

Inhibition of LOX-1 alleviates the proinflammatory effects of high-mobility group box 1 in *Aspergillus fumigatus* keratitis

Jia-Qian Jiang, Cui Li, Cong-Xian Cui, Yu-Na Ma, Gui-Qiu Zhao, Xu-Dong Peng, Qiang Xu, Qian Wang, Guo-Qiang Zhu, Chen-Yu Li

Department of Ophthalmology, the Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, China

Co-first authors: Jia-Qian Jiang and Cui Li

Correspondence to: Gui-Qiu Zhao. Department of Ophthalmology, the Affiliated Hospital of Qingdao University, No.16 of Jiangsu Road, Shinan District, Qingdao 266003, Shandong Province, China. Zhaoguiqiu_good@126.com

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Abstract

• **AIM:** To investigate the inflammatory amplification effect of high-mobility group box 1 (HMGB1) in *Aspergillus fumigatus* (*A. fumigatus*) keratitis and the relationship between lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) and HMGB1 in keratitis immune responses.

• **METHODS:** Phosphate buffer saline (PBS), and Boxb were injected into BALB/c mice subconjunctivally before the corneas were infected with *A. fumigatus*. RAW264.7 macrophages and neutrophils were pretreated with PBS and Boxb to determine the HMGB1 inflammatory amplification effects. Abdominal cavity extracted macrophages were pretreated with Boxb and Poly (I) (a LOX-1 inhibitor) before *A. fumigatus* hyphae stimulation to prove the the relationship between the two molecules. LOX-1, interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), macrophage inflammatory protein-2 (MIP-2) and IL-10 were assessed by polymerase chain reaction and Western blot.

• **RESULTS:** Pretreatment with Boxb exacerbated corneal inflammation. In macrophages and neutrophils, *A. fumigatus* induced LOX-1, IL-1 β , TNF- α and MIP-2 expression in Boxb group was higher than those in PBS group. Poly (I) treatments before infection alleviated the proinflammatory effects of Boxb in abdominal cavity extracted macrophages. Pretreatment with Boxb did not influence Dectin-1 mRNA levels in macrophages and neutrophils.

• **CONCLUSION:** In fungal keratitis, HMGB1 is a proinflammatory factor in the first line of immune response. HMGB1 mainly stimulates neutrophils and macrophages to

produce inflammatory cytokines and chemokines during the immune response. LOX-1 participates in HMGB1 induced inflammatory exacerbation in *A. fumigatus* keratitis.

• **KEYWORDS:** *Aspergillus fumigatus* keratitis; high-mobility group box 1; LOX-1

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INTRODUCTION

Fungal keratitis is an infectious keratitis with a high rate of blindness caused by pathogenic fungi^[1]. Agricultural trauma, contact lens abrasion, broad-spectrum antibiotics, glucocorticoids or immunosuppressants for systemic or local long-term usage are increasing the occurrence of fungal keratitis^[2]. Fungal keratitis, a serious infectious corneal disease, is not satisfactory for medical and surgical treatment^[3]. It is important to investigate the pathogenesis of fungal keratitis.

High mobility group box1 (HMGB1), which was known as a proinflammatory cytokine, is secreted by innate immune cells when the cells are stimulated with pathogenic microorganisms, and it plays a central role in immunity^[4-5]. When an appropriate external signal stimulates neutrophils and macrophages, HMGB1 is released into the extracellular milieu and recognized by TLR2, 4, and 9, thus promoting the secretion of proinflammatory factors^[1,6]. The structure of HMGB1 from the amino terminus to the carboxy terminus includes an A box, a B box (Boxb) and a C-terminal domain that contains only glutamic acids and aspartic acids^[4]. As structure function analysis showed that the B box of HMGB1 is a functional region that enhances inflammation^[6]. Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), a C-type lectin family member, is a key receptor located in human corneal epithelial cells (HCECs), neutrophils and macrophages^[7]. Previous studies found that *A. fumigatus* stimulation upregulates the expression of LOX-1

in HCECs and *A. fumigatus* infected corneas and that the inhibition of LOX-1 decreases inflammatory cytokines and chemokines expression, suggesting that LOX-1 is a proinflammatory receptor in fungal keratitis^[8].

Our previous study found that HMGB1 participates in the immunity of fungal keratitis and that TLR4/MyD88 is an important signaling pathway for HMGB1 to induce inflammation^[1]. The proinflammatory role of LOX-1 in *A. fumigatus* keratitis is clear in our previous studies^[3,7]. And LOX-1 and TLR4 could influence each other in this model^[3]. It is not known whether LOX-1 participates in HMGB1-induced proinflammatory effects in fungal keratitis. This study investigated the expression of LOX-1, Dectin-1 and inflammatory factors after pretreatment with Boxb, and the function of LOX-1 in the proinflammatory effects HMGB1 in *A. fumigatus* keratitis.

MATERIALS AND METHODS

Ethical Approval The study was conducted according to the Declaration of Helsinki and approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University. All mice were treated abided by the RAVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mice and Corneal Infection Specific pathogen-free (SPF) BALB/c mice (8-week-old females) were purchased from Jinan Pengyue Laboratory Animal Co., Ltd. (Jinan, China). Eight percent chloral hydrate was intraperitoneally injected into mice for anesthesia. A stereoscopic microscope ($\times 40$ magnification) was used to amplify the eyes. The left eye of each mouse was chosen as experimental eye, and scrapped the central corneal epithelium softly. The corneal surface was covered with a 5- μ L aliquot [1×10^8 colony forming units (CFU)/mL] of *A. fumigatus* (strain3.0072, China General Microbiological Culture Collection Center) and a sterile contact lens, then gently sutured the eyelids. Mouse corneas were collected at 1, 3 and 5d after infection.

Macrophages and Neutrophils Extraction For macrophage extraction, 1 mL of 3% thioglycollate medium was intraperitoneally injected into mice. After 7d of stimulation, the mice were sacrificed. For neutrophil extraction, 1 mL 9% casein (Sigma, Shanghai, China) was intraperitoneally injected into mice. After 24h, the mice were given a similar intraperitoneal injection, and 3h after injection, the mice were sacrificed. After being wiped with 75% alcohol, 10 mL Dulbecco's modified Eagle's medium (DMEM) (Gibco, San Diego, CA, USA) was injected into the abdominal cavity to collect cells. After centrifugation, purification and suspension, the cells were cultivated in culture plates.

Cell Culture and Stimulation RAW264.7 macrophages were obtained from the Shanghai Chinese Academy of Sciences (Shanghai, China), and the cells were grown in DMEM with

Table 1 Nucleotide sequences of mouse primers for RT-PCR

Gene	GenBank No.	Primer sequence (5'-3')
β -actin	NM_007393.3	F: GAT TAC TGC TCT GGC TCC TAG C R: GAC TCA TCG TAC TCC TGC TTG C
LOX-1	NM_138648.2	F: AGG TCC TTG TCC ACA AGA CTG G R: ACG CCC CTG GTC TTA AAG AAT TG
Dectin-1	NM_020008.3	F: GAC CCA AGC TAC TTC CTC R: GCA GCA CCT TTG TCA TAC T
IL-1 β	NM_008361.3	F: CGC AGC AGC ACA TCA ACA AGA GC R: TGT CCT CAT CCT GGA AGG TCC ACG
TNF- α	NM_013693.2	F: ACC CTC ACA CTC AGA TCA TCT T R: GGT TGT CTT TGA GAT CCA TGC
MIP-2	NM_009140.2	F: TGT CAA TGC CTG AAG ACC CTG CC R: AAC TTT TTG ACC GCC CTT GAG AGT GG
IL-10	NM_010548.2	F: TGC TAA CCG ACT CCT TAA TGC AGG AC R: CCT TGA TTT CTG GGC CAT GCT TCT C

RT-PCR: Reverse transcriptase polymerase chain reaction; IL: Interleukin; TNF: Tumor necrosis factor; MIP: Macrophage inflammatory protein.

10% fetal bovine serum (FBS; Gibco), then, they were cultured at 37°C, with 5% CO₂. When the cell density reached 80%, the cells were pretreated with or without Boxb (1 μ g/mL) (HMGBiotch), and with phosphate buffer saline (PBS; 1 μ g/mL) for 2h Poly (I) (250 μ g/mL; Sigma) was added prior to Boxb, then, cells were stimulated with *A. fumigatus* (at a final concentration of 5×10^6 CFU/mL) for 12 or 24h.

Boxb Treatment of BALB/c Mice One day before infection, Boxb (0.5 μ g/5 μ L) or control PBS was administered to the experimental eyes ($n=6$ /group) of BALB/c mice by subconjunctival injection. An additional 0.5 μ g/100 μ L Boxb or control PBS was injected intraperitoneally 1 and 3d after infection.

Real-Time Reverse Transcriptase Polymerase Chain Reaction RNAiso plus reagent (TaKaRa, Japan) was used to separate cornea and cell total RNA, which was rapidly quantified by spectrophotometry. Complementary DNA was obtained through reverse transcription of 1 μ g RNA. Next 2 μ L cDNA was diluted in 23 μ L diethylpyrocarbonate-treated water. Reverse transcriptase polymerase chain reaction (RT-PCR, 20 μ L reaction volume) was performed using a 2- μ L cDNA aliquot and SYBRgreen. β -actin was used as control. The oligonucleotide primers in this study are shown in Table 1.

Western Blot Analysis Cells were collected after 24h of infection. For protein extraction, the cells were lysed in radioimmunoprecipitation assay (RIPA; Solarbio) lysis buffer with PMSF (Solarbio) (100:1) for 2h. Twelve percent polyacrylamide SDS-PAGE was used to separate the total protein, and the separated proteins were transferred onto PVDF membranes (Solarbio). Five percent BSA (Beyotime, China) was used to block the membranes at 37°C for 2h. The membranes were incubated with primary antibodies to β -actin (Bioss, Beijing, China), LOX-1 (Abcam, USA), interleukin-1 β (IL-1 β ; Bioss) and tumor necrosis factor- α (TNF- α ; Cell

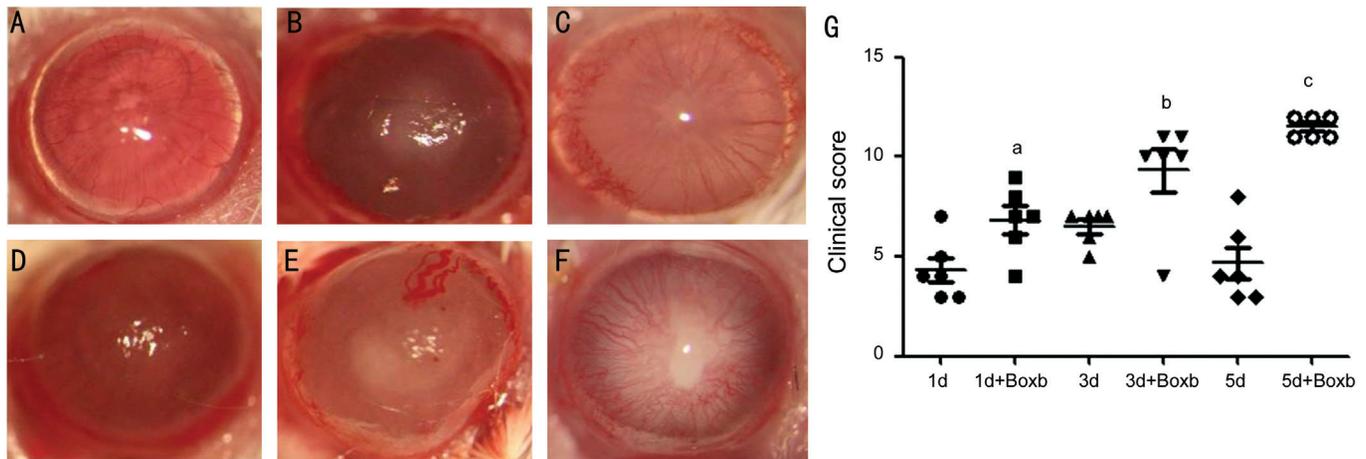


Figure 1 Boxb exacerbated inflammation and elevated the clinical score in *A. fumigatus* keratitis of BALB/c mice. Slit lamps were used to photograph PBS (A, B, C) and Boxb (D, E, F) pretreated corneas. The clinical score (G) at 1, 3, 5d were also illustrated the disease severity. ^a $P < 0.05$ vs 1d *A. fumigatus* group; ^b $P < 0.05$ vs 3d *A. fumigatus* group; ^c $P < 0.01$ vs 5d *A. fumigatus* group.

Signaling Technology, Massachusetts, USA) at 4°C for 12-16h. Then, the corresponding peroxidase-conjugated secondary antibodies (Elabscience; Wuhan, China) were used at 37°C for 1.5h, and chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) was finally used to develop the immunoreactive bands.

Statistical Analysis One-way ANOVA was used to analyze statistical significance with GraphPad 5.0 software. $P < 0.05$ was considered to indicate significantly differences between comparisons. To ensure reproducibility, all experiments were repeated at least three times.

RESULTS

Boxb Exacerbated Inflammation and Elevated the Clinical Score in *A. fumigatus* Keratitis in BALB/c Mice To investigate the effects of HMGB1 in *A. fumigatus* keratitis, we subconjunctivally injected Boxb (0.5 μg/5 μL) or PBS into the corneas 1d before infection. A slit lamp was used to take photographs. Mild corneal edema, and less than 25% ulcerative area could be observed 1d after *A. fumigatus* stimulation (Figure 1A), and the iris could be seen through the cornea. After 3d of stimulation, the corneal edema and ulcerative area were more severe than after 1d of stimulation (Figure 1B), and the iris could not be observed; however, the ulcerative area was improved 5d after stimulation (Figure 1C). In the contrast, the Boxb-pretreated group had higher clinical scores than the PBS control group, and perforated ulcers were observed in some Boxb group corneas after 5d of stimulation (Figure 1D-1F). Figure 1G indicates the corneal clinical score^[9].

Boxb Up-regulated LOX-1 Expression in the Corneas of BALB/c Mice To investigate the effects of Boxb in *A. fumigatus* keratitis, the corneas were treated with Boxb (0.5 μg/5 μL) or PBS 1d before infection. The results demonstrated increased LOX-1 mRNA levels in the Boxb pretreated groups compared with those in the PBS control groups ($P < 0.01$; Figure 2).

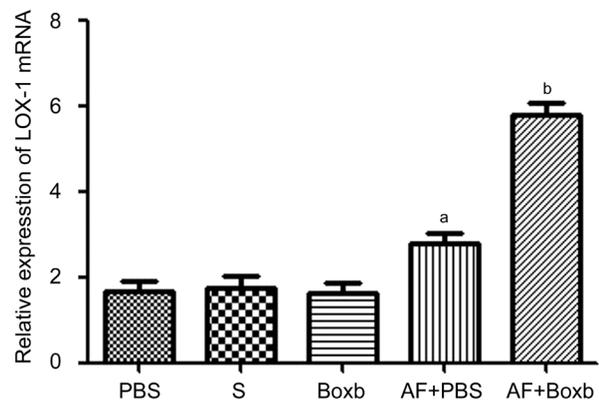


Figure 2 Effects of Boxb treatment on LOX-1 in corneas. Compared with infected PBS control, pretreated with Boxb significantly increase the LOX-1 mRNA levels. ^a $P < 0.01$ vs PBS group; ^b $P < 0.01$ vs *A. fumigatus* +PBS group.

Boxb Up-regulated LOX-1, IL-1β, TNF-α, MIP-2 and IL-10 Expression in RAW264.7 Macrophages To investigate the effects of Boxb on RAW264.7 macrophages, cells were preconditioning with Boxb or PBS for 2h, and stimulated with *A. fumigatus* for 12 or 24h. RT-PCR showed that compare with PBS groups, the mRNA levels of LOX-1 ($P < 0.05$; Figure 3A), IL-1β ($P < 0.001$; Figure 3C), TNF-α ($P < 0.001$; Figure 3D), macrophage inflammatory protein-2 (MIP-2, $P < 0.001$; Figure 3E) and IL-10 ($P < 0.001$; Figure 3F) were increased in Boxb pretreated cells. To confirm these data, WB was used to examin LOX-1, IL-1β and TNF-α expression. Dectin-1 ($P > 0.05$; Figure 3B) relative mRNA levels were unchanged in the Boxb pretreatment group compared with those in the PBS control groups.

Boxb Upregulated LOX-1, IL-1β, TNF-α, MIP-2 and IL-10 Expression in Neutrophils from BALB/c Mice We next sought to investigate whether Boxb has the same effect on neutrophils from mice. The cells were pretreated with Boxb or PBS for 2h, and stimulated with *A. fumigatus* for 12h. The

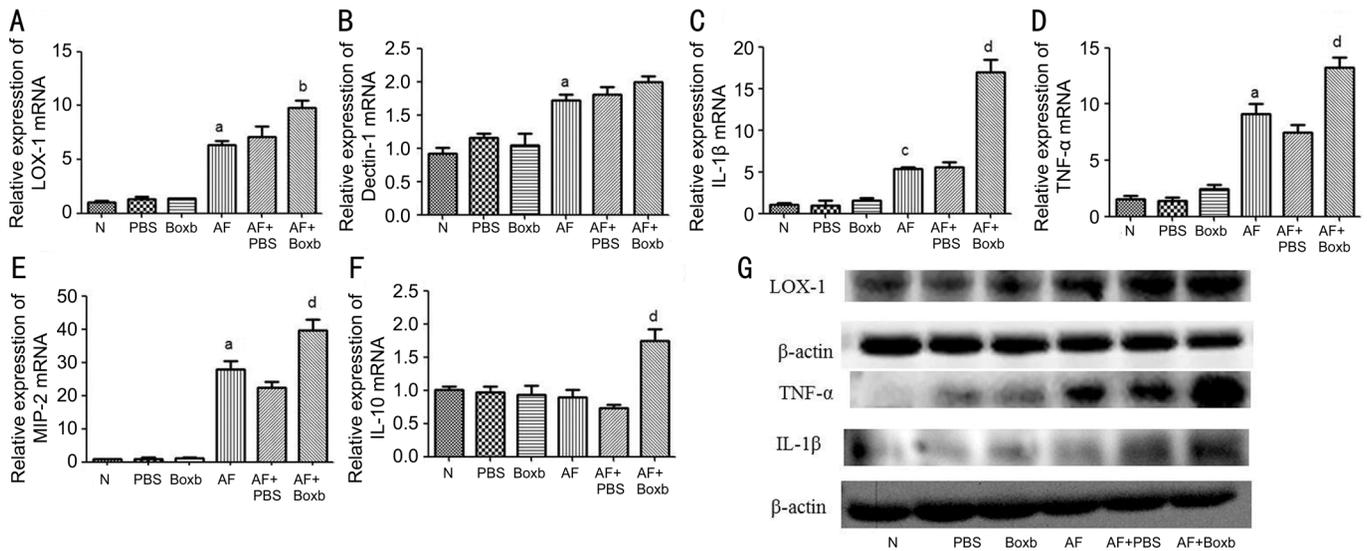


Figure 3 Effects of Boxb treatment on LOX-1, Dectin-1, IL-1 β , TNF- α , MIP-2 and IL-10 in RAW264.7 cells Compared with infected PBS control, pretreated with Boxb significantly increase the mRNA levels of LOX-1 (A), IL-1 β (C), TNF- α (D), MIP-2 (E) and IL-10 (F). While Dectin-1 (B) mRNA levels did not change significantly. Pretreated with Boxb significantly increased the protein levels of LOX-1, IL-1 β , TNF- α (G). ^a P <0.001; ^c P <0.01 vs N group, ^b P <0.05; ^d P <0.001 vs PBS+A. *fumigatus* group.

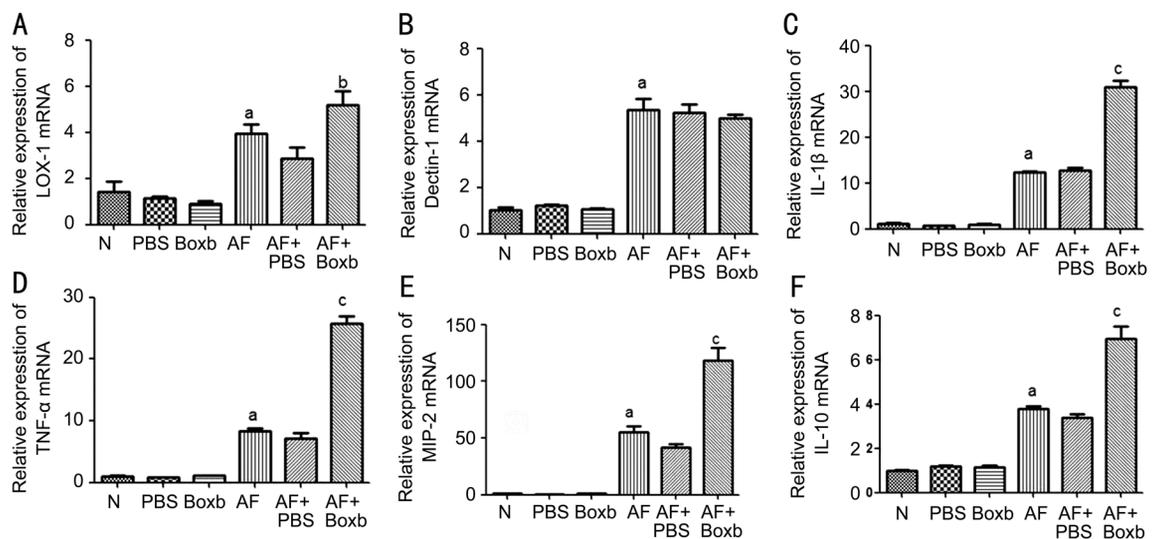


Figure 4 Effects of Boxb treatment on LOX-1, Dectin-1, IL-1 β , TNF- α , MIP-2 and IL-10 in neutrophils of BALB/c mice Compared with infected PBS group, pretreated with Boxb increase the mRNA levels of LOX-1 (A), IL-1 β (C), TNF- α (D), MIP-2 (E) and IL-10 (F). While Dectin-1 (B) mRNA levels did not change significantly. ^a P <0.001 vs N group; ^b P <0.01; ^c P <0.001 vs PBS+A. *fumigatus* group.

results showed that the mRNA levels of LOX-1 (P <0.01; Figure 4A), IL-1 β (P <0.001; Figure 4C), TNF- α (P <0.001; Figure 4D), MIP-2 (P <0.001; Figure 4E) and IL-10 (P <0.001; Figure 4F) were increased in Boxb pretreated cells compared with those in PBS control cells. Dectin-1 (P >0.05; Figure 4B) relative mRNA levels were unchanged in the Boxb pretreated group compared with those in the PBS control groups.

Inhibition of LOX-1 Alleviated the Proinflammatory Effect of Boxb in Macrophages from BALB/c Mice To investigate the influence of LOX-1 on HMGB1 proinflammatory effect, macrophages were preconditioning with Boxb and Poly (I), and stimulated with *A. fumigatus* for 12 or 24h. Compare with Boxb group, the mRNA levels of LOX-1 (P <0.05; Figure

5A), TNF- α (P <0.001; Figure 5B), MIP-2 (P <0.05; Figure 5C), IL-10 (P <0.01; Figure 5D) were decreased in Poly (I) pretreated group. The protein levels of LOX-1, IL-1 β and TNF- α (Figure 5E) were also decreased in Western blots in Poly (I) pretreated group compared with those in the Boxb group.

DISCUSSION

As one of the danger associated molecular patterns, HMGB1 is an important proinflammatory factor in innate immunity. HMGB1 is recognized by TLR2, 4, and 9 and functions as a proinflammatory factor^[1,10]. HMGB1 is secreted by neutrophils, macrophages, natural killer cells and dendritic cells^[11]. HMGB1 is a target to elicit inflammatory effects in ocular

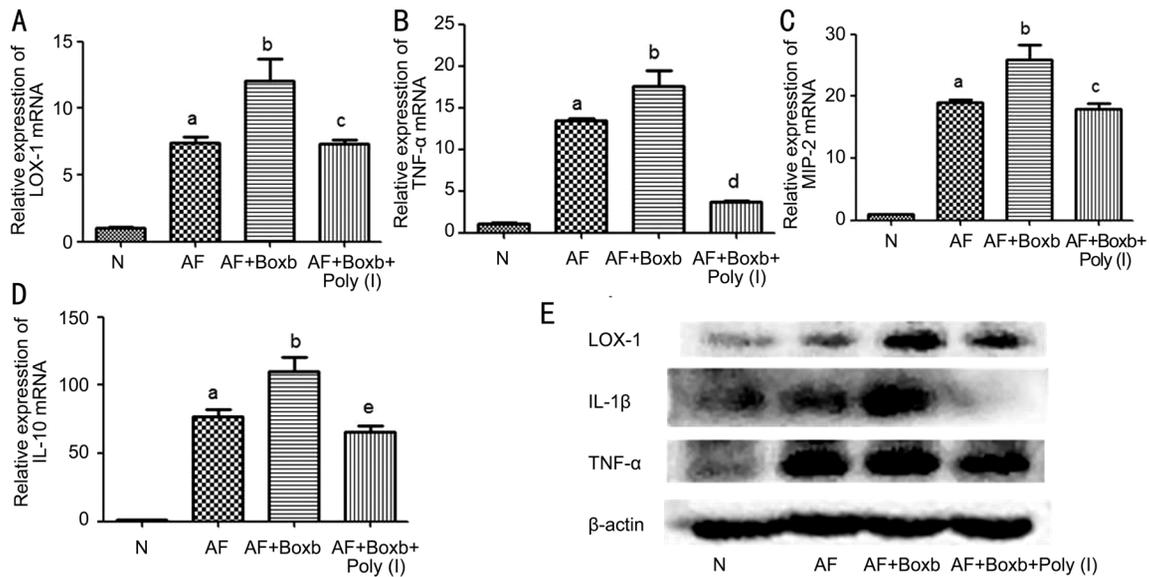


Figure 5 Effects of LOX-1 on HMGB1 proinflammatory effects in macrophages In Boxb pretreated group, the mRNA levels of LOX-1 (A), TNF-α (B), MIP-2 (C), IL-10 (D) were elevated, in Poly (I) pretreated group, the mRNA levels of LOX-1 (A), TNF-α (B), MIP-2 (C), IL-10 (D) were declined. In Boxb pretreated group, the protein levels of LOX-1, IL-1β, TNF-α (E) were elevated, in Poly (I) pretreated group, the protein levels of LOX-1, IL-1β, TNF-α (E) were declined. ^a*P*<0.001 vs N group; ^b*P*<0.05 vs *A. fumigatus* group; ^c*P*<0.05; ^d*P*<0.001; ^e*P*<0.01 vs Boxb+ *A. fumigatus* group.

surface inflammation^[12]. In a mouse model of *Pseudomonas aeruginosa* keratitis, blocking HMGB1 reduced clinical scores and a decreased of inflammatory cytokine expression^[13-15]. Liu *et al*^[11] indicated that in *A. fumigatus* keratitis, the pretreatment of corneas with Boxb led to severe clinical manifestations and upregulation of inflammatory cytokine expression after 1d of stimulation. Our data show that in *A. fumigatus* infected groups, the clinical scores were higher after pretreated with Boxb, which are consistent with previous data, explaining that additional HMGB1 plays a proinflammatory magnification role in *A. fumigates* keratitis.

HMGB1 participates in the inflammatory response by selectively activating multiple receptors on macrophages, neutrophils, eosinophils, fibroblasts, NK cells, T cells and endothelial cells to produce inflammatory cytokines^[16]. In chronic obstructive pulmonary disease (COPD) immunity, HMGB1 siRNA reduced proinflammatory cytokine expression in *A. fumigatus*-infected COPD alveolar macrophages compare with control alveolar macrophages^[5]. Neutrophils were also stimulated by HMGB1 to produce more cytokines^[17]. Previous studies showed that in fungal keratitis, neutrophils, macrophages, and less T cells are composed of the infiltrating cells^[18]. To fully confirm the function of HMGB1 in *A. fumigates* keratitis, *in vitro* studies were performed in macrophages and neutrophils. The results indicated that compared with the control group, the Boxb pretreatment group demonstrated upregulated IL-1β, TNF-α, MIP-2 and IL-10 expression. These data are consistent with *in vivo* results^[11]. Lu *et al*^[19] showed that the macrophage inflammatory response is enhanced by

HMGB1 in systemic lupus erythematosus and corresponds to proinflammatory cytokine production. Our results also corroborate those of previous studies showing that HMGB1 promotes inflammatory responses in macrophages and neutrophils.

Studies have shown that LOX-1 and Dectin-1 are important C-type lectin-like receptors in *A. fumigates* keratitis. Both of these proteins play proinflammatory roles in innate immunity^[7-8,20]. To conform whether HMGB1 induced proinflammatory cytokine production is related to LOX-1 in fungal keratitis, we detected LOX-1 expression after pretreatment with Boxb. Our study showed that in *A. fumigatus* corneas, LOX-1 expression was upregulated in Boxb pretreated group, this was further proved in macrophages and neutrophils studies. In a rat model of COPD, activated HMGB1/TLR4/MyD88 signaling pathway leading to the activation of TRAF6, thus leading to an upregulation of LOX-1, indicated the relationship between HMGB1 and LOX-1^[21]. And our previous studies have proved the function of HMGB1/TLR4/MyD88 signaling pathway in *A. fumigates* keratitis^[11]. Our results were consistent with this passage. In addition, when pretreated with Poly (I), the expression of IL-1β, TNF-α, MIP-2 and IL-10 decreased compared with the expression in the Boxb group. These results indicate that LOX-1 alleviated the HMGB1-induced proinflammatory effects in fungal keratitis. In contrast, the relative mRNA levels of Dectin-1 did not show any change in the Boxb pretreated groups compared with the PBS group both in macrophages and neutrophils, suggesting that Dectin-1 may not participate in the proinflammatory effects of HMGB1 in *A. fumigates* keratitis.

In summary, our study demonstrates that HMGB1 promotes inflammation in BALB/c mice corneas as well as in RAW264.7 macrophages and neutrophils. In addition, inhibition of LOX-1 alleviates the proinflammatory effect of Boxb on macrophages in BALB/c mice. Whether LOX-1 participate in HMGB1-induced proinflammation effect directly or through HMGB1/TLR4 signal pathway, further studies will be expanded. These results indicate that HMGB1 exacerbate inflammation mainly through neutrophils and macrophages and that LOX-1 functions in HMGB1-mediated inflammation in *A. fumigatus* keratitis.

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