CD23 mediated the induction of pro-inflammatory cytokines Interleukin-1 beta and tumor necrosis factors-alpha in *Aspergillus fumigatus* keratitis

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To the Editor: Fungal keratitis is primarily caused by pathogenic fungi species, such as *Fusarium solani* and *Aspergillus fumigatus*. The incidence of *A. fumigatus* has been increased worldwide.[1] Although new therapies have been used for fungal keratitis, the efficacy of antifungal drugs remains unsatisfactory at present.[2] Fungal keratitis is still a challenge for ophthalmologists because of its difficult and delayed diagnosis, as well as the lack of effective drugs and treatment.

After corneal damage occurs and infection sets in, the innate immune response is stimulated. It is the first line of defense against invasive fungal infections. Pattern recognition receptors (PRRs) have been reported to trigger immune defenses to fight off foreign pathogens.[3] CD23, a typical type II C-type lectin receptor (CLR), is expressed in a variety of lymphocytes and phagocytes. CD23 can identify α-mannan, as well as β-glucan, on the cell wall of *Candida albicans* and *A. fumigatus*. In addition, the production of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) can be upregulated by increased levels of CD23 via activating the nuclear factor kappa B (NF-κB) in innate immunity against fungal infection.[4] Zhao *et al*[5] reported that the expression of CD23 increased significantly in c-Jun N-terminal kinase 1 (JNK1) deficient mice after *C. albicans* infection, and it can negatively regulate innate immunity against invasive fungal infections in JNK1-integrity experimental subjects. Based on these findings, we can conclude that CD23 is an important component in response to fungal infections. However, the functions of CD23 in *A. fumigatus* keratitis are undefined.

This study was approved by the ethics committee of the Affiliated Hospital of Qingdao University and in accordance with the *Helsinki Declaration* of 1975, as revised in 2000. We informed the participating patients of the purpose and methods of the study in detail. After obtaining an informed consent, we began to manage the specimens. Patients with other inflammatory diseases and immune diseases were not allowed to enroll in this study. In total, six healthy donor corneas (six eyes) were used for keratoplasty, and the rest of the peripheral corneal tissues were collected. Six patients (six eyes) suffering from *A. fumigatus* keratitis who underwent penetrating keratoplasty and corneas with lesions were recruited. Half of the corneal epithelium was scraped off and placed in a 500 μL RNAiso (Takara, Dalian, Liaoning, China) and used for mRNA extraction and PCR reaction. The rest were used for immunofluorescence staining for CD23. The results of the immunofluorescence staining revealed that CD23 protein was highly expressed in *A. fumigatus* keratitis corneas, especially in the epithelial cells (a, epithelium; b, stroma) [Figure 1A]. Therefore, we chose human cornea epithelial cells (HCECs) for our study. Furthermore, in CD23 mRNA expression, a marked increase was observed in comparison with healthy corneas [Figure 1B].

Meanwhile, we performed mice experiments. All mouse treatments were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were anesthetized using 8% chloral hydrate (0.08 mL/mouse) administered through intraperitoneal injection. Then, the *A. fumigatus* conidia (0.5×10^5/μL) was injected into the corneal stroma using a micro-injector. The opacity and ulceration in the mouse corneas were recorded daily using a slit-lamp microscope (Keeler Ltd, Windsor, United Kingdom). The corneas and eyes were collected at particular time points after the establishment of the mouse models. In order to visually observe the corneal lesions, we used clinical scores to grade the level of ocular disease. Corneal opacity was recorded at 12 h and 1, 2, 3, 5, 7, 10, and 14 days after infection. The

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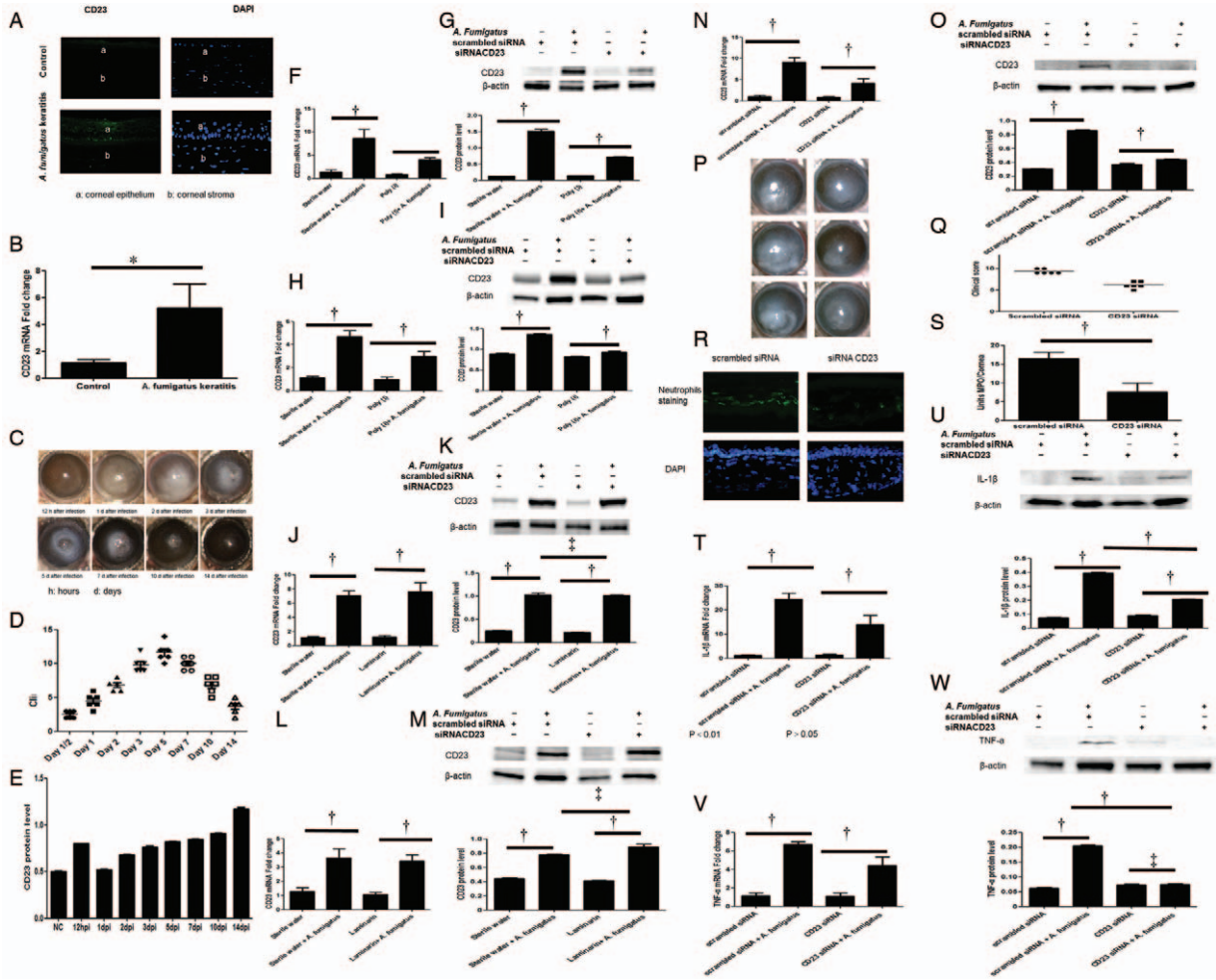

Figure 1: CD23 was involved in human *A. fumigatus* keratitis. (A) The protein level of CD23 in healthy and fungal keratitis patient corneas. CD23 is shown as green, and DAPI is shown as blue (original magnification ×400). (B) CD23 mRNA expression in healthy and fungal keratitis patient corneas. $^{*}P < 0.05$. (C) Representative corneal images of *A. fumigatus* murine models at 12 h and 1, 2, 3, 5, 7, 10, and 14 days after corneal fungal infection. (D) Clinical scores increased with fungal keratitis. (E) Changes of CD23 protein expression after infected with *A. fumigatus* keratitis. LOX-1 inhibitor can suppress. (F) CD23 mRNA and (G) protein expression upon *A. fumigatus* infection in the mouse model. $^{\dagger}P < 0.01$. (H) CD23 mRNA and (I) protein levels can be suppressed by LOX-1 inhibitors in HECEs. Dectin-1 inhibitor cannot suppress. (J) CD23 mRNA and (K) protein expression upon *A. fumigatus* infection in the mouse model. $^{\ddagger}P > 0.05$. (L) CD23 mRNA and (M) CD23 protein levels cannot be suppressed by Dectin-1 inhibitors in HECEs. (N) CD23 mRNA and (O) protein levels at 1 day after CD23 siRNA treatment compared with scrambled siRNA treatment. (P) Pictures were taken by slit-lamp 1 day after CD23 siRNA treatment compared with scrambled siRNA treatment. (Q) The clinical grade in CD23 siRNA pretreated mice compared with scrambled siRNA pretreated mice. (R) Immunofluorescence staining shows the activity of neutrophils in the corneas, 1 day postinfection. Green fluorescence represents neutrophils, and DAPI is shown as blue (original magnification ×400). (T) IL-1β mRNA expression in CD23 siRNA or scrambled siRNA pretreated mouse model 1 day postinfection. (U) IL-1β protein expression in CD23 siRNA or scrambled siRNA pretreated mouse model 1 day postinfection. (V) TNF-α mRNA expression in CD23 siRNA or scrambled siRNA pretreated mouse model 1 day post-infection. (W) TNF-α protein expression in CD23 siRNA or scrambled siRNA pretreated mouse model 1 day postinfection. DAPI: 4′,6-diamidino-2-phenylindole; HCECs: Human cornea epithelial cells; IL-1β: Interleukin-1 beta; LOX-1: Lectin-like, oxidized low-density lipoprotein receptor-1; TNF-α: Tumor necrosis factors-alpha; siRNA: Small Interfering RNA.

corneal ulcer presents a gradual process of aggravation and remission [Figure 1C]. Clinical scores can also clearly show this trend [Figure 1D]. After Western blotting, we found that CD23 increased at the initial stage of *A. fumigatus* keratitis and persisted for a long time [Figure 1E].

Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1) and Dectin-1 are PRRs, and it is essential for innate immunity activation because PRRs can recognize and interact with pathogen-associated molecular patterns (PAMPs). In order to identify the PRRs involved in CD23 expression following *A. fumigatus* infection, we used the LOX-1 inhibitor (poly I; SM528906, Sigma, St. Louis, Missouri, USA) or Dectin-1 inhibitor (laminarin; SCH772984, Selleck Chem, USA) to pretreat the mice 1 day and 2 h before fungal infection. HCECs (provided by Zhongshan Ophthalmic Center, Guangzhou, Guangdong, China) were incubated at 37°C in 5% CO_2 until near 80% confluency. Then, HCECs were pretreated with the LOX-1 inhibitor or the Dectin-1 inhibitor, in comparison to sterile water for 2 h before receiving conidia treatment. The cells were cultured in another incubator for 16 h under the same environmental conditions. Finally, they were harvested and used for real-time quantitative PCR (qRT-PCR) or Western blotting analysis.

Compared with the sterile water control group, the mRNA [Figure 1F] and protein [Figure 1G] levels of CD23 expression decreased significantly after LOX-1 inhibitor treatment 2 days after mice corneal infection. Therefore, it is likely that the expression of CD23 mRNA [Figure 1H] and protein [Figure 1I] expression in the HCECs also

decreased. However, after Dectin-1 inhibitor treatment, the expression of CD23 mRNA [Figure 1J] and protein [Figure 1K] did not decrease. Similar to the murine model, the expression of CD23 mRNA [Figure 1L] and protein [Figure 1M] was not downregulated in the HCECs. In this study, elevated CD23 production upon *A. fumigatus* keratitis was significantly suppressed by the LOX-1 inhibitor, but was not affected by the Dectin-1 inhibitor. This finding suggested that activated LOX-1 upregulated the CD23's expression, and contributed to the inflammatory response in *A. fumigatus* keratitis. Unlikely, Dectin-1 did not participate in the generation of CD23.

For small interfering RNA (siRNA) treatments, mouse eyes were administered a subconjunctival injection (5 μL) of 8 μmmol/L CD23 siRNA or scrambled siRNA controlled the same way. After siRNA treatment, we performed further experiments, such as clinical evaluation, myeloperoxidase assay and immunofluorescence assay of neutrophils. In order to determine whether CD23 siRNA treatment was effective, we examined CD23 mRNA [Figure 1N] and protein [Figure 1O] levels, one day after infection. The results demonstrated the efficiency of gene knockdown ($P < 0.01$) in the corneas of mouse fungal keratitis models. The severity of fungal keratitis was shown using slit lamp pictures [Figure 1P] at 1 day after infection in the CD23 siRNA pretreated control using scrambled siRNA pretreated mice. After the CD23 siRNA treatment, mouse corneal clarity and clinical scores increased during early fungal keratitis [Figure 1Q]. The reduction of inflammation during the initial stage of infection may be related to the decrease in the recruitment of neutrophils [Figure 1S]. Furthermore, immunofluorescence staining [Figure 1R] also proved the activity of neutrophils in the corneas 1-day post-infection. In contrast with scrambled siRNA pretreated mice, the number of neutrophils decreased significantly in the corneas of CD23 siRNA pretreated mice. (Green fluorescence represented neutrophils, while DAPI (4',6-diamidino-2-phenylindole) was marked as blue) The recruitment of neutrophils during the initial stage upon fungal infection, which subsequently killed the fungi, and can also contribute to processes that impair normal tissues under special circumstances.[6] Severe tissue damage can lead to a decrease in corneal clarity and diminution of vision. Vision greatly depends on corneal transparency, hence it is essential for the cornea to effectively kill invasive fungi, while avoiding excessive neutrophil infiltration. This makes us look forward to the rational use of CD23.

Similar to immune cells recruitment, CD23 knockdown could obviously impair the generation of pro-inflammatory cytokines after fungal infection. Pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factors-alpha (TNF-α), play significant roles in host antifungal immune responses. IL-1β is produced by many types of cells, such as endothelial, fibroblasts and monocytes, in response to corneal fungal infection. It has been widely regarded as a crucial inflammatory factor involved in the corneal antifungal immune response. Apart from killing and inhibiting the action of tumor cells, tumor necrosis factors can trigger a series of inflammatory responses. In this study, after the CD23 siRNA treatment, the upward trend in the expression of IL-1β and TNF-α was markedly inhibited, 1 day post-infection [Figures 1T–1W]. These findings indicated that CD23 contributed to the production of IL-1β and TNF-α in response to *A. fumigatus* infection.

The results revealed the roles of CD23 in fungal keratitis. CD23 was significantly activated in response to fungal keratitis, but was inhibited by the LOX-1 inhibitor. CD23 induced the production of pro-inflammatory cytokines, IL-1β, and TNF-α, following the onset of *A. fumigatus* keratitis. Thus, we can conclude that CD23 is an indispensable component for antifungal innate immunity.

In conclusion, our findings give us novel insights into the function of CD23, as a critical component for the response toward fungal keratitis. This response is regulated by LOX-1 and can mediate the induction of pro-inflammatory cytokines, IL-1β, and TNF-α with fungal keratitis.

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Conflicts of interest

None.

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