European Review for Medical and Pharmacological Sciences 2018; 22: 8063-8075

NLRP12 promotes host resistance against *Pseudomonas aeruginosa* keratitis inflammatory responses through the negative regulation of NF-κB signaling

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Abstract. – OBJECTIVE: To investigate the role of NLRP12 in regulating Pseudomonas aeruginosa *(P. aeruginosa)* keratitis.

MATERIALS AND METHODS: Real-Time-PCR and Western blot were performed to measure the NLRP12 level in corneas and bone marrow-derived macrophages (BMDMs) of C57BL/6 (B6) mice. B6 mice received a subconjunctival injection of lentivirus expressing active NLRP12 (NLRP12-lentivirus) or Ctl-lentivirus (as control), followed by infection of *P. aeruginosa*. The clinical score, slit lamp and bacterial plate count of mice were evaluated. In addition, myeloperoxidase (MPO) was detected to assess the infiltration of polymorphonuclear neutrophil (PMN). Cytokine levels were measured by Real Time-PCR and ELISA. Meanwhile, the bacterial burden was also evaluated. The activation of NF-κB signaling was determined by pIκBα/IκBα levels based on Western blot and NF-κB-dependent Luciferase activity on the basis of Luciferase assays using 293T cells.

RESULTS: NLRP12 mRNA and protein levels were decreased in B6 corneas and BMDMs after *P. aeruginosa* infection. The over-expression of NLRP12 in B6 corneas significantly ameliorated the severity of corneal disease, bacterial burden, PMN infiltration and pro-inflammatory cytokine expression. *In vitro* analysis demonstrated that the up-regulation of NLRP12 suppressed pro-inflammatory cytokine production and enhanced bacterial clearance in RAW264.7 cells. The protein levels of pIκBα and IκBα were significantly decreased after NLRP12-lentivirus treatment compared with that of Ctl-lentivirus. NF-κB-dependent Luciferase activity was potently inhibited by NLRP12 infected with *P. aeruginosa* or cotransfected with the downstream signaling molecules including IKKα and IKKβ in 293T cells.

CONCLUSIONS: NLRP12 decreases the severity of *P. aeruginosa* keratitis, reduces corneal inflammation and bacterial burden through the down-regulation of the NF-κB signaling pathway.

Key Words:
NLRP12, Pseudomonas aeruginosa, Keratitis, Regulatory mechanism.

Introduction

As a common type of Gram-negative bacteria, Pseudomonas aeruginosa *(P. aeruginosa)* is associated with microbial keratitis, especially in individuals with soft contact lenses[1]. *P. aeruginosa* keratitis represents a progressive corneal disease which might lead to inflammatory epithelial edema, stromal infiltration, corneal ulceration, tissue destruction, as well as blindness[2]. The pathogenesis of *P. aeruginosa* keratitis is complicated and involves interactions between host and invading pathogens. Beyond bacterial virulence factors, host inflammation-mediated immunopathologic damage is reported to be another major contributing factor to the pathogenesis of *P. aeruginosa* keratitis[3]. The host inflammatory response is a self-protective response to bacterial invasion, and is characterized by an accumulation of inflammatory cells and secretion of cytokines. In the process of infection, inflammatory cells such as polymorphonuclear neutrophils (PMNs) and monocytes/macrophages are activated and recruited to the infected cornea to eliminate invading pathogens[4]. Once activated, inflammatory cells also secret va-

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rious pro-inflammatory cytokines such as interleukin 6 (IL-6), interleukin 1 beta (IL-1β), tumor necrosis factor α (TNF-α) or macrophage inflammatory protein 2 (MIP-2)[5]. These inflammatory cytokines promote bacteria clearance. Nonetheless, the aberrant expressions lead to tissue damage and corneal perforation[2]. Therefore, the regulation of both bacterial burden and ocular inflammation is important for the treatment of *P. aeruginosa* keratitis. Nod-like receptors (NLRs) represent a large family of cytoplasmic PRRs which are characterized by a conserved nucleotide-binding and oligomerization domain (NOD) and a leucine-rich repeat (LRR) region[6]. The function includes pathogen/damage sensing, modulation of inflammatory signaling transduction[7-9]. NLR proteins, such as NLRP1, NLRP3, and NLRC4, activate caspase-1 through the assembly of a complex which is termed as inflammasome[10]. These inflammasome-forming NLRs mediate the processing and maturation of the proinflammatory cytokines pro-IL-1β and pro-IL-18 into IL-1β and IL-18. On the other hand, non-inflammasome-forming members regulate other key inflammatory pathways; for example, NOD1 and NOD2 activate NF-κB and MAPK pathways[11-14], NLRP6, NLRC3, NLRC5 and NLRX1 have been reported involved in the regulation of inflammation[15-18]. NLRP12 (known as RNO, PYPAF7, and Monarch-1), a member of the family consisting of an N-terminal PYD, an NBD, and C-terminal LRR regions, is one of the first described NLR proteins and its function remains controversial. Because of the typical tripartite domain structure similar to NLRP3, NLRP12 was considered to have functions in the inflammasome assembly. Recent findings indicate the involvement of NLRP12 in colon cancer[19,20]. A previous study showed that NLRP12-deficient mice were more susceptible to Yersinia pestis infection, with reduction of IL-1β and IL-18 levels in serum and growing bacterial load in spleen[21]. NLRP12 is also reported to suppress colon inflammation and tumorigenesis through the negative regulation of NF-κB signaling[19,20,22]. However, the potential role of NLRP12 in microbial keratitis remains poorly understood. NF-κB family is involved in the regulation of inflammatory and immune responses. It locates in the cytoplasm as an inactive form by the inhibitor IκB. Proteasome-mediated degradation of IkB causes its phosphorylation by IκB kinase (IKK) complex, IKKα-IKKβ-IKKγ (NEMO). The subsequent polyubiquitination and degradation of IκB lead to the release and nuclear translocation of NF-κB, which stimulates the transcription and expression of various inflammatory chemokines, cytokines and cell surface proteins. *In vitro* analysis of NLRP12 in human monocytic cell lines THP-1 suggests silencing of NLRP12 promoted NF-κB activation and the secretion of proinflammatory cytokines in response to TLR agonists, TNF-α and Mycobacterium tuberculosis[23,24]. In the present study, we aimed to detect the level of NLRP12 in mouse corneas after *P. aeruginosa* infection and determine its effect in diseases caused by *P. aeruginosa* keratitis.

Materials and Methods

Ocular Infection and Clinical Evaluation

Female C57BL/6 (B6) mice aged 8 weeks were purchased from the Animal Supply Center of Guangdong Medical University. The left cornea of B6 mice was infected by *P. aeruginosa* stain (ATCC 19660), as described previously[25-27]. Corneal disease was graded in scale as follows: 0, clear or slight opacity, partially or fully covering the pupil; +1, slight opacity, partially or fully covering the anterior segment; +2, dense opacity, partially or fully covering the pupil; +3, dense opacity, covering the entire anterior segment; and +4, corneal perforation or phthisis. The clinical score was recorded for each mouse after infection and photography with a slit lamp was used to illustrate the disease response. All procedures involving animals were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal Ethics Committee approval was granted by our institution.

Isolation of Murine Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages (BMDMs) were isolated as described previously[28]. Then, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 30% (vol/vol) L-929 conditioned medium which was used as a source of macrophage colony-stimulating factor. BMDMs were collected as a homogeneous population of adherent cells after culturing for 7 days. Cultured cells were stained with Alexa Fluor 488 conjugated anti-F4/80 (Invitrogen, Carlsbad, CA, USA) to measure the purity, which was more than 95%.

Cell Culture

Murine macrophage-like RAW264.7 cells (ATCC; Manassas, VA, USA) were cultured in DMEM media supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), and 1% L-glutamine (Invitrogen, Carlsbad, CA, USA) at the permissive temperature of 37°C. Human embryonic kidney (HEK) 293T cells (ATCC, Manassas, VA, USA) were cultured in DMEM media supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and maintained in 5% CO_2 at 37°C.

Recombinant Lentivirus Packaged and Titered

Lentivirus vector encoding NLRP12 was bought from Addgene (Seattle, WA, USA) and prepared as described previously[29]. In brief, cells were seeded into 10 cm dishes (4×10^6 cells per dish) 1 day before transfection and cultured overnight in DMEM containing 10% (v/v) FBS, followed by fresh media containing 25 mM chloroquine (Sigma-Aldrich, St. Louis, MO, USA) during transfection. For each dish, 10 mg lentivirus vector with 3 mg VSV-G envelope plasmid and 10 mg pCMVΔR8.2 packaging plasmid were adjusted to 450 ml in water followed by mixing with 50 ml 2.5M CaCl2 (Sigma-Aldrich, St Louis, MO, USA). Then, 500 ml of 2×HBS buffered-saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.05) was added for precipitation. At 12 h after transfection, cells were cultured with complete fresh media. After another 36 h, the conditioned medium was added and subsequent centrifugation was performed at 4000 g for 5 min. Then, lentivirus particles were concentrated through centrifugation at 50000 g for 140 min and the pellet was resuspended in 400 ml phosphate-buffered solution (PBS) with 0.1% bovine serum albumin (BSA). The viral titer was measured using the HIV p24 Antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix, Buffalo, NY, USA).

Subconjunctival Injection of Lentivirus

Lentivirus expressing NLRP12 (referred as NLRP12-lentivirus below) or control (referred as Ctl-lentivirus below) was administrated subconjunctivally into the left eye of mice (5 ml/mouse at a viral titer of 10^8, n=5/group/time) once a week three times before ocular infection.

Lentivirus Transduction

RAW264.7 cells (2×10^5/well) and 293 T cells (8×10^4/well) were seeded into a 12-well plate. To over-express NLRLP12, cells were infected with NLRP12-lentivirus or Ctl-lentivirus at MOI 10 for 6 h in the presence of 8 mg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). After that, the infected cells were cultured in complete culture media for another 24 h for detections of PCR, ELISA or killing assays.

Real Time-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and quantitated by a NanoDrop 2000C Spectrophotometers (Thermo Scientific, Waltham, MA, USA). Total RNA (1 mg) was reversely transcribed into cDNA, which was used for amplification using SYBR Green Master Mix (TaKaRa Bio, Otsu, Shiga, Japan) in accordance with manufacturer's instructions. Quantitative Real Time-PCR reactions were performed using the CFX96 Real Time-PCR System (Bio-Rad, Hercules, CA, USA). The relative mRNA levels were quantified via normalization with β-actin. Primer sequences were listed in Table I.

Western Blot

Whole corneas (n=5/group/time) were collected and pooled from normal uninfected and infected mice eyes at 1, 3, 5 days post-infection followed by being lysed and homogenized in a 1 ml glass tissue homogenizer with lysis buffer containing 1mM phenylmethylsulfonyl fluoride, 1% (v/v) protease inhibitor cocktail, and 1 mM DTT (Sigma-Aldrich, St. Louis, MO, USA). Cultured cells were washed three times using ice-cold PBS followed by treatment with lysis buffer. The protein concentration was determined by Quick Start Bradford protein assay (Bio-Rad, Hercules, CA, USA). 30 mg of each sample was loaded, separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI, USA) followed by blockage with Tris-Buffered Saline and Tween-20 (TBST) containing 5% non-fat dry milk. Then, NLRP12 polyclonal Ab (1:100, Abcam, Cambridge, MA, USA), κBα (44D4)/Phospho-IκBα (Ser32) (14D4) Rabbit mAb (1:1000, Cell Signaling Technology, Danvers, MA, USA) was added and incubated overnight at 4°C, respectively, followed by incubation with secondary IRDye 800CW Donkey anti-rabbit IgG (H+L) Ab (1:5000, LI-COR

Table I. Nucleotide sequence of the specific primers used in PCR amplification.

Gene	Primer Sequence (5'-3')	
β-actin	GAT TAC TGC TCT GGC TCC TAG C	F
	GAC TCA TCG TAC TCC TGC TTG C	R
NLRP12	AAG ACC GCA ATG CAC GAT TAG	F
	TGG AGC GTT CCC ACT CTA CA	R
IL-1β	CGC AGC AGC ACA TCA ACA AGA GC	F
	TGT CCT CAT CCT GGA AGG TCC ACG	R
MIP-2	TGT CAA TGC CTG AAG ACC CTG CC	F
	AAC TTT TTG ACC GCC CTT GAG AGT GG	R
IL-6	CAC AAG TCC GGA GAG GAG AC	F
	CAG AAT TGC CAT TGC ACA AC	R
TNF-α	CAC AGA AAG CAT GAT CCG CGA C	F
	TGC CAC AAG CAG GAA TGA GAA GAG	R

Biosciences, Lincoln, NE, USA) for 1 h. After that, blots were detected using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) according to the manufacturer's protocol. Semi-quantitative analysis of WB bands was performed using Image J software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Corneas and cell supernatants were collected and analyzed for secreted IL-1β, MIP-2, IL-6 and TNF-α using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Each sample was assayed in duplicate of three separate experiments. The reported sensitivity of these assays is <3.0 pg/mL for IL1β, <1.5 pg/mL for MIP-2, 1.3 to 1.8 pg/mL for IL-6, and <5.1 pg/mL for TNF-α.

Myeloperoxidase (MPO) Assay

Corneas were collected at 1, 3, 5 days post-infection (n=5/group/time) and homogenized in PBS followed by measuring the MPO concentration using Zen Myeloperoxidase ELISA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations.

Bacterial Plate Counts

Collected individual corneas were homogenized in sterilized water containing 0.85% (wt/vol) NaCl and 0.25% bovine serum albumin (BSA). Serial 10-fold dilutions of the samples were plated on Pseudomonas isolation agar (BD Biosciences, Franklin Lakes, NJ, USA) in triplicate followed by incubation at 37°C overnight. The results are reported as 10^6 colony-forming unite (CFU) per cornea ± SEM.

Bacterial Killing Assay

The intracellular bacterial killing was evaluated by plate count as described previously[30]. Briefly, RAW264.7 cells were treated with NLRP12- or Ctl-lentivirus for 24 h, and challenged with *P. aeruginosa* at an MOI of 25. After 1 hour, cells were treated with gentamicin (Sigma-Aldrich, St. Louis, MO, USA) at 300 g/mL for 30 minutes to eliminate the extracellular bacteria, followed by washing with PBS three times. Afterward, one of the duplicate wells was lysed in 0.1% Triton-X, and the other duplicate well was incubated at 37°C for 1 hour followed by lysis in 0.1% Triton-X. Serial 10-fold dilutions of each sample were plated on *Pseudomonas* isolation agar in triplicate and incubated at 37°C overnight. For intracellular bacterial killing, the efficiency was calculated as follows: intracellular bacterial killing=(CFU[1h]–CFU[2h]) /CFU (1h)×100%.

Luciferase Assays

HEK293 cells were transfected with NF-κB Luciferase plasmids and exogenous IKKα, IKKβ plasmids used as stimulators. Dual-Luciferase kits (Promega, Madison, WI, USA) were used for

subsequent analysis according to manufacturer's instructions.

Statistical Analysis

The difference in clinical score between NLRP12- or Ctl-lentivirus-treated mice corneas at 1, 3, and 5 days after *P. aeruginosa* infection was assessed by the Mann-Whitney U test. The significance of other assays were evaluated by unpaired, two-tailed Student's *t*-test. Continuous data from multiple groups were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. $p<0.05$ was considered statistically significant.

Results

NLRP12 Expression Was Decreased in Mouse Corneas and BMDMs Challenged With P. Aeruginosa

To investigate whether NLRP12 is involved in ocular inflammation, a murine model of *P. aeruginosa* keratitis[31] was established to mimic clinical ocular infection. The mRNA and protein expressions of NLRP12 in normal uninfected and infected corneas of B6 mice were measured by Real Time-PCR and Western blot, respectively. It was shown that NLRP12 was expressed in the normal mice cornea and gradually decreased at 1, 3, and 5 days post-infection (Figure 1A, 1C). We also examined the expression of NLRP12 in normal uninfected and infected BMDMs from B6 mice. Inconsistent with the front results of mice corneas, NLRP12 in BMDMs was expressed in the normal macrophages and gradually decreased at 12 h, 24 h, and 48 h after *P. aeruginosa* infection (Figure 1B, 1D).

NLRP12 Delayed the Disease Progression of P. Aeruginosa Keratitis

To evaluate the potential role of NLRP12 in *P. aeruginosa* keratitis, B6 mice received a subconjunctival injection of NLRP12-lentivirus Ctl-lentivirus, followed by infection with *P. aeruginosa* routinely. Clinical scores showed that the overexpression of NLRP12 reduced disease severity of B6 mice at 1, 3 and 5 days post-infection (Figure 2C, $p<0.01$). The efficacy of *in*

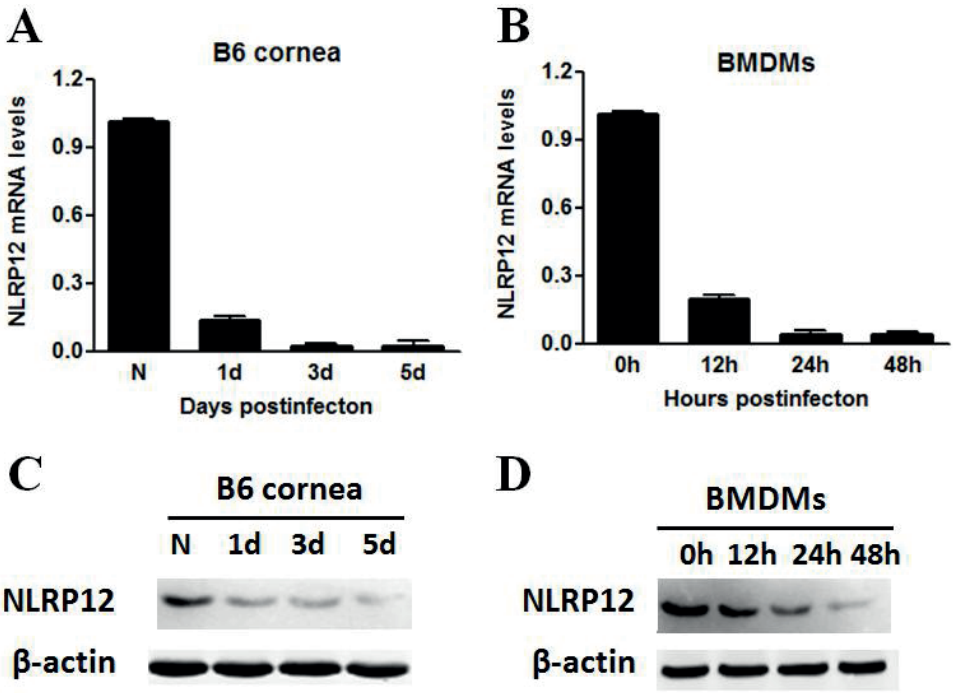

Figure 1. NLRP12 expression in B6 cornea and BMDMs. ***A,*** NLPR12 mRNA levels in normal (N) and infected cornea of B6 mice at 1, 3 and 5 days post-infection were tested by Real-Time RT-PCR. ***B,*** NLPR12 mRNA levels were examined in BMDMs infected with P. aeruginosa at 12 h, 24 h and 48 h post infection. The values represent means ± SEM of mRNA levels after normalizing to β-Actin. ***C,*** NLPR12 protein levels in normal (N) and infected cornea of B6 mice at 1, 3 and 5 days post infection were tested by Western blot. Data represent one of three similar experiments each using 5-pooled corneas per time. ***D,*** NLPR12 protein levels were examined in BMDMs infected with P. aeruginosa at 12 h, 24 h and 48 h post-infection.

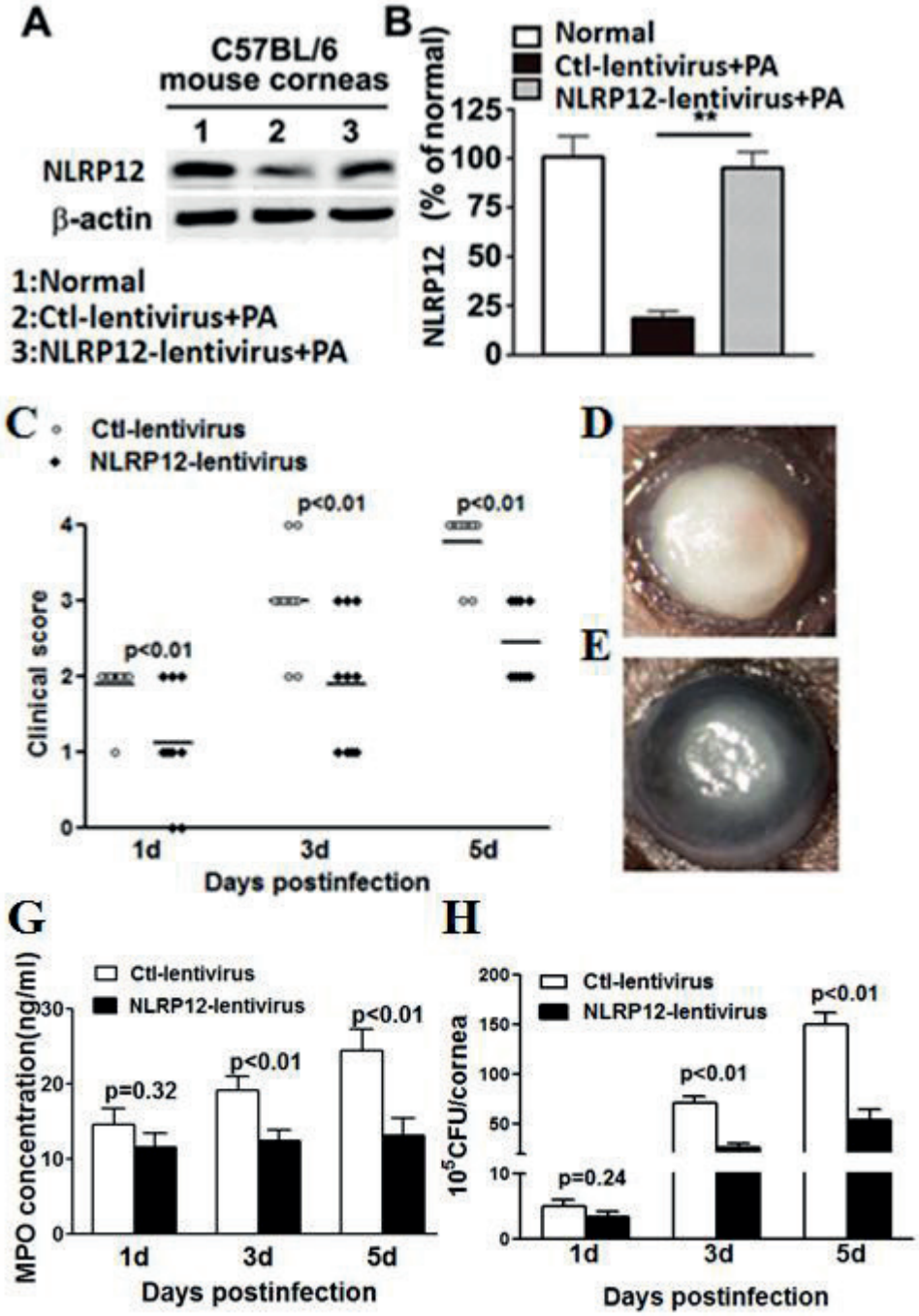

Figure 2. NLRP12 promoted host resistance against *P. aeruginosa* keratitis. NLRP12 protein levels were tested by Western blot ***A-B.*** Clinical scores C, and slit lamp at 3 days post infection reduced disease severity in control ***D,*** or NLRP12-lentivirus treated ***E,*** B6 mice. NLRP12-lentivirus led to reduced recruitment of PMNs as detected by MPO activity ***G,*** and decreased bacterial counts ***H,*** at 3 and 5 days post infection (p<0.01). Data are the means ± SEM and represent two individual experiments each with five animals per group/time/assay. PA stands for P. aeruginosa; **p<0.01.

vivo use of NLRP12-lentivirus was confirmed by Western blot, and showed that *P. aeruginosa* infection reduced NLRP12 expression in B6 corneas (Figure 2A), but the subconjunctival injection of NLRP12-lentivirus significantly enhanced NLRP12 expression (Figure 2B). Treatment with NLRP12-lentivirus resulted in a decrease of corneal opacity in *P. aeruginosa* infected B6 cornea

at 3 days post-infection (Figure 2D, grade = +3), while in the control group, the grades of the infected corneas at 3 days post infection were +2 (Figure 2E). Since both host inflammation and bacterial virulence contribute to the pathogenesis of *P. aeruginosa* keratitis, we further investigated the effect of NLRP12-lentivirus on PMN infiltration and bacterial load. The PMN infiltration was significantly reduced in mice infected by NLRP12-lentivirus compared with that by Ctl-lentivirus ($p<0.01$) (Figure 2G). Of note, NLRP12-lentivirus infection reduced the bacterial load after 3 and 5 days ($p<0.01$).

NLRP12 Inhibited Pro-Inflammatory Cytokine Expression in P. Aeruginosa Keratitis

To uncover the mechanism by which NLRP12 delayed the disease progression of *P. aeruginosa* keratitis, we first examined the expression of pro-inflammatory cytokines by Real Time-PCR and ELISA in groups of NLRP12-lentivirus and Ctl-lentivirus before and after *P. aeruginosa* infection. The PCR analysis showed that the over-expression of NLRP12 suppressed the mRNA expression of IL-1β (Figure 3A), MIP-2 (Figure 3B), IL-6 (Figure 3C), and TNF-α (Figure 3D) in infected corneas. Consistently, the protein expression levels of IL-1β, MIP-2 (Figure 3E), and IL-6, TNF-α (Figure 3F) measured by ELISA were significantly downregulated due to over-expression of NLRP12. These data suggested that NLRP12 decreased secretion of pro-inflammatory cytokines induced by *P. aeruginosa* infection.

NLRP12 Promoted Intracellular Bacterial Killing in P. Aeruginosa-Challenged Macrophages

In addition to *in vivo* murine model, we also evaluated the role of NLRP12 on the inflammatory response *in vitro*. RAW 264.7 cells were infected with NLRP12-lentivirus or Ctl-lentivirus, followed by *P. aeruginosa* challenge at MOI 25. Our result showed that *P. aeruginosa* infection reduced NLRP12 expression in RAW 264.7 cells, but the infection of NLRP12-lentivirus apparently enhanced NLRP12 expression (Figure 4A, 4B). Plate count analysis showed that the over-expression of NLRP12 significantly reduced the intracellular bacterial load in RAW264.7 cells at 1 and 2 h post-infection (Figure 4C, $p<0.01$ and $p<0.001$, respectively), compared with that of control. The result of bacterial clearance indicated that the over-expression of NLRP12 significantly enhanced intracellular bacterial killing in RAW264.7 cells (Figure 4D, $p<0.01$).

NLRP12 Inhibited Inflammatory Cytokine Expression in P. Aeruginosa-Challenged Macrophages

Consistent with *in vivo* studies, we also found downregulation of inflammatory cytokines *in vitro*. Macrophages were infected with NLRP12-lentivirus or Ctl-lentivirus, followed by PA challenge. The levels of inflammatory cytokines IL-1β (Figure 5A), MIP-2 (Figure 5B), IL-6 (Figure 5C) and TNF-α (Figure 5D) were significantly downregulated due to the overexpression of NLRP12. Meanwhile, ELISA data indicated that the protein levels of IL-1β, MIP-2 (Figure 5E), IL-6 and TNF-α (Figure 5F) were significantly downregulated after overexpression of NLRP12. Both *in vivo* and *in vitro* studies indicated that NLRP12 inhibited inflammatory response by suppressing inflammatory cytokines production during *P. aeruginosa* infection.

NLRP12 Negatively Regulates NF-κB Signaling

Since stringent control of the NF-κB signaling pathways is critical to effective host immune responses, we next assessed the potential role of NLRP12 in regulating the activation of NF-κB signaling in macrophages infected with *P. aeruginosa*. Murine macrophage-like RAW264.7 cells were infected with NLRP12-lentivirus or Ctl-lentivirus, followed by *P. aeruginosa* challenge. The level of phospho-IκB was gradually upregulated at different time points after *P. aeruginosa* infection (Figure 6A). However, phospho-IκB levels were significantly decreased after treatment with NLRP12-lentivirus compared with Ctl-lentivirus after 1 and 2 h (Figure 6B, $p<0.05$ and $p<0.01$, respectively). To determine whether NLRP12 was involved in *P. aeruginosa*-mediated NF-kB activation, we transfected human HEK293T (293T) cells with NF-κB-Luciferase reporter DNA, with or without the NLRP12 plasmid, followed by treatment with *P. aeruginosa*. The result of Figure 6C showed that NLRP12 potently inhibited *P. aeruginosa*-induced NF-κB activation. The expression of IKKα (Figure 6D) or IKKβ (Figure 6E) significantly induced NF-κB-Luciferase activity, but this activity was inhibited when NLRP12 was cotransfected. These data suggested that NLRP12 might be a negative regulator of NF-κB activation induced by *P. aeruginosa*.

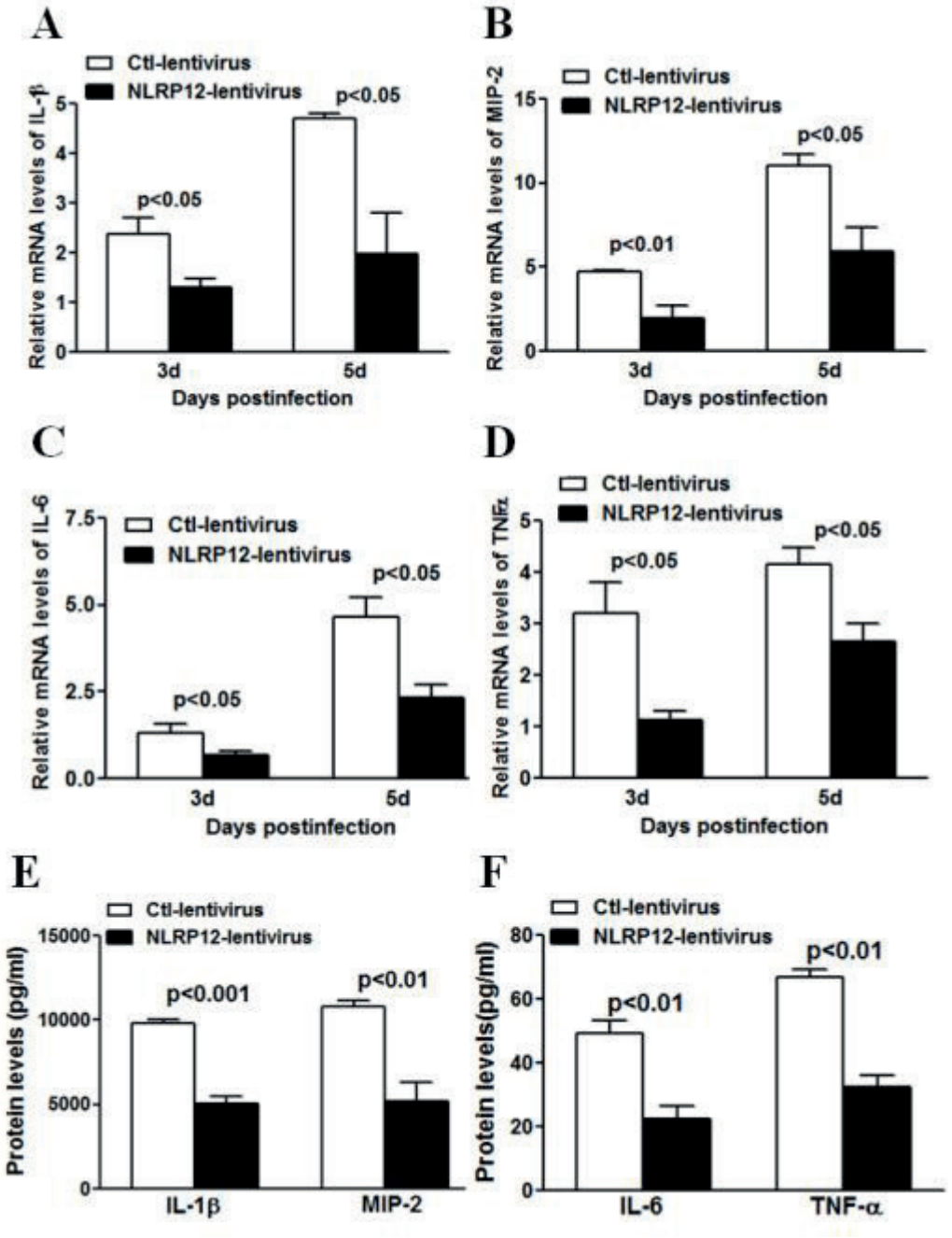

Figure 3. NLRP12 regulated inflammatory cytokine expression in *P. aeruginos*a-infected corneas. The mRNA expression levels of IL-1β ***A,*** MIP-2 ***B,*** IL-6 ***C,*** TNF-α ***D,*** were examined by Real Time-PCR, and the protein expression levels of IL-1β, MIP-2 ***E,*** IL-6, TNF-α ***F,*** were tested by ELISA in the uninfected and infected corneas at 5d post infection after treatment with NLRP12-lentivirus or Ctl-lentivirus. Data were the mean ± SEM and represent three individual experiments each with 5 mice/group/time.

Discussion

NLRP12 is a less characterized member of the NLR family. Recent studies have shown that NLRP12 played inflammasome-dependent and inflammasome-independent roles in the development and pathogenesis of infectious diseases[21,32,33]. However, NLRP12's role in ocular infection remains unknown. Our data demonstrated that the NLRP12 expression was decreased in B6 corneas as well as BMDMs after *P. aeruginosa* infection in a time- and dose-dependent manner. Interestingly, It was shown

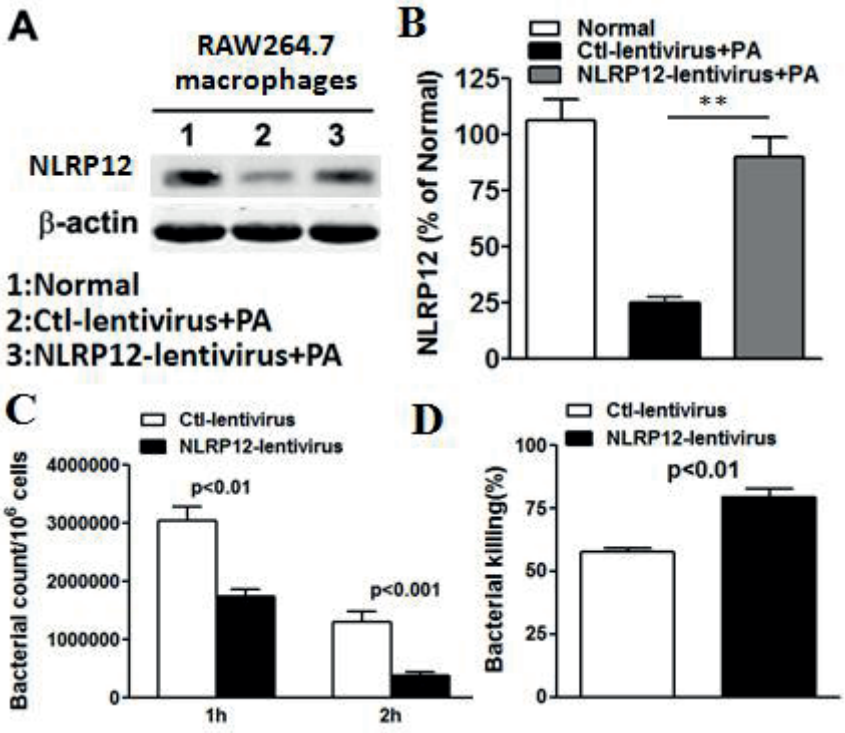

Figure 4. NLRP12 promoted bacterial killing in *P. aeruginosa*-infected macrophages. NLRP12 protein levels were tested by Western blot ***A-B.*** Bacterial counts ***C,*** and bacterial killing ***D,*** were tested by plate counts in control vs. NLRP12-lentivirus treated macrophages. Data were the mean ± SEM and represent three individual experiments. PA stands for *P. aeruginosa*; ***p*<0.01.

that *Salmonella (S.) Typhimurium* infection increased the mRNA level of NLRP12 in macrophages from wild-type mice, whereas M. tuberculosis infection downregulated NLRP12 transcription in THP-1 monocytic cell line[23,32]. The downregulation of NLRP1by infection of *P. aeruginosa* 2 suggested a potential role in ocular immunity. We indicated that the subconjunctival injection of NLRP12 lentivirus decreased disease severity in the infected corneas, suggesting that NLRP12 delayed the disease progression of *P. aeruginosa* keratitis. Vladimer et al[21] showed that Nlrp12-/- mice are hypersusceptible to Yersinia pestis infection, whereby NLRP12 was required as an inflammasome component to promote caspase-1 activation and IL-1β/IL-18 release. Zaki et al[32] indicated that Nlrp12-/- mice were highly resistant to *S. Typhimurium* infection by reducing bacterial burdens and increasing the production of proinflammatory cytokines, while another study showed that WT and Nlrp12-/- mice exhibit similar host innate responses to lung infections induced by Mycobacterium tuberculosis or *Klebsiella pneumoniae*[33]. These conflicting results might arise from the fact that its role can be pathogen-specific as well as time and cell type. Our *in vivo* and *in vitro* studies showed that the overexpression of NLRP12 decreased proinflammatory cytokine levels in *P. aeruginosa*-infected mouse corneas and macrophages, indicating the anti-inflammatory activity of NLRP12 in *P. aeruginosa* keratitis. The pathogenesis of *P. aeruginosa* keratitis is largely attributed to inflammation-induced tissue damage. In the process of infection, corneal resident cells were first activated, resulting in secretion of cytokines like IL-12 and the subsequent sequential influx of PMNs and macrophages[4]. These infiltrating leukocytes, in turn, produce a large amount of pro-inflammatory cytokines such as IL-1β, IL-6, MIP-2, and TNF-α, which can recruit infiltration of more inflammatory cells, and thus form positive feedback of local inflammation[2] which contribute to the pathogenesis of corneal disease[34,35]. Inflammatory mediators are important for host defense through the clearance of infectious pathogens[4]. However, in our study, we found that NLRP12 decreased the bacterial load both *in vivo* and *in vitro*. In this situation, NLRP12 may be involved in restricting uncontrolled inflammation, which is ultimately beneficial for anti-infection immunity. In the context of *P. aeruginosa* infection, the present study identified NLRP12 as an NLR member that inhibits NF-κB activation. NLRP12-lentivirus treatment significantly dampened the activation of NF-κB signaling in RAW264.7

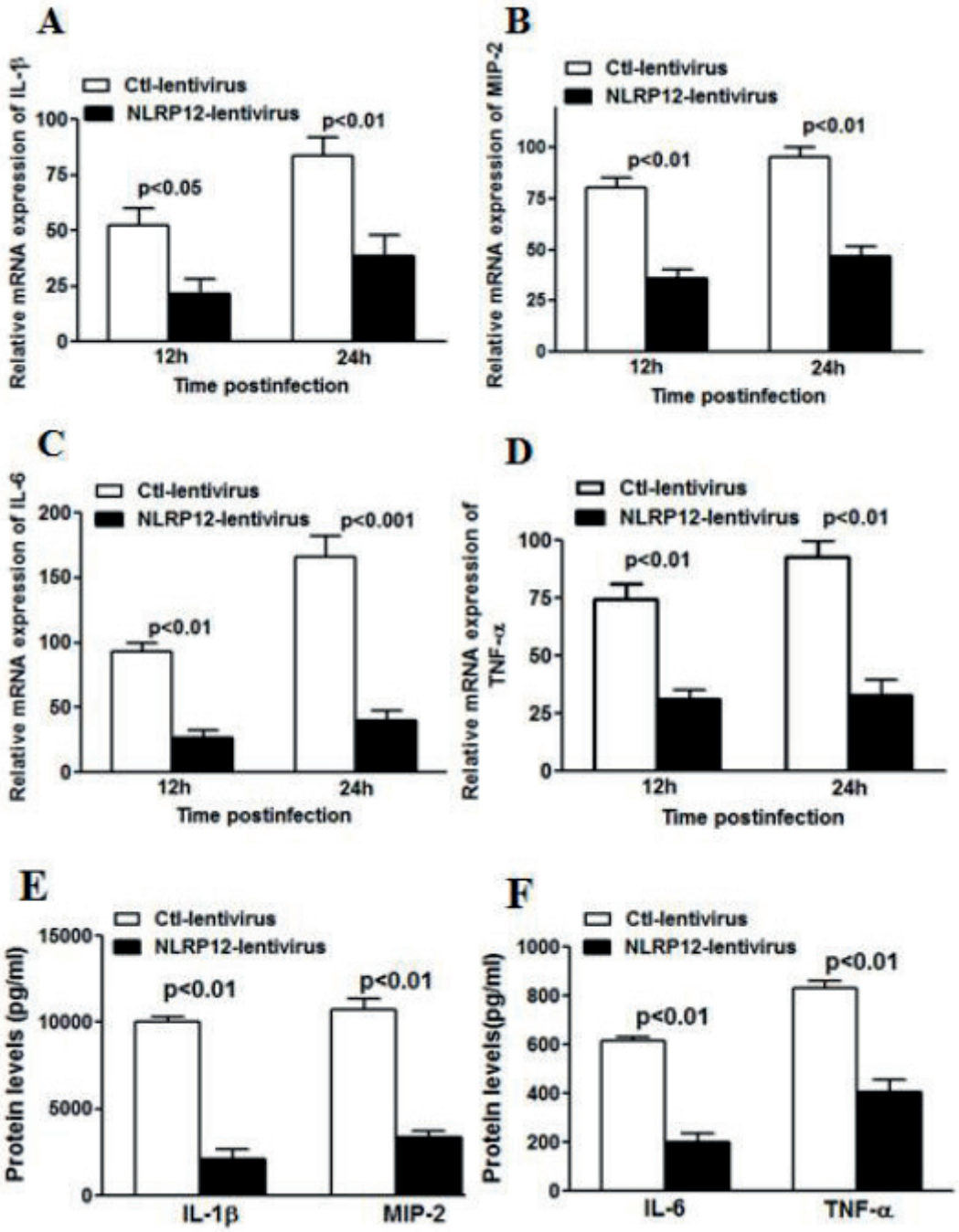

Figure 5. NLRP12 regulated inflammatory cytokine expression in *P. aeruginosa*-infected macrophages. The mRNA expression levels of IL-1β A, MIP-2 B, IL-6 C, TNF-α D, were examined by Real Time-PCR, and the protein expression levels of IL-1β, MIP-2 E, IL-6, TNF-α F, were tested by ELISA in the uninfected and infected macrophages at 24 h post infection after treatment with NLRP12-lentivirus versus Ctl-lentivirus. Data were the mean ± SEM and represent three individual experiments.

cells and Luciferase assays confirmed the interaction between NF-κB IKKα/IKKβ and NLRP12. Previous evidence demonstrated that a synthetic analog cord factor, trehalose-6,6-dimycolate (TDP) from *Mycobacterium tuberculosis* and LPS from *K. pneumoniae* significantly increased the levels of TNF-α and IL-6 in Nlrp12$^{-/-}$ bone marrow-derived DCs compared with those in WT cells, indicating that NLRP12 plays a role in suppressing NF-κB signaling in response to these bacterial components[33].

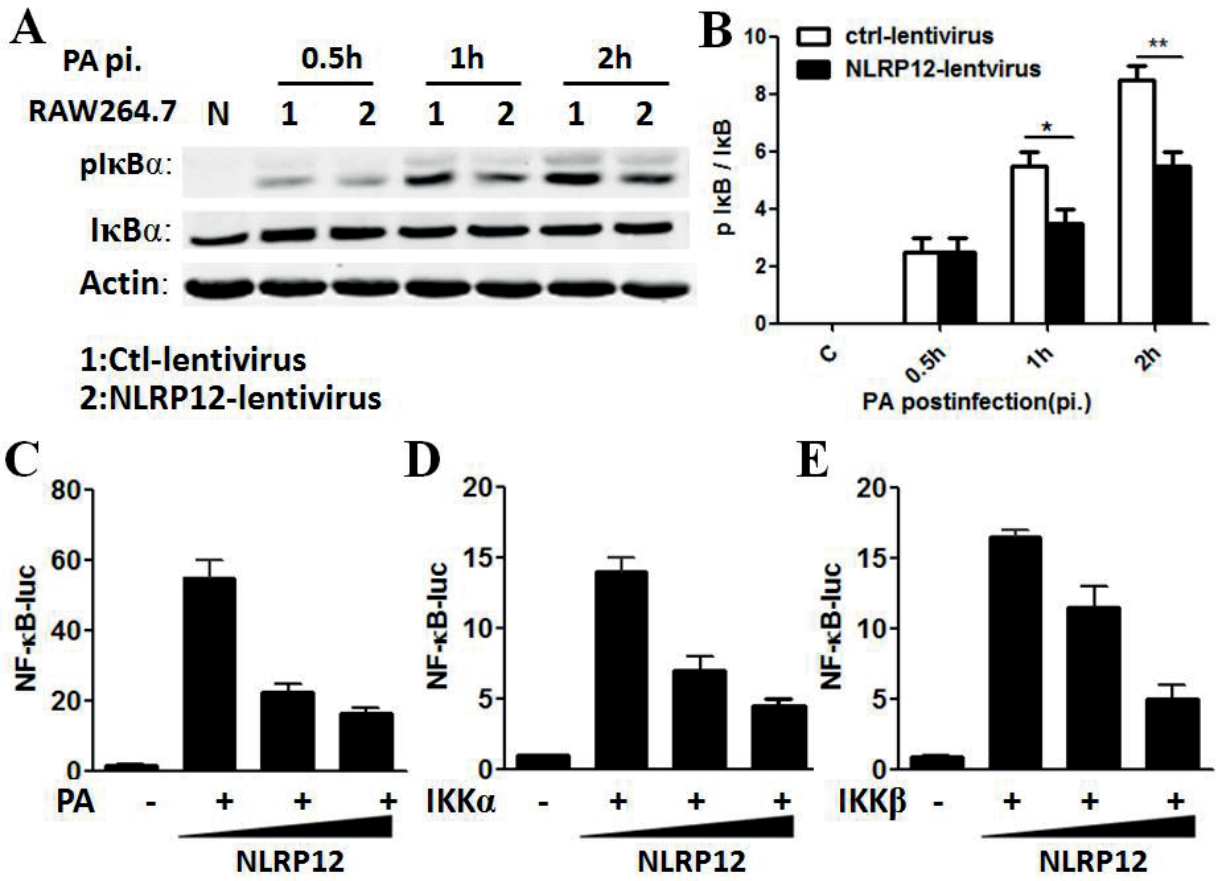

Figure 6. NLRP12 negatively regulates NF-κB signaling. ***A,*** pIκBα levels was evaluated in macrophages from different groups. ***B,*** Bar graphs showed pIκBα and IκBα levels. ***C,*** 293T cells were transfected with an NF-κB-Luciferase reporter plasmid, together with an empty vector or NLRP12 construct. NF-κB-dependent Luciferase activity (fold induction) was detected after treatment with P. aeruginosa. ***C-D,*** 293T cells were transfected with IKKα and IKKβ, along with NF-κB-Luciferase. PA stands for P. aeruginosa; **p*<0.05; ***p*<0.01.

Similarly, during the efficient control of *S. Typhimurium* infection, NLRP12 was shown to suppress the activation of NF-κB and ERK signaling and to inhibit the production of inflammatory cytokines and NO[32]. However, whether NLRP12 suppresses the NF-κB activation (e.g., *Salmonella*, *Mycobacterium,* and *Klebsiella* infections) or activates caspase-1 (e.g., Yersinia infection) remains unclear and requires further investigations.

Conclusions

We demonstrated that NRP12 reduced the severity of *P. aeruginosa* keratitis via inhibiting corneal inflammation and enhancing bacterial killing, which provides a potential therapeutic strategy for *P. aeruginosa* keratitis.

Acknowledgments

This study was supported by National Natural Science Foundation of China (81601382), Guangdong Medical Science Foundation (A2016543), Shenzhen Science and Technology Plan Project (JCYJ20160428141830465), (JCYJ20160428135756806) Shenzhen Longhua District Science and Technology Innovation Fund Project (20160523A1030137).

Conflict of Interest

The Authors declare that they have no conflict of interest.

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