

Boxb mediate BALB/c mice corneal inflammation through a TLR4/MyD88-dependent signaling pathway in *Aspergillus fumigatus* keratitis

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Abstract

• **AIM:** To investigate whether high-mobility group box 1 (HMGB1) Boxb exacerbates BALB/c mice corneal immune responses and inflammatory through the Toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88)-dependent signaling pathway in *Aspergillus fumigatus* (*A. fumigatus*) keratitis.

• **METHODS:** The mice corneas were pretreated with phosphate buffer saline (PBS), Boxb before *A. fumigatus* infection. The abdominal cavity extracted macrophages were pretreated with PBS, Boxb, TLR4 inhibitor (CLI-095), Dimethyl sulfoxide (DMSO) separately before *A. fumigatus* hyphae stimulation. HMGB1 was detected in normal and infected mice corneas and macrophages by real-time reverse transcriptase polymerase chain reaction (RT-PCR), the TLR4, MyD88, interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) were detected by Western blot and PCR.

• **RESULTS:** In BALB/c mice corneas, the expressions of TLR4, HMGB1, IL-1 β , TNF- α were increased after *A. fumigatus* infection. While pretreatment with Boxb significantly increased the expressions of TLR4, HMGB1, MyD88, IL-1 β , TNF- α compared with PBS control after infection. In BALB/c mice abdominal cavity extracted macrophages, pretreatment with Boxb increased the expressions of TLR4, HMGB1, MyD88, IL-1 β , TNF- α , while pretreatment with CLI-095 and Boxb significantly decreased the expressions of TLR4, HMGB1, MyD88, IL-1 β , TNF- α .

• **CONCLUSION:** In *A. fumigatus* keratitis, Boxb play a pro-inflammatory role in corneal anti-fungi immune response through the HMGB1-TLR4-MyD88 signal pathway.

• **KEYWORDS:** high-mobility group box 1; *Aspergillus fumigatus* keratitis; Toll-like receptor 4

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INTRODUCTION

Fungal keratitis is a severe suppurative ocular disease. It is caused by pathogenic fungi and has a gradually increased incidence in China^[1-3]. Trauma, corticosteroid or antibiotics abuse, extended usage of contact lens are the predisposing factors of fungal keratitis^[4]. The innate immunity of cornea play an important role in defense against pathogenic fungi infection. It is regulated by different pattern recognition receptors (PRRs)^[5-7]. PRRs could recognize the damage-associated molecular patterns (DAMPs).

Toll-like receptors (TLRs) play an indispensable role in the early detection of DAMPs and orientation of adaptive immunity^[8-9]. Fungal keratitis can stimulate the expression of various endogenous TLR ligands, especially TLR4, in the damaged corneal tissue^[10]. The endogenous molecule high-mobility group box 1 (HMGB1) was known to interaction with TLR4, and was regarded as a proinflammatory cytokine that mediates local inflammation, endotoxin lethality, and macrophages activation^[11-13]. More recently, HMGB1 was recognized as DAMP^[14-15]. HMGB1 is released by activated monocytes and macrophages after exposure to lipopolysaccharide (LPS), interleukin-1 β (IL-1 β) or tumor necrosis factor- α (TNF- α), and acted back on macrophages and monocytes by stimulating the synthesis of additional proinflammatory cytokines^[12,16]. HMGB1 has two homologous DNA-binding motifs, addressed as HMG A and HMG B boxes^[17-18]. And Boxb is identified as the proinflammatory domain, which is sufficient to recapitulate the cytokine activity of full-length HMGB1^[19-20]. There is no evidence showed the role of HMGB1 Boxb in *Aspergillus fumigatus* (*A. fumigatus*) infected BALB/c mice corneas. So, we explored the role of Boxb in BALB/c mice corneal innate immunity and the signal pathway of HMGB1/TLR4 in *A. fumigatus* keratitis.

MATERIALS AND METHODS

Corneal Infection BALB/c mice (8-week-old female) were purchased from the Changzhou Cavens Laboratory Animal Ltd. The left eyes were selected as experimental eyes. Mice were anesthetized by 8% chloral hydrate and magnified 40× using the stereoscopic microscope, and made a diameter 2 mm scratch on the left eyes central corneal epithelium. Covered the corneal surface by *A. fumigatus* (No.3.0772, China General Microbiological Culture Collection Center), and contact lens were placed. Eyelids were sewed up. Mice corneas were harvested at 1d after infection. The use of animals in Ophthalmic and Vision Research conformed to the ARVO Statement.

Boxb Treatment Boxb (0.5 µg/5 µL) or control phosphate buffer saline (PBS; 0.5 µg/5 µL) were given subconjunctivally the day before infection.

Macrophages Extraction Thioglycollate powder (3 g) was suspended in 100 mL dH₂O, stirred on heating plate to boiling for 1min. Stored at 4°C. Intraperitoneal (i.p.) injected 1 mL thioglycollate medium and 7d after injection, mice were sacrificed by cervical dislocation. The abdominal was swabbed by 75% alcohol and cut at the middle line through the skin. Slowly injected 6 mL harvest solution with syringe into mice abdominal cavity. Gently massaged the abdomen. Drew out as much of the harvest solution as possible.

Cells Culture and Stimulation Macrophages cells were seeded in Dulbecco's modified eagle medium (DMEM) and 10% fetal bovine serum (FBS) growth medium. Cells suspensions (1×10⁶/mL) were seeded in 12-well and 6-well culture plates, then cultured at 37°C in a humidified atmosphere containing 5% CO₂. Macrophages were pretreated with Boxb (1 µg/mL) or control PBS (1 µg/mL), CLI-095 (InvivoGen) (1 µg/mL) or control DMSO (1 µg/mL) for 2h before infection, then stimulated by *A. fumigatus*. for 12h and 24h.

Real-time Reverse Transcriptase Polymerase Chain Reaction Total RNA was isolated from corneas and cells by using RNAiso plus reagent (TaKaRa, Dalian, Liaoning Province, China) and quantified by spectrophotometry rapidly. Use total RNA of 2 µg to produce the complementary DNA by reverse transcription and SYBR Green was used for the quantitative polymerase chain reaction (PCR) reactions. The real-time reverse transcriptase PCR (RT-PCR) primer pair sequences are shown in Table 1. The housekeeping gene β-actin was used as an internal control. Used the 2^{-ΔΔCt} method to quantified.

Western Blotting Corneas and macrophages were used to detect the expressions of TLR4, HMGB1, TNF-α and IL-1β protein levels. Harvest the corneas 1d after infection with Boxb or PBS pretreatment. Harvest the macrophages 24h after infection with PBS, Boxb and CLI-095 pretreatment. Corneas and macrophages were lysed in RIPA (Solarbio) lysis buffer

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

Gene (5'-3')	GenBank No.	Primer sequence
β-actin	M_007393.3	F: GATTACTGCTCTGGCTCCTAG C R: GACTCATCGTACTCCTGCTTGC
TNF-α	M_013693.2	F: ACCCTCACACTCAGATCATCTT R: GGTTGTCTTTGAGATCCATGC
TLR4	NM_021297.3	F: CCTGACACCAGGAAGCTTGAA R: TCTGATCCATGCATTGGTAGGT
IL-1β	NM_008361.3	F: CGCAGCAGCACATCAACAAGAGC R: TGTCCTCATCCTGGAAGGTCCACG
HMGB1	NM-000071.6	F: GGCGAGCATCCTGGCTTATC R: GGCTGCTTGTTCATCTGCTG

contain phenylmethylsulfonyl fluoride (PMSF; 100:1). Total protein was separated on 12% acrylamide SDS-PAGE and transferred onto PVDF membrane (Solarbio). Then blocked the membranes at 37°C for 2h, and incubated with antibody to mouse anti-TLR4, anti-myeloid differentiation primary response 88 (MyD88), anti-TNF-α (Proteintech, Wuhan, China), anti-IL-1β (CST, MA, USA) and β-actin (Bioss, Beijing, China) at 4°C. Then incubated with secondary antibodies at 37°C for 1h. The blots were detected with chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis The data was expressed as the mean± standard deviation (SD) of at least three independent experiments. Data analysis of cells stimulation was performed by One-way ANOVA test using Graphpad Prism 5.0 software. To analysis the difference of clinical score between two groups, the Mann-Whitney *U* test were tested. Values were considered to be significant at *P*<0.05.

RESULTS

Boxb Exacerbated Corneal Inflammation in *A. fumigatus* Keratitis In order to observe the function of Boxb in *A. fumigatus* keratitis, BALB/c mice models were used to induce *A. fumigatus* keratitis. The corneas stay normal transparent status after 1d Boxb (0.5 µg/5 µL) or control PBS (0.5 µg/5 µL) were given subconjunctivally into the left eyes of BALB/c mice. But 1d after *A. fumigatus* infection, significant corneal edema and an irregular whitish mass could be seen (Figure 1B, 1C). And the Boxb group were more severe than the PBS group, as the corneal clinical score indicated in Figure 1A.

Boxb Up-regulated TLR4, HMGB1, IL-1β, TNF-α Expressions To investigate the function of Boxb in mice corneas after *A. fumigatus* induced corneal infection, we tested the expressions of TLR4, HMGB1, TNF-α, IL-1β. The real-time RT-PCR results showed that the relative mRNA levels of TLR4, HMGB1, TNF-α, IL-1β were significantly higher in Boxb pretreated mouse corneas than control PBS group at 1d after infection (*P*<0.05, *P*<0.05, *P*<0.01, *P*<0.05, respectively; Figure 2A-2D). To confirm these data, TLR4, IL-1β and

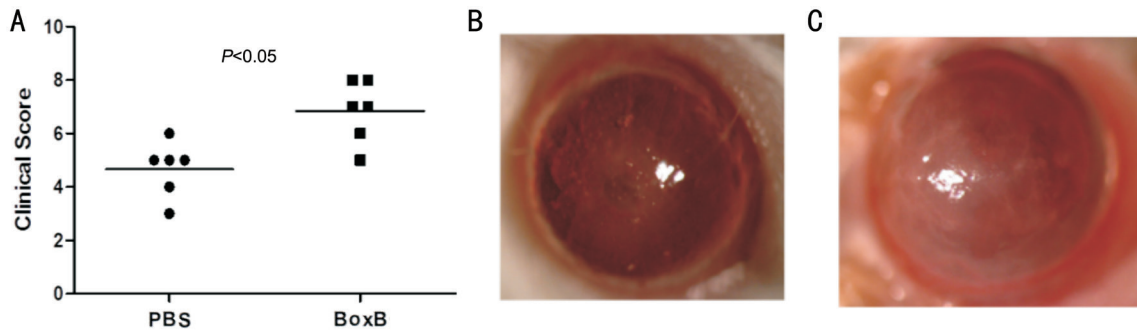


Figure 1 Corneal infection by *A. fumigatus* in BALB/c mice A: Clinical scores both increased in corneas infected by *A. fumigatus* pretreated with PBS and Boxb. Clinical score was shown as mean±SD. PBS (B) and Boxb (C) pretreated corneas were photographed under slit lamp 1d after infection.

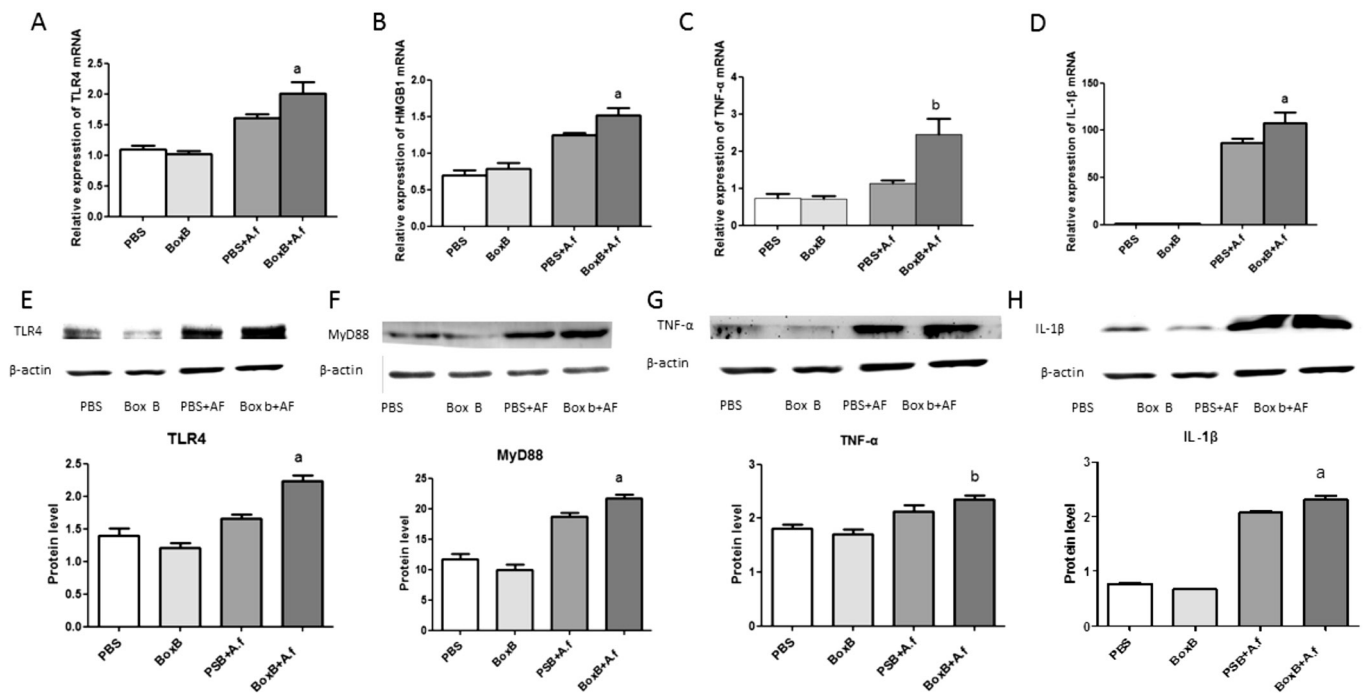


Figure 2 Effect of Boxb treatment on TLR4, MyD88, HMGB1, IL-1β and TNF-α in BALB/c mice corneas Corneas of BALB/c mice pretreated with Boxb compared with PBS at 1d. The mRNA expressions of TLR4 (A), HMGB1 (B), TNF-α (C), IL-1β (D) were significantly increased. And the protein levels of TLR4 (E), MyD88 (F), TNF-α (G) and IL-1β (H) were also increased. ^a*P*<0.05, ^b*P*<0.01 vs PBS+*A. fumigatus* group.

TNF-α protein levels were examined by Western blot. We found that TLR4, IL-1β and TNF-α protein expressions were low in control group, but increased significantly after *A. fumigatus* infection. The Boxb pretreated group can apparently increased the protein expressions of the TLR4, TNF-α and IL-1β (*P*<0.05, *P*<0.01, *P*<0.05, respectively; Figure 2E, 2G and 2H).

Boxb Exacerbates Inflammation Through the TLR4/MyD88-dependent Signaling Pathway in Macrophages of BALB/c Mice Macrophages are crucial immune cells in *A. fumigatus* keratitis, and it play a crucial role in the innate immunity. In Figure 2E and 2F, TLR4 and MyD88 both increased in Boxb pretreated corneas. Then in order to explore whether Boxb aggravate fungal keratitis inflammation through the TLR4/MyD88 pathway, CLI-095 and DMSO was added to the abdominal cavity extracted macrophages for 2h,

then stimulated with *A. fumigatus* hyphae and Boxb for 12h. Relative mRNA levels of TLR4, HMGB1, TNF-α and IL-1β were significant higher in Boxb treated group than PBS group (*P*<0.05, *P*<0.05, *P*<0.001, *P*<0.01, respectively) (Figure 3A-3D). In CLI-095 pretreated group, the relative mRNA levels of TLR4, HMGB1, TNF-α and IL-1β decreased dramatically compared with the Boxb group (*P*<0.001, *P*<0.001, *P*<0.001, *P*<0.001, respectively) (Figure 3A-3D). TLR4 protein level were tested by Western blot to confirm these data. TLR4 protein levels increased in Boxb group and decreased in CLI-095 group (Figure 3E). And the MyD88 protein level have the same tendency (Figure 3F).

DISCUSSION

HMGB1 was not only acted as a inflammatory responses promoter, but also recognized as a pro-inflammatory cytokine^[12]. HMGB1 enlarges and prolongs the inflammatory

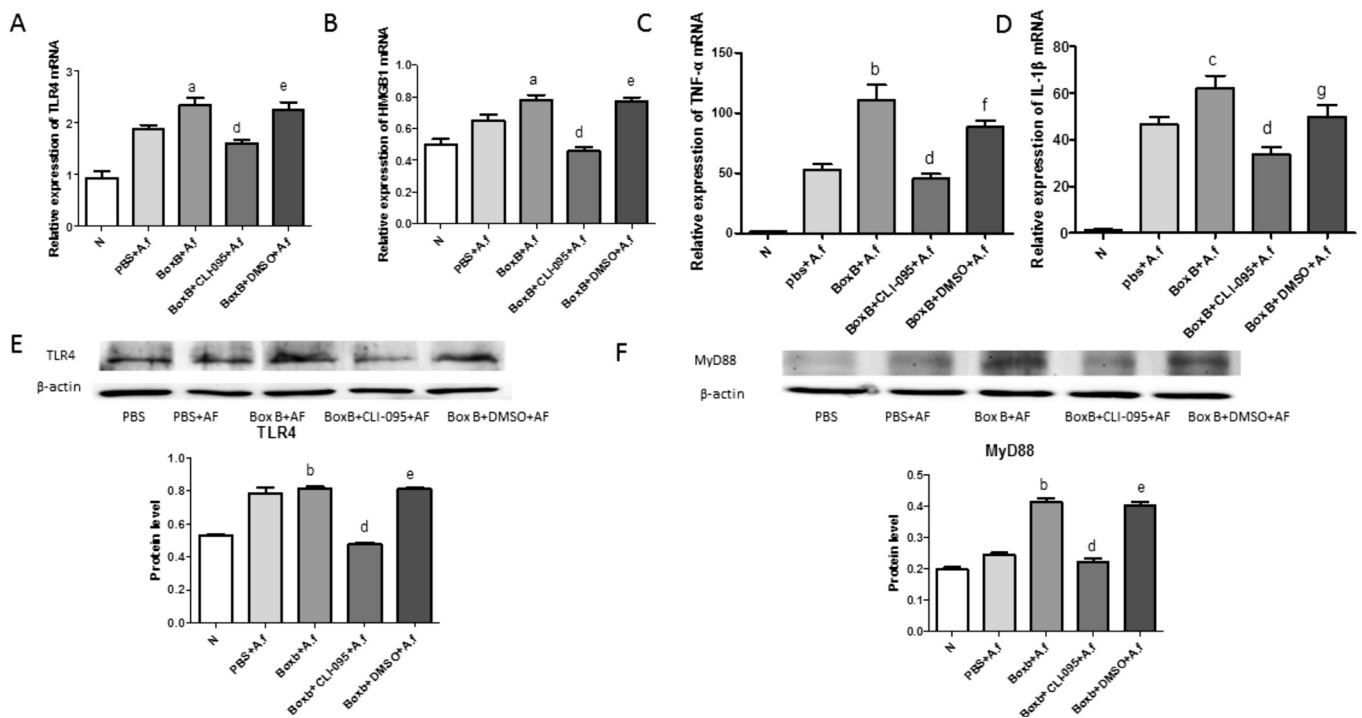


Figure 3 Effects of Boxb treatment on TLR4, MyD88, HMGB1, IL-1β and TNF-α in macrophages of BALB/c mice. Macrophages treated with Boxb compared with normal, PBS, CLI-095+Boxb, and DMSO+Boxb at 12h. A: The level of TLR4 mRNA was elevated in Boxb+A. *fumigatus* group, and in the CLI-095+Boxb+A. *fumigatus* group the TLR4 mRNA was declined; B: The mRNA level of HMGB1 was elevated in Boxb+A. *fumigatus* group, and the CLI-095 inhibited the HMGB1 mRNA expression; C: The level of TNF-α mRNA was significantly higher in Boxb+A. *fumigatus* group, and the CLI-095+Boxb+A. *fumigatus* group the expression was declined; D: The mRNA level of IL-1β was elevated in Boxb+A. *fumigatus* group, and declined in the CLI-095+Boxb+A. *fumigatus* group; E: The protein level of TLR4 was elevated in Boxb+A. *fumigatus* group, and declined in the CLI-095+Boxb+A. *fumigatus* group; F: The protein level of MyD88 was increased in Boxb+A. *fumigatus* group, and the CLI-095+Boxb+A. *fumigatus* group was significantly decreased. ^a*P*<0.05; ^b*P*<0.001; ^c*P*<0.01 vs PBS+A. *fumigatus* group; ^d*P*<0.001 vs Boxb+A. *fumigatus* group; ^e*P*<0.001; ^f*P*<0.01; ^g*P*<0.05 vs CLI-095+Boxb+A. *fumigatus* group.

by mediating cytokine release and regulating anorexia^[21], acute lung injury^[22], as well as the tissue necrosis^[23]. HMGB1 binds to cell surface receptors, such as TLR4, to activate the innate immune cells product pro-inflammatory factors, and HMGB1/TLR4 signaling pathway was also found in many inflammatory diseases, like sepsis, rheumatoid arthritis (RA), and arteriosclerosis^[24-25]. Fungal keratitis is an ulcerative disease of the cornea with severe inflammatory response^[26]. But in fungal keratitis, the role of HMGB1 is still not clear. In this study, we investigated the effect of HMGB1 Boxb in *A. fumigatus* induced corneal infection, and explored its signal pathway.

In severe sepsis and infection caused lethal inflammation syndrome, HMGB1 acted as a necessary mediator. It secreted by innate immune cells, like monocytes, macrophages, neutrophils, and dendritic cells. LPS, TNF-α or IL-1β stimulate the innate immune cells secrete HMGB1, and then stimulate additional pro-inflammation cytokines^[12,16]. In our study, the TNF-α, IL-1β expressions increase both in BALB/c mice fungal keratitis models and its abdominal cavity extracted macrophages. Boxb pretreated mice corneas infected with *A. fumigatus* have more severe clinical manifestations. The result suggested that fungal keratitis can promote the

expression of HMGB1, and the additional HMGB1 may play an proinflammatory role in the progression of *A. fumigatus* keratitis.

TLRs are important pattern recognition receptors in fungal keratitis^[27]. The engagement of TLRs can trigger the innate immune response and induce the adaptive immune response. Interestingly, it has been reported that HMGB1 can stimulate cellular signaling through TLR2, TLR4 and TLR9^[28-29]. Based on the former studies, we particularly explored the mechanism of how HMGB1 exacerbates fungal keratitis inflammatory through TLR4 signaling pathway. In Boxb subconjunctivally BALB/c mice, the protein levels of TLR4, MyD88, IL-1β and TNF-α increased. And in BALB/c mice abdominal cavity extracted macrophages, when we pretreated with CLI-095, the TLR4 inhibitor, the protein levels of TLR4, MyD88 decreased compared with the Boxb group. So we indicated that in *A. fumigatus* keratitis, HMGB1 can exacerbate corneal inflammation through TLR4/MyD88 signaling pathway. This finding corroborates the results of those studies HMGB1 binds to TLR4 to trigger proinflammatory cascade.

In conclusion, HMGB1 participates in the innate immune response of *A. fumigatus*, and acts as a proinflammation cytokine through TLR4/MyD88 signaling pathway.

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