·Basic Research·

Dectin-1 agonist curdlan modulates innate immunity to *Aspergillus fumigatus* in human corneal epithelial cells

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Abstract

• AIM: To explore the immunomodulatory effects of curdlan on innate immune responses against *Aspergillus fumigatus* (*A. fumigatus*) in cultured human corneal epithelial cells (HCECs), and whether C-type lectin receptor Dectin-1 mediates the immunomodulatory effects of curdlan.

• METHODS: The HCECs were stimulated by curdlan in different concentrations (50, 100, 200, 400 μg/mL) for various time. Then HCECs pretreated with or without laminarin (Dectin-1 blocker, 0.3 mg/mL) and curdlan were stimulated by *A. fumigatus* hyphae. The mRNA and protein production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined by real-time quantitative polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. The protein level of Dectin-1 was measured by Western blot.

• RESULTS: Curdlan stimulated mRNA expression of TNF-α and IL-6 in a dose and time dependent manner in HCECs. Curdlan pretreatment before *A. fumigatus* hyphae stimulation significantly enhanced the expression of TNF-α and IL-6 at mRNA and protein levels compared with *A. fumigatus* hyphae stimulation group (P<0.05). Both curdlan and *A. fumigatus* hyphae up-regulated Dectin-1 protein expression in HCECs, and Dectin-1 expression was elevated to 1.5- to 2-fold by curdlan pretreatment followed hyphae stimulation. The Dectin-1 blocker laminarin suppressed the mRNA expression and protein production of TNF-α and IL-6 induced by curdlan and hyphae (P<0.05).

• CONCLUSION: These findings demonstrated that curdlan pretreatment enhanced the inflammatory response induced by *A. fumigatus* hyphae in HCECs. Dectin-1 is essential for the immunomodulatory effects of curdlan. Curdlan may have high clinical application values in fungal keratitis treatment.

• **KEYWORDS:** curdlan; Dectin-1; innate immunity; *Aspergillus fumigatus*; corneal epithelium

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INTRODUCTION

Fungal keratitis is a severe corneal disease causing visual disability. In China, fungal keratitis has a gradual increasing incidence mainly because of traumatic injury associated with agricultural work in recent years [1]. *Aspergillus fumigatus* (*A. fumigatus*) is one of the main pathogens of fungal keratitis [2]. Due to low efficacy of anti-mycotic therapy, fungal keratitis can result in corneal perforation, permanent vision loss, or even loss of the eyeball [3,4]. Although the potential use of collagen crosslinking for management of fungal keratitis has been highlighted, its effect may be limited to the anterior layers of the cornea [5]. Therefore, a deeper understanding of the immunologic mechanisms for host defense against fungal infection is requisite for developing new target therapies.

The corneal epithelium serves as the frontline of cornea. Once its integrity is compromised, there is more opportunity for inoculation of fungal conidia into the corneal stroma, which may develop into fungal keratitis subsequently. After being invaded by a fungus, the corneal epithelium identifies fungus and triggers the host innate immune response by specific recognition of pathogen associated molecular pattern on fungal surface with pattern recognition receptors (PRRs), mainly including C-type lectin receptors, toll-like receptors (TLRs) and NOD-like receptors [6-9]. These receptors induce activation of signal molecules and ultimately lead to secretion of pro-inflammatory cytokines, cellular infiltration and fungal clearance.

Dectin-1 is a C-type lectin receptor, firstly found in dendritic cell, and specifically recognizes fungal β-glucans [10]. It has been discovered that Dectin-1 is essential for initial wave of cytokine production, neutrophil and monocyte recruitment

and fungal killing in fungal keratitis [7]. We have confirmed that Dectin-1 is expressed on corneal epithelial cells in fungal keratitis [6,11]. Curdlan, a β- (1,3)-glucan without side chains, has been identified as a Dectin-1 specific agonist[12,13] and has gained attention for its immunomodulatory properties on innate immune system. It is interesting that whether curdlan modulates antifungal innate immunity of corneal epithelial cells. In the present study, we examined whether curdlan modulate the innate immune responses against *A. fumigatus* in cultured human corneal epithelial cells (HCECs) and identified the role of Dectin-1 in curdlan-mediated immunomodulatory effect.

MATERIALS AND METHODS

Materials *A. fumigatus* strains (NO3.0772) was purchased from China General Microbiological Culture Collection Center; HCECs was a gift from Zhongshan University; Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin and Ham F-12 were from Gibco (America); Curdlan and laminarin were from Sigma (America); Rabbit anti-human Dectin-1 antibody was from Cell signaling (America), RNAiso Plus, PrimeScript RT reagent Kit With gDNA Eraser (Perfect Real Time), primers and SYBR ® Premix Ex Taq™ were all from TAKARA (Dalian, China); enzyme-linked immunosorbent assay (ELISA) MaxTM Deluxe Set was from BioLegend (America).

Preparation of Aspergillus Fumigatus Hyphae Antigens *A. fumigatus* conidia grew in Sabouroud liquid medium, 37℃ for 5d. Then hyphae were grinded, washed twice by sterile phosphate buffer saline (PBS) and inactivated by 70% ethanol at 4℃ for 12h. Inactive *A. fumigatus* hyphae was washed and added in PBS. The hyphae suspension was quantified using a hemacytometer, and saved at -20℃[14].

Human Corneal Epithelial Cells Culture and Stimulation HCECs were cultured and maintained in HCECs growth medium in a humidified 5% CO_2 incubator at 37℃. HCECs growth medium contains 1:1 DMEM/Hams F12 supplemented with 5% fetal bovine serum (FBS), 10 ng/mL human epidermal growth factor (EGF), 5 mg/mL insulin, and 50 mg/mL penicillin and streptomycin [15,16]. For stimulation, HCECs were seeded in 6 or 12-well plates. HCECs pretreated with or without curdlan and laminarin (0.3 mg/mL) were stimulated by *A. fumigatus* hyphae. Total RNA and supernatant were collected for real-time quantitative polymerase chain reaction (RT-qPCR) and ELISA.

Real -time Quantitative Polymerase Chain Reaction Assays Total RNA from HCECs were extracted, quantified by its absorption at 260 nm and stored at -80℃ according to the manufacturer's protocol. The total RNA extracted were reverse transcripted and conducted RT-qPCR by using SYBR at the same time. The thermocycler parameters were 95℃ for 30s, and followed by 40 cycles of 95℃ for 5s and 60℃ for 30s. The results of RT-qPCR were analyzed by the comparative threshold cycle (C_T) method [17,18]. The cycle threshold (Ct) was determined using the cycle at which the primary (fluorescent) signal crossed a user-defined threshold. Quantification was normalized by the Ct value of β-actin by using the $2^{-\Delta\Delta Ct}$ formula. The double-stranded probes used are as follow: (5'-3'): TGCTTGTTCCTCAGCCTCTT (forward) and CAGAGGGCTGATTAGAGAGAGGT (reverse) for tumor necrosis factor-α (TNF-α; human); AAGCCAGAGCTGTGCAGATGAGTA (forward) and TGTCCTGCAGCCACTGGTTC (reverse) for interleukin-6 (IL-6; human); TGGCACCCAGCACAATGAA (forward) and CTAAGTCATAGTCCGCCTAGAAGCA (reverse) for β-actin (human) as housekeeping gene.

Western Blot Assays Cells were lysed in RIPA buffer for 1h, and then were centrifuged to remove cellular debris. After estimation of protein content, addition of SDS sample buffer, and boiling, total protein was separated on 12% acrylamide SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with confining liquid at 37℃ for 2h, and then were incubated with a monoclonal antibody to human β-actin, and an antibody to human Dectin-1 (1:1000) at 4℃ overnight. After washed in PBST for three times, the membranes were incubated with corresponding peroxidase-conjugated secondary antibodies at 37℃ for 1h. Then mixture with equal chromogenic agents A and B were added and the blots were developed using chemiluminescence (ECL, Thermo Scientific).

Enzyme-linked Immunosorbent Assays The concentrations of TNF-α and IL-6 in culture supernatants were measured by double-sandwich ELISA according to the manufacturer's instructions. Culture supernatants were collected and centrifuged to remove cellular debris. The 96-well plate was coated with diluted capture antibody (100 μL) at 4℃ overnight. Then after blocked non-specific binding by diluent, 100 μL diluted standards and samples were added to the plate. After incubated at room temperature for 2h with shaking, the plate was washed for 4 times, and then diluted detection antibody (100 μL) was added to the plate. After incubated at room temperature for 1h with shaking, the plate was washed for 4 times, and then avidin-HRP solution (100 μL) was added. After incubation at room temperature for 1h, the plate was washed for 5 times, and then freshly mixed TMB substrate solution (100 μL) was added. After incubation in the dark for 15min, the stop solution (100 μL) was added and absorbance was read at 450 nm within 30min by an appliskan multimode microplate reader (Thermo Scientific, USA).

Statistical Analysis All data of three independent experiments were expressed as mean ±SD. The data were analyzed using SPSS19.0. Statistical analyses were performed by one-way ANOVA test, followed by Dunnett's

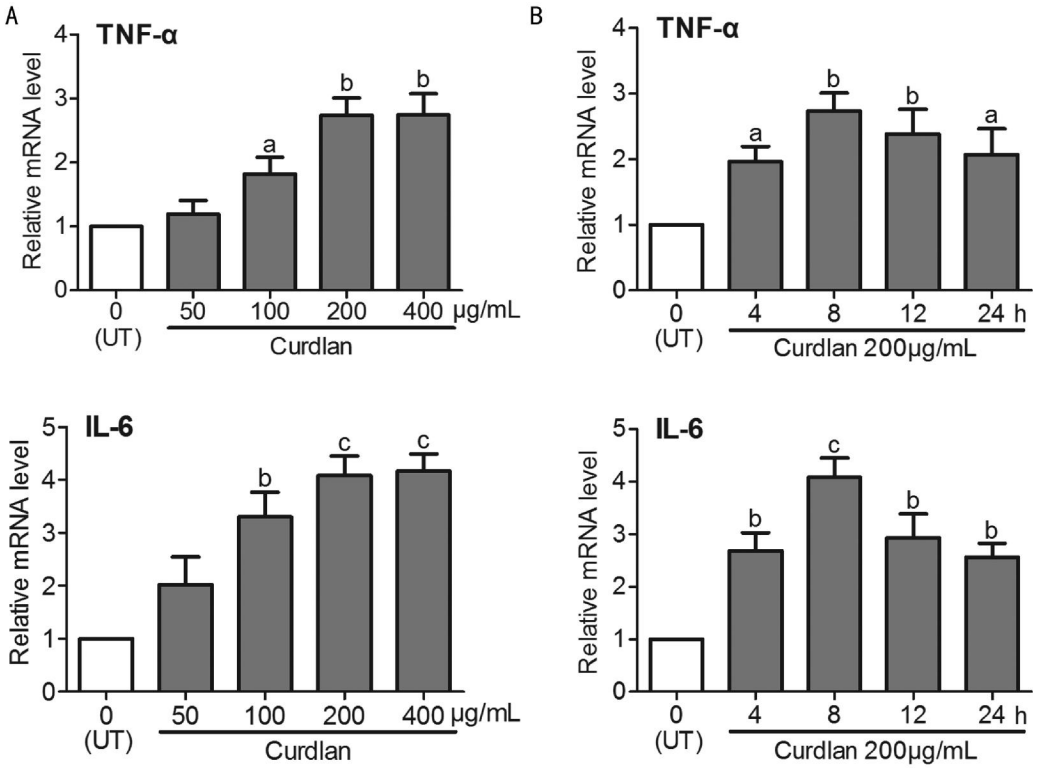

Figure 1 Curdlan–stimulated TNF–α and IL–6 mRNA expression in HCECs A: TNF-α and IL-6 mRNA expression of HCECs challenged with 50, 100, 200, 400 μg/mL curdlan for 8h was analyzed by RT-qPCR. B: TNF-α and IL-6 mRNA expression of HCECs challenged with 200 μg/mL curdlan for 4, 8, 12 and 24h were analyzed by RT-qPCR. $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$ compared with untreated controls.

test for multiple comparisons or by Student's t-test for unpaired data. $P<0.05$ was considered to be statistically significant.

RESULTS

Curdlan Induced mRNA Expression of Pro–inflammatory Cytokines To determine the response of HCECs to curdlan, we used RT-qPCR to measure the mRNA expression of TNF-α and IL-6. HCECs were stimulated with various concentrations of curdlan (50, 100, 200, 400 μg/mL) for different hours. Compared with untreated HCECs, the mRNA expression of TNF-α and IL-6 were significantly stimulated (up to 2- to 4-fold) in HCECs exposed to 50-400 μg/mL of curdlan for 8h (Figure 1A). The mRNA levels of TNF-α and IL-6 reached the peak at 8h after curdlan stimulation (Figure 1B). Curdlan induced both TNF-α and IL-6 mRNA secretion in a dose and time-dependent manner.

Curdlan Pretreatment Enhanced Pro–inflammatory Cytokines Production Induced by *Aspergillus Fumigatus* To investigate whether curdlan can modulate the innate immune response to *A. fumigatus* in HCECs, we pretreated HCECs with curdlan (200 μg/mL) for 1h, followed by *A. fumigatus* hyphae for 4, 8, 12 and 24h. The mRNA and protein levels of TNF-α and IL-6 were measured by RT-qPCR and ELISA, respectively. The mRNA expression of TNF-α and IL-6 were increased in HCECs after hyphae stimulation for 4-24h, but the levels were elevated to 2- to 3-fold by curdlan pretreatment (Figure 2A). Similarly, compared with HCECs exposed to hyphae, the protein levels of TNF-α and IL-6 secretion in supernatant were also elevated to 2- to 2.5-fold by curdlan pretreatment (Figure 2B).

Dectin–1 Mediated the Immunomodulatory Effects of Curdlan To investigate that whether Dectin-1 was essential for enhanced production of pro-inflammatory cytokines by curdlan pretreatment in *A. fumigatus* -stimulated HCECs. HCECs were pre-treated with or without laminarin (Dectin-1 blocker, 0.3 mg/mL) for 30min, subsequently incubated with curdlan or *A. fumigatus* hyphae. The Dectin-1 protein was measured by Western blot. The mRNA and protein levels of TNF-α and IL-6 were measured by RT-qPCR and ELISA. Both curdlan and *A. fumigatus* stimulated Dectin-1 expression in HCECs, while Dectin-1 expression was higher (up to 1.5- to 2-fold) by curdlan pretreatment followed *A. fumigatus* hyphae stimulation (Figure 3). When treated with curdlan and hypae, the production of TNF-α and IL-6 was significantly increased at both mRNA (Figure 4A) and protein (Figure 4B) levels. Pre-treatment with 0.3 mg/mL of laminarin significantly suppressed the expression of TNF-α and IL-6 induced by curdlan and hyphae at both mRNA (Figure 4A, $P<0.05$) and protein (Figure 4B, $P<0.05$) levels. These findings suggest that Dectin-1 plays an essential role in immunomodulatory effect of curdlan in HCECs against *A. fumigatus*.

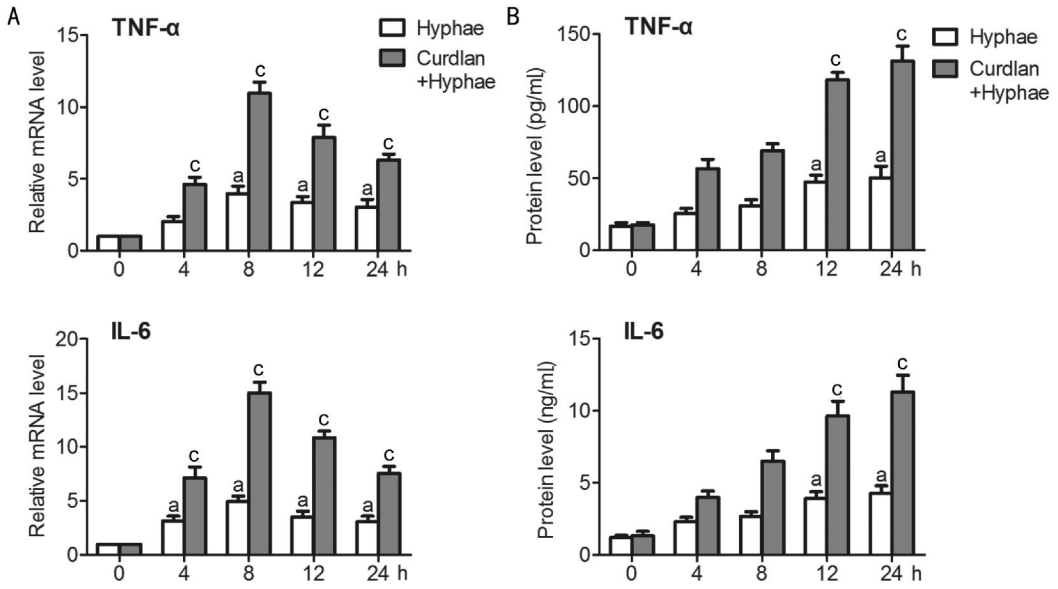

Figure 2 Curdlan enhanced production of TNF-α and IL-6 induced by *A. fumigatus* in HCECs The HCECs were cultured without or with curdlan 200 μg/mL for 1h. After being washed twice with serum-free medium, the cells were stimulated with *A. fumigatus* hyphae (5×10^6/mL) for 4, 8, 12 and 24h. The mRNA and protein levels in supernatant of TNF-α and IL-6 were assayed by RT-qPCR (A) and ELISA (B). [a] $P<0.05$ compared with untreated controls. [c] $P<0.05$ compared with HCECs stimulated by hyphae without curdlan pretreatment.

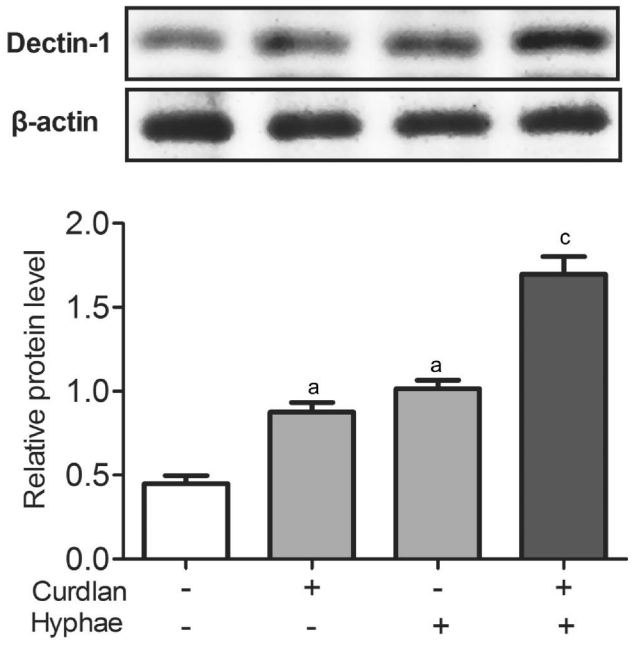

Figure 3 Curdlan and *A. fumigatus* stimulated Dectin -1 expression in HCECs HCECs were incubated with curdlan (200 μg/mL) or *A. fumigatus* hyphae (5×10^6/mL) for 24h, or pretreated with curdlan (200 μg/mL) for 1h followed by hyphae for 24h. The Dectin-1 protein expression was measured by Western blot. [a] $P<0.05$ compared with untreated controls. [c] $P<0.05$ compared with HCECs stimulated by hyphae without curdlan pretreatment.

DISCUSSION

Fungal keratitis is particularly a public health concern in China where the majority of population engages in agriculture and industry production. Corneal trauma especially plant trauma gives greater chance of fungal keratitis. Fungal infection of cornea leads to corneal ulcer or perforation, loss of vision even eyeballa. Early treatment of fungal keratitis is thus necessary to prevent threatening complications. Voriconazole and natamycin are the most common anti-mycotic agents, and voriconazole has been proved more efficient in tunnel fungal infection and other difficult cases with fungal infections [19,20]. However, these agents also have some limitations, including low water-solubility, poor bioavailability, limited ocular penetration and high cost[21,22]. Keratoplasty is another option for patients who are refractory to medical treatment or severe fungal infections, but it is limited by insufficient supply of corneal donors and graft rejection reaction. Additionally, some new therapies, like corneal collagen cross-linking, need to be clinically proved [23,24]. Therefore, it is urgent to understand the immunologic mechanisms of fungal keratitis to find new therapeutic targets.

Previous studies have implicated the essential roles of Dectin-1, TLR2, TLR4 and IL-1β in the pathogenesis of fungal keratitis [9,25,26]. Among these receptors, Dectin-1 is the first characterized PRR for β-glucans. Studies suggest that Dectin-1 is expressed in corneal eptithelial cells, in addition to macrophages, dendritic cells and neutrophils. Once β-glucans on the surface of fungi bound to Dectin-1 on resident corneal macrophages or corneal epithelial cells, Dectin-1 signals activate NF-κB and transcription of pro-inflammation cytokines through Syk and CARD9 [25]. A murine model of fungal keratitis has verified the vital role for Dectin-1 [7]. Besides, Dectin-1 polymorphism was associated with β-glucans binding capacity and Dectin-1 early stop codon increased susceptibility to *Candida albicans*

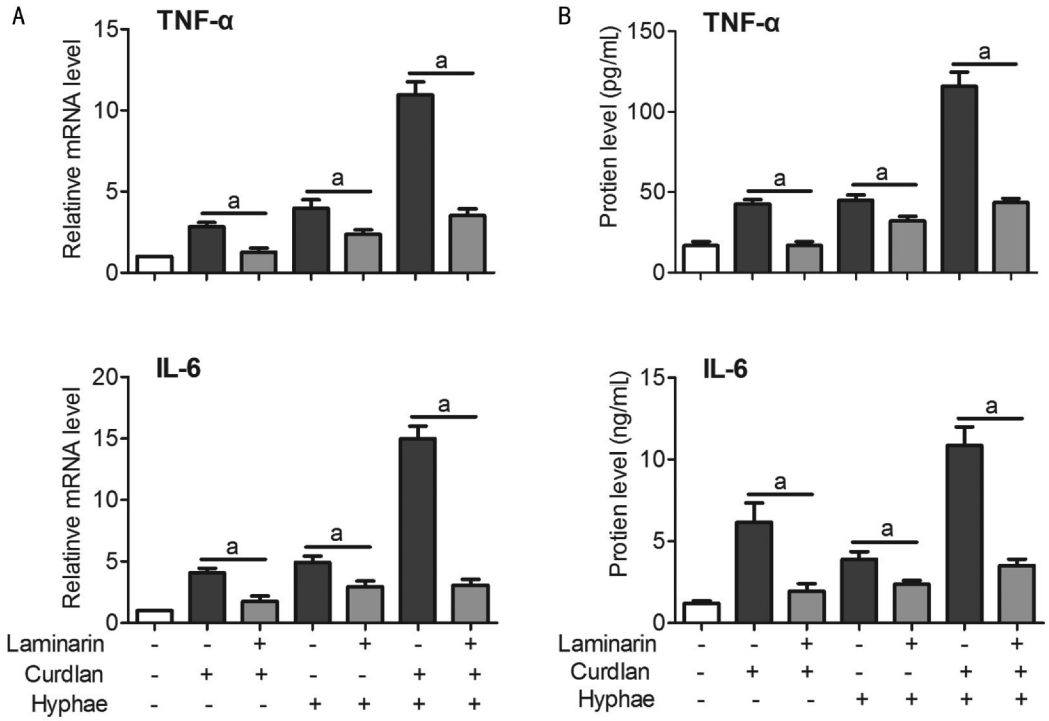

Figure 4 The immunomodulatory effects of curdlan were blocked by laminarin The HCECs were cultured without or with laminarin (0.3 mg/mL) for 30min, then stimulated with curdlan (200 μg/mL) or *A. fumigatus* hyphae (5×10^6/mL). The HCECs treated by curdlan or hyphae for 8h were subjected to RT-qPCR to measure mRNA (A), the HCECs treated for 24h were used to evaluate protein in medium supernatants by ELISA (B). $^aP<0.05$.

infections [27,28]. Together, human genetics and animal model studies demonstrate that Dectin-1 have an essential role in fungal keratitis, and therefore is a potential target for immunotherapy in prevention and treatment of fungal keratitis.

Curdlan, a pure linear β- (1,3)-glucan, is similar to the glucans in the majority of fungi surface and has been identified as a selective Dectin-1 agonist. It has been applied as an ingredient of various foods for many years. In recent years, its immunomodulatory effects attract more and more researchers [12,29]. Recent studies revealed that low curdlan concentrations leads to significant transcriptional modulation of TH1-type cytokines in lungs [30]. It also has been verified that curdlan enhances the production of TH1 or TH17 type cytokines, and induces the activation of cytotoxic T lymphocytes in macrophages and dentritic cells [31,32]. In the current study, we showed that curdlan activated transcription of TNF-α and IL-6 in HCECs, which is consistent with reports in macrophages and other immune cells. Additionally, we further identified that curdlan pretreatment enhanced *A. fumigatus* -induced TNF-α and IL-6 transcription and secretion in HCECs. These findings implied that curdlan pretreatment can strengthen the innate immune response of HCECs when confronted with *A. fumigatus*, which has potential clinical application.

Based on previous *in vitro* studies, β-glucans can act on several immune receptors including Dectin-1 and TLR-2/6, and trigger the immune response in a group of immune cells including macrophages, neutrophils, monocytes, and dendritic cells [31,33,34]. Recent Studies with β-(1,3)-glucans indicated that curdlan activates the transcription of the pro-inflammatory cytokine IL-1β and NLRP3 inflammasome activation through a dectin-1 dependent pathway in human macrophages and dentritic cells [35,36]. Studies using mouse models also have shown that curdlan targets Dectin-1 on the surfaces of alveolar macrophages and bronchiolar epithelium in lungs [37]. In this report, we found that both curdlan and *A. fumigatus* hyphae stimulated expression of Dectin-1 in HCECs, while Dectin-1 expression was higher by curdlan pretreatment followed *A. fumigatus* hyphae stimulation. Furthermore, blockage of Dectin-1 significantly suppressed the expression of pro-inflammatory cytokines induced by curdlan, indicating that Dectin-1 is essential for the immunomodulatory effect of curdlan on innate immune response in HCECs infected by *A. fumigatus*. In conclusion, results of the current studies demonstrated that curdlan pretreatment activates Dectin-1, stimulates pro-inflammatory cytokines production and enhances the innate immune response to *A. fumigatus* hyphae in HCECs. Therefore, curdlan may help cornea to resist *A. fumigatus* infection.

Researchers have made an understanding to the relationship between the expression of Dectin-1 in corneal epithelial cells and pathogenesis fungal infections. The idiographic role of Dectin-1 in resistance to fungal infection in innate immune

stage as PRRs need further study. Thus our study illustrates the effects of curdlan pretreatment on Dectin-1 and Dectin-1-conducted innate immune response in HCECs. These findings revealed the indispensible role of Dectin-1 in fungal keratitis, and intervention on Dectin-1 may enhance the innate immunity to fungi. Curdlan is proved to have the immunomodulatory effects through Dectin-1 in fungal keratitis and may have high clinical application values. Future efforts should direct at verifying the actual efficacy of curdlan by animal models and clinical trials.

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Correction

The paper "A comparative study on the transplantation of different concentrations of human umbilical mesenchymal cells into diabetic rat" DOI: 10.3980/j.issn.2222-3959.2015.02.08 was published in No.2 issue of IJO on 18th April.

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