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Celastrol ameliorates *Aspergillus fumigatus* keratitis via inhibiting LOX-1

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ABSTRACT

Purpose: To investigate the effect of Celastrol (CLT) on *Aspergillus fumigatus* (*A. fumigatus*) keratitis.
Methods: Primary peritoneal macrophages of C57BL/6 mice were pretreated with CLT before *A. fumigatus* hyphae stimulation. C57BL/6 mice were infected with *A. fumigatus*. Mice corneas were treated with CLT from 1 day post infection. Clinical score, PCR, ELISA and Western blot were used to test expression of anti-inflammatory mediators, proinflammatory mediators and Lectin-like oxidized low-density lipoprotein receptor 1(LOX-1). The protein levels of p38MAPK after pretreated with CLT in macrophages of C57BL/6 mice challenged with *A. fumigatus* were tested by Western blot.
Results: C57BL/6 mice treated with CLT from 1 day post infection showed decreased disease, IL-1β, TNF-α, IL-10, TGF-β, MIP-2 and LOX-1 levels. CLT treatment markedly inhibiting mRNA and proteins levels of anti-inflammatory mediators, proinflammatory mediators and LOX-1 in macrophages of C57BL/6 mice compared with control group. CLT pretreatment before *A. fumigatus* stimulation obviously inhibiting protein levels of p38MAPK versus DMSO pretreated group in macrophages of C57BL/6 mice challenged with *A. fumigatus*.
Conclusion: These data provide evidences that CLT ameliorates *A. fumigatus* keratitis of C57BL/6 mice via inhibiting LOX-1. CLT pretreatment before *A. fumigatus* stimulation decreased levels of inflammation in macrophages of C57BL/6 mice, which may be regulated by p-p38MAPK.

1. Introduction

Fungal keratitis (FK), caused by fungal infections, is a type of corneal disease with high rate of blindness. FK usually occurs in agricultural countries and tropical areas [1–3].For patients, inaccurate treatment and delayed diagnosis often results in poor vision [4]. Eye trauma, long-term usage of antibiotics or overuse of contact lenses and corticosteroids are the principal causes for the ascending occurrence rate of FK in Chinese mainland [5]. *A. fumigatus*, one of the crucial pathogens causing FK, can be recognized and eliminated by the host through innate immunity and acquired immunity [6].

The first line defending against fungal infection is innate immune system. Immune system needs to recognize fungi through pattern recognition receptors (PRRs) or other ways and then transmit relevant immune signals [7,8]. C-type lectin-like receptors (CLRs), and scavenger receptors (SRs) mentioned below belong to PRRs. LOX-1, a lectin-like 52-kD type II membrane receptor for the oxidation of low density lipoproteins (ox-LDLs), mainly expressed in endothelial cells, macrophages, vascular smooth muscle cells, neutrophils and platelets. LOX-1 belongs to the SR family, but also belongs to the CLR family in structure [9,10]. LOX-1 plays a proinflammatory role in *A. fumigatus* keratitis [11,12], and *A. fumigatus* keratitis can be ameliorated by downregulated LOX-1 expression [13].

Due to poor prognosis of FK, it is urgent to further explore relevant mechanisms and develop new ideas for treatment of FK. The prognosis of FK can be affected by the equilibrium between immunity systems of host and virulence of fungal pathogens. Innate immunity plays a primary role in the early stages of infection, while it may cause excessive inflammatory reactions that can lead to tissue damage and other unpredictable consequences [14–16]. A recent study has shown that suppression of excessive inflammatory response may contribute to healing of corneal wound, which provides a theoretical basis for our study [17].

CLT (3-hydroxy-9β,13α-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid), a promising bioactive compound extracted from the root of *Tripterygium wilfordii* (TW) plant, is a pentacyclic triterpenoid that belongs to a small sort of natural products of triterpene quinine methides [18]. Subsequent studies revealed that CLT exhibit potent pharmacological activities against inflammation [19], cancer [20], viral infection [21] and oxidative stress [22]. It was found

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that CLT can control cytokine-induced inflammation in influenza A virus infection, and it can be used with current antiviral drugs [21]. CLT can protect against inflammatory damage of retinal photoreceptor cells induced by intense light [23]. CLT affects the growth and virulence of *A. fumigatus* through influencing siderophore A, a key enzyme in iron metabolism [24]. Related literature shows that CLT restrains atherosclerotic developing in mice via inhibiting oxidative stress and LOX-1 [22]. Macrophages, expressing LOX-1 receptor, play a basic role in host resistance to fungal infection in cornea [8,25]. The relationship between CLT, LOX-1 and macrophages during *A. fumigatus* keratitis, remains untested to date, which may provide a new perspective for treatment of FK.

We found that treatment of CLT of C57BL/6 mice from 1 day post infection may ameliorate *A. fumigatus* keratitis and improve prognosis. CLT pretreatment before *A. fumigatus* stimulation decreased levels of inflammation in macrophages of C57BL/6 mice. The effect of CLT on *A. fumigatus* keratitis may be regulated by p-p38MAPK.

2. Materials and methods

2.1. Animals and corneal infections

C57BL/6 mice (8-week-old female) were purchased from Jinan Pengyue Laboratory Animal Co., LTD. (Jinan, China). All C57BL/6 mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The method to establish C57BL/6 mice model of *A. fumigatus* keratitis was based on Niu et al. [26]. Clinical score of cornea was assessed according to a 12-point scoring system [27]. In brief, the disease was scored according to area of corneal opacity, density of corneal opacity, and surface regularity, each of which was given a grade of 0–4, with the highest score for uniform opacity in over three-quarters of the corneal area, perforation and descemetocele [28]. Mice corneas were collected for Real-Time PCR (RT-PCR) and Western blot at 3 and 5 days after infection. Eyeballs were removed at 3 and 5 days post infection for hematoxylin and eosin (H&E) staining.

2.2. Preparation and preservation of CLT

CLT (Sigma-Aldrich,St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to generate a stock concentration of 100 mg/ml, stored at −20 °C. CLT was diluted with PBS or cell culture solution to the required concentration before usage.

2.3. CLT treatment of C57BL/6 mice

The left eyes of C57BL/6 mice (n = 6/group/time) were injected with 5 μl of CLT 1 μg/μl or DMSO (control) subconjunctivally at 1 day post infection. The right eyes of C57BL/6 mice are the normal group. Eyes of C57BL/6 mice were treated tropically with 3 μl of CLT 1 μg/μl or DMSO (control) twice daily from 2 day post infection. We used DMSO as control for all these experiments.

2.4. Isolation of mouse peritoneal primary macrophages

Thioglycollate powder (3 g) was dissolved in 100 ml ddH_2O. The thioglycollate solution was stirred on a heating plate until it dissolved completely. The thioglycollate medium was autoclaved at 121 °C for 15 min, then stored at 4 °C before 1 ml was injected intraperitoneally into each C57BL/6 mouse. Seven days after injection, the mice were sacrificed by cervical dislocation and were wiped with 75% alcohol. Then the abdominal wall was cut along the midline of the abdomen and 5 mL of DMEM was slowly injected into the abdominal cavity of the mouse with a syringe. The abdomen was softly massaged for 2 min. This was repeated once more to extract additional macrophages.

2.5. Cell counting Kit-8 assay

The Cell Counting Kit-8(CCK-8) assay was used to detect the toxicity of CLT to cells.

The cells (1×10^4) were seeded into 96 wells plate, treated for 24 h with different concentrations of CLT and incubated with 10 μl CCK-8 (MCE, New Jersey, America) for 2 h. The final production was evaluated by measuring the absorbance of the *OD450* on a microplate reader.

2.6. Culture of macrophages and A. fumigatus stimulation

Macrophages were cultured based on previous methods by He et al. [12] and Liu et al. [29]. Macrophages were stimulated with *A. fumigatus* hyphae (5×10^6 CFU/ml) after pretreatment with CLT of 0.1 μM or 0.3 μM for 2 h. After incubated with *A. fumigatus* hyphae for 4, 8, 12 and 18 h, primary peritoneal macrophages were collected for RT-PCR. Western blot detected LOX-1 protein of macrophages at 24 h after stimulation of *A. fumigatus*. The supernatant of macrophages was collected to test the protein levels of IL-1β, TNF-α, IL-10 at 24 h after *A. fumigatus* stimulation.

2.7. Real-time PCR

Total RNA extracted from C57BL/6 mice corneas or macrophages. The required experimental reagents and method steps was based on Peng et al. [30]. The specific primers were shown in Table 1.

2.8. Western blot

Corneas and macrophages were lysed on ice for 2 h in radioimmunoprecipitation assay (Solarbio, Beijing, China) lysis buffer containing phenylmethylsulfonyl fluoride (PMSF, 100:1; Solarbio) and phosphatase inhibitor cocktail (100:1; MCE). After centrifuged, mixed with SDS sample buffer and boiled, total protein was separated on 12% acrylamide SDS-PAGE and transferred onto PVDF membrane. After blocked at 37 °C or 2 h, membranes incubated with primary antibody at 4 °C overnight. Primary antibodies used in our study includes antibody to LOX-1 (1:1000; Abcam, Cambridge, MA, USA), β-actin (1:2000; Bioss, Beijing,China), p38MAPK(1:500; Elabscience, Wuhan, China) and p-p38MAPK(1:500; Elabscience). After washed with PBST for three times, membranes were incubated with secondary antibodies at 37 °C for 1 h. The blots were tested with chemiluminescence (ECL; Thermo Fisher Scientifc, Waltham, MA, USA).

2.9. Enzyme-linked immunosorbent assay

Corneas of C57BL/6 mice for Enzyme-linked immunosorbent assay

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

Gene	GenBank no.	Primer sequence (5′–3′)
mβ-actin	NM_007393.5	F:GATTACTGCTCTGGCTCCTAGC R:GACTCATCGTACTCCTGCTTGC
mIL-1β	NM_008361.4	F: CGCAGCAGCACATCAACAAGAGC R: TGTCCTCATCCTGGAAGGTCCACG
mTNF-α	NM_013693.3	F: ACCCTCACACTCAGATCATCTT R: GGTTGTCTTTGAGATCCATGC
mIL-10	NM_010548.2	F: TGCTAACCGACTCCTTAATGCAGGAC R: CCTTGATTTCTGGGCCATGCTTCTC
mLOX-1	NM_138648.2	F: AGG TCC TTG TCC ACA AGA CTG G R: ACG CCC CTG GTC TTA AAG AAT TG
mMIP-2	NM_009140.2	F: TGT CAA TGC CTG AAG ACC CTG CC R: AAC TTT TTG ACC GCC CTT GAG AGT GG
mTGF-β	NM_011577.2	F: TGCGCTTGCAGAGATTAAAA R: AGCCCTGTATTCCGTCTCCT

(ELISA) were taken at 3 and 5 days after infection, and cell supernatants were collected at 24 h after *A. fumigatus* stimulation. Preparation of mouse corneal homogenate and cell supernatant was based on manufacturer's instructions (Elabscience). A 100 µl aliquot of each cornea and each supernatant was tested in duplicate for IL-1β, IL-10, and TNF-α protein. Operating procedures followed the manufacturer's instructions (Elabscience).

2.10. Hematoxilin and eosin staining

The degrees of inflammatory cell infiltration, corneal cell edema and corneal structural destruction were assessed by H&E staining. After fixed in formalin, corneas of the mice taken from the different groups were embedded in paraffin. After the sections were fixed on glass slides, they were stained with hematoxylin and eosin. Their pathological changes were observed by optical microscope.

2.11. Statistical analysis

All data are shown as the means ± SD. The clinical score in different groups was determined by Mann-Whitney *U* test. An unpaired, two-tailed Student's *t*-test was used to analyze the significance of the RT-PCR, ELISA and Western blot data.

Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Effect of CLT on clinical score

To explore the effect of CLT on disease response in *A. fumigatus* keratitis of C57BL/6 mice, we used a slit lamp to capture images at 3 and 5 days post infection. The clinical score was used to assess disease response. Clinical scores in DMSO treatment group (Fig. 1A, C and E) were significantly higher than those in CLT treatment group (Fig. 1B, D and F) at 3 and 5 days after infection (Fig. 1G; $p < 0.001$, $p < 0.01$, respectively). H&E staining showed the pathological condition of inflammatory cell infiltration in the cornea of C57BL/6 mice infected with *A. fumigatus* in CLT-treated group (Fig. 1I) or DMSO-treated group (Fig. 1H). CLT treatment markedly restrained the infiltration of inflammatory cells in the cornea infected with *A. fumigatus* (Fig. 1I). These results indicated CLT might alleviate the damage degree of cornea of C57BL/6 mice with *A. fumigatus* keratitis.

3.2. CLT treatment of C57BL/6 mice alleviates the severity of A. fumigatus keratitis in model by inhibiting proinflammatory mediators and LOX-1

To further explore the mechanism by which CLT alleviated the severity of *A. fumigatus* keratitis in mice model, we detected the effects of CLT treatment on production of LOX-1 and inflammatory mediators in *A. fumigatus*-infected cornea. Firstly, we examined the effects of CLT treatment on inflammatory mediators. CLT treatment decreased mRNA levels of IL-1β at 3 and 5 days post infection. (Fig. 2A; $p < 0.05$, $p < 0.05$, respectively). TNF-α and MIP-2 mRNA was declined in the CLT treatment group at 3 days after infection (Fig. 2B and C; $p < 0.05$, $p < 0.05$, respectively). Their mRNA was decreased in the CLT treatment group at 5 days after infection; the difference with DMSO treated group was not significant (Fig. 2B and C). Then we found that CLT treatment down-regulated expression of anti-inflammatory cytokine IL-10 (Fig. 2D; $p < 0.001$) versus DMSO treatment at 3 days post infection. TGF-β mRNA was decreased in the CLT treatment group (Fig. 2E; $p < 0.05$) at 3 days after infection. No differences of mRNA levels were detected between normal corneal groups (Figs. 2A–F). Finally, compared with DMSO treated group, LOX-1 mRNA levels were down-regulated by CLT treatment at 5 days post infection (Fig. 2F; $p < 0.05$). To further confirm the above results, we detected the protein levels of IL-1β, TNF-α, IL-10 and LOX-1. After CLT treatment, IL-1β protein at

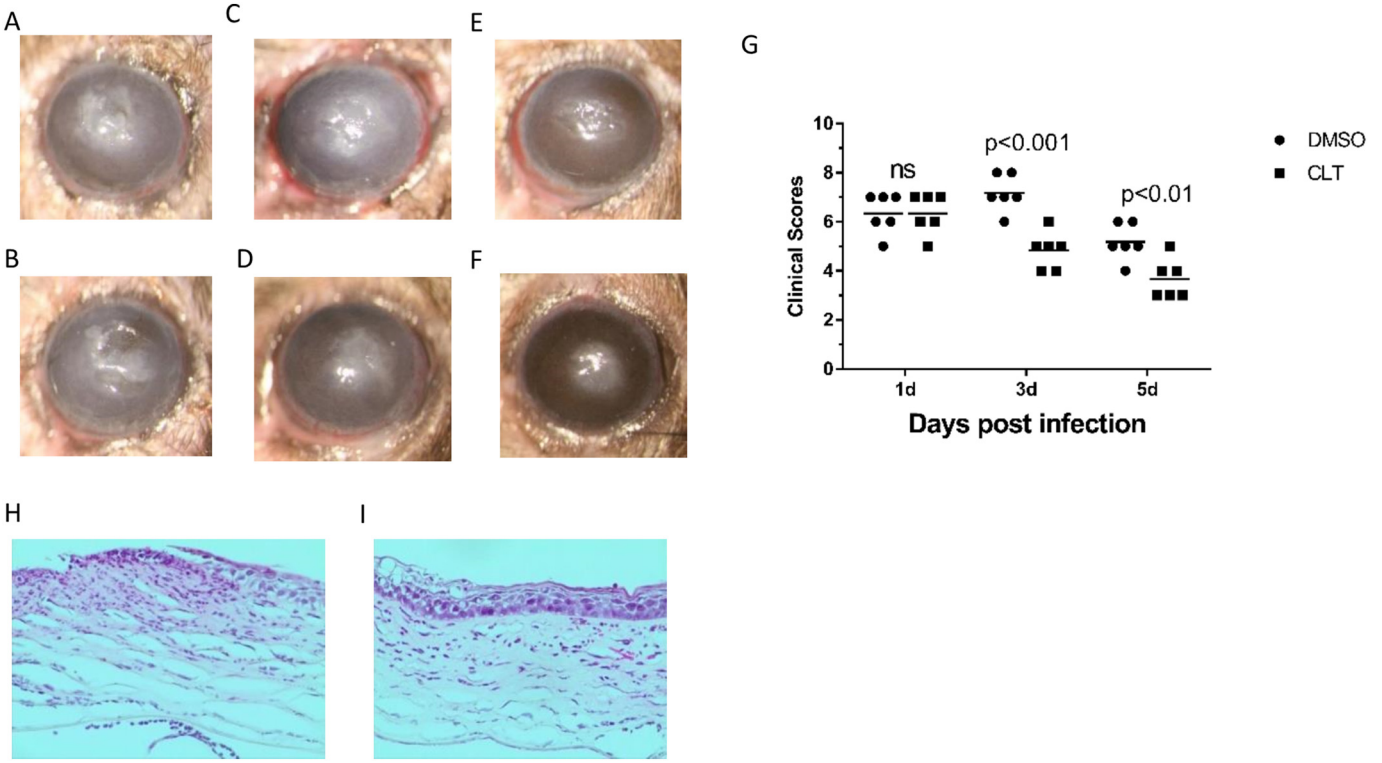

Fig. 1. (A-I) Effects of CLT on Clinical Score of C57BL/6 mice. Photographs were taken by a slit lamp at 1, 3 and 5 days post infection. CLT (D and F) significantly decreased clinical score versus DMSO treated group (C and E) at 3 and 5 days after infection. (H–I) Light microscopic histopathology of *A. fumigatus*–infected corneas from C57BL/6 mice. The infiltration of inflammatory cells was significantly reduced in CLT treatment group (I) compared with DMSO treatment group (H) at 3 days post infection. Magnification (H–I): ×200.

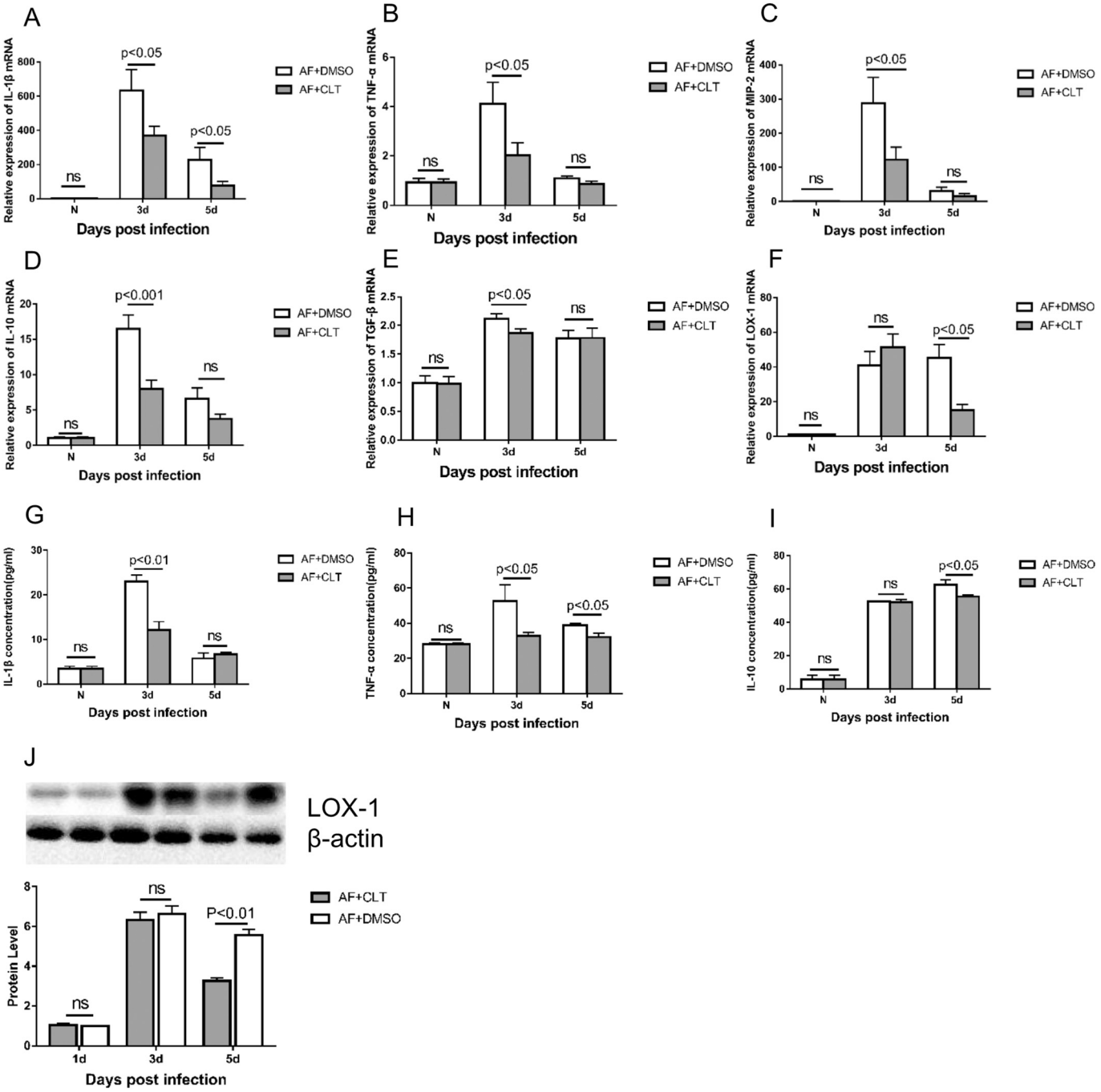

Fig. 2. (A–J) CLT treatment of C57BL/6 mice alleviates the severity of *A. fumigatus* keratitis in model by inhibiting inflammatory mediators and LOX-1. After CLT treatment, mRNA levels of IL-1β(A) were significantly decreased in the infected cornea of C57BL/6 mice at 3 and 5 days after infection; TNF-α (B) and MIP-2 (C) mRNA was declined at 3 days after infection, and no differences in mRNA levels of TNF-α and MIP-2 were detected between two groups at 5 days post infection; IL-10 (D) and TGF-β (E) mRNA was markedly declined at 3 days after infection, and no differences in mRNA levels of IL-10 and TGF-β were detected at 5 days post infection; LOX-1 (F) mRNA was declined at 5 days after infection, and no differences in mRNA levels of LOX-1 were detected at 5 days post infection. (G–J) Effects of CLT treatment on protein levels of IL-1β, TNF-α, IL-10 and LOX-1 in corneas. The IL-1β protein was significantly decreased (G) at 3 days after infection in CLT-treated over DMSO-treated mice. No differences in protein levels of IL-1β were detected at 5 days after infection. In addition, TNF-α protein (H) was significantly decreased at 3 and 5 days after infection. The IL-10 (I) and LOX-1 (J) protein levels were significantly decreased after CLT treatment at 5 days after infection, while there was no difference of their protein levels between two groups at 3 days after infection. (N: cornea of C57BL/6 mice in normal group without infection; AF + DMSO: cornea of C57BL/6 mice in DMSO-treated group post infection; AF + CLT: cornea of C57BL/6 mice in CLT-treated group post infection.)

3 days after infection were markedly reduced (Fig. 2G; $p < 0.01$), but it was not obviously decreased at 5 days post infection (Fig. 2G); TNF-α protein at 3 and 5 days after infection were decreased (Fig. 2H; $p < 0.05$, $p < 0.05$, respectively); IL-10 protein was decreased at 5 days after infection (Fig. 2I; $p < 0.05$), but no difference of its protein levels were detected at 3 days post infection between two groups (Fig. 2I). Consistent with PCR data of LOX-1, CLT restrained protein levels of LOX-1 at 5 days post infection (Fig. 2J; $p < 0.01$). No differences of its protein levels at 3 days after infection were detected between two groups (Fig. 2J). These results suggested that CLT might reduce disease response by down-regulating production of LOX-1 and proinflammatory mediators.

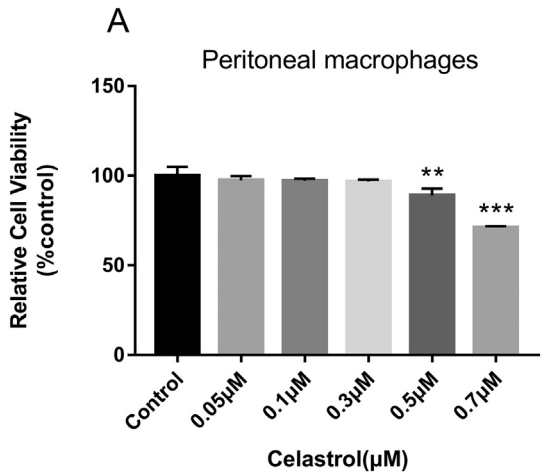

Fig. 3. (A) Effect of CLT on cell viability in primary peritoneal macrophages of C57BL/6 mice. Peritoneal macrophages of C57BL/6 mice were seeded onto 96-well cell culture plates and treated with different concentrations of CLT, as indicated, for 24 h. (**$p < 0.01$, ***$p < 0.001$).

3.3. Effect of celastrol on cell viability in primary peritoneal macrophages of C57BL/6 mice

In order to select the effective concentration of CLT for cell experiments, cell cytotoxicity of CLT on primary peritoneal macrophages of C57BL/6 mice (Fig. 3A) was evaluated by the CCK-8 assay. Results showed that 0.1 μM and 0.3 μM of CLT did not affect the viability of peritoneal macrophages (Fig. 3A). The concentration of CLT at 0.1 μM and 0.3 μM were selected as application concentrations for the next in vitro experiments.

3.4. Effect of CLT on proinflammatory mediators in peritoneal primary macrophages of C57BL/6 mice challenged with A. fumigatus

To confirm whether CLT could downregulate the expression of IL-1β, TNF-α and MIP-2, RT-PCR was used to evaluate the mRNA expression in primary peritoneal macrophages of C57BL/6 mice at 4, 8, 12 and 18 h after *A. fumigatus* hyphae pre-incubation with different concentrations of CLT. As shown in Fig. 4, the mRNA expression of proinflammatory mediators such as IL-1β (Fig. 4A–D), TNF-α (Fig. 4E–H) and MIP-2 (Fig. 4I–L) declined at 4, 8,12 and 18 h after pretreated with different concentrations of CLT during *A. fumigatus* infection. The data further demonstrated that mRNA expression of IL-1β, TNF-α and MIP-2 regulated by CLT in a dose-dependent manner (Fig. 4A–D, F–H, J and K). The most intuitive data was shown in Fig. 4K. To confirm above, we also tested the production of IL-1β and TNF-α in peritoneal primary macrophages pretreated with 0.3 μM CLT challenged with *A. fumigatus* by ELISA. ELISA data showed that CLT did inhibit IL-1β (Fig. 4M; $p < 0.01$) and TNF-α production (Fig. 4N; $p < 0.0001$) compared with control group. These results confirmed that CLT inhibited production of proinflammatory mediators in macrophages of C57BL/6 mice challenged with *A. fumigatus*.

3.5. CLT inhibited the expressions of IL-10 and TGF-β in primary peritoneal macrophages of C57BL/6 mice during A. fumigatus infection

To further explore the effect of CLT on production of IL-10 and TGF-β in peritoneal primary macrophages challenged with *A. fumigatus*, mRNA levels of IL-10 and TGF-β were assessed by RT-PCR; protein levels of IL-10 was assessed by ELISA.

In peritoneal primary macrophages stimulated with *A. fumigatus* for 4, 8, 12 and 18 h, compared with DMSO pretreated group, CLT dramatically inhibited the mRNA expression of IL-10 (Fig. 5A–D) and TGF-β (Fig. 5E–H) in a concentration-dependent manner. To confirm these data, protein levels of IL-10 in peritoneal primary macrophages were examined by ELISA (Fig. 4I). ELISA showed that protein levels of IL-10 was markedly decreased in 0.3 μM CLT pretreatment group (Fig. 5I; $p < 0.0001$). These results indicated that expression of IL-10 and TGF-β in macrophages of C57BL/6 mice challenged with *A. fumigatus* before we tested was markedly inhibited by pretreatment of CLT.

3.6. CLT exert anti-inflammatory effect by inhibiting production of LOX-1 in peritoneal primary macrophages of C57BL/6 mice during A. fumigatus infection, which may be regulated by p-p38MAPK

After CLT pretreatment in macrophages, mRNA levels of LOX-1 (Fig. 6A–D) were suppressed in a concentration-dependent manner. To confirm this, protein levels of LOX-1 was assessed by Western blot. LOX-1 protein levels (Fig. 6E and F) was significantly decreased in macrophages pretreated with 0.3 μM CLT versus DMSO pretreated group. It can be concluded that CLT likely suppresses inflammatory response by reducing the expression of LOX-1. To explore the underlying mechanisms of CLT on anti-inflammation, we studied the role of p38MAPK signaling pathway in the regulation of inflammatory mediators. As shown in Fig. 6E and H, the protein expression of p-p38MAPK were upregulated in DMSO pretreated group, while the activation of p38 MAPK were downregulated following pretreatment with CLT. Data showed that CLT exert anti-inflammatory effect by suppressing LOX-1, which may be regulated by p-p38MAPK.

4. Discussion

FK, a primary cause of corneal ulcer, is a severe disease which may result in blindness. 20% of FK patients who received a reasonable treatment may suffer corneal perforation, which may be caused by excessive inflammatory responses [31,32]. A certain degree of inflammatory response can defend against *A. fumigatus*, but excessive inflammatory response may be detrimental to its clearance [33]. The above evidence suggests that rational regulation of excessive inflammatory response may improve the prognosis of patients with FK.

CLT, an activated natural products derived from the medicinal plant *Tripterygium wilfordii Hook F*, displays powerful pharmacological activity against inflammation [34], which provides a possibility to regulate excessive inflammatory response in FK. A previous study has reported that CLT can suppress excessive inflammatory response in atherosclerosis by inhibiting the effect of LOX-1 [22]. LOX-1 plays a proinflammatory role in the development of *A. fumigatus* keratitis [11,12]. We explored the effects of CLT on LOX-1 and its regulation of excessive inflammation in FK. Results demonstrated that clinical scores were lower in group with CLT treatment of C57BL/6 mice versus control group, which was consistent with slit lamp photographs. More interestingly, mRNA levels of proinflammatory mediators, anti-inflammatory mediators and LOX-1 were decreased in CLT treatment group, which demonstrated that CLT treatment may significantly suppress the excessive inflammatory response during *A. fumigatus* infection. Our results support ideas of recent studies, showing that CLT ameliorated murine colitis [35] and experimental colitis of IL-10 deficient mice and restrained the mRNA levels of colonic proinflammatory cytokine, such as IL-1β and TNF-α [36].

Acute inflammation is an essential process in the host defending against infection; While excessive inflammatory responses is harmful to corneal healing [37]. Immune cells release excessive inflammatory cytokines and various proteinases that harmful to corneal epithelial wound healing [37–39]. Results indicated that the downregulation of inflammatory cytokines and LOX-1 by CLT treatment might restrain excessive inflammation in *A. fumigatus* keratitis, which may contribute to corneal healing.

Previous studies have reported that rational regulation of excessive inflammatory response is beneficial to corneal wound healing in FK

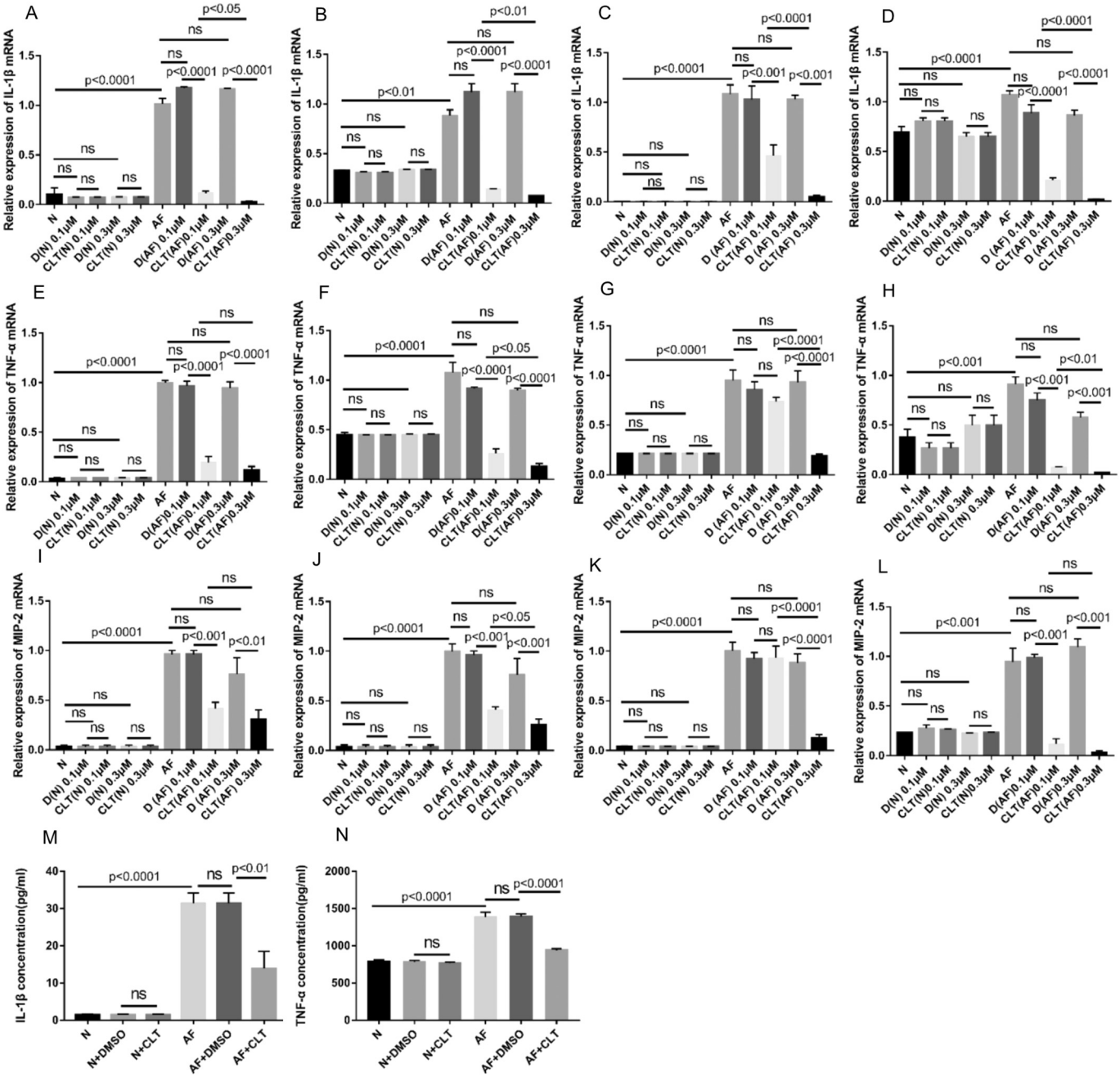

Fig. 4. (A–N) Effect of CLT on proinflammatory mediators in peritoneal primary macrophages of C57BL/6 mice challenged with *A. fumigatus*. Primary peritoneal macrophages were cultured without any pretreatment or *A. fumigatus* stimulation (N), with 0.1 μM or 0.3 μM CLT pretreatment for 2 h and without *A. fumigatus* stimulation (CLT(N)0.1 μM, CLT(N)0.3 μM), with DMSO (DMSO contained in 0.1 μM or 0.3 μM CLT, respectively) pretreatment for 2 h and without *A. fumigatus* stimulation (D(N)0.1 μM, D(N)0.3 μM). Macrophages were cultured with *A. fumigatus* stimulation for different hours and without any pretreatment (AF), with 0.1 μM or 0.3 μM CLT pretreatment for 2 h and *A. fumigatus* stimulation for different hours (CLT(AF)0.1 μM, CLT(AF)0.3 μM), with DMSO (DMSO contained in 0.1 μM or 0.3 μM CLT, respectively) pretreatment for 2 h and *A. fumigatus* stimulation for different hours (D(AF)0.1 μM, D(AF)0.3 μM). The mRNA expression of pro-inflammatory mediators such as IL-1β (A-D), TNF-α (*E*-H) and MIP-2 (I-L) was declined at 4, 8,12 and 18 h after pretreated with concentrations of CLT in a dose-dependent manner during *A. fumigatus* infection in primary peritoneal macrophages. CLT(0.3 μM) did inhibit IL-1β (M) and TNF-α (N) protein levels compared with DMSO-pretreated group after stimulation with *A. fumigatus* for 24 h.

[37,40,41], which can support our results.

Cell experiments were used to further explore the effects of CLT on LOX-1 and inflammatory mediators in vitro. Recent studies have demonstrated that macrophages play primary roles in host resistance to fungal infection in cornea [8,25], and they can remove fungal spores and suppress propagation of spores in vitro or in vivo [42]. Related literature shows that CLT suppressed the production of IL-1β in macrophage after LPS stimulation [43]. CLT inhibits oxLDL-induced oxidative stress in RAW264.7 cells [22]. Recent research has shown that concentrations of CLT below 1 μM hardly affected the growth and virulence of *A. fumigatus*. In this experiment, growth and virulence of *A. fumigatus* was hardly affected by 0.1 and 0.3 μM CLT [24].

We observed that CLT markedly decreased the mRNA levels of IL-1β, TNF-α, IL-10, MIP-2 and TGF-β in macrophage versus DMSO treated group. Results of ELISA were consistent with these. These results indicated that CLT may restrain excessive inflammation in

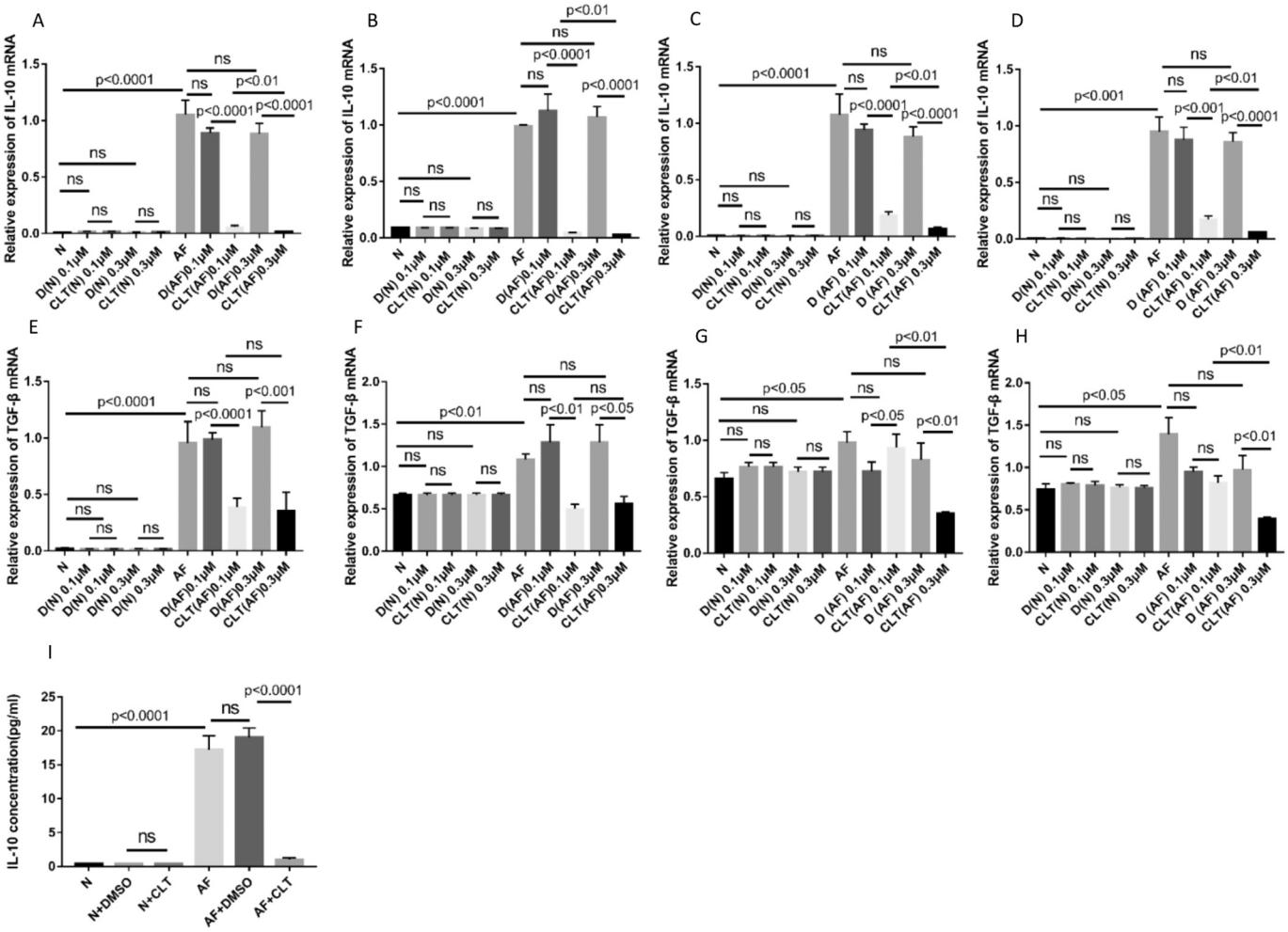

Fig. 5. (A–I) CLT inhibited the expressions of IL-10 and TGF-β in peritoneal primary macrophages of C57BL/6 mice during *A. fumigatus* infection. The mRNA expression of anti-inflammatory mediators such as IL-10 (A–D) and TGF-β (E, F and H) was declined at 4, 8 and 18 h after pretreated with different concentrations of CLT during *A. fumigatus* infection in peritoneal primary macrophages. TGF-β (G) increases significantly at 12 h after pretreated with 0.1 μM of CLT, decrease significantly at 12 h after pretreated with 0.3 μM of CLT. After CLT(0.3 μM) treatment, IL-10 (I) protein was declined compared with DMSO-pretreated group after stimulation with *A. fumigatus* for 24 h.

macrophage stimulated with *A. fumigatus* keratitis. Our study is corresponding with recent reports, showing that CLT alleviates injuries of kidney and liver in mice and LPS-induced inflammation by inhibiting the expression of IL-1β, TNF-α and related inflammatory markers [44]. CLT might offer a promising adjunct treatment for rheumatoid arthritis through downregulation of IL-1β and TNF-α [45]. However, the regulation of IL-10 by CLT in different disease models may be various. CLT ameliorates murine colitis via increasing IL-10 levels in the colon [35], and CLT attenuates acute hepatic dysfunction without affecting IL-10 expression levels [46]. Production of IL-10 were declined in macrophages after CLT pretreatment at different hours after stimulation with *A. fumigatus*. Inflammatory response in macrophages before tested challenged with *A. fumigatus* may have been completely inhibited by pretreatment with CLT, which may lead to results as we saw. It is interesting that at 12 h, at a concentration of 0.1 μM CLT there is an different trend compared with 4, 8 and 18 h after stimulation of *A. fumigatus*. We detected that the concentration of CLT at 0.1 μM markedly decreased the mRNA levels of IL-1β and IL-10 in macrophage versus DMSO treated group at 12 h after stimulation of *A. fumigatus*. No differences between 0.1 μM CLT-treated and DMSO-treated groups in mRNA levels of TNF-α, MIP-2 and LOX-1 were detected. We suspected that quantified mRNA level of TNF-α, MIP-2 and LOX-1 may peak at 12 h after stimulation with *A. fumigatus*. Their peak of mRNA expression cannot be suppressed by 0.1 μM CLT. The peak of LOX-1 and TNF-α mRNA expression has been confirmed by research [12].

These results further demonstrate that CLT may restrain excessive inflammatory response via downregulation of proinflammatory mediators and LOX-1 in macrophages during *A. fumigatus* infection, which were consistent with our results of mice model.

Although rational treatment with CLT can alleviate the excessive inflammatory response in *A. fumigatus* keratitis via inhibiting inflammatory mediators and LOX-1, the precise mechanism of its effect still needs to be explored. Protein levels of LOX-1 and p-p38MAPK was down-regulated after CLT pretreatment in our study. It indicated that appropriate treatment of CLT can ameliorate excessive inflammatory response in *A. fumigatus* keratitis, which may be regulated by p-p38MAPK. These findings also are consistent with this previous study showing that LPS induced LOX-1 and p-p38MAPK expression in mice aorta tissues [47]; Anti-LOX-1 is capable of inhibiting LPS-induced inflammatory response, including NF-KB activation, p38MAPK activation and apoptotic signaling in mouse lung [52]. LOX-1 plays a pro-inflammatory role in *A. fumigatus* keratitis through the p38MAPK pathway [11], and *A. fumigatus* keratitis can be ameliorated by down-regulated LOX-1 expression [13].

Besides innate immunity, acquired immune response also plays an important role in fungal clearance. Th1 and Th17 responses participated in splenic and lung cell responses to nonfatal systemic aspergillosis in mice [48]. A recent study identified an essential role Th17 cells

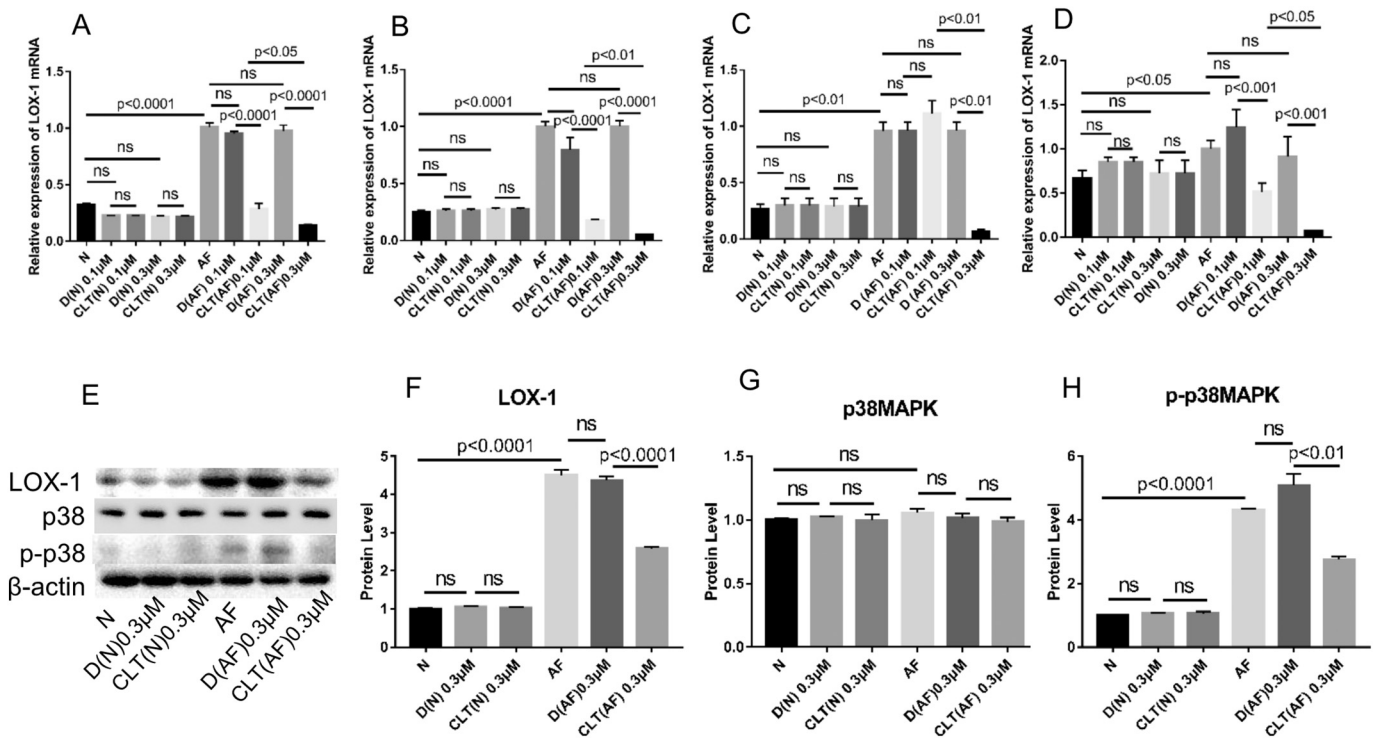

Fig. 6. (A–H) CLT exert anti-inflammatory effect by inhibiting production of LOX-1 in peritoneal primary macrophages of C57BL/6 mice during *A. fumigatus* infection, which may be modified by p-p38MAPK. After CLT pretreated, LOX-1 mRNA levels (A–D) were downregulated in the infected macrophages at 4, 8, 12 and 18 h post infection compared with DMSO-pretreated group. CLT(0.3 μM) significantly inhibited LOX-1(E and F) and p-p38MAPK (E and H) protein expression compared with DMSO-pretreated cells at 24 h post infection.

in regulating the growth of fungal hyphae and the severity of corneal disease [49]. A lack of Th17 cells increases susceptibility to invasive fungal infection, but excessive immune responses can cause severe tissue damage [6].

CLT suppress Th17 cell induction , while promoting the generation of Treg cells that have a central role for keeping the balance between pro- and anti-inflammatory immune responses against *A. fumigatus* [50,51]. Our results suggest that excessive inflammatory response can be suppressed by rational use of CLT. We speculated that excessive Th17 response may be inhibited by CLT. This speculation requires further experimentation to explore.

In summary, appropriate CLT treatment may ameliorate *A. fumigatus* keratitis by downregulating production of LOX-1, which may be modified by p-p38MAPK.

This research has shown that expression of LOX-1, playing a proinflammatory role in *A. fumigatus* keratitis, can be inhibited by CLT treatment, which may provide a new idea for the treatment of *A. fumigatus* keratitis.

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References

[1] S.E. Nielsen, et al., Incidence and clinical characteristics of fungal keratitis in a danish population from 2000 to 2013, Acta Ophthalmol. 93 (1) (2015) 54–58.
[2] P. Garg, A. Roy, S. Roy, Update on fungal keratitis, Curr. Opin. Ophthalmol. 27 (4) (2016) 333–339.
[3] C. Li, et al., Effect of corneal graft diameter on therapeutic penetrating keratoplasty for fungal keratitis, Int. J. Ophthalmol. 5 (6) (2012) 698–703.
[4] Y. Sharma, S. Jain, Jayachandran, Keratomycosis: etiology, risk factors and differential diagnosis- a mini review on trichophyton spp. J. Clin. Diagn. Res. 8 (10) (2014) Dd01–2.
[5] L. Xie, et al., Spectrum of fungal keratitis in north China, Ophthalmology 113 (11) (2006) 1943–1948.
[6] F.L. van de Veerdonk, et al., Aspergillus fumigatus morphology and dynamic host interactions, Nat. Rev. Microbiol. 15 (11) (2017) 661–674.
[7] A.M. Kvarnhammar, L.O. Cardell, Pattern-recognition receptors in human eosinophils, Immunology 136 (1) (2012) 11–20.
[8] T.K. Means, Fungal pathogen recognition by scavenger receptors in nematodes and mammals, Virulence 1 (1) (2010) 37–41.
[9] J.L. Mehta, et al., Lectin-like, oxidized low-density lipoprotein receptor-1 (lox-1): a critical player in the development of atherosclerosis and related disorders, Cardiovasc. Res. 69 (1) (2006) 36–45.
[10] H. Moriwaki, et al., Expression of lectin-like oxidized low density lipoprotein receptor-1 in human and murine macrophages: upregulated expression by tnf-alpha, FEBS Lett. 440 (1–2) (1998) 29–32.
[11] C. Li, et al., The role of lox-1 in innate immunity to aspergillus fumigatus in corneal epithelial cells, Invest. Ophthalmol. Vis. Sci. 56 (6) (2015) 3593–3603.
[12] K. He, et al., The role of lox-1 on innate immunity against *Aspergillus* keratitis in mice, Int. J. Ophthalmol. 9 (9) (2016) 1245–1250.
[13] W. Zhao, et al., Fenretinide inhibits neutrophil recruitment and il-1beta production in *Aspergillus fumigatus* keratitis, Cornea 37 (12) (2018) 1579–1585.
[14] M. Slowik, et al., Mycotic infections of the eye, Adv. Clin. Exp. Med. 24 (6) (2015) 1113–1117.
[15] N.V. FlorCruz, J.R. Evans, Medical interventions for fungal keratitis, Cochrane Database Syst. Rev. 4 (2015) Cd004241.
[16] L. Bajracharya, R. Gurung, Outcome of therapeutic penetrating keratoplasty in a tertiary eye care center in Nepal, Clin. Ophthalmol. 9 (2015) 2299–2304.
[17] J.E. Lee, et al., Inhibition of corneal inflammation by the resolvin e1, Invest. Ophthalmol. Vis. Sci. 56 (4) (2015) 2728–2736.
[18] R. Cascao, J.E. Fonseca, L.F. Moita, Celastrol: a spectrum of treatment opportunities in chronic diseases, Front. Med. (Lausanne) 4 (2017) 69.
[19] D.H. Kim, et al., Suppression of inflammatory responses by celastrol, a quinone methide triterpenoid isolated from celastrus regelii, Eur. J. Clin. Invest. 39 (9) (2009) 819–827.
[20] H.Y. Li, et al., Celastrol induces apoptosis and autophagy via the ros/jnk signaling pathway in human osteosarcoma cells: an in vitro and in vivo study, Cell Death Dis. 6 (2015) e1604.
[21] N. Khalili, et al., In vitro immunomodulatory activity of celastrol against influenza a virus infection, Immunopharmacol. Immunotoxicol. 40 (3) (2018) 250–255.
[22] L. Gu, et al., Celastrol prevents atherosclerosis via inhibiting lox-1 and oxidative stress, PLoS One 8 (6) (2013) e65477.
[23] M. Bian, et al., Celastrol protects mouse retinas from bright light-induced degeneration through inhibition of oxidative stress and inflammation, J.

Neuroinflammation 13 (2016) 50.
[24] J.S. Martin Del Campo, et al., Inhibition of the flavin-dependent monooxygenase siderophore a (sida) blocks siderophore biosynthesis and aspergillus fumigatus growth, ACS Chem. Biol. 11 (11) (2016) 3035–3042.
[25] J. Hu, Y. Wang, L. Xie, Potential role of macrophages in experimental keratomycosis, Invest. Ophthalmol. Vis. Sci. 50 (5) (2009) 2087–2094.
[26] Y. Niu, et al., Aspergillus fumigatus increased par-2 expression and elevated proinflammatory cytokines expression through the pathway of par-2/erk1/2 in cornea, Invest. Ophthalmol. Vis. Sci. 59 (1) (2018) 166–175.
[27] T.G. Wu, K.R. Wilhelmus, B.M. Mitchell, Experimental keratomycosis in a mouse model, Invest. Ophthalmol. Vis. Sci. 44 (1) (2003) 210–216.
[28] N. Jiang, et al., Indoleamine 2,3-dioxygenase is involved in the inflammation response of corneal epithelial cells to aspergillus fumigatus infections, PLoS One 10 (9) (2015) e0137423.
[29] M. Liu, et al., Boxb mediate balb/c mice corneal inflammation through a tlr4/myd88-dependent signaling pathway in aspergillus fumigatus keratitis, Int J Ophthalmol 11 (4) (2018) 548–552.
[30] X. Peng, et al., Interaction of mannose binding lectin and other pattern recognition receptors in human corneal epithelial cells during aspergillus fumigatus infection, Int. Immunopharmacol. 63 (2018) 161–169.
[31] L.J. Keay, et al., Clinical and microbiological characteristics of fungal keratitis in the United States, 2001–2007: a multicenter study, Ophthalmology 118 (5) (2011) 920–926.
[32] J. Zhong, et al., Inhibition of trem-1 and dectin-1 alleviates the severity of fungal keratitis by modulating innate immune responses, PLoS One 11 (3) (2016) e0150114.
[33] P. Bonifazi, et al., Intranasally delivered sirna targeting pi3k/akt/mtor inflammatory pathways protects from aspergillosis, Mucosal Immunol. 3 (2) (2010) 193–205.
[34] S.R. Chen, et al., A mechanistic overview of triptolide and celastrol, natural products from *Tripterygium wilfordii* hook f, Front. Pharmacol. 9 (2018) 104.
[35] M.E. Shaker, S.A. Ashamallah, M.E. Houssen, Celastrol ameliorates murine colitis via modulating oxidative stress, inflammatory cytokines and intestinal homeostasis, Chem. Biol. Interact. 210 (2014) 26–33.
[36] J. Zhao, et al., Celastrol ameliorates experimental colitis in il-10 deficient mice via the up-regulation of autophagy, Int. Immunopharmacol. 26 (1) (2015) 221–228.
[37] Y. Zhang, et al., Important role of epiregulin in inflammatory responses during corneal epithelial wound healing, Invest. Ophthalmol. Vis. Sci. 53 (4) (2012) 2414–2423.
[38] M.D. Wagoner, et al., Polymorphonuclear neutrophils delay corneal epithelial wound healing in vitro, Invest. Ophthalmol. Vis. Sci. 25 (10) (1984) 1217–1220.
[39] M.J. Chusid, S.D. Davis, Experimental bacterial keratitis in neutropenic guinea pigs: polymorphonuclear leukocytes in corneal host defense, Infect. Immun. 24 (3) (1979) 948–952.
[40] H. Guo, J. Gao, X. Wu, Toll-like receptor 2 sirna suppresses corneal inflammation and attenuates aspergillus fumigatus keratitis in rats, Immunol. Cell Biol. 90 (3) (2012) 352–357.
[41] X. Hu, et al., Sirt6 deficiency impairs corneal epithelial wound healing, Aging (Albany NY) 10 (8) (2018) 1932–1946.
[42] V.P. Kurup, Interaction of *Aspergillus fumigatus* spores and pulmonary alveolar macrophages of rabbits, Immunobiology 166 (1) (1984) 53–61.
[43] W. Xin, et al., A new mechanism of inhibition of il-1beta secretion by celastrol through the nlrp3 inflammasome pathway, Eur. J. Pharmacol. 814 (2017) 240–247.
[44] M. Wu, et al., Celastrol aggravates lps-induced inflammation and injuries of liver and kidney in mice, Am. J. Transl. Res. 10 (7) (2018) 2078–2086.
[45] S.H. Venkatesha, et al., Suppression of autoimmune arthritis by celastrus-derived celastrol through modulation of pro-inflammatory chemokines, Bioorg. Med. Chem. 20 (17) (2012) 5229–5234.
[46] G.S. El-Tanbouly, et al., The nf-kappab inhibitor celastrol attenuates acute hepatic dysfunction induced by cecal ligation and puncture in rats, Environ. Toxicol. Pharmacol. 50 (2017) 175–182.
[47] W. Zhao, G. Ma, X. Chen, Lipopolysaccharide induced lox-1 expression via tlr4/myd88/ros activated p38mapk-nf-kappab pathway, Vasc. Pharmacol. 63 (3) (2014) 162–172.
[48] I. Mirkov, et al., Splenic and lung response to nonlethal systemic *Aspergillus fumigatus* infection in c57bl/6 mice, Med. Mycol. 48 (5) (2010) 735–743.
[49] P.R. Taylor, et al., Aspergillus and fusarium corneal infections are regulated by th17 cells and il-17-producing neutrophils, J. Immunol. 192 (7) (2014) 3319–3327.
[50] J. Zhang, et al., Celastrol mediates th17 and treg cell generation via metabolic signaling, Biochem. Biophys. Res. Commun. 497 (3) (2018) 883–889.
[51] P. Bacher, et al., Antigen-specific expansion of human regulatory t cells as a major tolerance mechanism against mucosal fungi, Mucosal Immunol. 7 (4) (2014) 916–928.
[52] P. Zhang, M.C. Liu, L. Cheng, M. Liang, H.L. Ji, J. Fu, Blockade of LOX-1 prevents endotoxin-induced acute lung inflammation and injury in mice, J. Innate Immun. 1 (2009) 358–365.