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Lipoxin A4 alleviates inflammation in *Aspergillus fumigatus*–stimulated human corneal epithelial cells by Nrf2/HO-1 signaling pathway

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Purpose: To investigate the therapeutic effect of lipoxin A4 (LXA4) on *Aspergillus fumigatus (A. fumigatus)*–stimulated human corneal epithelial cells (HCECs).

Methods: The cell counting kit-8 (CCK-8) was performed in HCECs to evaluate the toxicity of LXA4. A cell scratch test was used to assess the impact of LXA4 on the migration of HCECs. Enzyme-linked immunosorbent assay (ELISA), quantitative real-time polymerase chain reaction (qRT-PCR), and western blot were applied to examine the expression of inflammatory mediators in *A. fumigatus*–stimulated HCECs. The nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation and expression in HCECs were detected by immunofluorescence staining.

Results: LXA4 at 0–10 nmol·L^{-1} (nM) had no significant cytotoxic effect on HCECs. LXA4 at a concentration of 1 nM and 10 nM significantly promoted the migration rate of HCECs. The mRNA and protein levels of pro-inflammatory mediators, including IL-1β, TNF-α, and IL-6, were remarkably lower in the LXA4-treated group. LXA4 promoted the expression of Nrf2 and heme oxygenase 1 (HO-1) in *A. fumigatus*–stimulated HCECs compared with the PBS control group. Pretreatment with brusatol (BT, Nrf2 inhibitor) or Zine Protoporphyrin (Znpp, HO-1 inhibitor) receded the anti-inflammatory ability of LXA4.

Conclusions: LXA4 plays a protective role in *A. fumigatus*–stimulated HCECs by inhibiting the expression of pro-inflammatory mediators through the Nrf2/HO-1 signaling pathway.

Fungal keratitis (FK), usually associated with corneal trauma, is the leading cause of blindness in Asia, contributing to nearly half of the world's FK cases [1,2]. FK has high morbidity (about 1,000,000 new corneal infections per annum) and a high rate of blindness, accounting for 1%–45% of infectious keratitis [3]. The most common etiologic agents embrace *Aspergillus* and *Fusarium* species [4].

As an indispensable component of the ocular surface, the corneal epithelium not only serves as the first structural barrier directly fighting against invading pathogens but also plays a non-negligible role in triggering and modulating the immune response [4,5]. The disruption of the structural integrity of the epithelium caused by corneal trauma facilitates the invasion of hyphae [6]. Pattern-recognition receptors (PRRs) expressed by the epithelial cells interact with pathogen-associated molecular patterns (PAMPs) presented on the fungal cell walls, activating the aggregation of immunocytes and the secretion of pro-inflammatory mediators [7,8].

Although inflammatory response is responsible for the elimination of fungal pathogens, exaggerated inflammation could result in further tissue destruction [9,10]. Therefore, the resolution of inflammation, an intricate clearance process comprising the biosynthesis of a superclass of lipid mediators [11-13], is the key process involved in the repair of and recovery from corneal tissue damage. Lipoxin A4 (LXA4), one of the most significant physiologic lipid mediators, is derived from the successive actions of lipoxygenases and is predominantly produced by biosynthetic circuits engaged in cell-cell interactions during the onset of inflammation [14-16]. As an endogenous inflammation-braking signal, LXA4 has been demonstrated to possess an anti-inflammatory property in a series of experimental models of inflammation in vivo and in vitro [17-19]. For example, LXA4 decreased plasma levels of pro-inflammatory mediators in murine sepsis models and suppressed NF-κB activity in peritoneal macrophages [20]. Recently, Ye et al. [21]. found

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that LXA4 exerts anti-inflammatory effects via the Nrf2/HO-1 pathway to mitigate acute lung injury. The nuclear factor erythroid 2–related factor 2 (Nrf2) is a cytoprotective transcription factor that could initiate the expression of various types of genes, including heme oxygenase-1 (HO-1), to boost anti-inflammatory and antioxidant responses during endogenous or exogenous stress [22-24]. As a rate-limiting enzyme involved in heme catabolism, HO-1 engages in the degradation of heme to carbon monoxide (CO), which exerts its anti-inflammatory effect via inhibiting the NF-κB pathway [25,26].

As the role of LXA4 in *A. fumigatus* keratitis remains undisclosed, in this study, we investigated whether LXA4 could attenuate inflammation in *A. fumigatus*–stimulated HCECs and the underlying mechanisms, which may provide a novel therapeutic approach for FK.

METHODS

LXA4 preparation: LXA4 stock (25 μg in 250 μl ethanol) was purchased from Cayman Chemical (Ann Arbor, MI) and stored at −80 °C. When biologic experiments were performed, the ethanol was evaporated under a gentle stream of nitrogen, and the solvent of PBS (1X; 120 mM NaCl, 20 mM KCl, 10 mM NaPO4, 5 mM KPO4, pH 7.4) was immediately added to dilute LXA4 to the corresponding concentration.

Cell culture and stimulation: Human corneal epithelial cells (HCECs; Zhongshan Ophthalmic Center, Guangzhou, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Solarbio, Beijing, China) supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2 (Appendix 1). When approximately 80% confluence was reached, the cells were pretreated with PBS or different concentrations of LXA4 for 2 h, followed by *A. fumigatus* stimulation (6×10^6 CFU/ml). After 8 h, 20 h, or 24 h, HCECs were collected for qRT-PCR, immunofluorescence staining, western blot, ELISA, and CCK-8 assay, respectively.

For Nrf2 and HO-1 inhibitor treatments, HCECs were incubated with the Nrf2 inhibitor brusatol (BT; 15 nM; Master of Bioactive Molecules, NJ) or HO-1 inhibitor ZnPP (10 μM; Sigma, St Louis, MO) for 2 h before LXA4 treatment. Then, cells were challenged with *A. fumigatus* (6×10^6 CFU/ml) for 8 h or 20 h for qRT-PCR, western blot, or ELISA.

Cytotoxicity assay: HCECs were seeded into 96-well plates and treated with LXA4 (0, 0.1 nM, 1 nM, 10 nM, 100 nM, and 1000 nM) for 24 h. The cell viability was accessed by adding 10 μl of CCK-8 (MCE) to each well and incubating for 2 h at 37 °C. The absorbance at 450 nm was measured using a microplate reader.

Quantitative real-time polymerase chain reaction (qRT-PCR): HCECs were collected for total RNA extraction with the RNAiso Plus reagent (Takara, Dalian, China). Spectrophotometry (Thermo Fisher Scientific, MA) was used for the quantification of purified RNA [27]. The cDNA was produced from each RNA sample using the HiScript III-RT SuperMix with a gDNA wiper (Vazyme, Nanjing, China). The qRT-PCR was performed with a 20-μL reaction system (2 μl of diluted cDNA [1:12.5], 10 μl of ChamQ SYBR Color qPCR Master Mix [Vazyme], 1 μl of diluted primers [1:9], and 7 μl diethyl pyrocarbonate [DEPC]-treated water) in accordance with the manufacturer's instructions. The mRNA levels of the target genes were analyzed relative to the housekeeping gene β-actin via the ΔCT method [28]. Primer pair sequences are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA): HCECs treated with PBS or LXA4 were exposed *to A. fumigatus* hyphae for 20 h and collected for centrifugation. Supernatants were used to detect the protein levels of IL-1β, TNF-α, and IL-6 via human ELISA kits in conformity with the manufacturer's instructions (Elabscience, Wuhan, China); 100 μl of standard or supernatant was added to the wells and incubated at 37 °C for 90 min. After this, the liquid was discarded; 100 μl of biotinylated detection antibody working solution was added to the wells and incubated for 60 min at 37 °C. The liquid was aspirated, and the plate was washed three times with PBS. Then, 100 μl horseradish peroxidase (HRP) conjugate working solution was added to the wells and incubated for 30 min at 37 °C. The liquid was aspirated, and the plate was washed five times with PBS. After this, 90 μl substrate reagent was added to the wells and incubated for 15 min at 37 °C; 50 μl stop solution was added to the wells. The absorbance was measured at 450 nm with a microplate reader.

Immunofluorescence staining: HCECs plated on the poly-L-lysine-coated glass coverslips were treated with LXA4 for 2 h, followed by *A. fumigatus* stimulation for 8 h. Immunofluorescence staining was performed as previously described [29]. Briefly, cells were fixed with 4% paraformaldehyde and blocked using goat serum (1:100), followed by incubation with an anti-Nrf2 antibody (Abcam, MA; 1:100) at 4 °C overnight and being washed three times with PBST. After the cells were incubated with Cy3-conjugated goat anti-rabbit secondary antibody (Abcam; 1:200) in the dark for 1 h, the cells were washed three times with PBST and washed one time with PBS. Finally, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min and photographed by a confocal laser scanning microscope (Leica SPE).

Western blot analysis: As outlined in previous publications [30-32], HCECs exposed to *A. fumigatus* for 8 h were lysed in

a mixture of RIPA buffer (Solarbio, Beijing, China), phenylmethanesulfonyl fluoride (Solarbio), and phosphatase inhibitor (MCE; mixing ratio: 98:1:1) for 2 h. The protein concentration was quantified by bicinchoninic acid (BCA) assay (Elabscience, Wuhan, China). The protein samples separated by 10% sodium salt -polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto polyvinylidene difluoride membranes. The membranes blocked with blocking buffer were incubated with anti-β-actin antibody (1:3000; Elabscience) and anti-HO-1 antibody (Abcam;1:10000) at 4 °C overnight. After being washed in PBST and incubated with the corresponding secondary antibodies for 1 h, the bands on the membranes were detected with enhanced chemiluminescence (ECL) reagents (Biorad, CA, America). A quantitative grayscale analysis of the western blot bands was performed using Image J.

Cell scratch test: HCECs seeded in the 6-well plates were incubated overnight at 37 °C. Three parallel lines were scratched on the cell layer with a sterile 200-µl pipette tip. Wells were rinsed 3 times with PBS. The cells were cultured with LXA4 (0, 0.1, 1, and 10 nM) for 24 h. Three images were taken along each scratch at 100X magnification using the optical microscope (Axio Vert; Zeiss, Jena, Germany). The width of the scratches were measured by drawing a perpendicular line across the scratch in Image J.

Statistical analysis: All data were displayed as mean ± standard deviation (SD) via GraphPad 7.0 software. An unpaired, two-tailed Student *t* test (for comparison between two groups) or one-way ANOVA (for comparison among three or more groups) was performed for the analysis of RT–PCR, western blot and ELISA data. $P<0.05$ was considered significant (ns: no significance). All experiments were repeated three times independently.

RESULTS

LXA4 Cytotoxicity Test: A CCK-8 assay was performed to test the cytotoxicity of LXA4 in HCECs. After 24 h of incubation, LXA4 less than 10 nM had no significant cytotoxic effect on HCECs, while it began to suppress the viability of HCECs at 100 nM compared with PBS (Figure 1A). Unexpectedly, treatment with 1 and 10 nM LXA4 enhanced the migration rate of HCECs in a dose-dependent manner (Figure 1B, C). Hence, LXA4 with a concentration of 1 nM and 10 nM was adopted for the following experiments.

LXA4 inhibits the expression of pro-inflammatory mediators induced by A. fumigatus: To investigate whether LXA4 plays an anti-inflammatory role in FK in vitro, HCECs were incubated with LXA4 at 1 and 10 nM for 2 h and stimulated by *A. fumigatus* for 8 h. Compared with the PBS control, the LXA4 pretreatment drastically downregulated *A. fumigatus*–induced elevated mRNA and the protein levels of TNF-α (Figure 2A, B) and IL-1β (Figure 2C, D), as well as the protein levels of IL-6 (Figure 2F) in a dose-dependent manner. The mRNA level of IL-6 was significantly suppressed by LXA4 at 10 nM but not at 1 nM (Figure 2E).

LXA4 activates Nrf2/HO-1 signaling pathway in A. fumigatus–stimulated HCECs: We investigated whether this signaling pathway involved the mechanism by which LXA4 alleviates inflammation in *A. fumigatus*–stimulated HCECs. The location of Nrf2 and the expression of HO-1 were analyzed in *A. fumigatus*–stimulated HCECs. Immunofluorescence staining showed that *A. fumigatus* triggered the production of Nrf2 (red), and the LXA4 pretreatment facilitated its nuclear translocation (Figure 3A). In addition, the LXA4 treatment increased the mRNA (Figure 3B) and protein (Figure 3C D) levels of HO-1 induced by *A. fumigatus*

Table 1. Nucleotide Sequence of Human Primers for qRT-PCR .

Gene	Primer	Nucleotide	Sequence GenBank
β-Actin	F	5′ - GCT CCT CCT GAG CGC AAG-3′	NM_001101.3
	R	5′- CAT CTG CTG GAA GGT GGA CA-3′	
IL-1β	F	5′- CCACAGACCTTCCAGGAGAATG-3′	NM_000576.3
	R	5′- ACA AAG GAC ATG GAG AAC ACC-3′	
TNF-α	F	5′- CTCTTCTGCCTGCTGCACTTTG-3′	NM_000594.4
	R	5′- ATGGGCTACAGGCTTGTCACTC-3′	
IL-6	F	5′- AGACAGCCACTCACCTCTTCAG-3′	NM_000600.5
	R	5′- TTCTGCCAGTGCCTCTTTGCTG-3′	
Nrf-2	F	5′- TCAGCGACGGAAAGAGTATGA −3′	NM_001313901.1
	R	5′- CCACTGGTTTCTGACTGGATGT −3′	
HO-1	F	5′- AAGACTGCGTTCCTGCTCAAC-3′	NM_ 002133.2
	R	5′- AAAGCCCTACAGCAACTGTCG-3′	

F, forward; R, reverse

in HCECs. These results indicated that LXA4 may exert its anti-inflammatory role through not only promoting the nuclear aggregation of Nrf2 but also enhancing the production of HO-1 in HCECs.

LXA4 attenuates inflammatory response by activating Nrf2 and HO-1: To further validate the involvement of the Nrf2/HO-1 signaling pathway in the anti-inflammatory effect of LXA4, we treated HCECs with BT (Nrf2 inhibitor) or ZnPP (HO-1 inhibitor) before LXA4 administration and *A. fumigatus* stimulation. Pretreatment with BT or ZnPP partially reversed the decreased mRNA and protein expression of TNF-α (Figure 4A, B), IL-1β (Figure 4C, D), and IL-6 (Figure 4E, F) elicited by LXA4 in infected cells. Furthermore, BT suppressed the increased HO-1 production induced by LXA4 in HCECs (Figure 4G, H). Therefore, LXA4 played its anti-inflammatory role in *A. fumigatus*–stimulated HCECs through activating the Nrf2/HO-1 signaling pathway.

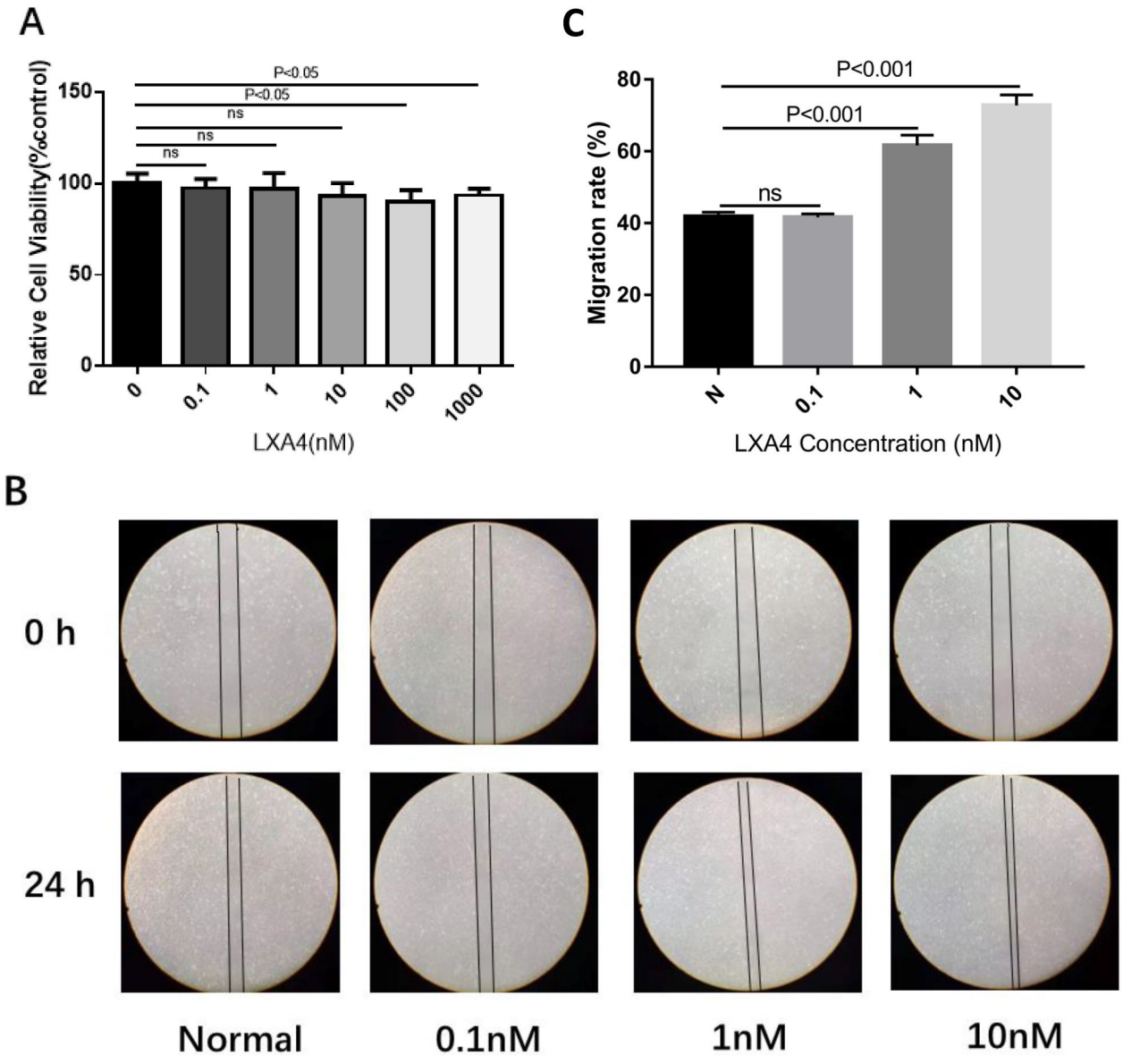

Figure 1. Effects of LXA4 on cell viability and migration. HCECs were incubated with LXA4 at 0, 0.1, 1, 10, 100, and 1,000 nM for 24 h. CCK-8 assay showed the viability of HCECs was inhibited by LXA4 at concentrations of 100 nM and 1,000 nM. **A**: Cell scratch test and **B**: quantitative analysis **C**: of LXA4 on cell migration ability. 1 nM and 10 nM LXA4 promoted the migration of HCECs. The data were analyzed by unpaired, two-tailed Student *t* test and presented as mean ± SD (ns: no significance).

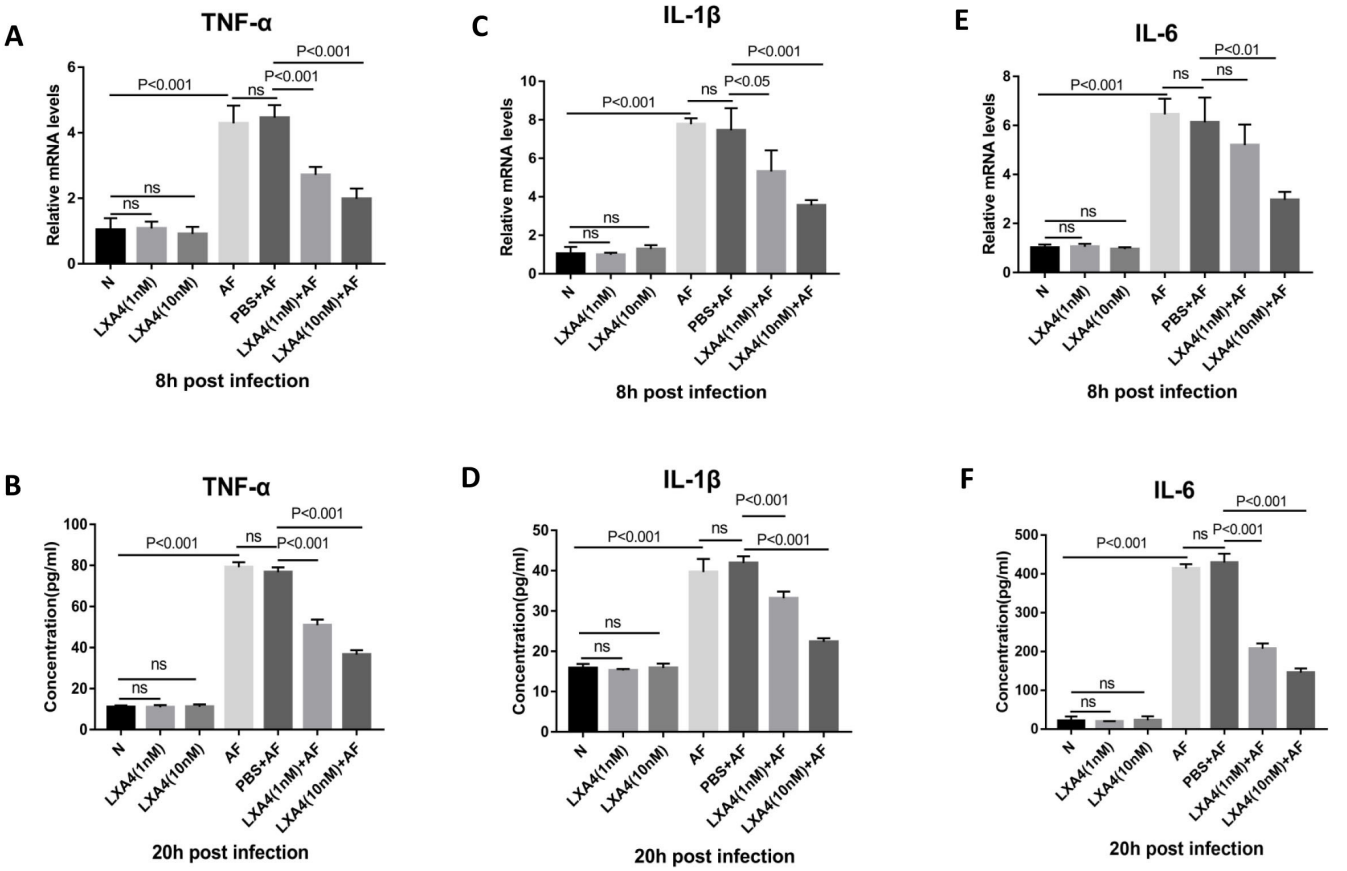

Figure 2. Effects of LXA4 on the expression of pro-inflammatory mediators in *A. fumigatus*–stimulated HCECs. Cells were pretreated with 1 and 10 nM LXA4 for 2 h, followed by 8 h or 20 h *A. fumigatus* stimulation. qRT-PCR quantification of mRNA levels of **A**: TNF-α , **C**: IL-1β, and **E**: IL-6. ELISA analysis of protein levels of **B**: TNF-α, **D**: IL-1β, and **F**: IL-6. All data are presented as mean ± SD and evaluated by an unpaired, two-tailed Student *t* test (ns: no significance).

DISCUSSION

FK is a destructive ocular infection that may lead to perpetual visual impairment and even blindness [33]. Since there are neither blood vessels nor full-time immune cells in the cornea [34,35], the corneal epithelium, consisting of five layers of epithelial cells, is both a physical barrier and a crucial participant involved in the initiating of the immune response to fight against fungal invasion [36,37]. However, the excessive generation of cytokines and inflammatory factors elicited by the immune response may result in tissue destruction, leading to the penetration of fungal hyphae [9]. LXA4 has been reported to antagonize the production of a range of pro-inflammatory mediators such as TNF-α, IL-1β, and IL-6, suppressing the expression of the IL-8 gene in human leukocytes [38], blocking acid-triggered IL-6 release in human bronchial epithelial cells [39], and inhibiting the secretion of TNF-α in human T cells [40]. Furthermore, Gao et al. [41] demonstrated that the LXA4 circuit regulated by estrogen engaged in the inducement of cornea wound healing. In this case, LXA4 may exert a protective effect in HCECs challenged with *A. fumigatus* through repressing the expression of pro-inflammatory mediators.

In this study, we first examined the cytotoxicity of LXA4 using a cell viability assay and migration test. Both 1 nM and 10 nM LXA4 promoted the migration of HCECs, consistent with previous studies indicating that LXA4 facilitates corneal wound healing [42,43]. Next, we found that 10 nM LXA4 potently inhibited the expression of IL-1β, TNF-α, and IL-6 at both the gene and protein levels. IL-1 is a master mediator in the inflammatory response, which appears early in corneal infection models, and blocking IL-1 ameliorates corneal inflammation during keratitis [44]. TNF-α promotes the adhesion of leukocytes via stimulating the expression of E- and P-selectins and regulates the activity of NF-κB in the inflammatory process [45]. Therefore, the administration of

LXA4 could play an anti-inflammatory role by repressing the expression of these pro-inflammatory cytokines.

LXA4 can protect the vascular endothelial cells [18] and lung epithelial cells [32] against oxidative stress-induced injury by activating the Nrf2-HO-1 axis. As an important transcription factor, Nrf2 plays various miscellaneous biologic functions. Under normal physiologic conditions, Keap-1 is associated with Nrf-2 to keep it in the cytosol and guide it to proteasomal degradation. Upon endogenous or exogenous stress induction, Nrf-2 dissociates from Keap-1 and translocates to the nucleus to interact with antioxidant responsive element (ARE) sequences, which then switches on the transcription of a series of related genes, including HO-1 [46,47]. HO-1 is a type of antioxidant enzyme that has been demonstrated to have anti-inflammatory, antiapoptotic, and antiproliferative capabilities [48]. Regulatory functions of HO-1 are intermediated via the catalytic degradation of heme, a complex with potential pro-inflammatory effects [49]. HO-1 contributes to the anti-inflammatory effect of IL-10 [50]. Furthermore, recent studies have revealed the anti-inflammatory targets of HO-1/CO (a product of heme degradation), including the modulation of both the autophagy and inflammasome pathways [51]. Notably, β-glucan, the main component of fungal cell walls, can activate the Nrf2/HO-1 signaling pathway in oral keratinocytes infected with *Candida albicans* [52]. Moreover, both Nrf2 [53] and HO-1 [54] have been verified to promote corneal epithelial wound healing. In the light of all these results, we hypothesized that the Nrf2/HO-1 signaling pathway participates in the pathogenesis of *A. fumigatus* keratitis. *A. fumigatus* stimulation elicited a marked increase in HO-1 expression and Nrf2 nuclear translocation compared with the normal group, which was further facilitated by the LXA4 treatment. In addition, LXA4 and its analogs have been shown to mitigate the inflammatory response through the Nrf2/HO-1 signaling pathway in lung epithelial cells [32], pulmonary microvascular endothelial cells [19,21], and rat glomerular mesangial cells [23]. These results strongly suggested the essential role of the Nrf2/HO-1 signaling pathway during the anti-inflammatory processes of LXA4. To further validate this hypothesis, BT (the inhibitor of Nrf2) and Znpp (the inhibitor of HO-1) were administrated to the HCECs before the LXA4 treatment. The decreased levels of pro-inflammatory cytokines were partially reversed by the BT and Znpp pretreatment. This suggests that aside from the Nrf2/HO-1 signaling pathway, other signaling pathways contribute to the anti-inflammatory effect of LXA4. Intriguingly, Biteman et al. [55]. disclosed the interdependence of LXA4 and HO-1 in counter-regulating inflammation in corneal wound healing, indicating that LXA4 may exert various effects on corneal diseases via the Nrf2/HO-1 signaling pathway.

In conclusion, our research revealed that LXA4 may ameliorate the prognosis of *A. fumigatus* keratitis by suppressing the inflammatory response via regulating the Nrf2/HO-1 signaling pathway. Therefore, the Nrf2/HO-1

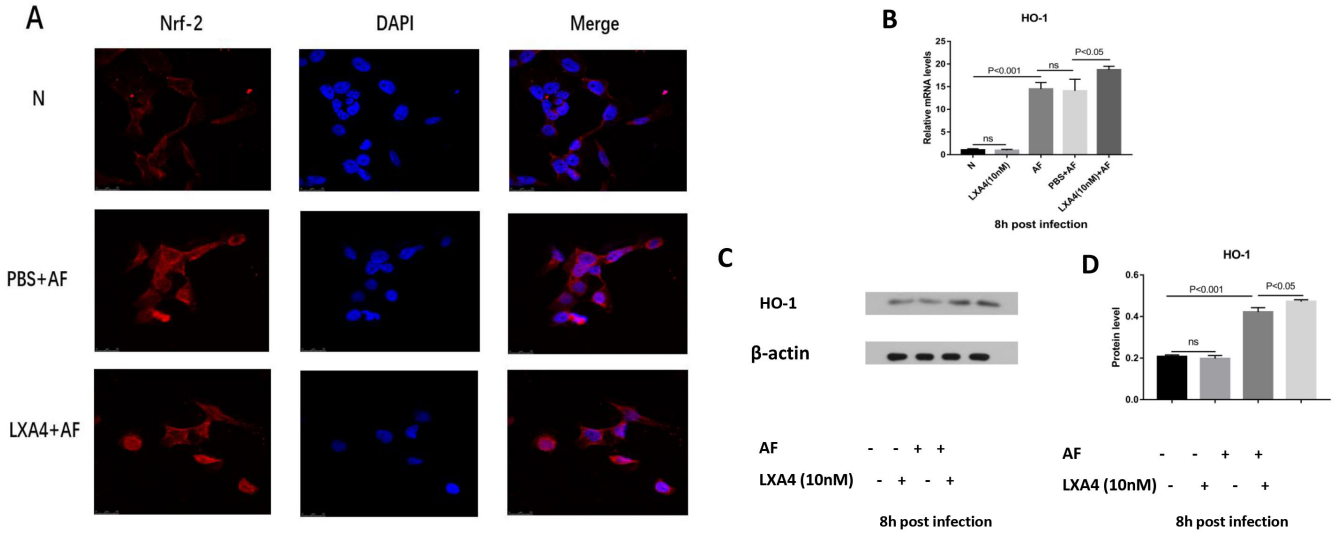

Figure 3. LXA4 treatment activates Nrf2/HO-1 signaling pathway. Immunofluorescence staining demonstrated that LXA4 treatment triggered the nuclear aggregation of Nrf2 in infected HCECs at 8 h post-infection in comparison with PBS treatment. **A**: Magnification, ×600. **B**: The mRNA and **C**, **D:** protein levels of HO-1 were increased in infected groups treated with 10 nM LXA4 compared with PBS control group. All data are presented as mean ± SD and evaluated by an unpaired, two-tailed Student *t* test (ns: no significance).

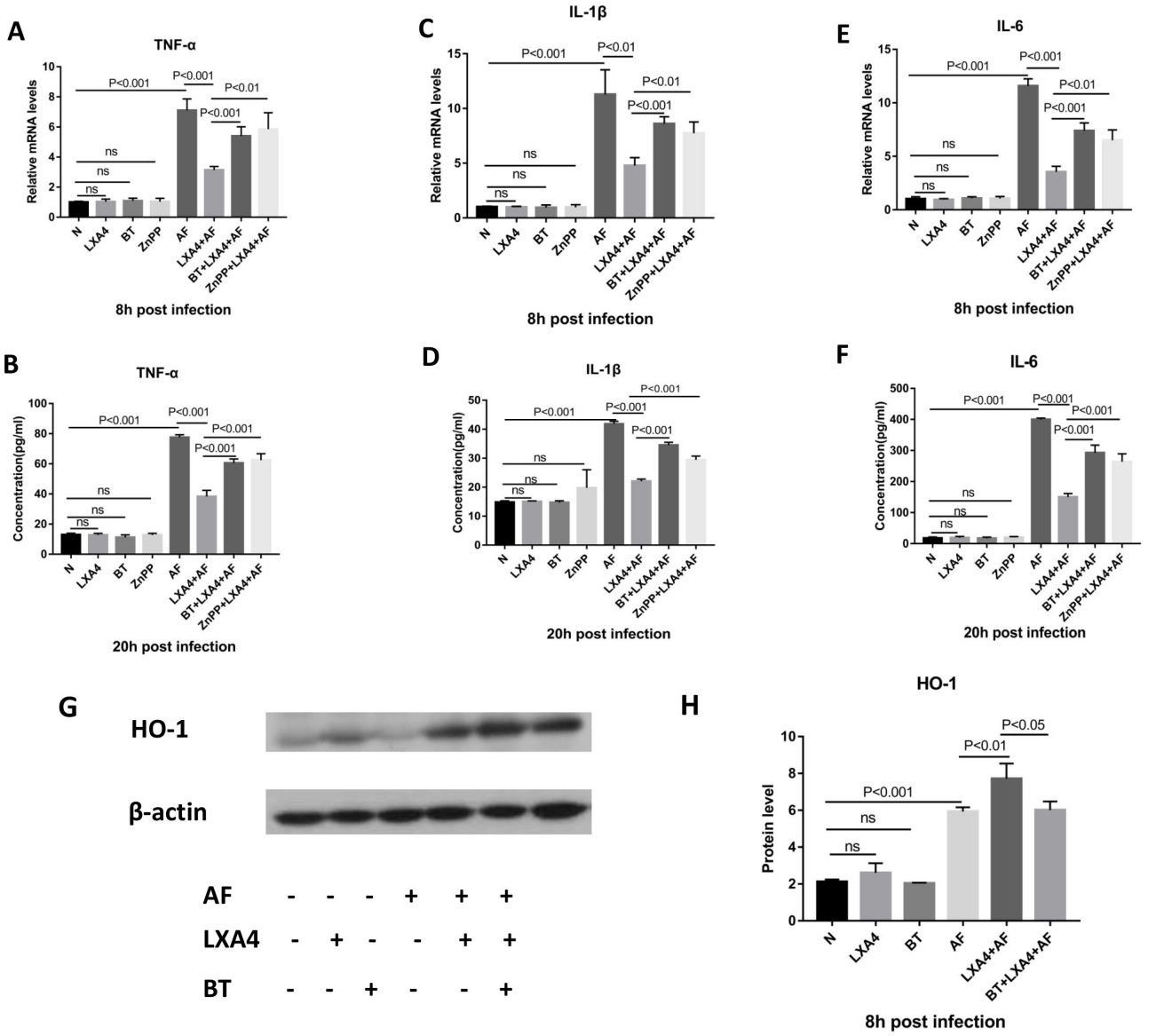

Figure 4. HCECs were pretreated with BT or ZnPP for 2 h, followed by 2 h incubation with 10 nM LXA4 and *A. fumigatus* stimulation. qRT-PCR and ELISA results showed that the LXA4-induced downregulation of **A**,**B**: TNF-α, **C**, **D**: IL-1β, and **E**,**F**: IL-6 mRNA and protein levels at 8 and 20 h post-stimulation in HCECs was partially blocked by BT or ZnPP pretreatment. The increment of HO-1 protein level elicited by LXA4 was suppressed at 8 h post-infection by **G**,**H**: BT pretreatment. All data are presented as mean ± SD and evaluated by an unpaired, two-tailed Student *t* test (ns: no significance).

signaling pathway could be a new therapeutic target for *A. fumigatus* keratitis.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words "Appendix 1."

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