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LOX-1 and TLR4 affect each other and regulate the generation of ROS in *A. fumigatus* keratitis☆

Xinran Gao, Guiqiu Zhao *, Cui Li, Jing Lin, Nan Jiang, Qian Wang, Liting Hu, Qiang Xu, Xudong Peng, Kun He, Guoqiang Zhu

Department of Ophthalmology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China

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ABSTRACT

Purpose: To explore the relationship between LOX-1 and TLR4 in *Aspergillus fumigatus* (*A. fumigatus*) keratitis. To determine LOX-1 and TLR4 can affect each other and regulate inflammation through regulation of the generation of reactive oxygen species (ROS) in *A. fumigatus* keratitis.

Methods: The cornea and abdominal cavity extracted neutrophils of susceptible C57BL/6 mice were infected with *A. fumigatus*. The cornea and neutrophils were pretreated with LOX-1 neutralizing antibody, Polyinosinic acid (Poly(I)) (the inhibitor of LOX-1) or CLI-095 (the inhibitor of TLR4) separately before infection. LOX-1, TLR4 and IL-1β expression were detected in normal and infected cornea by PCR and Western Blot, while ROS was detected in the neutrophils by flow cytometry.

Results: LOX-1, TLR4, IL-1β mRNA and protein levels were up-regulated in C57BL/6 cornea after infection. LOX-1 neutralizing antibody or Poly(I) pretreatment decreased the expression of LOX-1, TLR4 and IL-1β in C57BL/6 cornea after infection and CLI-095 pretreatment decreased the expression of LOX-1, TLR4 and IL-1β in C57BL/6 cornea after infection. ROS generation was increased in C57BL/6 neutrophils after infection, however, ROS generation was decreased in C57BL/6 neutrophils after infection by LOX-1 neutralizing antibody or Poly(I) or CLI-095 pretreatment.

Conclusion: LOX-1, TLR4 and IL-1β expression and ROS generation are increased after infection. LOX-1 and TLR4 can affect each other and regulate the generation of ROS in *A. fumigatus* keratitis. Inhibition of LOX-1 and TLR4 can reduce ROS generation.

1. Introduction

Fungal keratitis is a severe corneal disease caused by fungal infection with a high rate of blindness [1]. However, medical and surgical treatment is not so satisfactory [1–6]. Investigation of the key pathogenic mechanism and effective treatment methods are very important. The innate immune response mediates the recognition and eradication of fungi through infiltration of neutrophils. It also mediates the production of cytokines and chemokines, and constitutes the first line of defense to identify and resist the fungal infection [7]. The highly conservative consensus sequence called "pathogen-associated molecular patterns (PAMP)" can be immediately identified by immune cells through pattern recognition receptors (PRRs). PRRs such as Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), and scavenger receptors (SRs) participate in the immune response to fungi [8,9]. SRs play an important role in host defense against a variety of pathogens. SRs can ingest and clear polyanionic ligands of both pathogen and self-origin [9].

Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), is a type II membrane receptor for oxidized low-density lipoproteins (oxLDLs). LOX-1 belongs to the SRs with a typical CLRs structure, is mainly expressed in endothelial cells, macrophages, neutrophils, vascular smooth muscle cells, and platelets [10,11]. Previous studies have provided evidence that LOX-1 is also expressed in human normal corneal epithelium, and the expression of LOX-1 is increased after *A. fumigatus* stimulation [12]. Toll-like receptor 4 (TLR4) is a type I transmembrane protein. As a kind of PRRs in innate immunity, TLR4 has a crucial role that mediates signal pathway, and produces inflammatory cytokines, as well as promotes neutrophil chemotaxis [13–16]. Studies [17–19] have provided evidence that LPS can up-regulate the expression of TLR4 and LOX-1. The TLR4/NF-kB signaling pathway has an important role in the oxLDL-induced up-regulation of LOX-1 expression in human umbilical vein endothelial cells (HUVECs). LOX-1 together

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* Corresponding author.
E-mail address: zhaoguiqiu_good@126.com (G. Zhao).

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with TLR4 participates in the inflammatory response and the process of atherosclerosis. All above suggests that TLR4 closely correlated with LOX-1.

ROS is a double-edged sword because on the one hand it is beneficial to the host released by neutrophils for destructing exogenous pathogens and modifying various signaling molecules [20–23], on the other hand, it is harmful for damaging mitochondria, inducing cellular dysfunction and inflammation [24]. Previous studies showed that LOX-1 may regulate ROS in vascular smooth muscle cells [25]. Generation of cellular ROS plays an important role in many of the pro-inflammatory signals activated by LOX-1 activation including MAPK and NF-kB activation [26]. ROS can induce the production of IL-1β [27,28]. Meanwhile, external stimuli such as IL-1β and hypoxia also stimulate the formation of ROS [29,30]. As important pro-inflammatory cytokine participates in the immune defense process of corneal resistance to fungal infection, IL-1β is produced by mucosal epithelial cells of the ocular surface and immune cells. The expression level of IL-1β can reflect the severity of inflammation [31].

Studies have shown that LOX-1 and TLR4 were expressed in cornea [12,32]. We want to further explore the relationship between LOX-1 and TLR4 in fungal keratitis, and whether LOX-1 and TLR4 can regulate the generation of ROS in fungal keratitis. Our data provided evidence that LOX-1, TLR4, IL-1β mRNA and protein levels were up-regulated in C57BL/6 cornea after infection and the generation of ROS was increased in neutrophils after infection. In addition, LOX-1 inhibition significantly decreased LOX-1, TLR4, IL-1β expression and ROS generation in response to fungal infection, similarly, TLR4 inhibition significantly decreased LOX-1, TLR4 and IL-1β expression and ROS generation in response to fungal infection. Above provided evidences prove that LOX-1 and TLR4 can affect each other and LOX-1, TLR4 can regulate inflammation through regulation of generation of ROS in *A. fumigatus* keratitis. LOX-1 and TLR4 play a role of pro-inflammatory during *A. fumigatus* keratitis. Our results provide new ideas for the further study of fungal keratitis pathogenesis and looking for effective treatment methods.

2. Materials and methods

2.1. Animals and corneal infection

Eight-week-old female C57BL/6 (susceptible) mice were purchased from Changzhou Cavens Laboratory Animal Co., LTD. (Jiangsu, China). Mice were anesthetized with 8% chloral hydrate, and placed beneath a stereoscopic microscope at 40× magnification and the cornea of the left eye wounded (three 1-mm incisions) using a sterile $25^{5/8}$ gauge needle. A 5 μL aliquot containing 1×10^8 CFU/mL of *A. fumigatus*, strain 3.0772 (China General Microbiological Culture Collection Center, Beijing, China), prepared as described before [12], was topically applied to the corneal surface, the latter was covered with a soft contact lens before the eyelids were sutured. Mice corneas were harvested for real-time RT-PCR and western blot at 12 h, 1 and 3 days after infection. All animals were treated in accordance with the Chinese Ministry of Science and Technology Guidelines on the Humane Treatment of Laboratory Animals (vGKFCZ-2006–398) and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Isolation of neutrophils

C57BL/6 mice were given intraperitoneal injection of sterile 3% thioglycollate (Sigma); 12 h later, mice were sacrificed. Then, 10 mL of Dulbecco's modified Eagle's medium (DMEM; Gibco, San Diego, CA, USA) was injected into the abdominal cavity and fluid aspirated. After centrifugation for 5 min at 300*g* and 4 °C, neutrophils were collected and used for studies.

2.3. Neutrophils culture and A. fumigatus stimulation

Neutrophils were cultured in DMEM with 10% fetal bovine serum (Gibco) for 3 h and treated with *A. fumigatus* hyphae (to the final concentration of 5×10^6 CFU/mL) for 0, 4, 8, 12 and 16 h in 12-well plates. Cells were used for real-time RT-PCR.

2.4. LOX-1 neutralizing antibody and Poly(I) treatment

Goat anti-mouse LOX-1 neutralizing antibody (R&D Systems) (5 μg/5 μL) or control goat IgG (R&D Systems) (5 μg/5 μL) were given subconjunctivally into the left eyes of C57BL/6 mice ($n = 6$/group/time) the day before infection. On 1 day after infection, an additional 2 μg/100 μL was injected intraperitoneally; controls were similarly injected with IgG. Polyinosinic acid (Poly(I))(Sigma) (2 μg/5 μL) or control sterile water were given subconjunctivally into the left eyes of C57BL/6 mice ($n = 6$/group/time) the day before infection. On 1 day after infection, an additional 2 μg/100 μL was injected intraperitoneally; controls were similarly injected with sterile water.

2.5. CLI-095 treatment

CLI-095 (InvivoGen) (0.5 μg/5 μL) or control DMSO (0.5 μg/5 μL) were given subconjunctivally into the left eyes of C57BL/6 mice ($n = 6$/group/time) the day before infection. On 1 day after infection, an additional 2 μg/100 μL was injected intraperitoneally; controls were similarly injected with DMSO.

2.6. Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

After sacrifice, normal (uninfected) and infected corneas were removed at 12 h, 1 and 3 days after infection in C57BL/6 mice ($n = 6$/group/time) for detection of LOX-1, TLR4 and IL-1β mRNA levels. Corneas were harvested at 12 h, 1 and 3 days after infection after LOX-1 neutralizing antibody- or IgG-treatment, after Poly(I)- or sterile water-treatment and after CLI-095- or DMSO-treatment. The mRNA levels of LOX-1, TLR4 and IL-1β in neutrophils were detected after stimulation with *A. fumigatus* hyphae at 0, 4, 8, 12 and 16 h. Total RNA was extracted by using RNAiso plus reagent and quantified by spectrophotometry. RNA (2 μg) was used to produce a cDNA template for PCR reaction through reverse transcription. cDNA products were diluted 1:25 with diethylpyrocarbonate (DEPC)-treated water and a 2 μL cDNA aliquot was used for real-time RT-PCR (20 μL total reaction volume). Real-Time SYBR® Green was used for the PCR reaction with primer concentrations of 5 μM. All reactions were performed with the following cycling parameters: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and a final stage of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Relative mRNA levels of LOX-1, TLR4 and IL-1β, were tested by real-time RT-PCR. β-Actin was used as an internal control. The primer pair sequences used for real-time RT-PCR are shown in Table 1.

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

Gene	GenBank No.	Primer sequence (5′–3′)	Size (bp)
β-Actin	NM_007393.5	F – GAT TAC TGC TCT GGC TCC TAG C R – GAC TCA TCG TAC TCC TGC TTG C	147
LOX-1	NM_138648.2	F – AGG TCC TTG TCC ACA AGA CTG G R – ACG CCC CTG GTC TTA AAG AAT TG	273
TLR4	NM_021297.3	F – CCT GAC ACC AGG AAG CTT GAA R – TCT GAT CCA TGC ATT GGT AGG T	71
IL-1β	NM_008361.3	F – CGC AGC AGC ACA TCA ACA AGA GC R – TGT CCT CAT CCT GGA AGG TCC ACG	111