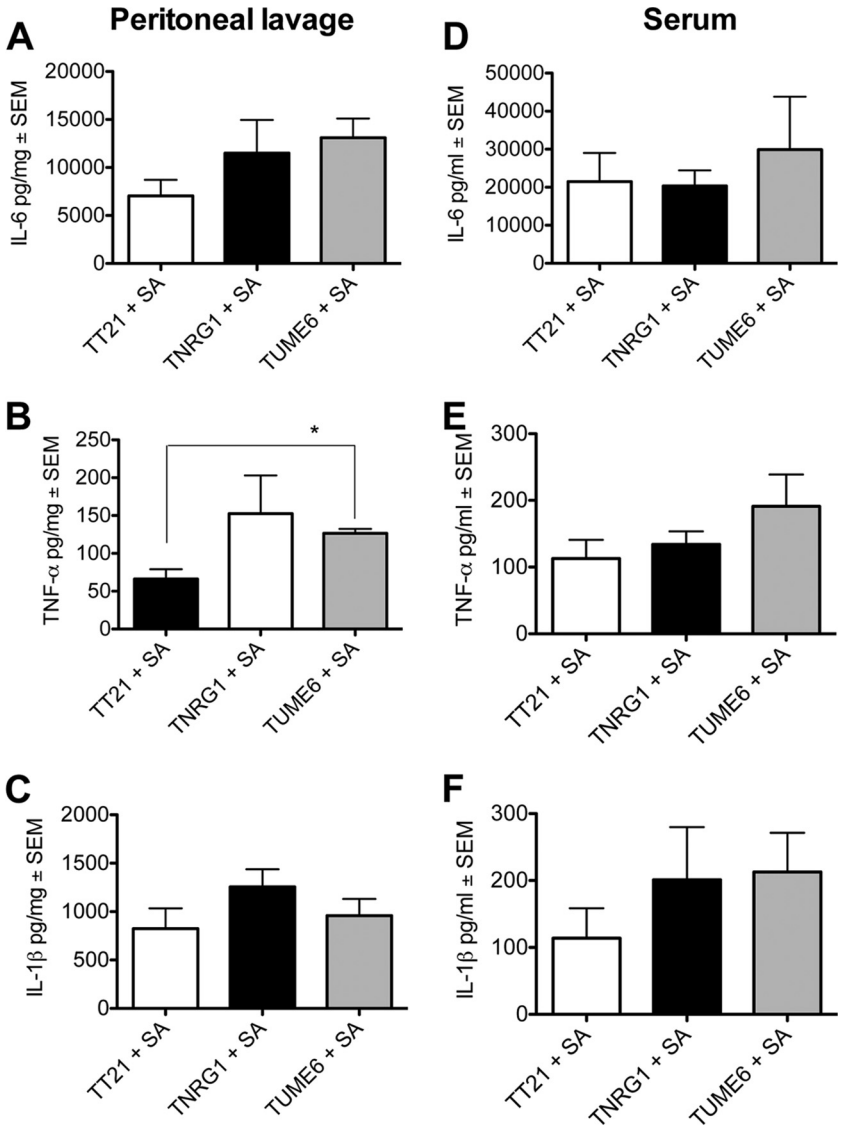

FIG 9 *C. albicans* morphogenesis is not required for induction of local and systemic inflammation. Mice ($n = 5$ mice/group) were inoculated with *S. aureus* (SA) and either *C. albicans* (CA) strain TNRG1, TUME6, or isogenic control strain TT21 at the 5× inoculum as described in the legend to Fig. 7. At 24 h p.i., peritoneal lavage fluid (A to C) and serum (D to F) were collected and analyzed for IL-6 (A and D), TNF-α (B and E), and IL-1β (C and F). Results are expressed as the mean cytokine level ± SEM. Shown are cumulative data from two repeat experiments, except TUME6 and TUME6-*S. aureus* inoculation, which was performed once. Data were analyzed using the unpaired Student's *t* test. *, $P < 0.05$.

as similar organ burdens were observed in control and yeast-locked strains. Because TNRG1 was capable of causing synergistic mortality when coinfected with *S. aureus*, we inoculated 1×10^6 CFU of *C. albicans* TNRG1 i.v. immediately followed by 8×10^7 CFU of *S. aureus* i.p. Surprisingly, mice appeared healthy, with no morbidity or mortality observed through day 10 p.i. (data not shown). *S. aureus* burden was confirmed in the peritoneal cavity, spleen, and brain of coinfected mice, but without the characteristic increases shown when both organisms originated from the peritoneum (see Fig. S1 in the supplemental material). *C. albicans* burden was present in the spleen and brain, but not in the peritoneal cavity (see Fig. S1). Finally, local and systemic cytokine production was present but similarly not enhanced during coinfection (see Fig. S2 in the supplemental material).

DISCUSSION

The synergistic mortality induced during the experimental model of fungal polymicrobial IAI is a striking phenotype that required further dissection to begin to understand the mechanisms involved. Through previous studies, we had determined that mortality induced during polymicrobial infection with *C. albicans* and *S. aureus* is associated with host and microbial factors that could both potentially cause damage. Thus, the main objective of this study was to characterize the early local and systemic inflammatory events during polymicrobial IAI to further characterize and identify the host and microbial factors that may contribute to mortality.

Overall, our data support the hypothesis that the inflammatory host response is primarily responsible for mortality during poly-

microbial infection. This is supported by equivalent fungal and bacterial burdens observed in the peritoneal lavage fluid and spleen of mono- and coinfected mice at early time points prior to the onset of any morbidity and/or mortality in coinfected mice, together with elevated local cytokines (IL-6, TNF-α, and IL-1β) during coinfection. This is further supported by previously published data (21) demonstrating that inoculation of either organism alone at doses equivalent to the polymicrobial inocula used does not result in mortality.

The cytokines detected locally during polymicrobial infection (IL-6, TNF-α, and IL-1β) are considered major contributors to sepsis (39, 40). Accordingly, kinetic analysis of inflammatory cytokines in serum resulted in the elevated detection of these septic markers early during coinfection. Heightened inflammation in the periphery is also a sign of dissemination, and *C. albicans* and *S. aureus* were equally present in the brains of mice during polymicrobial and monomicrobial infections, surprisingly as early as 4 h p.i. Interestingly, coinfected animals always developed elevated levels of *S. aureus* in the brain by 24 to 48 h p.i. The significance is not known but may contribute to the lethal sepsis. Elevated *S. aureus* dissemination during coinfection may be a consequence of heightened peritoneal inflammation leading to peritoneal membrane damage and enhanced leakage of microbes. Previous studies demonstrate that inhibition of this inflammation through the use of a nonsteroidal anti-inflammatory drug (NSAID) during coinfection counteracts the pathological inflammation, resulting in little to no mortality (21, 46). This supports the concept that the exacerbated inflammatory responses, which are initiated early after infection both locally and systemically, are associated with mortality. This together with the present data suggests that treatment for IAI should potentially include both anti-inflammatory agents in conjunction with antimicrobial therapy.

Our polymicrobial IAI model has many similarities to other established IAI models. Cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP) are well-accepted IAI models that both involve a surgical procedure in which the cecum is perforated to release the cecal contents into the normally sterile peritoneal cavity. Studies using these models reported TNF-α, IL-6, and IL-1β in the peritoneal lavage fluid and serum after surgery, albeit with different kinetics from those in our model (38, 47–51). Differences are likely attributed to the continuous leakage of diverse bacterial species from the cecum of mice that have undergone CLP or CASP, compared to the bolus injection of organisms used in our model. While these models are commonly used, they are limited by their inability to distinguish the contributions of individual microbes to disease, do not utilize a standardized inoculum, and are dominated by Gram-negative bacteria, which predominate in the cecum (52).

Data from another model of fungal polymicrobial IAI were recently published which involved injecting mice i.p. with sterilized mouse feces and live *Candida* cells (53). This resulted in early local fungal burden and polymorphonuclear leukocyte (PMN) infiltration to the peritoneal cavity but no mortality. This is likely because the bacterial component of infection is not alive, and the sterilization process disrupts pathogen-associated molecular patterns (PAMPs) that are necessary for recognition by pattern recognition receptors (PRRs) on innate immune cells. Both fungal IAI models will likely be important in dissecting mechanisms of polymicrobial IAIs involving fungi via alternate but complementary approaches.

The ability of *C. albicans* to switch between yeast and hypha is a well-established virulence factor (reviewed in references 23 and 24), as demonstrated in several animal models of candidiasis (34, 54, 55). Additionally, this morphological transition can modulate the host immune response, as exemplified in a murine model of vulvovaginal candidiasis (33), as well as *in vitro* models of reconstituted human or murine epithelium (56). Furthermore, *S. aureus* is known to preferentially bind to the hyphae of *C. albicans*, which could affect the virulence of either organism. Based on results from these models, we expected *C. albicans* morphogenesis to be required for lethality and the amplified inflammation observed during the *C. albicans-S. aureus* polymicrobial IAI model. However, surprisingly the putative avirulent yeast-locked *C. albicans* strain resulted in equivalent mortality, inflammation, and microbial burden to those of the wild type. Importantly we confirmed that the mortality using the higher inocula (5×) in these experiments was due to the synergistic effect of *C. albicans* and *S. aureus* during coinfection. The inoculation of either *C. albicans* TNRG1 or *S. aureus* alone at the combined coinfection inoculum concentration was not lethal, similar to what we previously reported with the lower inocula (21). Cumulatively, these results suggest that morphological transition is not required for IAI sepsis/mortality. This is supported as well from results with the hypha-locked *C. albicans* mutant, which was not inherently more pathogenic *in vivo*.

The results from the morphogenesis experiments prompted two other important questions. (i) Are the local and systemic inflammatory events induced by both organisms sufficient to induce mortality? (ii) Does mortality require the organisms to originate in the same anatomical space? We reasoned that both questions could be addressed by introducing the organisms via different routes (i.p. or i.v.). The avirulent property of the yeast-locked *C. albicans* strain given i.v. together with the virulent property of the same strain coinfected i.p. with *S. aureus* made this experimental design feasible and informative. Results showing that both organisms must originate from the peritoneal cavity to cause lethality suggest that local inflammation induced by both organisms together in close proximity (synergistic) greatly contributes to the pathogenic response. The microbial burden in the spleen supports this conclusion. Although both organisms are present in the spleen during coinfection, they are likely not occupying the same anatomical space in the spleen (*C. albicans*, internal; *S. aureus*, external) and thus are not costimulating innate immune cells to induce pathological inflammation. This leads to yet another question—whether a strict physical interaction between *C. albicans* and *S. aureus* is required. Previous studies using this IAI model from our laboratory and others have demonstrated the presence of polymicrobial biofilm-like structures on target organs (21), and several staphylococcal and fungal virulence factors are modulated during coculture *in vitro* (20). Therefore, physical interactions between *C. albicans* and *S. aureus* may modulate the degree of host immune activation. Given the fact that *S. aureus* preferentially binds to *C. albicans* hyphae (20), together with the yeast-locked strain resulting in similar lethalities during coinfection, it will be interesting to determine if and what type of interactions (physical and/or chemical) occur between the two *C. albicans* morphotypes and *S. aureus* during the coinfection. While we have clearly demonstrated that *C. albicans* and *S. aureus* coinfections in the peritoneal cavity lead to synergistic mortality, we have not considered other gastrointestinal bacteria or fungi that could coinfect with

Candida during clinical infections that also may result in mortality. Peritoneal infections with *C. albicans* and *Escherichia coli* have been shown to be lethal in mice (57), thus to broaden the use of this model, studies are also under way to identify combinations of enteric bacteria and pathogenic *Candida* species that result in synergistic lethality. Overall, the ability of *C. albicans* and *S. aureus* to amplify the host inflammatory response to a point of sepsis and death without requiring morphological transition stresses the need to fully dissect the pathogenesis during polymicrobial IAI involving fungi, which will lead to better diagnosis and targeted therapeutics. Accordingly, the model will also be used to further determine the role of fungal and bacterial PAMPs using killed organisms, define inflammatory response through inhibition of PRR signaling on innate immune cells, and to evaluate the role of the inflammasome as well as other inflammatory mechanisms via genetic or pharmacologic inhibition/manipulation.

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