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• Basic Research •

Regulation of lipoxygenase-1 and Dectin-1 on interleukin-10 in mouse *Aspergillus fumigatus* keratitis

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Abstract

• AIM: To investigate the regulation of lipoxygenase (LOX)-1 and Dectin-1 on interleukin-10 (IL-10) production in mice with *Aspergillus fumigatus* (*A. fumigatus*) keratitis.

• METHODS: The corneas of C57BL/6 mice were pretreated with LOX-1 inhibitor Poly(I) or Dectin-1 siRNA separately before the infection of *A. fumigatus*. Polymerase chain reaction (PCR) and Western blot were used to detect the expression of IL-10.

• RESULTS: The mRNA and protein expressions of IL-10 were significantly increased in mice with *A. fumigatus* keratitis. Compared with the group pretreated with sterile water before infection, Poly(I) pretreatment suppressed IL-10 expression significantly. Compared with the group pretreated with scrambled siRNA before infection, Dectin-1 siRNA pretreatment significantly reduced IL-10 expression in response to *A. fumigatus* infection.

• CONCLUSION: LOX-1 and Dectin-1 regulate IL-10 production in mouse *A. fumigatus* keratitis.

• KEYWORDS: keratitis; *Aspergillus fumigatus*; Dectin-1; lipoxygenase-1; interleukin-10

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INTRODUCTION

Fungal keratitis is an infectious corneal disease common in agricultural areas that can cause visual impairment and blindness[1-3]. Pattern recognition receptors (PRRs) are triggered by pathogen-associated molecular patterns (PAMPs) derived from microorganisms, leading to the production of many pro/anti-inflammatory cytokines[4-5]. The immune response protects the host from pathogenic microorganisms, but the strict regulation of the induced response is a necessary condition for limiting bystander damage[6]. But how to strike a balance between infection/inflammation and bystander damage? Interleukin-10 (IL-10) is a general anti-inflammatory cytokine, it mediates anti-inflammatory response and limits bystander damage[6-7]. Previous studies have shown that the recognition of PAMPs by Toll-like receptors (TLRs) induces IL-10 production in macrophages[8-9] and B cells[10-11] and dendritic cells (DCs)[12]. Dectin-1 is one of C-type lectin receptors (CLRs) that participates in many activities in protection against fungi by inducing cytokine production, reactive oxygen species[13-14]. Lipoxygenase-1 (LOX-1) is expressed in many types of cells, and our previous studies have proved that LOX-1 was expressed in human corneal epithelial cells and was up-regulated after *Aspergillus fumigatus* (*A. fumigatus*) stimulation[15]. What's more, LOX-1 was involved in production of pro-inflammatory cytokines in mice with fungal keratitis[3,16]. However, whether or not LOX-1 and Dectin-1 are involved in the regulation of IL-10 production in mouse with *A. fumigatus* keratitis is rarely studied. Our study showed that IL-10 was up-regulated upon fungal stimulation and was regulated by LOX-1 and Dectin-1 in *A. fumigatus* keratitis.

MATERIALS AND METHODS

Mice and Corneal Infection Eight-week C57BL/6 female mice (Changzhou Cavens Laboratory Animal Ltd., Jiangsu, China) were used in experiments. *A. fumigatus* strain (No.3.0772) was provided by the China General Microbiological Culture Collection Center (Beijing, China). After anesthesia, 2 μL conidial suspension (5.0×10^4 conidia/μL PBS) was given into the mice corneal stroma with a No.33 Hamilton syringe. All animal experiments were in accordance with Statement on the Use of Animals in Ophthalmic and Vision Research announced by Association for Research in Vision and Ophthalmology (ARVO).

Down-regulation of Lipoxygenase-1 and Dectin-1 A total of 5 μL LOX-1 inhibitor Poly(I) (2 μg/5 μL; Sigma) or sterile water was given into the left eyes of C57BL/6 mice (n=6/group/time) by subconjunctival injection 1d before or at the time of *A. fumigatus* infection. The 5-μL Dectin-1 siRNA (40 μmol/L; Santa Cruz) or scrambled siRNA (Santa Cruz) was given into the left eyes of C57BL/6 mice (n=6/group/time) by subconjunctival injection 1d before or at the time of *A. fumigatus* infection.

Table 1 Nucleotide sequences of mouse primers for real-time RT-PCR

Gene	GenBank No.	Primer sequence (5′–3′)	Size (bp)
β-actin	NM_007393.5	F-GATTACTGCTCTGGCTCCTAG C	147
		R-GACTCATCGTACTCCTGCTTGC	
IL-10	NM_010548.2	F-TGCTAACCGACTCCTTAATGCAGGAC	126
		R-CCT TGATTTCTGGGCCATGCTTCTC	

RT-PCR: Reverse transcription-polymerase chain reaction; IL-10: Interleukin-10.

Real-time Reverse Transcription-polymerase Chain Reaction After sacrifice, the corneas of C57BL/6 mice were removed. Total RNA was extracted using RNAiso plus reagent (TaKaRa) and rapidly detected using spectrophotometry (Eppendorf). Of 2 μg total RNA was reverse transcribed using the PrimeScript RT Reagent Kit (TaKaRa). Real-time PCR was carried out using SYBR green and Eppendorf Mastercycler. All values obtained were normalized to β-actin. The sequences of oligonucleotide primers are listed in Table 1.

Western Blotting Radioimmunoprecipitation assay (RIPA; Solarbio) mixed with PMSF (Solarbio) and phosphatase inhibitor cocktail (MCE) (100:1:1) was used to extract total protein from the corneas of C57BL/6 mice. Total protein was run on 8%-16% SDS-PAGE gels (GenScript, China) and transferred to PVDF membrane (Millipore). Membranes were blocked with blocking buffer (Beyotime, China) at room temperature for 2h and incubated with IL-10 primary antibody at 4℃ overnight. Horse radish peroxidase-linked anti-mouse was used for secondary antibodies. Electrochemical luminescence reagents (BIO-RAD) were used to detect the signal of the bands.

Statistical Analysis Data are expressed as mean±SD and were analyzed by GraphPad 5.0 software (USA). Statistical significance of real-time RT-PCR, Western blotting data was determined by unpaired, two-tailed Student's *t*-test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Increase of IL-10 in mouse *A. fumigatus* keratitis In order to facilitate the follow-up study, the time-dependent expression of IL-10 was examined in mice with *A. fumigatus* keratitis. PCR and Western blot were done to examine the expressions of IL-10 in normal and *A. fumigatus* infected mouse corneas. Compared with the normal control, infection of mice corneas by *A. fumigatus* significantly increased the mRNA level of IL-10 at 12h ($P<0.001$; Figure 1A), 1d ($P<0.001$; Figure 1A) and 2d ($P<0.001$; Figure 1A). IL-10 mRNA expression increased gradually from 12h after *A. fumigatus* infection (Figure 1A).

The result of Western Blot showed that there is no significant difference in IL-10 protein expression level between the normal control and 12h p.i (Figure 1B), but it was significantly increased at 1d ($P<0.001$; Figure 1B) and 2d ($P<0.001$; Figure 1B).

Regulation of LOX-1 on IL-10 in mouse *A. fumigatus* keratitis Since LOX-1 is involved in the defense of fungal infection, we set out to investigate the regulation of LOX-1 on IL-10 production in mice with *A. fumigatus* keratitis. The corneas of C57BL/6 mice were challenged with LOX-1 inhibitor Poly(I) before *A. fumigatus* infection. Compared with uninfected sterile water control, IL-10 mRNA expression in response to *A. fumigatus* infection was significantly increased ($P<0.001$; Figure 2A). However, compared with the group pretreated with sterile water before infection, Poly(I) pretreatment suppressed IL-10 mRNA expression significantly ($P<0.001$; Figure 2A).

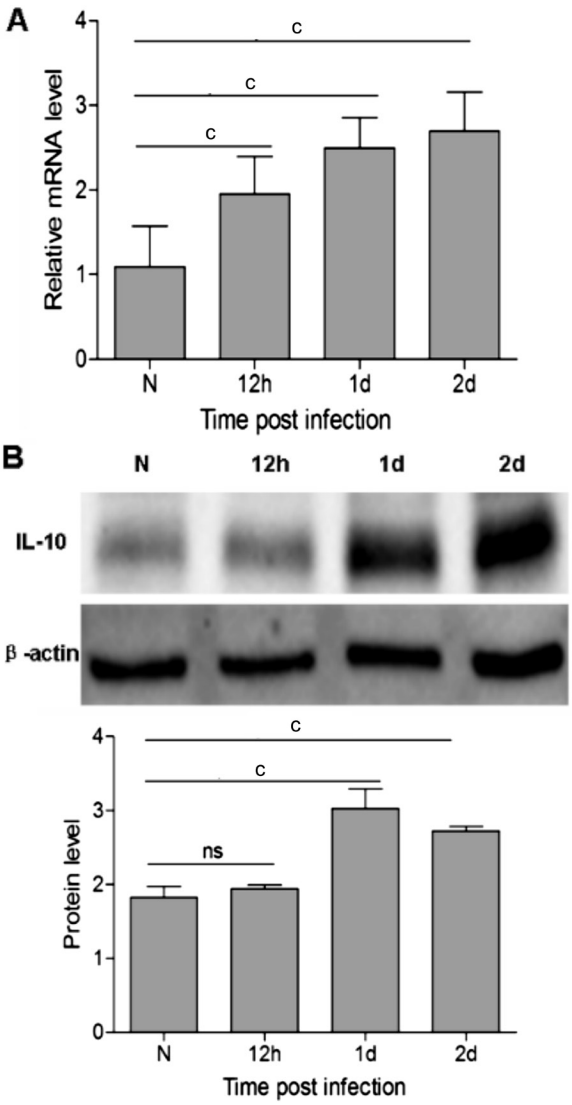

Figure 1 IL-10 expression was upregulated in mice with *A. fumigatus* keratitis A: IL-10 mRNA fold change in C57BL/6 mice corneas infected by *A. fumigatus* at 12h, 1d and 2d; B: IL-10 protein fold change in C57BL/6 mice corneas infected by *A. fumigatus* at 12h, 1d and 2d. °$P<0.001$.

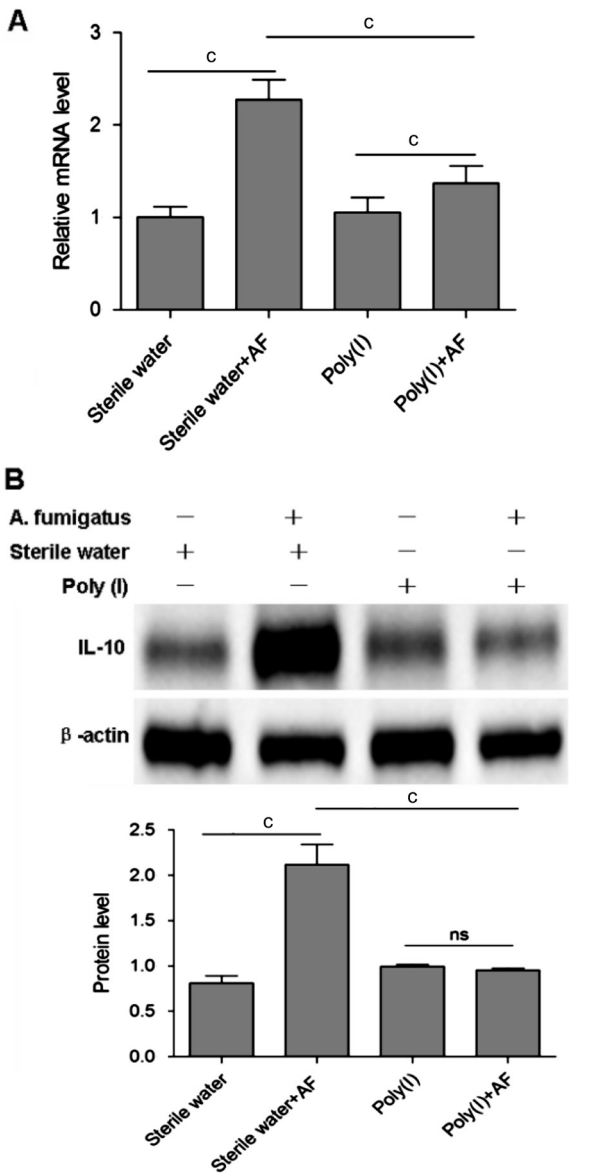

Figure 2 The change of IL-10 expression when C57BL/6 mice corneas were pretreated with LOX-1 inhibitor Poly(I) before *A. fumigatus* infection Compared with the group pretreated with sterile water before infection, Poly(I) pretreatment suppressed IL-10 mRNA (A) and protein (B) expression significantly. $^{c}P<0.001$.

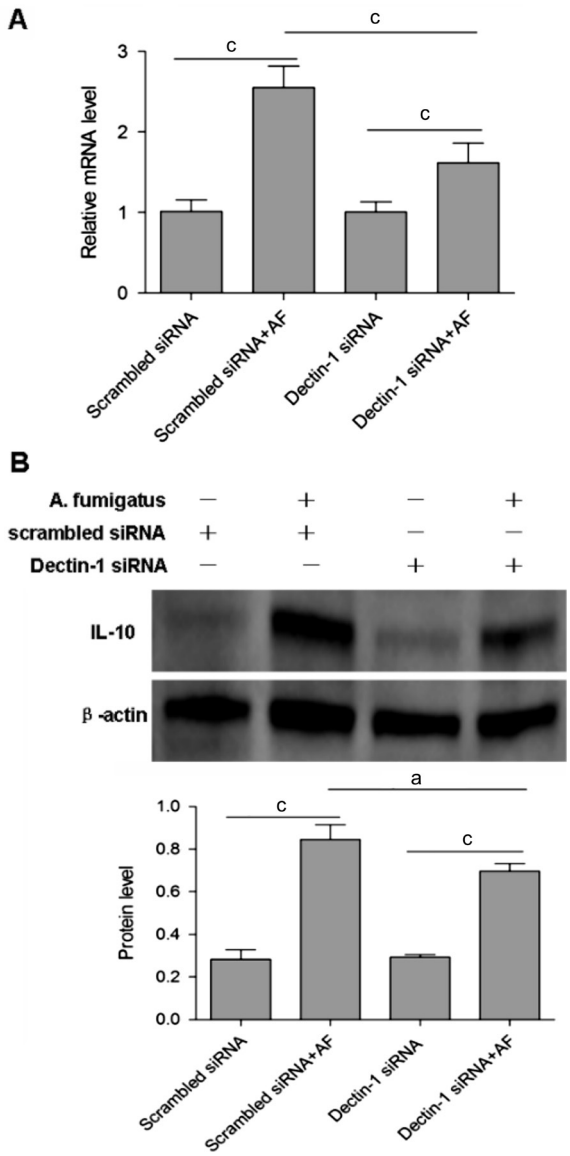

Figure 3 The change of IL-10 expression when C57BL/6 mice corneas were pretreated with Dectin-1 siRNA before *A. fumigatus* infection The group pretreated with Dectin-1 siRNA before *A. fumigatus* infection had a lower IL-10 mRNA (A) and protein (B) expression compared with the group pretreated with scrambled siRNA before *A. fumigatus* infection. $^{a}P<0.05$, $^{c}P<0.001$.

The IL-10 protein expression was also detected. The change of IL-10 protein expression showed that IL-10 protein level was significantly increased after *A. fumigatus* infection compared with uninfected sterile water control ($P<0.001$; Figure 2B). But LOX-1 inhibitor Poly(I) suppressed the up-regulation significantly ($P<0.001$; Figure 2B). The results suggested that IL-10 was regulated by LOX-1 in mouse *A. fumigatus* keratitis.

Regulation of Dectin-1 on IL-10 in mouse *A. fumigatus* keratitis Upon *easts* and *zymosan* stumulation, Dectin-1 was involved in IL-10 production, to determine the role of Dectin-1 in *A. fumigatus* induced IL-10 production, special siRNA targeting Dectin-1 was used in this study. A higher level of IL-10 mRNA expression was detected in scrambled siRNA pretreated mice with *A. fumigatus* infection than uninfected scrambled siRNA control ($P<0.001$; Figure 3A). Dectin-1 silencing significantly reduced IL-10 mRNA expression in reponse to *A. fumigatus* infection when compared infected Dectin-1 siRNA pretreated group with scrambled siRNA control ($P<0.001$; Figure 3A).

The result of Western Blot showed that the group pretreated with Dectin-1 siRNA before *A. fumigatus* infection had a lower IL-10 protein expression compared with the group pretreated with scrambled siRNA before *A. fumigatus* infection ($P<0.05$; Figure 3B). which is consistent with the fold change of mRNA. The results suggested that Dectin-1 induced the production of IL-10 in mouse *A. fumigatus* keratitis.

DISCUSSION

Fungal recognition and uptake by the cornea induces the production of inflammatory response, which can remove

fungi on the one hand and, cause corneal tissue damage on the other hand. How to limit tissue damage caused by inflammatory response while removing the fungus? Our study found that IL-10 expression was elevated in *A. fumigatus* keratitis. IL-10 was initially classified as a Th2-type cytokine, it functions to mediate anti-inflammatory response and limits bystander damage[6]. For example, it can inhibit the synthesis and release of inflammatory cytokines TNF-α, IL-1, IL-6, IL-12 and so on[17], suppress antigen presenting cell function[18-19], and inhibit Th1 type immune response[6]. However, on the other hand, IL-10 mediated anti-inflammatory response can be exploited by pathogens, which contributes to establish pathogenic persistence and chronic infection status[6,20-22]. For example, many chronic infection of bacteria and viruses were associated with elevated IL-10 expression[23]; DCs and macrophages induced IL-10 production can serve as an immune evasion mechanism for many pathogens; IL-10 and IL-10 induced Treg cells impairs the control and clearance of *Candida albicans*[20], *Mycobacterium tuberculosis*[24], and *Schistosoma mansoni*[25] in these infection models. Given that the role of IL-10 in the host inflammatory response may not show redundancy. It's important for us to investigate the regulation of IL-10 production in mouse with *A. fumigatus* keratitis. Our study showed that IL-10 was regulated by LOX-1 and Dectin-1 in *A. fumigatus* keratitis.

PRRs detect PAMPs of the pathogens in the first line of host immunity and, then trigger an immune response[26-28]. TLRs and CLRs expressed on DCs and other immune cells are a good example of PRRs[29-31]. DCs, as an immune cell, were initially considered to be important in starting immune response, but more recent evidence suggests that they play a key role in regulating quality of the immune response and in inhibiting the immune response[26-27]. PRRs are essential for immune cells to perform their functions. LOX-1 and Dectin-1 belong to CLRs. LOX-1, one of the major receptors of oxidized low density lipoprotein (oxLDL), plays a role of promoting atherosclerosis through activating NF-κB to promote gene expression of pro-inflammatory cytokines[32]. However, it shows an atheroprotective aspect by receptor-mediated oxLDL uptake in the presence of anti-inflammatory cytokines such as IL-10[33]. IL-10 increases the expression of LOX-1, and IL-10-induced LOX-1 contributed to scavenging of oxLDL without affecting the pro-inflammatory signaling[34]. LOX-1 induces increased expression of pro-inflammatory cytokines in mice with fungal keratitis[3,15-16], while can it also induce the expression of IL-10? Our study showed that LOX-1 enhanced the expression of IL-10 in mice with *A. fumigatus* keratitis.

Dectin-1 recognizes the β-glucans on the surface of fungal cell walls and is involved in anti-fungal immunity[35-37]. A variety of cellular functions can be mediated by Dectin-1, including identification and combination of fungi, uptake and killing, inducing a protective Th1 cells response and the production of cytokines and chemokines[9,38-39], these all contribute to anti-fungal immunity. Studies have shown that Dectin-1 is also involved in the production of IL-10. Recognition of *yeasts* and *zymosan* by Dectin-1 induced IL-10 production, which could suppress inflammatory cytokines production and contribute to the Treg cells development, thereby limiting inflammation as well as contributing to fungal tolerance and long-term infection[38,40-43]. Our study showed that Dectin-1 induced the expression of IL-10 in mice with *A. fumigatus* keratitis, which may play a role in rebalancing the inflammatory response and contributing to fungal tolerance.

Taken together, IL-10 was upregulated in mice with *A. fumigatus* keratitis, and it was regulated by LOX-1 and Dectin-1. This suggests that inflammation evoke by LOX-1 and Dectin-1 contributes to fungal clearance, at the same time LOX-1 and Dectin-1 can induce the production of IL-10, which may play a role in rebalancing inflammation and limiting tissue damage, even in immune evasion.

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