·Basic Research·

Co-regulation of Dectin-1 and TLR2 in inflammatory response of human corneal epithelial cells induced by *Aspergillus* fumigates

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Abstract

• AIM: To investigate the co-regulation of dendritic cell-associated C-type lectin-1 (Dectin-1), Toll-like receptor 2 (TLR2), and relative chemotactic factors in the Telomease-immortalized human corneal epithelial (THCE) cells after exposure to *Aspergillus* fumigatus (Af) hyphae.

• METHODS: The normal THCE cells were investigated as control. After cultured *in vitro* with Af hyphae, with or without laminarin and anti-TLR2 antibody for 4, 8, 16 and 24h, THCE cells were harvested. The expression of Dectin-1, TLR2, CXCL1 and CXCL8 mRNA were measured by real-time quantitative polymerase chain reaction at the stimulation of 4, 8 and 16h separately. The protein expression of Dectin-1 and TLR2 were analyzed at 8, 16, and 24h by Western blot.

• RESULTS: The mRNA expression of CXCL1 and CXCL8 increased in THCE cells after stimulated by Af hyphae. The stimulatory effects on these inflammatory chemokines were shown in a dose-dependent manner and reached the peak at 8h. Af hyphae significantly stimulated the production of Dectin-1 and TLR2 in THCE cells at both mRNA and protein levels. The protein of Dectin-1 and TLR2 gradually increased till 16h. While pretreated with laminarin (a Dectin-1 inhibitor), the expression of TLR2, CXCL1 and CXCL8 all decreased dramatically at the peak point. Interestingly, when pretreated with TLR2 neutralizing antibody, the expression of Dectin-1, CXCL1 and CXCL8 also decreased dramatically at the peak point.

• CONCLUSION: These findings suggest that Dectin-1 and TLR2 co-regulated with each other after treated with inactive Af hyphae in the THCE cells, and they contribute together to the inflammatory responses by induction of chemokines CXCL1 and CXCL8.

• **KEYWORDS:** dendritic cell-associated C-type lectin-1; corneal epithelial cell; Toll-like receptor 2; chemokines

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INTRODUCTION

Fungal keratitis (FK) is a common eye disease in developing countries. Survey showed that the incidence rate of FK increases gradually over time [1]. In some areas of China, it had become the primary reason of blindness caused by infection[2-3]. FK always occurs after the epithelial integrity of cornea has been breached and the underlying fibroblasts were exposed to a wide array of micro-organisms [1,4]. *Fusarium* and *Aspergillus* fumigatus (Af) are the two main pathogenic fungi in China [1]. In addition to serving as a protective barrier, the corneal epithelium participates in the host innate immune response to microbial infection[5].

The first step for macrophage and neutrophils activation is the recognition of fungal components by pattern recognition receptors (PRRs) in FK patients [6]. The most important classes of PRRs are the Toll-like receptors (TLRs) especially TLR2, which expressed in corneal epithelial cells[7]. Dendritic cell-associated C-type lectin-1 (Dectin-1), also known as CLEC7A is a small type II transmembrane receptor containing one extracellular lectin-like carbohydrate recognition domain, as well as an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail[8]. It is an another critical kind of innate immune receptor apparently involved in chronic inflammatory conditions, such as autoimmunity, inflammatory bowel disease and diabetes. Dectin-1 plays an important role in the recognition of β-glucans of *Aspergillus* [9], and can induce phagocytosis, phospholipase A2, cyclooxygenase 2, the respiratory burst and the production of numerous cytokines and chemokines, including tumor necrosis factor, macrophage inflammatory protein 2, interleukin (IL)-12, IL-2, IL-10, IL-6 and IL-23[10].

Our research group had proved that Dectin-1 is expressed in the corneal epithelium of FK patients. For the purpose of further investigation, this study was conducted to demonstrate

the co-regulation of Dectin-1 and TLR2 in Telomease-immortalized human corneal epithelial (THCE) cells in inflammatory response of corneal epithelium in FK patients.

MATERIALS AND METHODS

***Aspergillus* Fumigatus Antigens** Af (strain CCTCC 93024, China General Microbiological Culture Collection Center, Beijing, China) was cultured in Sabouroud fluid medium (Sigma, Louis, MO, USA) at 37℃ for 5d in a shaking table with a rotating speed of 500 rpm. It was ground and centrifuged at 3000 rpm for 5min after 70% alcohol inactivated for 30min, and then it was washed for three times by phosphate buffer saline (PBS)[5]. The above antigens stimulation were stored in -20℃, and these antigens stimulation liquid would be used up in 2wk.

Telomease -immortalized Human Corneal Epithelial Cells Culture and *Aspergillus* Fumigatus Challenges THCE cells (kindly provided by Eye Institute and Affiliated Xiamen Eye Center) were cultured in Delbeccon's modified Eagle's medium (DMEM; Sigma-Aldrich St. Louis, MO, USA) with 15% fetal bovine serum (FBS; Invitrogen Gibco, Carlsbad, CA, USA), 100 U/mL penicillin G and 100 μg/mL streptomycin sulfate at 37℃ in a humidified atmosphere containing 5% CO_2. The medium was replaced every 2d. THCE cells suspensions of 2×10^5/mL were seeded into 6-well tissue culture plates and grew to 70% -80% confluence. Before challengement THCE cells were incubated in serum-free medium for 12h. Then some THCE cells were added with serum-free medium as control, and others were added with inactive Af hyphae. After 4, 8, and 16h incubation, THCE cells were harvested, and the culture medium was collected for total RNA detection and 8, 16, and 24h, cells were harvested for protein extraction. TLR2 blocking experiments was conducted by incubating THCE cellswith LEAF™ purified anti-human CD282 (TLR2) antibody (biolegend, San Diego, CA, USA) and Dectin-1 blocking experiments was conducted by incubating THCE cells with laminarin (Sigma-Aldrich, St. Louis, MO, USA). THCE cells were incubated at 37℃ with purified anti-TLR2 or laminarin for 1h[5,11].

Quantitative Real -time Reverse Transcription Polymerase Chain Reaction Total RNA was isolated from THCE cells with RNAiso plus reagent (TaKaRa, Dalian, Liaoning Province, China), Trizol reagent (Invitrogen, USA), and quantified by Spectrophotometry. To measure the relative concentration of gene expression, RNA (1 μg) was used for first-strand cDNA synthesis according to the protocol for a reverse transcription system. Then cDNA (2 μL) was used for quantitative real-time polymerase chain reaction (PCR) in 20 μL reaction volume following the manufacturer's instructions. The accession number, primer sequence, and PCR amplification product size for each gene were shown in Table 1. PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) kits and SYBR Premix Ex Taq (Tli RNaseH Plus) kits were purchased from TaKaRa (Dalian, Liaoning Province, China). PCR cycling conditions were as follows: an initial denaturation step of 95℃ for 5min, followed by 40 cycles of 95℃ for 30s, 60℃ for 30s, 72℃ for 60s for Dectin-1, TLR2, CXC chemokine ligand (CXCL)1, CXCL8 and β-actin. A non-template control was included to evaluate DNA contamination. The results were analyzed by the comparative cycle threshold (CT) method and normalized by β-actin[12].

Table 1 Primer sequences used for PCR

Gene	Primer sequence	Product size
Dectin-1	F-5'CGACTCTCAAAGCAATACCAGGA3' R-5'GTACCCAGGACCACAGCTATCAC3'	138 bp
TLR2	F-5'TGCTGTGCTCTGTTCCTGCT3' R-5'TTCCTGGGCTTCCTTTTGG3'	116 bp
CXCL1	F-5'AGG GAA TTC ACC CCA AGA AC3' R-5'CAC CAG TGA GCT TCC TCC TC3'	193 bp
CXCL8	F-5'TTTCAGAGACAGCAGAGCACACAA3' R-5'CACACAGAGCTGCAGAAATCAGG3'	145 bp
β-actin	F-5' TGGCACCCAGCACAATGAA 3' R-5' CTAAGTCATAGTCCGCCTAGAAGCA3'	186 bp

Western Blot Analysis THCE cells were lysed with RIPA containing 1mmol/L PMSF (100:1). Cell lysates were mixed with loading buffer and boiled for 10min. The cell lysates were then assayed on an SDS-polyacrylamide electrophoresis gel (12%) and transferred onto polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). This membrane was subsequently blocked with 5% non-fat milk solution. Western blot analysis was performed following standard protocols using relevant antibodies, followed by incubation with relevant horseradish peroxidase-conjugated secondary antibodies. Beyo ECL Plus reagents (Beyotime, Shanghai, China) were used for the development of colorimetric signals on the membrane. The membrane was also blotted with a monoclonal anti-GAPDH antibody (Santa Cruz, Texas, USA) as a loading control. The band image was visualized using a gel documentation system (UVP Biospectrum HR410, USA).

Dectin -1 and TLR2 Functional Blocking Analysis THCE cells suspensions of 2×10^5/mL were seeded into 6-well tissue culture plates and grew to 70% -80% confluence. Then THCE cells were pre-incubated with laminarin (300 μg/mL)[11] or anti-human TLR2 (200 μg/mL)[13] antibody for 30min. Some THCE cells were cultured with serum-free medium as control, and then inactive Af hyphae was added into the culture medium. After 16h incubation, THCE cells were harvested and the culture medium was collected for detection.

Statistical Analysis All data were shown as the mean±SD from at least three independent experiments. Data analysis was done by one-way ANOVA test and further comparison in pairs was analysed by LSD test using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistical significance.

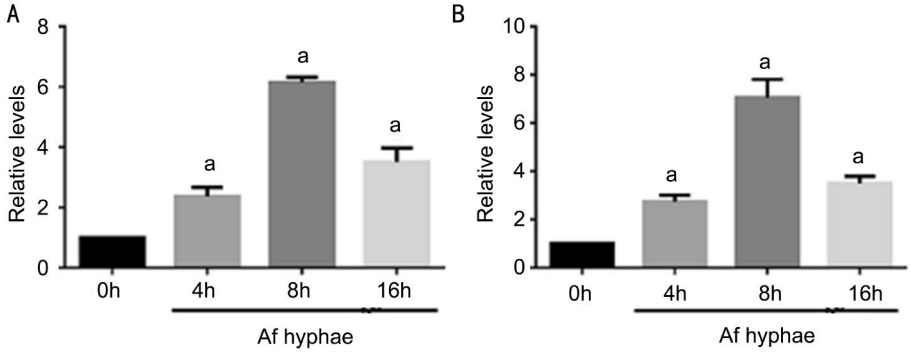

Figure 1 The expression of mRNA production of CXCL1 (A) and CXCL8 (B) in Af hyphae treated THCE cells The THCE cells were cultured with Af hyphae antigens stimulation liquid for 4, 8 and 16h. Data were presented as mean±SD. n=6. ^aP <0.05 *vs* control group.

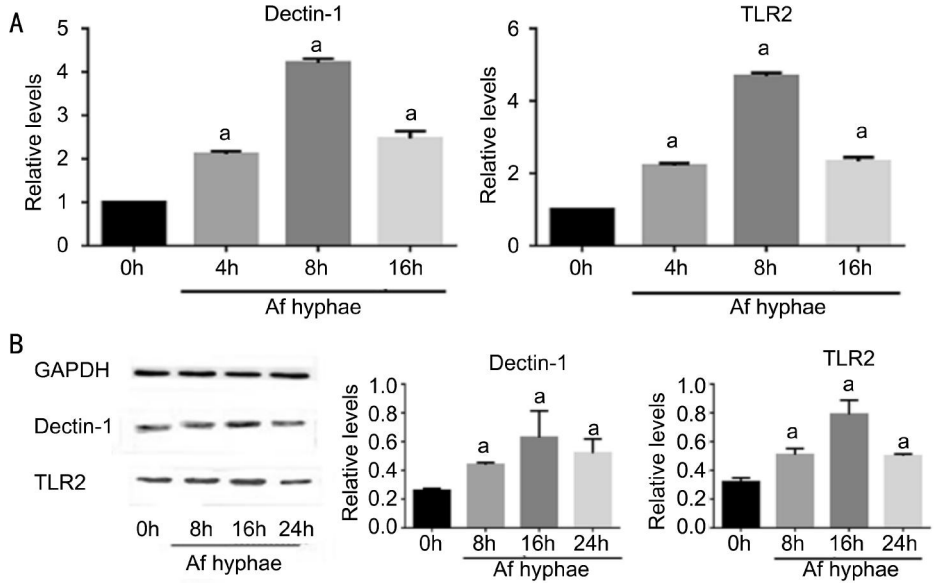

Figure 2 The expression of mRNA and protein of Dectin-1 and TLR2 in Af hyphae treated THCE cells The THCE cells were cultured with Af hyphae antigens stimulation liquid for 4, 8, 16 and 24h. A: The mRNA expression of Dectin-1 and TLR2; B: The protein expression of Dectin-1 and TLR2. Data were presented as mean±SD, n=6. ^aP<0.05 *vs* control group.

RESULTS

***Aspergillus* Fumigatus Hyphae Upregulated CXCL1 and CXCL8 in Telomease-immortalized Human Corneal Epithelial Cells** To explore the expression of CXCL1 and CXCL8 and identify its potential role in corneal epithelial cells during Af infection, we treated THCE cells with Af hyphae. As shown in Figure 1, the expression of CXCL1 and CXCL8 mRNA in THCE cells with Af hyphae significantly increased at 4h (P<0.05), and in a time-depended manner. The differences in the amount of secreted CXCL1 and CXCL8 between Af cells and control were more apparent after prolonged incubation. The mRNA expression of CXCL1 and CXCL8 began to increase at 4h and reached peak at 8h (P<0.05; Figure 1).

***Aspergillus* Fumigatus Hyphae Induced the Production of Dectin-1 and TLR2 in Telomease-immortalized Human Corneal Epithelial Cells** After stimulated by Af hyphae, Dectin-1 and TLR2 mRNA expression was significantly increased and in time-dependent manner, up to the peak point at 8h (P<0.05; Figure 2A). The group of Af stimulate 8h group compared with 4h and 16h group, the differences of Dectin-1 and TLR2 mRNA expression were statistically significant (P<0.05; Figure 2A).

Af hyphae upregulate the protein expression of Dectin-1 and TLR2 in a time-depended manner too. Increased Dectin-1 and TLR2 were detected as early as 8h (P<0.05), after stimulation with Af hyphae, Af hyphae significantly upregulated the protein expression of Dectin-1 and TLR2 at 16h (P<0.05; Figure 2B).

***Aspergillus* Fumigatus Hyphae Upregulated CXCL1 and CXCL8 by Dectin-1 or TLR2 Signaling** In order to investigate whether Dectin-1 invovled in CXCL1 prodution stimulated by with Af hyphae in THCE cells, specific inhibitors were used. As shown in Figure 3, Dectin-1 and TLR2 expression was significantly reduced after pretreatment with laminarin (P<0.05) or TLR2 neutralizing antibody (P<0.05). These results showed that the specific inhibitors of Dectin-1 and TLR2 can efficiently inhibit the expression of Dectin-1 and TLR2 respectively.

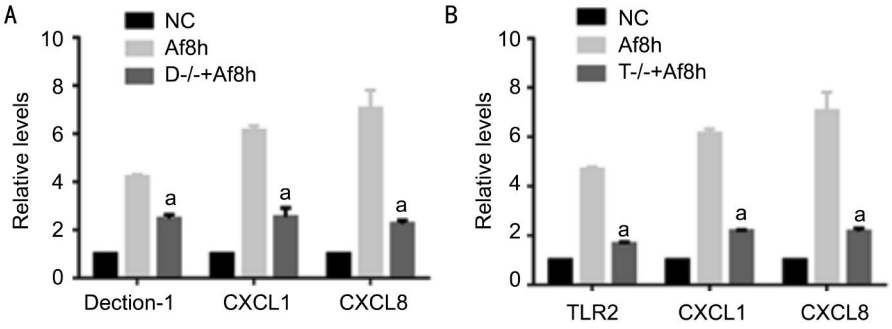

Figure 3 The expression of CXCL1 and CXCL8 blocked with Dectin-1 or TLR2 inhibitor in Af hyphae treated THCE cells A: The mRNA expression of Dectin-1, CXCL1 and CXCL8 in THCE cells blocked with laminarin and treated with Af hyphae; B: The mRNA expression of TLR2, CXCL1 and CXCL8 in THCE cells blocked with TLR2 neutralizing antibody. $^{a}P<0.05$ *vs* Af hyphae treated group.

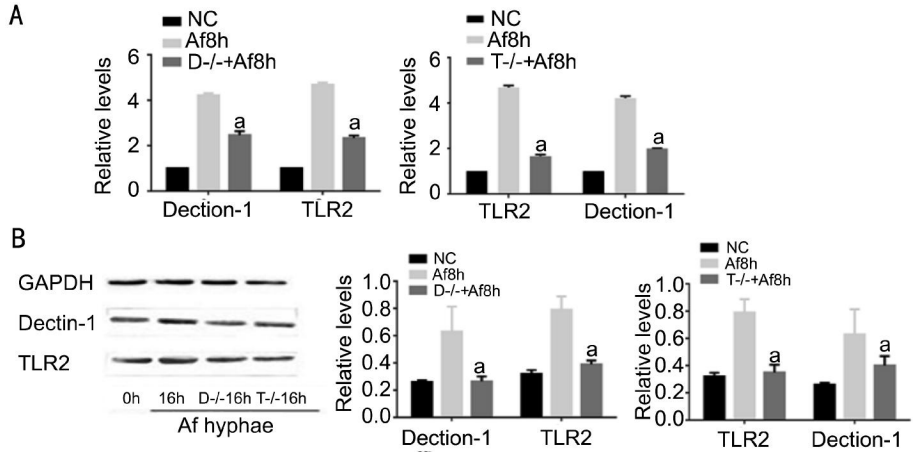

Figure 4 The expression of Dectin-1 and TLR2 blocked with inhibitor in THCE cells treated with Af hyphae A: The mRNA expression of TLR2 and Dectin-1 in THCE cells blocked with laminarin or TLR2 neutralizing antibody; B: The protain expression of TLR2 and Dectin-1 in THCE cells blocked with laminarin or TLR2 neutralizing antibody. $^{a}P<0.05$ *vs* Af hyphae treated group.

THCE cells were cultured with laminarin to inhibit Dectin-1 before they were stimulated by Af hyphae. As shown in Figure 3A, the levels of CXCL1 and CXCL8 expression were high in response to Af hyphae stimulation. However, they were significantly inhibited in cells treated with laminarin ($P<0.05$), indicating that Dectin-1 can mediate the production of CXCL1 and CXCL8 in THCE cells trigged by Af hyphae.

THCE cells were cultured with TLR2 neutralizing antibody to inhibit TLR2 before they were stimulated with Af hyphae. As shown in Figure 3B, the mRNA expression of CXCL1 and CXCL8 were high in the response to Af hyphae stimulation. However, they were significantly inhibited in cells treated with TLR2 neutralizing antibody ($P<0.05$), indicating that TLR2 can mediate the production of CXCL1 and CXCL8 in THCE cells trigged by Af hyphae.

Interaction Between Dectin-1 and TLR2 As shown in Figure 4A, the levels of TLR2 expression were high in response to Af hyphae stimulation ($P<0.05$). However, they were significantly inhibited in cells treated with laminarin ($P<0.05$), indicating that Dectin-1 can influence the expression of TLR2 in THCE cells trigged by Af hyphae.

THCE cells were cultured with TLR2 neutralizing antibody to block TLR2 before they were stimulated with Af hyphae. As shown in Figure 4B, the levels of Dectin-1 expression were high in response to Af hyphae stimulation ($P<0.05$). However, they were significantly inhibited in cells treated with TLR2 neutralizing antibody ($P<0.05$), indicating that TLR2 can influence the expression of Dectin-1 in THCE cells trigged by Af hyphae.

DISCUSSION

Fungal keratitis is a very common and serious infective corneal disease in many developing countries. Human epidermis plays an important role in the immune system[14]. The innate immune response against Af plays a crucial role in controlling infection. PRRs are crucial to provide protection during fungal infection. The epithelial expression of TLRs is of great importance in inflammation and immunity in response to pathogenic agents [15]. The immune reaction system plays the leading role through the cooperation of many PRRs, not by a single PRR. Among the TLRs, TLR2 have been reported to mediate host innate immune responses against various pathogens such as *Staphylococcus* aureus [16], *Aspergillus* [17] and *Cryptococcus* neoformans[18]. C-type lectin receptors (CLRs) can recognize and discriminate between nonpathogenic and pathogenic microorganisms. Among the CLRs, Dectin-1 is thought to be involved in innate immune responses to fungal pathogens[19]. Wang *et al*[11] verified that the expression of Dectin-1 rely on

the upregulation of TLR2. While, whether there is interaction between Dectin-1 and TLR2 in the FK?

In the pathogenesis of FK, chemokines can recruit neutrophils and macrophage, help the body remove pathogenic fungi. The CXCL1/GRO-α, is a key chemoattractant for neutrophils in inflamed corneas [20]. CXCL1 is a major chemoattractant for polymorphonuclears (PMNs). Lipopolysaccharides binds to TLRs, and induces the release of CXCL8 [21]. CXCL8 was significantly increased after stimulation with *Aspergillus* or *Fusarium*[22]. The present study produces much more data pointing towards the co-regulation of Dectin-1 and TLR2 in inducing the chemokines of THCEs. Studies have shown that the expression of Dectin-1 increases in corneal epithelium during the innate immune stage against fungal infection, as one of the PRRs participated in the antifungal response. Dectin-1 is mainly activated Syk/CARD9, but TLR2 is mainly activated myeloid differentiation factor88 [22-23]. Our findings showed that Af hyphae significantly stimulates production of Dectin-1 and TLR2 in THECs at both mRNA and protein levels. This transmembrane signaling receptor mediates various cellular functions, including fungal binding, uptake and killing and inducing the production of cytokines and chemokines[10].

In our study, the CXCL1 and CXCL8 in the THCE culture increased after stimulated by inactive Af hyphae in mRNA level (Figure 1). Recent studies have demonstrated that the expression of CXCL1 plays an important role in chemokine expression and neutrophil infiltration following adenoviral corneal infection[24]. Laminarin, a Dectin-1 inhibitor, blocked the stimulated induction of TLR2 and inflammatory chemokine CXCL1 and CXCL8 by Af hyphae. The induction of Dectin-1 and chemokine (CXCL1, CXCL8) by Af hyphae were also markedly blocked by TLR2 neutralizing antibody (Figure 3). These findings confirmed that both Dectin-1 and TLR2 involved in the inflammatory response of THCEs induced by Af hyphae. It indicated that corneal epithelial cells could recognize fungi antigen and induce the increase of inflammatory chemokines to defend fungi and recruit lots of PMNs, which could start stronger immune response to clear pathological fungi by Dectin-1 and TLR2 signalings. Interestingly, these findings also indicated that there were some interactions between Dectin-1 and TLR2.

Some experimental results verified that the expression of Dectin-1 rely on the upregulation of TLR2, they both depended on each other and induced high expression of each other in in human bronchial epithelial cells [25]. As shown in Figure 4, Laminarin significantly inhibited the expression of TLR2 both in mRNA and protein levels, and the expression of Dectin-1 reduced accordingly after inhibited by TLR2 neutralizing antibody. These data indicated that the Dectin-1 and TLR2 signaling pathways played an important role in the inflammatory response of THCEs stimulated by inactive Af hyphae, which can defend fungi at an early stage and start the following immune response.

In conclusion, Dectin-1 and TLR2 exist and express in THCE cells. They co-regulated with each other after treated with inactive Af hyphae in the THCE cells, and contributed together to the inflammatory responses by induction of chemokines CXCL1 and CXCL8. In addition, we speculated that these such reciprocal regulation of PRR may be a common mechanism to induce specific inflammatory responses in corneal epithelial cells.

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