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Candida albicans Airway Colonization Facilitates Subsequent *Acinetobacter baumannii* Pneumonia in a Rat Model

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The objective of the study was to determine the effects of *Candida albicans* respiratory tract colonization on *Acinetobacter baumannii* pneumonia in a rat model. Rats were colonized with *C. albicans* by instillation of 3×10^6 CFU into their airways, while sterile saline was instilled in the control group. The colonized rats were further divided into two groups: treated with amphotericin B or not. The rats were subsequently infected with *A. baumannii* (10^8 CFU by tracheobronchial instillation). *A. baumannii* lung CFU counts, cytokine lung levels, and rates of *A. baumannii* pneumonia were compared between groups. *In vitro* expression of *A. baumannii* virulence genes was measured by reverse transcription (RT)-PCR after 24-hour incubation with *C. albicans* or with Mueller-Hinton (MH) broth alone. Rats with *Candida* colonization developed *A. baumannii* pneumonia more frequently and had higher *A. baumannii* CFU burdens and heavier lungs than controls. After *A. baumannii* infection, lung interleukin 17 (IL-17) concentrations were lower and gamma interferon (IFN-γ) concentrations were higher in *Candida*-colonized rats than in controls. *Candida*-colonized rats treated with amphotericin B had a decreased rate of *A. baumannii* pneumonia and lower IFN-γ levels but higher IL-17 levels than untreated rats. Expression of *basC*, *barB*, *bauA*, *ptk*, *plc2*, and *pld2* was induced while expression of *ompA* and *abaI* was suppressed in *A. baumannii* cultured in the presence of *C. albicans*. *C. albicans* colonization facilitated the development of *A. baumannii* pneumonia in a rat model. Among *Candida*-colonized rats, antifungal treatment lowered the incidence of *A. baumannii* pneumonia. These findings could be due to modification of the host immune response and/or expression of *A. baumannii* virulence genes by *Candida* spp.

Candida albicans is a commensal of the human skin and mucosae but also the most common fungal human pathogen (1–3). Recent data are suggestive of clinically significant interactions between *C. albicans* and bacteria, potentially affecting their virulence (2, 4, 5) and propensity to develop antibacterial resistance (6–9).

Specifically, clinical and laboratory investigations have focused on the interplay between *C. albicans* and *Pseudomonas aeruginosa* (10). In animal models and observational studies, *C. albicans* airway colonization has been associated with increased incidence of *P. aeruginosa* pneumonia (11, 12). Prior antifungal treatment was found to reduce the risk of *P. aeruginosa* pulmonary infection (11, 13). However, in burn patients, *P. aeruginosa* inhibited the growth of *Candida* spp. on the wound surface (4).

Acinetobacter baumannii is an emerging nosocomial pathogen associated with significant morbidity and multidrug resistance (14–16). *A. baumannii* can firmly adhere to the hyphae of *C. albicans* and inhibit their growth via production of the OmpA protein (17). Nevertheless, *A. baumannii* exhibited enhanced growth in the presence of another yeast, *Saccharomyces cerevisiae* (18).

These observations indicate complex, possibly both synergistic and antagonistic yeast-bacterial interactions that have not been adequately investigated. Therefore, we aimed to study the relationship between *C. albicans* respiratory tract colonization and bacterial pneumonia caused by *A. baumannii* in a rat model, as well as the effect of culture in the presence of *C. albicans* on the expression of *A. baumannii* virulence genes.

MATERIALS AND METHODS

Animals. We used 2.5- to 3-month-old pathogen-free male Wistar rats, weighing 250 to 275 g (11), purchased from the Laboratory Animal Center (Southern Medical University, Guangzhou, China). All experiments were approved by the Ethics of Animal Experiments Committee of Nan Fang Hospital, an affiliate of the Southern Medical University, Guangzhou, China. The rats were housed under standard conditions (12 h light/12 h dark; 22 to 24°C) in the Animal Care Facility Service (Southern Medical University, Guangzhou, China). The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

***C. albicans* respiratory tract colonization.** *Candida* colonization was achieved by transglottal instillation of 3×10^6 CFU *C. albicans* SC5314 on the first day of experiments, as previously described (11). To establish the model, 4 animals were sacrificed at each time point, 4, 24, and 72 h and 7 and 14 days after instillation, and their lungs were removed. The lungs from one animal were homogenized and used for *Candida* CFU counts, whereas those from the other three were used for histopathologic analyses. We compared lung cytokine levels between *Candida*-colonized and con-

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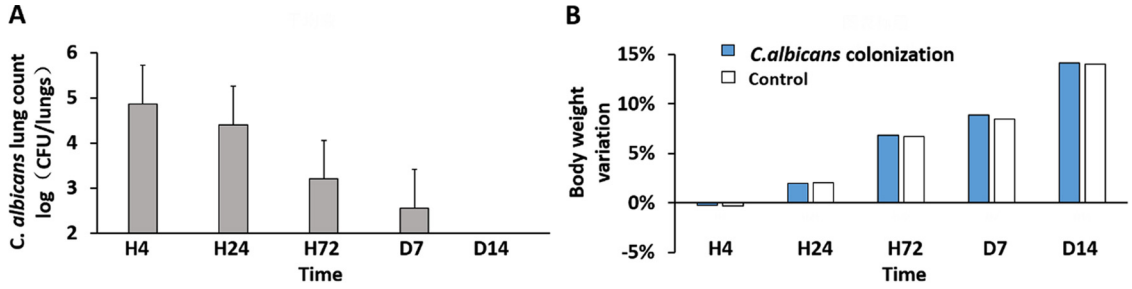

FIG 1 Rat respiratory tract colonization with *C. albicans*. A total of 3×10^6 CFU of *C. albicans* was transglottally instilled into rats. At different time points, the rats were sacrificed, and their lungs were homogenized. (A) *C. albicans* CFU counts at 4 h, 24 h, 72 h, 7 days, and 14 days after instillation. (B) Animal weight variation compared with that of noncolonized rats (control) at each time point. The error bars indicate SD.

trol rats without *A. baumannii* infection 48 h after *Candida* or saline instillation, respectively.

Assessing the effect of *C. albicans* respiratory tract colonization on *A. baumannii* pneumonia. Rats were divided into *C. albicans* respiratory tract colonization ($n = 16$) and saline control ($n = 18$) groups. A suspension of 10^8 CFU of *A. baumannii* ATCC 19606 was transglottally instilled in each animal on the second day of the experiment. At this inoculum, *A. baumannii* pneumonia develops in <50% of immunocompetent rats. The animals were sacrificed on the third day. Lung weights, *A. baumannii* CFU counts, and lung cytokine concentrations were compared between the two groups. Pneumonia was defined as macroscopic and/or microscopic lung inflammation with a bacterial burden of $>10^4$ CFU per lung (11).

Antifungal treatment. The *Candida*-colonized rats were divided into those treated with 1 mg/kg of body weight/day of intraperitoneal amphotericin B (treatment group; $n = 19$) or normal saline intraperitoneal injections (control group; $n = 16$), as previously described (11), for 3 days after airway colonization with *Candida*. On the 4th day, the rats were infected with *A. baumannii* as described above, and on the 5th day, they were sacrificed for lung tissue bacterial CFU and cytokine measurements. In a separate experiment, we compared lung cytokine levels between *Candida*-colonized rats treated for 3 days with amphotericin B or not and without *Acinetobacter* infection.

Cytokine measurements. Levels of interleukin 2 (IL-2), IL-5, IL-6, IL-10, IL-17, and gamma interferon (IFN-γ) were determined using the Milliplex Map Rat Cytokine/Chemokine Magnetic Bead panel (Millipore Corporation, USA) on a Luminex (Austin, TX) 100 IS system according to the manufacturer's instructions to evaluate the host local immune response.

In vitro experiments, RNA isolation, and real-time PCR analysis. For *in vitro* experiments, 1 ml of 5×10^8 CFU/ml *A. baumannii* in Mueller-Hinton (MH) broth suspension was cultured with 1 ml of *C. albicans* MH broth suspension (3×10^7 cells/ml) (*Candida*) or 1 ml of MH broth (control). After a 24-hour incubation at 37°C, 1 ml was moved to a 1.5-ml tube and centrifuged for 10 min at $5{,}000 \times g$ to obtain bacterial pellets; 100 μl of TE buffer (10 mM Tris · Cl, 1 mM EDTA, pH 8.0) with 1 mg/ml lysozyme was added to the precipitate, which was resuspended and incubated for 5 min at room temperature.

Total *A. baumannii* mRNA was extracted using an RNeasy Mini RNA isolation kit (Qiagen, Shanghai, China). RNA integrity, concentration, and purity were assessed using a Nanodrop spectrophotometer. Samples with a 260/280-nm ratio between 1.9 and 2.1 were used for further analyses. Any potential carryover genomic DNA (gDNA) contamination was removed using gDNA Eraser (TaKaRa, Dalian, China); 1 μg of total RNA was used to synthesize cDNA with the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time; TaKaRa, Dalian, China). Quantitative analyses of virulence genes of *A. baumannii* (*ompA*, *pgaC*, *lpxA*, *basC*, *basD*, *barB*, *bauA*, *ptk*, *plc2*, *pld2*, and *abaI*) were performed on a LightCycler 480 real-time PCR system (Roche, Switzerland) using the SYBR Premix *Ex Taq* II (Tli RNaseH Plus) kit (TaKaRa, Dalian, China).

Using 16S rRNA as the reference gene (19), reverse transcription (RT)-PCR data from at least 3 independent experiments were analyzed by the $2^{-\Delta\Delta Ct}$ method. Changes in the expression of *A. baumannii* virulence genes in the presence of *C. albicans* were expressed as a ratio relative to the control group.

Statistical analysis. Data were analyzed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as means and standard deviations (SD) unless otherwise specified. Normality of distribution was tested with the Kolmogorov-Smirnoff test. We determined statistical significance ($P < 0.05$) by *t* test, analysis of variance (ANOVA), or Mann-Whitney U test, where appropriate.

RESULTS

***C. albicans* respiratory tract colonization model.** On the basis of the model provided by Roux et al. (11), the respiratory tracts of Wistar rats were colonized with *C. albicans*. The 14-day survival of the colonized rats was 100%, and there were no differences in activity, fecal shape, and average daily weight gain between animals colonized with *C. albicans* and controls (airway instillation of normal saline) (Fig. 1). We found no histopathologic evidence of pneumonia in *Candida*-colonized rats.

***Candida* colonization of the respiratory tract facilitates the development of subsequent *A. baumannii* pneumonia.** Twenty-four hours after lung instillation of *A. baumannii*, rats with prior *C. albicans* respiratory tract colonization had higher *A. baumannii* lung CFU counts ($P = 0.026$) and lung weights ($P = 0.034$) than controls (Fig. 2). Ten of 16 rats with *C. albicans* airway colonization developed *A. baumannii* pneumonia as opposed to 4/18 in the control group ($P = 0.017$). Two lungs infected with *A. baumannii* are shown in Fig. 3. Consolidation is evident in the lung of a *Candida*-colonized rat (Fig. 3B). Microscopically, animal lungs colonized with *C. albicans* prior to *A. baumannii* infection demonstrated heavier infiltration of inflammatory cells and alveolar damage (Fig. 4D) than lungs with *A. baumannii* infection in the absence of prior *C. albicans* colonization (Fig. 4C).

***C. albicans* colonization downregulated the effect of *A. baumannii* on host IL-17 production and upregulated its effect on IFN-γ production.** We measured lung IFN-γ, IL-2, and IL-17 levels in *C. albicans*-colonized rats and controls in the absence of (48 h after *Candida* or saline instillation) and 24 h after *A. baumannii* infection. Levels of IL-17 (41.4 ± 7.0 versus 17.7 ± 4.2 pg/ml; $P = 0.01$) (Fig. 5A), IL-2 (84.6 ± 8.3 versus 54.3 ± 7.1 pg/ml; $P = 0.02$) (Fig. 5B), and IFN-γ (130.2 ± 33.9 versus 54.3 ± 10.8 pg/ml; $P = 0.005$) (Fig. 5C) were higher in rats colonized with *Candida* than in controls in the absence of bacterial infection.

In the control group, IFN-γ ($P = 0.002$), IL-17 ($P < 0.001$),

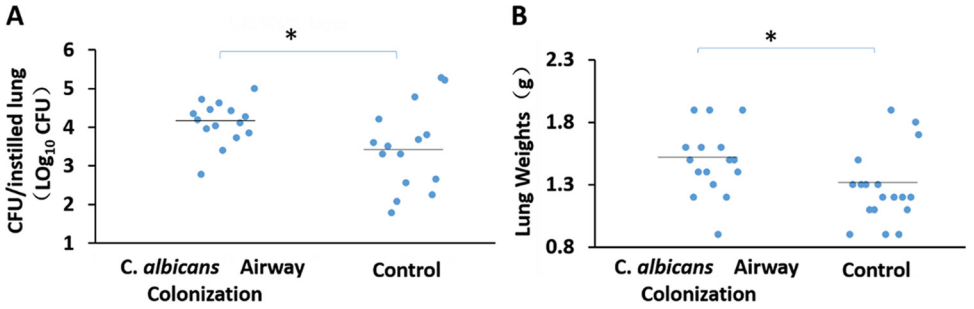

FIG 2 Fungal colonization facilitates development of subsequent *A. baumannii* pneumonia. Median *A. baumannii* CFU counts per lung (log transformed) (A) and lung weights (B) (horizontal lines) are shown. Mann-Whitney test; *, $P < 0.05$.

and IL-2 ($P = 0.02$) concentrations were significantly increased with *A. baumannii* instillation, whereas in *Candida*-colonized rats, only IFN-γ levels were increased with bacterial infection ($P < 0.001$). *A. baumannii*-infected rats with prior *C. albicans* airway colonization had significantly lower IL-17 (34.9 ± 6.53 versus 47.8 ± 0.7 pg/ml; $P = 0.029$) (Fig. 5A) and higher IFN-γ (480.5 ± 11.7 versus 301.8 ± 97.4 pg/ml; $P = 0.011$) (Fig. 5C) concentrations than *A. baumannii*-infected controls. There was no difference in IL-2 levels (79.8 ± 19.4 versus 67.8 ± 2.8 pg/ml; $P = 0.265$) (Fig. 5B).

Effect of antifungal therapy. In rats with *C. albicans* airway colonization, we studied the effects of systemic amphotericin B (1 mg/kg daily), starting on the day of *Candida* instillation and administered for 3 days, on the lung fungal burden, subsequent *A. baumannii* pneumonia, and host cytokine production.

The pulmonary fungal burden was significantly decreased in amphotericin B-treated rats compared to those that did not receive antifungal treatment ($P = 0.015$) (Fig. 6A). Fewer rats in the treatment group developed *A. baumannii* pneumonia (5/19 versus 10/16; $P = 0.031$). Lung weights ($P = 0.027$) (Fig. 6B) and lung tissue *Acinetobacter* CFU counts ($P = 0.038$) (Fig. 6C) were also significantly lower in *Candida*-colonized rats that were treated with amphotericin B than in those that were not.

In *Candida*-colonized rats without *A. baumannii* infection, IL-2 (Fig. 7B) and IFN-γ (Fig. 7C) lung concentrations were decreased with antifungal treatment ($P < 0.05$), whereas there was no significant difference in IL-17 levels (Fig. 7A). After *A. baumannii* instillation, IL-17 levels were higher ($P = 0.005$) (Fig. 7D) whereas the concentration of IFN-γ was lower ($P = 0.032$) (Fig. 7F) in *Candida*-colonized rats that received antifungal treatment than in those that did not. IL-2 levels were similar in the two groups ($P = 0.693$) (Fig. 7E). We did not find any significant effects of *Candida* colonization or antifungal treatment on IL-5, IL-6, or IL-10 levels (see Fig. S1 and S2 in the supplemental material).

Effect of *C. albicans* on expression of *A. baumannii* virulence genes. The expression of *A. baumannii* virulence genes was assessed by RT-PCR after 24 h of culture with *C. albicans* compared to MH broth alone. The primers used in this experiment are listed in Table S1 in the supplemental material. In the presence of *C. albicans*, the expression of *basC*, *barB*, *bauA*, *ptk*, *plc2*, and *pld2* was upregulated, whereas the expression of *abaI* and *ompA* was suppressed (Table 1).

DISCUSSION

We studied a simple, reproducible rat model of *C. albicans* respiratory tract colonization previously described by Roux et al. (11) and extended their findings for the evaluation of *C. albicans*-*A. baumannii* (an emerging opportunistic pathogen) coinfection. After instillation, *Candida* organisms were retrieved from animal lungs with slow clearance over time, 14-day survival was 100%, and there were no differences in weight or health status between *Candida*-instilled animals and controls (Fig. 1). These findings are consistent with colonization rather than clinical infection. How-

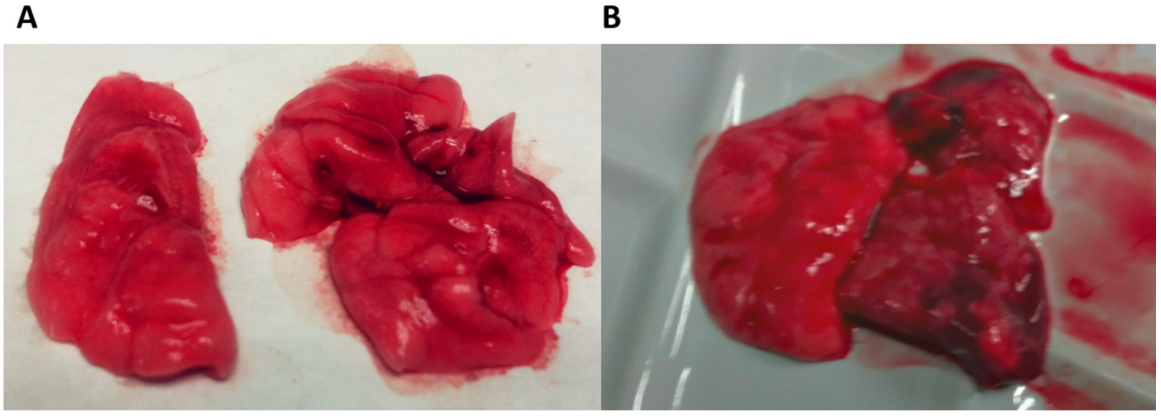

FIG 3 Macroscopic appearance of *A. baumannii* pneumonia. (A) Normal macroscopic appearance of the lungs from a rat that was not colonized with *Candida* and did not develop pneumonia after transglottal instillation of 10^8 CFU of *A. baumannii*. (B) Marked consolidation of the upper and middle lobes in the right lung of a rat colonized with *C. albicans* that developed pneumonia after instillation of the same *A. baumannii* inoculum.

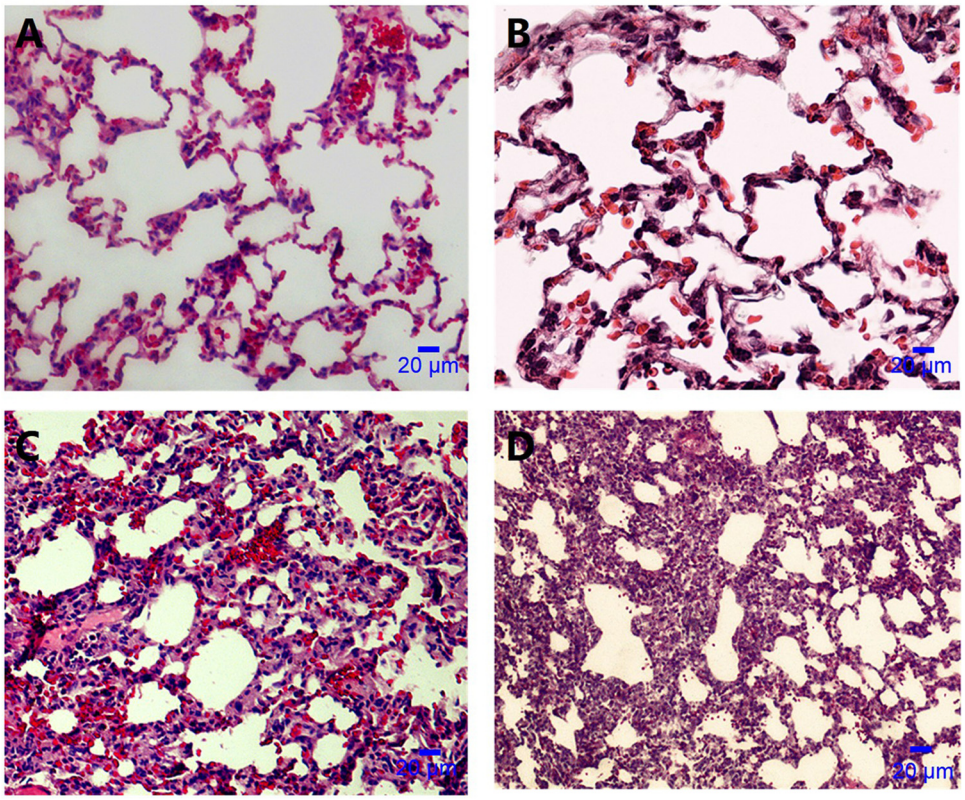

FIG 4 Microscopic appearance of an *A. baumannii*-instilled lung by light microscopy (hematoxylin-eosin stain; magnification, ×400). (A) Normal microscopic appearance of lungs after saline instillation. (B) *C. albicans* airway colonization; lungs appear similar to those in the control group. (C) *A. baumannii* infection in the absence of prior *Candida* colonization; inflammatory-cell infiltration and alveolar damage. (D) *C. albicans* plus *A. baumannii* infection; heavier infiltration of inflammatory cells and alveolar damage than with *A. baumannii* infection in the absence of prior *C. albicans* colonization (panel C).

ever, instillation of *C. albicans*, but not of normal saline, elicited a strong host immune response (Fig. 5) similar to that observed previously (11). Therefore, even though there was no evidence of clinical disease, it seems that *C. albicans* in the respiratory tract is not just a commensal bystander.

Importantly, we found convincing evidence that *C. albicans* respiratory tract colonization in rats facilitated the subsequent development of severe *A. baumannii* infection, as evidenced by higher bacterial CFU counts, lung weights, histopathology (Fig. 2 to 4), and percentages of animals that developed pneumonia than for controls. To our knowledge, this is the first report of an association between *Candida* airway colonization and *Acinetobacter* pneumonia, which is consistent with animal studies (11) and clinical data (12, 13) for other pathogens. Such observations could be due to (i) modification of the host immune response by *C. albicans* and/or (ii) fungal-bacterial interactions resulting in enhanced bacterial virulence.

As noted above, *C. albicans* colonization increased IFN-γ secretion, in agreement with a previous report (11). In both studies, this effect was completely reversed by administration of antifungal agents (Fig. 7), which also led to significantly lower rates of bacterial pneumonia, lung weights, and *A. baumannii* CFU counts (Fig. 6). These effects were previously attributed to a strong T_H1 response to the presence of *C. albicans* (11), leading to increased levels of IFN-γ. However, it should be noted that innate immune cell populations, such as NK cells, also produce IFN-γ in the context of pulmonary infection and likely contribute to cytokine production, especially at early time points before priming of T_H cells.

IFN-γ has been shown to hamper host antibacterial defenses. Specifically, overnight incubation with IFN-γ decreased phagocytosis of *Escherichia coli* and *Staphylococcus aureus* by alveolar macrophages by >90% (11). Moreover, IFN-γ downregulated the expression of a class A scavenger receptor on the surfaces of alveolar macrophages, thus impairing phagocytosis of *Pneumococcus*, which was restored to normal by anti-IFN-γ specific antibodies or opsonization, which bypasses scavenger receptors (20). Therefore, upregulation of IFN-γ production is a potential determinant of the increased susceptibility to bacterial infections associated with *C. albicans* airway colonization.

IL-17 levels were significantly lower in *Candida*-colonized rats than in controls after *A. baumannii* instillation, in agreement with previous observations in a rat model of *P. aeruginosa* pneumonia (11). Similar to the study by Roux et al. (11), we found no difference in IL-17 lung concentrations between *Candida*-colonized rats treated with amphotericin B and untreated rats in the absence of *A. baumannii* instillation. However, in the prior study, the effect of antifungals on IL-17 production in *Candida*-colonized rats as a response to bacterial infection was not evaluated (11). Interestingly, in our study, treatment of *C. albicans* colonization with amphotericin B led to a significant increase in post-*A. baumannii* instillation IL-17 levels. These findings support the well-described role of IL-17 in host defenses against extracellular bacteria and

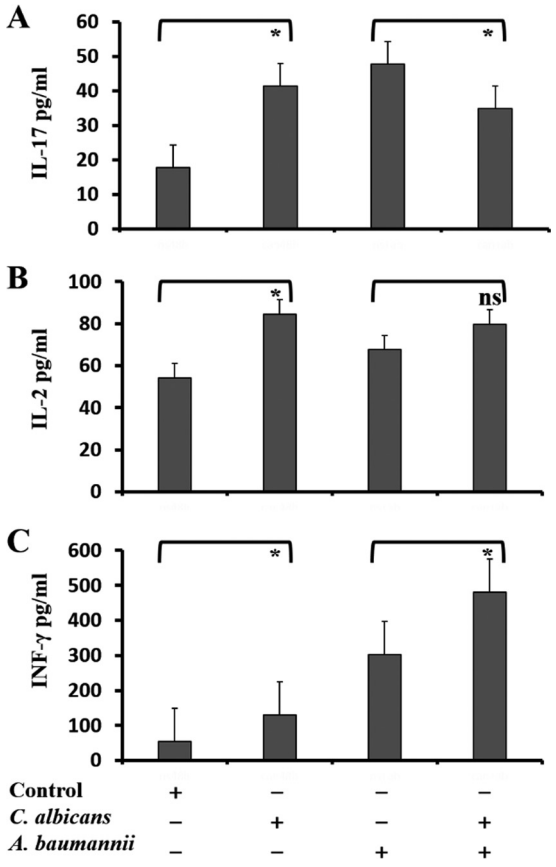

FIG 5 *C. albicans* respiratory tract colonization modulates lung immune response to *A. baumannii* infection. Production of IL-17 (A), IL-2 (B), and IFN-γ (C) in rat lungs was measured 48 h after *Candida* or saline instillation in the absence of *A. baumannii* instillation (left) or 24 h after *A. baumannii* instillation (right) in *C. albicans*-colonized rats versus controls (normal saline instillation). The data are presented as means and SD. Student's *t* test; *, $P < 0.05$; ns, not significant.

fungi (21, 22). Notably, amphotericin B has antifungal as well as immunomodulatory effects, and further studies are needed to determine if our observations are the result of either or both (23).

In previous studies using the same rat model (11, 24), *C. albicans* airway colonization primed the host for development of *S. aureus* and *P. aeruginosa* pneumonia; however, the T_H2 lineage did not seem to be activated, in agreement with our results (see Fig. S1 and S2 in the supplemental material). On the other hand, in BALB/c mice, *C. albicans* intranasal instillation had a beneficial effect on *Pseudomonas* pneumonia, with increased T_H2 cytokine levels (25). Therefore, it seems that the interplay between *Candida* airway colonization, host immune response, and subsequent development of bacterial pneumonia is host dependent. In human cells, where a T_H1-T_H17 response is protective against *Candida* infections (26), IL-26 was recently identified as a potent mediator of the T_H17 axis antibacterial properties (22). Since the gene for IL-26 is absent in rodents (21, 22), other mechanisms are likely implicated in our rat model observations, and it is not known whether they are conserved in humans, as well.

We demonstrated induction of the *A. baumannii* phospholipase (*plc2* and *pld2*) and *basC*, *basD* ($P = 0.066$), and *bauA* genes in the presence of *C. albicans* (Table 1); *basC*, *basD*, and *bauA* are essential for the biosynthesis and utilization of acinetobactin, the main siderophore in *A. baumannii*. Furthermore, we observed induction of *barB*, also part of the siderophore-mediated iron acquisition system (27, 28). Siderophores facilitate iron acquisition from the host and consequently enhance bacterial fitness and virulence, given its limited availability as free iron in mammalian tissues (28). Therefore, the intensification of (i) iron uptake and (ii) use of carbon as a source of energy from host phosphatidylcholine breakdown by *Acinetobacter* phospholipases (29) are potential mechanisms through which *C. albicans* enhances the ability of *A. baumannii* to cause pneumonia. On the other hand, suppression of the *ompA* and *abaI* genes could be indicative of an antagonistic relationship between the two organisms. The *ompA* product has been found to adhere to hyphae, inhibiting *C. albicans* growth (17), whereas *abaI* encodes an autoinducer synthase that contributes to quorum sensing and biofilm formation (30, 31).

Our study has limitations that should be taken into consideration and addressed in future experiments. First, we used only one *A. baumannii* and one *C. albicans* strain. Also, we studied a model of acute infection in previously healthy animals, and it is difficult to draw firm conclusions that will be applicable to intensive care unit (ICU) patients, who are frequently immunosuppressed, are hospitalized for days or weeks, have structural lung abnormalities, and have received multiple antibiotics. Third, we studied cytokine levels in lung homogenate tissue, consisting of diverse immune and other cell populations. Fourth, we assessed only the short-term *in vitro* effect of *A. baumannii* coincubation with *C. albicans*

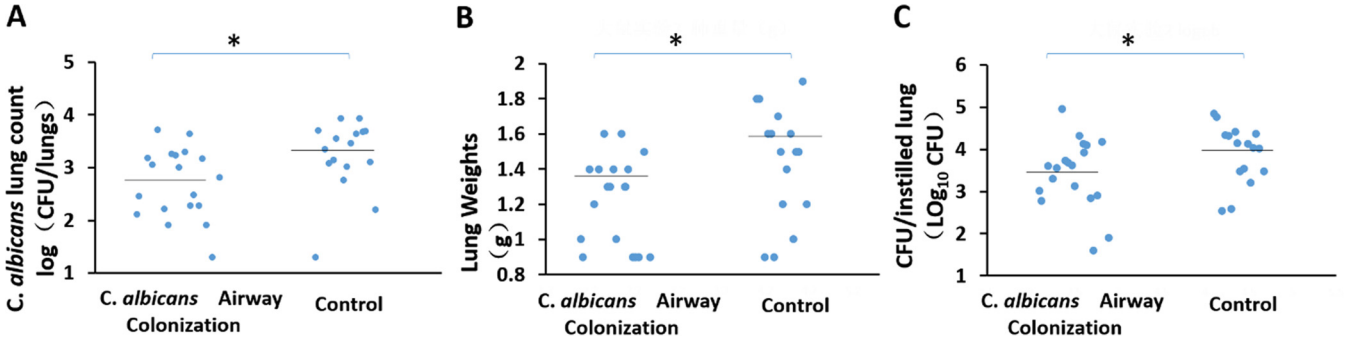

FIG 6 Effects of antifungal treatment on respiratory tract fungal colonization and *A. baumannii* pneumonia. (A) *C. albicans* lung CFU counts were significantly decreased after 3 days of antifungal therapy (amphotericin B). (B and C) Bacterial counts (C) and lung weights (B) were significantly lower in *Candida*-colonized rats treated with amphotericin B than in those that received no treatment. *, $P < 0.05$.

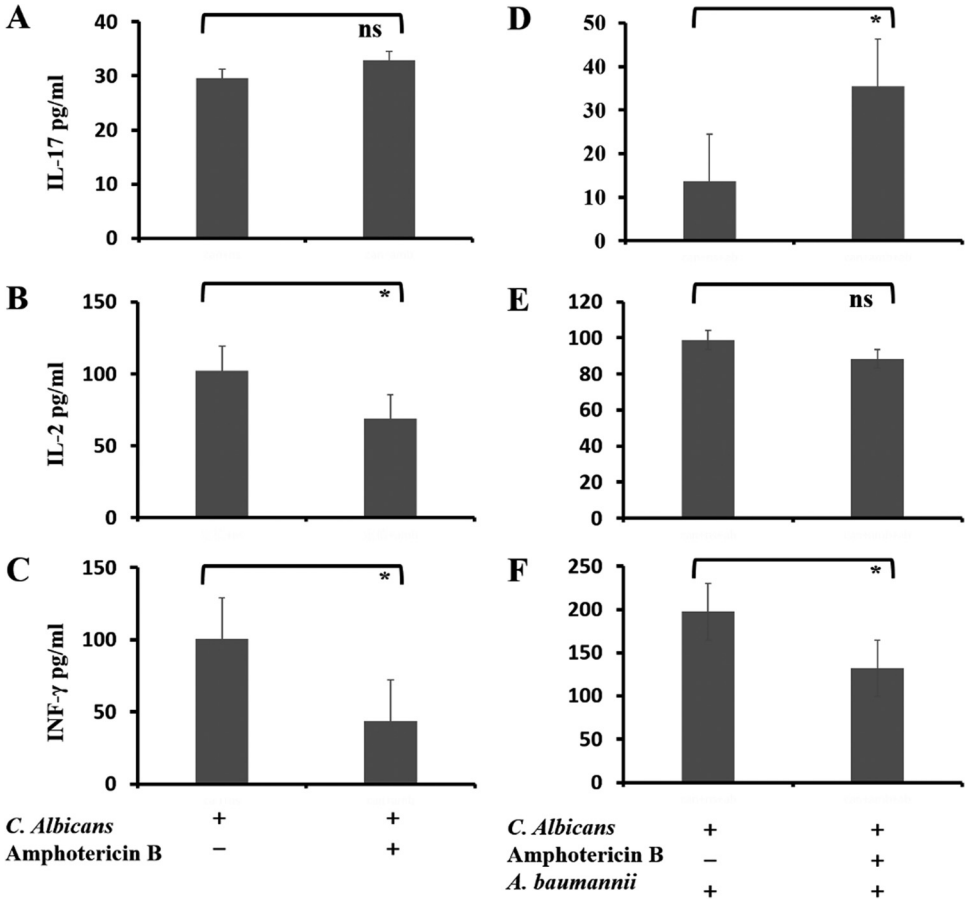

FIG 7 Effects of antifungal treatment on cytokine production. Colonized rats received either amphotericin B or saline by intraperitoneal injection for 3 days and were subsequently infected with *A. baumannii* and sacrificed after 24 h. Concentrations of IL-17 (A and D), IL-2 (B and E), and IFN-γ (C and F) were measured in rat lung homogenates. The data are presented as means and SD. We compared lung cytokine levels between *Candida*-colonized rats treated for 3 days with amphotericin B (right bars) or not (left bars) without (A to C) or after (D to F) *Acinetobacter* infection. Student's *t* test; *, $P < 0.05$; ns, not significant.

on the expression of *A. baumannii* virulence genes, which might not adequately simulate the complex *in vivo* bacterial-yeast interactions in the setting of chronic airway colonization and biofilm physiology. Notably, though, in a recent clinical study of 618 intubated patients, we found that *Candida* colonization was significantly associated with the subsequent development of *A. baumannii* ventilator-associated pneumonia, after robust adjustment for all other confounders and propensity score matching (32).

TABLE 1 *In vitro* expression of virulence genes relative to housekeeping gene 16S rRNA in *A. baumannii* ATCC 19606

Gene	Relative expression		*P* value
	C. albicans	Controls	
lpxA	0.47 ± 0.23	1	0.300
basC	8.49 ± 4.43	1	0.006
pgaC	1.01 ± 0.11	1	0.935
ompA	0.55 ± 0.06	1	0.006
basD	1.98 ± 0.87	1	0.066
barB	2.30 ± 0.64	1	0.010
bauA	2.60 ± 0.56	1	0.003
ptk	1.24 ± 0.15	1	0.024
plc2	1.31 ± 0.20	1	0.023
pld2	2.60 ± 0.77	1	0.004
abaI	0.47 ± 0.22	1	0.016

In conclusion, we found that respiratory tract colonization with *C. albicans* facilitated the development of *A. baumannii* pneumonia in a rat model by modulating the expression of *A. baumannii* virulence genes and/or through modification of the host immune response. In the setting of established *Candida* colonization, antifungal treatment lowered the incidence of *A. baumannii* pneumonia, potentially by suppressing IFN-γ production and enhancing IL-17 production. Prospective clinical studies on the association between *Candida* sp. airway colonization, preemptive antifungal treatment, and subsequent *A. baumannii* ventilator-associated pneumonia should be considered.

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