The Role of LOX-1 in Innate Immunity to Aspergillus fumigatus in Corneal Epithelial Cells

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PURPOSE. To determine the role of lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) in corneal epithelial cells exposed to fungus.

METHODS. Thirty corneas with fungal keratitis composed the experimental group. Ten healthy donor corneas were the control group. Corneal epithelium was scraped and LOX-1 mRNA tested. Immunostaining was used to detect LOX-1 protein. Human corneal epithelial cells (HCECs) were treated with 75% ethanol-killed Aspergillus fumigatus. LOX-1 was detected, and CXCL1, TNF-α, IL-6, and dendin-1 levels were detected with or without neutralization of LOX-1. Rat fungal keratitis was established and LOX-1 was detected by PCR, immunostaining, and Western blot. Phosphorylated-p38MAPK (p-p38MAPK) protein; CXCL1, TNF-α, and IL-6 mRNA levels; and myeloperoxidase (MPO) were tested before and after LOX-1 neutralization.

RESULTS. LOX-1 was expressed in corneal epithelium. In vitro cellular experiment showed that LOX-1 was detected in normal HCECs, and LOX-1 mRNA increased after stimulation of A. fumigatus and peaked at 8 hours; LOX-1 protein expression increased after stimulation at 24 and 48 hours. Neutralization of LOX-1 decreased expression of CXCL1, TNF-α, but did not change IL-6 or dendin-1 expression. Aspergillus fumigatus keratitis developed in rats activated p38MAPK and elevated the expression of CXCL1, TNF-α, and IL-6 through LOX-1. LOX-1 neutralization reduced MPO levels.

CONCLUSIONS. LOX-1 was detected in normal corneal epithelium and HCECs and A. fumigatus elevated the expression of CXCL1 and TNF-α through LOX-1. Rat A. fumigatus keratitis activated p38MAPK and elevated the expression of CXCL1, TNF-α, and IL-6 through LOX-1.

Keywords: LOX-1, corneal epithelial cells, Aspergillus fumigatus

Fungal keratitis is a severe corneal disease with high rate of blindness.1 The innate immune response is the first line of defense to identify and resist the pathogen infection. It can immediately identify the highly conservative consensus sequence called “pathogen-associated molecular patterns” through pattern recognition receptors (PRRs).2 Then it mediates the adhesion, absorption, and eradication of the pathogen through infiltration of neutrophils, macrophages, and the production of cytokines and chemokines. The PRRs that participate in the immune response to fungi mainly include Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), and nucleotide-binding oligomerization domain-like receptors.3

Another class of PRRs is the scavenger receptor (SR) family. Scavenger receptors function in the uptake and clearance of polyanionic ligands of both pathogen and self-origin. In addition to their involvement in lipid metabolism, SRs also bind and internalize microbial organisms and their products, including gram-negative bacteria (lipopolysaccharide), gram-positive bacteria (lipoteichoic acid), and CpG DNA.4,5 Scavenger receptors play a critical role in host defense against a variety of pathogens.

Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), a lectin-like 52-kD type II membrane receptor for oxidized low-density lipoproteins (ox-LDLs), was originally identified from vascular endothelial cells as a cell-surface endocytosis receptor for ox-LDL. LOX-1 is mainly expressed in endothelial cells, macrophages, neutrophils, vascular smooth muscle cells, and platelets.6,7 LOX-1 belongs to the SR family and also structurally belongs to CLRs, with a short intracellular N-terminal hydrophilic domain and a long extracellular C-terminal hydrophilic domain separated by a hydrophobic domain of 26 amino acids.8 The unique lectin-like structure enables LOX-1 to recognize a wide range of substances, including ox-LDL, damaged or apoptotic cells, endotoxins, activated platelets, and pathogenic microorganisms, suggesting that it has diverse activities.

LOX-1 plays a proinflammatory role in host defense. LOX-1 induction in arthritic joints can promote joint inflammation and cartilage destruction by mediating leukocyte infiltration into the arthritic joints, and LOX-1 neutralizing antibody treatment can significantly suppress leukocyte infiltration, TNF-α production, and ameliorate joint swelling and cartilage degradation induced by zymosan, indicating that LOX-1 plays a major proinflammatory role in articular inflammation.9 Blockade of LOX-1 prevents animal death in a rat endotoxemia model, and it suppresses leukocyte infiltration and inhibits leukocyte–endothelium interaction in retinal blood vessels in low-dose endotoxin-induced uveitis.10 Deletion of LOX-1 or inhibition of LOX-1 function by a blocking antibody prevents proinflammatory, pro-oxidant responses in endothelial cells and reduces atherogenesis in mouse atherosclerosis models.11,12 LOX-1 also acts as an adhesion molecule in the leukocyte recruitment and platelet–endothelium interaction.13 The multiple functions of LOX-1 support the idea that LOX-1 might be working as a member of innate immunity.
Recent studies have shown that LOX-1 is rapidly upregulated in mouse lung following lipopolysaccharide (LPS) challenge, and pretreatment of mice with anti-LOX-1-blocking antibody significantly inhibits LPS-induced lung inflammation, as indicated by decreased neutrophil accumulation in the lung. LOX-1 deletion prevents neutrophil overreaction after sepsis induction. Anti-LOX-1 inhibits LPS-induced inflammatory responses, including NF-κB activation, intercellular adhesion molecule-1 (ICAM-1) expression, and apoptotic signaling in mouse lung, and decreases the influx of neutrophils into the lung and inhibition of lung vascular leakage. LOX-1 might serve as a valuable target in the prevention of inflammatory lung injury in sepsis.

The expression and function of LOX-1 during *Aspergillus fumigatus* keratitis remain to be determined. Thus, the current study investigated the expression and function of LOX-1 in innate immunity to *A. fumigatus* infection in corneal epithelial cells. Our data provided evidence that corneal epithelium can express LOX-1, and LOX-1 mRNA and protein levels were upregulated after *A. fumigatus* infection. In addition, human corneal epithelial cells (HCECs) were treated with LOX-1 neutralizing antibody and LOX-1 small interfering RNA (siRNA). Data from these studies provided evidence that inhibition of LOX-1 results in decreased expression of CXCL1 and TNF-α mRNA and of protein levels. Furthermore, *A. fumigatus* keratitis developed in Wistar rats also activated p38MAPK and produced CXCL1, TNF-α, and IL-6 through activation of LOX-1. LOX-1 inhibition can reduce the infiltration of polymorphonuclear neutrophilic leukocytes (PMN).

**MATERIALS AND METHODS**

**Clinical Specimens**

In total, 10 cases of healthy donor corneas were used for keratoplasty and the rest of the peripheral corneal tissues were collected. Thirteen patients (15 eyes) with fungal keratitis (seven cases of *Fusarium*, six cases of *Aspergillus*) underwent penetrating keratoplasty, and corneas with lesions were collected. Half of the corneal epithelium was scraped and scrapings were put in 500 μl RNAiso (Takara, Dalian, Liaoning, China) and used for mRNA extraction and PCR reaction. Immunohistochemical staining for LOX-1 was carried out on the left region. Research adhered to the tenets of the Declaration of Helsinki. For the management of specimens, the agreement of patients was obtained and informed consents were signed. The experiment had the approval of the hospital’s ethics committee.

**Aspergillus fumigatus Culture**

*Aspergillus fumigatus* strain 3.0772 (China General Microbiological Culture Collection Center, Beijing, China) were inoculated in 150-ml Erlenmeyer flasks containing Sabouraud liquid medium (4% glucose, 1% Mycoprotein). Flasks were shaken at 37°C and 110 rpm for 48 hours. Then, the mycelia were disrupted into 20- to 40-μm pieces in tissue grinder, followed by sterile PBS wash ×3 times, 4000g/min ×5 minutes, after which the supernatant was discarded—without inactivation (for animals) or inactivated overnight in 70% alcohol (for HCECs). Phosphate-buffered saline (for animals) or Dulbecco’s modified Eagle’s medium (DMEM; Gibco, San Diego, CA, USA) (for HCECs) was used as solution for *A. fumigatus* and yielded 1 × 10⁸ CFU/mL.

**Human Corneal Epithelial Cell Culture and *A. fumigatus* Stimulation**

Human corneal epithelial cells (kindly provided by Ocular Surface Laboratory of Zhongshan Ophthalmic Center, Guangzhou, Guangdong, China) were cultured in DMEM with 10% fetal bovine serum (Gibco), 0.075% growth factor (Gibco), 0.075% insulin (Solarbio, Beijing, China), 1% penicillin G (Gibco) and streptomycin sulfate (Solarbio) at 37°C, and 5% CO2. Near 80% confluence, the cells were cultured in serum-free DMEM for 24 hours and treated with *A. fumigatus* hyphae (to the final concentration of 5 × 10⁶ CFU/mL) for 0, 4, 8, 16, 24, and 48 hours in 12-well plates. Cells were used for real-time RT-PCR, immunocytochemistry, and Western blot, and supernatant was collected for ELISA. The mRNA levels of LOX-1, CXCL1, TNF-α, and IL-6 in HCECs were detected by real-time RT-PCR after stimulation at 0, 4, 8, and 16 hours. LOX-1 protein levels of HCECs were detected by immunocytochemistry and Western blot at 0, 24, and 48 hours. CXCL1, TNF-α, and IL-6 protein levels of supernatant were detected by ELISA at 0, 24, and 48 hours.

**LOX-1 Neutralizing Antibody Treatment of HCECs**

LOX-1 blocking experiments were conducted by incubating HCECs with polyclonal antibodies against LOX-1. Human corneal epithelial cells were incubated with anti-LOX-1 (final concentration 10 μg/mL; Abcam, Cambridge, MA, USA) or IgG control antibody (final concentration 10 μg/mL; Bioss, Beijing, China) for 2 hours. They were then treated with *A. fumigatus* hyphae (final concentration of 5 × 10⁶ CFU/mL) for 8 hours or 24 hours. The supernatants were collected to evaluate the expression of CXCL1, TNF-α, and IL-6 protein at 24 hours and cells were harvested to estimate the expression of decin-1, CXCL1, TNF-α, and IL-6 mRNA at 8 hours.

**LOX-1 siRNA Treatment of HCECs**

Human corneal epithelial cells were incubated with LOX-1 siRNA (final concentration 200 nM; Santa Cruz Biotechnology, San Jose, CA, USA) or scrambled control siRNA (final concentration 200 nM; Santa Cruz Biotechnology) 1 day before infection. They were then treated with *A. fumigatus* hyphae (final concentration of 5 × 10⁶ CFU/mL) for 8 hours or 24 hours. The supernatants were collected to evaluate the release of CXCL1, TNF-α, and IL-6 protein at 24 hours and cells were harvested to estimate the expression of decin-1, CXCL1, TNF-α, and IL-6 mRNA at 8 hours.

**Animals and Corneal Infection**

Wistar rats (female) were purchased from Qingdao Institute of Drug Control (Qingdao, China) and weighed between 200 and 300 g. The animals were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The left eyes were chosen as experimental eyes. Rats were anesthetized with 10% chloral hydrate, placed beneath a stereoscopic microscope at ×25 magnification, and the central corneal epithelium (2-mm-diameter range) of the left eye was removed. A 5-μL aliquot (1 × 10⁶ CFU/mL) of *A. fumigatus* was topically applied to the ocular surface; the latter was covered with a soft contact lens and the eyelids were sutured. For the control eye, the central corneal epithelium was removed, the eye was covered with a soft contact lens, and the eyelids were sutured, without infection. Rat corneal epithelium was harvested for real-time RT-PCR at 4, 8, and 16 hours after the experimental model was...
established. The method to obtain rat corneal epithelium for real-time PCR was in accordance with Zhao et al.16 Eyeballs were removed at 48 hours for immunohistochemistry.

**LOX-1 Neutralizing Antibody Treatment**

LOX-1 neutralizing antibody (20 μg/10 μL; Abcam) or control IgG (20 μg/10 μL; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was given subconjunctivally into the left eyes of rats (n = 5/group/time) the day before infection. On 1 day post infection (p.i.), an additional 200 μg/100 μL was injected intraperitoneally (i.p.); controls were similarly injected with IgG. Rat corneal epithelium was harvested for PCR and Western blot at 48 hours after infection.

**Real-Time RT-PCR**

Total RNA was isolated from cells by using RNAiso plus reagent and quantified by spectrophotometry. RNA (2 μg) was used for first-strand cDNA synthesis according to the protocol for a reverse transcription system. Then cDNA was used for PCR in 20-μL reaction volume following the manufacturer’s instructions. Real-Time PCR Master Mix (Takara) was used for the PCR reaction with primer concentrations of 5 μM. All reactions were performed with the following cycling parameters: 95°C for 10 minutes, followed by 40 cycles of 95°C for 20 seconds, and 65°C for 45 seconds. Relative transcription levels were calculated by using the relative standard curve method that compares the amount of target normalized to an endogenous reference gene, β-actin. Data are shown as the mean ± standard error of the mean for relative mRNA levels. The primer pair sequences used for real-time RT-PCR are shown in the Table.

**Enzyme-Linked Immunosorbent Assay**

The supernatant was collected and centrifuged at 12,000g for 5 minutes. A 50-μL aliquot of each supernatant was assayed in duplicate for CXCL1, TNF-α, or IL-6 protein according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

**Immunofluorescent Staining**

Human corneas and infected rat eyes were enucleated (n = 3/group/time), immersed in 0.01 M PBS, embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN, USA), and frozen in liquid nitrogen. Twelve-micrometer sections were cut, mounted to poly-L-lysine–coated glass slides, and stored at 37°C overnight. After a 2-minute fixation in acetone, slides were blocked with 0.01 M PBS containing 2.5% BSA, goat IgG (1:100) for 30 minutes at room temperature. Then, sections were incubated for 1 hour with 1:100 dilution of rabbit anti-human LOX-1 (Abcam) or 1:100 dilution of rabbit anti-rat LOX-1 (Bioss). This was followed by secondary antibodies, FITC-conjugated goat anti-rabbit antibody (1:100; Bioss) or Alexa Fluor 546-conjugated goat anti-rabbit antibody (1:1500; Life Technologies, Carlsbad, CA, USA). Finally, sections were visualized and digital images captured with fluorescence microscopy.

**Immunohistochemistry**

After fixation and removal of endogenous peroxidase with 3% hydrogen peroxide, cells were incubated with goat-blocking antibody at 37°C for 20 minutes. After that, cells were reacted with LOX-1 antibody (1:100; Abcam) for 2 hours, then with a biotin-conjugated anti-rabbit secondary antibody (ZSGB Bio,
Beijing, China) at 37°C for 40 minutes, followed by peroxidase-conjugated streptavidin (ZSGB Bio) for 10 to 15 minutes. Slices were developed with diaminobenzidine (ZSGB Bio). The reacting time was controlled under microscope. Then cells were counterstained with hematoxylin for 1 minute, dehydrated, and finalized with neutral balsam.

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay lysis buffer (RIPA; Solarbio) buffer for 1 hour, and then were centrifuged. After testing the protein concentration, adding SDS sample buffer, and boiling, total protein was separated on 10% acrylamide SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Solarbio). The membranes were blocked with 5% BSA, and then were incubated with a monoclonal antibody to β-actin (1:2000; Beyotime, Suzhou, Jiangsu, China), and primary antibody to LOX-1 (1:1000; Abcam) or p38MAPK, phosphorylated-p38MAPK (p-p38MAPK, 1:1000; Beyotime) at 4°C overnight. After washing in PBS containing 0.05% Tween 20 (Bio-Rad, Hercules, CA, USA) for three times, the membranes were incubated with corresponding peroxidase-conjugated secondary antibodies (1:2000, Beyotime) at 37°C for 1 hour. Then the blots were developed by using chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Quantitation of Corneal PMN

A myeloperoxidase (MPO) assay was used to quantitate the PMN number. Corneas (n = 5/group/time) were removed at 2 days p.i. and homogenized in 1.0 mL phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide (Sigma-Aldrich Corp., St. Louis, MO, USA). Samples were
freeze-thawed four times and after centrifugation, 100 μL supernatant was added to 2.9 mL phosphate buffer (50 mM) containing o-dianisidine dihydrochloride (16.7 mg/100 mL) and hydrogen peroxide (0.0005%). The change in absorbency (460 nm) was monitored for 5 minutes at 30-second intervals. The slope of the line was determined for each sample and used to calculate units of MPO/cornea. One unit of MPO activity is equivalent to ~2 × 10⁵ PMN.

Statistical Analysis

An unpaired, two-tailed Student’s t-test was used to determine the statistical significance of the real-time RT-PCR, ELISA, and MPO assays. Values were considered significant at P < 0.05.

RESULTS

LOX-1 Expression in Human Corneal Epithelium

Messenger RNA and protein expression of LOX-1 in normal uninfected and fungi-infected human corneas were tested by real-time RT-PCR and immunostaining. Results indicated that relative LOX-1 mRNA levels were significantly higher in Aspergillus fumigatus–stimulated HCECs at 24 hours (C) and 48 hours (D). Isotype showed negative staining (E). After A. fumigatus stimulation, LOX-1 protein expression was significantly greater than in normal HCECs (F).

Figure 2. (A–F) Expression of LOX-1 in normal HCECs and A. fumigatus–stimulated HCECs. Relative mRNA levels of LOX-1 were significantly greater in A. fumigatus–stimulated HCECs at 8 and 16 hours than in normal HCECs (A). LOX-1 protein expression in normal HCECs (B). Aspergillus fumigatus–stimulated HCECs at 24 hours (C) and 48 hours (D). Isotype showed negative staining (E). After A. fumigatus stimulation, LOX-1 protein expression was significantly greater than in normal HCECs (F).
infected corneas (Fig. 1D). LOX-1 protein was expressed in epithelium and stroma of fungal keratitis (Figs. 1C, 1D). Isotype showed negative staining (Fig. 1E). Hematoxylin and eosin (HE) staining showed stroma infiltration in Fusarium- and Aspergillus-infected cornea (Figs. 1F, 1G).

**Effect of A. fumigatus on LOX-1 Expression in HCECs**

Messenger RNA and protein levels of LOX-1 were tested by real-time RT-PCR, immunostaining, and Western blot in both normal control and A. fumigatus–stimulated HCECs. Results indicated that relative LOX-1 mRNA levels of HCECs were significantly higher after stimulation of A. fumigatus for 8 and 16 hours than in controls (Fig. 2A; \( P < 0.01, P < 0.05 \), respectively). LOX-1 was detected in normal HCECs. To confirm these data, LOX-1 protein was examined by immunostaining. LOX-1 protein expressions were higher after stimulation of A. fumigatus for 24 (Fig. 2C) and 48 (Fig. 2D) hours than in normal controls (Fig. 2B). Isotype showed negative staining (Fig. 2E). Western blot results showed that LOX-1 protein levels were elevated in the infected HCECs at 24 and 48 hours, compared with normal control (Fig. 2F).

**Upregulation of Cytokines in A. fumigatus–Challenged HCECs**

Concomitant with LOX-1 expression, we used real-time RT-PCR and ELISA to examine CXCL1, TNF-\( \alpha \), and IL-6 mRNA in HCECs and protein levels in supernatant after stimulation with A. fumigatus. The CXCL1 (Fig. 3A; \( P < 0.05, P < 0.001, P < 0.05 \)), TNF-\( \alpha \) (Fig. 3C; \( P < 0.01, P < 0.001, P < 0.001 \)), and IL-6
LOX-1 neutralizing antibody and LOX-1 siRNA treatment of HCECs. LOX-1 neutralizing antibody and LOX-1 siRNA significantly inhibited *A. fumigatus* hyphae–induced secretion of CXCL1 (A) and TNF-α (C) mRNA at 8 hours and inhibited CXCL1 (B) and TNF-α (D) protein at 24 hours when compared with control cells. Interleukin-6 mRNA (E) and protein (F) levels did not change significantly after LOX-1 neutralization. LOX-1 inhibition did not change dectin-1 mRNA levels significantly (G).

**FIGURE 4.** (A–G) LOX-1 neutralizing antibody and LOX-1 siRNA treatment of HCECs. LOX-1 neutralizing antibody and LOX-1 siRNA significantly inhibited *A. fumigatus* hyphae–induced secretion of CXCL1 (A) and TNF-α (C) mRNA at 8 hours and inhibited CXCL1 (B) and TNF-α (D) protein at 24 hours when compared with control cells. Interleukin-6 mRNA (E) and protein (F) levels did not change significantly after LOX-1 neutralization. LOX-1 inhibition did not change dectin-1 mRNA levels significantly (G).
mRNA levels were elevated at 4, 8, and 16 hours. CXCL1 (Fig. 3B; \( P < 0.01 \)), TNF-\( \alpha \) (Fig. 3D; \( P < 0.05 \)), and IL-6 (Fig. 3F; \( P < 0.05 \)) protein were elevated at 24 hours when compared with untreated control cells. CXCL1 (Fig. 3B; \( P < 0.05 \)) and IL-6 (Fig. 3F; \( P < 0.001 \)) protein were elevated at 48 hours when compared with untreated control cells. Tumor necrosis factor-\( \alpha \) protein did not differ between the two groups at 48 hours (Fig. 3D).

**LOX-1 Neutralizing Antibody and LOX-1 siRNA Treatment of HCECs**

We next sought to determine whether the expression of cytokines induced by *A. fumigatus* hyphae in HCECs depended on LOX-1 expression. LOX-1 neutralizing antibody and LOX-1 siRNA significantly inhibited *A. fumigatus* hyphae-induced mRNA secretion of CXCL1 (Fig. 4A; \( P < 0.001 \), \( P < 0.001 \), \( P < 0.001 \), respectively). Low levels of LOX-1 mRNA could be detected in the scraped corneal epithelium of normal control rats (Fig. 5A). Immunostaining demonstrated that LOX-1 protein expression markedly increased in the infected corneal epithelium (Fig. 5F) versus controls (Fig. 5C) at 48 hours post infection. There is positive staining in the stroma of infected cornea (F). Isotype showed negative staining (D, G). Hematoxylin and eosin staining showed normal (B) and *Aspergillus*-infected (E) corneas. Magnification (B-G): \( \times 400 \).

**Expression of LOX-1 in Rat Corneal Epithelium**

We next tested LOX-1 mRNA expression in the epithelium of infected corneas of rats. Real-time RT-PCR demonstrated increased LOX-1 mRNA expression in corneal epithelium of infected rat cornea at 4, 8, and 16 hours (\( P < 0.001 \), \( P < 0.001 \), \( P < 0.001 \), respectively). Low levels of LOX-1 mRNA could be detected in corneal epithelium of normal control rats (Fig. 5A). Hematoxylin and eosin staining showed the normal cornea (Fig. 5B) and the infected cornea (Fig. 5E). Expression of LOX-1 mRNA had no change after inhibition (Fig. 4E). LOX-1 neutralizing antibody and LOX-1 siRNA significantly inhibited CXCL1 (Fig. 4B; \( P < 0.05 \), \( P < 0.05 \)) and TNF-\( \alpha \) (Fig. 4D; \( P < 0.01 \), \( P < 0.001 \)) protein at 24 hours when compared with control cells, while IL-6 (Fig. 4F) protein levels did not change significantly. LOX-1 neutralizing antibody and LOX-1 siRNA did not change the expression of dectin-1 mRNA (Fig. 4G).
FIGURE 6. (A–C) LOX-1 neutralizing antibody treatment of rat cornea. LOX-1 neutralizing antibody significantly inhibited LOX-1 protein expression (A, B). LOX-1 neutralizing antibody significantly inhibited p-p38MAPK protein expression (C) at 48 hours post infection.

FIGURE 7. (A–D) LOX-1 neutralizing antibody treatment of rat cornea inhibited A. fumigatus hyphae–induced secretion of CXCL1 (A), TNF-α (B), and IL-6 (C) mRNA at 48 hours and inhibited PMN infiltration (D).
was also confirmed by immunostaining. Results showed that LOX-1 protein expression markedly increased in infected corneas of rat (Fig. 5F) compared with control corneas (Fig. 5C). Isotype showed negative expression (Figs. 5D, 5G).

**LOX-1 Neutralizing Antibody Treatment of Rats**

To investigate the role of LOX-1 in vivo, we next gave rats the treatment with LOX-1 neutralization. After LOX-1 neutralization, the expression of LOX-1 protein was significantly downregulated (Figs. 6A, 6B; *P* < 0.01). LOX-1 neutralization decreased p-p38MAPK protein levels at 2 days’ infection (Fig. 6C). LOX-1 neutralization decreased the mRNA expression of CXCL1 (Fig. 7A; *P* < 0.05), TNF-α (Fig. 7B; *P* < 0.01), and IL-6 (Fig. 7C; *P* < 0.05), and MPO levels (Fig. 7D; *P* < 0.05) at 2 days post infection.

**DISCUSSION**

LOX-1 is emerging as a key player in immune responses. LOX-1 is expressed in endothelial cells, neutrophils, macrophages, dendritic cells, vascular smooth muscle cells, and platelets. Results presented in this study revealed that LOX-1 was detected in human normal and fungi-infected corneal epithelium and that, after *A. fumigatus* infection, its expression is significantly upregulated. These data, suggesting a potential role for LOX-1 in corneal *A. fumigatus* infection, are consistent with results of a previous study from another laboratory showing that renal epithelial luminal cells can express LOX-1 and its activation has proinflammatory function. This was further confirmed by an in vitro study, showing that relative LOX-1 mRNA and protein levels of HCECs are significantly higher after stimulation of *A. fumigatus* than in controls. Our findings also are consistent with those previous studies showing LOX-1 is rapidly upregulated in mouse lung after LPS challenge. Previous studies have reported that LOX-1 plays a role in host defense after articular inflammation. LOX-1 might play a role in promoting joint inflammation and cartilage destruction by mediating leukocyte infiltration and TNF-α production after treatment with zymosan. LOX-1 is increased in brain abscesses of mice compared to uninfected animals, and LOX-1 has recently been shown to collaborate with TLR2 to regulate innate immune responses to various pathogens. Anti-LOX-1 antibody reduces the degree of leukopenia and completely rescues the animals after injection of a high dose of endotoxin. In a model of low-dose endotoxin-induced uveitis, anti-LOX-1 antibody efficiently suppresses leukocyte infiltration. To fully determine the role of LOX-1 in corneal epithelium during corneal *A. fumigatus* infection, LOX-1 neutralizing antibody was given to HCECs; results showed that treatment with LOX-1 neutralizing antibody significantly decreased mRNA levels of proinflammatory molecules CXCL1 and TNF-α compared with control. We also found lower CXCL1 and TNF-α protein levels after LOX-1 neutralization. LOX-1 might be working as a member of innate immunity, though excess activity of LOX-1 might result in damage to organs. We conclude that manipulation of the activity of LOX-1 at appropriate levels would be beneficial for the control of inflammation-related diseases. Our findings are consistent with studies showing that deletion of LOX-1 or inhibition of LOX-1 function by a blocking antibody is able to prevent proinflammatory, pro-oxidant responses in endothelial cells and reduce atherosogenesis in mouse atherosclerosis models. Blockade of LOX-1 inhibits leukocyte-endothelium interaction in retinal blood vessels in low-dose endotoxin-induced uveitis. Our findings also are consistent with studies showing that pretreatment of mice with anti–LOX-1-blocking antibody significantly inhibits LPS-induced lung inflammation as indicated by decreased neutrophil accumulation in the lung.

LOX-1 represents a novel target for the modulation of the inflammatory response within the microcirculation in sepsis, and antibodies against LOX-1 significantly reduce endotoxin-induced leukocyte adherence in intestinal submucosal vessels. Anti-LOX-1 antibody inhibits *Candida pneumoniae* infectivity. Anti-LOX-1 antibody inhibits attachment of *C. pneumonia* to endothelial cells. Anti-LOX-1 is capable of inhibiting LPS-induced inflammatory responses, including NF-kB activation, p38MAPK activation, ICAM-1 expression, and apoptotic signaling in mouse lung. Our studies showed that LOX-1 expressed on rat corneal epithelium and fungal infection upregulate the expression of LOX-1. Moreover, p38MAPK is downstream of LOX-1 as shown in our study. LOX-1 can induce p38MAPK activation; LOX-1 can also induce the production of CXCL1 and TNF-α and infiltration of PMN. Dectin-1 and LOX-1 both belong to C-type lectin-like receptors. Neutralizing antibody or LOX-1 siRNA did not change the mRNA expression of dectin-1 significantly. Further in vivo studies will help to fully understand the role of LOX-1 and the relationship of LOX-1 and dectin-1 in fungal keratitis.

In summary, the data presented herein indicated that LOX-1 was expressed in corneal epithelium. In vitro cellular experiment showed that LOX-1 was detected in normal HCECs, and expression of LOX-1 mRNA and protein increased after stimulation of *A. fumigatus*. In addition, we provided evidence that neutralization of LOX-1 decreased expression of CXCL1 and TNF-α. *A. fumigatus* keratitis developed in Wistar rats activated p38MAPK through LOX-1. These data suggest that LOX-1 plays a proinflammatory role during fungal keratitis. Further studies need to be done to investigate the role of LOX-1 in the pathogenesis of fungal keratitis.

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**References**


