Perillaldehyde Ameliorates *Aspergillus fumigatus* Keratitis by Activating the Nrf2/HO-1 Signaling Pathway and Inhibiting Dectin-1-Mediated Inflammation

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Received: November 15, 2019
Accepted: March 30, 2020
Published: June 24, 2020

Citation: Fan Y, Li C, Peng X, et al. Perillaldehyde ameliorates *aspergillus fumigatus* keratitis by activating the Nrf2/HO-1 signaling pathway and inhibiting Dectin-1-mediated inflammation. *Invest Ophthalmol Vis Sci.* 2020;61(6):51. https://doi.org/10.1167/iovs.61.6.51

Purpose. The purpose of this study was to investigate the therapeutic effect of perillaldehyde (PAE) on *Aspergillus fumigatus* (*A. fumigatus*) keratitis.

Methods. Human corneal epithelial cells (HCECs) were pretreated with PAE and stimulated with *A. fumigatus* mycelium. C57BL/6 mice were infected with *A. fumigatus* and treated with or without PAE 1 day after infection. Clinical scores, PCR, ELISA, and Western blotting were used to detect the expression of pro-inflammatory mediators, dendritic cell-associated c-type lectin-1 (Dectin-1), nuclear factor (erythroid-derived 2) like 2 (Nrf2), and heme oxygenase (HO-1). Nrf2 expression in HCECs pretreated with PAE was observed by immunofluorescence. NIMP-R14 protein expression and localization in mouse corneas were observed by immunofluorescence staining after treatment with PAE. Corneal colony counting, time-kill tests, and mycelial transformation inhibition tests were used to evaluate the antifungal effect of PAE.

Results. C57BL/6 mice treated with PAE at 1 day after infection had a lower clinical score and decreased IL-1β, TNF-α, IL-6, Dectin-1, and MPO levels. PAE treatment significantly reduced neutrophil recruitments to the corneal stroma. Compared with the DMSO-treated group, PAE treatment significantly decreased mRNA and protein levels of pro-inflammatory cytokines and Dectin-1 in HCECs. PAE pretreatment before *A. fumigatus* stimulation obviously elevated the mRNA and protein levels of components of the Nrf2/HO-1 axis. HCECs pretreated with PAE before infection showed a weakened ability to inhibit inflammation in the presence of brusatol (BT; an Nrf2 inhibitor) or ZnPP (an HO-1 inhibitor). PAE treatment significantly reduced the fungal load of C57BL/6 mouse corneas and inhibited fungal growth in vitro.

Conclusions. These data proved that PAE may ameliorate *A. fumigatus* keratitis by activating the Nrf2/HO-1 signaling pathway and inhibiting the Dectin-1 mediated inflammatory response and neutrophil recruitment. Furthermore, PAE exerts direct fungicidal activity on *A. fumigatus*.

Keywords: perillaldehyde, innate immune, fungal keratitis, Nrf2/HO-1, inflammation

Fungal keratitis (FK), which poses a threat to vision, has become increasingly common, and most cases are due to filamentous fungus species, such as *Aspergillus fumigatus* (*A. fumigatus*) and *Fusarium*.[1] The incidence of FK has been increasing due to the increased prevalence of agricultural ocular trauma, the long-term use of contact lenses and antibiotics, and the excessive use of corticosteroids.[2] At present, the first-line treatment for FK is the regular use of antifungal drugs, such as natamycin and voriconazole.[3] However, the antifungal drugs in clinical use have disadvantages, including low bioavailability, poor pharmacokinetic properties, and cornea toxicity,[2] that limit their application; thus, there is an urgent need to identify the new antifungal compounds for FK.

A. fumigatus can cause a strong host immune response.[3] Innate immunity is the first line of defense against fungal infections; the innate immune system can rapidly identify conserved structural motifs expressed by microbial pathogens or pathogen-associated molecular patterns(PAMPs) via pattern recognition receptors (PRRs).[4] Dendritic cell-associated c-type lectin-1 (Dectin-1), a β-glucan receptor, is widely known as the most specific PRR in FK.[5] Dectin-1 plays an important role in the defense against *A. fumigatus* keratitis by recruiting immune cells, releasing inflammatory cytokines, and initiating an adaptive immune response.[6] However, Dectin-1 always evokes an excessive inflammatory response that leads to tissue damage, which is detrimental to tissue repair and fungal clearance.[7] Recent studies have demonstrated that the inhibition of Dectin-1 can alleviate the inflammatory response and decrease the fungal load in FK[8]; these data, provide the theoretical basis for our study.

Perillaldehyde (PAE), a promising natural monoterpene substance, is extracted from *Perilla frutescens*,[9,10] which

was once widely used as a constituent of essential oil and Asian Cuisine; PAE has strong antifungal, anti-inflammatory, antitumor, and anti-oxidant activities, as well as many other biological activities.[11–16] Studies have pointed out that PAE can inhibit the excessive inflammatory response in inflammatory diseases and infectious diseases in vivo and in vitro.[11–14,17,18] Previous studies have also shown that PAE exerts remarkable fungicidal effects on filamentous fungus species, such as *Aspergillus flavus, Aspergillus niger*, and *Candida albicans*.[15,16,19] Recently, Fuyuno et al.[20] found that PAE can activate the nuclear factor-erythroid 2-related factor 2 (Nrf2)/heme oxygenase (HO)-1 pathway and reduce the oxidative-stress-mediated innate immune response in human keratinocytes. Transcription factor Nrf2, widely recognized as an important regulator of anti-oxidant events, has been shown to be an important regulator that minimizes inflammation in various infectious diseases.[21–24] It is reported that Nrf2 plays an important role in promoting corneal wound healing and activated Nrf2 emerged as a protective role in traumatic corneal disease.[25] Under nonstress conditions, Nrf2 is located in the cytoplasm,[26] but in response to stress, free Nrf2 translocates to the nucleus, where it dimerizes with members of the small Maf family and binds to ARE-containing genes, such as HO-1.[27] HO-1 is one of the rate-limiting enzymes in heme catabolism that catalyzes the stereospecific degradation of heme to biliverdin, carbon monoxide (CO), and iron[28]; therefore, the upregulation of HO-1 leads to the increased conversion of heme into CO, bilirubin, and free iron. CO acts as an inhibitor of the NF-κB pathway, leading to the decreased expression of pro-inflammatory cytokines.[28,29] HO-1 is highly expressed in the cornea of humans and mice and deficiency of HO-1 shows exacerbated injury-induced corneal inflammation and repair.[30] Recently, studies have pointed out that β-glucan, the intrinsic component of fungal cell wall, induced HO-1 production via Nrf2-dependent manner and played a protective role in oral keratinocytes infected by *Candida albicans*,[31] indicating that fungus-induced Nrf2/HO-1 expression may also play a role during *A. fumigatus* infection. However, no study has investigated the application of PAE in *A. fumigatus* keratitis. Furthermore, the mechanism underlying the anti-inflammatory and antifungal effects of PAE during *A. fumigatus* infection in the cornea remains unknown.

We found that treatment with PAE could alleviate the inflammatory response during *A. fumigatus* infection by inhibiting Dectin-1 expression, neutrophil recruitment, and activating the Nrf2/HO-1 signaling pathway. In addition, PAE inhibited the growth of *A. fumigatus*, further ameliorating the inflammatory response in FK and perhaps providing a new candidate for the treatment of FK.

Materials and Methods

Materials

PAE (CAS-No.18031-40-8) was purchased from TCI Co. Ltd. (Tokyo, Japan), and prepared as a stock solution in 0.1% DMSO; this stock solution was diluted to create working solutions to achieve various final concentrations. Brusatol (BT; an Nrf2 inhibitor) was obtained from MCE. Curdlan and ZnPP (an HO-1 inhibitor) were purchased from Sigma-Aldrich. Anti-Nrf2, anti-HO-1, anti-Dectin-1, and anti-TNF-α were purchased from Abcam. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Solaibio. Human IL-6 and TNF-α ELISA kits were obtained from Elabscience, and IL-8 ELISA Kits were obtained from R&D Systems (San Diego, CA, USA). Anti-IL-1β and anti-IL-6 were obtained from Elabscience.

A. fumigatus Culture

A. fumigatus strain 3.0772 (China General Microbiological Culture Collection Center, Beijing, China) was inoculated in 150 mL Erlenmeyer flasks containing Sabouraud liquid medium (4% glucose and 1% Mycopeptone). Flasks were shaken at 37 deg Celsius (°C) and 110 rpm for 48 hours. Then, mycelia were disrupted with a tissue grinder into 20 to 40 um pieces, that were washed 3 times with sterile PBS, and centrifuged at 4000 g/min for 35 minutes. The supernatant was discarded, and the resulting fungi were stored—without inactivation (for animal experiments) or were inactivated overnight in 70% alcohol prior to storage (for human corneal epithelial cell [HCEC] experiments). PBS (for animal experiments) and Dulbecco's modified Eagle's medium (DMEM; Gibco, San Diego, CA, USA for HCECs experiments) were used to dilute *A. fumigatus* to 1 to approximately $3*10^8$ CFU/mL.

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

HCECs (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% CO_2. Near 80% confluence, the cells were cultured in serum free DMEM for 48 hours and treated with *A. fumigatus* hyphae (to the final concentration of $5*10^6$ CFU/mL) for 8 hours in 12-well plates or 16 hours in 6-well plates. Cells were used for real-time RT-PCR, and Western blot, and supernatant was collected for ELISA. The mRNA levels of Dectin-1, Nrf2, HO-1, IL-1β, TNF-α, IL-8, and IL-6 in HCECs were detected by real-time RT-PCR after stimulation at 8 hours. Dectin-1 and Nrf2/HO-1 protein level of HCECs were detected by Western blot at 8 or 24 hours. IL-8, TNF-α, and IL-6 protein levels of supernatant were detected by ELISA at 24 hours.

Animals and Corneal Infection

Female C57BL/6 mice were purchased from Qingdao Institute of Drug Control (Qingdao, China) and weighed between 20 and 30 grams (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 right eyes were chosen as the experimental eyes including PAE-treated eyes, DMSO-treated eyes, and the sham control group eyes. The left eyes were used as normal control without any treatment. Mice were anesthetized with 8% chloral hydrate and placed beneath a stereoscopic microscope at 325× magnification. A sterile surgical blade was used to scrape an approximately 2-mm-diameter piece of the epithelial tissue of the right cornea and the stromal layer was exposed under the microscope. A 5 uL aliquot ($1*10^8$ CFU/mL) of *A. fumigatus* was topically applied to the ocular surface, which was covered with a soft contact lens and the eyelids were sutured.

Table. Primer Sequences Used for Real-Time RT-PCR

Gene	GenBank No.	Primer Sequence (5′-3′)
Mouse β-actin	NM_007393.5	F: GATTACTGCTCTGGCTCCTAGC R: GACTCATCGTACTCCTGCTTGC
Mouse IL-1β	NM_008361.4	F: CGCAGCAGCACATCAACAAGAGC R: TGTCCTCATCCTGGAAGGTCCACG
Mouse TNF-α	NM_013693.3	F: ACCCTCACACTCAGATCATCTT R: GGTTGTCTTTGAGATCCATGC
Mouse IL-6	NM_001314054.1	F: TGATGGATGCTACCAAACTGGA R: TGTGACTCCAGCTTATCTCTTGG
Human GAPDH	NM_001101.5	F: TGGCACCCAGCACAATGAA R: CTAAGTCATAGTCCGCCTAGAAGCA
Human IL-1β	NM_000576.3	F: GCTGATGGCCCTAAACAGATGAA R: TCCATGGCCACAACAACTGAC
Human IL-8	NM_000584.4	F: TCTTGGCAGCCTTCCTGATT R: AACTTCTCCACAACCCTCTG
Human IL-6	NM_000600.5	F: AAGCCAGAGCTGTGCAGATGAGTA R: TGTCCTGCAGCCACTGGTTC
Human Nrf2	NM_001313901.1	F: GGTTGCCCACATTCCCAAATC R: CAAGTGACTGAAACGTAGCCG
Human HO-1	NM_ 002133.2	F: TTCAAGCAGCTCTACCGCTC R: GAACGCAGTCTTGGCCTCTT

The soft contact lenses used in the experiment were homemade. Their diameter was 3 mm and they were made of colorless, translucent, and nontoxic sealing film Parafilm M (BEMIS, Sheboygan Falls, WI, USA) with 127 µm thick.[32,33] Parafilm M has good ductility, unique permeability, excellent moisture permeability, and strong corrosion resistance. The eyelids were sutured after soft contact lenses were worn for prevention of further fungi leakage. For the sham control group eye, the central corneal epithelium was removed as described above, the eye was covered with a soft contact lens, and the eyelids were sutured, without infection. On the first day after modeling, the sutures and contact lens were removed and the mice were subconjunctivally injected with 6 mM PAE or 1% DMSO. A 5 uL aliquot PAE (6 mM) or 1% DMSO was topically administrated to the mice cornea three times a day from 2 days post infection and subconjunctival injection was performed every other day. Mice corneal epithelium was harvested for real-time RT-PCR and Western blot at 1, 3, and 5 days after establishing the experimental model. The method used to obtain the mouse corneal epithelium for real-time PCR and Western blot was in accordance with Zhao et al.[4]

Real-Time PCR

Total RNA was isolated from cells using RNAiso plus reagent and quantified by spectrophotometry. RNA (2 ug) was used for first-strand cDNA synthesis according to the reverse transcription protocol. Then cDNA was analyzed by PCR in a 20 uL reaction volume following the manufacturer's instructions. Real-time PCR Master Mix (Takara) was used with primers at 5 uM. 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 β-actin, an endogenous reference gene. Data (relative mRNA levels) are shown as the mean standard error of the mean (SEM). The primer sequences used for real-time RT-PCR are shown in the Table.

Western Blot

HCECs were observed under different inverted microscopes. Cells were seeded for adherent culture in 6-well plates, and allowed to reach approximately 80% confluence. The original culture solution was discarded. Three milliliters of precooled PBS were added to each well to wash the cells. Then, cell lysis buffer was prepared with PMSF and 150 uL of this buffer was added to each well. Corneal tissues from B6 mice were obtained for Western blot assay. The membranes containing separated proteins were incubated with primary antibodies against Dectin-1 (1:1000), HO-1 (1:250), Nrf2 (1:1000), TNF-α (1:500; Abcam, Cambridge, UK), IL-1β (1:1000), IL-6 (1:1000), β-tubulin (1:1,000; Elabscience, Wuhan, China), and β-actin (1:1000) (Elabscience). Goat anti-rabbit (1:5000) and goat anti-mouse (1:800) secondary antibodies were also used. Next, the bands were detected with ECL Western blot detection reagents (Biotime, China) in accordance with standard protocols.

ELISA

HCECs were detached with 0.05% trypsin and seeded onto 12-well plates. Cells were pretreated with BT (20 nM) or ZnPP (20 uM) for 1 hour or with PAE (600 µM) for 4 hours followed by exposure to *A. fumigatus* hyphae for 24 hours. After the media were collected and centrifuged, the released levels of IL-8, TNF-α, and IL-6 were assessed by human ELISA kits according to the manufacturer's protocols.

Immunofluorescence Assays

An immunofluorescence assay was performed to observe the expression and nuclear translocation of Nrf2. HCECs were seeded onto poly-L-lysine-coated slips in 24-well plates and cultured overnight, followed by treatment with PAE (600 µM) or *A. fumigatus* hyphae. The expression and nuclear translocation of Nrf2 were analyzed with anti-Nrf2 antibodies (1:50; Abcam, Cambridge, UK).

Quantification of Corneal Polymorphonuclear Neutrophils

NIMP-R14 protein expression and localization in mouse corneas were observed by immunofluorescence staining. The eyeballs were removed from C57BL/6 mice 3 days after infection, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in liquid nitrogen. Then, 10 μm slices were fixed in acetone for 5 minutes and blocked with 10% sputum serum (Solarbio) for 30 minutes at room temperature. For polymorphonuclear neutrophils (PMN) labeling, sections were incubated with 1:100 dilution of rat anti-mouse NIMP-R14 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. After being washed with PBS, the sections were stained with goat anti-rat IgG (1:100; Elabscience) for 1 hour. Finally, the sections were observed and digital images were captured at 200× magnification using a Zeiss Axiovert

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microscope. The slope of the line was used to calculate the units of myeloperoxidase (MPO) activity in each cornea.

Cornea Colony Count

A mouse model of FK was established. The corneas from the DMSO control group and the PAE treatment group were placed in PBS on the third day after infection, and the cornea was ground with a grinding rod. The ground fungal solution was evenly spread on agar medium and placed in a 37°C incubator.

Induction of Filamentous Structures

A. fumigatus spores ($5*10^3$/mL) were cultured in liquid medium containing 10% fetal bovine serum and PBS. Spores were divided into several groups: DMSO, 0.2 mM PAE, 0.6 mM PAE, 0.8 mM PAE, 1.0 mM PAE, 1.2 mM PAE, and 1.8 mM PAE. DMSO and PAE at above concentrations were separately added into these liquid culture mediums for 2 hours, then each group of *A. fumigatus* spores was added into 6 wells of 96-well plates at 37°C for 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours. The absorbance at different time point was measured. The absorbance at 540 nm of each well represented the amount of precipitation in every well.[34] Calcofluor white stain (18909-100ML-F; Sigma Corp. USA, Ronkonkoma, NY, USA) was used to observe fungal biomass intuitively. Another set of experiment was performed as above described for 48 hours. Supernatants were removed and the chitin-binding stain Calcofluor white (Sigma-Aldrich Biotechnology Company, St. Louis, MO, USA) was added at 50 uL per well for 10 minutes at room temperature.[35] Wells were washed three times in double distilled H_2O. After stained with Calcofluor white, the images were captured. Images were recorded using a Zeiss Axiovert microscope at 20× magnification. A range of 300 to 440 nm can be utilized for the emission wavelength, and the excitation occurs at approximately 355 nm. The formation of filamentous structures was quantitated.

Statistical Analysis

For comparisons of differences between two groups, a two-tailed Student's *t*-test (GraphPad Prism) was used to determine significance. One-way ANOVA followed by Bonferroni's multiple comparison test (GraphPad Prism) was used to analyze three or more groups. Any P value < 0.05 was considered significant, and the data are represented as the mean ± SEM.

Results

PAE Treatment Alleviates the Severity of *A. fumigatus* Keratitis in Mice Cornea

To investigate the therapeutic effect of PAE on *A. fumigatus* keratitis, we selected qualified mice for modeling, as described above. We used a slit lamp to record images at 3 and 5 days (Fig. 1A) after infection. Compared with the DMSO-treated group, the PAE-treated groups showed a significant reduction in corneal opacity, ulcer area, and inflammation. Clinical scores are used to assess disease severity according to a 12-point scoring system.[36] Compared with the DMSO-treated group, the PAE-treated group had a significantly decreased corneal clinical score at both 3 and 5 days after infection (Fig. 1B). The corneal fungal load was significantly lower in PAE-treated mice with FK than in DMSO-treated mice with FK (Figs. 1C–E). MPO measurements (Fig. 1F) and NIMP-R14 staining (Fig. 1G) in infected eyes were performed to assess whether PAE reduces PMN infiltration. Compared with DMSO-treatment, PAE treatment significantly reduced MPO levels in infected corneas at 3 days post-infection. Immunostaining revealed that fewer PMNs (green) accumulated in the stroma of infected corneas after PAE treatment than after DMSO treatment.

PAE Suppresses the *A. fumigatus*-Induced Production of Pro-Inflammatory Cytokines

To further explore the anti-inflammatory effects of PAE in FK, real-time PCR (Figs. 2A–D) and ELISA (Figs. 2E–H) were used to examine the effect of PAE on the expression of pro-inflammatory cytokines in infected corneas and HCECs. Our results revealed that IL-1β, IL-6, TNF-α, and IL-8 expression was significantly increased in HCECs after fungal stimulation and was significantly lower in the PAE-treated group than in the DMSO-treated group; these changes occurred in a dose-dependent manner. A concentration of 0.6 mM PAE was selected for pretreatment of HCECs prior to ELISAs. PAE treatment significantly reduced the protein levels of IL-6, TNF-α, and IL-8 in HCECs. Similarly, IL-1β and TNF-α mRNA levels in the cornea were markedly decreased in the PAE-treated group at 3 and 5 days after infection. IL-6 mRNA expression was decreased in the PAE-treated group at 3 days after infection; the difference compared with the DMSO-treated group was not significant at 5 days (Figs. 3A–D). These results were consistent with the Western blotting results, which showed that PAE treatment significantly reduced the protein levels of TNF-α (Figs. 3E, 3F) and of IL-6 and IL-1β (Figs. 3G, 3H) at 3 days after infection.

Nrf2/HO-1 Signaling Pathway Contributes to PAE's Anti-Inflammatory Effect in *A. fumigatus* Keratitis

Our results demonstrated that fungal stimulation increased Nrf2 and HO-1 expression in HCECs. Moreover, PAE treatment increased the mRNA and protein levels of Nrf2 and HO-1 induced by *A. fumigatus* in HCECs, which was consistent with the results of our mouse model at 5 days (Figs. 4A–L). We determined whether PAE can increase the nuclear translocation of Nrf2 in *A. fumigatus* keratitis. Our results indicated that PAE upregulated Nrf2 expression and efficiently enhanced Nrf2 translocation in HCECs (Fig. 4M). At the same time, Nrf2 expression staining in the *A. fumigatus* group was increased, but there was no increase in Nrf2 translocation in HCECs. To further determine whether the anti-inflammatory effect of PAE is related to the Nrf2/HO-1 axis, we pretreated HCECs with BT (Nrf2 inhibitor) and ZnPP (HO-1 inhibitor), respectively. Our results indicated that the Nrf2 and HO-1 expression declined in normal HCECs pretreated with BT. In addition, BT partially inhibited PAE-induced Nrf2 and HO-1 expression in infected HCECs (Figs. 5A–D). Furthermore, BT pretreatment partially reversed IL-6, TNF-α, and IL-8 expression levels, which were inhibited by PAE in infected HCECs (Figs. 5E–J). When normal HCECs were pretreated with ZnPP, HO-1 expression was also decreased (Figs. 5K, 5L). ZnPP downregulated the HO-1 expression induced by PAE during *A. fumigatus*

Investigative Ophthalmology & Visual Science

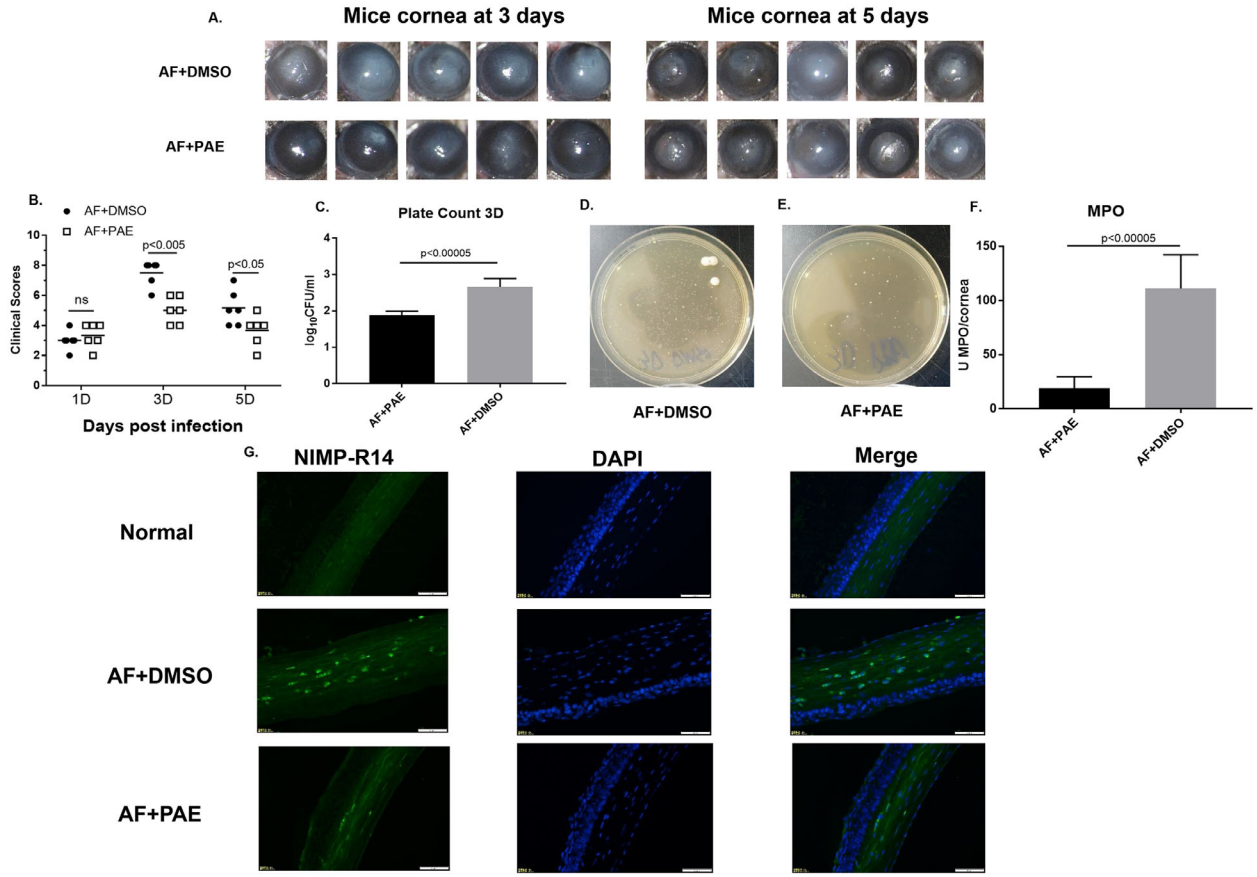

Figure 1. PAE treatment alleviates the severity of *A. fumigatus* (AF) keratitis in mice cornea. Photographs were taken by a slit lamp at 3 and 5 days post-infection (**A**). PAE significantly decreased the clinical score (**B**) compared with DMSO at 3 and 5 days after infection. (**C–E**) PAE treatment of C57BL/6 mice reduced the amount of live fungi in the corneal compared with DMSO treatment. (**F–G**) MPO levels and stained NIMP-R14 in infected eyes with or without PAE treatment are shown. Blue, DAPI. Green, PMNs.

infection and partially increased the expression of the pro-inflammatory mediators IL-6 and IL-8 (Figs. 5M–P).

PAE Treatment Attenuates Dectin-1 Expression in *A. fumigatus* Keratitis

To explore the effect of PAE in *A. fumigatus* keratitis, we examined the expression of Dectin-1 and downstream inflammatory cytokines by real-time PCR, Western blotting, and ELISA. Dectin-1 mRNA levels were significantly increased in the infected group and were significantly decreased in the PAE pretreatment group in a dose-dependent manner (Fig. 6A). Curdlan-induced Dectin-1 protein levels in HCECs (Fig. 6C) also significantly declined after PAE treatment. In addition, Dectin-1 mRNA (Fig. 6B) and protein level (Fig. 6D) were also markedly decreased in the PAE-treated mouse model at 3 days. We further selected two concentrations of PAE, 0.2 mM and 0.4 mM, to further analyze the expression of Dectin-1 and inflammatory mediators in HCECs. PAE treatment reduced curdlan-induced Dectin-1 expression (see Fig. 6C) and IL-6, IL-8, and TNF-α mRNA, and protein levels (Figs. 6E–J).

PAE Exerts Various Antifungal Activities

The results demonstrated that the 0.6 mM PAE caused 90% inhibition of fungal growth at 48 hours in vitro, and the inhibitory effect increased with the increase of the PAE concentration (Fig. 7A). The effects of drug concentration and time on the inhibition of *A. fumigatus* hyphal growth are shown in Fig. 7B. With the concentration of PAE increased, the absorbance was reduced at the same time point, indicating that the amount of *A. fumigatus* was decreased in a concentration-dependent manner. In addition, *A. fumigatus* was stained with Calcofluor white (Figs. 7C–I). Calcofluor white is a fluorochrome that binds with chitin contained in the cell walls of fungi. Calcofluor staining of hyphae intuitively demonstrated that as the concentration of PAE increased, the amount of *A. fumigatus* hyphae and the number of spores declined (see Figs. 7C–I). The *A. fumigatus* hyphae or spores almost could not be found in the field of view after treatment with 1.8 mM PAE.

Discussion

FK is a serious disease that can cause blindness; it is caused by infection with a pathogenic fungus that usually elicits ulceration or even perforation of the infected cornea.[1] The lack of high-efficiency, low-toxicity drugs for corneal fungal infections has made it very difficult to treat this disease.[2] PAE is a promising monoterpenoid extracted from *Perilla*, with strong anti-inflammatory and antifungal effects, especially against filamentous fungi.[11–18] Our results showed that PAE can reduce the inflammatory response of FK through both

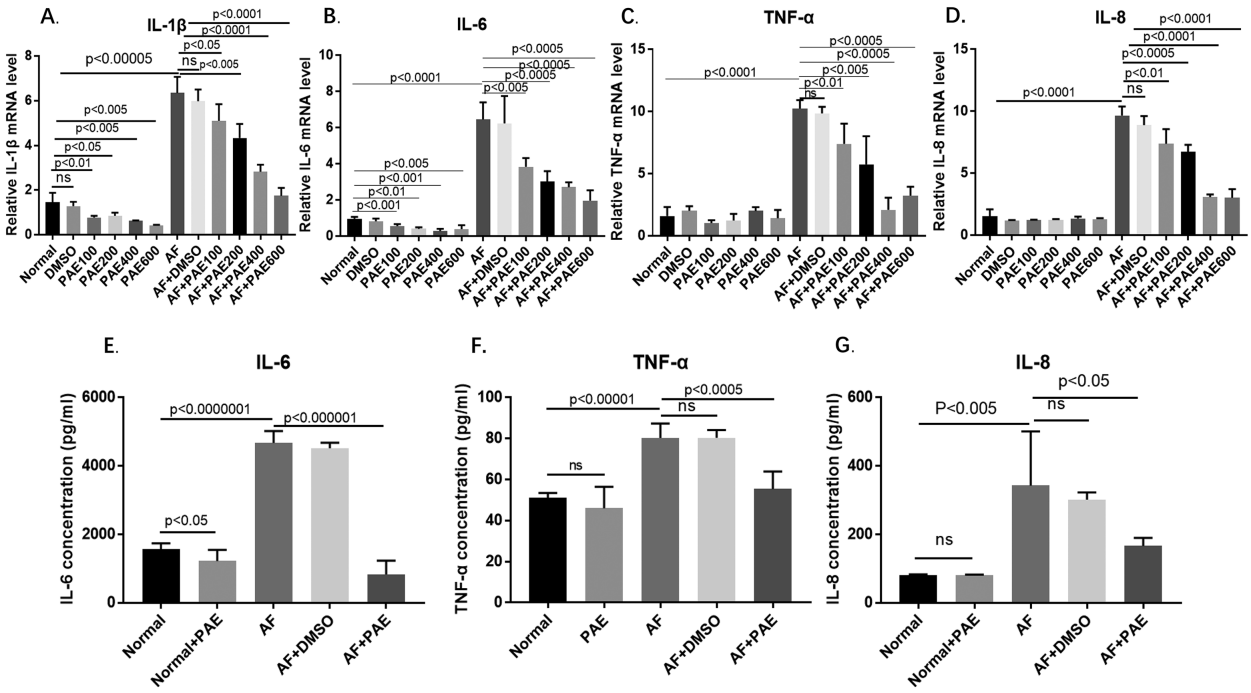

Figure 2. PAE decreases the *A. fumigatus*-induced production of inflammatory mediators in HCECs. HCECs were cultured without any pretreatment or *A. fumigatus* stimulation; pretreated with 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM PAE for 4 hours and no *A. fumigatus* stimulation (N, PAE100, PAE200, PAE400, and PAE600); or pretreated with DMSO (the corresponding amounts in the 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM PAE solutions) for 4 hours and no *A. fumigatus* stimulation. Another set of HCECs were not pretreated but were stimulated with *A. fumigatus* stimulation for 8 hours (AF); were pretreated with 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM for 4 hours and then stimulated with *A. fumigatus* for 8 hours (AF + PAE100, AF + PAE200, AF + PAE400, and AF + PAE600), or were pretreated with DMSO for 4 hours and then stimulated with *A. fumigatus* for 8 hours (AF + DMSO). The mRNA expression of the proinflammatory mediators IL-1β (**A**), IL-6 (**B**), TNF-α (**C**), and IL-8 (**D**) decreased in HCECs after pretreatment with different concentrations of PAE during *A. fumigatus* infection. PAE (0.6 mM) inhibited IL-6 (**E**), TNF-α (**F**), and IL-8 (**G**) protein levels compared with DMSO after *A. fumigatus* stimulation for 24 hours.

anti-inflammatory and antifungal effects. Moreover, studies have confirmed that the monoterpenoid perillaldehyde has been shown to have virtually no genotoxicity and does not pose any risk to the human body when used as a daily flavoring; therefore, it is safe to use as an alternative treatment for inflammatory diseases or disorders.[29,37]

In this study, after treating *A. fumigatus*-infected mice corneas with PAE, slit lamp images showed that corneal opacity was significantly reduced compared with DMSO treatment, the ulcer area was significantly smaller, and the corneal inflammation clinical score was significantly lower. Moreover, PAE significantly downregulated the expression of IL-1β, TNF-α, and IL-6, which are inflammatory factors induced by *A. fumigatus*, at both mRNA and protein levels in infected C57BL/6 mouse corneas. Corneal epithelial cells are the first physical barrier in the antifungal immune responses and play an important role in the immune response to fungal infection of the cornea.[38] Results also demonstrated that PAE also inhibited the expression of IL-1β, TNF-α, IL-8, and IL-6 induced by *A. fumigatus* in HCECs. It has been confirmed that an excessive inflammatory response can aggravate tissue damage in FK, which is detrimental to tissue repair.[7] Previous studies have shown that PAE inhibits the expression of TNF-α, IL-6, and IL-1β in LPS-induced colitis and has a therapeutic effect in colitis.[17] PAE can also significantly reduce TNF-α, IL-6, and IL-1β expression levels in LPS-treated macrophages,[17] which is consistent with our findings; these data suggest that PAE can alleviate the expression of pro-inflammatory cytokines in FK and, therefore, reduce the associated inflammatory response, which may reduce corneal tissue damage. To further investigate the mechanism by which PAE reduces the inflammatory response in *A. fumigatus* keratitis, we examined the MPO levels and the number of neutrophils in the corneal stroma on the third day post-infection. We found that, compared with the DMSO treatment, PAE treatment significantly reduced MPO levels in the infected mice cornea, and immunofluorescence showed that PAE application reduced the number of neutrophils in the corneal stroma during fungal infection. Neutrophils play a crucial role in the inflammatory response during the corneal antifungal immune response, whereas excessive neutrophil infiltration often results in severe damage to corneal tissue, which is detrimental to fungal clearance.[39–41] A previous study in the mouse model of *Candida albicans* (*C. albicans*) vaginitis have confirmed that as the PAE concentration increased, the neutrophil counts in the mouse vaginal tissue decreased significantly, and the tissue integrity was gradually restored[42]; *Uemura* reported that PAE can reduce the infiltration of inflammatory cells in colitis intestinal epithelial tissue,[17] suggesting that PAE can exert anti-inflammatory effects by inhibiting neutrophils recruitment to the corneal stroma in FK.

However, the mechanism by which PAE regulates the expression of inflammatory mediators in FK still remains unknown. Ishida et al.[31] found that β-glucan mediated HO-1 expression in the oral epithelium infected by *C. albicans* through Nrf2. β-Glucan is an intrinsic component of fungal cell wall[43] and mediates the innate immune response of

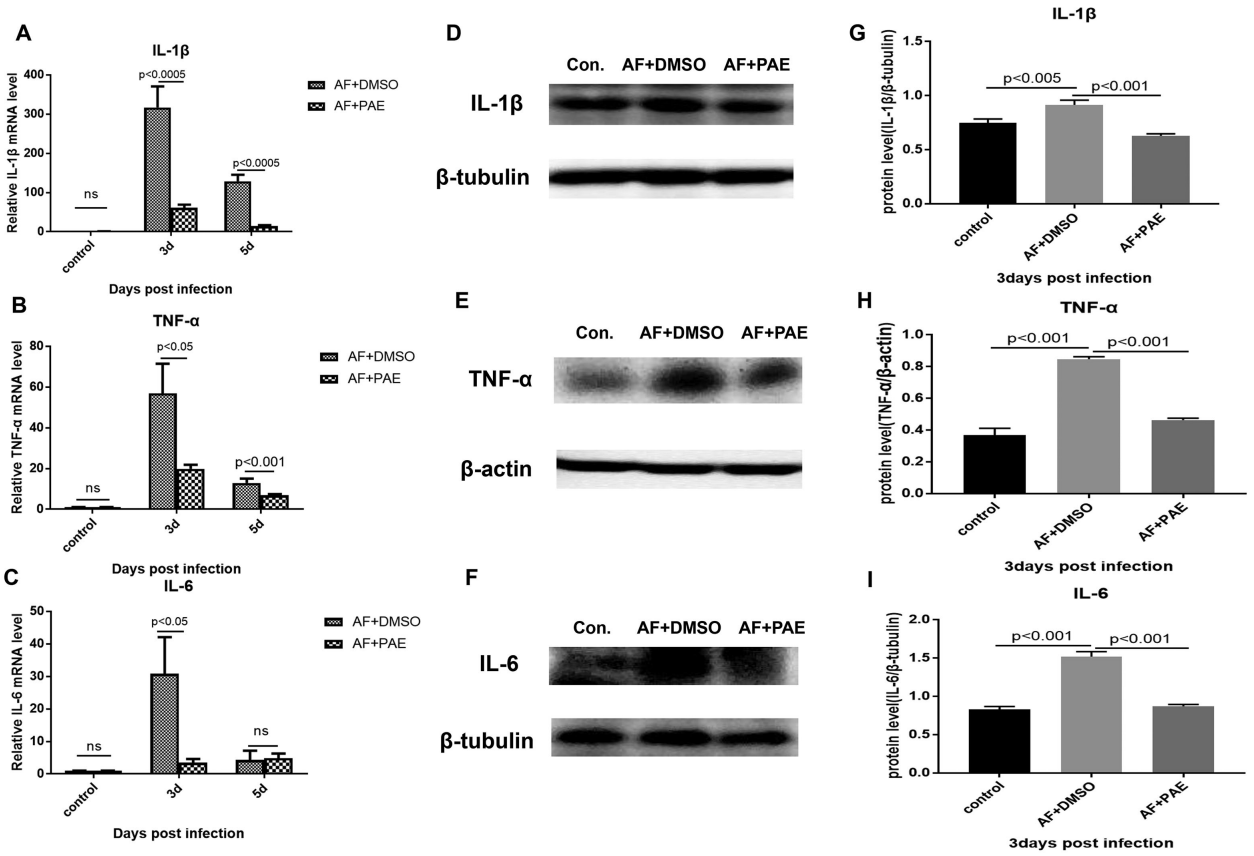

Figure 3. PAE suppresses *A. fumigatus*-induced production of inflammatory mediators in mice cornea. After treatment with 6 mM PAE, the mRNA levels of IL-1β (**A**) and TNF-α (**C**) were significantly decreased in the infected cornea of C57BL/6 mice at both 3 and 5 days after infection; IL-6 (**B**) mRNA was decreased at 3 days, but no differences was detected between the two groups at 5 days post-infection. IL-1β, TNF-α, and IL-6 protein levels (**D–I**) were decreased in the PAE-treated group 3 days post-infection.

the body against fungal infections,[44] which suggests that the Nrf2/HO-1 signaling pathway may play a role in the immune response to protect body against fungal infection. Ryuhei Hayashi has demonstrated that Nrf2 signaling was activated throughout the corneal epithelial wound-healing process and its activation plays a protective role in corneal wound healing.[25] It has been confirmed that HO-1 induction attenuated inflammation endangered by corneal epithelial injury, such as keratitis, and accelerated wound healing and represents a fundamental protective system in the cornea,[30] which collectively suggested that Nrf2/HO-1 may play a role in corneal defense to fungal infection. Moreover, It has been proved that PAE can activate Nrf2/HO-1 signaling pathway and exert antioxidant activities in human keratinocytes.[20] To determine the role of the Nrf2/HO-1 pathway, we first examined the expression of Nrf2 and HO-1 in corneas of mice 5 days post-infection. The expression of Nrf2 and HO-1 in the infected corneas increased after stimulation with *A. fumigatus*, which suggests that the Nrf2/HO-1 signaling pathway may participate in the immune response in FK. We then treated infected corneas with PAE and found that PAE treatment upregulated Nrf2 and HO-1 expression induced by *A. fumigatus* in infected C57BL/6 mice cornea, which confirmed that PAE could upregulate Nrf2 and HO-1 expression and Nrf2/HO-1 signaling pathway may participate in anti-inflammatory effect of PAE in FK. These results were also confirmed in HCECs. To further determine the mechanism by which PAE regulates the Nrf2/HO-1 signaling pathway, we detected the localization of Nrf2 in HCECs. Previous studies have pointed out that PAE activates the activation of Nrf2-KEAP1 system and leads to antioxidant response,[45] illustrating an activation effect of PAE on Nrf2 stimulation. Surprisingly, our immunofluorescence analysis showed that Nrf2 aggregated from the cytoplasm to the nucleus upon PAE treatment compared with fungal stimulation alone, suggesting that PAE could activate the anti-inflammatory effect of Nrf2 by promoting its nuclear translocation of Nrf2 in infected HCECs. The nuclear translocation of Nrf2 plays a key role in increasing its target gene HO-1 expression and inhibiting the subsequent inflammatory response.[46] Under circumstances of no stress, Nrf2 is located in the cytoplasm. When Nrf2 is activated, it translocates into the nucleus and binds with ARE to mediate the expression of HO-1. The HO-1 catalyzes heme to CO with anti-inflammatory effect, and also directly inhibits the expression of pro-inflammatory mediators.[47] Inhibition of Nrf2 by the use of a specific Nrf2 inhibitor, BT, has been reported to attenuate Nrf2-mediated defense mechanisms and impair their antioxidant capacity,[48] therefore, we next pretreat HCECs with BT (Nrf2 inhibitor) and ZnPP (HO-1 inhibitor) and found a sharp decrease in Nrf2 and HO-1 expression. In addition, PAE-induced Nrf2 and HO-1 expression were downregulated, and the production of inflammatory mediators, such as IL-6, IL-8, and TNF-α, was increased, indicating that the inhibition of Nrf2 or

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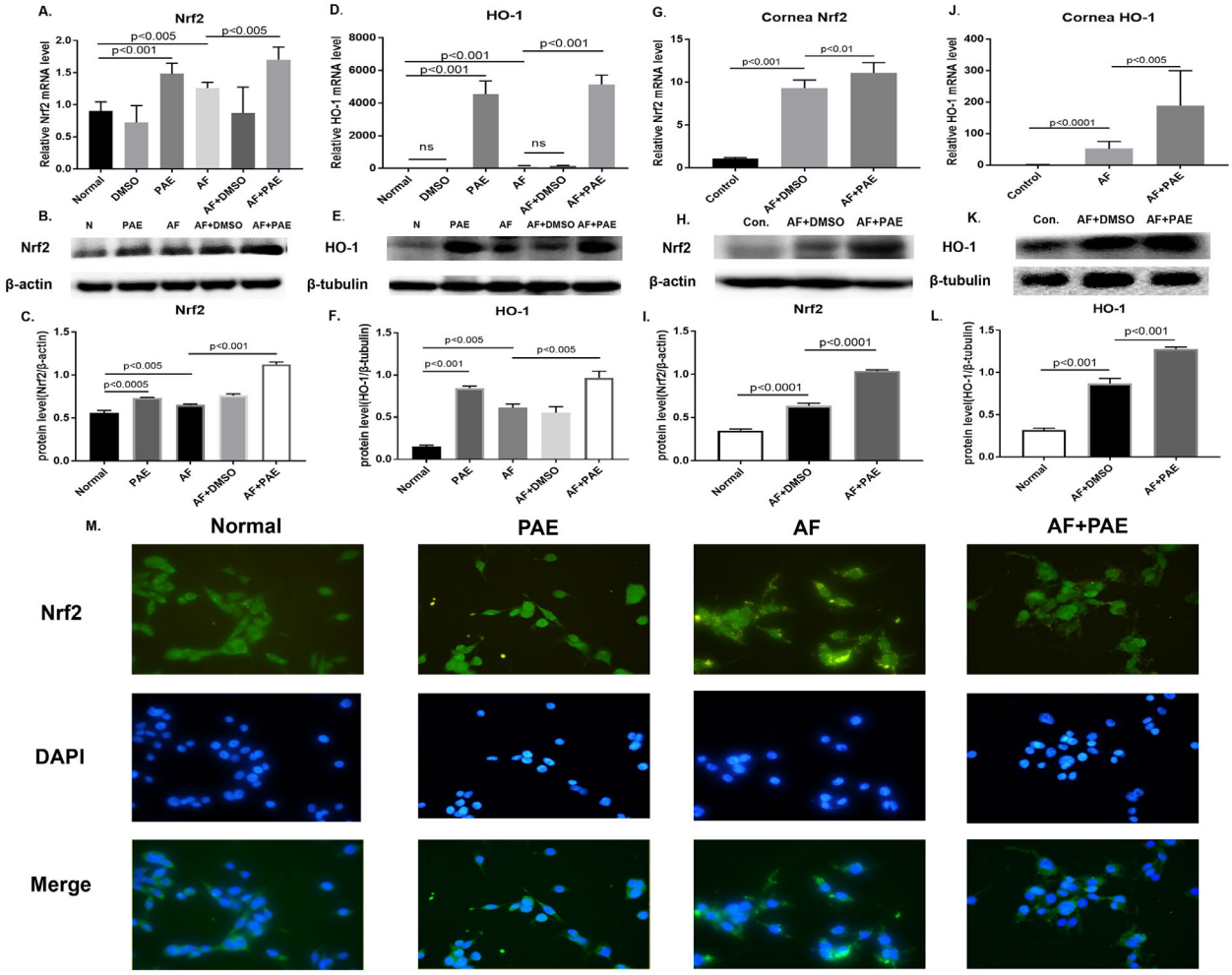

Figure 4. PAE treatment increases Nrf2 and HO-1 expression and enhances Nrf2 nuclear translocation during FK. HCECs were cultured without any pretreatment or *A. fumigatus* stimulation, pretreated with 0.6 mM PAE for 4 hours without *A. fumigatus* stimulation (Normal, PAE), or pretreated with DMSO (the amount corresponding to the 0.6 mM PAE solution) for 4 hours without *A. fumigatus* stimulation. Another set of HCECs were stimulated with *A. fumigatus* for 8 hours after no pretreatment (AF), pretreatment with 0.6 mM PAE for 4 hours, and then stimulated with *A. fumigatus* for 8 hours (AF + PAE), or pretreated with DMSO for 4 hours and then stimulated with *A. fumigatus* for 8 hours (AF + DMSO). (**A–L**) The mRNA and protein levels of Nrf2 and HO-1 after pretreatment with PAE during *A. fumigatus* infection in HCECs and mice cornea 5 days post-infection. (**M**) Immunofluorescence images show Nrf2 nuclear translocation in HCECs after pretreatment with PAE for 4 hours with or without *A. fumigatus* stimulation for 8 hours. Digital images were captured at 400× magnification using a Zeiss Axiovert microscope.

HO-1 with specific inhibitors BT or ZnPP, respectively, could partially weaken the suppressive effect of PAE on the expression of *A. fumigatus*-induced pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α. It was further suggested that the Nrf2/HO-1 signaling pathway participated in the anti-inflammatory effect of PAE during corneal fungal infection. The above evidence indicates that PAE suppresses the excessive inflammatory response partly by activating the Nrf2/HO-1 signaling pathway in *A. fumigatus* keratitis. However, after applying Nrf2 or HO-1 inhibitors to infected HCECs, the level of pro-inflammatory factors of PAE pretreatment group did not completely restore the inflammation to the levels seen in the infected HCECs with no treatment, which suggested that not all the anti-inflammatory effect of PAE was attributed to Nrf2/HO-1 signaling pathway during corneal fungal infection; there may also be other possible mechanisms participating in the anti-inflammatory effect of PAE in *A. fumigatus* keratitis.

Dectin-1 is a PRR that specifically recognizes β-glucan in FK and mediates the downstream inflammatory immune response.[49] To determine whether PAE affects Dectin-1 expression, we detected Dectin-1 expression in HCECs treated with different concentrations of PAE. The data showed that PAE inhibited Dectin-1 expression induced by *A. fumigatus* in a concentration-dependent manner, suggesting that PAE may inhibit excessive inflammation by inhibiting the expression of the PRR Dectin-1 during *A. fumigatus* infection. Moreover, Dectin-1 mRNA and protein levels significantly decreased after PAE treatment of fungi-infected mice corneas, which further confirmed our results. Curdlan-stimulated HCECs showed increased Dectin-1 expression at the mRNA and protein levels and increased expression of downstream inflammatory mediators. PAE treatment markedly decreased Dectin-1 and pro-inflammatory mediator expression, which indicated that PAE treatment can reduce the excessive inflammatory response in *A. fumigatus*

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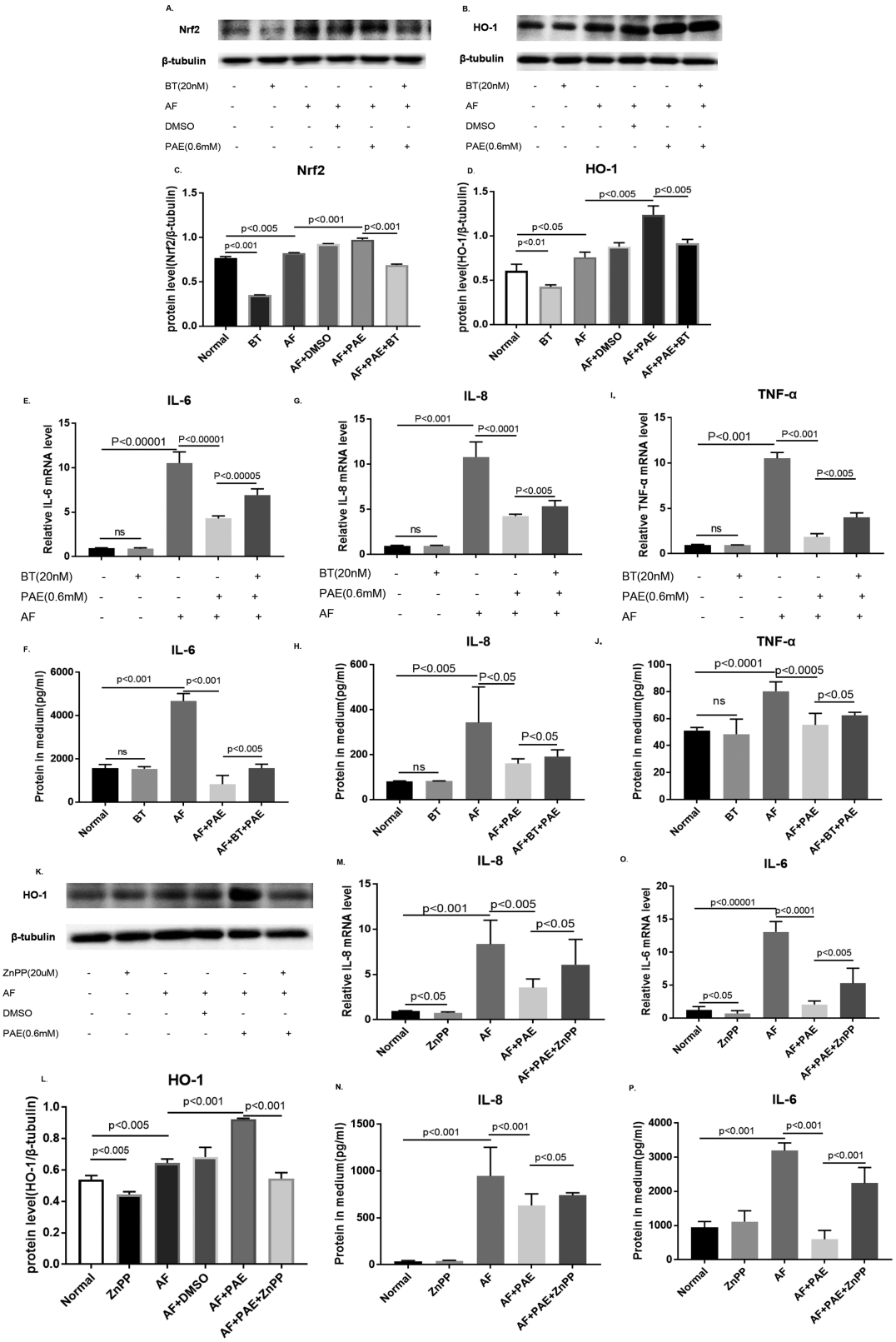

Figure 5. PAE exerts anti-inflammatory effects partially by activating the Nrf2/HO-1 signaling pathway. After pretreatment HCECs with 20 nM BT (Nrf2 inhibitor) for 2 hours, add 0.6 mM PAE or DMSO for 4 hours, and then stimulated with *A. fumigatus* for 8 hours or 24 hours. (**A–D**) Western blot analysis illustrates the expression of Nrf2 and HO-1 in HCECs after stimulated with *A. fumigatus* for 8 hours. (**E–J**) PCR and ELISA results show the mRNA and protein levels of IL-6, IL-8, and TNF-α after blocking Nrf2 activity. Pretreating another set of HCECs with 20 uM ZnPP (HO-1 inhibitor) for 2 hours, 0.6 mM PAE or DMSO for 4 hours, and then stimulated with *A. fumigatus* for 8 hours or 24 hours. (**K, L**) Western blot analysis illustrates HO-1 expression in HCECs after stimulated with *A. fumigatus* for 8 hours. (**M–P**) PCR and ELISA results show the mRNA and protein levels of IL-6 and IL-8 after the inhibition of HO-1 activity.

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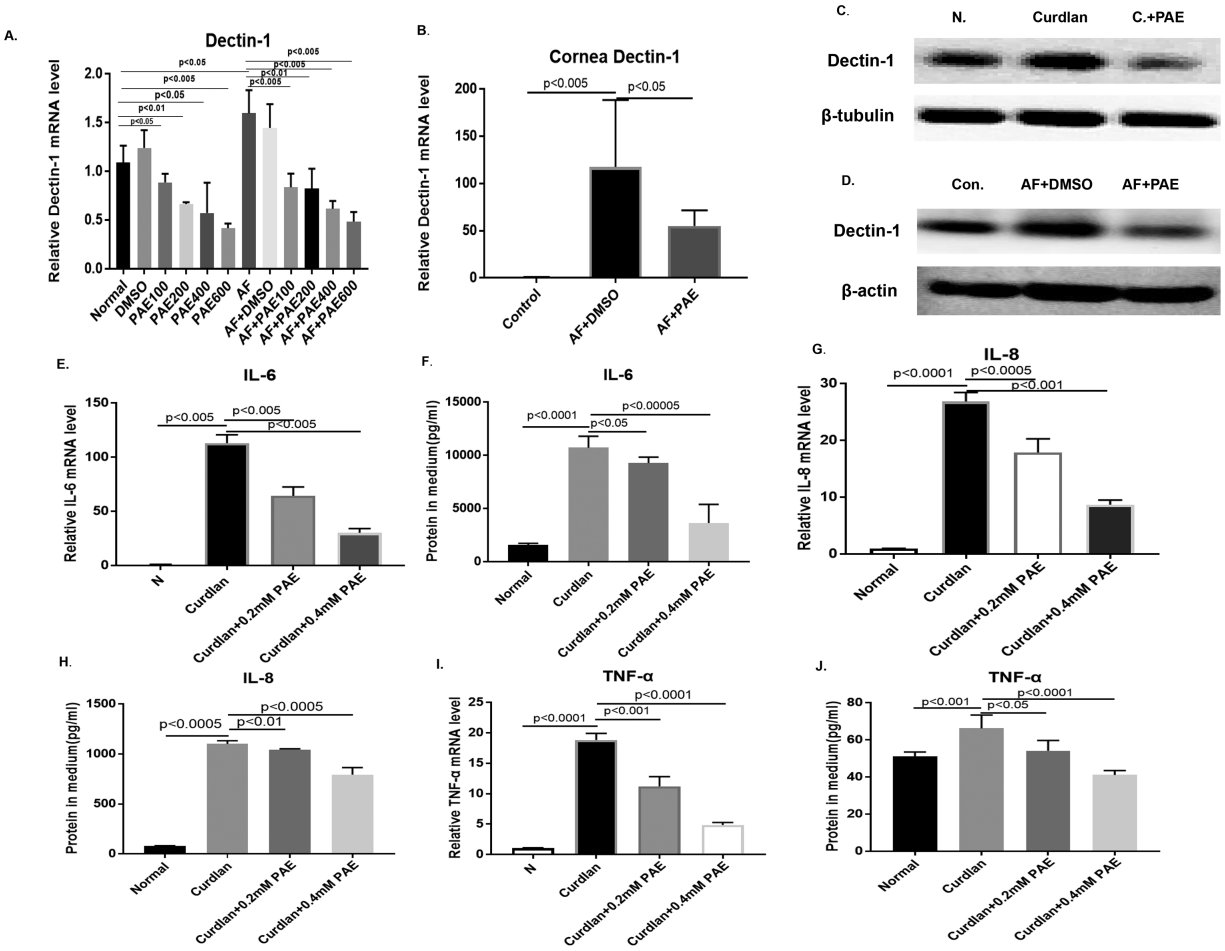

Figure 6. PAE treatment attenuates Dectin-1 expression in *A. fumigatus* keratitis. HCECs were cultured without any pretreatment or *A. fumigatus* stimulation; pretreated with 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM PAE for 4 hours and no *A. fumigatus* stimulation (Normal, PAE100, PAE200, PAE400, and PAE600); or pretreated with DMSO (the corresponding amount in 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM PAE solutions) for 4 hours and no *A. fumigatus* stimulation. Another set of HCECs were stimulated with *A. fumigatus* for 8 hours after no pretreatment (AF); pretreated with 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM for 4 hours and then stimulated with *A. fumigatus* stimulation for 8 hours (AF + PAE100, AF + PAE200, AF + PAE400, and AF + PAE600); or pretreated with DMSO for 4 hours and then stimulated with *A. fumigatus* for 8 hours (AF + DMSO). HCECs were also pretreated with 0.2 mM, 0.4 mM PAE for 4 hours, and then stimulated with Curdlan (200 ug/mL) for 8 hours or 24 hours. (**A**) Dectin-1 mRNA expression in HCECs decreased after pretreatment with different concentrations of PAE during *A. fumigatus* infection. The mRNA and protein levels of Dectin-1 declined in PAE-treated mice 3 days post-infection (**B, D**). (**C**) PAE inhibited Curdlan-induced Dectin-1 protein expression. (**E–J**) PCR and ELISA show the expression of proinflammatory cytokines.

keratitis also largely by inhibiting fungus-induced Dectin-1 expression. We speculate that Dectin-1 may emerge as a new therapeutic target of PAE in its anti-inflammatory effects in FK to reduce corneal tissue damage.

Previous studies have proven that PAE has a strong antifungal effect on *Aspergillus* species.[16,19] Our study found that the fungal load of *A. fumigatus* in the cornea of infected C57BL/6 mice was significantly reduced after PAE treatment. In vitro, *A. fumigatus* spores and PAE were cocultured in liquid medium, and the increasing concentrations of PAE showed stronger inhibition of spore germination and hyphal growth, which further indicated that PAE can efficiently inhibit the growth of *A. fumigatus*. Interestingly, the minimum inhibitory concentration (MIC) and anti-inflammatory concentration of PAE on *A. fumigatus* spores in vitro were quite low. At the low concentration of 0.6 mM, the PAE could inhibit both the inflammatory response and fungal growth. On the other hand, Calcofluor staining images intuitively showed that PAE significantly reduced *A. fumigatus* growth at a relatively low concentration of 0.6 mM in such medium. With an increase in PAE concentration, the number of *A. fumigatus* spores and mycelia decreased gradually. When the concentration reached 1.8 mM, the growth of *A. fumigatus* was almost completely inhibited in such medium. Previous studies have pointed out that at high concentrations PAE can inhibit the spore germination and kill fungus, whereas at low concentrations PAE can decrease spore germination and hyphal growth[50]; thus, PAE exerts antifungal activity and reduces fungal damage to corneal tissue.

Taken together, the data indicate that PAE possesses anti-inflammatory effect in *A. fumigatus* keratitis, which is attributed to inhibiting the Dectin-1-mediated inflammatory response, activating the Nrf2/HO-1 signaling pathway and decreasing neutrophil infiltration. Furthermore, PAE inhibits the growth and germination of *A. fumigatus*, suggesting that PAE may become a promising therapeutic agent for FK.

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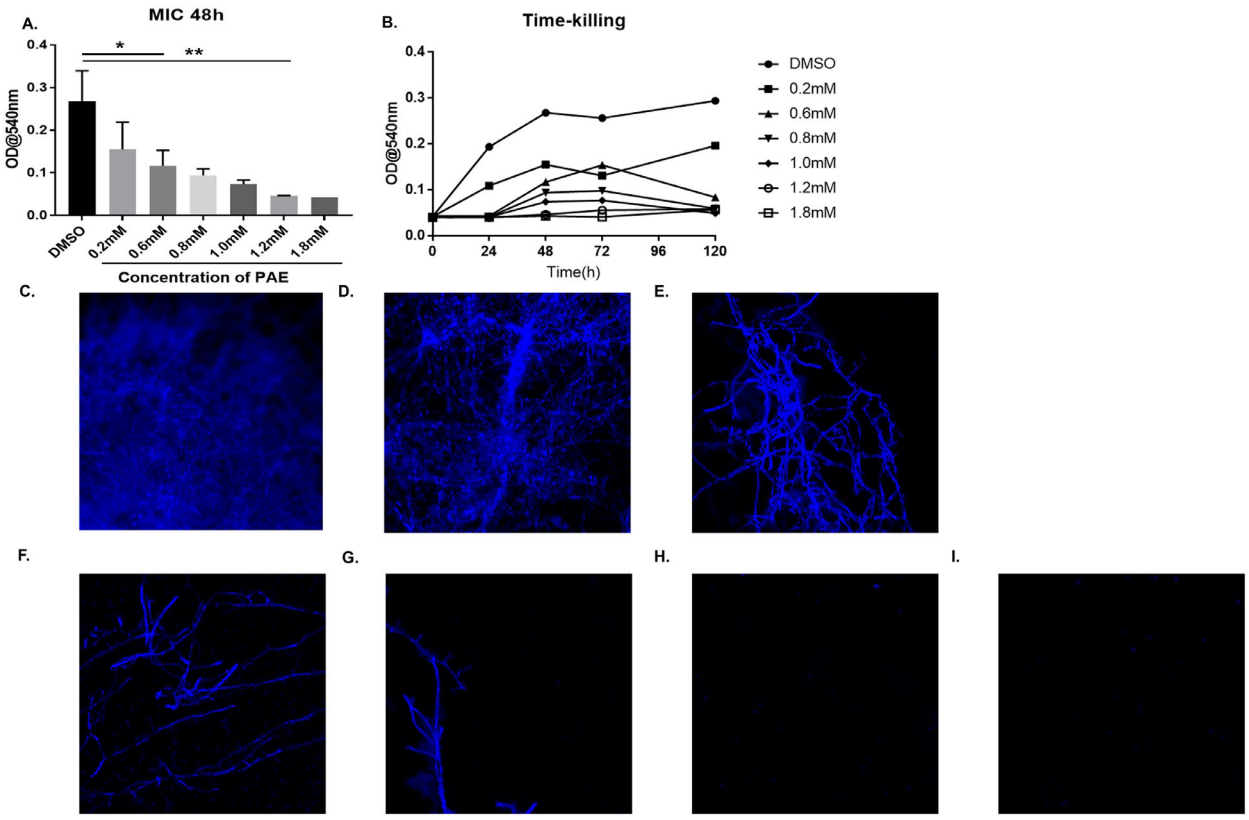

Figure 7. PAE exerts various antifungal activities. (**A**) MIC, (**B**) time-killing, (**C–I**) Calcofluor staining images were taken after separately treating *A. fumigatus* with DMSO **C**, 0.2 mM PAE **D**, 0.6 mM PAE **E**, 0.8 mM PAE **F**, 1.0 mM PAE **G**, 1.2 mM PAE **H**, and 1.8 mM PAE **I** for 48 hours in liquid medium containing 10% fetal bovine serum and PBS.

Acknowledgments

Supported by the National Natural Science Foundation of China (81470609); Youth Project of National Natural Science Foundation of China (81500695, 81300730); Natural Science Foundation of Shandong Province (ZR2012HZ001); Youth Project of Natural Science Foundation of Shandong Province (ZR2013HQ007); Doctor Foundation of Natural Science of Shandong Province (ZR2017BH025); and Key Research Project Foundation of Shandong Province (2019GSF107022). The authors alone are responsible for the content and writing of the paper.

Disclosure: **Y. Fan**, None; **C. Li**, None; **X. Peng**, None; **N. Jiang**, None; **L. Hu**, None; **L. Gu**, None; **G. Zhu**, None; **G. Zhao**, None; **J. Lin**, None

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