MicroRNA-mediated inflammatory responses induced by *Cryptococcus neoformans* are dependent on the NF-κB pathway in human monocytes

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Abstract. Cryptococcosis is a significant invasive fungal infection with noteworthy morbidity and mortality that is usually caused by either *Cryptococcus neoformans* (*C. neoformans*) or *Cryptococcus gattii* (*C. gattii*). Epidemiological studies have indicated that *C. neoformans* are more often reported in immunocompromised and immunocompetent patients. It has been well established that the cytokine profile of the host markedly affects the outcome of cryptococcal disease, and the negative regulators of microRNAs(miRs or miRNAs) are critically important for immunomodulation. However, the role of miRNAs and the molecular basis of the inflammatory response induced by *C. neoformans* in monocytes remain unknown. In this study, we identified 7 differentially expressed miRNAs in THP-1 cells exposed to *C. neoformans* by Illumina sequencing, and confirmed our findings by RT-qPCR. Furthermore, miR-146a was selected for further analysis to identify the regulatory mechanisms of inflammation induced by *C. neoformans*. An examination of the function of miR-146a in monocytes was performed by overexpressing and inhibiting miR-146a. In addition, we identified a pattern of induction in response to a variety of microbial components and pro-inflammatory cytokines. Our data suggested that the nuclear factor-κB (NF-κB) pathway was required for the induction of miR-146a, whereas miR-146a negatively regulated NF-κB activation by targeting interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6), then inhibiting NF-κB activation and the release of inflammatory cytokines in monocytes induced by *C. neoformans*.

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Introduction

Cryptococcosis, a potentially fatal infectious fungal infection, primarily caused by either *Cryptococcus neoformans* (*C. neoformans*) or *Cryptococcus gattii* (*C. gattii*), has gradually increased in incidence worldwide over the past 20 years, particularly in China (1,2). Infection proceeds via inhalation and subsequent dissemination to the central nervous system to cause meningoencephalitis. Worldwide, the most common risk for cryptococcosis caused by *C. neoformans* is immunodeficiency, such as AIDS, and infections caused by *C. gattii* are more often reported in immunocompetent patients (3). Epidemiological studies from far east Asian countries, particularly from China, have indicated that *C. neoformans* infects mostly HIV-uninfected patients for whom a predisposing underlying factor may or may not be apparent (4,5).

A previous study strongly suggested that anti-granulocyte-macrophage colony-stimulating factor autoantibodies are a risk factor for central nervous system infection by *C. gattii* cryptococcosis in otherwise immunocompetent individuals (6). Thus, this raises the question of whether the so-called healthy hosts of cryptococcosis caused by *C. neoformans* are, in fact, accompanied by other immunocompromising conditions that have not been identified.

The innate immune system, including cellular components, monocytes, macrophages and many other effectors, is the first line of defense against pathogens, and broadly protects against invading microorganisms. Monocytes are blood-borne cells that differentiate into macrophages within tissues, which are infiltrated following an inflammatory signal (7). Once within tissues, macrophages can further develop distinct functional phenotypes, which is determined by the cytokine milieu induced by pathogens (8,9).

Macrophages are important components with versatile functions prominently involved in host defense and immunity against foreign microorganisms, including bacteria, viruses, fungi and parasites (10). Macrophages possess a broad array of cell-surface receptors, intracellular mediators and essential secretory molecules for the recognition, engulfment and destruction of invading pathogens, and for the regulation of other types of immune cells (11). In light of the important role of monocytes in the immune response, additional investigations

of the immune mechanism in monocytes exposed to *C. neoformans* would be of interest.

Toll-like receptors (TLRs), are the first identified and the most well characterized pattern-recognition receptors (PRRs) that recognize components derived from a wide range of pathogens known as pathogen-associated molecular patterns (PAMPs). TLRs subsequently initiate an anti-infection innate immune response and help initiate and shape adaptive immune responses (12,13). Among the TLRs, TLR2 can recognize microbes, including components from Gram-positive bacteria in the presence of TLR1 or TLR6 (14). The importance of TLR2 in host defense against *C. neoformans* has been widely studied in animal experiments (15,16).

The activation of TLRs initiates a transmembrane signaling cascade and triggers intracellular signaling molecules, including interleukin-1 receptor-associated kinase (IRAK)1, IRAK4 and TNF receptor associated factor 6 (TRAF6), and then initiates a series of immune responses (17). Although the inflammatory response is valuable for dealing with pathogens, if the inflammatory response is unregulated, it can take a toll on the body and lead to serious disease. Thus, negative regulators of the response are critically important.

MicroRNAs (miRs or miRNAs) are a class of highly conserved small non-coding RNAs, 19-24 nucleotides in length, which post-transcriptionally regulate gene expression by targeting the 3' untranslated region (3'UTR) of target mRNAs (18). miRNAs prevent protein synthesis by degrading mRNAs and inhibiting their translation (19). Accumulating evidence has indicatd that miRNAs play a novel role in the regulation of the immune system, including the development and differentiation of immune cells, antibody production and innate immune regulation (20,21).

The abnormal expression of miRNAs is also involved in various diseases, ranging from the development and differentiation of cells to tumors (22), and inflammatory and autoimmune disorders (23,24). Recent evidence has indicated that miRNAs, such as miR-9, miR-21, miR-125a, miR-132, miR-146a/b and miR-155 are inducible by PAMPs, such as lipopolysaccharides (LPS). These miRNAs are also engaged in regulating innate immune responses (25-27). miR-146a is one of the most prominent miRNAs induced by TLR signals through nuclear factor-κB (NF-κB) activation and then feeds back to suppress TLR-triggered NF-κB activation (28).

It has been well established that the cytokine profile of the host markedly affects the outcome of cryptococcal disease, and the negative regulators of miRNAs are critically important for immunomodulation. However, the role of miRNAs and the molecular basis of the inflammatory response induced by *C. neoformans* in monocytes remain unknown. Thus, the aim of this study was to investigate the effects of the unique regulatory pattern of miRNAs in *C. neoformans*-exposed monocytic cells.

We analyzed the differential miRNA expression profiles in *C. neoformans*-exposed THP-1 cells, and predicted their target genes and function. Our data indicate that many miRNAs, including miR-146a, are altered in primary human macrophages exposed to *C. neoformans*. We demonstrate that *C. neoformans* components induce NF-κB activation through a TLR signaling pathway, resulting in the upregulation of the miR-146a, which, upon processing, downregulates the mRNA and protein levels of IRAK1 and TRAF6, reducing the activity of the NF-κB pathway. The interaction of miR-146a with NF-κB in *C. neoformans*-exposed THP-1 cells is thought to be crucial to the inflammatory response, determining Cryptococcosis progression and correlating with disease outcome in humans.

Materials and methods

Cells and bacterial culture. The human macrophage cell line, THP-1 (Shanghai Cell Bank, Chinese Academy of Sciences, Shanghai, China), was cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) (all from Beyotime Institute of Biotechnology, Shanghai, China), in a humidified incubator containing 5% CO_2 at 37˚C. *C. neoformans* (WN148; Center for the Preservation of Medical Mycology, Shanghai, China) was cultured in YPD broth (1% yeast extract, 2% peptone and 2% dextrose) for 3-5 days at 30˚C, then harvested, washed with sterile saline, and inactivated by heating at 56˚C for 60 min. The efficiency of heat-killing was assessed by culture in Sabouraud glucose broth (Shanghai Hao Yang Biotechnology Co., Ltd., Shanghai, China). The heat-killed *C. neoformans* number was counted and adjusted to the desired concentration. The THP-1 cells were incubated with the heat-killed *C. neoformans* WN148 for 0, 3, 6, 9 and 12 h. For all experiments, the cells were exposed to WN148 at an MOI of 1:5.

NF-κB inhibitor (PDTC) pre-treatment. The appropriate amount of THP-1 cells was planted according to the experiment. When the THP-1 cells were grown to 80% confluency, the fresh anti-culture medium was replaced. The cells were pre-treated with NF-κB inhibitor (PDTC, S1809; Beyotime Institute of Biotechnology) and the final concentration was 10 μM. After 12 h of pre-treatment, the cells of the experimental group were treated with *C. neoformans* (MOI = 5). After 3 and 6 h, the cells were collected.

In vitro exposure model. The THP-1 cells were seeded at $5x10^6$ cells/flask and grown to 70% confluency at 37˚C in 5% CO_2. THP-1 monolayers (containing approximately 10^7 cells) were incubated with $5x10^7$ *C. neoformans* (MOI of 5) for 6 h as the induction model. The exposed cells were lysed with 0.1% TRIzol (Invitrogen, Carlsbad, CA, USA), and CFU counts of the cell lysates were determined by RT-qPCR (7900 real-time PCR system; Applied Biosystems, Foster City, CA, USA).

Small RNA library construction and Illumina sequencing. miRNA expression profiling of *C. neoformans*-exposed THP-1 cells for 0 and 6 h (this is a representative experiment of 4). miRNAs were isolated by separating total RNAs on denaturing polyacrylamide gel electrophoresis and cutting a portion of the gel corresponding to the size 18-30 nucleotides. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and the RNA concentration and purity were determined using the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Small RNA library construction and sequencing, analysis of sequencing data, stem-loop RT-qPCR for miRNAs and bioinformatics analysis were carried out by the Amplicon Gene Biotechnology Companies (Shanghai, China).

Table I. Sequences of primers used for RT-qPCR.

Gene	Category	Primer sequences (5'→3')
Actin	Sense	ACAATGTGGCCGAGGCTTT
Actin	Antisense	GCACGAAGGCTCATCATTCA
IRAK1	Sense	GGACACGGACACCTTCAGC
IRAK1	Antisense	CAGCCTCCTCTTCCACCAG
TRAF6	Sense	TTGATGGCATTACGAGAAGCAG
TRAF6	Antisense	GCAAACAACCTTCATTTGGACAT
miR-146a	Sense	GCTACAAAAGCTGGGAA
miR-146a	Antisense	CTGATGCGTGAAGTGCTG
miR-146a	Reverse	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAACCCA

THP-1 cell transfection. miR-146a functional analyses were performed using synthetic miR-146a mimic, miR-146a inhibitor, relative controls, or FAM-negative control miRNA obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) and reconstituted in nuclease-free water at a concentration of 20 mM. Prior to transfection, the cells were transferred to fresh culture medium at a concentration of $1x10^6$ cells/ml. The following day, the THP-1 cells adjusted to $1x10^6$ cells/well were transfected with miR-146a mimic (20 nM) or inhibitor (40 nM) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Following transfection, the cells were allowed to recover for 6 h at 37˚C and fresh RPMI-1640 medium was changed thereafter, and the cells were then exposed to *C. neoformans* for 3 h. Supernatants from cell cultures were collected and assayed for cytokine secretion, and cell pellets were used for RNA isolation and RT-qPCR.

RT-qPCR. Total RNA of related factors in treated and untreated THP-1 cells was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. miR-146a expression was measured using a TaqMan MicroRNA reverse transcription kit (Takara, Dalian, China) and normalized to an internal actin control, as previously described (29). For the other genes, IRAK1, TRAF6 and actin, reverse transcription was carried out using 1 mg of RNA to produce cDNA. The primer pairs used are listed in Table I. RT-qPCR for miRNAs was performed using a standard SYBR Green PCR kit (Takara) in an 7900 real-time PCR (Applied Biosystems) according to the instructions from the respective manufacturers. Triplicate samples were analyzed in triplicate wells in each experiment. The $2^{-\Delta\Delta Cq}$ method was used to quantify the relative levels of gene expression.

Enzyme-linked immunosorbent assay (ELISA) for the determination of cytokine levels. Supernatants were collected from cell cultures at different time points following exposure to various stimuli. Secreted cytokines, such as tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) in the supernatants were measured using ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). The absorbance at 405 nm was measured using a microplate reader (Bio-Rad iMark microplate reader; Bio-Rad, Hercules, CA, USA). The absorbance at 405 was converted to protein concentrations (pg/ml) using standard curves of recombinant human or mouse cytokines.

Western blot analysis. The protein expression levels of IRAK1, TRAF6 and NF-κB p65 (p65) in treated and untreated THP-1 cells were detected by western blot analysis. Briefly, the THP-1 cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology). Supernatants were collected following centrifugation at 13,000 rpm for 20 min at 4˚C, and the protein concentration was determined using the BCA Protein assay kit (Beyotime Institute of Biotechnology). Total protein from the THP-1 cells was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon-P polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes. After blocking the membranes with 5% skim milk for 60 min, they were then probed with primary rabbit anti-IRAK1 (ab67841), anti-TRAF6 (ab13853; both from Abcam, Cambridge, UK), and anti-p65 (sc-109) antibodies at a concentration of 1:200 (Santa Cruz Biotechnology, Inc., Delaware, CA, USA). After washing the membranes, they were probed with the corresponding secondary antibodies [goat anti-rabbit secondary antibody (G1210-2); PB001; Shanghai Immune Biotech Co., Ltd., Shanghai, China] before developing them using an ECL western blot detection. The protein band intensities were measured using online ImageJ software provided by the Transformation Center of Changzheng Hospital. Background intensity was subtracted from each sample and then normalized to the actin loading control (sc-47778; Santa Cruz Biotechnology, Inc.).

Statistical analysis. The majority of the experiments were performed independently at least 3 times and yielded similar results. Data are presented in figures as the means ± SD using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). For multiple group comparisons, one-way ANOVA ($p<0.05$) was performed, followed by a two-sided, unpaired Student's t-test. An unpaired two-tailed Student's t-test was used to compare 2 independent groups. Differences at a level of $p<0.05$ were considered statistically significant.

Results

Identification of miRNAs with an altered expression in monocytes during C. neoformans infection. To identify the miRNAs in human monocytes whose expression was altered during *C. neoformans* infection, we performed all miRNA expression profiling in THP-1 cells post-infection with the standardized strain, *C. neoformans*, using the Illumina sequencing miRNA expression assay. The miRNAs in the THP-1 cells at 6 h following *C. neoformans* infection were compared with miRNAs from the control cells exposed for 0 h. The time point of 6 h post-infection was selected to reflect the phases of acclimatization to the extracellular environment and induction of the members of the innate immune system. This assay revealed that the expression of miRNAs was significantly altered during *C. neoformans* infection. Among the altered miRNAs, 7 miRNAs, miR-4792, miR-30b-5p (miR-30b), miR-30c-5p, miR-223-3p, miR-15b-3p, miR-146a-5p (miR-146a) and miR-155-5p were significantly upregulated compared to the control group. To validate the miRNA expression profiling

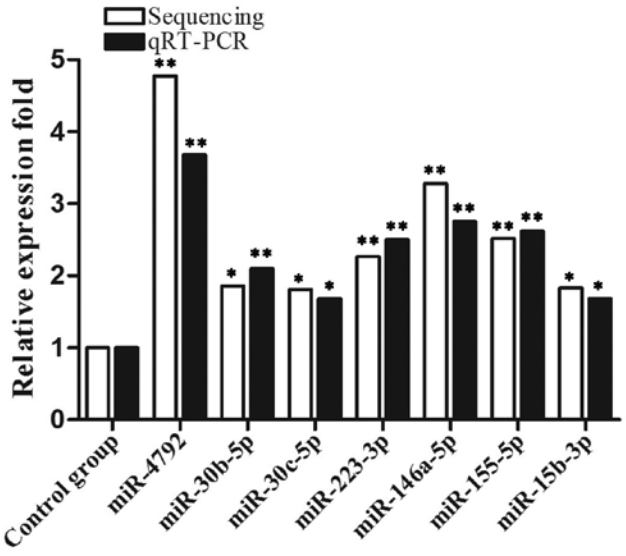

Figure 1. miRNAs altered inTHP-1 cells *in vitro* during *C. neoformans* infection. At 6 h post-infection, 7 differentially expressed miRNAs were identified. Differentially expressed miRNAs were validated by RT-qPCR. *p<0.05 and **p<0.01 compared with the control group.

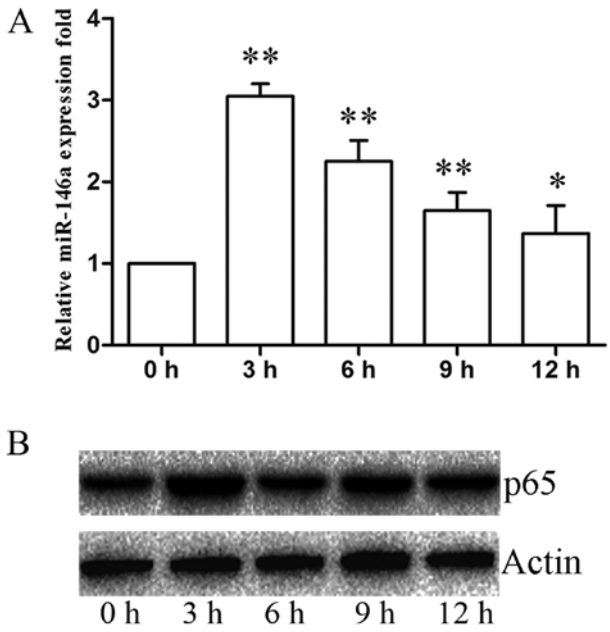

Figure 2. Expression pattern of miR-146a and the protein levels of p65 in THP-1 cells infected with *C. neoformans*. (A) Expression pattern of miR-146a was validated by RT-wPCR at the indicated time points. (B) The protein levels of p65 were determined by western blot analysis. Data are representative of at least 3 separate experiments. *p<0.05 and **p<0.01 compared with the control group.

results, we used RT-qPCR to assay the expression of several upregulated miRNAs (Fig. 1). When compared with the expression profiles established by Illumina sequencing, the RT-qPCR validation results demonstrated great congruence between the expression patterns determined using these two techniques for these miRNAs.

C. neoformans induces the expression of miR-146a and inflammatory cytokines in an NF-κB-dependent manner. The aforementioned findings together with related literature reports on the effect of miR-146a on the immune response (28), prompted us to select miR-146a for further investigation in our study. The THP-1 cells were stimulated with *C. neoformans* (MOI of 5) for 0, 3, 6, 9 and 12 h. The expression of miR-146a immediately increased in the early phase of incubation and reached peak levels at 3 h, then gradually decreased from 3 to 12 h (Fig. 2A). We first examined the levels of miR-146a during *C. neoformans* infection in the human macrophage cell line cells.

A previous study demonstrated that LPS stimulation led to NF-κB activation and an increase in miR-146a expression in THP-1 cells (30). To identify the NF-κB pathway involved in the regulation of miR-146a expression following *C. neoformans* infection, the THP-1 cells were exposed to *C. neoformans* and PDTC (NF-κB inhibitor) were used. The activation of NF-κB was determined by the presence of the p65 subunit of NF-κB in the nuclear compartment of the cells where it exerts its transcriptional activity.

The expression of miR-146a was rapidly induced by 3.5-fold at 3 h, and the induction of miR-146a then decreased with an increase of p65 expression, upon *C. neoformans* infection (Fig. 2). Although the THP-1 cells were pre-treated with the NF-κB inhibitor, PDTC, for 12 h, which cannot completely inhibit the p65 protein level (Fig. 3B, PDTC group).The *C. neoformans*-induced activation of NF-κB in the THP-1 cells was partially blocked by PDTC treatment at 3 h. However, the expression of NF-κB at 6 h was increased with the induction of *C. neoformans* (Fig. 3B, PDTC + *C. neoformans* group). Treatment with PDTC suppressed the *C. neoformans*-induced upregulation of miR-146a (Fig. 3A). The secretion of IL-1β and TNF-α into the extracellular medium was quantified by ELISA. The expression levels of IL-1β and TNF-α increased progressively in the THP-1 cells following incubation. PDTC treatment suppressed the expression levels of IL-1β and TNF-α (Fig. 3C and D). The results revealed that the miR-146a level was significantly increased by *C. neoformans* infection in the early phase, and was associated with the expression of inflammatory cytokines. These data indicated that *C. neoformans* infection increases miR-146a expression via a NF-κB-dependent mechanism.

NF-κB is negatively regulated by miR-146a in the miR-146a-NF-κB axis in THP-1 cells infected with C. neoformans. To provide additional evidence of the mechanisms of action of miR-146a as regards the regulation of the miR-146a-NF-κB axis, we examined the effects of miR-146a on NF-κB expression in THP-1 cells transfected with miR-146a mimics and miR-146a inhibitors. The efficiency of transfection is shown in Fig. 4A and B. When the THP-1 cells were transfected with miR-146a mimics and inhibitors they were allowed to recover for 6 h and subsequently infected by *C. neoformans* for 3 h. Transfection with miR-146a mimics significantly attenuated the expression of NF-κB, and transfection with miR-146a inhibitors elevated the expression of NF-κB compared to the miR-146a control (Fig. 4C, transfection group). The expression of NF-κB infected by *C. neoformans* was increased compared to the transfection group (Fig. 4C, transfection + *C. neoformans* group), which suggests that miR-146a significantly regulates the activation of NF-κB induced by *C. neoformans*. The changes

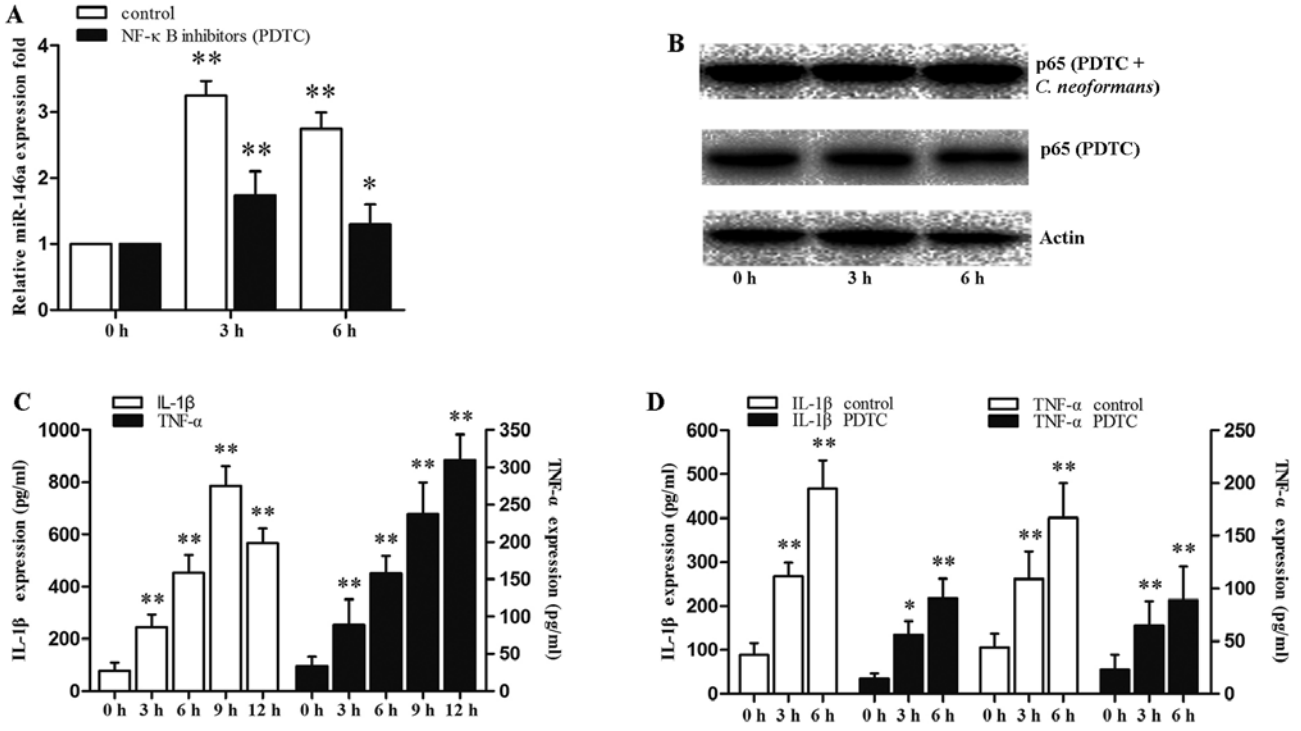

Figure 3. Nuclear factor-κB (NF-κB) protein, miR-146a expression and inflammatory cytokines secretion in THP-1 monocytes following *C. neoformans* induction. THP-1 cells were incubated with *C. neoformans* as indicated in the graphs. (A) miR-146a expression was suppressed in THP-1 cells were pre-treated with the NF-κB inhibitor, PDTC, for 3 and 6 h. (B) The protein levels of p65 were determined by western blot analysis. (D and C) Secretion of inerleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) into the extracellular medium was quantified by enzyme-linked immunosorbent assay (ELISA). All results are expressed as the means ± SD from 3 independent experiments. *p<0.05 and **p<0.01 compared with the control group.

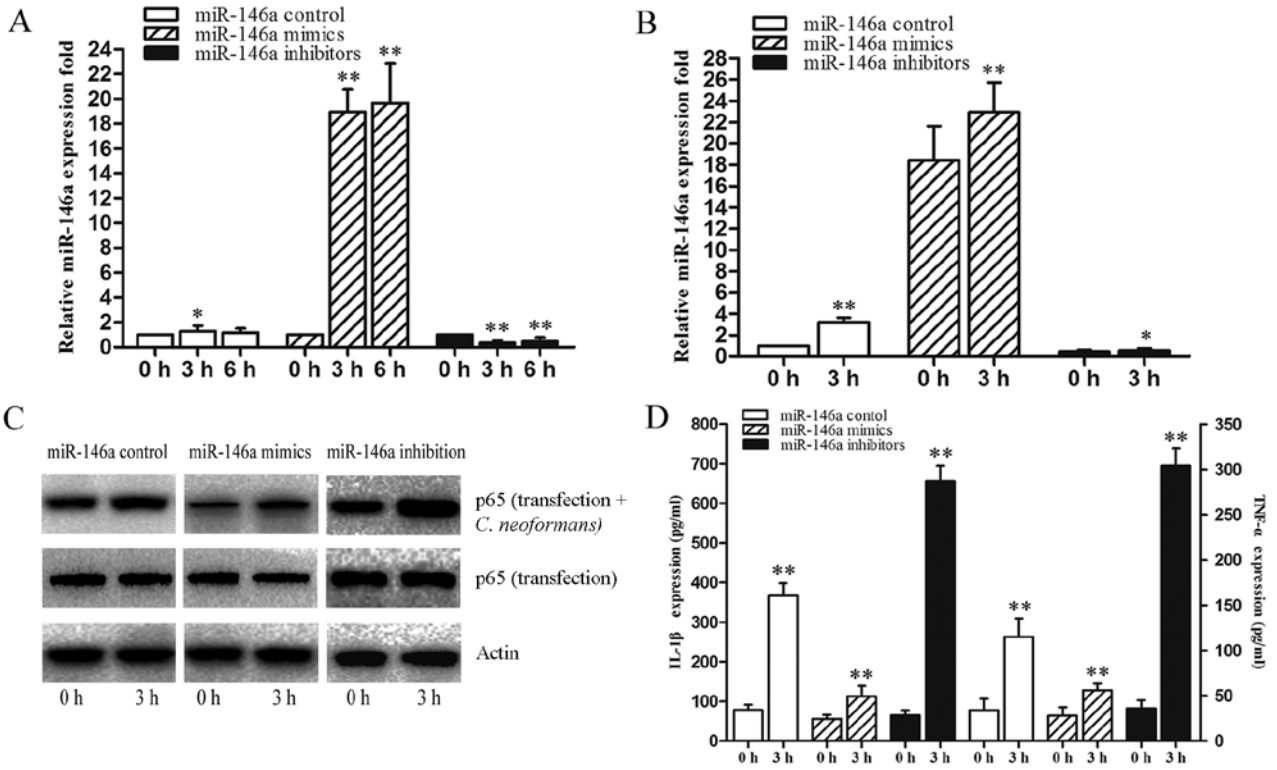

Figure 4. Regulatory effect of miR-146a on the miR-146a-nuclear factor-κB (NF-κB) axis in THP-1 cells infected with *C. neoformans*. THP-1 cells were respectively transfected with scrambled miR-control, miR-146a mimics or inhibitors for 3 or 6 h, and the transfection efficiency was measured in THP-1 cells. THP-1 cells were transfected with miR-146a mimics, inhibitors for 6 h, followed by *C. neoformans* infection. (A) Transfection efficiency was measured in THP-1 cells by RT-qPCR. (B) The expression of miR-146a was analyzed by RT-qPCR; miRNA expression was normalized to actin. (C) The protein levels of NF-κB were determined by western blot analysis following transfection of the THP-1 cells. (D) Secretion of inerleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) into the extracellular medium was quantified by enzyme-linked immunosorbent assay (ELISA). Data are representative of at least 3 separate experiments. *p<0.05 and **p<0.01 compared with the control group.

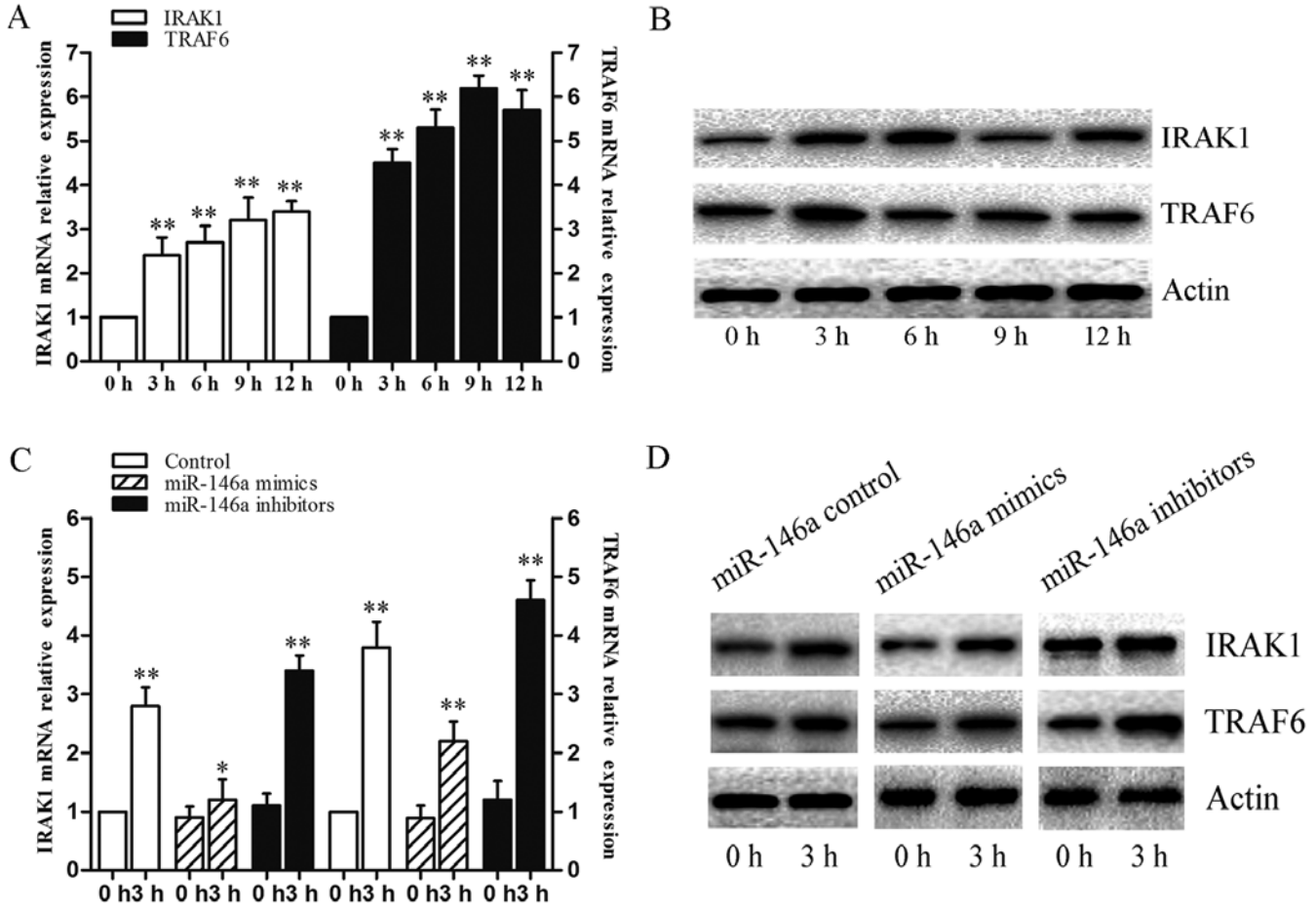

Figure 5. IRAK1 and TRAF6 are negatively regulated by miR-146a in THP-1 cells infected with *C. neoformans*. Western blot analysis of IRAK1 and TRAF6 protein levels in all groups. (A) mRNA expression of IRAK1 and TRAF6 in THP-1 cells in each group. (B) Protein levels of IRAK1 and TRAF6 inTHP-1 cells incubated with *C. neoformans* for different periods of time. (C) Effect of miR-146a mimics and inhibitors on the mRNA expression of IRAK1 and TRAF6. (D) Effect of miR-146a mimics and inhibitors on the protein levels of IRAK1 and TRAF6. Data are representative of at least 3 separate experiments. Data are the means ± SD of 3 assays. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the control group.

in the activation of NF-κB affected the release of IL-1β and TNF-α (Fig. 4D). The results revealed that NF-κB activation was regulated by miR-146a via transfection with miR-146a mimics and inhibitors. These data indicated that miR-146a exerts negative regulatory effects on the NF-κB pathway.

IRAK1 and TRAF6 are regulated by miR-146a in the miR-146a-NF-κB axis in THP-1 cells infected with C. neoformans. To further assess the function of miR-146a, it is important to determine the host mRNAs that are regulated by miR-146a. We selected two key signaling proteins, IRAK1 and TRAF6, whose 3'UTRs were previously confirmed to be complementary to miR-146a (28), as miR-146a primarily regulates these adaptor molecules via translational repression (31). To identify the function of miR-146a that results in the suppression of these target genes, we measured the mRNA and protein levels of IRAK1 and TRAF6 in THP-1 cells infected with *C. neoformans* for 0, 3, 6, 9 and 12 h. The mRNA and protein levels of IRAK1 and TRAF6 were significantly affected in these groups (Fig. 5A and B). To identify the mechanism that results in the suppression of these target genes, we measured the mRNA and protein levels of IRAK1 and TRAF6 in THP-1 cells transfected with miR-146a mimics or inhibitors. The results revealed that transfection with miR-146a mimics significantly decreased the mRNA levels of IRAK1 and TRAF6. Furthermore, the overexpression of miR-146a resulted in the downregulation of the protein levels of IRAK1 and TRAF6 (Fig. 5C and D). These data suggested that IRAK1 and TRAF6 are the main target of miR-146a on the NF-κB pathway during *C. neoformans* infection in THP-1 cells.

Discussion

Macrophages play important and indispensable roles in the anti-infection immune response. Studies have shown that macrophages are widely involved in the immune response to fungal infection (32,33). Insufficient macrophage activation may lead to the incomplete elimination of invading pathogens, but superabundant macrophage activation may result in tissue damage, inflammatory diseases, and even autoimmune disorders. Therefore, the inflammatory processes of macrophages in infection need to be accurately regulated. Over the past decade, research has focused on macrophage regulation by miRNAs and epigenetics-associated molecules (34,35).

As novel regulators of gene expression at the post-transcriptional level, miRNAs have emerged to play important roles in many biological processes ranging from cellular development and differentiation to tumorigenesis (22). miRNAs have also

been shown to be involved in innate immunity (36). During the activation of an innate immune response, the expression of some miRNAs, including miR-146a, miR-132, miR-155 and miR-125a, rapidly changes (28,37). Recent publications have indicated that miR-146a may play a key role in the innate immune response and also participates in the pathogenesis of immune diseases, such as infection, lupus and cancer (38-41).

In the present study, we observed that the expression of 7 miRNAs, miR-4792, miR-30b-5p (miR-30b), miR-30c-5p, miR-223-3p, miR-15b-3p, miR-146a-5p (miR-146a) and miR-155-5p was significantly upregulated compared to the control group (Fig. 1). We demonstrated that miR-146a expression was significantly induced by *C. neoformans* infection in THP-1 cells. Of note, in the control human monocytes, the basal expression levels of miR-146a were rapidly increased and reached a peak at 3 h, then gradually decreased from 3 to 12 h (Fig. 2A). This observation is in conflict with the expression pattern of miR-146a in THP-1 cells infected by other pathogens or bioactive components shown in another study which reported that miR-146a expression was gradually increased (42). The difference in miR-146a expression in THP-1 cells may be partially due to the differences in bacterial load or strains.

Activated signaling molecules induce the nuclear translocation of NF-κB and activator protein (AP)-1, resulting in the production of inflammatory cytokines, such as TNF-α, IL-1β and IL-6 (43,44). In a recent study, miR-146a expression induced by the activation of TLR signaling was NF-κB-dependent in human and mouse macrophages (45). The induction of miR-146a is known to be NF-κB-dependent and thus is a pivotal component of a negative feedback loop invoked by TLR signaling. The *C. neoformans*-induced expression of the miRNA, miR-146a, has been shown to be NF-κB-dependent, and the inhibition of NF-κB by PDTC, an NF-κB inhibitor, decreased miR-146a expression (34). We found that *C. neoformans* infection induced the upregulation of miR-146a in macrophages, and this enhancement occurred in an NF-κB-dependent manner (Fig. 2 and 3A and B). Furthermore, the negative regulatory effects of miR-146a on NF-κB activity in THP-1 cells infected with *C. neoformans* may be a secondary effect of the induction of inflammation (Fig. 3C and D).

We transfected macrophages with miR-146a mimic or inhibitor in the present study and found that the overexpression of miR-146a attenuated the activation of NF-κB. Moreover, miR-146a negatively regulated NF-κB expression in immunity against *C. neoformans* induction (Fig. 4B and D).

It is well known that miRNAs function by binding to the 3'UTR of target mRNAs to induce degradation or the suppression of translation. Thus far, many proteins have been identified as targets of miR-146a, including TRAF6, IRAK1, IRAK2, STAT1, TLR4 and Notch1 (41,46,47). Among these miR-146a targets, TRAF6 and IRAK1 have been demonstrated to be important molecules in the signaling pathway (48). More importantly, IRAK1 and TRAF6 are known to be part of the common signaling pathway derived from TLR-2, -4 and -5, and the IL-1β receptor, leading to speculation that increased miR-146a expression may act as a negative feedback pathway. Previously, Li *et al* (49) and Boone *et al* (50) observed LPS tolerance in monocytes caused by the impairment of IRAK1 and TRAF6 kinase activity, respectively. As discussed previously, miR-146a targets and suppresses IRAK1 and TRAF6 (51).

In this study, we also found that the overexpression of miR-146a in THP-1 cells resulted in the downregulation of the mRNA and protein levels of IRAK1 and TRAF6, which, in contrast, subsequently decreased NF-κB activity (Fig. 5). This suggests that miR-146a may exert a negative regulatory effect on NF-κB signaling via the negative regulation of IRAK1 and TRAF6, which play a critical role in immunity against *C. neoformans*.

In conclusion, we found that miRNAs were integrally involved in the inflammatory reaction in monocytes infected by *C. neoformans*. miR-146a was upregulated by the NF-κB pathway, whereas miR-146a negatively regulated NF-κB activation by targeting IRAK1 and TRAF6, and then inhibiting the activation of NF-κB and the release of inflammatory cytokines in monocytes, which helps to fine-tune the immune response. Taken together, our results suggest that miR-146a may represent a future therapeutic target for regulating the inflammatory response of the host innate immune response to *C. neoformans* infection.

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