Baicalein Protects Against *Aspergillus fumigatus* Keratitis by Reducing Fungal Load and Inhibiting TSLP-Induced Inflammatory Response

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PURPOSE. To investigate the antifungal and anti-inflammatory effects of baicalein on *Aspergillus fumigatus* (*A. fumigatus*) keratitis and the underlying mechanisms.

METHODS. The noncytotoxic antifungal concentration of baicalein was determined using CCK8, cell scratch assay, minimum inhibitory concentration, biofilm formation, scanning electron microscopy, propidium iodide uptake test and adherence assay in vitro and Draize test in vivo. In fungal keratitis (FK) mouse models, clinical score and plate count were used to evaluate FK severity, and myeloperoxidase assay and immunofluorescence staining were performed to examine neutrophil infiltration and activity. Real-time PCR, ELISA, and Western blot were performed to explore the anti-inflammatory activity of baicalein and the underlying mechanisms in vivo and in vitro.

RESULTS. Baicalein at 0.25 mM (noncytotoxic) significantly inhibited *A. fumigatus* growth, biofilm formation, and adhesion in vitro. In *A. fumigatus* keratitis mice, baicalein mitigated FK severity, reduced fungal load, and inhibited neutrophil infiltration and activity. Baicalein not only suppressed mRNA and protein levels of proinflammatory factors IL-1β, IL-6, and TNF-α, but also inhibited the expression of thymic stromal lymphopoietin (TSLP) and TSLP receptor (TSLPR) in vivo and in vitro. In HCECs, mRNA and protein levels of IL-1β, IL-6, and TNF-α were significantly lower in the TSLP siRNA–treated group, while higher in the rTSLP-treated group than in the corresponding control. Baicalein treatment significantly inhibited rTSLP induced the expression of IL-1β, IL-6, and TNF-α.

CONCLUSIONS. Baicalein plays a protective role in mouse *A. fumigatus* keratitis by inhibiting fungal growth, biofilm formation, and adhesion, and suppressing inflammatory response via downregulation of the TSLP/TSLPR pathway.

Keywords: Baicalein, keratitis. *Aspergillus fumigatus*, TSLP, TSLPR

Fungal keratitis (FK) is a blinding corneal disease caused by fungal pathogens especially *Fusarium* and *Aspergillus*,[1] which is becoming prevalent in developing countries associated with several risk factors such as trauma, long-term use of contact lenses, and corticosteroid abuse.[2,3] However, current therapies like polyenes (natamycin, amphotericin) and azoles (voriconazole) have significant limitations,[4] suggesting the need to explore new effective drugs.

Baicalein (5,6,7-trihydroxyflavone, BE) is a natural flavonoid, originally extracted from *Scutellaria baicalensis*, a Chinese herbal medicine that has been used for diarrhea, lung infections, and chronic liver disease for thousands of years.[5–8] Recently, baicalein has been reported to have antifungal,[9] antivirus,[10] anti-inflammatory[11] and anticancer[12] effects in diverse disease models including hematological malignancies,[13] neurodegenerative diseases,[14] and inflammatory disorders.[15] For example, baicalein has antifungal activity against *Candida albicans* and *Candida krusei* in vitro,[16] and a combination of baicalein and amphotericin B could accelerate *Candida albicans* apoptosis.[17] A recent preclinical study reported that baicalein inhibits SARS-CoV-2–induced lung injury by suppressing virus replication and inflammatory cell infiltration.[10] In addition, baicalein exerts an anti-inflammatory effect on activated macrophages by inhibiting nitric oxide and cytokines such as interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and growth factors through modulating the endoplasmic reticulum stress CHOP/STAT pathway, suggesting it might be useful for inflammation related diseases.[18] Moreover, baicalein inhibits non-small-cell lung cancer invasion and metastasis in the inflammatory microenvironment.[19] However, whether baicalein has antifungal effect in vivo and how baicalein plays a role in FK have not been studied yet.

Although baicalein has been studied in various inflammation-related pathways,[20] how it interacts with inflammatory mediators is still largely unknown. A recent study reported that baicalein is a novel thymic stromal lymphopoietin (TSLP) inhibitor through binding to the N-terminal helix in TSLP to break the interaction between

TSLP and TSLPR, inhibiting the TSLP/TSLPR signaling pathway.[21] TSLP is an epithelial cell-derived cytokine that acts as one of the central regulators of type 2 immunity and inflammatory diseases.[22] In response to various stimuli including microbial infection, mechanical injury, and endogenous cytokines, TSLP expression and release in epithelial cells are increased,[23] which in turn triggers the production of proinflammatory cytokines such as IL-4, IL-5, IL-9, IL-13, and TNF-α of the innate and adaptive immunity, driving various pathological inflammation.[24–26] Notably, previous studies have shown that TSLP is involved in FK progression. Qingshan et al. reported that TSLP was highly expressed and secreted in *Aspergillus fumigatus* (*A. fumigatus*) infected human corneal epithelial cells (HCECs), facilitating FK development via inducing caspase-1-dependent pyroptosis.[27] In addition, TSLP-activated dendritic cells could induce T helper type 2 (Th2) inflammation in mice with *A. fumigatus* keratitis.[28] Inhibition of TSLP could mitigate the severity of FK, and downregulate neutrophil infiltration and the expression of proinflammatory factors IL-6 and IL-8, controlling excessive inflammation caused by corneal injury,[29] which suggests TSLP could be a potential therapeutic target for FK treatment.

According to the existing evidence, we hypothesized that baicalein may exhibit antifungal and anti-inflammatory effects on FK, which could be associated with the TSLP/TSLPR pathway. This study investigated the role of baicalein in FK progression and its potential interactions with TSLP/TSLPR in *A. fumigatus* keratitis mouse models and HCECs.

MATERIALS AND METHODS

Baicalein Solution Preparation

Baicalein powder (10 mg) purchased from MedChem Express (Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) at 125 mM, and further diluted using required solvents, including Dulbecco's Modified Eagle Medium (DMEM) for cell culture experiments, Sabourand Medium for in vitro fungal culture assays and phosphate-buffered saline (PBS) for animal experiments to obtain the desired concentrations of baicalein solution.

Cell Viability (Cell Counting Kit-8, CCK-8)

HCECs (obtained from the Ocular Surface Laboratory Zhongshan Ophthalmic Center, China) were inoculated in 96-well plates and grown for one to two days to 80% confluence (37°C, 5% CO_2). Then HCECs were mixed with baicalein (0, 0.0625, 0.125, 0.25, 0.5 mM) or DMSO (0.2%) and incubated for 24 hours, followed by adding 10 µL of CCK-8 (Solarbio, Beijing, China) to each well and incubated for two hours. The absorbance at 450 nm was read using a microplate reader.

Cell Scratch Test

HCECs were seeded (3×10^5/mL) in 6-well plates with three parallel lines labeled by black marker on the bottom of each well. Then, three lines perpendicular to them were scratched using a 200 µL pipette tip in each well. HCECs per well were incubated with baicalein (0, 0.0625, 0.125, 0.25, and 0.5 mM) or DMSO (0.2%) for 24 hours. Cell migration was evaluated by measuring the width of the cell scratch at the same location at 0 and 24 hours under optical microscopy (Nikon, Tokyo, Japan, 100×). Experiments were repeated at least three times under the same conditions.[30]

Ocular Toxicology: The Draize Eye Test

Ocular toxicology study (Draize Eye Test) was performed to investigate the potential adverse effects of baicalein in normal mouse eyes. Baicalein (0.25, 0.4, and 0.5 mM) in dose of 5 µL was dropped into the conjunctival cul-de-sac of one eye in four mice. The contralateral eye treated with DMSO (0.2%) served as a control. Corneal fluorescein staining (CFS) was examined using slit-lamp microscopy under a cobalt blue light at one, three, and five days after corneal instillation in mice. CFS scores were evaluated according to the Organization for Economic and Cooperative Development (OECD) grading scale for ocular irritation. The scores involve weighting and summing six components of directly observable changes of the eye's anterior segment, including the density and area of corneal opacification, severity of iritis, conjunctival redness, edema, and discharge.[31–36]

Baicalein Minimum Inhibitory Concentration (MIC) on *A. fumigatus*

A. fumigatus conidia were prepared as described previously.[37] Briefly, the conidia (China General Microbiological Culture Collection Center, Beijing, China) were rinsed using PBS to adjust the concentration to 5×10^7/mL and dilute to 5×10^5/mL for MIC. Baicalein was diluted in different concentrations (0.0625, 0.075, 0.125, 0.15, 0.25, and 0.5 mM) with Sabouraud medium containing spores and distributed to 96-well plates, and incubated at 37°C for 48 hours. The absorbance at 570 nm was measured and analyzed. Then, the supernatant in 96-well plates were discarded, and 50 µL of Calcofluor White Stain (Sigma-Aldrich) was added and incubated at room temperature (RT) for 30 minutes. The staining pictures were photographed using the fluorescence microscope (Nikon, Tokyo, Japan, 100×).

Propidium Iodide (PI) Staining

The conidia suspension of *A. fumigatus* (2×10^6 CFU/mL) was poured on a 6-well plate and incubated at 37°C for 24 hours. Then, the hyphae were washed, centrifuged (12,000 rpm, 10 minutes), and transferred to a new 6-well plate, and then incubated with 0.25 mM baicalein or 0.2% DMSO at 37°C for 24 hours. After harvesting and washing with sterile PBS, the fungal hyphae were incubated with PI (Sigma-Aldrich) solution for 15 minutes at RT in the dark. Images were photographed with fluorescence microscope (Nikon, Tokyo, Japan, 200×).[38]

Scanning Electron Microscopy (SEM)

A. fumigatus conidia (2×10^6 CFU/mL) was cultured in a 6-well plate (1 mL per well) at 37°C for 24 hours to form hyphae. Hyphae were washed, centrifuged (12,000 rpm, 10 minutes), and transferred to a new 6-well plate, and then incubated with 0.25 mM baicalein or 0.2% DMSO at 37°C for 24 hours. Then, hyphae were collected in EP tubes and fixed with 2.5% glutaraldehyde at 4°C for two hours. The samples were washed with PBS, then mixed with 1% (v/v) osmium tetroxide in PBS at 4°C for one hour. Subsequently,

samples were gently dehydrated in graded ethanol, critical-point dried in CO_2, coated with gold, and observed under SEM (JSM-840, JOEL Company, Japan) at 2000× and 5000× magnification (bar = 20 or 10 μm).[9,38]

Biofilm Formation and Inhibition Test

Quantitative determination of biofilm forming capacity was tested by a colorimetric microtiter plate assay.[39] Briefly, the conidia of *A. fumigatus* were adjusted to a concentration of 5 × 10^5 CFU/mL. The suspension containing 90 μL Sabouraud medium with serial dilutions of baicalein (0.0625, 0.075, 0.125, 0.15, 0.25 and 0.5 mM) and 10 μL conidia were added to each well of a microtiter plate (in triplicate). Medium was added as the negative control for each plate. Subsequent to an incubation period of 48 hours at 37°C in aerobic conditions without shaking, wells were gently decanted and rinsed three times with 200 μL of sterile PBS (pH 7.2). After air drying, biofilms were fixed with 100 μL of 99% glutaraldehyde per well for 20 minutes, dried, and stained with 100 μL per well of 0.1% crystal violet (CV; Sigma-Aldrich) for 15 minutes. Unbound dye was rinsed with PBS. After air drying, dye bound to biofilm formed on the chamber was released with 100 μL of 33% acetic acid per well for 30 minutes at RT, without shaking. OD (Optical Density) of each well was measured at 620 nm using a microtiter plate reader.

Adhesion of *A. fumigatus* to HCECs

The chambered slides (four/slide) were inoculated with 1 mL complete medium containing HCECs and incubated overnight. Cell chambers were washed twice with sterile PBS, then 1 mL fresh media was added without antibiotics. Conidia suspension (2 × 10^5/mL) was combined with baicalein (0.25 mM) or DMSO immediately before application to the chambered slides. Each slide was incubated for three hours at 37°C under aerobic conditions. Then slides were washed three times with sterile PBS. After air drying, each slide was stained through hematoxylin and eosin (HE). The number of spores which adhered to the surface of HCECs were counted, averaged, and expressed as the number of adherent spores/cell. All specimens were observed and photographed by microscopy (Nikon, Tokyo, Japan, 400×).[40]

Cell Culture and *A. fumigatus* Stimulation

HCECs seeded in 12-well plates or 6-well plates were incubated at 37°C for 48 hours. Then, the cells were first stimulated with inactivated *A. fumigatus* hyphae (5 × 10^6 CFU/mL) for one hour, and treated with 0.25 mM baicalein or DMSO. After four hours of *A. fumigatus* stimulation, cells were collected for real-time PCR (RT-PCR) to detect mRNA levels of TSLP, TSLPR, IL-1β, IL-6 and TNF-α. After 24 hours of *A. fumigatus* stimulation, HCECs protein were collected for Western blot to detect TSLP protein level, and the supernatant were collected for enzyme-linked immunosorbent assay (ELISA) to detect protein levels of IL-1β, IL-6 and TNF-α.

TSLP RNA Interference (TSLP siRNA) and Recombinant TSLP (rTSLP) Treatment

TSLP siRNA was used to block TSP expression. HCECs suspensions of 1 × 10^5/mL seeded into 12- or 6-well plates were treated with TSLP siRNA (50 nM/mL, Ribobio, Guangzhou, China) or scramble siRNA (50 nM/mL, Ribobio, Guangzhou, China) for 48 hours. Cells were harvested for RT-PCR to evaluate transfection efficiency. HCECs were treated with human rTSLP (100 ng/mL, Peprotech, USA) or IgG (100 ng/mL, Bioss, China) for 12 hours. After transfection, cells were treated with *A. fumigatus* for one hour, then followed by 0.25 mM baicalein treatment. Then, cells were harvested after three hours, and cell protein and supernatant were collected 23 hours later for the future use.[41]

FK Murine Models

All treatments were performed on C57BL/6 mice (female, eight weeks, Jinan Pengyue Laboratory) according to the ARVO Statement regarding the Use of Animals in Ophthalmology and Vision Research. The method of establishing the FK mouse model was based on Che et al.[42] First, each mouse was abdominally anesthetized with 4 mL of 8% chloral hydrate. Each left eye was a blank control. The right corneal stroma was injected with 2.5 μL *A. fumigatus* conidia suspension (2.5 × 10^6 CFU/mL) with a micro syringe. In the baicalein treatment group, 5 μL baicalein (0.25 mM) was spotted on the right eye three times a day, and 5 μL DMSO (0.2%) was spotted on the right eye three times a day as control. Based on the observation under a slit lamp at one, three, and five days post infection (p.i.), the severity of keratitis was evaluated by clinical score referred to Wu et al.[43] The total score of 0 to 5 is mild infection, 6 to 9 is moderate infection, and 10 to 12 is severe infection. The corneas at days one, three, and five p.i. were removed for RT-PCR, ELISA, and Western blot. The corneas infected at three days were removed for Myeloperoxidase (MPO). The corneas infected at five days were used for plate count. The mouse eyeballs infected at three days were removed for immunofluorescence (IF).

Plate Count

The mice cornea infected after five days were taken (n = 6/group/time), and evenly ground with a glass. Each cornea was mixed with 1 mL PBS, then evenly dispersed on a Sabouraud agar plate. The plates were incubated under aerobic conditions at 37°C. The formation of aseptic colonies on the plate medium was observed every day. Then, the number of *A. fumigatus* colonies on the plate medium of the baicalein-treated group and the DMSO-treated group was counted.[44]

Myeloperoxidase (MPO) Assay

The mice corneas at three days p.i. (n = 6/group/time) were harvested and homogenized, then followed with the protocol of MPO test kit (Nanjing Jiancheng Institute of Bioengineering, China). MPO was measured spectrophotometrically at 460 nm at 37°C. The slope of the line was related to the MPO unit/g cornea.

Real-Time PCR (RT-PCR)

Total RNA of mouse cornea and HCECs were extracted using RNAiso Plus reagent (Takara, Dalian, China). RNA samples were reverse transcribed using HiScript III RT SuperMix-cjj (Vazyme, Nanjing, China) to produce cDNA template. RT-PCR was performed referring to previous experimental

TABLE. Target Gene Primer Sequence

Gene	Primer	Nucleotide Sequence	GenBank
β-Actin (mouse)	F	5′-GATTACTGCTCTGGCTCCTAGC-3′	NM_007393.3
	R	5′-GACTCATCGTACTCCTGCTTGC-3′	
IL-1β (mouse)	F	5′-CGCAGCAGCACATCAACAAGAGC-3′	NM_008361.4
	R	5′-TGTCCTCATCCTGGAAGGTCCACG-3′	
TNF-α (mouse)	F	5′-ACCCTCACACTCAGATCATCTT-3′	NM_013693.3
	R	5′-GGTTGTCTTTGAGATCCATGC-3′	
IL-6 (mouse)	F	5′-TGATGGATGCTACCAAACTGGA-3′	NM_001314054.1
	R	5′-TGTGACTCCAGCTTATCTCTTGG-3′	
TSLP (mouse)	F	5′-ACGGATGGGGCTAACTTACAA-3′	NM_021367.2
	R	5′-AGTCCTCGATTTGCTCGAACT-3′	
TSLPR (mouse)	F	5′-TCACGGGGTGATGTCACAGT-3′	NM_001164735.1
	R	5′-AGTGCCATAACGGAACTCCAG-3′	
GAPDH (human)	F	5′TGGCACCCAGCACAATGAA-3′	NM_001101.5
	R	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′	
IL-1β (human)	F	5′-GCTGATGGCCCTAAACAGATGAA-3′	NM_000576.3
	R	5′- TCCATGGCCACAACAACTGAC -3′	
TNF-α (human)	F	5′-TGCTTGTTCCTCAGCCTCTT -3′	NM_000594.4
	R	5′-CAGAGGGCTGATTAGAGA GAGGT-3′	
IL-6 (human)	F	5′-AAGCCAGAGCTGTGCAGATGAGTA-3′	NM_000600.5
	R	5′- TGTCCTGCAGCCACTGGTTC-3′	
TSLP (human)	F	5′-ACGGATGGGGCTAACTTACAA-3′	NM_033035.5
	R	5′-AGTCCTCGATTTGCTCGAACT-3′	

F, forward; R, reverse.

research.[45] The primer pair sequences used for RT-PCR are shown in the Table.

Western Blot

Mice corneas ($n = 6$/group/time) or HCECs were lysed in 196 μL RIPA (Solarbio) containing 2 μL PMSF (Solarbio) and 2 μL phosphatase inhibitor (MCE) for tow hours, and centrifuged at 12000 rpm for five minutes. BCA kit (Solarbio) was used for protein concentration determination. Then, total proteins were separated on SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Solarbio), which was then blocked in blocking buffer (Solarbio) for two hours. Subsequently, membranes were incubated with primary antibodies against TSLP (1:1000; Thermo), TSLPR (1:1000; Thermo), and β-actin (1:5000; Abcam) at 4°C overnight, followed by incubation with goat anti-rabbit secondary antibodies at 37°C for one hour. Then, the blots were visualized using enhanced chemiluminescence (ECL) (Thermo Fisher Scientific).

Enzyme-Linked Immunosorbent Assay (ELISA)

The HCECs supernatant and mouse corneas ($n = 6$/group/time) were collected and centrifuged. The ELISA kit (R&D Systems) was used to detect IL-1β, IL-6, and TNF-α protein levels according to the instructions. The absorbance was measured at 450 nm with a microplate reader.

Immunofluorescence Staining (IFS)

The immunofluorescence step refers to the previous study.[46] Briefly, the eyeballs of the euthanized mice ($n = 6$/group/time) were removed at three days p.i. and completely embedded in the optimal cutting temperature (O.C.T.) (Sakura Tissue-Tek, Torrance, CA, USA) and frozen with liquid nitrogen. The corneas were cut by microtome into 10 μm thick slices, and the slices were soaked in acetone for 30 minutes. After being blocked with goat serum (1:100) for 30 minutes, the sections were incubated with anti-NIMP-R14 antibody (1:200; Santa Cruz, CA, USA) or anti-mouse TSLP antibody (20 μg/mL, Thermo) at 4°C, overnight. Then, slices were stained with FITC-conjugated goat anti-rat (1:200; Abcam) or FITC-conjugated goat anti-rabbit (1:200; Abcam) secondary antibody at RT for one hour. Cell nuclei were stained with DAPI (1:100; Solarbio) for 10 minutes. The slices were observed under the microscope and captured at 400× magnification.

HCECs seeded in poly-L-lysine-coated slips were stimulated by *A. fumigatus* for one hour, and then were incubated with 0.25 mM baicalein for seven hours. Slips were rinsed three times in PBS, fixed by 4% paraformaldehyde at RT for 15 minutes and then blocked in goat serum (1:10) for 30 minutes at RT. Slips were incubated with primary antibody against human TSLP (20 μg/mL, Thermo) at 4°C overnight, followed by one hour incubation with secondary antibody FITC-conjugated goat anti-rabbit antibody (1:200; Abcam) at RT. After being rinsed three times in PBS, slips were stained with DAPI (1:100; Solarbio). Fluorescence images were obtained using optical microscopy at 200× magnification.

Statistical Analysis

The experimental data were expressed in the form of mean ± SEM and analyzed by GraphPad 7.0 software. The clinical score in different groups was determined by Mann-Whitney U test. MIC, plate count, RT-PCR, Western blot, ELISA, and IFS were analyzed by an unpaired, two-tailed Student's *t*-test (GraphPad Prism; GraphPad, San Diego, CA, USA). One-way ANOVA with post hoc analysis was used for CCK-8 test. $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) was considered as significant (ns = no significance). All experiments were repeated at least three times to ensure accuracy.
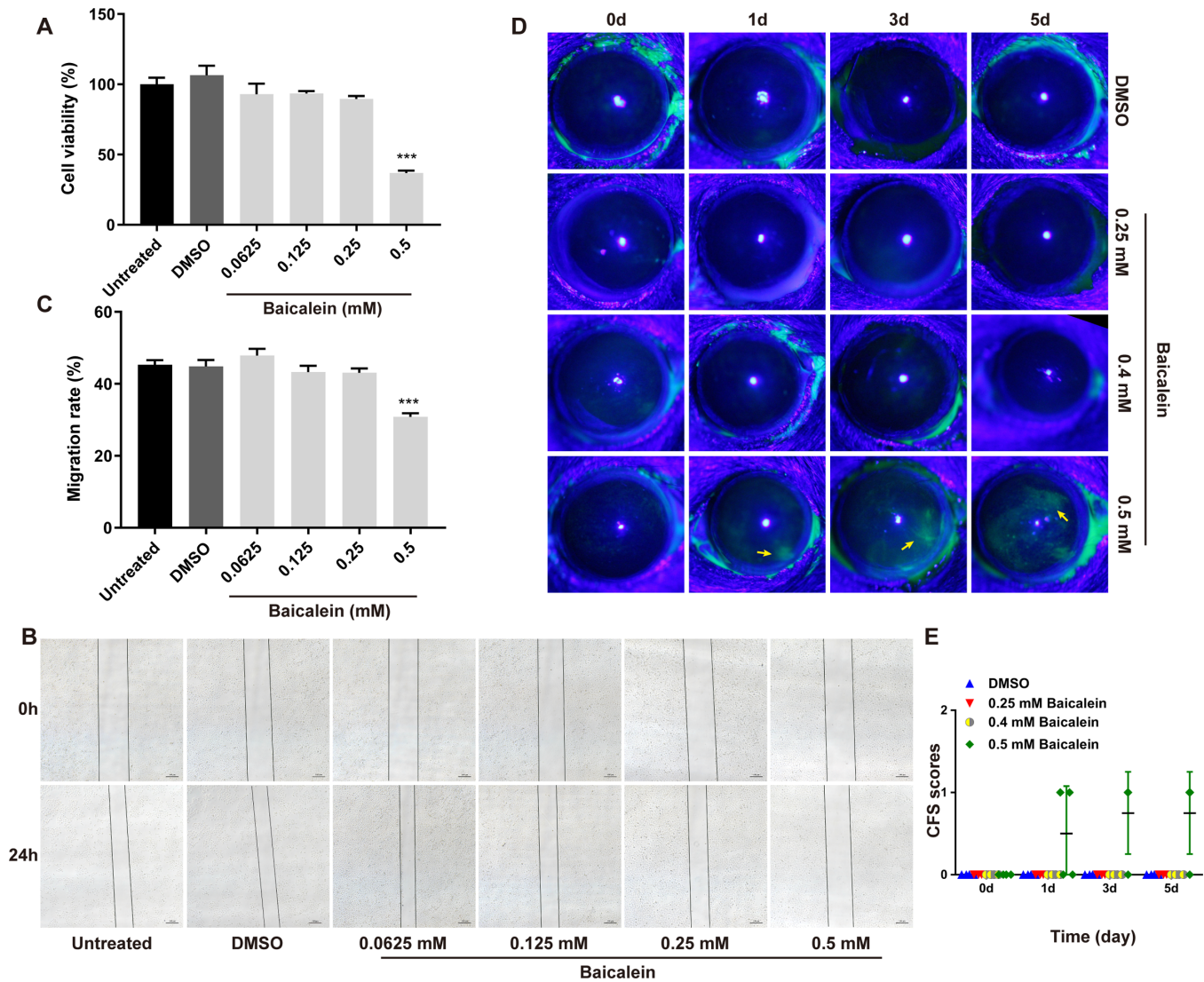
FIGURE 1. Toxicity evaluation of baicalein in vitro and in vivo. CCK-8 assay was performed on HCECs treated with DMSO or baicalein at 0.0625, 0.125, 0.25 and 0.5 mM for 24 hours **(A)**. Scratch assay **(B)** and quantitative analysis **(C)** showed baicalein ≤ 0.25 mM hardly affected HCECs migration at 24 hours (bar = 100 μm). Representative mouse corneas of Draize eye test **(D)** and CFS scores **(E)** before and after treatment with baicalein at 0.25, 0.4, 0.5 mM or DMSO for one, three, and five days. Ocular lesions were marked by sodium fluorescein staining (yellow arrows). All data were mean ± SEM. CCK-8 test was analyzed using One-way ANOVA with post hoc analysis, and all other data were analyzed by an unpaired, two-tailed Student's *t*-test (***P < 0.001).

RESULTS

Toxicity Evaluation of Baicalein in Vitro and In Vivo

To determine a nontoxic baicalein concentration, HCECs and mouse eyes were treated with different concentrations of baicalein for 24 hours and five days, respectively. CCK-8 test revealed that the cell viability of HCECs was not affected by baicalein when its concentration was less than or equal to 0.25 mM (Fig. 1A), and cell scratch assay showed that 0.25 mM baicalein did not affect HCECs migration within 24 hours (Figs. 1B, C). To estimate the toxicity of baicalein in vivo, Draize eye test was used to assess the ocular lesions marked by sodium fluorescein staining. Mouse eyes exposed to DMSO or 0.25 and 0.4 mM baicalein for one, three, and five days showed no sodium fluorescein staining (Fig. 1D),

and CFS scores were 0 (Fig. 1E). However, 0.5 mM baicalein treated mice displayed slight sodium fluorescein staining on the cornea at days one, three, and five (Fig. 1D) with higher CFS score (Fig. 1E), suggesting 0.5 mM baicalein could be toxic to mouse cornea, which is consistent with the adverse effects of 0.5 mM baicalein on HCECs (Figs. 1A–C). Thus, baicalein at 0.25 mM was considered as a nontoxic concentration and applied for the following in vitro and in vivo experiments.

Baicalein Inhibits the Growth, Biofilm Formation, and Adhesion Ability of *A. fumigatus*

MIC showed that baicalein at 0.0625 mM started to inhibit the growth of *A. fumigatus* and prevented 90% *A. fumigatus* growth (MIC$_{90}$) at 0.25 mM (Fig. 2A) at 48 hours. To
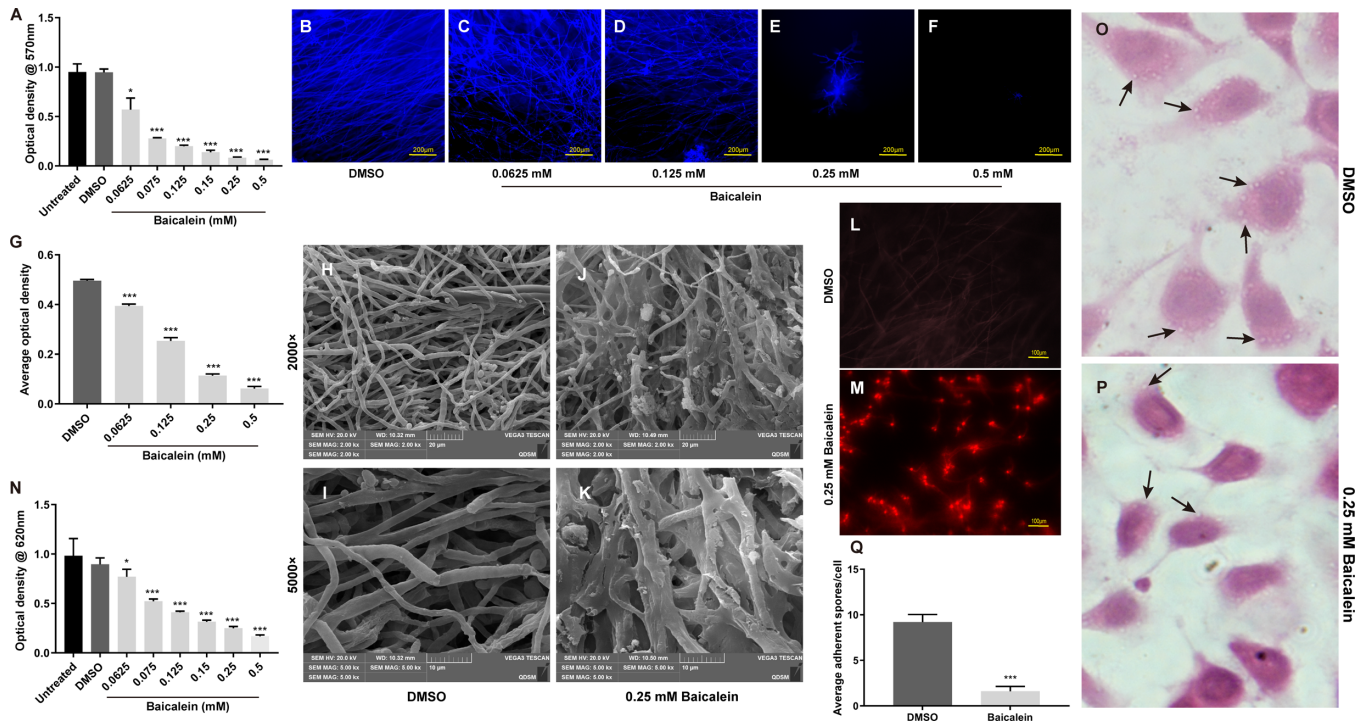
FIGURE 2. Baicalein inhibited the growth, biofilm formation, and adhesion ability of *A. fumigatus*. For *A. fumigatus*, the minimum effective concentration (MEC) of baicalein was 0.0625 mM. MIC_{90} was 0.25 mM, and the minimum fungicidal concentration (MFC) was 0.5 mM in *A. fumigatus* at 48 hours **(A)**. Fluorescence images of *A. fumigatus* hyphae (blue) treated with DMSO **(B)** or baicalein at 0.0625 **(C)**, 0.125 **(D)**, 0.25 **(E)** and 0.5 mM **(F)** for 48 hours ($n = 6$/group/isolate, bar = 200 µm) and quantitative analysis **(G)**. SEM images **(H–K)** of *A. fumigatus* hyphae exposed to DMSO **(H**, bar = 20 µm; **I**, bar = 10 µm) or 0.25 mM baicalein **(J**, bar = 20 µm; **K**, bar = 10 µm) for 24 hours at 37°C. PI staining of *A. fumigatus* hyphae treated with DMSO **(L)** or 0.25 mM baicalein **(M)** for assessment of hyphae membrane integrity (bar = 100 µm). Biofilm formation activity of *A. fumigatus* was inhibited at 0.0625 to 0.5 mM baicalein for 48 hours in a concentration-dependent manner when compared with no baicalein treatment **(N)**. HE staining images of HCECs treated with DMSO **(O)** or 0.25 mM baicalein **(P)** (magnification = × 40 µm) for three hours and quantitative analysis of spores on the cell surface **(Q)**. All data are mean ± SEM and were analyzed by an unpaired, two-tailed Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

detect how baicalein exerts its antifungal activity, mycelium growth, biofilm formation, and adhesion ability were tested at different concentrations of baicalein. Fluorescence staining showed that baicalein significantly inhibited hyphae growth in a concentration-dependent manner (Figs. 2B–G). Compared with the DMSO-treated tubular hyphae with smooth surfaces in control group (Figs. 2H, I), baicalein (0.25 mM) treated hyphae were shrunken and knotted, with evident efflux of a cotton-like substance (Figs. 2J, K). Additionally, the membrane integrity was examined by PI staining, in which nucleic acids exposed from damaged or compromised membranes were stained by red-fluorescent counterstain. An enhanced fluorescence intensity was observed in 0.25 mM baicalein-treated hyphae (Fig. 2M) in comparison to the DMSO-treated group (Fig. 2L), indicating baicalein may disrupt the membrane integrity of hyphae. Moreover, baicalein at 0.25mM significantly inhibited the biofilm formation of *A. fumigatus* at 48 hours (Fig. 2N). Compared with the DMSO group, HE staining showed a remarkable decreased number of conidia adhering to the surface of baicalein-treated (0.25 mM) HCECs, suggesting baicalein can inhibit the adhesion ability of *A. fumigatus* to HCECs (Figs. 2O–Q). These results demonstrated that baicalein may inhibit *A. fumigatus* growth through suppressing mycelium growth, biofilm formation, and adhesion abilities.

Baicalein Mitigates FK Severity, and Alleviates Fungal Load and Neutrophil Infiltration and Activity in Mice With *A. fumigatus* Keratitis

To evaluate the therapeutic effect of baicalein, 0.25 mM baicalein or DMSO were applied to an in vivo mouse model of *A. fumigatus* keratitis, and corneas were photographed under a slit lamp at one, three, and five days p.i. Compared with DMSO, baicalein treatment markedly reduced the ulcer area and corneal opacity, making the cornea more transparent (Fig. 3A). Consistently, clinical scores in baicalein-treated groups were much lower than DMSO-treated groups (Fig. 3B), which suggested baicalein treatment can improve the prognosis of mice with FK. In addition, plate count results showed that baicalein treatment significantly reduced the fungal load in *A. fumigatus* infected corneas in comparison to DMSO at five days p.i. (Fig. 3C). We then examined whether baicalein treatment could affect neutrophil infiltration and activity using immunofluorescence and MPO assay. Compared with DMSO, baicalein treatment not only reduced the number of infiltrated neutrophils in the infected corneal stroma (Figs. 3D, E), but also inhibited MPO activity level in corneas at three days p.i. (Fig. 3F). Thus, baicalein may mitigate the severity of FK in mice with *A. fumigatus* keratitis through decreasing fungal load and inhibiting neutrophil infiltration and activity.
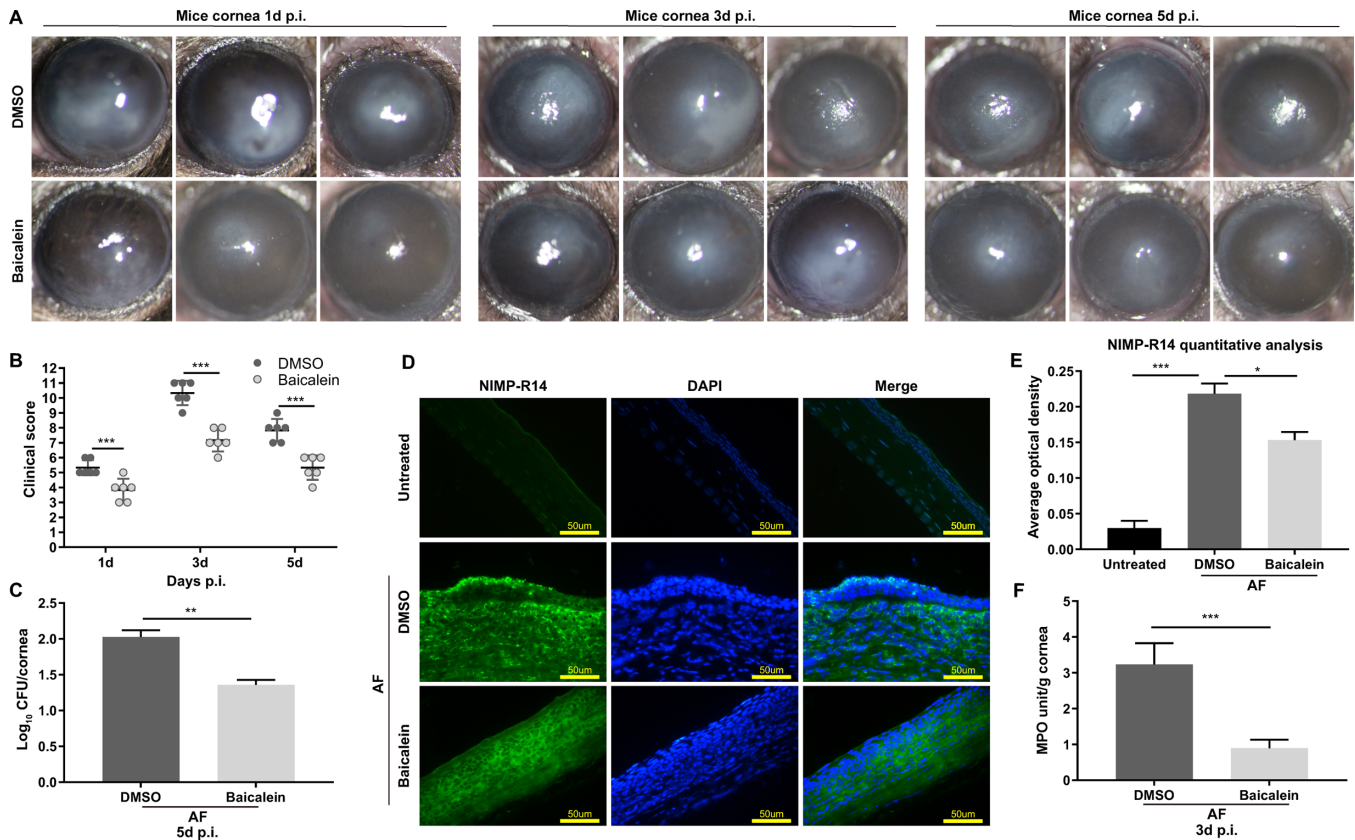
FIGURE 3. Effects of baicalein on *A. fumigatus*–infected mouse corneas. Photographs of mouse corneas in 0.25 mM baicalein-treated group and DMSO-treated group at one, three, and five days p.i. **(A)**. Compared with the DMSO, baicalein treatment significantly reduced clinical scores at one, three, and five days p.i. **(B)**. Quantitative analysis of fungal load in baicalein- or DMSO-treated mice cornea at five days p.i. **(C)**. NIMP-R14 labeled neutrophils (green) in baicalein-treated group and DMSO-treated group at three days p.i. (bar = 50 μm) **(D)** and quantitative analysis **(E)**. Green: NIMP-R14-FITC staining; blue: nuclear staining (DAPI); merge: neutrophil localization; magnification: 400×. MPO activity in baicalein-treated group was much lower than DMSO group at three days p.i. **(F)**. All data are mean ± SEM, in which clinical scores were analyzed by Mann-Whitney U test, and other data were analyzed by an unpaired, two-tailed Student's *t*-test ($n = 6$/group/isolate, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Baicalein Inhibits the Expression of Proinflammatory Cytokines Induced by *A. fumigatus* In Vivo and In Vitro

We then sought to detect whether baicalein could modulate the expression of proinflammatory mediators in *A. fumigatus* infected mouse corneas and HCECs. The mRNA and protein expressions of IL-1β, IL-6, and TNF-α were examined using RT-PCR and ELISA. In mice with FK, baicalein significantly reduced mRNA levels of IL-1β (Fig. 4A), IL-6 (Fig. 4C), and TNF-α (Fig. 4E) when compared with DMSO at one, three, and five days p.i. Consistently, treatment with baicalein versus DMSO showed decreased protein levels of IL-6 (Fig. 4D) and TNF-α (Fig. 4F) at three and five days p.i., but not for IL-1β (Fig. 4B),which was only significant at three days p.i. Similar results were obtained from *A. fumigatus* infected HCECs, in which baicalein remarkably inhibited mRNA levels of IL-1β (Fig. 4G), IL-6 (Fig. 4I), and TNF-α (Fig. 4K) at four hours after infection, and suppressed protein expressions of IL-1β (Fig. 4H), IL-6 (Fig. 4J), and TNF-α (Fig. 4L) within 24 hours following infection when compared with DMSO. Therefore, baicalein inhibited *A. fumigatus*–induced expression of proinflammatory cytokines including IL-1β, IL-6, and TNF-α in mouse corneas and HCECs, suggesting baicalein has anti-inflammatory activity in vivo and in vitro.

Baicalein Inhibits the Expression of Thymic Stromal Lymphopoietin (TSLP) and TSLP Receptor (TSLPR) In Vivo and In Vitro

As baicalein directly interacts with TSLP, we next explored whether TSLP is involved in the anti-inflammatory mechanism of baicalein in corneas of mice with *A. fumigatus* keratitis (Fig. 5) and HCECs (Fig. 6). In FK mouse models, baicalein significantly reduced *A. fumigatus*–induced elevated mRNA levels of TSLP (Fig. 5A) and TSLPR (Fig. 5B) at one, three, and five days p.i., and suppressed protein levels of TSLP (Figs. 5C, D) and TSLPR (Figs. 5E, F) at three and five days p.i. when compared with DMSO. Immunofluorescent assays revealed that TSLP was mainly located in corneal epithelium and stroma (Fig. 5G), and the secretion of TSLP was suppressed in baicalein-treated mice cornea versus The DMSO group at five days p.i. (Figs. 5G, H). These results suggested baicalein not only inhibited *A. fumigatus*–induced expression of TSLP and TSLPR in mouse
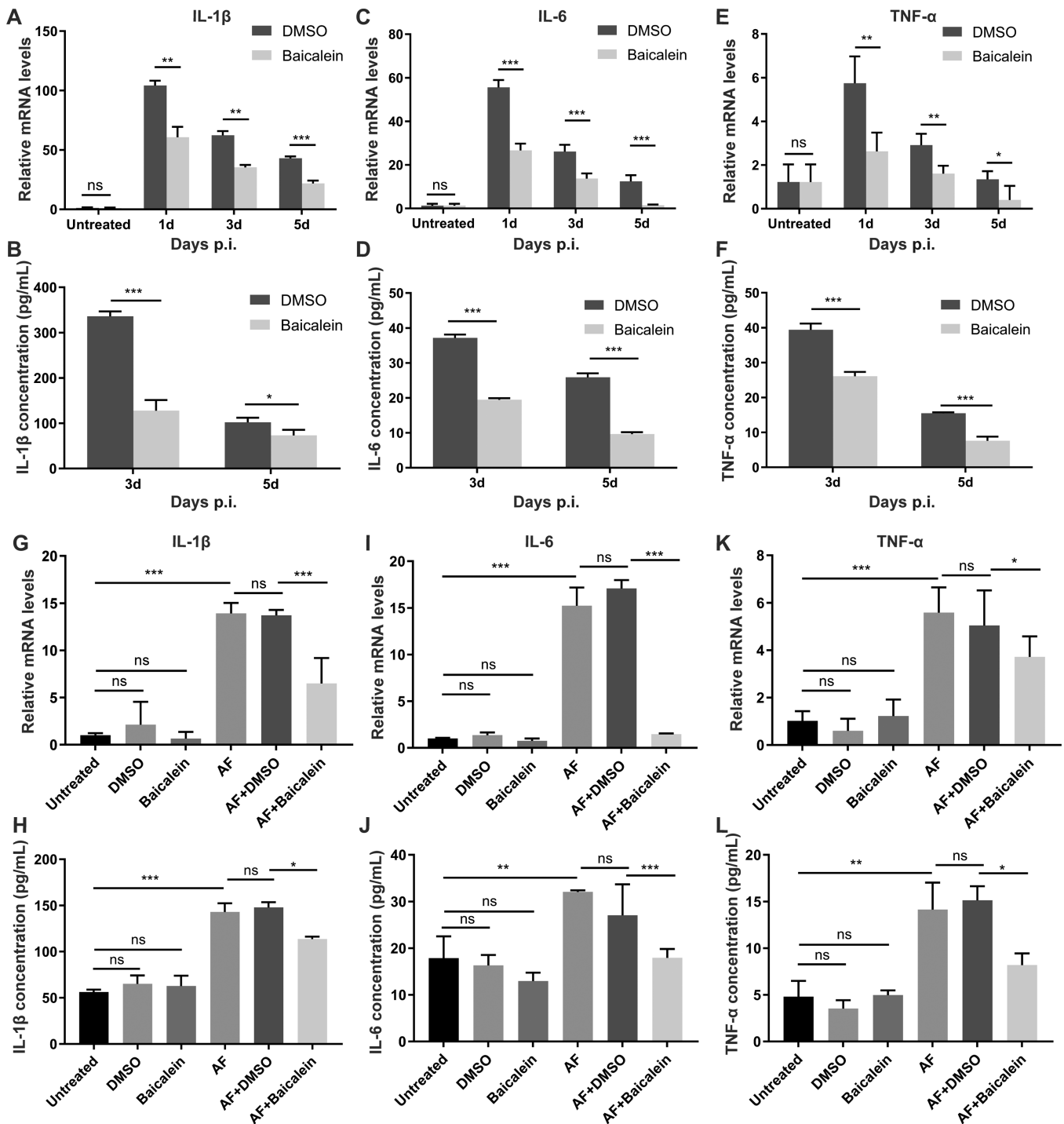
FIGURE 4. Baicalein inhibits the expression of proinflammatory mediators induced by *A. fumigatus* in vivo and in vitro. In mouse model of keratitis, RT-PCR showed the mRNA levels of IL-1β **(A)**, IL-6 **(C)**, and TNF-α **(E)** were reduced significantly in baicalein (concentration = 0.25 mM) vs. DMSO-treated corneas at one, three, and five days p.i. ELISA analysis for protein levels of IL-1β **(B)**, IL-6 **(D)**, and TNF-α **(F)** at three and five days p.i. in baicalein- versus DMSO-treated mice ($n = 6$/group). In HCECs, RT-PCR showed the mRNA levels of IL-1β **(G)**, IL-6 **(I)** and TNF-α **(K)** were reduced significantly in baicalein (concentration = 0.25 mM) vs. DMSO-treated cells at four hours after infection. ELISA analysis for protein levels of IL-1β **(H)**, IL- 6 **(J)**, and TNF-α **(L)** at 24 hours following infection in baicalein- versus DMSO-treated HCECs. All data are mean ± SEM and were analyzed using an unpaired, two-tailed Student's *t*-test (ns, no significance; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

corneas, but also alleviated TSLP secretion to corneal epithelium and stroma.

In addition, TSLP mRNA and protein expression levels were also tested in *A. fumigatus* infected HCECs (Fig. 6). Compared with DMSO, baicalein dramatically restrained the elevated TSLP mRNA (Fig. 6A) and protein levels (Figs. 6B, C) after infection, which is consistent with immunofluorescence staining that baicalein attenuated the
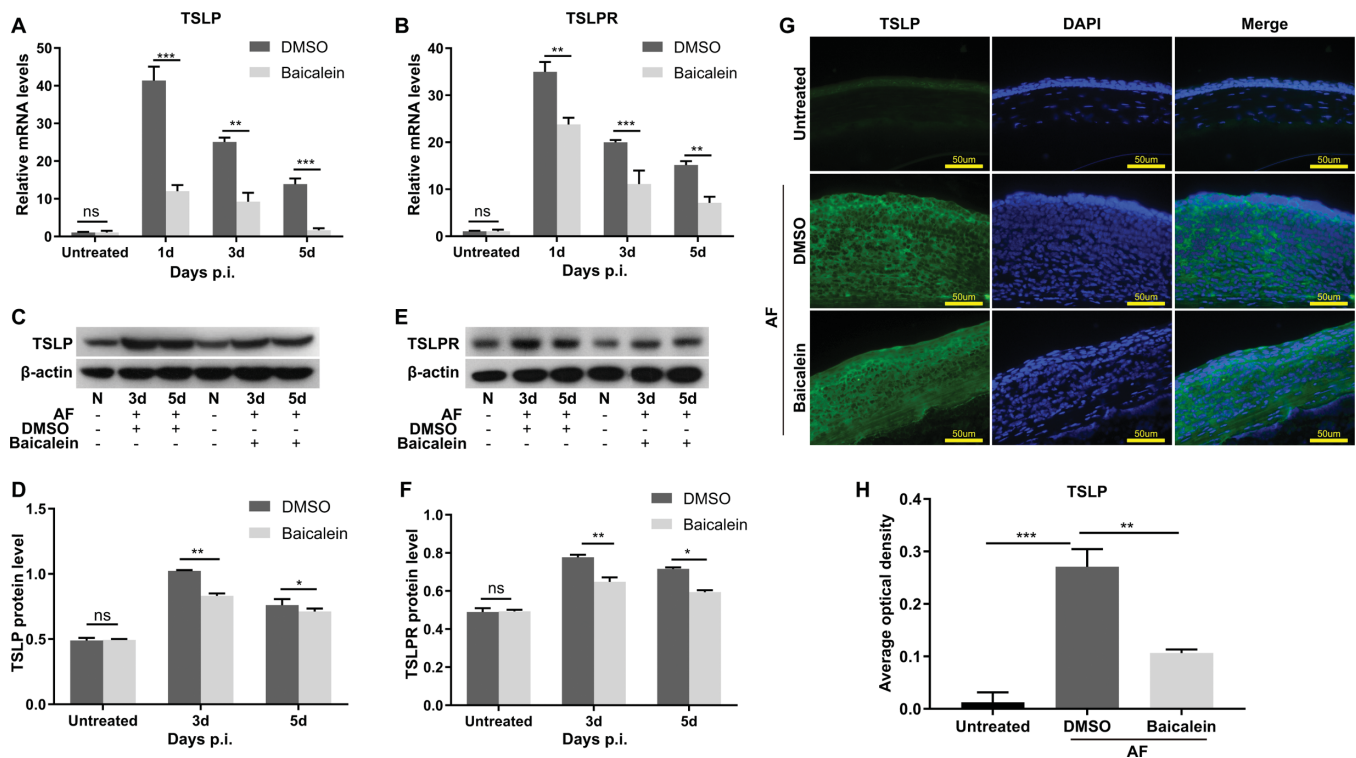
FIGURE 5. Baicalein inhibited the expression levels of TSLP and TSLPR in mouse models with *A. fumigatus* keratitis. RT-PCR showed mRNA expressions of TSLP **(A)** and TSLPR **(B)** in *A. fumigatus*–infected mouse corneas treated with 0.25 mM baicalein or DMSO at one, three, and five days p.i. Western blotting results demonstrated protein expression levels of TSLP **(C)** and TSLPR **(E)** in baicalein (0.25 mM) or DMSO-treated FK mouse models at three and five days p.i. and quantitative analysis **(D, F)**. Immunofluorescence images of TSLP (green) expression in corneal epithelium and stroma of FK mice treated with 0.25 mM baicalein or DMSO at three days p.i. Green: TSLP (FITC), blue: nucleus (DAPI), magnification $400\times$ (bar = 50 μm) **(G)** and quantitative analysis **(H)** ($n = 6$/group/isolate). All data were mean ± SEM and were analyzed by an unpaired, two-tailed Student's t-test (ns, no significance; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

overexpression of TSLP induced by *A. fumigatus* in HCECs (Figs. 6D, E). These data indicated that baicalein inhibited *A. fumigatus*–induced expression of TSLP in HCECs.

Baicalein Suppresses *A. fumigatus*–Induced Inflammatory Response by Inhibiting TSLP Signaling

HCECs were pretreated with TSLP siRNA or rTSLP followed by *A. fumigatus* stimulation, and then treated with baicalein or DMSO. RT-PCR and Western blot results showed, respectively, that TSLP mRNA and protein were markedly downregulated by TSLP siRNA (Figs. 7A–C) and were upregulated by rTSLP that partially counteracted the inhibitory effect of baicalein on the expression of TSLP (Figs. 7A, D, E). To further understand how TSLP is involved in FK-related inflammation, we examined the expression of proinflammatory mediators in response to TSLP knockdown and overexpression (Figs. 7F–K). The mRNA and protein levels of IL-1β (Figs. 7F, G), IL-6 (Figs. 7H, I), and TNF-α (Figs. 7J, K) were significantly lower in TSLP siRNA–treated group, while higher in rTSLP-treated group than in the corresponding control, indicating involvement of TSLP in *A. fumigatus*–induced inflammatory response. Baicalein treatment not only contributed to inhibit the expression of IL-1β (Figs. 7F, G), IL-6 (Figs. 7H, I), and TNF-α (Figs. 7J, K) in TSLP siRNA–treated groups, but also inhibited rTSLP-induced overexpression of these inflammatory mediators. These results

suggested that baicalein suppresses *A. fumigatus*–induced inflammatory response by inhibiting TSLP signaling.

DISCUSSION

Baicalein is a plant-derived flavonoid with various pharmacological properties including antifungal, anti-inflammatory, and antitumor effects.[15,47,48] However, it is still unclear whether baicalein has a therapeutic effect on FK. In this study, we demonstrated that baicalein improves *A. fumigatus* keratitis prognosis by inhibiting fungal load and suppressing inflammatory response via downregulation of the TSLP/TSLPR signaling pathway.

Baicalein has been reported to have potent antifungal activity against several fungal pathogens in vitro including *Candida albicans (C. albicans)*, *A. fumigatus*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*.[9,49] For example, baicalein effectively inhibited the total growth of *C. albicans* via suppressing biofilm formation and metabolic activity,[16] and restrained *A. fumigatus* growth by inducing deformation of the fungal surface structure and substance efflux in vitro.[9] Consistently, we showed baicalein not only inhibited *A. fumigatus* mycelium growth and biofilm formation, but also disrupted hyphae structure and membrane integrity, and the MIC$_{90}$ was 0.25 mM at 48 hours. Nevertheless, there is a lack of evidence to show the antifungal effect of baicalein in mammalian cells or animal models. In this study, we demonstrated that 0.25 mM baicalein inhib-
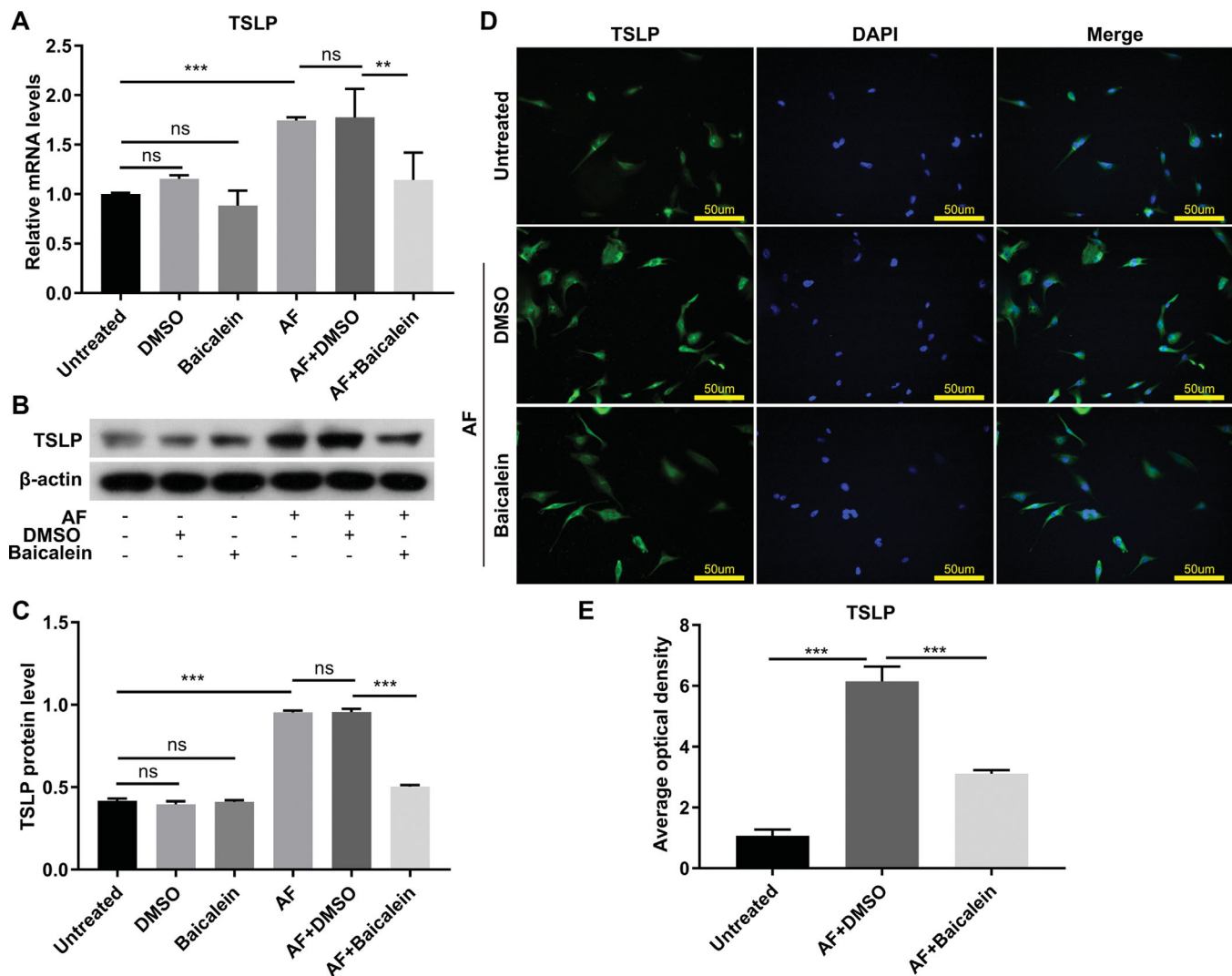
FIGURE 6. Baicalein inhibited *A. fumigatus*–induced expression of TSLP in HCECs. RT-PCR demonstrated mRNA expression level of TSLP in HCECs treated with 0.25 mM baicalein or DMSO at four hours after *A. fumigatus* infection **(A)**. Western blotting showed TSLP protein expression level in baicalein (0.25 mM) or DMSO-treated HCECs at 24 hours after infection **(B)** and quantitative analysis **(C)**. Immunofluorescence images of TSLP (green) expression in *A. fumigatus*–infected HCECs with or without 0.25 mM baicalein treatment at eight hours after infection **(D)** and quantitative analysis **(E)**. Green: TSLP (FITC), blue: nucleus (DAPI), magnification 200×. All data were mean ± SEM and were analyzed by an unpaired, two-tailed Student's *t*-test (ns, no significance; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

ited adhesion ability of *A. fumigatus* to HCECs and reduced fungal load in *A. fumigatus* keratitis mouse models, which further confirmed the antifungal activity of baicalein in vivo. According to cell viability, migration ability, and Draize eye test results, baicalein ≤ 0.25 mM was nontoxic to corneal epithelial cells and mice corneas. Thus, baicalein at 0.25 mM was considered as the nontoxic effective antifungal concentration and applied to the following in vitro and in vivo experiments.

On the other hand, we revealed baicalein inhibited neutrophil infiltration and MPO activity in the infected corneal epithelium and stroma, indicating baicalein could attenuate *A. fumigatus* keratitis–induced inflammatory response. Neutrophil infiltration is known to be essential in innate response against fungal infection,[50] promoting the release of various cytokines and chemokines to further attract immune cells to eliminate pathogens. However, the large number of proinflammatory factors and toxic substances released by neutrophils may result in continuous inflammation that causes cornea damage, even vision loss.[51] Thus, limiting the excessive inflammatory response is of significance for FK treatment. In the present study, we showed that baicalein treatment markedly mitigated the severity of FK in mice by reducing the ulcer area and corneal opacity, making corneas more transparent, which suggested baicalein may improve the prognosis of FK not only through inhibiting fungal load, but also via suppressing inflammatory response. Baicalein has been demonstrated to possess an anti-inflammatory effect in diverse diseases via various mechanisms.[52–54] For instance, baicalein inhibited LPS-enhanced neutrophil infiltration and MPO activity to protect rats from acute lung injury.[53] Moreover, baicalein alleviated acute pancreatitis–caused pathological injury through dampening the NF-κB, MAPK, and STAT3 signaling pathways.[55] Chen et al. reported that baicalein treatment improved histological and functional outcomes of traumatic brain
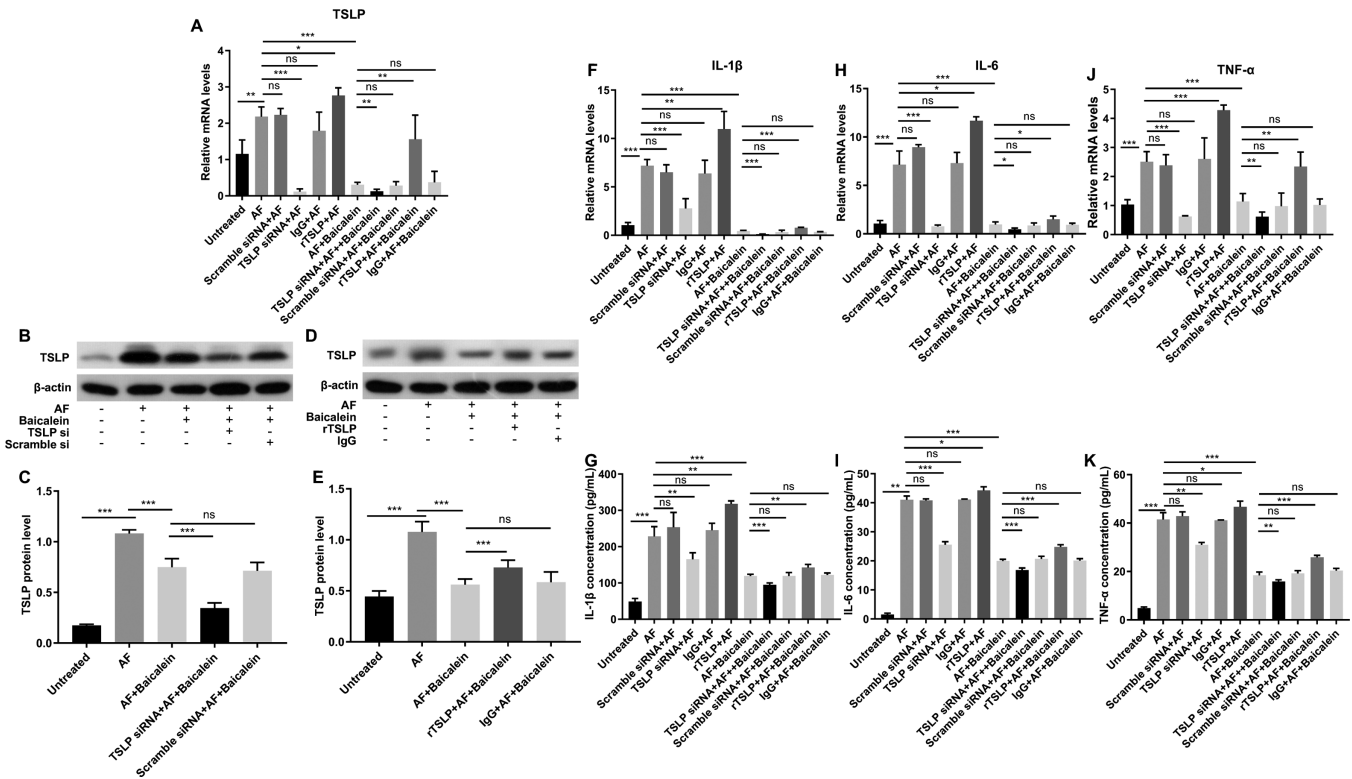
FIGURE 7. Baicalein suppresses *A. fumigatus*–induced inflammatory response by inhibiting TSLP signaling. HCECs pretreated with TSLP siRNA or rTSLP were stimulated by *A. fumigatus* for one hour, then incubated with DMSO or 0.25 mM baicalein for three hours or twenty three hours. RT-PCR showed TSLP mRNA expression level in HCECs with or without 0.25 mM baicalein treatment **(A)**. Western blot results of TSLP expression in TSLP siRNA **(B)** and rTSLP **(D)** treated HCECs and quantitative analysis **(C, E)**. Quantification of mRNA levels of L-1β **(F)**, IL-6 **(H)**, and TNF-α **(J)** in TSLP siRNA, rTSLP, or non-pretreated HCECs with or without 0.25 mM baicalein treatment. ELISA demonstrated protein expression levels of L-1β **(G)**, IL-6 **(I)**, and TNF-α **(K)** in TSLP siRNA, rTSLP, or non-pretreated HCECs with or without 0.25 mM baicalein treatment (N, no treatment; AF, *A. fumigatus* stimulation). All data were mean ± SEM and were analyzed by an unpaired, two-tailed Student's *t*-test (ns, no significance; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

injury by reducing the induction of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in rats.[56] Baicalein also inhibited vascular abnormality and neuron loss in diabetic retinas through ameliorating the expression of inflammatory factors including TNF-α, IL-1β, and IL-18.[54] In this study, we showed baicalein suppressed the mRNA and protein expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α in *A. fumigatus*–infected mouse corneas and HCECs, which further elucidated the anti-inflammatory activity of baicalein in FK.

Recently, baicalein was identified as a novel thymic stromal lymphopoietin (TSLP) inhibitor that blocks the interaction between TSLP and TSLPR, thus repressing the TSLP signaling pathway.[21] TSLP is an IL-7–like proallergic cytokine that plays a central role in dendritic cell (DC) activation, inducing Th2 immune responses and initiating allergic and inflammatory diseases.[57,58] Hiroki et al . showed that TSLP stimulated both DCs and innate lymphoid cells, facilitating the development of Th2 cell-mediated airway inflammation.[25] TSLP also promotes the expression of proallergic cytokines IL-4 and TNF-α, while downregulating IL-10 and interferon-gamma, mediating pathological inflammation.[26] Recent studies suggest TSLP may serve as a new target for FK treatment. TSLP knockdown reduced DC aggregation, maturation, and migration, and repressed Th2-attracting chemokines and cytokines to attenuate the inflammatory response in *A. fumigatus*–infected corneas, and alleviating the severity of FK.[28] In contrast, pretreatment of exoge-

nous TSLP with *A. fumigatus* resulted in severe keratitis with increased neutrophil infiltration and higher levels of Toll-like receptor (TLR)-2, TLR-4, IL-6, and IL-8.[29] TLRs are located on the cell surface, recognizing pathogen-associated molecular patterns (PAMPs) to initiate antimicrobial immune responses.[59] Our previous studies have shown *A. fumigatus* infection induced an elevated expression of TLRs,[37, 60] and TLR-dependent induction of TSLP has been confirmed in HCECs in response to microbial components,[29,58] which provides evidence for the TLRs mediated cross-talk between FK and TSLP.

Park et al. reported that the inhibition of TSLP signaling by baicalein could reduce eosinophilic airway inflammation, alleviating the severity of allergic reaction.[21] However, the effects of baicalein on TSLP/TSLPR signaling in FK has not been investigated yet. Our study demonstrated that baicalein not only inhibited *A. fumigatus*–induced overexpression of TSLP and TSLPR in mouse corneas, but also alleviated TSLP secretion to corneal epithelium and stroma. Similar results were obtained in corneal epithelial cells, in which baicalein inhibited the upregulated expression of TSLP in *A. fumigatus*–infected HCECs. These results indicate baicalein may exert its anti-inflammatory activity through inhibiting the TSLP/TSLPR signaling pathway. To further understand the regulatory role of baicalein on the TSLP/TSLPR pathway, HCECs were pretreated with TSLP siRNA or rTSLP followed by *A. fumigatus* infection. The rTSLP-induced upregulated TSLP partially counteracted the

inhibitory effect of baicalein on the expression of TSLP. Additionally, the mRNA and protein levels of proinflammatory factors IL-1β, IL-6, and TNF-α were significantly lower in the TSLP siRNA–treated group, while higher in the rTSLP-treated group than in the corresponding control, indicating the involvement of TSLP in *A. fumigatus*–induced inflammatory response. Notably, baicalein treatment significantly inhibited rTSLP-induced overexpression of IL-1β, IL-6, and TNF-α. These results suggested that baicalein suppresses *A. fumigatus*–induced inflammatory response by inhibiting TSLP/TSLPR signaling.

In summary, our study showed that baicalein plays a protective role in mouse *A. fumigatus* keratitis by inhibiting fungal growth, biofilm formation, and adhesion, and suppressing inflammatory response via downregulation of TSLP/TSLPR signaling pathway. Therefore, baicalein is expected to be a promising therapeutic drug or combined with other antifungal drugs in FK treatment.

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