Original Article

Upregulated *miR-155* inhibits inflammatory response induced by *C. albicans* in human monocytes derived dendritic cells via targeting p65 and BCL-10

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Background: *Candida albicans* (*C. albicans*) is one of the most common fungal pathogens causing superficial and systemic infections. The innate immune system is the first defense line against *C. albicans* infection. *MiR-155*, a multifunctional microRNA (miRNA), has been proved to be a crucial regulator in innate immune response against bacterial and virus. However, the biological function of *miR-155* in innate immune response against *C. albicans* infection remains unknown.

Methods: The expression *miR-155*, as well as inflammatory factors [interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)], in monocytes derived dendritic cells (DCs) during heat-killed *C. albicans* infection was detected by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). The biological functions of *miR-155* were investigated with "gain- and loss-of-function" experiments. Potential targets of *miR-155* were identified by bioinformatics analysis, luciferase assay and western blot. Small interfering RNA (siRNA) was used to validate the function of *miR-155* target.

Results: *C. albicans* increased the expression of *miR-155* and pro-inflammatory factors. *MiR-155* induced by *C. albicans* was depended on Dectin-1-spleen tyrosine kinase (Syk)/Raf-1-MAPK signaling pathway. Furthermore, *miR-155* suppressed the secretion of pro-inflammatory cytokines induced by *C. albicans* by targeting NF-κB p65 and B cell leukemia/lymphoma 10 (BCL-10).

Conclusions: In conclusion, up-regulated *miR-155* acts as a negative feedback regulator in the innate immune response against *C. albicans* infection.

Keywords: Dendritic cells (DCs); *Candida albicans* (*C. albicans*); *miR-155*; innate immune response

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Introduction

Candida albicans (*C. albicans*) is an opportunistic fungal pathogen commonly colonizing on the surface of skin and mucosa (oral cavity, intestinal tract and vagina). It rarely causes infection in immunocompetent individuals (1); however, in immunocompromised individuals, *C. albicans* is one of the most prevalent fungal pathogen. It causes various types of candidiasis, ranging from superficial

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infection to invasive systemic infection (2). In recent years, with the increasing number of immunocompromised patients [e.g., cancer therapy, transplantation and the human immunodeficiency virus (HIV) epidemics], the incidence of *C. albicans* infections has considerably increased (3). In addition, physical alterations of the anatomical barrier, such as surgery, indwelling medical devices or antibiotic treatment, facilitate the access of fungi to the bloodstream and lead to increased incidence of disseminated candidiasis (4). Noteworthy, the nosocomial bloodstream infection in immunosuppressive patients is life-threatening, with a mortality rate as high as 40% (5).

The innate immune response is the first defense line against *C. albicans* infection (6). It is initiated by the recognition of the conserved molecular components of *C. albicans*, termed as pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs depends on the pattern recognition receptors (PRRs) on innate immune cells (7). Dendritic cells (DCs), a kind of classical innate immune cells, plays an important role in anti-*C. albicans* immune response. With PRRs expressed on its surface, DCs senses the PAMPs of *C. albicans* and initiates the innate immune response rapidly. Many PRRs are involved in the interaction of *C. albicans*, such as toll-like receptors (TLRs), C-type lectin receptors (CLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (8). Dectin-1, a member of CLRs, can sense the β-glucan on the surface of *C. albicans*. The interaction between Dectin-1 and β-glucan triggers two intracellular signaling pathways named spleen tyrosine kinase (Syk)-dependent pathway and Raf-1 dependent pathway. The activation of these two pathways induces the immune response against *C. albicans*, which is characterized by the release of several types of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) to control *C. albicans* infection (9,10).

MicroRNA (miRNA) is a type of short non-coding RNA that regulates multiple target genes expression at post-transcriptional level (11). It is widely involved in the regulation of immune cell development and activation (12,13). *MiR-155* is an active immune regulator in innate immune response with dose-dependent effects (14,15). Moderate elevated *miR-155* expression is critical for pro-inflammatory response; however, extremely high level of *miR-155* starts to gradually terminate the response, due to its ability to target different transcripts (15). Previous studies have shown that *miR-155* was upregulated by heat-killed *C. albicans* in murine bone marrow-derived macrophages (BMDMs) (16) and human DCs (17). However, the biological function of *miR-155* in human monocytes derived DCs treated with *C. albicans* remains unknown. Hence, the aim of the present study was to investigate the biological function and underlying mechanisms of *miR-155* in *C. albicans* activated DCs.

Methods

Candida albicans (C. albicans)

C. albicans (SC5314) strain was grown in Sabouraud dextrose (SBD) agar plates at 37 ℃. For cells preparation, *C. albicans* cultures were incubated in Sabouraud broth at 37 ℃ overnight with shaking. Cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and heat-killed for 1 hour at 100 ℃ (18). *C. albicans* were counted and adjusted to the proper concentration before using.

Cell lines and culture

Healthy individuals' peripheral blood mononuclear cells (PBMCs) were isolated using density centrifugation with Ficoll (GE Healthcare, Sweden). $CD14^+$ monocyte subsets were positively selected using anti-CD14 MACS microbeads from freshly isolated PBMCs, according to the instructions of the manufacturer (Miltenyi Biotec, Germany). Purified monocytes were cultured at 37 ℃ in 6-well plates with complete RPMI 1640 medium at the concentration of 10^6/mL. The cells were induced into immature DCs with 50 ng/mL of human granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, USA) and 50 ng/mL human IL-4 (R&D Systems, USA) for 6 days and the culture was replaced at the third and fifth day of induction (19). This study was approved by the Ethic Board of Changzheng Hospital and written approvals were obtained from the subjects.

THP-1 cells, 293T cells and murine RAW264.7 cells were obtained from the Shanghai Institutes for Biological Sciences. All cells were cultured in either RPMI 1640 medium or DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/mL penicillin-streptomycin (Mediatech, USA).

Immature DCs were treated with heat-killed *C. albicans* at a ratio of 1:1 (20) or 100 ng/mL lipopolysaccharide (LPS, Sigma, USA). For inhibiting signaling pathways, DCs were pretreated with following inhibitors for 1 hour: Dectin-1

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inhibitor laminarin 100 μg/mL, Syk inhibitor R406 5 μM, Raf-1 inhibitor GW5074 10 μM, MEK/ERK inhibitor U0126 10 μM, JNK inhibitor SP600125 10 μM and p38 kinase inhibitor SB203580 1 μM. All these inhibitors, except laminarin, were purchased from MedChem Express (USA), while laminarin was purchased from Sigma-Aldrich (USA).

Transient transfection

Small interfering RNAs (siRNAs) for Dectin-1, NF-κB p65 and B cell leukemia/lymphoma 10 (BCL-10) were ordered from RiboBio Company (Guangzhou, China). The sequences of siRNAs were presented in *Table S1*. *MiR-155* mimics and mimic control, *miR-155* inhibitor and inhibitor control were purchased from RiboBio Company, and used at the concentration of 50 and 100 nM, respectively. SiRNAs (100 nM) were transfected into DCs using ribo*FECT*™ CP reagent from RiboBio according to the manufacturer's instructions. Twenty-four or forty-eight hours after transfection, cells and the supernatants were harvested for the subsequent experiments. The transfected miRNA negative control (NC) tagged by red fluorescence was imaged by fluorescence microscopy.

RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA, including miRNA, was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was assessed by NanoDrop 2000 (Wilmington, DE, USA). Total RNA was reverse-transcribed to complementary DNA (cDNA) with PrimeScript™ RT Master Mix (Takara, Japan). qRT-PCR for target genes was performed on the Applied Biosystems 7500 Sequence (Applied Biosystems, USA). The primers were presented in *Table S2*. The primers for *miR-155* and U6 small nuclear RNA were purchased from RiboBio Company (Guangzhou, China). Relative expression of messenger RNA (mRNA) and miRNA was calculated using the $2^{-\Delta\Delta CT}$ method (21).

Western blot

After stimulation and transfection, cells were harvested and centrifuged. Protein was isolated by RIPA Lysis Buffer containing protease inhibitor cocktail and phosphatase inhibitor. Protein concentration was quantified using BCA protein assay kit (Takara, Japan). Equal amounts of protein were separated by 10% SDS-PAGE (Beyotime Biotechnology) and transferred onto PVDF membranes (Millipore, USA). After blocking, the membranes were sequentially incubated with specific primary antibodies for 2 hours at room temperature. Antibodies against phospho-ERK (ab201015), total-ERK (ab184699), phospho-JNK (ab124956), total-JNK (ab179461) and BCL-10 (ab108328) were purchased from Abcam (USA); while phospho-p65 (3033S), Dectin-1 (60128S), p38 (9212S), phospho-p38 (4511S), c-Jun (9165S) and phospho-c-Jun (3270S) were from Cell Signaling Technology (USA); and total p65 [5006] was from Affinity Bioscience (USA). Immune-complexes were incubated with fluorescein-conjugated secondary antibodies and then detected using an Odyssey fluorescence scanner (Li-Cor, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of cytokines in cell culture supernatants were measured using specific commercial ELISA kits (Neobioscience, China) according to the manufacturer's instructions. All experiments were performed in triplicate.

Luciferase reporter assay

Luciferase reporter plasmids p65-3'UTR-Wt, p65-3'UTR-Mt, BCL-10-3'UTR-Wt, BCL-10-3'UTR-Mt were constructed from Obio Technology. Then these plasmids (0.2 μg) were co-transfected into 293T cells (2×10^5/mL) with a renilla control plasmid (0.01 μg) and *miR-155* mimics (100 nM), NC (100 nM) using 0.2 μL Lipo2000 transfection reagent (Invitrogen, USA) according to the manufacture's instruction. Medium contains transfection reagents was replaced with fresh and complete DMEM medium 24 hours after transfection. Reporter luciferase activities were measured using the Dual-Luciferase kit (E1910, Promega) according to the manufacture's instruction 48 hours later.

Statistical analysis

All experiments were performed in triplicate. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as mean and standard deviation (SD) from at least three independent experiments. Student's *t*-test was used to compare the mean values between two groups. Multiple comparisons were performed using one-way analysis of variance (ANOVA). A

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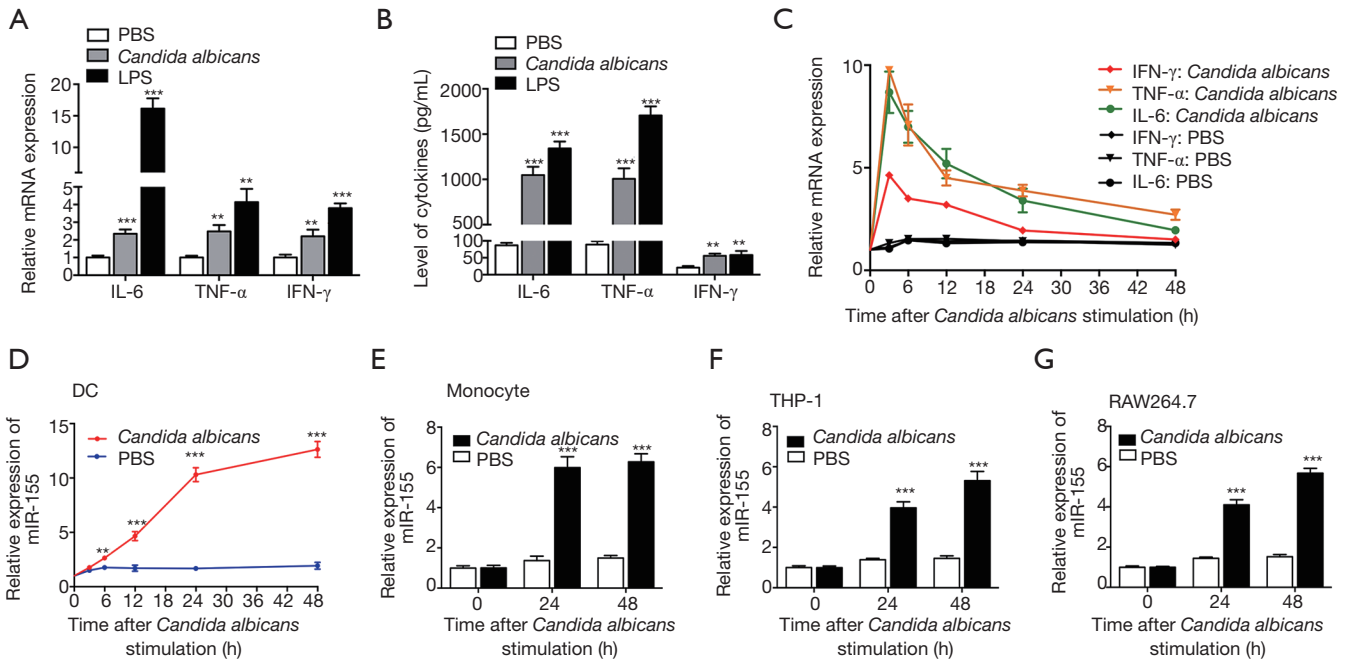

Figure 1 Heat-killed *C. albicans* promoted the expression of pro-inflammatory factors and *miR-155* in DCs. (A) Human monocytes derived DCs were treated with PBS (NC), heat-killed *C. albicans* (at a ratio of 1:1) or 100 ng/mL LPS (positive control) for 24 hours, and the intracellular IL-6, TNF-α and IFN-γ mRNAs were determined using qRT-PCR. β-actin was used as internal control; (B) 24 hours after stimulation, the concentrations of IL-6, TNF-α and IFN-γ in culture media were analyzed using ELISA; (C) time dependent expression of IL-6, TNF-α and IFN-γ mRNAs in DCs treated with heat-killed *C. albicans* was detected using qRT-PCR; (D) time dependent expression of *miR-155* in DCs treated with heat-killed *C. albicans* was detected using qRT-PCR, compared with control PBS. U6 was used as the internal control for *miR-155* determination; the expression of *miR-155* in (E) monocytes, (F) THP-1 cells and (G) RAW264.7 cells treated with heat-killed *C. albicans* for 24 and 48 hours was detected using qRT-PCR. Values are means ± SD from three independent experiments performed in triplicate. **, P<0.01; ***, P<0.001. *C. albicans*, Candida albicans; DCs, dendritic cells; PBS, phosphate-buffered saline; NC, negative control; LPS, lipopolysaccharide; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; mRNA, messenger RNA; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; SD, standard deviation.

P value <0.05 was considered statistically significant.

Results

Heat-killed C. albicans promoted the expression of pro-inflammatory factors and miR-155 in DCs

Heat-killed *C. albicans* significantly increased the expression of intracellular IL-6, TNF-α and interferon-γ (IFN-γ) mRNA as well as the concentration of these cytokines in culture media after 24 hours' stimulation (*Figure 1A,B*). We further detected the dynamic changes of these cytokine's mRNA with PCR, then found that their expressions were upregulated up to 3 hours after *C. albicans* stimulation and then decreased (*Figure 1C*). Meanwhile, *miR-155* was significantly upregulated in DCs by heat-killed *C. albicans* in time dependent manner (*Figure 1D*). In addition, a significant increase of *miR-155* expression was detected in human monocytes (*Figure 1E*), THP-1 cells (*Figure 1F*) and murine RAW264.7 cells (*Figure 1G*) in response to *C. albicans* for 24 and 48 hours.

Increased miR-155 expression in DCs induced by C. albicans was mediated via Dectin-1-Syk/Raf-1-MAPK pathway

To explore whether the upregulation of *miR-155* was relevant to Dectin-1, DCs were treated with Dectin-1 blocker laminarin for 1 hour before heat-killed *C. albicans*

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stimulation. As shown in *Figure 2A*, the upregulation of *miR-155* was significantly impaired by laminarin. Further, we specifically suppressed the expression of Dectin-1 in DCs using siRNA. Dectin-1 expression was significantly decreased by siRNA at mRNA and protein level (*Figure S1A,B*). The upregulation of *miR-155* induced by *C. albicans* was significantly impaired by siRNA of Dectin-1 (*Figure 2B*). A previous study has indicated that the interaction of *C. albicans* and Dectin-1 could further activate Syk-dependent signaling pathway and Raf-1-dependent pathway (22). To explore whether these two pathways were involved in the upregulation of *miR-155* upon Dectin-1 stimulation, DCs were pretreated with a specific inhibitor for Syk (R406) and Raf-1 (GW5074), respectively. As shown in *Figure 2C*, both R406 and GW5074 significantly attenuated the upregulation of *miR-155* induced by *C. albicans*. These results demonstrate that the increased *miR-155* expression in DCs induced by *C. albicans* is partially mediated via Dectin-1-Syk/Raf-1 pathway.

Next, we tested whether the induction of *miR-155* by *C. albicans* was dependent on MAPK pathway using inhibitors. First, we found that both ERK and JNK, but not p38, in MAPK pathway were significantly phosphorylated in response to *C. albicans* (*Figure 2D*). We used the U0126, SP600125 and SB203580 to in inhibit the phosphorylation of MEK/ERK, JNK and p38, respectively (*Figure S1C*). The MEK/ERK inhibitor U0126 and the JNK inhibitor SP600125 significantly suppressed the upregulation of *miR-155* by *C. albicans* stimulation, whereas the p38 inhibitor SB203580 had little influence on the expression of *miR-155* (*Figure 2E*). When both ERK and JNK were inhibited, the expression of *miR-155* was further declined (*Figure 2E*). These results imply that MAPK pathway is involved in the induction of *miR-155* upon *C. albicans* stimulation.

MiR-155 suppressed the upregulation of pro-inflammatory cytokines in DCs induced by heat-killed C. albicans

To investigate the function of *miR-155* on the production of inflammatory cytokines, synthetic *miR-155* mimics or scrambled control oligonucleotides were transfected into DCs. Transfection efficiency was detected by fluorescence microscope (*Figure S2A*) and qRT-PCR (*Figure S2B*) after 24 hours. Overexpression of *miR-155* significantly suppressed *C. albicans*-induced expression and secretion of IL-6, TNF-α and IFN-γ (*Figure 3A,B*); whereas inhibition of endogenous *miR-155* increased the expression and concentration of IL-6, TNF-α and IFN-γ induced by *C. albicans* (*Figure 3C,D*). These results indicate that the upregulation of *miR-155* has a negative effect on the production of pro-inflammatory cytokines in heat-killed *C. albicans* treatment DCs.

MiR-155 downregulated the expression of IL-6, TNF-α and IFN-γ in DCs against C. albicans through targeting NF-κB p65 and BCL-10

To identify underlying mechanisms of *miR-155* in inflammatory response induced by *C. albicans*, three types of bioinformatic prediction software, named Targetscan, miRanda and miRNA.org, were used to predict the potential targets of *miR-155*. Bioinformatics analysis showed that 3'UTR of NF-κB p65 contained *miR-155* binding sites at 197–204 nucleotides (*Figure 4A*). Luciferase activity was significantly reduced when co-transfected with wild type of p65 and *miR-155* (*Figure 4A*), indicating that *miR-155* can bind to the 3'UTR of p65 mRNA directly. Then, upon *C. albicans* stimulation, *miR-155* mimics reduced the protein level of p65, while *miR-155* inhibitor had an opposite effect (*Figure 4B*). The dynamic changes of p65 in *C. albicans* treated DCs were also investigated. We found that the expression of p65 was upregulated within 6 hours after *C. albicans* stimulation but then decreased (*Figure S3A*). Furthermore, we used siRNA to knock down the expression of p65 and determined the production of IL-6, TNF-α and IFN-γ induced by *C. albicans*. We found that p65 siRNA significantly inhibit the expression of p65 at both mRNA and protein level (*Figure S3B,C*). The production of IL-6, TNF-α and IFN-γ was impaired by p65 siRNA in *C. albicans* treated DCs, at both mRNA (*Figure 4C*) and protein levels (*Figure 4D*). These results indicate *miR-155* inhibits the production of IL-6, TNF-α and IFN-γ in *C. albicans* treated DCs partially by targeting p65.

We found that the 3'UTR of BCL-10 contained *miR-155* binding sites at 405–412 nucleotides (*Figure 4E*). Then luciferase activity was significantly reduced when co-transfected with BCL-10 wild type and *miR-155* (*Figure 4E*). Upon *C. albicans* stimulation, protein level of BCL-10 was reduced after transfection with *miR-155* mimics but increased with *miR-155* inhibitor (*Figure 4F*). We also determined the dynamic changes of BCL-10 in *C. albicans* treated DCs. Similar to p65, the expression of BCL-10 increased up to 6 hours after *C. albicans* stimulation but decreased then (*Figure S3D*). We used siRNA to knock down the expression of BCL-10 and found

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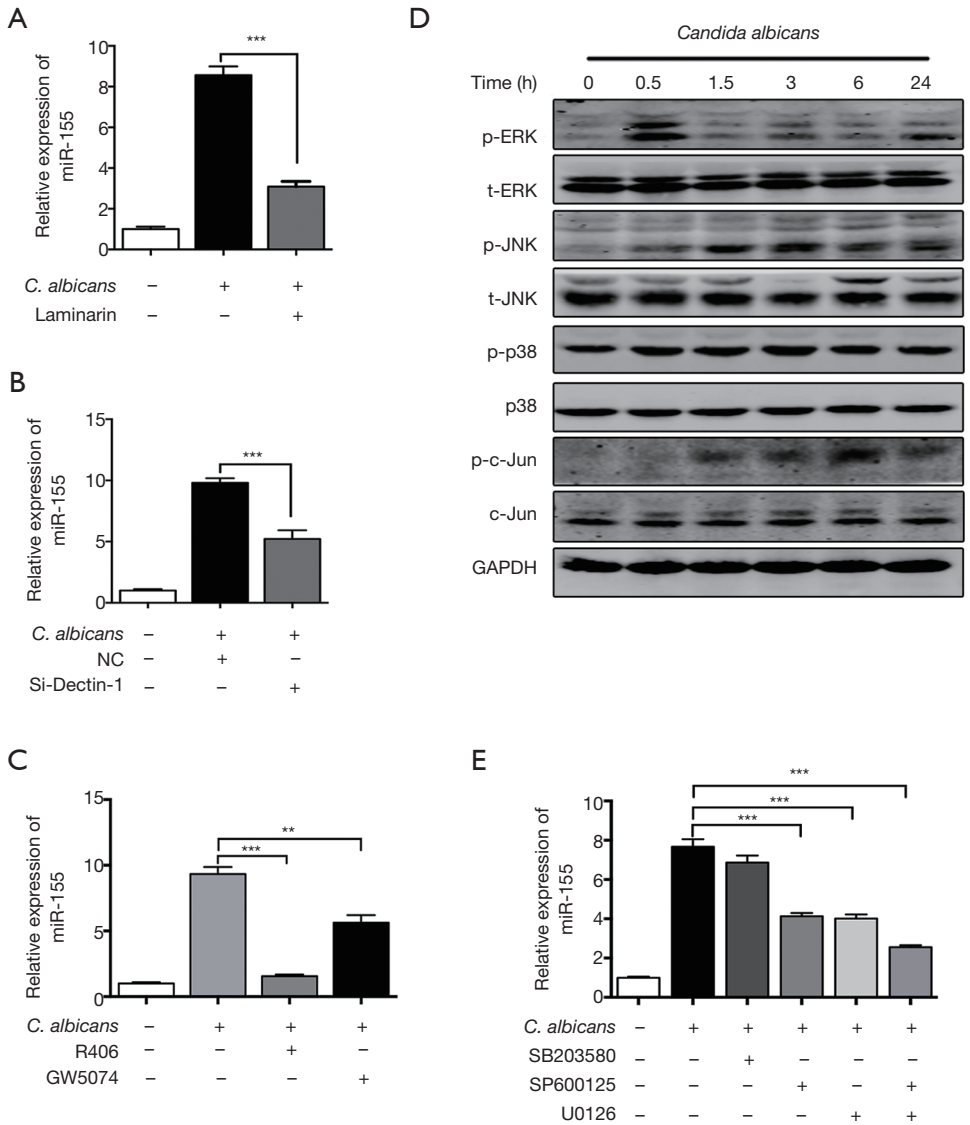

Figure 2 Increased *miR-155* expression in DCs induced by *C. albicans* was mediated via Dectin-1-Syk/Raf-1-MAPK pathway. (A) DCs were pretreated with DMSO or 100 μg/mL laminarin (Dectin-1 blocker) for 1 hour before *C. albicans* stimulation. Twenty-four hours later, *miR-155* expression normalized to U6 was determined using qRT-PCR; (B) DCs were transfected with 100 nM Dectin-1 siRNA (si-Dectin-1) or 100 nM NC. Twenty-four hours later, cells were exposed to *C. albicans* for 24 hours and the expression of *miR-155* was determined using qRT-PCR; (C) DCs were pretreated with DMSO (control), 5 μM R406 (Syk inhibitor) or 10 μM GW5074 (Raf-1 inhibitor) for 1 hour and then stimulated with *C. albicans* for 24 hours. *MiR-155* expression was determined by qRT-PCR normalized to U6 expression; (D) protein levels of p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-c-Jun and c-Jun in DCs treated with *C. albicans* for 0, 0.5, 1.5, 3, 6, 24 hours was detected by western blot; (E) DCs were pretreated with DMSO, 1 μM SB203580 (p38 inhibitor), 10 μM SP600125 (JNK inhibitor), or 10 μM U0126 (MEK/ERK inhibitor) for 1 hour and then stimulated with *C. albicans* for 24 hours. *MiR-155* expression was determined by qRT-PCR normalized to U6 expression. Values are means ± SD from three independent experiments performed in triplicate. **, P<0.01; ***, P<0.001. DCs, dendritic cells; *C. albicans*, Candida albicans; Syk, spleen tyrosine kinase; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; NC, negative control; SD, standard deviation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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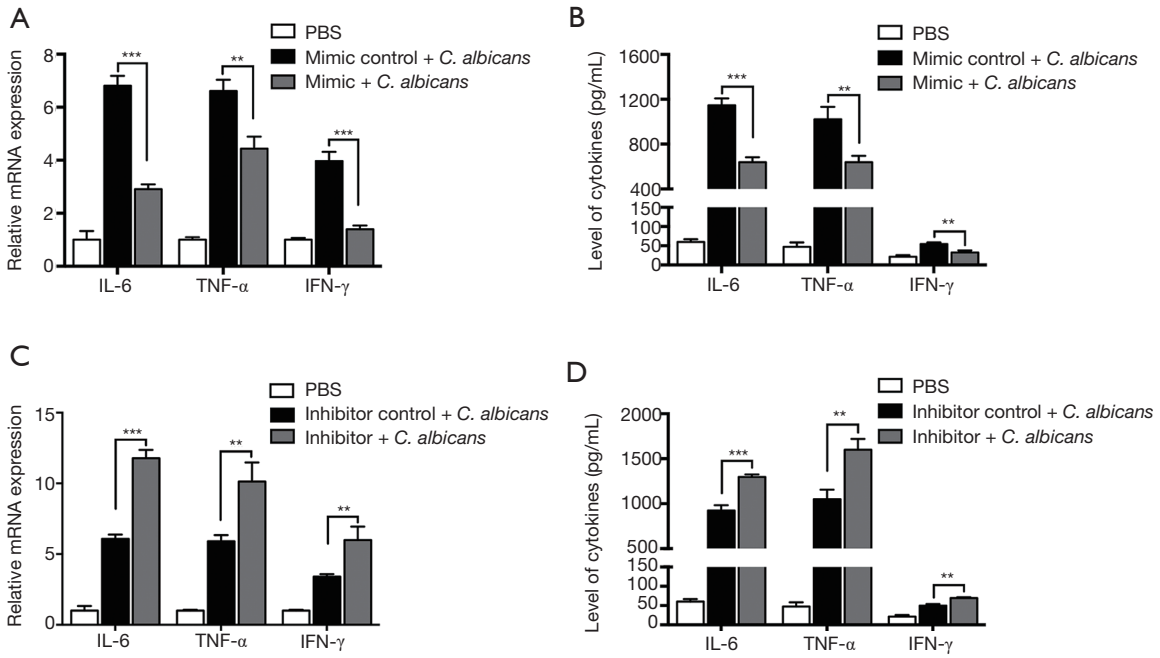

Figure 3 *MiR-155* suppressed the upregulation of pro-inflammatory cytokines in DCs induced by heat-killed *C. albicans*. DCs were transfected with (A,B) *miR-155* mimics (50 nM) or mimic control (50 nM), (C,D) *miR-155* inhibitor (100 nM) or inhibitor control (100 nM); after 24 hours, cells were exposed to *C. albicans* and the expression of IL-6, TNF-α and IFN-γ after 6 hours of stimulation was detected using qRT-PCR (A,C) and their concentration in culture media after 24 hours was measured using ELISA (B,D). Values are expressed as means ± SD from three independent experiments performed in triplicate. **, P<0.01; ***, P<0.001. *C. albicans*, Candida albicans; DCs, dendritic cells; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; PBS, phosphate-buffered saline; mRNA, messenger RNA.

that its expression was inhibited by siRNA (*Figure S3E,F*). Notably, siRNA of BCL-10 decreased the phosphorylation of p65 (*Figure 4G*). The levels of IL-6, TNF-α and IFN-γ induced by *C. albicans* were repressed significantly by siRNA of BCL-10 (*Figure 4H,I*). Taken together, these results indicate that *miR-155* inhibits phosphorylation of p65 and inflammatory cytokines secretion by targeting BCL-10.

Discussion

In this study, we found that heat-killed *C. albicans* could upregulate the expression of *miR-155* and inflammatory cytokines in DCs. Up-regulated *miR-155* negatively regulated the production of inflammatory cytokines by targeting p65 and BCL-10. These results indicate that *miR-155* plays a crucial role in negatively regulating the inflammation response against *C. albicans* in DCs.

Some previous studies have detected the miRNA profile of *C. albicans* treated cells. Using miRNA array and qRT-PCR, Monk *et al.* (16) reported that the expression of *miR-155* was increased in murine BMDMs stimulated with *C. albicans*, but the function of elevated *miR-155* was not illustrated. Similar to the study performed by Dix *et al.* (17), we observed the upregulation of *miR-155* in human DCs treated with *C. albicans*. Besides, we found that heat-killed *C. albicans* could also increase *miR-155* in human monocytes, THP-1 cells and RAW 264.7 cells. Interestingly, previous studies reported that *miR-155* could also be elevated by viral infection or LPS in monocytes, macrophages and DCs (23-28). Taken together, these findings revealed that *miR-155* may be upregulated in innate immune cells by various pathogens.

Dectin-1 was confirmed to bind β-glucans on *C. albicans* wall, playing an important role in the phagocytosis of fungi (29) and activation of immune response (30). In our study, the expression of *miR-155* was attenuated by Dectin-1 inhibitor laminarin and siRNA of Dectin-1, indicating that the induction of *miR-155* by *C. albicans* is partially through the activation of Dectin-1. A previous study has shown that the increased *miR-155* in murine macrophages exposure to

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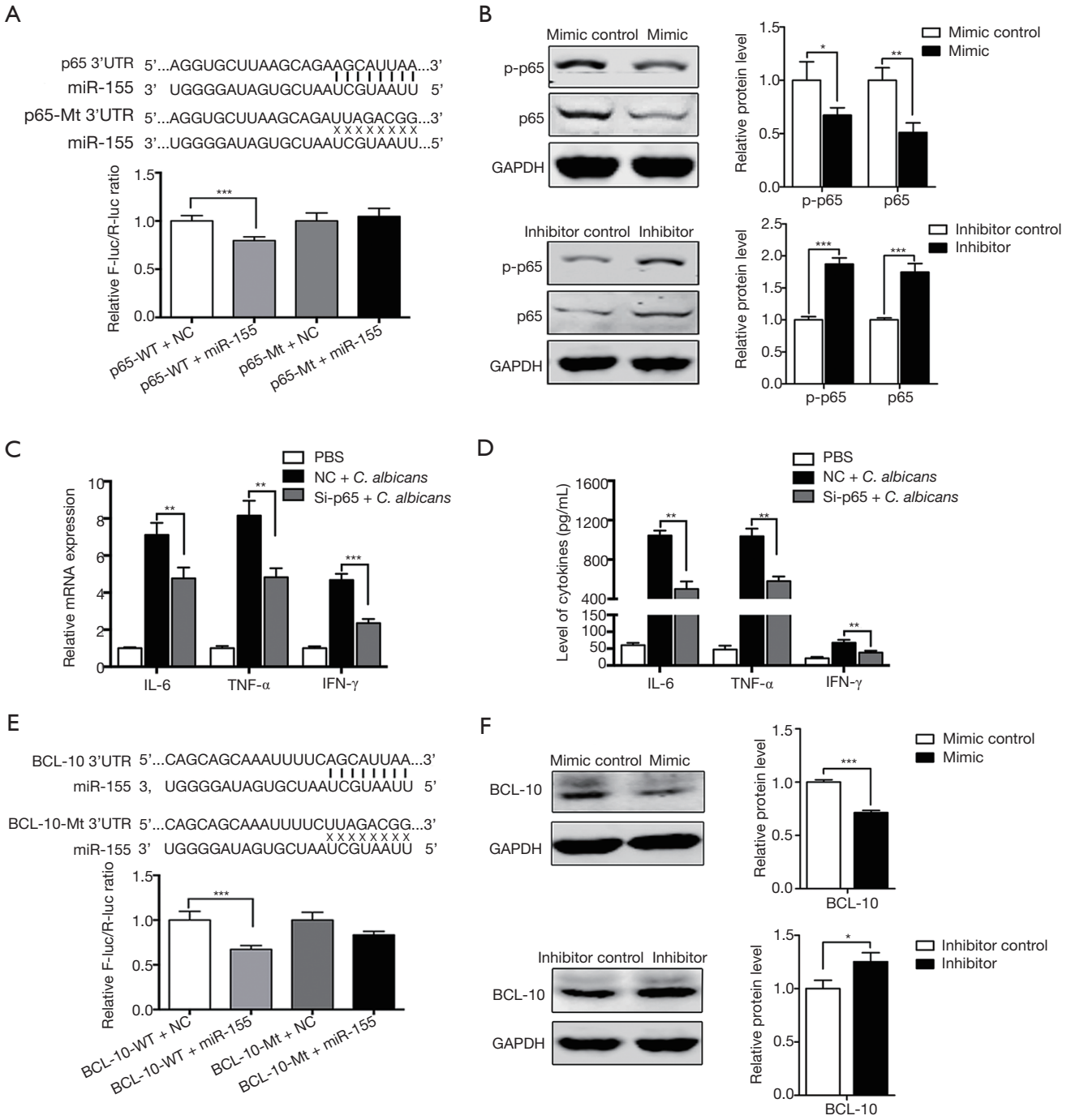

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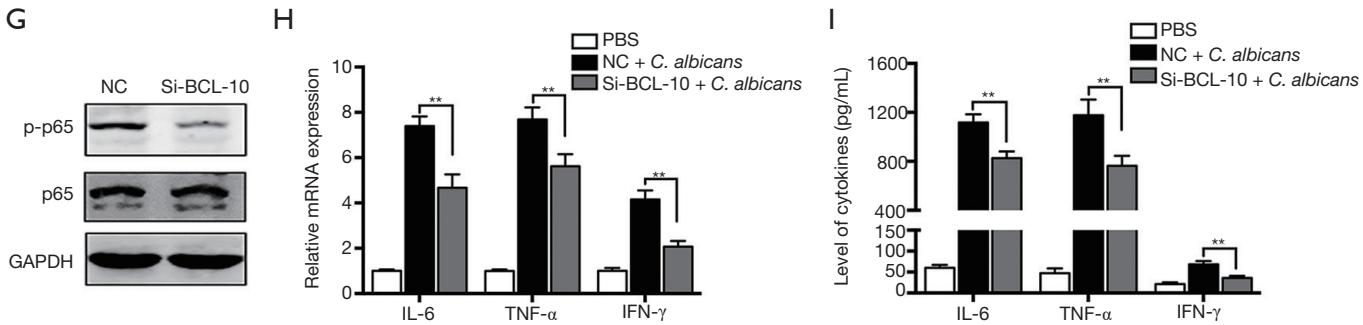

Figure 4 *MiR-155* regulated inflammatory response of DCs against *C. albicans* through targeting NF-κB p65 and BCL-10. (A) The sequence of *miR-155* and its potential matching sites in p65 3'UTR. Dual luciferase reporter assay was performed on 293T cells to determine targeting of p65 3'UTR by *miR-155*; (B) DCs were transfected with *miR-155* mimics, mimic control, *miR-155* inhibitor or inhibitor control for 48 hours. Protein levels of p-p65 and p65 in DCs exposed to *C. albicans* for 3 hours (mimics, mimic control) or 12 hours (inhibitor, inhibitor control) were detected using western blot. The levels of protein are normalized to GAPDH; (C,D) DCs were transfected with 100 nM siRNA of p65 or NC. After 24 hours, cells were exposed to *C. albicans* and the levels of IL-6, TNF-α and IFN-γ were detected 6 hours later using qRT-PCR and 24 hours later using ELISA; (E) the sequence of *miR-155* and its potential matching sites in BCL-10 3'UTR. Dual luciferase reporter assay was performed on 293T cells to determine targeting of BCL-10 3'UTR by *miR-155*; (F) DCs were transfected with *miR-155* mimics, mimic control, *miR-155* inhibitor or inhibitor control for 48 hours. Protein levels of BCL-10 were detected using western blot after the cell were treated with *C. albicans* for 3 (mimics, mimic control) or 12 hours (inhibitor, inhibitor control). The levels of protein are normalized to GAPDH; (G) DCs were transfected with 100 nM siRNA of BCL-10 or NC. After 24 hours, cells were exposed to *C. albicans* and the levels of p-p65 were detected 3 hours later using western blot; (H,I) after 24 hours' transfection with siRNA of BCL-10 or NC, cells were exposed to *C. albicans* and the levels of IL-6, TNF-α and IFN-γ were detected 6 hours later using qRT-PCR and 24 hours later using ELISA. Values are means ± SD from three independent experiments performed in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001. DCs, dendritic cells; *C. albicans*, Candida albicans; BCL-10, B cell leukemia/lymphoma 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; NC, negative control; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; mRNA, messenger RNA.

UV-killed *C. albicans* hyphae was associated with Dectin-1-Syk pathway, while Raf-1 pathway had no effect on *miR-155* expression (31). In contrast, using specific inhibitors for each pathway, our study demonstrates that both of Dectin-1-Syk pathway and Dectin-1-Raf-1 pathway are involved in the induction of *miR-155*. The inconsistence between our work and previous study might be attributed to the different strains of *C. albicans*, inactivated methods and cell lines used in experiments.

MAPK signal pathways are known to be triggered by inflammatory response induced by Dectin-1 (32,33). Notably, MAPK pathways were reported to modulate *miR-155* expression in murine macrophages by poly-I:C or TNF-α stimulation (34). In addition, the promoter regions of *miR-155* primary transcript, B-cell integration cluster (BIC) contain binding sites for activator protein-1 (AP-1) (35). In this study, ERK and JNK pathway were significantly phosphorylated when exposure to *C. albicans*. Inhibition of ERK or JNK significantly attenuated the enhanced expression of *miR-155* stimulated by *C. albicans*, whereas p38 inhibitor had no effect on *miR-155* accumulation. These might be attributed to that inhibition of ERK or JNK pathway could suppress the phosphorylation of c-Jun (AP-1 key component), influencing the transcription of *miR-155*. Since cross-talk is existed among MAPK pathways, inhibition of one pathway could activate another (36). Our study found that both ERK and JNK were inhibited, the expression of miR-155 was further declined. Previous studies have reported that *miR-155* could be induced by inflammatory cytokines, such as TNF-α or IFN-β (34,37,38). Therefore, we hypothesize that there might be two possible mechanisms responsible for the increased expression of *miR-155*. One is the direct activation of Dectin-1 and its downstream intracellular signaling pathways by *C. albicans*; another is the effect of inflammatory cytokines released by innate immune cells.

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As *miR-155* has been reported to be involved in regulating the strength and duration of innate immune response (14,39), whether *miR-155* could regulate the strength of innate immune response triggered by *C. albicans* in DCs remained unknown. We found that *miR-155* suppressed the production of inflammatory cytokines induced by *C. albicans*, including IL-6, TNF-α and IFN-γ, indicating that *miR-155* plays as a negative regulator in inflammation to *C. albicans*. To the best of our knowledge, this is the first study reporting that *miR-155* negatively regulate the releasing of IFN-γ in DCs. Then we identified the underlying mechanisms of *miR-155* in *C. albicans* induced inflammatory response. Previous studies have indicated that p65, a subunit of NF-κB, was the target of *miR-155* in endothelial inflammation (40) and gastric cancer (39,41). Therefore, we hypothesize that *miR-155* impairs the upregulation of inflammatory cytokines via targeting p65. Using reporter assay and western blot, we found that *miR-155* repressed the expression of p65 by direct targeting its 3'UTR. Furthermore, knockdown of p65 reproduced the effect of *miR-155* mimics. These results indicate that *miR-155* negatively regulates inflammatory cytokines producing via targeting p65.

Previous studies have indicated that BCL-10 is involved in the immune response against *C. albicans*. After the Dectin-1 activation by *C. albicans* and phosphorylation by Syk, caspase recruitment domain-containing protein 9 (CARD9) forms a complex with BCL-10 and mucosa-associated lymphoid tissue 1 (MALT1), mediating NF-κB activation and initiating the expression of several type of inflammatory cytokines (42). Furthermore, bioinformatics analysis indicated that BCL-10 was a potential target of *miR-155*. Therefore, we hypothesize that BCL-10 mediates the anti-inflammation effect of *miR-155*. In this study, we found that *miR-155* repressed the expression of BCL-10 induced by *C. albicans*, and BCL-10 was a direct target of *miR-155*. Furthermore, silencing BCL-10 by siRNA suppressed the phosphorylation of p65 and the secretion of pro-inflammatory cytokines, reproducing the anti-inflammation effects of *miR-155*. Accordingly, we deduce that *miR-155* negatively regulated pro-inflammatory cytokines in response to *C. albicans* by functionally targeting BCL-10.

Conclusively, our study demonstrated that the activation of Dectin-1 pathway in DCs upregulated the expression of *miR-155*. With targeting NF-κB p65 and BCL-10, increased expression of *miR-155* attenuated the excessive inflammation induced by *C. albicans*. *MiR-155* might "fine-tune" the innate immune response triggered by *C. albicans*.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are responsible for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethic Board of Changzheng Hospital and written approvals were obtained from the subjects.

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Supplementary

Table S1 Sequences of siRNA used in this study

Gene siRNA	Sequences
si-Dectin-1-1	CAGGATAGCTGTTGTTTCA
si-Dectin-1-2	GGATGAAGATGGATATACT
si-Dectin-1-3	GAAGATGGATATACTCAAT
si-p65-1	GCTGCAGTTTGATGATGAA
si-p65-2	CTTCCAAGTTCCTATAGAA
si-p65-3	GGACATATGAGACCTTCAA
si-BCL-10-1	GCCACCAGATCTACAGTTA
si-BCL-10-2	CACAGAACTTCCTGATACA
si-BCL-10-3	GGACACCCTTGTTGAATCT

siRNA, small interfering RNA; BCL-10, B cell leukemia/lymphoma 10.

Table S2 Sequences of primers used for qRT-PCR in this study

Gene name	Forward primers (5'-3')	Reverse primers (5'-3')
IL-6	CTCAGCCCTGAGAAAGGAGA	TTTCAGCCATCTTTGGAAGG
TNF-α	GGAGGGGTCTTCCAGCTGGAGA	CAATGATCCCAAAGTAGACCTGC
IFN-γ	TGAATGTCCAACGCAAAGCA	CTGGGATGCTCTTCGACCTC
Dectin-1	CGACTCTCAAAGCAATACCAGGA	GTACCCAGGACCACAGCTATCAC
p65	CCTCCACCTCGACGCA	GCCCAGAAGGAAACACCA
BCL-10	GCTTGGACACCCTTGTTGAATC	GGGCGTCGTGCTGGATT
β-actin	TACTGCCCTGGCTCCTAGCA	TGGACAGTGAGGCCAGGATAG
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAAT

qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; BCL-10, B cell leukemia/lymphoma 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

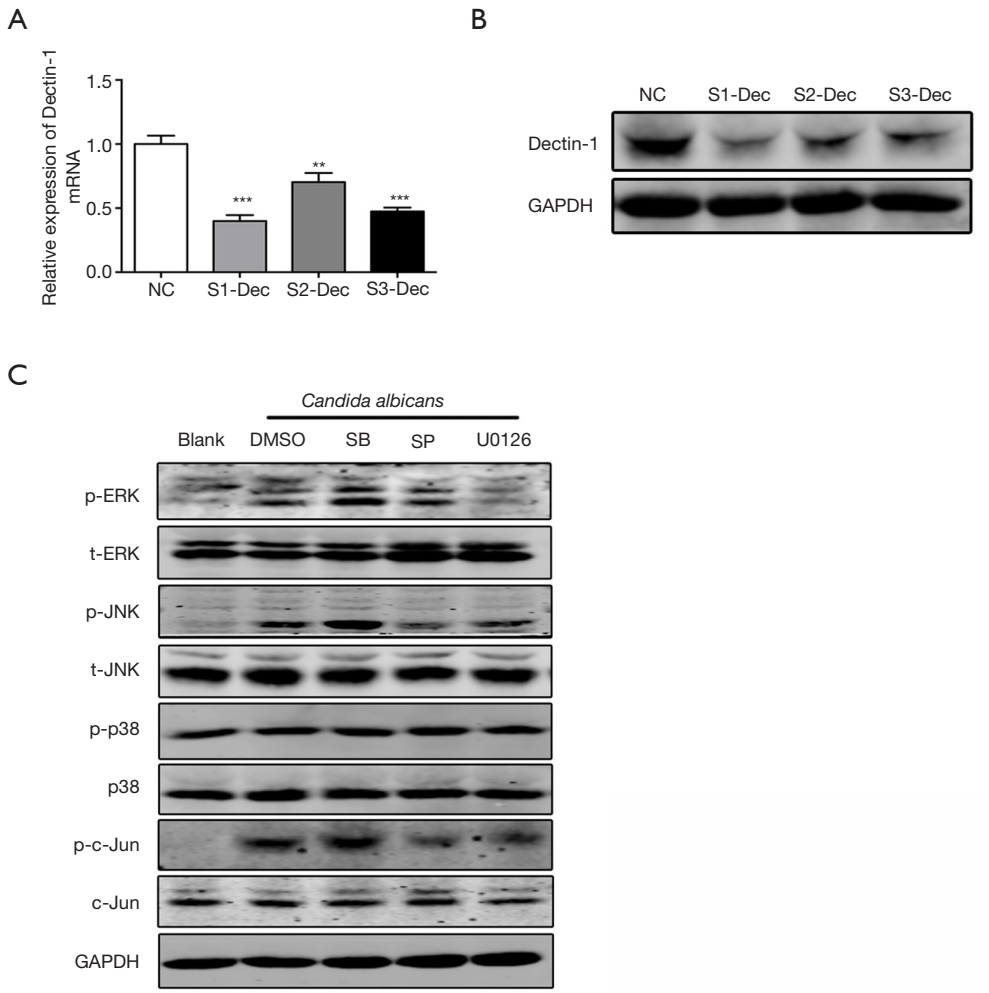

Figure S1 Identification of Dectin-1-siRNAs and inhibitors for MAPK pathway. (A) DCs were transfected with 100 nM Dectin-1-siRNAs (si-Dectin-1) or 100 nM NC. Twenty-four hours later, the expression of Dectin-1 mRNA was determined using qRT-PCR; (B) DCs were transfected with 100 nM si-Dectin-1 or 100 nM NC. Forty-eight hours later, the protein of Dectin-1 was determined using western blot; (C) DCs were pretreated with DMSO, 1 μM SB203580 (p38 inhibitor), 10 μM SP600125(JNK inhibitor), or 10 μM U0126 (MEK/ERK inhibitor) for 1 hour and then stimulated with *C. albicans*. The expression of p-ERK, p-JNK, p-p38 and p-c-Jun protein was determined using western blotting. Values are means ± SD from three experiments performed in triplicate. **, P<0.01; ***, P<0.001. DCs, dendritic cells; siRNA, small interfering RNA; NC, negative control; mRNA, messenger RNA; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; *C. albicans*, Candida albicans; SD, standard deviation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SB, SB203580; SP, SP600125.

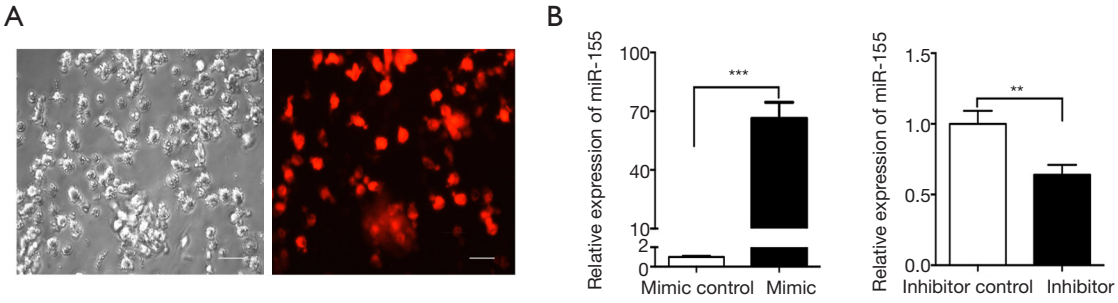

Figure S2 The transfection efficiency of *miR-155* in DCs. (A) Transfected *miR-155* negative control tagged by red fluorescence in DCs was detected by fluorescence microscope; (B) *miR-155* level of DCs after *miR-155* mimics (50 nM) or inhibitor (100 nM) transfection was measured by qRT-PCR. **, P<0.01; ***, P<0.001. DCs, dendritic cells; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction.

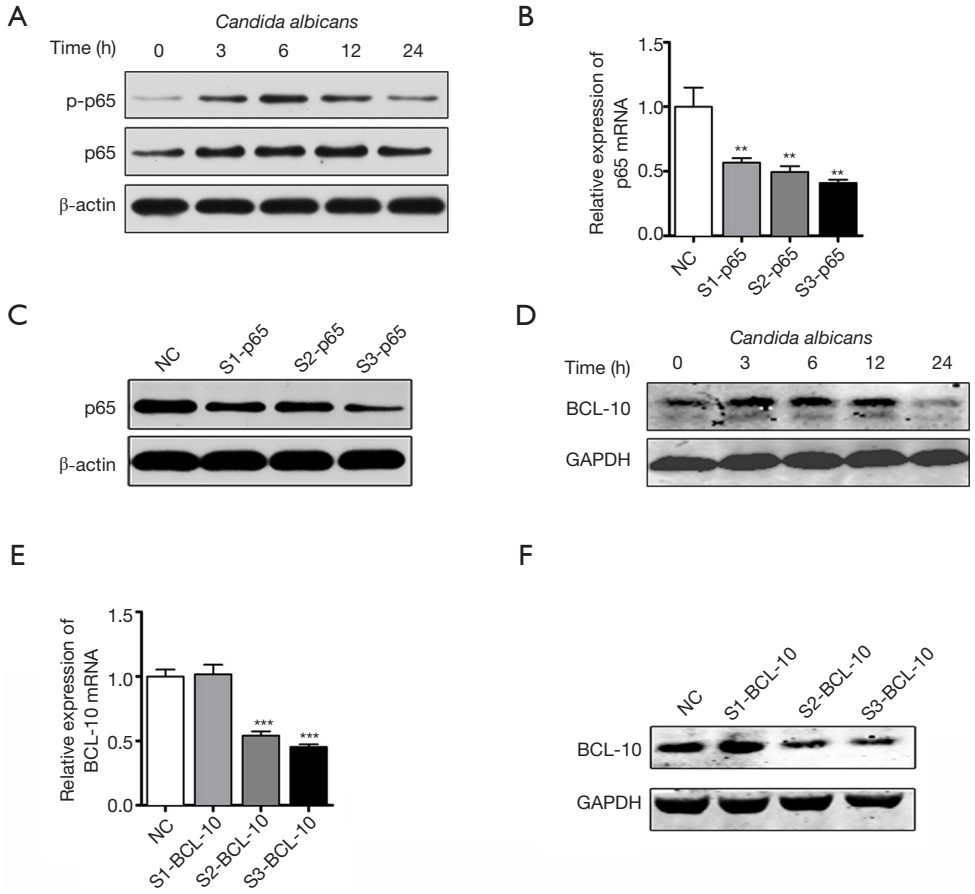

Figure S3 The inhibitory effect of p65 siRNAs and BCL-10 siRNAs. (A) Dynamic expression of p-65 protein in *C. albicans* treated DCs was determined using western blot; (B) DCs were transfected with 100 nM p65-siRNAs (si-p65) or 100 nM NC. Twenty-four hours later, the expression of p65 mRNA was determined using qRT-PCR; (C) DCs were transfected with 100 nM si-p65 or 100 nM NC. Forty-eight hours later, the protein of p65 was determined using western blot; (D) dynamic expression of BCL-10 protein in *C. albicans* treated DCs was determined using western blot; (E) DCs were transfected with 100 nM BCL-10-siRNAs (si-BCL-10) or 100 nM NC. Twenty-four hours later, the expression of BCL-10 mRNA was determined using qRT-PCR; (F) DCs were transfected with 100 nM si-BCL-10 or 100 nM NC. Forty-eight hours later, the protein of BCL-10 was determined using western blot. Values are means ± SD from three experiments performed in triplicate. **, P<0.01; ***, P<0.001. *C. albicans*, *Candida albicans*; DCs, dendritic cells; siRNA, small interfering RNA; NC, negative control; mRNA, messenger RNA; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; BCL-10, B cell leukemia/lymphoma 10; SD, standard deviation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.