• Basic Research •

Regulation of LOX-1 on adhesion molecules and neutrophil infiltration in mouse *Aspergillus fumigatus* keratitis

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Abstract

● **AIM:** To determine whether lectin-like ox-LDL receptor (LOX-1) regulates adhesion molecules expression and neutrophil infiltration in *Aspergillus fumigatus* (*A. fumigatus*) keratitis of C57BL/6 mice.

● **METHODS:** C57BL/6 mice were pretreated with a neutralizing antibody to LOX-1 (5 μg/5 μL) or control nonspecific IgG (5 μg/5 μL), LOX-1 inhibitor Poly-I (2 μg/5 μL) or PBS by subconjunctival injection. Fungal keratitis (FK) mouse models of C57BL/6 mice were established by scraping corneal central epithelium, smearing *A. fumigatus* on the corneal surface and covering the eye with contact lenses. The corneal response to infection was assessed *via* clinical score. The mRNA levels of the adhesion molecules intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-selectin were tested in control and infected corneas by reverse transcription-polymerase chain reaction (RT-PCR). The protein levels of ICAM-1 were evaluated by immunofluorescence (IF) and Western blot. Neutrophils were extracted from the abdominal cavity of C57BL/6 mice followed by pretreatment using antibody to LOX-1 (10 μg/mL) or control nonspecific IgG (10 μg/mL), the Poly-I (4 μg/mL) or PBS. The cells were then stimulated with *A. fumigatus* and tested mRNA and protein levels of lymphocyte function-associated antigen-1 (LFA-1) using RT-PCR and Western blot. IF and myeloperoxidase (MPO) assays were used to assess neutrophil infiltration in mice corneas.

● **RESULTS:** Pretreatment of LOX-1 antibody or the Poly-I reduced the degree of inflammation of cornea and decreased the clinical FK score compared with pretreatment of IgG or PBS (both $P<0.01$). And these pretreatment also displayed an obvious decline in the mRNA levels of ICAM-1, VCAM-1, P-selectin, E-selectin and LFA-1 expression compared with control groups (all $P<0.01$). Furthermore, pretreated with LOX-1 antibody or Poly-I, the protein levels of ICAM-1 and LFA-1 also decreased compared with control groups (all $P<0.05$). Neutrophil infiltration in the cornea was significantly reduced after pretreatment of LOX-1 antibody or Poly-I compared with control groups by IF and MPO assays (both $P<0.01$).

● **CONCLUSION:** Inhibition of LOX-1 can decrease the expression of adhesion molecules and reduce neutrophil infiltration in *A. fumigatus* infected corneas of C57BL/6 mice.

● **KEYWORDS:** fungal keratitis; lectin-like ox-LDL receptor; adhesion molecules; neutrophils; mice

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INTRODUCTION

Fungal keratitis (FK) is a serious purulent eye disease. In severe cases, it is likely to lead to loss of eye vision[1]. FK is very serious when infected, and the blindness rate of patients is also very high compared with other keratitis. The main fungus causing FK is *Aspergillus fumigatus* (*A. fumigatus*). External causes leading to FK infection, the more dangerous factor is agricultural trauma[2]. Without early diagnosis and effective treatment, the inflammatory response may cause stromal destruction, loss of vision and other significant corneal damage. In the process of FK, the immune system can significantly recognize and eliminate fungal pathogens by expressing pattern-recognition receptors (PRRs) on cells[3]. Moreover, the types of receptors are mainly classified into three types: C-type lectin receptor (CLRs), Toll-like receptor (TLRS) and nucleotide binding oligomeric domain-like receptor (NLRs)[4]. In addition, there is a type C receptor family: the lectin-like ox-LDL receptor (LOX-1).

LOX-1 was first confirmed by researchers as an endothelial-specific scavenger receptor and was then detected on monocytes, macrophages and platelets[5]. The previous studies showed that inhibition of LOX-1 can reduce the inflammatory reaction; even more important, blockade of LOX-1 has been shown to down-regulate adhesion molecules expression in pneumonia[6]. It is well known that adhesion molecules are involved in neutrophil recruitment[7]. Furthermore, LOX-1 inhibition is able to directly suppress leukocyte infiltration and protein exudation[8]. Polymorphonuclear neutrophilic leukocytes (PMNs) are key components of the innate immune system and can significantly eliminate infectious agents[9]. The intensity of the inflammatory reaction depends on the degree of neutrophil recruitment. However, tissue injury may be caused by the overreaction of neutrophils[10]. Granert *et al*[11] demonstrated that adhesion molecules are key regulatory molecules that are related to inflammation and PMN infiltration-associated tissue damage in infectious disease. A leaky barrier can be seen between the stroma and the aqueous humor due to the endothelium, through which essential nutrients can pass. Adhesion molecules can selectively open the passage for molecules and maintain physical integrity[12]. When inflammation occurs, adhesion molecules are modified, thus breaking the balance and allowing the inflammatory cells to transmigrate from the matrix into the infected or injured tissues[12]. Recently, Li *et al*[13] have found that the expression of LOX-1 in *A. fumigatus* infected mouse corneas was greatly increased after *A. fumigatus* stimulation. Previous research in our laboratory has confirmed that LOX-1 can affect the secretion of inflammatory factors in mice FK[14]. However, no study has examined the role of LOX-1 with adhesion molecules and neutrophil infiltration in *A. fumigatus* infected corneas. Therefore, the author of this article mainly studies the experimental hypothesis that LOX-1 regulates the expression of adhesion molecules and neutrophil infiltration in the corneas of C57BL/6 mice infected by *A. fumigatus*.

MATERIALS AND METHODS

Ethical Approval All the animals used in the above experiments are in accordance with the Guidelines for Human Treatment of Experimental Animals (vGKFCZ-2006-398) issued by the Ministry of Science and Technology of the People's Republic of China. And the treatment of animals after this experiment will be carried out in Vision and Ophthalmology (ARVO). Comply with the statement on the use of animals in ophthalmic and visual research. We carried out veterinary care regularly and cleaning each day and sterilization each week. We placed the experimental animals in a room with the adjustable climate where the light/dark cycle ratio is 14h:10h. Food and water were available to the animals.

Mice and Reagents The animals in this experiment are mainly provided by Cavens Experimental Animal Co., Ltd. in Changzhou, China. The uniform standard for C57BL/6 mice is female mice born 8wk. The *A. fumigatus* strains (No.3.0772) strains tested were provided by China's General Microorganism Culture and Collection Center. Babio biotech (Jinan, Shandong Province, China) supplied the Sabouraud medium; TAKARA provided RNAiso Plus, the PrimeScript RT reagent kit with gDNA Eraser, SYBR® Premix Ex Taq™ and primers (Dalian, Liaoning Province, China). In addition, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by the Gibco (San Diego, California, USA); R&D Systems (Minneapolis, MN, USA) supplied goat anti-mouse LOX-1 neutralizing antibody; goat anti-donkey intercellular cell adhesion molecule-1 (ICAM-1) antibody and goat anti-mouse lymphocyte function-associated antigen-1 (LFA-1) antibody were provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); the LOX-1 inhibitor Poly-I was purchased from Sigma (St. Louis, Missouri, USA); GADPH antibody was from Elabscience (Wuhan, China); Santa Cruz Biotechnology provided the primary rabbit anti-PMN antibody (neutrophil marker NIMP-R14); Nanjing Jiancheng Bioengineering Institute supplied the myeloperoxidase (MPO) detection kit.

Corneal Infection Anesthetize mice with 8% chloral hydrate and placed under a stereomicroscope. The magnification was adjusted to ×40; a section of 2 mm diameter epithelium was removed from the central corneas. The corneal surface was covered by *A. fumigatus*, and contact lenses were placed. Then sutured the experimental eyelids. In the control group, the same volume of physiological saline was used to replace the fungal suspension in the experimental group. The eyes of the mice were examined at 12h and on the 1 and 3d after infection in order to ensure that the corneas were infected. Corneal infection was illustrated by photography with a slit lamp.

Corneal Clinical Score The degree of corneal edema and turbidity was observed for 0.5, 3, and 5d. The observation tool was slit lamp. By setting the opacity area, opacity density and fractional grade of surface regularity (0-4). Therefore, the total possible score of the experiment is between 0 and 12[15]. The clinical score of each mouse was recorded three days after infection in order to make statistical comparisons in disease severity, and the corneal response was illustrated by photography with a slit lamp.

Neutrophil Extraction One hundred milliliters of distilled water was used to dissolve 3 g thioglycollate medium, and the liquid was stored at 4℃ followed by sterilization under high pressure. To extract neutrophils, we used an intraperitoneal injection (i.p.) injection of 1 mL 3% sodium thioglycollate in C57BL/6 mice to scarify the mice 12h later. We first used 75% alcohol to wipe the abdominal skin of the sacrificed mice, then opened the wiped abdominal skin along the middle line.

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

Gene	GenBank No.	Primer sequence (5' - 3')	Size (bp)
β-Actin	NM_007393.3	F-GAT TAC TGC TCT GGC TCC TAG C	147
		R-GAC TCA TCG TAC TCC TGC TTG C	
ICAM-1	NM_010493.3	F-GTCCGCTGTGCTTTGAGAACT	105
		R-CGGAAACGAATACACGGTGAT	
VCAM-1	NM_011693.3	F-TGCCGGCATATACGAGTGTGA	120
		R-ATGCGCAGTAGAGTGCAAGGA	
P-selectin	NM_011347.2	F-GATGAAGGCTCGCTCTTGGTG	147
		R-CATGAACTGGCATGTGGATTTGTAG	
E-selectin	M87862.1	F-GCC TGC AAT GTG GTT GAG TG	176
		R-ACG AAC CCA TTG GCT GGA TT	
LFA-1	NM_001253874.1	F-ATGTTCTTGCTGACCAATACCTTTC	127
		R-ATCACTTGCCTCCCCGTCT	

ICAM-1: Intercellular cell adhesion molecule-1; VCAM-1: Vascular cell adhesion molecule-1; LFA-1: Lymphocyte function-associated antigen-1.

We used a syringe to inject 5 mL DMEM into the abdominal cavity of the mice. Peritoneal lavage was used to extract the neutrophils. After centrifugation at 300 g for 4℃ and 10min, Using Percoll to purify the neutrophils.

Cells Culture and Stimulation We then resuspended the cells in the growth medium, which contained 5% FBS together with DMEM and seeded 1×10^6/mL cell suspensions in 12-well culture plates at 37℃ humidified 5% CO_2 incubator. When the cells became 80%-90% confluent, they were incubated in serum-free DMEM media overnight, followed by stimulation with the inactive *A. fumigatus* spores (final concentration of 5×10^7 CFU/mL) while only serum-free DMEM was added to the normal control group.

LOX-1 Neutralization and LOX-1 Inhibition The Subconjunctival injection was performed to administer control IgG (5 μg/5 μL) or LOX-1-neutralizing antibody (5 μg/5 μL) to the left eyes of mice one day prior to infection. Neutrophils were pretreated for 2h with LOX-1 neutralizing antibody (10 μg/mL) or control IgG (10 μg/mL).

The day before infection, LOX-1 inhibitor Poly-I (2 μg/5 μL) or control phosphate-buffered saline (PBS) was injected into subconjunctival of mouse left eye at the same time. Neutrophils will be treated with LOX-1 inhibitor Poly-I (4 μg/mL) for 2h in advance. LOX-1 neutralizing antibody concentration and inhibitor concentration are determined according to the previously published articles in our laboratory[14].

RNA Extraction and Real-time Reverse Transcription-Polymerase Chain Reaction Assay We removed the control corneas and infected corneas at 0, 0.5, 1, and 3d p.i. and collected neutrophils after 0, 4, 8, and 12h p.i. RNAiso plus reagent was used to separate RNA from the suspension. Then, spectrophotometry was used to quantify RNA obtained at a fast speed. For reverse transcription, we used 1 μg RNA for the first-strand cDNA synthesis. Then, based on the manufacturer's instructions, we used 2 μL cDNA for polymerase chain reaction (PCR) in a 20 μL reaction volume. Reverse transcription-polymerase chain reaction (RT-PCR) was used to measure the mRNA levels of β-actin, ICAM-1, VCAM-1, P-selectin, E-selectin and LFA-1 expression. Table 1 illustrates the primer pair sequences suitable for RT-PCR.

In this study, we used the threshold cycle method to analyze the mRNA expression.

Western Blot The protein from the corneas of each group (6 eyes pooled per group for each experiment) was extracted with cold lysis buffer. We collected the neutrophils and washed them in ice-cold PBS. Next, we used the RIPA buffer to lyse the cells on ice for 2h. SDS-PAGE was used to separate the prepared protein from corneas and neutrophils, which was the electroblotted onto a PVDF membrane (Millipore, USA). Following 1h of blocking in 0.05% PBS-T and 5% milk at room temperature, goat anti-donkey ICAM-1 (1:100) or goat anti-mouse LFA-1 (1:200) antibodies or GAPDH antibodies (1:3000) were incubated with the blots at 4℃ overnight. Then, the blots were washed in PBS-T for 5min, followed by 1h anti-goat secondary antibody incubation at 37℃. Electrogenerated chemiluminescence (ECL) reagent (Beyotime, China) was used to develop the signals.

Immunofluorescent Staining The eyes of C57BL/6 mice were enucleated 3d p.i., placed in 0.01 mol/L PBS solution, implanted in OCT compound and later frozen in liquid nitrogen. The cutting thickness of eyeball is 10 μm. Finally, it was installed on the glass slide coated with poly L- lysine. After a 2min fixation in acetone, normal goat serum (Solarbio, Beijing, China) was used to block the glass slides at room temperature for 30min. The protein levels of ICAM-1 were assessed using immunofluorescent dual-label staining and

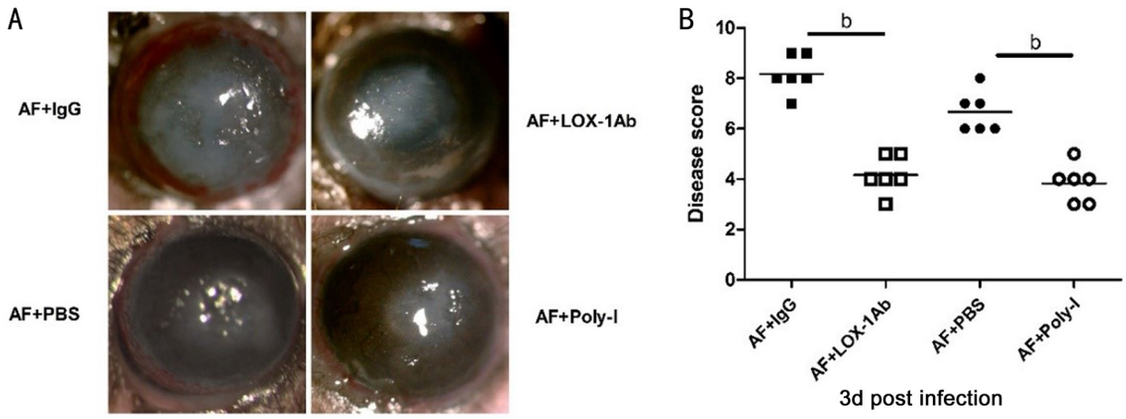

Figure 1 Disease response to LOX-1 neutralizing antibody and inhibitor treatment in *A. fumigatus* stimulated mice corneas A: Typical corneal image of mouse model of *A. fumigatus* keratitis after pretreatment of LOX-1 antibody or Poly-I and IgG or PBS respectively at 3d p.i. B: LOX-1 inhibition led to lower clinical scores after pretreated with LOX-1 antibody or Poly-I were euthanized 3d p.i. compared with pretreatment of IgG or PBS. The values are expressed as mean±SEM. $^{b}P<0.01$.

visualized on a fluorescence microscope. Corneal tissue sections were incubated for 1h using 1:100-diluted goat anti-mouse ICAM-1 (Santa Cruz, CA, USA) to label. Corneal tissue sections were then incubated for 1h with CY3-conjugated donkey anti-goat antibody (1:200). A primary rabbit anti-PMN antibody, NIMP-R14 (1:50), was used to incubate the sections for 1h. Then, the FITC-conjugated donkey anti-rabbit antibody (1:200), which is the corresponding secondary antibody, was incubated with the slides for 1h. Later, the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI) was used to incubate the sections for 10min. The controls were similarly treated, but the same host IgG was used to replace the primary antibodies. Finally, a fluorescence microscope was used to visualize the sections and capture digital images (Leica Microsystems).

Myeloperoxidase Assay As a constituent enzyme, MPO is a key component of neutrophils as it is beneficial for forming hypochlorous acid. MPO can be found in neutrophils, and thus it can reflect the neutrophil content of a tissue when it is extracted for quantification. The activity of corneal MPO was measured using an MPO detection kit. Briefly, we homogenized tissue in 1 mL potassium PBS (concentration of 50 mmol/L; pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide and then centrifuged the tissue at 4°C at 12 000 r/min for 20min. We transferred the 10 μL supernatant into PBS (pH 6.0) containing 0.0005% H_2O_2 together with 3,3'-dimethoxybenzidine (0.17 mg/mL). The MPO activity of the supernatant largely depends on the measurement of the H_2O_2-dependent oxidation of 3,3'-dimethoxybenzidine and can be represented as MPO/cornea units.

Statistical Analysis The results of this experiment need the difference of clinical scores between the two groups at each time point. The test method is mainly Mann-Whitney *U*; however, the difference test between the two groups is mainly to classify RT-PCR, Western blot, MPO and cell data by *t*-test of unpaired double-tailed students. When the experimental result $P<0.05$, the difference between the two groups of data were considered significant. All experiments need to be carried out twice to avoid errors caused by experiments. However, the data of the final experimental results must also be the average value ± the standard error of the average value.

RESULTS

Effect of LOX-1 Neutralizing Antibody and Inhibitor Treatment on the Severity of the Corneal Inflammation in C57BL/6 Mice A slit lamp was used to take photos 3d after infection to illustrate the disease response of *A. fumigatus* pretreated with LOX-1 antibody or Poly-I compared to those pretreated with IgG or PBS in mice corneas. The corneal area of opacity, the density of opacity, and surface regularity of the mice after LOX-1 neutralizing antibody or inhibitor pretreatment were milder than in IgG or PBS treated control mice at 3d p.i. (Figure 1A). At the same time, the clinical score of IgG and PBS treated mice at 3d p.i. was significantly higher than that of LOX-1 antibody and Poly-I pretreated mice (both $P<0.01$; Figure 1B).

Adhesion Molecules Gene Expression in the Corneas of C57BL/6 Mice To determine whether adhesion molecules are participated in *A. fumigatus* keratitis in mice, RT-PCR was used to detect the mRNA levels of adhesion molecules in the cornea of the mice. The results showed that the mRNA levels of ICAM-1 (both $P<0.01$; Figure 2A) and VCAM-1 (all $P<0.05$; Figure 2B) were increased significantly in infected C57BL/6 corneas at 0.5, 1, 3d p.i. The mRNA levels of P-selectin (all $P<0.01$; Figure 2C) and E-selectin (all $P<0.01$; Figure 2D) were also increased significantly in infected C57BL/6 corneas at 0.5, 1, 3d p.i.

The Effect of LOX-1 Neutralizing antibody on Adhesion Molecules in C57BL/6 Mice Corneas To investigate whether LOX-1 regulates adhesion molecules expression in

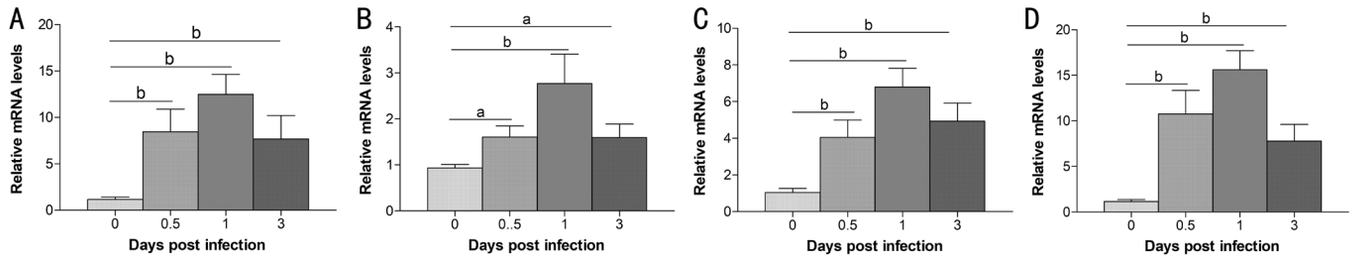

Figure 2 The expression of adhesion molecules in the cornea of C57BL/6 mice The corneas of C57BL/6 mice which were infected by *A. fumigates* at 0, 0.5, 1, and 3d p.i., the mRNA levels of ICAM-1 (A), VCAM-1 (B), P-selectin (C) and E-selectin (D) in control and infected corneas of C57BL/6 mice using RT-PCR. The values are expressed as mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$.

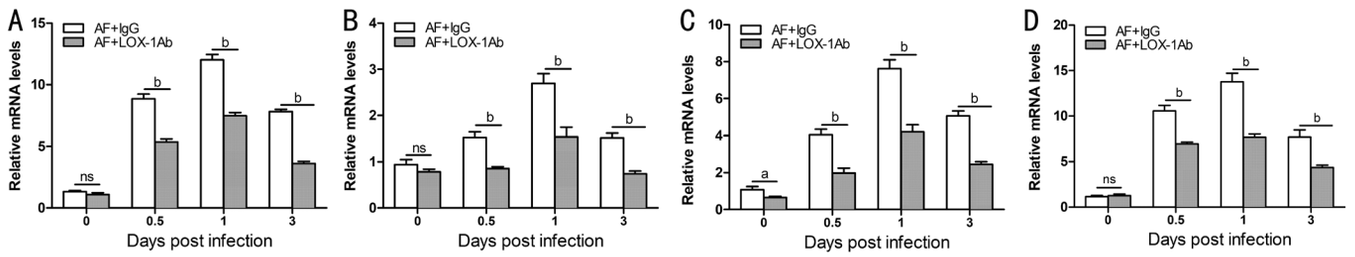

Figure 3 Effect of LOX-1 neutralizing antibody treatment on C57BL/6 mice corneas Before infection, C57BL/6 cornea was pretreated with LOX-1 neutralizing antibody or IgG, and then infected with *A. fumigatus* for 0, 0.5, 1, or 3d. The mRNA levels of ICAM-1 (A), VCAM-1 (B), P-selectin (C) and E-selectin (D) were measured by PCR. The values are expressed as mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$.

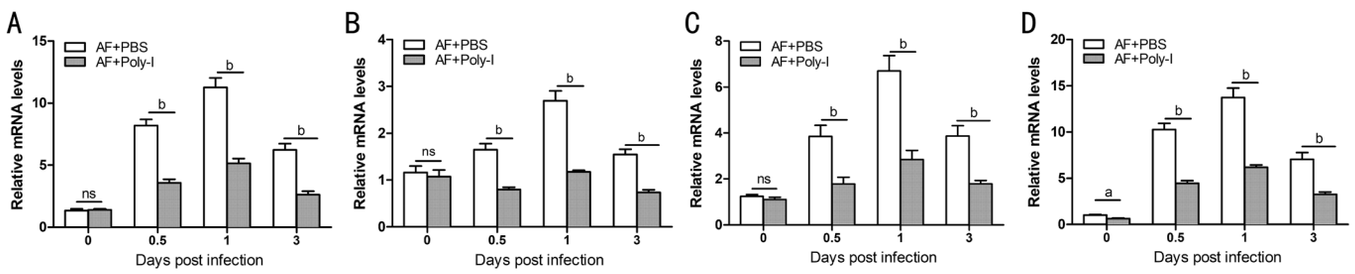

Figure 4 Effects of LOX-1 inhibitor Poly-I treatment on C57BL/6 mice corneas C57BL/6 corneas were pretreated with Poly-I before infection and then infected with *A. fumigatus* for 0, 0.5, 1, or 3d. The mRNA levels of ICAM-1 (A), VCAM-1 (B), P-selectin (C) and E-selectin (D) were measured by RT-PCR. The values are expressed as mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$.

A. fumigatus keratitis in mice, we used LOX-1 neutralizing antibody to down-regulate LOX-1 expression of mouse cornea, then we tested the mRNA levels of adhesion molecules in different groups cornea by RT-PCR. The data indicated that the mRNA levels of ICAM-1 (Figure 3A) and VCAM-1 (Figure 3B) were dramatically decreased after LOX-1 neutralizing antibody pretreatment compared with IgG pretreatment at 0, 0.5, 1, and 3d p.i. The mRNA levels of P-selectin (Figure 3C) and E-selectin (Figure 3D) were dramatically decreased after LOX-1neutralizing antibody pretreatment compared with IgG pretreatment at 0, 0.5, 1, and 3d p.i.

The Effect of LOX-1 Inhibitor on Adhesion Molecules in C57BL/6 Mice Corneas To confirm whether LOX-1 regulates adhesion molecules expression in *A. fumigatus* keratitis in mice, we used LOX-1 inhibitor to down-regulate LOX-1 expression of mouse cornea, and then used RT-PCR test to detect adhesion molecule mRNA levels in the cornea. The data indicated that the mRNA levels of ICAM-1 (Figure 4A) and VCAM-1 (Figure 4B) were significantly reduced after Poly-I pretreatment compared with PBS at 0, 0.5, 1, and 3d p.i. The mRNA levels of P-selectin (Figure 4C) and E-selectin (Figure 4D) were significantly reduced after Poly-I pretreatment compared with PBS at 0, 0.5, 1, and 3d p.i.

The Protein Levels of ICAM-1 Expression in Different Treatment Groups Cornea of C57BL/6 mice The results indicated that the protein levels of ICAM-1 were significantly higher in C57BL/6 mice corneas after 3d of *A. fumigatus* corneal infection ($P<0.01$; Figure 5A). Furthermore, the IF and Western blot results indicated that the protein levels of ICAM-1 were dramatically decreased after LOX-1 antibody pretreatment compared with IgG pretreatment at 3d p.i., meanwhile, the protein levels of ICAM-1 were also significantly reduced after Poly-I pretreatment compared with PBS pretreatment at 3d p.i. (both $P<0.01$; Figure 5A).

The Expression of the ICAM-1 ligand LFA-1 in Neutrophils of C57BL/6 Mice with Different Treatment Groups Compared

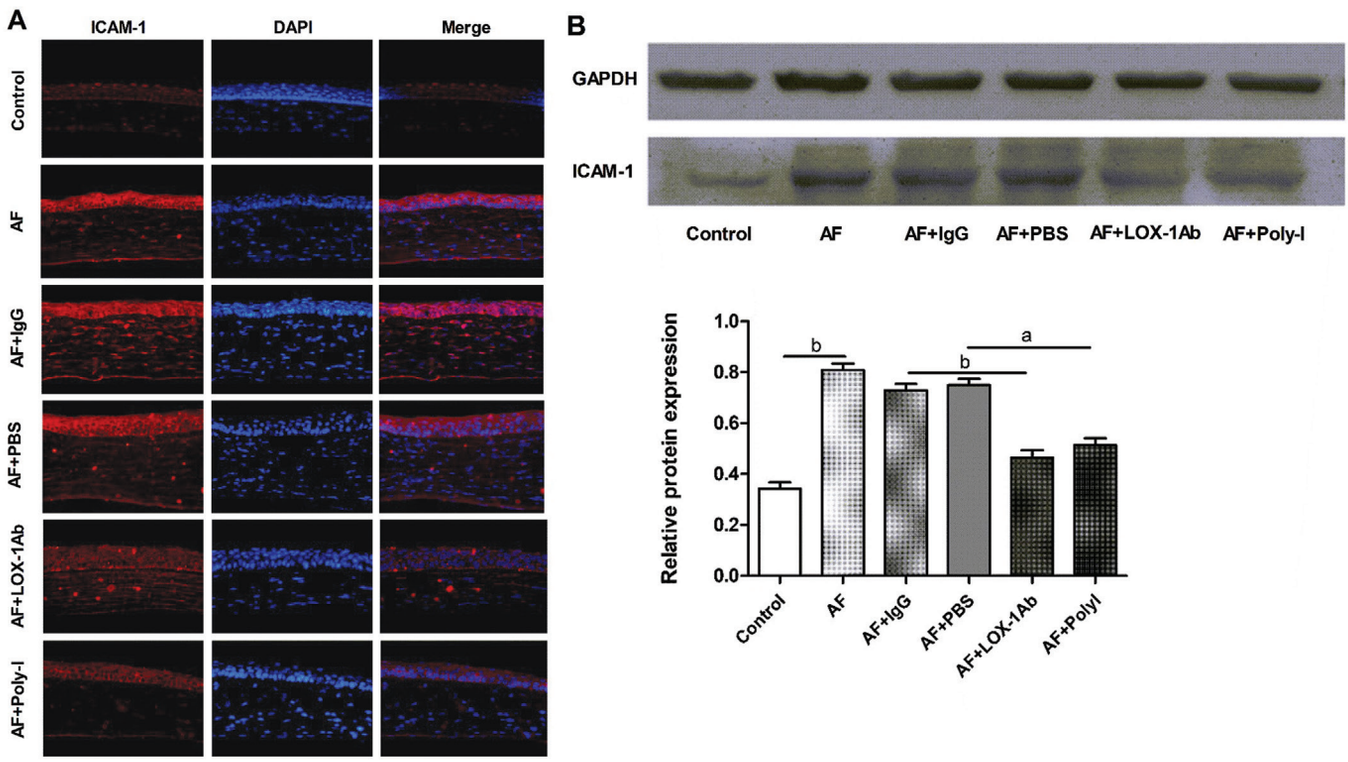

Figure 5 Protein Expression of ICAM-1 in the corneas of C57BL/6 mice Immunofluorescence of 10 μm frozen sections was used to observe the expression of corneal ICAM-1 protein in C57BL/6 mice of different treatment groups after 3d p.i. The protein levels of ICAM-1 expression using immunofluorescent staining (A) and Western blot (B). Green represents ICAM-1 and blue represents DAPI (nucleus). Magnification ×40. The values are expressed as mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$.

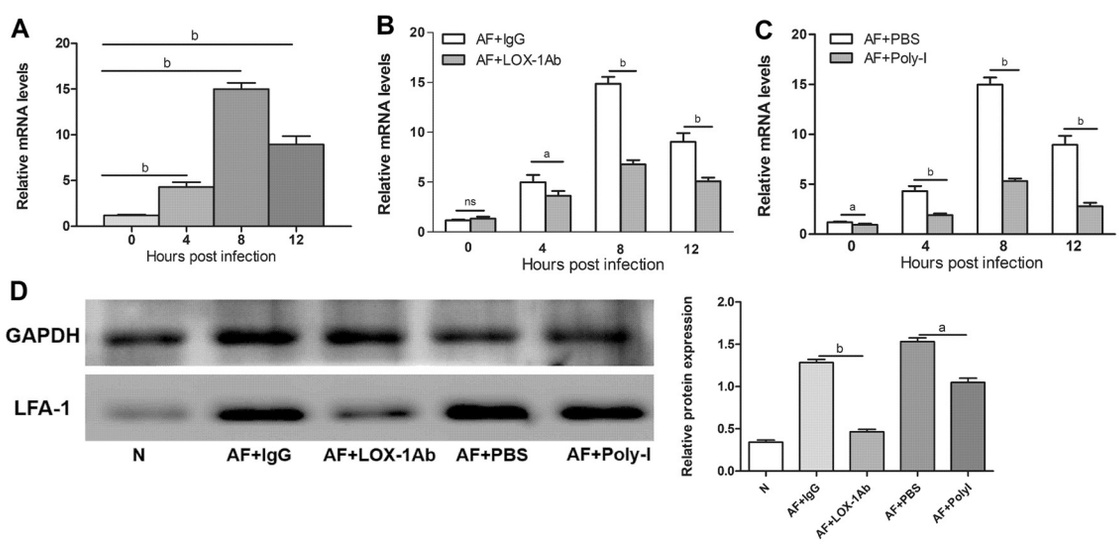

Figure 6 The expression of the LFA-1 in PMNs of C57BL/6 mice Before infection, PMNs were pretreated with LOX-1 neutralizing antibody or inhibitor, and then stimulated with *A. fumigatus* for 4, 8, 12h. The expression of LFA-1 in PMNs was analyzed by RT-PCR (A) and Western blot (B). The values are expressed as mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$.

with normal neutrophils, First, the experimental result showed that the mRNA levels of the LFA-1 (all $P<0.01$; Figure 6A) were significantly higher in mice neutrophils after *A. fumigatus* stimulation for 4, 8, and 12h. The mRNA levels of LFA-1 were dramatically reduced after LOX-1 antibody pretreatment compared with IgG after 0, 4, 8, and 12h p.i. (Figure 6B); the mRNA levels of LFA-1 were dramatically reduced after Poly-I pretreatment compared with PBS after 0, 4, 8, and 12h p.i. (Figure 6C). Moreover, pretreatment with LOX-1 antibody and the Poly-I decreased the protein levels of LFA-1 compared with pretreatment with control IgG or control PBS at 8h p.i. (Figure 6D).

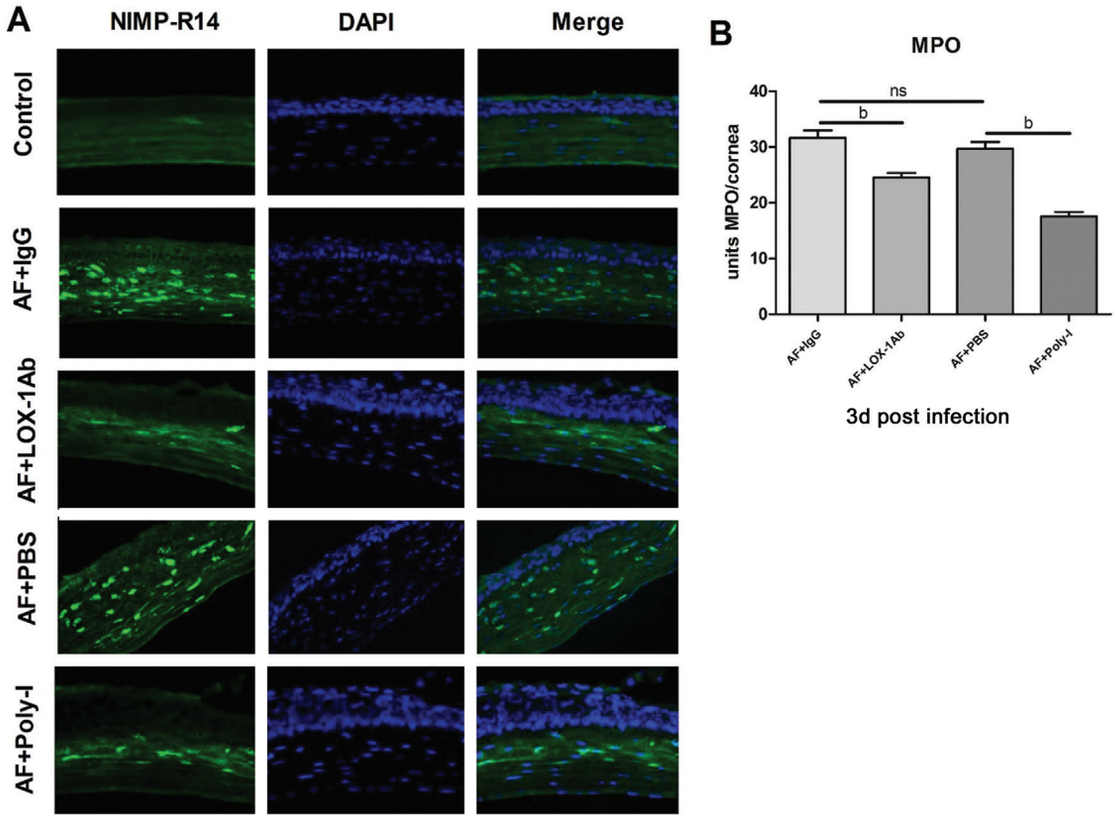

Figure 7 Effect of LOX-1 on neutrophil infiltration in mice corneas Mouse corneas were pretreated with LOX-1 neutralizing antibody or inhibitor and IgG or PBS before infection, then stimulate the corneas with *A. fumigatus* for 3d. A: Infiltrating neutrophils were directly observed and measured as a count per 10 μm cornea. NIMP-R14 (green) and DAPI staining (blue). Magnification: 40×. B: The levels of MPO expression after pretreatment of the LOX-1 neutralization antibody and inhibitor in *A. fumigatus* stimulated mice corneas compared with IgG and PBS. The values are expressed as mean±SEM. $^{b}P<0.01$.

Effect of LOX-1 on Neutrophil Recruitment to *A. fumigatus* induced Corneal Inflammation As shown in Figure 7, C57BL/6 corneas exposed to *A. fumigatus* had significantly more neutrophils per corneal section than control corneas (Figure 7A). Pretreatment with LOX-1 neutralizing antibody resulted in significantly decreased neutrophil infiltration compared with IgG pretreatment at 3d p.i. Next, C57BL/6 corneas pretreated with the LOX-1 inhibitor Poly-I before infection and then infected with *A. fumigatus* for 3d had significantly fewer neutrophils than pretreatment of PBS (Figure 7A). Also, the MPO levels, an indication of PMN infiltration, decreased at 3d p.i. after LOX-1 neutralization antibody or inhibitor pretreatment compared with IgG or PBS pretreatment (both $P<0.01$; Figure 7B).

DISCUSSION

FK is a serious corneal infectious disease that can cause severe vision loss[16]. At present, the host of corneal fungi is not aware of the biological reactions occurring in corneas. Studies have shown that innate immune response can induce effective anti-*Aspergillus* immune response[17]. Our previous studies have confirmed that LOX-1 as a family of PRRs may be involved in the innate immunity of corneal anti-*Aspergillus* [13-14]. However, it is not known in detail how LOX-1 affects the degree of inflammation of *A. fumigatus* keratitis.

First, we found that LOX-1 can affect the expression of adhesion molecules after infection with *A. fumigatus*. The results showed that down-regulating the expression of LOX-1 can reduce the expression of adhesion molecules. Meanwhile, other research found that the LPS-induced expression of ICAM-1 is significantly inhibited by LOX-1 blockade in the lungs[6], which is consistent with our data. Previous studies have indicated that LOX-1 knockout mice suffering endotoxemia have an obvious reduction in lung injury; NF-κB activation was decreased in the lungs of these mice. After LOX-1 is bound by pathogen-associated molecular patterns (PAMPs), NF-κB is triggered, which leads to adhesion molecules expression and plays an important role in the process of inflammation[18]. In our experiments, pretreatment of LOX-1 antibody and Poly-I significantly changed the disease outcome, the clinical score was significantly lower in LOX-1 antibody and Poly-I treated mice compared with the control group. These data indicate that LOX-1 can regulate the expression of adhesion molecules and then affect the degree of inflammation in mice of *A. fumigatus* keratitis.

In addition, LOX-1 also functions as an adhesion molecule; according to research found that PMNs infiltration in wild-type mice is more extensive than that in LOX-1 knockout mice[19]. Blocking LOX-1 can prevent the death of rats in the endotoxemia model and inhibit the leukocyte-endothelium interaction in low-dose endotoxin-induced uveitis[8]. These studies suggest that LOX-1 may affect the infiltration of neutrophils. The researchers have demonstrated that neutrophils recruited to the corneal stroma resulted in fungal killing, and early adhesion of PMNs leads to the rapid accumulation of inflammation[20-21]. PMNs can play an important role in host defense, but they may exert cytotoxic effects on the resident cells in the cornea and cause visual impairment, loss of corneal transparency, and even blindness[22]. In our study, pretreatment with LOX-1-neutralizing antibody or inhibitor followed by infection with *A. fumigatus* resulted in significantly fewer neutrophils than the control group. Besides, the MPO levels, indicative of PMN infiltration, also reduced after pretreatment of LOX-1-neutralizing antibody or inhibitor. At the same time, we found that the degree of inflammation of cornea was also reduced compared with pretreatment of IgG or PBS. These results demonstrated that the down-regulation of LOX-1 expression in mice *A. fumigatus* keratitis can reduce the number of neutrophils in mice corneas.

Currently, studies have demonstrated that neutrophil recruitment is a complicated process; the process involves a few groups of adhesion molecules, such as the immunoglobulin superfamily (VCAM-1 and ICAM-1, expressed primarily on endothelial cells), selectins (P-selectin and E-selectin expressed on endothelial cells) and the integrins αLβ2-integrin (LFA-1) and α4β1-integrin (VLA-4) expressed on PMNs. The extravasation cascade and neutrophil adhesion belong to a multi-step process[7]. In the first step, neutrophils are tethered and roll on the vessel wall; the selectin family mediates this transient adhesion interaction. In the second step, ICAM-1 binds the integrin ligands LFA-1 which are activated by chemokines, leading to transendothelial migration and firm adhesion[23-24]. PMN expresses LFA-1, which combines to ICAM-1; the absence of this interaction may impair neutrophils' migration and phagocytosis[25-27]. Moreover, researchers also have demonstrated that the initial recruitment of neutrophils to the lung may require adhesion molecules such as the expression of ICAM-1[28]. This suggests that ICAM-1 and LFA-1 are essential for neutrophil recruitment. Therefore, we selected ICAM-1 and LFA-1 to verify the regulatory effect of LOX-1 on adhesion molecules by Western blot. In our experiments, we found that the expression of adhesion molecules in the cornea of mice increased after fungal stimulation, meanwhile, the number of neutrophils in mouse cornea also increased. More importantly, LOX-1 inhibition decreased the expression of adhesion molecules, while reducing the numbers of PMNs in the cornea. Goldberg *et al*[29] research confirmed that sites of corneal inflammation have readily accessible adhesion molecules, which could obviously result in the recruitment of PMNs to these areas. Experiments in ICAM-1-deficient mice indicated that ICAM-1 recruits inflammatory cells into the infected eye and plays a vital role in the progression of *P. aeruginosa* eye infection with severe pathology characteristics[30]. Based on the above study, our results suggest that LOX-1 is involved in the neutrophil infiltration of mice *A. fumigatus* keratitis and this effect may be regulated by adhesion molecules expression.

In conclusion, these studies suggest that inhibition of LOX-1 can reduce the expression of adhesion molecules and thus decrease neutrophil infiltration in *Aspergillus* infected corneas of C57BL/6 mice after infection. Because neutrophil infiltration is key to the successful elimination of inflammation, further research on the role and mechanism of LOX-1-induced expression of adhesion molecules and signaling mechanisms may provide new methods to regulate the immune system to resist inflammatory and infectious diseases.

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