Calcineurin A Is Essential in the Regulation of Asexual Development, Stress Responses and Pathogenesis in *Talaromyces marneffei*

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*Talaromyces marneffei* is a common cause of infection in immunocompromised patients in Southeast Asia and Southern China. The pathogenicity of *T. marneffei* depends on the ability of the fungus to survive the cytotoxic processes of the host immune system and grow inside host macrophages. These mechanisms that allow *T. marneffei* to survive macrophage-induced death are poorly understood. In this study, we examined the role of a calcineurin homolog (*cnaA*) from *T. marneffei* during growth, morphogenesis and infection. Deletion of the *cnaA* gene in *T. marneffei* resulted in a strain with significant defects in conidiation, germination, morphogenesis, cell wall integrity, and resistance to various stressors. The Δ*cnaA* mutant showed a lower minimal inhibitory concentration (MIC) against caspofungin (16 µg/ml to 2 µg/ml) and micafungin (from 32 µg/ml to 4 µg/ml) compared with the wild-type. These results suggest that targeting calcineurin in combination with echinocandin treatment may be effective for life-threatening systemic *T. marneffei* infection. Importantly, the Δ*cnaA* mutant was incapable of adapting to the macrophage environment in vitro and displayed virulence defects in a mouse model of invasive talaromycosis. For the first time, a role has been shown for *cnaA* in the morphology and pathogenicity of a dimorphic pathogenic filamentous fungus.

**Keywords:** *Talaromyces (Penicillium) marneffei*, calcineurin, morphogenesis, cell wall integrity, immune escape, virulence

**INTRODUCTION**

Calcineurin is a Ca\(^{2+}\)/calmodulin (CaM)-dependent protein phosphatase that is ubiquitous and conserved among eukaryotes. The heterodimeric calcineurin protein consists of a catalytic subunit (A) that binds to the calcium sensor CaM and a regulatory subunit (B) that contains four Ca\(^{2+}\)-binding domains. The functions of calcineurin have been studied in a variety of fungal species, and it plays important roles in the regulation of cation homeostasis, morphogenesis, cell wall integrity, and pathogenesis (Rusnak and Mertz, 2000; Fox et al., 2001; Fox and Heitman, 2002). In filamentous fungi, calcineurin regulates conidial architecture, polarized growth extension and branching, sclerotial and appresorial development, cell wall integrity and stress adaptation (Fortwendel et al., 2009; Juvvadi et al., 2014; Juvvadi and Steinbach, 2015). Calcineurin activation...
leads to the dephosphorylation and activation of the transcription factor Crz1p/Tcn1p, which is involved in cell survival and calcium homeostasis in Saccharomyces cerevisiae (Cyert, 2003; Roque et al., 2016). It is involved in antifungal tolerance, cell morphogenesis (Sanglard et al., 2003; Bader et al., 2006; Cordeiro Rde et al., 2014), growth in an alkaline pH or high-temperature environment, membrane stress, mating, and virulence in Candida albicans (Cruz et al., 2002; Reedy et al., 2010; Liu et al., 2014). Previous reports on the dimorphic fungus Paracoccidioides brasilensis have implicated calcineurin in morphogenesis, environmental stress responses and mycelium-to-yeast dimorphism (Fernandes et al., 2005; Campos et al., 2008; Matos et al., 2013).

Talaromyces marneffei is an emerging opportunistic fungal pathogen that is endemic in southern China, Taiwan, Hong Kong, Thailand, Laos, Vietnam, and northeastern India (Supparatpinyo et al., 1994; Antinori et al., 2006; Vanittanakom et al., 2006). T. marneffei can cause a life-threatening systemic infection in immunocompromised individuals, especially HIV-positive patients (Woo et al., 2012). In recent years, T. marneffei has become a leading AIDS-defining diagnosis in Southern Asia, trailing only tuberculosis and cryptococcosis in incidence (Wu et al., 2008; Le et al., 2011; Hien et al., 2016; Lee et al., 2019). Furthermore, T. marneffei infection has recently been increasingly observed in HIV-negative adults with no reported immunosuppressive condition, but immunodeficiency is suspected to be the cause of these infections (Ramos-e-Silva et al., 2012; Kauffman et al., 2014). The mortality rate of T. marneffei infection exceeds 50% despite antifungal therapy (Le et al., 2011; Hu et al., 2013). Understanding the pathogenic mechanism is fundamental to combating T. marneffei infection.

Talaromyces marneffei is an intracellular pathogen; conidia are inhaled into a patient’s lungs and subsequently engulfed by alveolar macrophages, where the conidia transform into yeast cells and cause infection (Supparatpinyo et al., 1994). During this process, T. marneffei conidia will face a variety of stresses, such as heat, salt stress, oxidative substances, high osmolarity, nutrient deprivation and cytokine-mediated killing (Cao et al., 2009a; Wang et al., 2009; Kummasook et al., 2011). There are several important mechanisms in T. marneffei infection, including the conversion of conidia to the yeast phase, resistance to phagocytic killing and oxidative, and heat stress responses (Pongpom et al., 2017), that result in T. marneffei survival in macrophages. These strategies are the key processes of immune escape.

In our previous study, we found that the minimal inhibitory concentrations (MICs) of echinocandins were quite low for the T. marneffei hyphal form, but T. marneffei manifested resistance in its yeast forms (Cao et al., 2009b; Mo et al., 2014). The mechanism by which the T. marneffei yeast form is resistant to echinocandins is still unclear, but the cell wall composition is suspected to play a role. Echinocandins are antifungals that inhibit cell wall β-(1,3)-D-glucan synthesis (Douglas et al., 1997). It has been reported that β-(1,3)-D-glucan and chitin are two major components of the fungal cell wall (the other main components are 1,6-β-glucans and mannoproteins) (Klis et al., 2002). Reduced synthesis of β-(1,3)-D-glucan can result in reduced susceptibility to caspofungin, and elevated chitin content can reduce echinocandin efficacy in many fungi (Fortwendel et al., 2009; Cordeiro Rde et al., 2014). In a preliminary study, we found that T. marneffei yeast forms were more sensitive to calcium than the hyphal form (Cao et al., 2007). As calcium activates the calcineurin pathway, it is postulated that it may also affect the resistance of T. marneffei to echinocandins by regulating cell wall composition and could represent a potential drug target for augmenting echinocandin use in T. marneffei infection. Thus, in this study, we aimed to investigate calcineurin function by characterizing the cnaA gene and exploring the mechanism of immune escape in T. marneffei.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

Strains used in this study are listed in Table 1. T. marneffei FRR2161 is the type strain and was used as the wild-type for all experiments. T. marneffei G816 (AligD niaD− pyrG−) is a uracil/uridine auxotroph (pyrG) mutant of FRR2161 (Bugeja et al., 2012). Transformation was performed using the protoplast method (Borneman et al., 2000). The ΔcnaA mutant was generated by transforming strain G816 with a linearized ΔcnaA deletion construct and selecting for uracil/uridine (pyrG+) prototrophic transformants. The complemented strain ΔcnaA cnaA+ was generated by transforming the ΔcnaA mutant with the cnaA-ble-pKB plasmid and selecting for bleomycin (ble+) resistant transformants. T. marneffei strains were grown at 25°C in A. nidulans minimal medium (ANM) with 10 mM (NH4)2SO4 and supplemented appropriately as previously described (Borneman et al., 2000). T. marneffei strains were grown at 37°C in BHI medium. Escherichia coli DH5α (Invitrogen, United States) was used to clone and propagate the various constructs and was grown in Luria-Bertani broth at 37°C.

To test the radial growth and the spore-producing ability of the mutants, strains were grown in ANM for 14 days at 25°C, and conidia were harvested into sterile Stroke physiological saline solution. A suspension of 1 × 10⁵ conidia per milliliter was prepared. The wild-type, ΔcnaA mutant and complemented ΔcnaA cnaA+ strains were inoculated with a 5-µl drop of the 1 × 10⁵ conidia per milliliter suspension onto ANM with 1 µg/ml of amphotericin B.
10 mM (NH₄)₂SO₄ with or without uracil, BHI medium or SD medium supplemented with 10 mM (NH₄)₂SO₄. The conidia were incubated at either 25°C or 37°C, and radial growth was measured every day over a period of 14 days. For conidial counts, the number of spores per square millimeter were counted measured after 14 days at 25°C. The results were analyzed by the T-test analysis of variance.

To test for stress responses of the strains, a 5-µl drop of the 1 × 10⁵ conidia per milliliter suspension of each strain was inoculated onto agar-solidified ANM with 10 mM (NH₄)₂SO₄ and 5 mM uracil and supplemented as follows: 0.2, 0.4, 0.6, and 1 M KCl (salt stress); 2, 5 and 8 mM H₂O₂ (oxidative stress); 0.5, 1, and 1.5 M sorbitol (for osmotic stress); 2, 5 and 8 mM HCl (salt stress); 2, 5 and 8 mM H₄SO₄. The conidia were inoculated onto slides covered with a thin layer of agar-solidified BHI or SD medium supplemented with (NH₄)₂SO₄ and incubated at 37°C for 10 days (Borneman et al., 2000). For yeast growth, conidia were inoculated onto slides covered with a thin layer of agar-solidified BHI or SD medium supplemented with (NH₄)₂SO₄ and incubated at 37°C for 10 days.

Ultrastructure analysis was performed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM, strains were fixed with 2.5% glutaraldehyde for 2 h at 4°C, washed in 0.1 mol/L phosphate-buffered saline (PBS) three times for 10 min each time and fixed with 1% osmium tetroxide for 1 h. Then, they were washed in 0.1 mol/L PBS three times for 10 min each time and ethanol-dehydrated by sequential washing in 50, 70, 80, 90, and 100% ethanol. The samples were soaked in hexamethydisilane three times and dried under vacuum. Thin sections were examined with a Vega 3 LMU-apollo X Scanning Electron Microscope (Tescan, Czechia). For TEM, strains were fixed, washed and dehydrated as described for SEM. The samples were embedded in white resin, and thin sections were examined with a Hitachi H-7650 Transmission Electron Microscope (Hitachi, Japan).

For germination experiments, 10⁶ spores of each strain were inoculated into 30 ml of SD medium supplemented with 10 mM (NH₄)₂SO₄ and incubated for 4, 8, 16 or 24 h at 25°C and 37°C. The rates of germination were determined microscopically by counting the number of germinating conidia in a population of approximately 100 randomly selected spores. Three independent experiments were performed.

**Antifungal Susceptibility Testing**

Antifungal susceptibility testing was performed according to the standardized M27-A method approved by the National Committee for Clinical Laboratory Standards (NCCLS) and previously reported methods (Nakai et al., 2003). Caspofungin (CAS), micafungin (MCFG), amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), and vorconazole (VOC) were purchased from Med Chem Express (New Jersey, NJ, United States). Stock solutions were made with sterile distilled water (MCFG and AMB) or 100% dimethyl sulfoxide (CAS, FLC, ITC and VOC). The stock solutions were diluted in RPMI 1640 medium prepared according to the Clinical and Laboratory Standards Institute (CLSI) standards and then further serially diluted twofold. The final concentration ranges of the antifungals were 0.0625 to 32 µg/ml (CAS and MCFG), 0.0156 to 8 µg/ml (AMB), 0.125 to 64 µg/ml (FLC), and 0.0013 to 1 µg/ml (ITC and VOC). Wild-type and ΔcnaA mutant strains were incubated in the presence or absence of drug for 48 h at 25°C and 37°C, and all experiments were performed in triplicate. Candida parapsilosis ATCC22019 served as a control.

**Macrophage Assay**

RAW264.7 macrophages (1 × 10⁵) (InvivoGen, Hong Kong) were co-incubated with 1 × 10⁶ conidia in DMEM containing 10% fetal bovine serum (Gibco, United States) and 8 mM penicillin-streptomycin at 37°C for 2 h. The cells were then washed with PBS to remove unengulfed conidia and incubated for an additional 24 h at 37°C. Infected macrophages were harvested for microscopy or to determine fungal load. For microscopy, cells were either fixed and prepared for TEM or stained with 1 µg/ml calcofluor white (cell wall stress). All cultures were incubated for 14 days at 25°C.

**Murine Model of Talaromyces marneffei Infection**

Eight-week-old BALB/c mice (male and female) were immunosuppressed with intraperitoneal injections of cyclophosphamide (Sigma-Aldrich) at a dose of 200 mg/kg of body weight on days −4 and −1 and the day of infection, as well as triamcinolone acetonide (Sigma-Aldrich) at a dose of 40 mg/kg of body weight on the day of infection. To evaluate the histopathological progression of disease, four groups of 36 mice were infected with a sublethal dose (10⁸ conidia in 100 µl of physiological saline) of the wild-type, ΔcnaA, or ΔcnaA cnaA+ strains or a diluent control (0.9% physiological saline). The mice were sacrificed on days 3, 6, and 9 after inoculation, and their tissue was harvested under sterile conditions. The lung, hepatic and splenic tissues were removed to determine the number of T. marneffei by measuring CFU in YPD medium. The test was performed three times in triplicate, and the results were analyzed by T-test analysis of variance.
RESULTS

Calcineurin Genes in Talaromyces marneffei

The sequence of the T. marneffei cnaA gene was obtained from GenBank (GenBank accession no. XM_002147834.1, ATCC 18224). The gene encompasses 2261 bp and encodes a putative gene product of 557 amino acids. In searches against the GenBank database, the T. marneffei cnaA gene showed strong homology to the sequences in Talaromyces stipitatus (XM_002482031.1, 88% identity), Aspergillus aculeatus (XM_020205250.1, 79% identity), and Aspergillus nomius (XM_015546462.1, 78% identity).

Loss of cnaA Affects Colonial Morphology and Radial Growth

Wild-type T. marneffei growing at 25°C produces colonies comprised of vegetative hyphae that appear fluffy around the periphery and green in the center due to asexual development (conidiation) and the production of pigmented conidia. The colony edge is relatively uniform and compact. In contrast, colonies of the ΔcnaA mutant exhibited a significant reduction in radial growth rate with sparse growth and low aerial hyphae production. The colony surface was wrinkled, and the periphery was irregular. The colony also produced more red pigment that is characteristic of T. marneffei (Figure 1A). The ΔcnaA mutant colonies also readily detached from the medium, indicating a lack of invasive growth. The complemented strain (ΔcnaA cnaA+) displayed colony phenotypes that were very similar to that of the wild-type.

The radial growth rate of the wild-type, ΔcnaA and ΔcnaA cnaA+ strains was quantified during growth on solid medium at both 25°C and 37°C. At 25°C, the wild-type and ΔcnaA cnaA+ strains showed similar growth rates, while that of the ΔcnaA mutant was substantially reduced. Similarly, at 37°C, the wild-type and ΔcnaA cnaA+ strains showed similar growth rates, while that of the ΔcnaA mutant was reduced (Figure 1B).

ΔcnaA Mutant Displays Defects in Hyphal and Yeast Morphogenesis

To examine the cellular basis for the observed macroscopic growth defects observed in the ΔcnaA strain, all strains were grown on ANM or BHI medium for 10 days at 25°C and 37°C and either stained with calcifluor white (CAL) to visualize the cell walls by fluorescence microscopy or processed for SEM. The wild-type hyphal cells showed a smooth and uniform hyphal diameter with regular septation and uniform staining with CAL. In contrast, the ΔcnaA mutant exhibited irregularly shaped hyphal cells that were enlarged in diameter, particularly at septation sites, and showed abnormal CAL-stained chitin deposits along the hyphae. This result suggests that calcineurin is important for proper hyphal extension (Figure 3A). Compared with the wild-type, the poles of the yeast cell of the ΔcnaA mutant showed abnormal swelling and exhibited abnormal chitin deposits. Therefore, the cnaA mutant displayed defects in yeast morphogenesis (Figure 3B).

cnaA Is Required for Correct Cell Wall Biosynthesis in Talaromyces marneffei

The hyphal and yeast morphogenesis defects noted for the ΔcnaA strain were examined further by TEM. The strains were grown on agar-solidified ANM and BHI medium for 10 days at 25°C and 37°C and processed for TEM. For the wild-type strain, transverse sectioning of the hyphal cells showed a cell wall composed of three layers, namely, a thin inner membrane-proximal layer that was electron-dense, a thick middle layer that was electron-transparent and an irregular outer layer with protrusions. The wild-type yeast cells showed a similar cell wall architecture but with a smoother outer wall layer. In both cell types, the organelles could also be clearly seen. In contrast, the ΔcnaA mutant displayed cell wall perturbations in all of the layers for both hyphal and yeast cells, and the organelles appeared less distinct (Figure 4A).

The observed changes in the cell wall architecture were further examined by testing the sensitivity of the various strains to
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FIGURE 1 | Loss of cnaA affects colonial morphology and radial growth. (A) T. marneffei wild-type and ΔcnaA cnaA* strains exhibited highly vegetative hyphae that appeared fluffy and green and developed many conidiophores at 25°C when grown on ANM. The colony edge was relatively uniform and compact. Colonies of the ΔcnaA mutant showed thin growth and exhibited decreased aerial hyphae production, resulting in a film-like surface morphology that was wrinkled. (B) Conidia from each strain were inoculated on ANM or BHI medium and incubated for 1–14 days at 25°C and 37°C. The ΔcnaA mutant grew less than the wild-type and ΔcnaA cnaA* complemented strain.

cell wall-perturbing agents and a variety of antifungal drugs. Compared with the wild-type strain, the ΔcnaA mutant was more sensitive to Congo red at 5 mM and 25°C but equally sensitive to different CAL white concentrations (Figure 4B). When tested for sensitivity to various anti-fungal agents, the MICs for caspofungin (CAS) and micafungin (MCFG) for the mycelial form were much lower than those for the yeast form in the parental strain of T. marneffei. In contrast to the parental strain, the ΔcnaA mutant was more sensitive to Congo red at 5 mM and 25°C but equally sensitive to different CAL white concentrations (Figure 4B). The growth of the ΔcnaA mutant was similar to that of the parental strain at low concentrations of sorbitol, but mutant growth inhibition was obvious in 1M and 1.5 M sorbitol. In contrast to the parental strain, the ΔcnaA mutant was highly sensitive to oxidative stress; the growth of the mutant was inhibited in 5 and 8 mM H₂O₂ (Figure 5B).

cnaA Is Essential for Immune Escape in Macrophages

To assess whether the observed changes in the cell wall architecture and sensitivity to a broad range of stressors in the ΔcnaA strains affected its ability to interact with host macrophages, the various strains were co-incubated with RAW264.7 macrophages and examined after 12, 24, and 48 h of incubation. The wild-type conidia co-incubated with RAW264.7 macrophages were rapidly phagocytosed and germinated into ellipsoid-shaped yeast cells. By 24 h, these yeast cells had grown in size and were dividing by fission. This continued to 48 h, where macrophages were filled with dividing yeast cells. Conidia from...
Loss of cnaA affects conidiophore development and conidial germination. (A) Wild-type and ΔcnaA strains were grown on ANM + (NH₄)₂SO₄ for 14 days at 25°C and examined by SEM. The wild-type strain showed a conidiophore that was composed of four to seven phialides (red arrow) and long chains of conidia (white arrow), resulting in the production of abundant conidia. In the ΔcnaA mutant, the conidiophore displayed abnormal phialide and spore chains with decreased numbers of conidia. Scale bars, 10 µm. (B) Compared with the wild-type and ΔcnaA cnaA⁺ complemented strain, the ΔcnaA mutant showed a statistically significant decrease in conidial density (P < 0.01). (C) The kinetics of germination were measured at both 25°C and 37°C by counting the number of germinating conidia in a population of approximately 100 spores after incubation for 4, 8, 16, and 24 h in SD liquid medium. In contrast to the wild-type, the percentage of germination decreased in the ΔcnaA mutant.

The cnaA mutant displays defects in hyphal and yeast morphogenesis. (A) Wild-type and ΔcnaA strains were inoculated onto ANM medium and grown at 25°C for 10 days, followed by either staining with calcofluor to visualize cell walls by light microscopy or SEM. The wild-type hyphal cells showed a smooth and uniform hyphal diameter with regular septation and uniform staining with calcofluor white. In contrast, the ΔcnaA mutant showed irregular hyphal growth with enlarged cells (white arrow) and exhibited abnormal chitin deposits along the hyphae (red arrow). SEM images show that the mutant hyphal cells appear shrunken and irregularly shaped with very prominent septa. (B) The terminal ends of yeast cells of the ΔcnaA mutant were swollen (white arrow) and exhibited abnormal chitin deposits (red arrow). Scale bars, 10 µm.
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FIGURE 4 The cnaA gene is required for correct cell wall biosynthesis. (A) The morphology of the strains was observed by TEM after 10 days of growth in ANM medium at 25°C and 37°C. The cell wall of the wild-type strain was smooth and intact in the hyphal and yeast forms in vitro, and the organelles were clearly visible (white arrow). In contrast, the ΔcnaA mutant showed cell wall deformation with less distinct layering, and the organelles of the mutant appeared disordered (red arrow). Scale bars, 0.2 µm. (B) Strains were grown on ANM medium at 25°C after the addition of various concentrations of calcofluor white or Congo red. Compared with the wild-type, the ΔcnaA mutant showed greater sensitivity to Congo red at concentrations of 5 mM, but there was no difference in calcofluor white staining.

the ΔcnaA strain were also readily phagocytosed by RAW264.7 macrophages, and by 12 h, the conidia showed clear signs of germination into ellipsoid-shaped yeast cells, similar to the wild-type. However, by 24 h, the ΔcnaA yeast cells showed much slower growth than the wild-type cells, with only minimal signs of division. At 48 h, there were very few yeast cells remaining in macrophages, showing that most of the yeast cells had been killed and degraded (Figure 6A). TEM observations clearly showed that wild-type cells maintained normal cellular morphology and developed into yeast cells in macrophages. The septum was visible, the cell wall of yeast cells was complete and uniform, organelles were clear and the cytoplasm was uniform. However, most of the ΔcnaA conidia were atrophic and destroyed by the phagosome (Figure 7).

Conidia survival was measured by lysing a fixed number of macrophages infected with either the wild-type or ΔcnaA strain after co-incubation for 24 h and counting viable T. marneffei cells (colony forming units, CFU) on YPD medium after 72 h of incubation at 25°C. The number of surviving ΔcnaA mutant cells was drastically lower than the number of surviving wild-type cells, and over 60% of the ΔcnaA cells were killed at this time point (P < 0.01) (Figure 6B). Therefore, cnaA plays an important role in resisting macrophage killing.

Loss of cnaA Abrogates Virulence in a Murine Model of Invasive Talaromyces marneffei Infection

To examine the role of cnaA in virulence, a murine model of T. marneffei infection was utilized to mimic human disease. Four groups of 36 mice were infected by intraperitoneal injection with a sublethal dose (10^6 conidia in 100 µl of physiological saline) of the wild-type, ΔcnaA, ΔcnaA and cnaA^+ strains and a diluent control (0.9% physiological saline). The mice were sacrificed on days 3, 6, and 9 postinfection, and their tissues (lung, liver, and spleen) were harvested under sterile conditions. These tissues were macerated, plated on YPD medium and incubated at 25°C for 72 h for CFU assessment. Compared with the control group, the ΔcnaA mutant group exhibited sharply decreased CFU in the lung, hepatic, and splenic tissues (P < 0.001) (Figure 8). Infected mice displayed severe signs of invasive disease, including hunched posture, shivering, ruffled fur and emaciation. To evaluate the mortality rates, four groups of 36 immunosuppressed mice were challenged with 100 µl of suspensions containing 10^8 conidia/ml of each strain. The mortality rates of the mice infected with either the wild-type or ΔcnaA cnaA^+ strains were similar, and these mice all died by 14 days postinfection. In contrast, the mortality rate of the ΔcnaA mutant-infected mice was 45% at 14 days (Figure 8). These data

TABLE 2 In vitro assay of antifungal susceptibility.

| Antifungal drugs | Hyphal MIC (µg/ml) | Yeast MIC (µg/ml) |
|------------------|--------------------|-------------------|
|                  | wild-type | ΔcnaA | wild-type | ΔcnaA |
| Amphotericin B    | 0.5      | 0.5   | 0.5      | 0.5   |
| Itraconazole      | 8        | 8     | 8        | 4     |
| Fluconazole       | 0.03     | 0.03  | 0.03     | 0.01  |
| Voriconazole      | 0.06     | 0.03  | 0.06     | 0.03  |
| Caspofungin       | 2        | 0.5   | 16       | 2     |
| Micafungin        | 4        | 2     | 32       | 4     |
FIGURE 5 | The cnaA gene is required for adaptation to osmotic stress in vitro. To test the stress resistance of mutants, wild-type, and ΔcnaA strains were inoculated with a 5-µl drop of a 1 × 10^5 conidia/ml suspension onto ANM supplemented as follows: (A) for salt stress using 0.2, 0.4, 0.6, and 1M KCl; (B) for oxidative stress adding 2, 5 and 8 mM H_2O_2; and for osmotic stress adding 0.5, 1, and 1.5 M sorbitol, followed by incubation for 14 days at 25°C. The ΔcnaA mutant showed a gradual reduction in growth with increasing concentrations of the stress agent, especially for oxidative stress.

FIGURE 6 | The cnaA gene is essential for immune escape in macrophages. (A) Wild-type and ΔcnaA strains were co-cultured with RAW264.7 macrophages and observed by confocal microscopy. After 12, 24, and 48 h following phagocytosis, the ΔcnaA mutant conidia showed increased sensitivity to the cytotoxic activity of the macrophages and were killed and eliminated, compared with the wild-type, which germinated into yeast cells and replicated profusely intracellularly. Scale bars, 20 µm. (B) Conidial survival was measured by counting CFU on SD medium after lysis of T. marneffei-infected macrophages. The number of surviving ΔcnaA mutant cells was drastically lower than the number of surviving wild-type cells (P < 0.01).
FIGURE 7 | The cnaA gene is essential for survival in macrophages. Wild-type and ΔcnaA strains were co-cultured with RAW264.7 macrophages and observed by TEM. Wild-type conidia germinated into yeast cells and maintained cellular integrity and morphology after 24 h. The septum of dividing cells is clearly visible, the cell wall is complete and uniform, and organelles are distinct (white arrow). In contrast, most of the ΔcnaA mutant conidia failed to germinate or were clearly atrophic with little to no organelar integrity (red arrow). Scale bars, 2 µm.

DISCUSSION

This study investigated the roles of a calcineurin homolog (cnaA) in the dimorphism and pathogenicity of the opportunistic human fungal pathogen T. marneffei. We have shown that cnaA (i) is necessary for conidiation, germination, hyphal and yeast cell morphogenesis and growth; (ii) plays an essential role in cell wall integrity of both hyphal and yeast cell types; (iii) is required for stress adaptation for hyphal and yeast cell types; (iv) plays a unique role during immune escape; and (v) is required for full virulence in a murine model of invasive T. marneffei infection.

Conidia are an important cell type for almost all fungi and are often the infectious propagules of pathogenic fungi. Their production is tightly regulated, as is their capacity to sense the environment and germinate to initiate vegetative growth. Thus, conidiation and germination are central aspects of fungal cell survival and propagation and important pathogenicity determinants (Boyce and Andrianopoulos, 2007). Infection by T. marneffei is believed to occur by inhalation of conidia into the lungs, where they subsequently germinate and transform into yeast cells that cause disseminated infection. Deletion of the cnaA gene severely affected asexual reproduction, with the mutant showing defects in the development of the conidiophore, and this resulted in a sharp decrease in the number of conidia produced. It has been reported that calcineurin controls conidiation in Aspergillus fumigatus (Shwab et al., 2019), Aspergillus nidulans (Wang et al., 2012), and Penicillium digitatum (Zhang et al., 2013). In addition, a ΔcnaA mutant in Beauveria bassiana has been shown to have differential defects in conidial germination, vegetative growth and conidiation capacity (Huang et al., 2015; Wang et al., 2017). These findings show that cnaA plays important roles in both production of conidia and their ability to convert to vegetatively growing cells, both of which are likely to affect invasive infection in T. marneffei.

The cell wall is a physically rigid, yet plastic, structure that is responsible for the shape of the cell, protects the fungal cell from its environment, prevents killing by predators and mediates cell-cell interaction (Fontaine et al., 2000). Fungal cell walls are unique, and cell wall carbohydrates and proteins play important roles in cell physiology and disease pathogenesis (Mancuso et al., 2018). In Candida tropicalis, it has been shown that calcineurin is essential for tolerance of azoles, caspofungin, anidulafungin, and cell wall-perturbing agents (Chen et al., 2014). Similarly, in Cryptococcus neoformans, caspofungin tolerance is mediated by multiple pathways downstream of calcineurin function (Pianalto et al., 2019). This study showed that the T. marneffeiΔcnaA mutant displays defects in the hyphal and the yeast cell wall and that the cnaA gene is important for cell wall integrity. This was supported by the observation that yeast cells of the ΔcnaA mutant showed a lower MIC against caspofungin (CAS; eightfold) and micafungin (MCFG; eightfold) than wild-type cells (Table 2). CAS and MCFG are members of the echinocandin class of antifungal agents that inhibit fungal cell wall biosynthesis by inhibiting cell wall β-(1,3)-D glucan synthesis (Douglas et al., 1997). These results suggest that targeting calcineurin in combination with echinocandin treatment may be effective in T. marneffei infection, whereas echinocandins on their own are not.
The first line of defense in the human body against *T. marneffei* infection is the innate immune system (Romani, 2011). For *T. marneffei*, initial interactions are characterized by phagocytosis of the conidia by leukocytes in the lungs, followed by leukocyte-facilitated hematogenous dissemination (Vanittanakom et al., 2006). *T. marneffei* conidia face a variety of stresses, such as heat, salt, oxidative stress, osmolarity, nutrient deprivation and cytokine-mediated killing (Pongpom et al., 2017; Ellett et al., 2018). *T. marneffei* shows strong stress tolerance and the ability to resist the cytotoxicity of macrophages in the innate immune system (Pongpom et al., 2005; Vanittanakom et al., 2009). The ΔcnaA mutant showed increased sensitivity to salt, H$_2$O$_2$ and osmotic stress *in vitro* during hyphal growth. It has been reported that calcineurin is essential in stress resistance in *C. albicans* (Reedy et al., 2010; Liu et al., 2014). This stress adaptation not only helps *T. marneffei* survive in extreme environments but also plays important roles in resisting killing and replication inside macrophages. In fact, morphogenesis and survival of the ΔcnaA yeast was compromised inside host cells, but the mutant cells were still able to germinate and develop *in vitro*. During macrophage infection, the ΔcnaA mutant conidia hardly germinated or underwent yeast morphogenesis after being phagocytosed, and at longer incubation times, these conidia were killed and eliminated by the macrophages. Thus, the ΔcnaA mutant is defective in resisting killing by macrophages.

The phenotypes of the ΔcnaA mutant suggested that it would likely be compromised in virulence, and we showed that the deletion of cnaA resulted in a drastic increase in the mean survival time of systemically infected mice, with a substantially reduced fungal burden in the lung, hepatic and splenic tissues compared with the wild-type-infected mice. These results indicated that *T. marneffei cnaA* affects virulence in the murine model of invasive *T. marneffei* infection and that it is important for full virulence but does not block infection and dissemination. The capacity for the *T. marneffei* ΔcnaA mutant to disseminate despite showing severely compromised survival in an *in vitro* macrophage assay may suggest that there are additional routes of dissemination in an animal host. The importance of calcineurin in virulence has also been shown in *A. fumigatus*, *C. neoformans*, and *C. tropicalis* (Fox et al., 2001; Chen et al., 2014; Juvvadi et al., 2014).

In summary, our findings show that cnaA, and therefore calcineurin, plays a key role in controlling fungal morphogenesis and the response of *T. marneffei* to external stresses, including antifungal drugs as well as the host immune response and subsequent fungal pathogenicity. It is required for full virulence...
in a murine model of invasive T. marneffei infection. Moreover, cnaA could be a potential target for combinatorial antifungal therapy during life-threatening T. marneffei systemic infections.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/supplementary material.

**ETHICS STATEMENT**

This study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (ethics amendment dated 4/3/2012, approval number KY-074). All experiments in this study were conducted according to internationally accepted standards and regulations on the administration of experimental animals in China (8/1/2011 C-WISC).

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**AUTHOR CONTRIBUTIONS**

C-WC designed this study and drafted the manuscript. Y-QZ and K-SP performed the experiment and data analysis. Y-QZ calculated the statistics and edited the manuscript. J-PL and AA provided the valuable advice, supported the experiment protocol, and critically revised the manuscript. AA critically revised the manuscript. HL, R-FY, J-YW, and C-YH assisted in completing the experiment. All authors have read and approved the final manuscript.

**FUNDING**

This study was supported by grants from the National Natural Science Foundation of China (Nos. 81571971 and 81271804) and the Natural Science Foundation of Guangxi Province of China (AB18221017 and 2018GXNSFAA294090). The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Title:
Calcineurin A Is Essential in the Regulation of Asexual Development, Stress Responses and Pathogenesis in Talaromyces marneffei

Date:
2020-01-21

Citation:
Zheng, Y. -Q., Pan, K. -S., Latge, J. -P., Andrianopoulos, A., Luo, H., Yan, R. -F., Wei, J. -Y., Huang, C. -Y. & Cao, C. -W. (2020). Calcineurin A Is Essential in the Regulation of Asexual Development, Stress Responses and Pathogenesis in Talaromyces marneffei. FRONTIERS IN MICROBIOLOGY, 10, https://doi.org/10.3389/fmicb.2019.03094.

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