Effect of CAP10 gene on immune response in mice infected with *Cryptococcus neoformans*

Ni Lin

Fujian Medical University <https://orcid.org/0000-0003-3313-5289>

Liping Lin

Fujian Medical University

Xiaoyu Li

Fujian Medical University

Xianghui Li

Fujian Medical University

Juan Wu

Fujian Medical University

Donghong Lin

Fujian Medical University

Qishui Ou (✉ ouqishui@fjmu.edu.cn)

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Abstract

ABSTRACT Background Capsule is an vital virulence factor in *Cryptococcus neoformans* infection. Recent studies show CAP10 is a key gene in capsular formation. However, the role of CAP10 in the pathophysiology of cryptococcosis is still not well understood. This study aims to investigate the association of CAP10 expression with the immune responses to infected mice. Methods The shRNA expression plasmid was designed to interfere with the synthesis of CAP10. The animal model was established with *C. neoformans* wt strain H99, cap10-shRNA *C. neoformans* and PBS control in the respiratory tract. On the 7 days and 21 days after infection, mice lung histopathological examination and homogenate culture were performed, and cytokines expression level in the serum of mice were quantitatively detected. Results The lower degree of edema and infiltration of inflammatory cells were observed in cap10-shRNA group. The growth rate of cap10-shRNA strain was significantly reduced. In addition, interference with CAP10 altered the expression profile of Th1, Th2, Th17 and Treg type cytokine. Down-regulation of CAP10 was beneficial to the balance of Th1/Th2, Th17/Treg ratio. Conclusions Taken together, our results indicated the expression of the CAP10 was associated with an antifungal immune response to mice infected with *C. neoformans*. CAP10 might play an important role in regulating the inflammatory response, and could expected to be a new molecular therapeutic target in cryptococcosis.

Background

Cryptococcus neoformans is a clinically important pathogenic fungus which can lead to cryptococcal meningitis and cryptococcal pneumonia with severe symptoms, poor prognosis and high mortality. Host immunity to *Cryptococcus* includes cellular and humoral immunity, in which Macrophages, neutrophils, lymphocytes, natural killer cells, and Cytokines produced by T cells play an important role¹⁻³. The development and outcome of cryptococcosis is not only affected by the immune status of the host, but also depends on the expression differences in various virulence factors, such as polysaccharide capsule, phosphatidylserine synthesis⁴, laccase, urease and melanin. Among them, the high molecular weight polysaccharide capsule is the most Classical virulence factors, contributing approximately 25% of the total virulence composite⁵⁻⁷. However, the relationship between capsular virulence factors and its role in the pathophysiology of cryptococcosis is still unclear.

The capsule associated protein 10 gene (*CAP10*) is a key gene in capsular formation. There is a correlation between capsular thickness and *CAP10* expression⁸. Studies have shown that knocking out the *CAP10* in wild-type *Cryptococcus* can cause capsular loss, which shows no pathogenicity in animal models. If recomplemented with the *CAP10*, the strain forms a capsule and shows toxic in animal models⁹. Some metal ions, such as Mg²⁺ that have an effect on CAP gene expression, which are also important to capsule biosynthesis and virulence¹⁰. A mutation in the *CAP10* results in a pleiotropic phenotype with cell size, expression of virulence factors and size of extracellular vesicles¹¹. Our previous studies showed that the RNA interference on the expression of CAP10 gene can induce phagocytosis of *C. neoformans*. In this study, an animal model was established with inoculating *C. neoformans* wt strain

H99 or *cap10*-shRNA *C. neoformans* through respiratory tract. Our objective was to determine whether CAP10 expression affects the course of pulmonary infection and dissemination in infected animal. Understanding these functions of *CAP10* in host immune response may provide opportunities to improve the effective control and treatment of cryptococcosis.

Methods

Plasmid and strain

Psilencer4.1-CMV neo was purchased from Invitrogen Inc. *C. neoformans* strain (wt strain H99) was purchased from cryptococci professional laboratory of China Medical fungus preservation management center, *E. coli* DH5α was purchased from Tiagen Inc.

Construction, connection and transformation of shRNA expression vectors

According to the *CAP10* nucleotide sequence provided by the GenBank database, the targeting sequence was designed using Ambion's siRNA online design software. To clone the siRNA into the vector psilencer4.1-CMV neo and express the shRNA by this vector, the sticky end of HindIII and BamHI were used to form the expression cassette. The full sequences used were as follows: sense 5'-GATCCGGGCTTTGATGAATGGTACTTCAAGAGAGTACCATTTCATCAAAGCCCTTA-3' and antisense 5'-AGCTTAAGGGCTTTGATGAATGGTACTCTCTTGAAGTACCATTTCATCAAAGCCCG-3'. Two microliters of each oligo was combined in a 50 μL reaction system. The oligos were denatured at 95°C. The temperature was decreased by 5°C every 5 minutes until 45 °C, so that the single strand is anneal to form a double strand. Plasmid psilencer4.1 –CMVneo was digested by endonuclease HindIII and BamHI, and then Annealed oligos were ligated into the HindIII and BamHI sites of psilencer4.1-CMV neo. The recombinant plasmid was transformed to the competent *E. coli* DH5α, then the positive clones were selected, and verified by sequencing.

Transduction and selection of stable clones

The recombinant plasmid was transfected to *C. neoformans* using the LiAc lithium acetate yeast transformation kit. Stable *C. neoformans* expressing *cap10*-shRNA was selected using 100 μg/ml G418 in YPD. The culture medium was changed every 2~3 day, and transfected *C. neoformans* was harvested with G418 after 14 days of selection.

Establishment of animal models

Experimental animal

Inbred strain mice are the most sensitive animals to *Cryptococcus neoformans*. SPF graded female C57BL/6 mice were purchased from the Lecco Experimental Animal Center of Fujian Province. 96 mice were randomly divided into 3 groups, with 32 mice in each group. Mice were 8~12 weeks of age and the body weight was 16~22g at the time of infection. Rat group breeding cage was used and SPF grade mouse feed provided by Beijing Huayueyang Biotechnology Co., Ltd. All studies were conducted according to a protocol approved by the experimental animal ethics committee of fujian medical university. All studies were in line with the requirements of the “National Laboratory Animal Management Regulations” for the care and use of animals.

Preparation of fungal suspension

C. neoformans wt strain H99 group and the *cap10*-shRNA strain group were washed with PBS, then were centrifuged at 1200r /min for 5min and resuspended in sterile double distilled water. The number of *C. neoformans* were counted on the counting plate and adjusted to a final concentration of 5×10^6 CFU/mL.

Mouse model of cryptococcus neoformans infection

Sodium pentobarbital is formulated to a concentration of 3% before use. Injection dose is 30mg/kg. Then observe the anesthetic effect until the eyelash reflex disappears and the muscles relax, reaching the anesthesia requirements of this experiment. To simulate the natural infection process, the mice were quickly inoculated with 20μL of the fungal suspension through unilateral nostrils after anesthetized with Sodium pentobarbital intraperitoneal injection. Three experimental groups were inoculated separately with *C. neoformans* wt strain H99, *cap10*-shRNA *C. neoformans* or equal amounts of PBS solution as control.

Mouse lung histopathological examination and homogenate culture

Mice were euthanized with on 7th and 21th day post-inoculation in each group. We took the utmost care to alleviate suffering of the mice. Intraperitoneal injection of sodium pentobarbital, the dose is generally about 3 times the anesthetic dose. Judging the death of experimental animals: continued without spontaneous breathing for 2–3min and no blink reflex. Then lung tissues were excised using an aseptic technique. Lung tissues were fixed and immersed with neutral buffered formalin for 24h, and then placed into TS12C automatic dehydrator, embedded in paraffin at 60 °C. 5μm-thick slices of organs were cut and stained with PAS method. Slices were analyzed using light microscopy. In a separate experiment, lung tissues were homogenized in sterile PBS, followed by cultured on YPD agar. CFU were counted following incubation at 30°C.

Quantitative analysis of Cytokine in serum of mice by ELISA

The ELISA double-antibody sandwich method was used for the detection. The Mouse Quantikine ELISA Kit (R&D Systems, America) was purchased from Xiamen Taijing Biotechnology Inc. The test procedure is strictly followed according to the reagent instructions. Serum samples of three groups of mice were obtained in the same manner, and the levels of Treg/Th17 and Th1/Th2 related cytokines were determined by ELISA, including interleukin-2 (IL-2), gamma interferon- γ (IFN- γ), interleukin-4 (IL-4), transforming growth factor beta (TGF- β), and interleukin-17 (IL-17).

Statistical analysis

All data were expressed as mean $\pm$ SEM for each experimental group. Statistical significance was calculated using Student's *t* test of individual paired comparisons. A *P* value < 0.05 was considered to be statistically significant. Analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA).

Results

Construction and verification of stably expressing cap10-shRNA

Following transfection with *cap10*-shRNA plasmid, *C. neoformans* cells were selected using G418 at 100 μ g/ml. After 2 weeks, non-transfected cells were completely dead, and G418 resistant colonies were harvested and grown in culture medium containing 50 μ g/ml G418. The recombinant plasmid was sequenced by an automatic gene sequencer (Invitrogen company), and the results were consistent with the design sequence. FQ-PCR was performed to verify that *CAP10* gene expression in stably transfected cells was suppressed by *cap10*-shRNA. The experimental details were described previously¹².

The cap10-shRNA strain alleviated pathological lesions in pulmonary C. neoformans infection

In order to examine the levels of pulmonary inflammation in these three groups of animals, we picked the examination time points of day 7, and day 21 post-infection (The average survival time of mice with cryptococcosis was about 28 days, so we chose day 7 as early infection points and day 21 as later infection points).

The paraffin-embedded specimens and the pathological section of the lungs were observed. On day 7, compared with the control group, both the wt group and the *cap10*-shRNA group had swollen lung tissues. The wt group of *C. neoformans* showed obvious enlarged edema in the lungs. The degree of edema in the *cap10*-shRNA group was lower than that in the wt group (Fig.1A). Pathological sections of lung tissue showed that on the 7th day after infection, a large number of inflammatory cells infiltrated in the wt strain, and numbers of *C. neoformans* distributed among the whole lung. Some granuloma and tissue necrosis began to form. In the *cap10*-shRNA group, scattered Cryptococcus was observed, and the degree of infiltration of inflammatory cells was lower than wt group. No obvious granuloma and necrosis

was observed (Fig.1B). On day 21, the degree of edema in the wt group was further increased, while the change in the *cap10*-shRNA group was not significant compared with the time points of day 7 (Fig.1A). In the wt strain, the granuloma gradually enlarged to invade the surrounding tissues, and the fibrous tissue proliferated. Later, the lesions may be surrounded by fibrous tissue or fibrous scars. There were a large number of macrophages and lymphocytes inside. In the *cap10*-shRNA strain, only a few lymphocytes and neutrophils were infiltrated, while the necrosis and granuloma were still not obvious (Fig.1B).

Inhibition of *cap10* expression reduced lung fungal burden in infected mice

Pulmonary growth and clearance of wt strain were compared to those *cap10*-shRNA strain. Mice were inoculated intranasally with a final concentration of 5×10^6 CFU/mL, and the pulmonary fungal burden kinetics was evaluated. The wt strain showed continuous growth in lungs, resulting in the burden of *C. neoformans* expansion during 3 weeks [CFU(log10):week 1, 5.52; week 3, 6.20]. This was completely different contrast with the *cap10*-shRNA strain, which showed a slight decrease during 3 weeks (CFU(log10):week 1, 2.83; week 3, 2.68). Burden in the group infected with the *cap10*-shRNA strain was about 1000-fold lower than that of the group infected with the wt strain at the end of week 3 (Fig.2). These results may suggest that the growth and clearance of cryptococcus in the lungs is related to *cap10*

Inhibition of cap10 expression altered cell-mediated immune response in infected mice

IFN- γ and IL-2 are Th1-type cytokines, IL-4 is a Th2-type cytokine, IL-17 is a Th17-type cytokine and TGF- β is a specific cytokine produced by Treg cells. So we further observed these cytokine profiles in infected mice serum on day 7, and 21 post-infection. Compared with the PBS-control group, the Th1 type cytokine IFN- γ and interleukin-2 (IL-2) levels in mice infected with the *cap10*-shRNA strain and in those infected with the wt strain were significantly higher at early phase. The Th1-type cytokine IFN- γ and interleukin-2 (IL-2) levels in the *cap10*-shRNA strain still maintained a relatively high level in later phase. Compared with wt strain, the Th1-type cytokine levels showed significantly higher and Th2-type cytokine showed significantly lower in later phase in the *cap10*-shRNA strain (Fig.3A3B3C). It implied that inhibition of *cap10* expression might induce protective cell-mediated immunity in infected mice. The Th2-type cytokine interleukin-4 in the *cap10*-shRNA strain and in wt strain was significantly higher than control in early phase and later phase. It is suggested that interference with *CAP10* can alter the expression of Th1 and Th2 type cytokine. As for interleukin-17, it showed significantly higher in the *cap10*-shRNA strain and in wt strain compared with control group at each time point. As for transforming growth factor beta (TGF- β), it was significantly lower in the *cap10*-shRNA strain and in wt strain compared with control in early phase, but significantly higher in the *cap10*-shRNA strain compared with control in later phase (Fig.3D3E). It implied that in the later stage of infection, the higher concentration of TGF- β maybe induced the conversion of CD4+T cell into Tregs, which can alleviate the immune damage caused by Cryptococcus, thereby achieving the purpose of protecting the host.

Inhibition of cap10 expression altered the balance of Th1-Th2 and Th17-Treg responses

The immune response pattern is reflected by the ratio of Th1 to Th2 cytokines (Th1/Th2) and Th17 to Treg cytokines (Th17/Treg). Compared with the PBS-control group, the IFN- γ /IL-4 ratio and the IL-2/IL-4 ratio showed significantly lower in the *cap10*-shRNA strain and in wt strain at later time point. Compared with the *cap10*-shRNA strain, the IFN- γ /IL-4 ratio and the IL-2/IL-4 ratio showed significantly lower in the wt strain at later time point (Fig.4A4B). It indicated that Th2-based immunity should be the main way for *C. neoformans* to escape host clearance. As the disease progressed, the Th1/Th2 immunity imbalance became more and more serious in wt strain. As for the ratio of IL-17/TGF- β , compared with the PBS-control group, it was significantly higher in the *cap10*-shRNA strain and in wt strain at early time point, but only wt strain still showed significantly higher at later time point. Compared with the *cap10*-shRNA strain, the IL-17/ TGF- β ratio showed significantly higher in the wt strain at each time point (Fig.4C). It implied that the Th17 response is dominant in the early phase, but with the development of disease, the secreted TGF- β cytokine maintained high levels in *cap10*-shRNA strain.

Discussion

The capsule of *C. neoformans* regulates the host's immune response from many levels, which including changing the antigen presentation process to make the antibody non-responsive, cytokine secretion disorder, anti-phagocytosis, capable of absorbing microbicidal oxidative bursts, white cell migration inhibition^{13,14}. In this study, the effect of down-regulation of *CAP10* expression of the pathophysiological process of *C. neoformans* infection was observed. The *cap10*-shRNA strain was found smaller distributed among lung tissues, less inflammatory cell infiltration and no obvious necrotic lesions to be observed on 7 days (FIG.1). The wt strain was showed a sustained strong inflammatory response with the gradually enlarged granuloma and the fibrous tissue proliferated, even invaded into the surrounding tissues on 21 days. It demonstrates that the *cap10*-shRNA strain may alleviate pathological lesions in pulmonary *C. neoformans* infection. The *cap10*-shRNA strain was also found to exhibit a retained ability to grow on the YPD agar, though the growth rate was significantly reduced (FIG.2). In contrast, a progressive growth of the wt strain was found on the YPD agar.

Cytokine patterns of the serum may be related to clinical outcomes¹⁵⁻¹⁷. Gressler and colleagues reported an association between Th1/Th2 cells and fungal growth and dissemination in Cryptococcosis caused by *Cryptococcus neoformans*¹⁸. Th1-typed cytokines may induce prolonged STAT1 binding and protection against *C. neoformans*¹⁹. Additionally, Darin also reported that regulatory T cell induction and retention in the lungs drives suppression of detrimental Th2 cells during pulmonary cryptococcal infection²⁰. We therefore focused on the cytokine profiles after inoculated with pathogenic *Cryptococcus* strains. From the results of the cytokine profiles (FIG.3), we found the production of Th1 cytokines were reduced while Th2 cytokines were increased at later time point in wt strain, but Th1 and Th2 cytokines in *cap10*-shRNA strain both increased. Microbial infections such as bacteria, fungus and viruses may also cause Th1/Th2 imbalance in vivo. A significant drift of the Th1/Th2 cytokines ratio at later time point was observed in the WT group (FIG.4). It suggested that the Th2-type immune response might be the main way for *C. neoformans* to escape host clearance, which might make cryptococcal cells unable to be

effectively cleared by phagocytic cells. The fungus could further spread or even infect the central nervous system by means of the “trojan mechanism”.

Th17 cells also play an important role in infectious disease through their secreted molecules. Th17 cells and its secreted cytokines IL-17 are responsible for the immune surveillance, but also involved in the pathogenesis of many autoimmune diseases and in the mechanisms of fibrosis in many organs after the infection^{21–23}. Unlike Th17 cells, Treg cells are a subset of T cells with immunosuppressive effects, which control the intensity of immune responses and reduce the damage of inflammatory responses to tissues²⁴. The imbalance between Th17 cells and Treg cells is an important factor in the pathogenesis of many inflammatory and autoimmune diseases^{25–27}. From our results, we found IL-17 maintained a relatively high level in wt and *cap10*-shRNA strain at each time point while TGF- β showed significantly higher in the *cap10*-shRNA strain compared with control at later time point. As for the ratio of IL-17/ TGF- β , compared with the *cap10*-shRNA strain, the IL-17/ TGF- β ratio showed significantly higher in the wt strain at each time point. Compared with PBS control, only wt strain showed significantly higher at later time point. TGF- β plays a key role in the balance between Treg cells and Th17 cells. It has been reported that low concentration of TGF- β can induce the expression of ROR- γ t, but high concentration of TGF- β can inhibit the expression and function of ROR- γ t and facilitate the production of Treg cells²⁸. So that the increased Treg cells could regulate the balance between the response of effector T cells to remove pathogens and their over-immune response to severe inflammation and tissue damage. This observation matched well with pathological examination, less granuloma and necrosis were observed in the *cap10*-shRNA strain on 21 days.

Conclusions

This study clarifies the relationship between the immune response pattern and the CAP10 gene in mice infected with *C. neoformans*. The amount of *CAP10* expression can affect the secretion of cytokines. Knocking down the *cap10* gene contributes to the balance of Th1/Th2 and Th17/ Treg immunity during the development of cryptococcosis. It is also beneficial to controlling the spread of *C. neoformans*, which can affect the prognosis of cryptococcosis. A better understanding of the relationship between the immune response pattern and the CAP10 gene is helpful in finding a novel molecular therapeutic target to improve the outcomes of patients with cryptococcosis.

Abbreviations

CFU: Colony-forming units; ELISA: Enzyme-linked immunosorbent assay; IFN- γ : Interferon gamma; IL-4: Interleukin-4; IL-2: Interleukin-2; IL-17: Interleukin-17; TGF- β : transforming growth factor- β ; CAP10: The capsule associated protein 10 gene.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Fujian Medical University Ethics Committee.

Consent for Publication

The authors have stated that it is not applicable.

Availability of data and material

The data that support the findings of this study are included in the main manuscript and supplementary file.

Competing interests

The authors of this study declared that they have no competing interests.

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Authors' contributions

NL participated in designing and performing the experiments, writing the manuscript.

LPL participated in the construction of shRNA. JW carried out the immunoassays.

XYL and XYL participated in the animal experiments. JW and LPL carried out the statistical analysis. DHL performed the lunghistopathological examination. DHL and

QSO designed the study, directed the experiments and revised the manuscript. All

authors approved this manuscript.

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Figures

Figure 1

The CAP10-shRNA strain reduced inflammatory responses in pulmonary *C. neoformans* infection. (A) paraffin-embedded gross pathology of resected lungs of infected mice. (B) PAS staining of the lungs of mice infected with the wt strain or the cap10-shRNA strain (200×).

Figure 2

Views of fungal burdens during 3 weeks. Lung tissue were homogenized and cultured on YPD agar, then CFU was counted. Results are expressed as mean CFU per organ±SEM.

Figure 3

Inhibition of cap10 expression altered the expression profile of Th1, Th2, Th17, Treg cytokines. At 7 and 21 days, mice were sacrificed and serum of mice assayed by enzyme-linked immunosorbent assay (ELISA). (A) IFN- γ ; (B) IL-2; (C) IL-4; (D) IL-17; (E) TGF- β . Bars represent mean cytokine concentrations $\pm$ SEM (pg/ml; n= 16). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Figure 4

The ratio of Th1 to Th2 cytokines (Th1/Th2) and Th17 to Treg cytokines (Th17/ Treg) were altered through inhibition of cap10 expression. (A) IFN- γ /IL-4; (B) IL-2/IL-4; (C) IL-17/TGF- β . Bars represent the mean ratio of cytokine concentrations $\pm$ SEM (n= 16). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

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